7th CMAPSEEC

Conference on Medicinal and Aromatic Plants of Southeast European Countries

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7th Conference on Medicinal and Aromatic Plants of Southeast European Countries (7th CMAPSEEC)

Subotica (Serbia), May 27th-31st, 2012

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&
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Foreword

Medicinal and aromatic plants (MAP) have been essential resources for human health from ancient times to the present day. The majority of the world's population depends on traditional medicine for primary health care needs. More than 35.000 plant species are used in herbal medicine and as spices, out of the most are of a local importance due to traditional use. Because of their increasing appliance in pharmaceutical, food, cosmetic and beverage industry, as well as use in folk and official medicine, veterinary and plant protection, herbal industry has been recognized as an important element of global economy.

Together with growth in global demand for medicinal plants and in local demand for plant based traditional medicines, the pressure on the existing populations of medicinal plants has increased tremendously during the last few decades. The extinction or scarcity of these plants is not only a problem for conservation – it also results in serious problems for people's health and livelihoods. Cultivation may reduce harvesting pressure on some wild species, particularly rare and threatened species, and thus can also be an important production strategy that supports conservation.

South East Europe is particularly appreciated for richness in indigenous MAP resources and long tradition in use of MAP and their products. The region is known as one of the main suppliers of MAP raw material into EU and US. In addition, medicinal plants were being the subject of a great scientific interest in the SEE region, where significant contribution to understanding of various research aspects in number of MAP species was achieved by Conferences on Medicinal and Aromatic Plants of Southeast European Countries, under organization and support of the Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC) established in 2000 by the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, R. of Serbia.

We are very proud that our traditional, the 7th Conference on MAP of SEE countries took place in Serbia again, after the second held in Greece, the third in Slovakia, followed by Romania, Czech Republic and Turkey.

The 7th CMAPSEEC gathers together not only the researchers from SEE region, but from all over the world. At the Conference participated over 150 researchers, experts, company's representatives and guests interested in MAP diversity, biology, conservation, ecology, phytochemistry, pharmacology, breeding, cultivation and biotechnology. Near 200 of summaries of research contributions were presented, out of about 80 in a form of full papers. All contributions covered very different research areas, and were classified into to the three distinct groups: "*MAP diversity at all levels and tools for its evaluation", "Pharmacology and biological effects of active MAP compounds"* and *"Map cultivation, breeding and biotechnology"*.

The full papers, categorized as review papers, original scientific papers and professional papers, within this proceedings were reviewed by our Scientific Committee. In addition, a small group of papers was issued either without referring on reviewer's comments or without revision as submitted after the deadline. However, we decided to include them as well, considering the subjects and research approaches interesting.

We strongly believe that presented results will contribute to a general knowledge on MAP, and will encourage both young researchers and processing companies to deal with many species whose composition and biological effects were appointed as promising.

Moreover, we hope that pleasant and collegial atmosphere additionally contributed to establishing of the new professional and personal contacts and to strengthening of the ones already established. Finally, strong network on scientists and professionals interested in MAP under AMAPSEEC umbrella might bring new project ideas and new value in the near future.

Editors,

Prof. Dr Zora Dajić Stevanović and Prof. Dr Dragoja Radanović

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Introductory lecture

HYPHENATED TECHNIQUES IN ANALYSIS OF CRUDE PLANT EXTRACTS

Slobodan Milosavljević

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The isolation and structural elucidation of new natural products, *e.g*., from plants or microorganisms, is a rewarding – but often time-consuming – task, since it is a major effort to isolate each compound in a pure form, even the known ones. Furthermore, to obtain the required milligram quantities of all metabolites, even in the case of minor substances, one may need large amounts of the sometimes rare biological material and expensive tools and supplies, like *e.g*., adsorbents and eluents. Another problem, in particular when dealing with unstable compounds, is that they may decompose already during the preparative separation and thus may escape the analysis. The development of measurement techniques coupled (hyphenated) to high performance liquid chromatography (HPLC) in the past 20 years, such as LC-UV- photodiode array detection (LC-UV DAD), LC-mass spectrometry (LC-MS), LC-multiple stage MS (LC-MS^n), LC - infrared spectroscopy (LC-IR), LC - nuclear magnetic resonance spectroscopy (LC-NMR) and LC-circular dichroism (LC-CD), has provided powerful tool for direct analysis of complex mixtures. The combination of the high separation efficiency of HPLC with these different detectors has made possible the acquisition of on-line complementary spectroscopic data on an LC peak of interest within a intricate melange (Fig. 1).

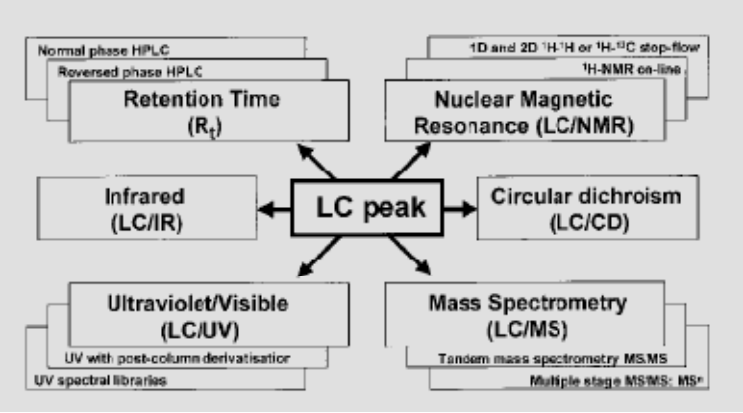

Figure 1. Type of information that can be obtained from a given LC peak using the different LC coupled (hyphenated) techniques available. [1].

The use of liquid chromatography/mass spectrometry (LC/MS) for these analyses can now be considered a standard analytical tool. A number of LC/MS interfaces are available to cope with a variety of compound types and a wide range of LC solvent systems. Most LC/MS interfaces involve the use of soft ionization techniques, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Consequently, the mass spectral data usually provide good molecular mass

information but limited structural information, as little fragmentation occurs. To obtain structural information, analyte ions are fragmented by colliding them with neutral molecules in a process known as collision induced dissociation (CID) or collisionally activated dissociation (CAD). Voltages are applied to the analyte ions to add energy to the collisions and create more fragmentation. CID is most often associated with multistage mass spectrometers where it takes place between each stage of MS filtering. Multiple-stage MS (also called tandem MS or MS/MS or MS^n) is a powerful way to obtain structural information. The most frequently used devices for LC-MS/MS are triple-quadrupole (Triple Quad) or quadrupole/quadrupole/time-of-flight (Q-TOF) mass spectrometers. The instruments which could carry out multistep MS, i.e. LC-MS^n, are those equpped with ion trap (ITD), ion cyclotron resonce (ICR) or orbitrap mass analysers.

However, the MS detection techniques mentioned above, do not allow a full on-line identification, except for some well-known natural products, and complementary on-line spectroscopic information is needed. In this regard, HPLC coupled to nuclear magnetic resonance (LC-NMR) represents a potentially interesting complementary technique to LC-UV-MS for detailed on-line structural analysis Indeed recent progress in NMR technology has given a new impetus to LC-NMR, which is now emerging as a powerful analytical tool. The hyphenation of NMR with high-performance liquid chromatography (HPLC), pioneered in the 1980s, found wide application from the mid-1990s and is now a well established analytical technique, especially in pharmaceutical and natural products research. However, partly owing to the intrinsic insensitivity of NMR and partly owing to practical limitations, the absolute sensitivity of the method has remained below that of other structural elucidation techniques (*e.g.* HPLC–MS). In recent years, some of this inherent insensitivity has been overcome by improvements in spectrometer hardware and solvent suppression pulse sequences which allow small molecules in the low to sub-microgram range to be analysed. Further improvements in spectrometer hardware such as cryogenic probe technology and miniaturized probes are now becoming widely available and these are leading to greater steps in sensitivity enhancement. Recently, a different approach to performing HPLC–NMR experiments has become available in which the separation step of HPLC–NMR has been modified so as to increase the absolute sensitivity of the technique. In this method, analytes eluting from theHPLC column are trapped on solid phase extraction (SPE) cartridges prior to subsequent elution into the NMR flow probe for analysis using an NMR-compatible solvent: this is HPLC–SPE–NMR. First published as a concept by Griffiths and Horton in 1998 [2]. and later applied using SPE cartridges in a custom set-up, this technique has recently become commercially available and its application to natural product analysis has been reported elsewhere [3]. The traditional use of SPE in analytical chemistry is as a sample purification and enrichment tool prior to HPLC analysis. However, in HPLC–SPE–NMR it is used as a means of trapping and concentrating analytes after HPLC separation and through the use of strong solvents the analyte can be eluted from the cartridge in a sharp, highly concentrated elution band, which results in an improved signal-to-noise ratio (S/N) compared with conventional HPLC–NMR.

The combination of liquid chromatography with detection methods such as NMR spectroscopy (HPLC-NMR) and tandem mass spectrometry (HPLC-MS/MS) has recently led to new strategies by which biological matrixes, e.g., crude plant extracts, are screened to

obtain as much information about known constituents as possible even with a minimum amount of material. By these techniques, even the discovery of new structures is possible, including the elucidation of the full constitution and, more recently, the relative configuration. But due to the intrinsic nonchiral character of NMR signals and mass fragments, no information concerning the absolute configuration is available unless by the use of chiral chromatographic phases, which requires prior possession of both configurationally known enantiomers. A method that allows the assignment of absolute configurations of compounds in an extract matrix would complete the structural elucidation without the necessity of isolation and purification. Widely used for the attribution of the absolute configuration is the application of circular dichroism (CD) spectroscopy. The application of CD spectroscopy, however, usually requires pure compounds. Although the on-line coupling of liquid chromatography with chiral detectors is well-known, applications are still rare. Circular dichroism, which is based on the absorption difference between right and left circularly polarized light is compatible with gradient elution. In the literature, most applications of HPLC-CD coupling have focused on the determination of the elution order of the enantiomers of given chiral compounds (e.g., amino acids or drugs) on chiral stationary phases using normal-phase chromatographic solvent conditions. The use of reversed-phase systems with nonchiral columns and CD detection has been reported previously only for the analysis of model mixtures of small proteins. The first applications of HPLC-CD for the profiling of crude extracts with standard C-18 stationary phases operating under reversed-phase conditions have been was reported in 1999 by G. Bringmann *et al*. [4]. Moreover, a strategy for the complete structural elucidation, including the absolute configuration of new alkaloids, the constituents of the tropical liana *Habropetalum dawei*, by the complementary use of the "HPLC-NMR/HPLC-MS/HPLC-CD triad" was presented.

The application of some hyphenated techniques mentioned above (LC-MS and LC-NMR), used for analysis of complex mixture of natural products is presented in this lecture through few selected examples.

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Plenary lecture
Review paper

CONSERVATION OF DIVERSITY OF MEDICINAL AND AROMATIC PLANTS IN SOUTHEAST EUROPE: CURRENT STATE AND FUTURE CHALLENGES

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SUMMARY

Southeast European countries due to position on the Balkans, as one of the world's biodiversity centres, are known for high floristic diversity, including richness of medicinal and aromatic plant resources that have been traditionally utilized by local population for long time in folk and veterinary medicine, as well as in different local products. Number of vascular species of the SEE region could be estimated as higher than 8000, out of at least 1000 is traditionally and/or officially used as MAP.

Sustainability of SEE herbal sector is depending on both size and structure of natural MAP populations and position of MAP collectors as key, but marginalized element within the whole value chain. However, the whole region faces with intensive depopulation of rural areas resulting in serious loss of MAP collectors, whose number dropped to about one third of former number in 1990s. Long term period of free access to MAP resources lacking in mechanisms of control of the wild collection in the past, followed with increased habitat alteration and remaining of much smaller collecting areas at present led to over-harvesting and decline in stocks of resources of many important MAP species. The endangered MAP species common for the SEE region includes *Gentiana lutea subsp. symphyandra, G. punctata, Adonis vernalis, Acorus calamus, Sideritis raeseri, Helichrysum plicatum, Arnica montana, Swertia punctata, Ilex aquifolium, Glycyrrhiza glabra, Arctostaphylos uva-ursi*, etc. In order to preserve the MAP biodiversity and develop the herbal sector in SEE there is a need for a long-term and complex strategy, able to ensure implementation of standards, certification and quality control system harmonized with those in EU. In medium term period, efforts put in strong increasing of activities on MAP collection, cultivation and processing could lead to establishment of a network of small and medium enterprises, farms, collecting centres and ethno-villages aiming to revive abandoned and insufficiently utilized rural landscapes of the region. High priority to programs for the conservation of medicinal plants should be justified on the basis of savings that the plants generate for national health expenditure and thus to the national economy. The concept of sustainable biodiversity use aiming at it conservation is the only model that could strongly contribute to the mountain rural development. Moreover, there is a need of upgrading of the existing regulatory framework and strengthening of its enforcement, especially in light of monitoring of MAP resources and evaluation of its ecosystem services.

INTRODUCTION

Together with growth in global demand for medicinal plants, the pressure on their existing populations has rapidly increased during the last few decades. About 15,000 species of medicinal plants are globally threatened. The key causes include loss of habitat and habitat fragmentation, over-harvesting, improper collecting practices and pollution.

In order to stop further biodiversity loss of natural resources of medicinal plants, it is needed to evaluate the remaining stocks of MAP populations and perform their sustainable and continuous use in order to conserve this essential part of our natural and cultural heritage [1]. The main general and long-term goal of conservation of target MAP species is to protect, manage and monitor the selected populations in the direction of maintenance of the natural evolutionary processes, thus allowing new variations in the gene pool allowing the species to adapt to changing environmental conditions [2].
There are three main conservation strategies of MAP species: *in situ* (protection of their habitats), *ex situ* (conservation at species and germplasm level through field collections, botanical gardens and gene banks out of their natural habitats) and domestication/ reintroduction and cultivation which could be conducted either *in situ* or *ex situ* [3].

PRESENT STATE ON MAP DIVERSITY AND KEY IMPACTS AND THREATS FOR ITS CONSERVATION

Biodiversity of MAP in SEE: quantitative and qualitative evaluation

The flora of the Balkan Peninsula is one of the most diverse floras in Europe, comprising more than 8000 species of vascular plants, out of about 2600-2700 are known as endemic species [4]. South East Europe, i.e. the Balkan Peninsula, is particularly appreciated by quantitative (number of species) and qualitative (endemic, relic and internationally important species and habitats) values of biodiversity, including a great variety of mosaic habitats within the mountains, forests, grasslands, river gorges, lakes and coastline. The Balkan and Rhodope Mountains are recognized as global Centers of Plant Diversity.
The region is also well known for richness in indigenous MAP resources and long tradition in use of MAP and their products. The total number of MAP species in SEE reaches at least 1000 species. In addition, the region is one of the main suppliers of MAP raw material into EU and US.
According to the recent surveys on quantities and trade of the wild collected MAP in SEE [5], [6], the most used species include: yarrow (*Achillea* ssp.), nettle (*Urtica dioica*), St. John's worth (*Hypericum perforatum*), marshmallow (*Althaea officinalis*), cowslip (*Primula* ssp.), elder (*Sambucus nigra*), rosehip (*Rosa* ssp.), linden (*Tilia* ssp.), wild thyme (*Thymus serpyllum*), savory (*Satureja montana*), hawthorn (*Crataegus* ssp.), plantain (*Plantago* ssp.), blueberry (*Vaccinium myrtillus*), sage (*Salvia officinalis*), oregano (*Origanum vulgare*), juniper (*Juniperus communis*), wild garlic (*Allium ursinum*) and some others. Despite the sufficient resources and generally high distribution of these species throughout the region, there is particular threat for maintaining of their genetic diversity and preservation of populations exposed to excessive use.
The special feature of the Balkan's flora is high endemism, also referring to MAP species. In general, endemic MAP species of SEE are not sufficiently researched and many of them are in fact unknown for their chemical profiles and related biological activity. Knowing the importance of searching for new phytochemicals and natural sources of high biological effectiveness, much more attention should be paid on comprehensive and coordinated research of MAP endemics, out of some have already shown promising performances (Tab. 1).

Tab.1. Endemic MAP species of SEE: phytochemistry and biological activity

Species	Distribution Habitat	Active compound	Activity	Reference
Achillea alexandri-regis	KOS Alpine shrub vegetation	Triterpenoids, flavonoids, phenolic acids, lignans	Cytotoxic , antioxidant, anti-inflammatory, anti-ulcer activity	Kundaković et al, 2005 [14]
Helichrysum plicatum	SRB, ALB High mountain grassland vegetation	Phenolic compounds, flavonoids, triterpenoids, diterpenoids, steroids, hydroxyisopentenyl acetophenone, and phloroglucinol	Cytotoxic, antidiabetic, antiviral antimicrobial, antimutagenic antioxidant activity	Bigović et al., 2011 [15] Aslan et al., 2007 [16]
Centaurea kosaninii	ALB, MAC, SRB Grassland and rocky vegetation on serpentine	Sesquiterpene lactones	Cytotoxic activity	Janaćković et al., 2008 [17]
Hypericum rumeliacum	ALB, BUG, MAC, SRB Grassland and rocky vegetation	Phenolic compounds and flavonoids	Antimicrobial, antioxidant, anti-inflammatory activity	Danova et al., 2010 [18]
Micromeria dalmatica	BUG, SRB, MNE Dry rocky habitats	Phenolic compounds	Antimicrobial activity	Šavikin et al, 2010 [19]
Satureja cuneifolia	CRO, ALB, MNE High mountain vegetation	Phenolic compounds	Antimicrobial, analgesic, antioxidant activity	Šavikin et al, 2010 [19]
Sideritis raeseri	BUG, MAC, ALB Dry sub-alpine grasslands	Phenols and flavonoids	Antimicrobial, anti-inflammatory, analgesic, gastroprotective, antioxidant	Pljevljakušić et al., 2011 [20]
Swertia punctata	BUG, SRB, KOS Wet mountain terrains	Xanthones, flavone-C-glucoside	Hypoglycemic, hepatoprotective, antituberculous, antimalarial, anti-inflammatory	Menković et al, 2002 [21]

Apart of the high MAP diversity at the species level, there is significant diversity of habitats particularly rich in MAP. There are many plant communities of different vegetation types (forests, shrub and herbaceous vegetation) characteristic for predomination of one or two MAP species, thus forming characteristic "MAP plant community" (Tab.2). Among them, the highest distribution in the SEE region exhibit the communities of *Vaccinium* ssp. and *Juniperus* ssp. of the sub-alpine zone along with the high mountain conifer communities of *Abies alba* and *Pinus* ssp., as well as deciduous forests of linden (*Tilia* ssp.), birch (*Betula* ssp.) and poly-dominant oak forests with hawthorn (*Crataegus* ssp.) highly distributed within the hilly region. Such plant communities actually could be treated as "MAP vegetation" which needs to be further evaluated in terms of distribution, ecological features, floristic composition, as well as main factors affecting their existence.

Tab.2. Examples of plant communities dominated by MAP species ("MAP vegetation")

Forest MAP communities	Shrub MAP communities	Herbaceous MAP communities
Cotyno coggygriae-Quercetum petraeae Querco-Tilietum tomentosae Tilio-Orno-Quercetum roboris Rusco aculeati-Tilio-Quercetum Crataego nigrae-Populetum albae Corylo colurnae-Fagetum Betuletum verrucosae Pineto peucis-Piceetum excelsae-Vaccinio myrtyllae Cratagetum monogynae	Artemisio-Amygdaletum nanae Astero-Juniperetum oxycedri Hieracio-Juniperetum communis Coryletum avellanae Junipero oxycedri-Prunetum spinosae Pruno spinosae-Crataegetum Vaccinio-Juniperetum sibiricae Juniperetum nanae Vaccinietum uliginosi Daphno alpini-Juniperetum sibiricae Vaccinietum myrtilli Coryletum colurnae	Artemisio-Salvietum officinalis Calamintha acinos-Mentha thymifolia Gentiano-Anemonetum elatioris Equiseto-Eriophoretum latifolii Junco-Menthetum longifoliae Atropetum belladonae Thymo-Chrysopogonetum grylli Teucrio-Artemisietum camphoratae Hyperico-Trifolietum trichopteri Acoretum calami Pulicaria vulgaris-Mentha pulegium Agropyretum intermedio-repentis Leonurus cardiaca-Ballota nigra Marrubium vulgare-Atriplex rosea Tussilaginetum farfarae Polygonetum avicularis

Impacts and threats for MAP resources conservation in SEE

There are several factors strongly affecting the MAP biodiversity and further maintenance of MAP resources in SEE, which are mainly related to loss and/or habitat alterations, over-harvesting and improper use and management of natural resources which is all tightly linked with complex issues of social and economic period of transition.

Habitat loss and/or alteration

In addition to livestock production, in hilly-mountainous rural areas of SEE, the activities focused on wild collection of MAP are important tool for conservation of natural and semi-natural habitats, the grasslands and forests. Knowing that mountain forests and grasslands are the most valuable biodiversity pools and are of the highest conservation value, the sector of MAP production and processing should be thus much more supported and further developed. The identification and survey of Important Plant Areas (IPAs) in the SEE region [7] and many other studies aiming at protection and sustainable use of biodiversity in SEE (e.g. [8], [9]) underlined the land abandonment as key cause of biodiversity loss in High Nature Value Farmlands e.g. [10]. Traditional human activities in agriculture (mainly mowing and grazing), rural tourism and use of non-wood forest products (NWFP) and MAP, solely or combined, represent the only way for preservation of habitats rich in biodiversity and an efficient measure for prevention the loss of species exposed to spontaneous vegetation successions of the abandoned habitats. Grasslands as major sources of MAP species in SEE e.g. [11], are especially sensitive to changes in their use and management. Consequently, abandonment of grasslands directly causes loss of resources of medicinal and aromatic plants (e.g. *Hypericum perforatum*, *Sanguisorba* ssp., *Filipendula* ssp., *Gentiana* ssp., *Thymus* ssp., *Satureja* ssp., *Achillea* ssp., *Centaurium erythraea*, *Primula* ssp., *Origanum vulgare*, *Teucrium* ssp., *Polygala* ssp., *Geranium* ssp., *Colchicum* ssp., *Carlina acaulis*, etc.).

Habitat alteration also refers to former and current expansion of agriculture, industry, urbanization and tourism in particular parts of SEE, which additionally diminishes the resources of NWFPs and MAP. For example, some steppe species at the north of Serbia and Croatia become endangered because of habitat transformation into the farmland (e.g. *Glycyrrhiza glabra, Adonis vernalis, Paeonia tenuifolia, P. officinalis* subsp. *banatica, Iris pumila, Nepeta pannonica, Colchicum arenarium, Centaurium pulchellum, Gypsophyla*

paniculata, Ruta graveolens, Herniaria incana, etc). On the other hand, drying of swamps and building of irrigation systems reduced the populations of species adapted to conditions of wet habitats (*Acorus calamus, Equisetum arvense, Galega officinalis, Althaea officinalis, Mentha aquatica, Lythrum salicaria*, etc.). Moreover, construction of tourist and other infrastructure in SEE coastal countries such as Croatia, Montenegro and Albania, caused decrease in population diversity of (sub)Mediterranean species, including *Salvia officinalis, Hyssopus officinalis, Rosmarinus officinalis, Lavandula officinalis, Paliurus spina-christi, Silybum marianum, Helichrysum italicum, Punica granatum, Laurus nobilis, Vitex agnus-castus*, etc.

Over-harvesting

In addition to causes such as habitat alteration and habitat loss, stocks of many medicinal plant species in the Balkan countries (Albania, BiH, Macedonia, Kosovo, Serbia, Montenegro and Croatia) have declined in the past decades, whit some species becoming rare or endangered due to over-exploitation. Over-harvesting is also a consequence of not adequate position of collectors and not coordinated and weakly established system of retail centers. In order to gather as much as possible of a quantity of the species, the collectors perform unsustainable and improper harvesting practices. In case of collecting the underground plant organs (roots, rhizomes and tubers) the whole plant is pulling out, whereas parts containing buds are not returned back into the soil, to ensure the reproduction of the plant. Especially threatened are "root drug" species growing more or less solitary, i.e. those which not form abundant groups, such as: *Gentiana lutea, G. punctata, Carlina acaulis, Inula helenium,* etc. Although recognized as internationally important species, many orchids are still harvested for their roots known as "salep" in SEE (mainly in Macedonia, Kosovo and Albania).

The huge problem represents the illegal harvesting of protected species, mainly due to lack of control and inspection in wild collecting and trade. It is well known that many protected species are still harvested and traded through black market channels. This is true for *Gentiana lutea, Arnica montana, Adonis vernalis*, *Menyanthes trifoliata, Ruscus aculeatus, R. hypoglossum, Arctostaphylos uva ursi, Acorus calamus, Cetraria islandica, Cnicus benedictus, Ruta graveolens, Sideritis* ssp., etc.

Finally, due to the fact that MAP collection in SEE is performed only in few target areas of the country, which are thus exposed to risk of over-harvesting. This is mainly consequence of tradition and history of wild collecting, available collectors and existence of the active retailing centers. Therefore, genetic variability and size of population of most harvested species are at risk, because of high pressure on resources of limited capacity. In most of cases, the state or local authorities don't declare obligatory cessation of harvesting for particular or all species in the target area exposed to risk enabling recovery of the vegetation. Sudden high increase of market demands for particular herbs (which often happen in case of MAP) is especially risky for the plants collected from areas of long history of utilization. As a consequence of over-harvesting, there is a group of common endangered species in SEE region, including: *Gentiana lutea, G. punctata, Arnica montana, Arctostaphylos uva ursi, Acorus calamus, Adonis vernalis, Ruta graveolens, Ilex aquiflolium, Sideritis* ssp, *Salvia officinalis, Helichrysum* ssp.*, Paeonia officinalis*, *Swertia punctata, Glycyrrhiza glabra,* etc.

Improper management of plant resources

Improper use and management of plant resources, including wild MAP harvesting, is mostly related to underdeveloped legal framework, as well as weak legislation enforcement and inadequate mechanisms of control of use of bio-resources. In most of SEE countries the legal framework regulating wild collection of flora and fauna is insufficiently consistent and underdeveloped, and usually not fully harmonized with EU regulations. Laws, bylaws and

regulations concerning access and use of MAP are more or less similar, where Laws on Nature Protection (usually amended with Annexes on protected species) and Laws on Protected Areas (defining allowed activities within protected areas) are of the highest importance. Moreover, key international Directives and Conventions on biodiversity have been ratified and related national legislation was formulated, including strategic documents such as biodiversity conservation strategies with action plans. However, management of utilization of MAP differ much among the SEE countries. Red books and Red lists of endangered flora and fauna so far haven't been issued for BiH, Montenegro, Macedonia and Kosovo, whereas for Serbia there are only data on extinct and critically endangered species ("Red book of flora of R. of Serbia 1"). In addition, in front of all countries there is a need to prepare or update the existing Red books and Red lists due to new recommendations and criteria provided by IUCN. In most of cases, lists of endangered species are provisory, lacking in exact field data gathered upon application of regular methodology. Access and use of MAP in SEE countries varies from free access without any serious limits, in case of BiH for example, followed by limits related to protected areas only (Albania, Montenegro and Kosovo), towards the established quota system preventing excessive wild collecting of flora and fauna (Serbia, Croatia). However, the income from taxes and fees gathered from wild collecting usually goes to the state budget and despite the national regulations, it is not used for monitoring and conservation of natural populations. In fact, in all SEE countries the fee rates (licenses, taxes) are not adequate, whereas the newest concepts such as benefit and equitable share, fair trade, "biodiversity offset" and ecosystem services have not been recognized in order to valorise the real value of bio-resources. Although legal framework was much improved during last few years, its implementation in practice is very weak. Thus, the black market is very present, being worst in BiH and Kosovo and tolerable in Croatia. Moreover, there is poor system of recording of wild collected products and data bases often include inappropriate data. If exists, such information system is not transparent and not accessible for the public.Protected areas (PAs) ideally offer a solid basis for sustainable utilization of MAP, because of established system of control of access, use and management of natural resources. In PAs of most of SEE countries there is a proclamation of certain protection zones in which access and use of biodiversity and all other human activities are clearly defined. The major obstacle for future sustainable development of PAs in SEE is definitely associated with low state funding and, in most of cases, lack in management and action plans for promotion of PAs, as values of the highest environmental, economy, social and cultural importance.

In quite all of SEE countries, no monitoring and serious resources assessment is performed, as a necessary tool for establishment a more accurate and more sustainable system of use and management of MAP. Monitoring of bio-resources and state of the biodiversity is occasionally conducted only in some PAs, depending on existing management plans and available funds. Without doubt, regular monitoring is the imperative tool for setting up the permitted quantities of the area, i.e. to establish the proper and effective quota system and thus, the strategy and action plan for sustainable use of MAP.

The key common obstruction for preservation of MAP biodiversity in the frame of sustainable use of bio-resources and development of the herbal sector in SEE countries is definitely dramatic decrease of collectors, especially in light of loss of traditional knowledge on plant's diversity, their use in folk and veterinary medicine, as well as practices of wild collecting and primary processing. For example, in Serbia there are nowadays about 50,000 people involved in collecting of both NWFPs and MAP, comparing with about 150,000 people in the late 1980s. Together with definite loss of experienced collectors, the ethnobotanical knowledge, old recipes and many of local brands containing herbs, berries or mushrooms were irreversibly lost.

FUTURE CHALLENGES AND GOALS IN CONSERVATION OF MAP DIVERSITY

Biodiversity conservation in relation to sustainable use of MAP

Conservation *in situ* essentially means protection of MAP populations by habitat and landscape conservation regulations, mainly related to legislative on nature protection and protected areas (e.g. national parks, nature reserves, outstanding nature areas, monuments of nature, etc). Ideally, all of these species should be maintained and conserved as evolving populations in natural ecosystems. *In situ* conservation of species, populations, and genetic diversity is possible through appropriate natural resource management and protection of habitats. There are few new national and/or international concepts concerning the habitat and landscape protection and thus relevant for the *in situ* conservation of MAP resources. For instance, the Important Plant Areas are internationally important sites for wild plants, identified at a national level using standard criteria. IPAs support existing conservation programs, such as networks of protected areas: the EU Natura 2000 and Emerald, as well as the CBD Global Strategy for Plant Conservation. In addition, implementation and promotion of High Nature Value Farmlands (HNVF) would offer the chance for protection of natural and semi-natural grasslands and agro-forestry areas of high biodiversity and of high conservational importance. All of such international conservation programs and networks (Emerald, Natura, HNVF, etc) are focusing on conservational strategies tightly linked to sustainable use and management of bio-resources, including MAP.

It is quite clear and well accepted that conservation is not considered anymore as purely fundamental, biological approach, protecting the species and their habitats by strict preventing of use and exclusion of all human activities within the protected area. New conservation concepts, however, address much more attention to socio-economic issues and dependence of global, local and regional development on sustainable use of natural resources, aiming at both nature conservation and preservation of traditional knowledge and practices in use of bio-resources. Thus, there is very strong mutual relation between MAP resources conservation and their sustainable use, being dependent in much extent on human activities in PAs and other areas of conservation importance.

International Standard for Sustainable Wild Collection of Medicinal and Aromatic Plants (ISSC-MAP, 2007), developed by BfN, WWF, TRAFFIC and IUCN, provide a strong base of expertise, practical standards and criteria for sustainable MAP wild-crafting [12]. Together with WHO Guidelines on good agricultural and collection practices (GACP) for medicinal plants (WHO, 2003) these guidelines offer the background supporting documents for many national and international initiatives, programs and frameworks in order to improve the knowledge on distribution, abundance, sustainable management and use of medicinal plants worldwide. Finally, the newest standards related to conservation and sustainable use of MAP, developed by FairWild Foundation of WWF and Traffic, include several principles (the wild collection and conservation requirements, legal and ethical requirements, management and business practices, relationships with collectors, fair labor conditions, and obligations for companies and buyers of MAP) aiming at sustainability of the whole value chain of use of MAP.

Ex situ conservation through preserving the germplasm of threatened species and by supplying plant material for propagation, species re-introduction, ecosystem restoration, agronomic improvement, research and education by Botanic gardens, Gene banks, and other *ex situ* conservation facilities, can play an important role in MAP species conservation. In general, *ex situ* conservation should be seen as a complementary strategy to *in situ* conservation.

When considering *ex situ* conservation and use of MAP genetic resources, several relevant international agreements should be addressed: International Treaty on Plant Genetic Resources for Food and Agriculture (regulating conservation and sustainable use of PGR, the access and fair and equitable sharing of benefits derived from their use for sustainable agriculture, and food security), FAO's Global Plan of Action for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture, as well as programs implemented under international frameworks, such as European Cooperative Program for Crop Genetic Resources Networks (ECPGR) and Southeast European Development Network for PGR (SEEDNet). Both in ECPGR and SEEDNet, the MAP working group succeeded to realize several projects on mapping and conservation of particular MAP species (oregano, Dalmatian sage and yellow gentian). Thanks to the SEEDNet project, all SEE countries established and/or upgraded their Gene banks. Among SEE countries, the highest number of Gene bank MAP accessions was reported for Croatia and Serbia reaching about 900 and 389 accessions respectively [13].

Cultivation of MAP may reduce harvesting pressure on some wild species, particularly rare and threatened species, and thus can also be an important production strategy that supports conservation. However, cultivation must not be used as a reason for failing to safeguard viable wild populations of medicinal plant species and their natural habitats or undertaken without consideration of the impact on local users and rural harvesters. In many cases a mixture of production systems will be needed to satisfy the world's demands for herbal medicines. Reintroduction means a measure of (re)planting of the species into the same, similar or other appropriate habitat to renew endangered or even extinct populations of MAPs.

Economic valorisation of MAP resources in the context of ecosystem services

Apart of needs for ensuring the sustainable use of bio-resources, permanent monitoring, enforcement of control mechanisms of wild collection and promotion of effective *in situ*, *ex situ* and cultivation concepts and practices, the future challenge in proper use and management of MAP resources will be comprehensive analysis of their real value, which must be fairly higher than the market price of the plant drug. Economic valorisation should include an estimation of MAP ecosystem services (Tab.3) since plants and their habitats have much broader role for global functioning of the biosphere and thus for survival of the mankind. Valorization of MAP resources in such context would raise the public awareness on importance of its conservation; finally, the multipurpose use of MAP could be efficiently implemented for revitalization of huge abandoned rural areas in the hilly-mountainous region of SEE. Thus, the imperative is to recognize, document and conserve enormous MAP biodiversity and treasury. Projects concerned with the conservation of medicinal plants cannot concentrate solely on 'pure conservation' – they must address to make these plants of interest to the local people and sustainable development of the community.

Tab.3. Ecosystem services referring MAP resources

Value in use		**Value out of use**	
Direct	**Indirect**	**Heritage and supporting services**	**Existence**
Direct exploitation of MAP resources Wild collecting Processing/products Food supplements Food and nutraceuticals (fruits, mushrooms) Biomass and compost **Health and culture** Official medicine Folk medicine Veterinary medicine Pharmacy Cosmetics Eco-tourism Herbal tourism Aesthetic and spiritual value Science Education	**Regulatory services** Climate regulation Floods regulation Fires regulation Illnesses regulation Soil, water and air remediation	**Utilization by next generations** **Supporting services** Primary production Food chains Oxygen production Sequestration of carbon-dioxide Soil formation Matter circulation and energy flow	**Existence rights** Individual Species Population Biotope Phytocoenose Ecosystem Landscape Biosphere

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Original scientific paper

IN SITU AND *EX SITU* CONSERVATION OF RARE HIGH-MOUNTAIN MEDICINAL PLANTS IN BULGARIA

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SUMMARY

This study presents the results of our research on ***in situ*** and ***ex situ*** conservation of eight rare and valuable high-mountain medicinal plants. The object of our investigations are endemics, critically endangered and endangered species according to IUCN (2001) – *Alchemilla jumrukczalica*, *A. achtarowii*, *A. mollis*, *Sideritis scardica*, *Gentiana lutea* ssp. *symphyandra*, *Ribes nigrum*, *Rhodiola rosea* and *Arnica montana* (not native). In 2010 we started monitoring surveys of the species. The study ***in situ*** proved that some of populations were adversely probably affected by climate change, warming and drought. The results were submitted to the Park Directorates and Regional Inspectorates for Protection of the Environment. With regard to ***ex situ*** conservation in the period 2003-2011 two experimental collections were created - Western Rhodopes Mts. (Mountain experimental station "Beglika"- 1500 m a.s.l.) and Vitosha Mt. (Beli Rid locality - 1400 m a.s.l.). Seeds and rhizomes from plants of natural populations of the species were collected and planted in already mentioned *ex situ* collections. Results have been obtained for their particular requirements in ***ex situ*** conditions, production of seeds and commercial cultivation.

Key words: IUCN, habitats, monitoring, propagation, cultivations.

INTRODUCTION

The Balkan region is one of the main centers of biodiversity in Europe, notable for a high plant diversity and considerable number of endemic genera and species [1]. Regardless of its small territory, Bulgaria is among the countries with the richest plant diversity in Europe. Considerable is the number of the medicinal plants spread in Bulgaria: from a total of 3997 higher plant species, 768 are medicinal and 247 species being gathered frequently [2]. In the Red List of vascular plants in Bulgaria were included 89 medicinal plants with conservation status, Critically Endangered (21) Endangered (40) and Vulnerable (28) of which seventeen species are endemic - 8 Bulgarian and 9 Balkan endemics [3].

Medicinal plants are a raw material of national and international market as well as unique sources for obtaining valuable, biologically active substances. The annual harvest of medicinal plants in Bulgaria amounts to 15.000-17.000 tons, and 80% is exported [4]. In the last 15 years in Bulgaria was found to increase the quantity of medicinal plants collected from natural populations. All this led to the adoption of urgent measures for the conservation of medicinal plants as a national resource. Over the last years in Bulgaria were held several studies of *in situ* and *ex situ* conservation of endangered medicinal plants [5,6,7,8,9]. The studies continue to increase which is related with protection and sustainable use of rare and valuable medicinal plants in Bulgaria [10,11,12] and other countries exporting herbs [13,14,15,16].

A large number of valuable and sought Bulgarian medicinal plants grow at high altitude, but often in small populations. It is known, these species participate in the composition of plant

communities, occurring above 1500 m altitude. When a destruction of the populations occurs, their regeneration is extremely hard, due to the severe climatic conditions.
The fact that many rare medicinal plants could be used as raw material for pharmaceutical industry and commercial product the Institute of Biodiversity and Ecosystem Research, BAS has initiated the development of the project - *"Complex investigation of protected, endemic and rare high-mountain medicinal plants of Bulgarian flora – protection and sustainable use"* (DTK 02/38/2009) with financial support of the National Science Fund.
The object of this study are rare high-mountain medicinal plants Bulgarian (Bg) and Balkan (Bk) endemics, critically endangered (CR) and endangered (EN) species according to IUCN (2001)[17] – *Alchemilla jumrukczalica* Pawł.(Bg,CR), *A. achtarowii* Pawł.(Bg,EN), *A. mollis* (CR), *Sideritis scardica* (Bk,EN), *Gentiana lutea* ssp. *symphyandra* (Murb.) Hayek (EN), *Ribes nigrum* L.(CR), *Rhodiola rosea* L.(CR) and *Arnica montana* L. This valuable medicinal plants are widely used in traditional and official medicine [Table 1] [18,19,20,21,22].

Table 1. Studied species in accordance to their medicinal properties

Plant species	Secondary compounds	Therapeutic activity and use	Sources
Alchemilla mollis	gallotannins, flavonoids and saponins	regeneration of the skin's epithelium; haemostatic, anti-inflammatory action; antioxidant activity	Nikolov 2007[18] Hamad et al. 2007 [19]
Alchemilla achtarowii	gallotannins, flavonoids and saponins	regeneration of the skin's epithelium; haemostatic, anti-inflammatory action; antioxidant activity	Nikolov, St. (Ed.) 2007, Hamad et al. 2007
Alchemilla jumrukczalica	gallotannins, flavonoids and saponins	regeneration of the skin's epithelium; haemostatic, anti-inflammatory action; antioxidant activity	Nikolov, 2007, Hamad et al. 2007 Nikolova et al.2012 [20]
Gentiana lutea	secoiridoids	acute forms of anemia	Nikolov, 2007
Sideritis scardica	flavonoids, tannins, essential oils	bronchitis, emphysema, anti-microbial and anti-oxidant activity	Koleva et al. 2003, [21] Nikolov 2007, Kostadinova, 2008 [22]
Rhodiola rosea	salidrozid, essential oil	stimulating the central nervous system	Nikolov 2007
Ribes nigrum	vitamin C, flavonoids, essential oil	rheumatism, gout, hypertension	Nikolov 2007
Arnica montana	sesquiterpene lactones of helanine type, flavonoids, essential oil, phenolic acids	treatment of atherosclerosis, paresis, traumas, rheumatism	Nikolov, 2007

They are included in the Medicinal Plants Act (2000)[23], list of protected species Supplement 3 to the Biological Diversity Act (2002) [24] and the Act on Amending and Supplementing the Biological Diversity Act [25], Red List of Bulgarian vascular plants (2009)[3] and Red Book of R Bulgaria Vol.1 - Plants (under print, 2011)[26], excepting *A. montana*, reported to grow in the Rila Mt.[27], but so far its distribution has not confirmed.

The main goals of the study are several, including ***in situ*** conservation of natural populations of seven high-mountain species by starting the monitoring surveys; and valuation of the level of endangermeant of the species as a result of the anthropogenic activities and climatic changes. With regard to ***ex situ*** conservation the first stage aims to create two living collections in different regions of the country. The next step will be collection and storage of genetic material from different populations of the species. Particularly important is the accumulation of experience for their particular requirements in ***ex situ*** conditions, production of seed and plant material for research of the possibility to cultivation and reintroduction in the natural habitats.

MATERIALS AND METHODS

Through the application of routing and transects methods was examined distribution and current state of natural populations of the species. Method for monitoring of higher plants was applied [28]. National System for Monitoring Biological Diversity (NBMS) is a complex mechanism for tracking and summarizing the changes in the biodiversity of Bulgaria in the long run. During the field studies following information were obtained – GPS coordinates, altitude, size of the localities, bedrock, soil type, humidity, type of plant communities, population size, number of plants or projective cover, abiotic and biotic threats, age structure of populations, conservation measures. The data collected from the study were made on special forms in electronic format.

Ten populations of *Alchemilla achtarowii* and A. *jumrukczalica* and only known population of *A. mollis* in Bulgaria were examined. The study also included eleven populations of *Gentiana lutea* ssp. *symphyandra*, six of *Sideritis montana*, three of *Ribes nigrum* and four of *Rhodiola rosea.* Species occur in different habitat types listed by Kavrukova (2005)[29]. From natural populations were collected rhizomes and seeds for *ex sit*u propagation of the species, as well as herbarium specimens to be deposited in the herbarium (SOM) of the Institute of Biodiversity and Ecosystem Studies, BAS, Sofia. Part of the seeds were submitted to the National Gene Bank at the Institute of Plant Resources – Sadovo. The collected data during field studies were sent to the Directorate of National Parks and the Regional Inspectorates for Protection of Environmental and Water. The adult plants and seeds were collected from nature with the permissations of the Ministry of the Enviroment and Water.

In the period 2004-2010 two collections - Western Rhodopes Mt. (experimental station "Beglika"-1500 m a.s.l.) /Fig.1/ and Vitosha Mt. ("Beli Rid" area - 1400 m a.s.l.) were established. Species *Alchemilla achtarowii*, *A. mollis*, *A. jumrukczalica*, *Rhodiola rosea* were propagated by rhizome cuttings and *Ribes nigrum* by root cuttings. *Sideritis scardica* and *Arnica montana* by seedlings and *Gentiana lutea* ssp.*symphyandra* by direct (before winter) sowing of seeds. The rhizome and root cuttings, as well as seedlings were planted in the experimental areas on the plots measuring 2x2 m. In our study the seeds of *A. montana*, originating from natural populations of Carpathians /Ucraina/ were used.

Using biotechnological method the *in vitro* propagated plants of *Alchemilla achtarowii*, *A. mollis*, *Gentiana lutea* and *Arnica montana* were obtained and planted in experimental fields.

RESULTS AND DISCUSSION

In situ studies

Alchemilla achtarowii*, *A. jumrukczalica* and *A. mollis are distributed only in the National Park "Central Balkan" (Stara Planina Mt). Their clon-populations are located along the mountain streams in the altitudinal range of 1527 to 1775 m a.s.l. In our study areas in the

subalpine zone between huts Vezhen and Taja (40 km) in NP "Central Balkan" were inventoried.

A. achtarowii and *A. jumrukczalica* were found in habitat of European importance, according to Directive 92/43/EEC – 6430 *Hydrophilous tall herb fringe communities of plains and of the montane to apline levels*. The size of the populations was between 0.005 and 1 ha. The investigation of *A. achtarowii* and *A. jumrukczalica* proved the existence of sympatric populations. Both species inhabit the same areas but the number of *A. jumrukczalica* plants is significantly lower. The state of the natural clon-populations of Bulgarian endemics *A. achtarowii* and *A. jumrukczalica* was mainly influenced by anthropogenic activities and global warming. In the localities situated on the inaccessible terrains state of the clon-populations is good but in the areas around mountain huts the populations are negatively affected. The vitality and regeneration of the clon-populations in undisturbed localities allow resumption of the species.

Only known population of *A. mollis* in Bulgaria is located at 1148 m a.s.l., and occupies part of the dry eroded slope with inclination 25^{o} and area 200 m^2. The survey showed a predominance of immature individuals (85%), while 15-20% are generative. In dry and warm years generative plants dry out before formation of the seeds which negatively impact on the resumption of the population.

Gentiana lutea* ssp. *symphyandra occurs in the altitudinal range from 1589 to 2250 m a.s.l.l in Stara Planina Mt., Rila Mt., Vitosha Mt., Pirin Mt., Rhodopes Mt. Eleven populations were examined within National Parks – Rila, Pirin and Central Balkan, Nature park – Vitosha and, Nature Reserve "Shabanitsa"(Rhodopes Mt.). Populations of *G. lutea* ssp. *symphyandra* in Bulgaria can be found in the following types of habitats of European importance – 4060- *Alpine and Boreal heaths*; 4070 – *Bushes with Pinus mugo*; 9410 – *Acidophilous Picea forests of the montane to alpine levels*; 91BA – *Moesian silver fir forest*; 95AO- *Forests of black and white fir*; 91CA – *Rila-Rhodope and Balkan white pine forests*.

The populations of *G. lutea* occupy open spaces in spruce and dwarf pine communities. Their size was between 0.01 and 10 ha. The observed number of the plants in the populations allows efficient maintenance of genetic drift between fragments of meta population in National Parks – Rila, Pirin and Rhodopes Mt.. In the populations studied during the season 2010 were found approximately 100.000 individuals as flowering plants were 15-20%. There was a change in the number of flowering plants in different years. In terms of conservation regime in the parks, populations are not subjected to anthropogenic pressure.

Sideritis scardica populations occupy open illuminated parts of the slopes in the altitudinal range of 1326 to 1915 m. Six populations of *S. scardica* in the regions of NP "Pirin", Rhodopes Mt. and Slavjanka Mt. were examined. The size of the population area was between 0.001 and 50 ha. Terrains occupied by the species were dried with revelations of bedrock. *Sideritis scardica* occurs in the habitat of European importance according to Directive 92/43/EEC – 5130 *Juniperus communis formations on heaths or calcareous grassland*. We found that populations of the species were suppressed by both low germination of the seeds (5%), and excessive collection of herbal material for commercial purposes. A few years ago *S. scardica* was removed from the list of protected plants in the Biological Diversity Act, which led to a sharp deterioration of the populations. As a result of our research we made proposals to the Minister of Environment and Water to be back again *Sideritis scardica* under the protection of Biological Diversity Act

Rhodiola rosea is distributed in Stara Planina Mt., Rila Mt., Pirin Mt. in the altitudinal range of 2000 to 2600 m a.s.l. on stony and rocky places above the timberline. A large number of populations of the species are within National Parks "Rila" and "Central Balkan" and protected areas of the European NATURA 2000 network in Bulgaria. The area of the populations varies from 0.001 to 0.5 ha. In recent years the development of the species in

natural populations is suppressed probably by global warming and drying and also by collection of rhizomes for medicinal purposes.

Ribes nigrum is distributed in Bulgaria only in the Rhodopes Mt.(Western) from 700 to 1800 m a.s.l. Plants were found on rocky and sandy places along the mountain streams and rivers, marshy land on the brown forest soils in coniferous zone. The population is of low density, and consist of no more than 25-30 plants that grow mainly solitary. Negative factors acting on the size of the population are changes in the level of the flow of streams and rivers, construction of reservoirs and deforestation. Single plants fall within the reserve "Beglika" and in a protected area by the European NATURA 2000 network in Bulgaria. For cultivation in *ex situ* collections, the mature shoots with parts of the root were collected .

***Ex situ* studies**

Alchemilla achtarowii*, *A. jumrukczalica* and *A. mollis. In the summer of 2009 plant materials (rhizomes) from the species were collected of their natural populations in the NP"Central Balkan".The materials were planted in both experimental stations – Beglika (Western Rhodopes Mt.) /Fig.1/ and the Beli Rid (Vitosha Mt.).

Figure 1. *Ex situ* collection of high-mountain rare and threatened medicinal plants in experimental station Beglika /1500 m a.s.l. /(Rhodopes Mt.)

Rhizomes were cut into pieces of 4-5 cm length with 2-3 viable buds. During the first and second year in the both stations survived 100% plants of *A. mollis*, *A.achtarowii* 83%, *A. jumrukczalica* 67% /Table 2/. It was found that *A. achtarowii* and *A. jumrukczali* required constant high air and soil humidity and rich in organic matter soils. *A .mollis* is ecologically plastic species withstanding the periodic drought which grows well in poorer soils. In the second year *A. achtarowii* и *A. mollis* plants increased significantly the height at a 40-50 cm. The study includes attempts for *ex situ* cultivation by *in vitro* propagated plants of *A. mollis* and *A. achtarowii.* Preliminary results indicate successful adaptation of the plants to the field conditions and high survival (100%).

Table 2 *Ex situ* collections of rare high-mountain medicinal plants in both experimental stations

Species	Date of planting		Plant materials		Number of survival individuals		Bloomed		Duration of vegetation	
	B	V	B	V	B	V	B	V	B	V
Alchemilla achtarowii	27.08. 2009.	24.09. 2009.	rhizome cuttings 12	rhizome cuttings 10	10	10	July	June	V – XI	IV – XI
Alchemilla jumrukczalica	27.08. 2009.	24.09. 2009.	rhizome cuttings 15	rhizome cuttings 12	10	10	July	June	V – XI	IV – XI
Alchemilla mollis	04.11. 2009.	24.09. 2009.	rhizome cuttings 12	rhizome cuttings 10	12	10	July	June	V – XI	IV – XI
Gentiana lutea	04.11. 2010.	24.09. 2010.	seeds 2000	seeds 4000	36	30	_	_	V – IX	IV – IX
Sideritis scardica	01.10. 2009.	19.09. 2009.	seedlings 13	seedlings 22	13	22	July	June	V – X	IV – X
Ribes nigrum	10.09. 2004.	_	root cutting 2	-	2	_	July	July	IV – XI	_
Rhodiola rosea	10.05. 2008.	24.09. 2009.	rhizome cuttings 20	rhizome cuttings 12	20	12	July	June	V – X	IV – IX
Arnica montana	20.05. 2010.	27.05. 2010.	seedlings 40	seedlings 40	40	40	July	June	V – X	IV – IX

Gentiana lutea* ssp. *symphyandra. The collected seedlings of *G.lutea* from natural populations in the Rhodopes Mt. were planted in the experimental stations but even in the first month all seedlings died. According Gorgieva [30], *G. lutea* reproduces successfully by direct sowing before winter, and germination is 87%. In the autumn of 2010 in both experimental stations were sown of 1000 seeds from each origin. In spring 2011, 30% of the seeds germinated. At the end of the first season survived only 0.9-1.5% of the seedlings /Table 2/. The plants grow slowly, forming one or two pairs of leaves 1.5-2 cm long in the first year. In an experimental station Beglika grow some 8-year plants. The results show that species can be successfully developed under field conditions. In sowing the seeds should be taken into account the low percentage of the seedlings survived in the first year. *Ex situ* we store materials of natural populations of the species from Rila Mt., Pirin Mt., Rhodopes Mt. and Vitosha Mt. The results of tests for *ex situ* cultivation of *in vitro* obtained plants showed weak options for adaptation and low survival.

Sideritis scardica. In the autumn of 2009 were planted young plants obtained from seeds and were grown in a greenhouse in the both stations. Young plants grow very well in the field conditions and in the first year 50% passed in generative phase forming 1 to 3 flowering stems. The plants reach a height of 25-30 cm and a diameter of the tuff 35-40 cm are frost tolerant and drought resistant. In the second and third year was observed 100% survival of plants.The study showed that the species successfully developed in the terms of cultivation.

Rhodiola rosea. Rhizomes of *R. rosea* were transferred from natural populations of the species /Rila Mt. and Stara Planina Mt./ in the experimental stations. It was found 100% survival of the plants /Table 2/. An important factors for plant growth are air and soil humidity, slope, soil type. The area with *R. rosea* in the experimental station Belia Rid /Vitosha Mt./ has southwestern exposure and slope 5-10 degrees. In the summer the sun heats

very strongly and lower humidity of air and soil limited the development of the plants. In Beglika /Wester Rhodopes Mt./ experimental station, located among spruce forest, ecological conditions are very suitable for growing of *R. rosea* /Fig.1/. The species successfully developed under field conditions in providing a suitable environment.

Ribes nigrum. In the autumn of 2004 in the Western Rhodopes Mt. we have found several bushes of *R. nigrum* and few rooted shoots were removed. The material was transferred to the experimental station Beglika and planted in well treated soil. From first to third year the plants grew very slowly. During this period the plants formed a well developed root system. After rooting bushes began to grow, forming a large number of shoots with a height of 1.5 m. The plants began to bloom four years after their transfer from natural populations. The study shows that under appropriate conditions, plants grow successfully in culture. It is possible to make reintoroduction of the species in suitable natural habitat with growing materials in the collection.

Arnica montana. The seeds of *A. montana* collected from natural populations in the Carpathians /Ukraine/ were sown in a greenhouse in a special soil mixture, providing the necessary soil acidity. Seedlings grew slowly and in the fifth month they were ready for removal to the field. In the both experimental stations, it was observed 100% of survival of the young plants in the first year. Plants began to bloom in the second year. Preliminary studies showed that the survival of the plants planted in areas with altitude of 600 – 800 m is very low. *In vitro* propagated plants were planted in the both experimental stations. Survival of plants was 100% and their development in field conditions is successful.

CONCLUSIONS

In situ studies showed a good state of natural populations of *G. lutea* ssp. *symphandra* in Bulgaria. Plants vitality and reproductive capacity of populations was surveyed to ensure their successful survival in the future. The state of natural populations of Bulgarian endemics *A. achtarowii, A. jumrukczalica* and the rare species *A. mollis, R. rosea* and *R. nigrum* was determined to be affected by anthropogenic pressure and global warming. All populations of *S. scardica* in Bulgaria are in poor state as a cause of over-collection for commercial purposes. *S. scardica* should be returned in the list of protected plants from the Biodiversity Act and strictly guarded.

In two ***ex situ*** collections the genetic material of the eight studied species were stored. It was found that species *A. mollis, S. scardica, G. lutea* ssp. *symphyandra, R. nigrum A. montana* successfully grow and develop when being introduced in the field conditions. The Bulgarian endemics *A. achtarowii, A. jumrukcalica* and rare species *R. rosea* adapted much slowly to field conditions.

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Original scientific paper

MICROMORPHOLOGICAL AND ANATOMICAL ANALYSIS OF *SALVIA OFFICINALIS* FLORAL NECTARIES

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SUMMARY

The distribution, shape, size, superficial structure and anatomical structure of *S. officinalis* floral nectaries were investigated, using light and scanning electron microscopy, aiming to provide better understanding of the mechanism of nectar secretion in Lamiaceae, and importance of the species as aromatic, melliferous and medicinal plant. SEM investigation demonstrated that the nectaries are asymetrically four-lobed, and the lobe which is the closest to the lower corolla lip is the biggest, while the lobe opposite to it is the smallest one. The modified stomata are present only at the upper surface of the biggest nectary lobe. They are mostly individual or less often in pairs and irregularly distributed among epidermal cells. The nectariferous tissue is composed of the uniseriate epidermis overlying eight to ten layers of polygonal cells forming subepidermal secretory tissue. Epidermal cells are lined with very thin cuticle. The cells of the nectariferous parenchyma are smaller compared to parenchyma of the flower receptacle, and contain small vacuoles and dense cytoplasm. Branches of vascular elements consisting of xylem only which is separating from the flower receptacle vascular tissue, were observed near nectariferous tissue.

Key words: nectary, *Salvia officinalis,* SEM, LM

INTRODUCTION

Salvia officinalis (common sage), member of the Lamiaceae family, is a perennial, evergreen subshrub, native to the Mediterranean region and widely cultivated for use in traditional medicine. *S. officinalis* is also very important melliferous plant, due to high level of nectar production and high quality of honey.

Floral nectar is secreted by distinct glands, the nectaries, which vary widely among species or within species, in ontogeny, morphology, structure and position in flower [1]. Nectar production is confined to anthesis, and usually starts shortly before the flower unfolds [2]. Additionally to other morphophysiological flower characteristics, the nectar secretion has an important role in sexual reproduction of plants by attracting pollinators.

There is a considerable literature data on the structure and ultrastructure of nectaries in the Lamiaceae [3, 4, 5, 6, 7]. Members of the Lamiaceae usually have disc glands at the base of the ovary [3]. The structure of sage nectaries has been studied with the aim of better understanding of the mechanism of nectar secretion and to contribute to the investigation of melliferous aspect of medicinal plants.

MATERIAL & METHODS

Plant material

The plants from which flowers were taken for analysis were grown in the experimental outdoor plots of the Institute for Medicinal Plant Research "Dr Josif Pančić" in Pančevo.

Nectaries were investigated at the full flowering stage (two days after the bud opening). Five flowers in this growth phase from five individual plants were taken.

Light microscopy

Flowers were prepared by removing the floral parts near the gland (the apical portions of the sepals, petals, stamens). The remaining receptacles with the ovaries were fixed in FAA for 24 h and post-fixed in 70% ethanol, dehydrated through a graded series of ethanol and then xylol before embedding in paraffin wax and dissected to various degrees. Longitudinal sections (3–5 μm thick) were made on LEICA SM 2000 R microtome, mounted serially and stained with alcian blue-safranin. The samples were examined by light microscopy and documented by digital camera. Measurements of some nectary anatomical features were done using the software program IM 1000.

Fluorescence microscopy

To examine the cuticle and xylem, freshly sectioned material was monitored for autofluorescence using a LEICA DMLS epifluorescence microscope (filter I3 BLU 450–490 nm).

Scanning electron microscopy (SEM)

Receptacle bearing the pistil (ovary) only were studied on fresh material without prior preparation procedure using a JEOL JSM-35 scanning electron microscope.

RESULTS & DISCUSSION

Micromorphology of nectaries

As pointed out elsewhere for many other genera of Lamiaceae [3, 4, 5, 7], the floral nectaries of *S. officinalis* have a form of four-lobed asymmetrical annular disc that surrounds the base of the ovary. The nectary lobes prominently rise at the junction of the ovary lobes. SEM investigation demonstrated that the lobe, which is the closest to the lower corolla lip, is the biggest one (Fig. 1). Nectar is secreted through the specialized stomata lacking the ability to close and characterized as "modified stomata" [8, 5, 9]. Modified stomata are present at the upper part of the biggest nectary lobe as found in other Lamiaceae such as *Rosmarinus officinalis* [5], and *Ocimum basilicum* in which all three large lobes were found to be functional, except the smallest one [7] . The open stomata, observable by SEM, are irregularly distributed among epidermal cells and consisted of two guard cells (Fig. 2).

Nectar is transported from one to another nectar-producing parenchyma cell of nectariferous tissue towards the intercellulars and afterwords it is secreted outside through stomata. The secreted nectar is accumulated in the reservoir formed by the corolla basis, and the relatively large nectar drops could be observed on the lateral lobes.

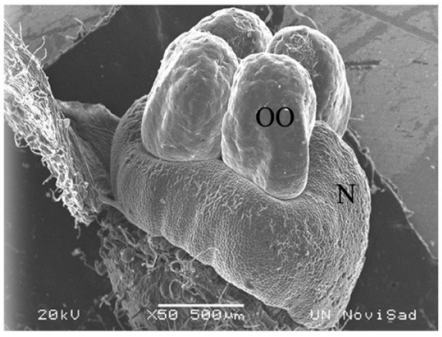


Fig. 1.: SEM of the floral nectary of *Salvia officinalis.* Abbreviations: OO- ovarian lobe; N- nectary

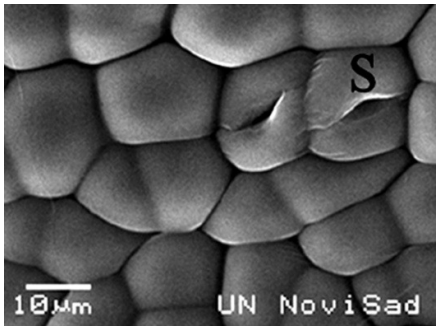


Fig. 2.: Modified stomata (S) surrounded by 5 to 8 epidermal cells

Anatomy of nectaries

The anatomical structure of nectaries, observed by LM, was analysed in the longitudinal sections of receptacle with ovary (Figs. 3,4). The nectariferous tissue is composed of a uniseriate epidermis overlying eight to ten layers of polygonal sub-epidermal secretory cells (Fig. 3). Epidermis is presented by one layer of oval to slightly rectangular cells and covered with very thin cuticle. The cuticle showed typical autofluorescence (Fig. 4). Secretory parenchyma cells were identified by their relatively small size and conspicuously dense cytoplasm, which made them easily distinguished from non-glandular receptacle cells. Although the vasculature within this tissue has not been visible in the longitudinal section anatomical preparations, the nectary appears to be supplied by the main receptacle vascular bundles through a band of large, tightly packed subglandular parenchyma cells. Unlike the phloem, the xylem tissue was visible near to nectariferous tissue (Figs. 3,4).

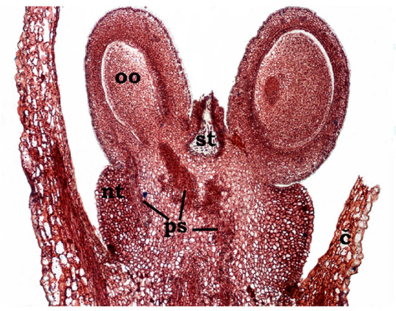
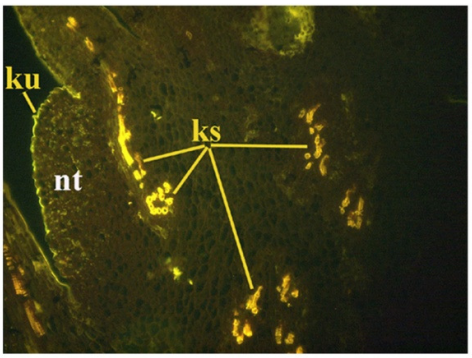


Fig. 3.: Anatomy of *Salvia officinalis* floral nectary: longitudinal section of receptaculum and ovary showing two opposite nectary lobes. Abbreviations: st- stylum; nt-nectariferous tissue; ks- xylem; OO- ovarian lobes; ps- vascular bundles

The general anatomical structure of floral nectaries of Lamiaceae family, including the analysed *S. officinalis* is similar to the some other angiosperms [3, 4, 5, 8, 9, 10]. Nectar producing parenchyma composed of few layers of small and densely packed secretory cells with small vacuoles has also been reported for floral nectaries of other plants during nectar secretion phase [3, 10, 11, 12].



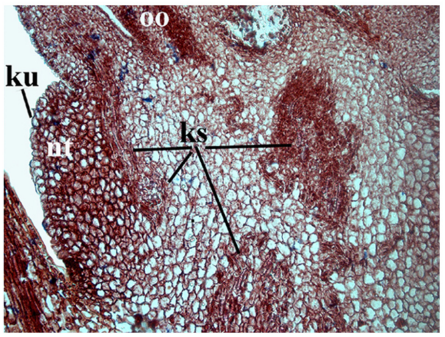


Fig.4.: Autofluorescence of nectary cuticle and lignified xylem. Abbreviations: nt-nectariferous tissue; ks-xylem, ku- cuticle; oo- ovarian lobe.

Unlike some other Lamiaceae species in which only phloem was observed in secretory tissue, or both xylem and phloem were found [4], the conducting elements in studied *S. officinalis* nectaries could not be distinguished with certainty. There is an option that phloem elements were not seen due to the failure of the applied method, or the presence of another type of flow - apoplastic, simplastic or vesicular transport [13]. Generally in flowering plants, vascularization of the floral nectaries is represented by conducting tissue consisted of xylem and phloem, only of phloem, or vascularization does not exist in secretory tissue [3, 4, 13].
The flowers of *Salvia officinalis* are well adapted for bee pollination by virtue of their blue color, strong scent and nectar, which is secreted in huge quantity [14] through a number of modified stomata and poured out into the bottom of the corolla tube. Given the morphophysiological flower characteristics, flower lifespan, time and length of flowering and growth requirements, sage can be considered as a significant source of food for honey bees, and its cultivation both for medicinal purposes and as a bee pasture is recommended.

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Original scientific paper

MODELING OF THE INDIVIDUAL LEAF AREA AND DRY WEIGHT OF HYPERICUM *(HYPERICUM PERFORATUM* L.) USING LEAF LENGTH, LEAF WIDTH AND SPAD VALUE

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SUMMARY

The study aimed at modeling of the individual leaf area and dry weight of St. John's Wort *hypericum* (*Hypericum perforatum* L.) using leaf length, leaf width and SPAD value. Non-destructive approaches of modeling can be very useful for plant growth estimation. Eight regression equations, commonly used for developing growth models, were compared for accuracy and adaptability. The two nonlinear models developed were as follows: individual leaf area $LA=0.1+0.36W+0.38L - 0.03W^2$ ($R^2 = 0.973$) and dry weight $DW = 1.8\ E^{-3}+1.02E^{-3}WS+3.45E^{-4}\ LWS$ ($R^2 = 0.928$), where *L* is the leaf length, *W* the leaf width, *S* the SPAD value, and *LWS = L x W x* S. For validation of the model, estimated values for individual leaf area and dry weight showed strong agreement with the measured values, Leaf dry weight, especially, was estimated with a higher degree of accuracy through the use of a SPAD value, as well as leaf length and width. Therefore, it is concluded that models presented herein may be useful for the estimation of the individual leaf area and dry weight of *hypericum* with a high degree of accuracy. The genus *Hypericum* has source of a variety of compounds including hypericin. Hypericin is found in dark glands of leaves the most intensively. That is why; leaves are the most important organs of these plants.

Keywords: *Hypericum perforatum* L., Modeling, SPAD value, Leaf length, Leaf width

INTRODUCTION

Hypericum perforatum L. is an herbaceous perennial plant, which has become a widely popular herbal remedy in recent years. It is commonly known as St. John's worth, and is native to relatively dry temperature zones of Europe and North America [1]. It was reported that its extract exhibited antiviral properties [2]. That is why is suggested that *H. perforatum* has a potential in Acquired Immuno Deficiency Syndrome (AIDS) treatment [3]. Use of the crude extract of this plant as an antidepressant is very popular today. This is evidenced by the fact that the market for *H. perforatum* has exceeded $210 million in the U.S.A and $570 million worldwide annually [4].

Developmental models are commonly explored using computational or simulation techniques [5]. The simulation software may be general-purpose, intended to capture a variety of developmental processes depending on the input files, or special-purpose, intended to capture a specific phenomenon. Input data range from a few parameters in models capturing a fundamental mechanism to thousands of measurements calibrated descriptive models of medicinal plants [6,7].

Leaf area has been measured in experiments concerning some physiological phenomenon such as light, photosynthesis, respiration, plant water consumption and transpiration In addition, leaf number and area of a plant have an important role in some cultural practices

such as training, pruning, irrigation, fertilisation, etc [7,8,9]. The leaf area estimation models aiming to predict leaf area non-destructively can provide researches with many advantages in agricultural experiments [10,11,12,13]. Leaf area plays an important role in photosynthesis, light interception, water and nutrient use, crop growth and development. Non-destructive method for the estimation of leaf area may be useful to determine the relationship between leaf area and plant growth rate. Simple regression models, related to leaf area and crop growth rate, were applied to estimate crop yields [14,15,16]. Since leaf development has a strong relationship with crop growth, knowing the change in leaf area may be useful for estimating crop growth. Considering that leaf area and crop growth are both affected by nutritional conditions, more reliable results may be obtained through the addition of nutritional factors to the models [17,18]. A chlorophyll meter is also useful for the prediction of crop production. SPAD values are indirectly related to nitrogen concentration. For simple, rapid, and accurate estimation of leaf dry weight, various non-destructive tests, measurable with ease, should be added [19].

Common measurements for prediction equations in some models carried out previously have included leaf width, leaf length, petiole length, main and/or lateral vein length, and different combination of these variables. Some researchers have tried using new equipment and tools such as hand scanner or laser optic apparatuses for predicting plant growth non-destructively, but these are very expensive investments for basic and simple research [20]. The objective of this study was to develop models of estimating leaf area using leaf length, leaf width and SPAD values.

MATERIAL AND METHOD

Plant Material and Experimental Conditions

This research was conducted in outdoor and greenhouse at Ondokuz Mayis University, Vocational School of Bafra in Turkey, from April 1 to September 1 2008. Dimension of the greenhouse length; width and height were separately 15m, 8m and 3m. The Sti John's wort seeds were handpicked from at least ten randomly selected *Hypericum* plants growing wild in the Cakalli district of Samsun province (41□04' N; 36□01' E); 470 m above sea level) in Turkey. St. Hohn's Wort seeds were sown in viols. The viol has got 380 *Hypericum* seeds. After germinated, each seedling was transferred to (40cm x 30cm x 15cm) each pod. In the present study the *Hypericum perforatum* L. seeds were used and set up in 100 pots. Hundred pods have got 100 *Hypericum* plants. The growing period average temperature was 27°C and it was measured with a Sato Keiryoki MFG R-704 thermo hydrograph (0°C with 50°C ±1), humidity 55%, Greenhouse and outdoor were separated shaded and un-shaded parts, however, 50% transparent polyethylene cover was used for shading. The peat used in the research and had a pH value of 7. Plants were watered daily until they reached maturity, then three times a week. Main chemical and physical properties and average amount of added nutrients for the peat are shown in Table 1.

Table 1. Main chemicaland physical properties and average amount of added nutrients for peat "Tray Substrate.

Peat type	Blend of white and frozen though black sphagnum peat
pH	5.5
Nutrients	Medium level, plus additional trace elements 160-260 mg/l N (CaCl2 - extract) 180-280 mg/l P2O5 (CAL - extract) 200 -350 mg/l K2O (CAL – extract) 80-150 mg/l Mg (CaCl2 - extract)

Model construction.

The general purpose of multiple regression is to learn more about the relationship between several independent or predictor variables and a dependent or criterion variable. Given a data set $\{y_i, x_{i1}, \cdots, x_{ip}\}_{i=1}^{n}$ of n statistical units, a linear regression model assumes that the relationship between the dependent variable y_i and the p-vector of regressor's x_i is linear. This relationship is modelled through a so-called "disturbance term" ε_i — an unobserved random variable that adds noise to the linear relationship between the dependent variable and regressors. Thus the model takes form

$$y_i = \beta_1 x_{i1} + \cdots + \beta_p x_{ip} + \varepsilon_i = x_i'\beta + \varepsilon_i, \quad i = 1, \cdots, n,$$

where $'$ denotes the transpose, so that $x_i'\beta$ is the inner product between vectors x_i and β. Often these n equations are stacked together and written in vector form as $y = X\beta + \varepsilon$, where

$$y = \begin{pmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{pmatrix}, x = \begin{pmatrix} x_1' \\ x_2' \\ \vdots \\ x_n' \end{pmatrix} = \begin{pmatrix} x_{11} \cdots x_{1p} \\ x_{21} \cdots x_{2p} \\ \vdots \ddots \vdots \\ x_{n1} \cdots x_{np} \end{pmatrix}, \beta = \begin{pmatrix} \beta_1 \\ \vdots \\ \beta_p \end{pmatrix}, \varepsilon = \begin{pmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{pmatrix}$$

Some remarks on terminology and general use:

y_i is called the regressand, dependent variable. The decision as to which variable in a data set is modelled as the dependent variable and which are modeled as the independent variables may be based on a presumption that the value of one of the variables is caused by, or directly influenced by the other variables. Alternatively, there may be an operational reason to model one of the variables in terms of the others, in which case there need be no presumption of causality. x_i is called independent variables. Usually a constant is included as one of the regressors.

The relationship between the error term and the regressors, for example whether they are correlated is a crucial step in formulating a linear regression model, as it will determine the method to use for estimation [21]. Multiple regression analysis was carried out until the least sum of square (R^2) was obtained.

Randomly selected *Hypericum* leaves were taken from all plants from April to September 2008 at a five time intervals. In this period, 10 leaves were collected for each plant within the first three-day of April, May, June, July and August to catch the different phases of leaf morphogenesis. Thus, 30 leaf samples for each plant and a total of 3000 leaves for all plants

were processed at the same day as they were collected in the following manner. First, they were placed on the photocopier desktop by holding flat and secure and copied on A3 sheet (at 1:1 ratio). Second, a Placom Digital Planimeter (Sokkisha Planimeter Inc., Model KP-90) was used to measure actual leaf area of the copy. Selection of leaf dimensions for measurement was governed by variation in leaf characteristics (e.g., size, shape, and symmetry) and practical constraints (e.g., ease and accuracy of measurements under field conditions).

Given these concerns, we chose maximum leaf width (W) and leaf length (L) to correlate with leaf area. Leaf width (cm) was measured from tip to tip at the widest part of the lamina and leaf length (cm) was measured from lamina tip to the point of petiole intersection along the midrib. The leaf positions were selected with regard to points that could be easily identified and used to facilitate the measurement of leaf length and width.

Dry weights were determined after drying for 72 h at 70°C. The SPAD readings were taken with a chlorophyll meter (Apogee Model CCM200) and recorded as a mean of 10 measurements for each intact individual leaf. The most common regression equations used to develop plant growth models were evaluated for accuracy and adaptability. All equations were composed of various subsets of independent variables, such as leaf length (L), leaf width (W), SPAD values (S), L^2, W^2, LW, *LS,* and *LWS*. The eight models determined to be the most suitable for estimating total leaf area (LA), fresh weight (FW) and dry weight (DW) of St. John's Worth were selected. All variables in the models below were significant at P = 0.05 level.

$$LA = a + bLW \quad \text{(eq.1)}$$
$$LA = a + bW + cLW \quad \text{(eq.2)}$$
$$LA = a + bL + cW + dLW \quad \text{(eq.3)}$$
$$LA = a + bL + cW^2 + dLW \quad \text{(eq.4)}$$
$$LA = a + bW + cL^2 + dW^2 \quad \text{(eq.5)}$$
$$LA = a + bL + cL^2 + dW^2 \quad \text{(eq.6)}$$
$$LA = a + bLW + cLWS \quad \text{(eq.7)}$$
$$LA = a + bLS + cLWS \quad \text{(eq.8)}$$

Where LA is the leaf area, fresh or dry weights; *L* the leaf length, *W* the leaf width, *LW* = *L* x W, *LS* = *L* x SPAD, and *LWS* = *L* x *W* x SPAD; a, b, c and d are the constant. All data was analyzed using the R-Program. Slopes, intercepts and regression coefficients of the models were compared using the R-Project software. Correlation coefficients were calculated between measured and estimated data [18].

RESULTS

Out of the all models, four consisting of leaf length (L) and leaf width (W) were selected for estimation of the leaf area (LA) of hypericum (Table 2). Equation 5 had a higher R^2 value with a lower root mean square error (RMSE) than other equations tested.

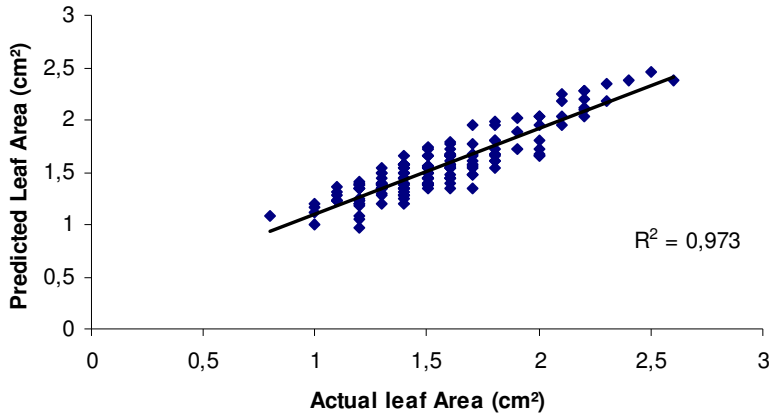
Table2. Regression models for the estimation of leaf areas (LA) of *Hypericum perforatum*

Regression model	Eq.	R^2	RMSE	Pr > *F*
LA=1.47 + 0.04LW	1	0.95	42.33	<0.0001
LA=1.61-0.72W+ 0.4LW	2	0.97	41.64	<0.0001
LA=-0.63+1.3L-0.04W +0.03LW	3	0.97	41.26	<0.0001
LA=0.1+0.36W+0.38L – $0.03W^2$	5	0.97	41.11	<0.0001

All variables in the models above are significant at P = 0.05. LA is leaf area; L is leaf length; Eq. is equation; W is leaf width; LW is L x W.

Figure 1 shows that both leaf length and leaf width were highly related to leaf area of hypericum. Equations with $P > 0.05$ and lower R^2 values were eliminated at the beginning of this study. To estimate leaf fresh weight (FW) of St. John's Wort, 3 models using *L* and W, and one model using L, W, and SPAD were selected (Table 1). Of the three models, Equation 5 showed the highest R^2 with a lower RMSE. By adding the SPAD value, accuracy of the model was slightly increased but not significantly.

Figure 1. Relationship between actual and predicted leaf areas (cm²) of Hypericum



Using the same method as in DW, 3 models for estimating leaf dry weight (DW) were selected (Table 3). Equation 7 showed the highest R^2 value with the lowest RMSE among the models using *L* and W. However, the addition of SPAD values resulted in an increase in the R^2 of the model from 0.893 to 0.928, a significant difference.

Table 3. Regression models for the estimation of dry weight (DW) of hypericum.

Regression model	Eq.	R^2	RMSE	Pr > *F*
$DW = 1.2E^{-3}+2.4E^{-3}$ LW	1	0.89	0.233	<0.0001
$DW = 2.5\ E^{-3}+1.1.\ E^{-3}L+4.3\ E^{-3}W + 3.1\ E^{-3}LW$	3	0.91	0.226	<0.0001
$DW = 1.8\ E^{-3}+1.02E^{-3}WS+3.45E^{-4}$ LWS	7	0.92	0.199	<0.0001

All variables in the models above are significant at $P = 0.05$. DW is leaf dry weight; *L is* leaf length; W is leaf width; LW = *L* x W; LS= *L* x SPAD value; LWS= *L* x *W* x SPAD value

According to these results, leaf length and width contribute to accurately determine leaf area of the plant, but not dry weight. However, SPAD values enabled us to increase the accuracy of the model for estimation of leaf dry weight.

DISCUSSION

Leaf area is routinely measured in experiments where some physiological phenomenon such as light, photosynthesis, respiration, plant water consumption and transpiration is being studied [22]. In addition, leaf number and area of a plant are important in terms of cultural practices such as training, pruning, irrigation, fertilization etc. The leaf area estimation models that aim to predict leaf area non-destructively can provide researches with many advantages in agricultural experiments. Moreover, these kinds of models enable researchers to carry out leaf area measurements on the same plants over the course of a study, resulting in

reduced experimental variability [23]. Leaf area can be determined by using expensive instruments and/or predictive models. Recently, new instruments, tools and machines such as hand scanners and laser optic apparatuses have been developed for leaf and fruit measurements. These are very expensive and complex devices for both basic and simple studies. Furthermore, non-destructive estimation of leaf area saves time as compared with geometric measurements [24]. For this reason, several leaf area prediction models were produced for some plant species in previous studies. But, to the authors' knowledge, there are no published reports related to leaf area prediction model for any medicinal plant.

In this study, the individual leaf area was well correlated with leaf length and leaf width, with high R^2 values (Tables 1), whereas leaf dry weight had a relatively low R^2 value, likely due to differences in specific leaf area (SLA) (Table 2). Although the shape of a leaf was not a significant factor in the estimation of leaf area and leaf fresh weight, an additional factor, SPAD, was required to estimate leaf dry weight more accurately. Non-destructive and rapid estimation of leaf area, many methods have been applied. This method was rapid and relatively accurate but it required trained operators. In our experiment, rulers were used directly to measure the leaf length and leaf width. According to the results of the current study that the leaf area and the dry weight of St. John's Wort may be estimated by nonlinear regression models including leaf length, leaf width, and SPAD values. For more precise modeling, environmental factors and computer systems as well as growth factors should be included in the models.

Hypericum plants are characterised mainly by the presence of three types of secretory structures, light glands, dark glands and secretory canals [25]. The dark glands on the vegetative organs of many species of *Hypericum* have been used by botanists as an important distinguishing mark for the classification of this genus [26]. In addition, there is a marked evidence and general agreement about localisation of hypericins in the dark glands. The presence of dark glands in *Hypericum* species is expressed as its density in leaves for which it is necessary to calculate the leaf area. Therefore, leaf area has been routinely measured in morphological and taxonomic studies on *Hypericum* genus [27]. There is a positive and significant relationship between dark gland density of leaves and both leaf and whole plant contents of hypericin in *H. perforatum* and other species. For this purpose, we measured leaf areas. Thus, we developed very useful, simple and accurate models for estimation leaf area for *Hypericum perforatum* L. in the present study.

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Original scientific paper

ON THE EMBRYOLOGY OF *SIDERITIS SCARDICA* GRISEB. (*LAMIACEAE*)

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SUMMARY

This work presents results of the first embryological study on the Balkan endemic species *Sideritis scardica* – known and used under the name "Pirin mountain tea" or "Mursalitsa tea" in the Bulgarian traditional medicine. The features of the embryological structures and processes in the male and female generative sphere were established. The anthers are tetrasporangiate. The anther wall develops according to the Dicotyledonous-type and consists of an epidermis, a fibrous endothecium, one ephemeral middle layer and a secretory tapetum. Meiosis in the microspore mother cells runs normally. The simultaneous microsporogenesis leads to the formation of tetrahedral and isobilateral tetrads. The mature pollen grains are predominantly three-colpate, rarely tetra-colpate, and two-celled at the time of shedding. The ovule is anatropous, tenuinucellate and unitegmic. The chalazal megaspore of the linear tetrad that forms after meiosis in the ovule functions as an embryo sac mother cell. After three mitotic divisions an eight-nucleate embryo sac of the *Polygonum* (monosporic)-type forms. Embryogenesis runs according to the Onagrad-type. The endosperm is *ab initio* cellular. The results of this study reveal that *Sideritis scardica* is a sexually reproducing species.

***Key words**: anther, embryo sac, pollen, ovule, reproductive sphere*

INTRODUCTION

Sideritis scardica Griseb. is a Balkan endemic species, distributed in Albania, Bulgaria, Greece, Macedonia, Serbia and Turkey [1]. It has limited spreading in Bulgaria, growing at dry rocky and grassy limestone places [2] in the Mt Slavyanka**,** Pirin Mts and Rhodopi Mts (Western and Central) from 1000 to 2200 m a.s.l. This species is included under category "endangered" in Red list of Bulgarian vascular plants [3] and Red Data Book of the Republic of Bulgaria [4]. Known under the name "Pirin mountain tea" or "Mursalitsa tea" in the Bulgarian traditional medicine, *S. scardica* is used in the treatment of gastrointestinal complaints, inflammation and rheumatic disorders. The excessive exploiting of this valuable medicinal plant affects the state of its natural reserves and reproductive capacity. Mainly, the wrong manner of gathering and the lack of enough long restoration period destroy the normal sexual and vegetative reproduction of the native populations of *S. scardica* – all that usually reduces the capacity of the natural habitats to serve as reserves.

Hitherto, *S. scardica* in Bulgaria was an object of chorological, karyological and phytochemical studies [5, 6, 7, 8, 9]. Karyological studies on the Rhodopi Mts populations of this species reveal a chromosome number 2n = 32 [7, 8] but up to now its ploidy level is not categorically determined. *Sideritis scardica* is considered as a tetraploid with x = 8 [7] while other authors not comment upon its ploidy level [8]. A recent karyological study on section *Empedoclia* of the genus *Sideritis* (including *S. scardica*) from Turkey reveals that all taxa are diploid (2n = 32) with basic chromosome number x = 16 [10].

So far, no data exist on the embryology of *S. scardica*. Only fragmentary embryological data are given for other species of the genus *Sideritis* L. [11].

The present embryological study on *S. scardica* is undertaken for the first time. The aim of the investigation is to establish the features of the processes and structures in its reproductive sphere and to reveal the mode of reproduction.

MATERIAL & METHODS

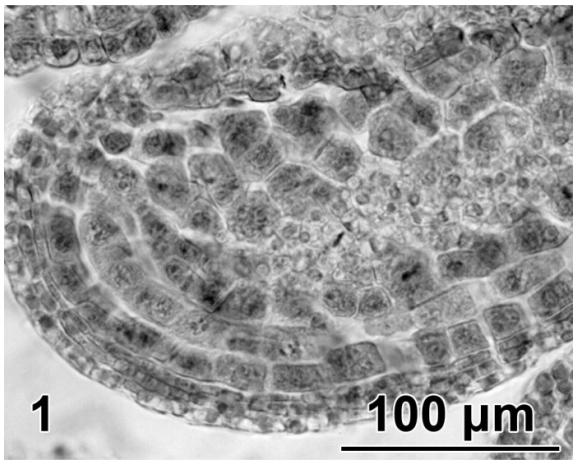
The embryological study was carried out on individuals presenting a population from Pirin Mts, below the peak "Orelek" (about 2000 m a.s.l.), growing at an experimental field outside of the native population and in greenhouse. The material of the study (buds and flowers in various developmental stages) were collected and fixed in FAA (5 parts formalin: 5 parts glacial acetic acid: 90 parts 70% ethanol). After, it was treated according to the classical paraffin methods. Sections 8-12 μm thick were cut with rotary microtome, stained with Heidenhain's haematoxylin and mounted in Entellan. Observations on permanent slides were carried out with the light microscope "Olympus CX21" and micrographs with "Infinity lite" 1,4Mpx Digital camera.

RESULTS & DISCUSSION

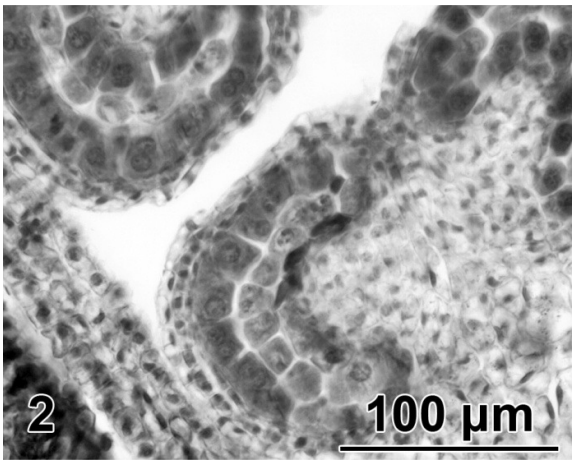
Anther and development of the male gametophyte

Anthers are tetrasporangiate. The formation of placentoids (sterile protuberances) between anther locules were observed, like in other species of the genera of *Lamiaceae* (*Labiatae*): *Lavandula* L., *Salvia* L., *Stachis* L., *Hyssopus* L., *Agastache* Clayt. [11, 12, 13, 14].

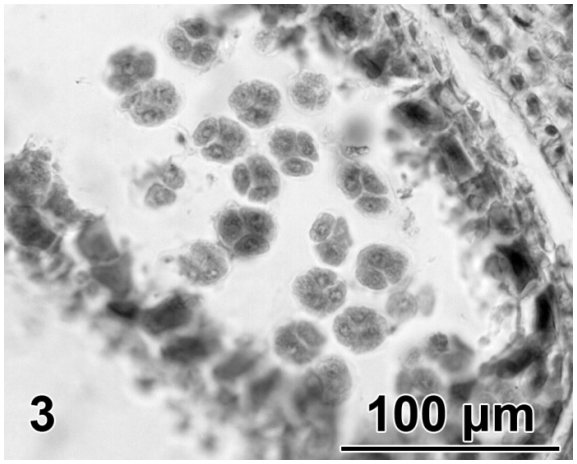
The anther wall develops according to the Dicotyledonous-type [15, 16]. It is four-layered (Figs 1, 2) consisting of an epidermis, fibrous endothecium, one ephemeral middle layer and secretory tapetum that is typical for the representatives of the family *Lamiaceae* [11, 17].



Four-layered anther wall and sporogenous tissue

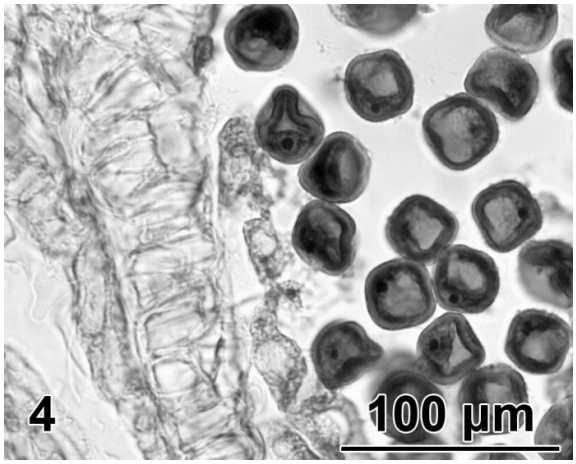


Anther wall with two-nucleate tapetum cells and MMCs

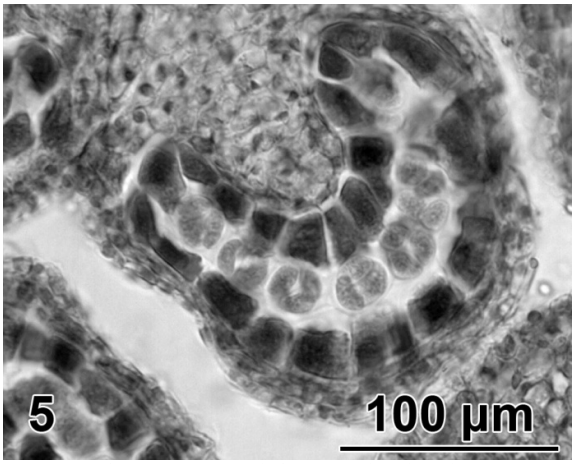


Anther wall and different type microspore tetrads

Initially, the epidermis, endothecium and middle layer are almost similar in shape (Fig. 1). Later, after the stage of microspore tetrads, they begin to distinguish (Fig. 3). The epidermal layer in the mature anther consists of one row of one-nucleate almost rectangular cells. Endothecium cells are bigger than the cells of epidermis, radially elongated and after one-celled pollen stage they develop fibrous thickenings. The middle layer is ephemeral as in the most representatives of the family *Lamiaceae* [11]. The tapetum is secretory (cellular), characteristic for *Lamiaceae* [17], consisting of one row of one-nucleate cells and remains cellular up to the maturity of the anther. As result of mitosis a multiplication of the nuclei in the tapetum cells passes and they become from one-nucleate to two- (Fig. 2), rarely four-nucleate. At the stage of mature pollen from the layers of the anther wall only fibrous endothecium retains its entirety while the epidermis and tapetum have already been partially destroyed (Fig. 4).
The sporogenous tissue is one-layered (Fig. 1). During the anthers ontogenesis the sporogenous cells become bigger, rounded up and function as microspore mother cells – MMCs (Fig. 2). The meiosis in MMCs runs almost normally. After the simultaneous microsporogenesis predominantly tetrahedral tetrads and a small number of isobilateral ones form (Figs 3, 5).

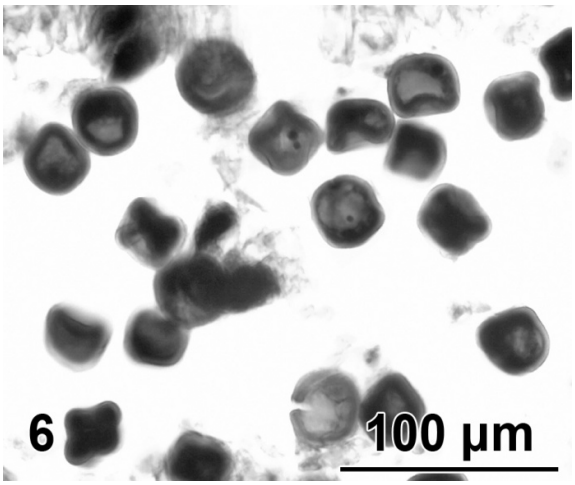


Anther wall with fibrous endothecium, destroyed epidermis and tapetum, and mature pollen



Microspore tetrads in an anther locule

The mature pollen grains are three-, rarely four-colpate, 2-celled at the time of shedding (Fig. 6). For representatives of the genus *Sideritis* predominantly four-colpate pollen grains were announced [18]. According to [19] in *Labiatae* tri-colpate pollen grains are sheded at two-celled stage that is also reported by [20]. Our observations not confirmed "the 6-pantocolpate pollen type" shown for the representatives of section *Empedoclia* of the genus *Sideritis* [21] in which *S. scardica* belongs too.

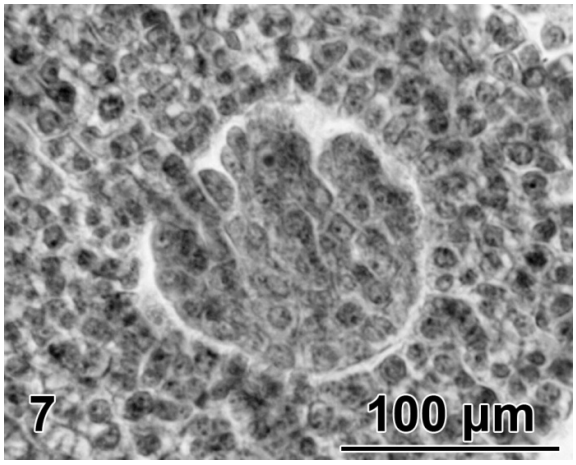


Mature pollen grains

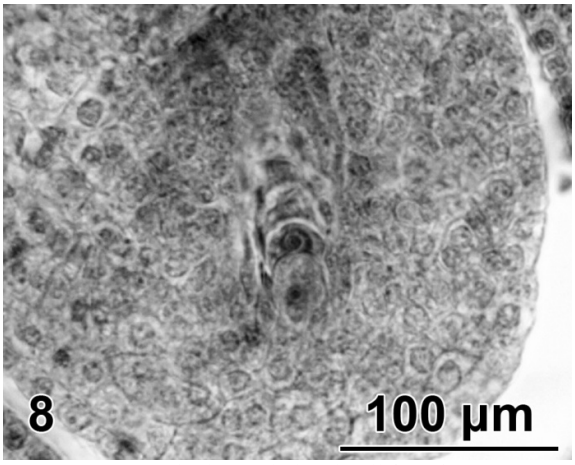
Ovule and development of the female gametophyte

Regarding the female reproductive sphere of *S. scardica*, the gynoecium is sincarpous. The ovary is superior and bilocular. Each locule has a single ovule on axile placentation. The well-developed ovule is anatropous, tenuinucellate, unitegmic. The innermost layer of the single integument differentiates into endothelium during ovule ontogenesis.

Archesporium is one-celled (Fig. 7) and the single archesporium cell functions directly as a megaspore mother cell like in the most *Lamiaceae*, in particular *Agastache foeniculum* [14]. No parietal cells form. As result of the meiosis in the megaspore mother cell, a linear tetrad of megaspores forms in the ovule and the chalazal one functions as an embryo sac mother cell (Fig. 8).

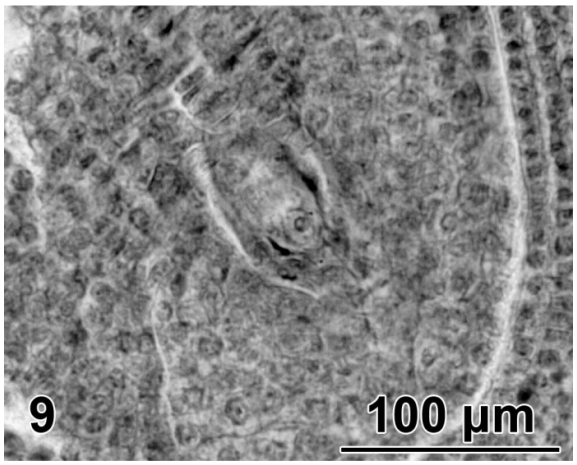


Archesporium cell in the ovule

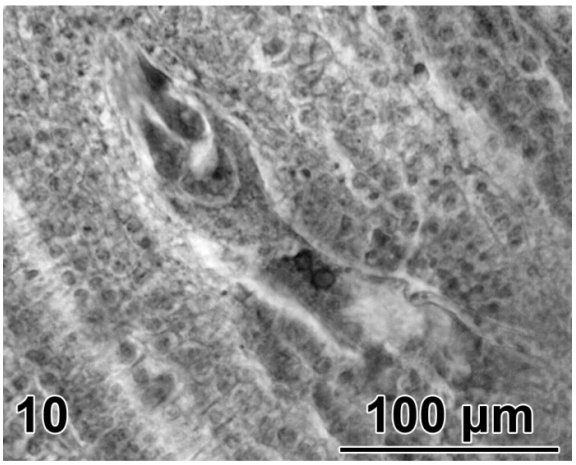


Megaspore tetrad whose chalazal cell functions as ES mother cell

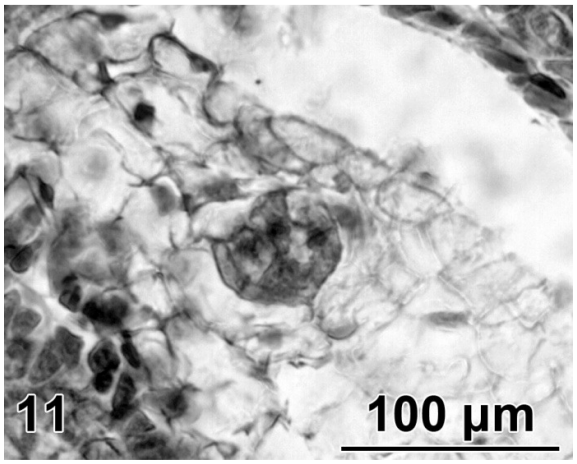
The one-nucleate embryo sac is clearly vacualized (Fig. 9). After three successive mitotic divisions two-, four- and finally eight-nucleate embryo sac (ES) form according to the basic *Polygonum*(monosporic)-type – typical for the family *Lamiaceae* [11, 15, 17]. The mature ES consists of three-celled egg apparatus (one egg cell and two synergids), two polar nuclei (after their fusion the central cell forms) and three antipodals in the chalazal pole (Fig. 10). Usually, the synergids degenerate after fertilization. Quite often, the antipodals did not observed as cells but as three free nuclei deeply in the chalazal part of the ES. The three antipodals are ephemeral and usually degenerate after the formation of the central cell of ES. The embryo and endosperm develop as result of a porogamous double fertilization. The endospermogenesis begins before the embryogenesis.



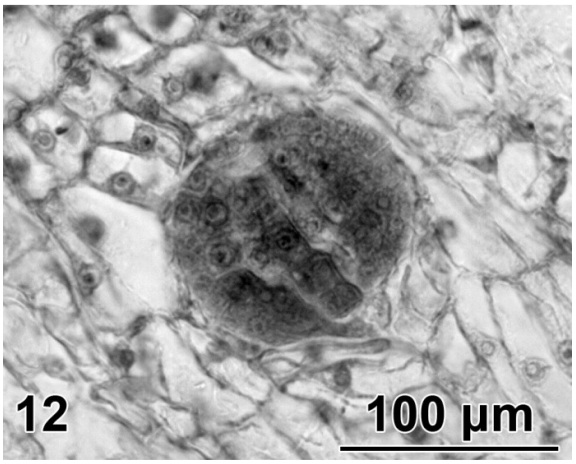
One-nucleate embryo sac



Egg apparatus and two polar nuclei in the ES



Four-nucleate Onagrad-type embryo and cellular endosperm



Globular Onagrad-type embryo and cellular endosperm

The embryogenesis runs after the Onagrad-type (Figs 11, 12) like in the representatives of the most genera belonging to the family *Lamiaceae* [11, 17]. Endospermogenesis follows the *Stachis*-type that was also established for the genera *Sideritis*, *Ajuga* L., *Marrubium* L., *Nepeta* L., *Stachis* L., *Ocimum* L. [11]. The endosperm is *ab initio* cellular (Fig. 11, 12) with terminal haustorium.

CONCLUSION

The embryological study carried out on the Balkan endemic *Sideritis scardica* shows that it is a sexually reproducing species. The embryo and endosperm develop after double fertilization. During the present study apomixis was not registered. The established embryological features such as: almost normally run of the meiosis in the anther and ovule, the stable structures in the male and female generative sphere, balanced embryological processes and the lack of apomixis reveal that the studied *S. scardica* from Bulgaria is rather a diploid than tetraploid taxon. Nevertheless, if the second assumption be accepted, on the base of the results obtained during the present investigation, this species can be exactly characterized as an autotetraploid.

As result of the embryological study carried out, the mode of reproduction, reproductive capacity, character and state of the populations of the Balkan endemic species *Sideritis scardica* were established in connection with its preservation for the Bulgarian flora.

ACKNOWLEDGEMENTS

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Original scientific paper

DIVERSITY AND RESOURCE ASSESSMENT OF ALCHEMILLA SPECIES IN OSOGOVO MOUNTAIN (BULGARIA) USED AS MEDICINAL PLANTS

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SUMMARY

Research concerning the *Alchemilla* ssp. resources along the ridges of the northern part of Osogovo Mountain has been done. The main purpose of the study was to evaluate the distribution of species of the genus *Alchemilla,* to map the abundant populations, and to determine their available stocks. Two methods have been used: the transect method for estimation of species' distribution; and setting up the plots for yield measurement. Seven *Alchemilla* species have been found in the studied region. They are from Section *Alchemilla* (*Brevicaulon* Rothm.), Subsection *Heliodrosium* Rothm. Three species are reported for the first time for the floristic region "Western Border Mountains" in Bulgaria. The plant communities involving *Alchemilla* ssp. were found in habitats of European importance, according to Directive 92/43/EEC - 6520 *Mountain hay meadows* and 6430 *Hydrophilous tall herb fringe communities of plains and of the montane to apline levels.* Four regions rich in *Alchemilla* ssp. have been identified which are located in the northern part of Osogovo Mt. The location, area, ecological and geographic characterization, type of the plant community, resource characterization and evaluation of the *Alchemilla* reserves, have been surveyed in the studied regions. Recommendations for the sustainable use and protection of the populations have also been provided.

Key words: *Lady's mantle, operating reserves, sustainable use.*

INTRODUCTION

Genus *Alchemilla* L. included about 118 species in Europe, which are divided in two sections [1]. The number of known and already reported species in Bulgaria is 35 [2]. They are divided in three subsections of Sect. *Alchemilla* (*Brevicaulon* Rothm.). Three species are from Subsect. *Chirophyllum* Rothm. The most abundant subsection in Bulgaria is Subsect. *Heliodrosum* Rothm. - 18 species. There are 14 species in Subsect. *Calycanthum* Rothm. Six species are Bulgarian endemics and six Balkan endemics. Six *Alchemilla* species /Lady's mantle/ are protected under the Biological Diversity Act [3]. Twelve species are from the Red List of Bulgarian vascular plants [4] and Red Data Book of the Republic of Bulgaria [5] (8 are endangered and 4 are critically endangered). Thirty-one species were included in the Medicinal Plants Act [6]. Every year the Ministry of Environment and Water sets the annual quotas for collection, according to the distribution and abundance of *Alchemilla* species in different areas of Bulgaria. Five species were included in IUCN Red List of Threatened Plants, 2001 [7].

The species of genus *Alchemilla* are herbaceous perennial plants, mostly having woody rhizomes. The basal leaves are long-stalked and clustered in a rosette. The leaf blades are roundish, palmate and jagged. The blossoms have short stems and compound cymose inflorescense. The flowers are small, green or yellowish, more or less aggregated into distinct clusters (glomerules).The hypanthium is urceolate, the sepals are 4 or 5, the epicalyx is developed but petals are not, the stamens are 4 or 5 and they are placed along the outer margin of the disc. The fruit is a single achene, entirely or partially enclosed in the thin, dry hypanthium, [1]. The species reproduce by seeds through facultative apomixes, though hybridization and/or vegetative reproduction which occasionally take place, forming clone-populations.

Alchemilla species grow in all Bulgarian high mountains at altitude between 800 and 2500 m. They are members of communities located along the rivers and streams or mountain hay meadow communities. They are heliophytes and do not grow under the canopy of the forest.

Lady's mantle are medicinal plants which contain tannins, flavonoids and saponins. They possess anti-inflammatory and skin epithelium regenerating effect. The herbs are used in gynaecology and as a medical treatment for diseases of the digestive and excretory systems [8]. A number of *Alchemilla* species are harvested as medicinal herbs. *Alchemilla vulgaris* complex is considered as their collective name. Annually about 3-4 tons of row material in the country were harvested from the wild populations.

This study is a part of the project supported by the Ministry of Environment and Water. The project aims to determine the distribution and diversity of the *Alchemilla* species and to evaluate their abundant populations, outside of protected areas, such as Natural or National parks. The whole project's scope covers Western Balkan Mt., Sredna Gora Mt., western and central parts of the Rhodopes Mt., and Osogovo Mt.

The aim of the presented study is to evaluate the number of species *Alchemilla*, to map their populations and to determine the available stocks of the species growing in the northern part of Osogovo Mt.

Study area

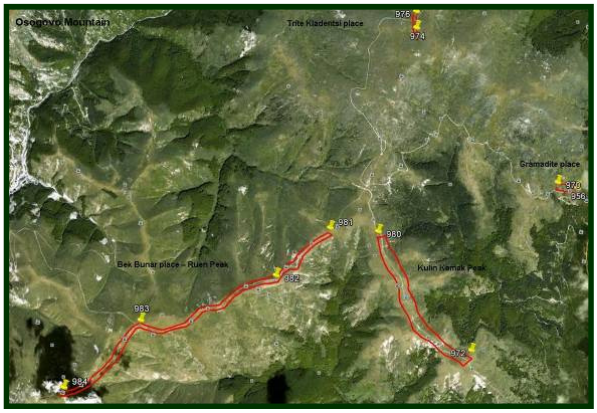
The study has been performed in July, 2011, within the floristic region 'Western Border Mountains' in Bulgaria. The Osogovo Mt. is the northernmost one, out of the Osogovo-Belasitsa group. Nearly the entire mountain ridge has been studied, from Kyunets Peak to the NE, through the peaks of Choveka, Tsarni Kamak, Shapka, Mali Ruen, to Ruen Peak in the SW. The areas called Gramadite and Kulin Kamak, located at the East of the main ridge have also been studied, as well as the catchment areas of the rivers Eleshnitsa and Bistritsa. The latter falls within Trite Kladentsi area, where Ruen Peak (2251 m) is located (Fig. 1). The climate of the Osogovo Mt. is apparently influenced by the Mediterranean. May and June are the months with the highest precipitation [9]. Osogovo Mt. is an important hydrological source – many rivers start out of its central parts. The most abundant type of rock is granite and the main soil types are dark-brown and chromic cambisols. All these mountain features favour the *Alchemilla* ssp. development in the region.

MATERIAL AND METHODS

The distribution of the *Alchemilla* species is evaluated by transects, which is suitable for sub-alpine terrains, mountain meadows and river valleys. The studied areas for evaluation of species' reserves are delineated by GPS co-ordinates over satellite images. The habitat description consists of: 1) Borders (GPS co-ordinates); 2) Altitude; 3) Habitat characterization – slope direction, slope inclination, relief type, main rock, soil (type, thickness, erosion, humidity); 4) Generally estimated vegetation cover; 5) Projected cover of *Alchemilla* spp.; 6) Relevé. The determination of the taxonomic status of *Alchemilla*

species was performed after 'Flora of NRBulgaria' [6]. The reserves of *Alchemilla* species are estimated after Shreter et al.[10]. This method is based on the plots for yield measuring. The 1 m^2 yield, the yearly yield, and the available stocks are given in kg of fresh material (FW). The yield for each population is calculated after Shreter et al. [10]. The error of the average of projected cover of the species, as well as the yield of 1 m^2, are in the range of 10-15 %, rarely 20-25%. The allowable yearly yield, as well as the turnover yields is determined by Ordinance № 2/20.01.2004 [11] concerning the rules and requirements for medicinal herbs and genetic material gathering.

Fig. 1 Abundant populations of *Alchemilla* species in Osogovo Mt.

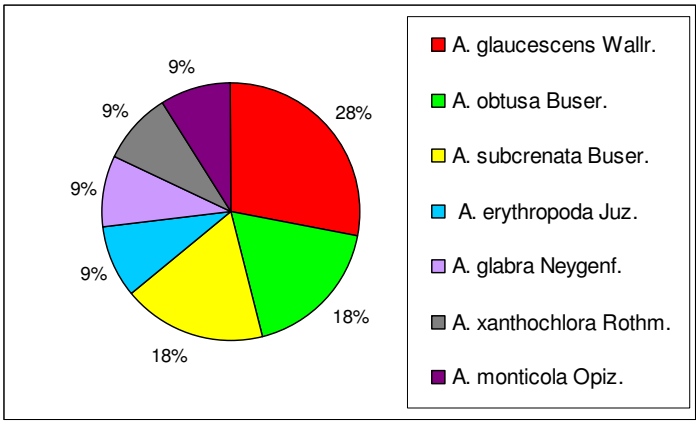


RESULTS AND DISCUSSION

The species of genus *Alchemilla* are mainly distributed in the river catchment basins of Osogovo Mt. Those regions provide sufficient air and soil humidity, critical for plants development.

Alchemilla species are found in two types of habitats. The first type is determined as the open mountain grassland, grouped into the habitat type 6520 *Mountain hay meadows* according to Directive 92/43/EEC. Generally, these are drier habitations, characterized by less diverse vegetation. The other type consists of herbaceous communities along the stream banks, which are prevailing in the region. The latter is habitat type 6430 *Hydrophilous tall herb fringe communities of plains and of the montane to apline levels.*

Fig. 2. Percentage contribution of *Alchemilla* in Osogovo Mt.



Seven species are found in the studied region. All of them are from Section *Alchemilla* (*Brevicaulon* Rothm.), Subsection *Heliodrosium* Rothm.:

- Series *Pubescentes* Buser. - *Alchemilla glaucescens* Wallr., *A. erythropoda* Juz.;
- Series *Vulgares* Buser. - *A. glabra* Neygenf., *A. obtusa* Buser., *A. xanthochlora* Rothm., *A. subcrenata* Buser., *A. monticola* Opiz.

The most occurring species in Osogovo Mt. are *A. glaucescens* (28%), *A. obtusa* (18%) and *A. subcrenata* (18%) (Fig. 2).

The species *A. subcrenata*, *A.obtusa* и *A. xanthochlora* from Osogovo Mt. are reported for the first time. It was found that in one population there are often more than one *Alchemilla* species (Table 1).

Table 1 Distribution of *Alchemilla* ssp. in Osogovo Mt.

Species	Date	Studied areas	Initial and end points of the transect (GPS)	Altitude [m.a.s.l.] min-max
A. glaucescens Wallr. *A. glabra* Neygenf. *A. obtusa* Buser. *A. monticola* Opiz	12.07.2011	Gramadite region	N 42,1666/ E 22,5991 N 42,1889/ E 22,6139	1626 - 1642
A. glaucescens Wallr	13.07.2011	Kulin Kamak Peak	N 42,1886/ E 22,6169 N 42,1869/ E 22,5794	1684 - 1815
A. obtusa Buser. *A. subcrenata* Buser	13.07.2011	Trite Kladentsi region	N 42,2079/ E 22,5883 N 42,1773/ E 22,5947	1667 - 1958
A. erythropoda Juz. *A. xanthochlora* Rothm *A. glaucescens* Wallr. *A. subcrenata* Buser.	14.07.2011	Bek bunar region – Ruen Peak	N 42,1741/ E 22,6099 N 42,1843/ E 22,5787	1413 - 2251

Description of the most important sites - habitat features and resources characterization

Gramadite region

The Gramadite region central GPS spot N 42,188952; E 22,615681, 1621 m.a.s.l. The slope is west, inclined at 1^{o} - 5^{o}.

It is characterized by medium thick, semi-humid, mountain meadow soils. The main habitats are mountain hay meadows, having diverse plants richness. The vegetation cover is 95 %. The trees and shrubs cover is 35 %, with main species - *Betula pendula*, *Pinus sylvestris*, *Salix caprea*, *Rosa canina*, *Rubus idaeus*, *Chamaecytisus absinthioides*. The grass cover is 65 %. The dominant species is *Deschampsia caespitosa* and the subdominant species are *Festuca rubra* and *Agrostis capillaris*. The other involved species are *Achillea collina*, *A. distans*, *A. lingulata*, *Angelica pancicii*, *Anthoxanthum odoratum*, *Armeria rumelica*, *Asperula capitata*, *Bistorta major*, *Bruckenthalia spiculifolia*, *Campanula sparsa*, Carex *otrubae*, *Carum carvi*, *Cerastium arvense*, *Chamaespartium sagittale*, *Cirsium ligulare*, *Cruciata glabra*, *Cuscuta sp.*, *Dactylorhiza cordigera*, *Dianthus superbus*, *Epilobium angustifolium*, *Euphorbia cyparissias*, *Galium palustre*, *G. verum*, *Geum coccineum*, *Hypericum perforatum*, *Juncus effusus*, *Juniperus communis*, *Lathyrus pratensis*, *Lerchenfeldia flexuosa*, *Linum capitatum*, *Luzula campestris*, *L. luzuloides*, *Mentha longifolia*, *Myosotis scorpioides*, *Nardus stricta*, *Potentilla erecta*, *Ranunculus acris*, *Rumex acetosella*, *Scabiosa columbaria*, *Senecio nemorensis*, *S. subalpinus*, *Sieglingia decumbens*, *Silene frivaldskyana*, *S. roemeri*, *Stellaria graminea*, *Thymus serpyllifolius*, *Trifolium repens*, *T. velenovskyi*, *Urtica dioica*, *Veratrum lobelianum*, *Verbascum densiflorum*, *Veronica*

chamaedrys, *V. officinalis*. Established species inhabit the habitat of European importance 6520 *Mountain hay meadows*, described in the Appendix I of the Habitat Directive 92/43/EEC.

Four *Alchemilla* species have been found at this site: *Alchemilla glabra, A. glaucescens*, *A. monticola* and *A. obtusa.*

The spatial structure of the population is characterized by a mosaic situated individuals. They form larger or smaller spots and are irregularly distributed within the habitation of 2.5 ha. The *Alchemilla ssp*. project cover varies from 1 % to 39 %, in different parts of the population. But average cover of the species in all population is relatively low /6.9 %/. The yield is 16.8 kg/ha. The available stocks of the herb in this habitation are 40.28 kg; and the possible yearly yield – 28.05 kg (Table 2). The population is in good condition, while the available stocks are relatively limited.

The area of Kulin Kamak Peak

The habitat is located between Kulitsite Peak (N 42,181979; E 22,583296; 1817 m.a.s.l.) and Dolna Chuka Peak (N 42,16669; E 22,59916; 1706 m.a.s.l.), having an area of 30 ha, with east exhibition, inclined at 11^o - 15^o.

It is characterized by medium thick, semi-humid, mountain meadow soils. The vegetation cover is 95 %. Projective cover of trees and shrubs is 5 %, where *Chamaecytisus absinthioides*, *C. sagittale*, *Juniperus communis*, *Rubus idaeus*, *Vaccinium* m*yrtillus*, *V. uliginosum* grow. The herbs cover is 90 %. Dominant species are *Lerchenfeldia flexuosa* and *Luzula luzuloides*having, *Alchemilla glaucenscens* had the abundance 2. The other species were *Agrostis capillaries*, *Achillea distans*, *Antennaria dioica*, *Anthriscus sylvestris*, *Armeria rumelica* (Balkan endemic), *Asperula capitata*, *Botrychium matricarifolium*, *Calamagrostis arundinacea*, *Campanula sparsa*, *C. velebitica*, *Carum carvi*, *Cerastium fontanum*, *Cerastium decalvans* (Balkan endemic), *Cirsium ligulare*, *Cruciata glabra*, *Festuca rubra*, *F. valesiaca*, *Galium album*, *Galium verum*, *Genista depressa*, *Hieracium pilosella*, *Hypochaeris maculata*, *Hypericum perforatum*, *Jasione montana*, *Koeleria eriostachya*, *K. splendens, Leontodon hispidus*, *Linaria dalmatica*, *Luzula campestris*, *Nardus stricta*, *Plantago subulata*, *Peucedanum oligophyllum* (Balkan endemic), *Primula veris*, *Ranunculus acris*, *Rhinanthus wagneri*, *Rumex acetosella*, *Silene frivaldskyana* (Balkan endemic), *Stellaria graminea*, *Thymus sp*., *Thlaspi praecox*, *Trifolium heldreichianum* (Balkan endemic), *Trifolium hybridum*, *T. velenovskyi* (Balkan endemic), *Veratrum lobelianum*, *Verbascum densiflorum*, *Veronica chamaedrys*, *V. officinalis*, *Potentilla ternata*, *Viola reichenbachiana*. Relatively high rate of endemism is peculiar for this area – 12 %.

The habitat type is characterized as 6520 *Mountain hay meadows*. That habitation is drier than those in Gramadite place. In case of *Lerchenfeldia flexuosa* dominance, the *Alchemilla* ssp. cover is higher. In case of *Luzula luzuloides* dominance, the *Alchemilla ssp*. cover is lower or the species does not grow. That could be explained by the fact that *Luzula luzuloides* develops large tufts and limits the vegetative or seed reproduction of *Alchemilla* species.

Only *Alchemilla glaucescens* is found within this habitat. The species is relatively evenly distributed, as its average cover is 19 %, although it reaches 40 – 50 % at some places.

The yield is 81.2 kg/ha. The available stocks in this localities are 2436.18 kg; the possible yearly yield – 1705.76 kg (Table 2). The population is in very good condition. It is valuable for wild harvesting.

Trite Kladentsi area

The Trite Kladentsi central spot co-ordinates are N 42,208806; E 22,588389, 1935 m.a.s.l., having an area of 1 ha, The slope is with west exhibition, inclined at 11^o - 15^o.

It is characterized by medium thick, overly wet, not eroded soil, having high grass growing along the streams. The vegetation cover is 95 %. The shrubs cover is 5 %, where *Vaccinium myrtillus* and *V. uliginosum* are present.
The grass cover is 90 %. Dominant species is *Deschampsia caespitosa*, having abundance 2. Subdominant species is *Festuca rubra*. The other species are: *Caltha palustris*, *Geum coccineum*, *Carex acuta*, *Rumex alpinus*, *Senecio nemorensis*, *S. subalpinus*, *Veratrum lobelianum*, *Luzula luzuloides*, *L. spicata*, *Myosotis scorpioides*, *M. ramosissima*, *Hypericum perforatum*, *Verbascum longifolium*, *Peucedanum oligophyllum*, *Thlaspi perfoliatum*, *Silene roemeri*, *Jasione montana*, *Epilobium montanum*, *Nardus stricta*, *Potentilla erecta*, *Trifolium hybridum*, *Cirsium appendiculatum*, *Genista depressa*, *Campanula sparsa*, *Sesleria fleoides*, *Phleum alpinum*, *Ajuga pyramidalis*, *Bruckenthalia spiculifolia*, *Angelica pancicii*, *Stellaria graminea*, *Acinos alpinus*, *Leontodon hispidus*, *Eriophorum latifolium*, *Dianthus microlepis*, *Jasione bulgarica*, *Antennaria dioica.*
Two species *Alchemilla* have been found in this localities - *Alchemilla obtusa* and *A. subcrenata.* The average projected cover of the species is 80 %.
The yield is 1276.0 kg/ha. The available stocks are 893.20 kg (Table 2). The habitation is suitable for wild collection of the herb.

Table 2 Estimation of stocks of natural popualtions of *Alchemilla* ssp. in Osogovo Mt.

Species	Studied areas	Ave-rage cover [%]	Area [ha]	Yield [kg/ha]	Operative reserves [kg]	Wet/ dry ratio [kg]	Turnover yields [years]	Possible yearly yield [кg]
A. monticola *A. glabra* *A. obtusa* *A. glaucescens*	Grama-dite area	6,9	2,5	16,8	40,28	4,10	2	28,05
A. glaucescens	Kulin Kamak Peak	19,0	30,0	81,2	2436,18	3,05	2	1705,76
A. obtusa *A. subcrenata*	Trite Kladentsi area	80,0	1,0	1276,0	1276,00	4,50	2	893,20
A. subcrenata *A. xanthochlora* *A. glaucescens* *A. erythropoda*	Bek Bunar area - Ruen peak	10,1	19,6	15,6	306,00	2,94	2	214,51

Bek Bunar place – Ruen Peak
The habitat is located between Bek Bunar shelter (N 42,182470; E 22,574290; 1831 m.a.s.l.) and Ruen Peak (N 42,162170; E 22,530870; 2182 m.a.s.l.), having an area of 19.6 ha, South-looking, inclined at $11^o – 15^o$.
The mountain meadows cover both slopes of the ridge. The vegetation cover is 95 %. The shrubs cover is 10 %, where *Chamaecytisus sagittale*, *Vaccinium* m*yrtillus*, *Juniperus communis*, and *Bruckenthalia spiculifolia* grow.
The grass species cover is 85 % with the dominant *Lerchenfeldia flexuosa*, having abundance 2. The other founded species are: *Anthoxanthum odoratum*, *Galium album*, *Festuca valida*, *Luzula luzuloides, L. campestris*, *Agrostis capillaris*, *Ligusticum mutellina*, *Asperula capitata*, *Rumex acetosella*, *Hieracium pilosella*, *Senecio nemorensis*, *Nardus stricta*, *Plantago subulata*, *Cirsium ligulare*, *Geum montanum*, *Campanula sparsa*, *Genista depressa*, *Campanula jordanovii* (Bulgarian endemic), *Stellaria graminea*, *Leontodon hispidus*, *Potentilla ternata*, *Acinos alpinus*, *Cruciata glabra*, *Asperula cynanchica*, *Botrychium matricarifolium.*

Four *Alchemilla* species have been found in this habitation - *Alchemilla erythropoda, A. xanthochlora, A. glaucenscens* и *A. subcrenata*. The average cover is 10 %.
The yield is 15.6 kg/ha. The available stocks are 306.44 kg. The possible yearly yield is 214.51 kg (Table 2). The population population is suitable for the collection of economically significant amounts of herb.

CONCLUSIONS

For the first time distribution and operating reserves of *Alchemilla* ssp. were cared out in Osogovo Mountain. The results show that in the northern part of the mountain there are populations that can be use for collection of the herbs. The studied region is of an area of 53.1 ha. Seven species of *Alchemilla* have been found. All of them have not any conservation status. *Alchemilla subcrenata*, *A.obtusa* and *A. xanthochlora* are described for the first time in Osogovo mountain. The dominant species for Osogovo mountain is *Alchemilla glaucescens.* The average cover of *Alchemilla* species was from 7 % up to 80 %. The estimated available stocks for the studied region are 4058,46 kg (fresh weight) or 1000 kg (dry).
The northern part of Osogovo is characterized by considerable diversity of species of *Alchemilla* spp. and significant operational stocks of herbal. This allows the area to be used for collection of plant material for commercial purposes according to appropriate rules.

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Original scientific paper

RESOURCE ASSESSMENT OF WILD GARLIC (*ALLIUM URSINUM* L.)

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SUMMARY

The production, consumption and international trade in MAPs and phytomedicines, are growing and are expected to grow in future significantly. With this growth in global demand for MAPs and a large base of local demand for plant based traditional medicines, the pressure on the existing populations of MAPs has increased tremendously during the last few decades. Considering the number of species and the relatively small land area, Bosnia and Herzegovina (B&H) is among the five richest countries in Europe in terms of species density and diversity. While B&H is an important center of biodiversity for the region, it has the highest proportion of threatened plant species of any country in Europe. The region of Vlasenica was selected for the implementation of the FairWild Standard in Bosnia and Herzegovina, to develop an approach on how to harmonize the commercial utilization and conservation of the regional *Allium ursinum* population in the wild. Main purpose of this study is to demonstrate effective management and sustainable use of wild-collected plant, ensuring thereby the long-term survival of the natural population and contributing substantially to local livelihoods. The resource assessment (RA) was implemented as required by the FairWild standard. RA is an essential component of an adaptive management process and includes gathering information on distribution, identification of its target populations, inventory and total natural harvestable stock in the selected area. RA included data collection, resource inventory, yield and regeneration study, harvest impact assessment and monitoring and harvest adjustment. Results show that *Allium ursinum* is not in a danger of overexploitation in surveyed region due to the abundance of this plant species and proper management performed by local forest authorities. Conducting RA, jointly with local forestry authorities, it was found useful to test the resource assessment methodology, and further develop the species management plan. Appropriate recommendations for local forest authorities, companies, experts and collectors are included in RA.

Key words: *resource assessment, Allium ursinum, wild collection*

INTRODUCTION

The production, consumption and international trade in medicinal and aromatic plants (MAPs), are continuously increasing. With this growth in global demand for MAPs and a large base of local demand for plant based traditional medicines, the pressure on the existing populations of MAPs has increased tremendously during the last few decades. Over-collection of MAPs and habitat destruction are among the factors creating pressure on populations plants, and potentially threaten their survival in the wild. Implementation of measures for conservation of natural resources of MAPs and their habitats is therefore urged to ensure the continued availability and use of MAPs. Considering the number of species and the relatively small land area, Bosnia and Herzegovina (BiH) is among the five richest countries in Europe in terms of species density and diversity [1]. While BiH is an important

center of biodiversity for the region, it has the highest proportion of threatened species of any country in Europe [2]. After the official launch of the International Standard for Sustainable Wild Collection of Medicinal and Aromatic Plants (ISSC-MAP) version 1.0 (now FairWild Standard) in 2007, implementation through selected projects was conducted around the globe (including in India, Nepal, Cambodia, Lesotho, Brazil and Bosnia-Herzegovina) to test its applicability in different geographic, ecological and socio-economic conditions of wild plants collection. Within the BiH-subproject of "Saving plants that save lives and livelihoods" project, supported by BMZ and implemented by TRAFFIC, wild garlic (*Allium ursinum* L.) was chosen as target plant in the Vlasenica region. Vlasenica region was selected for the implementation of the Fair Wild Standard in Bosnia-Herzegovina, to develop an approach to sustainable harvesting of the *Allium ursinum* population in the wild. Main purpose of this project was to demonstrate effective management and sustainable use of wild-collected plant, ensuring thereby the long-term survival of the natural population and contributing substantially to local livelihoods. The resource assessment (RA) was implemented within the project as required by the implementation of FairWild Standard. RA is an essential component of an adaptive management process and includes gathering information on distribution, identification of its target population, inventory and total natural harvestable stock in the selected area.

MATERIAL AND METHODS

Background information

According to the Flora Europaea [3], *Allium ursinum* L. is present from Norway to Mediterranean and from Russia in the East to the Atlantic Ocean in the West. The Middle European species *Allium ursinum* L. is divided in two subspecies: subsp. *ursinum* that is present in west and central Europe, and subsp. *ucrainicum* which is characteristic for east and south Europe. On flat land and in valleys it creates a dense population. The plant favors humus, loose soil, alkaline, neutral or moderate acid soil, and places where the snow stays longer. It is possible to find it on sandy, rocky or slightly loamy surface. Wild garlic could rise from river planes till high mountainous places; up to 1900 m [4]. Usually it covers big surfaces in beech forest associations [5]. In Bosnia and Herzegovina, *Allium ursinum* can be found on mountains Sara (near city Kljuc), Osjecnica, near Banja Luka, on Vlasic, Kruscica, Vranica, near Sarajevo, Fojnica and Vares cities, on Bjelasnica, Treskavica, Romanija, around Bugojno and Kupres, near Vlasenica and Han Pijesak. *Allium ursinum*. is wide spread all over Bosnia and partly in Herzegovina [6]. The biggest area with *Allium ursinum* in Bosnia and Herzegovina is found in the Vlasenica region. According to data gathered from Trade chamber of Bosnia and Herzegovina, more than 200 tons of dried *Allium ursinum* leaves from that region was exported to the European Union during the harvesting seasons 2007 and 2008. More than ten companies purchase and export *Allium ursinum* from Bosnia and Herzegovina [1]. Collection of *Allium ursinum* takes place during the period of vegetation (April-October). Collection of young leaves should be in April and May, before flowering. Collection of the bulbs should be in places where the population of *Allium ursinum* is noticed; before leaves comes out (April) or at the end of vegetation, when leaves are dried (October). Recommendation in guidelines related to organic collection is that 80 % of the bulbs and 30 % of the plants at one area need to stay untouched to ensure regeneration of populations [7].

Selection of survey area was done upon information provided by local forest authorities, local communities, companies that collect in that region and MAPs collectors. Total surface covered by this Forest authority is 40 817 ha. The forest area is divided on organization units and in four of them the *Allium ursinum*. is present (Studeni Jadar, Tisca, Drinjaca Donja,

Gornji Jadar). These organization units are also divided on smaller blocks that are numbered. All four units were surveyed in the present project. *Allium ursinum* is present in so called "belt" that comes from Serbia and mountain Tara, it cover parts of community forest "Birac" and continues further to central Bosnia. The width of "belt" is between 300 and 500 m and it is located in different altitudes. In the present research, *Allium ursinum* populations start growing from 772 m to 1263 m above sea level. All maps for conducting RA were provided by State Forest Company of Republic of Srpska named "Srpske Sume". They have precise digital maps of every forest region under their responsibility. Company has maps of organization units as well as precise maps with blocks within units. Collectors and companies that buy medicinal plants use experience and local knowledge about terrain in this area.

Resource assessment methodology

Work on resource assessment was completed in several phases. First of all, primary data collection was done on the location. After that, secondary data collection was followed. The present study followed the resource assessment methodology as suggested by Leaman and Cunningham (2008) *Resource Assessment: A Guide to Implementing Principle 1; Maintaining Wild MAP Resources* [8]. Primary collection of data was conducted in April 2009 and secondary collection of data was completed during May 2009.

Primary data, that included information about the availability of *Allium ursinum*, its density, distribution within different organization units, present vegetative stages of *Allium ursinum*, availability of the locations, condition of the roads in the area, hardness of the terrain, mine fields and all other relevant information, were collected from local forest authority, forest keepers, owners of the collection companies, collectors and all persons that are connected with collection of *Allium ursinum*. through interviews. Guided by collected information, resource inventory was conducted in four organization units (30 blocks) jointly with the persons from Forestry authority. Resource inventory was done by simple random sampling method due to the size of area, complexity of the terrain and heterogeneity in distribution of resource. Simple random sampling is the basic sampling technique where a group of subjects (a sample) is selected for study from a larger group (a population). Each individual is chosen entirely by chance and each member of the population has an equal chance of being included in the sample. Every possible sample of a given size has the same chance of selection.

Using this method, sampling was done in each block within organizational unit where *Allium ursinum*. is present. Plots of 1x1 m were used as frame for measurements and counting. Number of plots placed in each block varies according to the distribution and availability of *Allium ursinum*. in a specified surveyed area. Two size classes of wild garlic were determined and for each plot the following data were collected: (1) number of leaves in class 1, (2) number of leaves in class 2, (3) total number of plants, (4) number of plants in juvenile stage, (5) number of plants with inflorescence , (6) mass of class 1, (7) mass of class 2, (8) average length of class 1, (9) average length of class 2, (10) average width of class 1, (11) average width of class 2, (12) GPS data, (13) photos, (14) comments about surrounding environment.

Secondary data were collected from state, municipality, forest and university documents, reports, articles, maps, official records and other published and unpublished materials. Data collected in the field were processed and analyzed to obtain the following information: density of *Allium ursinum* in the region, estimation of the total yield, abundance, ratio between different classes of wild garlic, ratio between fresh and dry matter. Statistical data processing was done using Microsoft Excel program. Density was calculated as number of plants per square meter and per hectare. Also, density of first and second class and juvenile stage was calculated. Flower buds were counted and expressed as number of plants with inflorescence out of total number of plants. Yield was calculated as total yield of *Allium ursinum* in the region (stock) as well as yield per hectare. Ratio between fresh and dry matter was done after drying of collected leaves.

RESULTS AND DISCUSSION

Resource inventory

Resource inventory provides information about the quantity (standing stock) of the target resource by estimating both resource density (number per unit area) and abundance (total number in a specified area). An inventory of the target resource provides a base line for monitoring changes in resource quantity in the collection as a result of collection management or other impacts [8]. Collection of *Allium ursinum* leaves takes place in the spring time when the leaves are young and fresh and there are flower stems but they are small and collectors does not pick them. Smaller leaves have lower price on the market. Because of that, two size classes were set up:

1. Class 1 includes leaves with lenght between 10 and 15 cm
2. Class 2 includes leaves with length over 15 cm

Additionally, density of juvenile stage (seedlings or plants with length under 10 cm) and density of plants with inflorescent was counted.

Table 1. Density of different classes of *Allium ursinum* in organization units

	Total surface (ha)	**Area covered with *Allium ursinum* (ha)**	**Class 1 (N°/m²)**	**Class 2 (N°/m²)**	**N° of plants**	**Juveniles (N°/m²)**	**Flowers (N°/m²)**
Studeni Jadar	7308	489	108.00	382.95	201.05	189.89	77.05
Gornji Jadar	5339	763	189.20	253.40	179.60	161.60	87.40
Drinjaca Donja	2978	296	149.45	289.45	175.64	177.09	52.00
Tisca	3027	152,8	176.80	235.20	158.00	326.80	46.00

As it can be seen from Table 1, the two groups of major differences are visible, the Class 2 and Juveniles. The highest average value was found for Class 2 in organization unit Studeni Jadar. This characteristic is not caused by human factor because there is no difference in collection methods and number of collectors in different units. Difference is caused by local characteristics, such as, soil, micro climate, surrounding vegetation and related interactions. Second distinction in quantity of Juveniles could be related to human impact, because Tisca has the lowest frequency of collection and collection is mostly non-commercial.

Table 2. Estimated Yield of *Allium ursinum* L. in different organization units

	Area of unit with garlic (ha)	**Estimated total mass per m² (1+2) (g)**	**Mass (t /ha)**	**Estimated total stock of unit (t)**
Studeni Jadar	489	754	7.54	3687.06
Gornji Jadar	763	663	6.63	5058.69
Drinjaca Donja	296	719	7.19	2128.24
Tisca	153	565	5.65	864.45
TOTAL	1701	675.25	6.75	11 738.44

It may, thus, be assumed that there are less injures of plants and destruction of habitat and vegetation. In terms of the number of plants of *Allium ursinum*, there are no significant differences among the surveyed units. They are relatively equal with unit Tisca at last position, regarding harder terrain conditions for collection in that unit.

Yield and regeneration study

Yield and regeneration study estimates the total and sustainable harvest yield of a target resource in a determined area and time required for seedlings to replace harvested individual plants [8]. Total yield of *Allium ursinum*. was calculated by analyzing the data of the weight of leaves per square meter and collection area per block and percentage of the blocks covered with the plant.

From Table 2 it can be seen that the total estimated mass in grams per m² varies from 565 g to 754 g. This variation is not high and could be explained by different soil characteristics, micro climate, surrounding vegetation, population dynamics and interaction between above-mentioned factors. Same conclusion is valid regarding total stock of the units, which follows size of the units. Total stock of the *Allium ursinum*. in the Vlasenica region can be estimated to be over 11000 tons.

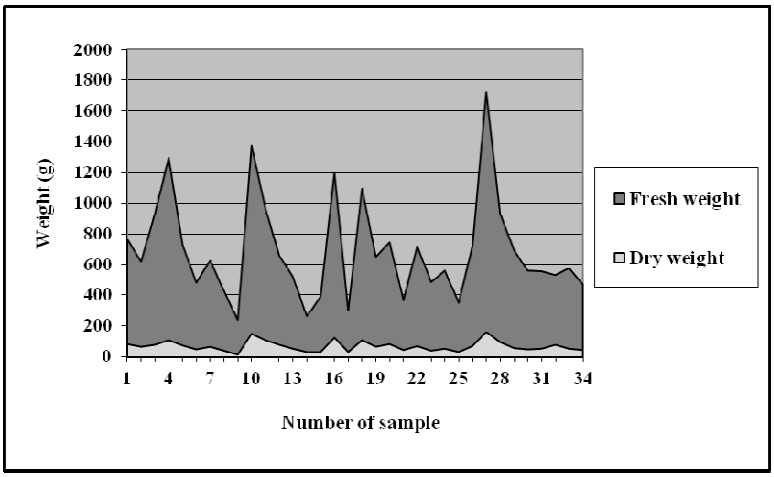

Chart 1. Fresh and dry weight of samples

Average length of leaves of Class 1 for the whole region is 11, 61 cm, and average length of the leaves of Class 2 for the whole region is 16, 99 cm. Average width of the leaves of Class 1 for the whole region is 4,14 cm, and average width of the leaves of Class 2 for the whole region is 6,55 cm. In 2007, according to official records collected from the local forest authority (Vlasenica region), 120 t of dry *Allium ursinum* was exported. In 2008, this amount was significantly lower with only 80 t exported. It is suspected, through the information gathered from collectors and from forest engineers and purchase managers, that real figures of export were 40% higher for dried *Allium ursinum* exported in these two years, which means around 170 t in 2007, and around 110 t in 2008. This 40% of dried *Allium ursinum* is collected without permission of the forest authority.

Amount of fresh matter per sample is between 240 g to 1724 g. Amount of dry matter per sample is between 16 g to 156 g (Chart 1). Average fresh/dry ratio is 9,7 and this is in conformity with literature data where it can be found that 8-10 kg of fresh *Allium ursinum* gives 1 kg of dry matter [4].

Combined with data from the resource inventory and yield data, gathering information on the species' regeneration provides the basis for estimating the sustainable harvest limit of the target resource. It is also used to estimate the recovery time needed to ensure sustainable harvesting [8].

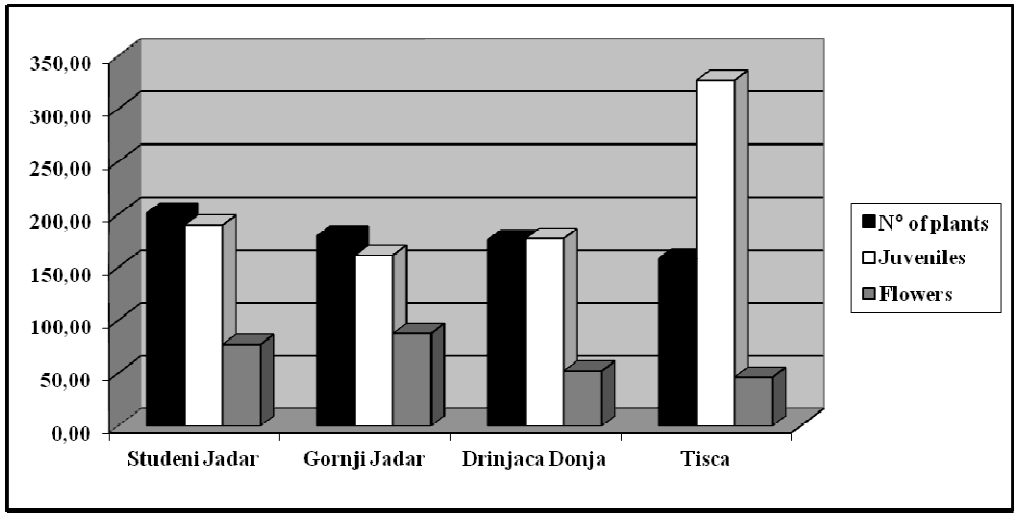

Chart 2. N° of plants, juveniles and flowers per organizational units

From Chart 2 it can be seen that the number of juveniles is very high, particularly, in the units Studeni Jadar and Gornji Jadar more than 90 % of total number of plants are juveniles. In units Drinjaca Donja and Tisca there is more plants in juvenile stage than mature plants. Additionally, number of flowers in samples is quite high. Number of mature plants bearing the flowers out of the total number of mature plants is around one third or more in all surveyed areas. Reproduction of *Allium ursinum* is mainly generative (with seeds), and seeds have good germination, hence it is assumed that the reproduction and regeneration of *Allium ursinum* in this region is secure.

Assessment of harvesting impact

Harvest impact assessment provides information about the effect of specific harvest treatments (different intensities, frequencies, and methods) on the target resource (reproduction, growth, survival, vigor, yield, quality). This information is needed to define a sustainable harvest protocol for the target resource that takes into account site-specific variables. Harvest impact was assessed to determine whether current harvest levels, technique and control are adequate for resource regeneration and productivity. Nature of harvest was studied and it was assessed whether the harvesting technique is destructive or not. Wild-harvesting of MAPs is largely carried out by the local population, mainly people over 40 years of age, predominantly women. Most harvesters belong to poorer or under-privileged groups in society and quite often depend on the additional income generated by wild-harvesting of MAPs. To some collectors, the wild-harvesting of medicinal plants provides a much needed additional income, to others it is the sole source of income. It is estimated that one collector can collect between 100 and 200 kg of fresh *Allium ursinum* leaves per day (150 kg on average). This quantity depends on density of *Allium ursinum* in collection area, size of leaves, slope of the terrain, density of the surrounding vegetation, and the age of collector, etc. Collection of *Allium ursinum* leaves takes place during second part of April and it continues in first part of May. In that period *Allium ursinum* leaves are young and fresh and plants are not in the flowering phase. Collectors pick *Allium ursinum* leaves by hand or they cut it with knifes. They choose healthy, long, leaves without damage and without petiole. They do not collect seedlings or the flowers because buyers request pure leaves. Collection of this type does not harm plants and does not disturb its regeneration. *Allium ursinum* blooms after harvest and produces seeds that ants disseminate around the

forest. Collection takes place in every surveyed organization unit every year. In some areas these activities are more intensive, and in some, like Tisca, are less intensive and collection is mainly performed for personal consumption. Every block in the unit is not equally exposed to collection. Local forest authority that gives permission for collection, change blocks permitted for collection every year, but they do not have long term plans for these activities.

According to collected data, 170 t of dry *Allium ursinum* was sold in 2007, which means that collectors collected between 1600 and 1700 t of fresh garlic and in 2008 the amount was around 1000 t of raw leaves. It can be seen that in 2007, around 15 % of total stock available in the region, was collected. In 2008, that percentage was under 10 %. These percentages don't represent serious treat for *Allium ursinum* population in the region.

For estimation of sustainable harvest quantities, it is necessary to take into consideration that in the region *Allium ursinum* is present in big quantities, that terrain does not allowed collection on every block, and that Forestry Authority rotate collection blocks, as well as that collection never exceeded 15 % of the total quantity. Sustainable harvest quantity for all units could be 60 % of the present stock of *Allium ursinum.* In the Table 3 the data on sustainable harvest quantities were given.

Table 3. Estimated/suggested sustainable harvest quantity per units

Organization unit	Estimated total stock of unit (t)	Suggested Sustainable Harvest Quantity (t) (60 % of total)
Studeni Jadar	**3687,06**	**2212,24**
Gornji Jadar	**5058,69**	**3035,21**
Drinjaca Donja	**2128,24**	**1276,94**
Tisca	**864,45**	**518,67**

Resources of *Allium ursinum*. in the Vlasenica region should be stable if the demand and current amount and intensity of collection remain unchanged.

CONCLUSIONS

Resource assessment was completed in Vlasenica region, which is main region with Wild garlic (*Allium ursinum* L.*)* in Bosnia and Herzegovina, using the internationally developed methodology [8]. The study was conducted in the frame of implementation of the FairWild Standard. In chosen region, four organization units (Studeni Jadar, Tisca, Drinjaca Donja, Gornji Jadar) were selected for implementation, and within those units, the study was conducted in 30 blocks. The results of resource assessment demonstrate that the current stocks of *Allium ursinum*, combined with high regeneration rates, current level of wild harvesting, are unlikely to be unsustainable. The authors recommend this resource assessment methodology to be applied for other MAP species in Bosnia and Herzegovina and region.

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Original scientific paper

EVALUATION OF HEAVY METALS CONTENT IN SELECTED MEDICINAL PLANTS COMMONLY USED AS COMPONENTS FOR HERBAL FORMULATIONS

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SUMMARY

For the majority of the world plant population medicinal plants represent the primary source of the health care. Although the effectiveness of medicinal plants is mainly associated with their constituents such as are essential oils, vitamins, glycosides, etc., it is considered that prolonged intake can cause health problems due to the possible presence of heavy metals, since the plants can easily be contaminated by heavy metals in the course of cultivation or later during the processing stage. Therefore determining the content of heavy metals accumulated in medicinal plants is of high importance. Thus, the aim of this research was to evaluate the content of heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Zn) and metalloids (As and Sb) in selected medicinal plants, that are traditionally used in alternative medicine, including *Matricaria chamomilla* L., *Melissa officinalis* L., *Mentha piperita* L. and *Foeniculum vulgare* Mill. The plant material was collected from an experimental field of Institute for Medicinal Plants Research "Dr Josif Pančić" (*M. officinalis*, *M. piperita*, *F. vulgare*) and wild habitats in Pančevo (*M. chamomilla*). Plant analyses were done according to ICP methodology, using ICAP 6300 ICP optical emission spectrometer. The obtained results show that the content of the potentially toxic heavy metals in the investigated medicinal plant specimens was below the recommended limits. These results impose that medicinal plants from the studied growing sites are appropriate for preparation of teas and medicinal extracts. Hence, it has been concluded that a determination of heavy metals content in medicinal plants must become a standard criterion for evaluation of their quality. Also, an appropriate choice of growing sites could greatly reduce the problem of heavy metal accumulation in medicinal plants.

Key words*: heavy metals content, Matricaria chamomilla, Melissa officinalis, Mentha piperita, Foeniculum vulgare.*

INTRODUCTION

For the majority of the world plant population medicinal plants represent the primary source of the health care. According to the World Health Organization (WHO) report, almost 80% of people in marginal communities use only medicinal plants for the treatment of various diseases [1, 2]. Nowadays, increased scientific interest and consumer demand have promoted the development of herbal products as dietary supplements [3]. Using herbs in medical treatment of various illnesses one should be aware that apart from the pharmacological effect they could turn out to be toxic because of the presence of heavy metals.

Although the effectiveness of medicinal plants is mainly associated with their constituents such as are essential oils, vitamins, glycosides, etc., it is considered that prolonged intake can cause health problems due to the possible presence of heavy metals like Pb, Cd, Zn, Ni and other impurities, since the plants can be easily contaminated by heavy metals in the course of

cultivation or later during the processing stage [4]. Together with other pollutants, heavy metals are discharged into the environment through industrial activity, automobile exhaust, heavy-duty electric power generators, municipal wastes, refuse burning and pesticides used in agriculture [5]. The content of heavy metals is one of the criteria for the use of plant material in the production of traditional medicines and herbal infusions. Therefore, control of heavy metals in medicinal plants and their products should be made such to ensure safety and efficacy of herbal products [6]. It has been reported that whatever is taken as food could cause metabolic disturbance subject to the allowed upper and lower limits of trace metals [7]. Both the deficiency and excess of essential micronutrients and trace of toxic metals may cause serious effects on human health [8, 9]. WHO recommends that medicinal plants, which form the raw materials for the finished products, may be checked for the presence of heavy metals, further it regulates maximum permissible limits of toxic metals like arsenic, cadmium and lead, which amount to 1.0, 0.3 and 10 ppm, respectively [10].

Regarding the above mentioned, the aim of this research was to evaluate the content of heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Zn) and metalloids (As and Sb) in selected medicinal plants, that are traditionally used in alternative medicine, including *Matricaria chamomilla* L., *Melissa officinalis* L., *Mentha piperita* L. and *Foeniculum vulgare* Mill.

MATERIALS & METHODS

Collection of the plant material: The plant material was collected from an experimental field of Institute for Medicinal Plants Research "Dr Josif Pančić" (*Melissa officinalis* L., *Mentha piperita* L., *Foeniculum vulgare* Mill.) and wild habitats in the city of Pančevo (*Matricaria chamomilla* L.), during summer 2011. The following parts of plants were sampled: *Melissae folium*, *Menthae piperitae folium*, *Chamomillae flos* and *Foeniculi fructus*.

The study area (Pančevo) is located about 20 km north-east from Belgrade in Serbia, with highly developed industry.

Preparation and analyses of the plant material were carried out in a laboratory under controlled conditions.

Preparation of the plant material: Analyzed aboveground parts of the study plant species were dried at 105^{o}C for a period of 2 hours, using gravimetric method for determination of dry matter content of plant tissue. The plant biomass was weighed and expressed in g per pot. This method quantitatively determines the dry-matter percentage in plant tissues based on the gravimetric loss of free water associated with heating to 105^{o}C. The dry-matter determination is used to correct the sample element concentration to an absolute dry-matter basis [11]. Water was removed from plant tissue to stop enzymatic reactions and to stabilize the sample. Removal of combined water also facilitates complete particle size reduction, thorough homogenization, and accurate weighing. Plant material was then reduced to 0.5 to 1.0 mm particle size to ensure homogeneity and to facilitate organic matter destruction. During this sample processing, necessary measures were taken in order to avoid any loss or contamination of heavy metals.

Analysis of the plant material: The content of heavy metals (Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Zn) and metalloids (As and Sb) in selected medicinal plants was determined with an inductively coupled plasma optical emission spectrometer ICAP 6300 (ICP-OES), after the samples were digested with concentrated HNO_3 / H_2O_2 for total forms extraction [12].

ICP-OES is a multi-element analytical technique that offers fast sample throughput, high sensitivity and a wide dynamic range. In daily operation, the ICP-OES instrument is started, brought to operation conditions and let stabilized. The sample introduction system is checked and the wavelengths are tuned. The intensity of the light emitted at specific wavelengths is measured and used to determine the concentrations of the elements of interest. The

instrument is standardized with the five working standard solutions (multi-point linear fitting). Samples are measured with standardization blanks, other kinds of blanks, drift control samples, and quality control samples. After a batch of samples is measured, the data are downloaded to an Excel spreadsheet. The data are corrected in terms of standardization blanks, other relevant blanks, drift correction, and dilution factor application [13].
The ICP working range and wavelength are given in Table 1.

Table 1. Main analytical characteristics of the ICP determination

Heavy metals	Working range, ppm	Wavelength, nm
Cadmium (Cd)	0.00-10.00	214.4
Cobalt (Co)	0.00-10.00	228.6
Chromium (Cr)	0.00-10.00	267.7
Copper (Cu)	0.00-10.00	327.3
Iron (Fe)	0.00-10.00	259.9
Manganese (Mn)	0.00-10.00	257.6
Molybdenum (Mo)	0.00-10.00	202.0
Nickel (Ni)	0.00-10.00	231.6
Lead (Pb)	0.00-10.00	216.9
Zinc (Zn)	0.00-10.00	213.8
Metalloids		
Arsenic (As)	0.00-10.00	189.0
Antimony (Sb)	0.00-1.00	206.8

Statistics: The data shown in Tables below are arithmetic means of three replicates of each treatment, namely, of corresponding number of analyzed samples. Standard deviation value and intervals are stated with these data in the Tables.

RESULTS & DISCUSSION

The human body requires both metallic and non-metallic elements for healthy growth and development within certain allowable limits. Using herbs and their extracts in medical treatment of various illnesses, besides the soughtafter pharmacological effect, could be dangerous because of heavy metals and other impurities. For this reasons it is important to control the level of contaminants in medicinal raw materials [14].
Table 2 summarizes pharmacognostic features of the studied medicinal plants used as herbal remedies. *M. chamomilla* is used in the folk medicine mainly as an antiinflammatory and antiseptic, also antispasmodic and mildly sudorific [15], whereas the leaves of *M. officinalis* are traditionally used because of their sedative, aromatic, digestive and antispasmodic properties [16]. Similarly, herbalists consider *M. piperita* an astringent, antiseptic, antipruritic, antispasmodic, antiemetic, carminative, diaphoretic, mild bitter, analgesic, anticatarrhal, antimicrobial, rubefacient, stimulant and emmenagogue [17, 18]. Furthermore *F. vulgare* is used in folk medicine as carminative, digestive, lactagogue and diuretic [19].
In the recent years, human activities, such as industry and agriculture, promote heavy metal release into the environment. Thus, the analytical determination of metals in medicinal plants has become a part of quality control in order to establish their purity, safety and efficacy.

Table 2. Pharmacognostic features of the study plants

Plant species	Family	Common name	Medicinal properties
M. chamomilla	Asteraceae	Chamomile	antiinflammatory, antiseptic, antispasmodic, mildly sudorific
M. officinalis	Lamiaceae	Lemon balm	sedative, aromatic, digestive, antispasmodic
M. piperita	Lamiaceae	Peppermint	astringent, antiseptic, antipruritic, antispasmodic, antiemetic, carminative, diaphoretic, mild bitter, analgesic, anticatarrhal, antimicrobial, rubefacient, stimulant, emmenagogue
F. vulgare	Apiaceae	Fennel	carminative, digestive, lactagogue, diuretic

Table 3 displays the results of the content of heavy metals and metalloids in the study medicinal plants. The concentrations of the analyzed heavy metals were within the allowed limits [20], while the certain metals (Pb and Cd) and two studied metalloids (As and Sb) did not detected.

Table 3. The content of heavy metals and metalloids (in ppm) in studied medicinal plants

Total content (ppm)	Plant species			
	M. chamomilla	*M. officinalis*	*M. piperita*	*F. vulgare*
Heavy metals				
Cadmium (Cd)	nd*	nd	nd	nd
Cobalt (Co)	0.005±0.002**	0.076±0.011	0.193±0.029	0.031±0.009
	0.004-0.007***	0.069-0.089	0.161-0.219	0.024-0.041
Chromium (Cr)	0.431±0.026	0.734±0.044	0.820±0.069	0.354±0.099
	0.411-0.461	0.701-0.784	0.753-0.891	0.243-0.431
Copper (Cu)	7.960±0.464	12.080±0.187	13.660±0.622	9.744±0.828
	7.492-8.420	11.880-12.250	12.990-14.220	8.820-10.421
Iron (Fe)	71.527±1.475	241.250±5.961	233.790±3.929	62.882±1.812
	70.52-73.22	235.51-247.41	230.98-238.28	60.79-63.94
Manganese (Mn)	35.907±1.633	37.853±1.215	24.090±0.903	35.617±1.743
	34.170-37.410	36.530-38.920	23.230-25.030	34.080-37.510
Molybdenum (Mo)	0.112±0.031	2.890±0.201	1.532±0.526	0.064±0.012
	0.091-0.147	2.680-3.080	1.119-2.124	0.054-0.078
Nickel (Ni)	3.050±0.525	0.977±0.083	2.095±0.491	1.853±0.547
	2.570-3.610	0.881-1.032	1.532-2.431	1.326-2.418
Lead (Pb)	nd	nd	nd	nd
Zinc (Zn)	24.290±1.102	23.490±1.146	15.487±0.571	14.070±0.475
	23.230-25.430	22.370-24.660	14.990-16.110	13.640-14.580
Metalloids				
Arsenic (As)	nd	nd	nd	nd
Antimony (Sb)	nd	nd	nd	nd

*not detected; **means ± standard deviation; ***intervals

Pb and Cd are non-essential trace metals having functions neither in humans body nor in plants. They induce various toxic effects in humans at low doses. The typical symptoms of Pb poisoning are colic, anemia, headache, convulsions and chronic nephritis of the kidneys, brain damage and central nervous system disorders. Pb accumulates in human body and damages mainly the kidneys and liver. WHO prescribed limit for Pb contents in herbal medicine is 10 ppm, while the dietary intake limit for Pb is 3 mg per week [10]. The lowest level of Cd which can cause yield reduction is 5 to 30 ppm, while the maximum acceptable concentration for food stuff is around 1 ppm [21]. Hence, no detection of Pb and Cd content in selected medicinal plants (below detection limit) in this study is highly acceptable.

Due to decreased electronegativity, proper metallic character has not been bestrode on As and Sb for which they are often referred to as metalloids, which means that these elements have both properties of metals and non-metals.

Arsenic is a common constituent of most plants, but little is known about its biochemical role. Apparently, plants take up As passively with the water flow. Contents of As in food plants vary highly, most commonly in the range from 10-60 μg kg^{-1}. No detection of As content in tested medicinal plants in this study is highly acceptable since arsenic is known to be highly toxic to humans and animals. It is reported by the WHO that 1.0 mg of inorganic As per day may give rise to skin lesions within a few years. The daily acceptable maximum intake of As by a healthy 70 kg BW person from food is 0.8-120 μg kg^{-1} [22, 23]. Unlike arsenic, antimony is not essential to plants, although it is known to be easily taken up by plants if present in soluble forms in growth media. Commonly reported Sb contents in agricultural plants range from <2 to 29 μg kg^{-1} [24]. According to some authors [25], antimony is a cumulative poison. Average contents of Sb in food products are between 0.2 and 1.1 μg kg^{-1}. Normal daily intake of Sb by adults from food is calculated for the USA to be 5 μg [24].

Regarding the other analyzed metals, it could be noticed that there are certain differences in the contents depending on the studied plant species, as well as the parts of the plants (Table 3).

Results in Table 3 reveal that high concentration of Cu was found in *M. piperita*, followed by *M. officinalis*, *F. vulgare* and *M. chamomilla*. Copper is consider to be an essential element for various metabolic processes. Its content of the majority of plant species varies between 20 and 30 ppm of dry weight. The critical copper deficiency level in vegetative plant parts is generally 3 to 5 ppm of dry weight [26]. Because it is required only in trace amounts, Cu becomes toxic at high concentrations [27]. Phytotoxicity can occur if its concentration in plants is higher than 20-100 ppm of dry weight [28]. The concentration of Cu in the study plants is high but it is below the critical level [29]. High levels of Cu may cause metal fumes fever with flue like symptoms, hair and skin decoloration, dermatitis, irritation of the upper respiratory tract, metallic taste in the mouth and nausea. WHO has recommended the lower limit of the acceptable range of Cu as 20 μg mg^{-1} of body weight per day [30]. Copper deficiency results in anemia and congenital inability to excrete copper resulting in Wilson's disease [31].

Similarly to the content of Cu, average maximum concentration of Co was found in *M. piperita*, followed by *M. officinalis*, *F. vulgare* and *M. chamomilla* (Table 3). Contents of Co in plants are highly controlled by both soil factors and the ability of plants to absorb this metal. Contents of Co in plant foodstuffs vary from 8 to 170 μg kg^{-1}. According to the previous studies, cobalt is consider to be toxic at elevated concentration, although the body needs it in trace amount. Cobalt is essential for humans as a component of the vitamin B_{12}. The deficiency of Co may affect anemia and anorexia. The excessive ingestion of Co may cause increased red blood cells, cardiomyopathy, hypothyroidism, pancreas failure, bone marrow hyperplasia, and some types of cancer. Human dietary intakes of Co vary from 5 to 40 μg per day [23, 32].

The toxic effects of Cr intake is skin rash, nose irritations, bleeds, upset stomach, kidney and liver damage, nasal itch and lungs cancer, while the deficiency of chromium is characterized by disturbance in glucose lipids and protein metabolism [33]. According to the results

obtained in this study (Table 3), average maximum concentration of Cr was found in *M. piperita*, followed by *M. officinalis*, *M. chamomilla* and *F. vulgare* (Table 3). As it has been recommended by US National Academy of Sciences, the daily intake of Cr for adults is 50-200 μg [30].

Iron is an essential element for human beings and animals and is an essential component of hemoglobin. It facilitates the oxidation of carbohydrates, protein and fat to control body weight, which is very important factor in diabetes. Results in Table 3 reveal that average maximum concentration of Fe was found in two Lamiaceae species - *M. officinalis* and *M. piperita*, followed by *M. chamomilla* and *F. vulgare*. The results suggest that high amount of Fe in plants may also be due to the foliar absorption from the surroundings air. The dietary limit of Fe in the food is 10-60 mg per day [34]. Low Fe content causes gastrointestinal infection, nose bleeding and myocardial infarction [35].

Average maximum concentration of Mn was found in *M. officinalis*, followed by *M. chamomilla*, *F. vulgare* and *M. piperita* (Table 3). Manganese concentration is high in all plants, however it is within normal background level for the element in plants under the critical concentration of 300-500 ppm of dry weight. Mn deficiency in plants causes chlorosis. The estimated safe and adequate daily dietary intake in adults is 11 mg per day [36]. Deficiency of Mn in human causes myocardial infarction and other cardiovascular diseases, also disorder of bony cartilaginous growth in infants and children and may lead to immunodeficiency disorder and rheumatic arthritis in adults [32, 37].

Molybdenum is an essential micronutrient, but the physiological requirement for this element is relatively low. Requirements for Mo are generally met at concentrations within the range of 0.2-5.0 mg kg^{-1} [38]. Results in Table 3 reveal that average maximum concentration of Mo was found in two Lamiaceae species - *M. officinalis* and *M. piperita*, followed by *M. chamomilla* and *F. vulgare*. As summarized by previous researches [39], variations of molybdenum concentrations in foodstuffs, especially plants, are greatly dependent on species and soil characteristics. A low order of toxicity of molybdenum compounds has been observed in humans. Possible reasons for the low degree of toxicity are the facts that molybdenum is a necessary trace element in the body, functioning in conjunction with some flavoprotein enzymes (xanthine oxidase, aldehyde oxidase, sulphite oxidase), and it is rapidly eliminated in the urine. The Food and Nutrition Board of the Subcommittee on the Tenth Edition of Recommended Dietary Allowances has established estimated safe and adequate daily intake values for Mo of 1.5-3.6 mg kg^{-1} per day for adolescents and adults [40].

In the case of Ni, average maximum concentration was determined in *M. chamomilla*, followed by *M. piperita*, *F. vulgare* and *M. officinalis* (Table 3). The most common ailment arising from Ni is an allergic dermatitis known as nickel itch, which usually occurs when skin is moist, further more Ni has been identified as a suspected carcinogen and adversely affects lungs and nasal cavities. On the other hand, its deficiency results in the disorder of liver [36]. EPA has recommended that daily intake of Ni should be less than 1 mg beyond which it is toxic [33]. Zinc is an essential trace element for plant growth and also plays an important role in various cell processes including normal growth, brain development, behavioural response, bone formation and wound healing. The dietary limit of Zn is 100 ppm [35]. According to the results obtained in this study (Table 3), high average concentration of Zn was found in *M. chamomilla* and *M. officinalis*, followed by *M. piperita* and *F. vulgare*.

CONCLUSIONS

From the present research it was concluded that the studied medicinal plants (*Matricaria chamomilla* L., *Melissa officinalis* L., *Mentha piperita* L. and *Foeniculum vulgare* Mill), growing in the area with highly developed industry, had the concentrations of analyzed heavy metals within the allowed limits, while the certain metals (Pb and Cd) and two studied metalloids (As and Sb) were below the detection limits. It could be also noticed that there are certain differences in the heavy metal contents depending on the studied plant species, as well as the parts of the plants.

Although our findings impose that medicinal plants from the studied growing sites are appropriate for preparation of teas and medicinal extracts, they should be collected from an area not contaminated with heavy metals, meaning that an appropriate choice of growing sites could greatly reduce the problem of heavy metal accumulation in medicinal plants. In addition, it was also concluded that a determination of heavy metals content in medicinal plants must become a standard criterion for evaluation of their quality.

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Original scientific paper

INFLUENCE OF ECOLOGICAL FACTORS ON THE ESSENTIAL OIL COMPOSITION OF *SIDERITIS SCARDICA* GRISEB.

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SUMMARY

Essential oil composition of six samples of *Sideritis scardica* was studied: one sample was collected from wild population and five were cultivars. The oils were analyzed by GC and GC/MS. After analyses it can be concluded that the variability in essential oil composition is closely related to climatic and environmental conditions. Thus, one cultivar which was growing close to the natural habitat, produces the essential oil quite similar in composition to the oil of wild growing plants. In addition, the essential oil profiles of the cultivars planted under the same environmental conditions in the same experimental field exhibited that the flowering stage does not cause significant qualitative differences.

***Key words**: Sideritis scardica, wild growing plants, cultivars, essential oils, composition.*

INTRODUCTION

The genus *Sideritis* comprises of numerous species distributed mainly in temperate and tropical regions of the Northern Hemisphere - Mediterranean area, together with Canary and Madeira islands. Diterpenes, flavonoids and essential oils, which occur in almost every *Sideritis* species, are responsible for their pharmacological activities. *Sideritis scardica* is alpine endemic plant for the Balkan Peninsula [1]. This herb is very popular in traditional medicine for different purposes – treatment of inflammations, gastrointestinal disorders, cough, etc. It is used as herbal tee for treatment of bronchitis, lung emphysema etc. Distribution of this species is quite limited in Bulgaria because of its intensive collection from the natural habitats for years. One of the most difficult problems is to control and stop this practice in order to protect this endangered species, included in the Red Data Book of Bulgaria [2]. It is under governmental protection and collection from the native habitats is prohibited. This fact imposes the necessity of its cultivation. *S. scardica* essential oil composition from native Bulgarian populations is discussed so far in two reports [3, 4].
The aim of this investigation was to analyze the influence of the ecological factors in the extreme cultivation conditions on the essential oil composition of *Sideritis scardica.*

MATERIAL & METHODS

Plant material

Flowering parts of the plants of *S. scardica* were collected in July 2011 from 3 experimental fields: Sofia, Beglika locality (Rhodope Mountain), Goce Delchev (Pirin Mountain), and one from native habitat – under the peak Orelek (Pirin Mountain). The samples S1 and S2 were cultivated in Sofia and were in initial and last stage of selection, respectively. Samples GD, B1 and B2 originated from S2. Details on the studied plant material are presented in Table1.

Table 1. Plant samples

Sample	Origin	Characteristic of sample	Altitude (m)	Soil	Climatic zone[5]*
OR	Pirin Mt	wild, full flowering	1990	alkaline	A
GD	Goce Delchev	cultivated, full flowering	550	alkaline	A
S1	Sofia	cultivated^{+}, full flowering	760	neutral	B
S2	Sofia	cultivated^{++}, full flowering	760	neutral	B
B1	Beglika	cultivated, beginning of flowering	1500	acidic	C
B2	Beglika	cultivated, full flowering	1500	acidic	C

$^{+}$ initial step of selection; $^{++}$ final step of selection

*A – Continental Mediterranean climatic zone; B – Temperate continental climatic zone;
C – Transitional continental climatic subzone of the European continental zone.

Preparation of essential oil

Essential oils were prepared by micro steam distillation-extraction of the air-dried plant material for 2h in Lickence-Nickerson apparatus, modified by Godefroot, using diethyl ether as a solvent [6].

Analysis of essential oils

GC analyses were performed on HP 5890 gas chromatograph (FID), carrier gas nitrogen, linear velocity 25 cm/s, split ratio 1:100, fused silica capillary column HP-5MS (poly-5%-diphenyl-95%-dimethylsiloxane), 30m x 0.25 mm, 0.25μm film thickness. The injector and detector temperature was 260ºC, column temperature was programmed from 50º-240ºC at rate of 4ºC/min, and 10 min at 240ºC. The quantitative estimation was determined by relative peak area (electronic integration). An internal standard has not been used.

GC-MS analyses were performed on HP 6890 instrument. All chromatographic conditions and the column were as described above, but the carrier gas was helium. The oil components were identified by comparison of their retention indices and mass spectra, with those published in the [7, 8] and presented in NIST 98 as well as a library developed by us. A homologues series of *n*-alkanes under the same conditions were used as reference points for calculation of RI. Compounds with RI 2325 and 2349 are reported previously by Kostadinova et al. [4] as components of *S. scardica* essential oils. MS (EI, 70 eV) *m/z* (%) of unidentified diterpenoids:

RI 1980: 272 (19), 257 (21), 229 (47), 201 (17), 189 (30), 161 (50), 147 (50), 133 (45), 133 (45), 93 (100), 81 (95), 69 (60), 67 (63);

RI 2325: 288 (20), 270 (100), 255 (49), 242 (10), 199 (14), 187 (20), 145 (15), 131 (20), 107 (20), 105 (20), 94 (43), 79 (20), 69 (16);

RI 2349: 288 (29), 273 (15), 257 (30), 161 (47), 145 (30), 133 (30), 131 (30), 119 (50), 105 (100), 91 (70), 81 (20), 79 (22), 67 (25).

RESULTS & DISCUSSION

The results from GC and GC-MS analyses are presented in Table 2. Thirty seven components registered as chromatographic peaks in concentration more than 0.5% at least in one of the samples, were taken into consideration for comparison of the studied oils. As it can be seen from the obtained results, there were no significant qualitative differences between the investigated samples of essential oils. Although in traces, all substances present in all six oils. Sesquiterpenoids were presented in low concentration in the essential oils obtained from OR and GD and appeared to be 2.5-3 times less then monoterpenoids. These oils were characterized by high concentration of diterpenoids. OR and GD samples differed from the others by α-pinene/β-pinene ratio. Thus, in GD dominated α-pinene, while β-pinene prevailed

in OR. Besides, high concentration of eugenol, benzyl benzoate, and 1-octen-3-ol distinguished the sample GD from OR. On the other hand, the amount of sesquiterpenes was twice larger in OR.

Table 2. Chemical composition of essential oils from *S. scardica*

RI	Compounds	OR	S2	S1	B2	B1	GD
932	α-pinene	13.7	4.6	10.6	9.4	8.8	14.6
952	benzaldehyde	+	+	+	+	+	0.6
969	sabinene	1.8	+	+	2.5	2.3	+
975	β- pinene	18.0	18.4	31.7	24.4	23.7	6.9
979	1-octen-3-ol	3.2	0.4	1.1	3.7	3.4	10.2
988	myrcene	0.6	0.6	1.2	0.9	0.8	+
1024	limonene	2.4	2.4	6.6	5.1	4.8	1.7
1036	phenyl acetaldehyde	1.9	1.1	1.0	0.7	0.6	2.0
1060	octen-1-ol (2e)	0.7	0.5	0.5	0.5	+	+
1080	terpinolene	+	0.5	0.5	0.5	+	+
1095	linalool	0.8	+	+	+	+	+
1106	phenyl ethyl alcohol	1.4	0.7	1.5	2.2	2.1	0.8
1135	trans-pinocarveol	+	1.7	1.6	+	+	2.9
1140	trans-verbenol	+	1.0	0.9	+	+	+
1160	pinocarvone	+	1.2	1.1	+	+	+
1194	myrtenol	+	1.1	1.0	+	0.4	+
1195	myrtenal	+	0.5	0.7	+	+	+
1204	verbenone	+	+	+	+	+	1.7
1356	eugenol	0.7	+	+	0.5	0.5	7.8
1374	α-copaene	0.6	2.1	3.6	+	+	+
1389	β-elemene	+	0.6	+	+	+	+
1417	β-caryophyllene	5.5	4.1	0.6	+	+	3.2
1454	trans- β-farnesene	1.1	11.0	5.0	12.8	12.9	1.3
1480	germacrene D	0.5	16.1	2.6	8.2	7.7	0.8
1500	bicyclogermacrene	1.7	2.1	1.2	3.0	2.8	2.0
1505	β-bisabolene	4.2	1.1	0.3	0.4	0.4	+
1522	δ-cadinene	1.0	2.6	3.6	0.4	0.4	+
1577	spathulenol	0.6	+	+	4.0	4.6	+
1582	caryophyllene oxide	0.6	0.7	+	+	+	+
1759	benzyl benzoate	1.3	0.8	1.0	1.4	1.3	3.0
1846	hexahydrofarnesyl acetone	+	1.0	+	+	+	+
1856	phenylethyl benzoate	+	0.5	+	0.8	+	+
1980	M=272	2.8	3.1	2.4	5.4	2.6	7.1
2237	7α-hyrdorxy-manool	2.4	0.2	0.8	0.4	0.7	5.0
2269	sandaracopimaradiene-3β-ol	1.0	0.8	0.3	0.5	0.4	1.8
2325	M=288	3.5	0.9	0.5	0.5	0.6	2.7
2349	M=288	0.8	0.8	0.7	0.3	0.2	4.4
	Monoterpene hydrocarbons	**36.5**	**26.0**	**50.6**	**42.3**	**40.4**	**23.2**
	Oxygenated monoterpenes	**0.8**	**5.5**	**5.3**		**0.4**	**4.6**
	Sesquiterpene hydrocarbons	**14.6**	**39.7**	**16.9**	**24.8**	**24.2**	**7.3**
	Oxygenated sesquiterpenes	**1.2**	**1.7**		**4.0**	**4.6**	
	Diterpenoids	**10.5**	**5.8**	**4.7**	**7.1**	**4.5**	**21.0**
	Aromatic compounds	**5.3**	**3.1**	**3.5**	**5.6**	**4.5**	**14.2**
	Others	**3.9**	**1.0**	**1.6**	**5.4**	**3.4**	**10.2**
	Total	**72.8**	**82.8**	**82.6**	**89.2**	**82.0**	**80.5**

+ - compounds in amounts less than 0.1%

The two samples originating from Beglika (beginning of flowering-B1 and full flowering-B2) were very similar qualitatively and quantitatively, but differed from the other oils. Thus, while only traces of sabinene were detected in the other samples, with exception of OR, in B1 and B2 reached 2.3% and 2.5%, respectively. Another distinctive feature was that, β-caryophyllene was almost absent from B1 and B2 essential oils. Furthermore, principal constituents in the oils from Beglika (B1 and B2) were β-pinene, trans-β-farnesene and germacrene D. Total amounts of monoterpenoids, sesquiterpenoids, diterpenoids, and aromatic compounds were close in both oils.

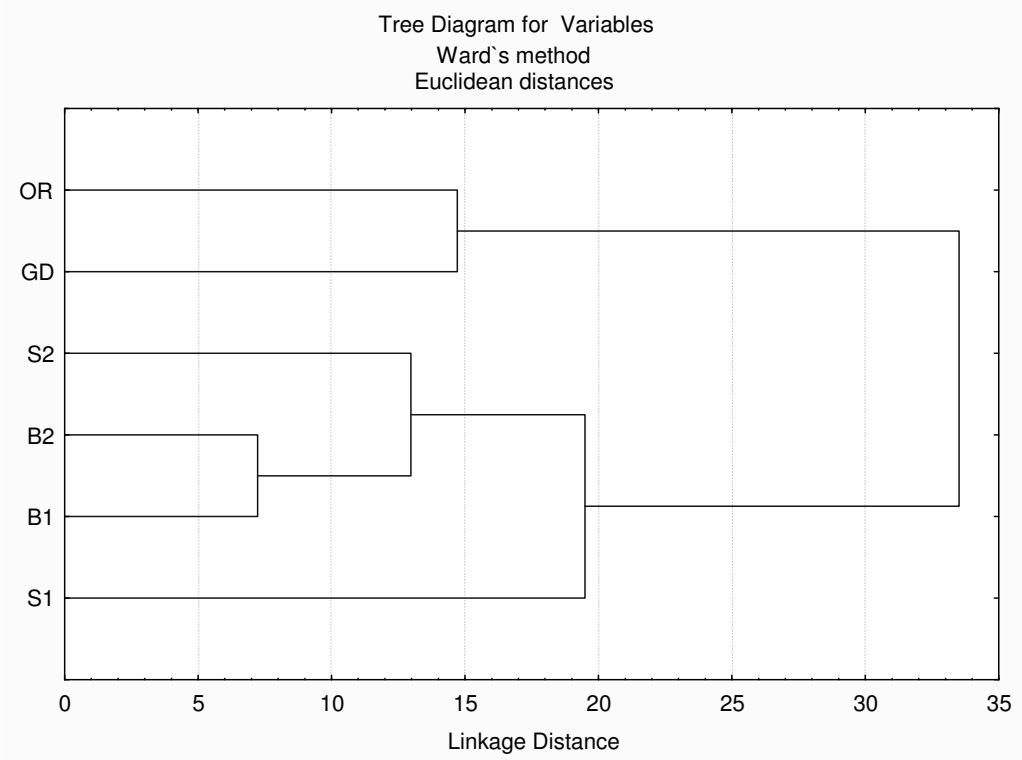

Figure 1. Dendogram. Tree clustering was made by Statistic 6.0 for 6 cases using Euclidean distance and Word's method as linkage rule.

Samples S1 and S2 (initial and final step of selection) were a pair of oils with very similar chemical composition. On the other hand, 1-octen-3-ol and eugenol were lowest in S1 and S2, while pinocarvone, myrtenol, α-copaene and δ-cadinene were highest in comparison with the other samples. Further, samples S1 differed from S2 in content of some components. Thus, the concentrations of β-caryophyllene, trans-β-farnesene and germacrene D were several times higher in S2, while those of α-pinene, β-pinene, limonene, and copaene were higher in S1. Cluster analysis shows that the studied samples were combined in two groups. One of them included samples OR and GD and the second one consisted of B1, B2, S1, and S2. The highest degree of similarity is established between the samples B1 and B2 among the samples in the second group. Further, close to these two oils was located sample S2, while S1 differed significantly. The observed difference between S1 and S2 is due to the step of selection (initial and final, respectively).

CONCLUSION

The obtained results established that the essential oil composition is determined mainly by ecological factors. Oils obtained from plants growing under similar ecological conditions are qualitatively and quantitatively comparable, independently of the origin – wild or cultivated (OR and GD) as well as of the flowering stage - initial and full flowering (B1 and B2). Oils from plants at the same stage of development (GD, B2 and S2), but growing under different ecological conditions (soil, climate) differed considerably. This statement is confirmed by cluster analysis.

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Original scientific paper

ROOT AND LEAF MINERAL CONTENT OF WILD GROWING YELLOW GENTIAN (*GENTIANA LUTEA* L) FROM NATURAL HABITATS IN WESTERN PART OF BOSNIA AND HERZEGOVINA

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SUMMARY

The content of macroelements (N, P, K, Ca, and Mg) and microelements (Fe, Mn, Zn, Cu) was examined in root and aboveground part of yellow gentian (*Gentiana lutea* L.) from the natural habitats of the mountains Klekovača, Osječenica and Vranica, Bosnia and Herzegovina. Plant samples were taken in the mid-August 2009 at three replications from each location were surveyed. Nitrogen was determined using Kjeldahl method, phosphorus by spectrophotometry and Ca, K, Mg, Fe, Mn, Zn, Cu AAS in the solution, after the destruction of samples in acid mixture

Yellow Gentian's leaves contained in first the Ca (1,3 - 1,9 %) and N (1,1 - 1,9 %) , followed by K (0,7 - 1,3 %) and Mg (0,2 - 0,8 %), whereas the lowest content expressed the phosphorus (0,03 - 0,11 % P). Among microelements, the leaves contained mostly the Fe (46 - 213 mg kg^{-1}) followed by Mn (23 - 82 mg kg^{-1}) and Zn (32 - 55 mg kg^{-1}), and finally the Cu (13 - 21 mg kg^{-1}).

The content of mineral elements in the root varied in significantly narrow range than in the leaves. Among the macroelements in the yellow gentian root, the most present were Ca (0,9 - 1,1 %) and N (0,8 - 1,2 %), while the lowest content had the phosphorus (0,01 - 0,02). Root bark had a similar content of macroelements as the inner part of the root, except for calcium which was for 45% higher in the cortex. Root bark contained 5 times higher content of Fe then inner root parts, while the content of Mn in the bark was 2 times higher than in the inner part of the root. The content of Zn and Cu was similar in both parts, the inner part of the root and root bark.

Different soil and climate characteristics at the study sites affected the most of identified differences in the content of Fe, Mg and Mn in gentian leaves, and in less extent had the impact on its content in the roots. The content of Zn and Cu in gentian parts was the least dependent on the site features.

Key words: yellow gentian, mineral content, root, leaves

INTRODUCTION

Yellow gentian, *Gentiana lutea* L. subsp. *symphyandra* (family *Gentianaceae*), is a perennial herbaceous plant that grows in mountainous regions, on meadows and open slopes from the Pyrenees to the Carpathian mountains and from Alps to the Balkan Peninsula [1]. Natural habitats of Yellow Gentian in the Balkans are recorded at the mountains of Montenegro, Serbia, Bosnia and Herzegovina [2]. Significant populations of the yellow gentian in Bosnia were recorded at the Mountain Treskavica (alt. 1.900 m) in 1952 [3], whereas many other

populations were found on many Bosnia and Herzegovina mountains (Jahorina, Zelengora, Šator, Vlašić, Kruščica, Klekovača), known for permanent and excessive exploitation.
Although it is stated that gentian inhabits ecosystems of mountainous areas on calcareous substrates [4], later studies showed that soils, on which the species grows, could also be a limestone substrates, silicate rocks and serpentine [5]. Wild growing gentian prefers meuble soils that are, generally, very humic, containing more than 6% of humus [6].
The underground organs, rhizome and roots (*Gentianae radix),* are used as a remedy in traditional medicine, and moreover, the roots of *Gentiana lutea* L. are official drug in many world's pharmacopoeias: Eur. 6; DAB 10, ÖAB 9, Ph. Jug. IV [1]. The active substances are bitter substances, amarogentin and gentiopicrin, that determine the therapeutic application area [7]. As a drug it is used for digestive disorders such as loss of appetite, flatulence, etc. Recent research has shown that the aboveground parts of the plant could be used in medical purposes [8]. The rhizome and root of yellow gentian (*Gentiana lutea* L.) have been used for a long time in pharmaceutical industry in larger quantities, but also in industrial production of bitter alcohol beverages: brandies, aperitives, liqueurs, etc. [9].
Unsustainable exploitation in the last century has put in danger the survival of the gentian in many of Bosnian mountains, similar as in other SEE countries. Therefore, in last decades of 20th century and beginning of 21st century many European countries invest their efforts to develop gentian cultivation technology [10], [11], [12], [13], [14]. In the Balkans these researches have been conducted in mountainous areas with the altitude above 1.000 m.
Defining of growing conditions of the gentian is particularly important for successful cultivation, as different areas exhibit specific ecological conditions. Growing conditions, the soil characteristics and specificities of the plant species determine the content of components in plants that represent its quality.
With this work we wanted to show how natural climate and soil conditions at natural habitats in Western part of Bosnia and Herzegovina affect the content of mineral nutrients and heavy metals in underground and aboveground organs of the yellow gentian.

MATERIAL AND METHODS

Plant material, such as root, rhizome and rosette leaves of yellow gentian was collected from three natural habitats at the mountains of Central and Western Bosnia and Herzegovina. All samples were taken in period from 12th to 14th August 2009, at the following sites: 1. Mountain Klekovača, N= 44º, 24', 03"; E= 16º, 26', 23"; 1106 m alt. 2. Mountain Osječenica, N= 44º, 27', 47"; E= 16º, 19', 57"; 1012 m alt. 3. Mountain Vranica, N= 43º, 59', 17"; E= 17º, 40', 48"; 1670 m alt. From each site three replicates of plant material were collected. At all three localities the soil was very shallow and mixed with the skeleton.
After digging, the root was washed out with tap and distilled water, peeled and dried. Peeled roots were cut into 1 cm pieces and naturally dried on air. Root bark was dried as a separate part of the sample. Rosette leaves were also dried on air. After drying, samples were floured and prepared for laboratory analysis.

Analysis of plant material

In the samples of gentian leaves and roots it was analyzed the content of macroelements (N, P, K, Ca, Mg) and microelements (Fe, Mn, Zn, Cu).
Total nitrogen (% N) was determined according to the method of Kjehldal (Tecator, Unit System I), after destroying sample in concentrated $H_2SO_4 + H_2O_2$.
Total concentration of other macro and microelements was determined from the common solution, wet combustion with the mix of acids HNO_3, H_2SO_4, $HClO_4$ in relation 3:2:1.
Phosphorus (% P) was measured by spectrophotometry, upon coloring method with ammonium vanadate.

Total K, Ca, Mg and microelements Fe, Mn, Zn, Cu were determined by AAS technique.

RESULTS AND DISCUSSION

The content of nitrogen (N) was the highest in the gentian leaves, in average of 1,35%, with the variation interval 1,14 - 1,56 % (Table 1), while the content of nitrogen in the root was lower, on average 0,91 % in inner part of the root, and in a bark of the root it was 0,94 % with the variation interval between 0,55 – 1,19 % (Tables 2 and 3). Considering physiological role of nitrogen in plants it is known that plants contain more nitrogen in aboveground organs than in the underground organs [15]. The lowest content of nitrogen was in the gentian samples from the Osječenica site in both analyzed parts of the plant, the leaves and the root, which indicates that environmental factors significantly determine the content of nitrogen in ther plant.

The content of phosphorus (P) was very low in gentian from natural habitats in Bosnia and Herzegovina. The content of phosphorus was the lowest in inner part of the gentian root, on average 0,01% P, while in the leaves was 0,03 – 0,10 % P, and in the bark of the root 0,05 – 0,08% P. Generally, low level of phosphorus could be caused by low content of physiologically active P_2O_5 of the soils from this part of Bosnia and Herzegovina [16]. Based on research of Radanović et al. [17], the gentian leaves showed symptoms of phosphorous deficiency already at the level of 0,28 % P_2O_5 in the leaves.

The content of potassium (K) was the highest in the gentian leaves, on average 0,86 % K, with variation interval between 0,70 – 1,11 %. Potassium in the root was almost three times lower, on average 0,33 % , while the content of K in the inner root part and in the bark of the root varied in a lower interval (Tables 2 and 3). Generally, we can say that the phosphorus content is low in all of gentian samples from the studied natural habitats in Bosnia and Herzegovina, which can be related to very poor soil conditions in all three analyzed sites. Similarly, low potassium content in the leaves (0,70 - 1,17% K) was determined for the cultivated gentian exhibiting a physiological symptoms of disease, while the healthy gentian plants contained 1,12 – 2,03 % K [17].

Table 1. Content of macro and micro elements in the gentian leaves from natural habitats in Bosnia and Herzegovina

	% (D.M.)					**mg kg^{-1} (D.M.)**			
	N	**P**	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Zn**	**Cu**
Klekovača	**1.56**	**0.10**	**1.11**	**1.77**	**0.27**	**130.0**	**40.0**	**43.5**	**15.0**
Stdev	*0.370*	*0.015*	*0.150*	*0.140*	*0.035*	*83.00*	*17.00*	*11.50*	*2.00*
Osječenica	**1.14**	**0.06**	**0.76**	**1.44**	**0.30**	**56.5**	**73.5**	**42.0**	**15.5**
Stdev	*0.050*	*0.015*	*0.035*	*0.040*	*0.000*	*2.50*	*8.50*	*2.00*	*1.50*
Vranica	**1.35**	**0.03**	**0.70**	**1.33**	**0.60**	**46.0**	**28.0**	**42.0**	**21.0**
Stdev	*0.210*	*0.015*	*0.093*	*0.090*	*0.017*	*42.75*	*12.75*	*6.75*	*1.75*

The content of calcium (Ca) in the gentian leaves was on average 1,51 % with the variation interval per sites from 1,33 to 1,77 % , and the highest content on Klekovača site. Inner root parts contained on average 1,03 % Ca, with small differences between sites (Table 2). The bark of the root contained significantly higher content of Ca (on average 1,44 % Ca) with the variation interval between sites from 1,38 to 1,50 % (Table 3). Higher calcium content in the bark of roots can be effect of its greater presence in the apoplast as well as because of

possible restraints of fine soil particles that contain calcium in the outer pores of the root bark.

Magnesium content in the gentian leaves varied between 0,27 and 0,30 % at the Klekovača and Osječenica sites, up to 0,60 % at the Vranica site. The Vranica site showed higher content of magnesium in the roots too, comparing with other two sites (Tables 2 and 3), which can be assumed as an impact of soil and environmental conditions, possibly more favorable for higher accumulation of magnesium in gentian plant material. The average magnesium content at all three sites was 0,39% in the leaves compared to 0,16 % found for the inner and outer part of the gentian root.

Table 2. The content of macro and microelements in pealed Gentian roots from the natural habitats in Bosnia and Herzegovina

	% (D.M.)					mg kg^{-1} (D.M.)			
	N	**P**	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Zn**	**Cu**
Klekovača	**0.99**	**0.01**	**0.30**	**1.02**	**0.15**	**73.5**	**11.5**	**23.0**	**13.5**
Stdev	*0.215*	*0.000*	*0.040*	*0.090*	*0.010*	*9.50*	*3.50*	*4.00*	*2.50*
Osječenica	**0.55**	**0.01**	**0.31**	**0.94**	**0.15**	**94.5**	**16.5**	**26.0**	**22.0**
Stdev	*0.315*	*0.003*	*0.010*	*0.030*	*0.010*	*4.50*	*2.50*	*1.00*	*0.00*
Vranica	**1.19**	**0.02**	**0.37**	**1.13**	**0.19**	**131.0**	**13.0**	**30.0**	**28.0**
Stdev	*0.265*	*0.035*	*0.025*	*0.060*	*0.010*	*7.00*	*3.00*	*2.50*	*1.25*

The content of iron (Fe) in gentian plant material is characterized by large variability of data, especially in root bark and leaf (Tables 1 and 3). The content of iron in the leaves was the highest at the Klekovača site: 130 mg kg^{-1} Fe, compared to other two sites where the level of iron in gentian leaves was 2.5 to 3 times lower (Table 1). In clean, peeled root the highest content of iron was found at the Vranica site (131 mg kg^{-1}), compared to 73,5 mg kg^{-1} found at the Klekovača site and 94,5 mg kg^{-1} at the Osječenica site. The content of iron was the highest in the root bark, on average 517,5 mg kg^{-1}(Table 3) which is 5 times higher than in the inner part of the root (99,7 mg kg^{-1}). Such a large difference in the content of iron between the inner and outer part of the root probably is the result of soil particles that remained at the bark of the root, which may contain iron in large percentage, up to 10 % [18]. Very fine soil particles remain at the root pores although the roots was repeatedly washed with water, which probably caused increased content of elements, especially those whose content in the soil was high.

Manganese content was the highest in the gentian leaves, on average 47,2 mg kg^{-1} , with large variations per sites from 28 mg kg^{-1} at the Vranica site to 73,5 mg kg^{-1} at Osječenica site. The content of manganese in peeled gentian root was in the range from 11,5 to 16,5 mg kg^{-1} , which is very low concentration in comparison with 108,8 mg kg^{-1} of Mn found by Radanović et al . [19] in the gentian root from Suvobor, Serbia. The content of manganese in the root bark was twice higher than in inner root part (Table 3), and on average its content ranged from 30 to 39 mg kg^{-1} at all three sites.

Table 3. The content of macro and microelements in the bark of the yellow gentian root from natural sites in Bosnia and Herzegovina

	% (D.M.)					mg kg^{-1} (D.M.)			
	N	**P**	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Zn**	**Cu**
Klekovača	**0.88**	**0.07**	**0.27**	**1.50**	**0.15**	**865.5**	**39.0**	**26.5**	**14.5**
Stdev	*0.095*	*0.035*	*0.020*	*0.075*	*0.005*	*100.50*	*2.00*	*2.50*	*1.50*
Osječenica	**0.86**	**0.05**	**0.26**	**1.45**	**0.14**	**334.0**	**34.0**	**31.5**	**23.0**
Stdev	*0.010*	*0.035*	*0.020*	*0.200*	*0.010*	*83.00*	*5.00*	*2.50*	*0.00*
Vranica	**1.09**	**0.08**	**0.28**	**1.38**	**0.20**	**353.0**	**30.0**	**33.0**	**27.0**
Stdev	*0.028*	*0.035*	*0.020*	*0.138*	*0.007*	*91.75*	*3.50*	*2.50*	*0.75*

Zinc content was the highest in gentian leaves (on average 42,5 mg kg^{-1} Zn), compared with the root (Tables 2 and 3). Slightly higher zinc concentration was found in the root bark (on average 30,3 mg kg^{-1}) than in the root inner part (on average 26,3 mg kg^{-1}). Differences between the sites regarding zinc content were very small and it could be considered that adoption of zinc is a relatively stable trait. A similar level of zinc in the gentian root was found by Radanović et al [19]. The content of copper in the gentian leaves ranged from 15 – 21 mg kg^{-1}, and in the roots varied between 13,5 and 28 mg kg^{-1} . The content of copper is very similar in inner and in outer part of the root (Tables 2 and 3). The highest level of copper was found in samples from Vranica site: in leaves (21 mg kg^{-1}) and root (27-28 mg kg^{-1}). It can be considered that quantities of copper found in wild growing gentian from all studied sites in Bosnia and Herzegovina are in a range quoted for natural plant materials with the most frequent concentration interval between 5 and 20 mg kg^{-1} [20].

CONCLUSION

Different climatic and soil conditions at the mountains of Central and Western Bosnia and Herzegovina have mainly influenced the content of iron, magnesium, and manganese in the leaves of wild growing yellow gentian.

Differences in the content of nitrogen in leaves and roots and content of calcium in the leaf samples indicate the influence of soil and other environmental factors on level of their uptake by the Gentian plants. Content of Fe, Ca, and Mn in the root bark was significantly higher than in the inner part of the gentian root.

The content of zinc and copper in samples of the yellow gentian was the least affected by ecological factors of the habitat.

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Original scientific paper

CONTRIBUTION TO THE STUDY OF THE POLYPHENOLIC COMPOUNDS OF *AGASTACHE RUGOSA* KUNTZE PLANTS IN *IN VITRO* AND CONVENTIONAL CULTURES

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SUMMARY

Agastache rugosa Kuntze (*Lamiaceae* family) is a perennial plant widely spread in Eastern Asia, being traditionally used in China. Recent studies mention anti-tumour, antifungal, antiviral and cytotoxic activities of the species. The aim of our research was the evaluation of the biosynthetic capacity of the *A. rugosa* species adapted to the environmental conditions of Romania, to achieve field cultures developed under the principles of organic farming .

We studied 2 varieties of *A. rugosa* – with blue and white flowers, and the *in vitro* multiplication was made for the blue flowered variety.

To achieve tissue cultures, the explants were made up of plantlets obtained by aseptic seed germination. For shoot multiplication we used MS supplemented with BAP and NAA, an optimal multiplication ratio being obtained by adding 2.0 ml/l BAP and 0.3 ml/l NAA to the culture medium. 95% of the shoots regenerated *in vitro* developed a vigorous root system, . The surviving ratio of the regenerants was of 85% after the transfer into the field culture.

The analysis of the absolute methanolic extracts for the antioxidant compounds was made by TLC and HPLC; we noticed a raised biosynthetical capacity for the compounds of the polyphenolic type (rosmarinic acid, chlorogenic acid, caffeic acid and cinnamic acid), out of the rosmarinic acid was determined in high quantities in all parts of the plant. As our study was achieved both on conventionally cultivated and on *in vitro* propagated plants, it was worthwhile to evaluate the polyphenolic compound accumulation (especially the rosmarinic acid) at different plant parts and to highlight the optimum of vegetal material to be used as raw material for food supplements. The highest quantity of the polyphenolic compounds, especially the rosmarinic acid, followed by the chlorogenic acid was found in leaves, both for *in vitro* regenerated plants and for the cultivated ones. .

***Keywords**: Agastache rugosa Kuntze, in vitro, conventional cultures, polyphenols, HPLC*

INTRODUCTION

Generally, the *Agastache* species are meliferous and ornamental species, out of which *A. rugosa, A. foeniculum, A. scorphulariifolia* and *A. Mexicana* are especially cultivated. From the ones mentioned, the first three species are generally described as having a smell of anason, but anetol, the main compound in anason, is not to be found in raised quantities in *Agastache.*

The *Agastache rugosa* was named as such due to the rugged leaves. The flowers are pink to violet. The essential oil has an antifungal effect on the *Trichophyton* species that cause skin infections and it has been proved that it selectively inhibits the *in vitro* proliferation of human cancer cells [1]. The main constituent of the volatile oil is estragol in a proportion of 20 -

96%, followed by isomenthona 0 – 45%, limonen 4 – 12%, and we found a variant with a content of 48 – 92% metil-eugenol, and only 2 – 6% estragol [2].
A. rugosa contains a great quantity of rosmarinic acid. It is used in Chinese traditional medicine to treat fever, stomach affections, angina aches. This species enters the composition of a mixture of Chinese herbs to treat fever and diarrhoea caused by SIDA [3]. In Romania, the species is found only in culture, especially as a decorative plant [4]. Recent studies on the *A. rugosa* species aimed both the active principle content – mainly the essential oil and the rosmarinic acid, the anti-apoptotic biological activity, antioxidant, antifungal, antiviral [5], and the capitalization of the biosynthetic potential by means of biotechnologies [6, 7, 8].

MATERIAL AND METHOD

Medium and culture conditions

The basic medium was made up by Murashige and Skoog (1962) (MS) [9] salts and vitamins, plus 2.5% sucrose, solidified with 0.80 – 0.85% agar. Previous to autoclavation (120°C for 15 min), the pH of the medium was adjusted to 5.5 with NaOH. To initiate caulogenesis, we used variants of MS, supplemented with BAP (benzylaminopurine) and NAA (naphthaleneacetic acid): the BN media (1.0 mg/l BAP and 0.3 mg/l NAA), B_{20} (2.0 mg/l BAP) and B_{10} (1.0 mg/l BAP). To induce risogenesis, we used the MS variants: MS without phytohormons, MS supplemented with 0.5 mg/l NAA and MR (MS medium variant for rooting , poorer in salts) - supplemented with 1 mg/l NAA. The cultures were maintained in the half-climatized room for experimental cultures, at a temperature of 22 $\pm$ 1°C and a light regime of 16 hours/8 hours of darkness.

The vegetal material

We studied 2 *A. rugosa* varieties – with white and blue flowers (obtained from the experimental field of the Vegetable Research and Development Station Bacau); the *in vitro* multiplication was achieved for the variety with blue flowers and thus we obtained 2 experimental variants for the plants obtained *in vitro* after acclimatization and transfer in the experimental field (the flowering and fruiting phenophases), and other 2 variants for the 2 varieties originated from conventional cultures (flowering phenophase).
The explants were plantlets obtained by aseptic seed germination. The seeds were collected from donor plants of *Agastache rugosa* from the experimental field of the mentioned station.
Our study aimed the comparative phytochemical analysis for polyphenolic compounds only for *A. rugosa* plants with blue flowers – obtained through generative propagation, respectively *in vitro* culture. In order to assess the phytochemical diversity of *A. rugosa* varieties, we also analyzed the variety with white flowers - plants obtained through generative propagation (from the collection of Vegetable Research and Development Station Bacau).

The phytochemical analysis of the experimental material

a) Thin Layer Chromatography (TLC)
The preliminary identification of the absolute methanolic extracts for the polyphenolic and flavonoidic compounds, of the dichlormethanic extracts for the triterpenic and phytosterolic compounds, respectively, was made by TLC. As standards we used caffeic acid, rosmarinic acid chlorogenic acid, quercetin, rutin, luteolin and apigenin (for polyphenols and flavons), β-sitosterol, stigmasterol, oleanolic acid and ursolic acid (for the triterpenic acids and phytosterols).
b) High Performance Liquid Chromatography
For the HPLC analysis, we used an Agilent 1200 series system, equipped with a diode-array UV detector (DAD) and autosampler. To separate the compounds, we used an analytic chromatography column with reverse phase of the Zorbax Eclipse XDB-C18 type (granulation 5 µm, 150 x 4.6 mm d.i.). The column temperature was maintained at 30°C. For

the elution we applied a gradient with two solvents: acetonitryl (solvent A) and an acetic buffer solution (solvent B), prepared starting from a watery solution of sodium acetate (2mM) adjusted to a pH=3.5 with acetic acid of 99% purity. The initial conditions were 2%A and 98%B. The linear gradient programme was of 2-14-20-30-25% solvent A for 0-20-40-50-60 min, after which we switched back to the initial conditions. The discharge used was of 1.0 mL/min. The sample injecting (100 µL) was done by help of the autosampler.
As standard substances we used chlorogenic acid, caffeic acid, rosmarinic acid cinnamic acid (furnished by the LG-Standards company). The stock solutions of the standards were diluted with methanol and analyzed in the same conditions to achieve the standard curves.
To identify the peaks we compared both the values of the retention time (RT) and the UV absorption spectra of the compounds identified in the analyzed samples, with those of the standards used. For the quantitative results calculation we used the peak areas in the standard curve method.

RESULTS AND DISCUSSIONS

Determining the germination capacity

The germination capacity of the seeds collected from the donor plants was made on three variants: on filter paper, on (in aseptic conditions) and in soil, obtaining a germination degree between 30 and 70%. To aseptically isolate the seeds, we tested 3 sterilizing agents (different action times).

Table 1. The effect of the sterilizing agents tested for aseptical seed isolation

Sterilizing Agent	Action Time (min.)	Aseptic Isolation Degree	Germination Degree
Mercury chloride (0,1%)	25 35	+ + + + +	+ + +
Sodium hypochlorite (3%)	25 35	+ + +	+ + +
Chloramine T (5%)	35	-	+ +

Plant regeneration and multiplication

Plant regeneration and inducing risogenesis for inoculated explants, after 4 weeks of culture, was of 95% for all the medium variants. The best multiplication ratio was obtained by using MS supplemented with 1 mg/l BAP and 0.3 mg/l NAA (BN), obtaining 4-5 plantlets per explant in 4 weeks. The media supplemented with 1 mg/l BAP, 2 mg/l BAP respectively, lead to more reduced multiplication ratios. The subcultivations were achieved ar 4 weeks each.
To induce risogenesis, a good morphogenetic answer was obtained by using MS supplemented with 0.5 mg/l NAA, in 4 weeks, 95% of the plantlets forming a vigorous radicular system.

Acclimatization of the in vitro regenerated plants

It was achieved in 2 steps: in a hydroponic system for 2 weeks, the plants being later transferred into sterile soil pots. To assure a vapour saturated atmosphere, the plants were covered with transparent glasses, gradually removed. After 4-5 weeks of accommodation, the plants were moved into the field, were they obtained maintenance activities characteristic to the species. The survival ratio of the regenerated plants was of 85%.

Aspects of the *in vitro* of *Agastache rugosa* culture


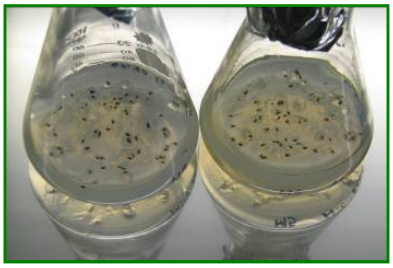


Fig. 1, 2, 3: Germination test

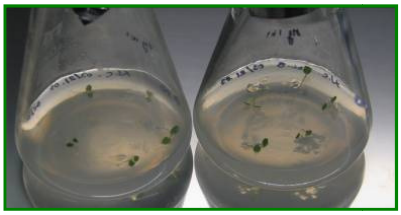

Fig. 4: Culture Initiation


Fig. 5, 6: Multiplication and growth

Fig. 7: Inducing risogenesis

Fig. 8: Accomodation to the septic medium

Phytochemical characterization

The analysis of the absolute methanolic extracts for the compounds with antioxidant action was made by TLC and HPLC showing the fact that the *A. rugosa* species has a raised potential for the synthesis of the polyphenolic type (chlorogenic acid, caffeic acid, rosmarinic acid and cinnamic acid). Along these, the rosmarinic acid is present in all the parts of the plant. As our study was made both on the plants obtained through generative propagation and on those obtained by *in vitro* multiplication, it was important for us to evaluate the accumulation of the rosmarinic acid from different plant parts (floriferous stems, stems and leaves), to highlight the vegetal material best suited to be used as raw material for food supplements. Our data show that at the level of the leaves there accumulates the highest quantity of polyphenolic compounds, and especially of rosmarinic acid (3275.5 mg/100g d.w.), followed by chlorogenic acid (231.8 mg/100g d.w.).

Due to the standards used by us and depending on the Rf, in the dichlormethanic extracts we identified β-sitosterol, oleanolic and ursolic acids, and the number of the fractions shown by TLC are variable, depending on the plant organ originating the extract obtained.

Table 2. Samples of *Agastache rugosa* phytochemically analyzed

Sample no.	Sample description		
1	*In vitro* multiplied plants	Flowering phenophase (July 2011)	floriferous stem / floriferous stem ear
2			Stem
3			Leaves
4		Fruiting phenophase (November 2011)	floriferous stems / floriferous stem ear
5			Stem
6			leaves / young shoots
9	Generative propagated plants	Blue flowers	floriferous stems / floriferous stem ear
10			Stem
11			Leaves
12		White flowers	Floriferous stems / floriferous ear
13			Stem
14			Leaves

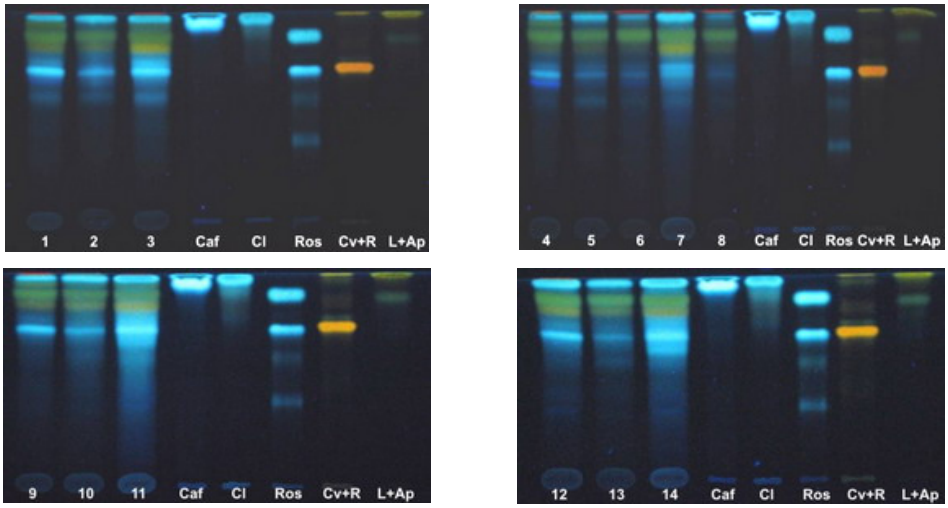

Fig. 9, 10, 11, 12: TLC for polyphenolcharboxylic acids and flavonoids (samples 1-6, 9-14)
Standards: **Caf =** Caffeic acid; **Cl.**= Chlorogenic acid; **Ros** = Rosmarinic acid; **C+R**= Quercetin + Rutin; **L+Ap** = Luteolin + Apigenin

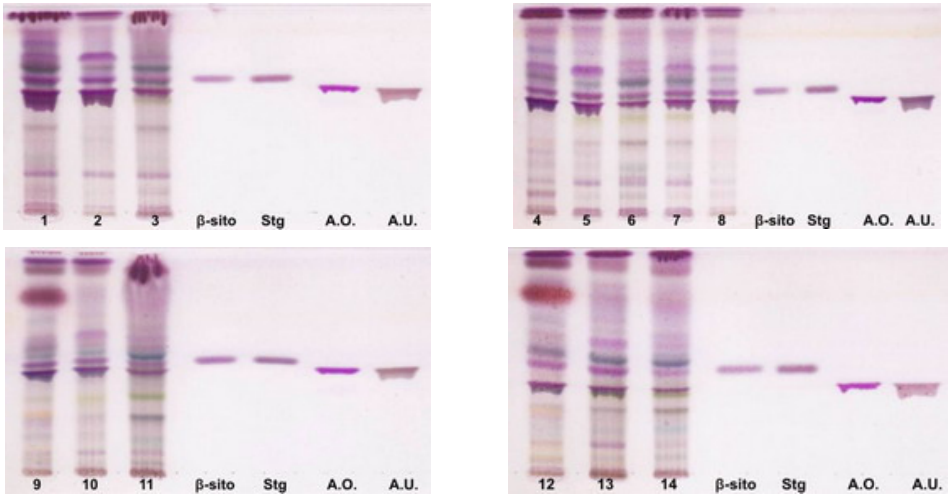

Fig. 13, 14, 15, 16: TLC for triterpenic acids and phytosteropls (samples 1-6, 9-14)
Standards: **β-sito** = β-sitosterol; **Stg** = Stigmasterol; **A.O.** = Oleanolic acid; **A.U.**=Ursolic acid;

Table 3. HPLC analysis of the absolute methanolic extracts for the polyphenolic compounds

Sample no	mg/100g dry vegetal material			
	Rosmarinic acid	*Cinnamic acid*	*Chlorogenic acid*	*Caffeic acid*
1	1297.1	714.3	185.3	5.1
2	190.3	101.6	9.1	0.0
3	2945.7	193.8	231.8	28.1
4	219.5	128.7	13.2	2.5
5	290.8	151.5	0.0	3.6
6	242.1	99.3	0.0	4.5
9	908.5	334.6	15.7	2.8
10	953.1	177.7	0.0	3.2
11	3221.2	324.8	66.8	4.7
12	1708.4	68.6	52.4	6.5
13	637.0	0.0	4.2	5.6
14	3275.5	106.7	57.7	3.5
Mind: <l.d.* = under the detection limit				

We analyzed the plant material collected in full flowering and fructification phenophase, because we wanted to evaluate the optimum harvest period in order to obtain a high content in polyphenols, which is important in using the plant material in food supplements or as seasoning.

The comparative evaluation of the biosynthetic capacity of the plants from the conventional culture and those from *in vitro* multiplication shows that the multiplied plants have the same biosynthetic model. The rosmarinic acid was the best represented compound, with the highest values at full flowering, the amount being comparable for the plants with blue flowers, respectively white flowers. Our result are in correlated those from literature where the variability of the rosmarinic acid content is mentioned as depending on the cultivated variant [10].

The analysis of the polyphenols for the studied variants aimed to advance the cultivation of *A. rugosa* species in Romania, especially as medicinal and aromatic plant; *A. rugosa* was taken in the collections of Vegetable Research and Development Station Bacau mainly as an ornaments species. In *A. rugosa* was also highlighted a content in flavonoids that have a variety of physiological and pharmacological activities on inflammatory immune responses [11].

The content in polyphenols, especially in rosmarinic acid gives the analyzed plant material an antioxidant potential with potential in the use of the species as medicinal and aromatic plant. Thus, further phytochemical studies will focus on volatile oil content for *A. rugosa* plants adapted to the climatic conditions of Romania.

CONCLUSIONS

The study shows the possibility of using *in vitro* multiplication in the propagation/conservation of the chemotypes/chemovarieties valuable from the point of view of the active principles content (antioxidant compounds), the plant multiplied *in vitro* preserving the biosynthetical potential. We intended an as complete as possible phytochemical analysis of the active principles characteristic to this species as, being an autochtonous species, the information regarding its chemical composition, after the adaptation to the pedo-climatic conditions of Romania, were not found.

With this species, the *in vitro* multiplication is a non-conventional way of multiplication/conservation of some chemotypes/chemovarieties with a raised biosynthetic potential.

ACKNOWLEDGEMENTS

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Original scientific paper

ESSENTIAL OIL POLYMORPHYSM IN *THYMUS PULEGIOIDES* L. FROM SERBIA

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SUMMARY

The genus *Thymus* L. is one of the most important genera regarding number of species within the Lamiaceae family. In the whole area of distribution, *Thymus* species are widely used as medicinal and aromatic plants. In this work we included seven populations of *Th. pulegioides*, species which is very common in flora of Serbia and widely used in traditional medicine. Dried leaves of each sample were examined by thermal desorption-gas chromatography-mass spectrometry. The essential oil compounds were identified by comparing retention indices (calculated against an *n*-alkane series) and by comparing mass spectra with published data. Three major groups of oil compounds were identified, such as monoterpene hydrocarbons, oxygenated monoterpene hydrocarbons and sesquiterpene hydrocarbons. In three populations the dominant compound was Geraniol with concentrations of 87.92%, 87,12% and 86.97%. Thymol was major compound in two populations (62.92% and 52.95%). In one population major compounds were Cymene and Thymol (48.59% and 22.12% respectively) and in one major compounds were Cymene and Carvacrol (42.59% and 39.95% respectively). In order to establish relationship between examined populations of *Th. pulegioides* five main compounds (in concentration higher than 10%) of essential oils were statistically analyzed through cluster analysis and PCA.

***Key words**: Thymus, Essential Oils, Chemotypes*

INTRODUCTION

Genus *Thymus* L. is one of the most important genera as regards number of species within the Lamiaceae family. *Thymus* belongs to tribe Mentheae, subfamily Nepetoideae. Although the number of species within this genus varies depending on taxonomical viewpoint, there is more than 200 species. *Thymus* is distributed throughout the arid, temperate and cold regions of the Old World north of the Equator and on the coasts of Greenland [1]. However the central area of this genus surrounds the Mediterranean Sea.

Thymus species are used as medicinal and aromatic plants, as well as in cosmetics and perfumery, throughout their range. Most aspects of their medicinal use are related to the essential oil which contains various levels of thymol and/or carvacrol, phenolic derivatives with strong and wide-spectrum of antimicrobial activity [2- 5]. Species such as *Thymus vulgaris* L., *Thymus zygis* Loefl. ex L. and *Thymus serpyllum* L. *sensu lato* are the biological sources of herbal drugs such as *Thymi herba, Thymi aetheroleum* and *Serpylli herba,* officially recognized in many modern pharmacopoeias e.g. European Pharmacopoeia 6.0 (2007).

Thymus pulegioides L. is a suberect to procumbent plant, woody at base, with gonotricgous stem. with wide distribution in Europe [6]. In Serbia it is widely distributed in various habitats [7].

The existence of different chemotypes within the same population of *Thymus* species is very well documented [8]. Estimation of chemical variability, besides scientific importance has great impact in assessment of the herbal drug quality.

MATERIALS AND METHOD

Plant material of seven populations of *Th. pulegioides* was collected from their natural habitats in Serbia. A list of the populations analyzed with latitude and longitude is given in Table 1. The plant taxa were identified according to Flora Europaea and Flora of Serbia. Voucher specimens have been deposited at the Herbarium of Department of Botany, Faculty of Agriculture, University of Belgrade.

Table 1. *Thymus pulegioides* populations included in the analyses

Population	Latitude (N)#	Longitude (E)	Altitude (m)
P01	43.84	21.68	592
P02	43.88	20.66	473
P03	43.45	21.47	648
P04	43.61	20.55	264
P06	44.17	21.12	877
P09	45.16	19.75	474

N - North; E - East; Coordinates are in degree decimal format.

GC-MS and TD-GC-MS Parameters:

The TD-GC-MS system consisted of an ATD400 thermal desorption unit, an AutoSystemXLGC, and a TurboMass quadrupole MS (Perkin-Elmer, Waltham, MA). Approximately 3 mg of plant material was held between glass wool in a glass tube insert and placed into a standard stainless steel desorption tube. The sample was desorbed at 150°C for 10 min in a flow of 60 mL/min helium that passed to a Tenax TA trap (80-100 mesh) held at 4 °C with no inlet split. Following desorption, the Tenax trap was heated ballistically to 300 °C under a helium pressure of 15 psi and with an outlet split flow of 18.75 mL/min. The volatile components passed through a deactivated glass capillary transfer line at 200 °C onto a 30 m x 0.25 mm i.d. x 0.25 μm DB-5MS capillary GC column (Agilent J&W, Santa Clara, CA), and chromatography proceeded using an oven temperature program of 60-300 at 6 °C/min under the pressure from the ATD. The MS was fitted with an EI source operated at 70 eV with a source temperature of 180 °C, and mass spectra were recorded in the range m/z 38-600. The operating software was Turbomass version 4.1.1. Retention indices (RI) were determined in relation to a series of n-alkanes (C8-C20, Supelco, United Kingdom), and peak integration was performed to RI 1900 (i.e., prior to the elution of palmitic acid).Compounds were identified by comparing RI and/or mass spectra with published data [9, 10].

Statistical Analisys

The five main compounds (in concentration higher than 10%) were chosen for further analyses of chemotypes identification: geraniol, thymol, carvacrol, p-cymene and γ-terpinene

Correlations. The relationships among five main essential-oil compounds were assessed by Pearson's correlation coefficient using STATISTICA (data analysis software system), version 8.0. (StatSoft, Inc. 2007).

Principal-Components Analysis (PCA). The PCA based on five main essential-oil compounds was performed using STATISTICA (data analysis software system), version 8.0.

(StatSoft, Inc. 2007). The biplot was constructed by two principal components showing populations and essential-oil compounds (as vectors).

Cluster Analysis (CA). The standardized scores of the two principal components were multiplied by the root of their eigenvalues and the Euclidean distance matrix between all pairs of populations was calculated to be used in cluster analysis (CA). The Average linkage method (i.e. UPGMA) was applied in order to determine the optimal number of clusters.

RESULTS AND DISCUSSION

In essential oils of seven populations of *Th. pulegioides*, growing wild in Serbia, a total of 67 compounds was recognized (Table 2). Three major groups of compounds were present, monoterpene hydrocarbons (16 compounds), oxygenated monoterpene hydrocarbon derivatives (13 compounds) and sesquiterpene hydrocarbons (26 compounds). 12 compounds didn't belong to any of major groups. In essential oil of population P02 41compound was identified, in P01 and P05 39 compounds, in population P06 38 compounds, in population P03 32 compounds , in population P04 30 compounds and in population P09 26 compounds.

Table 2. Essential oil composition in populations of *Th. pulegioides* (MH – monoterpene hydrocarbon; OMHD – oxygenated monoterpene hydrocarbon; R – rest; SH - Sesquiterpene hydrocarbons)

Compound	KI	Type	**P01**	**P02**	**P03**	**P04**	**P05**	**P06**	**P09**
Tricyclene	927	MH	-	0.02	0.02	-	-	-	-
α-Thujene	930	MH	0.01	0.83	0.01	0.73	0.75	-	1.15
α-Pinene	939	MH	0.02	0.73	0.08	0.40	0.40	0.01	0.60
Camphene	954	MH	0.03	0.76	0.15	0.04	0.05	0.01	0.25
Sabinene	975	MH	-	0.02	-	0.02	0.02	-	-
β-Pinene	979	MH	-	0.11	0.01	0.09	0.09	-	0.14
Myrcene	991	MH	0.45	1.14	0.50	0.09	0.49	0.42	0.01
α-Phellandrene	1003	MH	0.03	0.19	-	0.04	0.09	0.04	-
α-Terpinene	1017	MH	0.02	1.45	0.02	0.51	0.73	0.02	0.10
Limonene	1029	MH	0.08	0.17	0.09	-	-	0.06	0.12
β-Phellandrene	1030	MH	-	0.08	-	-	-	-	-
Sylvestrene	1031	MH	-	-	-	-	0.14	-	-
(*Z*)-β-Ocimene	1037	MH	0.11	0.04	0.12	0.01	0.03	0.10	-
(*E*)-β-Ocimene	1050	MH	0.17	0.03	0.17	-	0.01	0.16	-
γ-Terpinene	1060	MH	0.02	21.39	0.01	1.51	3.71	0.03	0.27
Terpinolene	1089	MH	0.02	0.04	0.03	0.02	0.04	0.02	-
(*Z*)-Sabinene hydrate	1067	OMHD	-	-	-	-	-	-	0.11
Linalool	1097	OMHD	0.12	0.05	-	-	-	0.11	0.21
α-Pinene oxide	1099	OMHD	-	0.01	0.10	0.03	0.11	0.07	0.05
Camphor	1146	OMHD	-	-	0.01	-	-	-	-
β-Pinene oxide	1159	OMHD	0.02	-	-	-	-	-	-
Borneol	1169	OMHD	0.06	1.57	0.37	0.03	0.04	0.01	0.61
3-Thujanol	1169	OMHD	-	-	0.01	0.09	0.04	-	-
trans-Dihydro carvone	1209	OMHD	-	-	-	-	0.01	-	-
Thymoquinone	1252	OMHD	-	-	-	0.11	3.09	-	5.49

Compound	KI	Type	**P01**	**P02**	**P03**	**P04**	**P05**	**P06**	**P09**
Geraniol	1253	OMHD	87.12	-	87.92	-	-	86.97	-
Bornyl acetate	1289	OMHD	-	0.02	0.02	-	-	-	0.08
Methyl geranate	1325	OMHD	-	-	0.18	-	-	-	-
Geranyl acetate	1381	OMHD	-	-	-	-	-	0.31	-
Caryophyllene oxide	1583	R	0.02	0.02	-	0.44	0.08	-	-
p-Cymene	1025	R	-	4.89	-	42.59	17.36	0.01	48.59
m-Cymenene	1085	R	-	-	-	-	-	-	0.04
p-Cymenene	1091	R	-	-	-	-	0.03	-	-
3-Octanone	984	R	0.05	0.12	0.06	0.01	0.10	0.10	6.14
Thymol	1290	R	0.05	52.95	-	0.05	62.82	0.07	22.12
Carvacrol	1299	R	-	0.14	-	39.95	0.61	-	0.04
Thymohydroquinone	1555	R	-	-	-	2.72	0.46	-	-
Thymol methyl ether	1235	R	-	2.75	-	0.91	0.91	-	4.05
Carvacrol methyl ether	1245	R	-	1.14	-	-	0.93	0.03	2.68
3-Octanol	991	R	0.35	0.04	0.11	-	0.03	1.10	0.63
Oct-1-en-3-ol	979	R	0.39	1.19	0.26	0.20	1.14	0.05	0.95
α-Cubebene	1351	SH	0.01	-	-	-	-	0.03	-
α-Copaene	1377	SH	0.02	0.01	-	-	-	0.01	-
β-Bourbonene	1388	SH	-	-	0.03	-	-	-	0.09
α-Bourbonene	1390	SH	0.14	0.02	-	0.02	0.01	0.15	-
β-Elemene	1391	SH	0.01	-	-	-	-	-	-
(*E*)-Caryophyllene	1419	SH	2.71	0.86	1.87	6.00	0.66	0.19	0.19
β-Copaene	1432	SH	0.03	0.01	-	0.01	-	0.02	-
trans-α Bergamotene	1435	SH	0.02	0.02	0.03	-	0.01	0.03	-
Aromadendrene	1441	SH	-	-	-	-	-	0.02	-
trans-β-Farnesene	1443	SH	-	-	-	-	0.16	0.01	-
trans-Muurola-3,5 diene	1454	SH	0.01	-	0.01	-	-	-	-
α-Humulene	1455	SH	0.27	0.04	-	0.14	0.04	-	-
Alloaromadendrene	1460	SH	0.01	-	0.08	-	-	0.05	-
γ -Muurolene	1480	SH	0.04	0.06	-	-	-	-	-
α-Amorphene	1485	SH	-	0.01	-	0.01	-	-	-
Germacrene-D	1485	SH	1.69	0.02	0.08	0.02	-	0.55	-
γ-Amorphene	1496	SH	-	-	-	-	-	0.02	-
Bicyclogermacrene	1500	SH	0.01	-	0.10	-	0.01	0.46	-
α-Muurolene	1500	SH	0.02	0.01	-	-	0.01	-	-
β-Bisabolene	1506	SH	3.40	6.47	4.24	0.08	3.70	7.28	3.41
δ-Amorphene	1512	SH	-	-	-	-	-	0.02	-
γ-Cadinene	1514	SH	0.02	-	-	-	-	-	-
β-Sesquiphellandrene	1523	SH	0.05	-	0.06	-	0.04	0.07	-
trans-Cadina-1,4 diene	1535	SH	0.01	-	-	-	-	-	-
α-Cadinene	1539	SH	0.02	-	-	-	-	-	-
β-Atlantol	1608	SH	-	-	-	-	0.01	0.02	-
Procenat determinacije			97.62	99.43	96.75	96.84	98.97	98.63	98.13

Monoterpene hydrocarbons: 16 different monoterpene hydrocarbons were determined and their percentage ranged from 0,86%, in population P06, to 27,01%, in population P02. In essential oil of population P01 the percentage of monoterpene hydrocarbons was 0,95%, in population P04 it was 3,44%, in population P03 1,21%, in population P09 2,64% and in population P05 it was 6,56%. In essential oil of population P02 only γ-terpinen, from this group of compounds, had significant concentration of 21,39%.

Oxygenated monoterpene hydrocarbons: In total 13 different oxygenated monoterpene hydrocarbon derivatives were determined and their percentage ranged from 0,27%, in population P04 to 88,60% in P03. This was, as well, the dominant group of compounds in essential oil of populations P06 (87,47%) i P01 (87,32%). In these three populations the major compound of essential oils was geraniol with concentrations of 87,92% in population P03, 87,12% in population P01 and 86,97% in population P06. In other four populations none of the compounds from this group was present in sikgnifikcant percentage.

Sesquiterpene hydrocarbons: 26 compounds from this group were determined, and none of compounds from this group wasn't present in significant percentage. In total their percentage ranged from 3,69% in population P09 to 8,93% in P06.

Rest: In the essential oils included in this study 12 compounds didn't belong to any of the major groups. Among these, two phenolic compounds, thymol and carvacrol were present in high percentages, as well aromatic hydrocabon *p*-cymene. In the essential oils of P02 and P05 thymol was the major compound with concetrations of 52,95% and 62,92% respectively. In population P09 thymol had significant percentage of 22,12%, while *p*-cymene was the major compound with 48,59%. *p*-Cymene was the major compound in the essential oil of population P04 with concentration of 42,59%, while carvacrol was als present in high percentage of 39,95%.

Pearson's correlation coefficients among the five main essential oil compounds showed that concentration of any of the main compounds is not in significant (P>0.5) correlation.

Figure 1. Biplot of the principal-components analysis based on the five main essential-oil compounds of *Thymus pulegioides*

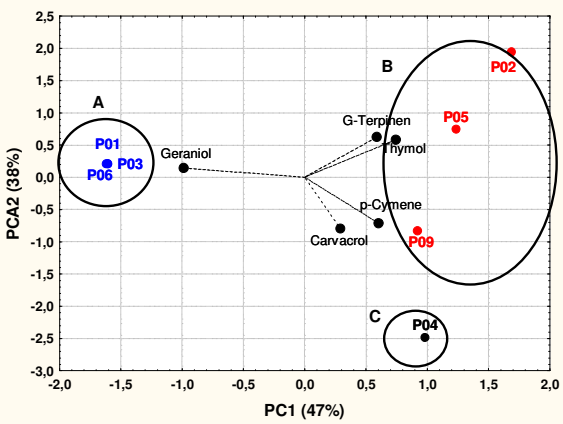
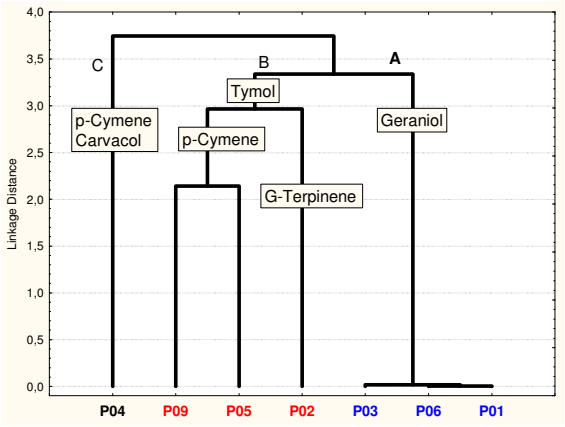


Figure 2. UPGMA dendrogram of cluster analysis of *Th. pulegioides* populations



Analysis of main components (PCA) revealed that first two components had eigenvalues higher than one, and they were responsible for 84.23% of total variability. PCA diagram (Fig 1) on the basis of five main compounds and correlations among five main compounds and first two components apparently show that: first main component is separating populations with high geraniol from other populations; second component is separating populations rich with thymol and γ-terpinene from populations rich in p-cymene and carvacrol. Euclidian

distance between populations was calculated using eigenvectors of first two components. Average Euclidian distance was 2.89, in the range between 0.001 (P01/P06) to 4.51 (P02/P04). Separation of populations into three clusters is presented on UPGMA (Unweighted pair-group method using arithmetic averages) dendrogram (Fig 2) where are: cluster A with three geraniol populations (P01, P03 and P06), cluster B with three thymol populations (P02, P05 and P09) and C with one population (P04) rich in carvacrol.

The existence of chemotypes in wild populations of *Thymus* taxa is well known. For example, in populations of *Th. vulgaris* from southern France it is established existence of six chemotiypes. They are named after dominant monoterpen in the essential oil as follows: Geraniol, Linalool, α-terpineol, Thuyanol-4, Carvacrol and Thymol [11, 12].

In populations of *Th. pulegioides*, included in this study, two well defined chemotypes can be identified. In populations P01, P03 and P06 geraniol was present in high percentage, 87.12%, 87.92% and 86.97%, respectively. Geraniol as a major compound in the essential oil of some *Th. pulegioides* populations was described previously [13-15].

In essential oils of populations P02, P04, P05 and P09 there is a high concentration of *p*-cymene, γ-terpinene, carvacrol and thymol, terpenes that are closely connected by biogenetical processes. These compounds always occur in essential oil simultaneously, since *p*-cymene and γ-terpinene are the precursors in the biochemical pathway of the phenols [16]. Total content of those compounds ranged from 71.02%, in population P09, to 84.50%, in population P05, in P02 it was 79.37% and in P04 it was 84.09%, so these populations can be treated as phenolic chemotypes. Phenolic terpenes thymmol and carvacrol are most important constituents of the essential oil considering their abundance in *Thymus* taxa [16]. Many of previously investigated *Th. pulegioides* populations belong to phenolic group. In essential oils of *Th. pulegioides* from Lithuania, both thymol and carvacrol chemotype were described [17]. Thymol and carvacrol chemotypes were described in populations from British Isles [18], as well in populations from Croatia [19].

CONCLUSION

Essential oils containing large amounts of phenolic compounds have been shown to possess high antioxidant activity [20-22], and our further research will therefore be focused on biological activity of the essential oil of *Th. pulegioides* as well as analysis of flavonoids, since they have been reported to be an important taxonomic character of the tribe Menthae [23].

ACKNOWLEDGEMENTS

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Original scientific paper

CONTENT AND COMPOSITION OF ANTHOCYANINS IN BLUEBERRY, VACCINIUM CORYMBOSUM

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SUMMARY

The fruits of blueberries are rich in anthocyanins. In those plant species there are six most important anthocyanidines: cyanidine, delfinidine, malvidine, pelargonidine, peonidine and petunidine. Anthocyanins are heteroglycosides, which are composed of sugar and aglycone – anthocyanidine. They have hydroxyl group position on C-3. Pelargonidine has sugar -3-O-arabinoside and -3-O-xyloside. The five other anthocyanidines have -3-O-arabinoside, -3-O-glucoside and -3-O-galactoside. The blueberry has 15 anthocyanins in the fruits. Total anthocyanins content depending on variety, ecological conditions, standard of agricultural technology, and particularly on the temperature and solar radiation. We were evaluated variety Berkeley from experimental field Regional Research Station Krivá located in Orava, Slovakia. In our study was found 0.62 % content of anthocyanins in fresh blueberry fruits. Literature sources indicate 0.300 – 0.698 % content of this compounds. Content of selected anthocyanins reached these amounts: cyanidine-3-O-glucoside 33.772 $mg.kg^{-1}$, cyanidine-3-O-galactoside 64.465 $mg.kg^{-1}$, malvidine-3-O-glucoside 36.052 $mg.kg^{-1}$, and malvidine-3-O-galactoside 230.037 $mg.kg^{-1}$.

Key words: *anthocyanidines, anthocyanins, blueberry (Vaccinium corymbosum), content, ecological conditions*

INTRODUCTION

Vaccinium L. (*Ericaceae*) is a morphologically diverse genus of terrestrial or epiphytic shrubs and lianas, comprising approximately 450 species. Blueberry (*Vaccinium corymbosum* L.) is one important species of this genus. The fruits have positive effect on the human and they belong to the healthiest type of fruits. Genus *Vaccinium* L. is the best known for the production of bioactive anthocyanins and flavonoids. More than 116 anthocyanins and flavonoids compounds have been isolated and identified primarily from the fruits and leaves of *Vaccinium* (Zushang Su, 2012). These bioactive substances are important source for food and pharmaceutical ingredients coupled with their high antioxidant potential. Above all anthocyanins are important plant pigments visible to the human eye.

The aim of our research will be the mixture of pure anthocyanins which can be used as food supplements. Based of this goal we tried to find the most optimal methods for the extraction anthocyanidins without their demages or degradation and their identification. Among the few different methods we choosed one described below which is the most suitable to our future goals.

MATERIAL AND METHOD

Plant material

Plant material was obtained from experimental planting Centre of Agriculture Research Piestany - Research Station Kriva in Orava in Slovak Republic. The locality was situated in North part of Slovakia, altitude 700 m up to see level, average temperature 6°C during the year and precipitation 800 - 900 mm. The blueberry fruits where harvesting on august 2011. For our experiment there were use the fruits from variety Berkeley.

Chemicals and analysis

The analytical procedure includes five successive steps: homogenizing of frozen fruits, extraction, concentration, qualitative and quantitative analysis. Homogenization was carried out on partially melted fruits. Fruits of homogenized samples were extracted with 70 vol. % methanol, 29 vol. % water and 1 vol. % formic acid to achieve the low pH of the solution and the stability of extract. The extraction mixture was cooled to -10 ° C. Concentrating the extract was carried out in stages on a rotary evaporator. Evaporation started at a pressure of 100 to 150 mbar. Then the pressure was reduced to 50 mbar. The final residual solvent evaporation was carried out at a pressure of 25 mbar to a final volume of extract.

Amount identification of anthocyanins components were determined by liquid chromatography on reverse phase in conjunction with mass detection. There were used technology of hybrid mass spectrometer consisting of an ion trap (IT) mass analyzer and time-range particles (TOF), which provides a highly differentiated mass spectra to determination the composition and content of anthocyanins. It is also possible to do the fragmentation experiments (MS^n, n = 1-10), which is essential for the structural analysis of unknown substances. Measured exact molecular weight and fragmentation experiments (MS^n), resulting in the calculation of the summary formula discovered chemical compound.

There was developed gradient chromatographic method of reversed-phase using an external standard for the quantitative determination. Commercially obtained pure compounds (98% HPLC purity) of selected anthocyanins were used to prepare standard in three concentration levels. Mixed standards included 13.34 $mg.l^{-1}$, 95.20 $mg.l^{-1}$ and 667.00 $mg.l^{-1}$ cyanidin-3-O-galaktoside, cyanidin-3-O-glucoside, malvidin-3-O-galaktoside, malvidin-3-O-glucoside. Gradient elution was optimized for the separation of the two isobaric pairs of cyanidin-3-O-galaktoside, cyanidin-3-O-glucoside and malvidin-3-O-galaktoside, malvidin-3-O-glucoside. As the mobile phase used was 0.12 $mol.dm^{-3}$ aqueous solution of formic acid (pH = 2.35) and acetonitrile at a flow rate 0.3$ml.min^{-1}$ and a temperature of 40°C.

RESULTS AND DISCUSSION

Fruit extraction was done in triplicate. In the first extraction there was achieved 59.6% efficiency. Second time was 26.9% and a third extraction reached 13.5% amount of anthocyanins. Total content of anthocyanins from the fresh fruits was 0.62%. The available literature sources say 0.300 to 0.698% of the total content of biologically active substances. In the blueberry fruit (*Vaccinium corymbosum* L.) was identified delphinidin-3-O-galactoside, delphinidin-3-O-glucoside, delphinidin-3-O-arabinoside, cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, petunidin-3-O-galactoside, petunidin-3-O-glucoside, cyanidin-3-O-arabinoside, petunidin-3-O-arabinoside, peonidin-3-O-galactoside, malvidin-3-O-galactoside, malvidin-3-O-glucoside, malvidin-3-O-arabinoside. It was detected also the presence of delphinidin + 3-hexose. The results correspond with findings reported in the literature. Zushang Su (2012) presents the contents of 15 anthocyanins in this plant species. These findings corresponded with our results. They also found the presence of peonidin-3-O-arabinoside and peonidin-3-O-glucoside, which were detected by our experiments. Similar composition anthocyanins declare other literature sources (Kader et al., 1996; Scibisz and

Mitek, 2006, Faria et al., 2010; Spela et al., 2011). Quantitative determination gave the following results: cyanidine-3-O-glucoside 33.772 $mg.kg^{-1}$, cyanidine-3-O-galactoside 64.465 $mg.kg^{-1}$, malvidine-3-O-glucoside 36.052 $mg.kg^{-1}$, and malvidine-3-O-galactoside 230.037 $mg.kg^{-1}$ (tab. 1).

Table 1: Total content of selected anthocyanins in blueberry fruits, variety Berkeley

Samples	Cyanidin-3-O-galaktoside	Cyanidin-3-O-glucosside	Malvidin-3-O-galaktoside	Malvidin-3-O-glucoside
VC2 (mg/kg)	63.55	39.00	200.13	37.79
VC3 (mg/kg)	61.15	36.38	210.64	38.10
VC4 (mg/kg)	61.70	30.92	227.05	32.99
VC5 (mg/kg)	71.46	28.74	282.33	35.33
X (mg/kg)	**64.465**	**33.772**	**230.037**	**36.052**
S	**2.75**	**4.73**	**36.90**	**2.39**
RSD (%)	**4.26**	**14.00**	**16.04**	**6.62**

*VC2 – VC5 – samples of Vaccinium corymbosum L., X-average, S - standard deviation, RSD – relative standard deviation

Statistical set of 4 samples does not allow a correct interpretation of the results obtained, but shows some trends. The relative standard deviation (RSD) ranged from 4.26 to 16.04 %. High levels were found in cyanidine-3-O-glucoside and malvidine-3-O-galactoside. The present results arising from the method of homogenization respectively of small number of analyzed samples, which may be limited by LC / MS quantitative method.

CONCLUSION

The study of anthocyanins content in selected plant species was focused on amount and its composition within research project *"Isolation of Natural Plant Substances by Lyophilisation and Change of their Qualitative and Quantitative properties"*.

Partial results of the work are described above by the methods and analysis optimalized for the aims of the main research project.

In next phase of our research we will use developed methods for purpose of anthocyanins extraction, evaluation of their quantitative and qualitative characteristics before and after lyophilisation, while in parallel evaluation of their biological and microbiological characteristics. As a plant material will be used more varieties and samples of fruits of blueberry (*Vaccinium corymbosum* L.) for statistical analysis with significant values.

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Original scientific paper

THE COMPARATIVE TLC ANALYSIS OF THEETHANOLIC EXTRACT OF *BIDENS TRIPARTITAE HERBA* COLECTED IN ROMANIA AND SOME TRADE PRODUCTS

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SUMMARY

In Romania the *Bidenstripartita L.* grows spontaneously, forming significant populations. This is very common in moist places, along rivers. Unfortunately in our country the *Bidenstripartita* isn't studied and used, is practically unknown. For this reason the purpose of this study was to identify the plant, the global chemical analysis for the identification of the main groups of compounds and the TLC analysis for comparison with some commercial products.

Two samples of *B.tripartita* from Romania collected in budding and flowering phase were analyzed. For comparison we used three commercial products representing *Bidenstripartitaeherba* tea from different geographical areas, one from Republic of Moldova and two from Russia (Moscow Region and Republic of Karaceaevo-Cerkesia).

Samples from Iasi fits very well in the global image of the product*Bidenstripartitaeherba*.

Among the analyzed samples were noted differences that we suppose to be due to differences in the habitat of the plants.

***Keywords*:** *medicinal plants, Bidenstripartita L.,phytochemical composition, TLC, flavones,*

INTRODUCTION

Bidenstripartita L. (family *Asteraceae*) is an erect annual plant, growing 15 to 100 cm high. The stem is erect, heavily branched, and often brownish-red. The leaves are opposite, 3 to 5 lobed, and narrow to a short, winged petiole. The flower heads are usually solitary, erect or inclined, with brownish-yellow petals. Fruit,glabrous achene, distinctly compressed, with 2, sometimes 3–4 (2 longer), thorns ascending on the angles [1,2,3,4,5,6].

The usual names are Burr marigold and Water agrimony.

*Bidenstripartita*is a species found throughout the world wide. It is found in North America, Europe, Asia and Australia. The areal stretches from temperate areas to the Polar Circle. This plant is common for wet places [1,2].

The dried aerial parts contains: flavones (luteolin, cynaroside), tannins, polyacetylenes, coumarins (umbelliferone, scopoletin and aesculetin), 0.05-0.11% essential oil (eugenol, ocimene and cosmene), 4.51-4.65% saccharides (arabinose, galactose, glucose, rhamnose and xylose), organic acids, carotene, vitamin C and microelements (manganese) [1,3,4,7,8].

The plant is known and used since ancient times, especially for treatment of dermal diseases and wound healing [2]. In the popular medicine it is orally administered as a diuretic, diaphoretic, febrifuge, antidiarrhoeal, antiallergic, anti-inflammatory, anthelminthic, choleretic and as a kidney tonic [1,5,6,9].

As a result of pharmacological tests or clinical studies, the following actions were confirmed: sudorific,diuretic, astringent, antimalarial, choleretic, antiulcer, photoprotective, anti-inflammatory, antioxidant, hepatoprotective, emenagoga and sedative action. Have been

reported good results in the treating of chronic dysentery, acute and chronic enteritis, diarrhea, ulcerative colitis, bladder and kidney problems and some forms of cancer [1,8].
The plant is often associated with other plants [10]. By far, the most famous combination is Averin's tea [2,6].
Even if its use in medicine is very old, there are many unsolved issues. No consensus regarding the optimum time for collection is established. Some authors indicate the period of flowering, others budding and/or flowering [1,3], or just budding [5,6,9]. Some of them admit the later collection, but only the side branches, with flowers buds [11], others are very strict about insisting that the product collected after the beginning of flowering not possesses curative properties [4]. The pickers from Moldova, which we managed to speak, testified that the product was collected during flowering. This was evident by the aspect of plant product which they sell.
In Romania the plant grows spontaneously and forms significant populations. It is very common in moist places, on rivers and streams. But unfortunately in our country is practically unknown. It is not enough studied and used.
For this reason we decided to collect native plant (during budding and flowering) to analyze and compare the results with literature data and vegetable products from the pharmaceutical trade of countries where it is used in therapeutic.

MATERIAL AND METHODS

The vegetal product was collected in Iasi in the summer of the year 2011. The plant was identified after macro- and microscopic characters [1,3]. The microscopic analysis was performed with a microscope Celestron digital LCD Model #44340. The drying was performed in our laboratory and, after that the vegetal product was subjected to successive extractions with solvents of different polarities (chloroform, methanol and water). The obtained extracts were analyzed using specific reagents [12]. To have a better view on the chemical composition of the collected plant product we carried out a comparative analysis by TLC. We prepared a 1:10 ethanolic extract for each analyzed sample. The extracts were subjected to chromatographic separation. Two samples of *Bidenstripartita* from Romania collected in budding and flowering phase were analyzed. For comparison we used three commercial products representing *Bidenstripartitaeherba* tea from different geographical areas (one from Republic of Moldova and two from Russia – Moscow Region and Republic of Karaceaevo-Cerkesia) and the results were compared.
The samples were noted as follows:

1. *B. tripartita* from Republic of Moldova (Depofarm)
2. *B. tripartita* from Moscow Region (Zdorov'e)
3. *B. tripartita*from Republic of Karaceaevo-Cerkesia (Fito-Bot)
4. *B. tripartita*from Iasi collected during budding
5. *B. tripartita* from Iasi collected during flowering

From each sample on the chromatographic plate were applied 10 µlfrom the extractive solution. For saponins and volatile oils the analyzed volume is 20 µl. The working conditions are:

For coumarins:

- stationary phase - Silica gel G F254-precoated TLC plates (Merck, Germany);
- mobile phase: toluene / ether (1 / 1, v / v), saturated with 10% acetic acid;
- detection: UV (365 nm) before and after spraying the plate with 10% ethanolic solution of KOH[13].

For flavones:

- stationary phase - Silica gel G F254-precoated TLC plates (Merck, Germany);

- mobile phase: ethyl acetate / formic acid / glacial acetic acid / water (100 / 11 / 11 / 26, v/v/v/v);
- detection: UV (365 nm) before and after spraying the plate with natural product reagent (1% methanolicdiphenylboric acid-β-ethylamino ester) [14].

For anthracene derivatives:

- stationary phase - Silica gel G F254-precoated TLC plates (Merck, Germany);
- mobile phase: ethyl acetate / methanol / water (100 / 13.5 / 10, v/v/v);
- detection: UV (365 nm) before and after spraying the plate with 10% ethanolic solution of KOH[15].

For polyacetylenes:

- stationary phase - Silica gel G F254-precoated TLC plates (Merck, Germany);
- mobile phase: n-hexane / ethylacetate / acetic acid (85 / 25 / 5, v/v/v);
- detection: UV (365 nm) examination [8].

For tannins:

- stationary phase - Silica gel G F254-precoated TLC plates (Merck, Germany);
- mobile phase: n-buthanol / glacial acetic acid / water (40 / 12 / 28, v/v/v);
- detection: spaying with 1% vanillin in concentrated HCl [16].

For saponins:

- stationary phase - Silica gel G -precoated TLC plates (Merck, Germany);
- mobile phase: chloroform / methanol / water (61 / 32 / 7, v/v/v), [16];
- detection: visual examination of the plate, after spraying with anisaldehyde-sulphuric acid reagent, and heated at 100°C for 5-10 min [15].

For essential oil compounds:

- stationary phase - Silica gel G -precoated TLC plates (Merck, Germany);
- mobile phase: hexane / ethyl acetate (9 / 1, v/v);
- detection: visual examination of the plate, after spraying with anisaldehyde-sulphuric acid reagent, and heated at 100°C for 5-10 min [15].

RESULTS AND DISCUSSION

The main microscopic characteristics are shown in Fig 1.

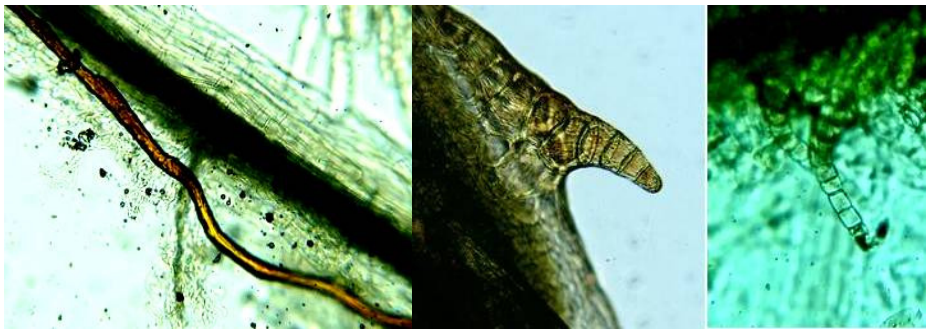


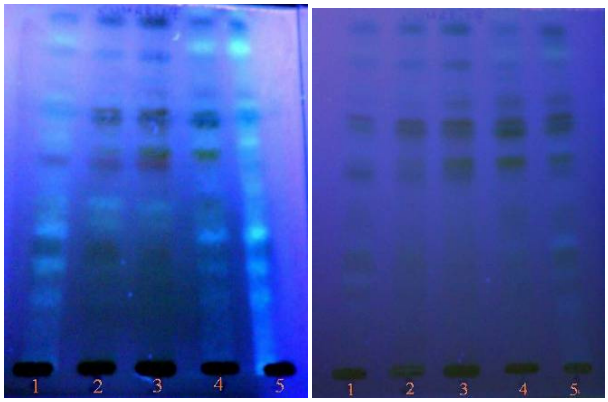
Figure 1. The main microscopic diagnostic characters of leaf of *Bidenstripartitae L.* Secretion ducts filled with dark red liquid (a), Simple hairs with thick cell walls (b), Simple hairs with thin cell walls

The compounds found after the identification reactions are presented in Table 1.

Table 1. Active compounds identified in *B. tripartitaeherba* collected in Iasi

Phase	Identified compounds
Chlorophormic	volatile oils, triterpenes, resin acids, flavones, anthracene derivatives
Ethanolic	tanins, saccharides, anthracene derivatives,triterpenes,flavones,coumarins, antocianosides
Aqueous	anthracene derivatives,antocianosides,flavonosids,coumarins,saponosids,reducing compounds,tanins,proteic compounds, polysacharides

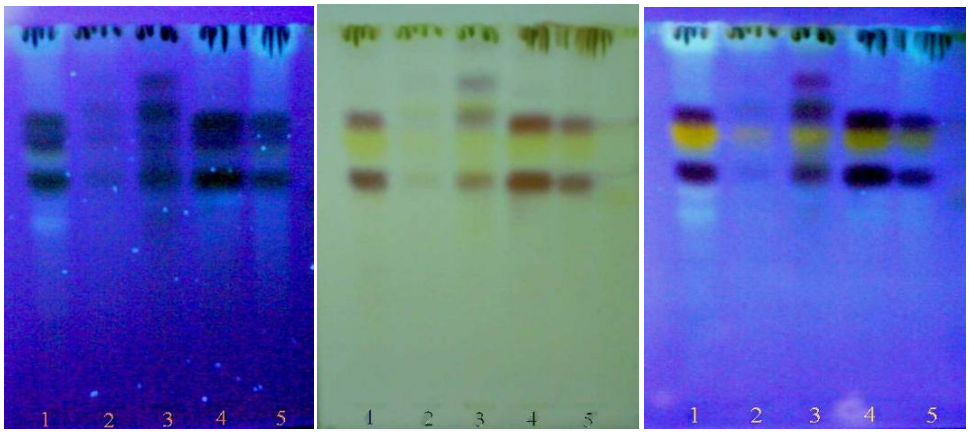
In the chromatograms obtained forcoumarins (Fig. 2) it can notice a slight resemblance to all samples (for nonspraying plate). Most of the spots were appeared after the spraying.A greater similarity is found for samples 1 and 5. Sample 4 have some compounds that are found in sample 1 and others in sample 3.



a b

Figure 2. The chromatogram for coumarins. a). before spraying. b) after spraying

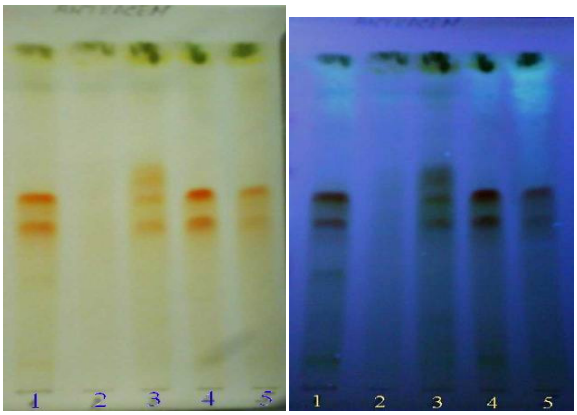
In the chromatogram obtained for flavonoids (Fig.3.) the presence of the same spotswas observed. Stands out the spot from the top of the plate at sample 3 (R_f= 0.84), not found in other samples. You may notice a small trace amount of flavones in sample 2 and a clearly higher concentration in the sample 4.



a b c

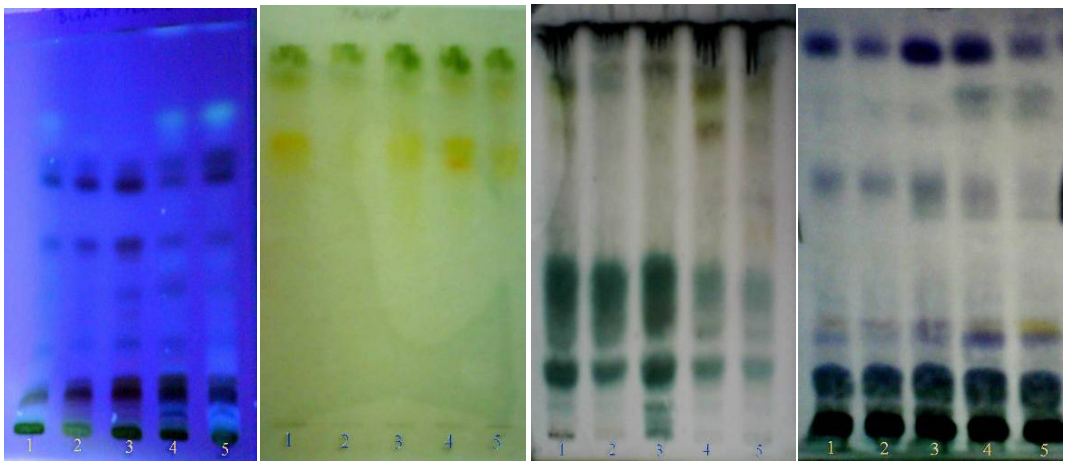
Figure 3. The chromatogram for flavones. a) before spraying-UV; b) after spraying-VIS; c) after spraying-UV.

After the chromatographic analysis for anthracene derivatives (Fig. 4) the similarity is observed for all samples (except sample 2). The sample 2 does not show any spot. In the sample 3 is a spot (R_f=0.69) not found in other samples.



a b

Figure 4. The chromatogram for the anthracenederivatives.a) after spraying-VIS; b) after spraying-UV



a b c d

Figure 5. The chromatograms for polyacetylenes- UV (a), tannins (b), saponins (c) and volatile oils (d).

In the chromatograms presented in Fig. 5.stands the differences in content of polyacetylenes (Fig.5.a) both qualitative and quantitative. The tannins (Fig.5.b) show only quantitative differences. The sample 2 is noticeably poor. The samples 4 and 5 are low in saponins (Fig.5.c). The samples 3 and 4 are richer in volatile oils (Fig.5.d). Smaller quantities of volatile oil from samples 1 and 2 would be due to the form of presentation (tea-bags); in the sample 5, probably due later collection (flowering stage).
The results showed the *Bidensherba* vegetal product collect from Romania, generally speaking, don't present major qualitative different comparing with trade vegetal products.
The differences between vegetal products collected in budding and flowering phase are more quantitative. Major differences present plant product derived from Moscow Region, Russia.
Given the assumption that all products marketed were collected and processed properly it can be concluded that the differences in chemical composition due to the climatic conditions of the habitats of plants. Plant product collected by us is much closer to the sample derived from Moldova (especially the one collected in budding phase); even if sometimes occur in common with that of Karaceaevo-Cerkesia.

CONCLUSION

The similarities of the preliminary results obtained in this study allow us to say that the *B.tripartitaeherba* collected in Iasi has a similar chemical composition like the commercial products we have found. So, this plant product can be used as a therapeutic for the same indication as the commercial ones.We suppose that the differences between the analyzed samples are due to climatic conditions of plants habitats. This encourages us to continue further studies.

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Original scientific paper

ESTIMATION OF THE PROTEIN QUALITY OF SOME MACEDONIAN EDIBLE TRICHOLOMATACEAE MUSHROOMS

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SUMMARY

The nutritional quality of mushroom protein varies and is strongly affected by the relative proportion of each amino acid. Thus, the purpose of this study was to estimate the concentration of the amino acids present in mushroom proteins in order to evaluate the protein nutritional value. In this investigation four field – collected mushroom samples of the *Tricholomataceae,* family from various parts of Macedonia were included.. After acid hydrolysis and pre – column derivatisation with phenyl isothiocyanate (PITC) determination of seventeen amino acids was carried out by the HPLC method. Tryptophan was determined spectrophotometrically in the alkaline hydrolisates. The dietary protein quality of the investigated mushrooms was evaluated by comparison of the essential amino acid content with the reference FAO/WHO pattern. Essential amino acids made up 45-74 % of all determined amino acids depending on the origin and the species of the fruit body. Lysine was the most often found limiting amino acid in the investigated mushrooms samples. The nutritional value of proteins calculated by biological value, protein ratio, chemical score and essential amino acid index was very high in the majority of mushrooms studied. The biological value of the mushroom protein varied from 54.01 to 64.52 %. Protein amino acids accounted for about 67.6 % of the total nitrogen, suggesting that practical nitrogen to protein conversion factor for this Macedonian edible mushroom may be considered to be about 4.23 on average. Upon to the analysis and the obtained results of edible mushroom samples of the *Tricholomataceae,* family, it can be concluded that edible mushrooms from genus *Marasmius* contained medium quality proteins and from genus *Tricholoma* contained proteins with low quality, required for the growth of a human organism.

Key words*: Tricholomataceae, mushrooms, protein quality, amino acid content, HPLC*

INTRODUCTION

Proteins are essential components of the diet needed for the survival of animals and humans. Proteins' basic function in nutrition is to supply adequate amounts of necessary amino acids. The protein quality, an important part of nutritional or nutritive value of a food, depends on its amino acids content. Most literature data consider edible mushrooms as a source of good quality proteins [1,2]. In Macedonia many kinds of wild *Tricholomataceae* mushrooms have acquired popularity as common food in addition to their usual use as condiments. No, information is available so far on the protein content and quality of a great number of the wide spread wild edible species in question. In order to establish the protein quality of Macedonian edible *Tricholomataceae* mushrooms, it is desirable to determine their protein content as well as to estimate the concentration of the amino acids present in their proteins. In this way this report will provide information on these mushrooms from Macedonia, distinguished for their protein content and great nutritional value.

MATERIAL & METHODS

Samples

The present study comprised four species of Macedonian edible mushrooms from the *Tricholomataceae,* family (of the *Marasmius* and *Tricholoma* genera). All the samples were field collected in different areas of Macedonia in the course of 2007/09. Identification [3] of the samples was confirmed by Prof Dr. M. Karadelev and voucher specimens were deposited at the Macedonian collection of mushrooms which belongs to the Institute of Biology at the Faculty of Natural Sciences in Skopje. After collecting, dry matter content was determined immediately by drying at 105 ^{o}C and the remaining part was stabilized, dried and milled to pass through a 0.2 mm screen. All the samples represent the whole mushrooms and were analyzed in triplicate for the determination.

Protein determination

The level of total nitrogen was determined by the micro - Kjeldahl method [4], and percent protein was calculated as % N x 6.25. By substraction of the water released during the amino acid condensation from the total sum, the net (pure) protein content was calculated [5].

Amino acid analysis

Seventeen amino acids were determined by HPLC after acid hydrolysis with constant boiling hydrochloric acid and pre – column derivatisation with phenyl isothiocyanate PITC [6,7]. A Perkin Elmer (USA) HPLC equipped with Binary LC Pump (model 250), UV diode array detector (model 235) set at 254 nm and Waters (USA) Pico-Tag column was used. A Mistral thermostated oven (type 880, Spark Holland, The Netherlands) was used for maintaining the constant column temperature at 38 ±1^{o}C. The amino acid content was calculated upon the standard curve of amino acid standard H (Pierce I, USA). Amino acid standard H was prepared and derivatised simultaneously with the samples.

The mobile phase consisted of aqueous buffer (0.14 mol L^{-1} sodium acetate, 0.5 mL L^{-1} triethylamine and titrated to pH 6.4 with glacial acetic acid) (Solvent A) and 60 % acetonitrile in water (B) and the flow rate was 1.0 mL min^{-1}. The gradient used for the separation consisted of 10 % B traversing to 51 % B in a ten – minute use of a convex curve (No. 5). A washing step was then programmed to 100 % B in order to clean any residual sample components from the column.

Tryptophan was determined spectrometrically (Perkin – Elmer UV/VIS Lambda 16 spectrometer), after hydrolysis with 5 mol L^{-1} NaOH according to Spies' and Chambers' method [8] modified by Shamanthaka [9].

Protein quality

The dietary protein quality of the investigated mushrooms was evaluated by comparison of the essential amino acid content with reference FAO/WHO pattern of amino acid requirements for pre-school children (two to five years) [10]. The lowest essential amino acid being deficient is marked as the limiting amino acid.

Statistical analysis

Statistical data processing was carried out on Microsoft Excel 2003 software.

RESULTS & DISCUSSION

The Kjeldahl nitrogen content, protein data and dry matter contents of the four Macedonian edible *Tricholomataceae* mushrooms, ranging from 6.80 (*Tricholoma georgii,* Berovo) to 10.33 % (*Marasmius oreades*, Veles), is relatively low when compared to other foods (11). The average dry matter value of 9.08 is comparable with the theoretical value of 10 % dry matter in mushrooms, which is always used in the literature data when this investigation is not done. Kjeldahl nitrogen varied from 3.36 (*Tricholoma terreum,* Probishtip) to 7.81 % (*Tricholoma georgii,* Berovo), which multiplied with the conversion factor 6.25 for proteins

amounts to a 21.00 - 48.81 % protein. The fruiting bodies of the mushrooms contain a number of unusual nitrogenous compounds which may interfere with the commonly used Kjeldahl nitrogen analysis and imply higher protein content values for mushrooms than given in the literature. The net (true) protein values were lower (14.88- 34.01 %), even through the obtained protein content was still higher than that of most natural products. *Tricholoma georgii* collected from Berovo (34.01 %) contained the highest net proteins levels, whereas *Tricholoma terreum* from Probishtip was the poorest in net proteins (14.88 %). Net protein nitrogen value compared with total nitrogen content was apparently different between species and ranged between 14.88 and 34.01 %. Since the Kjeldahl method does not distinguish between protein and non-protein nitrogen, the average value of 67.6 % protein nitrogen and practical nitrogen – protein conversion factor 4.23 might be used for evaluation of the net protein content in the Macedonian edible *Tricholomataceae* mushrooms. This coincides with Ogawa's [12] results of 65 % protein nitrogen in mushrooms from Japan and Stankeviciene's [13] value of 69.8 % protein nitrogen for Lithuanian mushrooms.
The dry matter content in the investigated mushrooms samples is not significantly correlated with the total nitrogen ($p>0.05$; $t=0.03$) and net proteins content ($p>0.05$; $t=0.02$), Mushrooms with closed values of dry mater very often differ in the proteins values.

Table 1. Dry matter, Kjeldahl nitrogen, crude and net protein content in the investigated mushrooms samples (% dry mass)

Sample (location)	Dry matter	Nitrogen	Crude protein (N x 6.25)	Net protein
Tricholoma albobruneum (Probishtip)	9.70	4.01	25.06	16.64
Tricholoma terreum (Probishtip)	9.50	3.36	21.00	14.88
Tricholoma georgii (Berovo)	6.80	7.81	48.81	34.01
Marasmius oreades (Veles)	10.33	6.43	40.19	25.85

The amino acid composition of the four investigated Macedonian edible mushroom samples from *Tricholomataceae,* family is presented in Table 2 and 3. All of the eighteen investigated amino acids, including the essential ones, were present in all mushroom proteins. Among amino acids, threonine alanine, arginine, cysteine and aspartic acid predominated. These data coincide with Fujita's [14] assumption that aspartic acids and alanine are the most abundant amino acids constitutive of mushrooms proteins. Among amino acids, tryptophan was present in the lowest amount, but still meeting the tryptophan requirements. Some authors [15] state that mushrooms are good sources of this amino acid and report the value of 11-20 g kg^{-1} protein, which is in agreement with our average tryptophan value of 11.35 g kg^{-1} protein. The lowest concentration of total amino acids of 17.30 % was found in *Tricholoma terreum* from Probishtip and the highest amino acids concentration of 39.53 % was found in *Tricholoma georgii* from Berovo. The amino acid pattern was different in all studied samples, but the quantitative ratios were identical in mushrooms of the same genus. The content of aromatic amino acids in all investigated samples was constistent at about 3.13 % (*Tricholoma albobruneum*, Probishtip) to 9.75 % (*Marasmius oreades,* Veles) of the total amino acids. The average value of 6.66 % aromatic amino acid found in this report was lower than that reported by Vetter [16] in mushrooms of various Russula and Agaricus species (10.8 % on average).

Table 2. Amino acid content (% dry mass)

Sample (location)	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys2	Ile	Leu	Phe	Lys	Trp
Tricholoma albobruneum (Probishtip)	0.37	0.67	0.18	0.23	0.39	0.65	0.40	2.19	0.70	0.42	10.64	0.22	0.85	0.29	0.42	0.18	0.31	0.23
Tricholoma terreum (Probishtip)	3.58	1.15	0.61	0.87	0.94	2.40	1.05	0.39	0.44	0.91	0.78	1.24	1.68	0.43	0.28	0.23	0.21	0.11
Tricholoma georgii (Berovo)	4.38	2.68	0.69	1.95	3.50	1.54	3.78	6.70	0.82	2.30	1.22	2.46	2.83	2.06	0.91	0.54	0.61	0.56
Marasmius oreades (Veles)	2.50	1.05	0.49	1.28	1.11	0.84	0.36	1.13	6.88	2.19	1.34	1.14	4.86	2.10	1.08	0.74	0.75	0.20

The average sulphur amino acids content of 14.4 % of the total amino acids in the mushrooms analyzed was higher if compared with average value of 4.6 % found by Vetter [16]. Acid amino acids accounted for 5.43 % of total amino acids in *Tricholoma albobruneum* from Probishtip to 27.34 % in *Tricholoma terreum* from Probishtip. The content of heterocyclic amino acids varied in concentration limits from 3.18 % (*Tricholoma terreum,* Probishtip) to 23.57 % *Tricholoma terreum* (Probishtip). Aliphatic monoaminomonocarbonic amino acids proved to be more abundant than other investigated amino acids and were present in 41.19 % on average. Overall differences in regard to the content of each amino acid per net protein (Table 3) could be noticed in different investigated samples. This confirmed to the fact that amino acid content depends on botanical origin. Comparison with other food proteins [17] of vegetable (wheat, potato, tomato, banana) and animal (beef, milk) origin indicated that mushroom proteins have a higher proportion of glycine, arginine, threonine, alanine, tyrosine, methionine, cysteine and isoleucine and a lower proportion of serine, phenylalanine and lysine. An increase in the proportion of eight amino acids in comparison with a decreased proportion of three amino acids still reflects the high nutritive value of mushroom proteins. The average net protein value of 22.84 % in Macedonian edible *Tricholomataceae* mushrooms (Table 1) was higher than that in vegetable food (3.9 % banana, 12.4 % tomato) and lower than that in food of animal origin (24.9 % milk, 70.0 % beef meat).

Table 3. Amino acid content in net proteins (g per 16 g of N i.e. per 100 g net protein)

Sample (location)	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Cys2	Ile	Leu	Phe	Lys	Trp
Tricholoma albobruneum (Probishtip)	2.22	4.03	1.08	1.38	2.34	3.91	2.40	13.16	4.21	2.52	63.94	1.32	5.11	1.74	2.52	1.08	1.86	1.38
Tricholoma terreum (Probishtip)	24.06	7.73	4.10	5.85	6.32	16.13	7.06	2.67	2.96	6.11	5.24	8.33	11.29	2.89	1.88	1.54	1.41	0.74
Tricholoma georgii (Berovo)	12.88	7.88	2.03	5.73	10.29	4.53	11.11	19.70	2.41	6.76	3.59	7.23	8.32	6.06	2.67	1.59	1.79	1.65
Marasmius oreades (Veles)	9.67	4.06	1.89	4.95	4.29	3.25	1.39	4.37	26.61	8.47	5.18	4.41	18.80	8.12	4.18	2.86	2.90	0.77

By comparing the obtained amino acid values from four species of Macedonian edible mushrooms from the *Tricholomataceae* family with the proposed FAO/WHO protein pattern [10], the following parameters were calculated and are presented in Table 4: E:N (ratio of essential to non essential amino acid), E:T (ratio of essential to total amino acids), E:P (ratio of essential to proteins), BV (biological value), PER (protein efficiency ratio), A/T (chemical score), EAAI (essential amino acid index), LAA (limiting amino acid) and X (percent limiting amino acid storage).

Table 4. Protein nutritional parameters

Sample (location)	E:N	E:T	E:P	BV	PER	A/T	EAAI	LAA	X
Tricholoma albobruneum (Probishtip)	2.97	0.74	0.86	58.96	1.59	32.12	0.84	Lys	67.88
Tricholoma terreum (Probishtip)	0.83	0.45	0.53	54.01	1.34	24.31	0.68	Lys	75.69
Tricholoma georgii (Berovo)	1.11	0.52	0.61	58.17	1.55	30.86	0.68	Lys	69.14
Marasmius oreades (Veles)	1.12	0.53	0.61	64.52	1.87	40.88	0.65	Thr	59.12
Literature data [10]									
Beaf meat 70-80 %				85.00	2.90	73.20			26.80
Wheat flour				57.50	1.50	29.80			70.20
soybean				59.90	1.60	33.50			66.50

*E:N (ratio of essential to non essential amino acid), E:T (ratio of essential to total amino acids), E:P (ratio of essential to proteins), BV (biological value), PER (protein efficiency ratio), A/T (chemical score), EAAI (essential amino acid index), LAA (limiting amino acid) and X (percent limiting amino acid storage)

The results show that E:N, E:T and E:P ratios were highest in *Tricholoma albobruneum* from Probishtip and lowest in *Tricholoma terreum* from Probishtip. The biological value of the investigated mushrooms fluctuated from 54.01 % (*Tricholoma terreum,* Probishtip) to 64.52 % *Tricholoma terreum* (Probishtip). Compared to the recent FAO reference pattern [10], lysine was the most limiting essential amino acid in the Macedonian edible *Tricholomataceae* mushrooms. According to the PER (1.5-2 PER) three samples of *Tricholomataceae* mushrooms have medium - quality proteins. Low - quality proteins (<1.5 PER) were estimated in *Tricholoma terreum* from Probishtip. Nutritional value parameters [10] for some other proteins such as that for 70-80 % wheat flour, soybean and beef meat were used comparatively (Table 4). According to the BV parameter, the data indicated one of four investigated mushroom proteins were of higher quality than the soybean protein, which is in all literature reports marked as having a nutritional value similar to that of animal proteins [2]. However, this did not apply to the EAAI value. *Marasmius oreades* from Veles showed the highest biological value, but the EAAI value is not highest, because the EAAI value calculation includes all the essential amino acids ratios compared to BV where only the lowest amino acid ratio is included. The EAAI values showed that in the investigated wild Macedonian edible *Tricholomataceae* mushrooms, all the essential amino acids, except the limiting one, were present in quantities meeting dietary requirements.

CONCLUSION

Due to their botanical origin, the amino acid content and protein nutritional parameters of the investigated wild Macedonian edible *Tricholomataceae* mushrooms showed considerable

differences. Macedonian edible mushrooms, when compared to other food sources, contain greater protein quality and represent a good protein food source.
Nitrogen to protein converting factor of 4.23 was obtained for the investigated Macedonian edible *Tricholomataceae* mushrooms.

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Original scientific paper

FLUORIMETRIC DETERMINATION OF ASCORBIC ACID USING METHYLENE BLUE

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SUMMARY

Vitamin C (L-ascorbic acid) is water soluble vitamin with strong reducing action and it is important coenzyme for internal hydroxylation reaction e.g. collagen.

The increasing use of pharmaceuticals and other natural samples containing vitamin C has meant that the practising chemists should develop analytical procedures for its determination that are simple to operate, rapid, accurate, sensitive and selective.

In our study determination of ascorbic acid (AA) is based on the measurement of decreasing of the fluorescence intensity of methylene blue (MB) due to the reaction between AA and MB, where MB is reduced to colourless leuco-lethylene blue (LMB) and AA is oxidized to dehydroascorbic acid (DHAA).

The fluorescence bands of MB were obtained at 635 nm for excitation and 694 nm for emission peaks. A linear relationship was obtained between the decreasing fluorescence intensity and the concentration of AA in the range of $1x10^{-6}$ – $5x10^{-3}$ mol/dm^3 with correlation coefficient of 0.9905. This method was applied in various farmaceutical samples.

***Keywords**: ascorbic acid, spectrofluorimetric method, methylene blue.*

INTRODUCTION

Vitamin C (L-ascorbic acid) is a water-soluble vitamin and a very important essential nutrient. This vitamin has a vital importance in processes of oxidation and reduction in human organism. Ascorbic acid (AA) is important coenzyme for internal hydroxylation reaction. AA is needed for the growth and repair of tissues in all parts of the body and it protects cell from oxidants attack, where AA reversibly oxidise to dehydroascorbic acid (DHAA).

AA is included in functions of central nerve system and has a role in other vitamins protection (vitamin E and vitamin A) from oxidants attack. Many analysis have shown that an adequate intake of vitamin C is effective in lowering the risk of developing cancer. A lack of vitamin C in the diet causes the deficiency disease, scurvy [1].

The increasing use of pharmaceuticals and other natural samples containing vitamin C has meant that there should be developed an analytical procedure for its determination which is simple to operate, rapid, sensitive and selective.

There are many methods that are used for the quantitative determination of vitamin C [2, 3, 4, 5]. Although some methods are available for determination of AA but just few methods are used for determination of both forms of ascorbic acid (AA and oxidized form, DHAA). This is because two forms of the vitamin C possess different chemical, optical and electrochemical properties. In the colorimetric and fluorimetric methods, AA is oxidized to DHAA and then reduced with a chemical reagent to form a colored or fluorescent compound. In this study, the determination of AA is based on the measurement of decreasing fluorescence intensity of

methylene blue (MB) due to reaction between AA and MB, where MB is reduced to colourless leuco-methylene blue (LMB) and AA is oxidized to DHAA.
This method allows us to determine the amount of AA in the purified materials, including vitamin C tablets.

MATERIAL & METHODS

Luminiscence Spectrometre LS 55 Perkin Elmer was used to record spectra and carry out fluorescence measurements. All used chemicals were of analytical reagent grade.
Preparation of AA: A stock solution of 0.1 mol/dm^3 AA was prepared daily no more then 3h before experiment by dissolving 440 mg of AA (Merck) in 25 mL distilled water. Working solutions of lower concentration were prepared by appropriate dilution with distilled water. All solutions were kept in dark.
Preparation of MB: Solution of $4x10^{-4}$ mol/dm^3 MB was prepared by dissolving the appropriate amount of the MB (Merck) in 100 mL distilled water.
Preparation of samples: Investigated samples were powdered, and appropriate amount of samples was weight accurately and dissolved in 25 mL distilled water. The final solution was centrifuged and supernatant was used for further analysis.
If there was an interference by the presence of citric acid it was carried away by reaction with calcium hydroxide.
Measuring: Appropriate amount of sample solution was diluted with 0.5 mL 1 M HCl, 125 µL $4*10^{-4}$ M MB and distilled water in 10 mL volume flask. The investigated samples were measured by fluorimetric method.

RESULT & DISCUSSION

The fluorescence bands for MB were obtained at 635 nm for excitation, and 694 nm for emission peaks (Figure 1.). Dilgin and Nisli also obtained excitation band at 664 nm and emission band at 682 nm [6].

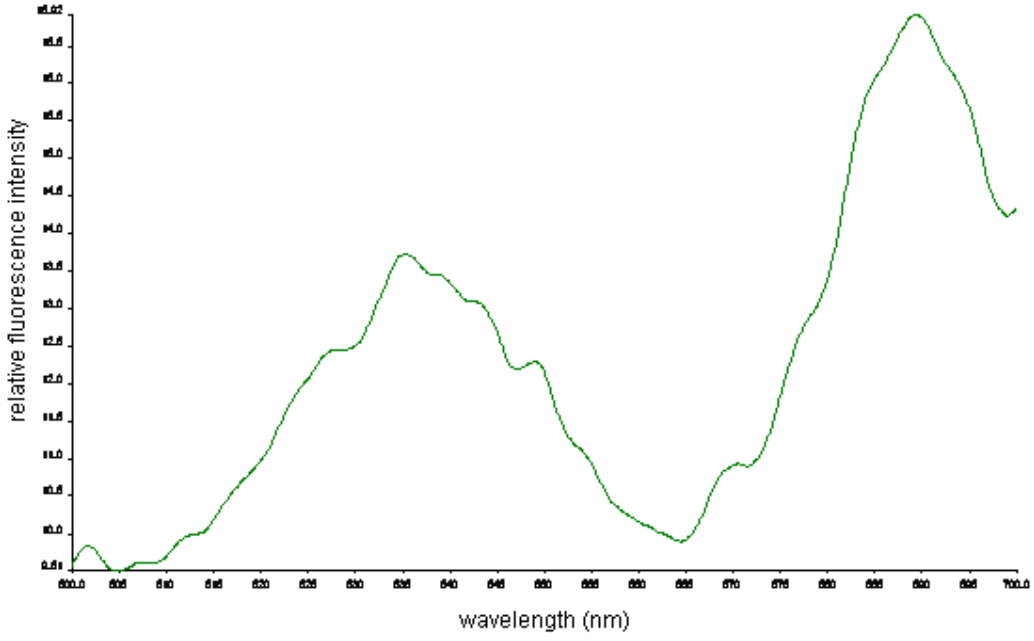

Figure 1. The fluorescence spectra of methylene blue.

Optimisation of concentration of HCl was investigated in range of 0.1 – 0.9 mol/dm^3 (Figure 2.).

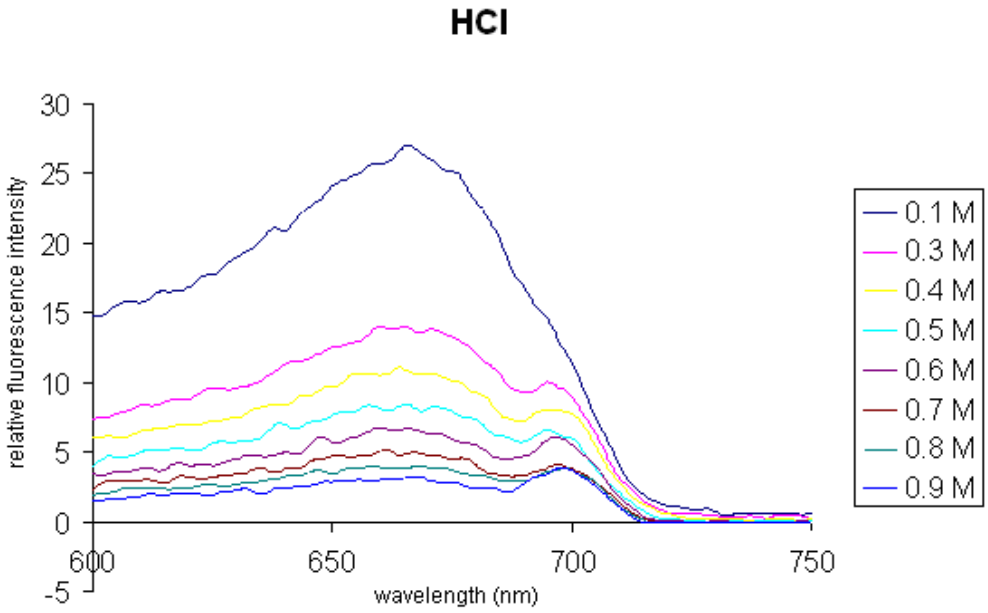

Figure 2. The fluorescence spectra of 10^{-4} M ascorbic acid and HCl (emission spectra λ_{em} = 694 nm) depending on HCl concentration.

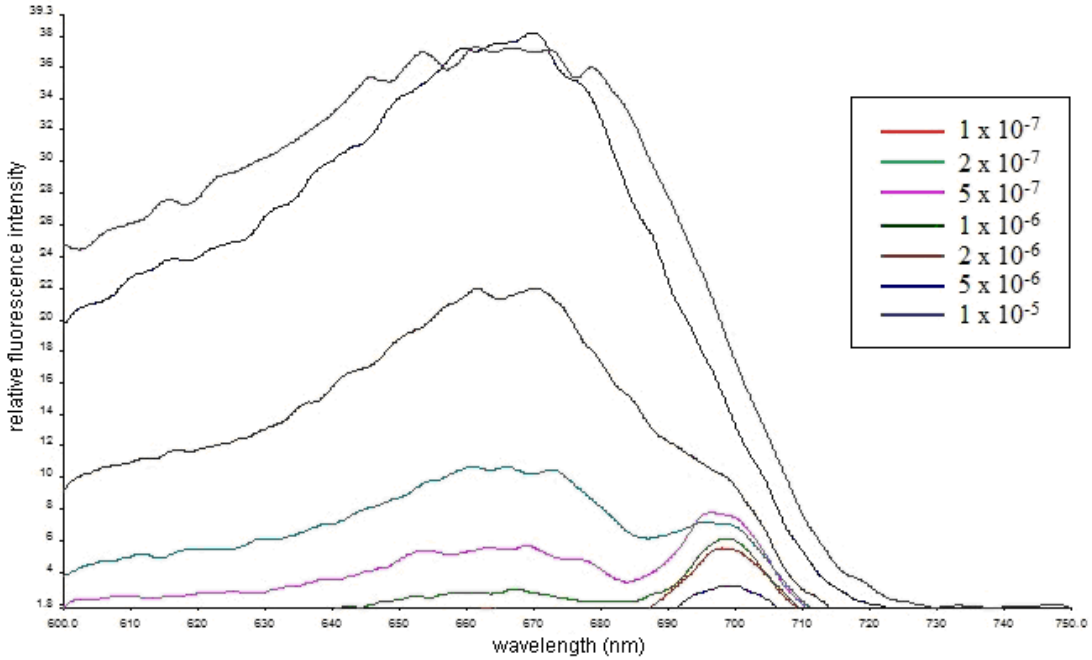

Figure 3. The fluorescence spectra of 10^{-4} M ascorbic acid and MB (emission spectra λ_{em} = 694 nm) depending on MB concentration.

From the Figure 2 it can be seen that fluerescence intensity increase with decrease of concentration of HCl. As optimum concentration 0.1 M HCl was chosen.
Optimisation of concentration of MB is chosen by recording spectra of MB in concentration range of $1x10^{-7}$ – $1x10^{-5}$ mol/dm^3 (Figure 3.).
From the Figure 3 it can be seen that fluorescence intensity increase with increase of MB concentration. As optimum concentration $5x10^{-6}$ M was chosen because it gave the highest fluorescence intensity.
Proposed method is based on the redox reaction between AA and MB, AA was oxidized to DHAA, while MB was reduced to colourless LMB (Figure 4).

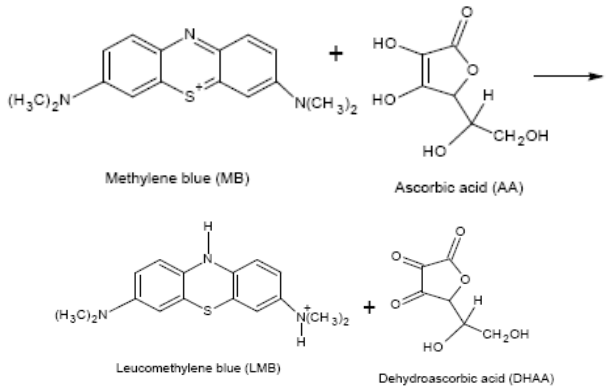

Figure 4. Reaction of MB with AA

The calibration curve for AA was establish by plotting the fluorescence intensity versus the concenration AA (Figure 5).

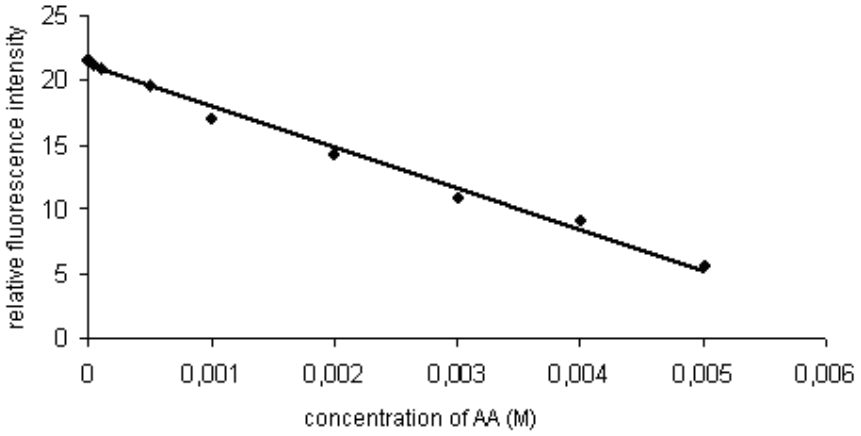

Figure 5. Calibration curve – relative fluorescence intensity was measured after 5 minutes for different concentrations of AA.

From the Figure 5 it can be seen that linear relationship was obtained between the decreasing fluorescence intensity and the concentration of AA in the range of $1x10^{-6}$ – $5x10^{-3}$ mol/dm^3 with correlation coefficient of 0.9905. Each spectrum was recorded at 5-min intervals.

Detection limit was $1.48x10^{-4}$ mol/dm^3. It was established measuring intensity 5 times using AA concentration $5x10^{-6}$ mol/dm^3. Reproducibility was 5.45 % and it was established measuring intensity 10 times using AA concentration $5x10^{-4}$ mol/dm^3.
This method was applied in various pharmaceutical samples of vitamin C.

Interference study was made by analysing the effect of citric acid on the determination of ascorbic acid (Figure 6.).

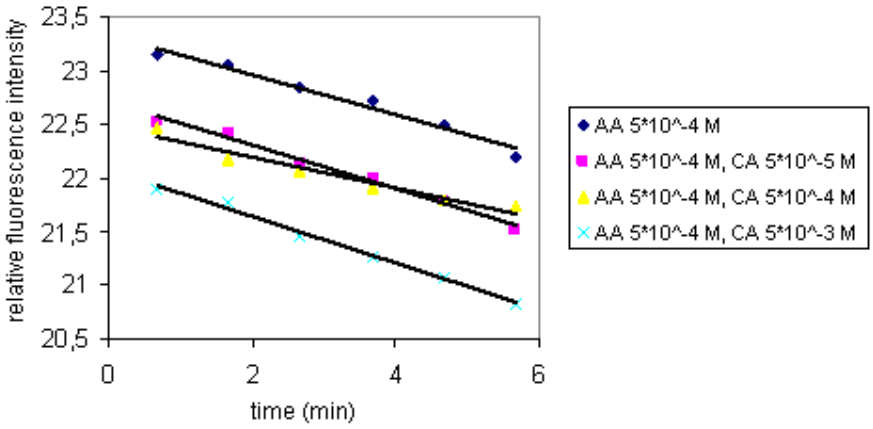

Figure 6. Interference study – Influence of different concentrations of citric acid on determination of AA

The experimental results showed that the presence of certain amount of citric acid had significant influence on the determination of AA. Considering that samples were pharmaceuticals, citric acid was separated as an interfering substance, using calcium hydroxide. After making final solutions, if there is presence of citric acid, calcium hydroxide was added, which with citric acid form a calcium citrate, which was removed from solution by filtration [7].
Results are shown in Table 1.
The results in Table 1 show good agreement between amount of vitamin C in the investigated samples that was established using proposed method and amount of vitamin C (mg) given on the lable. Recovery of the results obtained with proposed method was between 90.17 % and 104.93 % with RSD value ranging from 0.32 % to 9.93%. The results show that eight investigated samples show recovery values in the range of 94,81% and 104,93 %, and RSD in the range of 0.32 % to 5.22 %. Minor aberration of two investigated samples (Alkaloid and Gesunde Plus) with recovery value of 90.17 % and 92.24 %, could be caused by the product life of some samples. It is also known that AA concentration vary with conditions such as temperature and the storage period, and it could also be reason for lower content of vitamin C obtained by proposed method. Comparing to the some other methods such as titrimetric and spectrophotometric, this method is simple, sensitive, fast, and low cost and could be used for the determination of AA in tablets of vitamin C.

Tabele 1. Content of AA in pharmaceutical samples

Sample of vitamin C tabletes from different supliers	***Value of vitamin C (mg)/1 tablet***	***Amount of vitamin C mg/1 tablet on lable***
Krüger (180)	179.69 ± 0.57	180
Sensilab (180)	170.67 ± 3.58	180
SchneeKoppe (180)	183.07 ± 18.18	180
Gesunde Plus (240)	216.40 ± 9.87	240
Sunlife (180)	188.88 ± 9.87	180
Biofar (1000)	956.47 ± 27.19	1000
PlivitC (500)	479.53 ± 11.97	500
Alkaloid (500)	461.22 ± 44.31	500
Galenika (500)	519.83 ± 12.28	500
Cvit – Bosnalijek (500)	512.82 ± 17.89	500

CONCLUSION

The proposed method provides a simple and sensitive fluorimetric procedure for determination of AA with MB. This spectrofluorimetric method for determination of AA using MB is faster and more sensitive then the simple UV-spectrophotometric method [8]. The proposed spectrofluorimetric method can be used for determination of vitamin C in tablets as in other biological samples too.

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Original scientific paper

SPECTROPHOTOMETRIC AND TITRIMETRIC DETERMINATION OF ASCORBIC ACID IN SOME FRUITS AND VEGETABLES

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SUMMARY

Ascorbic acid (AA) occurs in different concentrations in a variety of natural samples. In this study we present spectrophotometric determination of AA in different fruits and vegetables. Method was based on the reaction of AA with highly coloured methylene blue (MB) where MB is reduced to colourless leucomethylene blue (LMB) and AA is oxidised to dehydroascorbic acid (DHAA). The reaction has been followed spectrophotometrically by measuring the decrease in the absorbance of MB at 665 nm. A linear relationship was obtained between the decreasing absorbance intensity and the concentration of AA in the range of 0.001–0.05 molL^{-1} with correlation coefficient of 0.9983. The proposed method is simple, safe, inexpensive and rapid. The obtained results were compared with titrimetric method.

Keywords*: ascorbic acid, spectrophotometric method, titrimetric method, methylene blue.*

INTRODUCTION

Ascorbic acid (AA, 2,3-endiol-L-gulonic acid-γ-lactone) or vitamin C is water-soluble vitamin that participate in many of the chemical reaction in the body. The principal compound with vitamin C activity that is found naturally is L-AA [1]. As humans are not able to synthesize ascorbic acid, they are dependent on their dietary intake. The dietary sources of vitamin C are fruits and vegetables, especially in fresh forms. The most important reducing property of ascorbic acid in biological systems is the radical chain terminating reaction Vitamin C's role as an *in vivo* antioxidant has received much attention over the past decades. Methodology has advanced from the bioassay to instrumentally advance spectrophotometric, fluorometric, electrochemical, and chemiluminescence methods.

The aim of this study was to establish spectrophotometric method for the determination of AA in some fruits and vegetables using methylene blue (MB), which is a water-soluble cationic dye molecule that has been widely studied since its synthesis. It is easely reduced to the colorless hydrogenated molecule leucomethylene blue (LMB), by a variety of agents and one of them is AA [2]. Results were compared with iodometric titration.

MATERIALS & METHODS

Samples

Fourteen different fruit and vegetable samples were analyzed. Samples were found in local supermarkets fresh and shade dried.

Reagents and Materials

L(+)-Ascorbic acid; citric acid monohydrate; starch and methylene blue were purchased from Merck, acetic acid (glacial) and potassium iodide from Semikem, iodine resublimisan from Zorka. All used chemicals were of analytical grade.

Preparation of solution

Iodine solution (0.005 mol L^{-1}). 2 g of potassium iodide and 1.3 g of iodine added into the 100 mL beaker and dissolved. This solution is diluted ten times. The concentration of prepared iodine solution was more accurately determinated by titration with a standard solution of AA.

Starch indicator solution (0.5%). 0.25 g of soluble starch added to 50 mL of near boiling water. It was stired to dissolve and cooled before using [3].

Preparation of Samples

Samples for the analysis were prepared the same way whether they're fresh or shade dried. Samples (5g) were coarsely powdered and it was added glacial acetic acid (2 mL) then filtered and the volume made up to 100 mL with distilled water. Samples were not centrifuged. The samples were analyzed both methods, spectrophotometric and titrimetric in very short time because of the AA instability.

Preparation of Standard Curve

Standard of AA was disolved in distilled water and concentration of stock solution was 0.1 $molL^{-1}$. This stock solution was used to prepare required dilutions containing 0.001, 0.005, 0.01, 0.025, 0.05 $molL^{-1}$.

Titration condition

The procedure for titration of AA: 10 mL of sample, 50 mL of water and 1 mL of 0,5% starch solution was titrated with iodine solution All samples were analised as triplicate.

Spectrophotometry conditions

The spectrophotometric study was carried out with Perkin Elmer UV/VIS Spectrometer to determine the amounts of AA in fruits and vegetables. Fifty microliters of sample and 125 μL of MB (c=0,004 mmol/L) solution were diluted up with distilled water to 10 mL. Decreacing of absorbtion was measuered at 665nm. A linear relationship was obtained between the decreasing absorbance intensity and the concentration of AA in the range of 0.001–0.05 mol L^{-1}. All analysis was carried out in triplicates. Reaction mechanism of MB with AA is given by Figure 1 [4].

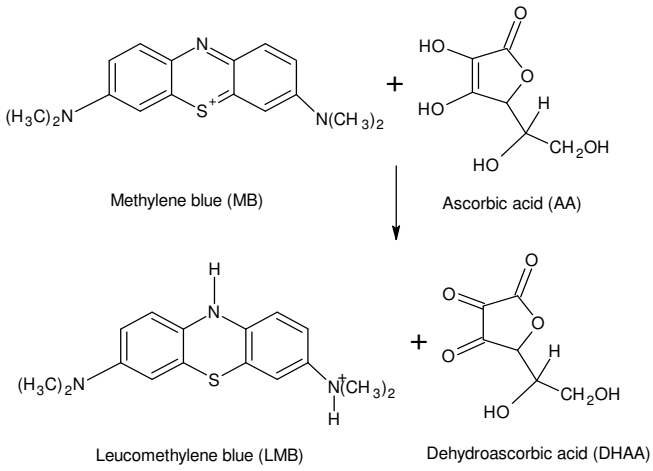

Figure 1. Reaction mechanism of MB with AA

From the Figure 1 we can see that this reaction is based on the reaction between AA and highly coloured MB where MB is reduced to colourless LMB and AA is oxidised to dehydroascorbic acid (DHAA).

RESULTS & DISSCUSION

For the investigation of absorption maximum, different concentration of MB in the range of 0.004 to 0.025 mmolL^{-1} were used. It was found λ=665 nm (Figure 2).

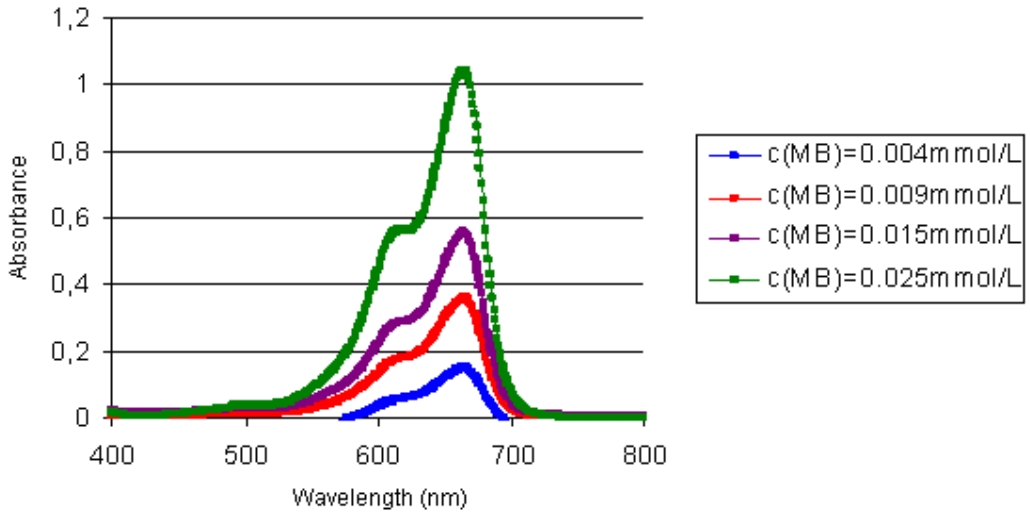

Figure 2. Absorption maximum

As it can be seen from the Figure 2 the intensity of absorbace increase with increase of concentration of MB. As optimum concentration of MB was chosen 0.004 mmolL^{-1} , because it gives the highest absorption intensity.
The standard curve for AA was prepared for concentration versus the decreasing absorbance intensity (Table 1. and Figure 3.)

Table 1. Concentrations and absorbances of standard solution of AA

c (mol L^{-1})	A
0.001	0.2646
0.005	0.2623
0.010	0.2543
0.025	0.2378
0.050	0.2096

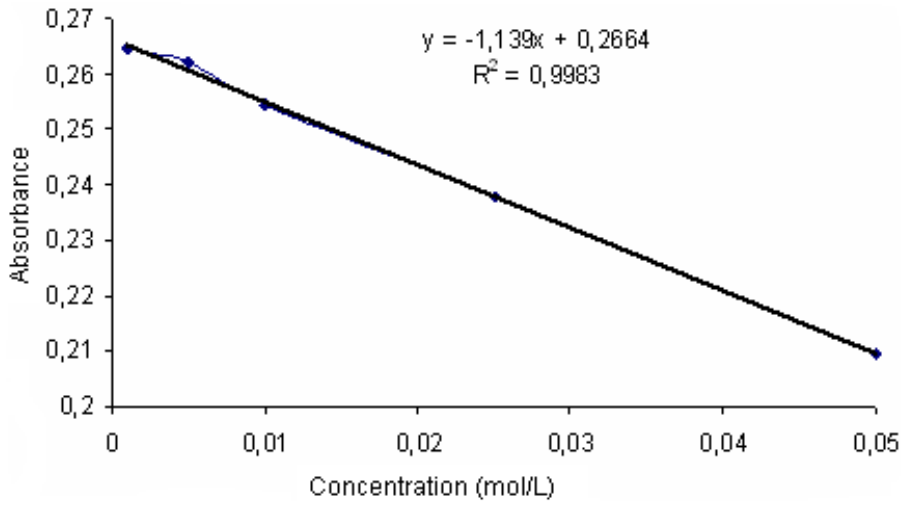

Figure 3. Standard curve of AA

The calibration curve for AA exhibite linear range from 0.001-0.05 mol L^{-1}, with good corelation coefitient of 0.9983.

In the Table 2. are given results of content of AA in different investigated samples. Content of AA in fourteen different samples of fruits and vegetables are ranged from 5.43 mg/100g to 51.24 mg/100g for the spectrophotometric method and from 5.35 mg/100g to 57.95 mg/100g for the titrimetric method. The highest content is in orange (both methods) and the lowest content is in the sample of banana (both methods).

Table 2. Quantitative results of AA in fourteen different samples by two methods.

	Samples	Spectrophotometric method (mg/100g of sample)	Titrimetric method (mg/100g of sample)
1.	Banana	5.43 ± 0.29	5.36 ± 0.21
2.	Pear	7.83 ± 0.19	12.46 ± 0.11
3.	Apple (1)	9.78 ± 0.04	9.46 ± 0.29
4.	Apple (2)	11.44 ± 0.07	9.38 ± 0.18
5.	Grapefruit	12.21 ± 0.89	14.68 ± 0.18
6.	Kiwi	14.37 ± 0.09	12.49 ± 0.30
7.	Lime	19.21 ± 0.69	17.35 ± 0.29
8.	Juniper (shade dried)	20.67 ± 1.96	19.58 ± 0.92
9	Lemon	23.89 ± 1.59	22.51 ± 0.22
10.	Potato	25.76 ± 0.07	8.83 ± 0.30
11.	Cranberry (candied)	27.05 ± 0.42	24.57 ± 1.22
12.	Cranberry (shade dried)	27.21 ± 0.59	23.33 ± 1.30
13.	Spinach	30.26 ± 1.16	24.03 ± 0.21
14.	Orange	51.24 ± 0.59	57.94 ± 0.17

A lot of scientific papers about investigation of content of vitamin C in different kind of fruits and vegetables were published. Okiei et al. (2009) were investigated AA levels in the red apple (12.72 mg/100 g), green apple (11.73 mg/100 g), yellow apple (11.73 mg/100 g), banana (15.00 mg/100 g), lime (56.57 mg/100 g) [5]. Pfendt et al. (2003) were publish determination of content vitamin C in some fruits and vegetables: orange (43.42 mg/100 g),

lemon (34.24 mg/100 g), grapefruit (43.33 mg/100 g), kiwi (42,26 mg/100 g) [6].
Aydogmus and Cetin (2002) reported the determination of AA in fruits and vegetables, and results were 48 mg/100 g in lemon, kiwi (79.9 mg/100 g), spinach- cultivated (80 mg/100 g), spinach- purchased (19.0-52.0 mg/100 g) [7].Accoding to Combs (2001) content of vitamin C in potato was 19-20 mg/100 g, juniper (25-30 mg/100 g), cranberry (10-12 mg/100 g), pear (4 mg/100 g) and banana (9 mg/100 g) ([8].
There are significant differences in the value of AA investigated and published by many researchers. Obtained value of AA are different since content depends of many factors, such as climate, conditions as light and temperature and the storage period on preservation. Vitamin C content slowly decreases with temperature and storage period of fruits and vegetables [9].
Citric acid that is present in many vegetables and fruits, and was interfered by the spectrophotometric determination of AA in lemon, grapefruit and lime. Citric acid stabilize ascorbic acid. Its influence on AA determination is shown in Figure 4.

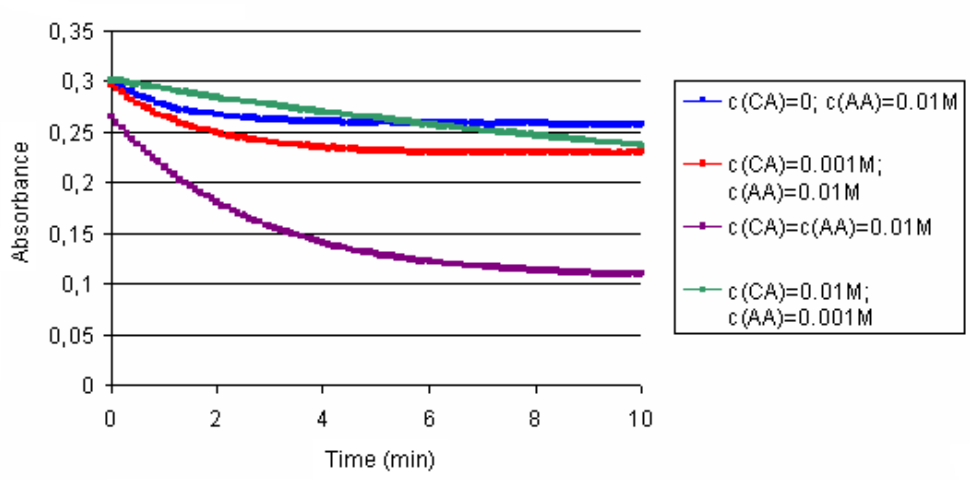

Figure 4. Influence of citric acid on determination of AA

That influence is more strongly expressed if the concentration of citric acid is approximately equal to the concentration of AA in the investigated solution. Citric acid has no influence on titrimetric determination of AA in vegetables and fruits. It is possible to remove citric acid from the solution using calcium hydroxyde. Citric acid with calcium hydroxyde combine to form a calcium citrate, which was removed from solution by filtration [10]. Because of the citric acid influence in three samples (grapefruit, lime and lemon) results were higher than those after citric acid was eliminated from solution. After citric acid was removed from solution results were allied to titration results. Results are given in Table 3.

Table 3. Quantitative results of AA before and after citric acid was removed

	Samples	Spectrophotometric method, before citric acid elimination (mg/100g)	Spectrophotometric method, after citric acid elimination (mg/100g)	Titrimetric method (mg/100g of sample)
1.	Grapefriut	22.39±1.36	12.21±0.89	14.68±0.18
2.	Lime	29.76±1.02	19.21±0.69	17.35±0.29
3.	Lemon	23.89±1.59	22.48±1.28	22.51±0.22

From the obtained results we can see good correlation between two methods for all investigated samples except for the potato. The reason for the low correlation between two methods used in potato sample could be high content of polysaharide starch. Since iodometric titration used starch as indicator, it could be possible that remove starch from the investigated sample was not completly and the end point in the titration method was achived much earlier. So obtained result of content od vitamin C was much lower that with spectrophotometric method.

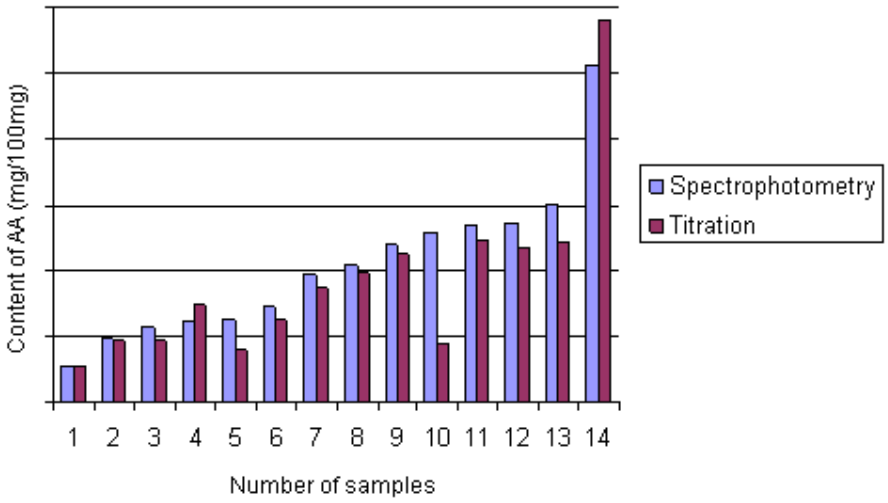

Figure 5. Results comparation by two different methods
1. Banana, 2. Pear, 3. Apple (1), 4. Apple (2), 5. Grapefruit, 6. Kiwi, 7. Lime, 8. Juniper, 9. Lemon, 10. Potato, 11. Cranberry (candried), 12. Cranberry (shade dried), 13. Spanach, 14. Orange.

CONCLUSION

Spectrophotometric method has been chosen for this study as it has been widely applied in determinig the AA contents in biological samples. Applied procedure detects L-ascorbic acid form of vitamin C. The results showed the fruits and vegetables are good source of vitamin C. Results obtained by two presented methods are in good correlation.

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Original scientific paper

DETERMINATION OF HEAVY METALS, PESTICIDE RESIDUES AND RADIOACTIVITY IN FRUITS OF SELECTED PLANT SPECIES

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SUMMARY

Plant species and its fruits choosed for our experiments (highbush blueberry - *Vaccinium corymbosum* L.; elderberry - *Sambucus nigra* L. variety Hachsberg; chokeberry - *Aronia melanocarpa* Wild. variety Nero and grapevine - *Vinis vitifera* L.) are rich in anthocyanins. Scientists have begun to realise the potential of anthocyanins as compounds of industrial importance, both as pigments in their own right and also as pharmaceuticals during last decades. Our research was focused on determination heavy metals, pesticide residues and radioactivity as an important environmental factors influenced development of selected fruits. Different ecological condition can caused differences in qualitative and quantitative properties of anthocyanins. Results of our investigation are as follows. Content of heavy metals at all samples determinated by AAS were under limit according to the Regulations from the Ministry of Healthy Service in Slovak Republic. Value of radioactivity was in hygienic convenient standard and pesticide residues reached minimum of the limit for Slovak Republic.

Key words: *Anthocyanins, heavy metals, radioactivity, pesticide residues, fruits*

INTRODUCTION

Xenobiotics have been the important and complex environment problems for the long time. They have caused a pollution of air, soil and water. Scientists divided them into two groups - inorganic (heavy metals) and organic components (pesticide residues). Usually they are not the natural origin and their quantity in ecosystem can operate from unhealthy to toxic. The largest catastrophe in history was in Chernobyl, Ukraine, on April 26th 1986. The radioactivity had been affected vegetation in the Slovak Republic even if the Chernobyl crash was many years ago. The agriculture commodities and medicinal plant exported to the West European countries was conditioned by the certificates of radioactivity determination (Šalamon – Habán, 2004). Measurments of ^{134}Cs mass radionuclide activity at medicinal plant have carried out by Šalamon (2001). It was determined that radioactivity of individual medicinal plant species are dependent on time and exposure in a space (Šalamon, 2001; Kirchner, Daillant, 2002). The medical quality and effect of selected plants depend on the environment conditions, which influence directly grow of plants and the process of creation secondary metabolites. At the same time, there are some changes in chemical composition (presence or absence of some component, or the content varies) (Veličković et al., 2002). It is very important to determine the hazard values and compare it with the regulation on the highest permissible toxic effects in food, which are notified by the Slovak legislation. Plants, selected for our research, are rich at antocyanins. They were collected in different localities in Slovak Republic. Key functional hypotheses of anthocyanins include protection of

chloroplasts from the adverse effects of excess light; attenuation of UV-B radiation; and antioxidant activity. Interactions between anthocyanin pigments and other flavonoids or other phytochemicals accumulating within a plant contribute significantly to the ability of natural plant extracts (ingested as food or pharmaceutical product) to protect human health or mitigate disease damage (Gould et al., 2009).
The aim of this contribution was to determinate heavy metals, pesticide residues content and radioactivity in fruits of selected plants, as a potential risks of environmental toxicity.

MATERIAL AND METHODS

Plant material

Plant material was collected in different localities - highbush blueberry (*Vaccinium corymbosum* L.) in location Krivá - Orava region, elderberry (*Sambucus nigra* L.) variety Hachsberg in cultivation area in the village Lesné, Michalovce, chokeberry (*Aronia melanocarpa* Wild.) variety Nero in cultivation area in the village Pozdišovce, Michalovce, grapevine (*Vinis vitifera* L.) varieties Frankovka and Cabernet Saugvinon. The fruits of this species were used for the analysis.

Chemicals and analysis

Chemical analysis required HNO_3 (65%) and H_2O_2 (30%) available in our laboratory purchased from Sigma Aldrich, Bratislava, Slovakia. Standards were supplied by Ultra scientific, Poland. 2 % solution of HNO_3 and deionized water with the conductivity <0,1µS was used for stabilization of extracts.
Mineralization for determination heavy metals in fruits was realized by Speedwave 2 Berghof (AAS), voltage ~ 230V, frequency 50/60 Hz, input power 1610W, magnetrone frequency 2450MHz. Pressure vessels DAP-60K, volume 60 ml, maximum pressure 40 bar, maximum temperature 230 °C, maximum amount <300mg, minimum volume of acids >5ml.
Atomic absorbtion spectrophotometer (AAS 7000) Shimadzu fully automatic dual-purposed instrument with 3D-optic system, automatic 6-lamps holder, background correction with D-lamp with SR-correction of spectral interferences. Software includes default operating parameters for oven and for all elements and standards. Thermal stablity of dual-purposed system is for 2 ppm Cu and 600 measurements relative standard deviation < 1 %. Low cross contamination, max. 10^{-4}, mikrosampling for lower than 100 µl.
For mineralisation in AAS there was weighted 0,3 g of each sample - blueberry, elderberry, chokeberry, grapevine. The pressure vessels DAP–60K was filled with this amount, than was added 5 ml HNO_3 (65%) and 2 ml H_2O_2 (30%). Temperature program beginning at 0 °C continue 8 °C/min to 75 °C (9 min), then temperature increased into 120°C (5 min) and 192 °C. The last fase was finishing mineralization by temperature decrease into 75 °C/10 min.
The samples were cooled in room temperature. After that 1 ml of mineralized samples was taken and refilled into 100 ml with 2% solution of HNO3 and deionized water. Finally, the measurements of Cd, Ni and Pb amount by AAS (λ = 226,502 nm for Cd, λ = 231,604 for Ni and λ =220,351 for Pb) was done. The pesticide residues were determined by the GC-method with an application of equipments type Varian Star, model 3,400, and Varian Star, model 3,800; the detector ECD after plant sample arrange. Most components were identified from their GC retention indices compared with standards. All these chemical analysis were carried out in the Ecological and Veterinary Laboratories in Spisska Nova Ves, Slovakia. The quantity values of pesticide residues obtained in plant materials were compared with the highest permissible concentrations according to the Regulation No. 14/1996 from the Ministry of Healthy Service in Slovak Republic. The fruits of selected plants were used to determination the mass radio-nuclide activities. The gama-spectrometric determination by the HPGe detector with using of Cesium (^{134}Cs) radio nuclide was carried out at the Special State

Health Institute, Department of Health Protection against the Radioactivity in Banska Bystrica, Slovakia. The quantity values of radioactivity obtained in these plant materials were compared with the highest permissible concentrations according to the Regulation No. 12/2001 from the Ministry of Healthy Service in Slovak Republic

RESULTS AND DISCUSION

Heavy metals have ecological toxic effect and high accumulation capacity. It presents one of the most important environmental problems than any other degradation. Determination contents of heavy metals and additional microelements in fruits of the selected plants are noted. Ministry of Agriculture and Ministry of Health Service in Slovak Republic in Regulation No. 608/3/2004-100 allowes the highest amount of chemical components in food. The highest allowed amount for Cd is 0,5 mg/kg, for Pb is 3,0 mg/kg and for Ni 0,5 mg/kg.

The very unfavourable comparative of heavy metal concentration (Cd and Pb) in medicinal plant rawmaterials, which were obtained from the cultivation in Scotland, Findland and in the Central Europe was published in 1994 by Svoboda and Gough. The medicinal plant drugs from our region (the Central Europe) were contamined by double quantities of cadmium and lead concentrations more than another rawmaterials.

Determination of heavy metals in the fruits of blueberry (*Vaccinium corymbosum* L.), elderberry (*Sambucus nigra* L.), chokeberry (*Aronia melanocarpa* Wild.) and grapevine (*Vinis vitifera* L.) by the AAS presented, that no one sample was over limit. The contents of heavy metals were determined by the force detection device, which was for nickel 0.009 mg .kg^{-1} and for lead and cadmium of 0.001 mg .kg^{-1} (Table 1).

Table 1: The contents [mg .kg^{-1}] of Ni, Pb and Cd in dry matter of selected fruits

Name of the sample	Ni concentration	Pb concentration	Cd concentration
elderberry 1	< 0.009	< 0.001	< 0.001
elderberry 2	< 0.009	< 0.001	< 0.001
chokeberry 1	< 0.009	< 0.001	< 0.001
chokeberry 2	< 0.009	< 0.001	< 0.001
blueberry 1	< 0.009	< 0.001	< 0.001
blueberry 2	< 0.009	< 0.001	< 0.001
grapevine 1	< 0.009	< 0.001	< 0.001
grapevine 2	< 0.009	< 0.001	< 0.001

The present production of the special crops is not possible without direct pesticide application. On the other hand, a control of pesticide residues in individual phases of ontogenetic plant development and into the final product or yield is inevitable (Oravec et al., 1981). The problematic question is a metabolism and degradation of pesticides in the single plant species. (Schuphan et al., 1990). Table 2 illustrates the values of determined residue pesticides in the selected plants.

Radioactivity was performed by artificial radionuclide 134Cs. All samples from fruits were under limit (Table 3). Contaminants in fruits were at a minimum levels. In any case exceed to the maximum allowable levels in accordance to the Decree of the Ministry of Health.

There are some plants, which are exposed to the radiation on the high places continuously several years. Icelandic Lichen, *Cetraria islandica* L. Ach., can be a good example.

Table 2: The contents [mg .kg^{-1}] of pesticide residues in dry matter of selected species

Pesticide residues	elderbery	chokebery	grapevine	blueberry
aldrine + dieldrine	< 0,005	< 0,005	< 0,005	< 0,005
endrine	< 0,005	< 0,005	< 0,005	< 0,005
heptachlorepoxide	< 0,005	< 0,005	< 0,005	< 0,005
4,4' DDT	< 0,005	< 0,005	< 0,005	< 0,005
alfa-hexachlorecyklohexane	< 0,005	< 0,005	< 0,005	< 0,005
beta-hexachlorcyklohexane	< 0,005	< 0,005	< 0,005	< 0,005
hexachlorbenzene	< 0,005	< 0,005	< 0,005	< 0,005
endosulfane I	< 0,005	< 0,005	< 0,005	< 0,005
endosulfane II	< 0,005	< 0,005	< 0,005	< 0,005
endosulfane sulfate	< 0,005	< 0,005	< 0,005	< 0,005
eldrine aldehyde	< 0,005	< 0,005	< 0,005	< 0,005
endrine ketone	< 0,005	< 0,005	< 0,005	< 0,005
metoxychlorine	< 0,005	< 0,005	< 0,005	< 0,005
heptachlorine	< 0,005	< 0,005	< 0,005	< 0,005
4,4' DDE	< 0,005	< 0,005	< 0,005	< 0,005
4,4' DDD	< 0,005	< 0,005	< 0,005	< 0,005
gama-chlordane	< 0,005	< 0,005	< 0,005	< 0,005
alfa-chlordane	< 0,005	< 0,005	< 0,005	< 0,005

This thallopatic plants is occurred on the rocks in the High and Low Tatras (the Slovak parts of Carpathian Mountains). In regard to radioactivity determination of Icelandic Lichen thallus were gotten from 350 to 600 Bq.kg-1 of ^{134}Cs mass radionuclide activity (Šalamon, 1998). Obtained data from different research works (Sloof et al. 1992; Steinnes et al. 1993; Kočiová et al., 2002) can serve as a model of the role of lichens in immobilization of radio strontium from contaminated environment and as a tool for radioactivity monitoring. The regulation on the highest permissible radioactivity in food was notified in the Slovak Republic in January 2001. The highest levels of food radioactivity after a nuclear crash are presented in Table 4.

Table 3. Determined radioactivity in the fruits of selected plants

Samples	**Fresh matter [Bq.kg^{-1}]**	**Dry matter [Bq.kg^{-1}]**
elderberry (*Sambucus nigra*)	0,05 ± 0,01	0,25 ± 0,06
chokeberry (*Aronia melanocarpa*)	< 0,06	< 0,31
grapevine (*Vitis vinifera*)	< 0,07	< 0,27
Blueberry (*Vaccinium corymbosum*)	< 0,07	< 0,52

Table 4. The highest permissible radioactivity of food after a nuclear breakdown in the Slovak Republic

Radio-nuclides	Baby food [Bq.kg-1]	Milk products [Bq.kg-1]	Basic foodstuffs [Bq.kg-1]	Liquid food [Bq.kg-1]
134Cs and 137Cs	400	1,000	1,250	1,000

CONCLUSION

Legislation of state in regard to hazardous interactions in environment is connected with human consciousness, control and measurement of chemical, physico-chemical and physical measurement and their influence for biological systems. At present it is very important to introduce the Europe environment recommendations.

Environmental risks (contents of heavy metals, pesticide residues and radioactivity) in regard to production and collection of plants was determined by the analytical methods. The results were compared by the Slovak Republic legislation. In spite of the results, the trace xenobiotic values and radioactivity of fruits of selected plants were stated.

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Section II

"Pharmacology and biological effects of active MAP compounds"

Original scientific paper

MORPHOLOGICAL AND CHEMICAL CHARACTERISATION OF HUNGARIAN WILD GROWING CHAMOMILE (*MATRICARIA RECUTITA* L.)

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SUMMARY

In our experiment we examined the genetic variability of 16 wild growing Hungarian chamomile populations by testing progenies of selected individuals as mother plants. In each population seeds were taken from 10-10 spontaneously pollinated individuals. In the next year, the 160 strains were propagated at the Experimental and Research Farm of the Corvinus University of Budapest in Soroksár. We measured 4 morphological features sampling 20-20 individuals from each progeny and 4 chemical characteristics from average sample of every strain. By detecting the variability among the progeny strains, assumptions could be made on the genetic variability of the original populations.

The results revealed that the diameter of the inflorescence and of the discus varied only on a very tight scale ($CV_{\%}$<10%) inside the progenies. Similarly, the plant height and size of the ray flowers exhibited a low level of variability, too ($CV_{\%}$=10-20%). The progenies originating from the same wild growing populations showed similar mean values and variability, therefore, it can be assumed that chamomile populations are characterised by relatively low individual genetic diversity referring to the morphological features. At the same time, differences among populations are more considerable.

Polysaccharide content (swelling index) and total-flavonoid content of the families were rather uniform. However, considering the essential oil content and composition, they showed large variances ($CV_{\%}$>20%) which reflects a great genetic diversity of the mother plants and significant polymorphism of the original wild-growing populations.

During our work we found several populations which are perspective based on their advantageous chemical properties for further breeding work.

***Key words**: essential oil, progeny, swelling index, total-flavonoid content, variability*

INTRODUCTION

Chamomile (*Chamomilla recutita* L.) is one of the most important medicinal plants in Hungary. It has been used for centuries in the Carpathian basin, and its usage still has great importance even nowadays. Beside its traditional application forms (internally the decoction is useful against inflammations in the throat and in the stomach, externally it is a well-know remedy against phtalmitis and skin problems) it is also used in the homeopathy, in several cosmetics; there is an increasing demand on chamomile also in the fields of domestic chemistry and food industry as well.

The inflorescence of chamomile contains 0.4-1.2% blue-coloured essential oil, where the main constituents are chamazulene, α-bisabolol, bisabolol-oxide A and B. Further components are cis- and trans-spiroethers, β-farnesene, bisabolon-oxide A and other minor sesquiterpenes (1). Among the non-volatile active agents, flavonoids and mucilage are the most important. Roemisch (2) examined the total flavonoid content of 102 commercially available chamomile drug samples and he measured 1.0-2.5% accumulation levels. Schilcher

(1) found 0,30-2.96% total flavonoid content in the flower drug of 12 chamomile populations. The total mucilage (polysaccharide) content may vary between 3-17% in the drug (3).
Higher ratio of the exported Hungarian chamomile is still coming from collection (wild growing populations of the Great Hungarian Plain), cultivation has less importance. However, the quality parameters of the collected populations have been hardly revealed.
The main aim of our research work was to make a survey on the morphological and chemical features of wild growing chamomile populations collected in the Great Hungarian Plain by applying modern examination techniques for getting appropriate additional information to develop raw material of extra high quality. Further goals were to determine the individual variability of chamomile populations based on the main quality parameters to avoid heterogeneity of the final product.

MATERIAL & METHODS

Basic plant material was collected in May (2006 and 2007), from 16 wild growing chamomile populations of the Great Hungarian Plain in Hungary. In each case seeds were taken from 10-10 cross pollinated individuals. All samples were stored individually in a cooled place until the seed sowing. During the spring of 2008 the propagated progenies (all together 160 strains) were grown in a glass-house and were transplanted into open field at the beginning of April in the Experimental and Research Farm of the Corvinus University of Budapest, in Soroksár.
In our study we compared these 160 strains of 16 original populations (further on: families) under the same environmental conditions in 2008 from morphological and chemical point of view. We investigated the following characteristics:
We measured the **plant height** from the soil surface till the shoot tip of 20, randomly chosen individuals during full flowering. **Diameter of the inflorescence** was measured at 25 randomly selected inflorescences at the end of the primary and secondary sprouts in every strain with ruler in full flowering. We determined the **diameter of the discus** (the size of the middle part of the inflorescence) in case of these flowers too. Difference between the diameter of inflorescence and diameter of discus resulted in the **size of ray flowers**.
For the analysis of chemical properties representative amount of samples with maximum 1 cm stem part, were collected in full flowering stage. The flowers were dried in natural way on trays; then the material was stored on a dry place until the chemical measurements.
Determination of the **essential oil content** was carried out by hydrodistillation using a Clevenger type apparatus. 20 g drug was distilled by 500 ml water for 3 hours. Since the essential oil is easily sticking on the wall of the cooling part of the apparatus, it was washed by hexane. The amount of the essential oil was measured after the evaporation of the solvent, and was expressed as ml/100 g dry material.
Essential oil composition was analysed by gas chromatograph (GC 6890 N) equipped with mass spectrometer (MS 5975), Agilent Technologies. Capillary columns: HP-5 MS (5% phenyl, 95% dimethyl polysiloxane, lenght: 30 m, film thickness: 0.25 μm, id. 0.25 mm). The instrument was programmed as follows: initial temperature 60 °C, then by rate of 3°C/min up to 204°C; the final temperature was kept for 5 min; injector temperature: 250°C, carrier gas: helium (constant flow rate: 1ml/min); split ratio: 30:1. Ionization energy was 70 eV. The mass spectra and linear retention indices (LRI) were compared with those of commercial (NIST) and home-made library mass spectra built up from data obtained from pure compounds.
For the characterisation of mucilage content the **swelling index** (SI) was determined according to the general and specified descriptions (for *Althaeae folium*) of the VIII. Pharmacopoeia Hungarica (4, 5), by using 0.2 dry, powdered drug.

Analysis of the **total flavonoid content** (TFC) was carried out following the specifications of the VIII. Pharmacopoeia Hungarica for *Crataegi folium cum flore* (6).
Because the limited quantity of drug, determination of the essential oil content and composition was made without replications, TFC and SI examinations, were carried out in triplicate for each population.
Data were evaluated using means, standard deviations and coefficient of variation (CV%). Variability of the measured characteristics was evaluated by grouping the values into the following clusters: very homogeneous (CV%<10.0%), homogeneous (CV%=10.0-20.0%), heterogeneous (CV%>20.0%).

RESULTS & DISCUSSION

Morphological features

The average **plant height** of the 16 families varied between 24.0-40.9 cm (St.d.: 4.3-7.0 cm) (Table 1), and in their progenies we found 14.8-48.2 cm mean values (St.d.: 1.5-7.5 cm) during the cultivation under the same environmental conditions. It may refer to the diverse genetic background of wild chamomile individuals. 13 families proved to be homogeneous in terms of the studied characteristics ($CV_{\%}$=11.6-19.4%), and 3 ones were heterogeneous ($CV_{\%}$=20.5-22.5%).
The average **diameter of inflorescence** was 16.5-17.9 mm as the families mean (St.d.:1.1-1.6 mm), and 14.9–18.8 mm as mean values of the progeny strains (St.d.: 0.6-2.8 mm). In point of individual variability we didn't experience significant difference between families, all of them were very homogeneous ($CV_{\%}$=6.2-9.1%) (Table 1). Therefore presumable, that wild chamomile populations have low individual genetic diversity in respect of flower size.
The mean values of the **diameter of discus** in the examined families changed between 5.8 and 6.5 mm (St.d.: 0.4-0.6 mm), while in their progenies we measured mean values between 5.3 and 6.9 mm (St.d.: 0.1-0.8 mm). Every family proved to be very homogeneous according to discus size ($CV_{\%}$=7.3-9.8%), except the family nr. 11 which showed a higher degree of heterogeneity ($CV_{\%}$=10.5) (Table 1).
Examining the **size of ray flowers** in the inflorescence the values varied from 10.5 to 11.7 mm (St.d.: 1.1-1.6 mm) as family means and from 9.5 and 12.8 mm as mean values of the progeny strains (St.d.: 0.7-3.0 mm). Regarding the individual variability each family proved to be homogeneous referring to the this characteristics ($CV_{\%}$=10.5-15.4%) (Table 1). Thus, it may be assumed that wild chamomile populations have a high degree of genetic uniformity with relevance to the flower structure and proportions, too.

Chemical properties

The average **essential oil content** of the examined families varied between 0.44 and 0.63 g/100g (St.d.: 0.05-0.19 g/100g), and in the progenies it's ratio changed between 0.26 and 1.01 g/100g during the investigation. Nine families proved to be heterogeneous ($CV_{\%}$=20.2-29.7%), six ones were homogeneous ($CV_{\%}$=15.2-19.5%) and one family was very homogeneous ($CV_{\%}$=9.9%) in terms of the essential oil content (Table 1). Because of the above mentioned variability of the progenies of the same origin, great genetic diversity of the mother plants and significant polymorphism of the basic, wild-growing populations can be assumed. However, huge differences may occur among the populations, as well.
The average ratio of **α-bisabolol** in the essential oil varied between 8.6-66.4% in the families (St.d.: 2.6-20.0%), while it showed an even larger diversity comparing the values of the progeny strains (4.6-88.3%). This component was detectable in each sample. Regarding the

amount of α-bizabolol, families proved to be heterogeneous ($CV_{\%}$=21.5-84.4%), except two of them (Nr. 10 and 12), which were homogeneous ($CV_{\%}$=11.4-16.7%) (Table 1).
The families' average **bisabolol-oxide A** ratio ranged from 0.5 to 52.5% in the essential oil (St.d.: 0.6-18.9%), and in their progenies we found values between 0.2 and 69.6%. In case of 10 strains we could not detect the presence of this component. It was established that each family was heterogeneous ($CV_{\%}$=22.7-258.1%), except two ones (Nr. 2 and 9) which was homogeneous ($CV_{\%}$=19.0%) (Table 1).
The average content of **bisabolol-oxide B** changed between 4.3 and 18.9% (St.d.: 2.9-15.9%) in the families, while in the progeny strains we measured 0.6-46.1% values. The component was found in each sample. Every group appeared to be heterogeneous, but there were differences in their rates. The family No. 10 was characterized by the lowest variability ($CV_{\%}$=53.2%), while the most variable family was the 15th ($CV_{\%}$=120.4%) (Table 1).
The families' average **chamazulene** ratio in the essential oil varied between 6.5 and 15.3% (St.d.: 2.6-6.7%), while in their progenies the amount ranged between 0.9 and 28.7%. This compound was detected in each samle, too. All of the 16 families proved to be heterogeneous according to the accumulation of chamazulene, but the degree of variability was different. The lowest variability (CV= 20.3%) was measured in family No. 2, and the highest ($CV_{\%}$=70.7%) was found in No. 8 (Table 1).
Families showed 8.3-19.3% mean accumulation level of **cis-spiroether** in the essential oil (St.d.: 1.1-8.9%), and that in their progenies was 2.9-25.5%. This compound was a uniform one too, found in each sample, except a single strain. 13 families were heterogeneous ($CV_{\%}$=21.4-81.0%) and three ones were homogeneous ($CV_{\%}$=13.3-16.5%) referring to the ratio of cis-spiroether (Table 1).
We established, that as the progenies within the families were very heterogeneous according to the ratio of their essential oil's components. Therefore it may be supposed that Hungarian wild growing chamomile populations have a high genetic diversity in this respect, too. The degree of variability, however, proved to be different in each population.
The **swelling index** refering to the polysaccharide content of chamomile inflorescences varied between 32.8 and 50.6 (St.d.: 3.2-16.1) in the examined families, and we measured mean values from 25.8 to 84.2 in case of their progenies cultivated during the same environmental conditions. More than half of families were homogeneous ($CV_{\%}$=11.2-19.9%), five ones were heterogeneous ($CV_{\%}$=22.3-31.8%) and one family was very homogeneous ($CV_{\%}$=8,4) according to the mentioned feature (Table 1).
The average **total flavonoid content** changed between 1.16 and 1.51% (St.d.: 0.09-0.31%) in the families, and we found mean values from 0.67 to 1.91% in the progenies. Seven families proved to be homogeneous ($CV_{\%}$=10.1-13.9%), six ones were very homogeneous ($CV_{\%}$=6.1-9.4%) while families Nr. 3 and 5 were heterogeneous ($CV_{\%}$=21.5-23.9%) based ont he total flavonoid content of the flowers (Table 1).
Based on the findling that only slight variability of swelling index and total flavonoid content was experienced among the strains of most examined familes, it seems that mother plants of the same origin might have similar genotype referring to these features. Presumable, wild chamomile populations may assure a relatively homogeneous drug quality from this point of view.

Table 1. Characteristics of the chamomile populations of wild origin according to the average values of their progenies under cultivation in Soroksár (2008)

Family	Plant height (cm)	Diam. of in-flores. (mm)	Diam. of discus (mm)	Size of ray flo-wers (mm)	EO content (g/100g)	α-bisa-bolol (%)	Bis. oxide A (%)	Bis. oxide B (%)	Cha-mazu-lene (%)	Cis-spiro-ether (%)	SI	TFC (%)
1. Mean	33.8	17.0	6.5	10.5	0.58	17.4	43.6	9.1	12.5	14.0	35.9	1.31
St.D.	5.7	1.2	0.6	1.3	0.12	10.8	13.8	5.5	3.9	4.4	6.0	0.18
CV%	16.9	7.2	9.6	12.4	20.2	62.5	31.6	59.8	31.5	31.8	16.6	13.9
2. Mean	32.3	17.1	6.3	10.7	0.63	13.9	45.1	11.7	13.0	11.7	48.3	1.35
St.D.	6.0	1.1	0.5	1.1	0.19	7.7	8.6	6.4	2.6	4.4	10.8	0.19
CV%	18.7	6.2	8.3	10.5	29.7	55.5	19.0	54.4	20.3	38.0	22.3	13.8
3. Mean	34.2	17.1	6.1	10.9	0.45	17.0	48.3	10.0	13.9	8.3	50.6	1.27
St.D.	5.3	1.4	0.4	1.5	0.07	13.9	12.1	7.6	6.7	1.1	16.1	0.27
CV%	15.6	8.2	7.3	13.4	16.2	81.9	25.0	75.6	48.4	13.3	31.8	21.5
4. Mean	31.3	17.3	6.2	11.1	0.45	11.5	40.6	15.2	15.3	13.1	-	-
St.D.	6.1	1.4	0.5	1.6	0.11	9.7	18.9	12.8	4.6	5.2	-	-
CV%	19.4	8.3	8.1	14.1	23.4	84.4	46.7	84.4	30.1	39.3	-	-
5. Mean	33.2	16.8	5.9	10.9	0.53	8.6	52.5	15.2	10.1	10.2	38.6	1.28
St.D.	5.0	1.4	0.5	1.4	0.12	2.6	11.9	10.5	3.7	3.1	10.0	0.31
CV%	15.0	8.6	9.2	13.2	23.0	30.6	22.7	69.0	36.5	29.9	26.0	23.9
6. Mean	35.4	17.2	6.4	10.7	0.48	35.8	21.3	18.9	10.4	10.2	41.1	1.51
St.D.	5.2	1.5	0.6	1.5	0.08	16.6	11.4	11.9	6.0	2.7	5.2	0.20
CV%	14.7	8.6	9.4	14.2	16.7	46.3	53.4	63.0	57.4	26.4	12.6	13.1
7. Mean	40.9	17.4	6.1	11.3	0.44	38.5	23.2	12.2	7.4	12.5	39.8	1.30
St.D.	4.7	1.4	0.4	1.5	0.11	12.2	9.8	7.5	4.0	3.1	5.6	0.10
CV%	11.6	8.3	7.3	13.4	23.8	31.7	42.4	61.4	54.7	24.9	14.1	7.7
8. Mean	30.8	17.2	6.1	11.1	0.54	38.4	23.5	15.4	8.4	10.7	32.8	1.20
St.D.	5.4	1.5	0.5	1.5	0.11	19.8	16.3	13.2	5.9	3.4	3.7	0.11
CV%	17.4	8.5	7.6	13.3	19.5	51.5	69.5	86.1	70.7	31.9	11.2	8.9
9. Mean	30.6	17.0	6.0	11.0	0.44	54.2	1.3	16.9	8.8	10.2	43.4	1.21
St.D.	4.9	1.3	0.6	1.3	0.11	20.0	1.7	15.9	5.6	4.6	6.5	0.12
CV%	15.9	7.9	9.2	12.2	25.5	37.0	1.28	94.0	64.4	44.9	15.0	10.1
10. Mean	24.2	17.0	6.2	10.9	0.50	60.4	1.7	4.3	6.9	9.5	38.3	1.34
St.D.	4.7	1.4	0.5	1.5	0.08	10.1	4.3	5.1	4.1	3.6	3.2	0.12
CV%	19.3	8.0	8.9	14.1	17.0	16.7	258.1	120.4	59.6	37.9	8.4	9.2
11. Mean	26.5	16.5	6.0	10.5	0.48	53.8	4.6	13.8	10.1	11.3	41.3	1.27
St.D.	4.3	1.5	0.6	1.6	0.07	14.3	5.2	12.7	3.8	2.0	5.4	0.16
CV%	16.1	9.0	10.5	15.4	15.3	26.5	114.4	92.1	37.2	17.5	13.2	12.4
12. Mean	24.0	16.8	5.8	11.0	0.45	66.4	0.5	4.7	9.9	11.0	36.5	1.32
St.D.	5.4	1.5	0.5	1.5	0.09	7.5	0.6	2.9	5.6	8.9	5.6	0.18
CV%	22.5	9.1	8.5	13.7	20.2	11.4	115.7	61.7	56.4	81.0	15.4	13.3
13. Mean	28.3	17.0	6.0	11.0	0.47	42.2	7.1	16.0	9.4	14.3	35.6	1.16
St.D.	5.3	1.3	0.5	1.3	0.08	13.1	6.8	10.7	2.7	2.4	8.0	0.11
CV%	18.8	7.4	8.4	11.9	17.2	30.9	95.8	67.1	28.7	16.5	22.3	9.4
14. Mean	30.2	17.9	6.3	11.7	0.47	44.0	3.5	16.1	6.5	19.3	41.8	1.36
St.D.	6.2	1.5	0.6	1.5	0.12	11.2	2.1	9.7	3.0	4.1	10.8	0.15
CV%	20.5	8.6	9.4	13.0	25.2	25.5	60.7	60.4	45.6	21.4	25.9	10.9
15. Mean	38.7	17.7	6.3	11.4	0.48	36.5	14.3	18.1	8.2	14.9	38.4	1.45
St.D.	7.0	1.6	0.6	1.6	0.10	11.5	8.1	9.6	3.8	4.3	4.6	0.09
CV%	18.2	9.1	9.2	14.2	20.4	31.6	56.7	53.2	46.3	28.7	12.0	6.1
16. Mean	24.7	17.0	6.1	10.9	0.50	62.6	1.6	6.6	10.3	13.5	41.3	1.34
St.D.	5.1	1.3	0.6	1.5	0.05	13.5	1.0	4.2	4.2	6.0	8.2	0.10
CV%	20.8	7.8	9.8	13.6	9.9	21.5	59.7	63.4	41.0	44.2	19.9	7.6
Average CV%	17.6	8.2	8.8	13.3	20.2	40.3	67.0	72.9	45.6	33.0	17.8	12.1

CONCLUSION

In case of the individual genetic diversity of chamomile populations we came to the conclusion that the progenies of spontaneously pollinated mother plants were rather homogeneous based on the diameter of the inflorescence ($CV_{\%}$=8.2) and the discus ($CV_{\%}$=8.8). With relevance to the plant height and size of the ray flowers they were also homogeneous ($CV_{\%}$=17.6 and 13.3). Progenies with the same origin showed the same variability, therefore, it can be ascertained that chamomile populations are characterised by relatively low individual genetic diversity referring to the morphological features; however, a slight variability still exists among the populations of different origin.

Referring to the chemical characteristics based on the SI and total flavonoid content the 16 families of different origin were homogeneous ($CV_{\%}$=17.8 and 12.1), however, with relevance to the essential oil content and composition they were more different ($CV_{\%}$=20.2-72.9). As the progenies of the same origin showed a high variability of their essential oil accumulation rate and compositional spectrum too, presumably the individuals of the wild-growing populations possess a considerable genetic polymorphism for these traits.

Summarizing our results we came to the conclusion that wild growing chamomile populations are rather variable especially referring to the chemical characteristics. Therefore good quality, homogeneous plant material is difficult to be produced by collection, which method is useful only for assuring common quality characteristics. The collection has to be restricted to those populations having appropriate chemical properties. In the bigger collection zones, based on the quality control done parallel with the primary processing, the different quality plant material needs to be handled as individual batch. Further solution can be that one part of the demand on the drug is satisfied by cultivation of those cultivars characterised by “traditional quality”.

For further breeding work 12 strains of the families signed 10, 14 and 16 can be perspective based on their essential oil content and composition, therefore, their seeds were taken into our gene-bank.

ACKNOWLEDGEMENTS

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Original scientific paper

ANTIMICROBIAL AND GENOTOXIC PROPERTIES OF ESSENTIAL OILS FROM *ACORUS CALAMUS* LINN.

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SUMMARY

Antimicrobial activity of the essential oils (EOs) obtained from the rhizome and roots of *Acorus calamus* Linn. against Gram-negative and Gram-positive bacteria, yeasts and fungi was investigated using disc diffusion method and microdilution methods. The EOs of both rhizome and roots exhibited a pronounced antifungal activity against tested dermatophytic and phytopathogenic fungi, mainly *Microsporum gypseum* and *Botrytis cinerea* as well as anti-yeast activity against *Rhodotorula glutinis*, *Candida albicans* and *Saccharomyces cerevisiae*. The rhizome essential oil has shown antibacterial activity against wider range of bacteria, but the oil from the roots was more effective in lower concentrations. In comparison, both EOs had stronger antifungal and anti-yeast activity than their antibacterial activity. Both EOs were non-mutagenic as determined by Ames test using *Salmonella typhimurium* test strains.

Key words: *Acorus calamus, antimicrobial, antifungal*, genotoxic

INTRODUCTION

Sweet flag (*Acorus calamus* Linn.) is a perennial plant belonging to the family of *Asteraceae* (Arum family). It grows up to 6 feet tall with sword-shaped leaves, small yellow/green flowers and branched rhizomes. The rhizomes, roots, leaves and essential oil distilled from these plant parts have been reported to posses important biological activities (1). Many authors described their antimicrobial properties in varying levels. Some authors described strong antifungal effect and low activity on bacteria (1-3), others described also antibacterial activities (4).

The purpose of this work was to estimate and compare the potential effect of the essential oils from the roots and rhizome of *Acorus calamus* on the growth of selected bacteria, yeasts and fungi, as well as to estimate potential genotoxic effect of these oils.

MATERIAL & METHODS

Essential oil isolation: The plants were harvested in the phase of full flowering and the roots and rhizomes were manually separated. The air-dried roots and rhizomes (50 g) were subjected to hydrodistilation for 4 h in distillation apparatus constructed in accordance with the European Pharmacopoeia (5). Isolated oil was diluted in n-hexane and dried over anhydrous sodium sulphate. Oil samples were analyzed using a Hewlett Packard HP 5971. A mass selective detector directly coupled to a gas chromatograph HP 5890 Series II FID was used. A capillary column DB-WAX/26m x 0.20 mm, 0.2 mm film thickness (Hewlett Packard, USA) was used.

Bacterial and fungal strains: Strains were obtained from Czech Collection of Microorganisms (Brno, Czech Republic). Four strains of Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium, Proteus mirabilis*, *Pseudomonas aeruginosa*) and two strains of Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) were used. For estimation of anti-yeast activity, *Candida albicans*, *Saccharomyces cerevisiae*, and *Rhodotorula glutinis* were used. For estimation of antifungal activity, filamentous fungi *Aspergillus flavus*, *Penicillium funiculosum*, *Fusarium nivale*, *Alternaria alternata*, *Microsporum gypseum*, and *Botrytis cinerea* were used. The Ames mutagenicity test was performed using *Salmonela typhimurium* TA98 and TA100.

Evaluation of antibacterial and anti-yeasts activity: Disc Diffusion Method – nutrient agar plates were swabbed with the respective broth culture of the organism (diluted to 0.5 McFarland standards with saline). Filter paper disc (6 mm in diameter) were impregnated with 5 µl of the oil and placed on the inoculated plates. These plates, after staying at 4°C for 2 h, were incubated at 37°C for 24 h for bacteria and at 28°C for the yeasts. The diameters of the inhibition zones were measured in millimeters. Minimum inhibitory concentrations (MICs) of the oils were assessed by microdilution method. Briefly, from the exponentially growing bacteria 2% inoculum was prepared. 180 µl of this suspension was added to 20 µl of increasing concentrations of oils *per* well in 96-well round bottom microplates. The final concentration of solvent in the culture medium was maintained at 0.1% (v/v) to avoid solvent toxicity. The assay plates were incubated at 37°C for 24 h and the growth kinetic assays for each strain were performed triplicate by growth curves observed as turbidity determined by a microplate reader (Varioskan Flash, Thermo) at 650 nm. MIC results for the oils were reported as %.

Antifungal activity: 300 µl of spore suspension were added to 15 ml of slightly cooled agar cultivation medium and poured in sterile Petri dishes. After solidifying, filter paper disc (6 mm in diameter) were impregnated with 5 µl of the oil and placed on the inoculated plates. After cultivation, the diameters of the inhibition zones were measured in millimeters.

Mutagenicity test: The plate-incorporation method (6) with *Salmonella typhimurium* TA98 and TA100 was used. 0.1 ml of cell suspension (16 h overnight culture, approximate cell concentration 2-5 × 10^8 cells *per* ml) and 0.1 ml of the oils were added to 2 ml of melted top agar containing 50 µmol/l L-histidine and 50 µmol/l biotin. The mixture was poured into plates containing minimal medium and incubated for 2 days at 37°C, then the number of histidine – independent revertants were counted. Positive and negative controls were included in every experiment. A positive response was defined as a reproducible two-fold increase of revertants with dose-response relationship and statistical evaluation using Student´s *t*-test.

RESULTS & DISCUSSION

Essential oils from rhizome and roots contained: Beta-asarone, camphor, borneol, spatulenole, geranyl-acetate, and other sesquiterpenes in small content. Chemical composition of essential oil was published in our previous work (7).

The essential oils from the rhizome and roots were screened for activity against Gram-positive bacteria, Gram-negative bacteria, yeasts and fungi by agar disc diffusion method. The obtained results are in Fig. 1. Of the 15 species of fungi, bacteria and yeasts tested, *Microsporum gypseum* and *Botrytis cinerea* were most susceptible to the essential oils. Both oils displayed remarkable antifungal activity, but differed in their activities against the various fungi tested. They had no inhibitory effect on the growth of *Aspergillus flavus*, but strongly inhibited sporulation of this mold. Anti-yeast activity was observed against all three tested yeasts in order from the most susceptible *Rhodotorula glutinis* > *Candida albicans* > *Saccharomyces cerevisiae*. There were only slight differences between the rhizome and roots

oils in point of view of anti-yeast and antifungal activity. The potential antibacterial activity of the oils was clearly lower. The rhizome oil had a moderate inhibitory effect on all tested Gram-negative and Gram-positive bacteria with the exception of *Proteus*. The highest inhibitory effect had on *Salmonella* Less effective was the roots oil, which had no inhibitory effect on Gram-negative bacteria *E. coli* and *Pseudomonas*, but on the other hand, had a more pronounced effect on Gram-positive bacteria *Bacillus* and *Staphylococcus* in comparison with the rhizome oil. In summary, both oils inhibited namely *Salmonella* and Gram-positive bacteria. For *Staphylococcus aureus*, minimum inhibition concentration (MIC) was estimated for the rhizome oil as 0.62% and for the roots oil as 0.31%. In the case of *Salmonella typhimurium*, MIC values was 1.25% for both oils.

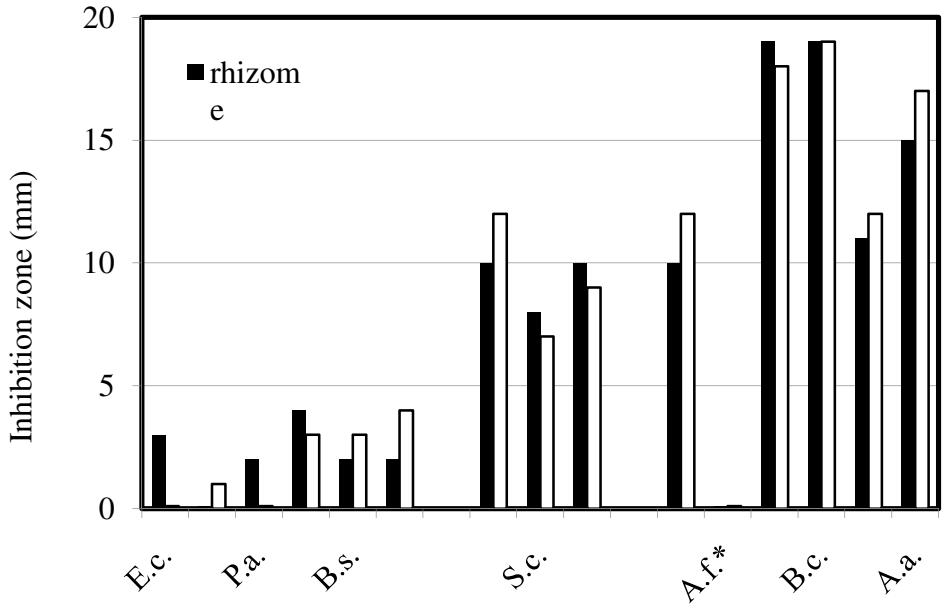

Fig. 1. Antimicrobial effect of the oils. E.c.-*Escherichia coli*, P.m.-*Proteus mirabilis*, P.a.-*Pseudomonas aeruginosa*, S.t.-*Samonella typhimurium,* S.a.-*Staphylococcus aureus*, R.g.-*Rhodotorula glutinis*, S.c.-*Saccharomyces cerevisiae*, C.a.-*Candida albicans*, P.f.-*Penicillium funiculosum*, A.f.-*Aspergillus flavus*, *inhibition of sporulation, M.g.-*Microsporum gypseum*, B.c.–*Botrytis cinerea*, F.n.-*Fusarium nivale*, A.a.-*Alternaria alternata.*

Table 1. Estimation of potential mutagenic activity of the oils

EO	Dose	TA98		TA100	
	(%)	M	±SD	M	±SD
Rhizome	0	24	1.37	150	6.11
	0.08	24	2.42	145	11.76
	0.16	23	2.17	156	7.25
	0.31	26	2.07	110	6.53
	0.62	14	1.03	69	12.14
	1.25	1	0.52	9	4.72
Roots	0	24	1.37	150	6.11
	0.08	25	2.66	149	7.99
	0.16	22	1.93	143	12.20
	0.31	20	2.5	129	6.06
	0.62	9	2.86	79	7.09
	1.25	0	0.60	0	0.82
	PC*	371	35.79	1163	114.02

*PC –positive control 3-(5-nitro-2-furyl) acrylic acid

Potential mutagenic activity of the oils was evaluated using plate incorporation assay according Maron and Ames (7). The oils were tested in concentrations, which were not toxic to *Salmonella typhimurium*. As is shown in Table 1, oils from rhizome or roots didn´t significantly increased the number of revertants in both *Salmonella* tester strain TA98 and TA100 and exhibited no mutagenic activity under the present experimental conditions.

CONCLUSION

This work was not aimed to estimate of chemical composition of essential oil but to compare susceptibility of bacteria, yeasts and fungi to essential oils from *Acorus calamus.* Both EOs have stronger antifungal and anti-yeast activity than antibacterial activity. Rhizome oil has shown antibacterial activity against wider range of bacteria, but oil from roots was more effective already in lower concentrations. Both EOs were nonmutagenic, determined by Ames test using *Salmonella typhimurium* test strains.

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Original scientific paper

QUALITATIVE PROPERTIES AND ANTIMICROBIAL ACTIVITY OF *CHAMAEMELUM NOBILE* (L.) All.

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SUMMARY

The content, composition and antimicrobial activity of essential oils (EOs) from the flowering parts of *Chamaemelum nobile* (L.) All., collected from three different locations in Slovakia were evaluated during four main phases of plant development.Content of essential oils in flower parts varied between 0.02-2.76% (v/w) and the highest was in second development phase in ligulate flowers. The location had no influence on the content of EOs. EOs of *Chamaemelum nobile* flower heads were assayed for *in vitro* antimicrobial activity against Gram-positive bacteria (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli* and *Proteus mirabilis*) and yeasts (*Saccharomyces cerevisiae* and *Candida albicans*) using disc diffusion method. The minimum inhibition concentrations (MICs) of oils in bacteria and yeasts were determined by the microdilution method.

***Key words**: Chamaemelum,, essential oils, antimicrobial activity*

INTRODUCTION

Chamaemelum nobile (L.) All., the so-called Roman chamomile, is a perennial herb of the Aster family. It is native to the Southwest Europe, but the plant is present in all over Europe, North Africa and Southwest Asia. Plant in nature grow on dry sunny places, especially on calcereous substrates, on wood-steppe hillsides, bushes, they are able to vegetate on infertile stands. It reaches the height of 15 to 30 cm and generally flowers from June to September. External morphologic signs are changing therefore, this plant is suitable for study of versatility and morphologic variability during ontogenesis. *Chamaemelum nobile* found broad use in the pharmaceutical and cosmetic industry and food industry as well. Beside many favoring properties, some authors described also antimicrobial activity of some kinds of extracts or of essential oils (1-3).

The purpose of this work was to estimate the content of essential oils in four main phases of plant development and to estimate their antimicrobial activity.

MATERIAL & METHODS

Collection of flowers was done in four main, according to the level of development of flower head. Material was continuously arranged, distributed into groups and processed.The content of EO was estimated by hydrodistilation with addition of *n*-heptane. Qualitative and quantitative analyses of EO were done using gas chromatography and gas chromatography and mass spectroscopy.

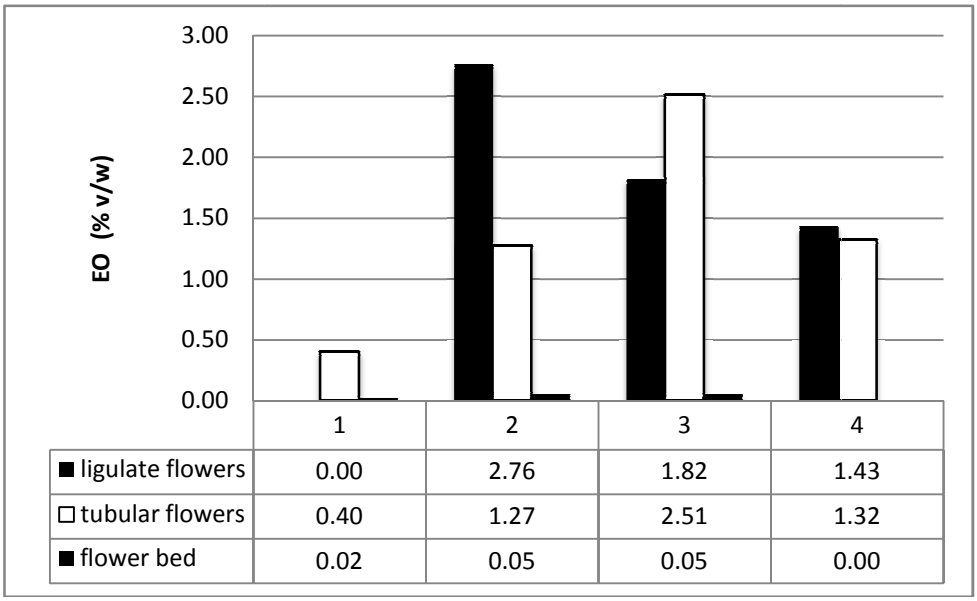
Bacteria *Staphylococus aureus, Bacillus subtilis*, *Escherichia coli, Proteus mirabilis* and yeasts *Saccharomyces cerevisiae* and *Candida albicans* were from Czech Collection of Microorganisms Brno, Czech Republic.

Antimicrobial activity was estimated using disc diffusion method and microdilution methods in 96 well plates. The growth kinetic assays for each strain were performed triplicate by growth curves observed as turbidity determined by a microplate reader (Varioskan Flash, Thermo) at 650 nm. MIC results for oils were reported as %.

RESULTS & DISCUSSION

In the work we have studied the content, composition and antimicrobial activity of the essential oil of *Chamaemelum nobile*. Plant were harvested from three different localities in Slovakia, however content of oils differed only slightly regarding to location. Changing climate and agro-technical conditions in given location influence the length of flowering. In the process of flowering occurs flowering of different parts of flower head during which time the appearance and position of ligulate flowers and the shape of the flower bed have been changing. The flower heads were harvested at four main phases of plant development and essential oils were isolated by hydrodistilation. The percentage of oil content in parts of the flower head is in Fig.1. Content of essential oil varied from 0.02 – 2.76% v/w. Low content of oil was regarded at the first development phase. At the second phase of flowering, percentage of oil was 1.53% in all flower head, ligulate flowers contained 2.76%, tubular flowers contained 1.27% and in flower bed we found only trace amount. Slightly different situation we regarded at the full flowering phase, when higher content of oil was in tubular flowers. In total, the highest content of oils reached ligulate flowers in the second development phase.

The essential oil obtained from the flowering parts of *Chamaemelum nobile* was analyzed by gas chromatography and gas chromatography and mass spectroscopy. In this study, 34 compounds representing 98.80% of the essential oil were identified. The main components were isobutyl butyrate, isobutyl angelate, methyl isobutyrate, α-pinene, myrcene, isoamyl angelate, and pinocarvone.



	1	2	3	4
ligulate flowers	0.00	2.76	1.82	1.43
tubular flowers	0.40	1.27	2.51	1.32
flower bed	0.02	0.05	0.05	0.00

Fig.1. Content of EO (%v/w) in some parts of flower heads of *Chamaemelum nobilis* during four main phases of flowering.

For the estimation of potential antimicrobial effects of essential oil from *Chamaemelum nobile* we used oils from ligulate flowers at the second development stage (L2) and from tubular flowers at the third development phase (T3). As seen in Table 1, oils possessed only very slight activity. The diameter of inhibition zone (IZ) including 6 mm disc, in Gram-positive bacteria and yeasts was 8-9 mm and in Gram-negative bacteria was only 6-7 mm. MIC (minimal inhibition concentration – the lowest concentration of compound, that fully inhibited growth of microorganism) of oils was in all tested microorganisms >2.5%.

Table 1. Antibacterial and anti-yeast activity of selected oils

	S. aureus		*B. subtilis*		*E. coli*		*P .mirabilis*		*S. cerevisiae*		*C. albicans*	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
L2	8	>2.5	9	>2.5	6	>2.5	7	>2.5	9	>2.5	9	>2.5
T3	9	>2.5	9	>2.5	6	>2.5	6	>2.5	8	>2.5	9	>2.5

IZ - inhibition zone (mm), MIC - Minimal inhibition concentration (%)

CONCLUSION

Content of essential oils in *Chamamelum nobilis* (L.) All. depends on the development stage of flowering and on the type of flower. The locality influenced the content of EO in lower ratio. Oils possess only very slight antimicrobial effect.

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Original scientific paper

PROTECTIVE EFFECT OF CAMPHOR, EUCALYPTOL AND THUJONE AGAINST UV- AND 4NQO-INDUCED GENOTOXICITY IN BACTERIA AND MAMMALIAN CELLS

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SUMMARY

It has been shown that active substances from medicinal and aromatic plants, especially terpenes, possess protective effects against environmental and endogenous genotoxic agents and might be used for prevention of important human diseases, including cancer.

In this work the dominant monoterpenes from sage (*Salvia officinalis* L.): Camphor (Cam), Eucalyptol (Euc) and Thujone (Thu) were investigated for their antimutagenic/ antigenotoxic properties. *Escherichia coli* K12 reverse mutation assay on repair proficient and nucleotide excision repair (NER) deficient strains was used to evaluate their antimutagenic potential against UV-induced mutagenesis. Comet test on repair proficient mammalian Vero cells was used to evaluate their antigenotoxic potential against UV-mimetic mutagen 4NQO. The cells were first treated with monoterpenes (pre-treatment) and then mutagen was applied.

Protective effect of monoterpenes was detected in both repair proficient bacterial and mammalian cells, with the highest inhibition of genotoxicity obtained at low doses. However, in NER deficient bacteria antimutagenic effect was diminished and Cam and Euc were even co-mutagenic. This was in line with our previously obtained data, when post-treatment with monoterpenes had strong protective effect at low doses and genotoxic effect at high doses. Taken together, the results indicate that Cam, Euc and Thu at low doses can stimulate error-free DNA repair processes, especially NER, leading to inhibition of mutagenesis.

Due to increasing destruction of atmospheric ozone layer which significantly increases the UV-index, obtained data encourage further research of monoterpenes as chemopreventive agents potentially useful in skin protection.

***Key words**: monoterpenes, antimutagenesis, UV, 4NQO*

INTRODUCTION

In order to protect human health, a relatively new area of research, designated as antimutagenesis/anticarcinogenesis, is continuously developing. The aim of antimutagenesis studies is to identify natural substances with antigenotoxic, antimutagenic and anticarcinogenic potential and to determine the cellular and molecular mechanisms of their action. Different tests, routinely used to detect environmental mutagens, are suitably adapted for antimutagenesis research. Possible application of plant antimutagens is in development of dietary and pharmaceutical supplements useful in primary prevention of mutation related diseases, including cancer [1, 2].

Terpenes $(C_5H_8)_n$ are the largest group of natural substances, biosynthetically derived from isoprene units [3]. They are abundantly found in fruits, vegetables, aromatic and medicinal plants, where their main function is protection against infections, parasites and other stress conditions [4, 5]. Moreover, they are endowed with many beneficial health effects and can be

used to treat different health disorders [6]. It has been shown that terpenes are important cancer chemopreventive and chemotherapeutic agents [7, 8].
Numerous studies indicate protective capacity of terpenes against endogenous sources of DNA lesions, as well as against environmental genotoxic agents, indicating their possible use in primary prevention of many mutation related diseases [2, 9].
The monoterpenes investigated in this study: Camphor (Cam), Eucalyptol (Euc), and Thujone (Thu) are widely distributed in essential oils of many medicinal and aromatic plants. They possess strong antimicrobial [10-14], and Euc also cytotoxic, anti-inflamatory, gastroprotective and hepatoprotective properties [15-18]. Cam is commonly applied to the skin for its antipruritic, analgesic and counterirritant properties [19] and used as a nasal decongestant and cough suppressant [20]. Euc is commonly used as a bronchodilator, to treat bronchitis and asthma, as well as for the treatment of sinusitis and chronic rhinitis [21]. Although the neurotoxic effect of Thu in mammals is well established [22, 23], reported data indicate that plants containing Thu, i.e. *Artemisia, Salvia, Thuja* spp. can be used for medical purposes [24-27]. In our previous work we have determined that Cam, Euc and Thu are potent antimutagenic agents which can reduce mutagenesis induced with UV and "UV mimetic" 4-Nitroquinoline-1-oxide (4NQO) and proposed molecular mechanism of their protective action [28, 29]. The aim of this work was to further investigate molecular mechanism of antimutagenic and antigenotoxic effect of Cam, Euc and Thu.

MATERIAL AND METHODS

Chemicals

D, L-Camphor (Cas No. 76-22-2, Alfa Aesar), Eucalyptol (Cas No. 207–431-5, Fluka) and α,β-Thujone (Cas No. 76231-76-0, Sigma Aldrich) were freshly dissolved in dimethyl sulphoxide (DMSO) for bacterial assay, or in ethanol for comet assay. 4-Nitroquinoline-1-oxide (4NQO, Cas No. N-8141, Sigma Aldrich) was dissolved first in DMSO and then, immediately before use, ten-fold diluted in distilled water for antimutagenicity assay, or in Dulbecco's Phosphate Buffered Saline without Ca and Mg (PBS buffer, PAA Laboratories GmbH, Austria) for comet assay.

Bacterial and eukaryotic cell cultures

E. coli K12 repair proficient strain SY252 (*argE3*) [30] and its NER deficient counterpart IB105 (*uvrA*::Tn*10*) [31] were used in this study. The Vero cell line obtained from the kidney of a normal adult African green monkey (ECACC No: 88020401) was a gift from the Institute of Virology, Vaccines and Sera - Torlak, Belgrade, Serbia.

Media and growth conditions

Media and growth conditions for both bacteria and Vero cells were as previously described by Nikolić et al. [29].

UV-irradiation conditions

UV-irradiation was carried out as previously described by Nikolić et al. [29].

E. coli K12 reversion assay

The overnight cultures of SY252 and IB105 strains were diluted 15× in LB medium and incubated for 120 min with a rising set of concentrations of test-substances at 37°C with aeration at 150 rpm. Afterwards, the cells were washed by centrifugation at 1700 g and re-suspended in the same volume of 0.01 M $MgSO_4$. For detection of UV-induced mutagenesis, the cell suspension was treated with appropriate UV dose (28 J/m2 for SY252 and 3 J/m2 for IB105). Samples (0.1 ml) of unirradiated and UV-irradiated cells, appropriately diluted for determination of cell survival and Arg^+ revertants, were added to 3 ml of molten top agar, mixed and poured in triplicates onto 3% SEM plates and incubated at 37°C for 48h. Two control groups were made: distilled water as negative control and DMSO as a solvent control.

Antigenotoxicity assay in mammalian cells

The Vero cells (5×10^4 cell/ml) were grown in adequately supplemented MEM medium in flasks (TC 25, NUNC, Germany) until cell monolayer became confluent (24h, at 37°C, in 5% CO_2 atmosphere, with 100% humidity). To evaluate antigenotoxicity, the cell monolayer was first incubated in a medium containing monoterpenes for 20h at 37°C. After that monoterpenes were removed, medium containing 4NQO (5 μM) was added and cells were incubated for 1h at 37°C in. The monolayer was harvested and cells were centrifuged at 100*g* for 10 min and resuspended in PBS buffer. Cell viability was measured by trypan blue dye exclusion method [32] and cell density was adjusted to 3×10^5 cell/ml. 2×1 ml of cell suspension was centrifuged at 100*g* for 5 min, pellet was re-suspended in 2×30 μl PBS buffer and used for the alkaline comet assay.

Comet assay

The alkaline comet assay was performed as previously reported by Nikolić et al. [29]. The microscopic slides were pre-coated with 0.5% NMP agarose (Eurobio, France) and air dried for 24h at room temperature. Cell suspension (30 μl) was mixed with 70 μl of 1% LMP agarose (Bio-Rad Laboratories, USA) and added to slides that had previously been coated with one layer of 1% NMP agarose. The slides were covered with a glass coverslips, placed at 4°C for 5 min and after that coverslips were gently removed, and slides were submerged into ice-cold lysing solution (2.5 M NaCl, 0.1M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10) and placed at 4°C for at least 1h. After lysis, the slides were placed in a freshly made ice-cold electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at 4°C to allow DNA unwinding and expression of alkali-labile sites. Following that, the samples were electrophoresed for 20 min at 25V and 300 mA, at 4°C and then were neutralized with 0.4 M Tris buffer (pH 7.5) for 15 min at 4°C. Each slide was stained with ethidium bromide (5 μg/ml) and visualized using fluorescence microscope (Leica, DMLS, Austria) with an excitation filter of 510-560 nm, barrier filter of 590 nm, at 400x magnification. Image analysis software (Comet Assay IV, Perceptive Instruments, UK) was used for comet analysis. Fifty nuclei per experimental point in each of the three independent experiments were analyzed; the tail intensity was scored as a reflection of DNA damage.

Calculations

The inhibition of mutagenesis in the bacterial assay was calculated according to the equation: %I (% of inhibition) = (1 - T/C) × 100, where T was the number of revertants *per* plate in the presence of test substance, and C was the number of revertants *per* plate in the solvent control. The inhibition of genotoxic effect in Comet assay was calculated according to the same equation, where T was tail intensity obtained in the presence of test substance, and C was tail intensity obtained for the solvent control. The antimutagenic/antigenotoxic effect was considered strong when inhibition of mutagenesis/genotoxicity was higher than 40%, moderate when it was in the range between 25% and 40%, and weak or absent when inhibitory effect was less than 25% [33].

Statistical analyses

The Student's *t*-test was used for statistical analysis of the data obtained in bacterial antimutagenicity assay. For the result of the Comet assay, the one-way analysis of variance (non-parametric ANOVA, Kruskal-Wallis test) was used to analyze differences between the treatments within each experiment. Statistical analyses were performed using SigmaStat 3.1 software. The significance was tested at $p<0.05$ level.

RESULTS AND DISCUSSION

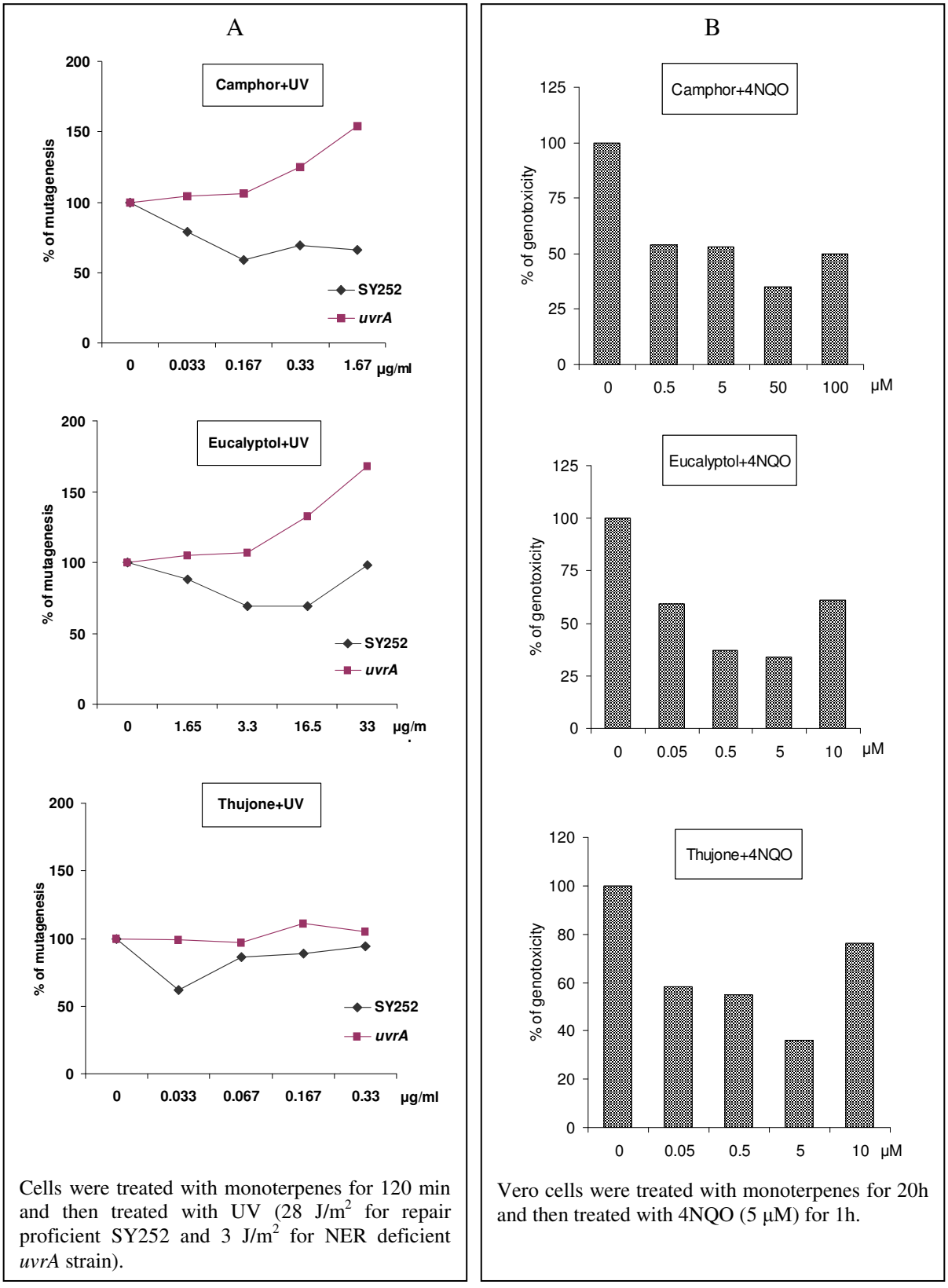
In our previous work, we have demonstrated that post-treatment with Cam, Euc and Thu strongly reduced UV- and 4NQO-induced mutagenesis in *Escherichia coli* K12 reversion assay, and showed significant protective effects against 4NQO-induced genotoxicity in Comet assay on Vero cells. We determined that they act as bioantimutagens – agents which modulate DNA replication and repair and prevent processing of premutagenic lesions into mutations [34]. Considering the experimental analysis of DNA repair mechanisms involved in antimutagenesis/antigenotoxicity of monoterpenes, we hypothesized that, by making a small amount of DNA lesions, low concentrations of monoterpenes stimulated error-free DNA repair, especially nucleotide excision repair (NER) and, therefore, reduced genotoxicity induced by UV or 4NQO [29].

This work was conducted in order to test proposed hypothesis. We analyzed if pre-treatment with low doses of monoterpenes could induce DNA repair mechanisms and protect from subsequent exposure to genotoxic agent. In reversion assay on repair proficient and NER deficient strain of *E. coli* K12 we evaluated antimutagenic effect of pre-treatment with Cam, Euc and Thu against subsequent UV irradiation. In alkaline comet assay on repair proficient Vero cells we monitored 4NQO-induced genotoxicity following pre-treatment with monoterpenes.

Results obtained in bacterial assay (Fig. 1A) indicated similar response obtained with all three monoterpenes. Cam, Euc and Thu reduced UV-induced mutagenesis only in repair proficient strain and maximum reduction was 41%, 31% and 38%, respectively. Moreover, protective effect against 4NQO-induced genotoxicity, with about 65% of inhibition, was obtained in Comet assay on Vero cells (Fig. 1B). Interestingly, the strongest protection in both prokaryotic and eukaryotic cells was obtained at low doses of monoterpenes resulting in U-shaped concentration-response curves. This kind of response, usually interpreted as indication of mutagenicity/genotoxicity of high concentrations of the agent, was expected since genotoxicity of high doses of monoterpenes was determined in our previous study [29]. As presented in Fig. 1A, none of the monoterpenes was antimutagenic in NER eficient genetic background; moreover, clear evidence of co-mutagenic effect was obtained with Cam and Euc. This result confirms the importance of NER proficiency for antimutagenic influence of monoterpenes.

In order to further strengthen our hypothesis, we evaluated if low concentration of positive genotoxic agent could induce DNA repair processes and protect from subsequent stronger genotoxic influence. The effect of 4NQO pre-treatment on UV-induced mutagenesis was monitored in repair proficient *E. coli* K12 strain. The results obtained (Fig. 2) provided evidence that even model mutagen, when it is applied in sufficiently low doses, can induce bioantimutagenic response. In our opinion, obtained result strongly supports proposed mechanism of bioantimutagenicity for Cam, Euc and Thu.

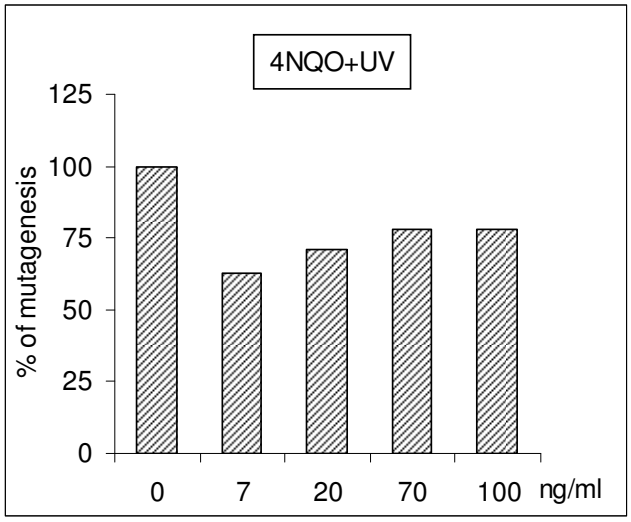
Figure 1. Antimutagenic (A) and antigenotoxic (B) effect of monoterpenes.



Cells were treated with monoterpenes for 120 min and then treated with UV (28 J/m^2 for repair proficient SY252 and 3 J/m^2 for NER deficient *uvrA* strain).

Vero cells were treated with monoterpenes for 20h and then treated with 4NQO (5 µM) for 1h.

Figure 2. Effect of 4NQO on subsequent UV irradiation in repair proficient SY252 strain



Taken together, our present and previously reported data support hormesis phenomenon, defined as beneficial response to a low dose of a stressor agent [35]. Hormesis is now generally accepted as a real and reproducible biological phenomenon, being highly generalized and independent of biological model, end-point measured and chemical/physical stressor applied [36].

Hormesis hypothesis could successfully explain controversial literature and our data concerning genotoxicity/antigenotoxicity of monoterpenes. No mutagenicity of Cam and Euc was detected in the *Salmonella*/microsome assay [28, 37, 38]. No DNA damage by Euc was observed in cultured Chinese hamster ovary cells [39], and in human leukemic K562 cells [40]. Cam did not induce significant mutagenicity in bone marrow cells of pregnant rats [41]. However, in SMART test Cam was genotoxic [42] and Euc induced apoptosis in two human leukemia cell lines and inhibited DNA synthesis in plant cells [43, 44]. Although no genotoxicity of Thu was detected in SMART test [42], Kim et al. [45] showed co-mutagenic effect of Thu on aflatoxin B1-induced mutagenesis in *S. typhimurium* TA100. On the other hand, Cam reduced γ-radiation-induced increase in SCE frequency in mice bone marrow cells [46] and Cam and Euc reduced mutagenesis induced by several model and environmental mutagens in *Salmonella*/microsome reversion assay [38, 45]. Besides our results, no antigenotoxic effect of Thu was reported, but there is evidence about antigenotoxicity of plant extracts containing high proportion of Thu [47, 48]. It is clear that antimutagenic and antigenotoxic features of tested monoterpenes depend on the cell type, genetic background, mutagen applied and other experimental conditions. Moreover, our results indicate the special importance of applied concentrations for antimutagenic response.

CONCLUSION

In conclusion, pre-treatment with low doses of monoterpenes Cam, Euc and Thu can stimulate DNA repair processes, mainly NER. Therefore, they can act as bioantimutagens and significantly protect from subsequent treatment with stronger genotoxic agent, such as UV and 4NQO. Taking into consideration that the increasing destruction of atmospheric ozone layer significantly increases the UV-index, obtained data encourage further research of monoterpenes as chemopreventive agents potentially useful in skin protection. However, their genotoxicity must be taken in consideration and carefully analyzed.

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Original scientific paper

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF *THYMUS PANNONICUS* SSP. *AUCTUS* (LYKA) SOÓ ESSENTIAL OIL

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SUMMARY

The aim of this study is to investigate the chemical composition of *Thymus pannonicus* ssp. *auctus* essential oil and to correlate the results with the antibacterial activity of the volatile oil. The vegetal material was collected in the flowering period, from the Suceava district, Romania. The volatile oil has been extracted using a Clevenger type apparatus based on a hydro-distillation process, with a plant material/water ratio being of approximately 1:3.The separation and the identification of the components have been carried out using a GC-MS (gas chromatography coupled with mass spectrometry) Agilant 6890 N. The antibacterial activity of the volatile oil of the analyzed species has been carried out using *Staphylococcus aures* ATCC-6538 and *Escherichia coli* ATCC-10536 test strains. The antibacterial activity was determined measuring the diameter of the inhibition area (in mm) around the cylinders containing the tested concentration, that were applied on the nutritive culture medium previously inoculated with the test microorganism. GC-MS analysis of volatile oils resulted in the identification of 20 compounds for *Th. pannonicus* ssp. *auctus,* the main constituent being the carvacrole (74.29%). After testing the antibacterial action of the volatile oil we found that this oil presents inhibitory effect on the growth and development of booth test strains and that the concentration of 50 mg\l produces an obvious growth and bacterial multiplication inhibition.

***Key words**: Thymus pannonicus ssp. auctus, essential oil, antibacterial activity, carvacrol*

INTRODUCTION

The genus *Thymus* L. contains about 350 species widespread in Europe, North Africa, and Asia. In the Romanian flora grow 17 species [1]. The various species of the *Thymus* are used all over the world as medicinal and ornamental, as spices and are sources of essential oils. *Thymus pannonicus* presents sturdy ramified stems, covered with tector hairs around the stem. The leaves are elliptic or lanceolated, 6-12 mm long and 3-5 mm wide, with long tector hairs on both sides, with the ribs less prominent. The inflorescence is capitated or elongated. The calyx is 3-4 mm long and the corolla is red-lilac, 6-7 mm long. *Thymus pannonicus* ssp. *auctus* present glabra leaves [3].

In recent decades, knowledge on the chemical composition of plants, especially aromatic compounds, has greatly expanded due to the identification of a large number of organic molecules through many sophisticated chemical techniques. Also a contributing factor is the realization that products of secondary metabolism have a significant role in plant-animal, plant-plant and plant-environment interactions [15]. A high chemical variability was observed in the volatile oils of the various species in the *Thymus* genus. Recent studies

revealed that the species of *Thymus* genus exhibit strong antibacterial, antifungal, antiviral, antiparasite, spasmolytic and antioxidant activity [2, 6, 10, 12, 14, 16].

MATERIAL & METHODS

The biological material is represented by *Thymus pannonicus* ssp. *auctus*, species collected from the Suceava district, Romania, during the flowering period. The volatile oil has been extracted using a Clevenger hydro-distillation process, with a plant material/water ratio being of approximately 1:3. The separation and the identification of the components have been carried out with GC-MS (gas chromatography coupled with mass spectrometry) Agilent 6890 N with a spectrometric mass detector 5973 and an auto sampler; the DB5 chromatographic column has a length of 25 m and an interior diameter of 0.25 m. The separated compounds were identified by means of the NIST spectra database, and the peaks position was confirmed by the Kovats indices.

The antibacterial activity of the volatile oil of the analyzed species was carried out with Kirby-Bauer disc diffusion method, using *Staphylococcus aureus* ATCC-6538 and *Escherichia coli* ATCC-10536 test strains. By placing the cylinders with different concentrations of volatile oils on the surface of a solid medium, inoculated with bacterial culture, the antimicrobial active substances will spread into the environment, presenting a constant decrease of the gradient concentration from the edge of the cylinder to the periphery. After certain incubation time, we can define two distinct areas: one in which the microbial growth is inhibited by the concentration of antimicrobial substances and an area of growth, where the concentration of oil is too low to inhibit growth.

RESULTS & DISCUSSION

Essential oil composition

The GC-MS essential oil analysis resulted in identification of 20 compounds. The main constituent of oil is carvacrol, present in a concentration of 74.29%. In lower percentages were identified thymol (11.84%), caryophyllene oxide (3.57%), β-caryophyllene (1.58%) and β-bisabolene (1.20%) (Figure 1).

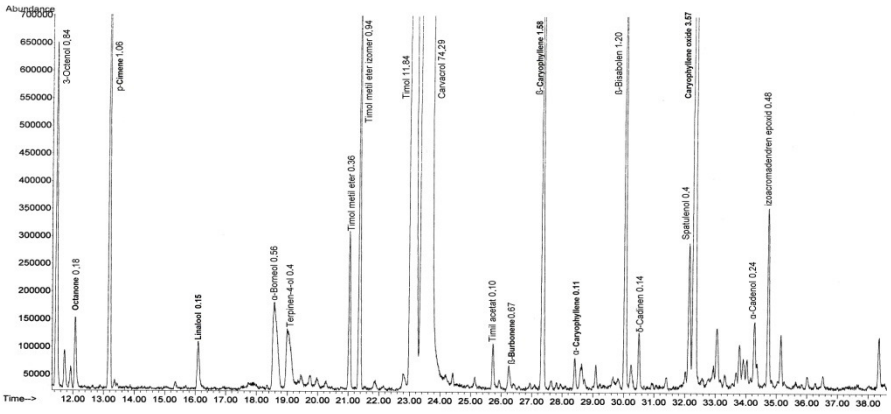

Figure 1. Chromatogram of essential oil extracted from *Thymus pannonicus* ssp. *auctus*

Carvacrol, the main constituent of essential oil of *Th. pannonicus* ssp. *auctus*, presents especially anthelmintic [4]), antibacterial [8] and antioxidant actions [5]. Also for this

constituent we found other activities such as antitussive [11], carminative [9], expectorant [9], antifungicidal [4] etc.

A high chemical variability was observed in the volatile oils of the various species of the *Thymus* genus. In 2008 Maksimovic Z. and his collaborators identified in the volatile oil of *Thymus pannonicus* All., harvested in northern Serbia, a total of 33 constituents. The main constituents were geranial (41.42%) and neral (29.61). Other researchers have identified in the volatile oil of this species large amounts of thymol (25-41%) and p-cimen (17-38%) [13]. According to Karuza-Stojaković and collaborators the principal constituents of *Th. pannonicus* essential oil from southern parts of Vojvodina province are terpinyl acetate, terpinen-4-ol, thymol, carvacrol and geranyl acetate (listed in order of descending quantity). The chemical composition of volatile oils of *Thymus* species presents a high variability and diversity, at least 20 different chemotypes been established so far. Regarding *Th. pannonicus* ssp. *auctus*, in a survey of available literature, we have not identified studies related to the chemical composition of the volatile oil.

Antimicrobial activity of essential oil

The sensitivity of *Staphylococcus aureus* and *Escherichia coli* to volatile oil (in decreasing concentrations) were tested in optimum cultivation conditions (growing medium, inoculum for incubation). For this purpose we used Kirby-Bauer disc diffusion method adopted by the NCCLS (National Committee for Clinical Laboratory Standards) in the U.S. On the surface of the culture medium (agar) we applied stainless cylinders. In each of this cylinders we distributed 200 µl from the essential oil dilutions performed (50, 100, 200, 400, 800 and 1600 mg/l). The antibacterial activity has been determined measuring the diameter of the inhibition area (in mm) around the cylinders containing the tested concentration that were applied on the nutritive culture medium previously inoculated with the test microorganism. The results were compared with the control sample. The analysis of the volatile oil extracted from *Thymus pannonicus* ssp. *auctus* showed an antimicrobial effect on the Gram positive bacteria – *Staphylococcus aureus*, and also on the Gram negative bacteria - *Escherichia coli,* for all dilutions (figure 2).

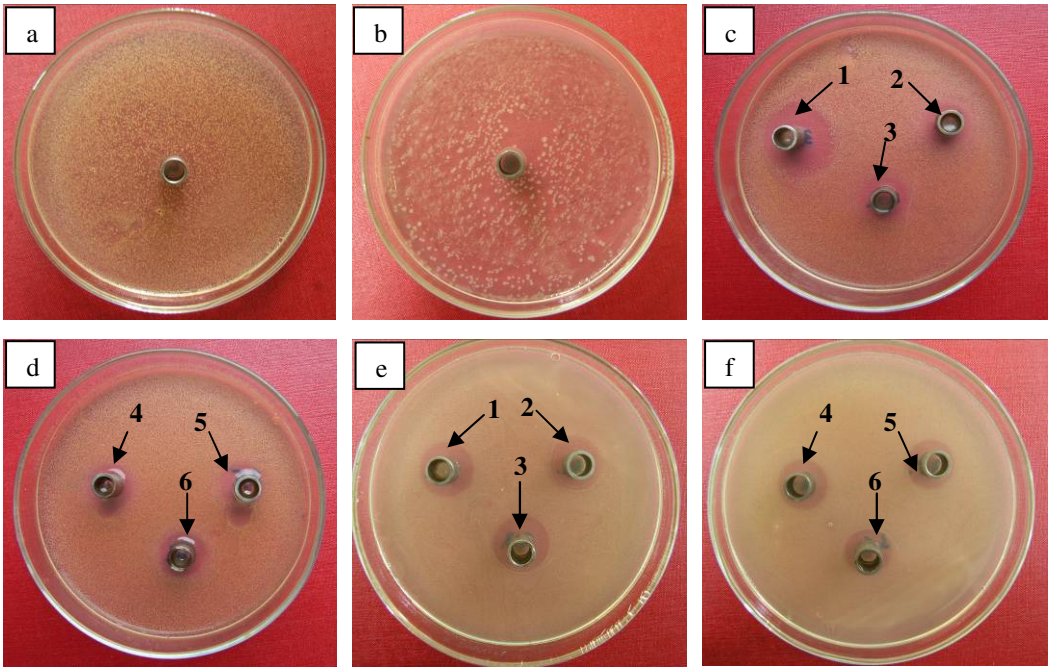

Figure 2. Antibacterial activity of essential oil to *Staphylococcus aureus* (c, d) and *Escherichia coli* (e, f), a – martor for *Staphylococcus aureus,* b - martor for *Escherichia coli,* 1 - concentration of 50 mg/l, 2 - concentration of 100 mg/l, 3 - concentration of 200 mg/l, 4 - concentration of 400 mg/l, 5 - concentration of 800 mg/l, 6 - concentration of 1600 mg/l.

Correlating these data with those regarding the chemical composition of the *Thymus pannonicus* ssp. *auctus* volatile oil, we can conclude that its strong antibacterial activity shown on the two test strains is due to the high concentration of carvacrol. Testing the 6 oil concentrations were performed to determine the optimal concentration which provides greater inhibition of bacterial growth and development. From the 6 concentrations tested a stronger inhibitor for the reference strains proved to be the concentration of 50 mg/l. As the concentration increases, the diameter of the inhibition zones decreases, so, the antibacterial activity is weaker.

CONCLUSION

In conclusion, we can affirm that *Thymus pannonicus* ssp. *auctus* volatile oil has a strong antibacterial effect on both Gram positive bacteria - *Staphylococcus aures* and on the Gram negative bacteria - *Escherichia coli.* Bacterial growth and development inhibition could be a result of the high quantity of carvacrol in the oil composition.

ACKNOWLEDGEMENTS

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Original scientific paper

EVALUATION OF ANTIOXIDANT POTENTIAL OF DIFFERENT TAXA OF GENUS *MENTHA* L.

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SUMMARY

The total content of flavonoids, phenols and antioxidant potential of methanol extracts of the herb of different taxa of genus *Mentha* L. were investigated. All investigated samples contained high amounth of phenols and flavononids, wich are the most important compounds for scavening of free radicals. The antioxidant activity ranged between 18.637 – 55.237 mM Trolox/ 100 g plant matter, and the best antioxidant activity showed *Mentha* x *piperita* 'Persephone' and the lowest *Mentha aquatica*.

***Key words**: Mentha L., TPC, flavonoids, TAC, DPPH*

INTRODUCTION

Plants are potential source of natural antioxidants [1]. A great number of aromatic, spicy and medicinal plants contain chemical compounds with antioxidant properties [2]. Plants belonging to *Lamiaceae* family are rich in polyphenolic compound and many of them are well known for their antioxidant properties [3,4]. Phenolic compounds act as free radical scavengers, as a chelators of metal ions and as a inhibitors of lipid peroxidation [5]. The major phenolic constiutents are caffeic acid derivates and flavonoids [6]. Flavonoids which are widely distributed in plants are higly effective antioxidants [7]. Major flavonoids in the genus *Mentha* L. are luteolin, apigenin, eriodictyol, heperetin and their glycosides [6].

Antioxidants are the subject of numerous medical studies to monitor the effect of antioxidants on oxidative stress, which causes serious damage to cells leading to severe diseases [8,9,10]. Another important part of using natural antioxidants is to protect food in food industry, which ensures oxidative stability [11]. Different taxa of *Mentha* L. was studied for their antioxidant potential, for example, antioxidant potential of *Mentha spicata* L. in radiation-processed lamb meat [12], activity of *Mentha* x *piperita* and *Mentha spicata* in sunflower oil [13] or antioxidant activity of *Mentha* x *piperita* in herbal tea [14,15].

The main goal of this study is to determine antioxidant activity of different taxa of *Mentha* L. which are commercialy available in Czech Republic or they are cultivated in botanical garden.

MATERIAL AND METHODS

Plant material

All analysed plant material was cultivated at Mendel University in Brno, Faculty of Horticulture. All samples were harvested in June – July 2011 in stage of full flowering and were naturally dried and stored in paper bags in dark and dry place. List of all analysed samples represented **Table 1**. Taxa names are written according to suppliers of plant material.

Table 1 List of taxa

Taxon	Supplier	Year of introduction to Lednice
Mentha aquatica 4	BG Praha [CZ]	2003
Mentha longifolia 1	Planta naturalis [CZ]	2003
Mentha longifolia 2	Jelitto [DU]	2003
Mentha longifolia 'Budleia'	BG Praha [CZ]	2003
Mentha spicata	BG Praha [CZ]	2003
Mentha spicata 2	BG Praha [CZ]	2003
Mentha spicata 'Marocan'	Pereny [CZ]	2007
Mentha suaveolens 'Variegata'	BG Praha [CZ]	2003
Mentha x *piperita*	Planta naturalis [CZ]	2003
Mentha x *piperita* 'Cinderella'	Kiepekierl [DU]	2008
Mentha x *piperita* 'Konfetka'	VNISSOK Moscow [RU]	2009
Mentha x *piperita* 'Krasnodarskaja'	BG Praha [CZ]	2003
Mentha x *piperita* 'Persephone'	BG Praha [CZ]	2003
Mentha x *piperita* 'Senior'	Pereny [CZ]	2007
Mentha x *piperita* var. *citrata*	Zahradnictvi Krulichovi [CZ]	2008
Mentha x *piperita* var. *crispa*	BG Praha [CZ]	2003
Mentha x *piperita* var. *piperita* 'Agnes'	BG Praha[CZ]	2003
Mentha x *piperita* var. *piperita* 'Eau Cologne'	BG Praha[CZ]	2003
Mentha x *villosa*	Pereny [CZ]	2007
Pulegium vulgare	Jelitto [DU]	2003
Pulegium vulgare 'Nanum'	Kotvičníková farma [CZ]	2011

BG - botanical garden

Preparation of extracts

The samples of the aerial parts of each taxon was groud in universal grinding mill (maximum grain size 3 mm). Small amount of each crushed sample of mint herb (around 2 g) was added to 75% methanol (50 ml), kept at the room temperature in digestor for 24 hours, occasionally mixed and filtered.

Determination of total phenols

The content of total phenols in each methanol extracts was determined according to the Folin-Ciocalteu procedure [16]. All used extracts were obtained from 2 g of plant material. Diluted methanol extract (250 µl) was mixed with 9 ml of distilled water and 1 ml of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min. Sodium bicarbonate solution (10 ml, 7%) was added to the mixture and incubated at room temperature for 90 min and the absorbance was measured at 765 nm, using spectrophotometer (JENWAY 6100). The total phenolic content was expressed as g Gallic Acid/100g plant matter (GAE/100 g). All samples were analysed duplicately and averaged.

Determination of total flavonoids

Aluminium chloride spectrophotometric method was used for total content of flavonoids determination [17] . All used extracts were obtained from 2 g of plant material. Diluted methanol extract (50 µl) was mixed with 1.5 ml of distilled water and 0.2 ml NaNO2 (5%) and kept in room temperature for 5 min. Aluminium chloride solution (0,2 ml, 10%) was added to the mixture, shaken vigorously and incubated for 5 min. And finally sodium hydroxide solvent (1,5 ml, 1mM) and 1 ml of distilled water were added to mixture, shaken vigorously and incubated for 15 min. The absorbance was measured at 510 nm, using spectrophotometer (JENWAY 6100). The total flavonoid content was expressed as g Catechin/100g plant matter. All samples were analysed in duplicate and averaged.

DPPH assay

Several type of free radicals, such as OH, O_2^-, LOO with different reactivity, can be formed during the pocess of lipid oxidatiton. Relatively stabile DPPH radical has been widely used to test the ability of compound to act as free radicals scavengers [18]. The antioxidant activity of methanol herb extract of different taxa *Mentha* L. were evaluated by their ability to scavening 2.2-diphenyl-1-picrylhydrazyl stable radicals.

Stock solution of DPPH – 0.079 g DPPH was added to 50 ml of methanol (75 %)

Reagent solution – 2.5 ml of DPPH stock solution was added to 100 ml of methanol solution (75 %), concentration of reagent solution is 100 $\mu M.l^{-1}$.

Suitable diluted (250 µl) methanolic extract (200 µl) was added to 3.8 ml DPPH reagent solution, mixed and incubated in the dark for 30 min. The absorbance was measured at 515 nm. All samples were analysed duplicate and averaged. The antioxidant acitivity was expressed as mM Trolox/100 g plant matter.

RESULTS AND DISCUSSION

Determination of total phenols

Results of determination total content of phenols are represented in **Table 2**. Total content of phenol in taxon of *Mentha* L. ranged between 2.765 – 5.798333 g GAE/100 g of plant matter and the highest content of phenols showed *Mentha* x *piperita* var. *piperita* 'Eau de Cologne'.

Table 2. Content of total phenols

Sample	g GAE/100g plant matter
Mentha aquatica 4	5.740000
Mentha longifolia 1	3.801111
Mentha longifolia 2	5.076111
Mentha longifolia 'Budleia'	4.326111
Mentha spicata	5.203889
Mentha spicata 2	3.940000
Mentha spicata 'Marocan'	3.781667
Mentha suaveolens 'Variegata'	2.765000
Mentha x *piperita*	5.470556
Mentha x *piperita* 'Cinderella'	3.812222
Mentha x *piperita* 'Konfetka'	4.398333
Mentha x *piperita* 'Krasnodarskaja'	4.851111
Mentha x *piperita* 'Persephone'	5.673333
Mentha x *piperita* 'Senior'	4.592778
Mentha x *piperita* var. *citrata*	4.909444
Mentha x *piperita* var. *crispa*	5.581667
Mentha x *piperita* var. *piperita* 'Agnes'	5.287222
Mentha x *piperita* var. *piperita* 'Eau Cologne'	5.798333
Mentha x *villosa*	4.001111
Pulegium vulgare	3.098333
Pulegium vulgare 'Nanum'	3.112222

Neugebauerová et Vábková (2011) reported that sample with the highest content of phenolics was *Mentha piperita* var. *piperita* 'Agnes' (6.10 g GAE/100 g plant matter) and sample with the lowest content was *Mentha longifolia* (2.26 g GAE/100 g plant matter) [19]. In this case the highest content of phenolics was found in *Mentha* x *piperita* var. *piperita* 'Eau de

Cologne' (5.79), but Neugebaurová et Vábková (2011) observed only 3.58 [19]. In this study was observed the lowest content of phenolics with *Mentha suaveolens* 'Variegata' (2.76), which is similar to result reported by Neugebaurová et Vábková (2011) [19]. Dorman et al.(2003) have reported total phenolic content in range 128-230 mg GAE/ g (DW of extract) from different *Mentha* plants [20]. Hajlaoui et al. (2009) compared content of phenolics in two taxa of *Mentha* and found, that content in *Mentha longifolia* (89.1 mg GAE/g DW) was doubled compared with *Mentha pulegium* (37.4 mg GAE/g DW) [21], but in this study result obtained for *Pulegium vulgare* and *Mentha longifolia* are very similar.

You can see the influence of total content of phenols to antioxidant capacity at **Fig. 1**. A correlation between total content of phenols and antioxidant activity (r^2= 0.58, $p < 0.05$) can be considered medium dependence.

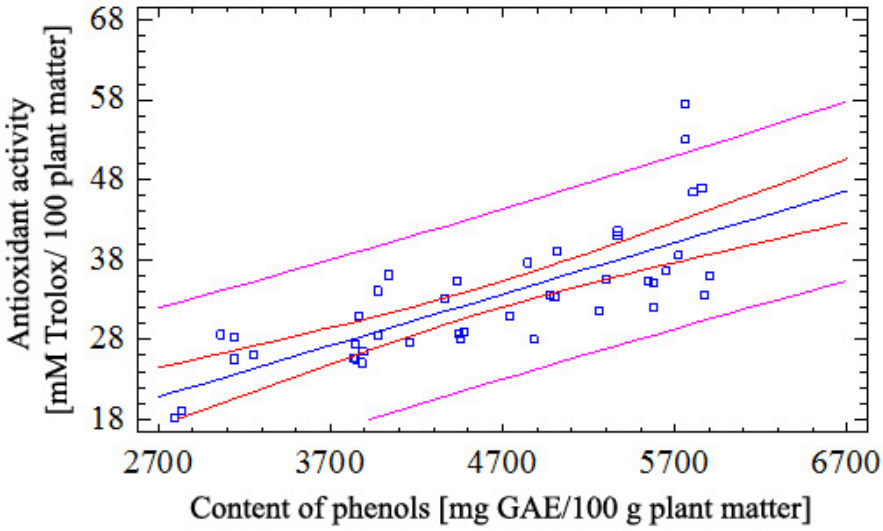

Fig. 1 Correlation betwen TPC and antioxidant activity

DPPH assay

Results for all analysed samples presented **Table 3**. The best antioxidant capacity showed the same sample with the highest content of flavonoids *Mentha* x *piperita* 'Persephone' (55.237 mM Trolox/ 100 g plant matter).

Neugebauerová et Vábková (2011) observed the highest antioxidant capacity with sample *Mentha* x *piperita* var. *piperita* 'Agnes' (35.31 mM Trolox/100 g plant matter) [19]. In this case this sample shows also high antioxidant capacity (41.32 mM Trolox/100g plant matter), but not the highest. The highest activity showed *Mentha* x *piperita* 'Persephone', which could be caused by high content of flavonoids and high content of essential oil (data not shown).

Neugebauerová et Vábková (2011) found *Mentha longifolia* (12.97) and *Mentha* x *piperita* 'Cinderella' (14.11) as samples with the lowest antioxidant capacity [19]. In this case samples above belongs to group with lower antioxidant capacity, but values are higher, *Mentha longifolia* 1 (26.12), *Mentha longifolia* 2 (31.46) and *Mentha* x *piperita* 'Cinderella' (28.05).

Table 3 Results of DPPH assay

Sample	mM Trolox/100 g plant matter
Mentha aquatica 4	46.747
Mentha longifolia 1	26.128
Mentha longifolia 2	31.463
Mentha longifolia 'Budleia'	34.182
Mentha spicata	33.526
Mentha spicata 2	35.197
Mentha spicata 'Marocan'	26.524
Mentha suaveolens 'Variegata'	18.637
Mentha x *piperita*	35.219
Mentha x *piperita* 'Cinderella'	28.055
Mentha x *piperita* 'Konfetka'	28.926
Mentha x *piperita* 'Krasnodarskaja'	38.333
Mentha x *piperita* 'Persephone'	55.237
Mentha x *piperita* 'Senior'	27.995
Mentha x *piperita* var. *citrata*	33.473
Mentha x *piperita* var. *crispa*	37.584
Mentha x *piperita* var. *piperita* 'Agnes'	41.326
Mentha x *piperita* var. *piperita* 'Eau Cologne'	34.731
Mentha x *villosa*	28.064
Pulegium vulgare	27.015
Pulegium vulgare 'Nanum'	27.373

Determination of total flavonoids

Total content of flavonoids in taxons of *Mentha* L. ranged between 5.623504 – 13.553607 g CA/100 g plant matter and the highest content of flavonoids show *Mentha* x *piperita* 'Persephone'. Result for all samples represented **Table 4**.

Table 4 Total content of flavonoids

Sample	g CA/100 g plant matter
Mentha aquatica 4	13.534019
Mentha longifolia 1	8.140025
Mentha longifolia 2	10.046403
Mentha longifolia 'Budleia'	9.433686
Mentha spicata	12.275057
Mentha spicata 2	9.404913
Mentha spicata 'Marocan'	8.372657
Mentha suaveolens 'Variegata'	5.623504
Mentha x *piperita*	13.264686
Mentha x *piperita* 'Cinderella'	8.347557
Mentha x *piperita* 'Konfetka'	9.750937
Mentha x *piperita* 'Krasnodarskaja'	10.718401
Mentha x *piperita* 'Persephone'	13.553607
Mentha x *piperita* 'Senior'	10.476013
Mentha x *piperita* var. *citrata*	11.156223
Mentha x *piperita* var. *crispa*	13.344977
Mentha x *piperita* var. *piperita* 'Agnes'	11.895558
Mentha x *piperita* var. *piperita* 'Eau Cologne'	13.436374
Mentha x *villosa*	7.984136
Pulegium vulgare	5.706437
Pulegium vulgare 'Nanum'	5.994432

Hajlaoui et al. (2009) reported also doubled content of flavonoids in *Mentha longifolia* (63.93 mg CA/g DW) compared with *Mentha pulegium* (33.83 mg CA/g DW) [21], which is result similar to our values.

Correlation between total flavonoid content is graphically expressed as **Fig. 2**. A correlation between total content of flavonoids and antioxidant activity (r^2= 0.59, p < 0.05) which is little bit more than influence of phenolics, but it is considered medium dependence

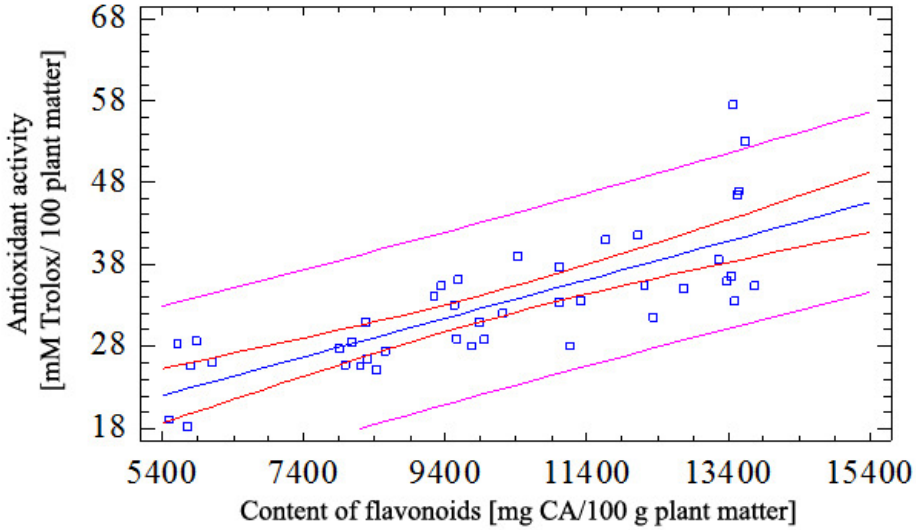

Fig. 2 Correlation between total content of flavonoids and antioxidant activity

ACKNOWDLEGEMENTS

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Original scientific paper

ANTIOXIDANT ACTIVITY OF THE METHANOL AND ETHANOL EXTRACTS OF *SATUREJA MONTANA* L.

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SUMMARY

Considering the importance of *Satureja montana* L.of Lamiaceae as a medicinal plant, in the present study we have compared possible antioxidant activity of the methanolic and 96% ethanolic extracts from winter savory, by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Free radical scavenging activity (expressed as EC_{50} - concentration which decrease absorption of DPPH for 50%) of methanolic extract was EC_{50} = 0.017 mg/ml, and for ethanolic extracts EC_{50} = 0.055 mg/ml. The results indicate that the methanolic extract from *S. montana* L. had a very potent antioxidant activity, stronger than 96% ethanolic extract.

***Key words**: S. montana, methanol, ethanol, extracts, antioxidant*

INTRODUCTION

Plant extracts are very useful for food industry as spices and additives and also are important in phytotherapy. The Lamiaceae family is well-known because of the antioxidant properties of its taxa. The genus *Satureja* L. of family Lamiaceae comprises more than 200 species of herbs and shrubs [1] often aromatic, widely distributed in the Mediterranean region, Asia and boreal America [2]. *Satureja* species are used widely as flavoring agents of food products and also as traditional herbal medicine. The glandular trichomes are the primary secretory organs of these plants [3]. The essential oil as well as the methanolic and ethanolic extracts from *Satureja montana* L. possess biological and pharmacological activities, such as antioxidant, antibacterial, antifungal, antiviral, and immune stimulating effects [4-6]. In the present study we have compared possible antioxidant activity of the methanolic and 96% ehtanolic extracts from winter savory, by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay.

MATERIAL & METHODS

The plant material (aerial parts) was collected at 15.07.2006. near Benkovac city (Croatia) (N 44° 02’ 87”; E 15° 36’ 10”). Voucher specimen is deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, BEOU 16496, Serbia.

The dried aerial parts of winter savory (5g) for methanolic and (5g) for ethanolic extracts, were previously homogenized in a Moulinex stirrer and then extracted with methanol (50 ml) and 96% ethanol (50 ml) in the dark at room temperature for 24h. The extracts were filtered through a filter paper and the filtrates were evaporated to dryness under vacuum. Then, stock solutions of 10mg/ml of both methanolic and ethanolic were prepared.

The antioxidant activity was determined by the DPPH radical scavenging method. For the methanolic extract a different dilutions experimentally found (40^x, 50^x, 60^x and 70^x*)* from the

stock (10mg/ml) were prepared. For the ethanolic extract dilutions of (20^x, 25^x, 30^x and 40^x) from the stock solution (10mg/ml) were used. Volume of 200 µl was placed in a cuvette, and 1800 µl of 0.04 mg/ml methanolic solution of DPPH was added. The decrease in absorbance at 517 nm after 30 min reaction in the dark was determined by UV-VIS spectrophotometer for all samples. The absorbance of the DPPH radical without antioxidant (with 200 µl of pure methanol) was the control (Ac). All determinations were performed in triplicate. The percentage of inhibition of the DPPH radical by the samples was calculated according to the equation:

$$\% \text{ inhibition} = ((Ac\text{-}Au)/Ac) \times 100$$

were Ac is the absorbance of the control and Au is the absorbance of the remaining DPPH radical after reaction with antioxidant at t = 30 min.

DPPH scavenging activity (EC_{50} values) of methanolic extracts and for ethanolic extracts were determined.

RESULTS & DISCUSSION

Recently, interest in plant-derived food additives has grown, mainly because synthetic antioxidants suffer from several drawbacks. In the present study we have compared possible antioxidant activity of the methanolic and 96% ehtanolic extracts from *S. montana* L., by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. In this test, the scavenging of DPPH radical is monitored by the decrease of absorbance at 517 nm, which occurs due to reduction by the antioxidant. The results indicate that both extracts from *S. montana* L. have potent antioxidant activity, and the methanolic extract was stronger than its ethanolic extract.

Free radical scavenging activity (expressed as EC_{50} - concentration which decrease absorption of DPPH for 50%) of methanolic extract was EC_{50} = 0.017 mg/ml (Fig. 1) and for ethanolic extract EC_{50} = 0.055 mg/ml (Fig. 2).

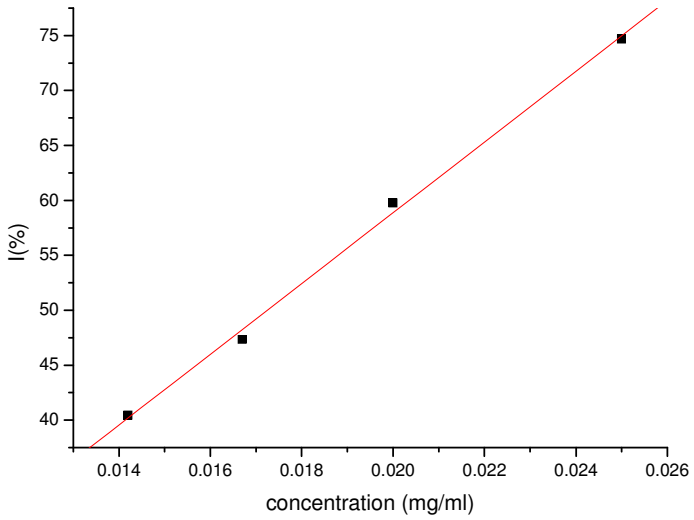

Figure 1. *Satureja montana* MeOH ekstract DPPH

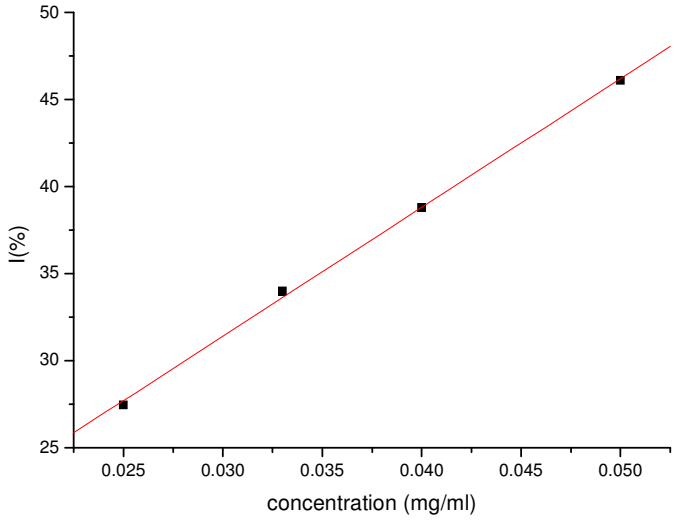

Figure 2. *Satureja montana* EtOH ekstract DPPH

CONCLUSION

The results indicate that both extracts from *S. montana* L. have a very potent antioxidant activity, and the methanolic extract was stronger than its ethanolic extract.

This investigation suggested that the plant extracts of *S. montana* L. have been shown to possess health-promoting properties and could be considered as a new potential source of natural antioxidants for food, pharmaceutical or cosmetics industries.

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Original scientific paper

ASSESSMENT OF POLYPHENOLS AND ANTIRADICAL ACTIVITY OF CALLUS CULTURE AND *IN VIVO* GROWN PLANTS OF *ARNICA MONTANA*

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SUMMARY

In present study extracts of callus cultures developed on nutrient media with different plant growth regulator combinations and *in vivo* grown plants of *A. montana* were analyzed for total contents of phenols and flavonoids, external flavonoid aglycones and antioxidant activity. Flavonoid aglycones - scutellarein 6-methyl ether (hispidulin) and scutellarein 6,4'-dimethyl ether (pectolinarigenin) were identified as the main components in the acetone exudates of all samples. It was found that the leaf extract of *in vivo* grown plants contain higher levels of flavonoids and phenols than the extracts of the calluses. The highest antioxidant activity was determined for the extract of *in vivo* grown plants. No differences regarding to the investigated parameters between calluses developed on both nutrient media have been found. Data will be used in future comparative studies with *ex vitro* adapted plants.

Key words: *surface flavonoids, phenols, DPPH*

INTRODUCTION

Arnica montana L. (Asteraceae) is a rare plant under strict protection in several European countries and the same time it is very valuable for pharmacy. The species contains several groups of active secondary compounds - sesquiterpene lactones (e.g. helenalin), phenolic acids (caffeic acid derivatives) and flavonoids with significant antiseptic, anti-inflammatory, antibacterial and antioxidant activity [1-3]. Phenolic compounds and flavonoids are the secondary metabolites of plants that cannot be synthesized by humans and they possess multiple beneficial effects for human health including antioxidant, anti-inflammatory, antibacterial, antiviral, anti-allergic, antiplatelet, antitumour etc. [4-5].

In Bulgarian Flora *Arnica montana* is a rare species and has been reported to grow only in Rila mountain [6], however so far its distribution has not been confirmed. Therefore the development of method for micropropagation of the species is essential for its cultivation and sustainable use. In the course of these studies it is important to examine and compare the content of bioactive compounds of micropropagated plants with wild or in vivo cultivated plants. Although there are detailed studies of phenolic compounds and flavonoids in *A. montana* extracts [7-10] information about antioxidant activity of *in vitro* cultured *A. montana* plants is insufficient.

The purpose of present study was to evaluate the contents of flavonoids and phenols as well as antioxidant properties on extracts of callus culture and in vivo grown A. montana plants.

MATERIAL AND METHODS

Plant material: Callus and *in vivo* grown plants of *A. montana* were studied. The origin of the samples is natural habitat in the Carpathians, Ukraine (AU). Two samples of callus cultures

developed on nutrient media with different plant growth regulator combinations were analyzed – CAU2 and CAU4 and one of *in vivo* sample AUG2 (leaves of cultivated plants from experimental station "Reli Rid" of the Vitosha Mt.).

Preparation of extracts: Acetone exudates. Air-dried (but not ground) plant material was briefly rinsed with acetone at room temperature to dissolve the lipophilic components accumulated on the surfaces. The acetone filtrate obtained was then dried using a rotary-evaporator to give a crude extract which was suspended in MeOH and future subjected on TLC. *Methanol extracts.* Dry, ground plant material (1g) was extracted with 80% methanol by classical maceration for 24 h. After evaporation of the solvent the crude extract was subject to subsequent analysis.

Biotechnological tools: Callus cultures were initiated from hypocotyls excised from fifteen-day old *in vitro* germinated seedlings. The calluses were developed on two different nutrient media: 1) Murashige and Skoog [11], 1962 medium (MS) supplemented with 0.1 mg/l 2.4-D and 0.2 mg/l IAA (CAU2) and 2) MS containing 0.1 mg/l 2.4-D, 0.2 mg/l IAA and 1 mg/l kinetin (CAU4).

Identification of surface flavonoid aglycones: The acetone exudates were screened for surface flavonoids by TLC analysis. Three TLC sorbents and three mobile phases were used for the analysis of the flavonoid exudates. Toluene-dioxan-acetic acid (95:25:4, v/v/v) were applied for the development of the aglycones mixture on silica gel plates Kiselgel 60 F_{254} (10x20 cm, 0.2 mm layer). Toluene-methylethylketone-methanol (60:25:15, v/v/v) were used for DC-Alufolien Polyamid 11 F_{254} plates (10x20 cm, 0.15 mm layer). Acetic acid–water (30:70, v/v) were used for cellulose plates DC-Alufolien Cellulose 5552 (10x20 cm, 0.1 mm layer). Chromatograms were viewed under UV light before and after spraying with "Naturstoffreagenz A'', 1% solution of diphenylboric acid 2-aminoethyl ester complex in methanol. The identification of the compounds was achieved by co-chromatography with authentic markers obtained from Prof. *Eckhard* Wollenweber.

Determination of total phenolic content: Total phenolic content of the methanol extracts was determined by Folin–Ciocalteu reagent [12-13]. Plant extracts were diluted to the concentration of 1 mg/mL, and aliquots of 0.25 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of Na_2CO_3 (6%). After 1 h of staying at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract) by the following formula:

$C = c \cdot V / m,$

where C - total content of phenolic compounds, mg/g plant extract, in GAE; c - the concentration of gallic acid established from the calibration curve, mg/mL; V - the volume of extract, mL; m - the weight of pure plant methanolic extract, g.

Determination of total flavonoid content: Total content of flavonoids was determined by spectrophotometric methods using aluminum chloride and rutin as a reference compound [14]. One ml of plant extract in methanol (10 g/L) was mixed with 1 ml aluminium trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at room temperature. Blank samples were prepared from1 ml plant extract and 1 drop acetic acid, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$C = A \cdot m_0 \cdot 10 / A_{0b} \cdot m,$

where C - flavonoid content, mg/g plant extract in RE; A - the absorption of plant extract solution; A_0 - the absorption of standard rutin solution; m - the weight of plant extract, g; m_0 - the weight of rutin in the solution, g.

DPPH radical scavenging activity: Free radical scavenging activity of plant extracts was evaluated using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [15,16]. Different concentrations of plant extract (50, 100, 200 and 300 μg/mL in methanol) were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:
DPPH antiradical scavenging capacity (%) = [1- ($Ab_{sample} - Ab_{blank}$) / $Ab_{control}$] . 100
Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, while DPPH solution plus methanol was used as a control. The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity (Software Prizm 3.00). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical.

Statistical Analysis

Statistical analysis was carried out using excel. All experiments were performed in triplicate. Results are presented as a value ± standard deviation (SD). Significant levels were defined at $p<0.05$.

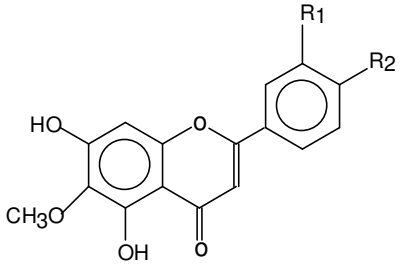
RESULTS AND DISCUSSION

Surface flavonoid aglycones

In the acetone exudates of leaf material of the studied samples two main flavonoid aglycones derivatives of 6-methyl apigenin were identified. Scutellarein 6-methyl ether (hispidulin) (**1**) and scutellarein 6,4'-dimethyl ether (pectolinarigenin) (**2**) were identified by TLC co-chromatographing with authentic sample. (Fig.1). It was found that there no difference in the synthesis of surface flavonoids between callus and *in vivo* grown plants.

Total phenolic content

The results of the total phenolic content determination in the methanol extracts of studied samples, evaluated using Folin - Ciocalteu method, are presented in Table 1. The content of phenols in extracts of callus culture – CAU2 and CAU4 expressed as gallic acid equivalents (GAE) was 22,45 to 22,68 mg/g of dry extract, respectively. No statistically significant differences in the content of total phenols among the methanol extracts of callus culture developed on nutrient media with different plant growth regulator combinations. The leaf extract of in vivo developing plants AUG2 contain higher levels of phenols- 39,78 mg/g of dry extract.



(**1**) R_1=H; R_2=OH scutellarein 6-methyl ether (hispidulin)
(**2**) R_1=H; R_2=OCH_3 scutellarein 6,4'-dimethyl ether (pectolinarigenin)

Fig. 1. Structures of the identified flavonoid aglycones (1) and (2)

Total flavonoid content

The results of the determination of flavonoid contents (expressed as rutin equivalents: mg RU/g of dry extract) are given in Table 1. No significant differences in the content of flavonoids between calluses developed on both nutrient media 0,632 and 0,611 mg/g of dry extract respectively for CAU2 and CAU4. However, it was found significant difference in the content of flavonoids between leaf extracts of callus and in vivo grown plants AUG2. The amount of flavonods is the highest in extracts of in vivo grown plants – 1,751 mg/g of dry extract.

Table 1. Polyphenol content and free radical scavenging activity of leaf samples of *callus culture* and *in vivo* grown *Arnica montana* plants

Samples	Total Phenols (mg/g extract) in GAE	Total Flavonoids (mg/g extract) in RE	DPPH scavenging activity IC_{50} (µg/mL)
CAU2 *in vitro*	22,45±0,98	0,632±0,27	>200
CAU4 *in vitro*	22,68±0,76	0,611±0,31	>200
AUG2 *in vitro*	39,78±0,89	1,75±0,58	62,97

Legend: CAU2 callus culture developed on MS medium supplemented with 0.1 mg/l 2,4-D, 0.2 mg/l IAA, CAU 4 callus culture callus culture developed on MS medium supplemented with 0.1 mg/l 2,4-D, 0.2 mg/l, 1 mg/l kinetin; AUG2 *in vivo* sample with Ukrainian origin cultivated on Vitosha mountain

DPPH free-radical scavenging activity

Results of the DPPH scavenging activity of studied samples, expressed as IC_{50} value that represents extract concentration providing 50% inhibition of the DPPH solution are given in Table 1. It was found that the leaf extracts of *in vivo* sample have the strongest radical scavenging activity. The extracts of callus samples shown low activity and their IC_{50} values were above 200 µg/mL.

Similar results such as the presented showing a greater amount of polyphenols and higher antioxidant activity in extracts of in vivo developing individuals compared with calluses cultures have been reported by other authors [17-20]. According to some authors, the increased synthesis of phenolics in plants growing under natural conditions is a defensive reaction to environmental stress [17]. An enhancement of phenolic compounds and flavonoids can be observed under different environmental factors and stress conditions [21-23].

CONCLUSION

In conclusion two surface flavonoid aglycones were identified in the acetone exudates of the studied samples of *Arnica montana*. No differences in synthesis of surface flavonoids among the extracts of callus and *in vivo* grown plants. The leaf extract of *in vivo* grown plants shown greater total polyphenol content and free radical scavenging activity than that of the extracts from callus cultures. Data will be used in future comparative studies with *ex vitro* adapted plants.

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Original scientific paper

POLYPHENOL CONTENT AND ANTIRADICAL ACTIVITY OF *GENTIANA LUTEA* SSP. *SYMPHYANDRA*: VARIATION AMONG PLANT PARTS AND POPULATIONS

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SUMMARY

In the present study comparative analysis on polyphenol content and antioxidant activity of methanol extracts of different parts (leaves, generative organs, stems and rhizomes) and populations (with origin of Rila and Pirin mountains) of *Gentiana lutea* ssp. s*ymphyandra* was carried out. The content of flavonoids in different plant parts showed greater variability than that of the phenols. The highest content of flavonoids was found in the extracts of generative organs whereas stems contained the lowest quantities. Maximum amount of phenols was established again in the generative organs, less in leaves, stems and rhizomes, in which the phenolic content was relatively uniform. The greater amount of phenolic compounds and flavonoids in generative organs leads to more potent radical scavenging effect of the extracts of these plant parts. No statistically significant differences ($p>0.05$) in the content of phenols and flavonoids between studied populations. The received data are basis for future comparison on materials from wild growing and cultivated plants of *Gentiana lutea ssp. symphyandra.*

***Key words**: flavonoids, phenols, DPPH*

INTRODUCTION

Gentiana lutea L. (Gentianaceae) is a perennial plant that grows in the alpine habitats of central and southern Europe. The herb is traditionally used for digestive disorders, such as loss of appetite, flatulence, anorexia, atonic dyspepsia, gastrointestinal atony [1,2]. Recently have been reported that extracts of the plant have radioprotective, choleretic and hepatoprotective activity [3,4]. Secoiridoid glycosides (gentiopicroside, swertiamarin, amarogentin, sweroside), xanthones (gentisine, isogentisine, methylgentisine, gentiseine), flavonoids (derivatives of C-glycoside), carbohydrates, loganic acid have been identified as main secondary metabolites of the plant [1]

Gentiana lutea ssp. *symphiandra* (Murb.) Hayek is spread in Bulgaria [5].The species was included in the Medicinal Plants Act 2000 [6], in the Biological Diversity Act (2002)[7], Red List of Bulgarian vascular plants [8] and Red Book of Bulgaria, in the category "Endegered Species"[9]**.** It is forbidden to collect the plant from its natural populations, which requires its cultivation. Introduction to culture is related to tracking changes in quantitative and qualitative composition of biologically active substances.

The aim of present study was to determine the total contents of phenols and flavonoids as well as free radical scavenging activity of extracts from different parts (leaves, generative organs, stems and rhizomes) and from different wild population of *Gentiana lutea* ssp. s*ymphyandra*.

MATERIAL AND METHODS

Plant material

The aerial parts of *Gentiana lutea* ssp. s*ymphyandra* were collected from natural populations of three localities at Rila and Pirin Mountains, Bulgaria (Table 2). Different plant parts (leaves, generative organs, stems and rhizomes) of studied species were collected at location "Rilska reka", Rila mountain (Table 2). Voucher specimens were deposited at the Herbarium of the Institute of Biodiversity and Ecosystem Research, Sofia (SOM).

Preparations of extracts

Air-dried, powdered plant material (1 g) was extracted with 80% methanol in an ultrasonic bath. After evaporation of the solvent the crude extract was subject to subsequent analysis.

Determination of total flavonoid content

Total content flavonoids was determined by spectrophotometric methods using aluminum chloride rutin as a reference compound [10]. One ml of plant extract in methanol (10 g/L) was mixed with 1 ml aluminium trichloride in ethanol (20 g/L) and diluted with ethanol to 25 mL. The absorption at 415 nm was read after 40 min at room temperature. Blank samples were prepared from1 ml plant extract and 1 drop acetic acid, and diluted to 25 mL. The absorption of rutin solutions was measured under the same conditions. Standard rutin solutions were prepared from 0.05 g rutin. All determinations were carried out in duplicate. The amount of flavonoids in plant extracts in rutin equivalents (RE) was calculated by the following formula:

$$C = A \cdot m_0 \cdot 10 / A_{0b} \cdot m,$$

where C - flavonoid content, mg/g plant extract in RE; A - the absorption of plant extract solution; A_0 - the absorption of standard rutin solution; m - the weight of plant extract, g; m_0 - the weight of rutin in the solution, g.

Determination of total phenolic content

Total phenolic content of the methanol extracts was determined by Folin–Ciocalteu reagent [11,12]. Plant extracts were diluted to the concentration of 1 mg/mL, and aliquots of 0.25 mL were mixed with 2.5 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 mL of Na_2CO_3 (6%). After 1 h of staying at room temperature, the absorbances of the samples were measured at 765 nm on spectrophotometer versus blank sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract) by the following formula:

$$C = c \cdot V / m,$$

where C - total content of phenolic compounds, mg/g plant extract, in GAE; c - the concentration of gallic acid established from the calibration curve, mg/mL; V - the volume of extract, mL; m - the weight of pure plant methanolic extract, g.

Free radical scavenging activity

Free radical scavenging activity of plant extracts was evaluated using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay[13,14]. Different concentrations of plant extract (100, 200, 300 and 500 μg/mL), in methanol were added at an equal volume (2.5 mL) to methanol solution of DPPH (0.3 mM, 1 mL). After 30 min at room temperature, the Ab values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation:

$$\text{DPPH antiradical scavenging capacity (\%)} = [\, 1- (\, Ab_{sample} - Ab_{blank}\,) / Ab_{control}\,] \cdot 100$$

Methanol (1.0 mL) plus plant extract solution (2.5 mL) was used as a blank, while DPPH solution plus methanol was used as a control. The IC_{50} values were calculated by sigmoid non-linear regression model using plots, where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of scavenging capacity (Software Prizm 3.00). IC_{50} values denote the concentration of sample required to scavenge 50% of DPPH radical.

Statistical Analysis

Statistical analysis was carried out using excel. All experiments were performed in triplicate. Results are presented as a value ± standard deviation (SD). Significant levels were defined at $p<0.05$.

RESULTS AND DISCUSSION

Different plant parts (leaves, generative organs, stems and rhizomes) of *Gentiana lutea* ssp. s*ymphyandra* collected at the same location were evaluated for their total content of phenols and flavonoids as well as for their antioxidant properties. With regard to the accumulation of flavonoids the extracts of separate parts differed significantly. The content of flavonoids in the studied extracts varied from 0,64 to 3,14 mg RU/g (Table 1). The extract of generative organs contain the highest flavonoids whereas the extract of stems contained the lowest. The extracts of leaves and rhizomes contain flavonoids in comparable amounts.

Table 1. Total content of phenols and flavonoids and free radical scavenging activity of extracts of different plant parts of *Gentiana lutea* ssp. S*ymphyandra*

Plant parts	Total flavonoids*	Total phenols**	Inhibition DPPH [%]			
			100	200	300	500
stems	0,64±0,146[a]	23,38±0,459[d]	14,00±1,2	20,21±1,4	25,46±0,5	36,26±1,6
leaves	1,61±0,506[b]	26,12±0,285[d]	11,07±3,5	19,46±1,9	25,40±1,6	40,20±5,3
generative	3,14±0,713[c]	37,02±0,105[e]	19,18±4,2	35,20±1,6	44,90±0,7	69,26±2,4
rhizomes	1,34±0,280[b]	20,74±0,535[d]	12,40±2,1	18,55±2,3	30,33±2,8	38,15±5,4

* value represent mean ±SD, mg RE/g extract

** value represent mean ±SD, mg GAE/g extract

Values with the same letter are not significantly different, $p \geq 0.05$

The total phenolic content in the extracts was determined using Folin-Ciocalteu reagent and its amount ranged between 20,74 to 37,02 mg GA/g extract. The phenol content of the generative organs was quite high compared to that of the leaves, rhizomes and the stem. Among the last extracts there no significant statistical differences. This was in accordance with reported previously results for higher amounts of phenolic compounds (xanthones and flavonoids) in flower than leaves [15].

Comparing our results with these reported in the literature it is observed that the accumulation of phenols and flavonoids in different plant parts varies for different species. In some cases the content of phenols has been highest in leaves [16] in others in flowers [17] and even the stems are the most rich in phenols [18]. Similar divergent trends have been reported for the accumulation of the flavonoids. In some plant species the flavonoid content is the highest in the flower [19] in other the leaves are the richest [20,21]. These examples demonstrate that each species has specificity in the accumulation of flavonoids and phenols in the various organs.

The antioxidant action of investigated plant extracts of *G. lutea* was assayed by scavenging of DPPH radicals and presented as IC_{50} values (μg/mL) - extract concentration providing 50%

inhibition of the DPPH solution. All tested extracts showed IC_{50} values higher than 200 μg/mL. Scavenging of DPPH radical was found to rise with increasing concentration of the extracts. The methanolic extract of generative organs showed the best inhibition of DPPH radical. The lowest scavenging activity was determined for the extract of rhizomes. This is in agreement with the previously reported differences in free radical-scavenging properties between leaves and roots of *G. lutea* by Kintziosa [22] which have been found that leaf extract is more active than root.

Correlation analysis showed a positive dependence between investigated indexes: R=0,9870 between phenols and antioxidant activity as well as R=0,9105 and R=0,8522 respectively between phenols:flavonoids and antioxidant activity:flavonoids. These results are in accordance of several reports for such positive correlation between phenols and antioxidant activity of plant extracts [23-25].

Total phenolic and flavonoid content, and antioxidant activity were determined for methanol extracts of generative organs of three populations of *G. lutea* (Table 2). The highest levels of flavonoids and phenols were detected in the extract of sample "Rila 2" respectively 4,35 mg RU/g and 37,41mg GAE/g extract. The extract of the same samples exhibited the highest free radical scavenging activity. Despite mentioned above observation the differences in the flavonoid and phenolic content of the extracts of studied populations were not statistically significant.

Table 2. Total content of phenols and flavonoids and free radical scavenging activity of extracts of generative organs of three populations of *Gentiana lutea* ssp. S*ymphyandra*

Sample	Locality	Total flavonoids[a]	Total phenols[b]	Antiradical activity[c], IC_{50}
Rila 1	Rila mountain, "Rilska reka" 1801 m a.s.l., Si, open places in *Picea abies*	3,83±0,1422	37,02±0,1053	467
Rila 2	Rila mountain, "Energoto" 1960 m a.s.l., Si, open places in *Picea excels*	4,35±0,6766	37,41±1,1808	264
Pirin	Pirin mountain, "Kazanite" 2220 m a.s.l., Ca, high mountain meadows with *Pinus mugo*	3,14±0,7133	35,05±0,3606	425

[a] value represent mean ±SD, mg RE/g extract
[b] value represent mean ±SD, mg GAE/g extract
[c] IC_{50} (μg/ml) extract concentration providing 50% inhibition of the DPPH solution

CONCLUSION

This study presents quantitative estimates of the contents of phenols and flavonoids as well as of free radical scavenging activity of extracts from the leaves, generative organs, stems and rhizomes of *Gentiana lutea* ssp. s*ymphyandra* by spectrophotometric methods. Comparative analysis of the flavonoid content in the different plant parts collected at the same location showed significant differences among plant parts, while significant difference in phenolic content among the samples was observed only between generative organs and other plant organs. The antioxidant properties of the studied extracts correlated positively with their polyphenol content and the highest activity was found for the extracts of the generative organs. There no statistically significant differences among different natural populations

regarding to the contents of phenolics and flavonoids as well as of free radical scavenging activity. The received data are basis for future comparison on materials from wild growing and cultivated plants of *Gentiana lutea ssp. symphyandra.*

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Original scientific paper

THE EFFECT OF THE ESSENTIAL OIL OF WILD-GROVING *SATUREJA MONTANA* L. FROM DALMATIA ON *SACCHAROMYCES CEREVISIAE* D7 AND *CANDIDA ALBICANS* ATCC10231 GROWTH

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SUMMARY

In this study we tested the influence of *Satureja montana* L. essential oil (EO) on *Candida albicans* ATCC10231 and *Saccharomyces cerevisiae* D7 growth by Macrodilution assay using Trypan blue. The effect of EO on growth inhibition was calculated by comparing CFU/ml of treated and CFU/ml of non-treated cultures. The toxic effect of 100% was considered as Minimal fungicidal concentration (MFC) of EO. In *S. cerevisae* the first concentration that had effect on growth rate was 0.0097µl/ml (21% of growth inhibition) while in the case of *C. albicans* the first effect on growth was detected at 0.039µl/ml (43% of growth inhibition). For both fungi species the toxic effect of 100% (MFC) was reached at 0.312µl/ml of EO. MFC: MIC ratio was 4, which according to literature indicates that EO of *S. montana* is considered to be fungistatic against *S. cerevisiae* and *C. albicans*.

Keywords: *S. montana; essential oil; Candida albicans ATCC10231; Saccharomyces cerevisiae; antifungal*

INRTODUCTION

Satureja montana L. (Lamiaceae), winter savory, is an aromatic species used as spice and as a traditional medicinal plant. It is a shrub typical of the sub-Mediterranean region. Due to the presence of phenolic compounds in the essential oil, *S. montana* is proved to have pharmacological activities [1] and has attracted the attention of many scientists in order to find natural antimicrobial and antioxidant agents [2-6].

In our previous work we showed antimicrobial activity of *Satureja montana* essential oil (EO) on *Escherichia coli*, *Pseudomonas aeruginosa*, *Bulcholderia cepacia*, *Streptococcus fecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Candida albicans* from ATCC collection, *Listeria innocua* and *Saccharamyces. cerevisiae* D7 [7] and determined minimum inhibitory concentration (MIC) for all tested strains. The components of EO were also examined by gas chromatography–mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy and obtained results showed that the main components of EO were carvacrol, *p*-Cymene, *γ*-terpinene, borneol and thymol [8].

Our previous results indicated the *S. montana* EO had most potent effect on fungi, for both tested fungi MIC was detected at 0.078µl/ml. We were continued analyses on *Candida albicans* ATCC10231 and *Saccharomyces cerevisiae* D7 growth and determined minimum fungicid concentrations (MFC). We also compare the effects of *S. montana* EO on growth rate of *C. albicans* and *S. cerevisae.*

MATERIAL AND METHODS

Overnight cultures were prepared freshly for every experiment by cultivation from frozen stock at 30°C for 24h in Yeast Extract Peptone Glucose Broth (YPD Broth: 1% Peptone, 0.5% Yeas extract, 2% Glucose, Difco & co, Corpus Christi, TX, USA).

Determination CFU/ ml with Trypan blue

Colony Forming Units per ml (CFU/ml) was determined by counting visible cells using Trypan blue assay. Cells were dyed with 0.4% solution of Trypan blue (0.2mg/ml Trypan blue; TCI Europe nv, Belgium), and numbers were assessed by haemocytometar. Blue cells were considered as non-viable.

Macrodilution assay

The impact of EO on growth rate of selected fungi species was carried out by methodology described by Sarker et al. [9] with slight modifications. Instead of 96 well plates we used sets of 2ml microtubes where each microtube represents one well, to enhance aeration. In the first tube the mixture of 1ml YPD Broth and 0.02ml of EO [8] was added. The volume of 0.5ml YPD Broth was added to seven test tubes and serial dilutions were performed by pipetting 0.5ml of the EO from first tube in serially decreasing concentrations. Finally, 0.1ml of cell suspension (5 x 10^7 CFU/ml, where is final concentration about 10^6 CFU/ml) and 0.4ml YPD Broth were added. The sets of controls included negative control (YPD Broth), positive control (serial gradient of antimycotic-cyclopiroxolamine, Jugomedija AD Zrenjanin, Serbia) and cell control (YPD Broth and cells suspension). Cultures were incubated in triplicate at 30°C for 24h. Each experiment was repeated three times. Growth rate was assessed by counts of viable cells. Concentration at which the decline in cells numbers appeared was considered as MIC.

Statistical analysis

The Student's *t*-test and Pearson's correlation were used for statistical analysis of the data. Statistical analyses were performed using STATISTICA 6.0 [10]. The significance was tested at $p<0.05$ level.

RESULTS AND DISCUSION

The effects of *S. montana* EO have been tested on *S. cerevisiae* D7 and *C. albicans* ATCC10231 growth (Tab.1).

Table 1. The influence of *S. montana* EO on on growth rate.

***S. montana* EO [µl/ml]**	***S.cerevisiae* D7 [CFU/ml]**	***C. albicans* ATCC10231 [CFU/ml]**
0.00487	2.50×10^7	3.50×10^7
0.00975	1.97×10^7	3.38×10^7
0.0195	1.79×10^7	3.30×10^7
0.0390	1.47×10^7	2.10×10^7
0.0780	1.44×10^6	4.91×10^6
0.0156	7.50×10^5	8.25×10^5
0.312	0	0
0.644	0	0

Results obtained in this study pointed that *S. montana* EO has effect on tested fungi growth rate. CFU/ml of both species showed negative correlation to tested concentrations of *S. montana* EO [10]. The effect of EO on growth inhibition was calculated by comparing CFU/ml in treated cultures with CFU/ml in non-treated control (2.5 x 10^7 for *S. cerevisiae*

and 3.5 x 10^7 for *C. albicans*). In *S. cerevisae* the first concentration that had effect on growth rate was 0.0097µl/ml (21% growth inhibition) while in the case of *C. albicans* the first effects on growth were detected at 0.039µl/ml (43% growth inhibition). For both fungi species the toxic effect of 100% (MFC) was reached at 0.312µl/ml of EO. (Fig.1.).
Considering that in the case of *S. cerevisiae* lower EO concentrations had effects, we assume that this species is more sensitive to *S. montana* EO comparing with *C. albicans*.

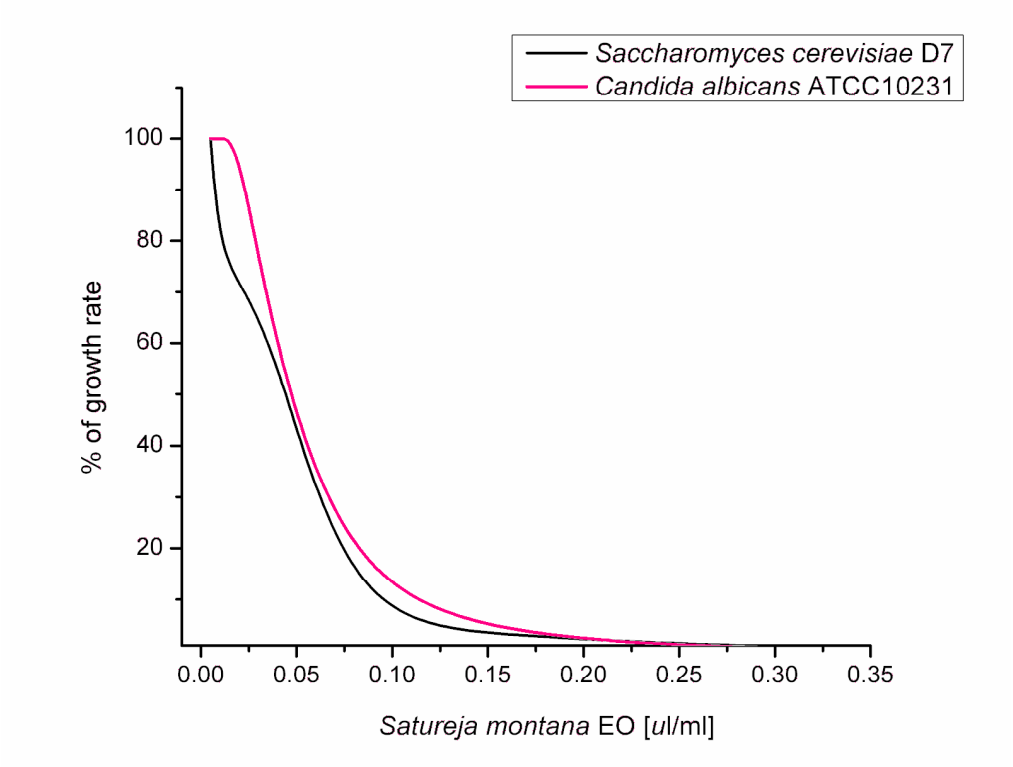

Figure 1. The effect of *Satureja montana* EO on growth rate of *Saccharomyces cerevisiae* D7 and *Candida albicans* ATCC10231.

For both fungi species, MIC was detected at 0.078µl/ml which confirmed our previous work [8], while the MFC was detected at 0.312µl/ml. The MFC: MIC ratio was used to specify the nature of the antifungal effect against selected fungi species. If the MFC: MIC ratio is between 1 and 2, the chemical substance is considered as fungicidal against selected pathogen, if the ratio is > 2, the mode of antifungal action is more likely to be fungistatic [11]. Considering that for both used species MFC: MIC ratio was 4, we can conclude that EO of *S. montana* has fungistatic effect on these two species (Tab.1.).

Table 2. MICs and MFCs of *Satureja montana* EO on *Saccharomyces cerevisiae* D7 and *Candida albicans* ATCC10231

	S. montana EO [µl/ml]			Cyclopiroxolamine [µg/ml]		
	MIC	MFC	MFC:MIC*	MIC	MFC	MFC:MIC
S. cerevisiae D7	0.078	0.312	4	0.2	0.4	2
C. albicans ATCC10231	0.078	0.312	4	0.1	0.2	2

*MFC: MIC ratio sowed that *S. montana* EO has fungistatic effect on tested fungi

CONCLUSION

Obtained results showed that *S. cerevisiae* seems to be more sensitive to *S. montana* EO with mainly fungistatic effect on investigated fungi species.

ACKNOWLEDGEMENTS

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Original scientific paper

ANTIBACTERIAL ACTIVITY OF *ZATARIA MULTIFLORA* AND *MENTHA ARVENSIS* ESSENTIAL OILS

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SUMMARY

In recent years, the quests for novel plant protectants with bactericidal potential have been increased because of development of resistance to bactericides and harmful effects of them on environment. The present study investigated antibacterial effects of *Zataria multiflora* and *Mentha arvensis* essential oils against *Pseudomonas syringae* pv *syringae, Brenneria nigrifluens, Pantoea agglomerans, Pseudomonas vesicatoria and Xanthomonas campestris* pv *campestris* bacteria isolates. Disc diffusion method was used to compare bactericidal activity of essential oils. Two loopfull suspensions were spread uniformly over the 6 cm (in diameter) plates containing nutrient agar (NA) medium. The filter paper discs (5 mm in diameter) were impregnated with 10 µL of essential oil placed on the NA surface under aseptic conditions. Paper discs with distilled water and Florfenicole were used as negative and positive controls respectively. After 24 h the inhibition zone (IZ) was measured by caliper. Results showed that both essential oils have exhibited significant inhibitory effects. Bactericidal activity of *Z. multiflora* was significantly more than *M. arvensis* and can be a good source of antibacterial agents.

Key words: *Essential oil, Mint, Thyme, Bactericide*

INTRODUCTION

Bacteria, as plant pathogenic microorganisms, haveimportantrole in decreasing agricultural products. Recently, using of bactericides and antibiotics leads to appearance of resistant isolates of bacteria [1]. There are many researches about the antibacterial activity of various plants [2].Plant essential oils have been known to show inhibition of proliferation or killing activity against a wide variety of micro-organisms[3]. *Zataria multiflora and Mentha arvensis* are valuable medicinal plants grown extensively in Iran and the chemical compositions of their essential oils have been extensively characterized.*Z. multiflora* With the local name of Avishan-e-Shiraziand *M. arvensis* L. are belonging to the *Lamiaceae* family [4] that geographically grow mainly in Iran, Pakistan and Afghanistan [5], extensively used as a flavor ingredient in a wide variety of food [6].

Z. multiflora and *M. arvensis* essential oils have also usedfor antimicrobial purposes in food [4] anda wide range of microorganisms have been subjected to investigations using essential oils as antimicrobial reagents [2]. The antimicrobial potential *of Z. multiflora* essential oil on food-borne pathogenic bacteria (*Escheraichia coli*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Bacillus cereus*) and probiotic bacteria (*Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* and *Lactobacillus casei* subsp. *casei*) was evaluated [7]. The effects of *Z. multiflora* essential oil, thymol and carvacrol, which are two main components of this essential oil, were assayed on growth of 10 *F. graminearum* isolates and reduction of deoxynivalenol production in PDA and PDB media. The results of this study showed that the essential oils and their main components had inhibitory activities against the

isolates of this fungus and decreased deoxynivalenol production of *F. graminearum* isolates [8].

The objective of this study was to evaluate the antibacterial effects of *Z. multiflora* and *M. arvensis* essential oils on the growth of *Pseudomonas syringae* pv *syringae*, *Brenneria nigrifluens, Pantoea agglomerans, Pseudomonas vesicatoria* and *Xanthomonas campestris* pv *campestris* by the disc diffusion method.

MATERIALS AND METHODS

The strains of plant pathogenic bacteria including: *Pseudomonas syringae* pv *syringae*, *Brenneria nigrifluens, Pantoea agglomerans, Pseudomonas vesicatoria* and *Xanthomonas campestris* pv *campestris* have been provided from department of plant pathology, Tarbiat Modares University, Tehran, Iran. All of them are pathogenic on their host plants and collected from various regions of Iran.The essential oilsof *Z. multiflora* and *M. arvensis* was provided from Barijessence co. Kashan, Iran. The qualitative screening of the susceptibility of different bacterial strains to the mentioned essential oils was performed by adapted disc diffusion techniques[2]. 24 h old bacterial inoculums was prepared andPetri dishes with Nutrient Agar (NA) medium were seeded with bacterial inoculums as the classical antibiotic susceptibility testing disk diffusion method (CLSI); 5 mm diameter paper filter disks were placed on the seeded medium at 60 mm distance by method Clinical and Laboratory Standards Institute [2].The Felorfenicole and water treatments used as positive and negative controls, respectively. The results of antibacterial test were recorded 24 and 48 hours after doing of the tests. The Inhibition Zone (IZ) in Petri dishes was recorded in millimeter scale. Experimental data were subjected to analysis of variance (ANOVA) and the Tukey's least significant difference multi-comparison test to determine significant difference among samples.

RESULTS AND DISCUSSION

According to the results shown in Table 1 and Figure 1, both essential oils are obviousantibacterial activity in comparison with negative control (water). The potential of growth inhibition in *Z. multiflora*was better than *M. arvensis*in all of bacterial isolates (Figure 1). The best control of bacterial growth was recordedin *P. agglomerans* in both of the essential oils as intense as in Felorfenicole treatment. In *P.vesicatoria* the essential oil of *Z. multiflora* inhibited the growth of bacteria as similar as the Felorfenicole but the *M. arvensis* essential oil slightly inhibited the growth of this bacterium (Figure 1). The inhibition zone of both essential oils in *B. nigrifluens* was similar.

In the case of *Z. multiflora*, comparison between all of bacteria showed that the lowest susceptibility to this essential oil was observed in *B. nigrifluens.* On the other hand, *P. agglomerans* and *P. vesicatoria* were the more susceptible bacteria to the mentioned essential oil (Figure 1). Additionally, except for the *P. agglomerans* and *P. vesicatoria,* there were significant difference between bacterial isolates in response to inhibitory activity of *Z. multiflora* essential oil ($p < 0.0005$).

The inhibition zone of *M. arvensis* was various in different bacteria. As seen in Figure 1, *P. syringae* pv *syringae*was the most susceptible bacteria, *B. nigrifluens* and *X. campestris* pv *campestris* both were in the second position and the more susceptible bacteria to this essential oil was P. *agglomerans.*

Table 1 Antibacterial activity of the essential oils of *Zataria multiflora* and *Mentha arvensis*

Bacteria isolates	*Zataria multiflora*	*Mentha arvensis*	Felorfenicole	Water
Pseudomonas syringae pv *syringae*	10.0±0.23*	5.4±0.24	25.0±0.00	5.0±0.00
Brenneria nigrifluens	8.5±0.41	8.4±0.34	25.0±0.00	5.0±0.00
Pantoea agglomerans	25.0±0.00	25.0±0.00	25.0±0.00	5.0±0.00
Pseudomonas vesicatoria	25.0±0.00	11.9±0.35	25.0±0.00	5.0±0.00
Xanthomonas campestris pv *campestris*	13.4±0.76	8.0±0.25	25.0±0.00	5.0±0.00

*Inhibition Zone± S.E.

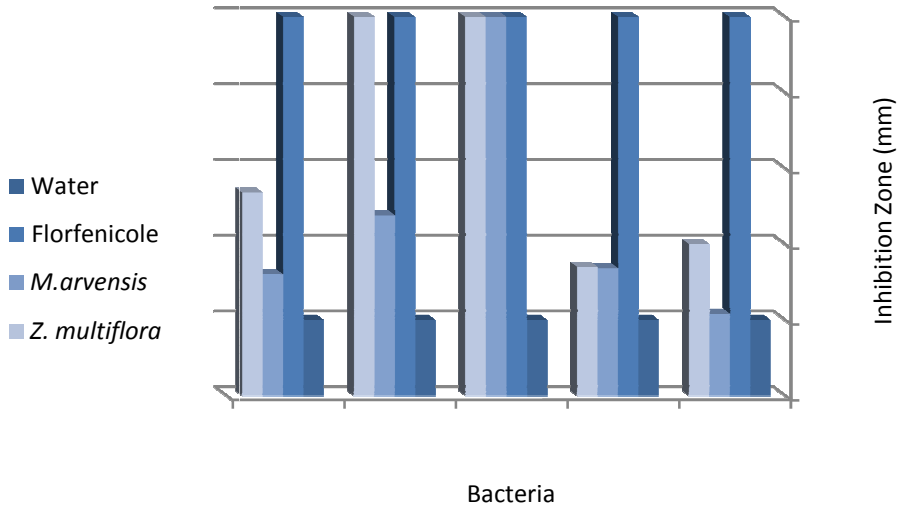

Figure 1: Antibacterial effect of *Zataria multiflora* and *Mentha arvensis* on examined bacterial isolates. Bacteria: 1- *Pseudomonas syringae* pv *syringae 2- Brenneria nigrifluens* 3- *Pantoea agglomerans*4- *Pseudomonas vesicatoria*5- *Xanthomonas campestris* pv *campestris*

CONCLUSION

A number of essential oils and several of their individual components exhibit antibacterial activity against plant pathogenic bacteria in vitro and, to a lesser extent, in vivo (Burt, 2004). According to the results obtained from this study, successful inhibitions of growth in several plant pathogenic bacteria were possible with *Z. multiflora* and *M. arvensis* essential oils. To achieve reliable and applicable results for using in agriculture, the antibacterial activity of mentioned essential oils against bacterial plant disease in the greenhouse and fieldare particularly appropriate subject for study in this area.

Overall, the results of the present work showed that there are strong bactericidal potential in plant essential oils and the future researches need to show the accuracy of this mention.

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Original scientific paper

FUNGICIDAL ACTIVITY OF *PELARGONIUM GRAVEOLENS*L. ESSENTIAL OILON THE PATHOGENIC FUNGUS *FUSARIUMOXYSPORUM* ISOLATED FROM HERBAL DRUGS

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SUMMARY

Depending upon the cultivation collection, harvesting, drying, fragmentation, and storage conditions, the medicinal herbs can be highly susceptible to microbial contamination, particularly to moulds. Presence of fungi in medicinal plants in addition to reducing their quality and usefulness could, under certain conditions, also lead to secretion the toxic metabolites, mycotoxins which posses vary powerful mutagenic and carcinogenic effect.
Species belonging to genus *Fusarium* and *Aspergillus* considered to be predominant in most of the herbal drugs. Among *Fusarium* species the most known as pathogen is *Fusarium oxysporum*. Because the *Fusarium* species are resistant *in vitro* to the majority of fungicides, new researches are oriented towards finding natural compounds with antifungal effect. It is recorded that great quantities of essential oils are known for exerting antifungal activity.
The aim of this study was to determine the fungicidal activity of the geranium essential oil (*Pelargonium graveolens*L., Geraniaceae) against *F. oxysporum* isolated and identified from corn silk and marigold flower. MIC's and MFC's of the oil were determined by dilution assay. Our investigation exhibited that geranium essential oil, with citronellol as main component, strongly inhibited the growth of this pathogen with 2,5 µl/ml as MFC. The commercial fungicide, fluconazole, used as a control, had much lower antifungal activity than the investigated oil. It could be concluded that geraniumessential oil possess great antifungal potential and could be used for the control of pathogenic fungi in medical and agricultural contexts.

Key words: *herbal drugs, fungal pathogens, essential oils*

INTRODUCTION

Since they are natural products, the herbal drugs are quite often deteriorated by microorganismsbefore harvesting, during handling and storage. The presence in sufficient number, the microorganisms can cause medicinal plants quality problems and may be harmless to consumers. The concern with the quality of the natural products is due to the potential fungal contamination and the risk of the presence of mycotoxins.
Species belonging to genus *Fusarium*, *Aspergillus*and *Alternaria*considered to be major fungal pathogens in most of the herbal drugsand are known to contain strains that could produce mycotoxins [1].The genus *Fusarium* collectively represents the most important group of fungal plant pathogens, causing various diseases on nearly every economically important plant species. Among *Fusarium* species the most known as pathogen of herbal

drugs,especially in agricultural settings, is *Fusarium oxysporum*, remarkably diverse and adaptable fungi [2,3].

F. oxysporum and its various forms have been characterized as causing the following symptoms: vascular wilt, yellows, corm rot, root rot, and damping-off. The most important of these is vascular wilt. Healthy plants could become infected by *F. oxysporum*if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the plant's roots. Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves stomata to close, the leaves wilt, and the plant eventually dies. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly [4]. The resulting spores can then be used as new inoculum for further spread of the fungus.

To control plant disease caused by this and other fungus plant should be treated with systemic fungicides before planting and/or after harvesting. The development of appropriate alternatives to chemical fungicides for the management of fungal disease would be useful inreducing the undesirable environmental effects, soil contamination with chemicals and public exposure to pesticides [5].

Because the *Fusarium* species are resistant *in vitro* to the majority of fungicides, new researches are oriented towards finding natural compounds with antifungal effect. It is recorded that large quantities of essential oils are known for exerting antifungal activity[6, 7].

The aim of this study was to determine the antifungal activity of the geranium essential oil (*Pelargonium graveolens*L., Geraniaceae) against *F. oxysporum* isolated and identified from corn silk and marigold flower, the herbal drugs that are mostly used in the manufacture of various products, especially teas.

MATERIAL AND METHODS

Herbal drugs

The molds used in this study were isolated and identified from dried medicinal herbal drugs marigold flowers (*Calendula officinalis* L.) and corn silk (*Maydis stigmata*). The drugs were obtained from warehouse of Institute for Medicinal plant Research "Dr Josif Pančić". The molds were maintained on potato dextrose agar (PDA), malt agar (MA) and Sabouraud agar (SBA). The cultures were stored at +4°C and subcultured once a month.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS)

Qualitative and quantitative analyses of geranium essential oil

Geraniumessential oil (*Pelargonium graveolens*L.) was investigated for their composition by the use of analytical GC/FID and GC/MS techniques. For this purpose a HP 5890 series II gas chromatograph, equipped with splitless injector, fused silica capillary column (25 m x 0.32 mm), coated with cross-linked methyl silicone gum (0.5 μm film thickness), and FID was employed. Essential oil solutions in ethanol (1%) were injected in split mode (1:30). Injector was heated at 250oC, FID at 300 °C, while column temperature was linearly programmed from 40-280 °C (4 °C /min). GC/MS analyses were carried out on a HP-GCD, equipped with split-splitless injector, fused silica capillary column (50 m x 0.2 mm) PONA, coated with cross-linked methyl silicone gum (0.5 μm film thickness). The chromatographic conditions were as above. Transfer line (MSD) was heated at 280 °C. EIMS spectra (70eV) were acquired in scan mode in m/e range 40-300. Identification of individual constituents was made by comparison of their retention times with those of analytical standards, and by computer searching, matching mass spectral data with those held in Wiley/NBS Library of Mass Spectra. For quantification purposes area percent reports obtained by FID were used.

Test for antifungal activity, Microdilution method

In order to investigate the antifungal activity of essential oil, a modified version of the microdilutiontechnique was used [8, 9]. The molds were maintained on potato dextrose agar (PDA), malt agar (MA) and Sabouraud agar (SBA). The cultures were stored at +4°C and subcultured once a month. Fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 105 in a final volume of 100 µL per well. The inocula were stored at 4°C for further use. Dilutions of the inocula were cultured on solid MA to verify the absence of contamination and to check the validity of the inoculum. Determination of MIC values was performed by a serial dilution technique using 96-well microtiter plates. The investigated essential oil was dissolved in MA or SDA broth containing fungal inoculum. The microplates were incubated for 72 h at 28°C. The lowest concentrations without visible growth (under a binocular microscope) were defined as the minimal concentrations which completely inhibited fungal growth (MIC). The minimal fungicidal concentrations (MFC) were determined by serial subcultivation of a 2-µL volume on micro titer plates containing 100 µL of broth per well and further incubation for 72 h at 28°C. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% killing of the original inoculum compared to fluconazole (the commercial antimycotic used as a positive control).

RESULTS AND DISCUSSION

In solid media culture, such as potato dextrose agar (PDA), the different special forms of *F. oxysporum* could have various appearances. In general, the aerial mycelium first appears white, and then may change to a variety of colors - ranging from violet to dark purple - according to the strain (or special form) of *F. oxysporum.* If sporodochia are abundant, the culture may appear cream or orange in color [10].

We have isolated and identified *F.oxysporum* from corn silk (*Maydis stigmata*) and marigold flower (*Calendula officinalis* L.). Colonies vary widely in appearance on PDA. Mycelium is abundant, woolly, whitish, turning to light to pale violet. The colour of mycelium and medium pigmentation varies depending on the isolates (Fig. 1a, 1b).

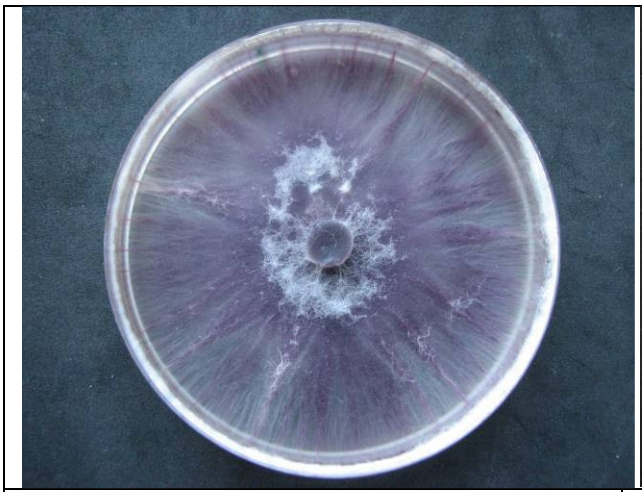

Fig. 1a. *F.oxysporum* isolated from corn silk

Fig. 1b. *F.oxysporum* isolated from marigold flower

The aim of our study was to determine the ability of geranium essential oil to inhibit the growth of *F.oxysporum* and to establish if there are differences of its activity on various fungal forms (isolated from corn silk and marigold flower).
The essential oil from *Pelargonium graveolens*L. (geranium oil) is composed of various chemical constituents. The dominant components arecitronellol (39.03%), geraniol (11.26%), phenethyl alcohol (7.45%), citronellylformate (5.08%), geranylbutanoate (4,81%), 6,9-guaiadiene (4,14%), isomenthone (3.48%); there are other less frequent compounds (Table 1.)

Table 1. Chemical composition of geranium essential oil

Components	RI	%	Components	RI	%
Tricyclene	906	-	Nerol	1231	-
α-Thujene	911	-	Carvone	1246	-
α-Pinene	919	0.20	Geraniol	1259	11.26
α-Fenchene	935	-	Geranial	1273	-
Camphene	938	-	Citronellylformate	1277	5.08
Sabinene	969	-	Nerylformate	1283	0.80
β-Pinene	975	-	Isobornyl acetate	1288	-
Myrcene	991	-	Bornyl acetate	1288	-
Octanal	1004	-	Geranylformate	1304	1.44
α-Terpinene	1017	-	Myrtenyl acetate	1328	-
p-Cymene	1024	-	Citronellyl acetate	1354	1.40
Limonene	1030	-	Eugenol	1359	-
1,8-Cineole	1032	-	Neryl acetate	1366	0.280
γ-Terpinen	1055	-	α-Copaene	1378	0.22
cis-Linalyl Oxide	1068	-	Geranyl acetate	1386	-
Terpinolene	1084	-	Geranyl acetate β-Bourbonene	1386	1.84
Linalol	1094	1.70	Phenyl ethyl isobutanoate	1397	2.77
cis -Thujone	1103	-	E-Caryophyllene	1422	0.86
cis-Rose oxide	1106	0.74	α-Ionone	1431	-
Phenethyl alcohol	1108	7.45	cis-Thujopsene	1433	0.87
trans-Thujone	1112	-	α-Guaiene	1442	0.26
dehydro-Sabinene ketone	1117	-	6,9-Guaiadiene	1447	4.14
trans-Rose oxide	1123	0.33	α-Humulene	1457	0.22
1-Terpinenol	1131	-	allo-Aromadendrene	1465	0.16
trans-Sabinol	1136	-	Geranylpropanoate	1478	0.40
Camphor	1142	-	Germacrene D	1485	0.55
Isopulegol	1143	0.26	β-Ionone	1490	-
Menthone	1151	1.82	Viridiflorene	1499	0.64
Isoborneol	1155	-	γ-Cadinene	1519	0.24
Isomenthone	1163	3.48	α-dehydro-Himachalene	1519	-
Borneol	1164	-	δ-Cadinene	1528	0.50
2-Phenyl ethyl formate	1176	0.32	Citronellylbutanoate	1533	0.65
Terpinen-4-ol	1177	-	Geranylbutanoate	1563	4.81
α-Terpineol	1192	0.13	Caryophyllene oxide	1586	-
Dihydrocitronellol	1197	-	2-Phenyl ethyl tiglate	1589	0.29
γ-Terpineol	1199	-	Cedrol	1604	1.26
trans-Carveol	1219	-	E-Citronellyltiglate	1670	0.54
Citronellol	1230	39.03	Geranyltiglate	1706	2.30
			Total		**99.24**

As it said, the present investigations were undertaken to find out effectiveness of geranium oil against *Fusarium oxysporum* isolated and identified from marigold flower (*Calendula*

officinalis L.) and corn silk (*Maydis stigmata*). Our results have shown that geranium essential oil, tested by microdilution method, completely inhibited the growth of tested fungi isolated from both herbal drugs with MIC 2.0 and 2.5 µl/mlrespectively (Table 2). *F.oxysporum* isolated from marigold flower was slightly more sensitive to essential oil. Its growth was completely inhibited at 2.0 µl/ml of essential oil. Minimal inhibitory concentration (MIC) was equal with minimal fungicide concentration (MFC). The results were compared with a commercial fungicide fluconazole which inhibited the complete mycelia growth at higher concentrations, so we can conclude that geranium oil possess very high antifungal activity against *F.oxysporum* isolated from both herbal drugs.

Table 2. Minimal inhibitory (MIC) and fungicidal (MFC) concentrations of geraniumessential oil

Fungi	**geranium essential oil**		**Fluconazole**	
	MIC (µl/ml)	MFC (µl/ml)	MIC (µl/ml)	MFC (µl/ml)
F.oxysporum-corn silk	2.5	2.5	15	20
F.oxysporum-marigold flower	2.0	2.0	15	20

In earlier studies, it was shown that citronellol, main component of geranium essential oil, completely inhibited mycelia growth of *Fusarium oxysporum*at 250 and 300 ppm, same as geraniol (at 200 ppm)[11]. According to other authors,the mycelia growth oftested fungal species (*Aspergillus flavus, A.niger, A. parasiticus, Fusarium moniliforme, Fusarium* spp., *Penicillium roqueferti, Penicillium* spp.)was totally inhibited bygeraniol and citronellol with MIC values ranged from 0.5 to 1.2 mg/ml [12]. Geraniol completely inhibited the growth of *A. flavus*and *A. niger*at 0.8 mg/ml,*A. parasiticus, Aspergillus* spp. and *Penicillium* spp. at 1.2 mg/ml and those of *Fusarium* species tested at 0.5 mg/ml and 0.6 mg/ml (*F. moniliforme* and *Fusarium* spp).Compared to our results it could be seen that the individual components were more active than the complete essential oil and that they inhibited the growth of fungi at lower concentrations.

There is possibility that interactive effects of other compounds present in smaller quantities may also contribute to antifungal activity of complete essential oil. Although in minor percentages, these compounds together with the main compounds identified can be considered as the antifungal constituents of the active essential oils.

CONCLUSION

Our results indicate that geranium essential oil could be useful as control agent forphytopathogenic fungi *Fusariumoxy sporum*. However, for the practical application of this oil and its single components as novel fungicides, further studies are necessary on the safety of these materials to humans and on the development of formulations to improve the efficacy and stability and to reduce cost.

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Original scientific paper

ANTIFUNGAL ACTIVITY OF INDIGENOUS *PSEUDOMONAS* ISOLATES AGAINST *ALTERNARIA TENUISSIMA* ISOLATED FROM *ECHINACEA PURPUREA*

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SUMMARY

Plant diseases caused by phytopathogenic fungi are one of the major problems in cultivation of medicinal plants in Serbia. Since the use of chemical agents is not allowed, biological control is becoming promising method in plant disease management. It has been showed that purple coneflower (*Echinacea purpurea, Asteraceae*) has medicinal properties which are significant for strengthening of the immune system. Therefore, in Serbia there is growing interest in cultivation of this plant. It has been showed that purple coneflower is host of *Alternaria tenuissima*, a very aggressive phytopathogenic fungus known for production of various toxins with negative effects on plant and human health. In search for ecological ways for prevention and reduction of disease symptoms, biological control with indigenous fluorescent *Pseudomonas* isolates has been offered as a possible solution.

The aim of this study was to examine antifungal activity of different indigenous fluorescent *Pseudomonas* isolates (Q16, B25 and PS2) against phytopathogenic fungus *A. tenuissima* which had infected *E. purpurea*.

Examined indigenous *Pseudomonas* isolates decreased conidial germination of *A. tenuissima* by 54.65 – 85.22 %. Isolate PS2 showed the greatest effect (85.22 % at 10^7 cfu/ml) on decreasing of *A. tenuissima* conidial germination. Among examined indigenous *Pseudomonas* isolates Q16 was the most efficient (74.62 % on King B agar plates) as the antagonist of *A. tenuissima* isolated from *E. purpurea*.

Examined *Pseudomonas* isolates exhibited the potential in controling *E. purpurea* disease caused by *A. tenuissima*. Therefore these isolates should be further analyzed in order to classify them as promising group of biocontrol agents against diseases of *E. purpurea* caused by *A. tenuissima*.

***Key words*:** *Pseudomonas*, *Echinacea purpurea*, *Alternaria tenuissima*, *plant disease management*

INTRODUCTION

Commercial cultivation of medicinal plants in Serbia is facing the problem of plant diseases caused by various plant pathogens. Among plant pathogens phytopathogenic fungi are very common. These organisms are able to cause diseases by infecting different types of plant tissues. This is causing a great damage in cultivation and growing of medicinal plants. Therefore the main interest is to find and apply efficient methods for their protection. Biological control (biocontrol) has been proven, in large number of research, as a promising strategy. It represents an ecological way for plant protection because it is based on use of natural antagonists of phytopathogenic fungi. [1]

There are many natural antagonists of phytopathogenic fungi and among them significant group are plant growth promoting rhizobacteria (PGPR) – bacteria that colonize the rhizosphere of plants and exibit beneficial effects on plant growth. [2] PGPR use various mechanisms: plant hormones production, fixation of N_2, antagonizing phytopathogenic organisms and solubilization of some nutrients, especially mineral phosphates. [3] In number of research different fluorescent *Pseudomonas* isolates showed the ability to stimulate plant growth by decreasing frequency of diseases (by competition and/or by antagonism). [4] Therefore fluorescent *Pseudomonas* species have been extensively studied in order to be classified as PGPR.

It has been shown that phytopathogenic fungus *Alternaria tenuissima* can be inhibited by fluorescent *Pseudomonas* species. *A. tenuissima* is common and very agressive plant pathogen which infects various crops: cereals, vegetables, fruits and medicinal plants. This phytopathogenic fungus is able to produce toxins (alternariol, alternariol monomethyl ether, tenuazonic acid, altertoxin and other metabolites) which are harmful for plant, animal and human health. [5] It causes infections and produces spores in suitable moisture and temperature (19–23°C) conditions. Infected plants are recognized by necrotic spots on leaves which often wilt and fall off. [6]

A. tenuissima is detected as pathogen of purple coneflower (*Echinacea purpurea* L., *Asteraceae*). *E. purpurea*, perennial wildflower which originates from North America, is widely cultivated in various parts of the world because of its medicinal properties. [7] It has been reported that liquid extracts and powdered forms of this plant exhibit immunostimulative effects, especially in activation of monocytes and macrophages – cells of innate immune response. [8]

Phytopathogenic fungus *A. tenuissima* is proven to be very harmful for health of large number of medicinal plants, including *E. purpurea*. Therefore, the aim of this study was to investigate potential antifungal activity of selected indigenous fluorescent *Pseudomonas* isolates (Q16, B25 and PS2) against *A. tenuissima* which infects *E. purpurea*.

MATERIAL & METHODS

Effect of different indigenous Pseudomonas isolates on the conidial germination of Alternaria tenuissima isolated from purple coneflower (Echinacea purpurea)

Determination of the effects on *A. tenuissima* conidial germination of investigated indigenous *Pseudomonas* isolates (Q16, B25 and PS2) was performed in *in vitro* experiment. *A. tenuissima* was grown on sterile seeds of *E. purpurea* on potato dextrose agar (PDA) at 23°C. Different concentrations of *Pseudomonas* isolates Q16, B25 and PS2 (10^8, 10^7,10^6 and 10^5 cfu/ml) were prepared from the liquid overnight culture in King B medium on the basis of OD_{600} measuring. One drop from each concentration was mixed with conidia of *A. tenuissima* (picked up from fungal culture with sterilized needle) on microscopic slides. Control variants included: the mixture of conidial suspension of *A. tenuissima* with sterile distilled water (1), sterile saline solution (2) and with fungicide DACOFLO 0.2% (3). After incubation of 24 h at 25°C, the conidial germination of *A. tenuissima* was determined microscopicaly. [9]

Decrease of conidial germination of *A. tenuissima* isolated from *E. purpurea* seeds was calculated by formula: $[(C-T)/C] \cdot 100$; C – germination of conidia in control and T – germination of conidia in treatment. Statistical analysis was performed by Duncan multiple test.

Test for antagonism of different indigenous Pseudomonas isolates toward Alternaria tenuissima isolated from purple coneflower (Echinacea purpurea)

The antagonistic effect of examined *Pseudomonas* isolates (Q16, B25 and PS2) on *A. tenuissima* was determinated on two nutrient media – King B and Waksman agar plates.

Overnight cultures of *Pseudomonas* isolates Q16, B25 and PS2, optimized to $1 \cdot 10^7$ cfu/ml, were used in the experiment. Afterwards these optimized cultures were used for derivation of two agents: 1) cells free of extracellular metabolites (1 ml of cultures was centrifuged at 13000 rpm for 10 min and resuspended in the same volume of sterile saline solution) and 2) termostable extracellular metabolites - heat stable antifungal factors (HSAF) (1 ml of cultures was centrifuged at 13000 rpm for 10 min, supernatant was filtered and filtrate was incubated at 70°C for 30 min). The influence of these two agents on mycelial growth of *A. tenuissima* was examined on King B and Waksman agar plates (four variants in total for every examined *Pseudomonas* isolate). 10 µl of these agents was spotted near the edges of agar plates. Mycelial disc of *A. tenuissima* was placed in the centre of every agar plate in Petri dish. There were two control variants on King B (1) and Waksman (2) agar plates, both contained only mycelia of *A. tenuissima*. All Petri dishes with inoculated plates were incubated at 25°C and measuring was conducted after 7 and 14 days. [10] The percentage of growth inhibition of *A. tenuissima* mycelia was calculated by the formula: % Inhibition = $[(R - r)/R] \cdot 100$;
R – growth of test fungus in control, r – growth of test fungus toward the inhibitory agent [11]

RESULTS & DISCUSSION

Effect of different indigenous Pseudomonas isolates on the conidial germination of Alternaria tenuissima isolated from purple coneflower (Echinacea purpurea)

The effects of examinated indigenous *Pseudomonas* isolates Q16, B25 and PS2 on decrease of *A. tenuissima* conidial germination are presented in table 1. *Pseudomonas* isolates Q16 and PS2 showed significant effect on inhibition of *A. tenuissima* conidial germination (70.80–85.22%). Isolate PS2 (at concentraton of 10^7 cfu/ml) showed the strongest effect on inhibition of *A. tenuissima* conidial germination (with statistically significant value of 85.22%). The least effect on decrease of *A. tenuissima* conidial germination showed isolate B25 (at concentration of 10^5 cfu/ml) which was less statistically significant (54.65%) by Duncan multiple test.

Our findings are consistent with inhibition of conidial germination reported by other researches [12,13]. Investigation of effects of the same indigenous *Pseudomonas* isolates (Q16, B25 and PS2) on conidial germination of *A. tenuissima* isolated from *Cynara cardunculus* shows their great ability in inhibition of germination of this phytopathogenic fungus (Josic et al., unpublished data).

Table 1. Decrease (%) of conidial germination of *A. tenuissima* isolated from *E. pupurea* under the influence of bacterial suspension

Pseudomonas Isolate	Concentration of Bacterial Suspension (cfu/ml)			
	10^5	10^6	10^7	10^8
Q16	76.28 a	74.25 a	70.80 b	80.07 a
B25	54.65 b	62.88 b	68.40 b	74.93 b
PS2	76.28 a	75.60 a	85.22 a	83.50 a

Test for antagonism of different indigenous Pseudomonas isolates toward Alternaria tenuissima isolated from purple coneflower (Echinacea purpurea)

Percentages of *A. tenuissima* mycelium growth inhibition depending on the type of inhibitory agent (cells free of extracellular metabolites or heat stable antifungal factors – HSAF) are presented in Tables 2 and 3. Mycelial growth of *A. tenuissima* was inhibited by examined

indigenous fluorescent *Pseudomonas* isolates (Q16, B25 and PS2) in range 7.02–74.62%. Isolate Q16 exhibited the strongest inhibitory effect (74.62% of *A. tenuissima* mycelial growth inhibition) among all three examined indigenous *Pseudomonas* isolates. Seetha et al., 2010 reported that *Pseudomonas fluorescens* inhibits *A. tenuissima* mycelial growth. Also research on the same indigenous fluorescent *Pseudomonas* isolates (Q16, B25 and PS2) confirmed antagonistic activity toward *A. tenuissima* isolated from *Cynara cardunculus* (Josic et al., unpublished data).

Cells free of extracellular metabolites showed higher inhibition in comparing with heat stable antifungal factors. This suggests that heat treatment may inactivate some of extracellular metabolites with antifungal potential. The research of Djuric et al., 2011 confirmed that indigenous *Pseudomonas* isolate PS2 has significant antifungal activity which relies on its ability to produce extracellular enzymes (chitinases and other lytic enzymes). Antifungal activity of indigenous *Pseudomonas* isolates Q16 and B25 indicates that investigation should be continued in order to identify potential production of extracellular enzymes as inhibitory agents.

Table 2. Inhibition of *A. tenuissima* growth under the influence of cells free of extracellular metabolites

Pseudomonas Isolate	7 Days			14 Days		
	r (mm)		Inhibition (%)	r (mm)		Inhibition (%)
Q16	WA	21.33 ± 6.43	60.49	WA	24.67 ± 5.77	67.55
	KB	10.67 ± 1.15	74.62	KB	12.67 ± 1.15	73.61
B25	WA	32.67 ± 4.16	39.51	WA	52.67 ± 1.54	30.70
	KB	28.00 ± 2.00	33.33	KB	28.67 ± 3.06	40.28
PS2	WA	32.00 ± 6.00	40.74	WA	47.33 ± 6.11	37.72
	KB	20.67 ± 1.15	50.81	KB	21.33 ± 1.15	55.56

* r - growth of test fungus toward the inhibitory agent; R – control growth of the test fungus (7 days: R_{WA}= 54 mm, R_{KB} = 42 mm; 14 days: R_{WA}= 76 mm, R_{KB} = 48 mm)

Table 3. Inhibition of *A. tenuissima* growth under the influence of heat stable anifungal factors (HSAF)

Pseudomonas Isolate	7 Days			14 Days		
	r (mm)		Inhibition (%)	r (mm)		Inhibition (%)
Q16	WA	34.00 ± 5.29	37.03	WA	60.00 ± 12.49	21.05
	KB	35.33 ± 3.06	15.87	KB	35.33 ± 3.06	26.39
B25	WA	40.00 ± 5.29	25.92	WA	70.67 ± 9.24	7.02
	KB	37.33 ± 3.06	11.11	KB	37.33 ± 3.06	22.22
PS2	WA	40.00 ± 4.00	25.92	WA	52.00 ± 14.00	31.58
	KB	36.00 ± 2.00	14.28	KB	36.67 ± 1.15	23.61

* r - growth of test fungus toward the inhibitory agent; R – control growth of the test fungus (7 days: R_{WA}= 54 mm, R_{KB} = 42 mm; 14 days: R_{WA}= 76 mm, R_{KB} = 48 mm)

CONCLUSION

A. tenuissima is a very common plant pathogen of various plant species in Serbia and among them there are number of medicinal plants. It causes serious problems in growing of these

plants which affect their health and yield. In order to protect medicinal plants from this aggressive fungus there is growing interest for finding solution for dealing with it. Biological control is a promising strategy for fighting plant diseases because it is an ecological way for plant protection. This research confirmed antifungal activity of indigenous fluorescent *Pseudomonas* isolates Q16, B25 and PS2 through inhibition of conidial germination and mycelial growth of *A. tenuissima*. In order to confirm the role of investigated fluorescent *Pseudomonas* isolates in biological control of *E. purpurea* disease caused by *A. tenuissima* it is necessary to conduct further research.

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Original scientific paper

INSECT REPELENCY ACTIVITY OF *ZATARIA MULTIFLORA* AND *SATUREJA HORTENSIS* (LAMIACEAE) ESSENTIAL OILS

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SUMMARY

Synthetic insecticides which used to increase yield and protect stored products are often associated with residuals that are dangerous for human being and the environment. Recently, researchers are focused their efforts on the search for active natural products as alternatives to conventional insecticides. *Zataria multiflora* and *Satureja hortensis* are members of Lamiaceae family are widely grown in different parts of Iran and use in traditional folk remedies. In this study, the repellency activity of essential oils extracted from *Z. multiflora* and *S. hortensis* was evaluated on a post-harvest pest of legumes in tropical and sub-tropical regions, *Callosobruchus maculatus* (Col: Bruchidae). Leaves were subjected to hydrodistillation for 3 hours, and the oils were collected by a modified Clevenger-type apparatus. Repellency was evaluated using the choice bioassay system in 8 cm diameter Petri dishes. The bottom of the Petri dishes were covered with filter paper and half of the filter paper was impregnated with 0.5 mL of acetone and another half were treated with different acetonic concentrations of essential oil (0.02 - 0.4 μL cm^{-2}). Twenty adults of *C. maculatus* were introduced into each Petri dish. Number of insects on each half of the filter paper was counted after 2 and 4 h of exposure. According to the results, the essential oils had an effective repellency against *C. macullatus*. The repellency increased with increasing the concentration of essential oils. After 2 and 4 h, *Z. multiflora* showed 84.6 and 91% repellency at highest concentrations of 0.4 μL cm^{-2}, respectively. In the case of *S. hortensis* there was no obvious repellency after 2 h, but after 4 h good repellent activity was seen. These observations suggest that the essential oils of *Z. multiflora* and *S. hortensis* essential oils may be useful in the protection of storage legumes against pests.

***Key words**: Repellency, Zataria multiflora, Satureja hortensis, Callosobruchus maculatus*

INTRODUCTION

Synthetic insecticides have a major role in pest control. However, in previous years the increasing use of them has caused some ecological and environmental problems such as bio-magnification, pest resurgence, insecticidal resistance of insect pest and dangers for human beings and non-target insects. Recently, researchers are focused their efforts on the search for active natural products as alternatives to conventional insecticides. *Zataria multiflora* and *Satureja hortensis* are members of Lamiaceae family which widely grown in different parts of Iran and use in traditional folk remedies. In this study, the repellency activity of essential oils extracted from *Z. multiflora* and *S. hortensis* was evaluated on a post-harvest pest of legumes in tropical and sub-tropical regions, *Callosobruchus maculatus* (Col: Bruchidae).

MATERIAL AND METHODS

Fresh leaves of *S. hortensis* and *Z. multiflora* were subjected to hydrodistillation in a Clevenger-type apparatus for 4 hours. The collected oil was dried over anhydrous sodium sulfate and stored at 4˚C until use.

Callosobruchus maculatus were obtained from our stock culture and reared in the laboratory on cowpea *Vigna unguiculata* in plastic containers at 25 ± 1ºC, 65 ± 5 % relative humidity and constant dark. Experiments were carried out under the same environmental conditions.

Petri dishes with 8 cm diameter were used to evaluate repellency of essential oils, using the choice bioassay system. The bottom of the Petri dishes were covered with filter paper and half of the filter paper was impregnated with 0.5 mL of acetone and another half were treated with acetonic solutions of different concentrations of essential oil (0.02 - 0.4 µL cm^{-2}) and dried for 5 minutes under a fume extractor. Twenty adults of *C. maculatus* were introduced into each Petri dish and the lid was sealed with parafilm. The experiment was replicated 5 times and the environmental conditions were the same as those described for the rearing of insects. The number of insects on each half of the filter paper was counted after 2 and 4 h of exposure. Percentage repellency (PR) values were computed as PR= [(NC-NT) / (NC+NT)] × 100, where NC= number of insects in the control area and NT= number of insects in the treated area [1].

RESULTS AND DISCUSSION

Table 1and 2 show the repellency activity of *Z. multiflora* and *S. hortensis.* According to the results, the essential oils had an effective repellency against *C. macullatus*. The repellency increased with increasing the concentration of essential oils. After 2 and 4 h, (Table 1) *Z. multiflora* showed 84.6 and 91.7 % repellency at highest concentrations of 0.4 µL cm^{-2}, respectively. In the case of *S. hortensis* (Table 2) there was no obvious repellency after 2 h, but after 4 h good repellent activity was seen.

Table 1: Repellent activity of essential oil from *Zataria multiflora* leaves against *Callosobruchus maculatus* at different exposure times.

Dose (µl/cm^2)	Repellency%	
	2h	4h
0.02	15.23 a	16.04 a
0.04	27.69 a	36.36 b
0.08	47.25 b	37.42 b
0.12	50.24 b	52.80 b
0.16	64.58 b	69.7 bc
0.2	84.64 c	91.7 c

Table 2: Repellent activity of essential oil from *Satureja hortensis* leaves against *Callosobruchus maculatus* at different exposure times.

Dose (µl/cm^2)	Repellency%	
	2h	4h
0.02	-	15.71
0.04	-	67.94
0.08	-	14.00
0.12	-	39.51
0.16	-	81.66
0.2	-	81.85

Some essential oils have been evaluated before for their repellent activity against stored product pests like *Acanthoscelides obtectus* [2], *Sithophilus oryzae* and *Bruchus rufimanus* [3], *Callosobruchus maculatus* [4] and *Tribollium castaneum* [4, 5]. All of above researches have demonstrated repellent activity of essential oils. According Zapata and Smagghe (2010), the repellency of essential oil were significantly influence by the concentration applied and the exposure time [5]. Based on our results, adults of *C. maculatus* were found to be repelled by essential oil tested even at low concentrations. Also an increase of repellent activity was found with increasing concentration of the oil and exposure time.

CONCLUSION

The results of this study suggest that the essential oils from *Z. multiflora* and *S. hortensis* have potential to use as repellent agents against stored products beetles.

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Original scientific paper

BIOCONTROL OF *ALTERNARIA TENUISSIMA* ORIGINATED FROM *OCIMUM BASILICUM* L. USING INDIGENOUS *PSEUDOMONAS* SPP. STRAINS

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SUMMARY

Basil (*Ocimum basilicum* L., Lamiaceae), an aromatic annual herb, is known as a plant with many benefits, including health, which are conferred by the essential oils it contains. Basil can suffer from several plant pathogens that can cause detrimental effects on plants and reduce yield. Phytopathogenic fungi *Alternaria tenuissima* penetrate the leaves during growth, causing black spots and remaining latent in the plant tissue until harvest. To avoid the unfavorable effects of this pathogen and the usage of chemical treatments, we applied plant growth promoting rhizobacteria (PGPR) as a biocontrol agent in order to achieve disease suppression in an environmentally friendly manner.

In order to estimate the capacity to reduce the disease symptoms on *Ocimum basilicum* L. caused by *A. tenuissima*, the indigenous plant growth promoting (PGP) *Pseudomonas* strains were used. During *in vitro* experiments, we selected strains with moderate (B25) and high (PS2 and Q16) fungal growth inhibition capacities. In greenhouse experiments with non-sterile soil the selected *Pseudomonas* strains reduced disease symptoms produced by *A. tenuissima* on *Ocimum basilicum* L. The disease incidence reductions of 90.6%, 86.5% and 81.5% were observed in the application of PS2, Q16 and B25 strains, respectively. Our results support the conclusion that the application of selected PGP *Pseudomonas* strains in basil rhizosphere during cultivation is a feasible alternative for management of *A. tenuissima* symptoms.

Key words: *Ocimum basilicum* L., *Alternaria tenuissima,* PGPR*, Pseudomonas*, biological disease control

INTRODUCTION

The genus *Ocimum* includes more than 150 species with large morphological variation among the different species. The diversity within genus increased due to the man selection, cultivation and hybridization within the genus. This medicinal plant is widely used for prophylaxis or treatment of many diseases, mainly due to the essential oil presence. The essential oils contents depend of environmental factors that may influence the plant chemical composition [1]. Cultivation in plant diseases suppressive environment may lead to production of plants materials without microscopic fungi contamination. Aldo the majority of *Alternaria* species are saprophytic fungi, some of them may be facultative pathogens and caused significant damage in plants cultivation, especially in stress growth conditions [2]. Fungus belonged to genera *Alternaria* is found in different medicinal plants in Serbia: St. John's wort seeds [3], peppermint [4], lemon balm [5], valerian [6], purple coneflower [7], sage seed [8], marshmallow [9] and yellow gentian [10]. *A. tenuissima* is found as one of the *O. basilicum* L. seed pathogens (unpublished data).

Bacteria in the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, *Agrobacterium, Lysobacter* are the most promising biological control agents - organisms capable of killing other organisms pathogenic or disease causing to crops. The mechanisms by which plant growth promotion rhizobacteria (PGPR) increase crop performance is not well understood, but the most important are: control of pathogens (bioprotectant role), increased nutrient acquisition (biofertilizers) and phytohormone production (plant stimulants). PGPR also can trigger a defense response in the plants as if attacked by pathogenic organisms [11].
The aim of this study was to estimate the capacity of indigenous plant growth promoting (PGP) *Pseudomonas* strains to reduce the disease symptoms on *Ocimum basilicum* L. caused by *A. tenuissima*.

MATERIAL & METHODS

In vitro assay of *Pseudomonas* isolates on growth of *Alternaria tenuissima* isolated from *Ocimum basilicum* L.

Antifungal activity of indigenous *Pseudomonas* isolates Q16, B25 and PS2 was determined by using a dual culture *in vitro* assay on Waksman agar (WA) [12]. Overnight cultures of bacteria were optimized to $1x10^5$ – $1x10^7$ cfu/ml and used for influence of: a) whole culture b) extracellular metabolites (supernatant) and c) heat stable antifungal factors (HSAF). HSAF represents the termostable extracellular metabolites obtained after heat treatment at 70°C during 30 min. Bacterial agents were applied near the edges of Petri dishes; 6 mm plugs of *A. tenuissima* mycelia were placed in the centre. Control variants contained mycelia of *A. tenuissima* on WA plates. After ten and fifteen days of incubation at 25°C, the fungal growth was measured and the percentage of growth inhibition was calculated [13].

The greenhouse experiment

Non-sterile soil was used in the greenhouse experiment for better simulation of field conditions. The disease incidence on *O. basilicum* caused by *A. tenuissima* and disease suppression with indigenous *Pseudomonas* isolates Q16, B25 and PS2 were estimated in ten replicates. Infected seedlings were planted ten days after the infection. As the control variants infected seedlings without bacterial inoculation were included. Two days after planting, the *Pseudomonas* isolates Q16, B25 and PS2 ($1x10^9$ cfu) was applied at the basis of the plant. Based on developed symptoms forty days after planting, the percentage of infected *O. basilicum* L. leaves by *A. tenuissima* was calculated by the formula:
% of leaf incidence = (no. of leaves infected with *A. tenuissima* / no. of leaves observed) x 100 [14]. Disease incidence reduction was calculated as: [(Control – Variant) / Control] x 100 [15].

RESULTS & DISCUSSION

Investigation of *Ocimum basilicum L.* seed infection revealed two dominant fungal genera: *Alternaria* (14% in 2010. and 23% in 2011.) and *Fusarium* (11% in 2010. and 14% in 2011.) (unpublished data). Because of significantly increasing percent of *Alternaria* spp. naturaly infection of *O. basilicum* L., we applied the indigenous *Pseudomonas* isolates on highly infected seeds to esstimate their biological control posibility.

In vitro assay of *Pseudomonas* isolates on growth of *Alternaria tenuissima* isolated from *Ocimum basilicum* L.

The minimal growth of *A. tenuissima* was observed in dual culture with the highest applied concentration of *Pseudomonas* Q16 strain overnight culture after ten days of cultivation (Table 1). The extracellular metabolites (supernatant) of the same strain showed no

statistically different results. The heat stable antifungal factors (HSAF) of PS2 and Q16 strains showed similar results at lower concentration, but the same effect at highest applied concentration. No additional fungal growths in dual culture were observed after fifteen days of cultivation, but fungal growth in control plate were maximal.

Percent of *A. tenuissima* growth inhibition caused by whole cells culture ranged from 27.50% (the lowest concentration of B25 strain) to 45.25% (the highest concentration of Q16 strain) after 10 days of cultivation (Table 2). The maximal growth of *A. tenuissima* was observed after 15 days in control variant; however the bacterial strains stopped fungal growth during the first time of observation. The inhibition after 15 days ranged from 36.26 to 58.21%. The significantly lower effects were caused by heat stable antifungal factors (HSAF) comparing to whole cells culture and supernatant fractions.

Table 1. Effects of indigenous *Pseudomonas* strains on growth (mm) of *A. tenuissima* isolated from *Ocimum basilicum* L.

bacterilal fraction	PS2			Q16			B25			Θ	
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7	10 days	15 days
cell culture	45.50* ±1.98	44.00 ±1.41	41.50 ±1.05	42.00 ±1.41	40.83 ±2.93	36.50 ±1.64	48.33 ±1.63	44.83 ±2.32	45.67 ±3.83		
Supernatant	45.67 ±1.97	44.33 ±1.97	42.00 ±1.79	40.33 ±1.51	39.67 ±1.97	37.67 ±2.34	49.00 ±2.45	46.33 ±3.83	44.00 ±2.83	66.67 ±3.27	87.33 ±1.33
HSAF	54.33 ±2.34	53.00 ±3.95	45.33 ±1.63	54.33 ±2.34	54.50 ±1.05	45.33 ±3.94	55.67 ±1.63	51.50 ±1.52	50.33 ±2.95		

* each number is the mean of six replicates (means ± SD)

Table 2. *A. tenuissima* growth inhibition (%) caused by indigenous *Pseudomonas* strains

Strains/ conc. (cfu/ml)	**PS2**			**Q16**			**B25**		
	10^5	10^6	10^7	10^5	10^6	10^7	10^5	10^6	10^7
bacterial fraction	incubation time: 10 days								
cell culture	31.75	34.00	37.75	37.00	38.75	45.25	27.50	32.75	31.50
supernatant	31.50	33.50	37.00	39.50	40.50	43.50	26.50	30.50	34.00
HSAF	18.50	20.50	32.00	18.50	18.25	32.00	16.50	22.75	24.50
	incubation time: 15 days								
cell culture	47.90	49.62	52.48	51.91	53.24	58.21	44.66	48.66	47.71
supernatant	47.71	49.24	51.91	53.82	54.58	56.87	43.89	46.95	49.62
HSAF	37.79	39.31	48.09	37.79	37.6	48.09	36.26	41.03	42.37

Antibiotic producing PGPR can release compounds able to prevent the growth of pathogens. Investigated *Pseudomonas* strain PS2 is good producer of lytic enzymes [16] and all investigated strains produce antibiotic phenazines: PCA and 2-OH- PCA (Josic et al., in press). The extracellular metabolites from cells culture and supernatants showed better potential for growth inhibition of *A. tenuissima* isolated from basil than heat stable antifungal factors. The HSAF involve production of antibiotics and HCN as the main antifungal factors. Concerning the differences of investigated fractions of bacterial cultures, the production of lytic enzymes, as heat non-stable substances, were significant in fungal growth inhibition.

Results in our investigation showed the significant effect of *Pseudomonas* spp. strains on growth inhibition of *A. tenuissima* originated from basil. The 36-55% in our investigation was still less than percents (50–80%) obtained by *P. fluorescens* for other phytopathogenic

fungi (*Pythium ultimum*, *Macrophomina phaseolina* and *Pyricularia oryzae*) [17]. However, different condition of bacterial cultivation and higher applied concentration may improve the level of fungal growth inhibition obtained in this investigation.

The greenhouse experiment

Seedlings infected with *A. tenuissima* were planted in non sterile soil during greenhouse experiment. Infected seedlings without *Pseudomonas* inoculation were included as a control. The same concentration of bacterial culture was applied once and developing of disease symptoms was observed. The applied *Pseudomonas* strains were competing with other microorganisms indigenous for non sterile soils, and to *A. tenuissima* added to *O. basilicum* seeds.

The percent of infected basil leaves by *A. tenuissima* ranged from 27.7% for control plants to 2.50% for plants with PS2 strain inoculation (Fig. 1). Percent of disease incidence reduction are shown on Fig. 2. The best results were obtained for PS2 strain, although disease incidence reduction for all three strains showed the same statistic significance.

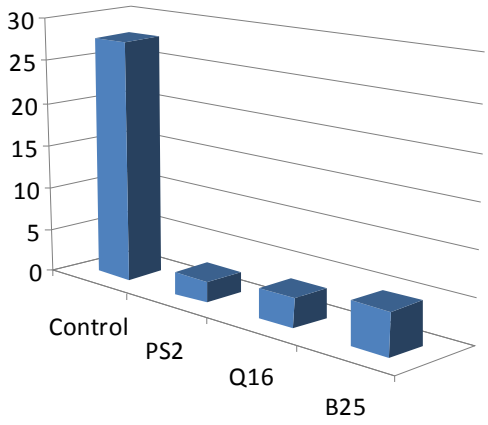

Figure 1. Infected *O. basilicum* L. leaves (%) by *A. tenuissima* with and without *Pseudomonas* strains inoculation

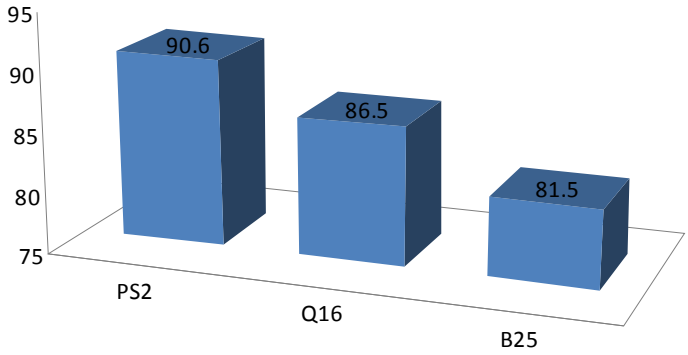

Figure 2. Disease incidence reduction (%) caused by *Pseudomonas* strains inoculation

Many medicinal plants are infected with *Alternaria spp.* in Serbia [18], but these pathogens are detected as phytopathogens in the other plants. After determination of *A. tenuissima* originating from soybean seed according to cultural, morphological and molecular characteristics, Jasnic et al. [19] reported the variability within the population and low pathogenicity on leaf, but their results indicate that, in favorable weather conditions, *A. tenuissima* could have economic significance in seed crop production.

To avoid spread of infection by phytopathogenic fungi and use of chemicals, PGPR application in plant production are recommended [20]. Production of lytic enzymes, antibiotics and HCN as fungal growth inhibition agents have bioprotectant role, which is the most important in biological control of pathogens [21]. Also, applications of PGPRs are available for increasing crop nutrient uptake. Species of *Pseudomonas* are known to produce phytohormones or growth regulators (indole-acetic acid, cytokinins, gibberellins, inhibitors of ethylene production) which have the effect of increasing the absorptive surface of plant roots for uptake of water and nutrients and acts as plant stimulants [11]. Most of the main PGPR treats are confirmed in *Pseudomonas* strains PS2, Q16 and B25 [16; Josic et al, in press]. Results of this investigation confirmed disease reduction after the application of selected PGP *Pseudomonas* strains, especially PS2 and Q16, in basil rhizosphere during cultivation. This can be a feasible alternative for management of *A. tenuissima* symptoms.

ACKNOWLEDGEMENTS

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Original scientific paper

BIOACTIVITIES OF ESSENTIAL OILS FROM ROSEMARY AND PENNY ROYAL ON *RHYZOPERTHA DOMINICA* (COLEOPTERA: BOSTRICHIDAE)

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SUMMARY

The lesser grain borror *Rhyzopertha dominica* is one of the most important insect pests of stored products such as cereals and cereal products. In the past decades, chemical fumigants like methyl bromide and phosphine have been used to control stored product pests. These insecticides have many problems such as development of resistance in pests and toxicity to human and environment, and there is an urgent need for development and use of safer crop protectants. In recent years many researches have been developed on use of plant secondary metabolites as new sources of insecticides. This research evaluates insecticidal activities of essential oils extracted from *Rosemarinus officinalis* and *Mentha pulegium* on *Rhyzopertha dominica*. The essential oils were obtained by hydrodistilation via a Clevenger type apparatus. To assess LC_{50} and LC_{95} of essential oils to *R. dominica*, twenty adults (1–7 days old) were placed in 50 mL plastic vials with screw caps, separately. Filter papers (Whatman No. 2, cut into 1 cm diameter discs) were impregnated with oils at doses equivalent to provide fumigant concentrations of 30 to 180 µL L^{-1} for *R. officinalis* and 150 to 800 µL L^{-1} for *M. pulegium*. These concentrations were selected after preliminary tests. Data obtained from dose response bioassays after 24 h were subjected to probit analysis to estimate LC_{50} and LC_{95} values using SAS software version 6.12. The calculated values for LC_{50} were 87.11 and 473.8 µL L^{-1} for *R. officinalis* and *M. pulegium*, respectively. The present study suggests that essential oil from these plants may be potential grain protectant as botanical insecticides and *R. officinalis* oil is more efficient than *M. pulegium*.

Key words: *Essential oil, Fumigant, grain, Rhyzopertha dominica*

INTRODUCTION

The lesser grain borror *Rhyzopertha dominica* (F.) is a major cosmopolitan pests of stored cereals and legumes throughout the world. Both adults and larvae of this insect are able to attack the grain [1]. Because the majority of the development occurs inside the kernel, *R. dominica* is difficult to kill with contact insecticides applied directly to stored wheat. In the past decades, chemical fumigants like methyl bromide and phosphine have been used to control stored product pests. These synthetic insecticides have many problems such as development of resistance in pests and residual toxicity to human and environment, and increasing costs of application, so there is an urgent need for development and use of safer crop protectants. In recent years many researches have been developed on use of plant secondary metabolites as new sources of insecticides. In this study insecticidal activities of essential oils extracted from *Rosemarinus officinalis* and *Mentha pulegium* were evaluated on *Rhyzopertha dominica*.

MATERIAL AND METHODS

Insects were obtained from our stock culture and reared in the laboratory on wheat in plastic containers at 25 ± 1ºC, 65 ± 5 % relative humidity and constant dark. Experiments were carried out under the same environmental conditions.

The leaves of *Rosmarinus officinalis* and *Mentha pulegium* were collected in April 2010 from Khuzestan province and subjected to hydrodistillation using a modified Clevenger apparatus for 4 h. Then collected oils were dried over anhydrous sodium sulphate and after filtration, stored at +4 °C until tested.

For bioassays twenty adults of *R.dominica* were placed in 50 mL plastic vials with screw caps. Filter paper (Whatman No. 2, cut into 1 cm diameter discs) was impregnated with oils at doses equivalent to provide fumigant concentrations of 30, 60, 90, 140 and 180 µL/L air for rosemary and 150, 200, 300, 600 and 800 µL/L air for Penny royal, respectively. These doses were selected after preliminary tests. The discs were attached to the vial caps and caps were screwed tightly on vials. Control insects were kept in the same conditions without any essential oil. Each dose was replicated five times. The number of dead beetles was counted after 24 hours of exposure to essential oils. The Abbott (1925) formula was used to correct for natural mortality in the controls [2]. Data obtained from dose response bioassays were subjected to probit analysis [3] to estimate LC_{50} and LC_{95} values using SAS software version 6.12[4].

RESULTS AND DISCUSSION

The results showed that the susceptibility of *R. domonica* adults differed according to the concentration of *M. pulegium* ($F = 132.2$; $df = 6, 14$; $P < 0.0001$) and *R. officinalis* ($F = 194.1$; $df = 6, 14$; $P < 0.0001$) (Fig. 1 and 2). After 24 h of fumigation, the mortality rate of adult beetles was found to increase as the essential oil concentration increased. At the highest concentrations, 800 µL/L air for *M. pulegium* and 180 µL/L air for *R. officinalis*, oils caused 80.0 and 88.7% mortality, respectively. This result was in accordance with a study by Ewete et al. (1996), which reported that the effectiveness of a substance for insecticidal purpose was related to its capacity to cause a strong significant mortality rate in a population of pests.

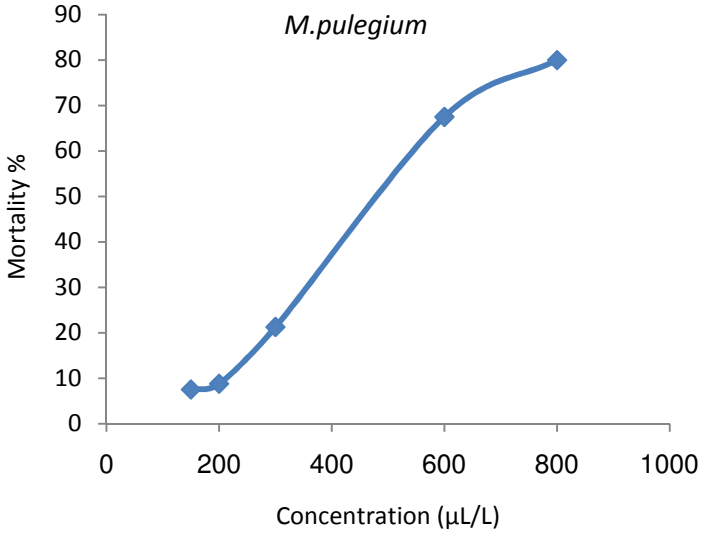

Figure1. Percent mortality of *Rhyzopertha dominica* adults at different concentration of *Mentha pulegium* essential oil.

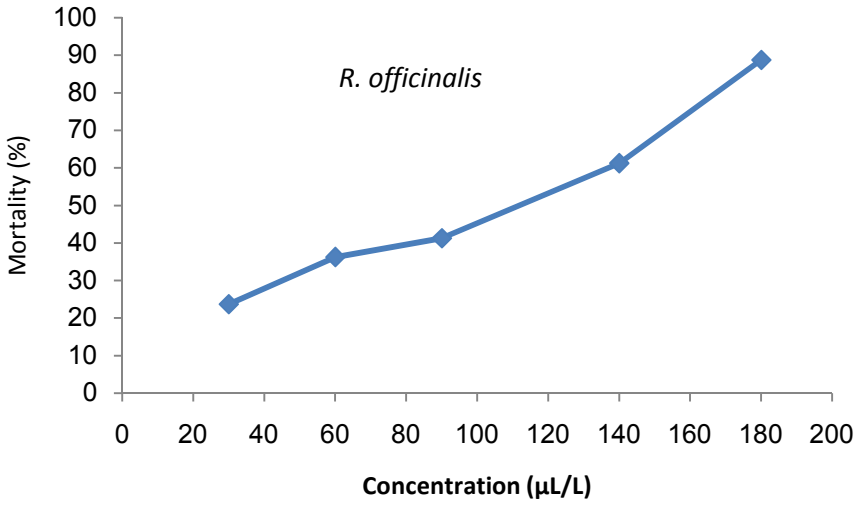

Figure2. Percent mortality of *Rhyzopertha dominica* adults at different concentration of *Rosemarinus officinalis* essential oil.

Table 1 shows the fumigant toxicity of essential oils to *R. dominica*. However, *R. officinalis* (LC50: 87.11) is more effective than *M. pulegium* (LC50: 473.8) in control of *R. dominica*. According to Lee et al. (2004) essential oils obtained from Myrtaceae family have fumigant toxicity to *R. dominica*. The LD_{50} varied from 7.8 to 17.6 µL/L air with plant species [5].Studies of Bekele and Hassanali (2001) indicated that the major component of Ocimum kilimandscharicum was largely responsible for the toxic action of its essential oil against *R. dominica* [6].

Table1 Fumigant toxicity of *Rosemarinus officinalis* and *Mentha pulegium* essential oil to *Rhyzopertha dominica* adults

Essential oils	LC_{50} (µL L^{-1})	Slope ± SE	Degree of freedom	Chi Square (χ^2)
R. officinalis	87.11† (27-225)	2.18±0.6	3	14.97
M. pulegium	473.8 (428-526)	3.66±0.3	3	1.5

† *Mean (minimum-maximum)*

CONCLUSION

This study demonstrate potential of *Rosemarinus officinalis* and *Mentha pulegium* essential oils as fumigant insecticides and Since this species is a common medicinal plants in Iran and, it can be used for cereal protection against the attack of infesting beetles like *R. dominica*. However, *R. officinalis* oil is more efficient than *M. pulegium*.

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Original scientific paper

ORGANIZATION MARKETING ACTIVITIES IN ENTERPRISES FOR BUYING, PROCESSING AND SELLING OF MEDICAL AND AROMATIC PLANTS IN SERBIA

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SUMMARY

The non-wood forest products (NWFPs) appear as one of the forestry products which meet the criteria of organic farming. The use of NWFPs in Serbia is still at a relatively low level for several reasons. The great problem is lack of information about the possibilities of their collection, processing technologies and marketing, the commercial importance of NWFPs, as well as informing the population of potential users of these products. The goal of this article is survey of marketing activities of chosen small and medium enterprises (SMEs) engaged in purchasing, processing and marketing of NWFPs, as well as presenting of current marketing activities of surveyed SMEs and providing of some recommendations for more efficient organization in the sector. The subject of the article is organization of marketing sector in each surveyed enterprise. By this study it has been tried to point out the role of marketing in the surveyed eneterprises, as one of the important instruments for achieving economic goals of business.

Key words: *non-wood forest products, medicinal herbs, organization, marketing, SME$_s$*

INTRODUCTION

Forests are the ecosystems containing a large number of medicinal plants of outstanding properties, valued in the market and frequently used by pharmaceutical and cosmetic industries. The main regions of collection of medicinal and aromatic plants in the Republic of Serbia are situated in the South-east. Region with the highest number of collectors of plants is the area of Sokobanja. It is estimated that there are about 4 000 collectors (about 12 000 family members) [1], [2]. Demand for NWFPs annually increases [3]. Many traditional products of the NWFPs, which were once associated with population of low-income, are today considered as a natural product or as specialty in the food industry, and represent a significant source of income for households [4]. NWFPs take significant place in the forest policy of many countries. The income from their use are approaching the use of revenues derived forest assortments [3], and they are available with significantly less financial investment, it can be assumed that the growing need is to promote these products in Serbia. The use of NWFPs in Serbia is still at a relatively low level for several reasons. The key problem is lack of information to citizens about the possibilities of the use, collection and processing technology and marketing activities of NWFPs [5].

The goal of this article is survey of marketing activities of chosen small and medium enterprises (SMEs) engaged in purchasing, processing and marketing of NWFPs, as well as presenting of current marketing activities of surveyed SMEs and providing of some recommendations for more efficient organization in the sector. There have been chosen enterprises with relatively different organisational structure and the size. The subject of the article is organization of marketing sector in each surveyed enterprise. By this study it has

been tried to point out the role of marketing in the surveyed enterprises, as one of the important instruments for achieving economic goals of business.

MATERIAL AND METHODS

The methodology used in the paper includes general scientific and special methods. The primary role in the research methodology takes the methods used in the study of marketing activities and case studies of SMEs [6]. The case study has been performed in the organizational and managerial process database created during three year period. It has been chosen 3 enterprises in the field of NWFPs which have different marketing activities related to organization. However, the sampling of cases from the chosen population is unusual when building theory from case studies. Such research relies on theoretical sampling (*i.e.,* cases are chosen for theoretical, not statistical, reasons [7]. For the purpose of creating a database in this case; the test method was used, as well as a survey research technique. It has been used data of representatives of SMEs involved in processing and place of NWFPs [8]. The enterprises have been chosen randomly from the database of surveyed enterprises of the group small, medium and big enterprises. There have been chosen enterprises with relatively different organisational structure and the size regarding number of employees. The identity of enterprises and participation in this research is voluntary. Their answers are used only for scientific purposes by assuring the respondents anonymity, because of that fact they are not named in the article. A case study protocol has only one thing in common with survey questionnaire, both are directed at a single data point - either a single case (even if the case is part of larger, multiple-case study) or a single respondent [9] [10]. Survey, as well as basic research techniques, includes six parts of questions: general information about the company, purchasing raw materials, processing them, the placement of the final product, the organization of enterprises and organizations of marketing activities in the enterprise. In order to get a clear environment in which companies from Serbia exist, PEST analysis was performed. During the survey companies were asked questions about the business environment of the surveyed enterprises.

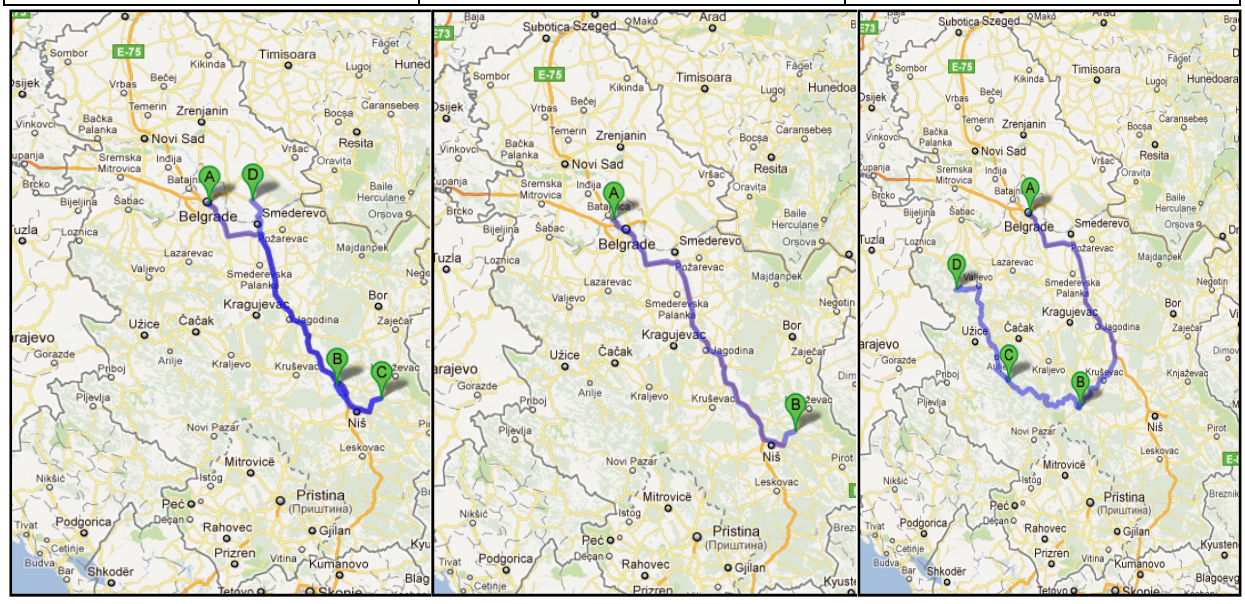
PEST analysis is a method of business environment analysis and a base for strategic planning. This type of analysis is focused on environment for emerging market and provides an overview of the external situation that could have an impact on the industry as a whole or to companies within the industry observed [11]. Political, Economic, Social and Technological (PEST, also often referred to as STEP) analysis as a tool to identify narrower contexts and focus research questions around feasible and meaningful regional contexts. The constituents of PEST can be considered as macro-environmental factors and its usefulness lies in the assumption that the success of a particular organization or management solution cannot be understood without having the information relevant to the specific business environment [12]. According to Ward and Rivani [13] PEST analysis assumes that specific external and indirect circumstances that characterize the business environment are able to influence organizational capacity to produce value. Hence, PEST analysis provides a "satellite view" to assess the external environment [14]. This is particularly relevant when trying to narrow very large business environments in order to study organizational marketing activities. Also, in the research is also applied and the SWOT analysis, which provides a balance between internal capabilities and external possibilities of the company [15]. The basis for this technique is the idea that successful companies are able to use their strengths and opportunities the environment, but also to recognize the weaknesses of their business, as well as threats from the environment and to respond to them [16]. Study is related to organization of marketing activities in 3 enterprises, and according to that analysis it has been created SWOT matrix.

RESULTS

First interviewed company has three full-time workers. The whole marketing department organizes the person who simultaneously performs the function of Deputy Director. The company advertising activities base on the flyers and posters, which share through distributors, mainly in pharmacies. They are also advertised through television and the partner who performed "on-line" sales and web-site. Currently, this marketing activity is performed only in Bosnia and Herzegovina. As part of the marketing activities, they have special design and packaging of medical and aromatic plants. Since the company has no sector that could respond to these requests, for this part of the job he was hired designer of another company. Minority of workers has caused that one person has multiple functions, *i.e.* perform multiple tasks. Therefore, it is assumed that the productivity of the enterprise is reduced. Organizational chart of the company is characterized by a small number of sectors. The company headquarters are in: Belgrade, Žitkovac, Svrljig and Bavanište (Fig. 1).

Another company interviewed has 40 full time employees and one person responsible for the marketing department. Marketing activities are carried out through newspapers and magazines. Also, the company participates in trade fairs of healthy food. The bigger number of employees has caused more efficient organization within the enterprise as well as better organization of marketing with a number of different forms of promotion. Medical plants and herbs are purchased from a company that is engaged in purchasing NWFPs with the center in Svrljig (Fig. 2).

FIGURE 1. Locations of purchasing and processing raw materials (A-Belgrade (processing), B-Žitkovac; C–Svrljig, D-Bavanište)	**FIGURE 2.** Locations of purchasing and processing raw materials (A-Belgrade (processing), B-Svrljig)	**FIGURE 3.** Locations of purchasing and processing raw materials (A - Belgrade (directorate), B - Brus, C - D - Vlasina)



Source: original

Third interviewed company has 170 employees and three people working in the marketing sector. Their marketing activities are: ATL[1] and BTL[2]. Regarding organisational aspect the most adequate structure is in the third enterprise. A number of workers allowed their specialization in a particular type of work, which certainly contributes to increased productivity, and greater number of people employed in the marketing sector. That provides a number of new marketing ideas, which led to different forms of promotion of their products. The enterprise buys herbs from collectors in the county of Brus, Ivanjica and Vlasina (Fig. 3). The first company's manufacturing section is situated in the base of the Kopaonik Mountain, where the purchase station is also located. Another is located in the area of Svilajnac. The best organization of production has the third company, because the production is very close to purchase stations. The company has organized its production in accordance with the requirements of HACCP system[3] and quality management system, in accordance with standard ISO 9001.

In order to better compare the results achieved with the direct survey of marketing organisation it has been conducted the SWOT and PEST analysis. PEST analysis tends to create a complete picture of conditions in which these 3 enterprises operate. Companies which have a better organizational structure also have a better market position and greater ability to resist the negative environmental factors.

Table 1. PEST ANALYSIS

Political and legal factors	Economic factors	Sociologic factors	Technological factors
Unstable political situation	High interest rates	The low standard of living	Increasing advances in IT technology
Weak law implementation	Increased risk of investment	Increased unemployment	Advanced payment techniques
Corruption	Application of international standards	Low awareness about sustainable collection practices	Departure of experts outside of Serbia
Long-lasting process of EU accession	The low purchasing power of the people		Sector that is poorly invested

Source: original

The respondent companies have to overcome many problems to be competitive in the market (Tab. 1). Important factors which influence business of enterprises are: unstable political situation, weak of law enforcement, high interest rates, low living standard, etc. Because of these facts it is important to have a good organization of the companies to overcome external factors.

[1] ATL – depersonalized, paid media communication, such as radio, TV, newspapers
[2] BTL – all promotional activities where there is no media
[3] HACCP - this system includes a series of procedures to control the process and sensitive points in the food chain.

Table 2. SWOT ANALYSIS OF ENTERPRISES

FIRST SURVEYED ENTERPRISE	
S (Strengths)	**W (Weakness)**
Long experience of employees	Insufficient marketing premise activity
	Non qualified stuff for marketing activities
Existence of a permanent market	High interest rates
Quality and wide range of the products	
Richness of raw material base	Small number of final products
Competitiveness at the price level in purchase stations	Lack of their own means of transport
	Final price of product is not competitive
	Distance of raw material base
	Insufficient number of employees
	Insufficient use of capacities
O (Opportunities)	**T (Threats)**
High natural potential	Weak state support
Favourable locations	Dependence on weather conditions
Increased demand	Strong competition
Certification of products	Fluctuations in product prices
Government subsidies and incentives	Unfavourable political and economic situation
SECOND SURVEYED ENTERPRISE	
S (Strengths)	**W (Weakness)**
Professional staff	Insufficient use of capacities
Acceptance of standards	Non qualified stuff for marketing activities
Price competitiveness of products	High interest rates
Own means of transport	Distance of raw material base
	Iinsufficient marketing activity
O (Opportunities)	**T (Threats)**
High natural potential	Weak support of state
Favourable locations	Dependence on weather conditions
Increased demand	Strong competition
Certification of products	Unfavourable political and economic situation
Government subsidies and incentives	
THIRD SURVEYED ENTERPRISE	
S (strengths)	**W (Weakness)**
Nearness of raw material base	Insufficient use of capacities
Wide range of products	High interest rates
Price competitiveness of products	Enterprises with dumping prices
Marketing activity	Uneven cash flow
Professional staff	Insufficient export orientation
Own means of transport	
Acceptance of standards	
Close to purchase stations	
O (Opportunities)	**T (Threats)**
Great natural wealth	Weak state support
Favourable locations	Dependence on weather conditions
Increased demand	Strong national competition
Certification of products	Unfavourable political and economic situation
Government subsidies and incentives	

Source: original

A small number of employees in the first respondent companies led to a poor division of labour within the enterprise. The situation that one person performs multiple tasks, led to the fact that this company has the greatest difficulties in the business. The company is largely dependent on other companies to perform these service activities. This leads to higher prices of their products over the competition. The higher prices are influenced by the distance from raw material source. If we add the lack of marketing activity and a small number of final products, it can be concluded that a great effort is necessary to get to the business forward. More efficient organization within the second surveyed company with more employees caused a smaller number of weaknesses that owns this company. Own means of transport contributed to more competitive prices in the market of final products. What might contribute to even lower real costs is to reduce the distance between the raw materials source and production section. It is necessary to increase marketing activity in this enterprise, which would perform better position of the enterprise in the market and more competitive relation. Many employees in the third company, good organization, close to the processing and purchase of NWFPs with well-organized marketing department are key factors of success. Because of that, it has many advantages over the other two respondent companies and very few weaknesses.

DISCUSSION

According Hegedűs in across Europe there is great potential for improving the market and the commercialization of NWFPs, which means that in this sector there are many business opportunities in the short and long-time [17]. In support of this conclusion is the fact that the growing demand for this group of products is present all around the world. Despite the overall economic importance of NWFPs, studies have shown that enterprises have relatively low returns. As the main reason is the lack of organized systems and insufficient marketing activity, which could help the individual producers in the organization of production and distribution, determining the appropriate price, choice of markets, and promotion of goods [18]. Even when there is information about the market, it is often not transmitted local small producers [19]. Marketing of NWFPs used as raw materials in industry is usually carried out in two main phases: the marketing of raw materials or the collection until it comes to industrial processors, and marketing of final or semi-finished industrial and consumer products [20].

Regarding the organization of purchasing, processing and marketing of NWFPs, the dominant position has the third interviewed company, because it buys NWFPs where the processing is performed. This significantly reduced transportation costs. When the final placement of the products in question, first surveyed company is at a disadvantage in the competition. The reason for this is the fact that this company does not own the means of transport. It distributes products through authorized distributors, which are further built in the final product price. To better organize the marketing sector requires better organization-wide enterprise. For the successful organization of marketing, it requires a good organization within the company, *i.e.* strict division of labour. Good organization of the company requires a greater number of experts who are closely specialized, especially in marketing division. The quality of the organization can be estimated based on the realization of economic principles of productivity, efficiency and profitability [21] [22]. Good organization within the company is transferred to the marketing department [23]. What third company makes more competitive compared to other companies is the increasing number of workers, which allowed more people working in the marketing sector. This makes it possible to implement a number of ideas into action. While his competitors has for just one person in the marketing sector, or

even one person is working on more functions in the third enterprise there are three persons working in the sector of marketing. Of course, this is just conclusion derived from the part of the research and the complete access to the topic is possible just if it is possible to analyse the majority enterprises in this sector.

CONCLUSIONS

It can be concluded following from the analysis:

- based on the number of employees[4] the first company was categorized as a micro (3 employees), the second as a small (40 employees), and the third as medium company (170 employees);
- in accordance with the size of respondent companies, marketing departments, either do not exist (the firs company), or are at different levels of development (the second, and the third companies). In the first studied company, one person performs multiple functions, also including performing marketing activities. The second respondent company have a separate marketing department with one permanent employee in the sector. The third company has a separate marketing department with a staff of three people;
- the first company purchases raw materials from the four buying centres, the second company from one, and the third company supplies raw materials from the three buying centres;
- the most common forms of promotional activities as advertising through the official website and print media, while only a third company uses more aggressive forms of promotion;
- two bigger companies have adopted the ISO 9001 and HACCP standards;
- the first company, although it has many weaknesses (such as distance from the raw material base lack of marketing activities, non-competitive prices, low capacity utilization, and many others), also has a long tradition in production and a limited but steady number of customers;
- the strengths of other companies may include skilled and specialized personnel, owning their own means of transport for deliveries of raw materials to processing and further distribution of final products, competitive prices, products, etc., although there is a series of weaknesses such as under-utilization of capacity, distance from buying centres, undeveloped marketing department, etc. The third company has a very small number of exceptional weaknesses and internal predispositions, such as a wide range of high quality products, good organization of the sector, the sector is also marketing, technical staffing structure is currently a very strong position in the market etc.;
- illiquid market and competition in general are threatening the environment for all three companies, but good natural qualities, the quality of raw materials and increasing demand in domestic and world markets for products of plant origin are also their developmental potential
- important factors which influence business of enterprises are: unstable political situation, weak of law enforcement, high interest rates, low living standard, etc.

The number of respondent companies is insufficient to be able to judge the marketing activities of companies engaged in purchasing, processing and marketing of NWFPs in Serbia. It can be concluded that there is a marketing activity of respondent companies, but insufficient. An exception might be the third enterprise, which could be as the pattern for other surveyed companies. It is necessary to structurally strengthen the marketing department in which will work closely qualified persons for that area, and organize production in close with raw material.

[4] Acording to the Republic Statistical Office the micro enterprises has 0-9 employees, the small one 10-49 employees, and the big one 50-249 employees.

It is important to mention that successful marketing departments in each company have to: be focused on the company, monitor the competition, own the brand, find and direct outside vendors, create new ideas, communicate internally, manage the budget, etc. To have complete insight to the situation of the enterprises dealing with NWFPs it is important to analyze all these facts and compare them mutually.

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Section III

"MAP cultivation, breeding and biotechnology"

Introductory lecture
Review paper

SOCIO-ECONOMIC EVALUATION OF THE INTRODUCTION OF NEW MEDICINAL AND AROMATIC PLANTS

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SUMMARY

During last decades long-term agronomical research and developmental efforts have been carried out for elaborating the production techniques of new aromatic and medicinal plants in Finland. The activities have been focused to the following species: *Agastache, Arnica, Carum, Drosera, Echinacea, Echium plantagineum, Gentiana lutea, Leuzea, Origanum, Rhodiola and Urtica.* The main aim of these introduction and acclimatisation studies were the diversification of the farm cultivation and supplying domestic raw materials directly to the consumers or for industrial processing. The total cultivated area during this time has increased from 100 ha in 1984 to 22.000 ha in 2006.

In this lecture we analyze the difficulties, bottlenecks and successes during this introduction process, especially from the industrial point of view. We analyze the motives of the introduction works, the national support of the research; evaluate the present state of art of their field production and industrial processing technologies. At the same time we try to take into account those socio-economic factors – the acquaintance of a new species, their economic importance in farm level, their national and international market potential- which finally determinate the present and the future existence of a given new species. Although the described activities and analyses geographically relates to a Nord-European country, the author believes, that the experiences obtained there can be useful in a wider sense.

Keywords: alternative crops, motives of introduction, acquaintance of new plants, economical importance

INTRODUCTION

Finland is situated between 60 and 70 degrees of the northern latitude. It is the most northerly country in the world to have an active commercial horticulture**.** Due to the climatic conditions, the spectrum of the cultivated useful plants is narrow, and historically the introduction of indigenous plants into Finland always had a great importance. The introduction of aromatic and medicinal plants into Finland is not a new activity. The only well-known herb, originating from the north, is angelica (*Angelica archangelica)*, whereas all other herb species have arrived from the central and southern parts of Europe. Herb cultivation had some significance in the monastery culture during the XIV-XV centuries of the Swedish Kingdom period, and during Linne's time in XVII century. Due to the changes in the agricultural policy, the introduction of new herbs and medicinal plants has been in focus again during the last three decades. In this lecture we try to analyze the difficulties, bottlenecks and successes during this introduction process, especially from the industrial point of view.

BACKGROUND AND ACTIVITIES

The motives of the introduction research. The interest for cultivation of new aromatic and medicinal plants may be due to several sociological factors. According to the intensive tourism during the 80s, the Finnish society became more open for the new tastes, new types of foods, new herbs, and the interest for herb consumption started to increase. The so-called green movement, interest in alternative medical treatments and alternative production systems, like organic or biological cultivation, became more well-known. The popularity of the aromatized local food specialties and new culinary experiments increased the interest for growing new spice species.
At the same time, the surplus of the traditional agricultural products forced both agricultural policymakers and farmers to find new, alternative crops. It was thought that part of the imported herb raw material could be replaced with domestic production of some herbs and medicinal plants. General belief (though weakly documented) in higher aromatic contents of plants grown in the north stimulated domestic herb cultivation as well. Additionally, the low level of environmental pollution in the Nordic environment was in favour of high quality raw material for the medicinal industry. During the last decades these factors increased the importance of domestic grown raw material and large-scale necessity occurred for developing domestic herb production.

Difficulties in the North. For the cultivation of MAP species in the North there seems to be three big difficulties: the climate, the geography and high costs. Shorter vegetation period, lower temperature sum in the growing season, the long and cold winters, as well as the mechanical injuries of snow and ice all limit the range of cultivated herb species and their biomass production. Therefore the spice consumption in Finland has traditionally been low and limited, and based on salt, pepper, dill and parsley only, whereas all other spices have been imported. The quantity of the imported spices and aromatic plants in Finland has ranged between 4-5000 t annually (3). Besides the climate, another important hindrance seems to be the Nordic socio-geography. The dispersed locations of the small farm units limit concentrated cultivation in the northern parts of the region. Due to the sparse population (5 million in 330 000 km²), the long distances lead to expensive transport and logistics. The high cost of living in the Nordic countries results in high production costs generally and in the agriculture as well.

The research activities. During 1984-2004 in Finland 143 R&D projects have been carried out, with the focus mainly on field cultivation and primary farm processing of MAPs with a total value of 22-24 million euro (4). In the beginning the research transfer from production countries had some importance and after gaining basic knowledge, some special Nordic questions were studied (aroma content, cleanness, indoor cultivation, etc).
One of the research subjects of this period was the introduction of new MAP species into Finland and elaborating their production techniques (5). The selection criteria of the new species were: their climatic suitability, existing use in other countries, evidence on the medical effects, industrial interest for using domestic raw materials, environmental aspects, popularity, decorability.
During the period of 1980-2010 this activity started with cultivation of *Echinacea purpurea,* followed by several warmth-requiring species, like *Agastache foeniculum, Perilla frutescens, Tagetes lucida* and *Origanum vulgare ssp. hirtum.* Cold tolerant, non-endemic medicinal plants were *Arnica montana*, *Gentian lutea, Leuzea carthamoides,* and endemic wild medicinal plants *Carum carvi, Drosera rotundifolia*, *Rhodiola rosea* and *Urtica dioica.* The production of fresh-cut herbs in greenhouses started by the initiative of private companies.

In this paper we evaluate the results of the introduction process of the most important new MAP species in Finland. We analyze the following main aspects: their acquaintance by consumers in the beginning and presently, their acceptance by the consumers and by the industry, the present state of art of the developed agro-technical and processing techniques, their cultivation area and their domestic and international market potential.

RESULTS

Introduction of warmth requiring species

***Echinacea purpurea* L.** Echinacea was the first medicinal plant to be cultivated during the 80-ies for industrial use in Finland, and its success was an inspiriting example in the country (6). The acclimatization and introduction work started in 1981 on the Frantsila Herb Farm and in 1984 in the Agrifood Research Finland MTT Mikkeli (61° N). The elaborated growing techniques have been passed on to the growers, who have agreements with industrial companies (7). Presently the area of cultivated Echinacea is 2-4 ha yearly and three products are prepared from domestic raw materials. The critical point of the plantations is the early spring late frosts, which may cause serious damages. Echinacea has been introduced successfully into Finland, and due to its decorative habit, it has gained great popularity in gardens. The raw material producers have industrial contracts and the consumers prefer domestic products. One company exports Echinacea products to 24 countries (www.hankintatukku.com)

***Agastache foeniculum* (Pursch)** The perennial plant originates from the northern states of America. The dry herb contains 0.07-2.5 % essential oils, with methylchavicol as a main compound. Despite that fact that it has long been on display in botanical gardens, it remained unknown to the public till 1981, when the Frantsila Herb Farm introduced it into Finland and started to trade it in various tea mixtures. During the last 30 years it has gained wide popularity among hobby gardeners, and presently one can find its seeds in all herb seed catalogues. The field cultivation technology using transplantation was elaborated (8) and given to special plant producers. Anise hyssop was successfully introduced into Finland. Due to its decorativity, pleasant anis like smell and easy cultivability, it has become a popular aromatic garden herb. At the same time, it has no significant market potential. Some special farms produce raw material for local products (tea mixtures) or it is used for aromatization of industrial herbal extracts.

***Tagetes lucida* Cav.** The species originates from Mexico and Texas, and due to its agreeable sweetish flavour it has been used for replacing the French tarragon. The dry herb contains 0.2-1.5 % of essential oils with methylchavicol (60 %) and trans-anetol (16 %) (9). Acclimatization and introduction experiments started in 1999 by the author, and the growing technology and yield potential have been studied in several agronomical experiments. As a warmth-requiring annual plant it can be cultivated using transplantation with black plastic mulch. Although this newly introduced herb species has been known by the public for only 10 years, it has become a fashionable garden herb due to its pleasant aroma, easy cultivability and wide possibilities for experiments and utilization in cooking. Preliminary hydroponic experiments were carried out using it as a fresh-cut herb as well. Presently there is no commercial cultivation of it yet.

***Perilla frutescens* (L.) Britton** A great number of clinical studies with *Perilla* have proved its extract to be effective against hay-fever and it has become a popular preventive anti-allergic medicinal plant (10). The acclimatization work for its cultivation was started based on demand from the medicinal industry in 1993 to replace imported raw material for producing Allermin® products (11). The disadvantageous characters of this annual plant include high ratio of useless stems (>50 %) and the complicated extraction methods of the dry raw material in the industry. During the last decade *Perilla* started to be cultivated hydroponically in greenhouses as a fresh-cut herb and it has gained some popularity with its delicate taste and aesthetic, varying green-red-ruffle leaf forms. Its acquaintance will increase among the professional and amateur cooks and gardeners, but presently it has no significant importance as an industrial medicinal plant.

***Echium plantagineum* L.** is a low, bushy annual species with purple, violet-blue, white and pink colour flowers. It originates from the coastal areas of southern and western parts of Europe. The seed oil (cc. 20-30 %) contains significant amounts of Gamma Linolenic acide (GLA) and some rarer stearidonic acid (SdA). The seed oil is used in cosmetic products because of its moisturizing and anti-inflammatory action, and used mainly in sun care and skincare products. *E. plantagineum* was introduced into cultivation in the UK as a specialty crop for its seed oil. Its cultivation area in the UK in 1997 was 20 ha and 1998 190 ha.
In Finland, the introduction experiments started based on the initiative of an industrial company. The greatest obstacle to its larger scale cultivation seems to be the short growing season in the north, because the plant biomasses do not reach the scale of ripeness suitable for mechanical harvest. As a final conclusion, *E.plantagineum* appears not suitable for large scale cultivation at this latitude (12). However, the achieved experience could be utilized in Central and South-European regions, where the climatic risks could be minimized.

Introduction of threatened medicinal plants
The initiative for studying the cultivation possibilities of the European endangered medicinal plants in Finland was put forward by the First International Symposium on the Conservation of Medicinal Plants in Trade in Europe, organized by TRAFFIC Europe, in 1998. According to the report, among the fifteen species being in the highest environmental danger there were several cold tolerant medicinal plants: *Arnica Montana, Cetraria islandica, Drosera rotundifolia, Gentiana lutea, Menyanthes trifolia, Primula veris*. (13). The report stimulated a research program in the institute Agrifood Research Finland MTT, Mikkeli, for elaboration of the basic growing techniques of some selected species. Due to the positive perception of environmental values in Finland, financial support for this program was found as well.

***Arnica montana* L.** Arnica is a non-endemic plant in Finland, originating from the high mountain areas of Europe, and therefore climatically well adapted to the cold climates. Its introduction experiments were carried out during 1990-2003 in Mikkeli (61° N) (14, 15). The basic result of these experiments was that arnica can be cultivated in Finland successfully from one transplantation for consecutive 6-7 years. The experimental dry flower yield ranged between 3.5-4.6 kg/100 m², and the hand harvest period lasted for three weeks.
For dissemination of the experimental results semi-large scale demonstration plantations were created for growers and additional on-farm cultivation was organized at ten farms (16). Moreover, a study trip was organized to the main buyer of arnica yield (Weleda Ag, Switzerland) and a long-term agreement was negotiated between the company and a Finnish cooperative. Unfortunately, during the following years the interest of the farmers in cultivation decreased, and the produced flower yields remained small, unsuitable for industrial processing, and the commercial arnica cultivation was finally stopped. The arnica-

based health care products are popular among the consumers, but the domestic cultivation in larger scale turned out unsuccessful.

***Gentiana lutea* L.** Yellow gentian is a perennial plant with aromatic and bitter roots, used in medicinal and alcohol industry. About 2000 tons of roots are collected from the wild in Europe annually, which has made yellow gentian a strongly threatened plant (13). The introduction experiment started in 1984. During the following 10 years of experimenting, the basic knowledge on cultivation was obtained (17), including mass propagation method (18) and semi-large scale demonstration plots, which were established for growers. The transplanted seedlings were grown 4-5 years in black plastic mulch with 9/m² plant density and after five years the dry root yields ranged between 130-170 kg/100 m², with high bittering power (19). The key obstacle to turning the research results into commercial production was the lack of local industrial processors in Finland. Additionally, the market price for the dry root yields in the traditional collection countries has been too low, in comparison with the high production costs in Finland.

***Drosera rotundifolia* L.** Extracts of *Drosera* species are used in a number of medicinal products for treatment of asthma and bronchitis, the necessary raw material originating from wild populations. During 1970-80's the collection of natural sundew yields was organized in northern Finland, and the exported fresh quantities (mainly to Bioforce AG, Switzerland) ranged between 400-2100 kg/ year. Due to the high collection cost and a highly threatened status in Europe, the introduction research was initiated by the company. During 1992-2002 a series of biological and agro-technical questions was studied in natural and artificial swamp environments. The experimental results indicated, that in a simple artificial peat beds, under pH 5, with continuous irrigation, sundew can be cultivated for industrial use. The direct dense autumn sowing (0.2 g/m² seed quantity) resulted in the 3rd-4th-5th-6th year of cultivation 76-489-212-69 g/m² collectable flowering plant yields, respectively. These results were not utilized immediately, because at the time there were several hundred educated collectors in Finland. However, in 2011-2012 new pilot fields have been created in northern Finland due to the lack of collectors and industrial raw material. The developed methods have been published in several papers (20, 21, 22, 23, 24) and the artificial peat beds allow good possibilities for production of sundew in a controlled system without a natural mire ecosystem in those countries, where there is a commercial need for sundew raw materials and sundews are protected plants.

Introduction of other cold-tolerant medicinal plants

***Leuzea carthamoides* DC.** (syn. *Rhaponticum carthamoides* Willd. Iljin, *Stemmacantha carthamoides*) is a perennial herb indigenous to Siberia, which has been used for a long time as an adaptogen medicinal and fodder plant. Due to its cold tolerance and adaptogen properties, it was introduced into Finland and its field cultivation methods were prepared (25). The plantations can be founded by direct seeding, and the fresh leave biomasses have ranged between 20-50 t/ha and the fresh root yield in the 2nd year between 0.8-2.2 t/ha. The disadvantages of the production are the high labour costs of the harvest and processing of the root yields. Presently Leuzea has no commercial importance, since after preparation of the first commercial root extract, the strict Finnish authorities prohibited its marketing.

***Urtica dioica* L.** Nettle is an endemic plant in Finland and it has good reputation among people, using it traditionally as a food and medicine. The popularity of natural raw materials inspirited the elaboration of its field production techniques for fibre production as well. The introduction works started from 1985. The best method for nettle cultivation seems to be the

use of potato ridges with transplantation of seedlings. The life cycle of the plantation is cc. 5-7 years, with one stem harvest or 2-3 leave harvests yearly (26). The experimental results were disseminated through several seminars and publications in Finland. Presently nettle is cultivated for domestic consumption on 3-5 ha for leaf raw material of different local products and for industrial use for extraction as well.

***Rhodiola rosea* L.** Roseroot is an endemic plant in the northern parts of Scandinavia. It is a valuable medicinal plant in Russia, and its adaptogenic properties were widely studied and utilized in the space programs of the former Soviet Union (27). The introduction experiments started in 1993 in our institute. The results of the long-term experiments have been disseminated in national seminars (28) and several international publications (29, 30, 31). Roseroot has been successfully introduced into cultivation in Finland with the support of medical industry. The critical points of the field production are the 5-year long field cultivation period and the root harvest with post-harvest processing requiring labour. These factors result in high production costs, weakening the competitiveness of the domestic production. Presently roseroot is cultivated in Finland on 2-4 ha, and several products exist mainly for domestic consumption. However, some of them are also exported to cc. 15 countries. (Dynaforce®)

***Carum carvi* L.** The biannual caraway is a common natural species in nearly all Finland. Since 1984 several agro-technical experiments have been carried out at the University of Helsinki and Agrifood Research Finland MTT (32, 33) and the commercial cultivation has been started by companies Actic Taste Ltd from 1981, Trans Farm Oy from 1990 and Caraway Finland Oy from 1994. In 2011, caraway was cultivated on 20600 ha and the estimated yield was 8400 t (2). Nearly all production is exported. Caraway is one of the good examples, where several crucial factors are in harmony and have resulted in successful production. The growers needed marketable alternative crops, and the field cultivation has been fully mechanized with the grain machine chain without climatic risks. The organiser companies supply the contract growers with seed-corn, production guides and new technological results. Transportation and logistics are well organized despite the size of the country ; the yields of several hundred growers are processed, controlled, finalized and marketed in centralized processing factories. Large quantities of aromatic waste obtained from cleaning are distilled for essential oil. It is estimated that 5-8 % of the caraway world consumption is originated from Finland.

Production of fresh-cut herbs in greenhouses. Due to the northern position of Finland, greenhouse production of vegetables and flowers has a significant role. The total acreage of greenhouses is 500 ha; tomato, cucumber and flowers being the main products. The interest in using fresh herbs among the consumers inspirited the greenhouse producers, and during 1987 two companies introduced the hydroponic cultivation system. Since then, production technology has been adapted, the spectrum of salad and herb species has widened and great effort has been put for the education of cooks and consumers. Presently the total production area of the hydroponic salads and herbs is over 22.5 hectares, 58 companies produce salads (63 million pots) and 35 companies herb pots (18 millions) (2). The main herbs are the traditionally used dill (4.9 mill.), parsley (2.9 mill.), basils (3.8 mill.), but the popularity of the other herbs (6.8 mill.) is continuously increasing. 22 different species, e.g. sage, rosemary, thyme, oregano, mints, lemon balm, cilantro, Asian leaf herbs, etc. are offered for consumers (www.jarvikyla.fi). The production of fresh-cut herbs is the other successful example in Finland of the utilization of high technology. The automatic hydroponic

production system has allowed all-year round supply of fresh-cut vegetables and herbs, and these products have responded to the consumer demand.

DISCUSSION

During the discussed period, the acquaintance of the use, health effects and cultivation of aromatic and medicinal plants have gained large popularity in Finland. From 1984 to 2006 the cultivated area has increased from 100 ha to 22.600 ha outdoors, and from a few hectares to 22.5 ha indoors. A totally new production technique – hydroponic cultivation of fresh-cut herbs – has been initiated into production, and the consumption of fresh-cut herbs has increased four times. These results are the outcomes of several factors: increasing interest of the society in these new products, informative activity of the media and several organisations, and long term institutional research activities, supported by the authorities. This advantageous social background has inspirited companies and several attempts have been made for utilizing this interest commercially. The atmosphere for introducing new herb species has been excellent. The catalogues of the seed companies are full of seeds and varieties of new species. New and new species have been tested in the institutional research fields, in thousands of home gardens and in the fields of some professional farmers. The media are full of articles and programs connected with the health effects and cooking arts of aromatic and medicinal herbs. In 2012 the horticultural organizations nominated fresh herbs as "*The plant of the year*".

At the same time, the evaluation of the present status of the new MAP species from the industrial perspective is more diverse and not so favourable. Some decisive socioeconomic factors have affected the fate and commercial utilization of the newly introduced species. The initial sentimental attitude of the small entrepreneurs has been changed after meeting the hard economical reality. Previously there were no sufficient information and consumption experiences of the new species. It took a longer period, until a new species became a well-known and widely accepted plant in the society, giving real preconditions for its commercial utilization by the companies. From the studied species *Agastache, Perilla, Tagetes* have gone through this process already during the last two decades.

It seems to have been a bit optimistic approach to introduce new species into the country based on environmental arguments only. The climatic suitability and agronomical preconditions for cultivation of endangered MAPs were good, but the utilization turned out a failure, due to the lack of cultivation traditions, lack of local processing industry and the price level of the international market (*Arnica, Gentiana).*

Transfer of the research results into commercial cultivation among the farmers is a really difficult issue. Generally the farmer's attitude is quite conservative to change the production of well-known plants, with existing farm infrastructure, for risky new ones. Presently the existing social conditions of the Finnish farmers are not so strict, that they urgently need new plants. This was the fate of the arnica cultivation attempt, where the farmers did not start the cultivation in areas large enough, as required by the medical company. Additionally the labour costs in the Finnish agriculture are very high and economically successful cultivation needs high level of mechanization. In the beginning these new plants did not have sufficient mechanization, which was an important factor of the farmers´ negative attitudes. The high production costs proved to be an important factor of the competition of the domestically grown *Rhodiola* roots.

The positive examples of some newly introduced MAPs reveal the importance of the above mentioned factors. Successful introduction of *Echinacea purpurea* into Finland was due to the existence of the basic agronomical research, venturesome farmers, and a medical company supporting the field cultivation with investment in special machinery and giving long term contracts for the farmers. The role of the industrial companies in the long-term

introduction and utilization process seems to be very important. The farmer produces only basic raw materials, which has always a low price and, respectively, low income level. The presence of a processing company with marketing experience and the need of sufficient volumes of raw material is a determining factor in the commercialization of a new species! The research projects supported by local, national or EU bodies always come to an end, and to finalize the introduction process there needs to be a dedicated company finalizing the new raw materials into marketable products. There are some positive examples in Finland with modern plant extract production (*Echinacea, Rhodiola* by Hankintatukku Ltd or with traditional Finnish herb products by Frantsila Organic Herb farm (www.frantsila.com).

Even if in the case of several plants the industry has played the initiative role and given financial support, other factors have determined negatively the utilization of a given new plant raw material. It soon became clear, that the commercial cultivation of the South-European origin *Echium plantagineum* has no perspective in the North. The cultivation of the annual *Perilla frutescens* seemed to be possible with sufficient biomass, but the complicated extraction methods in the factory was the impediment to the larger scale field production. During the time of the realization of the cultivation results of *Drosera rotundifolia*, there were sufficient numbers of collectors on the swamps in the northern Finland and there was no motivation to launch investments in cultivation.

Even if the interest in herbal products seems to be wide in the Finnish society, the real herb consumer segments are narrow among the population of 5 million, and this makes the marketing difficult. According to some analyses the main consumers of herbs are the young and highly educated people and middle-aged women. From this point of view the marketing focus of the herb-based products will still be only a special and small-sized market segment in Finland.

The caraway and fresh-cut herb production are those successful examples, in which the highly mechanized production technology has been met with real consumer demand. Even when the domestic caraway consumption is low, the world market offers endless possibilities. The fully mechanized production chain with effective marketing organization resulted in a real production alternative for more than 1500 growers in the countryside. Thus, caraway has fulfilled the agrarian policy expectations as a real alternative crop. The number of farms producing fresh herbs is much smaller (20-25 greenhouse companies), but the effective high technology connected with the transportation and distribution logistics result in final products, which are healthy and which demand is continuously increasing among the consumers.

CONCLUSION

As a final result, a new horticultural segment, "*The herb culture*", has been formed in Finland during the last 2-3 decades. Based on the raw material production systems and the market possibilities there are four herb production types, mainly for domestic consumption: 1. Hobby gardens grow large herb assortments for the family use; 2. Small entrepreneurs and family companies produce raw materials for local fresh consumption and for their own local processed products; 3. Some industrial companies produce high quality products mainly for domestic market, based on contacts with local growers. 4. Only a few industrial companies have large production, suitable for the international market. In this development the introduction of new MAP species into Finland has had an important role. Even if not all introduced new species can currently be utilized in industrial scale, these species have given important impulses for the development process of the domestic herb production, enriching and diversifying the traditional consumption palette.

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Plenary lecture
Review paper

FACTORS AFFECTING HEAVY METALS CONTENT IN MEDICINAL AND AROMATIC PLANTS AND RELATED PHARMACEUTICAL PRODUCTS

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SUMMARY

This paper summarizes the factors affecting content of essential and potentially toxic heavy metals (Fe, Zn, Cu, Mn, Co, Cd, Pb, Cr, Ni) in medicinal and aromatic plants (MAPs) and related pharmaceutical products. Metals in the MAPs and herbal products may be present in the concentrations that are beneficial to the users to correct micronutrient deficiencies or they may be present in concentrations that may pose threat to the consumer's health. Important factors affecting concentration of heavy metals in the MAPs are: growing site conditions and soil chemical characteristics, particularly soil pH. At acid conditions, mobility and availability of metals are generally high, which mainly result in their high uptake and accumulation in the MAPs (e.g. Cd accumulation in various plants, especially *Hypericum perforatum)*. Additionally, anthropogenic pollution of soil and air affect metal contents in the MAPs. The highest reported values of most toxic metals: Cd and Pb in the above ground parts (flowers, leaves and herb) of the MAPs are frequently associated with air contamination and depositions. Furthermore, the species and genotypes of a species differ greatly in their ability for metal uptake. Recent studies have shown that some MAPs, particularly *Hypericum perforatum*, may show higher Cd contents than other plants grown under the same conditions. Heavy metals content in the MAPs may also be influenced by fertilization mode and/or harvesting time. Finaly, MAPs and herbal preparations may (deliberately or accidentally) be contaminated with heavy metals during harvest, drying, storage and/or processing. Interaction of all the factors result in large differences in heavy metals content between species and even within varieties of the same species. Heavy metals content in a final pharmaceutical product (e.g. herb teas, ethanol extracts, and essential oils) is in addition affected by metals content in a plant organ for further processing and effectiveness of an extraction mode. Distilled essential oils and herb teas are generally found to be free or low in heavy metals, even prepared from the MAPs with high metals content. Thus, herbal based pharmaceutical products pose a low potential risk for human intoxication with heavy metals. However, there should be awareness that a good quality of the MAPs and that of the final pharmaceutical products (drugs) is of a primare importance. They should be free from potentially harmful constituents to human health, which can only be achived by: continuous monitoring of heavy metals in soils, MAPs and final products; carefull choise of growing site; selection of suitable genotypes and appropriate management of soil, crops, and herb processing.

Key words: soil, herb, essential oil, pollution, toxicity

INTRODUCTION

Interest in medicinal plants is increasing all over the world because of lesser side effects as compared to synthetic drugs besides cost effectiveness and easy availability. Promoters of natural products have the opinion that herbal medicines are safe enough and even if the desirable therapeutic response is not achieved, their use is not dangerous to health because of their natural origin. However, there are reports of human poisoning associated with heavy metal herbal medicaments in different parts of the world which have ruled out this principle of safety [1, 2]. The World Health Organization (WHO) prescribed limits for various medicinal plants of not more than 10 and 0.3 mg kg^{-1} Pb and Cd respectively in the final dosage form of the plant material [3]. Later, the WHO recommended tests for toxic metals to be included in the specification for herbal materials [4].

In the toxicological consideration, there are arguments that teas, tea products and fresh herbs represent less than 1% of the total food intake and that medicinal plants are used occasionally in the case of an illness [5]. However, consumers desire products free from potentially harmful constituents, especially in products related to health. Therefore, there is an increasing attention of both scientific and costumer's population in factors and pathways that might lead to elevated concentration of heavy metals in medicinal and aromatic plants (MAPs).

In a global market, MAPs mostly originate from spontaneous vegetation. Spontanious MAPs habitats are often located in non-cultivated and non-environment friendly areas, such as acid soils, alkaline soils and/or soils developed over ore deposits or metal-carrier minerals. Additionaly, MAPs are cultivated on a rather small scale in various regions. Consequently, growing/collecting sites significantly differ in soil chemical and physical properties that affect metal mobility and availability in soils, and accordingly, the metal content in plants. These are: soil pH, redox potential, adsorption/desorption processes, precipitation/solubility, salinity, sulphur content and chemistry, carbonates, cation exchange capacity, clay content, organic matter content, etc. Soil reaction (pH) is one of the most responsible factor moderating solubility, mobility and availability of heavy metals in the soil [6]. There is a general agreement that neutral to slightly alkaline soil reaction induces low mobility and availability of most metals [7].

Beside natural properties, many soils have been additionally loaded with heavy metals under an increasing anthropogenic influence (industrial centres, highways, smelters, etc) during last decades. The highest reported values of most toxic metals: Cd and Pb in the above ground parts (flowers, leaves and herb) of the MAPs are frequently associated with aero contamination and depositions [8, 9, 10, 11, 12].

Additionally, MAPs represent a multitude of species with differing genotypic abilities for metal uptake and mobilization in the rhizosphere. Thus, big differences in the heavy metals uptake are to be expected between species and even within varieties of the same species. Some MAPs (e.g. *Hypericum perforatum, Matricaria recutita*, *Artemisia absinthium*, *Papaver somniferum*) may show higher metal contents than other plants grown under the same conditions [5, 13].

Alongside during growth and development period, the MAPs are easily contaminated with heavy metals during harvesting operations and during drying, storage and processing of the produce [14, 15].

An extractability of metals and their transfer from raw material to a final product (e.g. herb teas, essential oils) is one more aspect that has to be considered in the terms of herbal potential risk for consumers. Available data imply to significant differences in extraction efficiency between common extractant such as: hot water, alcohol or propylene glycol [12]. Distilled essential oils are also found to be free or low in heavy metals, even prepared from MAPs with high metals content [16].

Content of heavy metals in the MAPs and possible consequences are very complex. There should be awareness that heavy metals may be deliberately or accidentally included in the MAPs and herbal preparations. Furthermore, metals in the MAPs may be present in the concentrations that are beneficial to the users to correct micronutrient deficiencies or they may be present in concentrations that may pose threat to the consumer's health. In this paper, we have discussed the factors affecting the uptake and accumulation of metals in the MAPs. Furthermore, risks of MAPs pollution from geogenic (natural) and anthropogenic sources are presented. We have also covered the quality aspect of a final product (essential oil, herb teas, etc.) and the potential risk for human intoxication with heavy metals after consumption of the herbal based pharmaceutical products.

SOIL REACTION AS A FACTOR AFFECTING HEAVY METALS CONTENT IN MEDICINAL AND AROMATIC PLANTS

Among many soil properties that influence heavy metal uptake by plants, soil pH is of fundamental importance. Soil reaction influences metals' solubility and their sorption on colloids, hence, influences their mobility and potential availability [6]. Mobility and availability of most metals (Cu, Zn, Mn, Cd, Pb, Ni) are generally low under neutral to alkaline soil reaction [7]. In soils with high pH and/or high content of lime (Ca-carbonates), availability of most metals decreases to such extent that, in a case of essential microelemets, it may lead to acute deficiency and retard in development of certain susceptible plants (e.g. Fe-chlorosis in *Arnica montana* and *Mentha arvensis* L.) [17, 18, 19]. In contrast, in acid conditions, the availability of most metals increases up to levels that cause their high accumulation in the MAPs. Evidently, plant uptake of metals can be affected by soil pH management practice or appropriate choice of wild MAPs collecting sites in the terms of soil pH.

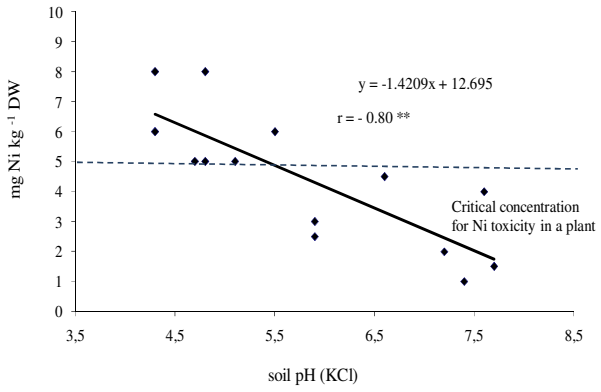

Figure 1. Nickel concentration in *Hypericum perforatum* as a function of soil reaction [20]

Nickel concentration in *Hypericum perforatum* (St. John's wort) was repored to significantly increase above the critical concentration in plants at soil pH < 5.5 (Figure 1) [20]. Additionally, negative correlation between soil reaction and metal concentration in *Hypericum perforatum* was also reported for Cd (Figure 2a) [20]. The same influence of soil pH on Cd plant concentration was manifested for *Achillea millefolium* (yarrow herb), however, it is interesting that St. John's wort take up more Cd than yarrow when grow/develop under the same conditions in the terms of soil pH (Figure 2b).

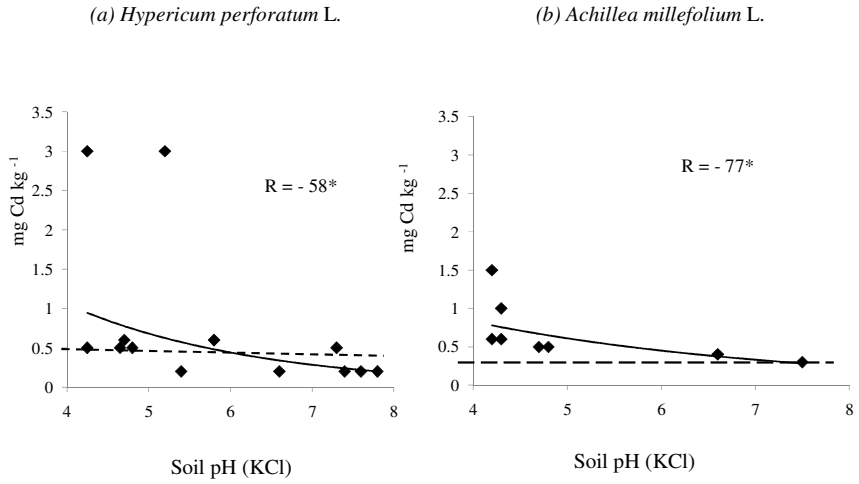

Figure 2. Cadmium concentration in *Hypericum perforatum* and *Achillea millefolium* at the same soil pH [20]; shorter dash pattern indicate proposed Cd reference value for St. John's wort (0.5 mg kg^{-1}), longer dash pattern indicate proposed Cd reference value for yarrow herb (0.3 mg kg^{-1})

GROWING SITE SPECIFICS - ANTHROPOGENIC/GEOGENIC POLLUTION

Heavy metals content might vary among different MAPs grown under the same environmental conditions; it can also vary among the same species grown under different environmental conditions. Beside the influence of a growing site soil characteristics, such as pH, the highest reported values in the above ground plant parts (flowers, leaves and herb) were mostly associated with aerocontamination and pollutant deposition [8, 9, 10, 12].

Zheljazkov et al. [12] showed that metal accumulation in the MAPs is not plant specific in the terms of polluted and unpolluted sites. They found similar zinc accumulation in *Mellisa officinalis* and *Origanum heracleoticum* grown on Zn polluted soil (Figure 3). Roots were generally higher in Zn than leaves for both species.The same species, when grown in unpolluted soils, exhibited Zn concentration within usual range for plants.

Radanović et al. [11] observed clear effect of growing site characteristics on metals concentration in the same species. They analyzed a peppermint (*Mentha piperita* L.) herb collected in the vicinity of fero-nickel smelter (at 1 km distance) and peppermint herb collected from the pot trial conducted with the soil from the same site, but relocated to an unpolluted location. The results of that study revealed up to 8-fold higher concentrations of Ni in the herb from polluted in comparison to the unpolluted location (Figure 4). The findings were attributed to metal deposition on peppermint leaves caused by a strong aeropollution. Significant influence of Pb aero deposition in the vicinity of Pb – Zn smelter on Pb concentration in the above ground plant parts was also recorded for *Lavandula angustifilia* Mill. [8]. It was estimated that even 95% of Pb in the aboveground plant organs originate from the air.

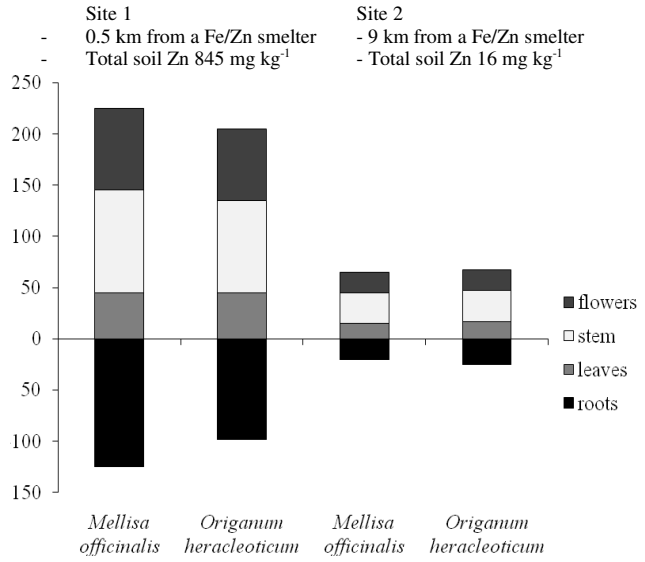

Figure 3. Zinc (Zn) content and distribution in different medicinal plants influenced by the distance from a Fe/Zn smelter [12]

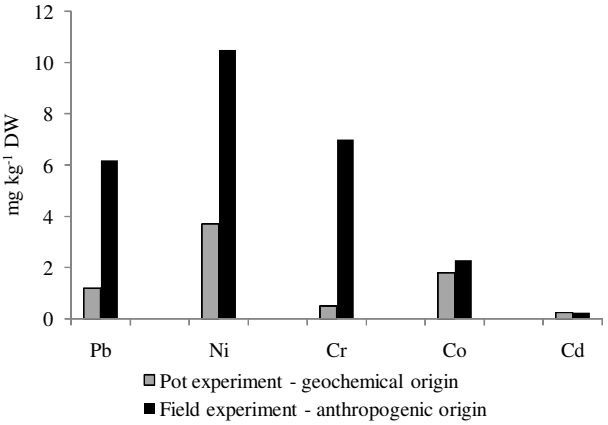

Figure 4. Heavy metals content in *Mentha piperita* L. leaves collected from the field trail near a fero/nickel smelter and the pot trail conducted at unpolluted location with the soil from the polluted field [11]

Soil contamination/pollution from geochemical sources might also lead to elevated concentrations of trace elements in the MAPs. High Ni (27 to 58 mg kg^{-1}) and Cr concentrations (11.5 to 16.5 mg kg^{-1}) were found in the gentian roots collected from an acid soil developed under the strong influence of serpentine minerals [16].

GENOTYPE AS A FACTOR AFFECTING HEAVY METALS CONTENT IN MEDICINAL AND AROMATIC PLANTS

MAPs represent a multitude of species with variable age or biomass, rooting depth, metal mobilization/immobilization by roots in soil, element specific uptake into roots, or transfer into shoots. All of those factors influence metal uptake by plants. Thus, big differences in heavy metals uptake are to be expected not only between species, but even within varieties of the same species.

Recent studies have shown that some MAPs, mainly yarrow (herb), St. John's wort (herb), poppy (seeds), absinth (herb) and chamomile (flowers) had enhanced Cd concentrations [5]. A specific regions where plants had enhanced Cd content could, however, not be found; the Cd level was more dependent on the plant species than on the origin. Still, there are reports [22] that increase of soil Cd would lead to an increase of plant Cd in species that preferentially take up this heavy metal (Figure 5).

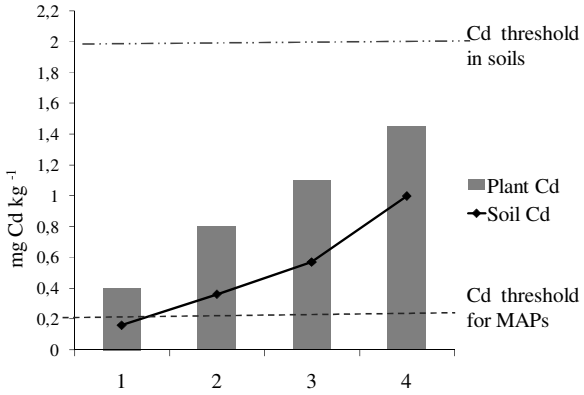

Figure 5. Cadmium (Cd) concentration in *Chamomila recutita* L. flowers influenced by soil Cd content [22]

Many studies revealed that *Hypericum perforatum,* in particular, may show higher Cd content than other plants grown under the same conditions [5, 13, 21]. Additionally, Cd concentrations in *Hypericum* herb has even been reported to be higher than soil Cd (Figure 6). Consequently, *Hypericum* sp. are considered to be Cd hyperaccumulators [23]. Several other MAPs, such as birch (*Betula* sp.), buckwheat (*Fagopyrum esculentum*), dandelion (*Taraxacum officinale*), mallow (*Malva silvestris*), willow (*Salix alba*), etc. have also been recognized as Cd accumulators [5]. Based on high Cd mobility and availability in soils and Cd accumulation ability of plants, the earlier proposed guide-value for Cd content in some of the MAPs has been risen from 0.2 to 0.3 mg kg^{-1} (*Crategus* sp*., Achilea millefolium*), and even up to 0.5 mg kg^{-1}, for Cd accumulating species (*Betula* sp*., H. perforatum, D. stramonium, Salicis* sp.) [24].

Various precautions may be suitable for minimization of the uptake of heavy metals by plants. Cadmium transfer from soil to plant may also be influenced by cutting time. In various herb species, the Cd content of later cuttings was higher than that of earlier ones [23].

Beside Cd, some MAPs might hyperaccumulate other metals. *Senecio coronatus* (Thunb.) Harv. is an extensively used medicinal plant in a certain part of the world (e.g. South Africa), yet it is known to hyperaccumulate nickel [26]. *Datura metal* L. is another example of metal accumulating medicinal plant. It is known as Ni and Co accumulator [28].

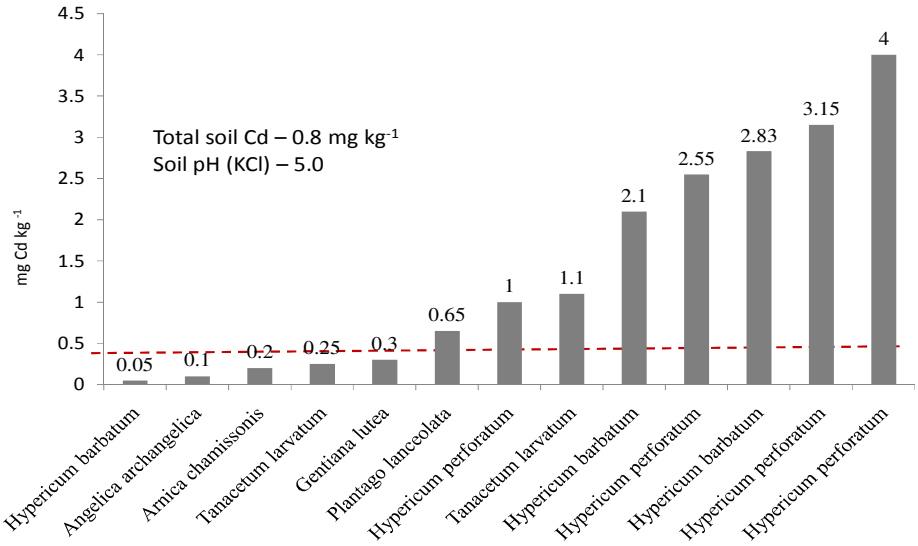

Figure 6. Cadmium (Cd) content in different medicinal plants grown on the same site in Serbia [21].

FACTORS AFFECTING METAL CONCENTRATION IN PHARMACEUTICAL PRODUCTS

Heavy metal concentrations in pharmaceutical products, such as: herb teas, alcohol extracts and essential oils, mostly depend on the efficiency of particular extractant or extraction mode, which, on the other side, depend on a metal binding form and place within a plant or plant organ.

On the basis on the metal concentrations extracted from the gentian roots with ethanol, Radanović et al. [16] divided metals in three groups: metals that are extracted in very low degree, less than 10 % of their total content in the plant tissue (Mn, Cr and Cu); metals extracted in medium degree (10-20%, e.g. Zn), and metals extracted in more than 30% of their total content in the plant tissue (Ni, Cd, Pb and Co). The low and medium degree of extraction was attributed to strong bound between particular metal and organic compounds which are not soluble in ethanol. Opposite, high extraction efficiency indicates presence of particular metal in inorganic compounds, which are easily soluble in ethanol.

Available data point to significant differences in extraction efficiency between common extractants such as hot water, alcohol (ethanol) or propylene glycol. Of them, hot water has been identified as the least effective metal extractant. Thus, herb teas are usually found to be free or low in heavy metals [16, 25]. The same is true for distilled essential oils, even produced from MAPs grown on the heavy metal contaminated soils [12].

However, some traditional medicine products like ayurveda was reported to contain heavy metals more than the safety limit as prescribed by the World health organization [28, 29]. Due to various factors such as soil, agroclimatic conditions, anthropogenic activities, storage and distribution conditions, enlarged heavy metals load in herbs is very likely. Consequently, users of ayurvedic medicine may be at risk for heavy metal toxicity. Therefore, testing of ayurvedic medicine products for heavy metals should be mandatory [29].

CONCLUSION

Major factors affecting the contents of heavy metals in the MAPs and the associated risks of contamination of the MAPs are: (1) soil and geoclimatic characteristic at growing/collecting site, particularly soil pH, (2) origin of a metal at growing/collecting site, particularly in the terms of soil and plants aeropollution, (3) genotypic characteristics of the species and genotypes of the species and (4) agro-technique. Many of the factors act simultaneously and might be mutually dependent, hence, a generalized approach in estimation of heavy metal load in the MAPs should be avoided and each medicinal plant should be tested separately [30].

In order to ensure a good quality of the MAPs and that of the final products (pharmaceutical drugs) so that they are free from potentially harmful constituents to human health, it is neccesary to:

- Monitor heavy metals continuously in soils and in the MAPs;
- Choose growing site carefully;
- Manage soil and crops appropriately;
- Choose suitable plant genotypes;
- Select and breed lines low in heavy metals and
- Implement Guidelines for Good Agricultural and Wild Collection Practice (GACP) of Medicinal and Aromatic Plants [31].

ACKNOWLEDGEMENTS

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Plenary lecture
Original scientific paper

EFFECT OF NUTRIENT SUPPLY ON PRODUCTION AND DRUG QUALITY OF FENNEL (*FOENICULUM VULGARE* MILL.)

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SUMMARY

Nutrient supply of several medicinal plants is often carried out according to empirical knowledge without proper data on specific requirements. In a recent long term (2008-2011) open field experiment we examined the effects of different fertilizers on drug yield and essential oil content of fennel (*Foeniculum vulgare* Mill.).

Beside unfertilized plots as control, the treatments consisted plots fertilized by nitrogen and phosphorous only, and ones getting different nutrition levels of N, P, K and/or Mg. Nitrogen was applied in form of ammonium-nitrate (34%), phosphorous in form of superphosphate (16% P_2O_5) while potassium and magnesium in form of the following preparations: Kieserite (magnesium-sulphate: 25% MgO), Potassium-suphate (50% K_2O), Patentkali (potassium and magnesium-sulphate: 30% K_2O+ 10% MgO).

A positive effect of the fertilization on overwintering could be proven only in the last year. The treatments assured an increased seed yield in each year. In the first year old stand, best treatments were the Kieserite and SOP fertilization while in the following years maximum seed yield was assured by the Patentkali treatment. The data show that well established potassium and magnesium fertilization in form of sulphates may increase the seed yield of fennel, especially in perennial stands. The increase of the production however, varied from 26-74% compared to untreated control. This indicates the great significance of year (weather) and/or plant age.

Fertilization by SOP decreased the size of the fruits in 2009. Essential oil accumulation was enhanced by the treatments only in two years. In first year old stand, best results were achieved by Patentkali, while in 2009 fertilization with only N and P assured the highest content. It seems, that potassium and phosphorous have less significance in essential oil accumulation of fennel.

Keywords: *fertilization, potassium, magnesium, frost tolerance, essential oil*

INTRODUCTION

Although there is a continuous increase in demand of medicinal and aromatic plants on the market and the spectrum of their products is widening enormously, these tendency is not followed by the agrotechnology. Up to date, environmentally and economically sustainable cultivation systems developed for special requirements of each species, or even varieties are available only in certain cases. In most part of the world, agrotechnology is still today based on the developments achieved 25-30 years ago. The elaboration of new products, demand of the processing industry for quality assurance and economical considerations necessitate research on the agronomical background. Recognising this fact, the Department of Medicinal and Aromatic Plants, together with K+S Kali GmbH started a long term project for

optimisation of nutrient supply of medicinal plants. This manuscript is presenting the results on fennel (*Foeniculum vulgare* Mill.).

Fennel is regularly cultivated in Europe providing raw material for phytotherapeutic products and spices. Till now, studies on nutrient requirements of fennel have been focused on the role of nitrogen. It had a superior effect on the seed yield and oil percentage under Egyptian conditions [1] and similar findings were described by some other authors, too. Ehsanipour et al. [2] mentioned 160 kg/ha N levels as optimal in Iran, while in India 100 kg/ha N assured highest yields [3]. In Pakistan increased N rates up to 120 kg/ha were suggested [4]. On the contrary, no effect of nitrogen was detected for essential oil yield and optimal composition of several fennel varieties in Greece [5]. Some data suggest, that optimal performance of various plant characteristics may require different levels of nutrient supply. According to the results of Khan et al. [6] drug yield and essential oil was improved by higher N, P and K dosages while it proved to be unadvantageous for accumulation of anethole. 40 kg/ha nitrogen proved to be the best for seed yield while 60 kg/ha of the same fertilizer assured the best essential oil yield in another trial in Turkey [7]. In Central European practice, fertilization by 80-100 kg/ha P, 40-60 kg/ha K is usually carried out in autumn before sowing year and 40-60 kg/ha P with 30-40 kg/ha K from the second year on [8]. Dachler and Pelzmann [9] suggest balanced levels (80-80-80 kg/ha) of the three macronutrients. The mentioned authors are warning of higher dosages of nitrogen in Hungary and Austria.

It can be concluded that reliable results are scarce and data are contradictious. Besides, most of the experimental results accumulated only for the effects of nitrogen and thus, under environmental and soil circumstances which are hardly comparable with the Central European ones, where both cultivars and anticipated yield levels are also different. Therefore sophisticated data based on long term study are needed for optimizing European cultivation for quality oriented production.

MATERIAL AND METHODS

The experiments were carried out at the Experimental Farm of the Faculty of Horticulture of Corvinus University, situated in South-Eastern district of Budapest, Soroksár.

The soil is sandy-loam (clay content below <15%) and basophyl (pH >7.0). The nutrient content of the soil was determined each year before fertilization (Table 1). 2008 and 2011 could be characterised by average weather conditions, while 2009 was an extremely arid one and 2010 a year with extraordinary high levels of precipitation (Table 2).

Table 1. Soil characteristics of the experimental plots

Plot	pH	Humus %	NO_3-N mg/kg	P_2O_5 m g/kg	K_2O mg/kg	Ca %	Mg mg/kg
2009	8.08	1.14	11.02	431	287	1.151	85.7
2010	7.98	1.24	11.05	408	275	1.120	82.2
2011	7.95	0.95	11.40	516	227	1.260	64.2

Seed material of fennel (*Foeniculum vulgare* Mill. var. *vulgare*) cultivar 'Soroksári' was obtained from the seed bank of the Department of Medicinal and Aromatic Plants. Sowing of fennel was carried out in March 2008 and the trial continued in perennial stands till 2011 (four years long). Row distance was 24 cm, plot size 20 m^2.

Beside unfertilized plots as control, the treatments consisted plots fertilized by nitrogen and phosphorous only, and ones getting different nutrition levels of N, P, K, Mg and S (Table 3). Nitrogen was applied in form of ammonium-nitrate (34%), phosphorous in form of superphosphate (monocalcium phosphate + calcium sulphate, 16% P_2O_5) while potassium and magnesium in form of the following preparations: Kieserite (magnesium-sulphate: 25% MgO+ 50% SO_3), Potassium-suphate (50% K_2O), Patentkali (potassium and magnesium-sulphate: 30% K_2O+ 10% MgO + 42% SO_3). Nitrogen was applied twice in spring, phosphorous at the beginning of vegetation while K and Mg containing fertilizers both at the end and at the start of growing seasons (Table 3). The fertilizers were dispersed by hand on the soil. Replication number was four in case of both species.

Table 2. The main climatic characteristics during the experiment

		2008				2009				2010				2011			
	Month	Decade			Mean	Decade			Mean	Decade			Mean	Decade			Mean
		I.	II.	III.		I.	II.	III.		I.	II.	III.		I.	II.	III.	
Temperature	III.	5.5	5.4	4.1	5.0	4.5	3.3	4.6	4.1	5.0	4.4	4.3	4.6	-1.3	6.7	6.3	3.9
	IV.	7.8	10.7	10.9	9.8	12.2	12.6	13.1	12.6	10.0	11.7	12.0	11.2	11.4	8.3	12.3	10.7
	V.	12.1	14.6	16.6	14.4	13.4	15.8	15.7	14.9	12.8	15.2	16.1	14.7	9.7	13.8	17.3	13.6
	VI.	18.2	16.5	21.3	18.7	15.4	17.8	16.6	16.6	16.8	17.2	18.9	17.6	19.7	16.9	17.0	17.9
	VII.	19.4	18.9	18.8	19.0	19.9	19.8	20.7	20.1	19.7	19.4	19.8	19.6	17.8	22.2	15.7	18.6
	VIII	19.9	18.9	17.6	18.8	21.4	19.3	18.7	19.8	20.6	19.1	18.2	19.3	19.0	21.9	21.0	20.6
	IX	18.4	10.8	10.0	13.1	16.6	17.3	15.3	16.4	9.7	12.2	8.8	10.2	18.3	17.7	15.0	17.0
Precipitation					Sum				Sum				Sum				Sum
	III.	22.6	15.6	48.4	86.6	15.8	8.6	16.8	41.2	25.0	6.4	6.4	37.8	3.4	20.4	3.6	27.4
	IV.	11.6	17.8	12.4	41.8	0.6	2.8	0.0	3.4	10.8	37.2	1.0	49.0	0.8	0.2	6.4	7.4
	V.	16.6	2.6	23.2	42.4	2.6	2.4	15.2	20.2	10.6	79.4	76.2	166.2	15.4	20.0	12.4	47.8
	VI.	3.8	54.8	16.2	105.8	24.2	10.6	82.6	117.4	47.6	78.6	42.0	168.2	58.0	1.2	17.2	76.4
	VII.	33.2	41.2	67.0	141.4	23.8	17.4	2.6	43.8	8.0	6.0	45.8	59.8	4.8	11.8	76.8	93.4
	VIII	22.2	0.0	19.2	41.4	11.6	3.6	10.4	25.6	32.4	19.4	29.8	81.6	2.0	0.2	0.0	2.2
	IX	24.2	34.8	8.4	67.4	14.2	12.4	31.2	57.8	58.6	78.8	15.8	153.2	1.8	5.4	0.4	7.6
					526.8				309.4				715.8				262.2

Irrigation was applied only in the arid year 2009 in May (40 mm). Plant protection was carried out against parasitic fungi by Topas 100 EC (penconazole) in May and July of each year. Weed control in the year of propagation was made by hand but after closing the stand it was not needed any more.

Table 3. Fertilization treatments and times (kg/ha fertilizer)

Nr.	Nutrient	N	P_2O_5	K_2O	MgO	S
		kg/ha				
1.	Control	0	0	0	0	0
2.	N:P	100	70	0	0	0
3.	N:P:Mg '*Kieserite*'	100	70	0	30	24
4.	N:P:K '*SOP'*	100	70	90	0	32
5.	N:P:K:Mg '*Patentkali*'	100	70	90	30	51
Time of application		Beginning March 70% Midest of May 30%	Beginning March 100%	End of Nov. 50% Beginning March 50%		

Harvesting of fennel happened after ripening of the fruits in October (2008) or in September (2009-2011) by hand, dried at 40 ^oC and cleaned by 'Petkus Mini 100'. Three times 100

seeds (achenes) were counted out and thousand seed mass calculated. Overwintering was assessed by estimating the casing of fennel plants on each plot after shooting in March.
The content of essential oil was determined according to the Ph.Eur. [10] by steam distillation of the fruits (fennel) in Clevenger apparatus and calculated to dry mass. Statistical evaluation of data was achieved by ANOVA in Statistica 9.0 software.

RESULTS AND DISCUSSION

Under the conditions of the experimental field, a significant effect of the fertilization on overwintering could be proven only in the last year (Table 4). The difference was established only between the untreated control and each of the fertilized plots, where frost damage decreased the coverage by 14-16 %. For the whole experiment however, it seems, that in contrary to some former reports in other crops breeding [e.g. 11], the studied levels of potassium has not considerably increased frost tolerance of fennel under the experimental conditions.

Table 4. Results of the fertilization treatments in fennel (*Foeniculum vulgare*)
(Treatments: see Table 3.)

Treat-ment	Frost damage (%)	Seed yield (g/m^2)	Thousand seed mass (g)	Ess. oil content (%)
		2008		
1	-	124 [a]	5.066	3.618 [a]
2	-	155 [a]	5.622	3.998 [b]
3	-	184 [b]	5.480	3.752 [a]
4	-	182 [b]	5.263	4.003 [b]
5	-	158 [a]	4.869	4.387 [c]
p		0.001	0.055	0.000
		2009		
1	20	159 [a]	4.12 [b]	6.416 [a]
2	17	153 [a]	4.11 [b]	6.760 [b]
3	22	189 [a b]	3.90 [b]	6.554 [a]
4	20	230 [c]	3.29 [a]	6.529 [a]
5	22	277 [d]	3.89 [b]	6.439 [a]
p	0.345	0.000	0.024	0.043
		2010		
1	12	167 [a]	7.043	5.653
2	15	194 [b]	6.675	5.950
3	8	197 [b]	6.830	5.789
4	10	201 [b]	6.503	6.001
5	11	210 [b]	6.923	5.806
p	0.896	0.020	0.087	0.484
		2011		
1	25 [a]	173	4.575	5.755
2	15 [b]	199	4.862	5.728
3	14 [b]	184	4.525	5.703
4	16 [b]	203	4.900	6.024
5	16 [b]	205	5.125	5.486
p	0.028	0.748	0.146	0.379

The same letters following the means within one year denote no significant difference at p=0.05

The treatments assured an increased seed yield in each year except the last one, although the tendency is obvious in this year, too (Table 4). In the first year, highest yields were achieved by Kieserite and SOP fertilization (treatments 3 and 4). However, in perennial stand, maximum seed yield was provided by the Patentkali (treatment 5) treatment. In 2009 Patentkali (K + Mg in form of phosphates) assured by 74% and 81% more fruits compared to the control and the plots received only N and P, respectively. In 2010 these values were 26% and 8%, respectively.

The size of the fruits was influenced by the treatments only in 2009 (Table 4). The fertilization exhibited a tendency to producing smaller seeds, especially in case of SOP (treatment 4). This tendency might be in connection with the yield increase achieved by the fertilizers. Recently, small seed size of fennel used to be an advantage in processing and an effort in breeding [12].

The data of the experiment show that well established potassium and magnesium fertilization in form of sulphates may increase the seed yield of fennel, especially in perennial stands. In addition to former findings which declared nitrogen as essential to optimal fruit development [1, 2], it could be proven that considerable surplus may be achieved by the mentioned nutrients in addition to nitrogen. The increase of the production is however, highly variable which indicate the great significance of year (weather) and/or plant age. Our data show, that in contrary to the results of Ayub et al. [4] the yield increase is not in connection with enlarged seed size.

Fertilization enhanced the accumulation of the essential oil only in two years (Table 4). In 2008 best results were achieved by Patentkali (treatment 5), while in 2009 fertilization with only N and P assured the highest content. In the last two years no significant differences were found among the experimental samples. The effect of fertilization on the essential oil accumulation seems to be contradictious which indicate the role of other factors in this feature. Our data are in coincidence and complete the results of Chatzopoulou et al. [5] who found that oil yield was affected mainly by cultivar and not by fertilization and those of Abdallah et al. [1] about the minor effect of potassium and phosphorous on the essential oil accumulation.

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Original scientific paper

PRACTICE ORIENTED INVESTIGATION OF HULL-LESS OIL PUMPKIN SEEDS (*CUCURBITA PEPO* L.) DRYING IN BATCH DRYERS

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SUMMARY

The production of hull-less oil pumpkin seeds can be profitable. One of most important activities is drying, which is in developing countries commonly performed in batch dryers.
In the period 2009 to 2011 investigation of drying hull-less oil pumpkin seed, variety Olinka, was performed in batch drier. The objective of the investigation was to optimize drying parameters in order to obtain good quality and to reduce energy input and drying time. Experimental drying was performed in semi laboratory drier with surface of grate 1.4x1.1 m and about 170 kg of fresh seeds batch. The drier was placed on weight cells which enabled continuous measuring course of material mass. Another electronic balance enabled continuous measuring of fuel consumption of light heating oil. Input temperature of drying air was controlled, and relative humidity of air was measured. Velocity of drying air was 0.2 ms^{-1}, and was adjusted during drying process. Drier enables performing in open and circulating mode of drying air flow. Open drying agent flow means single pass through material layer, and circulating mode means multi-passing till reaching upper limit value of agent relative humidity.
Drying was performed in three phases, first – till reduction of moisture content of dried material to about 32%, second – till reaching moisture content 18%, and third – till reaching final moisture content of about 7%. For the first phase open drying agent flow was always performed, and for the second and third alternately open and circulating mod. The drying agent temperature for the first phase varied in the range 60 to 80 °C. For the following phases it was reduced or remained same (for some experiments). As a control group drying at constant drying agent temperature 50 °C and open mode was used, as commonly applied in practice. For every setting five or more trials were performed.
Change of dried mass, i.e. moisture content, consumption of fuel, temperatures and humidity of drying agent were recorded. Specific drying energy, specific drying time and fuel consumption per kg of dried material were calculated.
The samples for measuring of microbial count and acidity of oil were taken, which have been performed using standard procedures. The results showed positive effects of higher drying agent temperature and application of alternate open and circulation drying agent flows resulted with reduction of specific drying energy up to 21%. Microbial counts were positive for all temperatures of 60 °C and above. Acidity of seed oils was in all cases lower than defined limit of 1%, but slightly higher for higher drying agent temperatures.

Key words: oil pumpkin seeds, drying, drying agent temperature, drying agent flow, specific drying energy

INTRODUCTION

The production of hull-less oil pumpkin seeds (*Cucurbita pepo* L.) is profitable activity, if following conditions are accomplished: the satisfactory yield, adequate seed quality and safety, and reasonable inputs level. The seeds are used for miscellaneous purposes, especially oil production, which has high nutritive and therapeutic properties.

Production of hull-less oil pumpkin seeds is mechanized to the high level. For the growing is mostly used common agricultural mechanization, but for the harvest special machinery [1]. The seed processing is also solved, by using of common machines for grains' purification, and some special for rinsing and polishing [2]. One of production steps is drying, with significant energy and labour inputs.

Hot air (drying agent), convective, continuous, semi-continuous and batch dryers are mostly used. Other drying methods are described in the literature, like use of microwaves and lyophilisation [3]. Wang et al. [4] presented results of microwaves drying and Que et al. [5] compared convective drying and lyophilisation. Sacilik [6] compared effects of conventional, solar and solar-tunnel hot air drying, and pumpkin seeds drying in laboratory at the temperatures from 40 to 60 °C.

The number of publications related to drying parameters is modest. Sito et al. [7] tested drying agent temperatures 40, 60, 80 and 100 °C. The seed quality was evaluated only organoleptic. It was concluded that the optimal drying temperature is 60 °C, while for the higher temperatures was recorded seed "roasting" effect. Wagner [1] named the drying agent temperature of 60 °C, as maximal. Only Rossrucker [8] has performed very practice oriented investigation. For the drying agent temperatures 40, 50, 60 and 70 °C was obtained drying time 11.3, 8.8, 7.0 and 5.5 h, respectively. As the maximal values the drying agent temperature of 60 °C for mercantile, and 40 °C for seeds aimed for propagation were declared. Kricka et al. [9] have provided laboratory tests, but no relevant results for practice have been obtained and presented.

Starting moisture content of seeds is commonly in the range 35 to 45%. After rinsing and straining it is 50 to 55%. Akritidis et al. [10] investigated equilibrium moisture content, (not of hull-less seeds), for seeds and hulls separately. The values 10.9 to 16%, depending on the relative air humidity were obtained. Lowest value corresponds with in practice accepted value, 7.5 to 8% [11], for oil pumpkin seeds.

In developing countries batch dryers with or without seed agitator are used dominantly. There is no uniformity concerning drying parameters. In the most cases drying agent temperature of 50 °C is applied, for whole process duration. Like in the case of drying of medicinal crops, farmers use to identify temperature of drying agent and dried material. It is well known that the temperature of material during evaporation of physical moisture is sometimes significantly lower than the temperature of drying agent [12]. For the further drying, lower temperature of drying agent is applied for medicinal crops, to prevent high losses of active ingredients, especially if they are essential oils [3]. It can be presumed that the same is valid for drying pumpkin seeds. From previous investigation of medicinal crops drying [13, 14] positive effects are reported for application of alternate use of open (only one pass of drying agent) and circulating (drying agent circulate inside the dryer till reaching certain relative humidity) working mode on specific drying energy (MJ per kg of evaporated water).

It is also known, for medicinal crops, that reduction of drying agent temperature is followed by higher microbial count [13, 14].

This leads to the conclusion that the temperature of drying agent should be as high as possible, concerning also influence on material quality.

The main objective of this investigation was to define optimal drying agent for drying of hull -less pumpkin seeds in batch driers, applicable in practice.

MATERIAL AND METHODS

The experimental drying was performed in September/October during three years 2009, 2010 and 2011. The drying started mostly at late afternoon, and prolonged during the nights.

The kernels of pumpkin variety Olinka, grown on the filed of Research Station for Hop, Sorghum and Medicinal Plants, in Backi Petrovac, vicinity of Novi Sad, Serbia, were dried. The mechanical harvest was provided, rinsing and gravity removal the part of physical moisture, straining. The material was transported and filled into dryer within maximum three hours after washing and straining. The samples of fresh material were taken for the moisture content measuring of the fresh material.

Batch dryer SD-16 MGA, manufactured by *Termoplin*, Mladenovac, Serbia, (www.termoplin.rs), Fig. 1, was used for the drying. The dryer was previously used for investigations of peppermint and chamomile drying [13, 14].

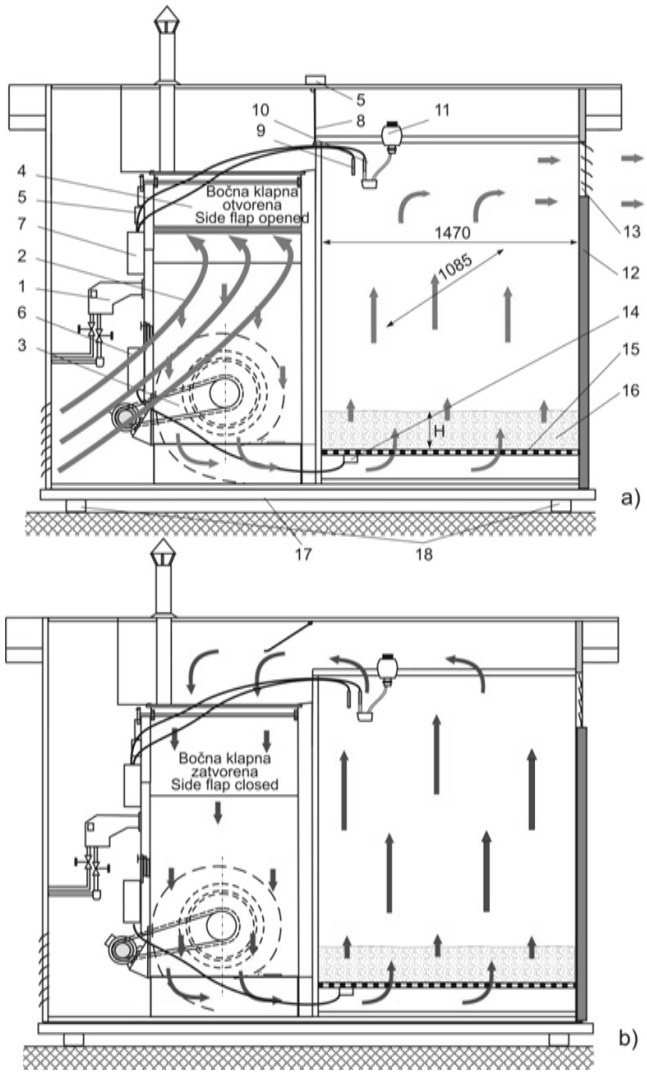

Figure 1 Experimental dryer, a) open mode, b) circulating mode
1– burner, 2– combustion chamber, 3– ventilator, 4– side flaps, 5– servo-motor, 6– electrical cabinet, 7– control unit, 8– circulation opening flap, 9– ”dry” bulb, 10– “wet” bulb, 11– water container, 12– door, 13– overpressure vents, 14– thermometer of drying agent, 15– grate, 16– dried material, 17– balance frame, 18– balance sensors

Electronic balance (17 and18), accuracy 2 N, enabled continuous measuring of dried material weight, i.e. mass. Before every test the balance was adjusted, tare weight zero, and measured

the weight of fresh material. The surface of dryer grate was 1.6 m^2, and height of fresh material layer 15 to 17 cm, i.e. 150 to 180 kg. Continuous measuring of batch weight enabled calculation of mass of evaporated water, i.e. moisture content of dried material.

Moisture content of fresh material was measured using microwave oven procedure, described by Martinov et al. [12], whereby average of five measuring was used as representative. The values of moisture content and mass of fresh material were used for the calculation of current moisture content during drying process, as well as to determine beginning of second and third drying phase. Finally, based on it, the end of drying was determined by calculation of end mass, when dried material reached final moisture content, 7.5%.

The temperature of drying agent was set-up as value on main control box, but monitored by thermometer –14 (accuracy ± 1 °C) as well. Relative humidity of drying agent was calculated from the temperatures of „wet“ and „dry“ bulbs, –9 and –10. For the additional control two psychrometers (accuracy ± 3-5%), were used.

For the heating of drying agent LHO burner was used, with 15 kW of nominal thermal power. Oil canister was placed on electronic balance, accuracy 1 N, what enabled continuous recording of its mass, and calculation of fuel consumption, i.e. energy inputs.

In the open mode, Fig. 1a, the drying agent passes through material and leaves the dryer through overpressure louvers, –13. The side flaps of hot air generator are opened, and circulation flap closed. For the circulation mode, Fig. 1b, side flaps are closed, and circulation flap opened. Drying agent passes through dried material and flows back to hot air generator. PLC –7 can be used for mode changing, via step engines for flaps rotation. Due to more precise process control and quicker response, manual mode changing was applied. The modes were changed depending of limit values of drying agent relative humidity.

In the reviewed publications different values of drying agent velocity though material layer were applied. Kricka et al. [9], used 0.8 ms^{-1}, and Sito et al. [7] performed the experiment with 0.8 do 1.6 ms^{-1}. Rossrucker [8] did practice oriented testing and proposed velocity in the range 0.05 do 0.13 ms^{-1}. Müller [15] investigated in details influence of drying agent velocity on progress of medicinal and aromatic plant drying, whereby the electricity consumption was measured as well. It was concluded that the increase of drying agent velocity over 0.2 m/s is not reasonable. This value was selected for the experiment. The anemometer was used to measure agent velocity on overpressure louvers opening, and ventilator inlet opening adjusted to achieve proper velocity through the material layer. Due to change of material air flow resistance, reduction of drying agent velocity was two times re-adjusted during dying process, keeping defined value.

Drying procedures

Drying in phases was performed as follow: started with open mode till reduction of moisture content to 32±2%. In the second phase alternately open and circulation modes were applied, whereby the switch to circulation mode was performed after reaching about 45% or relative humidity of drying agent, and return to open mode when 70% was achieved. This mode was applied till reduction of moisture content of dried material of 18±1%. Third phase was continued with the exchange of modes, whereby limits or relative humidity of drying agent were 40 and 60% respectively.

During the first phase the temperature of material remained lower, due to intensive water evaporation, and dried material was not overheated.

It was presumed that the drying agent temperatures in second and third phase should be reduced, to prevent losses of oil quality, as it is practiced for medicinal and aromatic plants [16, 13, 14]. As result of experiments in 2009 showed, there is no influence on content of free fatty acids *FFA* (% of oleic acid) of pumpkin seeds [17, 18]. That is why the temperature of drying agent remained same for the second and third phase for the experiments in 2010 and

2011, tab. 1. The only difference of drying procedure in second and third phase was in limits of drying agent relative humidity limits for changing of modes, as described.

Table 1 Applied temperatures of drying agent for drying phases in °C

Settings	1st phase	2nd phase	3rd phase
2009			
A	60	55	45
B	65	55	50
C	65	60	55
2010			
D	60	60	
E	80	60	
F	80	65	
2011			
G	70	60	
H	70	65	

As the control group, in all experiments, the temperature of drying agent was 50 °C and open mode was applied for whole drying duration.
For the experiments in 2009 was provided measurement of microbial count and content of free fatty acids *FFA* (% of oleic acid) of pumpkin seeds, as described in Martinov et al. [17, 18].

RESULTS AND DISCUSSION

The achieved drying characteristics are presented in tab. 3.

Table 3 Drying characteristics

Settings	Specific drying energy. MJ $(kg_{e.p.})^{-1}$		Specific drying time, 10^{-1} h $(kg_{d.p.})^{-1}$	Fuel per kg of dried product, L $(kg_{d.p.})^{-1}$
	M	SD	M	M
2009				
K1	6.4	0.9	1.1	0.17
A	5.6	0.5	1.1	0.14
B	5.5	0.3	0.9	0.13
C	5.3	1.7	0.9	0.13
2010				
K2	5.6	0.4	1.1	0.17
D	4.8	0.2	1.0	0.15
E	4.9	0.2	0.8	0.16
F	4.9	0.3	0.8	0.16
2011				
K3	6.4	0.6	1.2	0.17
G	4.9	0.2	0.7	0.11
H	4.7	0.2	0.7	0.10

M– median, SD– standard deviation

This is, as expected that the higher drying agent temperature results with lower specific drying energy. Exception is application of temperatures of 80 °C, settings E and F, as well as 70 °C, setting G. This may be the consequence of rapid water evaporation, especially during the first drying phase, and formation of some kind of waterproof film over seed lumps due to presence of sticky slime that remained after washing and rinsing of the seeds. This effect can be, potentially, eliminated by more frequent and intensive mixture of seeds, but this operation need additional agitator.

The results related to specific drying temperature are reflected in specific fuel consumption. By using temperature of 65 °C in the first phase reduces specific fuel consumption about 23%, and, if the temperature is in the first phase 70 °C, this reduction is even about 40 %. For the same settings is the specific drying time is reduced 19% i.e. 41%.

The obtained reduction of specific drying energy and fuel consumption are also result of application of alternation of open and circulation modes. The indicative, in that sense, is comparison of settings K1 and A.

The higher temperature, the less is the microbial count. This was proved for the experiments in 2009 and 2010 and reported by Martinov et al. [17, 18] and Matavuly at al. [19]. It seems that the drying agent temperature of 60 °C is the lower limit for achieving the seed belonging to 3B group according to European Pharmacopoeia.

For all drying agent temperatures the Content of free fatty acids *FFA* (% of oleic acid) of pumpkin seeds was under the limit of 1 %, defined by national legislation as upper limit, whereby the content increased with the drying agent temperature, from 0.18% for 50 °C to 0.31 for 80 °C [18].

For the user can be recommended to apply drying agent temperature up to 65 °C for all drying phases, and to apply alternateness of drying agent flow open-circulating modes, in the second and third phase. The recommended limits for relative humidity of drying agent, at which mode is changed, are 45/70%, i.e. 40/60% for second and third drying phases respectively.

CONCLUSIONS

As expected, higher drying temperatures and alternate use of open and circulating mode of drying agent has positive effects on drying characteristics. The specific drying time can be reduced up to 15%, and specific fuel consumption up to 24% (trials B and C compared with K1). This effect was not recorded for the drying agent temperatures of 80 °C, due to, creation of waterproof film over seed lumps, which presented obstacle for water evaporation. The same effect is recorded, but less intensive for the drying agent temperature 70 °C.

The positive effect of alternate use of open and circulation modes was proven, by comparison of K1, A and B settings for the second and third phase.

The rise of drying agent temperatures resulted with slight increase of free fatty acids, but the value was always under 1%, defined by national legislation as limit. Tests of microbial count shown that the drying agent temperature should be at least 60 °C, if it is applied for all phases.

Based on obtained results, following could be suggested to the practitioners which use batch dryers for hull-less pumpkin seeds: drying agent temperature 65 °C, same for all drying phases, application of mode alternation for second and third phase.

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Original scientific paper

STABILITY ESTIMATION OF POT MARYGOLD DRY FLOWERS AND PETALS YIELD

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SUMMARY

Calendula or pot marigold (*Calendula officinalis* L.) is well-known medicinal plant, producing large numbers of yellow-orange flowers over long vegetation. This specie apart from its use as an ornamental has traditional culinary and herbal uses. The aim of this paper was to find the best adapted variety in agro ecological conditions of Serbia, concerning dry flowers and petals yield.

A field experiments conducted in the 2006-2008 at the Institute of Field and Vegetable Crops, at Rimski Šančevi (φ 45°20 N, λ 19°51 E), Novi Sad, Serbia with 4 varieties: "Bački Petrovac", "Orange King" (originated from Serbia), "Plamen" and "Plamen Plus" (from Czech Republic). The yield data was processed by joint ANOVA and PCA analysis.

According to ANOVA year, varieties and interactions had very significant influence on both flowers and petals yield, except varieties on flowers yield. For both traits the highest yield was in 2008 (1237 and 475 kg ha^{-1} respectively) and between others two year there are no significant differences. Variety had very significant effect only on petals yield, and only significant differences were between Czech's and Serbia's varieties. Plamen Plus had the highest (495 kg ha^{-1}) and Bački Petrovac the lowest petal yield (323 kg ha^{-1}).

Due to high significant influence of interaction, we further analyzed it by AMMI (Additive Main Effects and Multiplicative Interaction) model. In the environmental condition of investigated year for both traits were significant only first PCI1, which explained respectively 93.5 % and 91.8% of total interaction variability. In 2008 yields of both traits were the highest and most stabile. In average for three years the variety Plamen Plus for both traits had yield high above overall average, but also was the most unstable variety. The most stabile varieties for both traits were Plamen and Bački Pertovac but with lower than overall average yields. The best solution for our environmental condition according to three year research is variety "Orange King", with medium stability and yield of both traits near overall average yield

***Key words**: Calendula officinalis, variety, dry flowers and petals yield, AMMI*

INTRODUCTION

Calendula or pot marigold (*Calendula officinalis* L.) is well-known medicinal plant, producing large numbers of yellow-orange flowers over a long vegetation This species apart from its use as an ornamental has traditional culinary and herbal uses. In Serbia and Vojvodina province pot marigold is used as a traditional medicinal plant – floral drug. The pharmacological activity of marigold, mainly from its flowers, is related to the content of several classes of secondary metabolites such as essential oils, flavonoids, sterols, carotenoids, tannins, saponins, triterpene alcohols, polysaccharides, a bitter principle,

mucilage, and resin [1]. Calendula had potential health benefits, including protective effects against development of cancer, inhibition of existing tumor cells, protection against chemotherapy and radiation therapy adverse effects, anti-inflammatory activity, antioxidant activity, cardiovascular protective effects and antiviral effects [2,3].

In environment of Serbia crop management of marigold was not study enough. For this reason, we decide to research one of the main management issues, it is variety recommendation. The aim of this paper was to find the best adapted variety in agro ecological conditions of Serbia, concerning dry flowers and petals dry yield, as the most important medical raw material of marigold. Cultivar performance is a function of a specific assortment of genes (G), the trait-associated factors of the environment in which it is grown (E), and their interaction (GE). Multi-environment trials (MET) which are usually conducted in multiple years and locations commonly use for evaluating genotypes [4]. The analysis of variance (ANOVA) used to analyze yield data and a comparison among genotypes could serves as the basis for making cultivar recommendations. Unfortunately the ANOVA is an additive model that describes main effects and determines if GE interaction is a significant source of variation, but it does not provide insight into the patterns of genotypic responses to changing environmental conditions. The presence of a large and significant GE limits data interpretation [5]. It makes that use of main genotype effects (e.g., overall means across environments) became questionable [6] and selection of the best cultivar, one that exhibits good performance regardless of the environment, less accurate [4]. It complicates the interpretation of the results and confounds the observed average performance of the genotypes with their true values [7] or it complicate effective identification of superior varieties because GE frequently result in changes in the ranking of genotypes when they are tested in different environments [8]. The degree to which genotypes and environments interact is usually attributable to both the adaptation of the genotypes and the range of conditions present in the environments. However, most of the biotic and abiotic characteristics of an environment cannot be controlled leaving the genotypes as the sole source for reducing GE.

Multiplicative models for MET have been used for studying G×E interaction, examining genotypic yield stability and adaptation and for developing methods for clustering sites or cultivars into groups with statistically negligible crossover G×E interaction. It models have an additive (linear) component (i.e., intercept, main effects of sites and/or genotypes) and a multiplicative (bilinear) component (G×E) and thus are also named linear-bilinear models [9]. Multiplicative models are joint use of analysis of variance (ANOVA) and singular value decomposition (SVD) which is also known as principal component analysis (PCA) [5]. PCA belong to ordination tools of multivariate data analysis, which aims at describing data by identifying a reduced data dimension of a few variables that account for the greatest amount of variability in the data [10]. It means that additive mains and multiplicative interaction (AMMI) model analysis combines the ANOVA (with additive parameters) and PCA (with multiplicative parameters) into a single analysis. In the AMMI model, the PCA is applied to the residual from the main effects of ANOVA model, that is, to the interaction. The interaction shows the influence of environmental factors on the stability and adaptability of genotypes, and it is a desirable feature only if is in connection with above average yield [11]. The AMMI model describes the G x E in more than one dimension, and it offers better opportunities for studying and interpreting G x E than regression on the mean [7], like Finlay and Wilkinson's [12] regression coefficient, Perkins and Jinks's [13] regression coefficient, and Eberhart and Russell's [14] sum of squared deviations from regression.

MATERIAL & METHODS

A field experiments conducted in the 2006-2008 at the Institute of Field and Vegetable Crops, at Rimski Šančevi (φ 45°20 N, λ 19°51 E), Novi Sad, Serbia. Weather conditions for these years and long term average values are present in Table 1. The experiment was on calcareous chernozem soil type, and each years in the autumn was applied 100 kg ha^{-1} NPK nutrients (15:15:15) and in spring 100 kg ha^{-1} of Urea (46% N). The trial each year sowed at the beginning of April in row distance of 50 cm and stand density of 27 plants per square meter. Four marigold cultivars were object of our investigations: „Bački Petrovac“ (cultivar made by Institute of Field and Vegetable Crops from Novi Sad), “Orange King” (originated from Pančevo, Serbia) and two foreign cultivars –“Plamen” and “Plamen Plus” (from Czech Republic). The trials were in randomized complete block design, and had four replications. Similar to commercial production fully opened flowers were plucked by hand once a week and air dry.

Table 1. Monthly precipitation sums (mm) and average monthly temperatures (°C) at Rimski Šančevi, Novi Sad

Year	X-III	IV	V	VI	VII	VIII	IX	IV-IX
Monthly precipitation sum (mm)								
2006	216	107	54	101	52	88	16	418
2007	209	0	81	49	31	101	50	312
2008	392	45	13	90	91	64	87	390
LTA*	261	50	58	86	65	56	52	367
Average monthly temperatures (°C)								
2006		12,7	16,5	19,7	23,6	19,7	18,0	18,4
2007		13,4	18,5	22,1	23,3	22,7	14,6	19,1
2008		13,0	18,4	21,9	21,7	22,2	15,7	18,8
LTA		11,8	17,2	20,1	21,9	21,6	17,0	18,3

*LTA – Long-Term average values for Rimski Sancevi (1964-2008)

In this paper it will be present totals of all harvests. .The yield data of dry flowers and petals yield was processed by AMMI.

The AMMI model combines the ANOVA and PCA and is expressed as

$$y_{ij} = \mu + \tau_i + \delta_j + \sum_{k=1}^{t} \lambda_k \alpha_{ik} \gamma_{jk} + \varepsilon_{ij} \qquad [1]$$

where μ is the overall mean, τ_i is the effect of the i^{th} genotype, δ_j is the effect of the j^{th} environment, λ_k's ($\lambda_1 \geq \lambda_2 \geq \ldots \lambda_t$) are scaling constants (singular values) that allow the imposition of orthonormality constraints on the singular vectors for genotypes, [$\alpha_k = (\alpha_{1k}, \ldots, \alpha_{gk})$] and for environments, [$\gamma_k = (\gamma_{1k}, \ldots, \gamma_{ek})$].and ε_{ij} is error.

To present results of Eq. [1] in a biplot, the singular value λ_k is often absorbed by the vectors of genotypic and environmental scores, that is, $\alpha_{ik}^{*} = \lambda_k^{f} \alpha_{ik}$ and $\gamma_{ik}^{*} = \lambda_k^{1-f} \gamma_{ik}$, with $0 \leq f \leq 1$. For the simultaneous interpretation of the impact of genotypes and envinonments $f = 0.5$ and then like in this paper, the genotypic and environmental scores are $\alpha_{ik}^{*} = \sqrt{\lambda_k} * \gamma_{ik}$ and $\gamma_{jk}^{*} = \sqrt{\lambda_k} * \gamma_{jk}$, respectively

To determine the significance PCI (principal component interaction) in AMMI analysis we used usual Gollob's *F*-test [11].

Biplot analysis is very useful for quick visualization and exploration of patterns inherent in the complex GE two way table [5]. One of the commonly used biplot in this category is an AMMI1-based scatter plot where main genotypic and environmental effects are provided for the abscissas and their PCI1 scores are the ordinates [15] and in this way to each varieties and

years belongs one point. On such biplot, comparison of varieties or years in the horizontal direction points to differences in the main effects, and comparison in vertical directions points to differences in the interaction effect. The variety or year with high value IPC1 components has large interaction effects and reverse. Years with IPC1 near zero values, since they have little interaction effects are suitable for all varieties. The low value of varieties on IPC1 axis indicates that they are under less influence of the environment (year) and more stabile [15]. It means that AMMI1 biplot enables a simultaneous view of the mean performance and the stability of the genotypes.

Although AMMI model analysis results are based only on yield data (not environmental data), Gauch [16] reported studies that showed that AMMI environmental scores were correlated with environmental factors, such as precipitation, mean daily maximum and minimum temperature, altitude, latitude, N fertilization, irrigation and clay content. This G x E structure allows interpretation of GE interaction in terms of genotypic trait x environmental factor if the genotypic and environmental PC scores can be related to genotypic and environmental covariates [17].

Using AMMI model analysis, we aimed at providing in-sight into the GE interaction effect on flowers and petiole yield as a means of determining the best performing genotype of Calendula for environmental conditions of Vojvodina.

RESULTS AND DISCUSSION

According to ANOVA year, varieties and interactions had high significant influence on both flowers and petals yield, except varieties (G) on flowers yield (Table 2, 4).

Table 2. Analysis of variance in AMMI model for dry flowers yield

Source of variation	d.f.	S.S.	M.S.	F values	F probability	Partitioning of S.S.
Replication	3	262649,0	87550,0	3,02		
E	2	735824,0	367912,0	12,68	<,001	40,0%
G	3	179681,0	59894,0	2,06	0,124	9,8%
G*E	6	925135,0	154189,0	5,31	<,001	50,3%
PCI1	4	867395,6	216848,9	7,47	<,001	93,8%
Residual PCI	2	57739,4	28869,7	0,99	0,381	6,2%
Error	33	957671,0	29020,0			

Table 3. Mean values of flowers yield (kg ha^{-1})

Variety	Year			Average
	2006	2007	2008	
B. Petrovac	952,5	889,5	1098,9	980,3
Orange King	1111,7	883,5	1272,0	1089,1
Plamen	989,9	1039,3	1151,1	1060,1
Plamen Plus	705,4	1321,8	1424,0	1150,4
Average	939,9	1033,5	1236,5	
LSD	Year	Varieties	Year * Varieties	
0,01	164,6	190,1	329,2	
0,05	122,5	141,5	245,1	

Table 4. Analysis of variance in AMMI model for dry petals yield

Source of variation	d.f.	S.S.	M.S.	F values	F probability	Partitioning of S.S.
Replication	3	38933,0	12978,0	3,69		
E	2	211293,0	105647,0	30,04	<,001	33,5%
G	3	220016,0	73339,0	20,86	<,001	34,9%
G*E	6	198607,0	33101,0	9,41	<,001	31,5%
PCI1	4	182315,8	45578,9	12,96	<,001	91,8%
Residual PCI	2	16291,1	8145,6	2,32	0,114	8,2%
Error	33	116046,0	3517,0			

Table 5. Mean values of petals yield (kg ha^{-1})

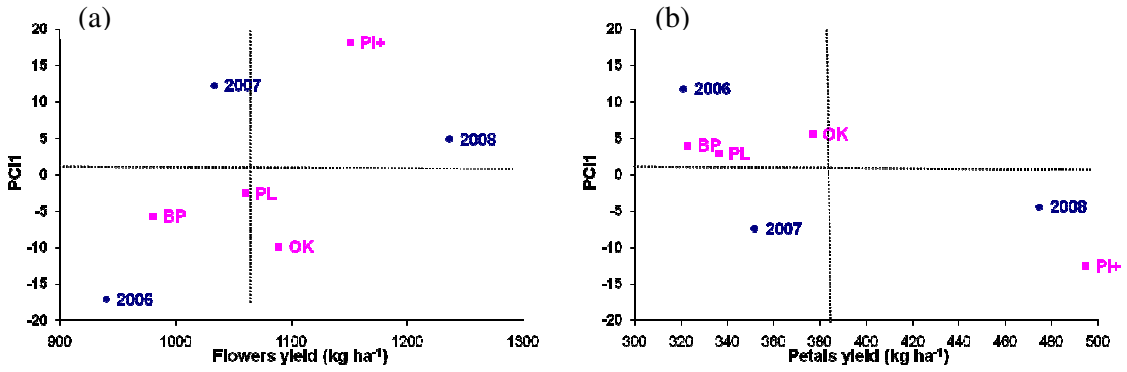
Variety	Year			Average
	2006	2007	2008	
B. Petrovac	310,4	271,0	387,0	322,8
Orange King	375,7	274,9	480,1	376,9
Plamen	314,4	309,5	384,5	336,1
Plamen Plus	284,2	552,4	647,4	494,7
Average	321,2	351,9	474,7	

LSD	Year	Varieties	Year*Varieties
0,01	57,3	66,2	114,6
0,05	42,7	49,3	85,3

For both traits the highest yield was in 2008 (1237 and 475 kg ha^{-1} respectively) and between others two year there are no significant differences (Table 3, 5). Variety had very significant effect only on petals yield. Plamen Plus had significantly the highest petal yield (495 kg ha^{-1}), and Orange King had significantly higher yield then the lowest yielding variety Bački Petrovac (323 kg ha^{-1}).

In variability of flowers yield the highest part had G*E (50,3%) and part of G was only 9.8%, while variability of petal yield were almost equally distributed between E, G and G*E, and the highest part had G (34.5%). Due to high significant influence of interaction, for both traits we further analyzed it by AMMI model. In the environmental condition of investigated year for both traits were significant only first PCI1, which explained respectively 93.8 % and 91.8% of total interaction variability (Table 2, 4). In 2008 yields of both traits were the highest and most stabile (Fig 1a, 1b). This year was characterized with highest winter precipitation (392 mm), average precipitation in April and the sum of precipitation in vegetation was higher than in LTA (Table 1). In average for three years the variety Plamen Plus for both traits had yield high above overall average, but also was the most unstable variety. This variety positively correlated with environment condition in 2007 and 2008 for both traits, while other 3 varieties positively correlated with environment condition 2006. The most stabile varieties for both traits were Plamen and Bački Pertovac but with lower than overall average yields. The best solution for our environmental condition according to three year research is variety Orange King, with medium stability and yield of both traits near overall average yield.

Figure 1. AMMI1 biplot of flower (a) and petal (b) calendula yields (Variety: BP - B. Petrovac, OK - Orange King, Pl - Plamen, PL+ -Plamen plus)



CONCLUSION

According to three years data and AMMI analysis for environment of Serbia the best calendula variety is Orange King, with medium stability and yield of dry flowers and petals near overall average.

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Original scientific paper

MINERAL CONTENT OF AUTOCHTHONOUS GENTIAN (*GENTIANA LUTEA* L) ROOT FROM NATURAL HABITATS IN NORTHERN PARTS OF MONTENEGRO

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SUMMARY

The content of biogenic elements (P, K, Fe, Mn, Zn, Cu, Co) and heavy metals (Ni, Cr, Pb, Cd) contained in yellow gentian root (Gentiana lutea L) has been examined from nine natural habitats on the mountains of Bjelasica (two locations: Strmenica and Kobilja glava), Prošćenske Mountains, Sinjajevina, Ljubišnja, Koritska plateau (two locations: Gutavica and Konjska reka), Durmitor and Štitovo in Montenegro. The root samples were taken in July and August 2009, in five repetitions for each location. Phosphorus was determined spectrophotometrically, and K, Fe, Mn, Zn, Cu, Co, Ni, Cr, Pb, Cd by AAS in a solution after the burning of the sample in an acid mixture (HNO_3 + $HClO_4$ + H_2SO_4). The soil samples were taken at the same time as the root samples. Primary analysis of soil pH, % of humus, and the content of P and K were done by standard methods for soil analysis. Content of pseudo total quantities of Fe, Mn, Zn, Cu, Co, Ni, Cr, Pb, Cd were done by AAS in 80% HNO_3. In most localities that were examined, the soil was medium acidic (from 4.80 - 5.22 pH/KCl). The high acid soils are on Bjelasica (Strmenica and Kobilja glava, pH KCl= 4.39 - 4.46) and on Sinjajevina (4.60), and the lowest acidity is on Prošćanske Mountains (average pH/KCl = 5.49). All soils are very rich with humus, with an average content in an interval from 5.59 % in Strmenica to 28.21 % humus on Ljubišnja.

The average content of phosphorus in gentian root was in an interval between 0.13-0.27 %, and potassium 0.34 - 0.46 %. The interval of variation of the average content of Fe in the gentian root is between 241.37 - 503.53 mg kg^{-1}, while the lowest is on Ljubišnja and the highest on Štitovo. The content of Mn in the root is not high (13.27 - 52.17 mg kg^{-1}), although the content in the soil in most locations is very high (703.00 - 1538.00 mg kg^{-1}). Furthermore, the average content of Cu (4.25 - 10.72 mg kg^{-1}) and Co (0.35 - 0.65 mg kg-1) in the gentian root was relatively low and it is similar with the content of Cu and Co in the soil. The average content of Zn in the root was several times higher (125.57 mg kg^{-1}) on Durmitor than in other localities (17.00 - 34.20 mg kg^{-1}), which could not be related to the content of Zn in the soil. The content of Cr in the root (0.40 - 2.00 mg kg^{-1}) and Pb (1.16 - 2.95 mg kg^{-1}) was in all localities lower than MPC. The content of Ni in the root compared to other localities was highest in the locality of Kobilja glava (7.62 mg kg^{-1}) as well as the content of Ni in the soil (78.5 mg kg^{-1}). In other localities the level of Ni in the root was lower than MPC (1.5 - 4.1 mg kg^{-1}). High content of Cd was found in gentian root from Prošćanske Mountains and Sinjajevina (0.55 and 0.35 mg kg^{-1}) and the lowest ones were in Gutavica and Konjska rijeka (0.07 and 0.06 mg kg^{-1}). Highest content of Cd in soil was also

on Prošćanske Mountains and Sinjajevina (0.55 and 0.35 mg kg^{-1}) and the lowest in Gutavica and Konjska rijeka (0.07 and 0.06 mg kg^{-1}).

***Key words**: Yellow gentian, mineral content, root, soils, Montenegro*

INTRODUCTION

Gentiana lutea L. – *Gentianaceae*, yellow gentian, is heliophyte, herbaceous, perennial, mountain plant, with wide ecological valence. It grows on mountain ridges, meadows, pastures, rocky slopes, forest clearings and in rare forests, on altitudes up to 2500 meters [1]. It inhabits the ecosystem of mountain grasslands on carbonate substrates [2]. The soil on which the wild gentian grows well is formed on carbonate base, as well as on silicate rocks and serpentinite [3]. Wild gentian grows on loose humus soils, which contain more than 6% humus [4]. It is widely spread in central and south Europe from Pyrenees to Caucasus Mountains. The richest localities in wild gentian populations, in Montenegro, are Sinjajevina and Bjelasica, but it has been recorded on almost every other Montenegro mountain like: Plavsko-Gusinjske Prokletije, Čakor, Hajle, Komovi, Durmitor, Ljubišnja, Moračke Mountains, Orjen, Lovćen, Rumija, Volujak, Vojnik, Golija, Koritska plateau and Štedim [5]. Rhizome and root of yellow gentian (*Gentiana lutea* L.) has been used even a long time ago in great quantities in pharmaceutical industry, and a lot more for industrial production of bitter alcoholic drinks: brandy, digestive, liqueur and such [6]. The dominant ingredients of underground gentian organs are the compounds of secoiridoid and xanthons structures, which determine the pharmacotherapeutic use of this drug [7]. Furthermore, the xanthon ingredients have a big chemotaxonomic significance. Underground parts of *Gentiana lutea* is official pharmaceutical drug *Gentiana radix* which properties has been described in many world pharmacopoeias such as: Eur.3; BHP.1990; DAB 10; ÖAB 9; Helv VII; Jug. IV [1, 8], and also it has found a wide use in traditional medicine.

Large quantities of gentian root, which participate annually on world market, cannot be gathered from natural localities without the risk of extermination of natural populations. Unreasonable exploitation in the last century has exterminated or very decreased the growth of gentian on many mountains of Montenegro. Consequently, in the last decades of twentieth, and in the beginning of twenty-first century, great efforts are invested in developing the technology of gentian cultivation [9, 10, 11] especially in high rural areas. By cultivation, the natural resources of gentian would be protected from careless collectors, besides the law regulations like endangered species, and the local population would increase their product assortment and increase their earnings as well.

In order to master the production and create a quality product of root of cultivated gentian *(Gentianae radix)* we have to know the characteristics of natural habitats of gentian as well as the characteristics of plants that grow spontaneous in our ecological conditions. With this work we wanted to show the soil characteristics of gentian in natural habitat of Montenegro and the content of mineral elements and heavy metals in wild gentian depending on soil characteristics.

MATERIAL & METHODS

Research has included yellow gentian (*Gentiana lutea*) from 9 natural habitats in mountains of northern Montenegro which are located at altitudes between 1400 and 2076 m a.s.l. (Table 1). Samples were taken during the summer of 2009. In each locality the samples of soil surface layers were taken simultaneously with the gentian roots samples, (0-20 (25) cm). All samples were taken in 5 repetitions.

Soil analysis

The soil samples were air-dried and milled to a particle size of <2 mm, in accordance with ISO 11464:1994. Standard methods were used for analyses of basic chemical properties of soil: pH values (active acidity in soil suspension with water and substitution acidity in soil suspension with 1M KCl, both 1:5 V/V) determined potentiometrically; humus, i.e., organic matter content, determined with a modified method of Tjurin based on the principle of soil organic carbon oxidation with $K_2Cr_2O_7$; available phosphorus P_2O_5 ammonium lactate extraction (AL method of Egner and Riehm), detection by spectrophotometry; available potassium K_2O, ammonium lactate extraction (AL method of Egner and Riehm), detection by flame photometry. The pseudo-total content of heavy metals (Fe, Mn, Zn, Cu, Co, Cr, Pb, Ni and Cd) was determined by the AAS method, in an acetylene/air flame after the following decompositions, according to EPA Method 3050:10. (digestion in conc. HNO_3 at 150 °C with adding H_2O_2).

Table 1. Localities of gentian samples from natural habitats of Montenegro, where the soil and herbal material was taken in summer 2009.

Mountain	Locality	Altitude	Sampling date
Bjelasica	Strmenica	1762 m a.s.l.	July – August 2009
Bjelasica	Kobilja glava	1624 m a.s.l..	July – August 2009
Prošćanske Mountains	Veliki do	1400 m a.s.l.	July – August 2009
Sinjajevina	Veliki Starac	1793 m a.s.l.	July – August 2009
Ljubišnja	Sedlo ispod vrha	2076 m a.s.l.	July – August 2009
Koritska plateau	Gutavica	1504 m a.s.l.	July – August 2009
Koritska plateau	Konjska rijeka	1511 m a.s.l.	July – August 2009
Durmitor	Žabljak	1450 m a.s.l.	August – September 2009
Štitovo (Nikšić)	Suvi vrh	1460 m a.s.l.	August – September 2009

Plant material analysis

Analyses have been carried out in dry samples of gentian root, which were washed with water before drying. Contents of macro and microelements: (K, Fe, Mn, Zn, Cu, Co) and heavy metals (Ni, Cr, Pb i Cd) were determined by using AAS technique after sample digestion by a mixture of acids (HNO_3 + $HClO_4$) in accordance with ISO 5515:1979. Content of Phosphorus (P) was detected using spectrophotometry.

Statistical Analyses

Data have been processed by one way ANOVA and Fisher's test of least significant difference (LSD) was used to distinguish significant differences between localities.

RESULTS AND DISCUSSION

Soil

Acidity (pH value) of the soil on which wild gentian grows in Montenegro was in a very wide range, from 4.39 to 5.49 (pH/KCl), or from 5.01 to 6.05 (pH/H_2O) and with small standard deviations (Table 2). A similar interval of soil pH was determined by [3] which also analyzed the soil with wild gentian in Serbia. Most of the studied soil sites in Montenegro belong to the class of medium acid soils (from 4.80 to 5.22 - pH/KCl). Very acid soil was on Bjelasica (Strmenica, Kobilja glava where the average was pH/ KCl = 4.39 to 4.46) and on the Sinjajevina (4.60). The highest average pH/KCl was on Prošćanske Mountains (5.49), what is the threshold between high and low acid soil (Table 2). All analyzed soils are very rich in

humus and very variable. The differences between the average humus contents of the sites ranged from 5.59% at Strmenica to 28.21% on Ljubišnja. The content of available phosphorus (P_2O_5) is extremely low and stabile. Average values in soil on which wild gentian grows were in the range 0.29 to 1.82 mg P_2O_5/100 g soil. Unlike phosphorus, the average content of available potassium (K_2O) is very unstable and varied in a wide range, from poor (8.87 mg/100 g of soil) to a very good supply (26.70 mg/100 g of soil). These differences are probably due to different mineralogical composition of the soil in this part of Montenegro, formed on various parent substrates [12]. The lowest content of available potassium was in Strmenica (Bjelasica) and the highest on Prošćanske Mountains.

Table 2. Basic soil chemical properties in gentian natural habitat of northern Montenegro

Locality	pH		%	%	mg / 100 g	
	H_2O	KCl	Humus	Total N	P_2O_5	K_2O
Strmenica	5.0* (0.04)**	4.39 (0.09)	5.59 (0.82)	0.35 (0.05)	0.31 (0.12)	8.87 (2.29)
Kobilja glava	5.08 (0.33)	4.46 (0.30)	7.08 (1.39)	0.39 (0.06)	1.39 (0.80)	16.64 (8.87)
Prošćanske M.	6.05 (0.64)	5.49 (0.63)	16.2 (11.67)	0.74 (0.57)	1.82 (1.95)	26.7 (17.45)
Sinjajevina	5.05 (0.25)	4.60 (0.19)	15.94 (3.50)	0.94 (0.22)	0.86 (0.47)	17.16 (3.90)
Ljubišnja	5.72 (0.69)	5.16 (0.88)	28.2 (19.99)	0.88 (0.39)	0.85 (0.45)	20.9 (11.53)
Gutavica	5.97 (0.63)	5.22 (0.62)	8.39 (0.40)	0.40 (0.02)	0.48 (0.22)	19.74 (6.07)
Konjska r.	5.40 (1.01)	4.80 (1.08)	11.79 (4.06)	0.54 (0.15)	0.83 (0.59)	19.1 (14.10)
Durmitor	5.59 (0.35)	4.85 (0.53)	13.33 (4.50)	0.71 (0.20)	0.90 (0.13)	13.15 (3.89)
Štitovo	5.97 (0.18)	5.31 (0.17)	14.86 (2.79)	0.65 (0.04)	0.29 (0.16)	16.40 (2.97)
CV%	9.7	11.7	66.8	45.7	94.4	54.7
$LSD_{1\%}$	1.10	1.18	18.45	0.58	1.75	20.21
$LSD_{5\%}$	0.82	0.87	13.68	0.43	1.30	14.98

* average values ** standard deviations

The content of trace elements and heavy metals in soils in gentian natural habitats in Montenegro is shown in Table 3. The determined content of pseudo-total amount of most of the metallic elements in the soil was within the normal for uncontaminated soils [13]. However, we can distinguish slightly higher content of certain metals, especially manganese (Mn) and cadmium (Cd) in some analyzed soils. Manganese content of the average for all sites was 1022.20 mg Mn kg^{-1}, with an interval of 333.50 mg kg^{-1} (Štitovo) up to 1538.00 mg kg^{-1} (Kobilja glava - Bjelasica). Content of Mn in the soil depends on the presence of particular ferromanganese minerals, and can reach up to 9000 mg kg^{-1} [14].

The confirmed differences in Mn content in soils of certain mountains in Montenegro, confirm the aforementioned hypothesis that it is a different mineralogical parent substrate on which soils covered by this study are formed of.

The average content of cadmium (Cd) in soils was 1.99 mg kg^{-1} with the interval of variation from 0.29 mg kg^{-1} in Gutavica (Koritska plateau) to 4.87 mg kg^{-1} on Prošćanske Mountains. Generally, cadmium content in soil has been estimated to range between 0.06 to 1.10 mg kg^{-1}, with an average of 0.50 mg kg^{-1} [13]. The content above 3 mg Cd kg^{-1} is considered the maximum allowable cadmium concentration (MAC) for a normal soil. The average Cd contents in soils on the Prošćanske Mountains and Sinjajevina are above the permissible limits, noting that the Cd content in individual samples at the sites varied in a wide interval from 0.50 to 8.30 mg kg^{-1}. Such high concentrations of Cd in the soil in the presence of low pH values can cause increased adsorption of cadmium by plants [15].

Table 3. Content of pseudo-total quantities micronutrients and heavy metals in soils in natural habitat of gentian in Montenegro

Locality	%	mg kg^{-1}							
	Fe	Mn	Cu	Zn	Co	Ni	Cr	Pb	Cd
Strmenica	3.73	1238.10	29.74	121.07	10.39	37.30	16.04	50.06	1.12
Kobilja glava	2.86	1538.00	39.46	91.81	11.56	78.51	25.47	31.05	0.95
Prošćanske M.	3.42	1155.00	24.05	108.49	10.67	33.00	25.77	51.45	4.87
Sinjajevina	4.65	969.80	24.25	150.47	13.45	78.89	33.91	48.22	3.60
Ljubišnja	4.43	975.60	23.92	149.20	11.66	65.02	33.10	62.28	2.17
Gutavica	3.92	820.00	24.07	103.82	11.11	35.75	28.33	46.30	0.29
Konjska r.	4.10	703.00	19.31	138.80	10.47	32.42	23.16	47.36	0.70
Durmitor	3.88	781.50	28.47	117.77	11.86	55.58	37.52	36.88	1.73
Štitovo	3.90	333.50	21.58	92.60	9.68	50.06	43.00	48.26	1.61
AVERAGE	3.86	1022.20	26.63	120.58	11.31	52.73	28.21	47.55	1.99
CV%	24.2	26.2	22.8	23.6	24.4	76.8	50.0	27.1	72.8
$LSD_{1\%}$	1.92	550.20	12.45	58.42	5.65	83.06	28.91	26.44	2.98
$LSD_{5\%}$	1.42	407.90	9.23	43.30	4.19	61.57	21.43	19.60	2.21

Besides these two elements, one could say that the content of Zn in the majority of soils was also slightly above the amount of 100 mg kg^{-1}, which is considered as the maximum for uncontaminated soil. The average content of Zn in all investigated soils was 120.58 mg kg^{-1} with the interval varying from 91.81 to 150.47 mg kg^{-1}. Similarly, the average nickel content in soil from four localities were little above the limit of 50 mg kg^{-1}, which is considered as the MAC in uncontaminated soil (Table 3)

Gentian root

Average content of nitrogen in gentian root (N) in all analyzed localities in Montenegro is 0.52 %. The gentian root found in localities of Gutavica and Konjska rijeka on Koritska plateau distinguishes itself by low average content of nitrogen (0.32-0.33 % N) compared to other localities, which have a high content, especially locality Kobilja glava on Bjelasica (0.69 % N) and Štitovo and Durmitor with 0.66 % N and 0.65 % N, respectively (Table 4).

Average content of phosphorus (P) in gentian root was in an interval between 0.13 – 0.27 %, while locality Strmenica on Bjelasica has the considerably highest content. Average content of potassium (K) in gentian root found in the examined root was in an interval between 0.34 – 0.46 % while the differences between localities were not significant (Table 4).

Variation interval of average iron content (Fe) in gentian root was between 241.37 mg kg^{-1} on Ljubišnja to 503.53 mg kg^{-1} on Štitovo, while there were no significant statistical differences between localities. The content of Fe in gentian root was very variable at some individual samples on same locality, which is best illustrated on Prošćanske Mountains with an interval between 188.20 to 738.10 mg kg^{-1} Fe. Considering the high content of Fe in the soil (Table 3) it is fair to presume that even small remains of soil which remained on the root after the washing, can have great effect on analytical information of iron content in the root, which can also be the cause of such big variations. Although the content of manganese (Mn) in soil in most localities is very high (Table 3), the average content of Mn in the root is not high and it is in an interval between 17.28 to 52.17 mg kg^{-1}, and only the locality Kobilja glava on Bjelasica has significantly higher content. Equal and relatively low was the copper (Cu) content (4.25 - 10.72 mg kg^{-1}) and cobalt (Co) (0.35 – 0.66 mg kg^{-1}) in gentian root (Table 4) and it is similar with the content of these two elements it the soil (Table 3). The average

content of Zn in gentian root in all examined localities is 27.51 mg kg^{-1}, while on Durmitor it was several times higher (125.57 mg kg^{-1}), than in other localities (17.00 – 34.20 mg kg^{-1}). In most localities the level of Zn was equal or lower than the one determined in gentian root from Suvobor in Serbia [16]. It could generally be said that the content of micronutrient in gentian root was not in correlation with pseudo total content of these elements in the soil, which means that other environment factors were decisive in intensity and the degree of uptake microelements by yellow gentian.

Table 4. Content of macronutrients (N, P, K) and micronutrients (Fe, Mn, Cu, Zn, Co) in gentian root from natural habitats in Montenegro

Locality	% (D.M.)			mg kg^{-1} (D.M.)				
	N	P	K	Fe	Mn	Cu	Zn	Co
Strmenica	0.55	0.27	0.34	308.51	22.87	5.39	18.11	0.36
Kobilja glava	0.69	0.21	0.44	353.99	52.17	10.72	22.81	0.65
Prošćanske M.	0.56	0.16	0.40	450.72	19.24	8.96	34.20	0.53
Sinjajevina	0.49	0.17	0.34	364.94	35.03	5.72	24.12	0.66
Ljubišnja	0.52	0.15	0.34	241.37	17.76	6.44	19.10	0.35
Gutavica	0.32	0.16	0.46	327.88	17.28	5.59	17.77	0.43
Konjska r.	0.33	0.13	0.41	329.20	19.13	4.25	17.00	0.46
Durmitor	0.65	0.14	0.40	412.00	21.78	7.35	125.57	0.55
Štitovo	0.66	0.14	0.35	503.53	13.27	7.04	17.67	0.54
AVERAGE	0.52	0.18	0.39	352.86	25.66	6.91	27.51	0.50
CV%	26.4	43.1	23.5	48.6	42.5	33.2	95.8	30.3
$LSD_{1\%}$	0.28	0.16	0.19	352.10	22.40	4.71	54.10	0.31
$LSD_{5\%}$	0.21	0.12	0.14	261.00	16.60	3.49	40.10	0.23

Average content of nickel (Ni) in gentian root in localities in Montenegro is 3.25 mg kg^{-1}, which is 15 times lower concentration than the determined concentration in gentian root in Suvobor, Serbia (54 mg kg^{-1}) [10]. The highest content of Ni in the root was in locality Kobilja glava (7.62 mg kg^{-1}) where the content of Ni in the soil was also the highest (78.51 mg kg^{-1}). In other localities the level of Ni in the root is significantly lower (1.49 - 4.14 mg kg^{-1}) (Table 5). Average content of chromium (Cr) in gentian root is in an interval between 0.40 - 2.00 mg kg^{-1}, the lowest one being in Strmenica (Bjelasica) and the highest one on Prošćanske Mountains, Sinjajevina and Štitovo. Average content of lead (Pb) in gentian root from all localities is 1.67 mg kg^{-1} with the variation interval of 1.16 to 2.95 mg kg^{-1}. Highest content is recorded in gentian root samples from Durmitor, but as all other, they were below MAC (5 mg kg^{-1}).

High content of cadmium (Cd) is found in gentian root from Prošćanske Mountains and Sinjajevina (0.55 and 0.35 mg kg^{-1}) and the lowest one were in Gutavica and Konjska rijeka (0.07 and 0.06 mg kg^{-1}). Content of cadmium was also highest in the soil on Prošćanske Mountains and Sinjajevina, and lowest in Gutavica and Konjska rijeka (Table 3). In other localities content of Cd in the root was between 0.10 to 0.27 mg kg^{-1}, while the gentian from Strmenica and Durmitor has > 0.20 mg kg^{-1} Cd (0.21 i 0.27 mg kg^{-1}, respectively. Considering that the maximum allowed concentration is 0.20 mg kg^{-1} Cd is frequent for most medicinal herbal raw materials, contents of Cd determined in gentian root in localities Prošćanske Mountains and Sinjajevina can be considerd too high from aspect of correctness of raw material *Gentianae radix* which originates from this mountains.

Table 5. Content of heavy metals (Ni, Cr, Pb, Cd) in gentian root from natural habitats in Montenegro

Locality	mg kg^{-1}			
	Ni	Cr	Pb	Cd
Strmenica	3.84	0.40	1.16	0.21
Kobilja glava	7.62	1.09	1.30	0.13
Prošćanske M.	1.49	2.00	1.94	0.55
Sinjajevina	3.79	1.85	1.60	0.35
Ljubišnja	2.00	1.32	1.45	0.11
Gutavica	2.22	1.38	1.91	0.07
Konjska r.	1.70	0.99	1.93	0.06
Durmitor	4.14	1.21	2.95	0.27
Štitovo	1.09	1.97	1.76	0.10
AVERAGE	3.25	1.34	1.67	0.21
CV%	73.3	54.0	44.4	54.5
LSD1%	4.89	1.48	1.52	0.24
LSD5%	3.62	1.10	1.13	0.18

CONCLUSION

In most localities of wild gentian in Montenegro soils belong to the class of medium acid. Soils are rich in humus and content of available phosphorus is extremely low while potassium varied from poor to very good supply. Content of the most trace elements and heavy metals in soil were within the normal for uncontaminated soils, except in some sites for Mn, Zn, Cd and Ni, what are probably due to different mineralogical composition of the soil formed on various rock parent substrates.

Generally, the content of micro and micronutrient in gentian root was not in correlation with content of these elements in the soil. Content of all heavy metals in gentian root were below MAC, except Cd which was more than MAC in 4 localities.

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Original scientific paper

VEGETATIVE CULTIVATION OF *ARNICA MONTANA* L. IN BULGARIA

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SUMMARY

Arnica montana L. is a herbaceous perennial medicinal plant from the *Asteraceae* family. It is distributed in the mountainous European regions from southern Iberia to southern Scandinavia and the Carpathians. It is a rare and endangered species in many countries. The aim of this work is to develop an appropriate model for the sustainable vegetative cultivation of *Arnica montana* as a valuable economic crop in Bulgaria.

The plant material of the species was obtained through *in vitro* germination of seeds obtained from a German population of *A. montana* (Botanical garden, Schmitz). After micropropagation *in vitro* the plants were cultivated in the experimental field in the Rhodopes mountains at an altitude.

At the third year of vegetation on the experimental field, the underground parts – the short rhizome and long cylindrical roots formed from 48 up to 62 (maximum 94) tips with roots and rosette of basal leaves. The new plantlets started to grow after a week of planting and 27% of them begun to bloom in the same year. This kind of propagation ensured development of flower stems with 1 to 4 flower heads in the first year and 13-17 flowers per plants in the second year. The morphological and productive parameters were recorded. The dry flower yields were in the range of 1.08-4.36 g/ m² in the first and 16.12-21.08 g/ m² in the second years.

The content of sesquiterpene lactones in the dry flowers was determined according to ^{1}H NMR Spectroscopy.The total sesquiterpene lactones content varied from 4.04mg/g to 3.14mg/g depending on the age of the plants. Helenanolides dominated significantly over dihydrohelenanolides.

Key words: *Arnica montana*, vegetative propagation, sesquiterpene lactones

INTRODUCTION

Arnica montana L. is a herbaceous perennial plant of the family *Asteraceae*. It is distributed in the mountainous regions from southern Iberia to southern Scandinavia and the Carpathians. It is a rare and endangered species in many countries. Many health benefits are related to *A. montana*. The flowers are used mainly as anti-inflammation drug and are applied externally for bruises, spains and muscular and rheumatic pain [1]. This activity is due to the sesquiterpene lactones of helenalin and 11α, 13-dihidrohelenalin type.

The aim of this work was the development of an appropriate model for the vegetative propagation as a sustainable cultivation practice of *Arnica montana* and a valuable economic crop in Bulgaria.

MATERIAL & METHODS

Plant material: The plant material for the present study was obtained from *in vitro* seedlings. The seeds from a German population of the species (Botanical garden, Schmitz) were sown in August 2007. The obtained seedlings were further micropropagated in Murashige and Skoog (MS) medium [2] supplemented with 1mg/l 6-benzylaminopurine and 0.1 mg/l indole-3-acetic acid. After *ex vitro* soil transplantation and subsequent adaptation in greenhouse conditions, the plants were planted in the experimental field in the Rhodopes mountain at an altitude of 1500 m asl in July 2008. Thus the whole process was developed within one year. The experiment was carried out on a small plot (10 m^2).

The Beglica region is characterized with continental-mediterranean climate. Environmental data show that the average annual temperature in this region is 3.8ºC. The coldest month in the year is January (-6º C), and July is warmest (12.6ºC average annual temperature). Temperatures very rarely drop below -30º C and rise above 25-30ºC (August). The total annual rainfall amount is about 1002 mm, with a maximum of 140 mm in June and a minimum of 46 mm in August. The Beglica region is characteristic with a lack of a dry period during the year. The relative air humidity is with an average value of 81.7%, reaching a minimum of 77% during the periods with the lowest rainfall (August). The experimental field is characterized with Fluvisols (the Alluvial Fluvisols and Mollis types). The highest layer is slightly sandy-loam, pH 5,5-6,5 and with a low content of N,P and K. That's why the soil of experimental field was fertilized by 3 kg/ m² compost before digging and planting.

Vegetative cultivation of Arnica montana: At the second year of planting in the experimental field (2010 year), the plantlets formed rosettes of basal leaves with rhizomes and roots (parent plants). Vegetative /clonal propagation was done by division of the clumps into individual rosettes (daughter plants) in May, 2010 (beginning of vegetation). They were transferred to soil at 60 cm spacing between rows and 40 cm between plants (the plant density was 4 plants per square meter). The soil was regularly weeded, dug and irrigated several times during the summer and manured in the autumn.

Analysis of the lactone content: The amount of sesquiterpene lactones in the dry flowers was determined by ^{1}H NMR Spectroscopy [3]. This method was used because authentic samples are not necessary for calibration curves and short duration of the time for measurement. The method allows the determination of the total lactones as well as two types of lactones without the need of their preliminary identification using common proton signals.

RESULTS & DISCUSSION

In 2010 (22 months after *ex vitro* soil transplanting to the experimental field) the parent plants formed from 48 up to 62 (maximum 94) rosettes of basal leaves with rhizomes and roots and 25-38 flower heads per clump.

All vegetative propagated plants started to grow after a week of their planting and 27% of them started to bloom in the same year (July), while 100% blossomed in the next year. This way of propagation ensured development of flower stems with 1 to 4 flower heads per plant during the first year (2010) and 13-17 during the second year (2011). In the first year the blossoming of the young plants started a week later than parent plants, while in the next year daughters and parent plants blossomed together. No significant differences were registered in the diameter of the main flower, number of ligulate flowers and the length of leaves.

Morphological characteristics of parent and daughter *A. montana* plants are given in Table 1.

Table 1. Morphological characteristics of cultivated *Arnica montana* in Bulgaria

A. montana sample	Flower stems per plant (min-max)	Plant height (cm)	Flowers per plant (min-max)	Main flower ∅(min-max) (cm)	Ligulate flowers (min-max)	Length leaf (cm)
Parent plants	18- 28	47.8	25-38	6.2-8.0	18-21	21.7
First year daughter plant	1-2	48.2	1-4	6.5 -8.2	15-20	22.5
Second year daughter plant	10-14	48.0	13- 17	6.2- 8.0	17-21	21.9

As can be seen in Table 2, good yield of daughter plants herb was obtained in the second year (2011) when the big roots and large leaf rosettes ensured more flower stems and flowers. The average yield of flower heads per 1 daughter plant in its second year of cultivation (2011) was 22.49-29.41 g fresh weigh or 4.03-5.27 g dry weight, or 89.96-117.64 g/ m^2 fresh and 16.12-21.08 g/m^2 dry yield. The drying proportion of fresh flowers was about 5.5:1. The flower yield obtained a one and two years old plants from the vegetative propagation compared with that reported by Galambosi [4] and Aiello et al. [5], for 2-4 years propagated by seeds is nearly the same.

Table 2. Productive characteristics of flowers in cultivated *Arnica montana* in Bulgaria

A. montana sample	Flower head (g)		Flowers/ plant (g)		Flower Yield (g/ m²)	
	fresh	dry	fresh	dry	fresh	dry
Parent plants	1.64	0.31	41,0-62.32	7.75-11.78	164.0-249.28	31.0-47.11
First year daughter plant	1.46	0.27	1.46-5.84	0.27-1.08	5.84-23.36	1.08-4.36
Second year daughter plant	1.73	0.31	22.49-29.41	4.03-5.27	89.96-117.64	16.12-21.08

On the basis of the experimental results it was established that about 4160 rosettes (derived from 20 m^2 blossoming plants after their third vegetation *ex vitro*) are necessary for a 0.1 ha cultivation field. Thus, the results show that the selected ecological conditions for cultivation in Bulgaria allow the successful development of this species.

The sesquiterpene lactones content in flowers from parent (3-years) plants as well as from 1-st and 2-nd year daughter plants are listed in Table 3. As can be seen the highest lactone content was obtained in the one year old plant (4.04mg/g), which is diminished to 3.44mg/g in the two years old plants. In the mother plants the amount of lactones is less – 3.14 mg/g. Helenanolides (H) dominated over dihydrohelenanolides (DH).

Table 3. Sesquiterpene lactones (mg/g dry wait)

Lactones	Parent plants	First year daughter plant	Second year daughter plant
H	2.98	3.60	3.32
DH	0.16	0.44	0.14
total (mg /g)	3.14	4.04	3.44

CONCLUSION

This experiment revealed that micropropagation combined with subsequent vegetative propagation by rhizome division allows successful cultivation of *A. montana* and a source of qualitative raw material for the industry. The vegetative propagation of *A. montana* gives an opportunity to conserve prospective genetically improved populations, which is an important component of the sustainable agricultural development.

According to our results, the climatic and soil conditions are suitable for the growth and the development of the species. The quality of the drug is good and can be used for commercial purposes.

Vegetative propagation of this species is economically effective, saving expenses and working area for seedlings production. In addition, the good development of the parents' rosettes guarantees the 100% successful rooting of the daughter plants and their subsequent flowering and sesquiterpenoid production.

The cultivation of *Arnica montana* by vegetative /clonal propagation is an practical, productive, and prospective for small-sized organic farms in the mountain areas in Bulgaria.

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Original scientific paper

EFFECT OF TWO IRRIGATION REGIMES ON THE PRODUCTION AND SECONDARY METABOLITES OF SWEET BASIL (*OCIMUM BASILICUM* L. 'GENOVESE')

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SUMMARY

As consequence of the documented climatic changes analysis of the effects of high temperature and drought stress on different plant species seems to be essential. In our present study sweet basil (*Ocimum basilicum* L. 'Genovese') was investigated in pot experiments. Each pot contained 10 L soil. 3 plants per pots were planted. Two irrigation regimes were set while natural precipitation was locked out. In the first treatment (T1) plants were irrigated with 1 L water when the soil volumetric water content (SVWC) decreased below 20% while in the second treatment (T2) the plants were irrigated with 0,5 L water if the SVWC decreased below 10%. To measure the SVWC HH2 moisture meter and ML2x theta probe were used. During the vegetation period the stomata conductance (Delta-T AP4 porometer) and the chlorophyll content (SPAD-502) were measured, too. The plants were harvested two times in full flowering phenophase. Fresh and dry mass was determined. The essential oil content was measured by hydro distillation from dry leaves. The essential oil composition was identified by GC-MS.

The effect of the different irrigation regimes was obvious to the majority of the examined traits. The stomata conductance decreased (T1: 353.4 $\mu mol*m^{-2}*s^{-1}$; T2: 22.04 $\mu mol*m^{-2}*s^{-1}$) while the chlorophyll content increased (T1:36.45 SPAD unit; T2: 49.30 SPAD) significantly with decreasing water supply. The dry weight of the plants decreased in the case of the firs t (T1: 16.63 g/pot; T2: 11.20 g/pot) and second harvest as well (T1: 14.09 g/pot; T2: 6.58 g/pot). Similarly, the essential oil accumulation of the drug from plants suffering by drought stress showed lower level at both harvests (first harvest: T1: 0.88%; T2: 0.66%; second harvest: T1: 0.60%; T2: 0,54%).

The main component of the essential oil was the linalool the ratio of which dropped as a reaction to lack of water, too (first harvest T1: 63%; T2: 43%; second harvest T1:65%; T2: 41%). The results show that the water supply of medicinal plants may significantly influence the efficacy of the production and quality of drugs as well.

Keywords: *basil, stress, drought, water supply, irrigation*

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is a well-known aromatic plant from the *Lamiaceae* plant family. Basil has many different uses: it is widely used as a fresh and dry spice, its pharmacotherapeutic effect is well-known and its value as ornamental plant is also important [1]. One of the newest field of use is the plant protection. The basil's inhibitory effect was proved against insects and fungi as well [2, 3, 4]. Basil is morphologically diverse regarding the size, shape and colour of the leaves and inflorescence. The main active constituent is the essential oil which varies between 0.2-5.2 ml 100g-1 dry weight (DW) [5, 6]. In the essential

oil more than 140 components have been identified till now [7]. The main components include *linalool, estragole, eugenol, methyl cinnamate* and *1,8-cyneole* [6,8]. One of the most popular basil cultivar is 'Genovese' which has green leaves and stem and the flowers are white [6]. Its main compounds are the *linalool, 1,8-cineol* and *eugenol* [6, 9, 10].

Basil is native to the subtropical areas, where it prefers humus rich, well drained, loamy soils. In the Central European region mostly the amount of natural precipitation and the irrigation possibilities determine the efficacy of the cultivation. In the case of the medicinal and aromatic plants (MAP) not only the biomass but the amount and ratio of the biologically active compounds are of major importance. The experiments are focused mainly on the cereals and plants which are cultivated in bigger volume. The effect of the water deficit on the MAP production has been rarely investigated and the results are often contradictory. The negative effect of the drought stress on the biomass yield of summer savory (*Satureja hortensis* L.) was detected while the essential oil concentration was increased [11]. Same tendency was described in the case of basil [9, 12, 13], but contradictory effect was found in the *Hysophus officinalis* L. where the abundant precipitation decreased the concentration of the essential oil [14].

Our climate is changing. For each species the optimal water supply needs to be determining if we want to maintain the effective MAP cultivation. Therefore our aim was to investigate the effect of two different water supplies on the biomass, drug production, essential oil content, composition and the stomatal conductance of sweet basil.

MATERIAL AND METHODS

The experiment was carried out in Soroksár (Hungary) in the experimental field of the Corvinus University of Budapest.

O. basilicum L. 'Genovese' cultivar was used. Seeds were sown under greenhouse. Three seedlings were planted into 10 L pots when they reached the 2 leaves phenophase. Pots (n = 5 per treatment) without drainage were filled with 10L sandy soil. The soil volumetric water content (SVWC) was determined by HH2 moisture meter and ML2x Theta probe (Delta-T, Cambridge, UK). Two irrigation regimes were set while natural precipitation was locked out. In the first treatment (T1) plants were irrigated with 1L water per pot when the SVWC decreased below 20%. In the second treatment (T2) the plants were irrigated with 0,5 L water when the SVWC decreased below 10%.

Measuring the stomatal conductance of the leaves AP4 porometer (Delta-T, Cambridge, UK) was used (25 records per treatment) while the chlorophyll content was determined (10 replication per treatment) by SPAD-502 chlorophyll meter (Konica Minolta Co., Osaka, Japan).

The plants were harvested two times in full flowering phenophase. The first harvest was carried out in July 2010 while the second was in October 2010. Fresh and dry mass were determined by digital scale. Fresh plant material was air dried in a shade. The essential oil content was measured by hydro-distillation from dried leaves (2 replications per treatment). The essential oil composition was identified by GC-MS using an Agilent Technologies 6890 N GC equipped with an Agilent Technologies MS 5975 detector using the method of Radácsi et al [9]. The results were analysed with a PASW.18.0 (Inc, Chicago, IL, USA) statistical program. Two independent sample t-test was applied.

RESULT AND DISCUSSION

Yield

The effect of the different irrigation regimes influenced the fresh and dry yield of the sweet basil. Table 1. shows that the lack of water caused significant decrease on the fresh and dry yield of sweet basil. Better water supply produced approximately double fresh mass at the first cut and almost four times higher at the second harvest. In the case of the higher water supply (T1) the fresh biomass of the basil plants was increased in the time of the second harvest, but in case of the T2 plants not even the yield of the first harvest could be achieved. The highest fresh biomass was measured in the second harvest at irrigation regime T1.

Table 1. Effect of the different irrigation regimes on the fresh and dry yield of sweet basil (*O. basilicum* L.)

Time of harvest		Irrigation treatment T1	Irrigation treatment T2
July 2010	Fress yield (g/pot) ± SD	102.00±11.60*	56.60± 14.57
	Dry yield (g/pot) ± SD	16.63 ± 3.26*	11.20 ± 2.60
October 2010	Fress yield (g/pot) ± SD	113.20 ± 14.70*	32.40 ± 3.58
	Dry yield (g/pot) ± SD	14.09 ± 2.19*	6.58 ± 0.78

* shows significant difference between the treatments at ($P < 0.05$)

Stomatal conductance and chlorophyll content

The stomatal conductance informs us about the transpiration rate of the plants. Higher stomatal conductance means that the stomas are opened and the transpiration rate is higher. Table 2 shows that the different water supply results in significantly different stomatal conductance. The average stomatal conductance of the T1 treated plants was sixteen times higher than that of the plants of the T2 treatment.

The plants of the T2 treatment were obviously dark greener than the plants of the T1. The visible signs were proved by the measurements of the chlorophyll content. The lack of water increased the chlorophyll content of the plants (Table 2).

Table 2. Effect of different irrigation regimes on the stomatal conductance and chlorophyll content of sweet basil (*O. basilicum* L.)

	Irrigation treatment T1	Irrigation treatment T2
Stomatal conductance ($\mu mol\ m^{-2}\ s^{-1}$) ± SD	353.40 ± 61.17 *	22.04 ± 7.83
Chlorophyll content (SPAD Unit) ± SD	36.45 ± 1.34	49.30 ± 5.07 *

* shows significantly higher results in rows ($P < 0.05$)

Essential oil content and composition

Different water supply modified the essential oil concentration (EOC) of the sweet basil plants (Figure 1). The highest EOC was measured in the case of the first harvesting time at T1 treatment while the lowest EOC was detected during the second harvest at the T2 treatment. Between the two values almost 40% difference was observable.

In the whole, 48 different essential oil compounds were identified (Table 3). No trends were observable between the different harvesting times while the different irrigation regimes modified the essential oil composition of the basil. The major compounds of the essential oil

were the *linalool*, *1,8-cineol* and *tau-cadinol*. The linalool concentration decreased by more than 20% when the irrigation regime decreased. In parallel, the concentration of the 1,8-cineol and *tau-cadinol* increased.

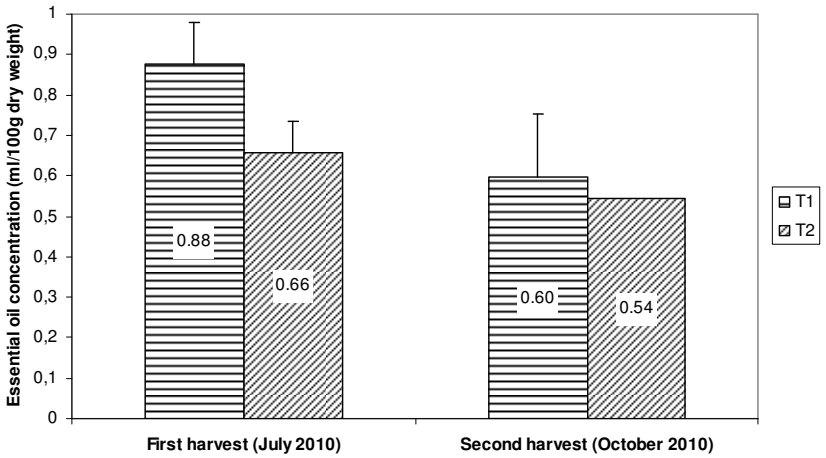

Figure 1. Effect of different harvesting time and irrigation regimes on the essential oil concentration of sweet basil (*O. basilicum* L.) Bars with horizontal lines indicate the T1 treatment while bars with leaning lines refer to T2 treatment

In the present study the effect of two different irrigation regimes on the production, physiological traits and chemical constituents of *O. basilicum* L. were investigated. The lower water supply had negative effect on the fresh and dry yield of the sweet basil. Similar tendency was observed by Simon et al. [12] and Khalid [13].
The stomatal conductance, which refers to the transpiration of the plants, showed that the well watered plants evaporated more than sixteen times more water than the less watered ones, indicating that the T2 treatments shows a sever lack of water for basil. The chlorophyll content showed contradictory values. The lower water supply resulted in higher chlorophyll content. However this behavior of the plants did not manifested itself in an elevated dry material production. It seems that increase of chlorophyll concentration rather shows a kind of stress effect for the basil plants.
In case of the essential oil concentration our results are in conformity with the previous studies [6]. However, there are in contradiction with some other findings like Simon et al. [12] and Khalid [13] who described negative correlation between the biomass yield and EOC. Our results showed that the water deficit decreased the fresh and dry biomass yield and the EOC alike. The phenomenon can be caused by the different plant growing conditions and variant water supplies.
Main components of the essential oils were the *linalool*, *1,8-cineol* and *tau-cadinol.* Our studies confute the results of Simon et al. [12] who described the increase of linalool in basil in consequence of increased the draught stress.

Table 3. Effect of different harvest time and irrigation regimes on the essential oil components of sweet basil (*O. basilicum* L.)

			July 2010		October 2010	
Component	**RT***	**RI****	**T1**	**T2**	**T1**	**T2**
α-pinene	5.56	938	0.00	0.00	0.02	0.00
sabinene	6.52	976	0.00	0.09	0.03	0.00
ß-pinene	6.64	981	0.06	0.22	0.08	0.17
ß-myrcene	6.99	995	0.05	0.40	0.13	0.41
limonene	8.19	1029	0.05	0.24	0.12	0.21
1,8-cineol	8.38	1034	**5.80**	**10.70**	**6.76**	**11.23**
(E)-ocimene	8.85	1046	0.00	0.00	0.02	0.07
γ-terpinene	9.20	1056	0.00	0.04	0.06	0.20
trans-sabinene-hydrate	9.73	1070	0.00	0.13	0.06	0.00
linalool	10.76	1097	**62.35**	**43.06**	**64.90**	**41.02**
camphor	12.68	1144	0.00	0.09	0.00	0.00
isoborneol	13.43	1162	0.00	0.08	0.05	0.00
α-terpineol	14.55	1189	0.46	0.96	0.48	1.16
octanol-acetate	15.38	1209	0.00	0.27	0.08	0.30
isobornil-acetate	18.41	1281	1.52	3.62	2.12	4.43
carvacrol	19.20	1300	0.21	1.41	0.91	0.00
α-cubebene	20.97	1348	0.00	0.00	0.04	0.10
eugenol	21.44	1361	0.07	0.07	0.52	0.51
α-copaen	22.03	1377	0.05	0.14	0.15	0.21
β-cubeben	22.47	1389	0.00	0.07	0.13	0.17
ß-elemene	22.55	1391	0.64	0.58	0.29	0.44
methyl-eugenol	23.31	1411	0.04	0.12	0.00	0.18
ß -caryophyllenne	23.68	1420	0.06	0.08	0.11	0.09
ß-gurjunene	24.06	1429	0.00	0.06	0.00	0.00
trans-α-bergamotene	24.36	1437	3.15	4.60	1.77	5.03
α-guajene	24.45	1439	0.00	0.51	0.58	1.20
aromadendrene	24.58	1442	1.08	0.55	0.30	0.00
α-humulene	25.07	1454	0.70	0.98	0.32	0.69
ß-farnezene	25.27	1459	0.00	0.25	0.24	0.26
alloaromadendrene	25.39	1462	0.32	0.55	0.28	0.48
germacrene-D	26.18	1482	3.13	3.41	2.22	3.28
ß-selinene	26.38	1486	0.09	0.28	0.08	0.28
bicyklogermacrene	26.81	1497	1.02	1.13	0.52	1.01
trans-beta-guajene	26.89	1499	0.08	0.17	0.11	0.17
α-bulnezene (δ-guajene)	27.16	1506	4.30	4.23	3.26	4.75
ß-bizabolene	27.23	1508	0.00	0.05	0.06	0.00
cisz-γ-cadinene	27.49	1515	4.27	4.66	2.99	4.84
δ-kadinén	27.80	1524	0.46	0.60	0.27	0.57
10-epi-cubenol	28.16	1534	0.00	0.24	0.10	0.18
ledol	29.51	1571	0.06	0.12	0.00	0.00
spatulenol	29.98	1584	0.44	1.01	0.42	0.71
viridiflorol	30.49	1598	0.00	0.05	0.08	0.00
1,10-di-epi-cubenol	31.36	1621	0.58	1.17	0.73	1.22
tau-cadinol	32.31	1646	8.76	11.94	8.20	13.63
ß-eudesmol	32.59	1653	0.00	0.14	0.12	0.21
α-cadinol	32.77	1658	0.21	0.44	0.24	0.42
α-bizabolol	33.84	1686	0.00	0.09	0.05	0.00
Total:			99.94	99.50	99.89	99.83

* RT= retention time

** RI= retention index relative to C_8-C_{21} *n*-alkanes on an HP-5 column

CONCLUSION

We can conclude that the lack of water has negative effect both on the essential oil concentration and plant biomass of sweet basil. These alterations have great influence in the agricultural practice. If our climate will be dryer the importance of irrigation will raise. That is why one of the major questions of the economic plant growing might be the possibility of irrigation. Our results contribute to the better understanding of stress responses of basil, however, show, that even more field and analytical work need to be done to get final conclusions.

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Original scientific paper

APPLICATION OF LOCAL PEAT IN THE LAVANDER NURSERY PRODUCTION (*LAVANDULA ANGUSTIFOLIA* MILL.)

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SUMMARY

The goal of this work was to apply domestic peat of Gaj and to estimate its appropriateness as the supstrate component in lavander nursery production. Peat was improved by adding different ratios of manure and water soluble mineral fertiliser.

Eleven substrates were tested. The control versions was pure peat (100%). Manure amounts of 10-50 vol % were added to the remaining variants. Water soluble mineral fertilizer formulations 20:20:20+ trace elments at the following rates 1.3; 1.9; 2.5; 3.1 and 3.7 g/l were added to peat. Examinations have shown that the best lavander nursery quality is obtained in case of production in the substrate containing peat of Gaj and manure at the proportion of 80%:20%. Among different dosages of used water soluble fertilisers, the 1.3 g L^{-1} dose had the best effect on the quality of lavander nursery production.

The obtained results are significant for the application and improvement of local peat as the main component in the nursery production of medicinal, aromatic and seasoning herbs in Serbia.

Key words: *lavander, manure, nursery, peat, fertiliser*

INTRODUCTION

Lavander (*Lavandula angustifolia* Mill.) is a medicinal, aromatic and seasoning herb species applied for therapeutical purposes and in perfume and cosmetic industry. The lavander flower (*Lavandulae flos*) and essential oil (*Lavandulae aetheroleum)* are used. Lavander reproduction takes place by older tillers division and by means of direct seeding on the production plot. The best and the safest manner to establish lavander crops is to produce seedlings [1].

Various substrates are used for the production of medicinal, aromatic and seasoning herb seedlings, most commonly: garden soil and other "cottage industry" substrates of indefinite chemical composition and inadequate quality. Various imported, commercial substrates, which make the production considerably more expensive, are also used.

Serbia is naturally rich in peats which are the main component of the seedling production substrate [2,3]. Peats are the basic component in the production of high-quality substrates used in vegetable, fruit, flower, mushroom, dendrological plant and lawn seedling production. There is a difference in terms of quality and reaction, which influences the quantity of easily accessible nutrients. Peats have small density (0.2-1 g/cm^3), good water-air capacity, high content of organic matters and humic acids. They are usually poor in easily accessible nutrients and therefore have to be mixed and improved [2,4,5]. Peat is easily mix by farmyard manure and vermicompost. Lately, various liquid or solid formulas of mineral fertilizers are also added to peat.

Many researches have confirmed that peat is relevant as the main substrat component in the production of particular kinds of vegetables, medicinal, aromatic and seasoning herbs [6-13]. Previous researches in the field of lavander seedling production dealt with the application of biostimulants and slowly-degradable fertilizers.

Based on the above, we have dedicated ourselves to discovering the most beneficial substrate for lavander seedling production, with the domestic peat of Gaj as its dominant component, which is simultaneously the objective of the present paper.

MATERIAL & METHODS

Researches with the above objective were carried out in the greenhouse of the Faculty of Agriculture of Belgrade during 2010. The experiment was carried out in two phases. Within the first phase, seeds of lavander cultivar Primorska were planted in polypropylene containers with 144 opening. Commercial seeding substrate *Stender* A – 250 was used to plant the seeds in the containers. The seeds were planted on February 20. When the first two pairs of permanent leaves appeared, the plants were picked to polypropylene pots type – V 9B (ø9 cm), which had previously been filled up with different substrate mixtures.

The main subtrate component was dark lowland peat originating from the South Banat region, namely village Gaj. The peat was improved by the mixed cow manure at different volume ratios (vol%) and the mineral fertilizer soluble in water with the following formulation: 20:20:20 + microelements at different weight rates - dosages (g/l). The controlled variant was pure peat (100%). In total, 11 final substrates were used.

The following substrate mixtures were used in the experiment (experiment variants):

1. Gaj peat (control) 100%
2. Gaj peat 90% + Cattle manure 10%
3. Gaj peat 80% + Cattle manure 20%
4. Gaj peat 70% + Cattle manure 30%
5. Gaj peat 60% + Cattle manure 40%
6. Gaj peat 50% + Cattle manure 50%
7. Gaj peat + Mineral fertilizer at the dosage 1.3 g/l
8. Gaj peat + Mineral fertilizer at the dosage 1.9 g/l
9. Gaj peat + Mineral fertilizer at the dosage 2.5 g/l
10. Gaj peat + Mineral fertilizer at the dosage 3.1 g/l
11. Gaj peat + Mineral fertilizer at the dosage 3.7 g/l.

Pursuant to the research objective, basic components of the substrates tested: Gaj peat and cattle manure (Table 1), underwent agrochemical analysis. Agrochemical properties were determined by usual methods at the Agrochemistry and Physiology Laboratory of the Faculty of Agriculture of Belgrade [14].

During the period of lavander seedling production on the abovementioned substrates, usual measures of tretment were applied: watering, shading and airing. The seedling production took 10 weeks. The plants were ”hardened“ before the analysis (measurement).

Thirty one plants were randomly selected from every of the above variants, and then they were measured to control the following parameters: plant height (cm), number of branches, plant weight (g) and root weight (g).

Table 1. Agrochemical properties of peat and cattle manure

Agrochemical properties	Gaj peat	Cattle manure
pH (H_2O)	7.44	6.98
pH (KCl)	7.03	6.95
$CaCO_3$ (%)	2.6	2.8
Humus (%)	23.0	23.9
Total N (%)	0.692	1.204
C/N (%)	19.3:1	11.5:1
NH_4-N (mg/kg)	9,8	30.1
NO_3-N (mg/kg)	108.5	2107
(NH_4+NO_3)-N (mg/kg)	118.3	2137.1
P_2O_5 mg/100g	20.0	2000
K_2O mg/100g	6.9	805
Water soluble P_2O_5 mg/100g	0.2	31.5
Water soluble K_2O mg/100g	0.8	4.0
EC mS/cm	0.380	5.81
Water soluble salts (%)	0.12	1.83

Results of the experiment have been presented through the basic indicators of descriptive and analytical statistics [15]. Central tendency indicators have been used to calculate arithmetic mean ($\overline{X}$). Variation of characteristics is expressed via the variation interval (I_v) and variation coefficient (C_v). Results of the experiment have been processed by the variance analysis method (31 plant) while the relevance assessment has been processed by the implementation of LSD-test.

RESULTS & DISCUSSION

Plant height. Results of the experiment from table 2 indicate to the highest average values of the plant height (17.22 cm) in the production on the substrate consisting of Gaj peat and cattle manure at the volume ratio 80%:20%. The least considerable lavander growth of 12.5 cm was realized in the controlled experimental variant on the pure peat.

Lavander seedling production on the substrates with farmyard manure resulted in statistically extremely relevant differences with regard to the controlled variant (pure peat).

Earlier researches proved the use of the substrate consisting of Gaj peat and farmyard manure at the ratio 80%:20% resulted in the most considerable influence on the sage plant height [12]. As for the seedling production of other medicinal herbs: basil, garden balm and thyme, the substrate consisting of Gaj peat and farmyard manure at the ratio 70%:30% turned out to be the best possible substrate combination [11,12,13].

Since Gaj peat was improved by various dosages of the mineral fertilizer soluble in water, it considerably influenced the average lavander plant height (Table 2). The highest average height (15.57 cm) was recorded with the plants grown on the following substrate: Gaj peat and mineral fertilizer soluble in water at the dosage 1.3 g/l.

Production of lavander seedlings on the substrates to which water-soluble fertilizers were added resulted in statistically extremely relevant differences in average values of the plant height when compared to the controlled variant.

The results comply with the researches of seedling production of other medicinal species. If you use more than 1.3 g/l of fertilizers you will not achieve the expected effect – increase in the plant height, but rather the decrease therein. Such a tendency has also been confirmed in the researches of seedling production of other medicinal species [11].

Table 2. Effect of substrates on plant height and number of branch

Trial variant	Plant height (g)			Number of branch		
	$\overline{X}$	Iv	Cv (%)	$\overline{X}$	Iv	Cv (%)
1. Gaj peat 100% (test)	12.15	9.5-13.2	10.54	3.22	2-3	12.45
2. Gaj peat 90% + Cattle manure 10%	16.36	14.7-17.2	8.42	4.65	4-5	9.85
3. Gaj peat 80% + Cattle manure 20%	17.22	15.9-17.9	7.44	4.85	4-5	8.44
4. Gaj peat 70% + Cattle manure 30%	15.94	14.8-16.4	6.21	4.22	4-5	7.56
5. Gaj peat 60% + Cattle manure 40%	15.11	14.2-15.9	6.22	4.11	4-5	7.88
6. Gaj peat 90% + Cattle manure 50%	15.45	14.5-16.1	6.01	3.65	3-5	9.74
7. Gaj peat + 1.3 Mineral fertiliser L^{-1}	15.57	13.9-16.1	7.45	3.62	3-5	9.11
8. Gaj peat + 1.9 Mineral fertiliser L^{-1}	14.81	13.4-15.2	8.11	3.25	3-5	9.54
9. Gaj peat + 2.5 Mineral fertiliser L^{-1}	14.67	13.1-15.4	8.54	3.11	3-5	10.11
10. Gaj peat + 3.1 Mineral fertiliser L^{-1}	14.44	13.2-15.8	9.22	3.09	2-4	10.22
11. Gaj peat + 3.7 Mineral fertiliser L^{-1}	13.92	13.1-14.7	8.41	3.01	2-4	10.14
LSD 0.05	0.55			0.20		
0.01	1.05			0.45		

Number of branches. Results of the examined substrates influenced the analyzed parameter of seedling quality – number of branches (Table 2).

The most branches (4.85) were noted when lavander was produced on the substrate consisting of Gaj peat and cattle manure at the ratio 80%:20% . The least branches (3.22) were noted when seedling was produced on pure peat. There were also statistically very importnt differences in the number of side branches between the experimental variants which included addition of farmyard manure and the controlled variant.

Earlier researches of the thyme seedling production indicated that the most branches were noted when the substrate consisting of Gaj peat and cattle manure at the ratio 70%:30% was used [13].

As for the part of the experiment which included application of various dosages of water-soluble fertilizer, the most branches (3.62) were noted when the 1.3 g/l dosage was used (Table 2). Such dosage of the fertilizer used makes the limiting quantity which influences production of the number of branches. The use of larger fertilizer dosages will not increase the number of branches.

Table 3. Effect of substrates on plant weight and root weight

Trial variant	Plant weight (g)			Root weight (g)		
	$\overline{X}$	Iv	Cv (%)	$\overline{X}$	Iv	Cv (%)
1. Gaj peat 100% (test)	2.95	2.61-3.05	11.47	1.01	0.84-1.06	15.43
2. Gaj peat 90% + Cattle manure 10%	4.65	4.35-4.75	10.22	1.26	1.12-1.35	12.24
3. Gaj peat 80% + Cattle manure 20%	4.75	4.43-4.84	9.44	1.42	1.31-1.47	9.41
4. Gaj peat 70% + Cattle manure 30%	4.22	4.01-4.36	9.11	1.32	1.23-1.39	9.25
5. Gaj peat 60% + Cattle manure 40%	4.11	3.95-4.28	8.97	1.31	1.20-1.37	8.11
6. Gaj peat 90% + Cattle manure 50%	3.25	3.05-3.41	8.38	1.29	1.25-1.35	7.44
7. Gaj peat + 1.3 Mineral fertiliser L^{-1}	3.42	3.11-3.54	9.44	1.33	1.22-1.38	8.41
8. Gaj peat + 1.9 Mineral fertiliser L^{-1}	3.25	2.95-3.34	9.14	1.20	1.09-1.28	8.55
9. Gaj peat + 2.5 Mineral fertiliser L^{-1}	3.11	2.88-3.24	8.48	1.14	1.05-1.26	8.66
10. Gaj peat + 3.1 Mineral fertiliser L^{-1}	3.05	2.81-3.11	8.25	1.11	1.01-1.22	9.11
11. Gaj peat + 3.7 Mineral fertiliser L^{-1}	3.00	2.74-3.05	8.22	1.09	0.98-1.18	9.02
LSD 0.05	0.15			0.10		
0.01	0.25			0.20		

Plant weight. The level of lavander seedling development is also reflected in the weight of its aboveground parts. Results of the research presented in Table 3 indicate that the biggest plant weight (4.75) is achieved when the plant is produced on the substrate: Gaj peat 80% + cattle manure 20%. The lowest average level of plant weight (2.95 g) is achieved when seedling is produced on the pure peat. Effect of the substrate: Gaj peat and farmyard manure at the ratio 80%:20% on the plant weight has also been confirmed in the sage seedling production [12].

As for the part of the experiment which included application of various dosages of water-soluble fertilizer, the biggest lavander weight (3.42 g) was noted when the 1.3 g/l dosage of the substrate composed of peat with fertilizer was used. Within this parameter, the 1.3 g/l dosage makes the limiting quantity.

Root weight. The substrate made of Gaj peat and cattle manure at the ratio 80%:20% proved to be dominant with the seedling quality parameter, too (Table 3). There was the biggest root weight of 1.42. The lowest average root weight value (1.01 g) was noted with the control variance. The 1.3 g/l fertilizer dosage proved to be the best quantity in the lavander seedling production. The use of such a dosage resulted in the highest average plant weight value of 1.33 g. The highest average root weight values were recorded in the experiments which included basil seedling production on the substrates the main component of which was Gaj peat, to which 1.3 g/l dosage of water-soluble fertilizer was added [13].

CONCLUSION

Results of the experiment indicate that application of the domestic raw material, treset of Gaj, considerably influences the lavander seedling quality. Improvement of the peat by cattle manure and water-soluble mineral fertilizer has resulted in the production of various substrate mixtures that considerably influence the examined parameters of lavander seedling quality.

Based on the researches that have been carried out, we can draw a conclusion that the lavander seedling is of the best quality when produced on the domestic substrate consisting of the peat improved by farmyard manure at the volume ratio of 80%:20%. When compared to other fertilizer dosages used, the best lavander seedling quality is achieved through the application of the water-soluble mineral fertilizer added in the 1.3 g/l dosage.

The obtained results are significant for the application and improvement of local peat as the main component in the nursery production of medicinal, aromatic and seasoning herbs in Serbia.

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Original scientific paper

INFLUENCE OF CONTAINER CELL CAPACITY ON THE PROPERTIES OF LEMON BALM NURSERY PRODUCTION

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SUMMARY

Until now the production of lemon balm nursery production in warm beds has been meeting the needs with regard to the herb. However, the ever-increasing interest for the plant has initiated the need to enhance the technology of seedling production.
Container nursery production being intensive has a number of advantages and is used in both vegetable and flower production. The "speedling system", i.e. the system of polystyrene containers with pots of different volumes for substrate and seed sowing is known to be the most rational and practical container production system.
The objective of the study was to develop a model for lemon balm nursery production under controlled conditions using containers of various sizes. The lemon balm nursery production was analyzed with respect to its growing under field conditions and consumption as a fresh spice.
The seedlings were grown in containers of seven different sizes. The most favorable lemon balm nursery production quality for field production was obtained in the largest sized containers cells 76 cm^3.The highest lemon balm fresh weight yield per m^2 was obtained in 22 cm^3 cell containers.

Key words: *conainer cell, lemon balm, nursery*

INTRODUCTION

Garden balm (*Melissa officinalis* L.) is a medicinal, aromatic and seasoning herb species used for therapeutical purposes and cookery. Garden balm reproduction takes place by older tillers division and by means of direct seeding on the production plot. Garden balm has tiny seeds, therefore it is usually produced through seedlings, since the production is safer that way [1].
Garden balm used to be produced in warm seedbeds, thus meeting the needs for this herb species. However, since interest for garden balm as raw material has increased lately, seedling production technology needs to be improved [2-8]. Container seedling production has been implemented in vegetable and flower production ever since. There are numerous container production systems; by far the most rational and practical one is certainly »speedling system«, i.e. polystyrene (polypropylene) containers with different cell capacities in which substrate is placed and seeding is carried out [9].
Container seedling production results in more plants per surface unit when compared to the traditional growing manner, which influences more economical usage of the space protected as well as energy saving [10,11]. The best polystyrene container model for basil production is the one with 76 cm^3 cell capacity [12].

Bearing in mind all the abovementioned, the objective of this paper is to make a garden balm seedling model, i.e. to opt for the most beneficial nursery production.

MATERIAL & METHODS

Researches were carried out in the greenhouse of the Faculty of Agriculture of Belgrade-Zemun during the year 2011. Garden balm seedling was produced in 7 different containers (Table 1).

Table 1. Properties of the containers tested

Cell size (cm^3)	Cells number	Cell form	Distance between cells (cm)	Material	Container size (cm)
14	230	square	2,5	Polietilen	49 x 27 x 3,5
22	144	cylinder	3,2	Polipropilen	53 x 31x 4,5
24	80	square	3,3	Polietilen	36 x 37 x 4
32	66	upside-down cone	4,5	Polietilen	50 x 28 x 4
38	84	upside-down cone	4,5	Polistiren	50 x 32 x 5
64	42	upside-down cone	6	Polietilen	50 x 28x5
76	40	upside-down cone	6	Polistiren	53 x 31 x 5,5

The containers examined were filled with a substrate the content of which was determined by standard methods [13] at the Agrochemistry and Physiology Laboratory of the Faculty of Agriculture of Belgrade (Table 2).

Table 2. Agrochemical properties of substrate

pH		%	% N	Ratio	mg/100 g		ppm		Total
H_2O	KCl	Humus	total	C/N	P_2O_5	K_2O	NH_4^+	NO_3^-	$NH_4^+ + NO_3^-$
5.88	5.66	68.37	1.034	38.3:1	94	64	60.2	97.3	157

Garden balm seed of the basil cultivar »Citron« was used for seeding. A few seeds were manually planted in each container cell on March 16. After the germination and cropping up, thinned to one plant in each cell. During the period of garden balm seedling production, usual measures of tretment were applied: watering, shading and airing. Seedling production took 52 days. 31 plants were randomly selected from every of the above variants, and then the observed seedling quality parameters were analyzed. Plant height, number of leaves, plant weight and root weight were the analyzed garden balm seedling quality parameters.
Analysis of variance (ANOVA) and lsd-testing were applied in order to examine the difference between treatments - containers with different cell capacity [14].

RESULTS & DISCUSSION

Plant height The highest average value of the plant height (12.84 cm) was achieved by the production in containers with the highest cell capacity of 76 cm^3. The lowest average value of the plant height (3.97 cm) was achieved by the production in containers with the lowest cell capacity of 14 cm^3. There were no statistically relevant differences in average garden balm heights among the containers with the cell capacities of 22, 24, 32 i 38 cm^3. Yet there were highly statistically relevant differences among other experimental variants.

Relevance of the container cell size was also confirmed in the production of vegetable, flower and other medicinal plant seedlings [2,4,7,12,15,16]. Plant species grown in containers with higher cell capacity achieved higher average height.

Table 3. Indices of lemon balm nursery quality

Cell volume (cm^3)	Plant height (cm)	Leaf number	Plant weight (g)	Fresh root weight (g)
14	3.97	6.10	0.23	0.31
22	9.88	8.42	0.76	0.57
24	9.76	9.45	0.79	0.77
32	9.33	12.40	1.11	0.99
38	9.41	12.75	1.08	1.08
64	11.69	16.14	1.52	1.30
76	12.84	18.28	1.96	1.33
$LSD_{0.05}$	0.75	0.25	0.10	0.15
0.01	1.10	0.48	0.20	0.25

Number of leaves is a very important indicator of the garden balm seedling quality. Research results (Table 3) indicate that the most leaves per plant (18.28) were achieved when garden balm seedling was grown in containers with the highest cell capacity. The least leaves (6.10) were achieved with the production in containers with the lowest capacity. Among the all containers examined, statistically relevant differences were noted in the number of garden balm leaves.

The fact that containers with the highest cell capacity can influence the number of leaves was confirmed in the examination of vegetable, flower and other medicinal plant species [2,4,7,12,15,16].

Plant weight (fresh mass) Garden balm seedling development reflected also in the weight of its aboveground parts. The most plant weight 1.96 g was achieved in the production container of the cell capacity 76 cm^3. The least weight 0.23 g was achieved in the production container of the cell capacity 14 cm^3. Among the all examination variants, highly statistically relevant differences were noted in average plant weight values.

In their experiments with other plant species, numerous authors confirmed that the container cell capacity influenced the plant weight [2,4,5,7,12,15,16].

Root weight. Container cell capacity influenced the garden balm weight as well (Table 3). Seedling quality parameter was also proved to be the most influenced by the highest cell capacity container. The biggest root weight of 1.33g was achieved in the highest cell capacity containers and with the »upside-down cone« cell shape, which stimulated development of side roots and the root system in whole. The smallest root weight of 0.31g was achieved when the production took place in containers with the cell capacity of 14 cm^3.

Other authors also found out that the container cell capacity considerably influenced the root weight of the relevant species.

Result of the present research have proven that polystyrene (styrofoam) containers are preferential to the plastic ones. Seedling production in polystyrene containers with the cell capacity of 76 cm^2 has came up with the best results, including all the examined garden balm seedling quality parameters. The use of polystyrene containers with the cell capacity of 38 cm^3 (Table 3) has shown good results in the seedling production as well. The use of polystyrene containers is preferential due to the fact that they make the seedling produced easy to handle and transport; on the other hand they require somewhat more storage space [17,18].

As for the production of garden balm as fresh spice, an important indicator, besides the plant raw material quality, is also the total production, i.e. fresh mass yield per surface unit. Results (Table 4) indicate that the highest yield per container of 109 g per surface unit 656.76 g/m^2 is attained in polypropylene containers with the cell capacity of 22 cm^3.

Table 4. Indices of fresh lemon balm yield

Cell volume (cm^3)	Fresh weight yield (g/container)	Number of plants/m^2	Fresh weight yield (g/m^2)
14	52.91	1209	278.07
22	109.00	876	665.76
24	60.00	600	450.00
32	73.26	471	522.81
38	90.72	525	567.00
64	63.84	300	456.00
76	78.40	243	476.28

Production of garden balm in the containers with bigger cell capacity (64 i 76 cm^3) has not resulted in the highest fresh mass values. Basil container production has similar results. The lowest capacity containers have had the highest fresh mass values per surface unit 955 g/m^2 [12].

CONCLUSION

We have to improve our traditional production technologies of garden balm seedling. Container production, which is multiply preferential to the old one, has been widely accepted in contemporary flower, vegetable, medicinal, aromatic and seasoning herb seedling production.

One of the most important moments in seedling production is appropriate container system selection. The most favorable lemon balm nursery production quality for field production was obtained in the largest sized containers cells 76 cm^3. For the production of garden balm as fresh spice, the highest yield per surface unit is achieved in production containers with the cell capacity of 22 cm^3.

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Original scientific paper

INITIAL GROWTH AND YIELD OF THE BLACK CHOKEBERRY (*ARONIA MELANOCARPA*) GROWN ON THE DYSTRIC CAMBISOL AND CALCAREOUS CHERNOZEM SOILS AND MINERAL COMPOSITION OF ITS FRUITS

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SUMMARY

In recent years, due to salutary effects of its fruits, the black chokeberry [*Aronia melanocarpa* (Michx.) Elliott] has gained popularity in Serbia and it has been grown on a variety of soil types. A three-year study was conducted in order to assess the establishment and early growth of the black chokeberry on an acid soil and a calcareous soil. Experimental plots were established in the spring of 2009. Young plants on their own rootstocks, which originated from the same source, were planted. The plants were grown as shrubs and they received conventional mineral and organic fertilizers. The following parameters were measured in five replications in the second and third years of the experiment: number of shoots, height and width of the shrub, and fruit yield. In the third growing season, the contents of following elements: P, K, Ca, Mg, Fe, Mn, Zn, Cu were analyzed in black chokeberry leaves and fruits. At the same time we analyzed chokeberry fruits from several locations in Serbia, grouped according to soil type: cambisols and chernozems. The soils were analyzed for pH and contents of humus, carbonates, available P and K. Plant development in the first three years of growing differed noticeably depending on soil properties. Plants grown on dystric cambisol achieved an average height of 147 cm, a diameter of 122 cm, and the yield of 4200 g of fruits per plant in the third year. Plants grown on calcareous chernozem had poor development, without forming a shrub and putting out new fertile branches. Symptoms typical of Fe chlorosis occurred on apical leaves in all three years, which was a great deterrent for plant growth. Only in their third year did the plants achieve an average height of the main stem of 95 cm and a shrub diameter of 43 cm, and they produced the first crop of fruits of only 79 g per plant, which was 50 times smaller than the yield of plants grown on cambisol. Potassium content was higher in black chokeberry fruits from dystric cambisol than from calcareous chernozem (1.86 % and 1.47%, respectively). Average contents P, Ca and Mg in black chokeberry fruits ranged from 0.21 to 0.27%, 0.43 to 0.49% and 0.12 to 0.15%, respectively, and, they seemed not firmly dependable on soil properties. Manganese content was several times higher in the fruits from dystric cambisol than in those from calcareous chernozem (66-96 mg kg^{-1} and 15-17 mg kg^{-1}, respectively). The contents of Fe, Zn and Cu in the fruits range from 34 to 40 mg/kg, 9 to 13 mg/kg and 5 to 7 mg kg^{-1}, respectively, and, they seemed not firmly dependable on soil properties. The results of the three-year study showed that the properties of dystric cambisol were more favorable for black chokeberry growing than the properties of calcareous chernozem.

Key words: *Aronia melanocarpa, soil types, yield, mineral content, berries*

INTRODUCTION

Black chokeberry [*Aronia melanocarpa* (Michx.)] is a deciduous shrub from the Rosaceae family originating from North America. It was spread long ago to the northern parts of Europe and Russia and its local common name is Siberian blueberry. The fruit is a black berry, for which there are great market demand in the recent years. The berries are processing for various products, such as: wine, jam, syrup, juice and tea [1]. It is also used for flavoring and coloring of beverages [2]. The berry has an astringent taste and it is highly valued for high contents of vitamin C and antioxidants. The latter feature makes it a useful dietary preventive for reducing risks of diseases caused by oxidative stress. The main active ingredients responsible for this effect are anthocyanins and proanthocyanidins [3]. Besides those ingredients, human health related properties are also attributed to organic acids, vitamins and minerals. Preliminary clinical studies have shown beneficial effect of black chokeberry in cases of colorectal cancer, cardiovascular diseases, chronic inflammations, stomach mucous membrane disorders and the inflammation of the liver [4,5].

Black chokeberry is already commercially grown in Europe, where its fruits are used as an ingredient in juices, alcoholic or energizing drinks, and as food colorant. In response to a high market demand, its production in Serbia has intensified in the past few years.

Ap to now, soil quality issues received a little attention when establishing aronia plantations, because of the widespread opinion that chokeberry grows well in all types of soil [2]. Bussieres et al. [6] have reported a high growth potential of aronia on soils damaged by peat exploatation in Canada. The annual precipitation of 700 mm and soil pH from 6 to 6.5 has been claimed as favorable for the growth of chokeberries [7]. The same authors stated that the light sandy soils, which are regularly subject to summer droughts, are not suitable for growing aronia.

The aim of this study was to examine the suitability of two diametrically different soil types, dystric cambisol and calcareous chernozem, for the growth and development of chokeberries in Serbia. The former soil type is typical for central Serbia; the latter is typical for the lowland region of Vojvodina. We also examined the effect of these soils on the content of minerals in aronia fruits.

MATERIAL AND METHODS

Sample collection and processing

A three-year study was conducted in order to assess the establishment and early growth of the black chokeberry on (1) an acid soil [dystric cambisol on Povlen Mountain (44º11'46˝N, 19º49'12˝E, altitude 600 m asl)] and (2) a calcareous soil [chernozem in Stara Pazova (44º59'47˝N, 20º08'25˝E, altitude 82 m asl)]. Experimental plots were established in the spring of 2009. Young plants on their own rootstocks, which originated from the same source (Suvobor Mountain), were planted in the 2 x 3 m arrangement. The plants were grown as shrubs. Before planting the experiment, the two locations received manure in the amount 30 t ha^{-1} and 200 kg ha^{-1} of mineral fertilizer NPK 15:15:15. The following parameters were measured in five replications in the second and third years of the experiment: number of new annual shoots, height and width of the shrub, and fruit yield. For the soil analysis, samples were collected from the topsoil (0 to 25 cm) at each location. The homogenized sample was air-dried and afterwards milled to a particle size of <2 mm, in accordance with ISO 11464:1994.

In the third growing season fruit samples were collected for the determination of macroelements (P, K, Ca, Mg) and microelements (Fe, Mn, Zn, Cu) content.

In addition to the two locations of study, soil and aronia fruit samples were taken in 2011 from four locations with soils similar to the studied ones: dystric cambisol in the locations

Velika Vrbnica (Aleksandrovac Župski) and Vrnčani (Gornji Milanovac), and chernozem in the locations Banstol (Fruška Gora Mountain) and Bavanište road (Pančevo).

Soil analysis

Standard methods were used for analyses of basic chemical properties of a soil: soil pH (active acidity in soil suspension with water and substitution acidity in soil suspension with 1M KCl, both 1:5 V/V) was determined potentiometrically; $CaCO_3$ content was determined volumetrically with a Scheibler calcimeter; humus content, i.e., organic matter content, was determined with a modified method of Tjurin based on the principle of organic carbon oxidation with $K_2Cr_2O_7$ in H_2SO_4; available phosphorus (P_2O_5) and available potassium (K_2O) were extracted by ammonium lactate extraction (AL method of Egner and Riehm), and measured by the means of spectrophotometry and flame photometry, respectively.

Plant material analysis

Contents of macro- and microelements (P, Ca, K, Mg, Fe, Mn, Zn and Cu) were determined by the ICP-OES technique (Vista Pro-Axial, Varian) after sample digestion with a mixture of acids (HNO_3 + $HClO_4$), in accordance with ISO 5515:1979.

RESULTS & DISCUSSION

Soil properties

The characteristics of dystric cambisol at the experiment locacion were: highly acidic soil, with medium humus content, medium provided with available potassium, and low provided with available phosphorus. At the chernozem location soil properties were: medium alkaline soil with 15% of Ca-carbonate and medium humus content, medium provided available potassium and well provided with available phosphorus (Table 1).

Table 1. Basic chemical properties of the soil layers 0 – 30 cm in the experiment locations and other locations in Serbia where Aronia is grown

	Location	pH		$CaCO_3$	Humus	AL- mg/100 g	
		H_2O	KCl	%	%	P_2O_5	K_2O
Dystric soils	**Povlen Mountain (experiment)**	**4.50**	**3.80**	**0.0**	**3.90**	**5.0**	**17.5**
	V. Vrbnica, Aleksandrovac *(only Aronia fruit sampled)*	4.57	3.92	0.0	3.17	6.0	12.1
	Vrnčani, G. Milanovac *(only Aronia fruit sampled)*	5.30	-	0.0	3.52	16.7	28.9
Calcareous soils (chernozem)	**Stara Pazova (experiment)**	**8.30**	**7.50**	**15.7**	**3.20**	**24.2**	**18.8**
	Banstol, Fruška Gora *(only Aronia fruit sampled)*	8.21	7.32	11.48	1.78	13.4	16.4
	Pančevo, Bavanište *(a recently established Aronia plantation)*	7.84	6.98	0.57	3.17	25.3	35.5

The properties of the soils at four locations where chokeberry is grown in Serbia are also presented in Table 1. Although there are some differences between the presented localities in regard of the certain chemical characteristic within the soils of the same type (e.g. available phosphorus), the soils within the group are, in general, in the same level of fertility.

Initial growth and yield of the black chokeberry

Plants development in the first three years of growing differed significantly depending on soil properties. There were no significant differences between the locations in the first year. The experimental plants developed only 2-4 lateral branches on the stem, with no new annual shoots from the roots.

Large differences in plant development occurred in the second and third growing seasons. Plants grown on dystric cambisol achieved an average height of 110 cm, a diameter of 70 cm and the average fruit yield per plant of 340 g already in the second year, and a height of 138 cm, a diameter of 146 cm, and the yield of 4200 g of fruits per plant in the third year (Tab. 2).

Table 2. Indicators of plant size and yield of berries in the second and third growing seasons

Soil type	Plant height		Bush diameter		Number of new annual shoots		Berry yield per plant	
	2nd year*	3rd year**	2nd year**	3rd year**	2nd year**	3rd year**	2nd year**	3rd year**
Cambisol	109.8	138	70.1	146.0	3.0	14.2	339.6	4200.0
Chernozem	72,0	95.4	25,0	43.2	0,2	2.8	11,4	79.0

*- difference at 95% significance level

** - difference at 99% significance level

Plants grown on calcareous chernozem had poor development, without forming a shrub or putting out new fertile branches.

Plants grown on chernozem were delayed in all growth parameters. Their height was 72 cm in the second and 96 cm in the third year, lower by 35% and 31%, respectively, compared with the plants grown on cambisol. The largest difference was observed in a very low annual production of new tillers, 0.2 on average in the second year and 2.8 in the third, which was 15 and 5 fold less, respectively, compared with the plants grown on cambisol (Table 2).

Picture 1. Aronia grown on cambisol, at the end of the third growing season

Picture 1a. Aronia grown on calcareous chernozem, at the end of the third growing season

The plants grown on chernozem formed very poor bush by the end of the third year, with the diameter 2.8 and 3.4 fold smaller in the second year and third year, respectively, than those of the plants grown on cambisol (Picture 1 and 1a).
A large number of shoots that emerged from cambisol included a significantly large number of fruiting branches (Picture 2) which resulted in 30 fold higher yield in the second year and 53 fold higher yield in the third year in relation to the yield of chokeberries obtained on chernozem (Table 2).
Symptoms typical of Fe chlorosis occurred on apical leaves in all three years, which was a great deterrent for plant growth (Picture 2a). As previously observed in the case of various plants susceptible to iron deficiency due to highly calcareous growing medium, iron deficiency in black chokeberry nutrition was manifested as the yellowing of youngest leaves due to a disorder in chlorophyll synthesis.

Picture 2. Fruiting branch of an Aronia plant grown on cambisol, in the third year

Picture 2a. Fruiting branch of an Aronia plant grown on calcareous chernozem, in the third year of growth, showing symptoms of Fe chlorosis

Prolonged absence of chlorophyll caused necrosis in young leaves which subsequently dried up. Further, the formation of new leaves was reduced and the growth of shoots was totally interrupted [8].
The plants reached an average height of the main stem of 95 cm and a shrub diameter of 43 cm only in their third year, and they produced the first crop of fruits of only 79 g per plant, which was 50 times smaller than the yield of plants grown on cambisol.

Content of mineral elements in fruit

Fruit mineral composition, in general, depends on several interactive factors; some of the most important being: soil conditions (pH, humus, clay, CEC, bioavailability of the particular mineral, etc.), climate, and plant genotypic characteristics.
The differences observed in black chokeberries mineral composition, as a result of a soil conditions in our research were the highest for potassium and manganese contents.

Potassium content was higher in the fruits of black chokeberries grown on dystric cambisol than in those grown on calcareous chernozem (1.86% and 1.47%, respectively). This might be explained by the competition between Ca and K in plant nutrition; the presence of Ca carbonate in the soil might induce poor potassium uptake for some plants [9]. Average contents of P, Ca and Mg in black chokeberry fruits ranged from 0.21 to 0.27 %, 0.43 to 0.49 and 0.12% to 0.15%, respectively, and they seemed not to depend on soil properties.
Manganese content was several times higher in the fruits from dystric cambisol than in those from calcareous chernozem (66-96 mg kg^{-1} and 15-17 mg kg^{-1}, respectively). Acidic soil reaction increases the Mn solubility, and consequently, availability [8] whichresulted in increased uptake of Mn by aronia plants grown in dystric soil compared with calcareous chernozem.

Table 3. Contents of macro- and micro-nutrients in fruits of Aronia plants grown on different soil types in Serbia

Soil type	Location	%				mg kg^{-1}			
		P	K	Ca	Mg	Fe	Mn	Zn	Cu
Calcareous chernozem	**Stara Pazova (experiment)**	**0.27**	**1.64**	**0.44**	**0.15**	**40.8**	**16.7**	**11.2**	**6.3**
	Banstol, Fruška Gora Mountain	0.19	1.30	0.54	0.13	27.6	15.0	7.0	8.5
	Average	**0.23**	**1.47**	**0.49**	**0.14**	**34.2**	**15.9**	**9.1**	**7.4**
	Standard deviation	***0.06***	***0.24***	***0.08***	***0.01***	***9.35***	***1.24***	***2.94***	***1.50***

Soil type	Location	%				mg kg^{-1}			
		P	K	Ca	Mg	Fe	Mn	Zn	Cu
Dystric cambisol	**D. Leskovice, Povlen Mountain (experiment)**	**0.21**	**1.71**	**0.43**	**0.12**	**31.3**	**95.9**	**12.6**	**5.6**
	Velika Vrbnica, Aleksandrovac	0.20	1.85	0.49	0.15	30.9	44.2	11.9	6.8
	Vrnčani, Gornji Milanovac	0.29	2.03	0.37	0.12	45.0	85.9	13.7	7.7
	Average	**0.23**	**1.86**	**0.43**	**0.13**	**35.7**	**75.3**	**12.7**	**6.7**
	Standard deviation	***0.049***	***0.160***	***0.060***	***0.017***	***8.028***	***27.42***	***0.907***	***1.054***

The contents of Fe, Zn and Cu in the fruits range from 34 to 40 mg/kg, 9 to 13 mg kg^{-1} and 5 to 7 mg/kg, respectively, and they seemed not strictly related to soil properties (Table 3.).

CONCLUSION

The results of the three-year study showed that dystric cambisol was more favorable for black chokeberry growing than the calcareous chernozem soil.
Black chokeberry plants grown on chernozem containing 15% Ca carbonate and pH/H_2O = 8.3, exhibited typical symptoms of Fe chlorosis. These symptoms significantly inhibited the growth and development of the black chokeberry plants.

Acidic soil reaction favored the uptake of Mn and K by chokeberry plants, which resulted in higher contents of these two elements in the berries of the plants grown on dystric cambisol than on calcareous chernozem.

The different properties of the tested soil types seemed to have small effect on the contents of P, Ca, Mg, Fe, Zn and Cu in the berries of cultivated Aronia, however, further research is needed for more information.

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Original scientific paper

YIELD AND MINERAL CONTENT OF YELLOW GENTIAN ROOT (*GENTIANA LUTEA* L.) GROWN ON BLACK WATER–PERMEABLE FOIL IN ORGANIC AND MINERAL MODEL OF FERTILIZATION

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SUMMARY

The influence of mineral NPK fertilizers and farm yard manure (FYM) on growth and yield of roots of Yellow Gentian (*Gentianalutea* L.) was examined in the field experiment conducted on dystriccambisol on the mountain Tara during 4 years period. Yellow Gentian was grown on black water-permeable foil. In the fourth vegetation, concentration of macroelements (P, K, Ca, Mg) and microelements (Fe, Mn, Zn, Cu) in the roots was analyzed.

Average losses of crop density in the first vegetation (the year of plantation establishment) ranged from 9.8 to 14.1% and were not dependent on the fertilization variants. By the end of the third vegetation, the average yield of fresh root ranged 84.6–115.9 g/plant while by the end of the fourth vegetation it ranged 182.5 – 227.5 g/plant. The highest yield in the third year was achieved in plots fertilized with organic fertilizer while in the fourth year in plots fertilized with mineral NPK fertilizer. The lowest yields were achieved in the control treatment (without fertilizer), being 27% and 20 % lower than the highest yield achieved in the third year and in the fourth year, respectively. The concentration of macro-and microelements in the roots were: 0.15 P %, 0.55– 0.60 K %, 1.15–1.24 Ca %, 0.17–0.20 Mg %, 251.1- 425.5 mg Fe kg^{-1}, 31.9-41.7 mg Mn kg^{-1}, 38.9-42.1 mg Zn kg^{-1} and 16.1-17.1 mg Cu kg^{-1}.The content of most metals was particularly high in the root cortex; concentration of Fe was 18 times higher in the cortex of the root then in the inner part of the root, while in the case of other tested metals the higher values recorded in the cortex varied from 12% (Ca) to 97% (Mn).However, this does not apply for the content of Zn (which was the same in the cortex and the inner root part) nor for the P and K, whose contents were higher in the inner part than in the cortex. Further research is needed to clarify an influence of fertilization model on root mineral composition.

Black water-permeable foil used in experiment was fully operational in the first and the second vegetation, while in the third one it started losing its strength and began to disintegrate so that in the fourth vegetation it lost its mulching function.

***Key words**: Yellow Gentian, cultivation, yield, fertilization, mineral content, root.*

INTRODUCTION

Gentianalutea is an herbaceous perennial and grows in mountainous regions, on meadows and open slopes from the Pyrenees to the Carpathian Mountains and from Alps to the Balkan Peninsula and Anatolia. In many countries a general decline in population size dedicated to commercial exploitation has lead to Red Data Book (in Romania, Portugal, Bulgaria, Bosnia and Herzegovina, Albania, Germany, Czech Republic, Poland etc.).

In Serbia, Yellow Gentian grows wild solely in mountainous regions (800 – 2500 m a.s.l.) and it may be found on following mountains: Šara mountain, Zlatibor, Tara, Kopaonik, Suvobor, Maljen, StaraPlanina etc [1]. Its natural stands in Serbia are soils of different types, formed equally on calcareous parent material and serpentine [2].

Gentianalutea is an officinal drug in European pharmacopoeia 5,0. The essential active principles are bitter substances amarogentin and gentiopicrin. Therefore, drug is used in digestive disorders, such as loss of appetite, fullness, flatulence. Among secondary metabolites, xanthoneisogentisin was also detected. Recent investigation suggested that the aerial parts of this plant could also be useful for medicinal purposes [3, 4]. Root of the Yellow Gentian is also used in large amounts by the industry of beverages, for liqueurs and as a base for various bitters, the most famous being the "Enzian schnapps".

Due to an excessive exploitation from the nature its survival in the nature is endangered. Thus, orientation to a large-scale production (cultivation) of this species is needed for both protection of its natural resources and satisfaction of market demands. During the last decenniums, there have been a lot of efforts to improve Yellow Gentian growing technology in many countries [5, 6, 7, 8, 9]. In the Balkans, research in introducing of Yellow Gentian into culture are quite new and refer to determination of optimal model for nursery plant production [10, 11, 12], or experimental cultivation of various autochthonous ecotypes of Yellow Gentian grown on different localities [13]. Based in the research in the past 6-7 years, the first technology of Yellow Gentian root production, suitable for agroecological conditions of the mountainous regions of Serbia has been published [14].

The aim of this study was to investigate the possibility of applying water-permeable black mulch in the production of Yellow Gentian root in dry farming conditions in the mountainous regions of Serbia, as a tool for weed control. In addition, the other objective was to examine the comparative efficiency of farm yard manure (FYM) and NPK fertilizers in Yellow Gentian root production with application of permeable mulch foil and to examine their impacts on the mineral composition of the roots.

MATERIAL AND METHODS

Description of experimental localities

Experimental fields of *G.lutea* were established on mountain Tara (Serbia), locality Tara - Kaluđerske bare (1004 m a. s. l., g. latitude 43°N, 53', 41" and g. longitude 19°E, 33', 41"), in the forest tree nursery of National park "Tara". The main soil and climate properties of the locality are given in Table 1.

Table 1. Main climate and soil properties on experimental locality Kaluđerske bare, mountain Tara, Republic of Serbia.

Mountain Tara, locality Kaluđerske bare (1004 m a. s. l.)		
Climate	Annual T/Precipitation	6.1 ºC / 900 mm
	IV-IX T/Precipitation	11.9 ºC / 448 mm
Soil *(Districcambisol)*	pH (H_2O)	5.5
	% Clay	7.0
	% Humus	5.0
	P_2O_5 mg/100g	5.3
	K_2O mg/100g	20.0

The influence of mineral NPK fertilizers and farm yard manure on growth and yield of roots of Yellow Gentian (*Gentianalutea* L.) was examined in the field experiment during the 4-year period. Organic fertilization mode (ORGANIC) was represented byfarm yard manure, applied at a dose 100 m^3 ha^{-1}. Chemical composition of the farm yard manure is shown in Table 2. Mineral fertilization (MINERAL) was carried out using solid mineral fertilizers in doses of N = 50, P_2O_5 = 150 and K_2O = 300 kg ha^{-1}. All fertilizers have been incorporated in the soil by tiller, before sowing, to a depth of 20 cm. The control treatment (Control) implies the cultivation of Yellow Gentian without application of any fertilizer. Indigenous Yellow Gentian population, originating from the mountain Suvobor (Serbia) was used in the experiments (the seed were multiplied at mountain Tara, Serbia).Yellow Gentian was grown on black water-permeable foil. Seedlings were produced outdoors in PVC containers, volume 180 ml (4x4x15 cm). Research was conducted in three replicates. The experimental plot was 10 m^2, with a plants schedule 25 x 30 cm (133 plants per plot). Planting was performed on May, 13th and 14th, 2008 (Photos 1 and 2). The experiment was conducted under natural moisture conditions, without irrigation.

Photo 1. Planting of Yellow Gentian seedlings in the water-permeable foil

Photo 2. Establishment of the experimental plots with Yellow Gentian

Table 2. Chemical composition of cattle farm yard manure used in the experiment (all data are expressed on fresh weight bases)

	The percentage on fresh weight % w/w				**Avaliable (AL method of extraction) mg / 100 g**		**Specific mass kg/lit**
pH	Dry matter	Organic matter	Total salts	Total N	P_2O_5	K_2O	
8.27	24.4	16.2	0.3	0.59	465	317	0.65

The percentage of plants receipt in the first year following planting and losses of crop density in the next vegetations were determined by counting plants at the beginning of each growing season.Also, each year, the number of plants with flowering stalks was determined. Root yield was determined at the end of the third and fourth growing season by measuring air dried root mass of 9 plants in each experimental plot. At the end of the fourth growing season (2011), the samples of Yellow Gentian roots were taken in six replicates, for the analysis of

mineral elements. The fresh root material was washed with water. Before the analyses, all plant material was oven dried (80 ^{o}C) and ground to powder.

Plant material analysis

In the fourth vegetation, concentration of macroelements (P, K, Ca, Mg) and microelements (Fe, Mn, Zn, Cu) in the roots was analyzed. Contents of the macroelements and microelements were determined using the ICP-OES technique (Vista Pro-Axial, Varian) after sample digestion by a mixture of acids (HNO_3 + $HClO_4$) in accordance with ISO5515:1979 [15].

The obtained results were statistically processed by the means of Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Growth and development of Yellow Gentian plantations

Average losses of crop density in the first vegetation (the year of plantation establishment) ranged from 9.8 to 14.1% and were not dependent on the fertilization model (Table 3). These losses were significantly lessin comparison to losses in the spring establishment under dry farming conditions without implemented mulch foils, which to account for, 20%, and in some vegetation even 40% ofthe experimental plantations established in Serbia [16].

The appearance of flowering stalks in Yellow Gentian depends on biological capabilities of the plants, and in agroecological conditions of Serbian mountains, it beginsfrom the third vegetation [14]. In our experiment with the water-permeable black foil, flowering stalks began to appear in the second vegetation (the year 2009.), and the highest percentage was recorded in the mineral fertilizer model (3.9%). The stalks appeared at a very low percentage in the organic fertilizer model and in the control treatment, (0.3% and 1%, respectively).Stems that have appeared in the second vegetation were very thin and low (65 - 90 cm).In the third vegetation (the year 2010.), the higher appearance of stemswas recorded forthe plants fertilized with organic and mineral fertilizers (7.6% and 5.9%, respectively), in comparison to the plants in the control (4.3%).In the fourth vegetation (in the 2011.) the stems appeared in a similar percentage as in the third one, for the plants fertilized with mineral and organic fertilizers, while in the control only 1.1% of plants had the stem (Table 3). The stems in the third and fourth vegetation were more robust than those of other vegetations and reached the height in the interval 120-160 cm.

Table 3. Losses of Yellow Gentian crop density in the first vegetationfollowing plantation establishment and incidence of flowering stalks in the second, third and fourth vegetation, depending on different fertilization model

Fertilization models	**The loss of crop density**		**Number of plants with flowering stalks in% of total plant**					
	Decayed plants (%)		II vegetation (2009)		III vegetation (2010)		IV vegetation (2011)	
	Beginning of 2009	*Stdev*	**%**	*Stdev*	**%**	*Stdev*	**%**	*Stdev*
MINERAL	**9.8**	*5.305*	**3.94**	*1.370*	**5.90**	*3.301*	**5.96**	*4.535*
ORGANIC	**14.1**	*5.675*	**0.32**	*0.550*	**7.65**	*2.174*	**6.46**	*4.939*
Control	**10.8**	*5.514*	**1.03**	*0.266*	**4.26**	*3.335*	**1.08**	*1.087*

Stdev – Standard deviation

Yellow Gentian root yield

By the end of the third vegetation, the average yield of fresh root ranged 84.6–115.9 g plant^{-1}, while it ranged 182.5 – 227.5 g plant^{-1} by the end of the fourth vegetation (Table 4). The highest yield in the third year was achieved in the plots fertilized with organic fertilizer while in the fourth year in plots fertilized with mineral NPK fertilizer. The lowest yields were achieved in the control, being in the third year and in the fourth year 27% and 20 % lower than the highest yield achieved, respectively. The average yield in the fourth year, achieved in all experimental models, was 2.1 times higher than the average yield achieved in the third vegetation (Table 4). The average effect of the organic and mineral fertilizers in the third and fourth vegetation on the yield of Yellow Gentian root was almost identical. In comparison to the control, the root yields increased for 18.5 and 19.2% after the application of organic and mineral fertilizers, respectively (Table 4).

Yield of fresh Gentian roots grown under water-permeable foil in the third year (84.6 to 115.9 g per plant) was similar to the yield previously achieved without the foil application (96.7 g plant-1) [17]. At the end of the fourth year the yield on the root decreased for 16 - 32%, depending on fertilization model applied, as compared to a yield achieved without the use of water-permeable foil, when the yield reached 270.1 g plant-1 [16]. The application of mulch foil had a positive impact on reducing weeds and water loss through evaporation from the soil, which improved soil water regime. However, it also increased the soil temperature, especially during the intense insolation in the summer. High temperatures under the foil during the summer adversely affected the development of Yellow Gentian, which was manifested by drying of lower leaves in the early stages of development, in July and early August (photo 3). The early loss of some leaves from the rosette certainly had a negative impact on development and yield of Gentian root. This may explain the obtained lower yields compared with the yields of Gentian grown in the same location but without application of the mulch foil. Thus, a positive effect of the foil on reduction of weeds together with better preservation of the soil moisturewas finally annulled by the negative impacts on plants due to high soil temperatures developed under the foil in agroecological conditions of the mountain region of Serbia.

Table 4. Yield of fresh Yellow Gentian roots (g per plant) grown with the use of permeable mulch films, achieved in the third (2010) and fourth (2011) vegetation, depending on different fertilization model applied

Years	Fertilization models	Mean (yields)	Mean (years)	Mean (models)		
				T1 (mineral)	T2 (organic)	T3 (control)
2010	T1 (mineral)	89.1 ^{c,d}	96.5	158.3 ^{a}	159.2 ^{a}	133.6 ^{b}
	T2 (organic)	115.9 ^{c}				
	T3 (control)	84.6 ^{d}				
2011	T1 (mineral)	227.5 ^{a}	204.2**			
	T2 (organic)	202.5 ^{a,b}				
	T3 (control)	182.5 ^{b}				
		$LSD_{0,05}$ =26,79	** F≤1%	$LSD_{0,05}$=18.95		

Photo 3. Damage of the lower leaf rosette of Yellow Gentian due to high temperatures (03. August 2009.)

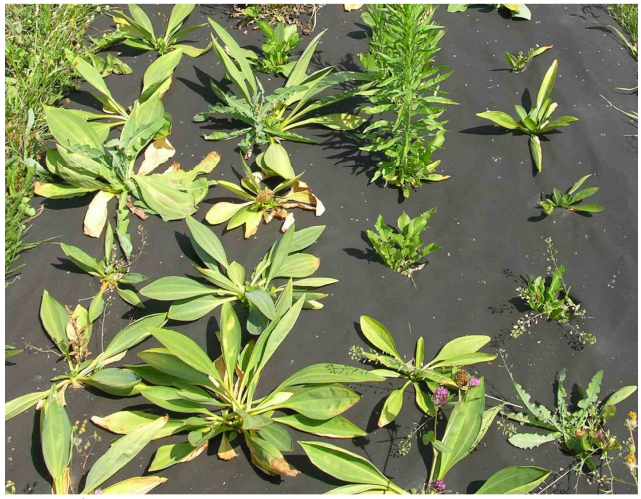

Photo 4. The emergence of weeds in the second vegetation and damage of Yellow Gentian leaf rosette due to high temperatures (03. August 2009.)

Analyses of mineral composition of Yellow Gentian roots

The concentration of macro-and microelements in the Gentian roots in the terms of the applied fertilization models are presented in Tables 5 and 6.For mostelementsthe differences areverysmall. The highest values are mostly measured in the plants from the organic fertilization model. However, in some cases (e.g. Mg, Zn, Cu) the highest concentration were found in the control. Since many factors simultaneously might influence plant mineral composition, further research is needed for determining the more suitable fertilization mode for gentian production in this geo-climatic conditions, both with and without mulch.

Table 5. The content of macroelements in the root of Yellow Gentian grown under the water-permeable mulch foil and with different variants of fertilization model applied

Fertilization model	**Content of macronutrient (% w/w D.M.)**							
	P	*Stdev*	**K**	*Stdev*	**Ca**	*Stdev*	**Mg**	*Stdev*
MINERAL	**0.15**	*0.012*	**0.57**	*0.045*	**1.15**	*0.125*	**0.17**	*0.007*
ORGANIC	**0.15**	*0.022*	**0.60**	*0.040*	**1.24**	*0.167*	**0.19**	*0.021*
Control	**0.15**	*0.015*	**0.55**	*0.059*	**1.16**	*0.096*	**0.20**	*0.015*

Stdev – Standard deviation

Table 6. The content of microelements in Yellow Gentian root grown under the water-permeable mulch foil and with different fertilization model applied

Fertilization model	**Content of micronutrient (mg kg^{-1} w/w D.M.)**							
	Fe	*Stdev*	**Mn**	*Stdev*	**Zn**	*Stdev*	**Cu**	*Stdev*
MINERAL	**251.1**	*128.07*	**31.9**	*1.761*	**39.8**	*3.964*	**16.1**	*3.020*
ORGANIC	**425.5**	*138.79*	**41.7**	*10.513*	**38.9**	*4.045*	**16.8**	*2.270*
Control	**404.2**	*84.31*	**38.7**	*6.622*	**42.1**	*6.802*	**17.1**	*1.635*

Stdev – Standard deviation

Due to the possible retention of very fine particles of soil at the surface and in the root cortex after washing, the content of mineral elements was analyzed separately, in the root cortex and in the inner part of the root. The content of most metals was particularly high in the root

cortex. Concentration of Fe was 18 times higher in the cortex of the root then in the inner part of the root (Table 7). In the case of other metals, the higher values wasrecorded in the root cortex as follows: 12% higher for calcium (Ca), 47% higher for copper (Cu),and up to 97% higher for manganese (Mn). However, this does not apply for the content of Zn, which was in the same order of magnitude in the root cortex and in the inner root part (Table 7).Apart from the fact that root pre-treatment for the analysis might have certain weaknesses (some metallic oxides might still be attached to the root surface), the higher content of metallic elements (Fe, Mn and Cu) and macronutrients (Ca and Mg) in the root cortexmight be a result of their relatively high content in the soil in relation to the amount which plants adopt for their own needs [18].The opposite is in the case of phosphorus (P) and potassium (K), which removal from the soils by the plants biomass is usually higher than their available concentration in the soil. Thus, their contents in the inner part of the Yellow Gentian root were higher than in the root cortex (Table 7).

Table 7. The average content of mineral elements in the root cortex and the inside of the Yellow Gentian root grown in the experiment

Yellow Gentian root part	%				mg kg^{-1}			
	P	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Zn**	**Cu**
Root (peeled)	0.16	0.50	1.12	0.17	59.0	30.8	32.1	15.0
Root cortex	0.14	0.41	1.26	0.22	1068.4	60.7	29.9	22.0

Applied water-permeable mulch foil has stopped the growth of weeds in a large extent, however, new weeds appeared very close to the plants in the each vegetation, and they had to be manually removed (photo 4). It can be concluded that in agroecological conditions of the mountain areas of Serbia, black water-permeable foil used in the experiment was fully operational in the first and second vegetation, while in the third one it started losing its strength and began to disintegrate, so that during the fourth vegetation it lost its mulching function. In addition, fertilization lead to an increase of Gentian root yield, without statistically significant differences between the organic and mineral fertilization model. Further research is needed to explain an affect of fertilization model on concentration of macro-and microelements in the roots.

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Original scientific paper

AGRONOMIC FACTORS AFFECTING YIELD AND ESSENTIAL OIL OF *OCIMUM BASILICUM* L.

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SUMMARY

The three replication field experiments with Serbian cultivar Sitnolisni (*Ocimum basilicum* L.) were conducted at the Bački Petrovac location (84 m elevation). Basic plot area was 7 m^2. Seed was sown continuously in rows at 70 cm distance between rows using standard agro technical measures. Experiment I included two sowing dates: early (April) and late sowing (July) while experiment II five variants of herb cutting height: 5, 10, 15, 20 and 25 cm above soil surface. In both experiments measurements included plant height, herb yield and calculation of essential oil yield. Essential oil content in dry herb was determined by using standard method. In the first experiment dry herb and essential oil yield in early sowing date were significantly higher (by 47 % and 42 %, respectively) than at late sowing date while the essential oil content was similar at both dates of sowing. Although significant yield reduction in later sowing date was recorded, its level was still economically sounded owing to the fact that this second crop yield coupled with the first crop yield increased profit per hectare in the same year of growing.

The second experiment showed a strong effect of cutting heights upon yield. The highest total yield of dry herb, essential oil yield and essential oil content in dry herb were recorded in 25 cm cutting variant. In this variant total yield of dry herb was higher by 11%, essential oil yield by 30 % and essential oil content in dry herb by 25 % when compared with 5 cm variant.

Key words: *Ocimum basilicum L., sowing date, cutting height, yield, essential oil*

INTRODUCTION

Generally, sweet basil (*Ocimum basilicum* L.) is most frequently grown in open field by applying early sowing (main crop), giving high yield of herb and essential oil. Rarely, later sowing is also applied while basil growing as the stubble crop is not performed under the conditions of Serbia [1]. Therefore, it was of interest to investigate comparatively the basil yield obtained in early and late sowing.

Literature data suggest cutting of sweet basil herb not more than 10 cm above soil surface, namely 10-15 cm, 15-20 cm and 20-25 cm [2, 3, 4, 5, 6]. Cutting variants including 5, 10 and 15 cm, and 0, 7.5 and 15 cm were also investigated [7, 8]. Significant effect of cutting height on basil regeneration and the outcome of second harvest was reported elsewhere. In the present paper we investigate the effect of cutting height ranging from 5 to 25 cm above soil surface upon yield and essential oil of the Serbian sweet basil cultivar Sitnolisni.

MATERIAL AND METHODS

Experiment I

The three replication field experiment with Serbian cultivar Sitnolisni of the species *Ocimum basilicum* L. was conducted at the Bački Petrovac location (84 m elevation). The experiment included two sowing dates: early (April) and late sowing (July). Basic plot area was 7 m^2. Seed was sown continuously in rows at 70 cm distance between rows using standard agro technical measures. In late sowing irrigation was applied after sowing and twice during vegetation period using 30 mm of water in each while early sowing was done without watering. In both sowing variants two harvests were obtained. Plant height was measured prior to harvesting. Collected plant fresh mass was dried in solar dryer at 45 ºC [9]. Dried samples were distilled after Ph. Jug. V, and quantity of essential oil was determined. Also, yield of dry herb and essential oil was calculated.

Experiment II

The experiment included five variants of herb cutting height: 5, 10, 15, 20 and 25 cm above soil surface. Three replication field experiments with Serbian sweet basil cultivar Sitnolisni was achieved in the April sowing. Basic plot area was 7 m^2. Seed was sown continuously in rows at 70 cm distance between rows using standard agro technical measures. In each cutting variant two harvests were obtained. Plant height was measured prior to harvesting. Harvested plant fresh mass was dried in solar dryer at 45 ºC [9]. Dried samples were distilled after Ph. Jug. V, and quantity of essential oil was determined. Yield of dry herb and essential oil was calculated.

RESULTS

Experiment I

In both harvests, dry herb and essential oil yield at early sowing date were significantly higher (in total yield by 47 % and 42 %, respectively) than at late sowing date (Tab. 1) while the essential oil content was almost equal in both sowings. Late sowing of sweet basil as a second crop produced economically sounded yield, especially calculated with the first crop yield obtained in the same year of growing.

Tab.1 Dry herb and essential oil yield and content of essential oil at early and late sowing date of *Ocimum basilicum*

Sowing date, harvest	Plant height, cm	Dry herb yield, t/ha	Essential oil yield, kg/ha	Essential oil content, %
Early				
I harvest	63	3.8	25.8	0.68
II harvest	45	1.5	10.7	0.77
total I+II		*5.3*	*36.5*	
Late				
I harvest	52	2.5	17.8	0.70
II harvest	40	1.1	7.9	0.72
total I+II		*3.6*	*25.7*	

Experiment II

The second experiment showed a strong effect of cutting height upon basil yield. In the first harvest the highest yield was obtained in 5 and 10 cm variants whereas the lowest in 25 cm variant (Tab. 2). On the opposite, in the second harvest the highest yield was achieved with 25 and 20 cm variants whereas the lowest with 5 and 10 cm cutting variants. In the first

harvest essential oil content ranged from 0.65 % to 0.88 % while in the second harvest from 0.60 % to 0.82 %. In both harvests cutting variant of 25 cm showed the highest content of essential oil whereas that of 5 cm the lowest. By comparing the total yield it may be concluded that cutting variant of 25 cm exhibited the highest essential oil yield – 49 kg/ha. In this variant total yield of dry herb was higher by 11%, essential oil yield by 30 % and essential oil content in dry herb by 25 % when compared with 5 cm variant.

Tab.2 Effect of cutting height upon dry herb and essential oil yield and content of essential oil in *Ocimum basilicum*

Cutting height, harvest	Plant height, cm	Dry herb yield, t/ha	Essential oil yield, kg/ha	Essential oil content, %
5 cm				
I harvest	62	4.3	28.9	0.65
II harvest	40	0.8	5.0	0.60
total I+II		*5.1*	*33.9*	
10 cm				
I harvest	62	4.2	31.1	0.74
II harvest	44	1.2	8.6	0.74
total I+II		*5.4*	*39.7*	
15 cm				
I harvest	62	3.5	27.6	0.80
II harvest	51	1.8	13.5	0.76
total I+II		*5.3*	*41.1*	
20 cm				
I harvest	62	3.2	24.0	0.73
II harvest	58	2.3	19.0	0.79
total I+II		*5.5*	*43.0*	
25 cm				
I harvest	62	3.0	26.6	0.88
II harvest	60	2.7	22.4	0.82
total I+II		*5.7*	*49.0*	

CONCLUSION

Although significant yield reduction of Serbian sweet basil cultivar Sitnolisni at later sowing date (July) was recorded, its amount was still economically sounded because this second crop yield coupled with the first crop yield increases profit per hectare in the same year of growing.

In two harvests 25 cm cutting variant of the sweet basil cultivar gave balanced yield of herb and essential oil and the highest total yield as compared with other variants of cutting height. Also, the content of essential oil was the highest in 25 cm cutting variant in both harvests.

ACKNOWLEDGEMENTS

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Original scientific paper

MYCOPOPULATION OF BASIL SEEDS

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SUMMARY

Basil (*Ocimum basilicum* L.) is a valuable multi-purpose medicinal plant which belongs to the family *Lamiaceae,* and is widespread in tropical and moderate continental climate regions. It is commonly used in the treatment of various diseases such as upper respiratory tract infections, diarrhea, headache, fever, ophthalmic and skin diseases as well pneumonia.
The pathogenic mycobiota of *O. basilicum* was studied on the commercial plantation of the Institute of Medicinal Plant Research, Belgrade at the localities Pancevo and Bavaniste during 2010 and 2011. Mycopopulation of basil were studied by seed incubation on filter paper, on potato dextrose agar (PDA) and water agar with leaf pieces of carnation (CLA) at 25°C. The active growing mycelia from the seeds were transferred on PDA. Monosporial isolates were used for further investigation. Identification of obtained isolates was based on the morphological and cultivation characteristics of isolated fungi.
Twelve different species were identified on collected seeds from both years. *Alternaria alternate* was a predominant pathogen species on the seeds, accounting for 14 and 23% in 2010 and 2011, respectively. Five *Fusarium* species were detected (*Fusarium oxysporum – 5%*, *F. proliferatum – 3%*, *F. verticillioides - 3%, F. solani – 2%* and *Fusarium spp.- 1%*). Species belonging to genera *Aspergillus*, *Penicillium*, *Cladosporium* and *Pleospora* were present in low percentage (1-2%). The percentage of infected seeds was 60% higher in 2011, compared with 2010.

***Key words**: Ocimum basilicum, basil, seed, mycopopulation*

INTRODUCTION

Medicinal plants are among economically most significant plants in Serbia. There is a long tradition of growing medicinal and aromatic herbs commercially such as mint, marxhmallow, sage, St. John's wort, coneflower, basil etc. in Serbia.
Basil (*Ocimum basilicum* L.) is a valuable multi-purpose medicinal plant which belongs to the family *Lamiaceae,* and is widespread in tropical and moderate continental climate regions. It is commonly is used in the treatment of various diseases such as upper respiratory tract infections, diarrhea, headache, fever, ophthalmic and skin diseases as well as pneumonia.
The experimental research of basil seeds' mycopoulation, conducted during 2010-2011 on the cultivating plantations of the Institute for Medicinal Plants Research 'Dr Josif Pančić' and on the cooperative fields in Bavanište. During the investigation of the basil seeds' health status, a number of different fungi species were found. The most dominant species was *Alternaria alternate,* then *Fusarium spp,* particularly *F.oxysporum.*

MATERIAL AND METHODS

During the seasons of 2010/2011, basil seeds were collected after harvesting from the commercial fields of the Institute for Medicinal Plant Research 'Dr Josif Pancic' and from the locality Bavaniste. Sampled seeds were analyzed for the presence of pathogenic fungal flora. An analysis of the health status of basil seeds was done by the incubation of seeds on the filter paper and on the potato dextrose agar (PDA) as well as water agar with carnation leaf piece (CLA).

Four hundred seeds (4 trials, each with 100 seeds) from each locality were sterilized with NaOCl for 3 minutes and then rinsed with sterile water and transferred to the filter paper on Petri dishes, 15 cm in diameter. Fifty seeds from each locality were transferred to the PDA medium following the seed surface sterilization. After the eight-day incubation at 25°C, parts of the mycelia taken from well-developed colonies was transferred to the PDA in order to be further examined [1].

Morphological examination of the isolated fungi was conducted using monosporial cultures. The following characteristics were monitored: speed of the growth on PDA at 25°C, the appearance of aerial mycelia, presence of pigmentation, the appearance of conidiophores and conidia, the manner of conidia formation, production of chlamidospores and sometimes sclerocia, and the formation of stroma. Hundred conidia were measured in every isolate.

Identification of the present fungi species was based on morpho-physiological characteristics and the cultivation of the fungi tested [2, 3, 4, 5, 6, 7].

The pathogenicity test was confirmed by the modified method of Molot and Simone (1967). A total of fifty basil seeds, surface-sterilized with sodium hypochlorite per isolate were inoculated in the sterile Petri dishes with 30 ml of spore suspension ($10^3 ml^{-1}$) *Fusarium oxysporum*. The pathogenicity of five isolates was investigated (B-3, B-5, B-14, B-19, B-22). The spore suspensions were prepared from 7 old isolates cultured on the PDA at room temperature. Inoculated and non-inoculated (control) seeds were incubated at 22°C for ten days. The level of root necrosis was calculated according to the scale from 0-3 (0 – health seedling, 1 – root tip necrosis, 2 – root and lower part of the stem necrosis, 3 –root and shoot completely rotted).

RESULTS AND DISCUSION

Collection of isolates

Analysing results of the collected basil seeds mycopopulation enabled twelve different species to be identified (Tab.1, Fig. 4). *Alternaria alternate* (Fig.1) was a predominant pathogen species on seeds, accounting for 14 and 23% in 2010 and 2011, respectively.

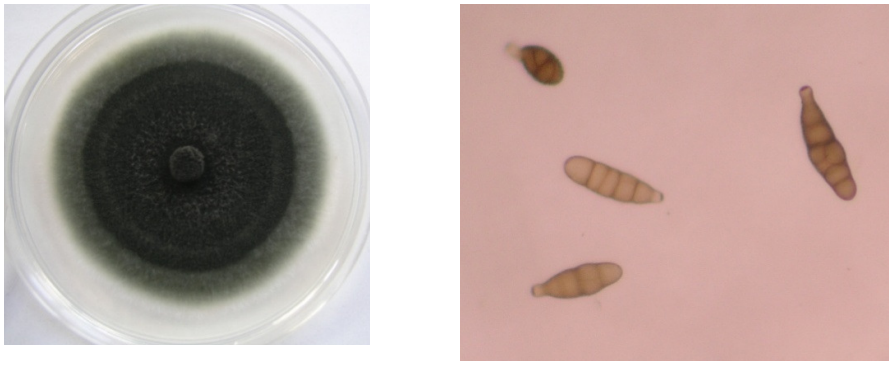

Fig. 1 *Alternaria* sp. : a) mycelia appearance on the PDA, b) conidia

Five *Fusarium* species were detected (*Fusarium oxysporum – 5%* (Fig.2), *F. proliferatum – 3%*, *F. verticillioides - 3%, F. solani – 2%* and *Fusarium spp.- 1%*). Species belonging to genera *Aspergillus* (Fig. 3), *Penicillium*, *Cladosporium* and *Pleospora* were present in low percentage (1-2%). The percentage of infected seeds was higher by 60% in 2011, compared with 2010.

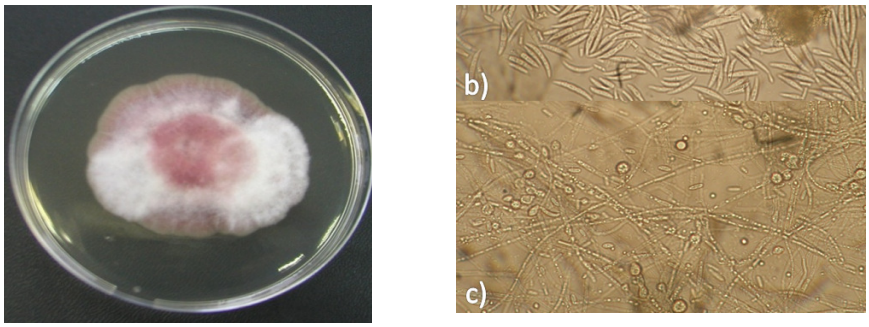

Fig. 2. *F. oxysporum*: a) mycelia appearance on the PDA b) macroconidia c) macroconidia and chlamidospora

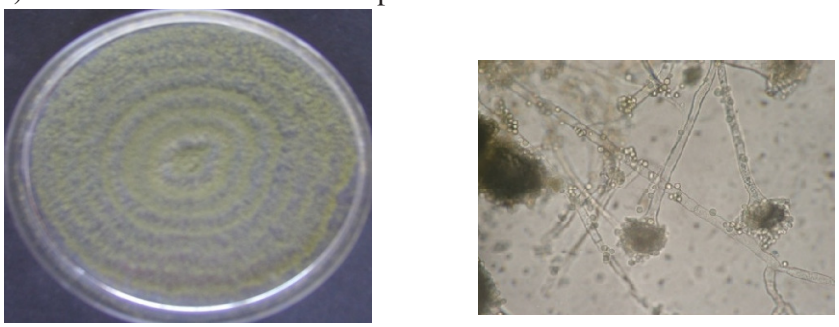

Fig. 3. *Aspergillus flavus*: a) mycelia appearance on the PDA b) conidiophorae and conidia

Table 1 Incidence of fungi (%) on the basil seeds in 2010 and 2011

Pathogen	Year	
	2010	2012
Alternaria spp.	14	23
Aspergillus flavus	2	0
Aspergillus niger	3	0
Cladosporium spp.	0	2
Fusarium oxysporum	5	3
Fusarium proliferatum	1	3
Fusarium semitectum	0	2
Fusarium solani	2	2
Fusarium spp.	1	1
Fusarium verticillioides	2	3
Penicillium spp.	2	1
Pleospora herbarum	2	2

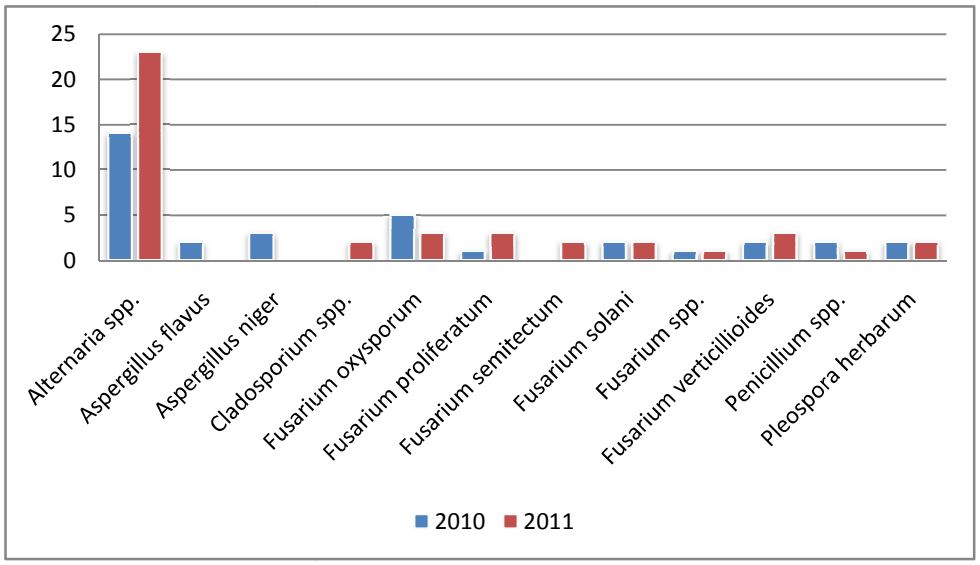

Figure 4 Incidence of fungi (%) on the basil seeds in 2010 and 2011

Alternaria alternata is the predominant fungus present on the basil seeds (14-23%). This fungus is also dominant on other medicinal plants such as camomile, salvia, marshmallow and ehinacea [8, 9]. The percentage of infection can be almost 100 % on gentian species [10]. From the five identified species from the genus *Fusarium*, the mot common one found on basil seed was *F. oxysporum* (5%). The diseased seed is small and wrinkled, with changed colour, wilting and seedling decay, commonly known as firing and melting of seedlings. *F. oxysporum* is found as a pathogen on seeds of a number of cultivated medicinal plant species such as sage [11], St.Jones' wort [12], lemon balm [13], marshmallow [14] and gentian [10] . Other identified species from genera *Aspergillus*, *Cladosporium*, *Penicillium* and *Pleospora* are commonly found on the seeds od cultivated plants. Althought they don't cause significant economical damages to the plants, they produce mycotoxins which can be harmful to health of people.

Pathogenicity of tested isolates of *F. oxysporum*

All five investigated isolates of *F. oxysporum* caused root necrosis of seedlings (Tab. 2).There were no significant differences between the isolates, but the isolates B-3, B-14 and B-22, showed highest pathogenicity in comparison with the other two (B-5, B-19). All isolates showed a degree of pathogenicity.

Table 2. A degree of pathogenicity of *Fusarium oxysporum* on the basil seedlings in laboratory conditions

Isolates	Pathogenicity
B-3	+++
B-5	++
B-14	+++
B-19	++
B-22	+++
Kontrola	-

- not pathogenic; ++ moderate pathogenicity; +++ very high pathogenicity

Figure 5 Pathogenicity test: a) healthy seedlings (negative control), b) seedlings containated with *Fusarium oxysporum* showing root necrosis

CONCLUSION

Twelve different species of fungi were identified in the mycopopulation of basil seeds in 2010/2011 seasons**.** *Alternaria alternate* was a predominant pathogen species on the seeds (14-23%). From all five identified species from the *Fusarium* genus, the most widespread species was *F. oxysporum* (5%). All investigated isolates of *F. oxysporum* caused root necrosis of seedlings (Fig. 5)

ACKNOWLEDGEMENTS

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Original scientific paper

FUSARIUM SP. CAUSING WITHERING OF NASTURTIUM IN SERBIA

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SUMMARY

Nasturtium (*Tropaeolum majus*) is important annual plant which is widely cultivated, both as an ornamental and as a medicinal plant. All parts of the plant are edible. The leaves and flowers contain lots of vitamin C and can be used as a salad. Nasturtium has for long time been used in traditional medicine as medicament. It is regarded as one of the most powerful natural antibiotics, and diuretic and laxative properties.

Little is known about the diseases in nasturtium. An intensive occurrence of chlorosis and mass wilting of plants in the flowering stage was noted in the experimental field of the Institute for Medicinal Plant Research “Dr Josif Pancic” in Pancevo, Serbia, during 2010 and 2011.

The infected plants were collected and pathogens isolation was performed by the standard procedure. More isolates were obtained from the infected plants, based on microscopic and macroscopic properties, and it was estimated that the pathogens belong to the *Fusarium* genus.

Eight isolates from leaves, stem and root of nasturtium were selected for further study. Colonies as well as conidia morphology were investigated on PDA. Two *Fusarium* species were identified based on the morphological characteristics: *F. oxysporum* and *F.solani*. Both species are well-known as typical soil pathogens that parasitize on a large number of hosts. Their appearance in the plantation of nasturtium is very important because all diseased plants were dead before completing thier life cycle, which caused significant loss of income.

Key words: *Nasturtium*, *Tropaeolum majus,* disease**,** *Fusarium withering*, ***Fusarium oxysporum, Fusarium solani***

INTRODUCTION

Nasturtium is an important annual plant which is widely cultivated, both as an ornamental and as a medicinal plant. Garden nasturtiums are growen for their flowers, and both their leaves and flowers are edible. They can be used in salads. The seeds are also, and can be used as a caper substitute [1]. All parts of the plant have antibiotic properities and increase the resistence of the organism. Nasturtium could be used as diuretic and laxative. Sulfur oil from nasturtium is healing in the case of emphyseum, bronchitis, improves mucus ejection. Extract from masturtium proved to be an exellent agent against aphids and other parasites [1].

Fusarium species have been isolated from over 100 plant species in Serbia [2]. From the economic aspect, they have been and still are the most important pathogens, affecting the production and storage of small grains and maize, and are exceptionally important for some other species [3]. Polyphagous and cosmopolitic fungi *Fusarium oxysporum* and *F.solani* are common pathogens on the cultivated medicinal plant in Serbia [4]. Both pathogens have been recently isolated from nasturtium plants. There is no any report on diseases of nasturtium in Serbia, so this report is dealing with the *Fusarium* species causing withering of this medicinal plant.

MATERIALS AND METHODS

The surveys on fungal disease of nasturtium were carried out in experimental plot of the Institute for Medicinal Plant Research in Pančevo (about 20 km north from Belgrade) from 2010 to 2011. For this purpose, plants were surveyed from March to Novemeber and samples of plants that showed symptoms of wilting, damping off, dwarhishm, chlorosis and laef shriveling were collected. All the semples with symptoms were collected in the separate plastic bags. Sampeles were taken into the lab as soon as possible in oeder to examination in due course time stored samples at refrigerator were used to isolation of fungal agents, and keep in the fridge.

For isolation of fungal agents, samples were washed thoroughly in running water for 30 min. Samples were dissected into small pieces from border of healthy and infected regions of the root and stem. Then, fragments were surface-sterilized with 2 % NaOCl for 30 sec, washed again with sterile distlled water and placed on Petri dishes containing potato dextrose agar (PDA) and carnation leaf agar (CLA) medium. The fragments of the diseased tissue were incubated at 25±1ºC and 12 h of illumination in incubator [5]. Sub cultures were obtained from hyphal tips of the developing fungal 2-4-days old colonies, hyphal tips were transferred on the 60 mm Petri dishes with PDA, to obtain a pure culture, and placed in incubator again

The morphological characteristics of isolates were studied on potato dextrose agar (PDA), synthetic nutrition agar (SNA), and carnation leaf agar (CLA), prepared according to directions supplied by the physiological characteristics (the growth of selected isolates). They were tested on PDA at 25 and 30ºC. The colony diameter was measured after 73 hrs of incubation and results represent the means of three replicates [6, 7, 8].

The pathogenic characteristics were confirmed by using the modified method [9]. Four hundred seeds of each medicinal plant were sown in plastic pots with sterile sand. The pots were watered with 100 ml of conidial suspension, which was prepared from 7-day-old culture on PDA. After 21 days the seedlings were picked up, washed in distilled water and level of root necrosis was calculated according to the scale 0-3.

The identification of the pathogens was done according to the morphological and physiological characteristics of the obtained isolates and several taxonomic keys [6, 7, 8].

RESULTS AND DISCUSSION

Infected nasturtium plants showed necrosis of seedlings and root rot, resulting in suppressed growth, leaf chlorosis and malformation (Fig. 1 and 2), and finally in withering of whole plants. A total of 19 isolates of *Fusarium* spp. were obtained: 8 from roots, 5 from stem and 6 from seedlings. Eight of them were selected for further investigations

Figure 1 and 2. Nasturtium: chlorosis of leaves (1); malformation of whole plants (2)

Colonies vary widely in appearance on PDA (isolates N° 1-4). Mycelium is abundant, woolly, whitish, turning to light to pale violet. The color of mycelium and medium pigmentation varies depending on the isolates (Fig. 3 a-d).

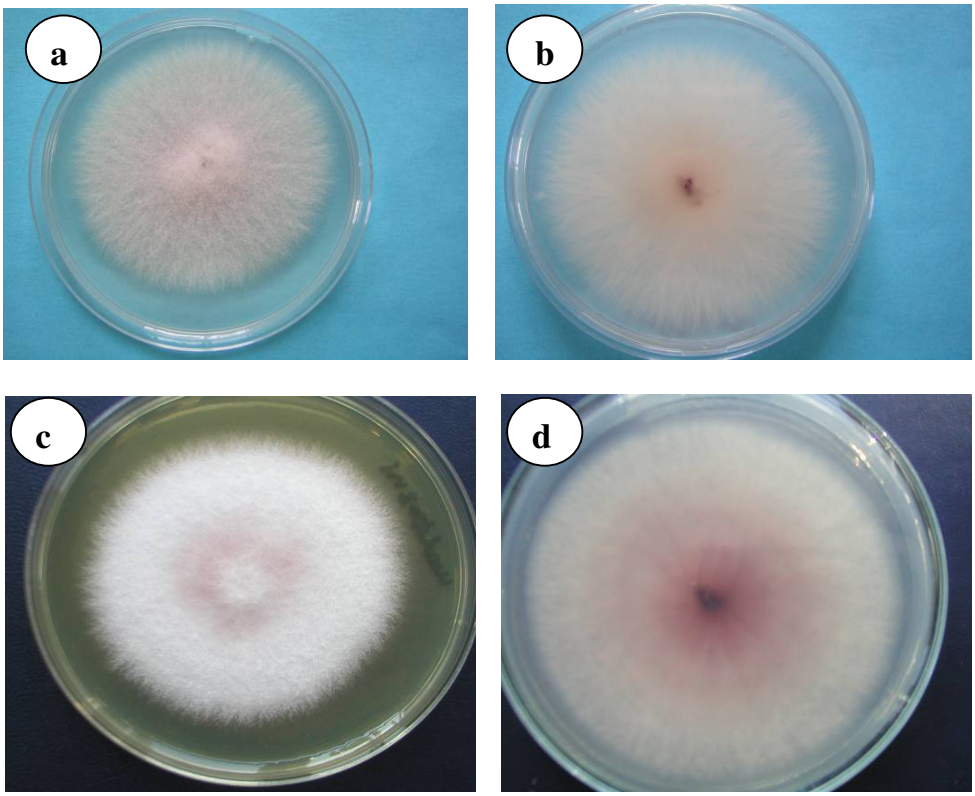

Figure 3. *F. oxysporum*: Areal mycelia (a, c) and substrate mycelia (b, d) of isolate from root (upper) and stem (down) of nasturtium

Microscopic characters on CLA showed that microconidia formed in "false heads" on short monophyalides. They are oval, elliptic, usually without septum. Macroconidia are formed on monophyalides on the branched conidiophores in sporodochia, and rarely on the monophyalides on hyphae. The abundant pale orange sporodochia are formed on CLA under combination of fluorescent and UV light. Macroconidia are slender, or straight, thin walled, mostly with 3-5 septa, the basal cell is notched or foot-shaped (Fig. 4 a-b). Chlamydospores are formed abundantly within hyphae after the 2-4 weeks growing on the CLA.

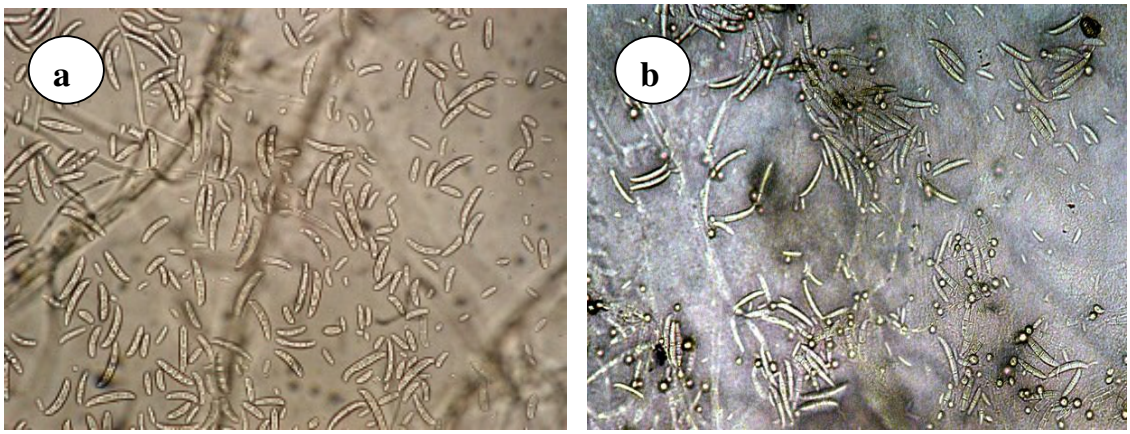

Figure 4. *F. oxysporum*: microconidia and macroconidia (a); macroconidia and chlamydospores (b).

Colonies of isolate N^{o} 5-8 produced white to cream colored, usually sparse, floccose mycelium. Sporodochia are often produced in abundance and may be cream, blue or green (Fig. 5 a-b). *F. solani* produces pigments in the agar violet or brown in color.

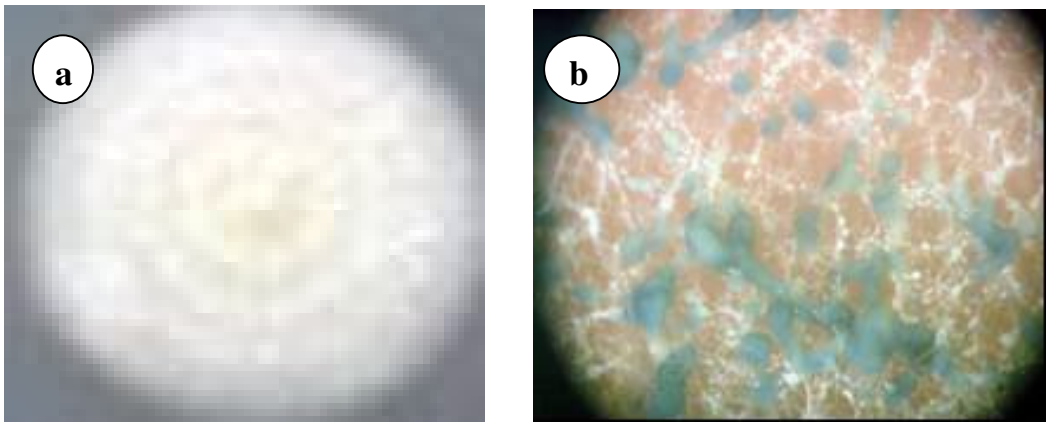

Figure 5. *F.solani*: aerial mycelia (a) and green sporodochia (b)

Microconidia has 0 or 1 septum, and are hyaline, oval, ellipsoidal, formed in round false heads on long monophialides. Macroconidia are produced abundantly in green sporodochia, they are 3-5 septate, hyaline, sometimes cell and notched base in the basal cell.

Chlamydospore formation abundant, usually within 2-4 weeks growing on the CLA (Fig.6 a-c). Mycelial growth of the tested isolates (isolate 1-8) at 25º and 30º C was almost identical. Morphological, physiological and growing characteristics of the tested isolates were in agiment with descriptions for *F. oxysporum* Schecht. Emend. Snyder & Hansen and *F. solani* (Mart.) Appel & Wollenw. Emend. Snyder & Hansen [6,7 8]. All isolates of both fungi were pathocenic to nasturtium and caused medium to severe root necrosis of seedlings.

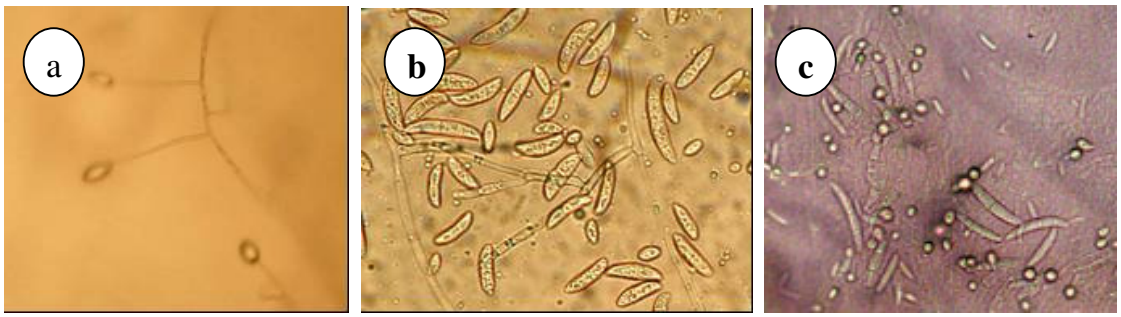

Figure 6. *F. solani*: long phialides *in situ* (a), microconidia (b), and macroconidia and chlamydospores (c)

F. oxysporum and *F. solani* as tipical soil pathogens are responsible for an enormous range of plant diseases on numerous plant species and are very important pathogens on wheat, maize, legumes, etc. [2]. Both fungi species were detected on some medicinal and aromatic plants. They were isolated from all green parts of valerian [10], from seeds and root of marshmallow [11], yellow gentian [12] and St. John's wort, from seed of lemon balm, lavender and chamomile, from seed, root and stem of basil, from stolons of mint, from stem of *Echinacea angustifolia* and from flower head of *E. purpurea* [4].
The application of pesticides is not allowed in the production of medicinal herbs according to low regulation [13] in Serbia. Therefore, it is need to look for alternative measures in controlling of pathogens, such as agrotechnical measures and biological control.

CONCLUSION

The nasturtium plants which showed necrosis of seedlings and root rot, resulting in suppressed growth, leaf chlorosis and malformation, and finally in withering of whole plants, were observed in Serbia in 2010 and 2011. These symptoms were caused by two fungal species (*Fusarium oxzsporum* and *F. solani*) which were isolated from leaves, stem and root of nasturtium. Both species formed macroconidia, microconidia and chlamidospores. The main morfological features distinguish these two species is that *F. oxysporum* microconidia in fase heads on short monophialides and *F. solani* forms it on very long monophialides.
As almoust all diseased plants died, severe incidence of these parhogens in the plantation of nasturtium could significantly reduce the yield.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Education and Science of the Repbulic of Serbia, through Projects TR-31018 and III46007.

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Original scientific paper

MYCOPOPULATION OF *LEUZEA CARTHAMOIDES* DC. CULTIVATED IN SERBIA

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SUMMARY

Maral root (*Leuzea carthamoides* DC.) is an endemic species in South Siberia, of the Altai and Sayan Mountains. It grows in the high alpine and sub-alpine meadows at 1200-1900 m.a.s.l.. Since 2010 it is grown in experimental field of the Institute for Medicinal Plants Research in Pancevo.

In the second year of cultivation the symptoms of wither, leaf spots and root rot were observed. Individual plants heavily infected didn't survived. The diseased plants were collected several times during the growing season. Isolation of pathogens from the collected samples was performed by the standard procedure. Isolation from seeds obtained in the experimental plantation was carried out by ISTA methods. Identification of pathogens was made on the basis of morphological and cultural characteristics.

The *Alternaria* and *Nigrospora* species were determined on infected leaves. Species from the genus *Fusarium* (*Fusarium oxysporum*, *F. verticillioides, F. proliferatum* and *F.equiseti*) were dominant at the root and seeds. From the seeds *Nigrospora oryzae, Phoma* spp. *Stemphylium botryosum, Epicoccum purpurescens, Chaetomium spp. Aspergillus flavus* and *Aspergillus niger* were also isolated.

***Key words**: Leuzea carthamoides,* maral root, mycopopulation

INTRODUCTION

Maral root (*Leuzea carthamoides* DC.) is an endemic, perennial medicinal plant of Siberian origin. It grows in the high alpine and sub-alpine meadows at 1200-1900 m.a.s.l. *Leuzea carthamoides* is usually found in high mountain meadows, in tundra brushwood and in the glades of coniferous forests [1]. *Leuzea carthamoides* was widely used in folklore medicine (usually roots) to treat overstrain and common weakness after illnesses. Since 2010 it is cultivated in experimental field of the Institute for Medicinal Plants Research in Pancevo.

As with many other agricultural products, maral root may be exposed to the wide range of microbial contamination during its vegetation and post harvest handling. The experimental research of the maral root mycopoulation was conducted during 2010-2011 on the cultivating plantations of the Institute for Medicinal Plants Research "Dr Josif Pančić". The aim of this research was to determine micopopulation of a new introduced plant species, in our country, as well as damages which pathogen microorganisam cause.

MATERIAL AND METHODS

During the seasons of 2010-2011 from April to October, in experimentally field in locality Pancevo, the samples of diseased plant were collected. Isolation of pathogens from infected

plant material was performed by standard procedure [2]. Fragments of diseased plants were washed with tap water, surface sterilized with 2 % NaOCl for two minutes, again rinsed with sterile distillate water and transferred on potato dextrose agar (PDA). Isolation from seeds of maral root was done by method of ISTA [3]. The total of 400 seeds were sterilized with 2 % NaOCl for two minutes, rinsed with sterile distillate water and transferred to wet filter paper in Petri dishes. Also, 50 seeds taken from each lot after surface sterilization were transferred to Petri dishes with PDA. Seeds were incubated 10 days at 25ºC. More than 100 isolates were obtained on these manner and 26 isolates were chosen for further study.
The pathogenicity of obtained isolates from the leaf of maral root: *Nigrospora* spp. and *Alternaria alternata* was performed by modified method of Molt and Simone [4]. The identification of pathogenic fungi was done based on morphophysiological and cultivation characteristics of isolated fungi [5, 6, 7, 8, 9].

RESULTS AND DISCUSSION

Symptoms

Two types of symptoms on diseased plants of the maral root were observed: leaf spot and plant withering. The leaf spot were appeared at any stage of plant development. Initially the pathogen formed small, water-soaked, roughly circular spots scattered randomly over the leaf and steam surface (Fig. 1A).
The symptom of withering has appeared during the middle of June, when climate was warm and moist. If the young plants were attacked, they died before completing its life cycle. (Fig. 1B).

Figure 1. Occurrence of *Alternaria* sp. manifested as black or brawn spots on leafs (arrowed) (A) and infection with *Fusrium* spp. causing decline of the young plants (B)

Pathogen isolation

From the collected diseased plants 12 different species were isolated and identified (Table 1). Species from the genus *Fusarium* (*Fusarium oxysporum*, *F. verticillioides, F. proliferatum* and *F .equiseti*) were predominant pathogens on the root of diseased maral root plant during the investigation period. As already published *Fusarium* species are also dominant pathogens in many other medicinal plants [10]. *F. oxysporum* was also isolated and identified from horsetail herb, corn silk and marigold flower [11].
The most important fungus isolated from maral root leaf was *Nigrospora oryzae, Alternaria alternata* (Fig. 2A ,B) and *Phoma* spp.

Table 1. Mycobiota of the maral root during the vegetation

Plant part	Fungus species	Time of isolation	Type of symptoms	No. of isolation
Stem and leaf	*Alternaria* spp.	Jun-July	spots	2
	Epicoccum purpurescens	July	spots	2
	Chetomium spp.	July	spots	1
	Alternaria alternata	September	spots	4
	Phoma spp.	August	spots	2
	Nigrospora oryzae	August	spots	2
Flower and root	*Fusarium* spp.	August	withering	2
	Fusarium oxysporum	August	withering	4
	Fusarium verticillioides	August	withering	3
	Fusarium proliferatum	October	withering	2
	Fusarium equiseti	October	withering	2

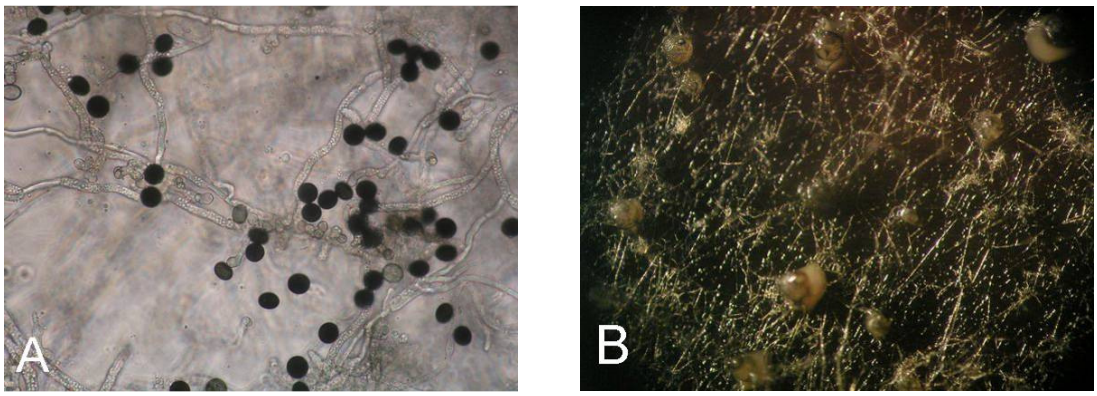

Figure 2. Maral root leaf fungus: *Nigrospora oryzae* (A) and *Phoma* spp. (B)

Analyzing results of the collected maral root seeds mycopopulation (Fig. 3) enabled 14 different species to be identified (Table 2).

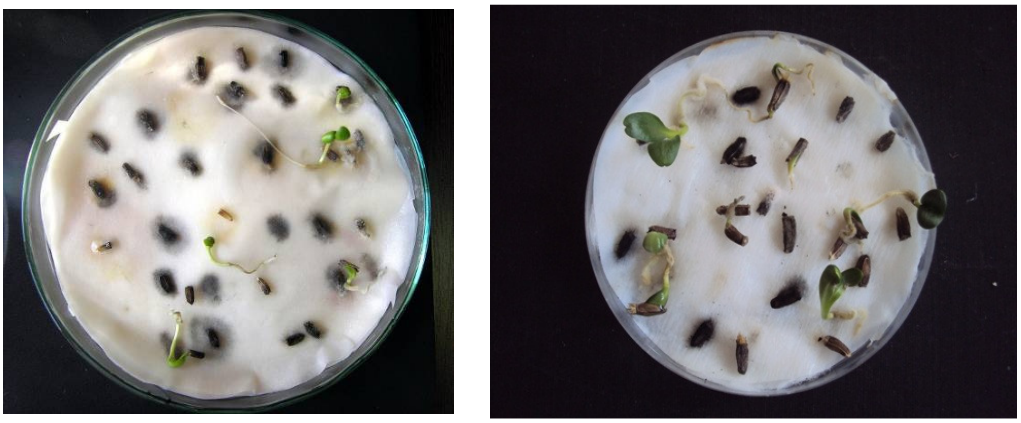

Figure 3. Maral root seeds mycopopulation tests on filter paper.

Table 2. Occurrence of fungi species on maral root seeds

Pathogen	2010 (%)	2011 (%)	Pathogen	2010 (%)	2011 (%)
Alternaria spp.	38	42	*Fusarium oxysporum*	4	4
Aspergillus ochraceus	1	-	*Fusarium verticillioides*	4	3
Aspergillus flavus	3	3	*Fusarium proliferatum*	2	3
Aspergillus niger	5	3	*Fusarium equiseti*	1	1
Botrytis cinerea	1	-	*Nigrospora oryzae*	3	3
Chetomium spp.	3	-	*Mucor* spp.	3	3
Epicoccum purpurascens	3	3	*Rhisopus* sp.	3	2

Alternaria alternata (Fig. 4A, B) was the most present fungus on the maral root seeds (38-42%). This fungus was also reported as dominant pathogen on many other medicinal plants such as chamomile, salvia, marshmallow and echinacea [12,13,14]. Four *Fusarium* species (*Fusarium oxysporum* (Fig. 5A, B) – 4%, *F. verticillioides* – 4%, *F. proliferatum* – 2–3%, *F. equiseti* (Fig. 6A, B) – 1%), and three *Aspergillus* species were detected. The species from genus *Fusarium* are very destructive causing rotting and decay of seedlings. The fungus from genus *Mucor*, *Rhisopus*, *Botrytis*, *Nigrospora, Chetomium* were present in low percentage (1-3%) and they should not be considered as significant economical pathogens, but since they produce mycotoxins an attention should be put on their occurrence as possible source of harmful compounds for humans [11].

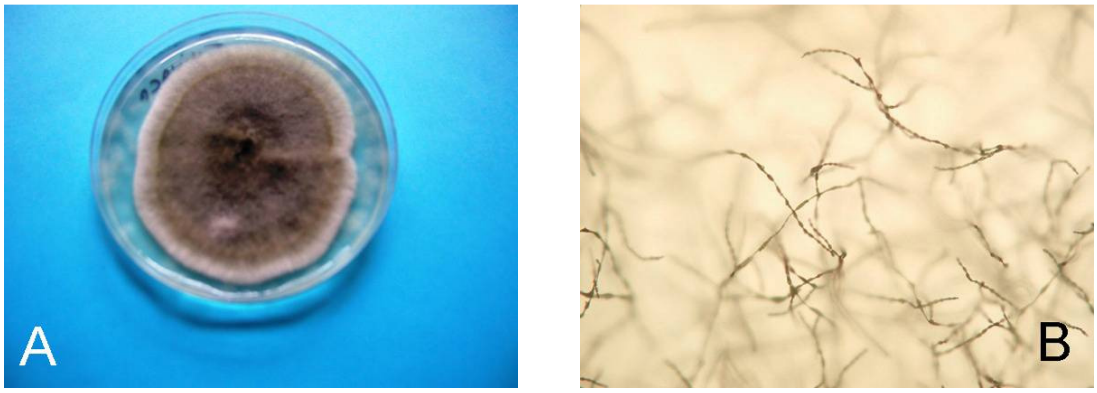

Figure 4. *Alternaria* spp. - colony growing on the PDA (A) and large chains of conidia (B)

Figure 5. *F. oxysporum*- colony growing on the PDA (A); microconidia (mi), macroconidia (ma) and intercalary chlamydospore (chl) (B)

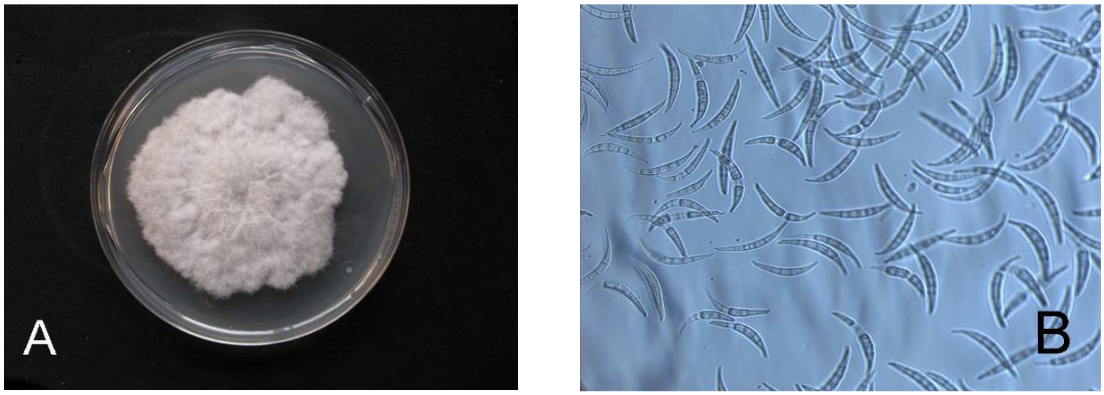

Figure 6. *Fusarium equiseti* - colony growing on the PDA (A) and macroconidia (B)

Pathogenicity tests

Six isolates of *Nigrospora* spp. and *Alternaria alternata* were tested in conducted pathogenicity tests and all of them caused leaf and necrosis of seedlings (Fig. 7A, B). There were no significant differences between the investigation isolates, as already detected in other medicinal plants [10, 12,14].

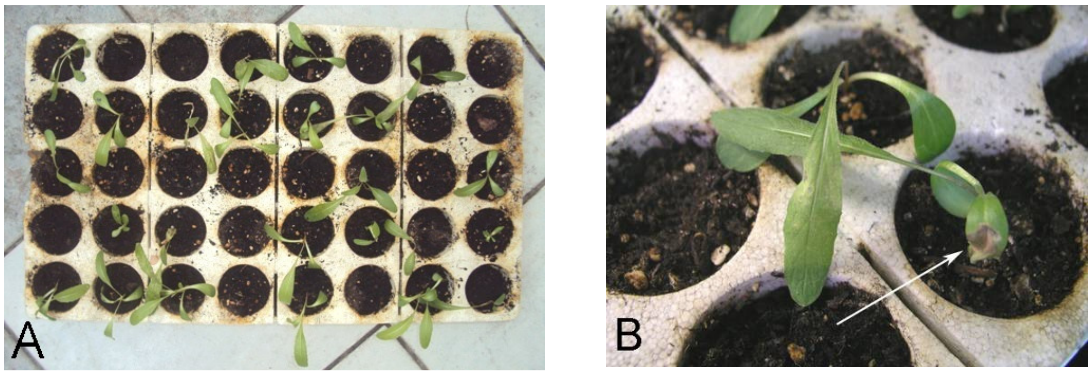

Figure 7. Pathogenicity test (A) with characteristic life spot symptoms of *Alternaria alternate* infection (arrowed) (B)

CONCLUSION

During the only two years of maral root grooving in our condition (2010/2011) a number of different fungi species were found. Fifteen different species of fungi were identified in the mycopopulation of maral root. The most dominant species was *Alternaria alternata* on the seeds, then *Fusarium* spp. and particularly *F. oxysporum* on root. These species are veru harmful owing to mycotoxin which they produce.

The most presented funguses on maral root leaf were *Nigrispora oryzae, Alternaria alternata* and *Phoma* spp., while on root there were *Fusarium oxysporum*, *F. verticillioides, F. proliferatum* and *F. equiseti* and finally the main fungus inoculating seed was *Alternaria alternata.*

All investigated isolates of *Nigrispora oryzae, Alternaria alternata* caused leaf necrosis of seedlings, which makes them descrutive.

Continuation of these researches will include detailed analysis of damages which identified pathogens cause as well as finding measures of fight.

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Original scientific paper

EFFECT OF SORBITOL ON GROWTH OF *GENTIANA LUTEA* PLANTS FOR *IN VITRO* CONSERVATION

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SUMMARY

Gentiana lutea L. is a valuable plant, included in the Red Book of Bulgaria in the category of threatened plants. Plant tissue culture is an alternative method for conservation of endangered and rare medicinal plants. The response to different concentration of sorbitol treatments (0, 1, 2, 3 and 4%) was examined using *in vitro* propagated *G. lutea* plants. *In vitro* cultures effectively were maintained on half strength Murashige-Skoog rooting medium (½MS) supplemented with indole-3-butyric acid (1 mg/l), sucrose (2%) and sorbitol (2%) at 22 °C temperature. It was found that this medium reduced microplant height, root length and number of formed roots under low light intensity. The survival rate was 50% after six months without subculture. The survival plants were subsequently recovered and multiplied on fresh MS medium supplemented with 2 mg/l zeatin and 0.2 mg/l indole acetic acid at 25 °C. The developed protocol for *in vitro* conservation of *G. lutea* under slow growth conditions allowing long-term storage of this endangered species.

***Key words**: Gentiana lutea, in vitro plants, storage, sorbitol, slow growth, plant grow regulator (PGR)*

INTRODUCTION

Gentiana lutea L. (Gentianaceae) is a valuable medicinal plant spread in the mountains of Central and Southern Europe. The species is included in the Red Book of Bulgaria in the category of threatened plants. It grows naturally on rocky slopes of Rila, Vitosha, Pirin and Rhodope Mountains [1]. Extracts of roots of *G. lutea* are widely used for treatment of digestive disorders and are applied as antivirus, antibacterial and antioxidant drug [2]. In the last years among different methods of preserving natural resources, *in vitro* propagation aroused great interest [3, 4]. Several micro propagation protocols of *G. lutea* have been reported [5, 6, 2, 7]. *In vitro* techniques are an alternative way to storage of rare and endangered species and could be used to complement conservation genetic diversity [8]. The growth rate of *in vitro* cultures can be limited by various approaches including incubation at reduced temperature and low light intensity, changes in some media components, additions of osmotic agents and growth retardants [9, 10]. The culture medium supplemented with osmoticum has proved efficient for reducing growth rate of different plant species [11, 12, 13].

The objective of present study was to develop of a protocol for *in vitro* conservation of *G. lutea* multiple plants under slow growth conditions.

MATERIAL AND METHODS

The *G. lutea* shoots were propagated from *in vitro* seedlings of Bulgarian population (the region of Vetrovala, Nature Park "Vitosha", Bulgaria). The influence of sorbitol as osmotic agent on survival rates of multiple plants was evaluated.

***In vitro* conservation of *G. lutea*:** Half strength MS medium [14] containing 2% sucrose, 1 mg/l IBA and different sorbitol concentrations (0; 1; 2; 3 and 4%) was used in order to retard the plant growth. The shoot buds were cultivated in the glass tube (150 x 20 mm), containing 8 ml of rooting medium under low light intensity. A survival of plants was monitored each month within six months without sub cultivation. The survival of prolonged storage *in vitro* cultures was determined by the presence of green plants with healthy growing tips without necrosis. The plant height and root length were recorded during storage.

Recovery of *G. lutea* plants after storage: The recovery of plants after long-term cultivation on medium with sorbitol was initiated by culturing under optimal conditions of growth and development. The plants were sub cultured on four different propagation media (full strength MS medium supplemented with 1 and 2 mg/l Zeatin alone and combination with 0.2 mg/l IAA). The mean number of shoots per explant and shoot height was recorded. To induce roots, two ½ basal MS media containing 0.5 and 1 mg/l IBA were tested. Data were recorded after three weeks of cultivation and mean number of roots per plant and plant height were established. Each experiment was performed twice with 20 replications per treatment.

Culture conditions: The culture media were adjusted to pH 5.8 before autoclaving at 120 ºC for 20 min at 1 atm. The cultures were stored at temperature 22±2 ºC under 16 h photoperiod with light intensity (20 $\mu Mm^{-2}s^{-1}$) for *in vitro* storage and temperature 25±1 °C in light intensity (40 $\mu Mm^{-2}s^{-1}$) for recovery of plants after prolonged cultivation.

Statictic analisys: The data were statistically analyzed using Sigma Stat computer package (Sigma Stat 3.1, Systat Software, San Jose, California, USA).

RESULTA AND DISCUSSION

***In vitro* conservation of *G. lutea* under slow growth conditions**

Multiple plants of *G. lutea* were subjected to slow growth medium supplemented with different concentrations of sorbitol (Table 1). The plants formed well developed root system when they were cultivated on ½ MS control rooting medium without sorbitol (Fig.1a), but they needed sub cultivation on fresh medium every month. The plants cultured in control medium survived three months without sub cultivation but with low survival rate (40%). High percentage of survival (100% and 80%, respectively) was recorded on medium containing 1% sorbitol after the first and third months of storage under low light intensity. However, survival of cultures decreased after six months of storage.

Table 1. The influence of different concentration of sorbitol on the survival of *G. lutea* plants

Concentration of sorbitol, %	Survival (%)		
	After 1 month	After 3 months	After 6 months
0	90	40	-
1	100	80	35
2	85	60	50
3	65	40	20
4	30	-	-

The survived plants were significantly less under osmotic stress conditions caused by sorbitol at 2% and 3% concentrations. For example, plants grown in a medium containing sorbitol at 2% had a survival rate (50%) after six months. The presence of sorbitol at this concentration

in culture medium had a retardant effect on the growth and development of *in vitro* cultures (Fig.1b). The concentration of 2% sorbitol caused growth reduction of vegetative part of plants (average height 1.2 cm) and root length (0.7 cm) compared with the same parameters of the control medium (Fig. 2). The further increased of sorbitol concentration lead to the yellowing of leaves and necrosis of plant tips. The content of 4% sorbitol in the medium blocked the growth and development of plants. The survival rate was low (30%) and plants died first month after cultivation. The optimal combination of 2% sucrose and 2% sorbitol in ½ MS rooting medium provided slow plant growth and increasing the storage period of six months under low light intensity. *G. lutea* plants can remain in this medium for six months without sub cultivation. Sharma [15] reported that the medicinal plant Gelltiana *kurroo* Royle could be storage at low temperature 4 °C over 30 months. After this period the cultures grew normally on multiplication medium. The effect of sorbitol for *in vitro* storage of various species as Globe Artichoke [11]; Strawberry [12]; Potato [13]; Garlic [16] was published.

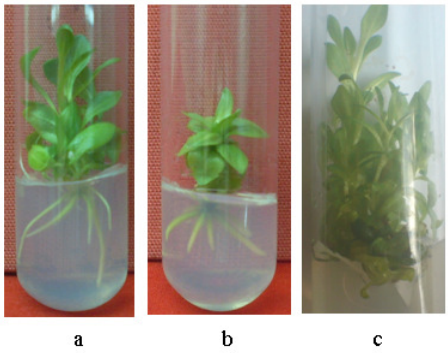

Fig. 1 *In vitro* storage of *G. lurea*: a) Rooted plant on ½ MS rooting medium (control) after first month; b) Rooted plant on ½ MS rooting medium with 2% sorbitol after six months of storage and c) Micropropagated plants after six months of storage on MS medium with 2 mg/l zeatin and 0.2 mg/l IAA.

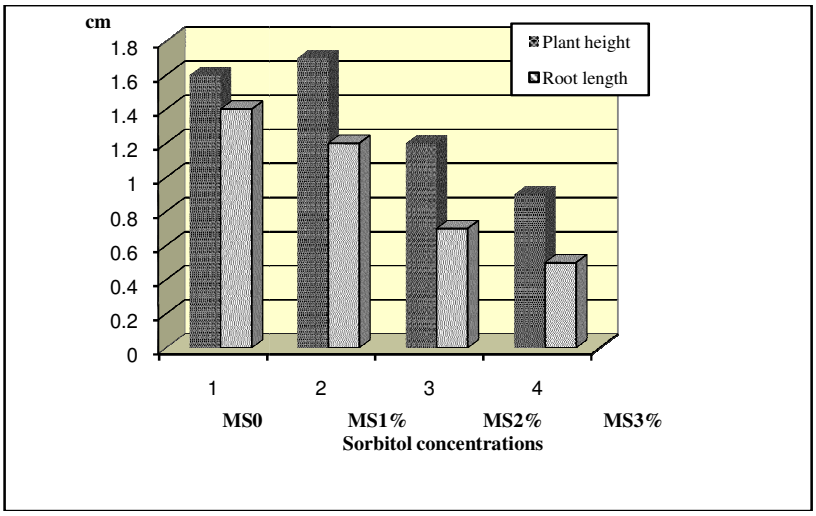

Fig. 2 The plant height and root length of *G. lutea* under slow growth conditions

Recovery of *G. lutea* plants after storage

The plants resumed normal growth when they were transferred in culture medium without osmotic agent at a temperature of 25±1 °C and a light intensity 40 $\mu Mm^{-2}s^{-1}$. It was necessary one or two passages of plants sub cultivation before started the process micro propagation. The plants were cultivated on MS media containing zeatin alone or in combination with 0.2 mg /l IAA (Table 2). Within four weeks of culture, plants cultured on MS medium containing 1 mg/l zeatin formed 1.4 buds per explant with mean height 1.5 cm (Fig. 1c) while these on MS supplemented with 2 mg/l zeatin developed more buds but smaller in height (Table 2). The formation of adventitious buds were more obviously in cultures grown on MS medium supplemented with zeatin and IAA. The addition of low level IAA to the medium containing zeatin had stimulatory effect on the frequency of shoot propagation and the number of shoots per explant. The plants produced mean 2.8 new shoots and their height increased by the addition of 2 mg/l zeatin and 0.2 mg/l IAA to MS medium after four weeks of cultivation (Fig. 1c). Thus, the combination Zea/ IAA increased the vegetative buds efficiency in comparison with media containing cytokinin alone. Root induction was conducted in ½ MS basal medium supplemented with tow concentrations of IBA (Table 2). Roots appeared during the first 10-12 days of culture. It seems that IBA have a great potential for rhizogenesis of *G. lutea* plants reported by us previously [17]. IBA at concentration 1 mg/l was effective for root induction producing healthy roots suitable for the subsequent stage of acclimatization in *ex vitro* conditions.

Table 2. Micro propagation and rooting of *G. lutea* plants after storage (six months under slow growth conditions induced by 2% sorbitol)

Plant growth regulator	Number of shoots/ explant	Plant height, cm	Number of roots/ plant	Root length, cm
1 mg/l Zeatin	1.4 ± 0.22	1.5 ± 0.23		
2 mg/l Zeatin	1.9 ± 0.25	1.2 ± 0.14		
1 mg/l Zeatin + 0.2 mg/l IAA	2.5 ± 0.34	1.9 ± 0.17		
2 mg/l Zeatin + 0.2 mg/l IAA	2.8 ± 0.52	1.5 ± 0.21		
0.5 mg/l IBA			2.7 ± 0.34	1.3 ± 0.15
1 mg/l IBA			3.6 ± 0.46	0.8 ± 0.12

CONCLUSION

In this study, we presented an experimental protocol for *in vitro* conservation of *G. lutea* as an alternative approach for protection of this endangered medicinal species. The presence of sorbitol (2%) and sucrose (2%) in half strength MS rooting medium retarded the growth and development of *in vitro* plants. They could be effectively maintained for six months under slow growth conditions without sub cultivation in the light (20 $\mu M\ m^{-2}s^{-1}$) at temperature of 22 °C. The plants were successfully recovered in 40 $\mu M\ m^{-2}s^{-1}$ light intensity at temperature 25 °C after six months of storage. However, the cultural conditions (nutrient media, temperature and light regimes) for *in vitro* conservation of *G. lutea* plants need to be improved.

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Original scientific paper

ORGANOGENESIS THROUGH CALLUS CULTURE OF *ECHINACEA PURPUREA*

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SUMMARY

Echinacea purpurea L. Moench is an important medicinal plant, widely used as immune-stimulant. The regeneration capacity through indirect shoot organogenesis from petiole and leaf derived callus were tested. Different combinations of plant growth regulators: 6-benzylaminopurine (BAP) with α-naphthalene acetic acid (NAA) or *indole*-3-*acetic acid* (IAA) were used in Murashige & Skoog (MS) medium to callus induction. The highest amount of callus (90%) was produced on MS medium containing 0.5 mg/l BAP and 1 mg/l NAA from leaf explants after three weeks. The highest percentage of regeneration (75%) was recorded in MS media containing BAP (1.0 mg/l) + Kinetin (1.0 mg/l) + Gibberellic acid (0.2 mg/l GA_3) + NAA (0.1 mg/l) from leaf-derived callus after four weeks. The callus from leaf segments gave the better results than petiole-derived callus for obtaining of organogenic callus. The regenerants were micro-propagated on MS medium with BAP (1.0 mg/l) and rooted in ½ MS medium containing indole-3-butyric acid (0.1 mg/l IBA). The developed efficient protocol in the study could be used for obtaining of new plant forms for breeding programs of *E. purpurea*.

***Key words**: plant growth regulators, petiole segment, leaf explant, callus culture, shoot organogenesis*

INTRODUCTION

E. purpurea L. Moench, (Asteraceae) is an important medicinal and ornamental plant widely used in the products to boost the immune system and prevent coughs and colds. Due to increased demand of this medicinal plant, different methods and strategies were developed, which include rapid multiplication of plants and introduction of new cultivars with desired traits. In this regard, *in vitro* tissue culture techniques are proved to be very valuable. *E. purpurea* plants were regenerated from a range of tissues from *in vitro* seedlings to mature, field-grown plants [1]. The juvenile tissues have high organogenic competence [2, 3]. In general, the explant type and plant growth regulator in the culture medium play a key role in regulating the differentiation process [3]. Koroch et al. [4] induced callus and indirect shoot organogenesis from leaf explants of *E. purpurea* with different auxin/cytokinin combinations. Bhatti et al. [5] found that combinations of BAP with NAA were effective in inducing shoot organogenesis from hypocotyl explants for *E. angustifolia, E. purpurea* and *E. pallida*. However, Coker and Camper [6] reported that the combination of NAA and Kinetin to be more effective than 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin. Shoot organogenesis was observed in different explant cultures of *E. purpurea*. Choice of explant varies with species and plays an important role in determining the efficiency of morphogenesis [7, 8].

The objective of present study was to develop of an optimized regeneration system in *E. purpurea* from petiole and leaf derived callus.

MATERIAL AND METHODS

Plant materials: Seeds of *E. purpurea* were purchased from Suttons Consumer Products Ltd., Woodview Road, Paignton, Devon TQ4 7NG, England and were stratified at 4°C for two weeks. The mature seeds were washed under running water, dipped in 70% (w/v) ethanol for 2 min, immersed in 15 % (w/v) commercial bleach for 15 min and rinsed in sterile, distilled water to remove bleach. Disinfected seeds were germinated aseptically on MS basal medium [9] supplemented with sucrose (30 g/l), BAP (0.1 mg/l), NAA (0.1 mg/l) and GA_3 (0.2 mg/l). The seedlings were sub cultured on same medium for *in vitro* obtaining stabile plants.

Callus induction: To induce callus, petiole and leaf explants were taken from 1 month-old plants grown at *in vitro* conditions. Petiole segments (0.5 mm) and leaf sections (0.6 x 0.6 mm) were placed on callus induction media MS supplemented with BAP and NAA or IAA (Table 1). Each treatment consisted of 50 segments. The callus induction frequency was estimated after 21 days of culture. The callus was transferred into the fresh medium after every three weeks. Brown, watery and dead calli were removed during each subculture. Friable, green callus was used for organogenesis.

Plant regeneration: The friable calli were transfered on MS medium supplemented with different concentration and combination of growth regulators (Table 2). The percentage of shoot regeneration and the mean number of shoots per callus were counted for each treatment after four weeks of cultivation. The regenerated shoots from the callus were excised and transferred on MS medium for further growth.

Micro propagation and rooting of regenerated plants: The plants from each individual callus were multiplied by shoot tip culture to obtain more plant material. The nutrient media used for the experiment were MS formulation containing 30 g/l sucrose, 7 g/l agar and supplemented with cytokinins BAP and TDZ (thidiazuron) alone at two concentrations (0.5 and 1.0 mg/l) or in combinations with low level of NAA (0.1 mg/l). The frequency of shoot formation, number of developed shoots and their length was determined after four weeks of culture. The microplants were placed on half strength MS basal medium supplemented with auxin IBA (0.1 and 0.5 mg/l) for *in vitro* rooting. An auxin-free medium was included as a control. Data were recorded on percentage rooting, mean number of roots per shoot and root length after three weeks of culture. Each treatment consisted of 20 replicate plants and each experiment was repeated two times. The media were adjusted to pH 5.8 and autoclaved at 121 °C for 20 min. All cultures were incubated at 22±2 °C with a 16-h photoperiod under 40 µmol $m^{-2}s^{-1}$ of cool white fluorescent light. The data were statistically analyzed using Sigma Stat computer package (Sigma Stat 3.1, Systat Software San Jose, California USA).

The rooted plants were transferred to small pots (8 cm diameter) containing peat and perlite at the ratio of 2:1 under *ex vitro* condition. After keeping three weeks in the growth culture room, the plants were moved to the room temperature for another two weeks. The acclimatized plants were transferred in a greenhouse. Finally, they were planted in field conditions.

RESULTS AND DISCUSSION

The surface sterilization provides about 90-95% contamination-free seed culture. The seed germination was stimulated from cold stratification for two weeks and cultivation on MS medium with BAP (0.1 mg/l), NAA (0.1 mg/l) and GA_3 (0.2 mg/l). The sub-cultivations of seedlings on the same medium had a positive effect on their stabilization in plants.

Callus development

In our study, the combination of cytokinin BAP and auxin NAA found to produce appreciable amount of callus. The highest percentage of callus induction (80% and 90%,

respectively) was observed on MS medium with BAP (0.5 mg/l) and NAA (1 mg/l) in two types of segments (Table 1). The explants formed callus at the cut surface of segments. These calli were green in colour and friable in nature. In BAP/ IAA combinations, two types of calli were occurred. These calli were greenish in colour and both compact and friable in nature. High frequency of callus induction (62% and 70%) was observed in BAP (0.5 mg/l) and IAA (1 mg/l) containing medium. The lowest percentage of callus formation was recorded on MS medium containing BAP (1 mg/l) and IAA (1 mg/l) by using leaf explants. The percentage of callus induction was also low when petiole segments were treated with the combination BAP/ IAA at the same concentration.

Table 1. Effect of cytokinin (BAP) in combination with auxins (NAA or IAA) employed in MS media on callus induction of *E. purpurea*

Medium	Plant growth regulators, mg/l			Explant type			
				Petiole		Leaf	
	BAP	NAA	IAA	Callus formation, %	Nature of callus	Callus formation, %	Nature of callus
MSC0	0	0	0	0	-	0	-
MSC1	0.5		1	62	HG	70	HG
MSC2	0.5	1		80	FG	90	FG
MSC3	1		1	42	FG	30	HG
MSC4	1	1		50	HG	40	HG

Callus type: Friable Green (FG) and Hard Green (HG)

Plant regeneration

The effect of different growth regulators on subsequent indirect organogenesis was tested (Table 2). When petiole and leaf derived callus was cultured in MSR3 medium, the highest shoot differentiation was observed (70% and 85%, respectively). The highest mean number of shoots per callus recorded in the same medium (Figures 1a and 1b).

Table 2. Effect of plant growth regulators on shoot regeneration from petiole- and leaf-derived callus of *E. purpurea*

Medium	Plant growth regulators, mg/l					Shoot regeneration, %	Number of shoots/callus $x \pm SE$
	BAP	Kn	GA_3	NAA	IAA		
	a/ from petiole-derived callus						
MSR0	0	0	0	0	0	0	0
MSR1	0.5	1	0.2	0.1	0	50	3.2 ± 0.91
MSR2	0.5	1	0.2	0	0.1	45	1.8 ± 0.46
MSR3	1	1	0.2	0.1	0	70	4.6 ± 1.52
MSR4	1	1	0.2	0	0.1	60	3.5 ± 1.17
	b/ from leaf-derived callus						
MSR0	0	0	0	0	0	0	0
MSR1	0.5	1	0.2	0.1	0	65	2.2 ± 0.75
MSR2	0.5	1	0.2	0	0.1	50	1.4 ± 0.32

Also, the callus from two tested explants regenerated into shoots on MSR4 medium. In this case, mean number of shoot per callus was lower than that on MSR3 medium. In combinations of BAP (0.5 mg/l) with NAA or IAA (1 mg/l), shoot differentiation rate was low. Among all combinations, the highest percentage of shoot regeneration was obtained on MSR3 medium from leaf derived callus which showed better shoot differentiation with a good degree of growth. Role of cytoknin in combination with auxin for *E. purpurea* callus formation and shoot regeneration from different explants of *E. purpurea* is well documented [7, 10, 4, 5].

Micro propagation of plants

The shoots were separated from callus cultures and transferred to multiplication medium to obtain a large number of plants. The results of this experiment indicated that BAP (1.0 mg/l) was more suitable than TDZ (1.0 mg/l) for shoot multiplication of regenerated plants (Table 3). Great number of shoots was obtained on MS medium with BAP (1.0 mg/l) with high multiplication frequency of plants regenerated from petiole derives callus. TDZ alone was less effective in shoot production compared to BAP. The most effective combination for shoots propagation was 1.0 mg/l BAP with 0.1 mg/l NAA (Fig. 1c). In this combination, the highest frequency of shoot formation (95%), the number of shoots per explant (9.2) and an average shoot length (0.9 cm) were observed (Table 3). MS medium supplemented with TDZ (1.0 mg/l) and NAA (0.1 mg/l) produces small number of shoots. MS medium has been the most appropriate choice in many studies on Echinacea micro propagation where the presence of BAP or in combination with an auxin was essential for shoot multiplication [11, 12, 13, 3].

Table 3. Micro propagation of *E. purpurea* plants regenerated from callus

Plant growth regulators, mg/l			Frequency of shoot formation, %	Number of shoots/explant $x \pm SE$	Shoot length, cm $x \pm SE$
BAP	TDZ	NAA	a/ from petiole-derived callus		
1.0			70	3.6 ± 0.18	1.7 ± 0.16
	1.0		65	1.8 ± 0.15	1.5 ± 0.13
1.0		0.1	90	6.1 ± 0.86	1.2 ± 0.12
	1.0	0.1	70	2.4 ± 0.16	0.8 ± 0.10
			b/ from leaf-derived callus		
1.0			70	4.7 ± 0.26	1.4 ± 0.22
	1.0		60	1.5 ± 0.21	1.2 ± 0.19
1.0		0.1	95	9.2 ± 1.18	0.9 ± 0.14
	1.0	0.1	65	2.1 ± 0.34	0.5 ± 0.11

***In vitro* rooting and *ex vitro* acclimatization of regenerated plants**

Root formation was induced in *in vitro* propagated shoots by culturing them on half strength MS medium with 0.1 and 0.5 mg/l IBA (Table 4). In the regenerated plants from petiole derived callus, the percentage of root induction (80%) and mean number of roots per plant (4.5) was recorded on ½ MS media containing 0.1 mg/l IBA. The highest frequency of rooting (95%) and mean number of roots per plant (5.2) was found in ½ MS medium containing IBA at the same concentration in the regenerated plants from leaf derived callus (Table 4; Fig. 1d). The parameters were lower value in media containing 0.5 mg/l IBA. The presence of the auxins IBA in ½ MS medium was found to be more effective than the control medium for rooting of Echinacea plants Different concentrations of auxins for plant rooting of *E. purpurea* were effectively applied [11, 14, 15]. The *in vitro* plants were acclimated

better under *ex vitro* condition when they transferred on plastic pots with potting mix containing peat and perlite in the volume ratio 2:1. The rooted plants survived about 90% during the acclimatization stage. The regenerated plants developed very well and flowered within 3-4 months after transplantation in the field (Fig. 1e).

Table 4. Rooting response of *E. purpurea* plants regenerated from callus

Medium	Root formation, %	Number of roots/plant $x \pm SE$	Root length, cm $x \pm SE$
a/ from petiole-derived callus			
MS (control)	40	1.2 ± 0.21	0.6 ± 0.15
MS + 0.1 mg/l IBA	80	4.5 ± 0.73	1.5 ± 0.31
MS + 0.5 mg/l IBA	60	2.4 ± 0.54	2.0 ± 0.52
b/ from leaf-derived callus			
MS (control)	45	1.5 ± 0.23	0.8 ± 0.16
MS + 0.1 mg/l IBA	95	5.2 ± 0.92	1.4 ± 0.22
MS + 0.5 mg/l IBA	70	1.6 ± 0.24	1.9 ± 0.25

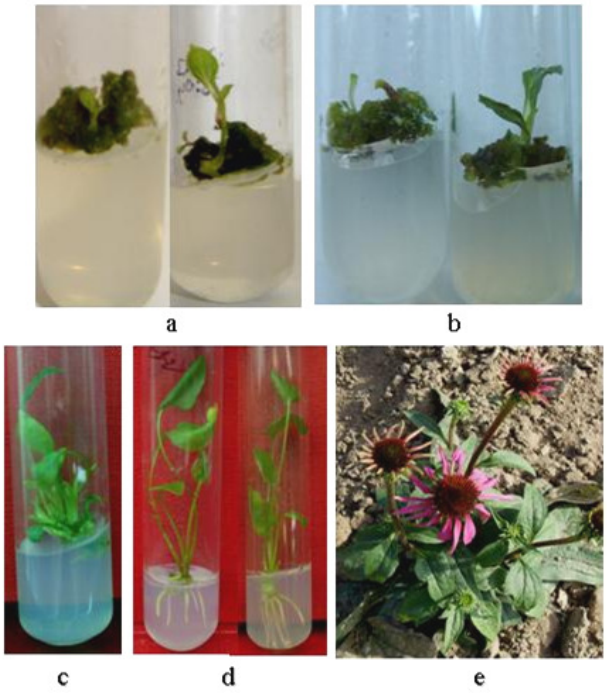

Figure 1 a) Organogenesis from petiole derived callus on MSR3 medium; b) Organogenesis from leaf derived callus on MSR3 medium; c) Micro propagated plants on MS medium with 1 mg/l BAP + 0.1 mg/l NAA; d) Rooted plants on ½ MS with 0.1 mg/l IBA and e) Adapted plants under field conditions.

CONCLUSION

An efficient plant regeneration system of *E. purpurea* that could be used for production of *Echinacea* plants was developed. In the present study, BAP (0.5 mg/l) and NAA (1 mg/l) was most effective in inducing callus culture. The data showed that MS medium containing BAP (1.0 mg/l), Kinetin (1.0 mg/l), GA_3 (0.2 mg/l) + NAA (0.1 mg/l) allow high percentage of regeneration from leaf-derived callus after four weeks of culture. These results demonstrate that *E. purpurea* have a good morphogenetic potential for shoot formation, however the response is highly sensitive and directly related to the combinations of exogenous growth regulators in the culture medium. The procedures described here could be useful for propagation of elite genotypes of *E. purpurea*.

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Original scientific paper

ENHANCED PRODUCTION OF FLAVONOIDS IN *ASTRAGALUS MISSOURIENSIS,* USING BIOREACTOR BY MODEL BASED CONTROL OF THE BIOPROCESS

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SUMMARY

Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control. Quercetin shows anti-proliferative effects against various cancer cell lines. These aspects made flavonoids an interesting object for industrial production. All in vitro cultures of *Astragalus missouriensis* produced flavonoids. A rapidly growing cell line of *A. missouriensis* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation for 24 days of culturing. This is the first report on the production of flavonoids from cell cultures of *A. missouriensis* in stirred tank bioreactor.

Our goal here was to improve the bioreactor-based production of flavonoids by suspension cultures of *A. missouriensis*, an endemic rare plant species, by model based control of the bioprocess**.** The manipulation of environmental factors such as pH, temperature, shear stress and O_2 supply are the keys to production of plant cell culture processes. This research presents the implementation of optimal control strategy to control a reactor in production of flavonoids in cell suspension of sensitive *A. missouriensis* with a high concentration, using a mathematical model. We have investigated the use of model control algorithms on batch fermentation processes with *Astragalus* cells, using a non-linear model in the controller. They operated the process with combination of operating regimes and simple local state-space model into a global model structure using an interpolation method. This approach requires all the process states to be available on line. Since direct measurement of these states is not possible, a state estimator - a Kalman filter was implemented. After this optimization, maximum biomass of 24.5 $g.l^{-1}$ dry wt was harvested from the bioreactor culture vessel (recording about 9 times increase over initial inoculum), with 1.63 % ± 0.12 Flavonoids, greater than yields from 300 ml flasks were obtained. In this way the batch reproducibility can be improved depending to the process control strategy. The cultivation of *A. missouriensis* in bioreactor, after using this control method resulted in an effective increase of biomass and flavonoid production compared with flasks cultivation.

Key words: *Astragalus missouriensis, in vitro* cell cultures, flavonoids, Quercetin, stirred-tank bioreactor

INTRODUCTION

Epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control. The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasm [1].

Due to the economic and medicinal importance of the *Astragalus* species, they have been investigated for their chemical compounds. The biologically active constituents of *Astragalus* plants represent mainly three classes of chemical compounds: flavonoids, saponins and polysaccharides and have been extensively studied [2-4]. A number of flavonoids and their glycosides have been isolated from *Astragalus* species [5].

Variable quantities and qualities of the plant material, plants that need to grow several years before they are ready for harvesting and over collecting of endangered species (*A.membranaceus*, *A.mongholicus, A. missouriensis, A. angustifolius, A. thracicus, A. aitosensis* etc.) are just a few of the problems connected with the production of these natural products. Therefore, cultured cells rather than plants are as a possible alternative production method. Northeastern North Dakota is home to the Missouri milk-vetch *A. missouriensis*. The plant obviously gets its name from the river. *A. missouriensis* is a local endemic plant whose global distribution is limited to the upper basin of the San Juan River in south-western Colorado and north-western New Mexico. In general Isoquercitrine and Quercitrine were the main flavonoid glycosides in the plant. Rutin, Hyperoside, Scopoletin and phenolcarbonic acids - p-coumaric and chlorogenic have been also detected [6].

Quercetin shows anti-proliferative effects against various cancer cell lines. These aspects made flavonoids an interesting object for industrial production. Very few plant cell cultures systems have been reported for *Astragalus* ssp. to date, there are only few reports available on flavonoids production in vitro. All in vitro cultures of *A.missouriensis* produced flavonoids. The main aglycon identified of the in vitro cell suspension was Quercetin in both free and bound forms (as glycosides). In general Isoquercitrine and Quercitrine were the main flavonoid glycosides in all tested cell lines. Rutin, Hyperoside, Scopoletin and phenolcarbonic acids - p-coumaric and chlorogenic have been also detected. After optimization of production medium maximum total amount of flavonoids 1.34% was achieved in the flasks [6].

In the current study, for the first times a rapidly growing cell line of *A. missouriensis* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation. Our goal here was to improve the bioreactor-based production of flavonoids in suspension cultures of *A. missouriensis*, by model based control of the bioprocess**.**

MATERIALS AND METHODS

Cell suspension culture in bioreactor

The induction of callus and suspension of *A. missouriensis* cultures were performed as described in [6]. A rapidly growing cell lines were selected for cultivation in 2-L stirred-tank bioreactor to increase the efficiency of the production of Quercetin derivatives. Ten day-old suspension (5.0 g fresh mass) from *A. missouriensis* (Chicago Botanic Garden, USA) were transferred to 2 l jacketed glass vessel, containing optimized MS liquid medium [6] enriched with 1.0 mg/l concentrations of 2,4-D and 1.0 mg/l 6-benzylaminopurine (BAP), sucrose concentration 5.5%, applying cultivation for 24 days. The flavonoid production was controlled by HPLC.

Quantitative analysis

The HPLC with a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a photodiode array detector was used. Separation was performed using a reversed phase Hypersyl C 18 column with guard column (Grom Company, Herrenberg, Germany) and a gradient program with 0.5% orthophosphoric acid p.a. in water (A) and acetonitrile/methanol 400/200 (B) as eluents as follows: 0 to 22 min from 40 to 27% B, from 22 to 24 min to 85% B, and until 30 min back to 27% B. The flow rate increased from 1.0 mL/min at temperature 25 ^{0}C, detection 335 nm, injection volume 20µl. The retention time for Rutin (quercetin -3-O-

rutinoside) is about 9,41 min, for Hyperoside (quercetin-3-O-galactoside) about 10,04 min, for Isoquercitrine (quercetin-3-O-glucoside) about 10,14 min, Quercitrine (quercetin-3-O-rhamnoside) about 11,64 min, for coumarine - Scopoletine about 10.38 min, Quercetin about 15,65 min.

Bioreactor equipment

Fermentations were carried out in 2 L jacketed glass vessel applying cultivation for 21 days. Equipment of the vessel includes: sensors for temperature, pH, dissolved oxygen concentration, foam, and speed of stirrer drive system; four integrated peristaltic pumps and one external pump for flow control and feeding. Bioreactor conditions: temperature - 26 ^{o}C, batch mode of cultivation, dissolved oxygen saturation (DO at 50%), marine-type impeller design with low-shear stress, speed 140 rpm. This impeller provides mixing and creates a higher oxygen mass transfer rate (Kla).

Control system

Control system has the following functions: display of all process values via schematic P&ID algorithms, digital calibration of sensors and pump dosing counters indication of sensor parameters, recalibration function of pH-probe, control loops for temperature, stirrer speed, pH, foam level, substrate, pO2 with two stage cascade control, set point profile for substrate pumps.

Data acquisition

Data acquisition system includes the following functions: data collection, data base maintenance, visualization of the process variables by several plotting functions. This software allows starting or finished process batches, exporting database in appropriate data formants and sample data configuration.

RESULTS AND DISCUSSION

The chemical investigation of cell extracts from *A. missouriensis* led to the isolation of different flavonoids by means of HPLC and TLC [6]. The common structural aspect of produced by cell cultures of *A. missouriensis* flavonoid glycosides is that Quercetin is common aglycon. With respect to the potential use of Quercetin as cancer-preventive or chemotherapeutic agents, it is worth mentioning that these aspects made Quercetin and its glycosides an interesting object for industrial production.

Bioreactor strategy for flavonoid production by A. missouriensis cells

The aim of this study was to establish a bioreactor culture of *A. missouriensis* (Fabaceae) as a renewable source of flavonoids. In order to reach effective production we have to combine two factors - both flavonoid content and biomass growth. In spite of the fact that stirred tank reactors exert more hydrodynamic stress on plant cells, they have great potential when used with low agitation speed and modified impeller. A low-shear marine-type impeller has been found to be effective for batch cultivation of shear-sensitive *A. missouriensis* cells in stirred tank bioreactor for production of flavonoids.

The manipulation of environmental factors such as pH, temperature, shear stress and O_2 supply are the keys to production of plant cell culture processes. This research presents the implementation of optimal control strategy to control a reactor in production of flavonoids in cell suspension of sensitive *A. missouriensis* with a high concentration, using a mathematical model. Using the optimum conditions of the shake flask system, the growth and product formation kinetics was investigated in a 2 L bioreactor. The success of this technology is dependent not only on the discovery of appropriate production plant system, but also from optimization of cultivation with control of various culture parameters.

Automatic control of bioreactor

The automatic control of the specific growth rate in cell cultivation processes has become a major issue in process control in the recent years. One of the important applications of advanced specific growth rate control schemes is the production of secondary metabolites for pharmaceuticals.

The model control approach is a theory that incorporates the process model directly in the control law; it has the distinctive characteristic that it solves an optimization problem in only one step of calculation [7]. This section shows the basics of the theory.

Suppose a process is described by equations (1) and (2):

$$\dot{x} = f(x,u,d,t) \tag{1}$$

$$y = g(x) \tag{2}$$

Where **x** is the state vector with dimension **n**, **u** is the manipulated variable and has dimension **m**, **d** is the vector of disturbances with dimension **l**, **y** is the vector of measurements with dimension **p**. In the general case, both **f** and **g** are nonlinear functions.

The classical approach in feedback control is to compare a set point desired for the output with its actual value, in order to form an error signal to be given as input to the controller. In Model Control, this error signal is also formed, but the control objective is expressed in terms of the value of the derivative of the output. The control scheme calculates the manipulated variable vector so that the derivative of the output follows an established pattern. To get the expression of this pattern, it must be considered that, when the error signal is zero, we want the system to remain steady (with null derivative), when the output is less than the set point we want the system to increase the output (positive derivative) and when the output value is greater than the set point we want the output to decrease.

So, it is desirable that the rate of change in the output to be proportional to the error signal. In addition, to have zero error in steady state, the output should also change its value in presence of an integral error. All these qualitative considerations lead to a desired expression for the system's output derivative as seen in equation (3):

$$\dot{y}_{system} = K_1(y_{set} - y) + K_2\int(y_{set} - y)dt \tag{3}$$

Where $\mathbf{K_1}$ and $\mathbf{K_2}$ are diagonal matrices that can be made to vary with time. The values for $\mathbf{K_1}$ and $\mathbf{K_2}$ must be determined during tuning procedures. The manipulated vector **u(t)** must be determined in order that the system follows (3) as closely as possible. Therefore the optimal control problem can be formulated as follow:

Using the model equations it is necessary to find a control profile

$u(t)$, $\quad |u(t)| \le \alpha$, witch minimizes the criteria

$$\int_0^{t_f}\left[h(x,u,d,t)^T.W.h(x,u,d,t)\right]dt \tag{4}$$

where **W** is a positive weighting matrix and

$$h(x,u,d,t) = \dot{y} - \dot{y}_{system}$$

$$h = \frac{\partial g}{\partial x} f(x,u,d,t) - K_1(y_{set} - y) - K_2\int(y_{set} - y)dt \tag{5}$$

if we estimate the derivative of the system's output $\dot{y}$ using the equations (1, 2)

$$\dot{y} = \frac{\partial g}{\partial x} f(x,u,d,t) \tag{6}$$

Now it is easy to become an analytical solution for optimal control profile **u(t)** using equation (4) and (5).

Practical aspects of the control algorithm

Several experiments were carried out in a bioreactor, the data sets obtained from online and offline measurements were used to identify the parameters of a model with the structure of equations (7) to (10), using also the Monod equation, (11) for the kinetics of the growth rate. The method used for identification was non-linear least squares.

$$\frac{dx}{dt} = \mu x - \frac{F}{W} x \quad (7)$$

$$\frac{ds}{dt} = -\frac{\mu x}{Y_{x/s}} + \frac{F}{W}(s_F - s) \quad (8)$$

$$\frac{dW}{dt} = F - F_{ev} \quad (9)$$

$$OUR = \alpha \mu x W + \beta x W \quad (10)$$

$$\mu = \mu_{\max} \frac{s}{K_s + s} \quad (11)$$

Where: α [g/g] -yield biomass/oxygen, β [g/g/d] - maintenance term for oxygen, s_F [g/l] - substrate concentration in feed, μ_{max} [l/d] - maximum growth rate, K_S [g/l] - saturation constant, $Y_{x/s}$ [g/g] - yield substrate/biomass, μ [1/d] - specific biomass growth rate, S [g/l] - substrate concentration, x_b [g/l] - biomass concentration, F [kg/d] - feeding rate, W [kg] - bioreactor weight, OUR [g/d] - oxygen uptake rate.

Kalman Filtering Scheme

For better control and optimization of cultivation processes of *A. missouriensis* cells it is essential to know on-line these physiological parameters. Measuring is difficult, if not impossible, for many of these parameters. Therefore, any control or optimization based on physiological parameters cannot be implemented unless values can be measured on-line to provide the necessary information required by the controller. In vitro cell cultures of *Astragalus* species are characterised by complex, non-linear relationships involving poorly identified parameters. This makes them likely candidates for applying adaptive algorithms. For instance, to avoid explicitly modelling the specific rates, these can be treated as parameters, and estimated along with the state variables. The dynamics of the latter can then be described by simple mass-balance equations. In order to implement the control law, all the states of the process, and also an estimation of the growth rate must be available to perform online calculations. For this purpose, the control scheme with the Kalman filter algorithms as featured in Figure 1 was investigated. The Kalman filter makes the prediction of the states in such way that the variance of the prediction error is minimized (Fig.1).

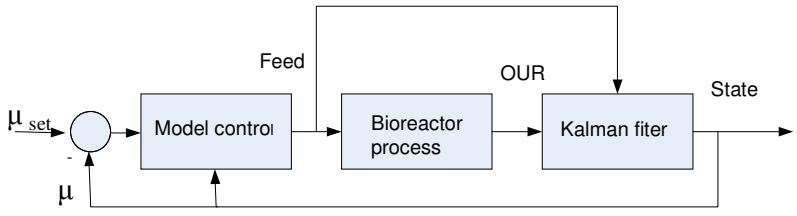

Figure 1 Block diagram of the control algorithm

We have investigated the use of model control algorithms on batch fermentation processes with *Astragalus* cells, using a non-linear model in the controller. They operated the process

with combination of operating regimes and simple local state-space model into a global model structure using an interpolation method. This approach requires all the process states to be available on line. Since direct measurement of these states is not possible, a state estimator - a Kalman filter was implemented. After this optimization, maximum biomass of 24.5 $g.l^{-1}$ dry wt was harvested from the bioreactor culture vessel (recording about 9 times increase over initial inoculum), with 2.97%± 0.08 total Flavonoids. The obtained production in bioreactor is with 1.63 % ± 0.12 greater than yields from 300 ml flasks.

In this way the batch reproducibility can be improved depending to the process control strategy. This applies not only to the cell biomass of *A. missouriensis* but, as the results clearly show, to the flavonoid production also.

CONCLUSION

For the first times a rapidly growing cell line of *A. missouriensis* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation. It was found that the designed and application of advanced control strategies in bioprocesses for production of flavonoids in cell suspension of *A. missouriensis* have been successfully achieved. The cultivation of *A. missouriensis* in bioreactor, after using this control method resulted in an effective increase of biomass and flavonoid production compared with flasks cultivation. In conclusion it can be stated that the future success of large scale cultivation of plant cell and tissue culture will depend on the collaborative efforts of researchers from different disciplines.

ACKNOWLEDGEMENTS

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Original scientific paper

LIGNAN PRODUCTION BY CELL SUSPENSION OF *LINUM TAURICUM*

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SUMMARY

Linum tauricum Willd. (Petrova), endemic to Eastern Europe, Balkan and Krim region, Turkey, belongs to the section *Syllinum* of the genus *Linum* (Linaceae). The podophyllotoxin derivatives 4'-demethyl-6-methoxypodophylotoxin (4'DM-6MPTOX) and 6-methoxypodophyllotoxin (6MPTOX) have been isolated and identified by NMR and UV as two main lignans in the aerial parts and cell cultures of the plant. The *in vitro* investigation of cytostatic properties of 4'DM-6MPTOX has demonstrated 2 to 3.5 times higher activity than that of the referent antineoplastic drug etoposide. The objective of this study is to investigate and improve the biomass growth and lignan production in *L. tauricum* cells by stirred-tank bioreactor. A rapidly growing cell line was selected for cultivation and using the optimum conditions of the shake flask system, the growth and product formation kinetics was investigated in a 2 L bioreactor. In order to give the best possible growing conditions the pH (5.4) and temperature levels (26 ^{o}C) are kept constant. An agitation speed of 140 rpm was sufficient to mix the culture broth in the bioreactor without causing any significant cell damage. Our goal here is to improve the bioreactor production of podophyllotoxin derivatives by suspension cultures of *L. tauricum.* A new control algorithm for biosynthesis of ariltetralin lignans was proposed. The control of bioreactor was synthesized by method combining sliding control, nonlinear control of the error and linear control of the space vector. This is the first report on the bioreactor production of ariltetralin lignans (podophyllotoxin derivatives) in batch mode of operation by suspension cultures from *L. tauricum* with a control approach for regulation of the specific growth rate of cultivation process. Quantitative profiles were obtained experimentally by means of a series of cultivation runs, where 4'DM-6MPTOX and 6MPTOX were produced. All experimental data confirmed the assumptions made in the robust process design study. After optimization, the maximum biomass reaching 19.3 gL^{-1} dry weight. An improvement in the lignan accumulation from 2.50±0.12 mgL^{-1} and 2.36±0.09 mgL^{-1} in the flasks for 6MPTOX and 4'DM-6MPTOX, to 25.35±0.05 mgL^{-1} and 19.17±0.03 mgL^{-1} respectively in the bioreactor by cell culture of *L. tauricum* for 24 days was achieved. The batch cultivation of *L. tauricum* suspension cultures in a 2-L stirred-tank bioreactor, using optimized medium and control system, based on new control algorithm leads to substantial increase (8-10 times higher than production in 300 mL flasks) of the levels of both lignans.

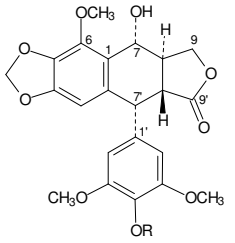
***Key words*:** *Linum tauricum,* stirred tank bioreactor, antineoplastic lignans, bioprocess control

INTRODUCTION

The aryltetralin lignan podophyllotoxin (PTOX) is currently being used as a lead compound for the semi-synthesis of anticancer drugs etoposide, teniposide, etopophos, which are used for the treatment of lung and testicular cancers and certain leukemias [1]. In the coming decades, several new enabling technologies will be required to develop the next generation of

advanced plant-based pharmaceuticals. With modern biotechnology, it has become possible to use plant cells for the production of specific pharmaceuticals.

Linum tauricum (Willd.) Petrova, endemic to Eastern Europe, Balkan and Krim region Turkey, belongs to the section *Syllinum* of the genus *Linum* (Linaceae). After recent taxonomic revision [2] of the four subspecies of *Linum tauricum* (ssp. tauricum, ssp. bulgaricum, ssp. linearifolium and ssp. serbicum) this subspecies is now know as distinct taxa at species level. The podophyllotoxin derivatives 4'-demethyl-6-methoxypodophylotoxin (4'DM-6MPTOX) and 6-methoxypodophyllotoxin (6MPTOX) have been isolated and identified by NMR and UV as the two main lignans in the aerial parts of *L. tauricum* ssp. *tauricum* [3] Fig. 1. The compound 4'DM-6MPTOX was for the first time isolated from wild medicinal plant by us and is interesting with its pharmacological activity. The *in vitro* investigation of its cytostatic properties has demonstrated 2 to 3.5 times higher activity than that of the referent antineoplastic drug etoposide [4]. Due to their restricted natural abundance and the important pharmacological application of these active compounds, identification of new sources or establishment of rational *in vitro* synthesis is very important for the production of therapeutic candidates for cancer chemotherapy.



1. R=H (4'-demethyl-6-methoxypodophylotoxin)
2. R= CH_3 (6-methoxypodophyllotoxin)

Fig.1. Chemical structures of isolated ariltetralin lignans.

Here we report the identification of the both derivatives as the main lignans in suspension cultures of *L. tauricum*, cultivated in 2L bioreactor. Our goal here is to improve the bioreactor production of podophyllotoxin derivatives by suspension cultures of *L. tauricum.* In order to give the best possible growing conditions, a new control algorithm for biosynthesis of ariltetralin lignans was proposed.

MATERIALS AND METHODS

Plant material

Linum tauricum was collected near Varna (Bulgaria) in July 2007. A plant specimen was deposited in the Herbarium of the Faculty of Pharmacy, Medical University of Sofia, Bulgaria (No FAF 0001). The seeds were germinated on modified MS medium, which consisted of half strength MS macro element.

Establishment of in vitro cultures

Seeds of *L. tauricum* were surface sterilized with absolute ethanol and chlorine-releasing disinfectant and germinated on hormone free MS medium in the dark at 25 ^{o}C. Shoot explants were placed on MS medium with 0.4 mgL^{-1} naftilacetic acid (NAA) solidified with 1% agar-agar. After 3 to 4 weeks, developed callus cells were subcultivated weekly by transferring 5 g wet cells to 50 mL fresh medium in 300 mL Erlenmeyer flasks. The suspension cultures were placed on a gyratory shaker (100 ppm) in the dark at 25 ^{o}C. Suspensions (5 g frwt) were transferred every 12 days into 50 mL fresh medium. Ten days-old suspension from selected line of *L. tauricum* was used for bioreactor cultivation.

Extraction of lignan aglycons

A fine powder (0.2 g) of the lyophilised plant material was extracted with methanol (2 mL) in an ultrasonic bath (two times for 30 s). Distilled water (6 mL) was added, and the pH was adjusted to 5.0 by *o*-phosphoric acid. After adding β-glucosidase (1 mg), the sample was incubated at 35 °C for 1 h. Methanol (12 mL) was added and the mixture incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation, the supernatant was directly used or stored at -18 °C.

Isolation of lignan aglycons using semi-preparative HPLC

A fine powder (1g) of the lyophilised plant material was analized. HPLC with a Thermo Quest HPLC system (Egelsbach, Germany) equipped with a photodiode array detector was used. Separation was performed using a GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 4.6 mm i.d. and 40 mm long, 4.6 mm i.d., respectively; Grom Company, Herrenberg, Germany) and a gradient program with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 40 to 67% B, from 17 to 18 min to 40% B, and until 24 min back to 40% B. The flow rate increased from 0.8 mL/min at 0 min to 1.0 mL/min at 17.0 min and decreased again to 0.8 mL/min between 18 and 24 min. A Waters/Millipore HPLC system equipped with a photodiode array detector and a fraction collector was used for the collection of the compounds. After the lignan extraction (mentioned above) the methanolic part of the supernatant was evaporated. The aqueous remnant was extracted twice with equal volumes of ethylacetate p.a. After the ethylacetate phases were combined and evaporated, the remains were dissolved in methanol p.a. Collection of the two main lignans was carried out by using a semi-preparative GROM-SIL 120 ODS-5ST column with guard column (250 mm long, 8 mm i.d. and 50 mm long, 8 mm i.d., respectively; 5 µM particle; Grom Company, Herrenberg, Germany) and the same gradient system as mentioned above, but the flow rate was doubled. Retention times for 4'-demethyl-6-methoxypodophylotoxin and for 6-methoxypodophyllotoxin were at 28.82 min and 39.74 min, respectively.

NMR spectroscopy

^{1}H-NMR spectra were recorded on a Bruker DRX 200 spectrometer at 200.13 MHz in $CDCl_3$. Respectively conditions and data, were as those reported earlier [3]. Fractions eluting at at 28.82 min and 39.74 min, respectively, were collected and analysed.

Bioreactor equipment

Ten days-old suspension from selected line of *L. tauricum* was used for bioreactor cultivation. Fermentations were carried out in 2 l jacketed glass vessel applying cultivation for 24 days. Equipment of the vessel includes: sensors for temperature, pH, dissolved oxygen concentration, foam, and speed of stirrer drive system; four integrated peristaltic pumps and one external pump for flow control and feeding. Bioreactor conditions: temperature 26 °C, batch mode of cultivation, dissolved oxygen saturation (DO at 50%), marine-type impeller design with low-shear stress, speed 140 rpm. This impeller provides mixing and creates a higher oxygen mass transfer rate (Kla).

Control system

Control system has the following functions: display of all process values via schematic P&ID algorithms, digital calibration of sensors and pump dosing counters indication of sensor parameters, recalibration function of pH-probe, control loops for temperature, stirrer speed, pH, foam level, substrate, pO_2 with two stage cascade control, set point profile for substrate pumps.

Data acquisition

Data acquisition system includes the following functions: data collection, data base maintenance, visualization of the process variables by several plotting functions. This software allows starting or finished process batches, exporting database in appropriate data formants and sample data configuration.

RESULTS AND DISCUSSION

Since plant cells produce unique pharmaceuticals, which may be harnessed, they need to be produced in large-scale bioreactors. Selection of the best performing cell line, and its maintenance and stabilization are necessary prerequisites for its production in bioreactors and subsequent scale-up of the cultivation process to the industrial level. Scale-up of growth and product yield depends on a multitude of factors, such as growth medium, conditions of cultivation, inoculum, type of reactor and processing conditions.

A rapidly growing cell line of *L. tauricum* was selected for cultivation in 2-L stirred-tank bioreactor, by batch mode of cultivation for 24 days of culturing. There is no report in the literature related to production of ariltetralin lignans from *L. tauricum* in bioreactor.

Our goal here was to improve the bioreactor-based production of ariltetraline lignans by suspension cultures of *L. tauricum*, by model based control of the bioprocess. Here we report a new control algorithm for biosynthesis of ariltetralin lignans in bioreactor by batch mode of cultivation for 24 days of culturing. The control of bioreactor was synthesized by method combining sliding control, nonlinear control of the error and linear control of the space vector.

A low-shear marine-type impeller has been found to be effective for batch cultivation of shear-sensitive *L. tauricum* cells in stirred tank bioreactor for production of ariltetraline lignans. The manipulation of environmental factors such as pH, temperature, shear stress and O_2 supply are the keys to production of plant cell culture processes. This research presents the implementation of combined strategy to control a bioreactor in production of cytotoxic lignans in cell suspension of *L. tauricum* with a high concentration, using a mathematical model. Using the optimum conditions of the shake flask system, the growth and product formation kinetics was investigated in a 2L bioreactor.

Bioreactor control algorithm

In this article an algorithm is proposed combining sliding control and a linear controller based on classical principle. State-space bioreactor representation of a linear system is:

$$\dot{x} = Ax + Bu + \xi \qquad (1)$$
$$y = Cx$$

where

x is state vector, y is output vector, ξ is disturbance.

To achieve robust tracking of the reference input, the control algorithm takes the following form:

$$u = v - Kx \qquad (2)$$
$$v = f_1(r - y) + f_2(\xi)$$

where $f_1(...)$ and $f_2(...)$ are nonlinear control laws (feedback and forward algorithm) which gave: $e = (r - y) \to 0$.

The structure of the system is presented in Fig. 2.:

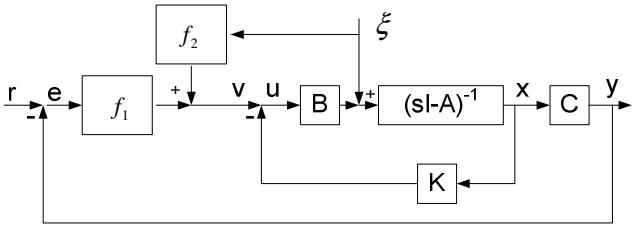


Fig. 2. Structure of the nonlinear control system

Sliding-mode control is capable of robust tracking with fast transient response, at the price of control chattering. It was shown that, combining sliding control and nonlinear control of the error and linear control of the space vector, it is possible to achieve robust tracking with satisfactory transient performance and a steady-state response without chattering.

Let $L = sign(e + \alpha\dot{e})$ (Fig.3.):

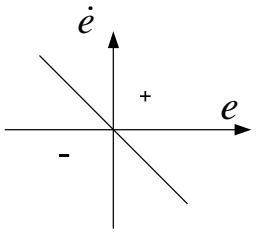


Fig. 3. Plane $(e, \dot{e})$ of the bioreactor system

The bioreactor control and training algorithm in the transient phase is

$$u^* = -\beta.sign(L) \tag{3}$$
$$v^* = f_1(r - y) + f_2(\xi) + g(u - u^*)$$

Combining the sliding control and the nonlinear controller, the overall control algorithm becomes:

$$U = u\varepsilon + u^*(1 - \varepsilon) \tag{4}$$
$$V = v^*(1 - \varepsilon) \tag{5}$$

where:

$\varepsilon = 1,\ \left\|e^2\right\| < \rho$

$\varepsilon = 0,\ \left\|e^2\right\| \geq \rho$

ρ - Reference accuracy of control

In this article it is shown how to connect two fundamentally different control laws with a nonlinear controller to achieve robust tracking with satisfactory transient performance in bioreactor. Combining the sliding control and the nonlinear controller, one can use a control action of high authority to force the system to approach the target states in a short transient,

and then use a controller of moderate gains to maintain a steady response of zero tracking/regulation errors with smooth control input.

L. tauricum cells have successfully been cultivated in a 2-L stirred-tank bioreactor in batch and fed-batch modes of operation. In order to give the best possible growing conditions the pH and temperature levels are kept constant. An agitation speed of 140 rpm was sufficient to mix the culture broth in the bioreactor without causing any significant cell damage.

This is the first report on the bioreactor production of ariltetralin lignans (podophyllotoxin derivatives) in batch mode of operation by suspension cultures from *L. tauricum* with a control approach for regulation of the specific growth rate of cultivation process. Quantitative profiles were obtained experimentally by means of a series of cultivation runs, where 4'DM-6MPTOX and 6MPTOX were produced. All experimental data confirmed the assumptions made in the robust process design study. An optimized culture medium MsLi-MOD [5] was used for the cultivation of *L. tauricum* suspension. Under optimum culture conditions of *L. tauricum* cells, the culture showed a growth-associated product formation. After a short lag phase of four days, the suspension grew rapidly, the biomass increased up to 19.3 gL^{-1} dry weight basis. After connection of two fundamentally different control laws with a nonlinear controller in bioreactor, an improvement in the lignan accumulation and biomass were achieved (see Table 1.).

Table 1. Lignans in suspension of *L. tauricum in vitro* cultures

Type of Cultures	**Medium**	**4'DM-MPTOX** (mgL^{-1})	**6-MPTOX** (mgL^{-1})
Suspension in 300 mL flasks	MsLi-MOD	2.36±0.09	2.50±0.12
Suspension in 2L Bioreactor, after optimization	MsLi-MOD	19.17±0.03	25.35±0.05

The batch cultivation of *L. tauricum* suspension cultures in a 2L stirred-tank bioreactor, using optimized medium and control system, based on new control algorithm leads to substantial increase (8-10 times higher than production in 300 mL flasks) of the levels of both lignans. This is the first report on the production of 4'DM-6MPTOX and 6MPTOX from suspension cultures of this plant species in stirred tank bioreactor.

CONCLUSION

Working with plant cells drastically reduces the preparation time, handling and storage costs associated with the traditional whole plant approaches. In our laboratory, we focus on the production of some important pharmaceuticals in plant cell cultures and have successfully established cell cultures for production of anticancer agents. The success of this technology is dependent not only on the discovery of appropriate production plant system, but also from optimization of cultivation with control of various culture parameters.

A new control algorithm for biosynthesis of ariltetralin lignans was proposed. The control of bioreactor was synthesized by method combining sliding control, nonlinear control of the error and linear control of the space vector. This is the first report on the bioreactor production of ariltetralin lignans in suspension cultures of *L. tauricum.*

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Original scientific paper

ESTABLISHMENT OF *ARNICA MONTANA* CELL SUSPENSION CULTURE

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SUMMARY

Arnica montana L. (Asteraceae) is a rare and endangered medicinal plant according to International Union for Conservation of Nature. This species contains valuable biologically active substances and largely applied in homeopathy and pharmacy. The effect of some plant growth regulators and their combinations on induction of suspension cultures of *A. montana* were examined. The suspensions were initiated from friable callus tissue developed from hypocotyls and cotyledon explants. The cultures were incubated in 200 ml flasks at 110 rpm on a horizontal shaker. Optimal cell growth (5 fold increase in biomass in a 30 day period) was achieved in liquid Murashige and Skoog medium, supplemented with adenine (20 mg/l), 2,4-dichlorophenoxyacetic acid (0.05 mg/l 2,4-D), gibberellic acid (0.2 mg/l GA_3), glutamine (100 mg/l) and casein hydrolysate (500 mg/l). The viability of cells was over 90%. These fast growing, fine aggregated suspensions were suitable for large-scale production of secondary metabolites.

***Key words**: Arnica, suspension culture, biologically active substances*

INTRODUCTION

Arnica montana L. (Asteraceae) is a perennial plant distributed in the high mountains of Europe at 1000 – 2500 m altitude. The species contains valuable biologically active substances. The most important among them are sesquiterpene lactones, flavonoids, phenolic acids and essential oils [1]. This plant is largely applied in homeopathy and pharmacy due to its antiinflammatory, antirheumatic, antiseptic and cardiotonic action. *A. montana* is endangered medicinal plant according to International Union for Conservation of Nature. The reduction of *A. montana* populations and enhanced commercial interest require the development of biotechnological methods for increasing biomass supply. Cell suspension cultures have several advantages over traditional cultivation of whole plant include high growth rates combined with high accumulation of biologically active compounds in short time, production of secondary metabolites under controlled condition independent of climate change and soil conditions, automated control of cell growth etc. [2, 3]. The suspension culture can be used for studying important biosynthetic pathways [4]. There are a number of protocols of *A. montana in vitro* multiplication [5, 6, 7, 8]. The publication related with the development of callus and suspension cultures of the plant are very limited. Only Kalynyak et al. [9] indicated some of conditions for callus induction and Puhlmann et. al. [10] isolated two homogeneous polysaccharides from the nutrition medium of *A. montana* cell cultures.

The purpose of the present study was to investigate the influence of different combinations and concentrations of plant growth regulators on establishment of *A. montana* suspension cultures for selection of fast growing lines which could be used for bioreactor cultivation and secondary metabolites production.

MATERIALS AND METHODS

Plant material: Seeds of *A. montana* used in this study were collected in the Botanical garden, Chemnitz, Germany.

Callus induction: Two types of segments - cotyledons and hypocotyls excised from fifteen-day old *in vitro* germinated seedlings were used as explants sources for callus induction. The nutrient media for callus development contained MS salts and vitamins [11] supplemented with growth regulators in different concentration (Table 1). Sucrose and agar concentrations were constant - 3% and 0.6%, respectively. Medium pH was adjusted to 5.7 before autoclaving at 1 atm (120 ºC for 20 min). Fifty explants per each nutrient media were utilized. To determine the optimum nutrient media for callus formation several parameters were measured: growth intensity, consistency, colour and structure of the tissue. Callus growth was expressed as fresh weight. Callus (0.3 g of fresh weight) was transferred to callus modified media and measured after 35 days cultivation. The experiments were carried out in two replications.

Suspension cultures: The cell suspension cultures were obtained from actively growing friable callus tissue isolated from four week old cultures. The calluses (approximately 1.5 g fresh weight) were aseptically transferred to 200 ml Erlenmeyer flasks containing 50 ml culture media with different combinations of plant growth regulators without agar. Three different nutrient media were tested. The composition of the first two nutrient media is the same as these applied for callus induction (MS1 and MS3). The third medium (MS4) contained adenine (20 mg/l), 2,4-D (0.05 mg/l), GA_3 (0.2 mg/l), glutamine (100 mg/l) and casein hydrolysate (500 mg/l). The cultures were incubated at 110 rpm on a horizontal shaker. The established primary cell suspension cultures were routinely subcultured in the same media (5 ml inoculum/45 ml fresh media) at 7 day intervals. After 30 day culture period the cells originated from the initial flask were gathered and the fresh weight was measured. For fresh weight determination the suspension was filtered through a pre-weighed Whatman No. 1 filter paper. For cell viability determination one ml of suspension was pipette out and mixed with one ml of Fluorescein diacetate at a final concentration of 0.01% [12]. After about 5 min of incubation the cells were examined under fluorescence microscope. Under UV illumination the living cells gave a green fluorescence.

***Cultural conditions*:** The callus and suspension cultures were grown in cultural room at temperature 25±1 ºC, relative humidity 65-70 % and photoperiod 16 h/8 h at 40 μMm^{-2} s^{-1} light intensity.

RESULTS AND DISCUSSION

Induction and growth of callus culture

The initial explants formed primary calluses on all tested nutrient media including plant growth regulators within six weeks of cultivation. Callus induction was not observed on MS control medium without phytohormones. The explants turned brownish and died 10 days after cultivation. The influence of kinetin (Kn), 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) applied at different concentrations in MS nutrient medium on callus induction were tested (Table 1). It was established that the frequency of callus formation depended on explant type and nutrient medium composition. Hypocotyls were found to be more responsive than cotyledons for the callus production. Friable pale yellow callus with high growth intensity was formed on MS medium containing 2,4-D (0.1 mg/l) and IAA (0.2 mg/l). The nutrient medium supplemented with kinetin and 2,4-D provoked formation of compact pale green callus with low growth intensity. The favorable effect of

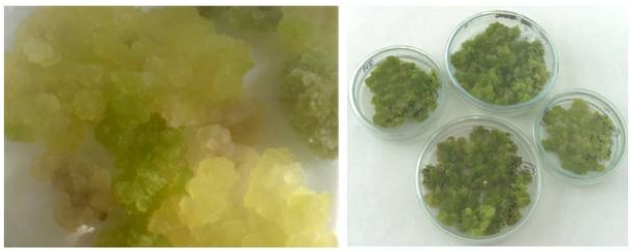
combination of 2.4-D and IAA indicates synergistic effect of auxins on callus culture development. This observation was confirmed also in other studies [13].

Table 1. Effect of different plant growth regulators employed in MS media on callus induction of A. montana

Media	Plant growth regulators, mg/l			Explant type					
				Cotyledon			Hypocotyl		
	Kn	2,4-D	IAA	Callus formation %	Growth intensity	Nature of callus	Callus formation %	Growth intensity	Nature of callus
MS1		0.1	0.2	66	+ +	FPY	78	+ + +	FPY
MS2	1	0.1		76	+	CPG	84	+	CPG
MS3	1	0.1	0.2	86	+ + +	FPG	92	+ + +	FPG

Note: Friable Pale-Yellow (FPY), Friable Pale Green (FPG) and Compact Pale Green (CPG)

The optimum response was achieved on MS nutrient medium supplemented with Kn (1 mg/l), 2,4-D (0.1 mg/l) and IAA (0.2 mg/l). The calluses developed on this medium were characterized with green colour, friable consistency and fast growth. The induction rate was 86% for cotyledons and 92% for hypocotyls. This medium was assessed as optimum for long term maintains and four year old callus culture, which retains its growth characteristics, was obtained (Fig. 1b).



a b

Fig 1. Callus culture of *A. montana* grown on MS medium supplemented with 1 mg/l Kn, 0.1 mg/l 2,4-D and 0.2 mg/l IAA. a) callus, suitable for initiation of suspension, b) four year old cultures derivate from hypocotyl explants.

After establishment of cultures, fresh weight of calli (0.3 g) was transferred onto the examined three nutrient media. The calluses were harvested at 35 day in order to measure its biomass. Callus culture grew slowly during 0-15 days and accelerated their growth from 28 to 35 days. After this period the growth of calluses was inhibited and started browning. Maximum fresh weight was achieved on MS3 medium (0.86 g measured callus originated from hypocotyls) which were 2.8 fold higher that of the initial callus (Fig. 2).

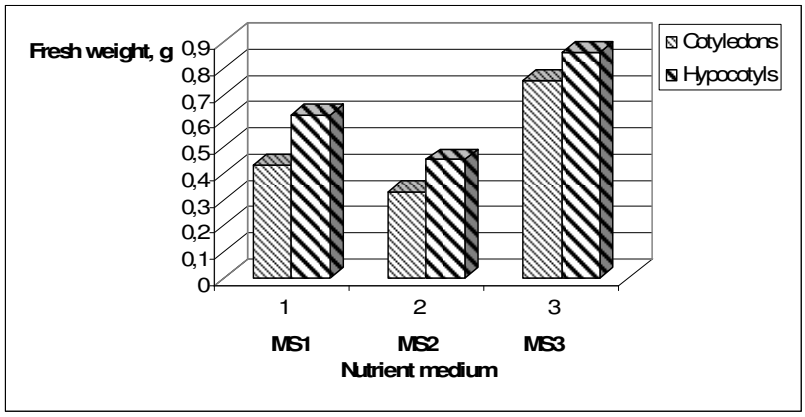


Fig 2. Growth of callus obtained from cotyledons and hypocotyls on three different nutrient media expressed by fresh weight (g)

Establishment of cell suspension culture

Cell suspension cultures were established by using four week old callus (Fig. 1a). After transferring the callus to the culture medium very little increase of cell biomass was observed during the first three days. After six days of cultivation the cells were rapidly divided. When the cultures were developed as fine aggregate suspensions, they need to sub-cultivation every 7 days. The effect of different nutrient medium containing plant growth regulators on cell biomass accumulation was tested. Cell culture were developed on all examined nutrient media, however they have different characteristics. The MS nutrient medium supplemented with 2,4-D (0.1 mg/l) and IAA (0.2 mg/l) induced dark yellow, slow growing suspension (Table 2). The fresh weight of cells reached to average weight of 3.62 g in culture derivate from cotyledon explants and 2.80 g from hypocotyls explants after 30 day cultivation. The growth of cells was improved when adding 1 mg/l kinetin in the nutrient medium with the same composition. The suspensions grown in this medium were light brown with high grow intensity. The cell increased approximately four fold then initial weight (fresh weight of hypocotyls was 6.28 g). The best response was observed in MS4 nutrient medium (Table 2, Fig 3). The composition of the medium was enriched with adenine (20 mg/l), 2,4-D (0.05 mg/l), GA_3 (0.2 mg/l), glutamine (100 mg/l) and casein hydrolysate (500 mg/l). The cells developed in this medium show best growth rate and the best accumulation of biomass. The fresh weight of cells originated from hypocotyls was 7.74 g which was five fold higher that of the initial weight of cells. The cell viability in this medium calculated by dividing the number of live cells of the number of total cells was 92 %.

Table 2. Effect of different plant growth regulators added in liquid MS media on cell suspension development of A. montana

Culture media	Explant type			
	Cotyledon		Hypocotyl	
	Fresh weight, g $x \pm SE$	Nature of suspension	Fresh weight, g $x \pm SE$	Nature of suspension
MS1	3.62±0.23	Dark yellow	2.80±0.42	Dark yellow
MS3	5.12±0.49	Light brown	6.28±0.43	Light brown
MS4	6.52±0.28	Pale yellow	7.74±0.25	Pale yellow

The established fast growing suspension cultures are suitable for further study the role of different culture conditions, elicitors - physical (light, UV-irradiation) and chemical (carbon source, growth regulators including jasmine acid) on the production of the respective secondary metabolites.

The suspension cultures capable for production of biologically active substances were developed in other medicinal plants [14, 15, 16]. Among the regulators added to the suspension medium the 2.4-D is a preferable synthetic auxin because of its capacity to efficiently stimulate the cell division of numerous plants [13, 17, 18]. Malarz et al. [6] also recommended using of 2.4-D in low concentration (0.1 mg/l) for callus induction of *A. montana*. The higher concentrations of auxin (0.5-2.0 mg/l) retarded growth and caused necrosis within four weeks of culture. To induce the growth of cells a cytokinin is frequently added in nutrient medium. The combination of auxin and cytokinin gave enhanced callus proliferation and maintenance [19, 20]. This is confirmed also in our investigation.

Fig. 3. Cell suspension culture developed in MS nutrient medium supplemented with adenine (20 mg/l), 2,4-D (0.05 mg/l), GA_3 (0.2 mg/l), glutamine (100 mg/l) and casein hydrolysate (500 mg/l).

CONCLUSION

The cultural conditions for induction and long-term maintenance of *A. montana* callus culture were elaborated. Due to this the four year old callus line which retains its growth characteristics was obtained. The developed friable callus tissue was suitable for induction of suspension culture. The optimum nutrient medium for obtaining of small, fine aggregated cell suspension was established. The developed fast growing suspension cultures could be used for cultivation in bioreactor and large-scale production of secondary metabolites.

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Original scientific paper

DISRUPTION OF ATTRACTANT PROPERTIES OF POTATO FOLIAGE ON *LEPTINOTARSA DECEMLINEATA* SAY BY THE USE OF *SALVIA OFFICINALIS* L. ESSENTIAL OIL

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SUMMARY

Colorado potato beetle has a very high reproductive potential in Serbia due to favorable climatic conditions and nutrition. Considering that it is an introduced species, the activity of limiting biotic factors seems negligible. With the time, it developed resistance on commercial insecticides, and consequently they cease to be used for this purpose. In recent years, not only in the EU, a strong public pressure has been posed on production of safe food, i.e. food with no pesticide residues which are harmful and dangerous to human health. For these reasons, today we examine several alternative routes, such as: the creation of resistant potato varieties (transgenic potatoes), joint cultivation of potatoes with plants with efficacy to repel or confuse the potato beetle. A great number of herbal extracts and essential oil has been studied so far in order to find natural solution that will replace conventional insecticides. In this study, the possibility to disrupt the attractant properties of potato leaf on Colorado potato beetles, by application of 95% ethanol solutions of sage essential oil and its fractions (F1-F5) (0.5% concentration) was examined. Chemical composition of sage essential oil and its five fractions used in experiment was presented. Tests were conducted at the Adult (female) *Leptinotarsa decemlineata* Say in the olfactometer. The experiments were set up in five replicates, in micro-climatic chamber, with following constant conditions: temperature 27°C ± 1°C, relative humidity 65% ± 5% and the illumination was 9400π cd. Results were processed by the use of Analysis of variance and Duncan's test. The most pronounced disturbance was recorded with application of sage essential oil and the least one was achieved with application of the fraction one (F1). The obtained results indicate possibility of using secondary metabolites of *Salvia officinalis* L. in protection of potato foliage of its major pest (*L. decemlineata*).

Keywords: *Leptonotarsa decemlineata* Say, sage essential oil, fractions, disturbance, olfactometer.

INTRODUCTION

Colorado potato beetle (*Leptinotarsa decemlineata* Say) has a very high reproductive potential in Serbia due to favourable climatic conditions and nutrition. Considering that it is an introduced species, impact of limiting biotic factors seems to be negligible. With the time, the pest has developed resistance on commercial insecticides, so they cease to be applied for this purpose. In addition, they proved to be highly toxic to non-target organisms, including humans and the environment [1, 2, 3], and since they are not easily biodegradable, they also leave dangerous residues in food products.

In recent years, not only in the EU, a strong public pressure has been posed on production of safe food, i.e., food without pesticide residues harmful to human health. For these reasons,

several alternative approaches have been examined, such as: the creation of resistant potato varieties (transgenic potatoes), joint cultivation of potatoes with plants with efficacy to repel or confuse the potato beetle.

A great number of herbal extracts and essential oil has been studied so far in order to find out natural solution that will replace conventional insecticides. In this study, the possibility to disrupt the attractant properties of potato leaf on *Leptinotarsa decemlineata* Say female adult, by application of *Salvia officinalis* L. essential oil and its fractions (F1-F5) was examined in olfactometer.

MATERIALS AND METHODS

Experimental design

Essential oil of *Salvia officinalis* L. was isolated by the Clevenger steam distillation apparatus. The oil and its five fractions (F1–F5) were subjected to detailed qualitative and quantitative analysis using the GC and GC/MS method. Chemical composition of sage (*Salvia officinalis* L.) oil and its five fractions (F1-F5) are presented in Kostic et al., 2007 [4].Prior to be applied, the oil and the fractions were dissolved in 96% ethanol. The repellent efficacy of 0,5% solutions of the oil and it's F1–F5 fractions on *L. decemlineata* was examined.

Insect Colorado potato beetle (Leptinotarsa decemlineata Say)

The insects originated from Dobanovci (Serbia). The insect population proved to be resistant to carbamates and ogranophosphorous compounds [4] was used.Insects were collected from the potato not treated with pesticides. The insect population was grown in the laboratory conditions. The experiments were conducted in five replications and for each replication new adults were used. Only adult female individuals (3 days old) of Colorado potato beetle were used, since they are responsible for discovering the host plants, enabling the survival of the species. In the experiment, opportunity to choose between the discs of potato leaf treated with 0,5 % solution (different variants) and untreated leaf disk (control treatment), was provided to female adults, in order to examine possibility to prevent the pest to discover potato leaf. Since the adults in experiment used to easily discover untreated leaf disks, time and speed they needed to complete the task were recorded.

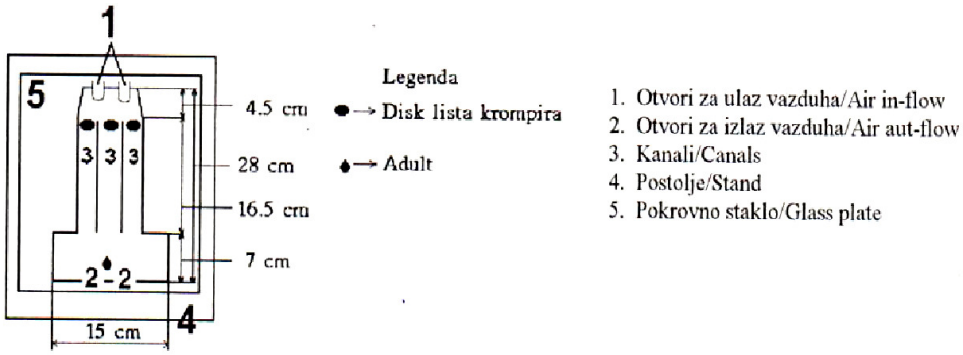
Potatoes

Potato cultivar "Dessire" was grown in greenhouse conditions, without application of pesticides. Fresh potato leaf disks, 2 cm diameter, were first immersed in 0,5 % solution (weather of sage essential oil or one of its five fraction), then dried for 20 minutes at 27°C, and finally placed in the olfactometer (in the left tunnel of the olfactometer).

Olfactometer

To test the repellent efficacy of the 0,5% solutions of sage essential oil and its fractions, olfactometer was used. The olfactometer is made of 3-5 mm thick glass, and consists of two entrances for the air intake and two exits of the exhausted air. The air passes through three tunnels (17cm long and 3,5cm wide). The air comes to the part where the olfactometer is expanded and it has two openings (related to the part with tunnels), in order to prevent the air turbulence (dimensions of this part of olfactometer are 15x7cm). The overall dimensions of usable space in the olfactometer are 28x15x5cm (length x width x height).

Figure 1. Schematic display of olfactometer used in experiment.



In order to function, the olfactometer uses the air pump and rubber-coated tubes (9 mm in diameter) for the inlet air to the manifold, the air flow regulator, the rotameter and the glass air hub with activated charcoal (which served to neutralize the odoriferous substances in the air). The air velocity through the rotameter was ca. 2000 l/ h (i.e. 0,55 l/s), while in the olfactometer itself the air speed was ca. 75 ml/s.

Distance between the potato leaf discs and the adults was 21 cm. In the left tunnel, treated potato leaf disk (various solutions) was placed while the untreated leaf disk was placed in the right tunnel. The adults' preferences towards treated/untreated potato leaf disks, as well as their timing to reach the leaf disks, was monitored.

Air Chamber

The experiments were placed in air-chamber in order to enable optimal conditions for the biological cycle of the Colorado potato beetle. Temperature and relative humidity was adjusted via control panel ("Danfoss, EKH 20"). All experiments were carried out in micro-climatic chamber under following stable condition: T = 27±1°C, Relative humidity (RH) = 65±5% and illumination 9400 candela π.

Statistical..analysis

Results were analyzed using Analysis of Variance and statistical significances determined by the Duncan test.

RESULTS AND DISCUSSION

Chemical composition of sage essential oil showed presence of 14 different compounds. The main ones were α–thujone (31.87%) and camphor (24.65%). Each of the sage oil fraction (F1–F5) showed less number of compounds with their different presence (%) in the fraction, in comparison to the entire sage essential oil. The F-1 fraction had 8 compounds, with dominant one being α–thujone (25.37%). The F-2 fraction had 7 compounds and the dominant one was also α–thujone (48.99%). Both fractions, F-3 and F-4 had 6 compounds, with dominant one being camphor (46.99% and 44.42%, respectively), while the fraction F5 had 7 compounds with two dominant compounds with similar presence; γ–selinen (19.57%) and α–humulene (18.27%). The only component that is present in the entire sage oil and in all five fractions of the sage oil was camphor and with the highest percentage detected in the F-3 fraction (46,99%). Chemical composition of the entire sage oil and the five sage oil fractions (F1-F5) used in experiment are presented in Table 1.

Analysis of variance revealed that very significant differences ($p \leq 1\%$) were recorded between the time length felame adults needed to reach untreated and treated leaf discs, depending on the applied treatment (Table 1).

Table 1. Analysis of variance for the time (in seconds) Colorado potato beetle female adults need to reach untreated potato leaf disks, depending on the examined variants.

Source of variation	Degrees of freedom	Mean square	F value
Repetitions	4	119.95	2.16
Variants	5	8394.85	151.34**
Error	20	55.47	
Total	29		

** ≤ 1%

The fastest average arrival time to the untreated potato leaf disk (35,6 s) was recorded for adults that had to choose between the untreated leaf disk and the leaf disk treated with sage essential oil. The average times of adults to reach untreated disks in experiment with fractions F3, F4 and F5 were similar (65.0–72.6 s), while in the experiment with fractions F2 and F1, adults needed much more time (85.8 and 157.6 s, respectively). The results are presented in Table 2.

Table 2. Time (in seconds) that Colorado potato beetle female adults needed to arrive to the untreated potato leaf disc, depending on the examined variants.

Treatments	Repetitions					Average values
	I	II	III	IV	V	
Sage ess. oil (*S. officinalis*)	26	35	38	37	42	35.6**a**
Fraction 1	158	155	158	164	153	157.6**d**
Fraction 2	88	80	84	88	89	85.8**c**
Fraction 3	52	74	70	84	83	72.6**b**
Fraction 4	67	68	60	72	84	70.2**b**
Fraction 5	68	50	75	67	65	65.0**b**

$LSD_{0.05}$ = 9.8 seconds

The process of selection of the host plant by insects is composed of three quite distinct phases: 1. Insects has to be attracted by potential host plants; 2. Insects has to reach the host plant; 3. Stimulation (prevention) of the insect to feeding on the host plant [5]. This confirms that there is a subtle interaction between the host plants and insects, mainly achieved by their sensory organs, especially those used to establish their contact with given environment and feel smell and taste (*sesilum basiconicum*).

Plant species are abundantly present on Earth and are preferred by many pathogenic organisms. It is assumed that there are 6 million species of organisms, 50% of them being harmful to plants [6]. In order to effectively fight against the invasion of microbial pathogens and insect herbivores, plants have developed sophisticated defensive strategy to "see" the attack of insects and other pathogens, and thus translate this "experience" in appropriate defensive response [7, 8]. These defensive responses helped plants to develop their defense by networking of interconnecting signals of different plant components produced by the plants in order to protect themselves from harmful pathogens [9, 10]. On the attacked places, the plant accumulate metabolites as a response to pathogen infection or damage caused by phytophagous insects, by activating different sets of defense associated with genetic inheritance [11]. For these reasons, scientists intensively search for natural substances that have possibility to deter insects not to come and feed on host plants (repellents and

antifeedants) nor to place their embryos (anti-oviposition), which also have no toxic effects (contact or gastrointestinal) so they could be used in the plant protection [12].

It was found that the main components, responsible for the attractive effect on the Colorado potato beetle, are present in the essential oil of potato leaves. The basis of this "grass-like" smell the green potato leaf volatiles represents a chain of saturated and unsaturated aldehydes and alcohols produced by oxidative degradation of plant lipids. The relative proportion of these final products varies among different plant species within the same genus, and also seasonally, within a single species, and due to plant aging or injuring, all of these affecting the degree of attraction of Colorado potato beetle by the plant [13, 14, 15, 16]. Visser et al. [17] determined following volatile components present in potato leaf that attract Colorado Potato beetle, *trans*-2-hexene-1-ol, 1-heksenol, *cis*-3-hexene-1-ol, *trans*-2-heksenol and linalool, present in potato leaves in following percentage proportion: 100: 17 : 17 : 7: 4, respectively.

In our experiment, all tested variants successfully deterred the arrival of female adults of Colorado potato beetle on the treated potato leaf disks. The difference between different variants applied was recorded in the time they needed for arrival to the untreated disk. The fastest arrivals to untreated leaf disk have had those that had to choose between the leaf disk treated with sage essential oil and untreated leaf disks (the average arrival time was 35.6 sec). The most of the measured time adults have spent on making correct choice between the untreated leaf disks and disk treated with fraction F1 (157.6 sec). Regarding the remaining fractions F2, F3, F4 and F5, the adults spent the shortest time to reach untreated leaf disks when fraction F5 is applied (65.0 sec), while when other fractions were applied the required time ranged between 70.2 and 85.8 seconds.

Despite the attempts to mask the attractant property of potato foliage on *L. decemlienata*, by the use of various herbal extracts and/or essential oils, in order to prevent them to find their host plant, a lot of efforts has beet input in order to find out how to prevent these pests to feed on [18] or how to prevent both, feeding and ovipositioning on potato foliage [19].

CONCLUSION

In this study the possibility of preventing the female adults of Colorado potato beetle to feed on their host plant potato by attempting to mask the attractant volatile components of potato foliage with 0.5% ethanol solution of sage essential oil (*S. officinalis*) and its five fractions (F1-F5) was examined The application of sage oil caused the most significant interference, followed by the application of the fraction F5. The obtained results encourage further researches on disturbance of the attractant properties of potato foliage volatiles on Colorado potato beetle female adults or prevention of the arrival of adults on the host plant (first phase), with the use of various concentrations of sage essential oil and its fraction F5.

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Original scientific paper

ALTERNATIVE CONTROL OF *ALTERNARIA ALTERNATA* USING ESSENTIAL OILS *IN VITRO*

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SUMMARY

Effectiveness of volatile phase of essential oils of *Pinus silvestris*, *Petroselinum crispum*, *Origanum vulgare* and *Pimpinella anisum* in control of *Alternaria alternata* were studied for inhibition of mycelium growth, *in vitro*. The oils were applied as a drop onto the inner side of the plate covers on the sterile filter paper (R=0,5cm), which was placed in the center on the cover glass, at the concentrations of 0.04; 0.06; 0.1; 0.15; 0.3 and 0.6 µl/ml of air inside the Petri plates. In order to enable the contact of volatile phase of oils and pathogen, the Petri plates were turned upside down. The plates were sealed with self-adhesive foil in order to prevent release of oil vapors out of the plates. Inhibitory effect of tested oils has been determined seven days after setting the trial by calculating percentage of inhibition of radial growth of pathogen mycelium (PIRG) and minimal inhibitory concentration (MIC) were determined. Minimal fungicidal concentration (MFC) has been determined fourteen days after essential oils were applied. For determining MFC, plates were ventilated in the sterile laminar flow for 30 minutes in order to remove volatiles of oils. Complete inhibition of mycelia growth (100%) had essential oil of *O. vulgare* applied in the lowest concentration of 0.04µl/ml of air followed by essential oil of *P. anisum* in 0.06µl/ml of air. Essential oils of *P. silvestris* and *P. crispum* were significantly less effective with PIRG values of 29,74% and 26,67% applied even at the highest concentration (0.6µl/ml of air).Seven days after exposure minimal inhibition concentration were determined (MIC). Lowest MIC value had oil of *O. vulgare* (0.04µl/ml of air), while MIC for *P. anisum* oil was 0.1µl/ml of air. Essential oils of *P. silvestris* and *P. crispum* did not expressed total inhibition of mycelium growth after seven days MIC were higher then 0,6µl/ml of air. Essential oil of *O. vulgare* expressed MFC at lowest applied concentration 0.04µl/ml of air. MFC for *P. anisum* oil was at highest applied concentration 0.6µl/ml of air. Our results proved that essential oils of *O. vulgare* and *P. anisum* could be an alternative approach for control of *A. alternate, in vitro*. These results will help in further testing of effectiveness of essential oils *in vivo*.

Key words: *Alternaria alternata*, seed pathogen, essential oils, effectivenes, control

INTRODUCTION

Aternaria alternata is one the most common pathogens of vegetables pre as well as post harvest [1]. This is pathogen that jeopardizes production of different vegetable crops in all phases of production, from seeds, plants in vegetation to fruits in storage [2,3]. As a postharvest pathogen, *A. alternata* causes Alternaria rot that causes great losses that can go up to 30% per year and even up to 43% of the total production of tomatoes [3]. It is important to note that storage conditions necessary to preserve the quality of fruits for a long time are also favorable for the development of this pathogen. Increased humidity needed to prevent the occurrence of fruit shrivel is important for development of pathogen [4,5].

Except for great losses that *A. alternata* can cause, this pathogen present a potential health risk for consumers. *A. alternata* can produce several types of mycotoxins in fruits of tomatoes, peppers, melons and also in processed tomato products [6]. The most important mycotoxins that can occure in the vegetable fruits are: alternariol ($C_{14}H_{10}O_5$), alternariol methyl ether ($C_{15}H_{12}O_5$) and altenuene ($C_{15}H_{16}O_6$), which are benzopirone derivates; also tenuazonic acid ($C_{10}H_{15}NO_3$), which is a tetramic acid derivate; and altertoxin-I ($C_{20}H_{16}O_6$), a perylene derivate [7]. Several synthetic fungicides are commonly used to control this pathogen. However application of same active ingredients in long terms can lead to resistance or can become ineffective [8]. Having in mind the impact of this pathogen and problems of continues application of some fungicides it is important to find new and environmental acceptable ways of control of *A. alternata* [9,10]. One of the possible solutions for control could be application of some be essential oils. Effect of these oils to pathogens was reported by several authors [1,11,12,13,14,15]. Researches indicate that plants are rich sources of antifungal compounds and that they could be an appropriate alternative to conventional fungicides if these compounds are formulated correctly [16]. Before any pesticide's application *in vivo*, biological or conventional, it is necessary to determine its toxicity, i.e. its efficiency *in vitro* [1].

The aim of this study is to determine antifungal effect of essential oils against *Alternaria alternata*, *in vitro*.

MATERIALS & METHODS

The pathogen isolated from tomato plants was identified as *A. alternata* based on microscopic examination.

The antifungal activity of essential oils of *Pinus silvestris*, *Petroselinum crispum*, *Origanum vulgare* and *Pimpinella anisum* was investigated, by expossing mycelium of pathogen to volatile phase of these oils [17]. Mycelial plug (5x5mm) was transferred to the center of the Petri plate (R=9cm). After that the plates were turned upside down. The oils were applied as a drop onto the inner side of the plate covers on the sterile filter paper (R=0,5cm), placed in the center on the cover glass, at the concentrations of 0.04, 0.06, 0.1, 0.15, 0.3 and 0.6 µl/ml of air inside the Petri plates using micropipette. In order to enable the contact of volatile phase of oils and pathogen, the Petri plates were kept upside down. The plates were sealed with self-adhesive foil in order to prevent release of oil vapors out of the plates. The Petri plates were also kept at 23°C. Petri plate with a drop of sterile distilled water instead of oil was used as a control. Radial growth of pathogen mycelium in the treated plates and control were measured after seven and fourteen days. Seven days from exposure to oil vapor, the percent of inhibition of radial growth of pathogen mycelium (PIRG) was calculated. The lowest concentration of oil which completely inhibited mycelium growth after seven-day exposure was considered as the minimum inhibitory concentration (MIC). After that, the plates were opened and ventilated in the sterile laminar flow for 30 minutes to remove volatiles of oils in order to determine fungicidal effect of oils. Concentrations that were consider fungicidal were the ones that suppress mycelial growth even seven days from ventilation. The lowest concentrations were considered as minimal fungicidal concentration (MFC). All experiments were performed twice with five replications of each oil concentration. The percentage of inhibition of radial growth of pathogens mycelia were calculated using the following formula:

$$PIRG(\%) = \{g_c - g_t / g_c\} \times 100,$$

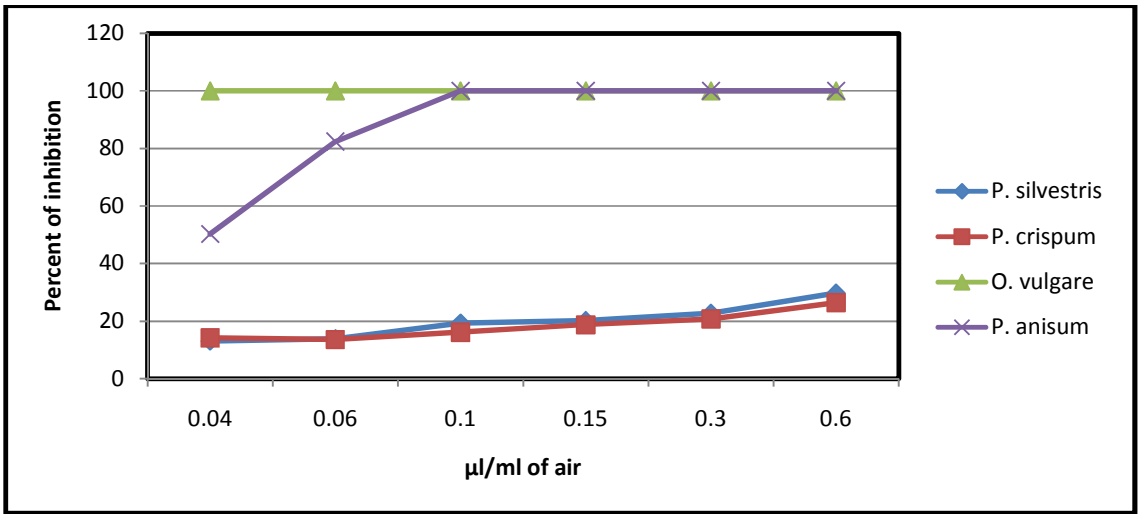
where ***gc*** is the growth of mycelium in control plates, ***gt*** the growth of mycelium in treated plates.

RESULTS & DISCUSSION

Essential oil of *Origanum vulgare* expressed total inhibition of radial growth of mycelia growth applied in all tested concentrations. Essential oil of *Pimpinella anisum* expressed total inhibition applied at 0.1 µl/ml of air. Essential oils of *Pinus silvestris* and *Petroselinum crispum* were less effective in inhibition of *A. alternata* with similar inhibition percent 26.14% and 26.42%, at highest concentration that was applied (Graph. 1).

Essential oil of *O. vulgare* expressed minimal inhibition concentration applied at 0.04 µl/ml of air which was the lowest applied concentration in this experiment. MIC of essential oils of *P. anisum* was expressed when applied in 0.1 µl/ml of air. Essential oils of *Pinus silvestris* and *Petroselinum crispum* did not express total inhibition of mycelia growth of observed pathogen after 7 days, but only 26.14% and 26.42%, we can say that the MIC was not observed in these concentration rates (Graph. 2.).

Gaph. 1. Inhibition of radial growth of mycelia of *A. alternata* by essential oils after 7 days



Since only essential oils of *O. vulgare* and *P. anisum* showed total inhibition seven days after exposure and therefore expressed MIC, and essential oils of *Pinus silvestris* and *Petroselinum crispum* did not, we examined only these two oils for possibility to have fungicidal effect (MFC) toward *A. alternata*. Essential oil of *O. vulgare* expressed minimal fungicidal concentration at lowest applied concentration of 0.04 µl/ml of air, and essential oil of *P. anisum* expressed MFC applied at 0.6 µl/ml of air (Table 1.).

Table 1. Effect of essential oils to *A. alternate*

Essential oils	MIC*	MFC**
Pinus silvestris	> 0.6 µl/ml of air	> 0.6 µl/ml of air
Petroselinum crispum	> 0.6 µl/ml of air	> 0.6 µl/ml of air
Origanum vulgare	< 0.04 µl/ml of air	< 0.04 µl/ml of air
Pimpinella anisum	0.1 µl/ml of air	0.6 µl/ml of air

* Minimal inhibitory concentration

** Minimal fungicidal concentration

Implementation of biological control of the pathogens is one of the most promising alternatives to fungicides [9,10]. Natural pesticides based on plant-essential oils may be that alternative, but this claim has yet to be proved through thorough scientific investigation [18]. We investigated effectiveness of volatile phase of essential oils of *Pinus silvestris*, *Petroselinum crispum*, *Origanum vulgare* and *Pimpinella anisum* in inhibition of mycelia growth of *Alternaria alternata*. Essential oil of *O. vulgare* expressed highest level of inhibition, both MIC and MFC applied at lowest tested concentration. Essential oil of *P. anisum* was also very effective in control of this pathogen with MIC at 0.1 µl/ml of air and MFC applied at 0.6 µl/ml of air. Both oils inhibited radial growth of mycelia of pathogen four days after exposure. Meanwhile essential oils of *Pinus silvestris* and *Petroselinum crispum* were not efficient in inhibiting mycelial growth of *A. alternata*. They had low percent of inhibition of mycelial growth even applied at highest tested concentration. Both oils had lower percent of inhibition applied at 0.06 µl/ml of air than applied at 0.04 µl/ml of air, especially essential oil of *P. crispum* that did not inhibit radial growth at all applied in this rate. Effectiveness of different essential oils in control of *A. alternate* was reported previously [1]. In that research essential oils of *Mentha piperita*, *Eucaliptus citriodora* and *Rosmarinus officinalis* were very effective in inhibition of radial growth of this pathogen, especially essential oil of *Mentha piperita* with total inhibition of mycelia growth applied at 0.15 µl/ml of air and MIC applied at 0.3 µl/ml of air. Some other researchers examined possibility of controlling *A. alternata* by using essential oils of medicinal plants [19, 20]. Essential oils of cassia and thyme were able to totally inhibit radial growth of this pathogen applied in concentration of 300-500ppm [19]. Essential oils of Halfa barr and ginger, followed by avocado, cinnamon and laurel were very effective in suppression of mycelia growth of this pathogen [20]. Essential oils of *O. vulgare* expressed total inhibition of *Verticillium fungicola* var. *fungicola*, *Mycogone perniciosa* and *Cladobotryium* sp. applied at 0.02 µl/ml of air and had MIC and MFC applied in that concentration as well [21]. In that same research essential oil of *P. anisum* expressed high antifungal effect to *Mycogone perniciosa* with MIC and MFC applied at 0.04 µl/ml of air, while oil of *P. crispum* had strong antifungal effect toward *M. perniciosa* (MIC 0.02µl/ml of air and MFC 0.04 µl/ml of air) and toward *Cladobotryum* sp. (MIC and MFC values were at 0.32 µl/ml of air). Compared to our results essential oils of *O. vulgare* and *P. anisum* can be used for control various fungal pathogens. Although these studies reported antifungal activity of essential oils, the mechanisms of action of such oils are poorly understood. However, some researchers reported that there is a relationship between the chemical structure of the most abundant compounds in the essential oils and the antimicrobial activity. According to [22], the antimicrobial activity of major oil compounds happens in the following order: phenols (highest activity) > alcohols > aldehydes > ketones > ethers > hydrocarbons. Taking in consider the research of [23], that indicated that composition of oil may vary on different localities, the analysis of composition of these oils should be the next step in further research.

CONCLUSION

The application of essential oils of some medicinal and aromatic plants can be used as alternative to chemical fungicides in control of fungal plant pathogens. Essential oils of *O. vulgare* and *P. anisum* expressed high efficacy in controlling *A. alternate*, *in vitro*.
Based on these encouraging results we can proceed with research to determine effectiveness *in vivo*.

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Original scientific paper

TOLERANCE OF HONEY BEES ON THREE COMMERCIAL ESSENTIAL OILS

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SUMMARY

The need to provide healthy and secure bee products, honey bees welfare and alternative systems of the fight against pests without application of chemicals influenced our study on possible effects of different substances of natural origin on the honey bee colony. In order to perceive honey bee tolerance, contact residual toxicity was examined in the laboratory conditions on following commercially available essential oils: thyme, hyssop and wintergreen. The chemical composition of essential oil was determined by standard GC and GC/MS methods. Different doses of the essential oils dissolved in acetone were applied in Petri dishes and left to dry for 20 minute at a room temperature. Following this time, ten honey bees were added in each Petri dish. The total number of the variants was 26 and they were all maintained in controlled conditions of temperature and humidity. Survival of honey bees was recorded after 24 h and 48 h. The obtained results were processed by analysis of variance. The most prominent toxic effect on the examined honey bees was observed after 24 h with the dosage of 20 µl of thyme oil (average value for dead individuals was 8.75), followed by the same dosages of hyssop oil (7.75) and finally with wintergreen oil (2.5). Similar results regarding contact residual toxicity of tested three essential oils were obtained after 48 h. Comparative chemical characterization of selected essential oils and recorded biological activities of the oils tested in different doses, revealed the opportunity to proceed with this important investigation by selecting the most appropriate variants for further investigation on both, honey bee and honey bee mite *Varroa destructor*.

***Keywords**: Apis mellifera, essential oils, residual contact toxicity, natural products, tolerance.*

INTRODUCTION

Beekeeping is a specific branch of animal husbandry where, during the honey harvest season, at the same place, a large number of bee colonies of different owners and different levels of infestation with *Varroa* mite may occur [1]. Difficult disease, varroa, caused by parasitic mite of *Varroa destructor* Anderson and Trueman, causes to beekeeping the greatest economic losses [2, 3]. The parasite makes direct damage to the bee brood and adult bees, by sucking their haemolymph. In the larval and pupal stage, the loss of hemolymph proved to have a negative impact on developing organs in bees [4]. In adult bees, varroa shortens the life of worker bees and reduces their ability to orient themselves and to return to the colony, while in drones it reduces bee reproductive capacity [5, 6]. Apart from its direct effect, the parasite also causes indirect damage to the bees, since it is a vector for viruses [7].

In order to suppress parasitic mites numerous synthetic chemicals proved to be more efficient in comparison to many natural organic compounds tested [2]. However, many synthetic acaricides, such as fluvalinate, flumethrine, coumaphos, which are liposoluble, retained in the

wax and they were frequently found in the honey. The occurrence of the residues in bee products makes the products unsafe for human consumption. On the other hand, exposure of parasites to low concentration levels of acaricides has developed resistance of parasite to fluvalinate [8, 9]. Due to many unfavourable properties of synthetic chemicals, alternative organic preparations, mainly based on essential oils and organic acids, began to be developed, tested and applied in the fight against these dangerous parasites.

Preparations created on the basis of essential oils and plants from the local area, might be the best substitute for synthetic chemical substances and pose less risk to human health and to bees. So far, over 150 different essential oils were tested, but only a few showed a good bee tolerability and contact residual toxicity to the mites [10, 11, 12].

The aim of the first phase of our research was to examine in the laboratory conditions tolerance of bees to contact residual toxicity of commercial essential oils of thyme, hyssop and wintergreen, of which the first two oils were isolated from the plant material of domestic origin (Serbia).

MATERIAL & METHODS

Examination of bees tolerance to contact residual toxicity of essential oils, was conducted under laboratory conditions (T = 30° C, Relative humidity = 60%), in Petri dishes, in four replications. Different doses of the essential oils dissolved in acetone (0.005–20 μl/Petri dish) were applied in Petri dishes (9 cm in diameter) and left to dry for 20 minute at a room temperature. Then, each dish was supplemented with 10 newly emerged adult bees (between 0 and 3 days old). Bees in dishes were fed with 3 g of candy and watered with water from a plastic micro tube (1.5 mL). Acetone was used as control. Survival of examined honey bees was recorded after 24 h and 48 h.

For all the examined characteristics, the assessment of significance is achieved on the basis of group F test, for the significance levels 5% and 1%.

Origin of the essential oils used in experiment

The essential oil of hyssop (*Hyssopus officinalis* L.) cultivated in Vojvodina was obtained from company "Herba d.o.o", (Belgrade, Republic of Serbia. The essential oil of common thyme (*Thymus vulgaris* L.) cultivated in Serbia was obtained from company "Beolab" (Belgrade, Republic of Serbia). The oil of wintergreen (*Gaultheriae procumbens* L.) of Chinese origin was obtained from the Institute for Medicinal Plant Research "Dr Josif Pančić" (Belgrade).

Essential oil analyses procedure

Analytical gas chromatography (GC-FID) was performed on the GC HP-5890 Series II apparatus, equipped with autosampler (ALS), split-splitless injector, attached to HP-5 fused silica capillary column (25 mm × 0.32 mm, 0.52 μm film thickness) and, fitted to flame-ionization detector (FID). Carrier gas flow rate (hydrohen) was 1 mL/min), temperatures of injector and detector were set to 250°C and 300°C, respectively, while the column temperature was linearly programmed from 40–260°C at rate of 4°C/min. Essential oil samples were diluted in ethanol and injected (1 μl) in split-mode (1:30, ALS). The percentage compositions of each sample were computed from the peak areas, without correction factors. For GC/MS, a HP G 1800 C Series II GCD analytical system equipped with HP-5 MS column (30 m × 0.25 mm, 0.25 μm film thickness) was used. Carrier gas was He (1 mL/min). Other chromatographic conditions were as those for GC-FID. Transfer line was heated at 260°C. Mass spectra were recorded in EI mode (70 eV), in a range of m/z 40–450.

Identification and quantification of the oil components

The identification of individual constituents was accomplished by comparison of their mass spectra with those from available MS libraries (NIST/Wiley) and by comparison of their

experimentally determined retention indices (calibrated AMDIS), with data from the literature [13].

RESULTS AND DISCUSSION

Results of GC analysis of commercial thyme, hyssop and wintergreen essential oils are presented in Table 1. For the essential oil of common thyme dominant components were *p*-cymene, thymol and α-terpineol. Hyssop oil was characterized by a high content of *trans*-pinocamphone, *cis*- pinocamphone and β-pinene. wintergreen oil predominantly contained methyl salicylate.

The essential oil of common thyme (*Thymus vulgaris* L., fam. Lamiaceae) is known for its representative component thymol, which is in beekeeping firstly used against *Acarapis woodi*, and later in fighting *Varroa destructor* [14, 15, 16, 17]. Since there is no ISO standard that prescribes the quality of common thyme essential oil, richness mainly in thymol and to a less extent in carvacrol, is frequently the decisive quality criterion for its commercial acceptance [18]. Imdorf et al. [12] reported existence of at least seven chemotypes of *Thymus vulgaris* essential oil, classifying them into two groups; the "strong chemotype" with a higher concentration of thymol and carvacrol, and the "mild chemotype" with a higher content of alcohols geraniol, linalool and thujanol. According to this division, the common thyme oil used in our study belongs to the "strong chemotype" (Table 1). Total of 25 compounds were identified in common thyme oil used in this experiment. The most abundant compounds were *p*-cymene and thymol (61,5% of the oil), while the following 8 components (present in the oil in the range between 9,3 and 1,0 %) and listed in descending order of their contribution to the oil, were α-terpineol, carvacrol, linalool, γ-terpineol, α-pinene, borneol, limonene and *cis*-β-terpineol), representing other 31,35% of the oil. Remaining 15 components, with their presence in the oil less than 1 %, together contributed to the oil with 5,84 % (Table 1).

Hyssop (*Hyssopus officinalis* L., fam. Lamiaceae), was known in the ancient times as a medicinal drug with a pleasant spicy scent. It grows wild in Eastern Serbia. In the herb, there is 0,3–1% of essential oil [19] that is obtained from the aboveground plant part by water distillation. According to ISO 9841:2007(E), which serves as a normative for the quality of the essential oil of *Hyssopus officinalis* L. spp. *officinalis*, the most abundant components, are supposed to be *iso*-pinocamphone (min 25% - max. 45%) and pinocamphone (min 8% - max. 25%). In addition, according to Imdorf et al [12], hyssop oil can be generally classified into two chemotypes, weather the most abundant component in the oil is eucalyptol or pinocamphon. For instance, the main component in hyssop oil originated from Spain was eucalyptol (syn. 1,8-cineole) while the main component in hyssop oil from Serbia was pinocamphone [21]. Based on GC/MS analysis, hyssop oil used in our experiment (Table 1) fit into proposed ISO standard and statement that oil from *Hyssopus officinalis* L. commonly cultivated in Serbia, belongs to “pinocamphone” chemotype. Total of 30 compounds were identified in the oil and the most abundant compounds (with their presence in the oil above 14%), were *cis*–pinocamphone (syn. *iso*-pinocamphone), followed by *trans*–pinocamphone (syn. pinocamphone) and beta–pinene, all together covering 65,03 % of the total oil. Eleven compounds present in the oil in the range between 8,35 and 1,09 %, and listed in descending order of their contribution to the oil, were: beta–phellandrene, germacrene-d, *trans*–beta-caryophyllene, beta-myrcene, bicyclogermacrene, alloaromadendrene, linalool, beta-bourbonene, sabinene, elemol and myrtenol, altogether representing other 28,04% of the oil. Remaining 16 components, with their presence in the oil with less than 1,00 %, accounted for the remaining 6, 24% of the oil (Table 1).

Table 1. Chemical composition of tested essential oils of thyme, hyssop and wintergreen.

RI*	Compounds	Thyme	Hyssop	Wintergreen
920	α-Thujene	0.05	0.34	*
925	α-Pinene	2.01	0.86	0.22
939	Camphene	0.66	0.15	*
962	Sabinene	*	1.65	0.08
967	β-Pinene	0.48	14.84	0.25
973	cis-m-Mentha-2,8 diene	0.02	*	*
974	p-Mentha-1(7),8-diene	0.07	*	*
978	trans-p-Menthane	0.13	*	*
986	β-Myrcene	0.85	2.60	0.09
1012	α-Terpinene	*	0.18	*
1019	p-Cymene	35.26	0.22	*
1019	Limonene	1.28	*	2.17
1021	β-Phellandrene	*	8.35	*
1023	1,8-Cineole	0.83	0.45	*
1031	trans-β-Ocimene	*	0.87	*
1046	m-Diethylbenzene	0.09	*	*
1049	γ-Terpinene	*	0.35	*
1084	Fenchone	*	*	0.17
1096	Linalool	5.26	1.75	*
1126	Terpineol	0.3	*	*
1131	trans-Pinocarveol	*	0.67	*
1136	cis-β-Terpineol	1	*	*
1147	iso-Borneol	0.53	*	*
1150	Menthone	*	*	0.12
1152	trans-Pinocamphone	*	16.17	*
1156	Borneol	1.96	*	*
1166	cis-Pinocamphone	*	34.02	*
1168	Terpinen-4-ol	0.09	*	*
1183	α-Terpineol	9.3	*	*
1189	γ-Terpineol	2.63	*	*
1190	Myrtenol	*	1.09	*
1198	Methyl salicylate	*	*	96.9
1241	trans-2-Hydroxypinocamphone	*	0.14	*
1250	Linalool acetate	*	0.04	*
1290	Thymol	26.24	*	*
1298	Carvacrol	7.91	*	*
1318	Myrtenyl acetate	*	0.12	*
1374	β-Bourbonene	*	1.74	*
1398	α-Gurjunene	*	0.48	*
1408	β-Caryophyllene	0.93	2.68	*
1418	b-Copaene	*	0.16	*
1442	α-Humulene	0.08	0.53	*
1449	Alloaromadendrene	*	1.87	*
1478	Germacrene D	*	2.91	*
1485	Bicyclogermacrene	*	2.27	*
1540	Elemol	*	1.14	*
1567	Spathulenol	*	0.67	*
1570	Caryophyllene oxide	0.73	*	*
Total compounds identified (%)		*98.69*	*99.32*	*100.00*

RI – Retention index*

Wintergreen (*Gaultheria procumbens* L., fam. Ericaceae) is a plant species whose essential oil is produced by steam distillation of the leaves, after they have been macerated for a certain period of time in warm water. Methylsalicylate is not present in the plant but it is present in the oil since it is formed by enzymatic action from a glycoside within the leaves during their maceration in warm water, prior to oil distillation. The oil is a pale yellow or pinkish liquid that is strongly aromatic and has distinctive "medicinal" smell. In the commercial sample originating from China, used in the experiment, methylalicylate represented almost 97% of the oil, and together with limonene, with its contribution to the oil above 2%, those two compounds covered 99,07% of the entire oil. Six other remaining components (bellow 1%) detected in the oil accounted for 0,93 % of the oil. In the literature, it is stated that in tests conducted with the application of wintergreen essential oil, brood and adult mortality of bees were small [10].

Table 2. Residual contact toxicity on bee adults caused by application of different variants (doses) of common thyme, hyssop and wintergreen essential oils.

No.	Variants	Dose in µl / Petri dish Ø 9cm (254.34 cm^2)	Mean number of dead bees after 24	Mean number of dead bees after 48h
1.	Common thyme	0.005	1.50	3.00
2.	Common thyme	0.01	.75	1.50
3.	Common thyme	0.05	0.25	3.25
4.	Common thyme	0.100	0.25	1.75
5.	Common thyme	0.500	0.50	0.75
6.	Common thyme	1.000	0.25	2.75
7.	Common thyme	2.500	0.25	2.50
8.	Common thyme	5.000	4.25**	5.50**
9.	Common thyme	10.000	8.25**	9.50**
10.	Common thyme	20.000	8.75**	9.75**
11.	Hyssop	0.005	0.25	1.25
12.	Hyssop	0.010	0.25	2.00
13.	Hyssop	0.050	0.25	1.50
14.	Hyssop	0.100	0.25	1.00
15.	Hyssop	0.500	0.25	1.25
16.	Hyssop	1.000	0.25	0.25
17.	Hyssop	2.500	0.25	2.25
18.	Hyssop	5.000	0.50	1.25
19.	Hyssop	10.000	6.75**	8.75**
20.	Hyssop	20.000	7.75**	8.25**
21.	Wintergreen	1.000	0.50	1.50
22.	Wintergreen	2.500	0.25	0.25
23.	Wintergreen	5.000	0.25	0.25
24.	Wintergreen	8.000	0.25	1.00
25.	Wintergreen	10.000	1.25	1.50
26.	Wintergreen	20.000	2.50*	3.75**
27.	Control	0.000	0.00	0.00
			LSD005= 2.18 *LSD001= 2.89*	*LSD005= 2.75* *LSD001= 3.64*

*** P<0.01; * P<0.05*

Table 2., presents the results of the residual contact residual toxicity of the essential oils of common thyme, hyssop and wintergreen, applied at different doses After 24 hours common thyme oil showed the most pronounced toxic effects on the surface of Petri dishes, applied at doses of 5, 10 and 20 µl. The average number of dead bee adults per replication for these

three doses was 4.25, 8.25 and 8.75, respectively, what made them significantly different in comparison to other variants. Hyssop essential oil applied at a dose of 10 µl and 20 µl also showed a strong contact residual toxicity, but slightly lower in comparison to thyme oil, and the average number of dead individuals per repetition was 6.75 and 7.75, respectively. The lowest contact residual toxicity among tested oils showed wintergreen oil, and significant contact residual toxicity compared to other variants was observed at its highest dose applied (average number of dead adults was 2.5 per replication). Following 48 hours toxic effects in almost all variants was even more pronounced. The highest and very significant contact residual toxicity showed thyme oil, applied at doses of 5, 10 and 20 µl, causing death of 5.5, 9.5 and 9.75 adults, respectively; hyssop oil applied at doses of 10 and 20 µl also had very significant contact residual toxicity, causing death of averagely 8.75 and 8.25 dead adults, respectively. Comparing to previous two oils, wintergreen oil is distinctly less toxic, and very significant contact residual toxicity was achieved only with the highest dose applied, where the mean value of the dead adults per replication was 3.75.

Compared with the chemical composition of the "Pinocamphone" chemotype of the thyme oils used in the work of Imdorf et al. [12], our oil, regardless it was a same chemotype, had different contents of *para*-cymene, thymol, linalool and alpha-terpineol and contained higher contents of beta-pinene, beta-phellandrene and *trans*- and *cis*-pinocamphones, while the wintergreen oil had a standard high content of methylsalicylate.

In our research, thymol showed the most pronounced toxic effects, as it was also confirmed in the work of Ellis and Baxendale [22], where the authors presented results of the toxic effects of thymol on honey bees and *M. domestica*. However, Imdorf et al. [12] state that the bees are tolerant at thyme essential oil, highlighting the tolerance of bees at para-cymol (syn. para-cymene), the most abundant compound in our common thyme oil. Although the bees showed good tolerance to hyssop oil of the "pinocamphone" type [12], in our study, significant mortality of bees was observed in the application of higher doses of hyssop oil. In relation to the oil used in experiment conducted by Imdorf et al. [12], our hyssop oil contained a lower percentage of pinocamphone and much more of beta-phellandrene. The bees showed the best tolerance at wintergreen essential oil, as in the work conducted by Imdorf et al. [12]. Applied on the surface of Petri dishes, doses of thyme oil (5, 10 and 20 µl), hyssop oil (10 and 20 µl), and wintergreen oil (20 µl), caused very significant contact residual toxicity. Rice and Coats in their paper [23] reported that monoterpenoids' potencies vary considerably and that minor structural differences can cause significant differences in the contact residual toxicity of essential oils. Imdorf et al. [10] stated that for their further study they used the oils that proved to cause less mortality to the bees (bellow 10%).

In our further research we will use the essential oils tested at doses that will meet all the above stated conditions and at the same time hindering presence and development *Varroa destructor*. Due to the occurrence of resistance in varroa mites in the application of synthetic chemicals and presence of their residues in the bee wax [9, 24, 25], it is important to implement an integrated approach to combat harmful organisms.

CONCLUSION

Common thyme essential oil applied at doses between 1 and 20 µl caused contact residual toxicity to bees at an unacceptable level. Hyssop essential oil applied at doses of 2.5 to 20 µl had unacceptable toxic effects to the bees.

Wintergreen essential oil applied 10 and 20 µl had toxic effects which caused an unacceptable level of mortality in bees. Further studies of toxic effects of these essential oils to the bees, will be conducted in laboratory conditions, and the doses applied in order to fight *Varroa destructor* will be such to provide a normal development of the bees.

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Original scientific paper

FIRST RECORD OF POWDERY MILDEW ON CAMOMILE IN SERBIA

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SUMMARY

German chamomile (*Matricaria recutita L.*) is a well-known medicinal plant species from the Asteraceae family which has been used since ancient times as folk drug with multitherapeutic, cosmetic, and nutritional values. On the plantation (14 hectares) located in northern Serbia (Pancevo), as well as on the wild plants in the vicinity of Belgrade, the powdery mildew was observed on all green parts of chamomile plants in spring during 2010 and 2011.

The first symptoms were manifested as individual, circular, white spots of pathogens mycelium formed on the surface of stem and both sides of the leaves. Later on, the spots merged and dense mycelia completely covered all parts of infected plants. The consequence of this disease is the destruction of foliage, which prevents obtaining of high-quality herbal products for pharmaceutical purposes. Based on the morhological characteristics the pathogen was determined as *Golovinomyces cichoracearum* (syn. *Erysiphe cichoracearum*). It is already known as a pathogen of chamomile, but for the first time is described in Serbia.

Key words: *chamomile*, *Matricaria recutita*, *disease, powdery mildew*, *Golovinomyces cichoracearum*

INTRODUCTION

German chamomile (*Matricaria recutita L*) is one of the most favored medicinal plants in the world. It is native to southern and eastern Europe, though naturalized as a weed throughout North America [1]. Chamomile has been used since ancient times as folk drug with multitherapeutic, cosmetic, and nutritional values. Over centuries, the popularity of these plants increased and spread to different parts of the world and it has been cultivated in many temperate countries including German, Hungary, Slovakia, Czech Republic, France, Russia, ex Yugoslavia, Brazil, Kashmir, Lebanon, Argentina and Colombia, northern Africa, Asia, and the United States [1, 2]. In Serba, chamomile is cultivated at more than 500 ha with tendency of increasing areas with this plant. At plantations of the Institute for Medicinal Plants Research, Belgrade, chamomile is cultivated at 14 ha in vicinity of the city Pancevo, (about 20 km north from Belgrade).

On the plantation in Pancevo, as well as on the wild plants in found in vicinity of Belgrade, the powdery mildew was observed on all green parts of chamomile plants in spring during 2010 and 2011. The disease symptoms and morphological characteristics of the pathogen are presented in this article.

MATERIAL AND METHODS

The chamomile plants with symptoms were collected from Apri, when the first symptoms appeared, till the end of the vegetation season. Microscopic examination of morphological characteristics was done using Olympus microscope at 1000 x magnification. Dimensions of the reproductive structures were obtained by measuring 50 chasmothecia and asci and 100 ascospores and conidia. Photos were made by Olympus digital camera.

RESULTS AND DISCUSION

The first symptoms were manifested as individual, circular, white spots of mycelium formed on the surface of stem and both surfaces of the leaves. Later on, white mycelial structure completely covered the whole plant including the inflorescence (Fig. 1-3). Infected leaves become chlorotic and wither. The consequence of this disease is the destruction of foliage, which prevents obtaining the high-quality herbal products for pharmaceutical purposes.

Figure 1-3: Stems and leaves of *Matricaria chamomilla* infected with *Golovinomyces cichoracearum*

Long conidiophores produced 2-(5)-6 ellipsoid, hyaline conida (Fig. 4), of 28- 44 x 15-25 µm, without distinct fibrosin bodies. Chasmothecia are formed in the mid-May,. mainly on stems and adaxial surface of the leaves. They are pale yellow in the beginning, later on turn to dark brown, scattered or grouped, sphaerical, of 94-144µm in diameter (Fig. 5). The appendages are mycelioid hyaline. Up to 12 asci (Fig. 6), of 52-68 x 30-46 µm, with two ascospores are formed in chasmothecia. Ascospores were ellipsoid, of 20−30 x 16−18 µm. Based on these characteristics, this fungus was identified as *Golovinomyces cichoracearum* (syn. *Erysiphae cichoracearum*) [3].

The following fungi are known to attack the chamomile: *Albugo tragopogonis*, *Cylindrosporium matricariae*, *Erysiphe cichoracearum*, *E. polyphage*, *Helicobasidium purpureum*, *Plysmopara leptosperma*, *P. radii*, *Phytophthora cactorum*, *Puccinia anthemedis*, *P. matricaiae*, *Septoria chamomillae*, *Sphaerotheca macularis, Stemphylium botryosum* and *Fusarium* spp [1, 2, 4]. Species from the genera *Fusarium* (*F. verticilliodes*), *Aspergillus, Alternaria* and *Penucillium* were identified on commercial chamomile seeds [5].

Besides damaging the cultivated crop of chamomile, fungi from the genera *Aspergillus, Penicillium, Fusarium* and *Rhizopus* also cause extensive damage to the dry flowers during

storage and reduce the quality of the dried raw product. Also, there is a risk that the stored product will be contaminated with mycotoxins, which are a health hazard [2].

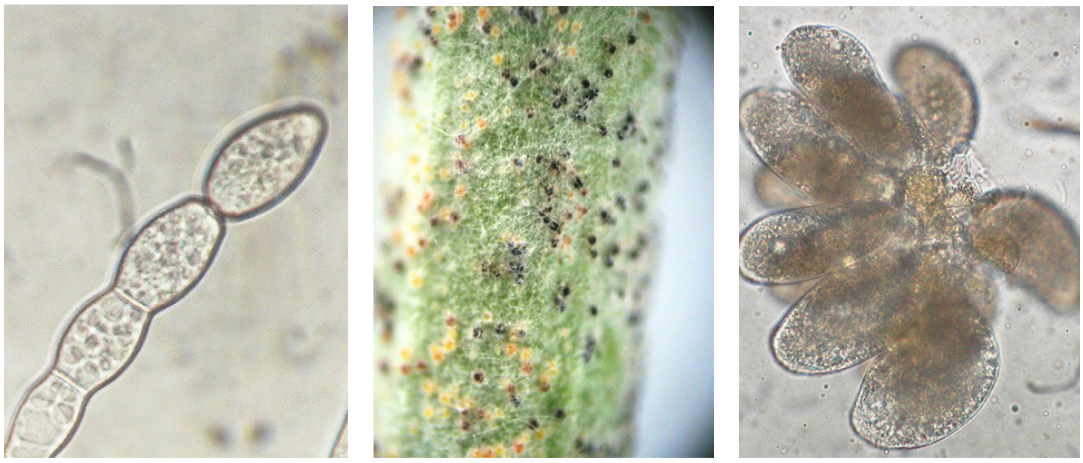

Fig. 4-6. *Golovinomyces cichoracearum.* Conidia (Fig. 4); Chasmothecia formed on infected stem (Fig. 5); Immature asci (Fig.6)

Two powdery mildew pathogens (*Podosphaera fusca /Sphaerotheca fusca, S. fuliginea/* and *Golovinomyces cichoracearum* /syn. *Erysiphe cichoracearum/*) have been reported on *Matricaria recutita* [3]. The first pathogen has been recorded in Canada, Egypt, Germany, Switzerland, USSR, Japan and another one is a rather common chamomile powdery mildew species in Europe and Japan [5]. This article is the first report of powdery mildew on chamomile caused by *G. cichoracearum* in Serbia.

CONCLUSION

The powdery mildew was observed on all green parts of cultivated German chamomile (*Matricaria recutita L.*) in Pancevo, as well as on the wild plants collected from sites in the vicinity of Belgrade, in the spring during 2010 and 2011. The first symptoms were manifested as individual, circular, white spots of pathogens mycelium formed on the surface of stem and both sides of the leaves. The consequence of this disease is the destruction of foliage, which prevents obtaining the high-quality herbal products for pharmaceutical purposes. Based on morhological characteristics, the pathogen was identified as *Golovinomyces cichoracearum* (syn. *Erysiphe cichoracearum*). This fungus species is already known as the pathogen of German chamomile, but for the first time is described for Serbia.

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Original scientific paper

THE EXPRESSION OF HETEROSIS IN THE PERSPECTIVE F_1 POLYCROSS HYBRIDS OF THE *LAVANDULA ANGUSTIFOLIA* MILL.

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SUMMARY

The quantitative traits and heterosis effect of F_1 polycross hybrids of *Lavandula angustifolia* Mill. has been studied and carried out the selection of the perspective hybrids. The height of a plant variety from 38.0 cm. to 75 cm, the length of inflorescence stem is ranged from 12.4 to 24.0 cm, the number of whorls ranges from 5 to 9. The content of essential oil varies between 3.150% and 5.790% (dry matter). The effect of heterosis in different hybrids were detected at different level, such as: the height of the plant from 1.9% to 14.0%, the number of inflorescences from 35.0% to 155.0%, from 1.3% to 39.0% for the length of inflorescences stems, and the content of essential oil is from 6.1% to 101%. The effect of heterosis in F_1 polycross hybrids of lavender is obviously expressed by the height of the bush, number of the inflorescences, and the content of the essential oil. The most perspective hybrid for selection of new variety-clone is hybrid polycross Fr.5S-8-24.

Key words: *Lavender, hybrid, heterosis, essential oil.*

INTRODUCTION

Lavender is the most important aromatic, medicinal and ornamental plant that, for long times, were used for different goals in the medicine and everyday life. The origin of Lavender is believed to be from the Mediterranean region – the South of France, Italy, Spain and North Africa [1, 2, 3]. In the 16th and 17th centuries lavender was grown in England and France as the cultural plant. In Bessarabia lavender has become known as ornamental plant in 1857, but the first industrial plantations were appeared since 1948 [1].

The genus Lavandula L. belongs to the Lamiaceae family, and includes 30 species [1, 3]. The most common are: *Lavandula angustifolia* Mill (*L. officinalis* Ch., *L. vera* D.C.) and *Lavandula latifolia* Will. (*L. spica* DC; *L. stoechas* L.) [4, 6]. Lavandula angustifolia Mill is characterized by long, narrow leaves, with pale green-grey color. The corolla may have different colors: white, blue, pink or violet [2, 3]. One of the most important lavender's properties is synthesis and accumulation of essential oil. The smell of L. angustifolia essential oil is considered more refined than other species of lavender [1, 2, 3, 4]. The main purpose of lavender cultivation is the essential oil. Lavender's oil largely is used in pharmacy and medicine. Pharmacological researches show that the essential oil has sedative, antiseptic and antioxidant effects [4,5,6,7].

The lavender breeding in the Republic of Moldova achieved high results. Thus, were created and homologated a lot of varieties as: Moldoveanca-4, Alba-7, Vis Magic-10 [3], De Chisinau-90, De Chisinau- 32 [8, 9]. For the following successful breeding is necessary to create new initial material, using the heterosis effect of F1 hybrids. The main goal of this research was studying of the heterosis effect on the policross F1 hybrids and selection of promising genotypes for future variety-clones.

MATERIALS & METHODS

The biological materials that are used in this research include 90 polycross F_1 hybrids of *L. angustifolia*. For polycross as maternal forms were served the French (Fr.-5) and Ukrainian genotype (Cr.-13; Cr.-26). The planting scheme was: 1 x 0,5 m. Researches were carried out in 2011 that corresponds to the third year of vegetation, when the bushes have already been formed and were visible differences between policross hybrids regarding the quantitative traits of the bush and inflorescence. In this period of time was possible to determine the effect of heterosis compared with maternal forms. The following characteristics were evaluated: plant height, the number of inflorescences per plant, length of peduncle and inflorescence, number of whorls in inflorescence.

The essential oil was separated from fresh collected material using hydrodistillation in Ginsberg apparatus and the oil composition was recalculate to dry matter. After distillation the essential oil was dried with Na_2SO_4 and was preserved in the freezer. Qualitative and quantitative composition of essential oil was determined by gas-chromatographic analysis in tandem with the mass spectrometry (GC-MS). The analysis equipment included: gas-chromatograph Technologies Agilent 7890 equipped with Selective Mass Detector with Quadruple MSD Agilent Technologies 5975C, capillary column (30 м/0.25 мм/0.25 μм) with non-polar stationary phase HP-5ms. Analysis was performed at a temperature of 250 ° C injector and detector - 280 ° C, using a temperature gradient from T1 = 70 ° (2 min), T2 = 200 ° C (5 ° C / min), T3 = 300 ° C (20 ° C / min, 5 min). Mobile phase: Helium 1ml/min, injected volume - 0.03 ml essential oil, split rate - 1:100. Identification of chromatographic peaks was performed using the software package AMDIS ™, coupled with the NIST database.

The heterosis effect is expressed on the *percentage,* compared with maternal form. For hybrids classification depending on the length of vegetation period, the phonological observations were carried out. Were observed the all phonological phases: growth, bud formation, flowering and technical ripeness.

REZULTS & DISCUSSION

The great importance in the creation of new varieties of *L. angustifolia* with the high yield of inflorescences and high content of essential oil has the use of heterosis hybrids of the first generation. During the heterosis was observed the increase of the power, viability and productivity of F_1 hybrids, compared with maternal forms.

The effect of heterosis expressed by the perspective F_1 policross hybrids, by the quantitative features of the bush, compared with maternal forms has been defined. (Table 1). Hybrids vary a lot by the quantitative traits of the bush, which determined the productivity of plantations and the quality of products. For example, the height of hybrid plant is about 50-75 cm. The heterosis effect of this trait varies widely: from negative to high positive (Table1). Regarding to the bush height, the heterosis effect is positive for 12 studied hybrids and is from +1,3% to +39,0%. The highest heterosis effect has the hybrids Cr. 13S-6-4, Cr. 13S-6-31, Cr. 13S-6-35, that is +14,0% and Fr. 5S-8-24, with +38,1%. The polycross hybrids Fr. 5S-8-16, Fr. 5S-8-3, Fr. 5S-8-24, Cr.26S -9-6, Cr. 13S-6-35 have been exceeded the maternal forms by the trait “number of inflorescences per plant”. The effect of heterosis for 10 polycross hybrids was from +35,6% (Cr.13S-6-41) to +155,0% (Fr. 5S-8-24). Analyze of the hybrids plants by the inflorescences length, was revealed that the half of hybrids has a positive heterosis effect compared with maternal forms Fr.-5 and Cr. -26. According to this trait, the highest heterosis effect (+28,3%) has the hybrid Fr. 5S-8-24 (Figure 1).

The main selection criteria of 19 hybrids included in Table 1, 2 was the high quantity of essential oil in inflorescences that is from 2,78% to 5,79% (dry matter), compared with

maternal forms, that is from 2,58% to 2,87% (dry matter) (Table 2). This trait determines not only the productivity of the plantations and the quality of initial materials, but also the profitability of culture. The heterosis effect on the content of essential oil in the percentage varies from +6,7% to +101,3%. F1 policross heterosis hybrids as well as Fr. 5S-8-3 (+53,7%); Fr.5S-8-2 (+80,1%); Cr. 26S-9-4 (+85%) and Fr. 5S-8-24 (+101,3%) are usually used for creation of clones with a high content of essential oil.

Figure 1. The best perspective F_1 polycross hybrid Fr. 5S-8-24

Table 1. The heterosis effect by quantitative characters of bush at policross hybrids F1 in relation with maternal forms

Maternal forms, hybrids	The height of the bush, см	The effect of heterosis, %	Number of inflorescences on plant	The effect of heterosis, %	Length of peduncle, см	The effect of heterosis, %
Fr.-5 m.f.	54.3	-	323.0	-	18.7±0.7	-
Fr.5S-8-5	50.0	-7.9	139.0	-56.9	13.9±1.4	-25.7
Fr.5S-8-8	55.0	+1.3	184.0	-43.0	17.3±1.4	-7.5
Fr.5S-8-12	55.0	+1.9	118.0	-63.0	21.4±2.1	+14.4
Fr.5S-8-13	38.0	-30.0	145.0	-55.1	19.4±2.2	+3.7
Fr.5S-8-16	60.0	+10.5	664.0	+105.6	21.7±3.7	+16.0
Fr.5S-8-19	58.0	+6.8	155.0	-52.0	10.6±1.9	-43.0
Fr.5S-8-24	75.0	+38.1	825.0	+155.0	24.0±3.7	+28.3
Fr.5S-8-3	55.0	+1.9	490.0	+53.1	12.5±1.9	-33.2
Fr.5S-8-2	50.0	-7.9	465.0	+49.9	16.5±1.4	-11.8
Cr.26 m.f.	55.4	-	320.0	-	13.3±2.9	-
Cr.26S-9-4	60.0	+8.3	258.0	-19.4	20.1±0.8	+13.6
Cr.26S-9-6	55.0	-0.7	538.0	+68.1	16.0±1.4	+20.3
Cr.26S-9-2	49.0	-11.6	144.0	-55.0	17.7±3.1	-5,3
Cr.26S-9-11	62.0	+11.9	478.0	+49.4	16.7±1.5	+27.1
Cr.13 m.f.	57.0	-	435.0	-	20.2±3.1	-
Cr.13S-6-4	65.0	+14.0	480.0	+10.3	18.5±1.7	-8.4
Cr.13S-6-31	65.0	+14.0	341.0	-21.6	16.0±1.3	-20.7
Cr.13S-6-35	65.0	+14.0	808.0	+85.0	17.8±1.5	-11.8
Cr.13S-6-41	60.0	+5.3	590.0	+35.6	16.2±0.9	-19.8
Cr.13S-6-43	55.0	-3.5	685.0	+57.5	13.2±1.4	-34.6

The analyses have shown that only Fr.5S-8-24 hybrid has significantly higher indicators, such as length of peduncle, number of whorls and essential oil content, than maternal form Fr.5 (Table 2).

The essential oil of lavender is frequently used in aromatherapy systems worldwide. The first detailed studies of chemical composition of essential oil of lavender were carried out in the 19th century. Pharmacological studies have shown that the essential oil of lavender has sedative, anticonvulsant and antispastic effect that promote active wound healing, with full regeneration of the epidermal cells, as well as chemical burns. As a result of today's researchers the oil revealed more than 50 organic compounds, most of which belong to the terpenes [4].

In time of studying of qualitative and quantitative composition of essential oil compared with maternal forms were established that qualitative and quantitative composition of essential oil of maternal forms and F_1 polycross hybrids differ (Table 3).

Maternal forms differ from policross hybrids on qualitative and quantitative composition of essential oil. So, in the maternal form Fr.5 was identified 26 compounds, and the main of them are linalool (34,196%), linalool-acetate (31,497%) and 4-terpenol (8,449%). In the essential oil of maternal form Cr-26 were identified 24 compounds, and the main are the same three compounds: linalool (39,554%), linalool acetate (35,265%) and 4-terpenol (9,721%).

Table 2. Heterosis effect of *Lavandula angustifolia* F_1 policross hybrids by quantitative characters of inflorescences in relation to the maternal forms

Maternal form/ hybrid	Length of inflorescences, см	The effect of heterosis, %	Number of verticiles	The effect of heterosis, %	Essential oil content, %	The effect of heterosis, %
Fr.-5 m.f.	9.4±2.7	-	7.2±1.3	-	2.876	-
Fr.5S-8-5	8.4±0,7	-13.4	8.0±0.6	+11.1	3.070	+53.7
Fr.5S-8-8	7.1±1.4	-26.8	8.0±0.8	+11.1	3.110	+6.7
Fr.5S-8-12	8.5±1.9	-12.8	5.7±0.7	-20.8	3.150	+8.1
Fr.5S-8-13	10.5±1.7	+8.2	5.8±1.8	-19.4	3.860	+9.5
Fr.5S-8-24	10.1±1.5	+4.1	7.0±0.7	-2.8	4.320	+34.2
Fr.5S-8-19	5.8±0.9	-40.2	6.6±0.5	-8.3	3.390	+50.2
Fr.5S-8-3	7.7±1.5	-20.6	6.9±0.6	-4.2	4.420	+38.7
Fr.5S-8-24	13.5±2.5	+39.2	9.9±0.7	+37.5	5.790	+101.3
Fr.5S-8-2	4.6±1.6	-52.6	7.0±1.6	-2.7	5.180	+80.1
Cr.-26 m.f.	11.4±2.7	-	6.9±1.1	-	2.588	-
Cr.26S-9-2	9.8±2.4	-14.0	6.8±0.5	-1.4	2.330	-9.9
Cr.26S-9-4	6.6±4.6	-42.1	5.8±0.8	-15.9	4.800	+85.4
Cr.26S-9-6	8.4±2.1	-26.3	5.5±0.9	-20.3	4.430	+71.2
Cr.26S-9-11	5.8±1.3	-49.1	4.9±0.3	-37.6	2.780	+7.4
Cr.-13 m.f.	7.5±1.0	-	5.9±1.2	-	2.763	-
Cr.13S-6-4	7.6±1.2	+1.3	5.9±0.6	-	4.770	+72.0
Cr.13S-6-11	4.9±1.2	-34.7	6.0±1.4	+1.6	3.980	+44.0
Cr.13S-6-31	4.6±0.9	-38.7	5.6±0.6	-5.1	3.310	+19.7
Cr.13S-6-35	5.9±1.3	-21.3	6.9±0.6	+16.1	3.390	+22.7
Cr.13S-6-41	9.1±3.2	+21.3	6.1±0.6	+3.4	3.860	+39.7
Cr.13S-6-43	8.7±2,7	+16.0	6.4±0,8	+8.5	3.620	+31.0

The analyze of the quantitative and qualitative composition of essential oil for policross hybrids, revealed that there are differences not only between them and maternal forms, but even between themselves. So, F1 hybrids, which maternal form is Fr.-5 have the most number of identified compounds in essential oil -26, then hybrids of Cr.-26 maternal form.
It is known that the main components of essential oil are linalil-acetate and linalool. Linalil-acetate gives an exquisite flavor to lavender oil, and his presence affects the perfume value. The higher percentage of linalil-acetate permit to obtain the higher quality of the essential oil is [6].

Table 3. The qualitative and quantitative composition of essential oil of polycross hybrids *Lavandula angustifolia* in relation with maternal forms

Components	Maternal forms		Polycross hybrid F_1			
	Cr.-26	Fr.-5	Cr.26S57	Cr.26S-9-4	Fr.5S-8-24	Fr.5S-8-2
n-Octene-l-ol	0.235	0.186	0.566	0.924	0.213	0.221
3-Octene		0.399				0.184
β-Mircene	0.265	0.365			0.249	0.365
3-Octanol		0.194				
3-Carene		0.35		0.601	0.157	
Limonene	0.197		0.351			
Eucaliptol	0.437	0.229	0.629	0.844		1.459
trans-Ocimene	1.599	1.501	2.394	1.196	0.517	0.301
cis-Ocimene	0.918	0.535	0.849	0.315	0.554	0.33
γ-terpinene	0.128					
Hexylacetat	0.145					0.124
cis-Linalool oxide		0.227				
α-Terpinene	0.128	0.31			0.17	0.198
Linalool	39.554	34.196	61.16	57.361	37.25	50.542
n-Octene-lyl- acetat	0.605	1.23		0.751	0.765	0.49
Camphor	0.166	0.753		0.453	0.186	0.524
Borneol	0.596	2.491	1.244	3.396	0.374	1.401
4-Terpineol	9.721	8.449	9.969	3.641	1.718	4.654
α-Terpineol						0.143
Nerol	3.551	4.11	2.529	2.433	3.899	3.691
Linalylacetate	0.432	0.47		0.246	0.494	0.421
Bornylacetate	35.265	31.497	15.507	20.68	44.713	27.676
Lavandulylacetate	0.135	0.918			0.271	0.825
Nerylacetat	0.981	4.511	0.704	3.051	0.707	0.789
Geranylacetat				0.205		
β-Caryophilene	0.555	0.74	0.374	0.353	0.699	0.704
Germacrene D	0.976	1.417	0.665	0.594	1.26	1.209
Caryophilene oxid	1.265	2.609	1.334	2.044	3.598	2.159
β-Cadinene	0.527	0.255	0.428		0.737	0.138
n-Octene-l-ol	0.201	0.89		0.345	0.551	0.648
3-Octene		0.552				0.186
Identified compounds	24	26	15	19	21	25
Total, %	98.582	99.384	98.703	99.433	99.082	99.382

There is an international gradation of the linalil-acetate content that is the main component of essential oil in Lavandula angustifolia. For perfumery purposes is recommended 50% of linalil-acetate, for cologne and toilet water- 40%, for production of soap-30% [6]. In this context, Fr.5S-8-24 policross hybrid is used in perfume industry, it contains -44,713% of linalil-acetate, and the concentration of this compound is higher than as at maternal forms as at other F_1 polycross hybrids.

According to already existed international gradation of essential oil of lavender by the main component linalil-acetate, can be affirm that the essential oil of F1 Frr.5S-8-24 hybrid can be used in perfumery industry for production of toilet water and cologne (linalil acetate higher than 44,713%). F_1 policross hybrid Fr.5S-8-2 and Cr.26S-9-4 have less linalil-acetate than maternal forms, and can be used in industry of soap production.

Accumulation of essential oil in inflorescences depends, first of all, on ripening time, that's why production needed new varieties and hybrids with different time of ripening that will permit to harvest each varieties at the optimal time, when the content of essential oil can achieve the highest rate. The time of harvest affect the quality of lavender essential oil too, the main indicator that is linalil-acetate.

The presence of the varieties with different period of ripening (earlier, medium and later), is an advantage also because it permit to extend the period of lavender harvest. So, you can increase the plantation area without increasing the production capacity for processing and apply the cleaning conveyor.

The study of phenological phases of development has shown that F1 hybrids are different not only on phases of growth and bud formation, but also on period of mass flowering. These differences allowed to dividing hybrids by ripening time. For example: early ripening hybrid - Fr.5S-8-24, middle ripening hybrid- Fr.5S-8-8 and late ripening hybrid – Fr.5S-8-3.

The study of the heterosis effect of selected hybrids remarked that all 19n F1 policross hybrids have a positive heterosis effects on different features compared with maternal forms. Only Fr.5S-8-24 hybrid exceeded maternal form by all quantitative traits of the bush and inflorescences. In this case the heterosis effect achieved the highest level on all above mention traits (Table 1, 2). The content of linalil-acetate for F1 hybrid- Fr 5S-8-24 is 44,713%. The effect of heterosis of the Fr.5S-8-24 hybrid by the height of bush is +38,1%, by the quality of inflorescences is +155% and by the length of peduncle is +28,3%. Regarding to the length of inflorescence heterosis effect is +39,2% and on quantity of whorls is +37,5%, at this hybrid the heterosis effect is positive too. The content of essential oil at F1 policross hybrid Fr.5S-8-24 of L. angustifolia Mill is 5,790%, and the heterosis effect achieved +101%. The content of linalil acetate of this hybrid is 44,713%.

The result of this study revealed that the best F1 policross hybrid is Fr.5S-8-24. All studied hybrids - 19 exceeded the maternal forms by the content of essential oil and have a positive heterosis effect. For the most of hybrids, the heterosis effect of main quantitative characteristics of bushes and blossoms is positive. According to the international gradation of essential oil, the content of the main component – linalil-acetate is higher only in the Fr.5S-8-24 hybrid.

CONCLUSIONS

- The quantitative traits and heterosis effect of F_1 polycross hybrids of *Lavandula angustifolia* Mill. has been studied and carried out the selection of the perspective hybrids. The height of a plant variety from 38.0 cm. to 75 cm, the length of inflorescence stem is ranged from 12.4 to 24.0 cm, the number of whorls ranges from 5 to 9. The content of essential oil varies between 3.150% and 5.790% (dry matter).

- The effect of heterosis in different hybrids were detected at different level, such as: the height of the plant from 1.9% to 14.0%, the number of inflorescences from 35.0% to 155.0%, from 1.3% to 39.0% for the length of inflorescences stems, and the content of essential oil is from 6.1% to 101%.
- The effect of heterosis in F_1 polycross hybrids of lavender is obviously expressed by the height of the bush, number of the inflorescences, and the content of the essential oil. The most perspective hybrid for selection of new variety-clone is hybrid polycross Fr.5S-8-24.
- The heterosis effect of Fr.5S-8-24 hybrid by the height of bush is +38,1%, by the quality of inflorescences is +155% and by the length of peduncle is +28,3%. For the features "length of inflorescence" the heterosis effect is +39,2% and "quantity of whorls" is +37,5%.
- Fr.5S-8-24 polycross hybrids has the highest content of essential oil – 5,790% (dry matter), and the heterosis effect by this feature is +101%. The content of linalil acetate of this hybrid is 44,713%.

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Original scientific paper

XANTHONE PRODUCTION IN *HYPERICUM PERFORATUM* HAIRY ROOT CULTURES TRANSFORMED WITH *AGROBACTERIUM RHIZOGENES* A4

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SUMMARY

Hypericum perforatum is a well-known medicinal plant which contains various secondary metabolites, including xanthones. Xanthones have a wide range of biological and pharmacological properties, such as antiradical, anti-inflammatory, cancer-chemopreventive, hepatoprotective, cardiovascular protective and cytotoxic activities. Investigations have been made to study the xanthone production in *H. perforatum* hairy root cultures genetically transformed with *Agrobacterium rhizogenes* agropine-type A4 strain (pRiA4). Hairy root induction with pRiA4 was effectively occurred at a transformation frequency of about 33%. The transgenic nature of the selected HR cultures was confirmed through PCR analysis by the presence of *rol*B sequences from T_L-DNA of *A. rhizogenes* Ri plasmid. *H. perforatum* hairy root lines showed a homogeneous morphology and similar growth patterns among individual root clones, even that each root clone arose from a separate transformation event. Transformed roots exhibited active elongation with high branching and plagiotropic growth when cultured on phytohormone-free medium. The HPLC/DAD/ESI-MS^n technique was used to analyze the xanthone production in *H. perforatum* hairy roots. Twenty eight xanthones were detected in the methanolic extracts from *in vitro* biomass of *H. perforatum* transformed and untransformed roots and 22 of them were fully identified by ESI-MS. HPLC chromatograms of xanthones in transformed roots confirmed the increase of ten xanthones (1,3,5,6-tetrahydroxyxanthone; 1,3,6,7-tetrahydroxyxanthone; 1,3,6,7-tetrahydroxyxanthone 8-prenyl xanthone; 1,3,7-trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone; toxyloxanthone; 1,3,7-trihydroxy-6-methoxy-8-prenyl xanthone; 1,3,6,7-tetrahydroxyxanthone 2-prenyl xanthone; 1,3,6-trihydroxy-7-methoxy-8-prenyl xanthone; trihydroxy-1-metohy-*C*-prenyl xanthone and garcinone E). Four major *de novo* synthesized xanthones in hairy roots have been identified as 1,3,5,6-tetrahydroxyxanthone dimer; 1,3,6,7-tetrahydroxyxanthone dimer; γ-mangostin isomer and garcinone C. Production of xanthones can also be affected by *Agrobacterium* mediated transformation as *rol* genes act as potential activators of secondary metabolism via transcriptional activation of defence genes in transformed plant cells. The present study demonstrates that *H. perforatum* hairy roots can offer a valuable source of xanthones that are useful as pharmaceuticals.

Keywords: *Agrobacterium rhizogenes A4*, *genetic transformation*, *hairy roots*, *Hypericum perforatum*, *xanthones*.

INTRODUCTION

Hypericum perforatum L. is an important medicinal plant that has been used since ancient times for the treatment of numerous ailments. This species is a natural herbal alternative used mainly for the treatment of mild to moderate depression [1] and was also shown to have potential as a novel anticancer drug [2]. The extract is also reported to possess antiviral [3], neuroprotective [4] and antioxidant [5] properties. Biological activities of *H. perforatum* are mainly attributed to compounds of phenolic extracts. *Hypericum* contains at least ten classes of biologically active detectable compounds: phenylpropanoids, flavonoids, procyanidins, tannins, phloroglucinols, xanthones, essential oils, amino acids, naphtodianthrones, and other water-soluble components [6]. Among them xanthones are a class of polyphenolics that exhibit well-documented pharmacological properties such as antiradical [7], anti-inflammatory [8], cancer-chemopreventive [9], hepatoprotective [10], cardiovascular protective [7], selective inhibition of cyclooxygenase-2 [11], inhibition of platelet activating factor-induced hypotension [12] and cytotoxic activities [13].

To date, commercial production of *H. perforatum* is generally based on field grown plant material but the quality of these products may be affected by different environmental conditions, pollutants, microorganisms, viruses, and insects which can alter the concentration of its secondary metabolites. Micropropagation method offers an opportunity to exploit cell, tissue, organ or entire organism by growing *in vitro* and the genetically manipulate them to get desired compounds. Recently, the production of secondary metabolites using plant cells and tissue cultures has been subject of extended research. It was expected that the biosynthetic capacity of plants could be exploited *in vitro* using plant cell and tissue systems. Biosynthesis of therapeutically useful compounds can be effectively improved in medicinal plants by altering the expression of transcription factors or structural genes through metabolic engineering [14]. As the pharmacological activities of *H. perforatum* extract are largely attributed to compounds like hypericin and hyperforin that are exclusively produced in this species, improving their production is an important target for genetic manipulation. In spite of the availability of excellent regeneration protocol [15], this goal is not realized satisfactorily so far because of the poor knowledge about the biosynthetic pathways involved and also because of the absence of a suitable genetic transformation system for the species.

Gene transfer by *Agrobacterium* is the method of choice for the genetic transformation of most plant species and a possible strategy to enhance production of secondary metabolites in plant cultures. *Agrobacterium rhizogenes* infection induces the production of hairy roots (HR) in plant cells, which hold high growth rates and genetic stability [16]. Nevertheless, several parameters are known to affect T-DNA transfer and integration into the plant genome. Thus, a transformation protocol depends on the establishment of a reliable plant regeneration system as well as on the efficiency of *Agrobacterium*-plant interaction. The transformation of *H. perforatum* mediated by *A. rhizogenes* was already reported [17, 18]. The most important characteristic of transformed roots is their capability of synthesizing secondary metabolites specific to that plant species from which they have been developed.

In spite of the interesting properties of the xanthones and the potentialities of *in vitro* cultures for production of secondary metabolites, information about how xanthones accumulate in HR cultures is scanty. Recently, the potential of *H. perforatum* root cultures for improving xanthone accumulation have been investigated [19, 20, 21]. Also, the capacity of *H. perforatum* cell suspensions and callus cultures to produce xanthones has been explored [22, 23]. Xanthone production in unorganized *in vitro* cultures of *H. perforatum* is very low because the xanthone pathway requires root organization in order to be developed completely. Therefore, attention is now being focused towards exploring the potential of *A. rhizogenes*-mediated transformed roots (hairy roots-HR) for large-scale production of

bioactive secondary metabolites [24]. However, the establishment of HR cultures of *H. perforatum* and production of different xanthone compounds have not been reported.
This study has been focused on two areas: - establishment of an efficient *A. rhizogenes* A4 mediated transformation protocol of *H. perforatum* and rapid induction of HR cultures; - development of high-performance liquid chromatography (HPLC) method coupled to diode array detection (DAD) and tandem mass spectrometry (MS^n) with electrospray ionization (ESI) for identification of xanthones (HPLC/DAD/ESI-MS^n).

MATERIAL & METHODS

Plant material

H. perforatum seeds were washed with 70 % ethanol for 30 sec., surface sterilized with 1 % NaOCl for 15 min., rinsed 3 times in sterile deionized water and cultured on MS macro and oligoelements [25], B_5 vitamin solution [26], supplemented with 3 % sucrose and solidified with 0.7 % agar [15]. No growth regulator was added. The medium was adjusted to pH 5.6 before autoclaving (20 min at 120°C). *In vitro* cultures were maintained in a growth chamber at 26±1°C under a photoperiod of 16 h light, irradiance at 50 μmol m^2 s^{-1} and 50 to 60 % relative humidity.

Establishment of hairy root cultures

A. rhizogenes A4 mediated transformation protocol was performed according to [17]. When the hairy roots reached about 4-5 cm in length, those were excised from the explant tissue and subcultured on fresh MS/B_5 medium in the dark at 25±1°C. Genomic DNA from hairy roots and non-transformed roots of *H. perforatum* was extracted with CTAB procedure [27] and subjected to PCR analysis. The presence of the integrated genes in the genome of the putative transformed roots was determined by PCR amplification of *rol*B gene, according to the sequence of *rol*B gene from *A. rhizogenes* strain A4 [28]. Bacterial contamination of plant tissue was excluded by testing the amplification of the *vir*C1 gene which is located outside the bacterial T-DNA and is not transferred to the plant genome [29]. Hairy root lines were selected according to [17] on the basis of their characteristic phenotype, rapidly branching and plagiotropic growth with a reduced geotropism on hormone free medium. The hairy roots were harvested, frozen in liquid nitrogen or lyophilized and stored at -80°C, until analysis.

HPLC/DAD/ESI-MS^n analysis of hairy roots

The HPLC system was equipped with an Agilent 1100 series diode array and mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v.08.03).
Chromatographic separations were carried out on 150 mm x 4.6 mm, 5 μm XDB-C18 Eclipse column (Agilent, USA). The mobile phase was consisted of two solvents: (A) water-formic acid (1 %) and (B) methanol in the following gradient program: 90% A and 10% B (0-20 min), 80% A and 20% B (20-30 min), 65% A and 35% B (30-50 min), 50% A and 50% B (50-70 min), 20% A and 80%B (70-80 min) and continued with 100% B for a further 10 min. Each run was followed by an equilibration period of 10 min. The flow rate was 0.4 ml min^{-1} and the injection volume 10 μl. All separations were performed at 38°C. Spectral data from all peaks were accumulated in range 190-600 nm, and chromatograms were recorded at 260 nm for xanthones.
The HPLC system was connected to the Agilent G2445A ion-trap mass spectrometer equipped with electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as nebulizing gas at pressure of 65 psi and the flow was adjusted to 12 L·min^{-1}. The heated capillary and the voltage were maintained at 350 °C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan mass

covered the mass range from *m/z* 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra was set at 300 ms and 3, respectively. Identification of the component peaks was performed by the UV/Vis, MS and MS^2 spectra and retention times of the available standards.

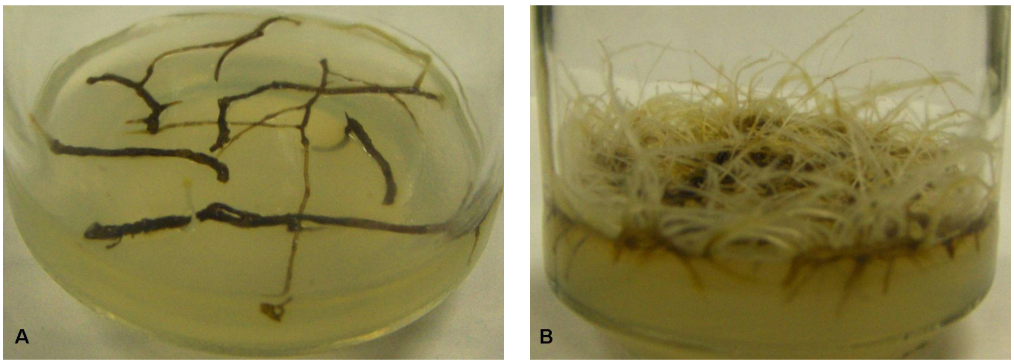
RESULTS & DISSCUSION

Hairy roots induction

In the present study, hairy roots (HR) induction was effectively achieved in *H. perforatum* root segments via infection with *A. rhizogenes* strain A4. HR of *H. perforatum* showed the characteristic traits of hairy roots previously described by [30]. They were highly branched, and showed a lot of root hairs with a plagiotropic growth habit (Fig. 1). Results from this study revealed that *H. perforatum* HR display most of these morphological properties and that transformed clones exhibited strong phenotype differences when compared with non-transformed root clones. All established HR lines showed a homogeneous morphology. HR cultures were subcultured and propagated on a MS/B_5 medium without phytohormones and usually display interesting growth capacities owing to the profusion of lateral roots (Fig. 1). Most of the reports display distinctive morphological characteristics associated with the HR phenotype, including a high degree of lateral branching, rapid growth, plagiotropism, numerous hairs and the capacity of hairy roots to grow when isolated from the mother plant in hormone-free medium [31].

In addition to their growth capacities, HR cultures display interesting properties regarding the production of secondary metabolites. The metabolite pattern found in HR is similar, if not always identical to that of plant roots [32]. In most cases, the differences are only on trace compounds. A major characteristic of HR is that they are able to produce secondary metabolites concomitantly with growth.

Figure 1. Morphological characteristics of 1-month-old (A) control roots and (B) hairy roots of *Hypericum perforatum* L. cultivated on solid hormone-free MS/B_5 medium.



Xanthone production in hairy roots

Twenty eight xanthones were detected in the methanolic extracts from *in vitro* biomass of *H. perforatum* transformed and untransformed roots and 21 of them were identified by ESI-MS (Fig. 2, Tab. 1). Compound **X1** was putatively identified as mangiferin. HPLC–MS/MS analysis of this compound gave a molecular ion *m/z* $[M–H]^-$ of 421 and major $–MS^2$

fragments at *m/z* 331 $[M–H–90]^-$ and 301 $[M–H–120]^-$, losses characteristics of *C*-hexosyl compounds [33]. Compounds **X4**, **X6**, **X11** showed UV spectral characteristics of the 1,3,5,6 oxygenated xanthones, with band IV reduced to shoulder [34] while most of the other identified xanthones had UV spectra similar to mangiferin typical of the 1,3,6,7 oxygenation pattern with a very well-defined band IV [35, 36, 37]. Compounds **X6** and **X7** were identified as 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone aglycones, respectively (single intense molecular ion $[M–H]^-$ at *m/z* 259).

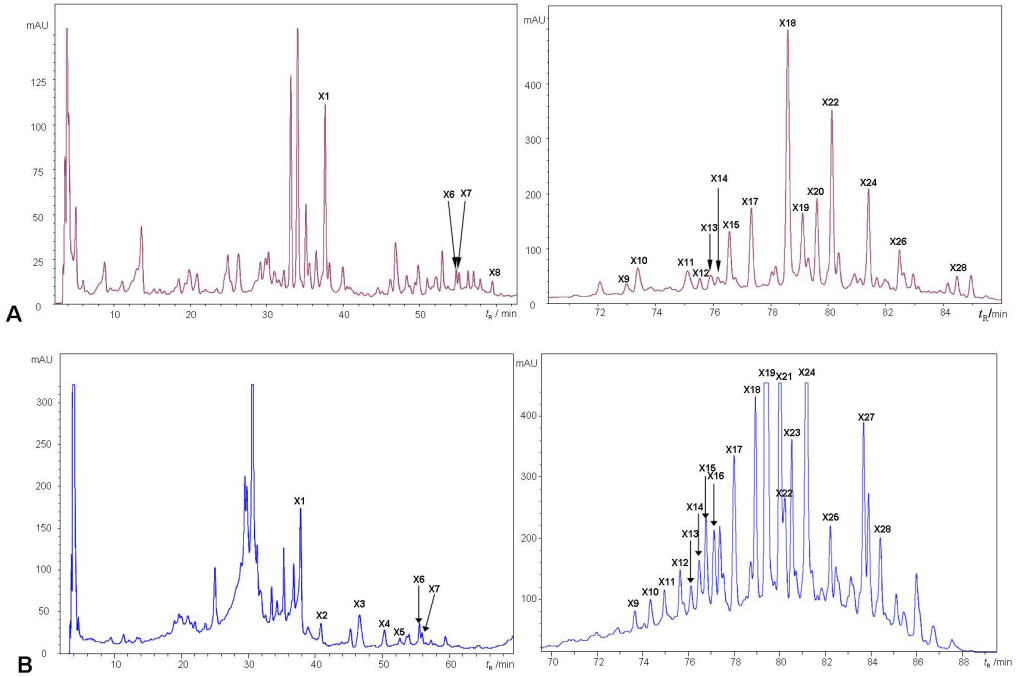
Table 1. HPLC-MS/MS data of the major identified xanthones in control and hairy root cultures of *Hypericum perforatum.*

Xanthones:		t_R (min)	UV (nm)	$[M–H]^-$ (*m/z*)	$MS^2[M–H]^-$ (*m/z*)
X1	mangiferin	37.3	238, 256, 312, 362	421	331, 301, **258**
X2	xanthone derivative 1	45.8	208, 257, 322, 374	441	423, 397, 373, 305, 257 229
X3	xanthone derivative 2	46.2	242, 306	367	**287**
X4	1,3,5,6-tetrahydroxyxanthone dimer	50.2	252, 284, 328	517	499, 468, 446, 391, **365**
X5	1,3,6,7-tetrahydroxyxanthone dimer	53.9	238, 254, 312, 364	517	517, 469, 447, 379, **257**
X6	1,3,5,6-tetrahydroxyxanthone	55.4	250, 282, 328	259	**229**, 213, 187
X7	1,3,6,7-tetrahydroxyxanthone	55.8	236, 254, 314, 364	259	231, **215**, 187, 147
X8	xanthone derivative 3	59.2	244, 280, 316	353	**273**
X9	mangiferin *C*-prenyl isomer	73.5	238, 260, 312, 372	489	399, **327,**
X10	1,3,6,7-tetrahydroxyxanthone 8-prenyl xanthone	73.9	248, 312, 366	327	325, **297**, 258,201
X11	1,3,5,6-tetrahydroxyxanthone 8-prenyl isomers	74.9	242, 260, 320, 368	327	325, **297**, 258, 201
X12	1,3,7-trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone	75.3	238, 260, 314, 388	327	**309**, 257
X13	toxyloxanthone	76.2	242, 262, 330, 384	325	307, 283, 272
X14	1,3,7-trihydroxy-6-methoxy-8-prenyl xanthone	76.5	240, 260, 318, 370	341	326, 311, **297**, 285
X15	1,3,6,7-tetrahydroxyxanthone 2-prenyl xanthone	76.7	248, 312, 368	327	325, 283, **271**
X16	γ-mangostin isomer	77.1	254, 286, 324	395	326, 283, **271**
X17	1,3,6-trihydroxy-7-methoxy-8-prenyl xanthone	77.2	240, 256, 312, 370	341	293, **256**
X18	γ-mangostin isomer	78.9	260, 316, 370	395	351, **339**, 326, 283
X19	trihydroxy-1-metohy-*C*-prenyl xanthone	79.4	260, 286, 314	341	**326**
X20	xanthone derivative 4	79.9	260, 308, 374	295	**277**, 251, 195, 171
X21	γ-mangostin	80.0	246, 262, 320	395	351, 339, 326, **283**
X22	banaxanthone D	80.2	244, 268, 332	461	393, 341, **297**
X23	xanthone derivative 5	80.5	254, 310	355	340, 325, **297**, 285, 271
X24	garcinone E	81.2	256, 286, 332	463	394, 351, **339**, 297, 285
X25	xanthone derivative 6	82.2	262, 288, 322	393	
X26	banaxanthone E	82.6	252, 302, 330	477	419, 393, **339**, 297
X27	garcinone C	83.9	286, 340	413	369, 344, **301**, 233
X28	xanthone derivative 7	84.4	254, 284, 326	481	**412**, 397, 327, 271, 234

Compounds **X4** and **X5** gave molecular ions $[M–H]^-$ at *m/z* 517. Major $–MS^2$ fragments at *m/z* 365 and 257, respectively, characterized them as dimers of 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone. Compound **X9** was putatively identified as mangiferin-*C*-prenyl isomer. HPLC–MS/MS analysis of these compound gave molecular ions $[M–H]^-$ at *m/z* 489 and major MS^2 fragments at *m/z* 399 $[M–H–90]^-$, 369 $[M–H–120]^-$ with losses

characteristics of *C*-hexosyl compounds [38] and 327 as a base peak (1,3,6,7-tetrahydroxyxanthone-*C*-prenyl residue). Compounds **X10** and **X15** had UV spectra characteristic of 1,3,6,7-oxygenated xanthones and molecular ions $[M–H]^-$ at 327. So, these compounds were identified as 1,3,6,7-tetrahydroxyxanthone-*C*-prenyl isomers. In the literature it is found that in some *Hypericum* species the *C*-prenyl moiety can be in position 2 or 8 [39]. They can be tentatively assigned as 1,3,6,7-tetrahydroxy-8-prenyl xanthone and 1,3,6,7-tetrahydroxy-2-prenyl xanthone. Compound **X11** had same fragmentation pattern as **X10** and **X15** but different UV spectra, characteristic of 1,3,5,6-tetrahydroxyxanthone, leading to its assignment as 1,3,5,6-tetrahydroxy-8-prenyl xanthone [40]. Compound **X12** gave molecular ion $[M–H]^-$ at *m/z* 327, but showed a different fragmentation pattern in comparison with the other compounds with the same mass. In the MS^2 it exhibited a loss of a hydroxyl group $[M–H_2O]^-$ to give the base peak at *m/z* 309, indicating that the OH group is not linked to the xanthone aglycone, but to the prenyl group. In the next MS^3 step, after the loss of the prenyl moity, the base peak at *m/z* 257 was detected. According to this behaviour and literature data, it is evident that this compounds is 1,3,7-trihydroxy-2-(2-hydroxy-3-methyl-3-butenyl)-xanthone [41]. Xanthones **X16** and **X19** were identified as 1,3,7-trihydroxy-6-methoxy-8-prenyl xanthone and 1,3,6-trihydroxy-7-methoxy-8-prenyl xanthone (molecular ions $[M–H]^-$ at *m/z* 341) using the previously published data [42, 43, 39]. Compound **X19** had similar fragmentation pattern as compound **X14**, and indicate that compound **X19** has similar nature as compound **X14**. So, we can tentative assigned compound **X19** as trihydroxy-1-metohy-*C*-prenyl xanthone.

Figure 2. HPLC–MS/MS analysis of xanthones in *Hypericum perforatum* extracts from 1-month-old (A) control roots and (B) hairy roots.



Comparison to previously published data for UV and MS spectra indicate that compound **X21** is γ-mangostin (molecular ion $[M–H]^-$ at *m/z* 395). Compounds **X16** and **X18** were putatively identified as isomers of γ-mangostin (1,3,6,7-tetrahydroxyxanthone-*C*-bis-prenyl), since they have a similar molecular ion $[M–H]^-$ of 395 but different UV spectra and retention times. Compound **X13** gave a $[M–H]^-$ peak at *m/z* 325. The UV spectrum was characteristic of 1,3,5,6-tetraoxygenated xanthone. A distinct shoulder at 365 nm revealed conjugation with

a pyran ring. MS^n and UV spectra were in complete agreement with those of toxyloxanthone, previously reported by [40]. Compounds **X22**, **X24**, **X26** and **X27** gave deprotonated molecular ions $[M–H]^-$ at *m/z* 461, 463, 477 and 413, respectively. Their MS^2 spectra were generated by the loss of a prenyl residue C_4H_8 (56 amu) and two prenyl residues (112 amu). So, compounds **X22**, **X24**, **X26** and **X27** were identified as banaxanthone D, garcinone E, banaxanthone E and garcinone C, respectively. Several other peaks (**X2**, **X3, X8**, **X20, X23 X25** and **X28**) were categorized as due to xanthone derivatives by HPLC-DAD-MS/MS analysis, but could not be fully identified.

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Original scientific paper

ESSENTIAL OIL ACCUMULATION DURING RIPENING PROCESS OF SELECTED APIACEAE SPECIES

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SUMMARY

The species of the plant family *Apiaceae* are frequently cultivated medicinal and culinary herbs in Hungary. Key factor of the economic viability is the appropriate drug quality, for which one of the basic influencing element is the harvest time. The lack of the appropriate data motivated us to examine the changes of essential oil quantity and quality during ripening in case of various *Apiaceae* species having different seed morphology and life cycle.

Angelica (*Angelica archangelica* L.), coriander (*Coriandrum sativum* L. *var. microcarpum* DC.) and fennel (*Foeniculum vulgare* Mill. *subsp.capillaceum var. vulgare* DC.) were grown at the Experimental Farm of Corvinus University of Budapest in Soroksár in 2006 and 2007. Sampling was carried out in the following phenological phases of the primarily and secondary umbels: 1. Green ripening; 2. Milky ripening, 3. Semi-mature seed and 4. Full ripened seed. In each phase stereo microscopic image was taken to detect the anatomical changes. Essential oil content (calculated to dry mass) was determined by water distillation and its composition analysed by GC-MS method.

In the first year the essential oil level decreased in each species during the fruit development. However, the tendency and rate of this decrease depend on the species. In angelica (2.61 ml/100g – 0.53 ml/100g) and fennel (10.0 ml/100g – 4.4 ml/100g) the highest loss could be measured between the green ripening and milky ripening phases, while in coriander (1.10 ml/100g and 0.88 ml/100g) it is more characteristic after the milky ripening stage. In the second year, a similar decreasing tendency was only observable in case of coriander, while in the other two species there is no firm trend concerning the essential oil accumulation level. Compositional changes are largest for anethole in fennel oil, showing a decrease from 83 till 62% during fruit development.

The results indicate that the studied trends of essential oil accumulation and compositional changes are not stable and family specific, but highly influenced by environmental conditions and exhibiting species specific traits. The results call the attention that changes during ripening process might be in connection also with seed morphology and have a big influence on the drug quality.

***Key words**: Angelica archangelica, Coriandrum sativum, Foeniculum vulgare, ontogenesis, oil duct,*

INTRODUCTION

The *Apiaceae* family is one of the most important medicinal plant family. In Hungary, four species (caraway, fennel, coriander, anis) are among the "top ten" cultivated herbs [1]. The quality of the harvested product is basically influenced by the content of essential oil. In the 7th *European Pharmacopoeia* it is an important requirement, too.

Angelica is a biennial plant that has been used in folk medicine. The most characteristic secondary metabolites are the essential oils and coumarins. The oil in the mature seeds contains maximum 65% β-phellandrene, [2,3], 5-15 % α-pinene and 11-20% sabinene. The

ratio of these latter ones often depends on the growing locality [4]. Thus, from France barely trace amounts of sabinene have been reported [2]. The essential oil from the angelica is used for flavoring beverages and liquors and has a highly value as a fragrance component in perfumery and cosmetics [5].

The coriander is an annual, culinary and medicinal plant that is used worldwide. From 11 infraspecific taxa [6] two have agricultural importance, the *Coriandrum sativum var. vulgare* ALEF. and *Coriandrum sativum var. microcarpum* DC.[7,8]. The essential oil level varies between 0.13 and 1.90 ml/100g [9,10,11]. The main component of the essential oil is linalool the proportion of which reaches 40.2 to 82.9 % [12,9,13]. Other components are γ-terpinen, e-2-decenal, camphor, geranyl acetate, geraniol, borneol, terpine-4-ol, and limonene [9,11]. Investigation of coriander fruit in different ripening phases (inmature, intermediate, mature) reveled, that the essential oil level increased from 0.01 to 0.35 ml/100g as did also the ratio of linalool in the oil (from 10.96 to 87.54 %). Other authors found however contrary results, the essential oil level decreased during ripening (from 0.50 to 0.39 ml/100g) but the ratio of linalool increased (from 35% to 65%)[8]. Domokos and Kiss described, that coriander fruits have two different types of essential oil ducts: peripheral ducts containing aldehyde type essential oil, with mainly e-2-decenal responsible for the typical coriander odor [8,14] and internal ducts with high amount of linalool [10].

Fennel is a perennial plant used widely as flavouring agent. It has several taxa among which *Foeniculum vulgare subsp. capillaceum var. vulgare* (MILL.) THELL. and the *Foeniculum vulgare subsp. capillaceum var. dulce* (MILL.) THELL. have practical importance concerning the essential oil production. Formerly it was shown that highest essential oil content is measured in the green seed stadium (5.8 – 14.9 ml/100g) [15,16,17,18,19], but opposite observation can be found, too [20]. The tendency of relative essential oil content is in tight connection with the increasing dry matter accumulation during ripening [21]. Compositional changes during the ripening seem to be less characteristic than those in oil level. In the practice, information about the dynamics and rate of accumulation as well as on changes in oil composition are of basic importance in order to optimize harvesting time and/or phase. Therefore the aim of our recent investigations has been the detection of the role of ontogenesis in essential oil characteristics of the fruits and comparision of the characteristics of the species.

MATERIAL AND METHODS

Plant Material

Angelica (*Angelicha archangelica* L. *subsp. archangelica var. sativa* (MILLE)RIKLI) seed was purchased through an exchange from Finland. Coriander (*Coriandrum sativum var. vulgare* ALEF.) seeds originated from the genebank of the Department without cultivar denomination. For fennel (*Foeniculum vulgare subsp. capillaceum var. vulgare* MILL.) the breeding strain 'SM-1' of the Department was used for the experiment.

Location

The experiment was carried out at the Experimental Farm of Corvinus University of Budapest suburb 'Soroksár' in 2006 and 2007. The character of the soil is loamy sand, the pH is slightly alkaline. Climate is arid- continental, mean annual temperature is between 10-11°C, the hours of sunshine is around 2100 hour/year. Average rainfall is 550 mm from which 360 mm fall in the growing season. The two experimental years proved to be different. The spring of the first year was cool, the summer and autumn warm. In the second year spring and summer was warm, the autumn cool. In 2006 [Fig.1.], average temperatures of eight weeks exceeded 20°C, three of them showed temperatures above 25°C. In 2007 [Fig.2.], the weekly average temperature was above 20°C during 13 weeks and only during a

single week temperatures over 25°C could be measured. In summary, weather conditions of 2007 were warmer and more balanced, but at the same time the amount of precipitation was much lower (118 mm) than in 2006.

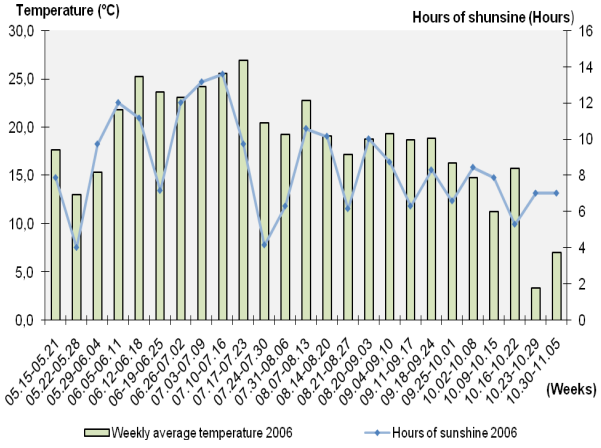

Fig.1.: Weekly average temperature and hours of sunshine in 2006

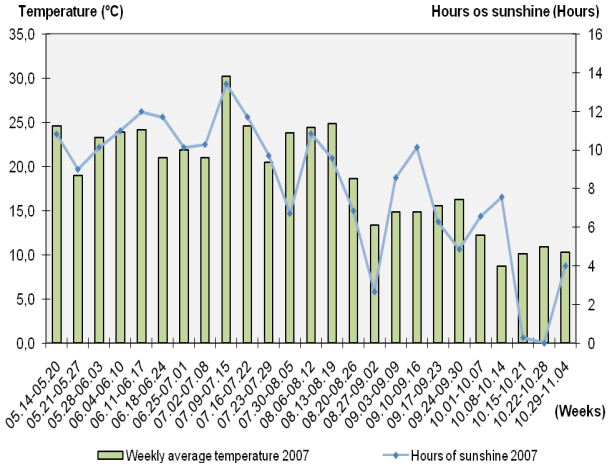

Fig.2.: Weekly average temperature and hours of sunshine in 2007

METHODS

Based on the work of CHUNG [22] we divided the fruit ripening period into 4 developmental phases (phenophases):

1. Green ripening: seed have the final shape and dark green color, easy to squeeze
2. Milky ripening: the color is greenish-yellow, grooves getting darker
3. Semi-mature seed: yellow or russet color, grooves are brown (collected only in 2007)
4. Full ripened seed: completely brown grooves, hard to squeeze.

At each phases, samples were taken from the primarily and secondary umbels to carry out the morphological and chemical investigations. For the morphological observations cross-sections were prepared from the fresh fruits and studied under stereo-microscope (BMS 143 Digital Zoom, Art Nr 74959) and characterised. Replication number was three pieces/phase/species.

The collected seeds where dried at room temperature and stored till the laboratory analysis.

The chemical investigations were carried out at the laboratory of the Department of Medicinal and Aromatical Plants. The essential oil content was determined in three replications/phase/species from 5 g grinded seed by steam distillation in a Clevenger-type apparatus according to the VII. Hungarian Pharmacopoeia. The oil content of the samples was calculated to the dry weight basis. In case of samples collected in 2007, the main components of the essential oil were determined by GC-MS method using the equipment 'Agilent Technologies' with a Colonna: HP-5MS (5 % phenyl-methyl-siloxane), length: 30 m, i.d.: 250. Carrier gas was helium, the flow was 0.5 ml/min. constant. Temperatures of the injector and the detector were 250 °C, the ionization energy was 70 eV. Detection of the components was by comparing the mass spectra and retention times to those of standard compounds as well as by using library database (NIST).

RESULTS AND DISCUSSION

Changes of essential oil content and composition in fruits of *Angelica archangelica* L.

In the first year a significant decrease of essential oil content could be registered during the maturation process, from 2.60 ml/100g till 0.53 ml/100g. The difference between the stages was significantly high ($p<0.001$). The largest difference was observable between the first and second stages (Fig.6.). In the second year however, a fluctuation of oil level was detected during the observed period with a maximum value at the full ripening stage (from 0.84 ml/100g till 1.74 ml/100g). Between phases 1 and 3 there was no significant difference, but it appeared between phases 2 and 4 ($p<0.001$).

It can be established that the results of the two years reflect inconsistent tendencies. In 2006 at the beginning of the fruit development the weather was cool and humid, after that warm and still rainy. In the second year because of the extraordinary warm weather, the seed development started 3 weeks earlier than in the previous year and it was accelerated.

The main compound was β-phellandrene in the essential oil, which did not changed significantly and stayed above 90 % in each phase [Fig.6.]. Proportion of other compounds fluctuated during fruit development. The level of α-pinene showed significant differences ($p<0.001$). The proportion of β-myrcene was different in each stage ($p<0.001$). Ratios of α-phellandrene at stages 1 and 4 are statistically identical while those at stages 2 and 3 are different ($p<0.001$). There are no standard regulations for minimum essential oil quantity in angelica seed and for the containing components.

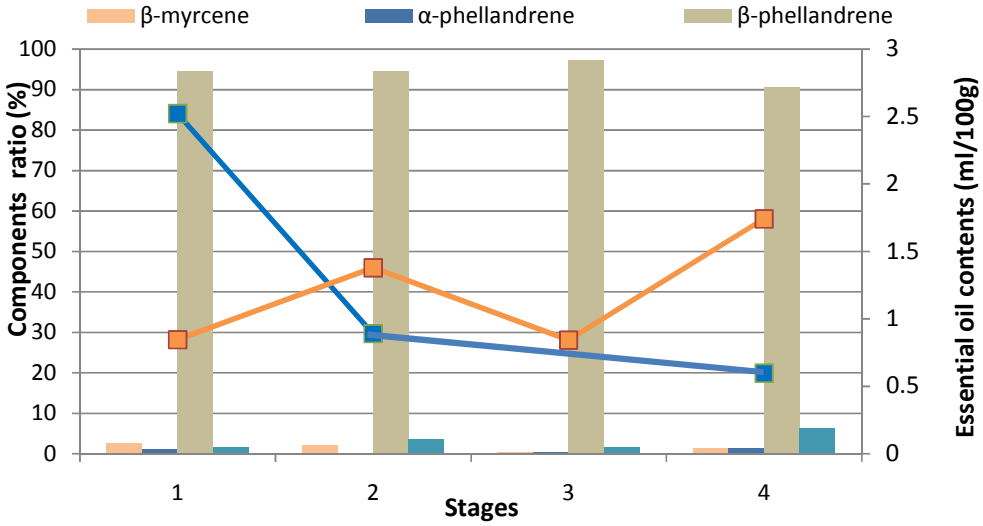

Fig.6.: Essential oil accumulation during the ripening of angelica fruits

Changes of essential oil content and composition in fruits of *Coriandrum sativum* L.
In the first year, after an insignificant increase (from 0.768 ml/100g to 0.795 ml/100g) of essential oil accumulation a considerable fall was detectable ($p<0.001$) resulting in more than 50% relative loss of the oil [Fig.7.]. In the second year the essential oil decreased significantly ($p<0.001$) after the first phenophase (1.10 ml/100g and 0.88 ml/100g), whereas the later differences were not significant. In both years the essential oil content of the ripen fruits was lower than in the green seed stage. This is similar to what TELCI ET AL. [8] observed and controversial to the results of MSAADA ET AL. [13].

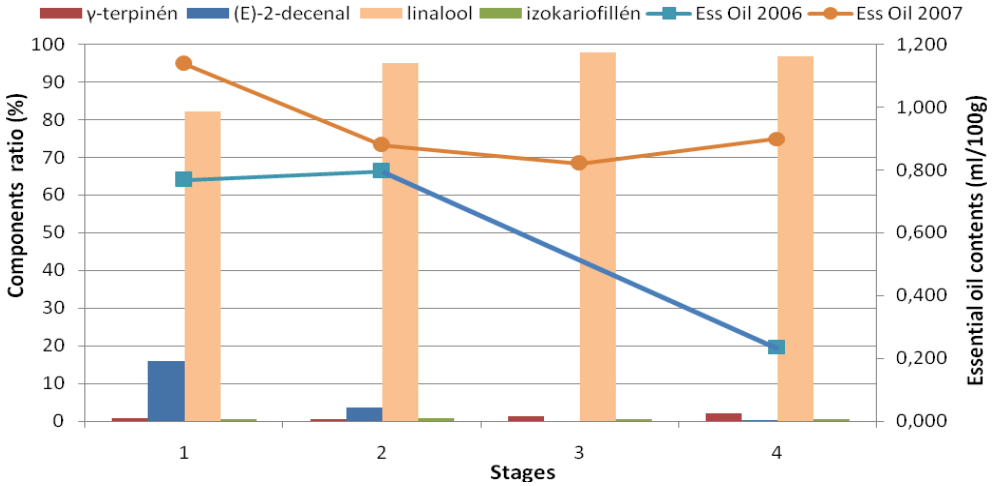

Fig.7.: Essential oil accumulation during the ripening of coriander fruits

The main compound in the oil was linalool, which increased from 82% to 96%. Significant difference was detected only after the 1 phenophase ($p<0.001$). The second most abundant compound, e-2-decenal which is responsible for the characteristic odor of the leaves -, decreased from 15.0 % to 0.3 % until the third phase; the most important difference was detected between the first and second phase ($p<0.001$). Thus, our results support the reports of DOMOKOS and KISS [10] and HÉTHELYI and NYÁRÁDI-SZABADY [14]. The compound γ-terpinene increased from 0.8 ml/100g to 2.2 ml/100g ($p<0.01$) [Fig. 7.].
The regulation standards for the coriander seed (*Pharmacopoea Hungarica VIII*.) require a minimum of 3.0 ml/1kg (0.3 ml/100g) essential oil content, and composition containing 65.0-78.0 % linalool and 1,5-8.0 % of γ-terpinene. The essential oil yield, except the last phase in 2006 in all samples was over the minimum, but the component ratio did not.

Changes of essential oil content and composition in fruits of *Foeniculum vulgare* Mill.
In the first year the relative oil content decreased from 10,1 ml/100g to 4,4 ml/100g between the first two studied phases which was significant ($p<0.001$); afterwards it remained constant [Fig.8.]. In the second year increase ($p<0.001$) can be observed during the first developmental phases (5.7 ml/100g up to 7.9 ml/100g), but the last 3 stages did not exhibit any significant difference among each other. The significant accumulation was recorded after the green stage ($p<0.001$) each year [Fig.8.]. The observed tendency is similar to that in angelica.
The main compound of the essential oil is anethole. The proportion of this compound decreased during ripening from 83% to 62%. This loss is significant ($p<0.01$). On the contrary, the ratio of fenchone did not change significantly except at the 3. phenophase when a slight decrease was detected. The third important compound, methyl chavicol was hardly

detectable in the 1 and 2 phenophase (1%) but after that its proportion increased significantly ($p<0.001$) to 12%.

The regulation standards (*Pharmacopoea Hungarica VIII.*) for the fennel seed require a minimum of 40.0 ml/kg (4.0 ml/100g) essential oil content; the anethole of minimum 60.0%, and fenchone of minimum 15.0%. Except the 2 phase in 2006 and 3 phase in 2007, all of the samples reaches these criteria.

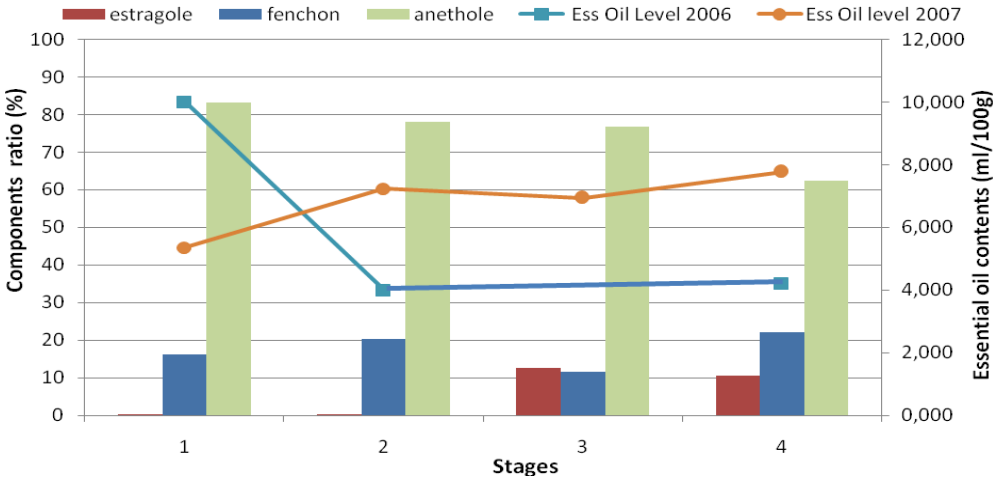

Fig.8.: Essential oil accumulation during the ripening of fennel fruits

Morphological changes

In the fruits of angelilca the endosperm with the oil ducts in the surface detaches from the pericarp during the ripening [Fig.3.].

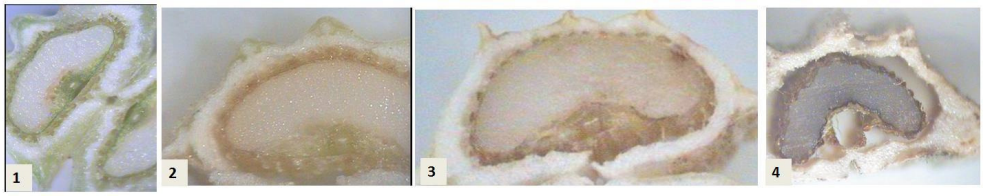

Fig.3.: Cross-sections of angelica fruits in different developmental phases
1.Green ripening, 2. Milky ripening, 3. Semi-mature, 4. Full ripened seed

The fruits of coriander have two different oil ducts: the one in the pericarpium and another attached to the endosperm. During ripening, the pericarp ducts loose [Fig.4.] their shapes which confirms the observations of DOMONKOS and KISS [10]. The endosperm decreases in size.

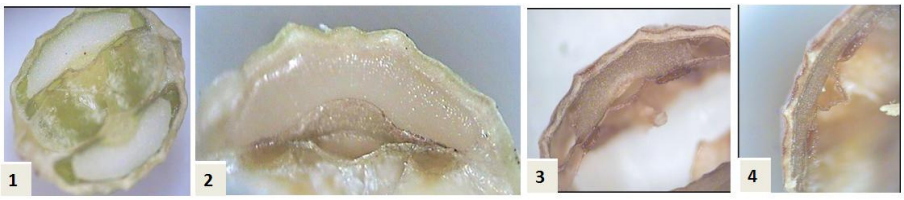

Fig.4.: Cross-sections of coriander fruits in different developmental phases
1.Green ripening, 2. Milky ripening, 3. Semi-mature, 4. Full ripened seed

The changes in fennel were similar as described by BERNATH ET AL. [18]. The ducts develop already at very early stages of fruit growth. Later, their shape changes presumably in consequence of the physical pressure that is caused by the growing endosperm during the fruit development [Fig.5.].

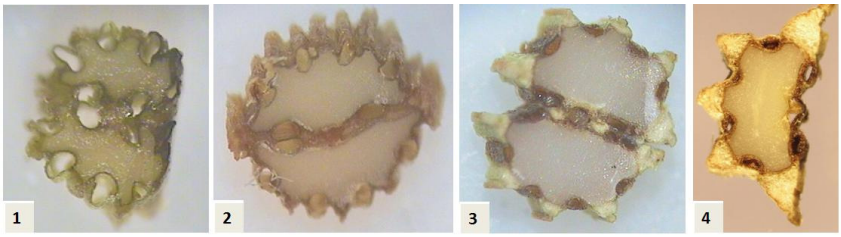

Fig.5.: Cross-sections of fennel fruits in different developmental phases
1. Green ripening, 2. Milky ripening, 3. Semi-mature, 4. Full ripened seed

CONCLUSION

In the first study year the essential oil yield decreased in each species during fruit development. However, the tendency and rate of this decrease depend on the species. In angelica and fennel the highest loss could be measured between the green ripening and milky ripening phases, while in coriander it is more characteristic after the milky ripening stage. The level of decrease is highest in angelica, reaching five fold differences, and the lowest in fennel, reaching 2.5 fold deviation.

In the second year, a similar decreasing tendency was only observable in case of coriander. However, the dynamics does not coincide with that of the previous year, because severe loss was measured between the first two phases. In the other two species there is no firm trend concerning the level of essential oil accumulation. Fluctuations are insignificant, thus, we could state that the oil level seems to be stable in this year.

Observing and comparing the weather characteristics of the two vegetation cycles, it may be supposed, that early hot weather and lack of precipitation in 2007 influenced unfavorably the essential oil accumulation and the regular changes during the fruit development. This presumption is supported also by the fact, that in angelica and fennel, the maximum oil levels were lower for about 1% and 2% respectively, compared to the previous year. Nevertheless, it should be considered that the mentioned decreases of the oil were relative when calculated upon dry mass and not necessarily affect the total accumulation level.

Seed morphological charcteristics of the studied species seem to be in connection with the chemical features. The oil ducts of the angelica are almost intact because they are divided from the pericarp and thus, no pressure is likely to appear from any side. In contrary, in coriander seeds the periferial ducts became smaller and deformed during the development, while the inner ducts stay intact. In fennel fruits we found only periferial ducts which are also exposed to the pressure of the endopserm, which was also mentioned. by SZÉKELY ET AL. [8] . This phenomenon in the two latter species might contribute both to quantitative and qualitative changes of the essential oil. Different seed morphology may be a reason for diffculties of exact determination of ripening phases as well. In the literature, the different system for division of characteristic fruit developmental stages can be found, out of some authors count the days after the bud stage [18], some others calculate from the flowering [8] or define the stages according to morphological features [22, 23] We recommend to create a uniform system for evaluation of fruit development which might be valid for the most important *Apiaceae* spices.

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Original scientific paper

GENOTYPES OF *SALVIA OFFICINALIS* L. WITH DIFFERENT ESSENCIAL OIL CONTENT AND COMPOZITION

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SUMMARY

The quantitative and qualitative analysis of essential oil, extracted through hydrodistillation, from different genotypes of *Salvia officinalis* L. cultivated in Republic of Moldova has been studied. The essential oil content, analyzed from 5 genotypes of *S.officinalis* is variable in function of genotype and collected phase. The highest content of essential oil was registered in the material collected after flowers and seeds fall: 1.455-1.823% (dry matter) in shoots with leafs and 1.408-1.749% (dry matter) in leafs. GC-MS analysis of essential oil revealed the presence of different number of components, which depend on genotypes. In this context in the shoots with leaf are registered 14-23 components, but in leafs - 17-25. The major components of essential oil are represented by monoterpene ketones: α-thujone (21.2-38.8), β-thujone (5.877-16.201%), camphor (17.5-24.6%), followed by eucalyptol (6.47-11.2%), therefore the whole of genotypes represents a single chemotype: α-thujone/β-thujone/camphor/eucalyptol.

***Key words**: Salvia officinalis, five genotypes, different developmental phases, essential oil, composition.*

INTRODUCTION

Salvia officinalis L. belongs to *Lamiaceae* family, a small evergreen bush used as medicinal, aromatic and spice plant from ancient times. Actually are used young shoots with leafs, flowers and essential oil. The pharmacological action of *Salvia officinalis* L. is attested as antiseptic and astringent, spasmolytic, anti-inflammatory haemostatic, expectorant, cicatrizing, heal wounds, antibiotic, bacteriostatic, antisudorific and tonic owing due to the essential oil of this plant [1]. The tea from leafs is used to treat different mouth diseases, as pharyngitis**,** atherosclerosis [2] and has antioxidant activity [3]. Also is used in case of stress, irritation, skin cancers, abundant transpiration, rheumatism and for memory improvement [4]. The *S.officinalis* leaf extracts are used in the treatment of Alzheimer disease in medium and moderate forms [5, 6] and for sure has an antihiperlipidemic effect [7]. Was noticed also an anticancer action [8, 9]. Largely are used antiviral and antifungal characteristics of essential oil [10] in perfumery and aromatherapy [11]. In the Republic of Moldova some *S.officinalis* plantations are used for leaf production as a pharmaceutical product, others – to separate essential oil, with steam distillation. Our past investigations demonstrated that in *S.officinalis* leafs, cultivated in Romania and Republic of Moldova, besides essential oil contains also flavones, triterpenes, phytosterols and polyholozides [12].

This research represented the study of the essential oil content of different *Salvia officinalis* genotypes, at different harvesting phases, as well as oil's quantitative and qualitative compounds at late harvesting phases, when the essential oil content is maximal. These results may indicate the most convenient phase for harvesting.

MATERIALS & METHODS

Was investigated 5 genotypes of *Salvia officinalis* of different origin: the variety Miracol, created in the Institute of Genetics and Plant Physiology and approved, registered in the State Register of Moldova Republic; two genotypes largely cultivated in the South of the Republic of Moldova, district Cahul - Cahul-D (a variety created by the Botanical Garden Nikita, Crimea) and Cahul-M (originating from Russia); another two genotypes (G-1 and G-2) from the collection of Centre Genetics and Breeding of Aromatic and Medicinal Plants Institute of Genetics and Plant Physiology Academy of sciences of Moldova.

The samples were collected in the mornings, corresponding to three developmental phases: before the formation of flower button (13 and 20 May 2011), in the flowering time (02 June 2011) and after flowers and seeds fall (29 July 2011).

The essential oil was separated from fresh collected material using hydrodistillation in Ginsberg apparatus and the oil composition was recalculate to dry matter. After distillation the essential oil was dried with Na_2SO_4 and was preserved in the freezer.

Qualitative and quantitative composition of essential oil was determined by gas-chromatographic analysis in tandem with the mass spectrometry (GC-MS) for the separated samples of shoots with leaf, harvested only after the shaking of flowers and seeds. In these samples the content of essential oil was highest. The analysis equipment included: gas-chromatograph Technologies Agilent 7890 equipped with Selective Mass Detector with Quadruple MSD Agilent Technologies 5975C, capillary column (30 м/0.25 мм/0.25 μм) with non-polar stationary phase HP-5ms. Analysis was performed at a temperature of 250 ° C injector and detector - 280 ° C, using a temperature gradient from T1 = 70 ° (2 min), T2 = 200 ° C (5 ° C / min), T3 = 300 ° C (20 ° C / min, 5 min). Mobile phase: Helium 1ml/min, injected volume - 0.03 ml essential oil, split rate - 1:100. Identification of chromatographic peaks was performed using the software package AMDIS ™, coupled with the NIST database.

REZULTS & DISCUTIONS

The evaluation of 5 genotypes of *Salvia officinalis* L. demonstrated the diversity of essential oil content at the different development phases. Was demonstrated that young shoots with leafs, for the majority of genotypes, accumulate a relatively low content of essential oil – 0.636 – 0.691% which depends of the shoot's growth stage (Table 1). The genotype Cahul-D contains only 0.511% of essential oil, but G-1 is the genotype with highest content of essential oil – 0.905% before the floral buttons appears (20 May).

Table1. Essential oil content in some genotypes of *Salvia officinalis* L., 2011

Variety, genotype	Essential oils content, % (dry matter)						
	13. 05.	20. 05.	2.06.2011			29.07.2011	
	young shoots	shoots with floral buttons	shoots with inflorescences	shoots with leafs	inflores-cences	shoots with leafs	leafs
soi Miracol	0.636	0.693	0.981	0.365	1.066	1.572	1.543
Cahul-D	0.691	0.511	0.682	0.646	0.644	1.619	1.536
Cahul-M	0.675	0.648	0.818	0.314	1.187	1.455	1.408
G-1	0,675	0.905	0.662	0.505	0,905	1.823	1.749
G-2	0,756	0.682	0.795	0.509	0.890	1.679	1.741

At the third evaluation term, when the shoots have inflorescences, the essential oil content increased considerably for the variety Miracol – (0.981%) and the genotype Cahul-M (0.818%). In this phase also increased the oil content for the genotype G-2 – 0.795 %.
The highest content of essential oil was registered in inflorescences - 0.905-1.187% with exception of Cahul-D genotype, where the oil content do not differ significantly in the shoots with inflorescences and leafs, shoots and leafs, inflorescences. The amount of essential oil increased in all genotypes after fall flowers – 29 May. The highest amount was registered for the genotypes G-1 and G-2, where the shots with leafs contain 1.823 and 1.679% (dry matter) respectively, but in leafs - 1.749 and 1.741%. Similar content of essential oil – 1.6% was described [13] for the variety Predgornyi, created in the Botanical Garden Nikita, Crimea.

Table 2. Qualitative and quantitative composition of the essential oil in the genotypes of *Salvia officinalis* L., (29 July 2011)

Component	Variety Miracol		Cahul-D		Cahul-M		G-1		G-2	
	shoots/ leafs	leafs	shoots/ leafs	leafs	shoots/ leafs	leafs	shoots/ leafs	leafs	shoots/ leafs	leafs
α-Pinene	2.591	2.565	0.913	3.799	1.249	1.352	2.998	6.632	3.248	3.826
Camphene	2.418	2.613	1.327	2.57	1.781	1.582	2.445	3.076	2.252	1.704
Sabinene	0.253						0.326			
β-pinene	1.365	1.68	0.831	1.41	0.982	1.029	1.243	1.419	1.133	0.849
β-Mircene	0.778	0.669	0.513	0.719	0.695	0.662	0.949	1.162	0.885	0.86
o-Cymene								0.26		
Limonene	1.505	1.302	0.955	1.406	1.248	1.306	1.486	1.899	1.536	1.68
Eucalyptol	8.416	10.372	6.472	11.203	11.203	10.91	7.162	9.275	8.301	9.781
γ-terpinene	0.311			0.307	0.385		0.444	0.491		0.331
α-terpinene	0.489	0.317		0.369	0.384		0.411	0.484		0.527
Linalool	0.497	0.483		0.463	0.515	0.488	0.593	0.511		0.384
α-thujone	33.791	21.239	35.035	22.415	35.134	32.492	36.605	34.793	38.818	34.179
β-thujone	5.877	16.201	13.559	13.328	11.19	10.783	12.119	10.936	9.863	3.19
Isothujol				0.267						
cis-Sabinol	0.345			0.408	0.256					
Camphor	24.59	19.144	19.753	21.138	21.297	21.461	18.996	17.508	20.065	19.995
Borneol	3.302	3.472	3.685	4.541	2.573	2.935	1.56	1.365	1.654	2.125
4-Terpineol	0.538	0.554	0.817	0.694	0.685	0.689	0.593	0.507		0.542
α-Terpineol	0.285			0.314						0.24
Myrtenol				0.281						0.316
Bornylacetat	2.324	3.856	3.535	2.934	2.390	2.512	1.543	1.223	1.818	2.546
α-terpinylacetat		0.27		0.204	0.324					0.322
β-caryophilene	3.929	5.44	2.75	1.703	3.875	4.048	3.191	2.152	2.706	2.221
α-caryophilene	2.617	3.367	3.305	4.745	3.607	3.527	5.074	4.795	5.808	7.998
Caryophilene oxide		0.42			0.331					
Viridiflorol	3.063	3.653	5.607	3.924	4.17	3.791	1.476	1.511	1.913	3.638
Aroma dendrenoxid				0.379	0.336					0.576
Labdatriene	0.716	0.968	0.934	0.477	0.499	0.431	0.453			0.742
Components identifiend	22	20	16	25	23	17	20	19	14	23
Total, %	100.0	98.585	100.0	99.998	99.772	99.998	99.67	99.999	100.0	99.277

GC-MS analysis of essential oil extracts from shoots with leafs, harvested at the end of July, revealed 14 – 23 components; in leaf samples – 17-25, which depend of genotypes (Table 2).

The major compounds are represented by monoterpenes: α-thujone (21.2-38.8%), β-thujone (5.877-16.201), camphor (17.5-24.6%), followed by eucalyptol (6.47- 11.2%).
Thus, the variety Miracol and another four analyzed genotypes have the same chemotype: **thujone/camphor/eucalyptol** while, the concentration of major components, especial the minor compounds in extracted oil from leafs and shoots with leafs substantially differ for all genotypes. Other researchers [13] described varieties with two major components: β-thujone (39.5%), camphor (17.4%) and with significant concentration of monoterpenoxides – 1.8-cineole (10.5%) that was not detected in our studied genotypes. In the *S.officinalis* cultivated in Estonia and other European countries, 1.8-cineol is the major component of essential oil [14]. Romanian genotypes contain 12 components in the essential oil and the major one is α-thujone in the concentration of 31.23-52.86% [15].
In our genotypes the number of minor components of the essential oil from shoots with leafs, with the concentration bellow 1% vary from 1 (β-mircene, genotype G-2) to 11 (genotype Cahul-M), in the essential oil from leafs – from 5 (genotype G-2) to 12 (genotype Cahul-D) (Table 2). The number of minor components with the concentration 1% to 5% varies from 6 to 9 in the essential oil from shoots with leafs and leafs. These facts demonstrate the high variability of chemical components of the essential oil in the studied genotypes. Strong differences exist in the number and concentration of minor and major components.
The concentration of major components detected in the shoots with leafs – α-thujone varies from 33.791%, in the variety Miracol, up to 38.818%, for the genotype G-2. The same major component concentration in the essential oil is maximum in leafs and shoots with leafs for the genotype G-1(34.793%). For all analyzed genotypes the concentration of α-thujone is higher in the shoots with leafs in comparison with leafs. Camphor concentrations vary from 18.996 % in the genotype G-1, up to 24.59% for the variety Miracol. For all genotypes, with exception of two genotypes from Cahul, the camphor concentration is higher in the essential oil extracted from shoots with leafs. The third major component – eucalyptol have the higher concentration (9.275-11.203) in the essential oil extracted from leafs. In the oil from shoots with leafs its concentration is 8.416 %, for variety Miracol and up to 11.203% for the genotype „Cahul-M".
The species *S.officinalis* is characterized through a high variability of chemical composition that depends of cultivation zone, variety, genotype as well as plant's developmental stage. These conditions influence especially the essential oil and the number, concentration of its components. The oil amount is accumulated gradually, with plant development, and increase the maximum in the phase of flowers and seeds fall. In our opinion this is the optimal harvesting phase.

CONCLUSIONS

Quantitative and qualitative analysis of essential oil extracted through hydrodistillation from genotypes of *Salvia officinalis* L., cultivated in Republic of Moldova was described.
The essential oil content evaluated in 5 *Salvia officinalis* L. genotypes varies in function of genotype and harvesting phase in the shoots with leafs, leafs and inflorescences.
The highest amount of essential oil was registered in the material harvested after flowers and seeds fall: 1.455-1.823% (dry matter) in the shoots with leafs and 1.408-1.749% (dry matter) in leafs;
GC-MS analysis of essential oil demonstrated that for different genotypes in the shoots with leaf are identified from 14 to 23 components, in leafs from 17 to 25.
Major components are represented by monoterpenes ketones: α-thujone (21.2-38.8%), β-thujone (5.877-16.201%), camphor (17.5-24.6%), followed by eucalyptol (6.47- 11.2%). The studied genotypes of *Salvia officinalis* belong to the thujone/camphor/eucalyptol chemotypes.

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Original scientific paper

CHANGES IN BIOCHEMICAL COMPOSITION OF THE BEVERAGE PRODUCED BY JAPANESE CRYSTALS DURING PROLONGED FERMENTATION

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SUMMARY

Changes in biochemical composition of the several different beverages produced by Japanese crystals during prolonged fermentation of up to 21 days, was investigated. To demonstrate the advantages of the production of Japanese crystals at the same time were examined and sugar aqueous solutions with raisins, which were used as a medium for cultivation of Japanese crystals in the beverages. Analyses were made with contemporary analytical methods that were used in analytics of food for testing of beverages and fermentative products. Enzymes of Japanese crystals hydrolyzed saccharose into glucose and fructose. For this purpose an analysis was performed for determination of sugars content of saccharose, glucose and fructose by HPLC method with Refractive index detector. After 24 hours fermentation at room temperature was spent 39-67% of saccharose, depending on the used amount of sugar and Japanese crystals. Glucose was completely consumed and fructose remained during the fermentative processes. Four hydro soluble vitamins were identified (nicotine amide, vitamin B_1, vitamin B_2, pantotenic acid) and determined by HPLC method of ion pairs in precisely defined concentrations in the beverages. Glucuronic acid content determined by HPLC method increased rapidly during the prolonged fermentation after 7 to 14 days. It is concluded that the desired quality or composition of the beverage prepared from Japanese crystals can be obtained through the proper control of fermentation time.

***Key words**: Japanese crystals, glucuronic acid, chemical content, HPLC*

INTRODUCTION

Refreshing and healthful beverage was obtained by cultivation of Japanese crystals in a sweet aqueous solution in the presence of raisins. Japanese crystals still popularly are so-called as crystals from the Caucasus, crystals of kefir, water kefir fungus, and sea algae, translated into different languages [1]. As a traditional medicine the beverage produced by Japanese crystals was used as healing liquor in the treatment of many diseases, and at present it is considered to be a folk remedy. Japanese crystals are not the only organism, but symbiotic community of different species of bacteria and yeast, with many diverse and complex metabolic pathways. The composition of symbionts of these and similar fermentative beverages varies depending on climatic and geographical conditions, which contributes beverage does not always to have the same chemical composition and taste [2-4]. The Republic of Macedonia is under continental and Mediteranian climate influence, with spring temperatures between 15 and 20 ^oC. These mild climate conditions are ideal for cultivating the Japanese crystals. Over the last few years there has been a great interest in the cultivation and application of this beverage in Macedonia. Because of the diversity of factors affecting the fermentative and oxidative processes, the purpose of this study is to investigate the changes in the chemical composition

in the several different prepared beverages during prolonged fermentation from Japanese crystals, under the Macedonian climate and geographical conditions.

MATERIAL & METHODS

Microbiological research

Japanese crystals were placed on Columbia, Schaedler and Calb agar (Oxoid, UK) directly and after 24 hours incubation in de Man Rogosa and Sharpe broth (MRS broth). The plates were incubated under aerobic and anaerobic conditions in 72 hours. The growth of microorganisms was first examined by microscopic and cultural characteristics. Identification of organisms was done by classical biochemical and automated methods. Vitek system (GP, ANC and YST card) was used to identify various microorganisms [5,6].

Samples

Several beverages obtained by cultivation of Japanese crystals in aqueous solution of varying quantities of sugar and raisins were prepared for this research as follows:

Beverage 1: 3 tablespoons (approximately 40 g) Japanese crystals were spilled over with 1000 ml 2.8% sugar aqueous solution and 6 grains raisins were added.

Beverage 2: 3 tablespoons (approximately 40 g) Japanese crystals were spilled over with 500 ml 5.6% sugar aqueous solution and 6 grains raisins were added.

Beverage 3: 6 tablespoons (approximately 80 g) Japanese crystals were spilled over with 1000 ml 5.6% sugar aqueous solution and 6 grains raisins were added.

At the same time were examined 2.8% and 5.6% sugar aqueous solutions that were used as a medium for cultivation of Japanese crystals in the analyzed beverages.

After filtration through Econofilters (0.20 μm) all the samples were analyzed in triplicate for the determination.

A culture of Japanese crystals was deposited and quoted with number 834, in the Macedonian collection of microorganisms, which belongs to the Institute of Biology on the Faculty of Natural Sciences in Skopje.

Biochemical composition analysis

After 24 hours, in the filtered beverages biochemical composition was investigated by determining the concentration of present sugars, hydro soluble vitamins and glucuronic acid. Analyses were made with contemporary analytical methods that were used in analytics of food for testing of beverages and fermentative products and in biochemistry for testing of biological fluids [7,8,9].

Hydro soluble vitamins analysis

The obtained filtrate was subjected to analysis of hydro soluble vitamins by HPLC, method of ion pairs. A Perkin Elmer HPLC series 250 equipped with UV/VIS detector and RP-select B column (250 mm x 4 mm, 5 μm) was used. The mobile phase consisted of 0.6 g sodium salt of hexane-1-sulfonic acid, 60mL methanol, 330 mL aqua with HPLC grade, 3.2 mL acetic acid, 0.6mL triethylamine, pH 3.2. The flow rate was maintained as 1.3 mL min^{-1}. Detection was carried out at 275 nm [10]. Extraction medium was acetic acid 1:4 water.

Pantotenic acid analysis

A Perkin Elmer HPLC series 250 equipped with UV/VIS detector and Purospher RPe-18 column (125 mm x 4 mm, 5 μm) was used. The mobile phase consisted of 5.1 g potassium dihydrogen phosphate adjusted to 150 mL aqua with HPLC grade and titrated to pH 2.5, with o-phosphoric acid. The flow rate was maintained as 1.5 mL min^{-1}. Detection was carried out at 205 nm.

Determination of reducing sugars and remained sucrose content

Reducing sugars and the remained sucrose content during the fermentation were determined by HPLC method. A KNAUER HPLC equipped with Ri detector and NH_2 column (250 mm

x 4 mm, 5 μm) was used. The mobile phase consisted of 22 % aqua with HPLC grade and 78 % acetonitrile.

Glucuronic acid analysis

Glucuronic acid was determined by HPLC method [10, 11]. An Agilent HPLC series 1100 equipped with UV/VIS detector and SUPELCOSIL LC-ABZ column (250 mm x 4.6 mm, 5 μm) was used.

The mobile phase consisted of aqueous buffer (50 mM sodium dihydrogen phosphate, and titrated to pH 2.58 with phosphoric acid). Detection was carried out at 210 nm. Column was at room temperature. The resolution peaks were recorded on the HPLC chart according to the retention time of glucuronic acid as standard (Fluka, Switzerland) [11,12].

RESULTS & DISCUSSION

In symbiotic community of analyzed Japanese crystals grown in the geographical climate conditions in Republic of Macedonia the following microorganisms *Lactobacillus spp., Leuconostoc mesenteroides, Streptococcus spp., Pantoea agglomerans, Candida pelliculosa, Candida spp (*not *C. albicans),* and *Saccharomyces cerevisiae* were identified. Algae were not found, and hence the folk name "sea algae" is unjustified.

Fermentative and oxidative processes begin immediately when the Japanese crystals are transferred into a freshly prepared aqueous solution of sugar in the presence of raisins. Japanese crystals grown in sugar medium as a small biochemical factory produce many other medicinal substances that contribute to beverage to have an unusual taste, aroma and medicinal properties. To demonstrate the advantages of the production of Japanese crystals at the same time were examined and sugar aqueous solutions with raisins, which were used as a mediums for cultivation of Japanese crystals in the beverages.

Table 1. Analysis of sugars (%)

	saccharose	fructose	glucose
beverage 1	0,94	0,51	0,42
beverage 2	3,78	0,55	/
beverage 3	2,97	0,62	/
medium 1	2,85	0,09	0,08
medium 2 & 3	6,24	0,16	/

Enzymes of Japanese crystals hydrolyzed saccharose into glucose and fructose. After 24 hours fermentation at room temperature was spent 39-67% of saccharose, depending on the used amount of sugar and Japanese crystals (Table 1). Metabolic fates of glucose and fructose produced as a result of hydrolysis of sucrose were different. Glucose was not loosed in parallel with fructose.

The loss of glucose in beverage 2 and 3 after 24 hours fermentation was complete. The same was noted in our previous investigations in Kombucha beverage where also glucose was completely consumed faster and fructose remained during the fermentative processes [13].

Hydro soluble vitamins were identified in the beverages and determined by HPLC method of ion pairs. Four hydro soluble vitamins were determined in precisely defined concentrations shown in Table 2. Studies show that the beverage 2 has higher levels of B vitamins. The same beverage 2 was lyophilized and in its lyophilizat only pantotenic acid was investigated. Caution should be used during determination because exposing to sunlight may adversely affect the processes.

Table 2. Analysis of hydro soluble vitamins (mg/L)

	nicotine amide	vitamin B_1	vitamin B_2	pantotenic acid
beverage 1	/	/	/	/
beverage 2	1,30	0,50	0,075	1,90
beverage 3	0,60	0,10	/	0,94
medium 1	/	/	/	/
medium 2 & 3	/	/	/	/

The recommended daily intake of 1 liter of the beverage 2 obtained from Japanese crystals significantly will improved the vitamin status of the human organism. The same was reported in our previous investigations in Kombucha beverage [13].
Glucuronic acid a derivative of glucose produced in the liver of humans and in most animals is also present in this kind of beverages [15]. It is a highly soluble compound that can bind to substances such as steroid hormones and other drugs, and toxins (i.e. phenols, camphor etc) forming glucuronides and facilitate their transport around the body. In this way glucuronic acid is largely responsible for the elimination of poisonous substances [16].
Four measurements of glucuronic acid quantity in the beverages prepared from Japanese crystals have been done with a time distance of 7 days, because of a small quantity of glucuronic acid in the beverage on the first day of fermentation. From the results shown in Table 3 could be noticed that glucuronic acid increased steadily with time for all samples till 14 day of fermentation. There after, it decreased, gradually.

Table 3. Analysis of glucuronic acid (mg / L)

	1 day	7 day	14 day	21 day
beverage 1	136	201	2797	3248
beverage 2	109	256	4066	3533
beverage 3	101	249	3847	2532
medium 1	119	138	489	718
medium 2 & 3	120	129	397	675

The glucuronic acid content from beverage 2 is sufficient a human body to be detoxified for a period of several weeks.
From the obtained values can be concluded that the best biochemical quality, smell, taste and content of the active ingredients has beverage 2 derived from Japanese crystals. The procedure for the preparation of this beverage should be exercised in the future.

CONCLUSION

Gram positive bacilli and yeast structures predominated in microscopic smear of analyzed Japanese crystals in the geographical climate conditions in Republic of Macedonia.
Studies show that the beverage, made from fermented Japanese crystals, has high levels of B vitamins and glucuronic acid.
The end of the fermentation processes during preparing the beverage produced by Japanese crystals can not be exactly determined, so we suggest it to be a compromise between the individual evaluation of the beverage, flavor, quantity of its biochemical constituents and medical application.

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Original scientific paper

DETERMINATION OF AFLATOXINS IN SPICE PLANTS

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SUMMARY

The occurrence of the aflatoxins B1, B2, G1 and G 2 in common spice plants was examined. The aflatoxins are toxic metabolites of the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus.* The method involves the implementation of high-performance liquid chromatography (HPLC) with postcolumn UV derivatization and fluorescence detection. The aflatoxins are extracted using a mixture of methanol and water and isolated and concentrated by means of immunoaffinity column chromatography. The aflatoxins are separated on Zorbax Eclipse XDB C18 column and detected by fluorescence detector. The method permits the detection of aflatoxins with a detection limit of 0.1 ppb and recoveries in a range 56-88% for a variety of spice plants. The results revealed that two (8%) out of twenty five of spice plants samples were contaminated with detectable amount of the AFB1 (22.3 and 59.0 µg/kg), both in *Foeniculi fructus*. The association between particular spice plant and the AFs contaminated could not be determined due to the low frequency of positive samples.

***Key words**: aflatoxin B1, B2, G1, G2, spice plants, HPLC-FLD*

INTRODUCTION

Medicinal plants, as raw material for herbal tea manufacturing, always contain a certain number of microorganisms that mainly originate from the epifit flora of the plant itself. The other part of the microflora occurs in medicinal plants during some stage of harvest, transportation or it develops during storage period. By processing medicinal plants, during the process of tea manufacturing, a substantial number of microorganisms is removed and destroyed. However, a part of microflora remains, as well as the products of their metabolism [1]. Over 220 species of toxic moulds have been discovered, around 300 of their metabolites, 60 of them real mycotoxins [2]. Among the known mycotoxins, the most toxic one is aflatoxin synthesized by species of *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare genera, and a minor number of other fungi [3,4]. Since mycotoxin producing fungi are quite widespread, they can be detected in various products intended for human diet. Therefore it is very important to discover the sources of contamination of raw materials, intermediate and final products with mycotoxins and mycotoxin producing moulds. This paper follows the frequency of medicinal plant contamination with moulds and mycotoxins. Aflatoxin analysis of medicinal plant is not simple because of interference of high colored materials that are co-extracted with aflatoxins. Acceptable levels of aflatoxins in spices vary in different countries; however, regulatory agencies are imposing uniformly rigorous standards on the level of acceptance in imported commodities. In the EU, as well as in Serbia, an acceptable level of aflatoxins for spices has been set at 5 µg/kg for aflatoxin B1 and 10 µg/kg for aflatoxins in combination (B1 + B2 + G1 + G2) [5,6]. About herbal infusions or med plants there are still not European Regulations. [7] Current analytical methods for the determination of aflatoxins include thin-layer chromatography (TLC), high-

performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA) [8] Although several of these methods achieve low levels of detection, in practice they consume large amount of time and solvent, and require one or more clean-up steps involving liquid–liquid partition or solid-phase extraction [9]. In this study, we applied the IAC method to analyze AFs in several medicinal plants using HPLC with fluorescence detection coupled with immunoaffinity column clean-up step. We investigated the contamination of AFs in mainly consumed herbs commercialized in Serbia.

MATERIAL AND METHODS

***Samples*:**

The following medicinal plant and herbal tea samples were screened for the level of aflatoxins contamination:

1. *Foeniculi fructus*, 2 samples,
2. *Majoranae herba*, 2 samples,
3. *Coriandri fructus*, 1 sample,
4. *Calami rhizome*, 1 sample,
5. *Melissae folium*, 3 samples,
6. *Origani herba*, 1 sample,
7. *Salviae folium*, 1 sample,
8. *Lavandualae flos*, 1 sample,
9. *Anisi fructus*, 1 sample,
10. *Semen sinapis*, 1 sample
11. *Petroselini folium*, 1 sample,
12. *Serpylli herba*, 1 sample,
13. *Satureja herba*, 2 samples,
14. *Carvi fructus*, 1 sample,
15. *Menthae folium*, 2 samples,
16. *Thujea herba*, 1 sample,
17. *Basilici herba*, 1 sample,
18. Herbal tea - *Salvia officinalis*,
19. Herbal tea-*Mentha piperita*.

Materials

(a) Aflatoxin columns were from AflaCLEAN, LC Tech, Germany.

(b) Phosphate buffer saline pH 7.4 (PBS) was prepared with 1.44g di-sodium hydrogen orthophosphate, 0.24 g potassium di-hydrogen phosphate and 8.0 g sodium chloride for each liter.

(c) Extraction solvent was methanol (70 %, vol/vol).

(d) LC mobile phase solvent: it was prepared with:

A) 60% water,

B) 40% acetonitrile–methanol (50-50, vol/vol),

(e) Mixed aflatoxins standard solution (2.02 µg/ml AFB1, 2.03 µg/ml AFG1; 0.50 µg/ml AFB2, and 0.54 µg/ml AFG2 in methanol), LC Tech (Germany).

Analytical reagent di-sodium hydrogen orthophosphate, potassium di-hydrogen phosphate, acetonitrile and methanol for HPLC, sodium chloride, were purchased from Merck (Darmstadt, Germany). All reagents were of recognized analytical grade.

Analysis

Samples were analyzed using the in house validated method.

Stock standard solution of aflatoxins (Aflatoxin B1 40.4 ng/ml, Aflatoxin G1 40.6 ng/ml Aflatoxin B2 10.0 ng/ml and Aflatoxin G2 10.8 ng/ml) was derived from mixed aflatoxins standard solution (e), by diluting it 50 times in mobile phase (d) Calibration was performed using 6 calibration standard mixtures. They were performed by diluting the stock standard solution in appropriate proportions:
Aflatoxin B1 (0.20; 0.40; 1.01; 2.02; 4.04; 10.10 ng/ml)
Aflatoxin G1 (0.20; 0.40; 1.01; 2.03; 4.06; 10.15 ng/ml)
Aflatoxin B2 (0.05; 0.10; 0.25; 0.50; 1.00; 2.50 ng/ml)
Aflatoxin G2 (0.05; 0.11; 0.27; 0.54; 1.08; 2.70 ng/ml)
All samples were finely grinded and thoroughly mixed. Five grams of test portion was blended with 2 g sodium chloride and 100 ml extraction solvent (c). After blending (1min), the sample extract was filtered. The immunoafnity column was washed prior to use with 10 ml phosphate buffer saline (b) at flow rate of 5 ml/min, then 10 ml of clear filtrate was diluted with 40 ml PBS (b) and applied to the conditioned column (2–3 ml/min). After that, the column was washed with 2x10 ml water (flow rate 5 ml/min) and dried by passing air through it. Finally, bound aflatoxins were eluted slowly with 2 ml methanol and pushing air through the column to collect the last drops of eluate in recipient. Methanol was evaporated almost to dryness under a gentle stream of nitrogen and the residue was reconstituted in 0.5 ml of mobile phase (d). The presence of aflatoxins was detected by high performance liquid chromatography (HPLC) using a post-column derivatization with photo-chemical derivatization reactor (LC Tech UVE, Germany) and a fluorescence detector (Agilent 1260 Infinity, with FLD) (λex=365nm and λem=455nm). Using this technique, we were able to determine all four aflatoxins simultaneously, by increasing of fluorescence of aflatoxin B1 and G1 with UV light. The HPLC column was a reverse phase Zorbax Eclipse XDB C18 (150x4.6mm, 5 µm particle size) and the flow rate was 1,5 ml/min. Aflatoxins are subject to light degradation, thus it was necessary to protect work from light by using amber vials or aluminum foil.

RESULTS AND DISCUSSION

Specificity

The specificity of the analysis is given, on the one hand, by the comparison of the retention times of the signals in the sample and the standard solution, and on the other, by the excitation and emission spectra of the signals. Fig. 1 shows as an example the FLD spectrum of aflatoxin B1.

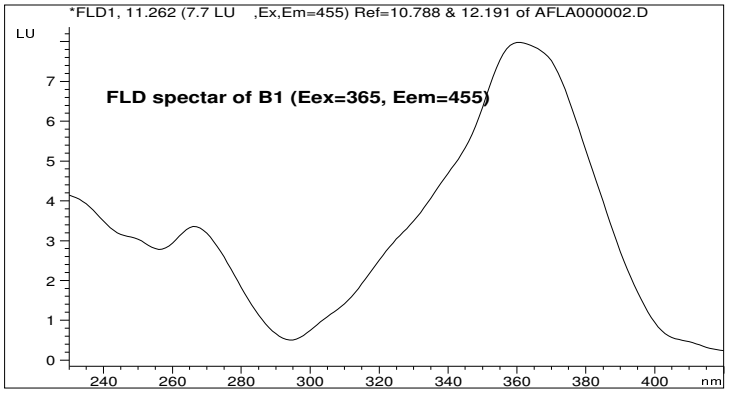

Fig. 1. Excitation spectrum of aflatoxin B1

Precision

The precision was determined by a multiple analysis of a spiked sample *Foeniculi fructus*. The precision is expressed by the relative standard deviation (%RSD) and the confidence interval of the mean value (C.I.; n=6; P=95 %) (Table 1).

Table 1: Intra-day precision of the analytical method (n = 6; P = 95%):

	AFB1	**AFB2**	**AFG1**	**AFG2**
Mean (µg/kg)	7.47	1.89	2.35	2.29
SD	0.57	0.35	0.62	0.26
%RSD (max 30%)*	7.6	18.4	26.5	11.5
C.I.	0.60	0.37	0.65	0.28

*Commission Regulation 401/2006

Accuracy

The accuracy of the method was examined by the determination of the recoveries of the aflatoxins. The recoveries of different matrices were determined by the addition of an aflatoxin standard to the blank sample Blank samples used were following herbs: *Basilici herba*, *Majoranae herba and Foeniculi fructus*. One-hundred microliters of concentrated mixture of afatoxins standard solution (AFB1 4.11 ng/ml, AFB2 1.56 ng/ml AFG1 6 ng/ml and AFG2 7.4 ng/ml) was added on the test material (5 g) before analysis. The results of the recovery tests and statistical data are given in Table 2.

Table 2: Recovery of aflatoxin B1, B2, G1 and G2 from blanks samples spiked with known concentration of toxin and statistical data

		AFB1	**AFB2**	**AFG1**	**AFG2**
Basilici herba	Recovery (%)	57.8	61.4	83.8	88.2
	Standard deviation (SD)	0.5	0.1	0.4	0.1
	Reproducibility (%RSD)	11.6	6.7	9.0	8.1
Majoranae herba	Recovery (%)	56	63	88	56
	Standard deviation (SD)	0.1	0.03	0.1	0.1
	Reproducibility (%RSD)	4.1	3.3	3.7	14.0
Foeniculi fructus	Recovery (%)	56.4	82.3	81.4	79.9
	Standard deviation (SD)	0.2	0.1	0.3	0.04
	Reproducibility (%RSD)	7.2	6.4	9.1	4.2

Linearity

The linearity of the measurements was checked for a standard solution containing aflatoxins in a range from the limit of: Aflatoxin B1 (0.20-10.10 ng/ml), Aflatoxin G1 (0.20-10.15 ng/ml), Aflatoxin B2 (0.05- 2.50 ng/ml) and Aflatoxin G2 (0.05- 2.70 ng/ml). The calibration graphs can be described by the following equations (Table 3) and curves (Fig. 2)

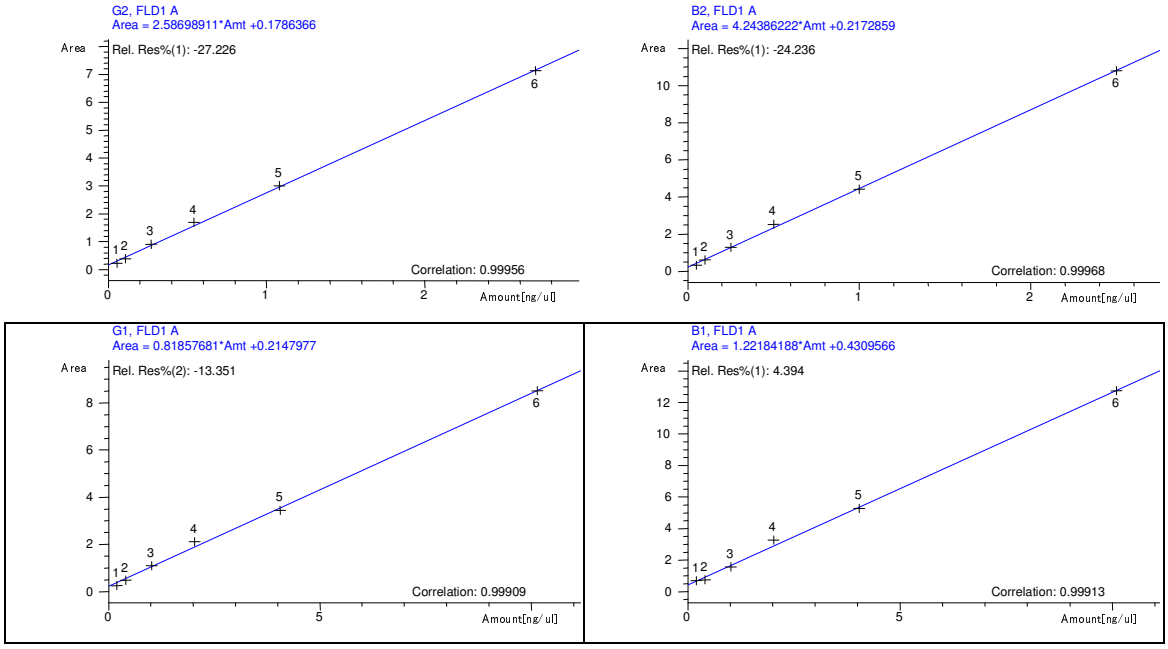

Fig. 2. Calibration curves of aflatoxins

Table 3. Equations, coefficients of correlation, LODs and LOQs

	y=ax+b	R^2	LOD (µg/kg)	LOQ (µg/kg)	%RSD
AFG1	y = 0.81857681x+0.2147977	0.99909	0.06	0.3	3.02 %
AFG2	y = 2.58698911x+0.1786366	0.99956	0.04	0.1	3.55%
AFB1	y = 1.22184188x +0.4309566	0.99913	0.05	0.4	8.67%
AFB2	y = 4.24386222x+0.2172859	0.99968	0.03	0.1	12.95 %

Limit of detection and limit of quantification

The limit of detection (LOD) for an analytical method is defined as the minimum detectable level of control sample under the conditions of a particular assay. The limit of quantification (LOQ) is defined as the minimum level of control sample that can reliably be quantified. The concentration (in µg/kg) with signal to noise ratio of at least 3 was taken as LOD and concentration with signal to noise ratio of at least 10 was taken as LOQ, which meets the criteria defined by ICH guidance [10]. The LOD and LOQ results are presented in Table 3.

Application to real samples

Developed method was successfully applied to real samples collected from retail market. The results revealed that two out of twenty five (8%) of spice plants samples were contaminated with detectable amount of the AFB1 (22.3 and 59.0 µg/kg), both in *Foeniculi fructus* (Fig. 3). These results are similar to those presented by Romagnoli et al. [7]. In this work none of the aromatic herb, herb-tea and medicinal-plant samples analyzed was contaminated, even if they are from tropical countries.

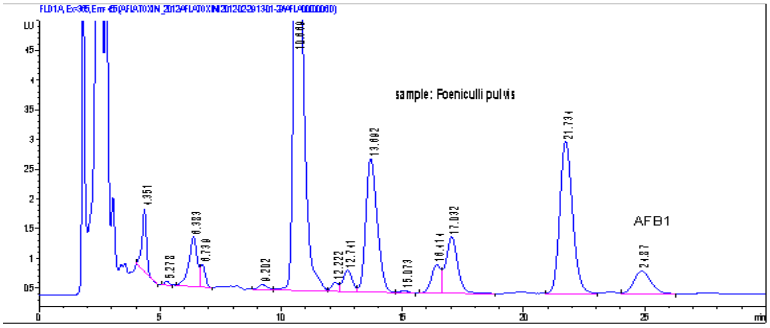

Fig.3. Chromatogram of contaminated sample *Foeniculli fructus* (59,0 µg/kg)

CONCLUSION

This study provides useful information about the risk of mycotoxin hazard and hopes to raise the consciousness among consumers, researchers, farmers and traders about the importance to improve processing methods (harvest, drying, transportation and storage) and to establish a monitoring program on the distribution and contamination levels of AFs in human food like spices, herbs and med plants.

A procedure for determination of aflatoxins in plant material using immunoaffinity columns clean up and photochemical derivatisation, with FL detection has been described. The sensitivity of the aflatoxin B1 and G1 is substantially increased with the use of UV derivatization (PHRED). The method can be applied to various plant materials and herbal remedies and with high selectivity for all aflatoxins. The procedure offers several advantages: sufficient selectivity, high reproducibility and good recoveries for a variety of plant materials, easy automation, no need to use aggressive or unstable reagents. For the investigation presented in this paper, the samples of spice from retail market were used. Out of 25 samples, it was find that only two were contaminated with AFB1 and did not comply with legislation.

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Original scientific paper

NUTRITIONAL PARAMETERS OF 4 BASIL CULTIVARS

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SUMMARY

In this study there were evaluated ascorbic acid content, total phenolic content, and total antioxidant capacity of 4 basil (*Ocimumbasilicum* L.) cultivars: ´Ohře´, ´Compakt´, ´Purple Opaal´ and ´Lettuce Leaf´. The plants were grown under field conditions and the samples were taken in 3 terms of harvest. The ascorbic acid content was determined by High Performance Liquid Chromatography (HPLC). The ascorbic acid content ranged from 156.9 to 314.1 mg/100g d.w.of plant material. The highest average ascorbic acid content was confirmed in the aerial part of basil cultivar ´Ohře´ in the second harvest (314.1 mg/100g d.w.) and the lowest in the aerial part of basil cultivar ´Lettuce Leaf´ collected in the third harvest (156.9 g/100g d.w.). Second harvest had the highest values of measured ascorbic acid in three of four tested cultivars. Significantly the highest ascorbic acid content was found at basil cultivar ´Ohře´. The total phenolic content (TPC) of each sample was determined by using the modified Folin-Ciocalteu method. The highest TPC was shown by ´Purple Opaal´ cultivar on the third harvest date (7.71 g gallic acid equivalents (GAE)/100g d.w.) and the lowest by ´Ohře´ cultivar on the third harvest date (4.58 g GAE/100g d.w.). The total antioxidant capacity (TAC) was measured using DPPH assay. TAC ranged from 13.01 - 27.72mM TE/100g d.w. in cv. ´Compakt´ (first harvest) and ´Lettuce Leaf´ (third harvest), respectively. The highest values of TPC and TAC were obtained by basil cultivar ´Purple Opaal´. Among harvest times in interaction with cultivar there were not found significant differences in contents of all measured parameters, but the highest values of the total phenolic content and the total antioxidant capacity were in most cases measured in the samples collected in the third harvest.

Key words*: total phenolic content, total flavonoid content, total antioxidant capacity, DPPH, basil cultivars*

INTRODUCTION

Ocimum L. is an important economic and medicinal plant. It belongs to *Lamiaceae* family. *Ocimum* requires warm ecological conditions for its growth and should be protected from the frost, because *Ocimum* is indigenous to tropical areas of Africa, America and Asia. Economically, the most important taxon within *Ocimum* is section *Ocimum*. The most frequently used species are *O.basilicum*, *O.americanum* and their hybrid *O.* x *citriodorum*. These species are used for essential oil production and also as pot herbs. [1] Sweet basil (*O. basilicum*) is frost sensitive herbaceous annual plant. It is naturalized almost all over theworld. Basil is reported to tolerate very variable soil conditions. The basic ecological requirements for basil cultivation are warm, light and moisture. [2] There are many different cultivars offered for the fresh basil production and for the garden cultivation. In the CzechRepublic there are companies that offer a lot of cultivars too. For example seed company Semoa.s. offers in 2012 12 sweet basil cultivars [3] and company Seva Seed 6 sweet basil cultivars [4].

Traditionally basil has been used as a medicinal plant for various ailments, such as headaches, coughs, diarrhoea, constipation, warts, worms and kidney malfunction [2]. It is also thought to be an antispasmodic, stomachic and carminative [2, 5].
Basil is used fresh, dried, or as a paste in oil. The dried form is less aromatic than the fresh form. The name basil is derived from the Greek word basileus, meaning "king" because of its wonderful "royal" fragrance [6]. Fresh basil contains ascorbic acid (vitamin C) as well. [7]. The fresh aromatic basil leaves are used as flavourings or spices in sauces, stews, salads, pickled vegetables, vinegar, aromatic oils as well as in "Bouquet garni" [2].
We do not eat herbs in such amounts that they could be a significant source of vitamins or minerals in our daily diet, but sweet basil is an effective antioxidant[8]. Its antioxidant effectiveness is more than that of BHA (butylhydroxyanisol) or BHT (butylhydroxytoluene)[8]. There are many substances responsible for the antioxidant effect of basil, such as β-carotene, tocopherol and eugenol, isoeugenol, linalool and linalyl acetate as well as polyphenols and flavonoids [2, 9].

MATERIAL & METHODS

Plant material

For the experiment 4 basil (*Ocimumbasilicum* L.) cultivars were used: ´Ohře´, ´Compakt´, ´Purple Opaal´ and ´Lettuce Leaf´. Seeds were obtained from Czech seed companies Seva Flora Valtice and SemoSmržice. Sowing was done on the 27th March 2009 in greenhouse of Mendel University, Faculty of Horticulture in Lednice. The seedlings were planted out on the 15th May 2009. The plants were grown in the open field of Faculty of Horticulture in conditions of Lednice. Aerial parts were harvested on the day of analysis: first time on the 9th July 2009, second time on the 6th August 2009 and third time on the 14th September 2009. The fresh plants were used for ascorbic acid evaluation and extraction. The contents of measured parameters were afterwards calculated on dry basis.

Evaluation of ascorbic acid content

The ascorbic acid levels in the basil cultivars were determined. 10 g of fresh herba were homogenised in a blender with oxalic acid from which the samples for analysis were prepared. The analyses were performed by RP-HPLC in a LCO-101 column at 254 nm using a UV-VIS detector. [10]
The amount of ascorbic acid was expressed as mg/100gd.w.

Extract preparation

Fresh matter was extracted using 75% methanol [9]. The mixture was filtered after 24 hours of extraction in room temperature through filter paper. The extract was stored in the refrigerator until further analysis. The extracts were used for evaluation of total phenolic content and total antioxidant capacity.

Evaluation of total phenolic content

The total phenolic content was estimated using the modified Folin-Ciocalteu photometric method [11]. The appropriate amount of filtered methanol extracts were oxidized with Folin-Ciocalteu reagents and after 5 minutes was the reaction neutralized with saturated sodium carbonate. The solution was then immediately diluted to the volume of 50 ml with distilled water. The absorbance was measured at 750 nm after 90 minutes of incubation at room temperature against the blank. As the standard was used Gallic acid. The total phenolic content is here expressed as g Gallic acid equivalents (GAE)/100g d.w.

Total antioxidant capacity - DPPH assay

The total antioxidant capacity of samples was determined using the modified DPPH (2,2-diphenyl-1-picrylhydrazyl) assay [12]. The DPPH radical ($DPPH^{\cdot}$) solution (100 $\mu M.l^{-1}$) was prepared in methanol. In 3.8 ml of the DPPH solution was added 0.2 mL of the tested extracts. After 30 minutes of incubation at room temperature was measured the reduction in absorbance at 515 nm. The standard curve was prepared using different concentration of Trolox. The results were expressed as mMTrolox equivalents (TE)/100g d.w.of plant material.

Statistical analysis

All determinations were conducted in triplicates, and all results were calculated as mean ± standard deviationin this study. Significance was evaluated by analysis of variance (Anova) followed by Tukey's HSD (Honestly Significant Difference) test using the PC software StatisticaCz v. 8 (Stat Soft). Probability value of $p<0.05$ was used as the criteria for significance differences.

RESULTS & DISCUSION

Ascorbic acid content

The highest ascorbic acid content was determined in basil cultivar ´Ohře´ in the second term of harvest (314.1 mg/100g d.w.) and the lowest in ´Lettuce Leaf´ cultivar in the third term of harvest (156.9 mg/100g d.w.). The results are shown in Tab. I. Among periods of harvest there were not find significant differences but significantly highest average ascorbic acid content was analysed in ´Ohře´ cultivar.

Table 1: Ascorbic acid content (mg/100g d.w.)

	Cultivar				
	´Ohře´	´Compakt´	´Purple Opaal´	´Lettuce Leaf´	average
harvest No. 1	289.1 ± 59.5	235.8 ±50.3	231.8 ± 15.3	188.2 ± 5.4	236.2 ± 50.5
harvest No. 2	314.1 ± 48.0	213.9 ± 28.6	271.7 ± 42.8	218.4 ± 69.0	254.5 ± 60.2
harvest No. 3	296.0 ± 27.3	238.5 ± 56.9	181.3 ± 22.8	156.9 ± 9.3	218.1 ± 63.2
average	299.7 ± 42.1b	229.4 ± 42.2a	228.3 ± 46.8a	187.8 ± 43.9a	

Values are mean ± standard deviation.Means followed by the same letter are not significantly different α = 0.05.

Total phenolic content

The results of evaluation of total phenolic content are shown in Tab. II.

The total phenolic content measured at 4 basil cultivars ranged from 4.58 g GAE/100g d.w. (´Ohře´, harvest No. 3) to 7.71 g GAE/100g d.w. (´Purple Opaal´, harvest No. 3). There were no significant differences found in total phenolic content among cultivars. In the case of basil cultivars Compakt´, ´Purple Opaal´ and ´Lettuce Leaf´ were measured the highest total phenolic contents in plants harvested in the third period. The plants from the third harvest contained significantly higher total phenolic content than those from the previous two harvests. Among cultivars the highest average total phenolic content (but not significantly) was found in red-leafed basil cultivar ´Purple Opaal´. This finding corresponds to the results of the study by Llorach et al. [13] who compared total phenolic content and TAC in green-leafed and red-leafed lettuce cultivars. The highest total phenolic content was found in red-leafed cultivars that contain anhocyanins. The strong antioxidant activity of anthocyanins

could be responsible for the high antioxidant activity of red lettuce [13]. This is in agreement with previous work showing that coloured varieties of vegetables (red onion, red cabbage, and red pepper) are especially rich in phenolic compounds [14]

Table 2: Total phenolic content (g GAE/100g d.w.)

	Cultivar				
	´Ohře´	´Compakt´	´Purple Opaal´	´Lettuce Leaf´	average
harvest No. 1	5.77 ± 1.64	4.59 ± 0.43	5.27 ± 0.76	4.70 ± 0.87	5.08 ± 1.00a
harvest No. 2	5.50 ± 0.94	4.97 ± 2.03	7.22 ± 0.45	4.84 ± 0.33	5.63 ± 1.39a
harvest No. 3	4.58 ± 0.79	6.28 ± 2.02	7.71 ± 1.07	7.60 ± 1.74	6.54 ± 1.83b
average	5.29 ± 1.16	5.28 ± 1.64	6.73 ± 1.32	5.71 ± 1.73	

Values are mean ± standard deviation.Means followed by the same letter are not significantly different α = 0.05.

In the study of Shan et al.[9] the extracts of 26 spices were measured. In basil there was measured the amount of total phenolic content of 3.64 g GAE/100g d.w.. Total phenolic contents of all tested basil cultivars were higher than that measured by Shan et al.[9]. In this study there were determined two times higher amounts of total phenolics in ´Lettuce Leaf´ basil cultivar in the first and second harvest. Shan et al. [9] analysed the total phenolic content in 6 species of *Lamiaceae* family. They measured amounts that ranged from 3.64 g GAE/100g d.w. (*Ocimumbasilicum* L.) to 10.17 g GAE/100g d.w. (*Origanumvulgare*L.). Thyme contained 4.52 g GAE/100g d.w., sage 5.32 g GAE/100g d.w., mint 5.15 g GAE/100g d.w.and rosemary 5.07 GAE/100g d.w.. In the study by Yi and Wetzstein[15] there were determined total phenolic contents of some culinary and medicinal herbs grown under greenhouse and field conditions. Thyme contained 6.90 g GAE/100g d.w. (greenhouse conditions) and 4.49 g GAE/100g d.w. (field conditions). The amounts reported byYi and Wetzstein[15] in thyme are similar to those measured in this study. Surveswaran et al [14] analysed in dried basil leaves much lower content of phenolics (2.63g GAE/100g d.w.).

Total antioxidant capacity (DPPH)

The results of evaluation of total antioxidant capacity by DPPH method are shown in Tab. III. Total antioxidant capacity ranged from 13.01 mM TE/100g d.w. (´Compakt´, harvest No. 1) to 27.72 mM TE/100g d.w. (´Lettuce Leaf´, harvest No.1). There were not found significant differences in TAC in particular cultivars in different periods of harvest. As well as in the case of the total phenolic content there were the highest TAC levels measured at the plant material confirmed on the third harvest in ´Compakt´, ´Purple Opaal´ and ´Lettuce Leaf´ basil cultivars. ´Purple Opaal´ showed the highest average TAC (not significantly) of tested basil cultivars. The plants from the third harvest contained significantly higher TAC than those from the previous two harvests. The correlation between total antioxidant capacity and total phenolic content was identifed as a highly significant linear regression (R = 0.95). Such high R value suggested that TAC (DPPH method) is significantly dependent on total phenolic content (modified Folin-Ciocalteu method). Phenolic compounds were responsible for antioxidant activity of tested basil cultivars. Similar result was obtained by Shan et al. [9] in 26 spices. In contrast to Total phenolic content, there was not find correlation between total antioxidant capacity and ascorbic acid content (R = -0.07).

Table 3 DPPH (mMTE/100g d.w.)

	Cultivar				
	´Ohře´	´Compakt´	´Purple Opaal´	´Lettuce Leaf´	average
harvest No. 1	19.41 ± 5.67	13.01 ± 2.61	15.34 ± 3.59	15.35 ± 3.19	15.78 ± 4.13a
harvest No. 2	16.63 ± 3.60	15.60 ± 7.08	22.02 ± 2.34	17.17 ± 1.18	17.85 ± 4.40a
harvest No. 3	18.32 ± 3.03	22.34 ± 8.04	26.12 ± 3.54	27.72 ± 7.78	23.62 ± 6.41b
average	18.12 ±3.88	16.98 ± 6.92	21.16 ± 5.47	20.08 ± 7.17	

Values are mean ± standard deviation.Means followed by the same letter are not significantly different α = 0.05.

Surveswaran et al. [16] evaluated natural phenolic antioxidants from 133 Indian medicinal plants. They choose 2 Ocimum species for TAC evaluation. The level of TAC (DPPH method) was 23.45 mM TE/100g d.w.in Ocimumbasilicum (dried leaf) and 7.18 mM TE/100g d.w.in Ocimum sanctum (dried leaf). In this study there were measured similar levels of TAC at Ocimumbasilicum, but higher than at Ocimum sanctum in Surveswaran´s study [16].

CONCLUSION

Basil (Ocimumbasilicum) belongs to the rich sources of bioactive compounds. In this study there were founddifferences between basil cultivars (´Ohře´, ´Compakt´, ´Purple Opaal´ and ´Lettuce Leaf´) in ascorbic acid content. ´Ohře´ contained significantly highest amount of ascorbic acid. Differences in TPC and TAC among the cultivars were also shown. The highest values of TPC and TAC were observed by red-leafed basil cultivar ´Purple Opaal´. There were found significant differences in TPC and TACamong harvests. The highest TPC and TAC were determined in plants harvested in late summer (14th September 2009).Moreover, linear regression between total antioxidant capacity and total phenolic content was found.

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Original scientific paper

INFLUENCE OF TILLAGE SYSTEM ON WEEDENESS OF SOME MEDICINAL PLANT CROPS

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SUMMARY

Survey of weed flora in four crops of medicinal plants (thyme- *Thymus vulgaris* L., sage - *Salvia officinalis* L., lemon balm- *Melissa officinalis* L. and lavender- *Lavandula angustifolia* L. and) was conducted at plantations of the Institute of Medicinal Plants "Josif Pancic" during 2009. Qualitative and quantitative evaluation of the presence of weeds was performed twice during the growing season. The first assessment (May 19) was obtained before and after hand hoeing. In each medicinal crop, by random sampling method, the plots of 5 x 1 m^2 were chosen. After visual observation and assessment of abundance and cover of weed species the plant material (individual weed species) were taken for measurements of its fresh and dry weight.. In surveyed crops the total of 35 different weed species was determined. The highest weed population diversity was found in sage field (35 species), followed by lavender (23), thyme (20), and the balm with only 16 species. Among the weed species the highest particpation exhibited annuals (13), while biannual as *hemicriptophytes*, *geophytes* and *tero-hemicriptophytes* were presented in number of 12, 5 and 12, respectively. The species of the highest abundance were: *Convolvulus arvensis*, *Agropyrum repens*, *Cirsium arvense*, *Erigeron canadensis*, *Lactuca serriola* and *Polygonum lapathifolium*. Species of the highest abundance (*C. arvensis* and *A. repens*) had also the highest fresh weight, followed by: *Sonchus arvensis, Sorghum halepense, L. serriola* and *C. arvense*.

Key words: *Thymus vulgaris* L., *Melissa officinalis* L., *Lavandula angustifolia* L., *Salvia officinalis* L., weed control.

INTRODUCTION

Conventional plant production includes pesticide (herbicide) applications, known as . not fully suitable approach for large scale production of medicinal plants. Although certain authors (1, 2) studied possibilities of herbicide applications in medicinal plant production, in general, the use of herbicides is not recommended for weed control in medicinal plant plantations. Non-chemical practices, mostly agro-technical measures are usually used for weed control in medicinal plant plantations (3). Best results for weed control in MAP crops are achieved by integrated control measures which combine preventative and direct (physical, agro-technical and chemical) control measures. In addition, comprehension of competition relationships and allelopathy effects among different groups of plants could contribute to better weed control. In the future, for organic production of medicinal plants the biological weed control measures should also be integrated with other measures of weed control.

Goal of this study was to determine the weed infestations in medicinal plant crops (thyme, sage , lemon balm and lavender), as well as influence of tillage on weediness of these crops.

MATERIAL AND METHODS

The experiment was conducted at the Institute of Medicinal Plants "Josif Pančić" in Pančevo (near Belgrade) in 2008 on alluvial black marsh soil (pH of soil 5.7-8.0, with 4.3% organic matter and 48% clay). In 2008 precipitation and GDD in period April to September in the experimental areas was 235.6 and 1361.35, respectively. Previous crop was wheat. The first evaluation of weediness was conducted before hoeing (19 May) and the second evaluation after hoeing (21 July) at the time when weeds reached flowering growth stage. Presence of weeds in MAP crops was estimated visually upon Vestoff and van der Marrel (4) scale: 1- individual examples present; 2- a few plants present, covering small areas; 3- a lot of plants, covering 1-10% of the area; 5- species present in high numbers, coverage 10-25%; 7- no concern on number of the plant species, coverage is 25-50%; 8- no concern on number of the plant, species covers 50-75% of the area; 9- no concern on number of the plant, species covers 75-100% of the area. In each crop the five plots of 1 m^2 were randomly chosen and all of the plant mass from those areas was collected. For each weed species we determined the numbers present, fresh and dry weight. All data were analysed using statistical software STATISTICA 5.0.

RESULTS AND DISCUSSION

In the thyme field before weed removal by hoeing we determined a presence of 20 weed species, and after hoeing the 13 weed species. This indicated that hoeing reduced weed population for 35% (data now shown). The species presented in the highest number were *Convolvulus arvensis* (with abundance 3 to 8) and *Cirsium arvense* (with abundance 2 do 7). These species are *geophytes* with strongly developed roots for vegetative reproduction which makes them difficult to control (5, 6). Other species like *Taraxacum officinale*, *Ereigeron canadensis, Lactuca serriola, Polygonum lapathifolium*, *Sorghum halepense* and *Agropyrum repens* were also presented with high abundance (2 to 5). Other weed species individually did not cover significant area. These species should not be underestimated because all together can create strong competition with the crop. After hoeing, weeds of the highest abundance and coverage were still the *C. arvensis* and *C. arvense*, but expressing lower abundance (3-5, and 2-3 respectively). Other 11 species were in low populations and had low coverage. In addition, certain species after hoeing did not come back (*S. arvensis, P. lapathifolium, C. bursa-pastoris, A. artemisifolia, Ch. album, S. halapense*, *C. biennis* and *L. viminea*). The highest total fresh weight had the species that were presented in the highest number and had the highest coverage: *C. arvensis* (165.4g at I assessment and 218.4g in the II assess.), *C. arvense* (116.6g at the I assess. and 107.2g at the II assess.) and *T. officinale* (124g at the I assess.) (Table 1). We also noted the species exhibiting low abundance and coverage which had high mass per plant (e.g. *Tragopogon major*).

In sage field, before hoeing we recorded 25 weed species (data not shown). Species presented in the highest number were *A. repens* (with abundance 7 to 8), *C. arvensis* (with abundance 7), *E. canadensis* (with abundance 5 to 7), *L. serriola* and *L. viminea* (with abundance 3 to 7). As in the hyme field, the species of the highest abundance and with greatest coverage were mostly *geophytes*, while *therophytes* (annual plants) and *hemicriptophytes* were present in a lower number. After hoeing we recorded 13 species, and weed presence was reduced by 48%. After hoeing, similarly as in thyme field, the *C. arvensis* and *A. repens* were the dominating species (5-7, and 3-5 respectively). Fifteen species did not return after hoeing, and those were mostly species of low abundance and coverage.. Furthermore, we recorded a presence of 3 new species: *Lolium temulentum*, *Sonchus oleraceus* and *Artemisia vulgaris.* The highest fresh weight had *A. repens* (690g) and *T. major* (395g) (Table 2). Significant fresh weight at second evaluation had *C. arvensis* (146,4g) and *S. halepense* (103,2g).

In the lemon balm field, before hoeing we recorded 14 weed species, out of the highest presence were: *C. arvensis* (with abundance 5 to 8), *L. serriola* (with abundance 7) and *A. repens* (with abundance 7 to 8*),* followed with *E. canadensis* and *P. lapathifolium* (with abundance 5 to 7), and *S. oleraceus*, *C. biennis* and *T. major* (with abundance 3 to 5). Other species were presented in lower number (data not shown). After hoeing we recorded 10 weed species, which mean that the weed presence after hoeing was reduced by 29%. Like in previous crops the most common weed species were: *C. arvensis, L. serriola* and *P. lapathifolium*. When compared with first evaluation there were 6 weed species absent and 2 new weed species were present (*Chamomilla recutita* and *Papaver rhoeas*). The *A. repens* (124.1g), *L. serriola* (218.6g) and *T. major* (128.3g with only 2 plants per m^2) had the highest weight at first evaluation (Table 3), followed by *C. arvensis* (96.9g) and *C. biennis* (69.3g). At the second evaluation the highest vegetative mass was recorded for *L. serriola* (180.4g) and *C. arvensis* (144.8g).

Before the land tillage, in lavender field we recorded 21 weed species. Species of the highest abundance and coverage were *C. arvensis* and *E. canadensis* (with abundance 7 to 8) followed by *A. repens* (with abundance 7). Next to these two *geophyte* species, an species of high frequency was a *therophyte* (*E. canadensis*). This species is spreading and has a status of invasive weed species in Serbia (7, 8). After hoeing we recorded 13 weed species indicating that the weed population was reduced by 38%. At the second evaluation we recorded 10 weed species less that at the first evaluation. In addition, we recorded 2 new weed species, the *Lolium perenne* and *Chamomilla recutita*. Weed species presented in the highest number were *A. repens* and *C. arvensis*, while instead of *E. canadensis* which was the third most present species at the first evaluation, at the second evaluation , the third most present species was *L. serriola* (data not shown). The *S. arvensis* (275.4g) and *C. arvensis* (232.8g) had the highest vegetative mass (before hoeing), while *R. crispus* had the highest average weight per plant (78.5 g) (Table 4). At the second evaluation species *L. serriola* had the highest average mass per plant and total mass per m^2, followed by *P. lapathifolium.*

CONCLUSION

In general, perennial weed species (*A. repens*, *C. arvensis, S. halepense*, and *S. arvensis*) before and after hoeing dominated in all 4 medical plant crops. The number and mass of these weed species was high even after hoeing (in some cases the values after hoeing or at the second evaluation were higher). This means that hoeing alone as a method for weed control in medical plant crops is not sufficient. These perennial weeds (life form *geophytes*) due to their high generative reproduction potential, represent a significant long term problem in medical plant crops. As a result, we believe that the use of herbicide (with stringent control) would be necessary to control weed species in medical plant crops in Pancevo district.

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Original scientific paper

VARIABILITY OF SIDERITIS RAESERI BOISS. & HELDR., ENDANGERED SPECIES, GROWING WILD IN GRAMOZI MOUNTAIN IN ALBANIA

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SUMMARY

Sideritis raeseri Boiss.et Heldr. (Çaji i Malit) is Balkan endemic species growing wild in Albania, which is included in the Red Book of Albania as an endagerend species. It is one of the most important tea plants, used as a medical herb which also contributes to the livelihoods of many people in region of the south and southeast of Albania. Ten samples of *S. raeseri* from various locations of the Gramozi Mountain were evaluated for their morpho-biometric data. In this investigation 8 quantitative and qualitative characters were evaluated using R statistics. Significant correlations among some important characters were observed. Principal Component Analysis (PCA) explained about 80% of the variation related to main effective characters such as height of plant, the length of stem, length and width of leaf, ration length/width leaf ratio and length of the flowering stem. Cluster analysis upon similarity coefficient distance divided populations into two main groups.

Key words*: *Sideritis raeseri, Albania, morphology, statistical analysis

INTRODUCTION

Sideritis raeseri Boiss.et Heldr. is the Balkan endemic specie growing wild in Albania, and included in the Red Book of Albania as an endangered species. In Albania, the mountain tea is known as "Çaj mali" and is commonly consumed as tea, which is also used in Albanian folk medicine [1,2]. *Sideritis* L., belongs to the family Lamiaceae (Labiatae). Over 150 species of the genus *Sideritis* are mainly found in the Mediterranean area [3].

S. raeseri is perennial herb that grows in the mountainous regions between Southern Europe and Eastern Mediterranean. There are 4 *Sideritis* species in Albania and endangered rate of this genus is high [2]. The aerial parts of *S. raeseri* have been widely used in folk medicine to treat diseases such as cough, common cold, gastrointestinal disorders [4] and therefore these species are used as herbal tea in Albania as well as in the other Mediterranean countries [5].

Sideritis species are spread widely through-out the world, because of their pharmaceutical effects, and are consumed in different countries [6,7]. These plants are known to have antispasmodic, antifeedant, carminative, analgesic, nervous system stimulant, sedative, antitussive, stomachic, and anticonvulsant, antibacterial, antinflammatory, antimicrobial and antioxidant activities [8].

"R" is widespread statistical software, which was invented by Robert Gentleman and Ross Ihaka and is currently maintained by the R core-development team [9].

The purpose of this study is to determine the variability of wild populations of *S. raeseri* in Southeast of Albania. In this study the bio-morphological data of *S. raeseri* using R Statistics were investigated for the first time.

MATERIAL AND METHODS

Plant material

The aerial parts of *Sideritis raeseri* Boiss. et Heldr. were collected from different area of Gramozi Mountain in South East of Albania from June to July 2011. The plant material were determined by Prof. A. Mullaj, Research Centre for Flora and Fauna, Faculty of Natural Sciences, Tirana University, Albania, The equal number of individuals (single plants) was taken (60) from six populations of *Sideritis raeseri*. grown in North-East line. In this investigation for evaluation of morphological diversity, 8 quantitative and qualitative characters were evaluated. The voucher specimens are deposited at the Herbarium of Botanical Garden, Crop Production Department, Faculty for Agriculture and Environment, Agricultural University of Tirana, Albania

Statistical Analysis

The data were compared with R statistical software. The morphological data were collected and stored in MS excel and imported easily from a basic core function of R. The data were compared with R statistical software using cluster and labDSV.

RESULTS AND DISCUSSION

From different area of Gramozi Mountain in South East of Albania from it was collected 60 individuals (single plants) from sixth population (Table 1).

Table1. Geographic positioning of studied populations

Locality No.	Altitude (m)	Latitude	Longitude	Exposition
1	1751 m	40°26'23.12"N	20°47'42.03"E	Eastern
2	1766 m	40°26'18.58"N	20°47'44.61"E	Eastern
3	1807 m	40°26'27.45"N	20°47'57.16"E	Southern
4	1816 m	40°26'18.68"N	20°47'59.14"E	Western
5	1845 m	40°26'10.72" N	20°48'1.96"E	Eastern
6	1751 m	40°26'7.61"N	20°47'51.75"E	Eastern

Figure 1. Site where populations were collected; southeast region in Albania (Gramozi Mountain)

By using R-package (cluster and labDSV), Cluster Analysis (CA) has been done in order to categorize the plants. In present study the highest positive significant correlations were observed between width and height of middle stem (0.58); height of plant and height of middle stem (0.48); height of plant and with stem (0.36). The lowest significant negative correlation was found between plant height and leaf length (-0.01) while the lowest significant positive correlation was found between height of middle stem and length (0.02) (Table 2). Principal component analysis revealed six components, which justified 100 percent of the total variation among traits (Table 3), Principal component 1 (PC1) justified 28 percent of the total variation and was equally associated with height, stem. PC2 mainly consisted of height middle stem and accounted for 15 percent of total variation. PC3 explained 13 percent of the total variation and was mainly associated with Length of leaf, No of flowering. (Fig.2.) PC4 accounted for 11 percent of the total variation and consisted of Length of leaf and flowering stem. PC5 accounted for 11 percent of the total variation and consisted of stem, length of leaf.

Table 2. Correlation data of 8 traits of 6 populations of *S. raeseri* in Southeast of Albania (Gramozi Mountain)

	Stem	H.mdl.stem	Length	Width	Flowering.stem	No.of.flowering	H.mdl.Flowering
Height	0,36	**0.46**	-0.01	0.16	0.33	0.33	0.18
Stem		0.11	-0.10	0.18	0.32	0.18	0.14
H.mdl.stem			0.02	0.04	0.16	0.33	0.18
Length				0.05	-0.6	0.41	0.17
Width					**0.58**	0.17	0.04
Flowering.stem						0.12	-0.15
No.of.flowering							-0.15

Table 3. Principal Component coefficients of 6 populations of *S. raeseri* in Southeast of Albania (Gramozi Mountain)

Characters	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**
Height	0.46	0.15	0.35	0.46	-0.07	0.30	-0.12	0.64
Stem	0.41	0.07	0.18	0.19	0.79	0.09	0.36	0.11
H.mdl.stem	0.25	-0.58	0.16	-0.25	0.35	0.13	0.38	0.20
Length of leaf	0.01	0.13	0.52	0.69	-0.41	0.18	0.08	0.18
Width of leaf	0.34	0.20	-0.52	0.25	0.24	0.35	0.01	0.56
Flowering.stem	-0.47	0.03	-0.38	0.43	0.03	0.14	0.08	-0.68
No.of.flowering	0.42	-0.03	-0.48	0.13	0.19	-0.29	0.28	0.02
H.mdl.Flowering	0.26	0.58	0.28	-0.33	-0.03	-0.31	0.44	-0.04
Percent variation	0.27	0.15	0.13	0.11	0.11	0.07	0.19	0.05
Cumulative %	0.28	0.43	0.56	0.67	0.76	0.85	0.91	0.69

PC6 explained 7 percent of the total variation and was mainly associated with width of leaf.

PC7 mainly consisted of height middle flowering for 19 percent of total variation and was mainly associated with height middle stem, height middle flowering. PC8 explained 5 percent of the total variation was mainly associated with height of plant. (Table 3)

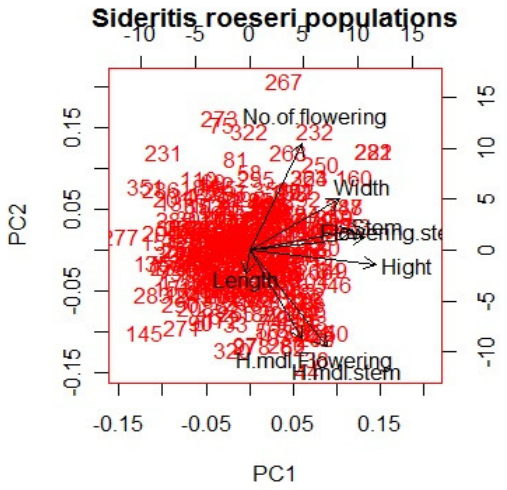

Figure 2. PCA of *Sideritis raeseri* populations

Cluster analyses and PCA (Fig. 2) show the genetics distance among six populations of *S. raeseri.* The strong relationship between stem and flowering stem and between height of middle stem and height of middle flowering stem was revealed.
Our results showed that *S. raeseri* grown in North-East line of Albania provides variability in morphological characteristics. Moreover, analysis of results shows the strong correlations among some traits and large morphological variability.

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Original scientific paper

EFFECT OF FERTILIZATION ON YIELD, SEED QUALITY AND CONTENT OF ESSENTIAL OIL OF ANISE (*PIMPINELA ANISUM* L.) AND DILL (*ANETHUM GRAVEOLENS* L.)

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SUMMARY

In the two years experiments, the effect of fertilization with NPK (15:15:15) on seed yield, quality and content of essential oil of Anise (cv. "N-210") and Dill (cv. "Domaća aromatična") were examined. The experiments were established in Pančevo, Serbia, and completely randomized block design with four replications was applied. In the first experiment, fertilization with 400 kg NPK (15:15:15) was performed both years (n 2009 and in 2010), before the autumn tillage. The second experiment was established without fertilization (control plot). Sowing of anise and dill was performed in continuous rows, both years in the second decade of March. Row spacing was set at 50 cm, sowing depth at 2 cm and the sowing rate was 6 kg ha-1. During the vegetation period, common agricultural measures were performed and weed was removed mechanically (without herbicide application). Harvesting of Anis was performed at the time when most of the inflorescences got a yellow color, i. e. when more than 60% of the fruits was ripe. Harvesting of Dill was performed when most of the inflorescences got waxy yellow color. Harvested fruits of both crops were dried up to 10% of the moisture content and following characteristics of the seeds were examined: seed yield (kg ha-1), seed germination (%), 1000 seeds weight (g) and essential oil content (%). In order to determine the seed essential oils quality of both crops, GC and GC/MS chemical characterization of the obtained oil samples was performed. The obtained results show that for all examined traits, significantly higher ($p \leq 1\%$) mean values were obtained in the plots with applied NPK fertilizer in comparison to plots without fertilization (control plot). In anis, significantly higher yield was achieved in 2011 compared to 2010 year, while in dill there was no significant difference of the seed yields between the respective two years. The oil content of anise and dill was higher in 2011 compared to 2010 year, while the overall seed germination rate of anise and dill did not significantly differ between the examined years.

***Keywords*:** anise, dill, seed, germination, essential oil.

INTRODUCTION

Anise is an annual herbaceous plant of the Apiaceae family. It is used as a carminative, to improve digestion, to regulate digestion, for treatment of asthma, to elevate coughing and to stimulate secretion of bile and saliva. As an aromatic spice anise fruit is used in bread and cakes [1]. In veterinary medicine the essential oil is used for stimulating the peristaltic motions.

The anise fruit contains from 1,5 to 4,0% essential oil (*Anisi aetheroleum*). The oil is obtained by steam distillation of fruits and it is colourless, pale yellow, sometimes lightly green, clear liquid or crystalline mass with pleasant odour recalling of anethole [2] and sweet

aromatic flavour. According to ISO3475:2002 [2], chromatographic profile of the aniseed oil oil should contain following 3 representative compounds with their minimal contribution to the oil above 0,5%: *trans*–Anethole (min. 87 %/max. 94%), γ–Himachalene (min. 1% / max. 5%) and Methyl chavicol (min. 0,5% / max. 3%).

Dill is an annual, herbaceous plant of the Apiaceae family. It is used in pharmacy as an ingredient in tea that improves digestion, prevents stomach cramps, insomnia, haemorrhoids and improves secretion of milk and urine. As a condiment, in households mainly dill leaves (rarely fruits) are used while in food industry, the entire plant is used. The use of dill essential oil of dill is primarily in the food industry, perfumery and medicine.

Dill fruit contains 16-18% protein, 15-20% fatty oil, about 6% pectin and 3-4% essential oil. The essential oil content in the plant significantly affects environmental conditions during the production year [3]. The dill seed oil is slightly yellow, almost colourless, easily movable liquid with specific pleasant and sharp smell. It is obtained by stem distillation and according to literature data [4, 5], the main constituents are carvone and limonene, accounting for ca. 96% of the oil. Bearing in mind that the root system in dill is poorly developed, with weak suction power, and that the vegetation period is relatively short, in order to establish an adequate biomass the crop should be provided with sufficient quantities of accessible nutrients, in the right time. Basic fertilization involves the application of NPK complex fertilizers as follows: 50-70 kg ha^{-1} of N, 60-80 $kgha^{-1}$ of P and 50-60 kg of Kha^{-1}[6]. These amounts should be increased if in the previous cultivation FYM is not applied.

The aim of this study was to determine the effect of application of mineral fertilizers on anise and dill fruit yield, seed quality and content of essential oils.

MATERIAL AND METHODS

The effect of NPK 15:15:15 fertilizer on yield, seed quality and essential oil content in both cultivated crops, anise (cultivar "N-210") and dill (cultivar "Domaća aromatična") was examined. Experiments have been conducted according to completely randomized block design with four replications, in the years 2010, and 2011, in Pančevo, Republic of Serbia. In the first experiment, fertilization with 400 kg NPK 15:15:15 was conducted before the autumn tillage, in the years 2009 and 2010. In the second experiment, no fertilization was conducted (control). Anise and fennel were sown both years in continuous rows (2 cm in depth), in the second decade of March. Space between the rows was set to 50 cm and the sowing rate was 6 kg ha-1. During the vegetative period, the common agricultural practices were performed.Weed was destroyed mechanically, without application of herbicides. Harvesting of anis was performed at the time when most umbels received a yellow colour, i.e., when more than 60% of the fruits was ripe. Harvest of dill was performed when the largest number of umbels got waxy yellow colour. The harvested anis and dill fruits were dry up to 10% moisture content.Following characteristics were tested in both crops: seed yields (kg ha-1), seed germination (%) and the yields of the seed essential oil (%).

In each experimental year, the study sample of 5 x 100 anise and fennel seeds were taken from each replication and tested for viability (germination). Tests were conducted in the laboratory conditions, in Petri dishes, on filter paper, at constant T =20° C, following ISTA methodology.

For all the examined characteristics, the assessment of significance is derived on the basis of group F test, for the significance level 5% and 1%.

Essential oil analyses procedure

All essential oil samples were diluted in ethanol (1µl) and injected in split-mode (1:30). The GC was performed on GC Agilent Technologies 7890A apparatus, equipped with the split-splitless injector attached to HP-5 column (30 m x 0.32 mm, film thickness 0.25 µm) and fitted to flame-ionization detector (FID). Operating conditions were as follows: carrier gas

was H2 (1 mL/min/210°C); temperatures were set as follows: injector at 250°C and detector at 280°C, while the column temperature was linearly programmed 40–260°C at 4°C/min. The percentage compositions of each sample were computed from the peak areas, without correction factors. The GC/MS was performed on HP G 1800C Series II GCD analytical system equipped with HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm). Carrier gas was He (1 mL/min). Other chromatographic conditions were as those for GC-FID. Transfer line was heated at 260°C. Mass spectra were recorded in EI mode (70 eV), in a range of *m/z* 40–450.

Identification and quantification of the oil components

The identification of individual constituents was accomplished by comparison of their spectra with those from available MS libraries (NIST/Wiley) and by comparison of their experimentally determined retention indices (calibrated AMDIS), with data from the literature [7].

RESULTS AND DISCUSSION

For all the examined characteristics, significantly very higher ($P \leq 1\%$) were the mean values obtained in the plots where the fertilizer was applied in comparison to control plots (without fertilization), which is consistent with the results of some other authors [8, 9]. In fennel, a very significantly higher yield was achieved in 2011 compared to 2010 year, while in dill, there were no significant differences in the fruit yieldsbetween the respective years. Yields of anis and dill essential oil were higher in 2011 compared to 2010 year. Total seed germination of the seeds of both crops did not vary significantly depending on tested years (Table 1 and 2) Out of 12 identified components composing the oils of anise seed (what accounted for 99.68% of the entire oil). The most abundant component was *trans*-anethole with an average content of 86,35%, while the four other components, each of them present in oil with 1-5% (Methyl chavicol, 1,8–Cineole, γ-Himachalene and Anisyl methyl ketone), represented approximately 10,55% of the oil. These five components accounted for averagely 96.90% of the total anise seed oil, while the remaining seven components of the oil (content below 1%) accounted for remaining 3.43% of oil (Table 4). Chemical composition of essential oil obtained by steam distillation of the fruits of *Pimpinella anisum* L., is defined by the standard ISO3475:2002(E)[2]. Compared to this standard, the average content of the most abundant component in the oil,*trans*-Anethole (86.35%), was slightly lower than required (min. 87%), while the next most abundant components of the oil, γ–Himachalene, with an average content of1.39%, fit into proposed limits (1-5%). These two components represented a total of 88-99% of the oil, while their average contents in the examined oil samples varied from 86,74-88.79%. Of the remaining six components of aniseed oil required by the standard, the content of Methyl chavicol (4.86%) was slightly higher than the proposed maximum (3.0%), *cis*–Anethole was detected in only one of the three tested oil samples, and was in the range below the prescribed boundary (min. 0.1%), while the two other components, Anisic aldehyde and Pseudoisoeugenyl2-methylbutyrate were not detected in anyof the three tested oil samples. The composition of the aniseed oil components is quite consistent in tested samples, with an exception of the component α–Copaene and *cis*–Anethole, present only in one out of three oil samples, while the other 10 components identified in these three aniseed oil samples are present with negligible variation between the samples.

Table 1. Analysis of variance for anise fruit yield, yield of aniseed essential oil and aniseed germination.

Source	Df	MS		
		Yield	Essential oil content	Germination
Replication	3	1385,1	0,023	7,7
Year (Y)	1	48841,0**	0,325**	36,0*
Treatment (T)	1	525625,0**	2,205**	144,0**
Y x T	1	272,2	0,013	0,25
Error	9	2404,2	0,016	7,81
Total	15			

* P≤5%
** P≤1%

Table 2. Analysis of variance for dill fruit yield, yield of dill seed essential oil and dill seed germination.

Source	Df	MS		
		Yield	Essential oil content	Germination
Replication	3	2552,1	0,001	0,56
Year (Y)	1	2889,1	0,017**	0,56
Treatment (T)	1	1521645,1**	0,656**	18,06**
Y x T	1	189,1	0,008**	1,56
Error	9	1150,5	0,001	0,73
Total	15			

* P≤5%
** P≤1%

For dill seed oil there is no ISO standard as a normative for the quality. In the analysed samples of dill seed oil, three components (Carvone, Limonene and α–Phellandrene) were present with the content over 10%, what representedin total averagely 85.07% of dill seed oil. Three other components (Dill ether, p–Cymene and *cis*–Dihydro carvone) were present in the oil with the content between 1 and 10%, what represented averagely other 10.48% of dill seed oil. These 6 components, altogether presented averagely 95.55% of the total dill seed oil, while the remaining 10 components of the oil, with their contribution to the oil with less than 1%, represented averagely about 3.82% of oil (Table 5). The composition of the oil components is quite consistent within the tested samples of dill seed oil, with negligible small variation between them. This can lead to the assumption that, in the absence of ISO standards proposing the quality of this oil, these data could serve as a reference for the chemical composition of dill seed essential oil cultivated in our agro-ecological conditions.

Table 3. Average values of fruit yields, essential oil yields and seed germination of anise and dill for the years and variants of mineral fertilizers application.

Pimpinella anisum L.			*Anethum graveolens L.*			
Variants	Yield of fruit (kg ha^{-1})	Average (Y)	Year (Y)	Variants	Yield of fruit (kg ha^{-1})	Average (Y)
Control	802.0	987.3	2010	Control	920.7	1097.8
NPK 400 kg ha^{-1}	1172.7			NPK 400 kg ha^{-1}	1275.0	
Control	920.7	1097.8	2011	Control	887.0	1071.0
NPK 400 kg ha^{-1}	1275.0			NPK 400 kg ha^{-1}	1255.0	
Average Control	861.3		Average Control		903.8	
Average NPK 400 kg ha^{-1}	1233.8		Average NPK 400 kg ha^{-1}		1265.0	

Variants	Yield of essential oil (%)	Average (Y)	Year (Y)	Variants	Yield of essential oil (%)	Average (Y)
Control	2.493	2.893	2010	Control	2.035	2.215
NPK 400 kg ha^{-1}	3.293			NPK 400 kg ha^{-1}	2.395	
Control	2.835	3.178	2011	Control	1.925	2.150
NPK 400 kg ha^{-1}	3.520			NPK 400 kg ha^{-1}	2.375	
Average Control	2.664		Average Control		1.980	
Average NPK 400 kg ha^{-1}	3.406		Average NPK 400 kg ha^{-1}		2.385	

Variants	Germination (%)	Average (Y)	Year (Y)	Variants	Germination (%)	Average (Y)
Control	72.7	75.6	2010	Control	91.5	92.0
NPK 400 kg ha^{-1}	78.5			NPK 400 kg ha^{-1}	92.7	
Control	75.5	78.6	2011	Control	90.2	91.6
NPK 400 kg ha^{-1}	81.7			NPK 400 kg ha^{-1}	93.0	
Average Control	74.1		Average Control		90.7	
Average NPK 400 kg ha^{-1}	80.1		Average NPK 400 kg ha^{-1}		92.8	

Table 4. Chemical composition of 3 samples of *Pimpinela anisum* L. seed essential oil, presented in descending order of the presence of their compounds in the oil.

RI	Compounds	1	2	3	Average
1280	Anethole <trans->	87.48	84.97	86.61	86.35
1193	Methyl chavicol	5.32	4.92	4.32	4.86
1024	Cineol <1,8->	2.35	3.35	4.15	3.29
1480	Himachalene <gamma->	1.31	1.77	1.1	1.39
1388	Anisyl methyl ketone	1.7	0.22	1.12	1.01
1097	Linalool	0.43	1.39	1.15	0.99
1366	Copaene <alpha->	*	0.86	*	0.86
922	Thujene <alpha->	0.61	0.53	0.38	0.51
1172	Terpinen-4-ol	0.05	0.68	0.49	0.41
1185	Terpineol <alpha->	0.22	0.52	0.16	0.3
1397	Longipinene <beta->	0.1	0.43	0.22	0.25
1248	Anethole <cis->	0.11	*	*	0.11
Total identified compounds		*99.69*	*99.65*	*99.71*	*99.68*

Table 5. Chemical composition of 3 samples of *Anethum graveolens* L. seed essentialoil, presented in descending order of the presence of their compounds in the oil.

RI	Compounds	1	2	3	Average
1238	Carvone	42.47	40.88	43.26	42.2
1021	Limonene	29.04	31.49	30.97	30.5
996	Phellandrene <alpha->	13.12	12.92	11.07	12.37
1176	Dill ether	6.52	6.6	7.7	6.94
1017	Cymene <para->	2.26	1.96	2.11	2.11
1189	Dihydro carvone <cis->	1.51	1.61	1.16	1.43
1196	Dihydro carvone <trans->	0.81	1.01	0.78	0.87
924	Thujene <alpha->	0.44	0.59	0.59	0.54
1146	Menthone	0.42	0.56	0.54	0.51
985	Myrcene	0.51	0.41	0.35	0.42
1166	Menthol <neo->	0.28	0.32	0.34	0.31
1156	Menthone <iso->	0.17	0.23	0.28	0.23
1470	Dauca-5,8-diene	0.24	0.29	0.27	0.27
919	Tricyclene	0.13	0.11	0.17	0.14
1210	Dihydro carveol <iso->	0.14	0.38	0.16	0.23
1223	Dihydro carveol <neoiso->	0.46	0.36	0.09	0.3
Total identified compounds		98.52	99.72	99.84	99.36

CONCLUSION

The application of mineral fertilizers NPK 15:15:15 in the crop of anise and dill very significantly increased the fruit yield, essential oil content and seed germination in both years.

Anise fruit yield was very significantly higher in 2011 compared to 2010 year, while in dill there were no significant differences in fruit yield between the respective years. The essential oil yields of anise and dill seeds were higher in 2011 compared to 2010 year, while the germination of the seeds of both crops varied significantly depending on the tested years.

Out of 12 identified components in the oils of anise seed the most abundant component was *trans*-Anethole. Compared to the ISO standard that prescribe quality of this oil, the average oil content of *trans*-Anethole was slightly lower that the limit (min. 87%)

For dill seed oil, there is no ISO standard that represents a normative for the oil quality. Composition of dill oil components was consistent in all tested oil samples with negligible variation between them, so that the results obtained may serve as a reference for the chemical composition of the essential oil obtained from dill cultivated in our agricultural conditions.

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Professional paper

WEED CONTROL METHODS IN CHAMOMILE PRODUCTION IN SERBIA

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SUMMARY

Chamomile is an important cultivated medical and aromatic plant in Serbia, which is relatively pest and disease free. However, weeds are the serious hygiene concern, since contaminants in the final product (flowers, oil or extract) will detract from the specified quality. Experience shows the chemicals to be useful, but none is registered for use on chamomile in Serbia. For prevention, some herbicides (i.e. glyphosate), which residuals in the soil weakly penetrate in chamomile plants, can be used before seeding. Cultivation maintenance is carrying out with fertilizing and weed control, depending on the kind of cultivation: organic or conventional. In conventional agriculture, suited herbicides are applied in rows plantation for medical and aromatic plants protection. In contrast, in organic agriculture, weeds control can be done in row cultivation by rod weeder and by hand pulling in plant rows, because organic agriculture avoids or excludes the use of synthetic preparations-artificial fertilizers, pesticides and growth accelerators. It is pointed out that the higher quality of this product in organic agriculture involves more both manual and mechanical workforce than in the conventional agriculture. Only minimum quantity of approved agrochemicals can be used, either to promote the growth or to protect (pesticides and herbicides) medical plants, and applied only when no alternative measures are available. In the paper are reviewed and discussed different techniques for chamomile protection, based on the application of mechanical and chemical methods.

***Key words**: Chamomile, weeds, herbicides, residues, environment*

INTRODUCTION

Chamomile (*Matricaria recutita* L./*Chamomilla recutita* L. Rauschert), family Asteraceae is highly appreciated as a medicinal plant, foodstuff and as raw material for the cosmetic industry. Different native and refined products, based on Chamomile crops, are available: chamomile flowers (*matricariae flos*), chamomile fines, Chamomile herb with flowers, chamomile herb, Chamomile for extraction (industrial Chamomile), Chamomile root, Chamomile oil (*matricariae aetheroleum*), Chamomile fluid extract and Chamomile tincture, Chamomile. Therefore, the Chamomile has been considered by many authors [1]. In Serbia, Chamomile is commonly used and traded medicinal plant. According to available data, the total area under cultivated medicinal plants in Serbia has been estimated to 1300-1350 ha, out of which Chamomile accounted for about 650 ha [2] and 1000 ha [3].

Many cultivated medicinal plants are considered as weak competitors towards weeds, during early growth stages, what results in high costs for weed control management. This problem is more expressed when desirable yield of medicinal plants should be achieved without application of herbicides. Actually, production of medicinal plant raw material requires either low input or preferably organic farming systems, as strict and precise standards are recommended for evaluation of drug quality [1] and their subsequent trade. More precisely, organic agriculture is a production system which avoids or excludes the use of synthetic

preparations - artificial fertilizers, pesticides, growth accelerators and fodder additives [4, 5]. Under such limitations, the plant receives nutrients by using natural organic or mineral fertilizers and weeds or pests are controlled and prevented by stimulating the population of useful insects. Organic farming generally demands more labor intensive than conventional farming, assuming that chemical inputs are substituted by factors such as increased management knowledge, new practices and techniques, capital and labor, which also refers to chamomile organic production [6].

SOME APPROACHES TO WEED MANAGEMENT

Weeds are undesirable intruder plants that reduce crop yields and quality, what obviously results in lower efficiency of land management. Certain plants have been legally declared as noxious weeds, which must be cut or controlled in order to prevent production of their seeds. The most serious problems are induced by weeds that resemble the crop in physical characteristics, growth habits, and requirements for soil water, nutrients, and light.

However, weeds control is a very complex problem. For example, it can be noted that some production methods, especially cultivation, even favor some weed species. A possible approach to weeds control is related to prevention weed seeds production. Unfortunately, weed seeds have certain characteristics, which make them very difficult to control: large numbers, tolerance with respect to extreme conditions, long-life, easy spreading, etc.

The most effective ways to control weeds assume mechanical and chemical methods. In contrast, biological control methods, based on the application of natural enemies of weeds (such as insects or diseases), have not been highly successful so far. However, they offer some potential for the future. Reproduction and survival of annual and biannual weed species depend exclusively on seed production. Therefore, an effective way to control them is by destroying the shoot of the plant - by mowing, tillage, or by herbicides. It is important to destroy the growing point to prevent seed production. Perennials are more difficult to control by simply destroying the top of the growth. In such cases, it is more effective to destroy the underground parts of the plants, either through tillage or through the herbicides application.

Mechanical weed control - hand weeding, plowing, harrowing, etc. - has been practiced for centuries. Many of the methods of weed control used today have been changed very little over the years. In addition to mechanical methods, they include: clean seed, clean feed, crop competition, rotating crops, fallowing fields, companion crops, etc. [7].

Chemical control, based on herbicides application, is the most common method of weed control in agriculture. Among many others, two main factors affect herbicides application: soil type (with respect to organic matter content, soil texture and soil acidity) and environmental conditions, related to soil moisture, i.e., rainfall, irrigation and flooding, humidity, dew, temperature and sunlight. However, some herbicides remain in the soil for a long time, causing injury in the following year's crop. Herbicide carryover is more likely to occur with unusually low rainfall, because dry soils limit the chemical and microbial activity needed to degrade herbicides. To minimize the herbicide carryover in soil, the following rules should be followed: apply the lowest effective rate, apply uniformly, select crop sequences that are tolerant to the herbicide used on the previous crop, rotate herbicides, apply spot treatment when using high rates of herbicide, etc.

PRECISION AGRICULTURE

If chemical approach to weed control is chosen, the use of precision agriculture will reduce negative effects of herbicide applications. Precision agriculture can be defined as a concept of agricultural management that relies on the existence of in-field variability. It requires the use of new technologies, such as GPS, sensors, satellites, and aerial images, as well as field management tools to assess and understand existing variations in the field, [8, 9]. Precision-

agriculture can include anything from a sprayer equipped with global positioning systems (GPS in further text), automatic steering, and automatic boom shut-off control, to simply a sprayer equipped with an automatic rate controller. Automatic rate controllers are capable to select and set-up the targeted rate of the herbicide application quantity. Such kind of a device automatically (independently) controls a flow meter, a control valve, and a speed sensor of applied sprayer machine. Automatic boom height leveling systems prevent the cut down and struck the field by sprayer boom, but also an operator fatigue. The auto height leveling system does not need GPS technology to operate, but is still an intricate piece of technology. At present, two basic styles of auto-height leveling are available today: one that uses ultrasonic sensors and one that utilizes a gauge wheel to detect ground height. Automatic steering control is a useful technique, commonly based on a GPS controller. It provides an advanced possibility to a user, to set up field guidance paths that would be able to be followed quite accurately. Automatic boom shut-off systems work with the GPS guidance and provide the advanced capability to operator to optimize their application out in the field.

APPLICATION TECHNOLOGY

Incorrect application of herbicides may result in wasted chemicals, poor or lost weeds control, as well as in crops and environmental contamination, yield decrease, etc. To avoid such kind of problems, each herbicide should be applied under specific and accurately controlled conditions, carefully chosen for any specified plant under protection, as well as for each soil and climate type, etc.

Fortunately, recent advances in equipment and control systems development can make this job much simpler, than it was the case in the paste. Innovations in developments of building sprayers, such as closed injection systems with herbicide concentrate carried separately from the water carrier, are now in use. Electronic flow rate controllers enable more accurate herbicide spray application by utilizing speed sensors, flow controllers and microprocessors to maintain the desired application rate. This technology has also included radar to accurately sense (measure) ground speed of the sprayer device.

Rate controllers are commonly used by professional applicators. GPS guidance control systems, with possible auto steer, allow sprayers to cover the field with minimal overlap swath to swath. Such kind of approach allows complete field spray coverage without double applying product in certain areas. Site-specific weed management can comprehend both limited herbicide application to areas of the field where weed density is above the economic level (patch spraying) [10].

The most commonly used type of sprayer in herbicide application is the boom sprayer, which has to satisfy the following requirements:

- uniform flow output rate of the nozzles across the whole boom;
- fairly constant forward speed of the sprayer in actual non-stationary field conditions;
- ability to adjust a stable boom height with respect to reference plane;

In order to provide uniform application quantity of a herbicide, the forward speed of a sprayer must be constant whenever the nozzles are delivering liquid. However, under conditions related to slipping of the driving tractor wheels over the soil surface, the tractor's speedometer is not capable to measure the forward speed of the sprayer accurately. Fortunately, this problem can be easily resolved by independent speedometer, powered by radar or GPS speed sensors.

Nozzles - Proper spacing and orientation of nozzles is essential to ensure adequate overlap of adjacent nozzle spray plumes.. The success of the spray application is dependent in part on the condition of the nozzle tips [11] and the uniformity of application across the whole spray boom. Flat fan nozzles are widely used on boom sprayers to apply herbicides. Poor timing of spray, reduced water volumes, spray pressures that are too low and difficult-to-wet weeds

may all contribute to poor control. Special "even flat fan" spray nozzles are available for band spraying of herbicides. These even flat fan nozzles deliver a uniform amount of spray over their sprayed area. Flooding nozzle tips are used at low pressures and, because of their wide spray angle, can be used closer to the ground surface, thus reducing the potential for drift. Full or hollow cone nozzle tips may be used for applying herbicides to the soil surface when the herbicide is mixed into the soil with a disk harrow, cultivator or similar tillage implement. These types of nozzles will not provide as uniform spray distribution as flat fan nozzles setup correctly. Herbicide application use a deflector nozzle (or if that is not available, a fan nozzle) with a flow rate of around 0.5 - 1 l/min. Calibration process is the most important step in any pesticide application. Calibration is the first essential step in any successful chemical weed control; it is a must before every spraying operation. Sprayers must be regularly calibrated to uniformly distribute the herbicide over the required area.

HERBICIDE APPLICATION

Reported studies of weed populations in chamomile crops in Serbia, indicate prevalence of biannual and annual life forms. In addition, the presence of perennial weeds has been also verified in the areas under extensive agricultural production. Among dominant species, a lot of highly-efficient competitors exist, which habitués obstruct the growth of chamomile plants and (obviously) significantly decrease the flower yields. Among the most frequently evidenced species, in the region of Serbia, the following weed species could be mentioned: *Lamium amplexicaule*, *Papaver rhoeas*, *Cirsium arvense*, *Galium aparine*, *Lactuca serriola*, *Polygonum persicaria*, *Sonchus asper*, *Sonchus oleraceus*, *Stellaria media*, *Veronica persica*, *Capsella bursa pastoris*, as well as *Sorghum halepense*, *Convolvulus arvensis*, *Agropyrum repens*, *Lolium perene* and *Rumex crispus* [6, 12].

The possibility of herbicide use for weed control in chamomile crops has not been adequately researched yet, in Serbia. According to the authors experience, it could be recommended to apply total herbicides (based on glyphosate as active ingredient) before the crop establishing, resulting in decrease of weeds potential. In addition, the land is cleaned from winter-spring weed species germinated during that period. However, this approach can be fully effective only if applied two or three weeks before crop establishing (minimum) and without any kind of additional tillage of the soil, previously treated by herbicides. Available literature data comprehend testing results of few herbicides, in the form of pre-planting applications in comparison with weedy check and weed-free check. However, having in mind the small distance between plants and habitués structure of chamomile, manual weed elimination suffers from serious damage of chamomile crops.

Up to date, fairly small number of studies related to herbicide use in chamomile production has been reported. According to Singh et al (1986) [13], the highest efficiency in decreasing the weeds population (herbs number and dry mass) has been evidenced under application of oxyfluorfen (0,5 kg/ha) and alachlor (1,5 kg/ha). However, the oxyfluorfen is characterized by the highest selectivity toward chamomile. This herbicide does not decrease the growth of chamomile and, obviously, the yield of chamomile flowers. In contrast, existing reports indicate inadequate selectivity of alachlor (resulting in plant height decrease). Strong phytotoxicity and, simultaneously, the weak control of weed species present (dominated by dicotyledonous: *Chenopodium album*, *Melilotus indica*, *Anagalis arvensis* and *Ranunculus acutus*) has been evidenced for chloramben (2 kg/ha), butachlor (1,5 kg/ha) and bentiocarb (1,5 kg/ha). Further studies have verified the superiority of oxyfluorfen in controlling the weeds population, including (besides the previously mentioned), grass weed: *Setaria glauca*, at application rates of 0,4 and 0,6 (kg/ha).

Table - 1. List of herbicides, tested for weeds control in the chamomile crop, according to published data *

Weeds controlled	Active ingredient and application rates**	Remarks
HERBICIDES SPECIFIED FOR APPLICATION PRIOR TO CROP ESTABLISHING		
Annual and some biannual grass and broadleaf weeds	Glyphosate (480 g/l) Large number of preparations 2-4 l/ha – for annual weeds control 4-8 l/ha – for perennial weeds control 8-12 l/ha – for control of highly resistant perennial weeds	To be applied before crops establishing, by treating the complete planted area. It is specified for controlling perennial weeds possessing deep roots and rhizomes. The best results can be achieved when the annual weeds are in the phase of intensive growth. For perennial weeds, the highest efficiency can be achieved during the blooming phase. To accomplish the so-called "chemical cleaning", the application rate depends on the type and development (growth stage) of specified weed under treatment. **Limitations:** Insufficiently effective for some perennial weeds in older growth stages. This herbicide is not sufficiently effective for *Convolvulus spp.*.
Control of annual broadleaf and grass weeds	oxyfluorfen (240 g /l) 1 – 1,25 l/ha	Any kind of tillage is not allowed after application, because this can decrease the herbicide effects. Besides residual, this herbicide is also characterized by post-em activity. Residual period: > 4 months (DT50 = 35 days).
Annual broadleaf and grass weeds	linuron (450 / 500 g/l) 2 – 3 l/ha	Treatment and application rate depends on the soil texture and humus content. To be applied before weeds germination, or during the early phases of weeds germination. Characterized by residual activity (DT50 = 30 – 60 days). **Limitations:** This herbicide should not be applied on sandy, sandy-clay and gravel soils, in the areas under erosion, as well as on the soils containing less than 1% or more than 5% of humus. Moisture is necessary for activation.
Annual broadleaf and grass weeds	trifluraline (480 g/l) 1 – 2,5 l/ha	The incorporation is obligatory after application of this herbicide. Possesses certain influence on the rhizomes of *Cynodon dactylon* and *Sorghum halepense*. Residual period: 6 - 8 months (DT50 =57 – 126 days).
Annual broadleaf and grass weeds	alachlor (480 g/l) 3 – 6 l/ha	Lower application rates are recommended for soils containing 1 – 3 % of humus, while higher rates are recommended for soils containing >3 % humus. Characterized by certain residual activity (DT50 =30 days). **Limitations:** Significant risk of phytotoxic effects toward crops exposed to strong rain and cold weather, planted on light soils.
Annual broadleaf and grass weeds	ethofumesate (200 g/l) : 5 – 10 l/ha (500 g/l) : 2 – 4 l/ha	Residual activity (DT50 = 94 days in average). **Limitations:** Applicable only for soils containing between 1% and 5% of humus.
Annual and some perennial broadleaf and grass weeds	propyzamide (500 g/kg) 3 – 4 kg/ha	Certain residual activity (DT50 = 30 days).
POSTEM HERBICIDES		
Most of annual and perennial grass weeds	fluazifop-P-butyl (125 g/l) : 1 – 4 l/ha (150 g/l) : 0,8 – 1,3 l/ha	The best results (i.e. the highest efficiency) are achieved if this herbicide is applied during specified growth stage, different for each weed (*Sorghum halepense*: 30-45 cm, *Cynodon dactilon*: 7 cm (or lateral branches 10-20 cm), and for annual grasses: 5-10 cm). **Limitations:** This herbicide should not be applied for grass weeds under stress – low effectivity. Residual period: less than month (DT50 = 3 – 25 days). Low effectivity against *Poa annua*.
Most of annual and perennial grass weeds	cycloxydim (100 g/l) 0,75 – 4 l/ha	Insufficiently effective for Poa spp.

* - herbicide active substances, registered for use in Serbia, but for crops different from chamomile
** - active substance content and recommended application rates for various crops, covered by registration license
DT50 – herbicide half-life

Higher application rate induced some phytotoxic symptoms visible on height, spread and chlorophyll content of plants, which disappeared within 3-4 weeks of herbicide application.
However, only oxyfluorfen application at 0,6 kg/ha proved economical over weed-free check. Significant, but still insufficient and not economically justified, suppression of weeds population was also achieved by application of thiobencarb (1,5; 2 and 2,5 kg/ha) and nitrofen (0,5 and 1,5 kg/ha) [14].
Examinations [15] have verified the selectivity and good efficiency of ethofumesate against the present weed species. In opposite, mecoprop manifested not only the high efficiency, but also the phytotoxic effect, followed by plants recovery during vegetation. Application of trifluraline and propyzamide was not successful enough in weeds suppression, what resulted in inferior crops development. Slightly different results reported in [16]: inefficiency of etofumesata, relatively high efficiency of propyzamide (except for the species *Senecio vulgaris* and *Galinsoga ciliata*), while the application of linuron was highly effective in controlling the existing weed species, but with initially decreased growth of chamomile plants. Besides efficiency in controlling existing weeds, and selectivity toward the protected crop (especially in the case of medical plants), the important aspect of herbicides application is the residual level of herbicide. Fortunately, the application rates of herbicides in chamomile crops are lower in comparison to other crops treated by the same active substances. However, some of these substances still induces higher level of residuals then those allowed by MRLs for chamomile flower [17, 18] (1,9 mg/kg for mecoprop [15]); 0,54 – 0,63 mg/kg for linuron; 0,78 mg/kg for fluazifop-P-butyl and 1,8 – 3,16 mg/kg for cycloxydim [19].
Starting from the verified data on the existing weed species in chamomile crops in Serbia, as well as on the properties of specific herbicides (table-1), the applicability of oxyfluorfen, linuron, and typical graminicides: fluazifop-P-butyl and cycloxydim need to be examined for so-called targeted control of weeds, but under careful monitoring of eventual presence of residuals in chamomile flower.
Table 1 given above represents our approximation of possible herbicide effects on already established weeds in chamomile crops in Serbia. It is based on the experience acquired during their application in different crops, as well as on herbicide selectivity and weed spectrum.

CONCLUSIONS

The chamomile is fairly resistant with respect to pathogens and weeds in the Serbian region. Therefore, the crop protection is not based on application of chemicals. Consequently, the crop quality is enchanted, with simultaneous decrease of environmental contamination. However, in some situations, weeds can seriously influence the chamomile crop growth and quality, what can be resolved by cultural control and, only if necessary, by careful application of herbicides. Verified data on the existing weed species in chamomile crops in Serbia, and reported properties of specific herbicides, indicate the applicability of oxyfluorfen, linuron, and typical graminicides: fluazifop-P-butyl and cycloxydim. Thus, these herbicides should be examined for so-called targeted control of weeds, under careful monitoring of eventual presence of residuals in chamomile flower.

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ADDITIONAL PAPERS*

***Note to Additional papers**

All papers within the Proceedings of the 7th CMAPSEEC were reviewed by the members of the Scientific Committee and kind assistance of some members of other Conference bodies. Reviewed papers were categorized into review papers, original scientific papers and professional papers.

However, the Scientific Committee of the 7th CMAPSEEC agreed on issuing of group of papers whose authors either haven't referred on reviewer's comments or those which weren't reviewed as submitted after the proposed deadline. However, it was decided to include them as well, considering the subjects and research approaches interesting.

ANALYZING CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM THE AERIAL PARTS AND ECOLOGICAL PROPERTIES OF MEDICINAL PLANT *TECURIUM POLIUM*

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SUMMERY

Tecurium polium plant is a medicinal species with wide distribution in different regions through Iran, Alborz region and suburbs of Tehran, particularly in semi-arid regions and semi-dry mountains. Plants belonged to this type come with medicinal properties and are used in traditional medicine. This study aims to investigate the chemical Composition of Essential Oils From The Aerial Parts of Medicinal plant and ecological properties this plant with scientific name of *Tecurium polium* from Lamiaceae. The aerial parts were hydrodistilled using a Clevenger-type apparatus. The essential oil was analyzed by GC and GC/MS. The habitats of this plant investigated included: slop percentage, geographical direction, soil texture, climate, average annual precipitation, mean temperature, as well as studying the geographical coordinate of related habitat and sea level using GPS and also the medicinal properties of above species was studied using literatures. Fifty seven constituents were found representing 85.75% of the essential oil. The main constituents of the essential oil were Caryophyllene oxide (13.08%), trans-Caryophyllene (66.6%), 7-epi-α-Selinene (6.09%), β-Eudesmol (4.90%) and Spathulenol (4.88%) respectively. The oil was characterized by large amounts of oxygenated sesquiterpenes (37.23%) and sesquiterpenes hydrocarbons (32.78%).Other components of this plant comprised included monoterpene hydrocarbons (6.87%), non-terpenoid components (4.57%), oxygenated monoterpenes (3.94%) and oxygenated diterpenes(0.40%) in further ranks.This plant indicates dry and cold climate developing in the sandy-silt soil beds, with slop of 10-30%, western south slop direction, height range between 1500 and 1600 from sea level, average precipitation of 229.4mm and minimum and maximum mean of annual temperature of 4.2 and 22.9^{o}C. based on therapeutic properties, the part of plant is used include its flowering shoots; therefore scientific studies indicated that this plant comes with advantages like: tonic, bracing, anti-convulsant, analgesic, anti-fever, anti- duodenal ulcer, anti-inflammation, anti-microbial, anti- appetite, anti-oxidant, reducing the serum cholesterol and tri glycerides. Further studies needed for recognizing the active chemical components in the plant and due to possessing the valuable medicinal properties, one can exploit it.

Keywords: *ecologic, chemical composition, medicinal species, Caryophyllene oxide, Tecurium polium*

INTRODUCTION

Plant of Lamiaceae family and some of its genus such as Teucrium , based on their essential oil were studied by researches in and out of country. Genus Teucrium from Lamiaceae with about 340 species through the world have relatively extended growth. This genus in Iran come with 12 species of herbaceous plant with annual and perennial dispersed through the country and 3 of its species is specific for Iran [10].

Herbal species of this genus like *Teucrium polium* and *Teucrium chamaedrys* are being used for 2000 years as anti-fever, diuretic, treating the nervous disorders, chronic headaches and epilepsy, analgesic, anti-inflammation as well as treating the rheumatism [1,12].
Botanical properties: *Teucrium polium* is a reliable , hairy, white colored, wooden based plant with height of 10-40cm with crouching or straight branches, mostly twisty, no petiole in its leave, with broad-leaved with toothed edges and 8-16mm in length, white , small flower collected in hill-like inflorescence and dense and semi-circular or ovule with 10-20mm in diameter, with some peduncle, brackets with 3-5mm in length, linear or spathe linear, unequal with flower, with folded edge, and calyx with 3.5- 4.5mm in length, to some extent tube- cup formed without peduncle[6].

MATERIAL & METHODS

Collecting the Studied Plant: For recognizing the habitat factors and lab studies for knowing the components of herb essence, there have been conducted field studies. In this habitat, the related species was collected. Habitat factors of this species include:
Slop percentage, geographical direction, soil texture, climate, average annual precipitation, mean temperature, as well as determining the geographical coordinates and height from the sea by GPS device. And pharmaceutical properties of this species were studied using related literatures. In the related habitat, the soil sample was prepared based on the soil horizons and its rooting and sent to the lab for being analyzed. The climate situation of habitat was extracted using statistics and data of synoptic stations in Karim Abad, Karaj. In order for preparing the essence, the plant sample was initially collected. For sampling from related plant, after recognizing the growing region and when the plant attained to its fruiting stage in related region, there was sampled.
Essence Extraction: Initially the shoot part of plants was dried under the shade. Then they were grinded by mill. Distillation by water and Clevenger-type Device were used for extracting the essence. Initially 100gr of dry plant powder added to a balloon with capacity of 1lit and distilled water added to. Flavoring took long about 4 hours. Because for this plant there was extracted very little essence, so there was used 0.5cc of hexane for separating the essential oil in essence collecting tube of Clevenger-type device. For this reason, the efficiency of flavoring could not be calculated.
Recognizing the essence components:The resulted essence was studied by analytical gas chromatography (GC) and gas chromatography linked to mass spectrophotometer (GC/MS). In all spectra of GC/MS, Coats index was calculated for all peaks in by pattern of exiting the normal alkanes and spectra inhibition index and by comparing them with related resources and books they was interpreted by library- computer data of Wiley 725 and book Adams (2004) and other resources and the components of essence and its chemical formula was recognized [3,5].

RESULTS & DISCUSSION

1-Plant Ecology: According to the data extracted, it was determined that species *Tecurium polium* is a plant from Lamiaceae and genus *Tecurium*. The geographical distribution of this herb, *Tecurium polium* in in arid regions, rocky shores and sand prairies in different regions of Europe, Mediterranean region, north Africa and western south Asia such as Iran. This plan is widely distributed in different regions, North, west, south and center of Iran, Alborz region, around Tehran, particularly in the semi-arid regions and semi-dry mountains [14]. This plant is a representative of dry and cold climate and growing in the sandy-silt soils, with slop of 10-30%, with eastern south slop direction, height of 1500 and 1600m of sea level, average

precipitation of 229.4mm and min and max mean annual temperature of 4.2 and 22.9°C. plant of this genus come with medicinal properties used in traditional medicine. Its medicinal usage goes back to Hippocrates and Galenic era and its medicinal part is its flowery branches. It has tonic effect, anticonvulsant and it is useful for diseases of genitourinary diseases and delay or lack of menstrual [14]. This plant is used in traditional medicine for its expectorant effects, anti-diabetes, anti-inflammatory, duodenal ulcer, anti-fever, anti-microbial, anti-appetite, anti-oxidant, reducing the cholesterol and serum tri glyceride, and anti- spasmodic [1,7,11,13]. The essence extracted from fruited shoots of this plant is scarce for this reason; its flavoring efficiency may not be measured.

2-Recognizing the chemical components :Totally 57 chemical components, equal with 85.75% have been recognized that have been reported based on quantitative percentage (GC) and inhibition index (KI) [Table 1].

Table1. Main Chemical Components of *Tecurium polium*

Item	Components	GC%	KI
1	Caryophyllene oxide	13.08	1582
2	trans-Caryophyllene	6.66	1418
3	7-epi-α-Selinene	6.09	1516
4	β-Eudesmol	4.90	1650
5	Spathulenol	4.88	1578

Main components comprising the essential oil of this plant include Cariophylen Oxide (13.08%), trans- Caryophyllene (6.66%), 7-epi-α-Selinene (6.09%), β-Eudesmol (4.90%), and Spathulenol (4.88%). Among them, highest size of components is related to oxygenated sesquiterpenes (37.23%) and sesquiterpene hydrocarbons (32.87%). Other components include monoterpene hydrocarbons (6.87%), non-therpenoid components (6.87%), and oxygenated monoterpenes (3.94%) and oxygenated ditherpens (0.40%).

Results of other studies as indicated below approve the main components extracted from *Tecurium polium* L include:

Babakhanloo et al (2006), chemical components of essential oil extracted from plant that most important of them include β- caryophyllene, β- pinene and pharnezen.

Mirza (2001), studied the quality and quantity of chemical components in the essential oil that most important of them include β- caryophyllene, β- pinene and pharnezen.

Moghtader (2009), Analyzed the chemical components of essential oil in *Teucrium polium* in Kerman province using GC/MS and GC methods.There was recognized 28 components equal with 99.75% of essential oil with percentage of 0.75%. its main components include:

α-pinene(12.52%), Llinalool (10.63%)،Caryophyllene oxid(9.69%)، β-pinene(7.09%)، β-caryophyllene(6.98%).

Aborja et al (2006), Analyzed the chemical components of essential oil in the shoots of *Teucrium polium* developed in Jordanian by GC/MS and GC. The essential oil extracted by water distillation was 0.8. there were recognized about 39 components the most important of them include:

8-cedren-13-ol (24.8%)، germacrene-D (6.81%)، β –caryophyllene (8.7%) and sabinene (5.2%).

According to the results of this study and other studies, there were components such as β-caryophyllene, β- pinene and caryophilene oxide in *Tecurium polium*, but their percentage content were different and it was related to the ecological and climate conditions.

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PHENOLOGY, ECOLOGY AND ANALYZING CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM THE AERIAL PARTS OF MEDICINAL AND WEEDY HERB, *BIDENS BIPINNATA* L.

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SUMMERY

One of the grassland species with much usability based on investigations and its multi-purpose usage is *Bidens bipinnata L* belonged to Compositae. This study aims to investigate the phenology, ecology and recognizing the chemical compounds in aerial parts of medicinal plant. The aerial parts were hydrodistilled using a Clevenger-type apparatus. The essential oil was analyzed by GC and GC/MS. The habitats of this plant investigated included: slop percentage, geographical direction, soil texture, climate, average annual precipitation, mean temperature, as well as studying the geographical coordinate of related habitat and sea level using GPS and also the medicinal properties of above species was studied using literatures. Thirty one constituents were found representing 91.92% of the essential oil. The main constituents of the essential oil were α-pinene (25.83%), β-myrcene (22.73%), germacrene-D (10.20%), bicyclogermacrene (7.61%), δ-elemene (3.50%), spathulenol (2.94%) respectively. The oil was characterized by large amounts of monoterpene hydrocarbons (57.82%) and sesquiterpene hydrocarbons (26.68%), small amounts of oxygenated sesquiterpenes(4.52%) and oxygenated monoterpenes (0.79%) respectively. Other components of this plant comprised 0.26%. the plant species *Bidens bipinnata* L is a plant from Asteraceae (compositae); it is an annual plant, almost herbaceous, with standing stems, trapezium, yellowish green and strong. Results indicate that this plant begins its growth in last April, and turns to a two leaved, four leaved and claw-form plant in early June, and flowers in mid-June with complete flowering in August and begins producing the seeds in last August and its seeds will be ripen in September and can be harvested. This plant represents dry and cold climate and developing in low slops between 0 to 10% and height range between 1300 and 1400m from sea level with average precipitation of 223mm, the average temperature of 13^{o}C and mean annual evaporation of 2072 and in recent years it entered to Iran as a weed. According to studies, it has many usages including: medicinal and edible usages and as a food flavor and forage usage. Based on the medicinal properties, different parts of this plant are used like seed, root and leave. Its seed is stimulant and laxative, expectorant, supplying blood, soothing, and promoting the nervous system. Its root is a strong expectorant is used in chronic colds. Its leaves are used as diaphoretic and antiemetic. This plant is also used for treating the conjunctivitis, shortness of breath, injuries, earache, and snake bite. Other species of this genus have also medicinal properties as indicated. According to the initial field studies and based on its high adaptation and tolerance to climate conditions as well as possessing valuable medicinal properties, it can be planted in Iran in mass production and exploited.

Keywords*: phenology, Essential oil composition, ecology, weedy herb, α – pinene, Bidens bipinnata L.*

INTRODUCTION

Using flavored plants in Iran has a long history and essences due to their roles that can be in drugs, have significant importance. So precise recognition and ecological investigation of industrial ,medicinal and flavored plants species which depending on climate conditions and the ecology of each region have various diversity, is necessary that considering the importance of such a plants it is obligatory to run a complex survey about various ecological aspects on them[9]. Global approach presents using medicinal plants and natural compounds in medic, sanitary and food and following it, People , suppliers and domestic industries attention to application of medicinal and aromatic plants relieves critical needs to basic and applicable studies in this field. Medicinal plants are one of the much valuable resources in Iran natural resources that if scientifically recognized, cultured, developed and benefited, can play important role in society's health, preoccupation making job and non-petroleum export[11]. *Bidens bipinnata* L. belongs to family and is of Bidens type. It is yearlong, static, almost grass, Green without fuzz. Stem: standup, single, firm, four -side, yellowish, with cross shoots and arms. Leaves: cross, leafy, without fuzz, with depth divisions. Flowers: yellow, usually all cylindrical. Fruit: hazelnut- shape, long and linear,4-corner,blackish with barbed fuzz. The root of this plant is vertical and flowering time is in July and September[7,11]. Basic origin of this plant is South America, Europe, Asia and Islands[13]. The geographical propagation of this species in Iran: In all north areas and Tehran suburb that found native state and distributed and almost grow as a weed in implanted regions and in farms, plains, forests, ruined areas, gardens and plains, wet settlements, and road ways[15]. This plant has a various usages from before that is as follow:

1. Medicinal usage: This plant's seeds are as a motive and lenient, the best one is mucus removal and is a very good provider of blood, also is soothing and supporter of nerve system and most of the doctors verify their effects on periodic pains and the root of this plant is a very good in chronic colds and also soothing used as an anti sweat and anti nausea drug. In Seiralean Republic, in the west of Africa the leaves of this plant are crushed and for the recovery of boils placed on them[4]. Also this plant used for boil pains remedy, asthma, insects sting, lesion, earache and snakebite[2].
2. Edible usages and as a food sauce: the leaves and young sprout of this plant is used as a food sauce or cook and also from its flowering head stems, the tea is provided[20]. In southern Africa, the young stems consider as a spicy sauce and is used dry or fresh. However this plant not be used as raw cause has a lot of Susanin[17].
3.Hrebal usage: Done surveys show that livestock eat young plants pleasantly but there are volatile oils on it that have undesired smell and maybe septic the milk[2].

The anti diabetes effect of *Bidens bipinnata*. L[10], anti ulcer effect and anti diarrhea in rat[3], anti squirt effect in rat[21], and sitotocksik effect of this plant is studied[18]. Three phenolic glycoside propanoidic from aerial parts is separated[19]. A new glycoside flavanony namely bidenoside F and a chalconi glycoside namely bidenoside G separated from aerial parts and by spectroscopy methods its molecular structure is determined[14]. Study about the essential oil of other types of Bidens done for example three types of this one includes *B.pilosa*, *B.alba* and *B.subalternans* that verified with gaseous chromatography. Totally 24 compounds recognized with contains Polyacetylene and sesquiterpene. Some of them were unknown. Five sesquiterpen in all three species recognized uncertaintly including E-caryophyllene, α -humulene, germacrene D,B-cyclo germacrene and α -morolen. The only Polyacetylene known compound is phenil hepta-1,3,5-3N that was the only one of *B.alba*. Based on done surveys yet have not run any scientific study on the essential oil and chemical compounds of *B.bipinnata* L.in Iran.

MATERIAL & METHODS

Collecting in question plant: After recognition of origin of *Bidens bipinnata* L. in Karaj, for collecting plant sample it is referred to original area and herbaceous samples for providing herbarium sample collected. Studied matters in this area were as follow:

The pendant percentage, geographical direction, soil context, climate, mean annual rainfall and temperature by GPS set determined and plant's features by resources verified. For phonological studying in growing period, each month records maintains and in this origin the soil sample considering soil horizons and plant roots from 0 up to 12 and from 12 up to 28 cm provided and in order to analysis referred to soil laboratory. The climate situation of this origin by using statistic and the information of Sinoptic stations of Karimabad in Karaj extracted.

Phenology: The phenomenology or phenology of plant *Bidens bipinnata* L from early May to September studied. During this period with frequent visits from in question region, the important events including flowering, and the changes of growing in this period studied.

Preparing essential oil: Firstly the aerial parts of fruity plant in outside temperature and in dried shade and by electrical mill were tiny. By longer set, 100 gram of this plant's powder, during 3 hours its essence extracted. This one by sodium sulfate without water, dried and in dark dishes without light in 4 c in refrigerator conserved.

Recognition of essence chemical compounds: Resulting essence with chromatography methods GC and gaseou chromatography GC/MS verified. In all spectrum of GC/MS from exit patterns of normal alkanes and inhibitory index of spectrums, Andis quats calculated for mercury which by their adjustment with books and references and computer library information namely Wiley 275 and the book of Adams and other resources, related spectrums to each body were explained and its essence compounds and chemical formula recognized[1,5].

RESULTS & DISCUSSION

1.Phenology and ecology of medicinal plant: The results show that growing considering annual weather of this plant is from early May onwards and in early Jun the plant is two, four and five-leaves and from the meddle of Jun get flowering and from last July, seed grow. Early September till last the seed grow completely and is collectable.

Table1: Plant scientific name and Medicinal features and application

Plant scientific name	Usage parts	Medicinal features and application
Bidens . tripartiataL	All parts of plant	Liver disease, removal insects bite, gastric blooding and bowel and ulcer
Bidens.angustata	Leaf	Energy making,for clear body ofsunburn
Bidens.magnifolia	root	Remedy pneumonia and cough
Bidens.schimperi	leaf	Removal chest pains
Bidens.pilosa	All parts	Eye tumor, remedy malaria, stomach pain, removal cylindrical worms and irregularity
Bidens bipinnata L	Seed, root, leaf	Mucus removal, soothing and reinforcement of nerves, antiasthma, earache, lesion and snakebite

Also this plant is symbol of cold-dry climate and grow in low gradient of 0 to 10 percent and height range is between 1300 and 1400 meter from sea surface, the mean rainfall is 223 mm,

mean temperature is 13 c and mean annual steam is 2072.The origin of this plant in Karaj with geographical coordinates includes :north wide 35° 48□ 26□□ and eastern long 51° 00□00□□ and also transmittal of this species in Tehran west-north by geographical coordinates includes north wide 35° 45□ 815□□ and east long 51° 19□ 710□□.Regarding the results of soil experiments the soil acidity mount is 0 to 12 depth is 8.1 and in 12 to 28 depth is 8 and electrical conduction in 0 to 12 depth is 35.5 and in the depth 12 to 28 is 34.2 decimeter which is of sandy soils so plain and non-direction lands is good for it. This species and other ones have also medicinal features that here it is mentioned to some of them[Table1],[4,12]:

Table2. The essential oil compounds and percentages of *Bidens bipinnata* L in Karaj

Row	Compound name	RI	GC%	SD
1	α-thujene	928	0.6	0.2
2	α -pinene	937	25.8	2.5
3	Camphene	951	0.5	0.1
4	Sabinene	975	0.5	0.2
5	β -pinene	978	1.9	0.1
6	β -myrcene	992	22.7	2.2
7	α -phellandrene	1006	0.3	0.1
8	β -phellandrene	1031	2.1	0.2
9	(Z)- β -ocimene	1031	2.1	trivial
10	(E)- β -ocimene	1048	2.7	0.2
11	γ -terpinene	1060	0.4	trivial
12	linalool	1100	0.2	0.09
13	Terpinen-4-ol	1181	0.2	trivial
14	Bornyl acetate	1290	0.2	0.05
15	δ-elemene	1344	3.5	0.3
16	β -elemene	1399	0.6	0.06
17	β -caryophyllene	1432	1.9	0.2
18	β -gurjunene	1440	0.7	0.1
19	(Z)- β -farnesene	1458	0.7	0.1
20	α -humulene	1466	2.5	0.3
21	germacrene D	1495	10.2	1.1
22	bicyclogermacrene	1511	7.6	0.8
23	epizonarene	1517	0.5	0.1
24	γ -cadinene	1525	0.1	0.1
25	δ-cadinene	1531	0.3	0.1
26	nerolidol	1566	0.1	trivial
27	spathulenol	1592	2.9	0.6
28	Caryophyllene oxide	1598	0.5	0.1
29	humulene epoxide II	1625	0.5	0.2
30	Cedr-8(15)-en-9-a-ol	1651	0.5	0.1
31	6,10,14-trimethyl-2-pentadecanone	1843	0.3	0.1
total				91.9

Trivial= less than 0.05%

2. Recognition of essence chemical compounds: Resulting essential oil of aerial parts of fruity yellow plant is 0.01 percent. Totally 31 chemical compounds equivalent 91.9% along with GC,RI and Scale deviance reported(Table2).Basic compounds including α -pinene (25.8%), β -myrcene (22.7%), germacrene D (10.20%), bicyclogermacrene (7.6%), δ-elemene (3.5%) and spathulenol (2.9%).Also in this plant's essential oil the monoterpene hydrocarbons (57.82%), sesquiterpene hydrocarbons (26.68%), oxygenated sesquiterpenes (4.52%) and oxygenated monoterpenes (0.79%) with lower mount founded, the rest of the compounds were 0.26%.

Related results to chemical compounds recognition(table1) show that 31 compounds that are totally in 91.9% essence in plants which hydrocarbon monotrapene with 57.8% is the most significant compound in Bidens bipinnata L essential oil. Among other important compounds of this essence can imply to α -pinene (25.8%) , β -myrcene (22.7), germacrene D (10.2%), bicyclogermacrene (7.6%), δ-elemene (3.5%) and spathulenol (2.9%).The other works about Bidens is mentioned follow. Deba and coworkers(2008)analyzes the essential oil chemical compounds in leaves and flowers of B.pilosa by GC/MS,GC.44 compounds determined in essence which basic ones were τ -cadinene and β -caryophyllene. Considering the results of current research and other works, 3 other compounds were also including α -humulene, germacrene D and bicyclogermacrene in *B.alba*,*Bpilosa*,*B.subalternans*,*B.bipinnata* but their percentages in these 4 species were various which depend on species and climate conditions.

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ENERGY-SAVING PROCEDURES FOR STEAM PROCESSING AROMATIC PLANTS

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SUMMARY

The climatic and soil conditions in the Republic of Moldova are favourable for cultivation of many aromatic plants and production of high quality essential oils and extracts. In 1980s Moldova was one of the biggest producers of these products in former USSR, but after 1990 this branch has degraded. After 2000s in Moldova have appeared favourable social and economic opportunities for economic agents to restore and develop this branch by implementing effective innovative technologies.

The steam distillation of aromatic plants is a high energy consumption process – it makes up 20-40% of the total essential oils costs depending on the processing type of aromatic plants. We have tested several technological procedures and the findings are very encouraging. The elaborated energy-saving procedures are based on some physical and chemical properties of the steam, the basic components of essential oils and the dewatered aromatic raw materials.

Low humidity (15-20%) raw materials were processed. There is an incontestable advantage for transportation of dried aromatic raw materials from plantations to the processing units. The energy expenditure for pre-distillation steam heating is 2-3 times lower than for the heating fresh materials. The distillation process occurs more than twice faster. Steam is twice lighter than air and that is why we pump it from the top to bottom of the processing containers. This procedure results profound elimination of the air from the processed material and considerably accelerates the distillation process. The effective heat insulation of the processing equipment has been done. The processed dried raw materials, without any supplementary drying were directly used for superheated steam production.

Molecular weights of more valuable components of essential oils are 8-15 times higher than of water vapor and their boiling points are at 200-270^{0}C. We have determined that the 12-22^{0}C increase of the processing temperature accelerates the distillation procedure of essential oils more that twice. At the same time, high-temperature superheated steam (150-175^{0}C) at low pressure (0.5-0.7bar) pumped from top to bottom through the dried aromatic raw materials considerably increased, for example, the percentage of sclareol (its molecular weight is 302daltons).

All of the discussed procedures permit us to reduce more than twice the costs of the steam processing the aromatic plants and to obtain the high-quality organic products.

***Key words**: essential oils, energy-saving steam distillation*

INTRODUCTION

Essential oil production in the Republic of Moldova was one of the most profitable branches of agriculture up to the end of the 1980. Almost 160 tons of high quality oil extracted from rose, lavender, Clary sage, peppermint, fennel, dill were produced and exported annually, which was about 35-55 % of the total essential oil production volume of each aromatic culture in the former USSR. The main aromatic culture was lavender (*Lavandula angustifolia*, Mill.), the annual production achieving 80 tons. The area cultivated with the enumerated aromatic plants was of 18.000 to 20.000 hectares. After 1990, this branch has

degraded. Nowadays the annual quantity of oil produced does not exceed 20 to 25 tons, while the surface cultivated with these plants does not exceed 1500-2000 hectares. There are some objective and subjective causes of this branch fault.

One of the principal causes of the decrease of the production of aromatic cultures both in Moldova, and in other countries was and still is a constant rise of prices of fossil fuel, whose share in raw materials processing makes up 20-40 % from the cost of manufacture of essential oils and extracts depending on processed culture. Existing processing technologies of aromatic raw materials and installations have become morally outdated and demand considerable expenses of energy, materials and manual skills. For this reason, some economic agents from the Republic of Moldova gain only minimal profit, and some cultures as dill, hyssop, coriander, etc. became unprofitable. It is necessary to notice that technological progress almost did not concern this branch because of its insignificant share in the global economy and consequently, in all countries-producers of essential oils and extracts the situation is similar with ours.

At the same time after 2000 last years in the Republic of Moldova in new social and economic conditions we have accumulated certain practical experience, a number of original energy-saving processing methods and installations have been developed and tested both in production and processing the raw materials of aromatic plants cultivated in Moldova. Therefore, for our economic agents there is a unique chance to restore and develop this branch on new principles of production organization by implementing effective innovative technologies and in this way in the nearest several years to achieve the level of manufacture of natural essential oils and extracts as it was in the 1980s.

MATERIAL & METHODS. RESULTS &DISCUSSION

The basic aromatic culture for the Republic Moldova was, is and will be lavender. Proceeding from this fact, we have made estimations on the basis of the results of economic activities in 2006-2011 of the join Moldavian-British enterprise "ResendJer", which shows the necessity of the implementation of some effective technological procedures to cut expenses under certain articles.

For six years, the average cost of manufacture of the lavender essential oil at the mentioned economic agent fluctuated for six years within 21-31 US dollars per 1 kg and consisted of the following expenses:

- Amortization of plantations – $ 6.0... 8.0 per 1kg;
- Care of plantings, taxes, land rent, etc. –$ 5.0... 6.0 per 1kg;
- Manual harvesting of inflorescences, loading, transportation – $4.0... 6.,0 per 1kg (the share of manual harvesting makes $3.5... 4.5 per 1kg and more than 20 persons-days per 1 hectare);
- Processing of raw materials by steam distillation method – $5.0... 8.0 per 1kg (the share of fuel for steam manufacture makes up $3.5... 4.5 per 1kg!);
- Amortization of installation – $3.0... 5.0 per 1kg.

This economic agent has the „SGS-Moldova" certificate for ecological production that allows it to realize lavender essential oil at negotiable price. At other economic agents- producers of lavender essential oil who have no given certificate the situation is difficult enough.

From these analyses, two basic directions result on elaboration and implementation of technological innovations – the mechanized harvesting, and the increase of the efficiency of installations for processing raw materials using biomass (inclusive the dried lavender wastes) as renewable energy source for steam production.

Harvesters in different countries for harvesting the lavender inflorescences do not meet the requirements of the biology of the culture and of the parameters of technological procedure of

processing. Machines strongly damage bushes, a significant amount of impurity gets in to raw materials (the leaves which are not containing essential oils, inflorescence stalks and woody stems), expenses of the thermal agent increases further at processing and worsens quality of essential oils. In order to overcome these lacks we develop a single-row lavender harvester which copies the profile of bushes without damaging them and arranges the cut off inflorescences on bushes for drying. Then the dried raw materials then are picked up and processed. The author has emphasized the description of a biphasic way of harvesting lavender inflorescences as the dried raw materials are the defining factor in energy-efficiency of the technological process of its processing by superheated steams at low pressure.

The aim of this work is to present the generalized results of the long-term scientific and technological investigations and practical experience of the author on processing lavender raw materials and others aromatic cultures. All that, has allowed to formulate and offer an energy effective technology and installations of various productivity for processing vegetable aromatic raw materials both in periodic and in continuous modes.

From the very beginning it was necessary to overcome the some stereotypes formulated in special literature – the statement that the harvested aromatic raw materials must be processed fresh, not later than 2-3 hours after harvesting [1].

Thereupon it is necessary to notice that the structure of glandules, inclusions or receptacles for essential oils allows to keep their integrity at physiological temperatures for a long time. The dried up plants or inflorescences of basil (the Moldavian national herb), lavender, *Salvia sclarea* and *officinalis*, hyssop, *Saturea montana*, peppermint and many other aromatic plants growing in Moldova keep the aromas for years. Besides, the basic most valuable components of essential oils of these plants have considerable molecular weights (150-300dalton) and high boiling temperatures (200-270^{0}C and even higher) that defines their small evaporation speed at ambient temperature.

Moisture content in fresh raw materials at technical ripeness of these cultures makes up 75-80 %. At hydro- or steam distillation at atmospheric pressure the raw materials, at first should be warmed up to 100^{0}C and only after that the process of distillation begins. To warm up fresh raw materials, up to ¾ thermal energy of distillation necessary for all process is spent. To warm up a mass unit of vegetable raw materials with the humidity of 15-20 % it require approximately 2.2 – 2.5 times less thermal energy in comparison with the fresh raw materials humidity of 75 % [1].

Now one of the most promising dehydration methods of vegetable products is drying by superheated steam at atmospheric pressure [2]. The processing by superheated steam of fresh aromatic raw materials the such research is only in the beginning [3,4,5]. Here, however, there is one essential obstacle – at the contact with the raw materials of high humidity the superheated steam quickly becomes saturated and the temperature is stabilized at 100^{O}C and the effect of superheating the steam is lost.

On the basis of temperature dependence and partial vapors pressure of the most important components of vegetable essential oils [6], we have constructed the diagram of the temperature dependence of component's vapor quantity in one cubic meter, from which it is clearly visible exponential character of such dependence (Fig.1.). For example, linalool and linalyl acetate are the basic components of lavender essential oil (in sum they make up to 80 %). It is possible to assume that at temperature 100^{0}C in 1 м^3 water steam or air they consist amount of 350-400g. The 15-16^{0}C temperature rising doubles this quantity. One tone of lavender dry raw materials at 15 % of humidity contains approximately 30kg of essential oil, i.e. at temperature 100^{0}C it is enough 80м^3 or 40kg of steam for its distillation. Let's add 80кg of steam necessary for the warming up 1000kg dried raw materials to 100^{0}C and the sum will be 120kg of steam for manufacture of 30kg essential oil. That is 8-10 time less, than for processing of the lavender fresh raw materials.

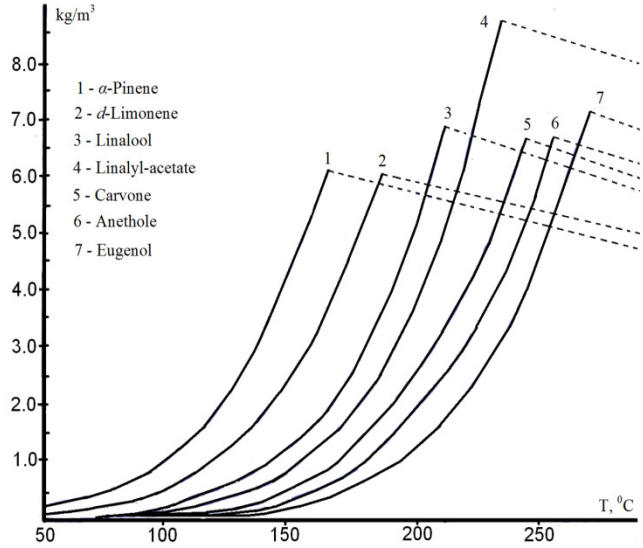

Fig.1. Temperature dependence of the vapor density of basic components of essential oils

Using the superheated steam at temperature up to 170^0C will be even more reduced the time and expenses of thermal energy for processing of the dried raw materials. The humidity of 15-20 % of lavender inflorescences has been determined from practice – the raw materials well keep within containers, do not crumble and are easier for steam to penetrate than fresh ones. The limit of superheated steam temperature at 170^0C was taken from [4], because at the distillation above this temperature in essential oil composition may appeared volatile products of thermal degradation of some components of vegetative tissues. At the same time the components of essential oils in the inert gas atmosphere (unsaturated water steam is inert too) are stabile at the temperatures much more above their boiling point – an example may be gas chromatographic component separation of essential oils.

Traditional installation on processing aromatic raw materials includes a steam generator at pressure 6-8bar at 160-170^0C, the mobile metal container with the punched pipes in the bottom for steam pumping, the heat exchanger and Florentine vessel, where the mixture of essential oil and water is separated [2].

We have established that at raw materials processing on traditional technology with steam pumping from below, air from raw materials leaves throughout all process of distillation. It occurs because the steam is twice lighter than air and the speed of its pumping through the container does not exceed 2-3см/sec. At the end of distillation process because of non-uniform packing of raw materials on container volume there are unprocessed zones, the mixture of steam and essential oil vapor at the entrance into the heat exchanger reaches 125^0C. It shows that steam rise through zones, where it does not encounter resistance and does not carry out its functions.

It is necessary to underline that speed of distillation at 100^0C for fresh vegetable raw materials is limited by diffusion of components of the essential oils and at atmospheric pressure in water environment this limit can not be exceeded. Increasing the pressure and the temperature of environment also does not solve the problem of process acceleration. As essential oils are thermally unstable substances in the air and water environment, it is recommended to reduce time of thermal influence during their extraction [3].

CONCLUSIONS

We carried out some investigations and tests under the production conditions of energy-saving processing methods and equipment. At insignificant improvement of existing processing installations these recommendations may have wide application. At the same time highly effective continuously operating installations with maximum recuperation of thermal energy as alternative of the container mode of processing in stationary and mobile variants were developed.

On the offered processing technology is carried out only at low humidity of aromatic raw materials and based on using the low pressure superheated steam, having following advantages in comparison with the traditional.

- Reduces fuel expenses up to 12-17 % for transportation and up to 50-60% at the stage of heating up raw materials before distillation, allows to produce more quality essential oils in comparison with processing fresh aromatic raw materials.
- The thermo insulated containers allow to reduce heat losses up to 30-40 % in comparison with traditional containers.
- The using of superheated steam accelerates not less than twice process of distillation of essential oils, allows to make more profound processing of raw materials with simultaneous decrease up to 50 % of expenses of thermal agent on a finished unit of product, and also allows to obtain valuable aromatic products, which usually are extracted by solvents.
- The top pumping of the steam in containers provides fast removal of the air and intensifies mass and heat exchange, prevents the oxidation and hydrolysis of some components, and also excludes unprocessed zones
- After processing, the humidity of wastes decreases a little, and they can be directly used for production of the superheated steam that may reduces up to 40 % the total cost of essential oils manufacture, depending on processed culture in comparison with the using of liquid fuel or natural gas, excepting environmental contamination.
- Thanks to the offered non-polluting technology, aromatic products may be certificated as organic, considering that demand for them continuously increases.

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EFFECT OF REGION ON MORPHOLOGICAL CHARACTERISTICS OF LICORICE(*GLYCYRRHIZA GLABRA* L.) COLLECTED FROM SOUTH OF IRAN

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SUMMARY

In order to investigate of region effect on morphological traits of licorice plant, a CDR experiment with 4 replications was conducted in Abadan division at 2009. Treatments were collection places including Bahmanshir river coast (p.1), Arvand river coast (p.2), place between p.1 and p.2 (p.3) and Minoo Island (p.4). Results showed that there were significant difference between plants in branch number and seed number in per capsule. Our results did not show that significant difference between plants in root diameter, Stem diameter and stem length. Root is the most important part of licorice that use as a medicine and this experiment showed that regions can change morphological traits of plant roots.

***Key words**: licorice, morphological characteristics, medicinal plant, Abadan.*

INTRODUCTION

Glycyrrhiza glabra, commonly known as licorice, has been used for centuries in the traditional and folk medicines of Asia and Europe to treat ailments ranging from the common cold to liver disease[1]. The plant is cultivated in Greece, Russia, Turkey, Iran, China and parts of Europe. It is commercially available in many forms virtually everywhere[2] .The licorice plant grows best in deep, fertile, well-drained soils with full sun, and is harvested in the autumn two to three years after planting. Licorice is a hardy, perennial shrub, which typically reaches a height of 3 to 7 meters. Its leaves are compound and alternate, having 4—7 pairs of oblong, elliptical or lanceolate leaflets. The lavender to violet flowers are narrow, growing in in axillary spikes. The calyx is short, campanulate, with lanceolate tips and glandular hairs. The fruit is a compressed legume or pod, up to 1.5 cm long, erect, glabrous, somewhat reticulately pitted, and usually contains 3-5 brown, reniform seeds. The licorice taproot is approximately 1.5 cm long and subdivides into 3-5 subsidiary roots, about 1.25 cm long, from which the horizontal woody stolons arise. These may reach 8 m and when dried and cut, together with the root, constitute commercial licorice. It may be found peeled or unpeeled[3].

Licorice has been widely used in European herbal medicine as a treatment for gastric ulcers. Modern use began in 1946, when the Dutch physician F. E. Revers demonstrated that licorice was the active ingredient in a domestic medicine used in the Netherlands. When the substance was studied for this use, good results were obtained in the treatment of stomach ulcers in 32 patients[2]. Licorice has been used traditionally in the prevention of liver diseases[4]. An increase was seen in the lag phase of oxidation of ascorbate free radicals in the liver and myocardia of experimental animals when licorice was administered. The antioxidant activity of the root powder was comparable to that of p-carotene, and caused markedly decreased lipid peroxides in liver[5]. Licorice potentiated the antitumor and antimetastatic activi cyclophosphamide when tested in metastasising Lewis lung carcinoma[6].

Varience of regions can be change morhpological characteristics, acording to this, were disided to study effect of regions on morphological characteristics of licorice(*Glycyrrhiza glabra* L.) collected from south of Iran.

MATERIAL & METHODS

In order to investigate of regions effect on morphological traits of licorice plant, a CDR experiment with 4 replications was conducted in Abadan division at 2009. Treatments were collection places including Bahmanshir river coast (p.1), Arvand river coast (p.2), place between p.1 and p.2 (p.3) and Minoo Island (p.4). there were some differences between regions that we showed in tabel 1,2. We did not show any varience between regions from a pharameter of climent poit of veiw(tabel 3). At the end, all results of experiment were assimilatd by SAS software analys.

Table 1 - Elements of soil regions

Discription	Na meq/l	Cl meq/l	T.N.V %	O.M %	O.C %	N %	P(Av.) p.p.m	K(Av.) p.p.m
P1	140	181	40	1.28	0.76	0.065	8	320
P2	125	89.4	33	0.71	0.43	0.035	2.2	944
P3	74	87	43	2	1.18	0.100	4	416
P4	226	266	34	1.60	1.04	0.080	4	493

RESULTS & DISCUSSTION

Results showed that there was significant difference between plant in branch number and seed number per capsul. The most seed and branch number was observed in collected plants from p.4 and the least was for p.2. Difference between plants of places was not significant for stem length. The highest stem length was observed in p.3 plants while the minimum was for p.4 plants. There was no significant difference between plants at p.1,2, 3 and 4 for stem diameter. Stem diameter in collected plants of p.1 was lower than p. 2, 3 and 4. Our results showed that root diameter of p.2 plants were higher than p.1, 4 and 3. Root is the most important part of licorice that use as a medicine and this experiment showed that regions can change quantity of plant roots.

Table 2 - Chemical and Physical analysis of soil regions

Discription	S.P %	E.C ds/m	PH	Sand %	Silt %	Clay %	Class
P1	68	19.74	7.45	5	51.5	43.5	Silty clay
P2	74	14.26	8	5	46	49	Silty clay
P3	64	11.16	7.50	3	50	47	Silty clay
P4	70	29.7	7.75	6	46	48	Silty clay

Table 3 -Pharameter of climent from1951-2005

Jan	Feb	Mar	Apr	May	June	July	Aug	Sep	Oct	Nov	Dec	
12.7	14.9	19.2	25.0	31.0	35.0	36.6	36.2	32.9	27.3	20.0	14.3	Average of mean daily temperature in C
70	61	52	44	33	27	28	31	34	45	58	70	Average of relative humidity in percent
35.6	20.6	20.4	14.2	3.5	0.0	0.0	0.0	0.1	4.0	20.3	37.3	Monthly total of precipitation in mm

Table 4. compar between morphological characteristics avarage of licorice in Duncan

Seed number	Branch number	Root diameter	Stem diameter	Stem length	
2.50 ab	2.00 ab	9.13 a	3.62 a	63.20 a	P 1
1.75 c	1.00 c	9.29 a	3.79 a	68.45 a	P 2
2.25 bc	1.57 b	8.81 a	3.38 a	69.85 a	P 3
3.00 a	2.50 a	8.65 a	3.29 a	56.30 a	P 4

In this study, all of the plants were glabra variety and there were not any genetic different among plants and the other hand were not watched any different betwen regions by climents parameters(Table3). Therefor physical and chemical soils parameters are important reason that were caused difference between regions (Table1,2). Presence of high quantity of Cl and Na in p.4 cause decrease amount of stem diameter and stem length but increace root diameter, branch number and seed number per capsul. Gathering of Cl and Na can decrease photosentetic so that all parameters that had direct conection with photoseentetic for example stem diameter and stem length were decrease [7]. Also Cl and Na cause kind of physiological drought, so plant can not add water, elements.., these conditions can decrease diameter and length of stem[8]. Finally root is the most important part of licorice that use as a medicine and this experiment showed that regions can change morphological characters of plants especially diameter of roots but did not significantgy.

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CONTENT AND COMPOSITION OF ANTHOCYANINS IN SELECTED PLANTS SPECIES

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SUMMARY

Anthocyanins are the final product of flavonoides production in secondary metabolism of plant cells and they have a positive effect on human organism because of antioxidant properties. The six most important anthocyanines (cyanidine, pelargonidine, peonidine, delfinidine, petunidine, malvidine) are present in large amounts in plant species *Vitis vinifera* L., *Vaccinum myrtillus* L. and *Sambucus nigra* L. In the berries of *Vitis vinifera* L. anthocyanins are accumulated in hypodermal cell layer of peel, or in the pulp of some cultivars. Except for pelargonidine, it contains all important anthocyanidines, with predomination of malvidine. In the literature, the following total content of anthocyanins in selected plant species are reported: in the fresh grape berries ranged from 0.50 to 4.99 $g.kg^{-1}$ and in peels from 20.7 to 66.6 $mg.g^{-1}$ of peels dry matter. In the bilberry *Vaccinum myrtillus* L. were identified five anthocyanidines: cyanidine, delphinidine, malvidine, peonidine and petunidine. Total determined content of anthocyanins was found in quantities from 0.115 to 0.133 $\mu g.ml^{-1}$, or 0.58 % in berry and 1.09 % in berry skins. Elderberry *Sambucus nigra* L. contains five important anthocyanins: cyanidine-3-O-sambubioside-5-O-glucoside, cyanidine-3-5-O-diglucoside, cyanidine-3-O-sambubioside, cyanidine-3-O-glucoside and cyanidine-3-rutinoside. The content of identified anthocyans in fruit of this species ranges from 602.9 to 1265.3 $mg.100g^{-1}$. In fresh black elder juice where found total anthocyanins content 6287.4 $mg.l^{-1}$. The amount of accumulated anthocyanins pigments depends on variety, ecological conditions standard of agricultural technology, and particularly on the temperature and solar radiation.

***Key words**: anhtocyanidines, Vitis vinifera* L.*, Vaccinum myrtillus* L.*, Sambucus nigra* L.*, content of anthocyanins*

INTRODUCTION

Anthocyanins occurred naturally are heteroglycosides which are composed of sugar moiety and aglycone – anthocyanidine. They are polyhydroxyderivate and polymethoxy-derivate 2-fenylbenzopyryl or also flavyliol cations. Only six of 15 the most important anthocyanins which contain hydroxyl group in position C-3 have importance in food. The highest occurrence in the different fruits and plants in nature has malvidine, lower amount presents petunidine than delfinidine, peonidine, pelargonidine and cyanidine (Balík, 2010). Anthocyanins are known as potent antioxidants. They have many positive effects on human organism.

ANTHOCYANINS IN *VITIS VINIFERA L.*

Anthocyanins in grape accumulate during fruit maturing in hypodermal cell layer of berry hull or also in the pulp of some cultivars and cause their specific colour as a pigments. There were identified five aglycone – anthocyanidines: delfinidine, cyanidine, malvidine, peonidine and petunidine in the fruits of plant species *Vitis vinifera* L. Second important component jointed in anthocyanins is sugar. There has been identified glucose and in rare cases more closely non-specified monopentosoid in *Vitis vinifera* L. (Mazzuca et al., 2005). Mazza (1995) mentioned total anthocyanins content in fresh berries ranged from 0.86 to 0.98 $g.kg^{-1}$ in cultivar Cabernet Sauwignon, from 0.27 to 0.59 $g.kg^{-1}$ in cultivar Gamay and 0.33 $g.kg^{-1}$ in cultivar Ruland blue. Cultivar Alicante-Bouchet contains 5.2 $g.kg^{-1}$, out of which 78.4% in hull, 18.7 % in pulp, 1.6 % in bunch and 1.3 % in seed. Cultivars Shiraz (2.2 $g.kg^{-1}$) and Cabernet Sauvignon (1.7 $g.kg^{-1}$) belong in cultivars group with high pigments potential. Kumšta and Balík (2008) evaluated anthocyanins composition in *Vitis vinifera* L. in nine cultivars and ten inter-specified crossbreds. They identified 22 different anthocyanins. The highest average occurrences in all evaluated cultivars were malvidine-3-O-monoglucoside (39.18 %), malvidine-3-O-monoglucoside acylated by kumar acid (9.03 %), peonidine-3-O-monoglucoside (8.00 %) and petunidine-3-O-monoglucoside (7.58 %), with total amount 72.07 % that shared all anthocyanins. In all cases, there was identified anthocyanidins glucoside only with glucose. In the case of cultivars Mo.1 a Mo.3 there were determined very low abundance of malvidine-3-O-monoglucoside compared with other cultivars (5.49 % and 7.03 %). Diglucoside anthocyanins were found in cultivars inter-specified top cross Golubek, Kaberon, Mo.1 and Mo.3 and in cultivars Regent and Peking that contained malvidine-3-5-O-diglucoside.

ANTHOCYANINS IN *VACCINIUM MYRTYLLUS* L.

According to Riihinen et al. (2008) blueberries are the most important source of anthocyanins. Their content of pigments make world interest about their fruits.

Burdulis et al. (2007) had been studied the biodiversity of anthocyanin content in blueberries (*Vaccinium myrtillus* L.). He compared fruits of this species in different regions o Lithuania, from Belarus, Russia and Sveden. Cyanidin, delphinidin, malvidin, peonidin and petunidin were dominant of all anthocyanins. There were identified 14 anthocyanins. Sugar components were 3-O-arabinosis, 3-0-glucosis a 3-O-galactosis (Burdulis et al., 2007; Gould et al., 2009). Content of anthocyanins ranged from 0.115 $\mu g.ml^{-1}$ to 0.133 $\mu g.ml^{-1}$ (Burdulis et al., 2007).

Burdulis et al. (2009) compared anthocyanins in bilberry fruits (*Vaccinium myrtillus L.*) and blueberry (*Vaccinium corymbosum L.*). The content of anthocyanins in bilberry was 0.58 % and in fruits of different species of blueberry 0.07 – 0.20 %. The highest content was in fruits peel, in bilberry 1.08 % and in blueberry 0.63 – 1.24 %.

Bolda et al. (2011) have been tested native populations of bilberry from three locations (Arieseni, Retezat and Sebes Valley district) of Carpatian mountains in western Romania. They were evaluated total content of anthocyanins from calluses and leaves of mother plants. In fresh material of mother plants there were determined content of anthocyanins from 280 $\mu mol.g^{-1}$ to 540 $\mu mol.g^{-1}$ (fresh weight).

ANTHOCYANINS IN *SAMBUCUS NIGRA* L.

Veberic et al. (2009) compared anthocyanins content in fruits of black elder (*Sambucus nigra* L.) in two varieties (Rubini and Haschberg) and three selections (Selection 13, 14 and 25). They determined five anthocyanidins: cyanidine-3-O-sambubioside-5-O-glucoside, cyanidine-3,5-O diglucoside, cyanidine-3-O-sambubioside, cyanidine-3-O-glucoside and cyanidine-3-O-rutinoside. Other anthocyanins occurred only in small amounts. As it

results black elder contains the most cyanidine-3-O-glucoside and cyanidine-3-O-sambubioside. The highest anthocyanins content was determined in cultivar Rubiny, 1265.3 mg.100 g^{-1}.

Lee and Finn (2007) have also studied anthocyanins content in black elder. They compared two cultivars Konsor (Denmark) and Haschberg (Austria). Total anthocyanins content was 400.2 mg.100g^{-1} (2004) and 806.1 mg.100 g^{-1} (2005) by cultivar Korsor. Cultivar Haschberg accumulated 391.0 and 656.5 mg.100g^{-1} (year 2004 and 2005). There were identified five anthocyanins in both cultivars: cyanidine-3-O-sambubioside-5-O-glucoside, cyanidine-3,5-O-diglucoside, cyanidine-3-O-sambubioside, cyanidine-3-O-glucoside and pelargonidine-3-O-glucoside. Cultivar Haschberg consist also cyanidine-3-O-rutinoside and delphinidine-3-O-rutinoside. It was the first case when there was identified delphinidine-3-O-rutinoside in black elder (only in cultivar Haschberg). There is significant interaction between genotype and growing season in anthocyanins content. The highest content is present cyanidine-3-O-glucoside and cyanidine-3-O-sambubioside (Lee and Finn, 2007). It is comparable with the results of Veberic et al. (2009), Kaack et al. (2008) and Jakobek et al. (2007). Jakobek et al. (2007) set down anthocyanins content at 6287.4 mg.l^{-1} in fresh black elder juice. Cyanidine-3-O-glucoside and cyanidine-3-O-sambubioside amount was 83.1 % (5226.6 mg.l^{-1}).

CONCLUSION

The study of anthocyanins content in selected plant species was focused on amount and composition detection of these compounds intra research project *"Isolation of Natural Plant Substances by Lyophilisation and Change of their Qualitative and Quantitative properties"*. From natural occurrence sources and from cultivation areas of selected species and intra those of selected cultivars there were obtained fruits for purposes of anthocyanins extraction, evaluation of their quantitative and qualitative characteristics before and after lyophilisation, while in parallel evaluation of their biological and microbiological characteristics.

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ANTIMICROBIAL ACITIVITIES OF SOME TRADITIONAL NATURAL OIL EXTRACTS

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SUMMARY

Checking of the antimicrobial activity of natural oil extracts is highly recommended, if they are to be used in pharmacy as antiseptic remedies. For that purpose eight oil extracts traditionally prepared from seven different plants and one from mice are used in this investigation.

Antimicrobial effect of eight oil extracts to seven microorganisms was examined on: six bacterial strains (*Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633, Enterococcus ATCC 29212, Klebsiella pneumoniae* - laboratory strain) and on yeast *Candida albicans ATCC 10231.* The antimicrobial test was done by agar diffusion of oil extracts on Mueller-Hinton agar. Inhibition of bacterial growth was registered for *Escherichia coli* and *Bacillus subtilis* only in the Hyperici Oleum (St John's wort oil) traditionally prepared.

Key words: *oil extract, antimicrobial activity, traditional medicine*

INTRODUCTION

Natural oil extracts from plant or animal origin have been used for centuries in traditional medicine for topical treatment of wounds, bruises, ulcers, cuts, burns, hemorrhoids, ear pain and also as an antiseptic. Biological sources of this kind of oil extracts are representatives of several herbs i.e. *Hypericum perforatum* L., *Salvia officinalis* L. *Calendula officinalis* L. *Juglans regia* L., *Arctium lappa* L. etc. The entire plant or parts of it, such as roots, leaves or seeds, were used in this preparation. Also, just born animals i.e. mice are used for preparing of these traditional remedies. The extracts were prepared according to the prescriptions from traditional medicine, with different vegetable oils used as an extractant, namely: olive and sunflower oil [1,2,3]. Formulae often do not give precise details as to quantity or weight, and instructions such as a handful, a bundle, a cup had to suffice [4].

Checking their antimicrobial activity is highly recommended, if they are to be used in pharmacy as antiseptic remedies. For that purpose eight oil extracts traditionally prepared from seven different herbs and one from mice are used in this investigation.

MATERIAL & METHODS

Samples

The present study comprised eight oil extracts traditionally prepared from seven different plants and one from mice in olive oil. Three plants samples were field collected in different areas of Macedonia in the course of 2010/11. Fresh aerial parts of *Hypericum perforatum* L., leaves from *Salvia officinalis* L. and flowers from *Calendula officinalis* L. separately were extracted with olive oil at room temperature for 40 days. The rest herb oil extracts were

manufactured by Galafarm from St. John's wort (*Hypericum perforatum* L.) herb, burdock (*Arctium lappa* L.) root, walnut (*Juglans regia* L.) leaves and marigold (*Calendula officinalis* L.) flowers in olive oil (1:4).

Mice oil extract was obtained by extraction of 5 just borne white mice in 1000 mL sunflower oil for 40 days.

All the samples were analyzed in triplicate for the determination.

Microorganisms tested

Antimicrobial effect of eight oil extracts to seven microorganisms was examined on: six bacterial strains (*Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, Bacillus subtilis ATCC 6633, Enterococcus faecalis ATCC 29212, Klebsiella pneumoniae* - laboratory strain) and one yeast *Candida albicans ATCC 10231.*

All tested microorganisms (3 species of Gram positive bacteria: *Staphylococcus aureus, Enterococcus faecalis and Bacillus subtilis;* 3 species of Gram negative bacteria*: Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae;* one species of fungus *Candida albicans*) were obtained from the Institute of Microbiology and parasitology, Faculty of Medicine, University of "Ss Cyril and Methodius" Skopje, Republic of Macedonia. All strains were referent strains, except *Klebsiella pneumoniae* which was clinical isolate.

Additionally the oil extracts obtained by maceration of mice was tested on the follow microorganisms that may cause otitis: *Streptococcus pyogenes, Moraxella catarrhalis, Haemophilus influenzae* and *Aspergillus niger.* Strains were clinical isolates from human respiratory tract.

Antimicrobial investigations

Agar diffusion method as well as agar dilution method were used for testing the antimicrobial activity of the investigated oil extracts.

Agar diffusion method – Microorganisms were suspended in sterile trypticase soy broth with turbidity corresponding to 0.5 Mc Farland (approximate by 10^8 CFU/mL) measured by densitometar. The suitable solid media (deep of agar 4 mm) were inoculated with suspensions of microorganisms: Columbia blood agar was used for *Streptococcus* and *Moraxella*, Chocolate agar – PVX (Oxoid, UK) for *Haemophilus*, Müeller Hinton (Oxoid, UK) for other bacteria and Subouraund (Calb) agar (Oxoid, UK) for *Candida and Aspergillus.* In each medium, after inoculation of microorganisms, wells of 6.5 mm in diameter were made by sterile glass pipette. The wells were filled with 0.1 ml of ethanolic solution of the oil extracts and one well only with ethanol (blank probe). The growth inhibition zones were measured after incubation of appropriate time. The plates with bacteria were incubated 24 hours and the plates with fungi were incubated 48-72 hours at 37^{o}C) [5,6].

Agar dilution method – This method was used to determine the minimal inhibitory concentration (MIC) of the oil extract (expressed in percent of oil extract), required to inhibit growth of microorganisms. Dilution of the oil extracts with ethanol was performed to obtain the twofold serial dilution from the basic solution. Such diluted oil extracts were mixed with Müeller Hinton agar in proportion 1:10 poured into 100 mm – diameter round sterile plastic plates and were allowed to solidify. As growth controls, plates containing oils free agar were also prepared with ethanol. Microorganisms were suspended in trypticase soy broth with turbidity that matches 0.5 Mc Farland standards. The microorganisms were inoculated on the surface of the media by a calibrated loop in manner to form macro colonies. After 24-48 h incubation, the growth was observed. The lowest concentration that inhibits visible growth of an microorganism was recorded as MIC [7].

RESULTS & DISCUSSION

Oil extract with animal origin

Therapeutic records in Macedonian folk medicine, where many century long experiences in traditional medicine and pharmacotherapy of the European and Asian civilization were transfused, contain medicines from animal origin. Hence, these records recommend crude pork blood and liver for anaemia, donkey skin against convulsion, secretions from frog skin for heart diseases, tortoise meat against snakes bite [1]. For some of them contemporary medicine discovered there effects [8].

In Struga and surrounding one interesting medicine from animal origin exist for healing an ear pains. This traditional medicine probably was prescribed by the physician Vladimir Kavaev (born in 1885) who studied a military medical academy in Russia. Doctor Kavaev and his wife Elena (also physician) were personal acquaintances and collaborators of Lenin [1]. To this day this medicine is prepared in Struga and surroundings as a very efficient and irreplaceable remedy for ear pain. No, literature data exist about preparing and usage of this remedy prepared with just borne mice in our country and other countries in the world. Because of that with our investigations of the antimicrobial activities of this oil extract we have tried to find an explanation of its usage for healing ear pains. But we were surprised when we don't observed inhibition on bacterial and fungal growth on the all tested microorganisms: 3 species of Gram positive bacteria: *Staphylococcus aureus, Enterococcus faecalis and Bacillus subtilis;* 3 species of Gram negative bacteria*: Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae;* one species of fungus *Candida albicans*. Additionally the oil extracts obtained by maceration of mice was tested on the follow microorganisms that may cause otitis: *Streptococcus pyogenes, Moraxella catarrhalis, Haemophilus influenzae* and *Aspergillus niger.* Strains were clinical isolates from human respiratory tract. But again the inhibition of none of these bacterial growths of the oil extracts obtained by maceration of mice was not observed. Further investigation of this oil extract prepared from mice should be performed with other kind of investigations in order to find an explanation of the widespread usage of this remedy to date in some areas in Republic of Macedonia.

Oil extracts with plant origin

The usage of oil extracts obtained from plants has been actual for centuries in traditional medicine in Macedonia. First written document for the use of plant oil extracts is the work of Eftim Sprostranov from 18th century entitled as "Folk medicine and its nomenclature in Macedonia" [1]. According to this work St. John's wort oil has been used for topical treatment of cuts, burns, hemorrhoids, as antiseptic because of its antibacterial action. The usage of St. John's wort oil is still so current in our country.

Also, in our drug stores could be found and other kinds of oil extracts obtained from various plants (i.e. St. John's wort, burdock, walnut and marigold) with declared antimicrobial action. They are prescribed externally for treatment of acne, dermatitis, burns, cuts, open wounds, bruises and also as an antiseptic. Some pharmaceutical manufactures imported this kind of final traditional remedies. We have noticed before that Macedonian companies have export of a good quality raw material to processing and import of final products with added value [9,10]. Because we are suspicious for the declared antibacterial and antifungal action of this imported oil plant extracts with our investigation we are trying to find a resolution. Unfortunately not one of the four imported oil plant extracts (Bardanae oleum, Hyperici oleum, Juglandis oleum and Calendulae oleum) showed antimicrobial action against 3 species of Gram positive bacteria: *Staphylococcus aureus, Enterococcus faecalis and Bacillus*

subtilis; 3 species of Gram negative bacteria*: Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae;* one species of fungus *Candida albicans*.
For comparison we have examined our traditionally home prepared St. John's wort oil from our Macedonian fresh aerial parts of *Hypericum perforatum* L., where we proved the antibacterial action. The inhibition of bacterial growth was registered for *Escherichia coli* and *Bacillus subtilis* only in the Hyperici Oleum (St John's wort oil) traditionally home prepared. Serial dilutions showed that the antimicrobial activity was present only in the basic solution (not diluted).
Small antimicrobial activity was registered in basic solution of home prepared oil from *Salviae officinalis* leaves to followed microorganisms: *Staphylococcus aureus, Escherichia coli* and *Candida albicans.*
No, activity against against 3 species of Gram positive bacteria: *Staphylococcus aureus, Enterococcus faecalis and Bacillus subtilis;* 3 species of Gram negative bacteria*: Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae;* one species of fungus *Candida albicans* was observed in the home prepared oil from *Calendula officinalis* flowers.

CONCLUSION

The antimicrobial activity of natural oil extracts prepared from animal or plant origin was tested on seven microorganisms in vitro.
The inhibition of the bacterial and fungal growth of the oil extract prepared from just borne mice was not observed.
Inhibition of bacterial growth was registered for *Escherichia coli* and *Bacillus subtilis* only in the Hyperici Oleum (St John's wort oil) traditionally prepared, not in the imported.
The home prepared oil from *Salviae officinalis* leaves inhibited the growth of *Staphylococcus aureus, Escherichia coli* and *Candida albicans.*

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GERMINATION RESPONSES TO SALT STRESS IN ST.JOHNS WORT (*HYPERICUM PERFORATUM* L.) GENOTYPES

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ABSTRACT

The response of ten st.johns wort (*Hypericum perforatum* L.) genotypes to NaCl salinity at germination life stage was investigated. This study conducted was carried out a factorial in a randomized complete design (CRD) with three replications in 2009 at seed technology laboratory of Islamic Azad university, Shahr-e-Rey branch. Four levels of NaCl (0, 100, 200, and 300 mM) and ten genotypes of st.johns wort were used as treatments. The experimental laboratory characters contains: percentage and speed of germination, Length of root and shoot, Length of seedling, ratio of Shoot/Root and seed vigour. The result of germination stress index (GSI) showed that salinity had significant different ($P<0.01$) on all of characters. Genotypes of Jannat in all the characters except ratio of Shoot/Root had higher resistance than other genotypes.

Key words*: st.johns wort, Germination, vigor, Salinity, seed.*

INTRODUCTION

If the value of salts existing in the environment of plant roots is to such extent that damages the germination, growth, development and fruiting of the plant, that environment (soil) is called as salty. These kinds of soils exist in dry and semidry regions of the country and various factors affected creation of which.

The salinity is considered as one of the major factors for reducing the crops performance. The salinity effects are not only referred to a special stage of plant growth, but are effective during the whole plant growth period, and finally lead to performance reduction. The most agricultural products even the species resistant to salinity are sensitive to salinity in germination stage and this problem avoids using resistant to salinity genotype.

The most common evaluation method of plants compatibility with the salinity environments is determining their viability and growth-ability in such environments and plant compatibility with the salinity in germination stage is deemed as the most important state. The most obvious effect of salinity on the crops is non-uniformity of seeds germination so that the resistance degree is different for various plants in this stage.

Increasing the salinity, the germination rate and rapidity of medicinal herbs' seeds is reduced like as the other corps and descending trend of germination due to the salinity increasing is most intensive than germination rate reductionB2X. In other study applied by Safarnezhad & Increasing the chloride sodium concentration, the rootlet and stemlet length of fennel is reducedB4X. Salinity had no effect on the resistant genotype germination of canola but in sensitive genotype, all studied traits were reduced and rootlet growth in all genotype less than stemlet growth was affected by the salinityB5X. The test results on sorghum confirmed the effect of chloride sodium on the germination, rootlet length, stemlet length, wet weight, abnormal sprout number, rapidity and power of germinationB6X. The cause of this effect as

reducing the water absorption by the plant and establishing facilities for input and absorption of ions to the extent of poisoningB1X. The cause of salinity effect on the germination reduction and growth of plant seeds' plantlet due to growth difference and wasting the photosynthesis of plant surfaceB2X.

Hypericum perforatum pertaining to Hypericaceae family, is a herbaceous and perennial plant which is grown at the margins of villages, mountains, farms, jungles and derelict areas and is reproduced in the nature through the seed. Floral branches of this plant have pharmaceutical virtue and evasive oily essence and about 5% to 7% of a type of glucoside named hyperion and a red material in the name of Hypericin has been specified particularly in its seed B5X.

The effort for breeding important medicinal herbs that in environment salinity conditions can have acceptable extracted performance and essence is very important. Therefore and towards these purposes, the extant study has been applied on 10 important genotypes of Hypericum perforatum of northern regions of Iran in relation to the resistance to salinity stresses in germination and plantlet establishment stage.

MATERIALS AND METHODS

The experiments used a factorial experiment based on randomized complete design with four replications at the laboratory of seed technology of Islamic Azad University Shahr-e-Rey, Iran in 2008. Ten genotypes of st.johns wort as first treatment included: Jannat Roodbar, Khalkhal, Arasbaran, Topaz, Gorgan-Derazno, Noshahr, Firoozkoh, Khalkhal-Asalem and Kordestan and four concentrations of salt as second treatment included: 0 (distilled water as control), 100, 200 and 300 millimolar (Na Cl).

All the seeds were surface-sterilized by fungicides of Vitavax (0.002) . Twenty-five seeds of each test genotypes wereplaced in separate Petri dishes that contained 2 mL ofthe salty liquid or distilled water.

Variance analysis of data was performed by SAS ver.9.2 Mean comparisons were carried out by Duncan's multiple range test at 0.01 probability levels.

RESULTS AND DISCUSSION

- Germination percentage: the resulted of this test indicated that a significant effect between the genotype and variety interaction in salinity so that the maximum germination rate referred to Jannat equal to 16.67% and the minimum rate referred to Kurdistan variety equal to 6.66%. also, regarding the interaction between species and salinity it was concluded that Gorgan variety has the maximum germination rate equal to 13.21 in salt concentration equal to 0 (control) and the minimum germination rate equal to 5.71 in salt concentration equal to 100 (table 1 & 2). The high value of chloride sodium concentration caused to germination rate reduction because upon salinity increasing, the water absorption by the seed is reduced and the salt may deter some enzymes that have critical effect on seed germinationB3X.
- Germination rate: variance analysis table indicates that a significant effect existed on the species as respect to the salinity on the germination rapidity and comparing the mean values of germination clears this difference (table 1 & 2), accordingly, the genotype positioned in six groups; the maximum germination rate was related to Jannat equal to 30.60% and the minimum was related to Kurdistan equal to 4.59%. variety interaction in salinity indicated that in two 0 (control) and 100 concentrations of salt, the variety had the maximum and minimum germination rapidity. The salinity stress increase the soil osmotic pressure and reduce the water absorption by the seed and leads to seed's water stress, also low and delayed germination is due to the toxic ionsB7X.

Rootlet length: the salinity has had significant effect on rootlet growth and upraising its length (table 1 & 2), so that Jannat variety equal to 14.90% and Noshahr variety equal to 3.72% were respectively the longest and shortest length of rootlet and the other species categorized between them.

- Stemlet length: the results of variance analysis indicate that as regard to the longitudinal growth of the stemlet, the tested genotype had significant difference with each other within the range of 1% (table 1) so that the longest value of stemlet longitudinal growth related to Arasbaran variety equal to 7.86mm and the shortest related to Khalkhal variety equal to 4.68mm (table 2). Disorder of stemlet growth and destruction of photosynthetic surface leads to such reduction.
- Seed vigor: the results indicated that the salinity had significant effect on the seed vigor within range of 1% (table 1) so that this effect is observing for the genotype in table 2. The highest rate of seed vigor equal to 3.80 is related to Jannat variety and the lowest equal to 0.90% is related to Firoozkouh variety.
- Stemlet to rootlet ratio: the effect of the salinity was significant on the ratio of stemlet to rootlet of the genotype within the range of 1% (table 1). In different applied salinity concentrations, Arasbaran variety equal to 2.47 and Jannat equal to 0.61 had the maximum and minimum value of this ratio (table 2). The number of chickpea's leaf, stalk and root is reduced upon salinity increasing and the reduction of stalk seiz is more than root sizeB3X.
- The salinity reduces the stalk to root ratio in some plants such as barley because of disorder in cells actions and reduction of physiologic processesB7X.
- Plantlet length: the results of tables 1 and 2 indicate that concurrent to chloride sodium increasing, the plantlet growth rate has been reduced. Transfer of toxic ions to the shoots and disorder of necessary nutrient transport avoids the production of new dry material and plantlet growth reduction, this reduction is due to the lack of photosynthesis and increasing the breath that leads to the plant growth disordering B4X.

Table 1: Analysis of variance for effect of salt stress on some traits of ten genotype st.johns wort seeds by Duncan test

Plantlet length	rootlet/ stemlet	Seed vigor	Stemlet length	Rootlet length	Germination rate	Germination percentage	d.f	S.O.V
147.98**	32.64**	10.68**	26.44**	95.64**	740.13**	182.62**	9	varity(A)
5380.80**	30.78**	117.75**	963.78**	1794.35**	6411.62**	1718.94**	1	Salt stress (B)
46.66**	01.58**	04.87**	09.23**	36.23**	200.34**	4.06**	7	Interaction A*B
5.64	0.361	0.248	1.82	6.60	6.82	3.77	-	Error
19.19	31.43	27.99	22.09	20.08	20.18	18.53	-	C.V

Ns (Non significant), * (Significant at 5%) and **, 1% (Significant at 1%) levels

Table 2: Mean comparison for effect of salt stress on some traits of ten genotype st.johns wort seeds

Plantlet length	rootlet/ stemlet	Seed vigor	Stemlet length	Rootlet length	Germination rate	Germination percentage	**genotype**
22.37a	0.61f	3.80a	7.47a	14.90a	3.60a	16.67a	Jannat Roodbar
12.4cd	2.2ab	1.97c	7.21a	5.18cde	14.96c	14.72b	Khalkhal
14.27bc	2.47a	2.68b	7.86a	6.40cd	23.39b	13.16bc	Arasbaran
14.68b	0.91ef	2.30bc	5.47bc	9.20b	10.77d	11.89cd	Topaz
9.11f	1.36cde	1.45d	4.92c	4.17de	12.98c	10.82d	Gorgan-Derazno
9.85de	1.75bcd	0.97e	6.12b	3.72e	8.94de	8.12e	Noshahr
9.15f	1.39cde	0.9e	4.86c	4.28cde	4.65f	7.73ef	Firoozkoh
11.27de	1.28de	1.21de	4.68c	6.57c	7.44e	7.18ef	Khalkhal-Asalem
11.05def	1.32cde	1de	5.25bc	5.80cde	4.59f	6.66ef	Kordestan
11.96d	1.86bc	1.06de	7.65a	4.30cde	7.72e	6.02f	Gorgan-Torkestan

In each column, means with similar letters do not differ significantly at 0.05 probability level

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FOREIGN TRADE TENDENCIES OF MEDICINAL, AROMATIC AND SPICE HERBS IN THE REPUBLIC OF SERBIA

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SUMMARY

Agricultural and food products have a significant role in the total foreign trade of the Republic of Serbia. After the changes in 2000 there was a liberalization of foreign trade and increased foreign competition at domestic market. Exports of agricultural products have been increased and there has been a surplus of trade.

Research of agricultural and food products' foreign trade is mainly concentrated on commodities with the highest export value. By contrast, this paper analyzes the commodity exchange of medicinal, aromatic and spice herbs. In the analyses the selected indicators of foreign trade have been used, such as unit values and revealed comparative advantage, based on the official trade data for the period from 2000 to 2010. Using those indicators, international comparisons are possible.

The analysis results show that in the mentioned period there were significant changes in the trade volume, unit value and comparative advantages. Based on the obtained results it can be concluded that there is a developing trend in foreign trade of medicinal, aromatic and spice herbs.

***Key words*:** *medicinal, aromatic and spice plants, foreign trade, Republic of Serbia.*

INTRODUCTION

Medicinal, aromatic and spice herbs have an important role in national culture and tradition in Serbia. Due to favourable climatic conditions, soil quality and unpolluted environment there are favourable natural conditions for wild plants and for growing.

In the Republic of Serbia there is a high diversity of flora with 3272 species and 390 subspecies. The diversity of flora applies to wild plants as well, which occurs at very different natural habitats, from wetlands to hilly mountainous areas [1]. The medicinal, aromatic and spice plants are considered over 700 species, of which 420 registered and 279 could be found on the market [2, 3].

Due to increased demand for these herbs in the last period, besides collecting wild plants, there are increasingly developing farm productions. It is estimated that the medicinal, aromatic and spice plants grown in Serbia every year an area of about 1550 ha, with significant deviations of sown area. The largest area planted was in 2002 and it was on 1832 ha, and minimum was in 2006 and amounted 1211 ha [4].

A maximum amount of herbs that can be collected in nature is determined each year. Institute for Nature Protection defines quotas for each species for each year. On the basis of the quotas collecting in each region is done. Thus, for example, in 2010 the amount of dried plants collected totalled 16 000 tonnes [8].

Previous studies of foreign trade of medicinal, aromatic and spice herbs are mainly focused on the total trade. In this paper, there is a more detailed analysis of this group of plants. It is important to emphasize that there is no single definition of medicinal aromatic and spice herbs, as no clear legal guidelines of what this term means. This statement applies to the

Republic of Serbia as well as the EU. The basic dilemma is the classification of this commodity, since these cultures can be treated like food supplements, but elsewhere like drugs, pharmaceutical [5]. It is known that herbal spices are used to improve taste, aroma, appearance, digestibility and utilization of food [6].
In this paper, the definitions are used according to the Customs Tariff from the year 2011 [7] and includes parts of chapter 9 and 12 of the Customs Tariff. From chapter 9 are included tea, maté and spices. This chapter includes products and mixtures in which resulting mixtures retain the essential character of the goods of those headings. Chapter 12 includes, among other, seeds and fruit; industrial or medicinal plants.
The central hypothesis of this paper is observation that there are positive changes in foreign trade. The research aims to show important changes in trade with this group of products based on selected indicators.

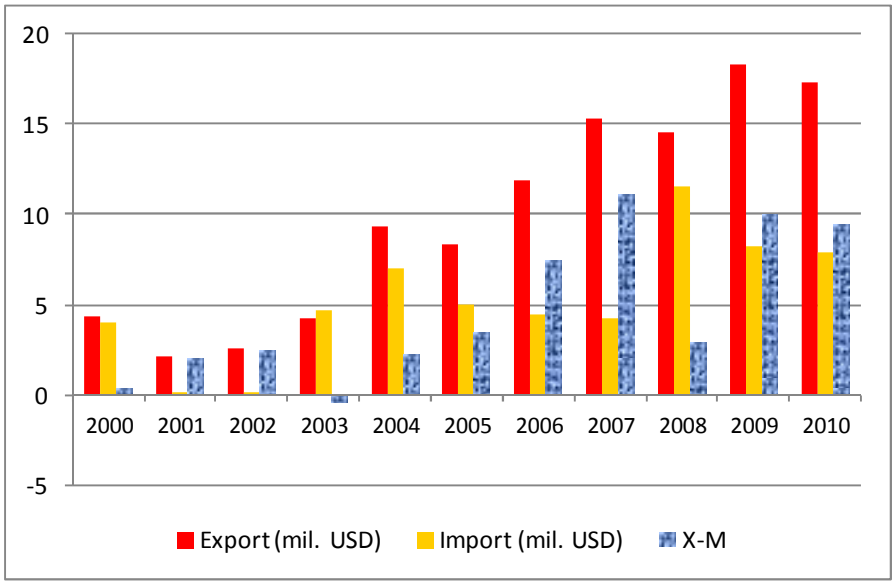
MATERIAL & METHODS

For the purposes of this study data are obtained from the Ministry of Agriculture, Trade, Forestry and Water Management on the basis of the Customs Tariff for the period from 2000 - 2010, and Chamber of commerce and industry of Serbia. In this paper is applied descriptive statistics and indicators used through the unit value and revealed competitive advantages.

RESULTS & DISCUSSION

The total volume of export of medicinal, aromatic and spice plants tends to increase and reached a value of $ 18 million in 2010. At the same time import also recorded increase in average in observed period, amounted 5 million USD.

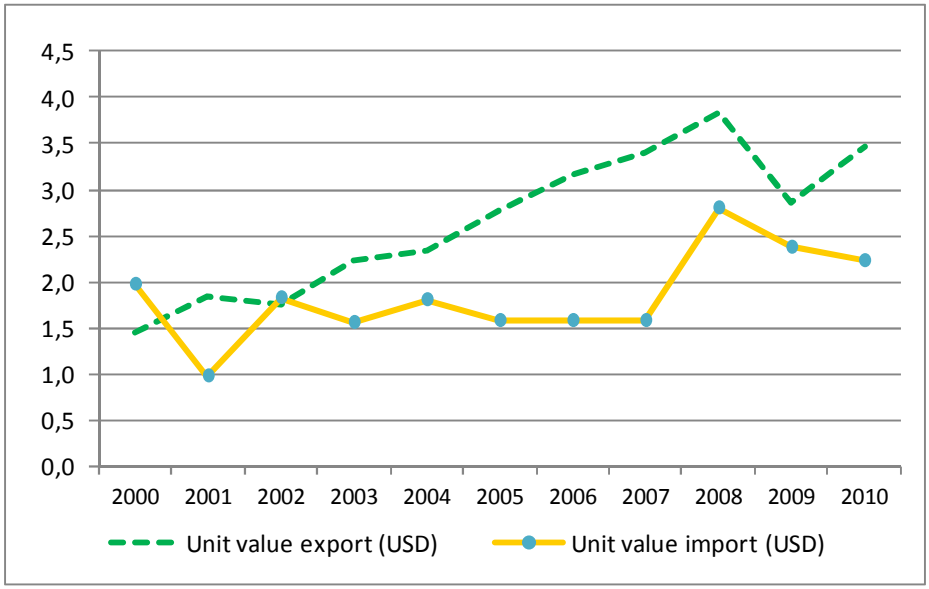
Chart 1: Foreign trade of the Republic of Serbia of medicinal, aromatic and spice herbs



Source: Ministry of Agriculture, Trade, Forestry and Water Management

Unit value of export is higher than the unit value of import, which suggests that the terms of trade of this group are improved. In 2010 the unit value of export amounted to 3.5 and of import 2.2 €/kg. The increase in export unit value tells us that likely here is an increase in product quality or Serbian traders are skilful.

Chart 2: Unit value of export and import (2000-2010)

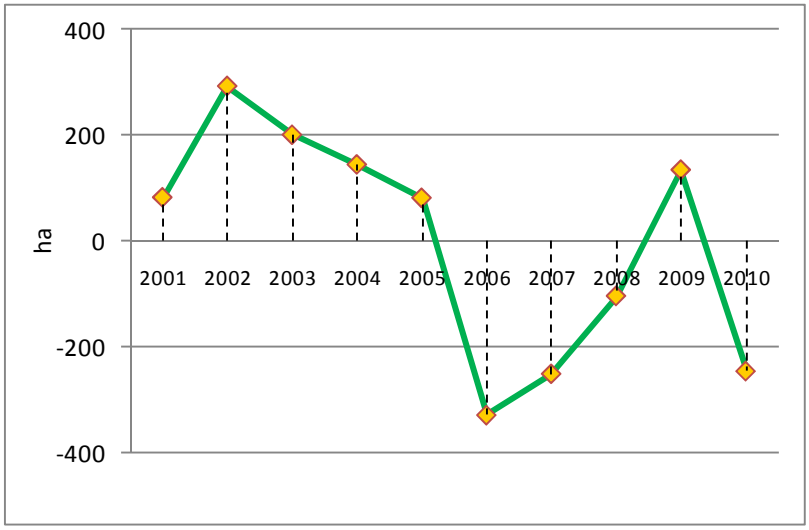


Source: Ministry of Agriculture, Trade, Forestry and Water Management

If we consider the vertical chain of trade which involves collector or producer of medicinal herbs, wholesaler, retailer and exporter, it is obvious that there are significant price differences at different levels of trade. As expected, the lowest share in the value chain belongs to collector or producer. On average they get about 0.99 € / kg for dried herbs. This price is dried plant material on the gate of warehouse or collecting centre.
Wholesale price is 2.5 times to 4.5 times higher than prices gained by collectors or producers. In export traders usually achieve about 20% lower prices than in case of selling on the domestic market. Consequently, their motivation is prior to the sale on the domestic market.
Revealed comparative advantage shows that in this period surplus share in the total volume of foreign trade had increased. This findings lead to the conclusion that there has been an international competitiveness growth of this product group. However, on the basis of this indicator it is not possible to conclude whether the source of international competitiveness improvement is result of price growth or increased products qualities.

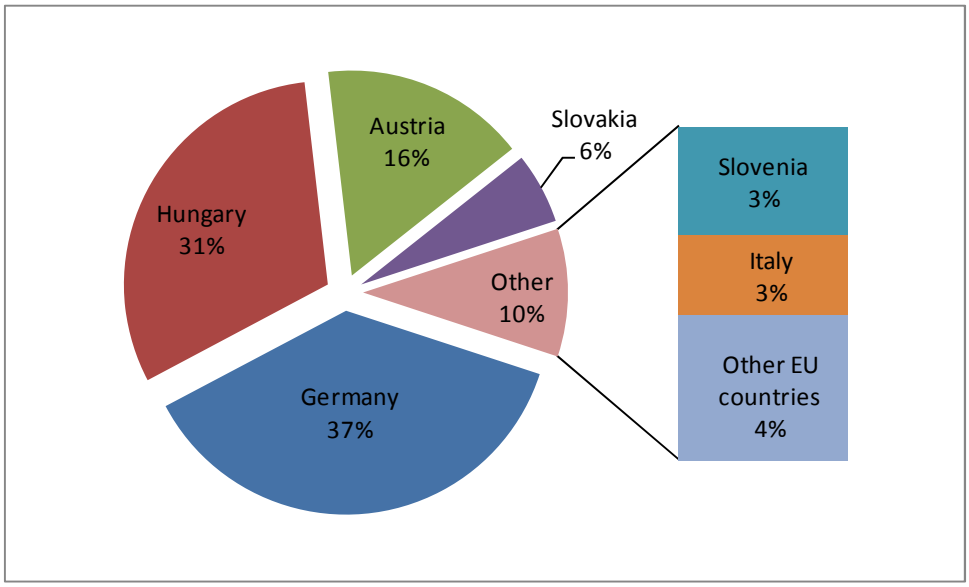
Production of this group of plants is not a subject of state restrictions. The annual production of dried herbs amounts about 4000 tons. Changes in planted area are not result of change in demand but in the supply and the value chain.
The largest area was sown in 2002 and then decreased by nearly one-third and reached a minimum in 2006. Increasing the area lasted for three years, and then decreased again in 2010. It seems that the biggest obstacle for increasing production is to find in lack knowledge of foreign customers' requirements, compliance with quality systems and the achievement of appropriate standards.

Chart 3: Yearly changes of sown area of medicinal, aromatic and spice herbs



Source: Ministry of Agriculture, Trade, Forestry and Water Management

Figure 1: Export of medicinal, aromatic and spice herbs from Serbia into EU countries



Source: Author's own research, unpublished

The most important export market for this product group is the European Union, in which the most important buyers are Germany, Hungary, Austria and Slovakia. Other countries have a share, which are individually less than 5% [4]. The EU market is the most dynamic world market, which annually imports about 120 000 tonnes, or some 200 million USD.
The relative importance of Serbia's total import of this product group in the EU is small and ranges between 0.4% and 2.0%. The main Serbian competitors in the South East Europe are Bulgaria, Poland, Hungary and Albania [9]. The main exporters to the EU market, measured as a share of total EU imports, are the USA (15.8%), India (8.0%), China (7.5%), Bulgaria (6.5%) and Egypt (5.5%).

CONCLUSION

In the Republic of Serbia, medicinal, aromatic plants and herbs play an important role in culture and tradition. The favourable climatic conditions, soil quality and unpolluted environment provide favourable conditions for different flora.

The total production consists of the collection of plants in nature and production on farms. The quantities that can be collected in nature are limited by quotas, while farm productions have a cyclical character.

This paper deals with the foreign trade of this group of products based on data from the Ministry of Agriculture, Trade, Forestry and Water Management. Coverage of products was determined on the basis of the Customs Tariff of the Republic of Serbia.

Research has confirmed the main hypothesis that there were positive changes in foreign trade, as terms of trade are improved. Unit value of exports is greater than the unit value of imports which indicates the quality of export products.

In the vertical chain the highest added value belongs to exporters and retailers, followed by wholesalers and processors, while persons involved in plant collecting have the smallest share. The most important export market is the EU, while Serbia is the marginal supplier to the EU, because Serbian exports accounts for less than two percent to the EU imports of this product group.

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ANALYZING CHEMICAL COMPOSITION OF ESSENTIAL OILS FROM THE AERIAL PARTS AND ECOLOGICAL PROPERTIES OF MEDICINAL PLANT *STACHYS INFLATE* BENTH

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ABSTRACT

Stachys inflate plant is a medicinal species distributed in the regions including Northern and Central Alborz, surrounding Tehran and Karaj, Qazvin, Qom, Isfahan, Tabriz, Uromieh, Hamadan and Shiraz. *Stachys* genus plants are used for treating the dermal injuries, kidney and liver diseases, sedative and reducing the blood pressure as well as are used as analgesic and anti-inflammation in traditional medicine. This study aims to investigate the chemical Composition of Essential Oils From The Aerial Parts of Medicinal plant and ecological properties this plant with scientific name of *Stachys inflate* from Lamiaceae. The aerial parts were hydrodistilled using a Clevenger-type apparatus. The essential oil was analyzed by GC and GC/MS. The habitats of this plant investigated included: slop percentage, geographical direction, soil texture, climate, average annual precipitation, mean temperature, as well as studying the geographical coordinate of related habitat and sea level using GPS and also the medicinal properties of above species was studied using literatures. Fifty three constituents were found representing 91.01% of the essential oil. The main constituents of the essential oil were α-pinene (19.02%), δ-3-Carene (10.18%), Limonene (8.27%), Spathulenol (6.13%) and germacrene-D (5.31%) respectively. The oil was characterized by large amounts of monoterpene hydrocarbons (63.17%) and oxygenated sesquiterpenes (19.46%).Other components of this plant comprised included sesquiterpenes hydrocarbons (11.37%), oxygenated monoterpenes (2.76%), and non-terpenoid components (1.24%). This plant represents dry and cold climate, in the sandy-silt soil beds, slop of 30-60%, western slop direction, height range of 1800 and 1600 m from sea level, average precipitation of 229.4mm and maximum and minimum mean annual temperature of 4.2 and 22.9°C. Based on therapeutic properties, the part of plant is used include its flowering shoots; therefore scientific studies indicated that this plant comes with advantages like: anti-inflammation, anti-microbial, and analgesic, and is used for treating respiratory diseases and arthritis. Other species of this genus also have medicinal properties as indicated to their properties. Therefore, due to the importance of this species based on its medicinal properties, further studies needed for recognizing its ecological properties and chemical components.

Keywords*: chemical composition, ecological, medicinal species, essential oils, Lamiaceae ,Stachys inflate*

INTRODUCTION

Among flora of Iran, there are more than 7500 plant species most of them comprised plant called herbs. Using flavoring plants in Iran has long history and due to their role in the medicines, essences play important role. Therefore recognizing and studying the ecology of herbs, industrial and flavoring plants depending on the climate- ecological conditions of any regions enjoys different species variety and according to the importance of such plant, there is needed to conduct general studies on different ecological facets.

Plant *Stachys inflate* from genus *Stachys* and family Lamiaceae. Most dispersion of this plan is in Europe and North America. In Middle Asia, Western South Asia like Middle East, there can be seen different species. Genus Stachys in Iran come with 34 species growing dispersedly in most points through Iran[4]. this species and other species of this genus have also pharmaceutical effects some of them indicated as below[Table1] ,[3, 5, 9, 10]:

Table1: Plant scientific name and Medicinal features and application

Scientific name	Pharmaceutical properties and its application
S.Sylvatica	Wound healing, treating the pains and abdominal spasms, disinfectant particularly in urinary ducts, anti-fever, anti-oxidant effects
S.Candida	Anti-inflammatory effects, anti-microbial effects
S.Chrysantha	Anti-inflammatory effects
S.inflata	Breath diseases, arthritis, anti-inflammatory effects
S.scardica	Anti-microbial effects
S.germanica	Anti-microbial effects
S.euboica	Anti-microbial and anti- fungi effects
S.recta	Wound healing, anti-inflammatory and anti-fungi effects
S.menthifolia	Anti-microbial and anti-fungi effects
S.cretica	Anti-microbial and anti-fungi effects
S.palustris	Wound healing, treating the pains, and abdominal spasms , disinfection particularly in urinary ducts, anti-fever
S.mucronata	Wound healing, anti-diarrhea, anti-inflammatory effects
S.lavandulifolia	Treating the gastrointestinal pains

Botanical Features: It is a reliable plant in the wooden based heighted to 15-40cm with simple or a little branched, white colored, hairy branches with fragile branches. Its leaves have almost no petiole and are long and linear with 1-2cm in length and in flowery branches are shorter than calyx. The flower of this plant has a red goblet coated with silky fibers in its end and its tubular part hidden in calyx. This plant has white hairy calyxes with short and a little sharp teeth with a ovule appearance and 1cm in length [6].
Terminology: Stachys in European encyclopedia, Chistetes, means cleaner and improver of sours indicating the extended usage of essence and or extract of species of such genus as antiseptic and treating the skin diseases [7].

MATERIAL & METHODS

Collecting the studied plant: For recognizing the habitat factors and lab studies for knowing the components of herb essential oil, there have been conducted field studies. In this habitat, the related species was collected. Habitat factors of this species include:
Slop percentage, geographical direction, soil texture, climate, average annual precipitation, mean temperature, as well as determining the geographical coordinates and height from the sea by GPS device. And pharmaceutical properties of this species were studied using related literatures. In the related habitat, the soil sample was prepared based on the soil horizons and its rooting and sent to the lab for being analyzed. The climate situation of habitat was

extracted using statistics and data of synoptic stations in Karim Abad, Karaj. In order for preparing the essence, the plant sample was initially collected. For sampling from related plant, after recognizing the growing region and when the plant attained to its fruiting stage in related region, there was sampled.

Essence Extraction: Initially the shoot part of plants was dried under the shade. Then they were grinded by mill. Distillation by water and Clevenger-type device were used for extracting the essence. Initially 100gr of dry plant powder added to a balloon with capacity of 1lit and distilled water added to. Flavoring took long about 4 hours. Because for this plant there was extracted very little essential oil, so there was used 0.5cc of hexane for separating the essential oil in essence collecting tube of Clevenger-type device. For this reason, the efficiency of flavoring could not be calculated.

Recognizing the essence components: The resulted essential oil was studied by analytical gas chromatography (GC) and gas chromatography linked to mass spectrophotometer (GC/MS). In all spectra of GC/MS, Coats index was calculated for all peaks in by pattern of exiting the normal alkanes and spectra inhibition index and by comparing them with related resources and books they was interpreted by library- computer data of Wiley 725 and book Adams (2004) and other resources and the components of essence and its chemical formula was recognized [1,2].

RESULTS & DISCUSSION

Ecology of herb

Based on extracted data, it was determined that *Stachys inflate* is a plant from Lamiaceae and of genus Stachys. Genus Stachys in Iran come with 34 species growing dispersedly in most points through Iran. Geographical distribution of this herb, *Stachys inflate* is in northern and central Alborz, surrounding Tehran and Karaj, Qazvin, Qom, Isfahan, Tabriz, Uromieh, around Hamadan and Malayer, Shiraz and Ahvaz [6]. This plant is a representative of cold and dry climate growing on the bed of sandy- silt soils with slop of 30-60%, western slop direction, in the height of 1600 and 1800m sea level, with average precipitation of 229.4mm and mean max and min annual temperature of 22.9 and 4.2^{o}C.

2.Recognizing the chemical components: Essence resulted from fruited shoots of yellowish plant is very scarce, for this reason the flavoring efficiency may not be measured. Totally, there are 53 chemical components equal with 91.01% that have been recognized and reported together with quantitative percentage (GC) and Inhibition index (KI)[Table2].

Table2.Main chemical components of plant *Stachys inflate* Benth

Item	Component	GC%	KI
1	α- pinene	19.02	930
2	δ-3-Carene	10.18	1008
3	Limonene	8.27	1026
4	Spathulenol	6.13	1576
5	Germacrene-D	5.31	1479

Main components comprising the plant essential oil include α- pinene (19.02%), δ-3-Carene (10.18%), limonene (8.27%), Spathulenol (6.13%), and Germacrene-D (5.31%). Among

them, most size of components is related to monoterpene hydrocarbons (63.17%) and oxygenated sesquiterpenes (19.46%). Other components include sesquiterpene hydrocarbons (11.37%), oxygenated monoterpenes (2.76%) and non-therpenoid components (1.24%).
Results of other studies as indicated below approve the main components extracted from *Stachys inflate:*
Sajadi et al (2004) analyzed essential oil extracted from *Stachys inflate* by GC/MS, GC. There were determined 39 components in the essence of *Stachys inflate* of most important of them include:germacrene-D(16.9%)،bicyclogermacrene(6.16%),α-Pinene(11.3%),β-hellandrene(9.8%) ، bicycloelemene(6.6%)، β-pinene(5.6%) , spathulenol(3.2%).
Morteza Semnani et al (2005) analyzed essential oil extracted from dried shoots of 4 species Stachys byzantina,s.inflata,s.laxa,s.lavandulifolia by GC/MS and GC.
The main components in species S.byzantina include ،piperitenon(9.9%)، 6,1014-trimethyl pentadecan-2-one (6.4%)، n-tricosane(6.4%) and main components of essential oil in species S.inflata include hexadecanoic acid(9.1%), germacrene-D(8.9%), α-pinene(5.8%), bicyclogermacrene (5.1%) and main components of essence in species S.lavandulifolia include , 4-hydroxy-4-methyl-2-pentanone(9.3%)، α-pinene(7.9%) and hexadecanoic acid(5.2%) and also the main components of essence extracted from S.laxa include ،germavrene-D(17/1%)، 4-hydroxy-4-methyl-2-pentanone(12.3%)، 7-epl- α-selinene(8.3%) ، bicyclogermacrene(6.7%)، β-caryophyllene(6.2%) و α-pinene(5.9%).
Noroozi Ersi et al (2006) analyzed the essential oil extracted from shoots of Stachys inflata by GC/MS and GC. There were recognized 51 components from essences extracted, most important of which include:α-pinene(15.21%)، δ-3-Carene(12.3%)، Limonene(11.6%)، β-pinene(7.25)، myrcene(6.5)، (z)-β-ocimene(5.9%)، germavrene-D(4.2%)، allo-ocimene(4.1%)، linalool(3.5%).
according to the results of this study and other studies, components such as α -pinen, germavrene-D, δ-3-Carene, limonene and spathulenol were present in species *Stachys inflata* but their percentage content were different and it was related to the ecological and climate conditions.

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