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Determination of D-saccharic acid-1,4-lactone (DSL) in fermentation tea (Kombucha) by capillary electrophoresis

Surapol Natakankitkul*[a], Panee Sirisa-ard[a], Sakunnee Bovonsombut[b], Chatchai Kitipornchai[b], Suwalee Kiatkarun[c]

[a]Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand 50200

[b]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200

[c]Five Star Herbal Gallery, 365/1 Moo1, Tambon Tha Sala, Muang, Chiang Mai, Thailand 50000

*surapol.nat@cmu.ac.th phone +66-53944342 fax +66-53222741

ABSTRACT

Kombucha or fermentation tea beverage produced from symbiosis of yeast species and acetic acid bacteria, is a popularly health tonic around the world today. D-saccharic acid-1,4-lactone (DSL), a component of kombucha, inhibits the activity of glucuronidase, known as an enzyme indirectly related with liver cancer. In this research, the tea fermentation was traditionally carried out by inoculating a previously grown culture into a freshly prepared tea decoction and incubated statically under aerobic conditions using 1.0% local tea from Northern Thailand, 10.0% sugarcane and 10.0% fungus broth at room temperature for 7-40 days, then filtered and pasteurization. After subsequent fermentation process procedure above, sampling was performed periodically in each jar. We founded various yeasts, acetic acid bacteria and lactic acid bacteria in floating cellulosic pellicle layer and the liquid broth after 7 days of fermentation. The ethanol content was determined by gas chromatography (GC) and Ebulliometer® was founded between 0.5-5.0% v/v. For safety of consumers, all samples were checked toxic substance such as methanol by using GC-MS. The total antioxidant of kombucha samples capacity was measured in terms of free radical–scavenging activity by the 1-diphenyl-2-picrylhydrazyl (DPPH) Radical decolorization method were between 0.221-0.512 mg gallic acid/ml and the total phenolic content was determined by the Folin-Ciocalteau method were between 0.291-0.854 mg gallic acid/ml. Using a capillary electrophoresis (CE) for the separation and determination of DSL in kombucha samples was carried out on CE System: PA 800 *plus* Pharmaceutical Analysis System (Beckman Coulter Inc., Brea, USA) with optimized conditions of 50 cm effective length capillary at a separating voltage of 30 kV in 40 mmol/l borax buffer (pH 6.5) containing 30 mmol/l SDS and 15% v/v methanol. The relationship between peak are and concentration of DSL was determined by UV absorption at wavelength 190 nm with the linear range of 25-200 µg/ml and a detection limit of 25 µg/ml. The electropherogram or fingerprint of the different fermented type's tea such as; green tea, oolong tea and black tea were established. We have successfully applied a simple CE method for quantitative evaluation of polyphenol or catechins and DSL in various fermented conditions and different kombucha products.

Keywords: D-saccharic acid-1,4-lactone, DSL, Fermentation tea, Kombucha, Capillary electrophoresis

1. INTRODUCTION

Kombucha, a fermentation tea beverage produced from a symbiosis of yeast species and acetic acid bacteria, has been favorite drinkand becoming increasingly popular around the world today [1]. The beverage has been claimed to be beneficial to human health [2–3]. Some studies have detected the presence of polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins and antibiotics in the brewed tea [4–5].

β-Glucuronidase is an enzyme responsible for hydrolyzing glucuronides in the lumen of the gut. This reaction generates toxic and carcinogenic substances. β-Glucuronidase promotes cancerous formations by hydrolysing conjugated glucuronides to carcinogenic aglyconic compounds and could be detoxified by glucuronide formation in the liver and then enter the bowel via bile. Thus, toxic aglycones are regenerated in situ in the bowel by bacterial β-Glucuronidase. d-Saccharic acid-1,4-lactone (DSL) is the competitive inhibitor of β-Glucuronidase[6]. DSL has been discovered in kombucha with varied concentrations and are considered to be the most healthful component in kombucha[7]. Effective identification and quantitative analysis of DSL in kombucha sample is of significance in explaining the beneficial effects of kombucha.In the past, several methods have been used in analyzing DSL and polyphenol components in brewed kombucha, such as; high-performance liquid chromatography (HPLC) [8], thin-layer chromatography (TLC) [9], mass spectrometry (MS) [10] and gas chromatography (GC) [11]. However, all these methods are required the complexities in sample pretreatment procedures. Up to today, there is onlyonereport on the determination and separation of DSL in brewed kombucha samples using CE [12]. In this paper, a simple and selective CE method is established for the separation and quantitative determination of DSL in our brewed kombucha.

2. MATERIALS AND METHODS

Materials

d-Saccharic acid-1,4-lactone monohydrate ($C_6H_8O_7 \cdot H_2O$, analytical grade, >99.7%) (DSL), of which the molecular structure was shown in Figure 1, was purchased from Sigma (Germany). Methanol (HPLC grade, >99.9% purity) and Sodium dodecyl sulphonate (SDS) were purchased from Merck, Darmstadt, Germany (analytical grade, >99.7%). Distilled, deionized water (Milli-Q Water Systems, Millipore Corporation, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals used were of analytical reagent grade. There were brewed kombucha samples from our laboratory (Lab of Microbiology, Faculty of Science, Chiang Mai University).

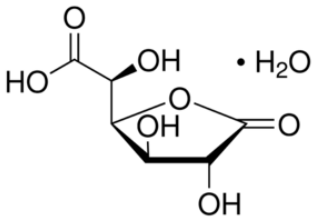

Figure 1. Chemical structure of d-saccharic acid-1,4-lactone monohydrate.

Instrumentation

All CE separations were conducted on a capillary electrophoresis system PA 800 *plus* Pharmaceutical Analysis System (Beckman Coulter Inc., Brea, USA) equipped with an autosampler and a DAD detector. The electrophoresis separation was performed on a fused-silica capillary of 75 μm ID (375 μm OD) × 50 cm effective length.

Cultivation method

The kombucha tea was prepared using kombucha culture obtained fromour laboratory. Tea samples were cultivated and received from Mae-sa-long highland, Chiang Rai, Thailand. Kombucha tea was prepared in a tea broth (1.0% w/v) supplemented with sucrose (10% w/v) by using our starter culture. Figure 2 showed the tea fungus in kombucha tea increased through 7 days of the fermentation. The subsequent fermentation process followed the procedure aboveup to 40 days. Sampling was performed periodically; each jar was sampled once only in order to avoid potential contamination. All analyses were carried out in duplicate.

Figure 2. Kombucha tea with tea fungus.

DSL Standard and Sample preparation

The DSL standard was prepared by quantitatively diluting the stock solution with pure water, and the stock solution was diluted to the desired concentrations just prior to use. Each kombucha sample was kept at −80 ºC. The samples were centrifuged at 10,000 rpm in 5 min. All samples were properly diluted, and duplicates were made.

Capillary electrophoresis

Running buffer was made up with 40 mM borax buffer ($Na_2B_4O_7$–KH_2PO_4), 30 mM SDS, 15% methanol (v/v), with pH of 6.52. Sample solutions, standard solutions and the running buffer were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to use. CE was performed at separation voltage of 30 kV with the running buffer. Sample injection was performed hydrodynamically for 10s. Capillary temperature was controlled at 25ºC and detection wavelength was set at 190 nm. The capillary was washed with 0.1 mol/L NaOH and pure water for 2 min, respectively, and then rinsed with running buffer for 5 min before the next run.

3. RESULTS AND DISCUSSION

Microscopic evaluation revealed that the tea fungus mainly contains various yeasts, acetic acid bacteria and lactic acid bacteria in floating cellulosic pellicle layer and the liquid broth after 7 days of fermentation. During the fermentation process, yeasts and bacteria metabolize sucrose into a number of organic acids, such as acetic acid and gluconic acid. Due to an increased concentration of these organic acids, the pH decreased from 5 to 2.5. The ethanol content was determined by gas chromatography (GC) and Ebulliometer® was founded between 0.5-5.0 %v/v. For safety of consumers, all samples were checked toxic substance such as methanol by using GC-MS. The total antioxidant of kombucha samples capacity was measured in terms of free radical–scavenging activity by the 1-diphenyl-2-picrylhydrazyl (DPPH) Radical decolorization method were between 0.221-0.512 mg gallic acid/mL and the total phenolic content was determined by the Folin-Ciocalteau method were between 0.291-0.854 mg gallic acid/mL. It should be mentioned that from the antioxidant perspective, green tea kombucha are, generally more effective than black tea kombucha due to the higher amount of polyphenols or catechins.

According to the procedure of experiment, DSL separation and quantitative determination of DSL in kombucha samples were investigated. Standard curves were established from serial dilution of the stock DSL solutions. The relationship between peak area and the amount of substance applied, in a specified working range with linear response, was determined by first-order polynomial regression over the range 25–200 μg/mL for DSL. The limit of detection (LOD) was at 25 μg/mL with a signal-to-noise ratio of 3 (S/N = 3). The regression equation was $y = 0.002x + 10.62$, where y is the peak area and x the concentration of standard DSL (μg/mL) ($n = 3$, $R^2 = 0.998$). The method of addition of standard DSL sample to kombucha was used to prove qualitatively the effective separation of DSL and to determine the exact retention time of the eluted DSL peak. Electropherogram of kombucha tea after 7 days of fermentationwas showed in Figure 3.Excellent baseline separation was obtained between DSL and other polyphenol components. Migration time for DSL was 14.6 min.

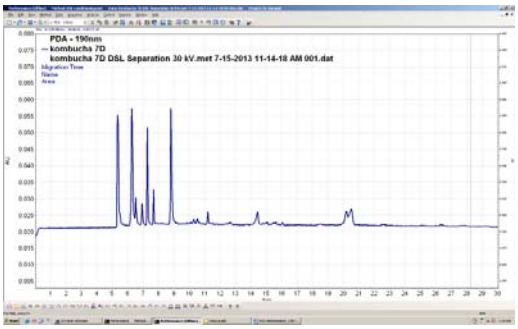

Figure 3. Electropherogramof kombucha tea after 7 days of fermentation.

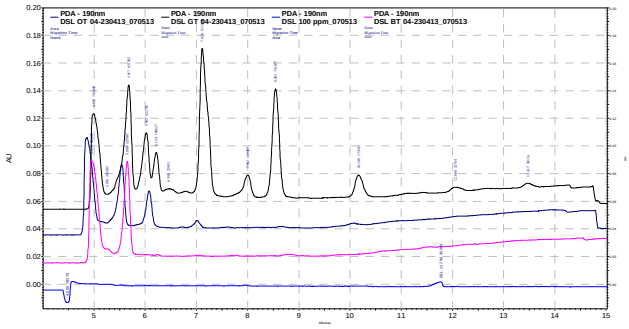

Figure 4. Electropherogram of green tea, oolong tea, black tea of kombucha samples and DSL standard.

In Figure 4, the electropherogram or fingerprint of the different fermented type's tea such as; green tea, oolong tea, black tea and DSL standard were established, from upper to lower, respectively.The separation performances under optimal conditions such as; pH, buffer, SDS concentration, and methanol concentration. In alkaline solutions (pH >8.0), the catechins or polyphenolswere unstable and completely degraded within a few minutes [13]. The stability of catechins was increased in acidic solutions, but the speed of analysis was reduced for the slower electroosmotic flow. The pH value of running buffer was therefore chosen in neutral region, pH 6.52. Borax-phosphate was added into the running buffer to improve the separation-based on its complexation with the hydroxyl groups of tea polyphenols [14]. The borate concentration was selected to be 40 mM for optimum peak resolution, theoretical plates and shorter migration times. The use of a surfactant (SDS) might increase the resolution between DSL and other similar molecules in sample and therefore the migration time because of the negative charge of sulfate group of the SDS. Higher SDS concentration (40–50 mM) did not improve the peak resolution, while the electrophoretic current and migration times of the analytes were remarkably increased. For shorter migration times, better peak resolutions and fewer joule-heatingproblems, SDS concentration was selected to be 30mM.Organic modifiers were usually used to improve the separation [15].On the other hand, an increase of concentration of organic solvent could enhance the solubility of DSL. Since organic solvents decrease the EOF and Joule's heat during electrophoresis, high voltage should be used to increase the resolution of electrophoresis. By adding methanol 15% (v/v), the resolution of polyphenols and DSL peaks were improved. With the optimized conditions, the kombucha samples can be analyzed within 15 min with a high theoretical plate number (>50,000) and a reduced electrophoretic current of about 30μA. More than 100 determinations were performed in the same capillary without significant changes in migration time, peak area and peak height. Compared with the running conditions of previous researches, the lower concentrations of SDS, phosphate and tetraborate buffer in this MEKC method were provided for a reduced the joule-heating problem and short the migration times of polyphenols and DSL[12,16-18]. Kombucha samples were determined by CE analysis; differences of DSL content between 25-75 μg/mL were shown in different kombucha samples after incubation periods. This difference may be due to the different origins of microbial strains and different incubation conditions [19].

4. CONCLUSIONS

The results of this paper showed that CE method for the separation and determination of DSL in kombucha samples was highly selective, reproducible and simple to use. The main DSL and polyphenol constituents can be well separated in a 50 cm length capillary at a separation voltage of 30 kV in a 40 mmol/L borax buffer (pH 6.52) containing 30 mmol/L SDS and 15% methanol (v/v).

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Proximate analysis, phytochemical screening, total phenolic and flavonoid content, and free radical scavenging activity of the Philippine bamboo "Buho" *Schizostachyum lumampao*

Jovale Vincent Tongco*[a], Ramon Razal[a], RemilAguda[b]
[a]Department of Forest Products and Paper Science, College of Forestry and Natural Resources, University of the Philippines Los Baños, College, Laguna, Philippines 4031
[b]Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños, College, Laguna, Philippines 4031

*jvvtongco@uplb.edu.ph phone +63-9263578337

ABSTRACT

This research is a pioneering attempt to undertake proximate analysis, phytochemical screening, total phenolic and flavonoid content determination, and quantification of the free radical scavenging activity of a native bamboo species *Schizostachyum lumampao*, locally known as buho in the Philippines. Preliminary phytochemical screening of endemic plants is very important in drug discovery. It will lead to potential sources of bioactive compounds for health and beauty, specifically phenolics and flavonoids which are natural antioxidants. Proximate analysis showed that the air-dried buho leaves contain 10% moisture, 30.5% ash, 22.1% crude protein, 1.6% crude fat, 28.7% crude fiber, and 7.2% total sugar (by difference). Qualitative phytochemical screening detected saponins, diterpenes, triterpenes, phenols, tannins, and flavonoids in both the ethanolic and aqueous leaf extracts, while phytosterols were only detected in the ethanolic extract. Using the Folin-Ciocalteu method, the total phenolic content in gallic acid equivalent (GAE) per 100 g dried sample, was 76.72 ± 9.06 for the ethanolic extract, and 13.48 ± 4.12 for the aqueous extract. The total flavonoid content in quercetin equivalent (QE), were 70.24 ± 7.52 and 17.86 ± 3.41 mg QE/100g dried sample for the ethanolic and aqueous extracts, respectively. The antioxidant activity of different concentrations of buho ethanolic extract was determined using the DOPH (2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl) free radical scavenging activity (FRSA) assay. The %FRSA values for the ethanolic extracts were 51.27 ± 1.50, 52.33 ± 3.00, 54.98 ± 0.75, 56.57 ± 1.50 and 50.21 ± 0.00 for 10, 50, 100, 200 and 400 ppm, respectively. These initial results suggest the potential of buho as a natural health and nutrition supplement.

Keywords: bamboo, Phenolics, Flavonoids, Proximate analysis, Phytochemical screening, Antioxidant activity

1. INTRODUCTION

The culms of bamboo have been widely used in Asia, Africa and Latin America as a construction material because of its high strength and versatility. However, the bamboo leaves are discarded or burned as fuel after separation from the culm. Ancient Indians and Chinese have used dried bamboo leaves as tea. Bamboo leaves are reported to contain polyphenols, which are secondary metabolites widely distributed throughout the plant [1]. Phenolics account for the antioxidant activity of the extracts of bamboo leaves as shown by previous studies [2]. These compounds showed significant inhibitory effects on superoxide radical, hydroxyl radical, DPPH radical, and ferrous metal-chelating capacities. Bamboo extracts also exhibited potential antihypertensive properties [3]. This points to the need for the chemical characterization (e.g., proximate analysis) and qualitative identification of phenolics and other bioactive phytochemicals in Philippine species of bamboo before conducting assays on their biological activity, such as antioxidant activity in living systems, for possible use as food supplement with potential health benefits.

Thus far, we have found no evidence in the literature on the screening of possible important bioactive phytochemicals and other novel compounds from the leaves of the endemic species [4], *Schizostachyum lumampao* locally known as "buho" in the Philippines. Thus, the present study is a pioneering attempt to characterize and quantify phytochemicals present in Philippine bamboo.

2. MATERIALS AND METHODS

Sample collection and preparation

Schizostachyum lumampao (buho) bamboo leaves were collected from the Bambusetum of the Ecosystems Research and Development Bureau (ERDB) of the Philippine Department of Environment and Natural Resources (DENR), located in the Mt. Makiling Forest Reserve, Los Baños, Laguna, Philippines. All bamboo species in the ERDB Bambusetum are properly labeled and identified by plant taxonomists. The study was conducted from August 2013 to October 2013 at the Institute of Chemistry, College of Arts and Sciences, University of the Philippines Los Baños and at the Wood Chemistry Laboratory, Department of Forest Products and Paper Science, College of Forestry and Natural Resources, University of the Philippines Los Baños. The bamboo leaves were washed and air-dried at room temperature for at least five days prior to solvent extraction.

Proximate analysis

Air-dried bamboo leaves were analyzed for their moisture, ash, crude fat, crude fiber and crude protein content using standard analytical methods [5].

Solvent extraction

Two solvents were used for extraction: water and ethanol. The bamboo leaves were ground using a blender. For ethanol extraction, the air-dried powdered bamboo leaves were mixed with 80% ethanol in water for 24 hours with frequent agitation. After ethanol extraction, the solution was concentrated by evaporating ethanol under reduced pressure. Water extraction was done by boiling air-dried powdered bamboo material in distilled water for 15 minutes and then cooled.

Phytochemical screening

The extracts were tested for the presence of certain phytochemicals. The procedure of Harborne and Tiwari were adopted for the detection of alkaloids, reducing sugars, cardiac glycosides, anthranol glycosides, cyanogenic glycosides, saponins, diterpenes, triterpenes, phenols, phytosterols, tannins, flavonoids, amino acids, and proteins [6,7].

Total phenolic and flavonoid content

The total phenolic and flavonoid content of the ethanolic and aqueous extract was determined using the Folin-Ciocalteu assay [8].

Free-radical scavenging activity (% FRSA) of ethanolic extract

The ethanolic extract of buho was subjected to DOPH (2,2-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl) free radical scavenging activity (FRSA) assay. The procedure of Burda and Oleszek [9] with slight modifications was employed for the free radical scavenging property of buhoethanolic extract. Gallic acid was used as standard.

3. RESULTS

Proximate analysis

The proximate analysis of buho shows a high amount of ash, crude fiber, and crude protein. The ash content, which is equivalent to the amount of inorganics in the leaves, is about 30%. The % total sugar was obtained by difference. The values for the proximate analysis of the leaves (reported as mean ± sd, n=3) are as follows: 9.99 ± 0.056 % moisture, 30.49 ± 0.58 % ash, 22.10 ± 1.26 % crude protein, 1.56 ± 0.97 % crude fat, 28.65 ± 0.09 % crude fiber, and 7.21 ± 0.79 % total sugar.

Phytochemical screening

Qualitative phytochemical screening methods detected the presence of particular phytochemicals in the ethanolic and water extracts. Natural products belonging to saponins, diterpenes, triterpenes, phenols, tannins, and flavonoids were shown to be present in both the ethanolic and aqueous extracts. However, phytosterols were only found in the ethanolic extract (Table 1).

Table 1. Qualitative phytochemical screening of ethanolic and aqueous extracts of Philippine bamboo, *Schizostachyum lumampao*, leaves.

Phytochemical test	ethanolic extract	water extract
Alkaloids (Wagner's test)	-	-
Carbohydrates - Reducing Sugars (Benedict's test)	-	-
Cardiac glycosides (Legal's test)	-	-
Anthranol glycosides (Modified Borntrager's test)	-	-
Cyanogenic glycosides (Picrate paper test)	-	-
Saponins (Froth test)	+	++
Diterpenes (Copper acetate test)	++	+
Triterpenes (Salkowski's test)	+	+
Phenols (Ferric chloride test)	++	+
Phytosterols (Liebermann - Burchard's test)	+	-
Tannins (Gelatin test)	+	+
Flavonoids (Alkaline reagent test)	+	+
Amino acids (Ninhydrin test)	-	-
Proteins (Nitric acid test)	-	-

(-) not detected/present (+) present in low amounts
(++) present in high amounts

Total phenolic and flavonoid Content

The determination of the total phenolic and flavonoid contents of buho leaves was undertaken by adding Folin-Ciocalteu reagent (FCR) to the sample and then measuring the absorbance of the treated sample at 725 nm through UV-Vis spectrophotometry (Table 2). The concentrations of the phenolic and flavonoid extracts were determined from a standard curve of FCR-treated solutions with known gallic acid or quercetin concentrations.

Table 2. Total phenolic and flavonoid content of *Schizostachyum lumampao* leaf extracts

Chemical test	ethanolic extract	water extract
Phenolic Content (mg GAE/100g air-dried sample)	76.72 ± 9.06	13.48 ± 4.12
Flavonoid Content (mg QE/100g air-dried sample)	70.24 ± 7.52	17.86 ± 3.42

Antioxidant activity (% FRSA)

The free radical scavenging activity of buho was undertaken by reacting various concentrations of ethanolic extracts with DOPH. The absorbance at 531 nm of resulting solutions after incubation in the dark for 30 minutes was obtained via UV-Vis spectrophotometry. The DOPH %FRSA values obtained for the resulting solutions (Table 3) were recorded and compared to the gallic acid standard. The equation below (eq. 1) was used to calculate for the %FRSA values from the absorbance values obtained from UV-Vis spectrometry readings.

$$\%FRSA = \left(1 - \frac{Abs_{sample}}{Abs_{ctrl}}\right) x\,100 \qquad \text{(eq. 1)}$$

Table 3. Total phenolic and flavonoid content of *Schizostachyum lumampao* leaf extracts.

Concentration, ppm	% FRSA of gallic acid Standard (n=1)	% FRSA of ethanolic extract (n=2)
10	56.57	51.27 ± 1.50
50	59.75	52.33 ± 3.00
100	67.16	54.98 ± 0.75
200	70.34	56.57 ± 1.50
400	73.52	50.21 ± 0.00

4. CONCLUSIONS

The moisture, ash, crude protein, crude fat, crude fiber, and total sugar content (proximate analysis) of *Schizostachyum lumampao* leaves were determined by using standard analytical methods. Phytochemical screening qualitatively assessed the phytochemicals present in *S. lumampao* leaves. The *S. lumampao* leaf extracts were negative for toxic compounds such as cardiac and cyanogenic glycosides, which means that the *S. lumampao* leaves are safe for human consumption and possesses great promise as herbal tea. Phenolics in the buhoethanolic extract were shown to be good free radical scavengers, making the bamboo a potent natural antioxidant supplement. Further studies are being done especially the determination of bioactivity of the specific phytochemicals in related Philippine bamboo species.

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The compositions of omega-3 and omega-6 polyunsaturated fatty acid in seven sea cucumbers species

Sabreena Safuan*[a]
[a]Department of Biomedical Sciences, School of Health Sciences, University Science Malaysia, Kubang Kerian, Kelantan

*sabreena@kk.usm.my phone +60-97677823 fax +60-97677515

ABSTRACT

Sea cucumbers have been used in many communities worldwide because of its various medicinal potential. Despite the widespread used, research of this holothuroid is scarce and lots of bioactive substances of sea cucumber believed to facilitate health and prevent diseases are yet to be identified. Polyunsaturated fatty acids of the class omega-3 and omega-6 play an active role in inflammation and wound healing in which these sea cucumbers extract is widely used for. Therefore, the objective of this study is to determine the fatty acids composition of seven species of sea cucumbers namely *Stichopus hermanii*, *Stichopus chloronotus*, *Stichopus badionotus*, *Holothuria atra*, *Holothuria tubulosa* and two *Molpadiia* sp. using gas chromatography. Recorded values were expressed as mean ± standard deviation. Analysis of variances (ANOVA) was used to determine statistical significant. All species show major differences in their polyunsaturated fatty acid compositions. *Holothuria atra* showed the highest percentage area of linoleic and linolenic acids which was 49.25 ± 2.73% and 23.16 ± 2.83% respectively. *Stichopus hermanii* showed the highest percentage area of arachidonic acid which was 26.73 ± 1.84%. Interestingly, the fatty acids composition of the two *Molpadiia* sp. taken from two different locations showed a significant different in term of their fatty acid profile. On the basis of this study, sea cucumbers could be used as a source of omega-3 and omega-6 fatty acids. The fatty acid compositions of *Molpadiia* sp. could be used as an indicator of scientific differentiation and classification of species.

Keywords: Polyunsaturated fatty acids, Omega-3, Omega-6, Sea cucumber

1. INTRODUCTION

Sea cucumber was used worldwide as a source of medicine and food since 300 years ago. They are well known for their ability to facilitate wound healing, reduce irritations and their ability to relieve arthritis, asthma and hypertension. Extract of sea cucumbers was reported to mend internal wounds after surgery, caesarean birth and normal labor [1]. The ability of a sea cucumber to regenerate and heal itself upon cutting proves its potentiality in medicine and healing.

Polyunsaturated fatty acids of the class omega-3 and omega-6 fatty acids are bioactive components believed to facilitate health and prevent diseases. Omega-3 fatty acids are linolenic acid (LNA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) while omega-6 fatty acids are linoleic acid (LA) and arachidonic acid (AA). Both LNA and LA are essential fatty acids which act as precursors for AA, EPA and DHA. The important functions of both polyunsaturated fatty acids in inflammation, thrombus formation, and cell-cell communication have taken center stage and lead to intervention studies in coronary heart diseases [2], hypertension [3], diabetes [4] and various autoimmune diseases [5].

Despite the traditional use of sea cucumber in medicine and healing, knowledge on the fatty acids profile of sea cucumbers was scare and not as established. Therefore, this study aims to determine the fatty acid profile of sea cucumbers species especially in term of omega-3 and omega-6 fatty acids. It is hoped that the medicinal value of these species will be enhanced if their fatty acids content are established.

2. MATERIALS AND METHODS

Sample collection

Fresh samples of *Stichopus hermanii*, *Stichopus chloronotus*, *Stichopus badionotus*, *Holothuria atra* and *Holothuria tubulosa* were collected off the coastal area of Terengganu, Malaysia. *Molpadiia* sp. was collected from two different areas; Langkawi Island and Port Dickson, Malaysia. All samples were separated according to their species, kept in plastic bags and stored at -80 ºC until further used.

Preparation of sea cucumber extract

Sea cucumbers were defrosted and the visceral organs and body fluid were removed. Only the body wall was used in this research. The sea cucumbers were then blended and homogenized until standard uniform phase achieved.

The lipid extract preparation was modified based on the Bligh and Dyer (1959) method [6]. 100 g of wet tissue were homogenized for 4 minutes in 100 ml chloroform and 200 ml ethanol (1:2 ratios). The mixture was filtered, the filtrate collected and the tissue residue was re-homogenized with 100 ml chloroform for 3 minutes. The resulting mixture was again filtered and combined with the first filtrate. If the resulting mixture has two liquid phases, more chloroform-methanol was added until a single phase was achieved. 100 ml of 0.88 % potassium chloride in distilled water was added and the mixture was shaken thoroughly and then allowed to settle. The upper layer including the interphase layer was removed by aspiration while the lower layer containing purified lipid was filtered to remove any insoluble substance. It was then evaporated at 50°C and 100 rotations per minutes. The resulting lipids were stored in chloroform at -30°C until further analysis.

Fatty acids methyl esters

Preparation of fatty acids methyl esters was carried out using modified method of Christie (1982) [7]. First, the stored lipid extract containing chloroform was evaporated under the nitrogen gas flow. 0.1 g of lipids was dissolved in 1.0 ml hexane. 1.0 ml of sodium methoxide solution was then added and this mixture was shaken with a vortex and was allowed to settle for 30 minutes until 2 phases of solutions resulted. The upper phase which contained the fatty acids methyl esters was removed by a pasteur pipette and analyzed using gas chromatography. The lower phase containing glycerol and non-methylated fatty acids was discarded.

Fatty acids analysis

The fatty acids methyl esters of sea cucumbers were analyzed using chromatography (Shimadzu) with the following set-up parameters

Detector: Flame ionization detector

Injector: Split 20:1 (250°C)

Column: BPX70, 60 m, 0.32 mm ID 0.25 film thickness

Operating condition: Temperature 80°C for 4 mins; 180°C 200°C, 5°C/min for 65 mins; 200°C 220°C, 20°C /min for 15 mins

Carrier gas: Nitrogen, 2 ml/min

Make up gas: Nitrogen, 30 ml/min, hydrogen, 30 ml/min and air, 300 ml/min

The fatty acids were identified by comparison with authentic standard (189-18 Sigma).

Statistical analysis

Recorded values were expressed as mean ± standard deviation. Statistical significance was determined using ANOVA one way analysis. *P* value less than 0.01 was considered significant.

3. RESULTS

The fatty acid compositions in six species of sea cucumbers are shown in Table 1. Palmitic acid is the major fatty acids in all species except for *Stichopus hermanii* and *Holothuria atra. Stichopus badionotus* shows the highest percentage area of palmitic acid which is 36.85 ± 2.45 %. Analysis shows that all saturated and monounsaturated fatty acids were significantly different ($p < 0.01$) in the methanol-chloroform extracts.

The comparison of major polyunsaturated fatty acids of omega-3 and omega-6 families was also shown in Table 1. Statistical analysis showed that the variance were significantly difference at $p<0.01$. In term of LA composition, *Holothuria atra* showed the highest percentage which was 49.25 ± 2.73 % followed by *Molpadiia* sp. (Langkawi) and *Molpadiia* sp. (Port Dickson) with 4.39 ± 0.63 % and 2.13 ± 0.19 % respectively. On the other hand, *Holothuria atra* contained the highest percentage area of LNA which was 23.16 ± 2.83 % followed by *Molpadiia* sp. (Port Dickson) and *Molpadiia* sp. (Langkawi) with 2.17 ± 0.82% and 1.64 ± 0.75%, respectively. In term of AA composition, the highest percentage area of AA detected was 26.73 ± 1.84% in *Stichopus hermanii.* No traces of AA were detected in *Stichopus badionotus, Holothuria atra, Holothuria tubulosa* and *Molpadiia* sp. (Port Dickson). Subsequently, all species show significant difference in the DHA composition with the highest percentage area detected in *Holothuria atra* (8.88 ± 1.83 %).

The order of Molpadiia from Langkawi and Port Dickson showed significant differences ($p < 0.01$) in their fatty acid compositions even though they were of the same species. Total polyunsaturated fatty acids detected in *Molpadiia* sp. (Langkawi) was 17.39 % compared to 7.11 % in *Molpadiia* sp. (Port Dickson).

Table 1. Composition of saturated, monounsaturated and polyunsaturated fatty acids in six species of sea cucumbers used in this study. Data showed the percentage area of each fatty acid profile from gas chromatography.

NAME	Percentage area (%)						
	Stichopus hermanii	*Stichopus chloronotus*	*Stichopus badionotus*	*Holothuria atra*	*Holothuria tubulosa*	*Molpadiia* sp. Langkawi	*Molpadiia* sp. Port Dickson
Saturated fatty acids							
Caprylic	0	0	0.77±2.10	0	0	1.14	0
Myristic	1.43±0.09	5.90±0.99	3.81±1.49	0	5.78±0.65	1.96±0.61	2.43±0.73
Palmitic	10.63±1.21	30.71±1.57	36.85±2.45	8.71±2.32	22.99±2.28	18.92±2.87	21.09±1.73
Margaric	0.71±0.06	0.85±1.21	2.02±1.88	0	2.23±0.56	4.29±1.13	3.76±1.00
Stearic	7.23±1.44	10.70±2.19	13.61±2.65	0	9.06±1.10	9.67±0.95	11.88±1.45
Arachidic	5.71±1.76	3.28±0.98	8.28±0.86	0	2.32±0.23	4.83±0.68	5.67±0.52
Henecosanoic	2.17±1.56	1.43±1.68	2.40±3.01	0	2.49±0.73	3.01±0.39	3.46±0.63
Behenic	1.12±0.08	5.28±1.89	1.62±2.65	0	1.98±0.13	1.24±0.02	1.60±0.73
Lignoceric	6.97±2.10	4.43±2.67	5.57±2.21	0	6.21±1.42	11.36±1.43	13.54±2.74
Monounsaturated fatty acids							
Myristoleic	0.50±0.01	1.04±0.09	1.41±0.32	0	18.18±2.64	3.95±0.63	3.88±0.85
Palmitoleic	2.81±0.21	9.23±1.52	9.58±1.29	0	10.84±2.11	6.92±0.86	7.00±0.35
Oleic	1.65±0.65	2.32±0.51	3.30±0.72	0	2.57±0.73	5.06±0.13	6.46±0.42
Gondoic	19.70±0.61	8.83±0.52	1.35±0.84	0	6.44±1.73	0	0
Erucic	0	0	0.49±0.13	0	0	0	0
Polyunsaturated fatty acids							
Linoleic	1.15±0.09	0.59±0.24	1.72±0.43	49.25±2.73	0	4.40±0.63	2.13±0.19
Linolenic	0.66±0.21	0.65±0.10	0.92±0.46	23.16±2.83	0	1.65±0.75	2.17±0.82
Arachidonic	26.73±1.84	7.82±2.62	0	0	0	9.47±0.72	0
DHA	7.80±1.52	4.30±1.63	0.49±0.16	8.88±1.84	2.87±0.11	1.88±0.12	2.81±0.83

4. CONCLUSIONS

A high percentage of LA, LNA, AA and DHA were detected in *Stichopus hermanii, Stichopus chloronotus* and *Molpadiia* sp (Langkawi). These species have that potential to be used as a source of omega-3 and omega-6 fatty acids in human diet thus having the potential as anti-inflammatory agents and assisting in wound healing process. The rate of wound healing in rabbit using methanol extraction of *Stichopus chloronotus* was shown to increase by 6.7 % [8]. Although no AA was detected in *Stichopus badionotus, Holothuria atra* and *Molpadiia* sp. (Port Dickson), these sea cucumber could also be used as a source of essential fatty acids in human because they have high content of LNA and LA, which could be converted to AA, EPA and DHA [9]. Conversion of these fatty acids to eicosanoids is believed to play an active part in inflammation and wound healing. Study has shown that the extract of *Stichopus badionotus* and *Holothuria atra* can heal wounds faster that the control [10]. *Molpadiia* sp. taken from two different location showed significant different of their fatty acids profile. It is suggested here that the fatty acids composition of this species be use as an indicator of scientific differentiation and classification of species next to the morphological differences.

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Dietary fiber from Cassava pulp

Pornariya Chirinang[a], Ratchadaporn Oonsivilai[*a], Natta Kachenpukdee[a]
[a]School of Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand 30000

*roonsivi@sut.ac.th phone +66-44224232 fax +66-44224387

ABSTRACT

Cassava pulp is a high value by-product from cassava starch industry that contain high amount of neutral detergent fiber (NDF) at 31.40% (w/w). Response surface methodology was applied for optimization of extraction parameters. Three parameters such as α-amylase concentration, protease concentration, and amyloglucosidase concentration including three levels for each parameter were studied. The optimum condition for the highest NDF preparation by enzymatic digestion was at 0.1% of α-amylase (w/v), 1% of protease (v/v) and 0.1% of amyloglucosidase (v/v). Dietary fiber from cassava pulp contained 40.24% (w/w) crude fiber, 79.03% (w/w) neutral detergent fiber (NDF), 70.14% (w/w) acid detergent fiber (ADF) and high content of cellulose at 58.55% (w/w). In addition, the hydration properties of dietary fiber prepared were investigated. The results showed that the dietary fiber prepared exhibited 4.82 ml/g swelling capacity, 8.36 g/g water retention capacity and 8.17 g/g water holding capacity. The major monosaccharide constituent of dietary fiber prepared was glucose, together with other neutral sugars. The FTIR spectrum of dietary fiber prepared was similar to cassava pulp spectrum with showing the sharp peak at 1005-1031 cm^{-1} that is usually the fingerprint of polysaccharides. Finally, scanning electron microscopy (SEM) of cassava pulp revealed a lot of starch granule embedded within cell wall material of cassava pulp. Otherwise, there was no starch granule appeared in dietary fiber prepared after enzymatic digestion. In conclusion, as physicochemical properties of dietary fiber prepared from cassava pulp by enzymatic digestion described above, cassava pulp could be used as a rich source of useful dietary fiber and could be applied to many food products.

Keywords: Cassava pulp, Neutral detergent fiber, Hydration properties

1. INTRODUCTION

Dietary fiber (DF) is defined as non-digestible carbohydrate and lignin that are the part of plants that have beneficial physiological effects in humans. The broader definition of dietary fibers may include fibers of animal origin and modified or synthetic non-digestible carbohydrate polymers (Polymerization Degree ≥ 3) [1]. Cassava (*Manihot esculenta*) is the third-largest source of food carbohydrates in the tropics.Thailand is the third largest producer of cassava starch, which yielded around 22.2 million tons per year.Consequently, there are a lot of by-products from the cassava processing which the important one is cassava pulp. Cassava pulp is fine and white mainly composed of carbohydrate, around 55-56%. Starch remaining in the pulp may approximately be 50-60% of its dry weight, in which the starch mostly be trapped inside ligno-cellulose [2]. Thus, the alternative way is to produce the value added cassava pulp is dietary fiber production.The purpose of this study was tostudy the optimize condition of NDF production by response surface methodologyandphysicochemical properties of dietary fiber prepared from cassava pulp.

2. MATERIALS AND METHODS

Materials and samples preparation

Cassava pulp was purchased from tapioca starch industry in the local area of NakhonRatchasimaprovince, Thailand. Heat-stable α-amylase (Cat. No. A3306), amyloglucosidase (Cat. No. A9913) from *Aspergillusniger*and protease (Cat. No. P1236) from *Bacillus amyloliquefaciens* (0.80 AU/g) were obtained from Sigma-Aldrich, Sigma Chemical, S. t. Louis, MO. All chemicals using were of reagent grade.

Extraction optimization

Determination of factors affecting the optimized yield of NDF was carried outwith Box-Behnken design. Response surface methodology was applied to obtain maximum extraction of NDF. All experimental data were statistically analysed with Design Expert® software (Version 8.0.7.1, Stat-Ease, Inc. Minneapolis, MN). Three level of three independent variables are α-amylase concentration, X_1 (0.1-0.3% w/v), protease concentration, X_2 (0.5-1.5% v/v) and amyloglucosidase concentration, X_3 (0.1-0.5% v/v) were applied. The selected dependent variables were cumulative neutral dietary fiber (NDF) percentage (*Y1*).

Compositional analysis

Dried cassava pulp and dietary fiber was analyzed for moisture, ash, protein, fat and crude fiber contents according to the methods of AOAC [3].The contents of neutral dietary fiber (NDF), acid detergent fiber(ADF) and acid detergent lignin (ADL) were investigated using the detergent method according to Van Soest[4]. Analyses were conducted using a Fibertec System 2010 apparatus(Foss Tecator, Höganäs, Sweden).Monosaccharide compositions were analyzed using a DionexHPAEC system (Archemica international, Co. Ltd., Sunnyvale, CA, USA) equipped with a pulsed amperometric detector (PAD).Gradient elution was carried using 250 mMNaOH and deionized water at the flow rate of 0.2 ml/min.

Hydration properties determination

Swelling capacity (SWC), water retention capacity (WRC) and water holding capacity (WHC) of NDF prepared from cassava pulp were determined using the method of Robertson et al. [5].

Fourier transform infrared spectroscopy

Cassava pulp and dietary fiber were dried and stored in desiccators prior to FTIR analysis. FTIR spectra were recorded using a golden-gate diamond single reflectance ATR on a Bruker T27/Hyp 2000 FTIR spectrophotometer. The spectra were recorded in the transmittance mode from 4000 to 400 cm^{-1} (mid-infrared region) at a resolution of 4 cm^{-1}; and 64 scans were collected. At least triplicate spectra readings for each sample were obtained [6].

Scanning electron microscope (SEM)

The cassava pulp and dietary fiber, spread on a double side conducting adhesive tape, pasted on a metallic stub, coated with gold in a sputter coating unit for 8 min and observed in a JEOL JSM-6010LV electron microscope (JEOL ltd., Tokyo, Japan).

Statistical analysis

All experiments were performed in triplicate and mean values (on dry basis) with standard deviations are reported. The experimental data were analyzed using an analysis of variance (ANOVA). SPSS® software (version 16, SPSS Inc., Chicago, IL, USA) was used to perform all the statistical calculations.In addition, response surface method was applied for optimization study.

3. RESULTS

Effect of enzyme concentration on NDF extraction

The effect of three independent parameters on the yield percentage of NDF from cassava pulp is investigated and data are shown in Table 1. The statistical analysis indicated that the second-order polynomial model correlated three variables in this study was obtained in Eq.(1) below:

$$NDF = +100.67969 - 257.69167 \times X_1 + 26.25667 \times X_2 - 160.17500 \times X_3 - 7.50000 \times X_1 \times X_2 + 108.50000 \times X_1 \times X_3 + 30.02500 \times X_2 \times X_3 + 490.54167 \times X_1^2 - 14.74833 \times X_2^2 + 163.44792 \times X_3^2 \quad (1)$$

Statistical significance of the model was checked by the F-test and model F-value of 59.79 implied that the model to be significant ($p < 0.05$). The regression coefficient ($R^2 = 0.9038$) of experimental model also indicated the suggested model to be a good fit for NDF extraction. The optimum condition was 0.1% w/v. 1% v/v and 0.1% v/v for concentration of α-amylase, protease and amyloglucosidase, respectively.

Table 1. Effects of extraction conditions on percentage NDF of dietary fiber from cassava pulp.

Trt.	Independent variables			%NDF
	α-amylase (%w/v) X_1	protease (%v/v) X_2	amyloglucosidase (%v/v) X_3	
1	0.2	0.5	0.5	58.53 ± 0.25^{fg}
2	0.3	0.5	0.3	54.22 ± 1.49^{g}
3	0.3	1.0	0.1	69.49 ± 1.96^{bc}
4	0.3	1.0	0.5	69.44 ± 2.26^{bc}
5	0.2	1.5	0.5	65.15 ± 2.62^{cde}
6	0.2	0.5	0.1	68.45 ± 1.10^{bcd}
7	0.2	1.0	0.3	57.50 ± 1.95^{fg}
8	0.2	1.5	0.1	63.06 ± 2.01^{def}
9	0.3	1.5	0.3	61.39 ± 1.51^{ef}
10	0.2	1.0	0.3	64.74 ± 1.51^{cde}
11	0.1	0.5	0.3	62.19 ± 4.95^{ef}
12	0.2	1.0	0.3	60.60 ± 4.44^{ef}
13	0.1	1.0	0.5	70.95 ± 0.42^{b}
14	0.1	1.5	0.3	70.86 ± 0.59^{b}
15	0.1	1.0	0.1	79.68 ± 0.55^{a}

Note: Each value is mean ± SD

[a,b] Data in the same row with different superscripts are significantly different ($p < 0.05$).

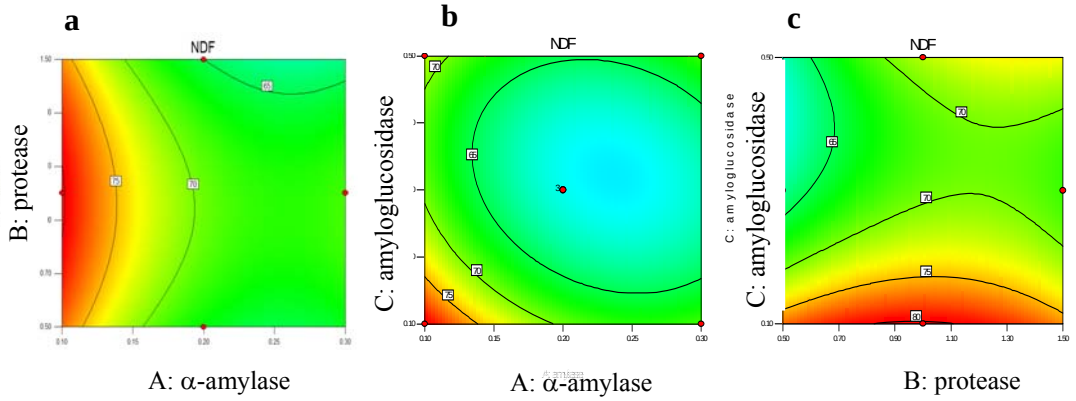


Figure 1. Contour plots of the effect of independent parameters on NDF yields percentage. (a) The effect of concentration of α-amylase and proteaseon the NDF yields percentageat concentration of 0.1% amyloglucosidase. (b) The effect of concentration of α-amylase and amyloglucosidaseon the NDF at concentration of 1% protease. (c) The effect of concentration of protease and amyloglucosidaseon the NDF yields percentageat concentration of 0.1% α-amylase.

Chemical compositions

The proximate analysis of cassava pulp powder and dietary fiber are shown in Table 2. The major composition of cassava pulp and dietary fiber was carbohydrate at 70.15 and 48.46% respectively. Cassava pulp still contains high starch contents while dietary fiber contains high cellulose with insoluble form. Moreover, dietary fiber showed higher contents of NDF, ADF, ADL, cellulose and hemicellulose when it passed through extraction process due tohydrolysis properties of enzymes applied. Kosoom et al.[8] reported that cassava pulp from many cassava starch manufacturing plants in Thailand contain average crude fiber, NDF, ADF, lignin and starch at 13.99, 40.62, 27.65, 3.60 and 50.19%, respectively. Oonsivilai et al. [7] studied on enzymatic digestion of dietary fiber from cassava pulp, the results showedthat NDF content of cassava pulp extraction was highest at 13.96%.

Sugar profile of cassava pulp and dietary fiber showed that glucose was the most abundant composition. In dietary fiber, glucose was present in the highest amounts followed by galactose, rhamnose, xylose, arabinose and mannose, respectively. The results showed that glucose contents of dietary fiber decrease perceptibly when compared with raw material. It might be due to starch contents decreased with enzyme activity.

Hydration properties correlate with both physiological and technological point of view and could influence the incorporation of fiber-enriched ingredients into food products**.** Hydration properties of dietary fiber such asswelling, water retention capacity and water holding capacity of dietary fiber showed the value of 4.82 mL/g, 8.36 g/g dry weight and 8.17g/g, respectively.

Fourier transform infrared spectroscopy

FTIR spectrum of dietary fiber exhibited similarities in absorption pattern to raw material. The sharp peak was appearing at around 1031 and 1005 cm^{-1} which showed the indication of stretching vibration of pyranose. The band at ~1239 cm^{-1} of dietary fiber was indication of acetyl group substitution of some of the –OH groups present [8].The bands at 1318 cm^{-1} indicated ring breathing with C–O stretching. Both cassava pulp and dietary fiber are composed of protein which usually has specific absorption bands in the 1700-1500 cm^{-1} region. The carbonyl (C=O) stretching was at 1736 and 1606cm^{-1}. The band at ~2936 and 2906 cm^{-1} was indeed characteristic of C-H stretches associated with the ring hydrogen atoms. An intense peak at 3336 and 3291 $cm^{-1}$was –OH stretching peak.

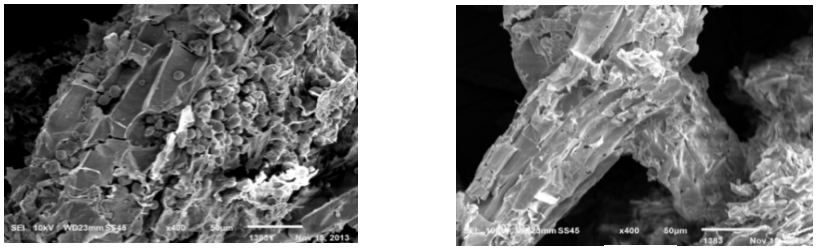
Table 2. Chemical compositions (%) of cassava pulp powder and dietary fiber (dry weight basis)

Components	Cassava pulp (%)	Dietary fiber (%)
Crude protein	2.02 ± 0.19	1.01 ± 0.10
Fat	0.21 ± 0.08	0.25 ± 0.06
Moisture	6.63 ± 0.11	5.52 ± 0.09
Ash	3.76 ± 0.05	4.52 ± 0.04
Crude fiber	17.23 ± 0.13	40.24 ± 2.22
Carbohydrate	70.15	48.46
Starch	58.11 ± 0.06	8.50 ± 0.31
Neutral detergent fiber (NDF)	31.40 ± 0.58	79.03 ± 0.51
Acid detergent fiber (ADF)	25.08 ± 0.17	70.14 ± 0.40
Acid detergent lignin (ADL)	4.16 ± 0.10	11.59 ± 0.01
Cellulose[a]	20.92	58.55
Hemicellulose[b]	6.30	8.89
Monosaccharide (mg/g dry sample)		
Galactose	65.12 ± 0.43	94.41 ± 0.21
Glucose	717.80 ± 1.76	309.49 ± 1.45
Xylose	21.91 ± 1.31	66.90 ± 0.67
Mannose	6.03 ± 1.09	17.81 ± 0.86
Rhamnose	74.17 ± 0.99	81.64 ± 1.33
Arabinose	51.37 ± 0.87	63.27± 1.03

[a]ADF-ADL, [b]NDF-ADF

Scanning electron microscope (SEM)

From the SEM results, the pulp showed hugely starch granules embedded well inside the matrix which related to starch contents as above (Figure 3a). For dietary fiber, after hydrolysis by α-amylaseand amyloglucosidase enzyme, the results showed that there was no starch granule embedded inside the fiber matrix (Figure 3b). This NDF produced from cassava pulp showed higher purification than starch. Its fiber structure seems to have high porosity. Sowhagya et al. [9] extracted dietary fiber from spent residue cumin. They found that the fiber matrix appeared to have a typical "honey comb" structure, almost avoid of starch granules.



a **b**

Figure 3. Scanning electron micrographs of cassava pulp (a) and dietary fiber (b) at 400x.

4. CONCLUSIONS

Cassava pulp could be the new source of NDF production by enzymatic extraction at the optimal conditions of 0.1% w/v, 1% v/v and 0.1% v/v for concentration of α-amylase, protease and amyloglucosidase enzyme, respectively. In addition, NDF prepared from cassava pulp contained 79.03%and high contents of cellulose. The physical properties as hydration properties of dietary fiber such as swelling, water retention capacity and water holding capacity of dietary fiber showed the value of 4.82 mL/g, 8.36 g/g dry weight and 8.17g/g, respectively.Finally, there was no starch granule embedded inside the fiber matrix that suggests high porosity of fiber structure. The application of dietary fiber in food products system should be interested topic for the future study.

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Phenolic compounds from the leaves of *Garcinia dulcis*

Arun Saelee*[a], Wilawan Mahabusarakam[a,b]
[a]Department of Chemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla, Thialand 90112
[b]Natural Product Research Center of Excellence, Prince of Songkla University, Hat-Yai, Songkhla, Thialand 90112
*arun_2532@hotmail.com

ABSTRACT

Garcinia dulcis Kurz. (Guttiferae) is known as Ma-Phut in Thailand. The fruit juice has been used in traditional medicine as an expectorant. In Indonesia, the leaves and seeds have been used for the treatment of lymphatitis, parotitis and struma. We therefore investigated the chemical constituents of this plant. Study on the chemical constituents from the acetone extract of the leaves of *G. dulcis* resulted in the isolation of five known biflavonoids: morelloflavone (1), morelloflavone-7-sulfate (2), volkensiflavone (3), amentoflavone (4), GB-2a (5) and three benzene derivatives: 4-hydroxybenzoic acid (6), 4-hydroxy-3-methoxybenzic acid (7), (Z)-methyl 5-(4-hydroxyphenyl)-3,4,4-trimethoxy-5-oxopent-2-enoate (8). Their structures were elucidated by analysis of 1D and 2D NMR, as well as comparison the data with those previously reported.

Keywords: Guttiferae, *Garcinia dulcis*, Biflavonoids, Benzene derivatives

1. INTRODUCTION

Garcinia species are well known to be rich in phenolic compounds, such as biflavonoids, flavonoids and xanthones. Over the decade, phenolic compounds have been reported in several literatures and almost they exhibited the biological activity, including antioxidant, anticancer, anti-HIV-1 and antibacterial. *Garcinia. dulcis* belongs to the family Guttiferae. It is distributed mainly in tropical area in the global. Fruits can be eaten raw or cooked. The leaves and seeds have been used in traditional medicine against diseases [1]. We thus interested in study on the chemical constituents of the leaves *G. dulcis*.

2. MATERIALS AND METHODS

2.1 Plant material

The leaves of *G. dulcis* were collected from Amphur Meuang, Songkhla province in the southern part of Thailand.

2.2 Extraction and isolation

The leaves of *G. dulcis* (800 g) were drenched in acetone at room temperature (3 days). After evaporation, a crude acetone extract (45.19 g) was obtained. The crude extract was dissolved in CH_2Cl_2 to give CH_2Cl_2 soluble (A 23.96g) and insoluble fractions (B 21.23g). Fraction B was subjected on sephadex LH-20 and eluted with MeOH:CH_2Cl_2 (7:3) to give fractions (B1-B4). A yellow solid **4** (40.0 mg) was crystallized from fraction B2, the filtrate (8.81g) after removed of **4** was further purified by CC on silica gel and eluted with a gradient of CH_2Cl_2 – MeOH to give nine fractions (C1 – C9). A yellow solid **2** (6.2 mg) was isolated from fraction C4 (362.4 mg) by CC on silica gel and eluted with acetone:CH_2Cl_2:hexane (3:2:5). Purification of fraction C6 (730.5 mg) by CC on silica gel and eluted with CH_2Cl_2:MeOH (9.7: 0.3) gave a yellow solid **3** (5.5 mg). Fraction B3 was subjected to CC on silica gel and eluted with MeOH:CH_2Cl_2 (1.5:8.5) to give **5** (9.0 mg). Fraction B4 (1.53 g) was purified by CC on reversed-phase and eluted with MeOH:H_2O (2:8) to give fifteen fractions (G1-G15). Fraction G14 (80.0 mg) was purified by CC on silica gel and eluted with MeOH:CH_2Cl_2 (7:3) to give **1** (1.2 mg).

(**1**): yellow solid, m.p. 247-250°C; UV (MeOH) λ_{max} (nm) (log □): 339 (3.17), 322 (3.21), 292 (3.34); FT-IR (neat) ν_{max} (cm^{-1}) 3215 (O-H stretching), 1622 (C=O stretching), ^1H and ^{13}C NMR spectral data ($CDCl_3$+DMSO-d_6), see Table 1 and 2

(**2**): yellow solid, m.p. 215-216°C; UV (MeOH) λ_{max} (nm) (log □): 291 (5.14), 201 (5.54); FT-IR (neat) ν_{max} (cm^{-1}): 3215 (O-H stretching), 1635 (C=O stretching), ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1 and 2

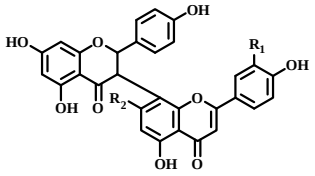
(**3**): yellow powder; UV (MeOH) λ_{max} (nm) (log □): 333 (4.05), 269 (4.09), 202 (4.28); FT-IR (neat) ν_{max} (cm^{-1})3402 (O-H stretching), 1649 (C=O stretching), ^1H and ^{13}C NMR spectral data ($CDCl_3$+DMSO-d_6), see Table 1 and 2

(**4**): yellow solid, m.p. 305-307°C; UV (MeOH) λ_{max} (nm) (log □): 344 (4.75), 289 (4.93); FT-IR (neat) ν_{max} (cm^{-1}) 3215 (O-H stretching), 1641 (C=O stretching), ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1 and 2

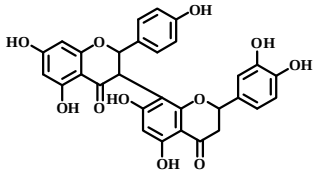
(**5**): yellow solid, m.p. 290-293°C; UV (MeOH) λ_{max} (nm) (log □): 348 (4.88), 289 (5.02), 201 (5.41); FT-IR (neat) ν_{max} (cm^{-1}): 3423 (O-H stretching), 1643 (C=O stretching), 1262 (S=O stretching), 1042 (C-O-S stretching), ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1 and 2

3. RESULTS

Investigation of the chemical constituents of the leaves of *G. dulcis* resulted in the isolation of five compounds. Structure elucidation using 1D and 2D NMR spectroscopic data as well as comparison the data with those previously reported indicated that they were volkensiflavone (**1**), GB-2a (**2**), amentoflavone (**3**), morelloflavone (**4**) and morelloflavone-7-sulfate (**5**).



1 : R_1 = H, R_2 = OH
4 : R_1 = R_2 = OH
5 : R_1 = OH, R_2 = SO_4H



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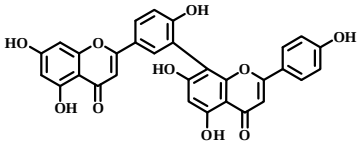

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Table 1. ^{1}H spectroscopic data of compounds **1-5**

Position	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
	δ	***δ***	***Δ***	***δ***	***δ***
2	5.75 (*d*, 12.0)	5.73 (*d*, 2.3)	-	5.73 (*d*, 12.0)	5.81 (*d*, 12.0)
3	4.65 (*d*, 12.0)	4.56 (*d*, 12.3)	6.39 (*s*)	4.92 (*d*, 12.0)	4.83 (*d*, 12.0)
6	5.95 (*s*)	5.90 (br *s*)	6.12 (*d*, 2.1)	5.99 (*s*)	5.92 (*d*, 2.1)
8	5.97 (*s*)	5.79 (br *s*)	6.25 (*d*, 2.1)	6.06 (*s*)	5.94 (*d*, 2.1)
2′	6.99 (*d*, 8.4)	7.13 (*d*, 8.5)	7.76 (*d*, 2.1)	7.17 (*d*, 8.4)	7.18 (*d*, 8.4)
3′	6.41 (*d*, 8.4)	6.79 (*d*, 8.5)	-	6.41 (*d*, 8.4)	6.31 (d, 8.4)
5'	6.41 (*d*, 8.4)	6.79 (*d*, 8.5)	7.05 (*d*, 8.7)	6.41 (*d*, 8.4)	6.31 (d, 8.4)
6'	6.99 (*d*, 8.4)	7.13 (*d*, 8.5)	7.67 (*dd*, 8.7, 2.1)	7.17 (*d*, 8.4)	7.18 (*d*, 8.4)
2″	-	5.43 (br *t*)	-	-	-
3″	6.23 (*s*)	2.90 (*m*) 2.60 (*m*)	6.39 (*s*)	6.60 (*s*)	6.60 (*s*)
6″	6.25 (*s*)	5.86 (*s*)	6.41 (*s*)	6.24 (*s*)	7.05 (*s*)
2‴	7.45 (*d*, 8.7)	7.10 (*d*, 2.0)	7.31 (*d*, 8.7)	7.45 (*d*, 3.4)	7.39 (*brs*)
3‴	6.86 (*d*, 8.7)	-	6.62 (*d*, 8.7)	-	-
5‴	6.86 (d, 8.7)	6.64 (*d*,8.5)	6.62 (*d*, 8.7)	6.92 (*d*, 8.4)	6.88 (*d*, 8.1)
6‴	7.45 (*d*, 8.7)	6.73 (*dd*, 8.5, 2.0)	7.31 (*d*, 8.7)	7.27 (*dd*, 8.4, 2.4)	7.40 (*d*, 8.1)
5-OH	12.20 (*s*)	12.19 (*s*)	12.67 (*s*)	12.30 (s)	12.19 (*s*)
5″-OH	12.20 (*s*)	12.15 (*s*)	12.86 (*s*)	13.10 (s)	12.95 (*s*)

Table 2. ^{13}C NMR spectroscopic data of compounds **1-5**

Position	Compound 1	Compound 2	Compound 3	Compound 4	Compound 5
	δ	*δ*	*Δ*	*δ*	*δ*
2	82.0	82.2	168.9	81.4	81.4
3	48.7	47.8	108.2	49.1	49.0
4	196.5	197.1	188.9	196.7	195.8
4a	102.3	101.8	109.2	102.1	101.9
5	164.4	165.3	166.7	164.3	164.3
6	95.8	96.5	104.2	95.8	96.6
7	167.2	166.9	168.9	167.0	166.9
8	96.8	95.9	98.9	96.7	95.6
8a	163.5	163.3	162.6	163.3	163.3
1′	128.8	130.2	126.9	128.6	128.9
2′	128.7	129.4	136.1	128.7	129.0
3′	115.3	114.4	124.8	114.9	114.6
4′	157.0	158.1	163.9	157.8	157.5
5'	115.3	114.4	120.9	114.9	114.6
6'	128.7	129.4	132.3	128.7	129.0
2″	164.3	79.0	168.9	164.1	164.4
3″	103.4	43.1	107.7	102.7	102.9
4″	182.2	196.6	187.4	182.1	182.4
4a″	104.2	102.1	109.6	103.6	105.5
5″	161.0	164.1	166.3	163.9	160.2
6″	99.0	95.5	104.2	99.1	102.8
7″	161.6	161.2	166.2	162.1	158.1
8″	101.2	101.8	108.4	101.0	104.6
8a″	156.0	162.5	159.8	155.7	154.8
1‴	122.5	128.4	126.7	121.5	121.4
2‴	128.8	129.0	132.7	113.7	113.8
3‴	116.5	145.7	121.6	161.0	146.1
4‴	161.0	146.2	165.8	150.1	150.4
5‴	116.5	115.3	121.6	116.6	116.6
6‴	128.7	118.2	132.7	119.8	119.9

Compound 1 is a yellow solid, m.p. 247-250°C. The ^{1}H and ^{13}C NMR spectrum in $CDCl_3$+DMSO-d_6 displayed duplicated signals (in a ratio of 1:0.67), suggesting the existence of two conformers at room temperature. The signals of major conformer (Table 1) were a singlet chelated hydroxyl proton 5-OH at *δ* 12.20, two siglets of *meta* aromatic protons H-6 and H-8 at *δ* 5.95 and 5.97, two doublets of *trans* methine protons H-2 and H-3 at *δ* 5.75 and 4.65 (*J* trans = 12.0 Hz), and AA′BB′ system of aromatic protons H-2′/H-6′ and H-3′/H-5′ at *δ* 6.99 and 6.41 (*d* each, *J* = 8.4 Hz). In HMBC experiment, protons H-6, H-8, 5-OH and H-3 all correlated to C-4a (*δ* 102.3). The chemical shift of *δ* 5.95 was assigned for H-6 due to HMBC correlation of 5-OH to C-6 (*δ* 95.8) and H-6 to C-5 (*δ* 164.4). The HMBC correlation of H-2′/H-6′ to C-2 (*δ* 82.0) and H-2 to C-2′/C-6′ (*δ* 128.7), suggested 1,4-disubstituted aromatic ring was connected to C-2. The ^{1}H NMR spectrum further showed singl aromatic proton H-6″ at *δ* 6.25, singlet olefinic proton H-3″ at *δ* 6.23 and AA′BB′ system of aromatic protons H-2‴/ H-6‴ and H-3‴/ H-5‴ at *δ* 7.45 and 6.86 (*d* each, *J* = 8.7 Hz). Proton H-3″ and H-6″ correlated to C-4a″ (*δ* 104.2). The HMBC showed the correlations of H-2‴/H-6‴ to C-2″ (*δ* 164.3), C-4‴ (*δ* 161.0) and C-6‴ (*δ* 128.7); H-3″ to C-1‴ (*δ* 122.5) suggested the *para*-disubstituted was connected to C-2″. The HMBC correlation of H-3 to C-8a″ allowed the two units of flavonoids connected via C-3 and C-8″. Thus **1** was assigned as volkensiflavone [2].

Compound 2 is a pale yellow solid, m.p. 215-216°C. The ^{1}H and ^{13}C NMR spectrum in DMSO-d_6 displayed duplicated signals (in a ratio of 1:0.4), suggested the existence of two conformers at room temperature. The major conformer showed the resonances of chelated hydroxyl proton 5-OH at *δ* 12.19, *meta* aromatic protons H-6 and H-8 at *δ* 5.90 and *δ* 5.79, trans methine protons H-2 and H-3 at *δ* 5.73 and *δ* 4.56 and aromatic protons H-2′/H-6′ and H-3′/H-5′ at *δ* 7.13 and 6.79. The HMBC correlation of H-6 to C-8 (*δ* 95.9), C-4a (*δ* 101.8) and 5-OH to C-6 (*δ* 96.5),

C-4a suggested H-6 was *ortho* to 5-OH. The HMBC correlation of H-2′/H-6′ to C-2 (δ 82.2) and H-2 to C-2′/C-6′ (δ 129.4) suggested 1,4-disubstituted aromatic ring was connected to C-2. The ^{1}H NMR spectrum further showed singlet of aromatic protons H-6″ at δ 5.86, oxy-methine proton H-2″ at δ 5.43, non equivalent methylene protons H-3″ at δ 2.90 and δ 2.60, and ABX pattern of aromatic protons H-2‴, H-5‴ and H-6‴ at δ 7.10, 6.64 and δ 6.73. Proton H-3″ and H-6″ correlated to C-4a″ (δ 102.1). The HMBC showed the correlations of H-2‴ to C-2″ (δ 79.0) and H-3″ to C-1‴ (δ 128.4) suggesting the trisubstituted benzene ring was connected to C-2″. The HMBC correlation of H-3 to C-8a″ allowed the two units of flavonoids connected via C-3 and C-8″. Therefor **2** was assigned as GB-2a [2].

Compound 3 is a yellow powder. The ^{1}H NMR spectrum suggested that it was a biflavonoid which the methine olefinic protons H-3 and H-3″ resonated at δ 6.39. The resonances of chelated hydroxyl proton 5-OH at δ 12.67, *meta* aromatic protons H-6 and H-8 at δ 6.12 and 6.25, aromatic proton H-2′, H-5′ and H-6′ as an ABX pattern at δ 7.76 (*d*, *J* = 2.1 Hz), δ 7.05 (*d*, *J* = 8.7 Hz) and δ 7.67 (*dd*, *J* = 8.7, 2.1 Hz) were determined for the first unit. The HMBC correlation of 5-OH to C-4a (δ 109.2), C-6 (δ 104.2) and H-6 to C-4a confirmed the location of H-6. The HMBC correlation of H-3 to C-1′ (δ 126.9) and H-6′ to C-2 (δ 168.9) suggested the trisubstituted benzene ring was at C-2. The resonances of chelated hydroxyl proton 5″-OH at δ 12.86, aromatic proton H-6″ at δ 6.41, aromatic proton H-2‴/H-6‴ and H-3‴/H-5‴ at δ 7.31 and δ 6.62 (*d*, *J* = 8.7 Hz each) were assigned for the second unit. The HMBC correlation of 5″-OH to C-6″ (δ 104.2), C-4a″ (δ 109.6) and H-6″ to C-4a″ confirmed the location of H-6″. The correlation of H-3″ to C-1‴ (δ 126.7) and H-2‴/H-6‴ to C-2″ (δ 168.9) suggested the *para*-disubstituted benzene ring was at C-2″. The HMBC correlation of H-2′ to C-8″ (δ 108.4) allowed the two units of flavonoids connected via C-6 and C-8″. Thus **3** was assigned to be amentoflavone [3].

Compound 4 is a yellow solid, m.p. 305-307°C. The ^{1}H and ^{13}C NMR spectrum in DMSO-d_6 displayed duplicated signals (in a ratio of 1:0.38), suggested the existence of two conformers of biflavonoid at room temperature. The ^{1}H and ^{13}C NMR and HMBC spectrum were similar to those of compound **1**. The ^{1}H NMR spectrum suggested that it was a flavonylflavanone which the *trans* methine protons H-2, H-3 resonated at δ 5.73 and 4.92 (*J* = 12.0 Hz) and the methine olefinic protons H-3″ resonated at δ 6.60. The ^{1}H NMR spectrum showed the resonances of chelated hydroxyl protons 5-OH (δ 12.30) and 5″-OH (δ 13.10), *meta* aromatic protons H-6 (δ 5.99) and H-8 (δ 6.06), *para*-disubstituted benzene H-2′/H-6′ (δ 7.17) and H-3′/H-5′ (δ 6.41), aromatic proton H-6″ (δ 6.24) and 1,3,4-trisubstituted benzene (δ 7.45, H-2‴; δ 6.92, H-5‴; δ 7.27, H-6‴). The spectrum pattern of **4** was similar to that of **1**, except for the presence of 1,3,4-trisubstituted benzene instead of 1,4-disubstituted benzene. In HMBC spectrum, the flavonyl moiety displayed the correlations of 5-OH to C-6 (δ 95.8), C-4a (δ 102.1); H-6 to C-4a, C-7 (δ 167.0), C-8 (δ 96.7); H-8 to C-4a, C-7; H-2′/H-6′ to C-2 (δ 81.4), and H-3 to C-8″ (δ 101.0). In HMBC spectrum, the flavanone moiety displayed the correlations of 5″-OH to C-6″ (δ 99.1), C-4a″ (δ 103.6); H-6″ to C-4a″ C-8″; H-2‴ /H-6‴ to C-2″ (δ 164.1). Compound **4** then was assigned as morelloflavone [4].

Compound 5 was a yellow solid, m.p. 290-293°C. The ^{1}H and ^{13}C NMR and HMBC spectrum were very similar to those of **4**. The ^{1}H NMR spectrum showed the resonances of *trans* methine protons H-2 and H-3 (δ 5.81 and 4.83), methine olefinic proton H-3″ (δ 6.60), chelated hydroxyl protons 5-OH (δ 12.19) and 5″-OH (δ 12.95), *meta* aromatic protons H-6 (δ 5.92) and H-8 (δ 5.94), *para*-disubstituted benzene H-2′/H-6′ (δ 7.18) and H-3′/H-5′ (δ 6.31), aromatic proton H-6″ (δ 6.24)**.** The proton resonances of **5** were almost identical to those of **4**, except for the H-6″ (δ 7.05) of **5** appeared at much lower field than that of **4** (δ 6.24). According to the IR spectrum that showed S=O stretching at 1262 and C-O-S stretching at 1042 cm^{-1} and the chemical shift of C-7″ at δ 157.1 allowed to assigned –OSO_3H at C-7. The structure of **5** was supported by HMBC correlation. Thus **5** was assigned to be morelloflavone-7-sulfate [4].

4. CONCLUSIONS

Five biflavonoids, known as volkensiflavone (**1**), GB-2a (**2**), amentoflavone (**3**), morelloflavone (**4**) and morelloflavone-7-sulfate (**5**) were isolated from the leaves of *G. dulcis.*

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Identification of new lipo-alkaloids from Aconiti Radix by UHPLC-Q-TOF-MS/MS approach

Na Li*[a], Ying Liang[ab], Jian-Lin Wu[a]
[a]State Key Laboratory of Quality Research in Chinese Medicine, Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macao,
[b]School of Chinese Medicine, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macao

*nli@must.edu.mo phone +853-8897-2405 fax +853-2882-2799

ABSTRACT

Aconiti Radix is widely used as anti-inflammatory and analgesic agent in Eastern Asia. Lipo-alkaloid is a type of alkaloids in *Aconitum* species, which usually contains an aconitane skeleton and one or two long-chain fatty acid residues. Due to the high similarity of the structures, it's difficult to obtain the pure lipo-alkaloids. Ultra-High Performance Liquid Chromatography-Quadrupole-Time of Flight-mass spectrometry (UHPLC-Q-TOF-MS) approach can provide excellent separation and structural elucidation, and thus was used to identify the lipo-alkaloids in Aconiti Radix in our research. The high resolution MS and characterizations of fragmentation ions in MS/MS were firstly investigated. Then, 46 lipo-alkaloids were identified based on their MS and MS/MS spectra, and 4 new compounds were identified as 14-benzoylhypaconine-8-dihydroxyoleate (**1**), 14-benzoylmesaconine-8-trihydroxyoleate (**2**), 14-benzoylmesaconine-8-dihydroxystearate (**3**), and 14-benzoylmesaconine-8-dihydroxyoleate (**4**), respectively, using this approach.

Keywords: Lipo-alkaloids, Aconiti Radix, UHPLC-Q-TOF-MS/MS

1. INTRODUCTION

Aconiti Radix is the roots of *Aconitum carmichaelii* Debx. and widely used as anti-inflammatory and analgesic agent in Eastern Asia [1]. Aconitine alkaloids were the main components, while more and more lipo-alkaloids were also reported in recent years [2-6]. Lipo-alkaloid is a type of alkaloids in *Aconitum* species, which usually contains an aconitane skeleton and one or two long-chain fatty acid residues. Due to the high similarity of the structures, it's difficult to obtain the pure lipo-alkaloids. Until now, this kind of alkaloid was mainly identified by high sensitive mass spectrometry approaches [2]. Ultra-High Performance Liquid Chromatography method (UHPLC) can provide excellent separation in short time, thus was applied to separate the lipo-alkaloid mixtures in Aconiti Radix extract. And, the identifications of lipo-alkaloids were conducted using Quadrupole-Time of Flight-mass spectrometry (Q-TOF-MS) approach in our research.

2. MATERIALS AND METHODS

Preparation of Aconiti Radix extract

Aconiti Radix was purchased from the market of Macao. One gram of herbs was powdered and extracted with 3 mL methanol for 60 min with the aid of ultrasonicator, and then centrifuged at 12,000 rpm for 10 min. The supernatant was collected and diluted for 500 times then followed by analyzing by UHPLC-Q-TOF-MS approach.

UHPLC-Q-TOF-MS analysis

Agilent 1290 UHPLC system hyphenated with Q-TOF 6550 mass spectrometer was applied. Agilent Eclipse C18 column (2.1 × 100 mm, 1.7 μm) was used. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with the following gradient: 0-0.5 min, 10% B; 0.5-10 min, 10-95% B; 10-20 min 95% B; 20-20.1 min, 95-10% B, and then maintained for 2 minutes. The flow rate was 0.35 mL/min, and the injection volume was 1 μL. The mass parameters were set as follows: dry gas temperature and flow were 250° and 15 L/min, sheath gas temperature and flow were 300° and 11 L/min, nebulizer at 20 psi, capillary and nozzle voltages were 3500 and 2000 V, respectively. The fragmentor and collision cell energy were set at 380 V and 50 eV, and positive mode was applied.

3. RESULTS

All lipo-alkaloids identified so far are based on C19 aconitine skeleton [2], and the main difference is the side chain, which is fatty acid with long chain in lipo-alkaloids, while is usually an acetic acid in aconitine alkaloids. Thus, the MS2 characterization of the skeleton was firstly studied using three aconitine alkaloids, aconitine (A), mesaconitine (MA), and hypaconitine (HA). The MS2 spectra of these three standards indicated that the main fragment ions were $[M+H-CH_3COOH]^+$, $[M+H-CH_3COOH-C_6H_5COOH-3CH_3OH]^+$, and $[C_6H_5CO]^+$ at *m*/*z* 586.3246, 368.1870, and 105.0338 for aconitine, *m*/*z* 572.2861, 354.1707, and 105.0339 for mesaconitine, and *m*/*z* 556.2939, 338.1763, and 105.0341 for hypaconitine, respectively, as shown in Figure 1A-1C.

Then the methanol extract of Aconiti radix was analyzed using the same method. The compounds were firstly extracted using the function of Find by Molecular Feature (MFE) in Masshunter software, and then the molecular formulae were generated from the mass spectra of the extracted compounds using the function of Generate Formula (MFG). The MS2 spectra were obtained using auto-MS2 or target-MS2 methods. By comparison with the MS2 spectra of three standards, 46 lipo-alkaloids were identified and most of them were the long-chain fatty acid ester of 14-benzoylaconine, 14-benzoylmesaconine, and 14-benzoylhypaconine. Among them, four lipo-alkaloids, **1**-**4**, were detected for the first time. The molecular formulae of compounds **1**-**4** were generated as $C_{49}H_{75}NO_{12}$, $C_{49}H_{75}NO_{14}$ $C_{49}H_{77}NO_{13}$, and $C_{49}H_{75}NO_{13}$ from the molecular ion peaks at *m*/*z* 870.5358, 902.5232, 888.5473, and 886.5291, respectively. Compound **1** had the characteristic fragment ions of hypaconitine at *m*/*z* 556.2912, 524.2638, 338.1746, and 105.0336 (Figure 1D), thus was deduced to be the derivative of 14-benzoylhypaconine. Moreover, the fatty acid chain should be dihydroxyoleic acid from the neutral loss of 314 produced from the ion at *m*/*z* 870.5358 to the ion at *m*/*z* 556.2912 ($C_{18}H_{34}O_4$). So, compound **1** was identified as 14-benzoylhypaconine-8-dihydroxyoleate. Compounds **2**-**4** had the similar fragment ions with mesaconitine at *m*/*z* 572.28, 540.26, 354.17, and 105.03 (Figure 1E-1G), and the side chains were deduced to be trihydroxyoleic acid, dihydroxystearic acid and dihydroxyoleic acid, respectively, from the neutral losses of 330, 316, and 314 produced from the molecular ions to the ion at *m*/*z* 572.28. Thus, compounds **2**-**4** were determined as 14-benzoylmesaconine-8-tirhydroxyoleate, 14-benzoylmesaconine-8-dihydroxystearate, and 14-benzoylmesaconine-8-dihydroxyoleate.

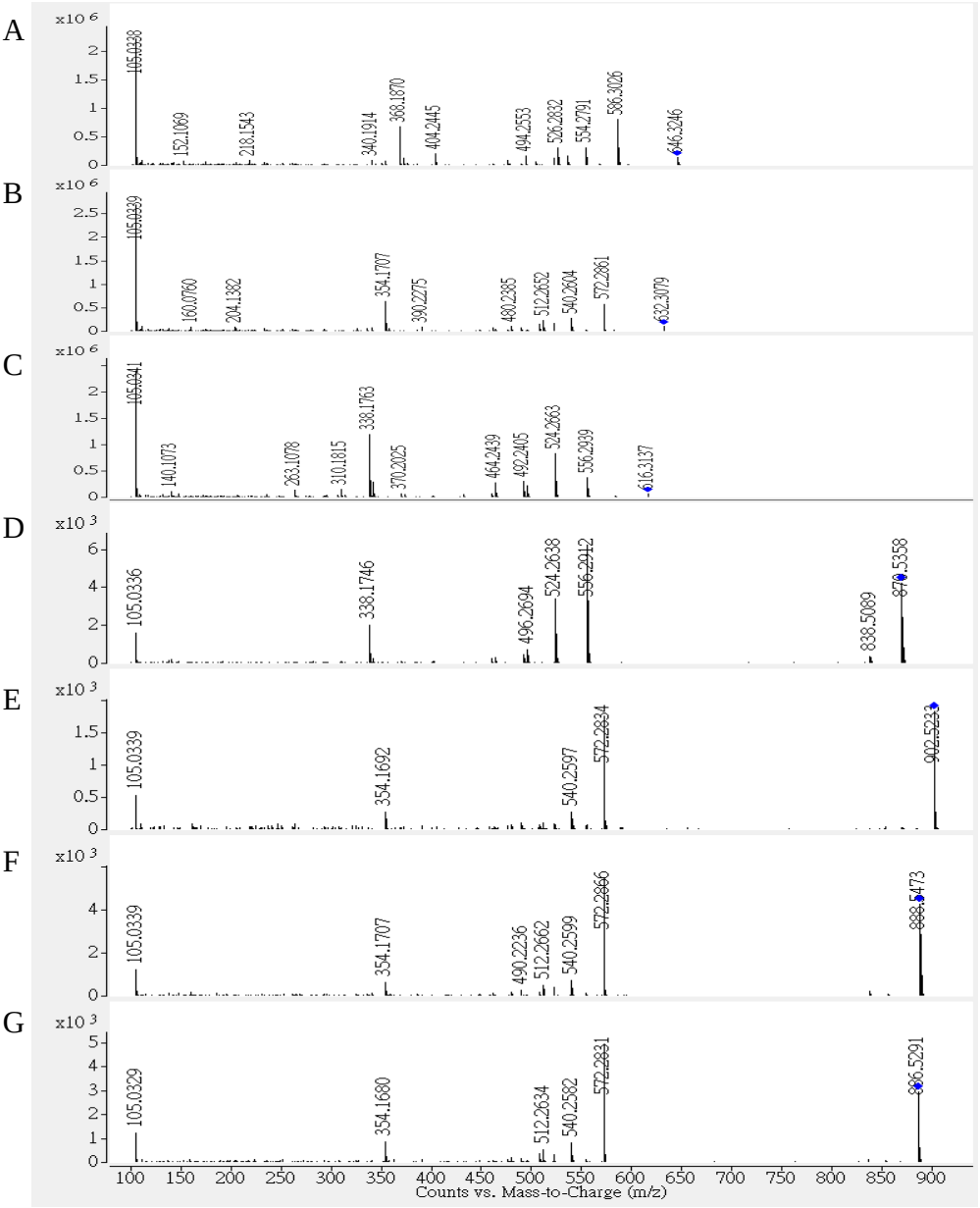

Figure 1. MS2 spectra of aconitine (A), mesaconitine (B), and hypaconitine (C), lipo-alkaloids **1** (D), **2** (E), **3** (F), **4** (G).

4. CONCLUSIONS

UHPLC-Q-TOF-MS approach was applied for the separation and identification of lipo-alkaloids in Aconiti radix. Based on the MS and MS/MS spectra, 46 lipo-alkaloids were identified and 4 new compounds were identified as 14-benzoylhypaconine-8-dihydroxyoleate (**1**), 14-benzoylmesaconine-8-trihydroxyoleate (**2**), 14-benzoylmesaconine-8-dihydroxystearate (**3**), and 14-benzoylmesaconine-8-dihydroxyoleate (**4**), respectively.

ACKNOWLEDGEMENTS

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Fast and standards free characterization of major flavanonols from *Smilax glabra* using integrated on-line ultra high-performance liquid chromatography-quadrupole-time of flight-mass spectrometry/solid phase extraction-nuclear magnetic resonance

Na Li[a], Wan-yi Gu[a], Hua Zhou[a], Zhi-hong Jiang[a], Liang Liu, Jian-lin Wu*[a]
[a]State Key Laboratory of Quality Research in Chinese Medicines, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macao, China

*jlwu@must.edu.mo phone +85-388972406 fax +85-328822799

ABSTRACT

Integrated ultra high-performance liquid chromatography-quadrupole-time of flight-mass spectrometry/solid phase extraction-nuclear magnetic resonance (UHPLC-Q-TOF-MS/SPE-NMR) is powerful platform, which was used to analyze the target components in complex system, for instance, bioactive compounds in herbal medicines, biomarkers in animal biofluids, nutrient contents in food and toxic components environmental samples. The rhizome of *Smilax glabra* Roxb. has been widely used in traditional Chinese medicines for the treatment of syphilis, acute bacillary dysentery, and acute as well as chronic nephritis. Its major active components are flavanonols. In this study, a standard free on-line UHPLC-Q-TOF-MS/SPE-NMR approach was firstly established to identify major flavanolols in several milligram level of raw material. Waters C18 column (250 × 4.6 μm, 5 μm) was used to separate the prepared sample within 30 min, 0.1% of formic acid and 5 mM ammonium format in water and methanol were used as mobile phase A and B, repectively. And the injection volume was 10 μL, among of them, 95% were split into SPE as well as 5% into MS. Seven major peaks at wavelength of 280 nm with accurate m/z for 449.1085, 433.1273 and 451.1062 were preliminary elucidated as $C_{21}H_{22}O_{11}$, $C_{21}H_{22}O_{10}$ and $C_{24}H_{20}O_9$ as well as mass differences were less than 5 ppm, combined to MS/MS spectra, they were elucidated as flavanonols. The each major peaks was also collected by cartridge of SPE, after multi-trapping for 5 times of each peaks, the cartridges were eluted by 250 μL of deuterated acetonitrile, then directly injected into CryoFit and detected by CryoProbe NMR, according to the ^{1}H NMR and ^{1}H-^{1}H COSY spectra, the major components were further identified as neoastilbin, astilbin, neoisoastilbin, isoastilbin, engeletin, isoengeletin and cinchonain, respectively. And their amounts were also estimated using tetramethylsilyl as internal standard.

Keywords: Flavanonols, *Smilax glabra*, Integrated UHPLC-Q-TOF-MS/SPE-NMR

1. INTRODUCTION

The rhizome of *Smilacis glabrae* acts as an important role in functional food and traditional Chinese mecicines, which was used for dissipating stasis, detoxification and diuresis [1]. Flavanonol, as the major components of *S. glabrae*, showed several biological activities, such as anti-oxidantion [2], anti-angiogenesis [3], anti-tumor [4], anti-inflammation [5] and anti-allergic activity [6]. In particular, astilbin 3'-O-methylastilbin, as the metabolite of the major component for astilbin of *S. glabrae*, was revealed its unique immunosuppressive activity against contact dermatitis [7]. According to their biological activities, it is of great significance to determine flavanonols in *S. glabrae*. The approaches applied to determine the major components in *S. glabrae* mainly focused on capillary electrophoresis [8], HPLC [9], HPLC-MS [10].

The HPLC-NMR or HPLC-SPE-NMR were applied to analyze plant and herbal medicines, for instance, Acevedo De la Cruz A1 *et al*. identified anthocyanin in *vitis* species using LC-MS and LC-NMR [11], LC-NMR, NMR, and LC-MS identification and LC-DAD approaches were used to identified and quantified of flavonoids and ellagic acid derivatives in *Drosera peltata* [12], Identification of lignans and related compounds in *Anthriscus sylvestris* by LC-ESI-MS/MS and LC-SPE-NMR [13].

In this study, an integrated platform for on line UHPLC-Q-TOF-MS/SPE-NMR presents a significant development and has several advantages compared to previous HPLC-MS, HPLC-NMR or HPLC-SPE-NMR systems:

1) The separation efficiency of UHPLC was higher than that of HPLC;
2) The separated fraction was split into two parts, one directly injected into high resolution Q-TOF-MS and another one accumulated on solid-phase extraction (SPE) cartridges;
3) Analytes eluting from the UHPLC were enriched on SPE cartridges under multiple trapping technology;
4) The SPE mediated solvent exchange from UHPLC grade solvents to the deuterated NMR solvent reduced the need of residual solvent suppression to a minimum;
5) The deuterated eluted from SPE were directly transferred into CryoFit and analyzed using NMR with CryoProbe.
6) Using integrated on line UHPLC-Q-TOF-MS/SPE-NMR was fast and high efficient, both of qualitative and quantitative analyses.

2. MATERIALS AND METHODS

Chemical and reagents

LC/MS grade methanol and water were purchased from J. T. Baker (USA). LC/MS grade formic acid and ammonium format were purchased from Sigma Aldrich Laboratories, Inc. (St. Louis, MO, USA). Deuterated acetonitrile was purchased from Sigma-Aldrich (USA). Other reagents were of analytical grade and purchased from ACS (1510 Eldridge Parkway, Houston, TX 77077, USA).

Instrumental

UHPLC-SPE-NMR-MS system: An Agilent 1290 liquid chromatography (Agilent Technologies, Germany) consisting of an autosampler, thermostatted column compartment and binary pump were equipped with an UV detector, Waters Symmetry C18 column (250 × 4.6 μm, 5 μm, Waters, USA) used as separation column, a maxis impact Q-TOF-MS with an electrospray ionization source (Bruker Daltonics, Germany). A Prospekt SPE system (Bruker Topspin, Germany) was hyphenated to UHPLC, and Bruker ASCEND 600 MHz spectrometer (Bruker Topspin, Germany) equipped with CryoProbe and CryoFit (Bruker Topspin, Germany).

Sample preparation

S. glabrae Roxb obtained from drugstore for Lei Sang Tong drugstore in Macau. The dried *S. glabrae* (8.6mg) was extracted with 60% aqueous methanol (3×1.0ml, 1hour each) under ultrasonic at room temperature. Then the extracts were combined. After being evaporated until remaining 1.0ml of water, and then extracted with petroleum ether and acidic ethyl acetate to obtain two fractions. And the flavonoids were mainly in the ethyl acetate fraction. Then, this fraction was dissolved in 100μl methanol and the solution was centrifuged at 15,000 rpm/min to injection for UHPLC-Q-TOF-MS/SPE-NMR analyses.

UHPLC-Q-TOF-MS/SPE-NMR analyses

Waters C18 column was used to separate the prepared sample within 30 min, 0.1 % of formic acid and 5 mM ammonium format in water and methanol were used as mobile phase A and B, respectively, the gradient program was as follow: 0-1min, 5% B; 1-1.5min, the linear gradient from 5% to 15% B; 1.5-30min, the linear gradient from 15% to 35% B; 30-35min, the linear gradient from 35% to 95% B; 35-36min, 95% B; 36-36.5min, return to 5% B.

The injection volume was 10 μL, among of them, 95% were split into SPE as well as 5% into MS after separated by UHPLC. The wavelength of UV detector set at 280nm, and high resolution ESI-Q-TOF-MS and MS2 was performed to analyze the major peaks under this wavelength. Meanwhile, the enriched separated fractions using SPE multiple trapping technology were dried under nitrogen evaporator for 45min, then, eluted by deuterated acetonitrile, and directly transferred into CryoFit and analyzed ^{1}H and ^{1}H-^{1}H COSY using NMR with CryoProbe.

3. RESULTS

Results of UHPLC-UV-SPE

The major bioactive components of *S. glabrae* are astilbin and its stereoisomers for neoastilbin, isoastilbin, neoisoastilbin, it's time-consuming to separate well [9]. In order to trap them well using SPE cartridge, these components should be separated by baseline. In UHPLC-UV spectrum (Figure 1), the ten major peaks were obtained under wavelength of UV at 280nm within 25min. The peaks were separated well by baseline, sequentially split into 10 partitions and multiple trapped for 5 times using SPE and analyzed using Q-TOF-MS, simultaneously.

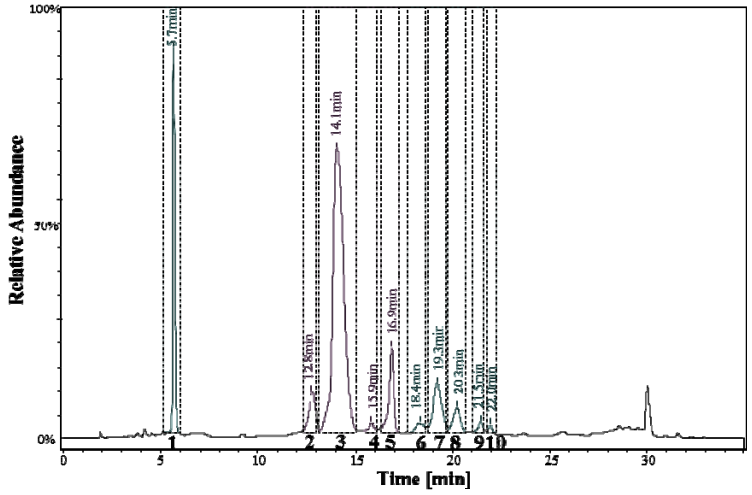

Figure 1. UHPLC-UV (280nm) chromatogram and its major 10 peaks.

Results of UHPLC-Q-TOF-MS and MS^{2}

The separated peaks were analyzed by Q-TOF/MS, the peak 2, 3, 4, and 5 with same m/z at 449.1310 and their retention time were 12.8, 14.1, 15.9 and 16.9min, respectively. It was elucidated as $C_{21}H_{22}O_{11}$ using Compass DataAnalysis software (Bruker Daltonics, Germany) and mass difference of the $[M-H]^{-}$ ions for 449.1310 was less than 1ppm (0.78ppm) between calculated and obtained m/z, as well as the other four isotopic ions were less than 4ppm. The MS/MS spectra at 303.0623, 285.0490 and 179.0044 were elucidated as loss of a [glycoside–O], [glycoside] and [glycoside+ring B], respectively (Figure 2). Thus the peaks of m/z at 449.1310 were preliminary elucidated as astilbin and its stereoisomers.

The peaks of retention time at 18.4 and 19.3 min with same $[M-H]^{-}$ ions at 433.1273 ($C_{21}H_{21}O_{10}$) in mass spectra, the characteristic neutral losses of 146 Da for a rhamnose at m/z 287.03 and other fragment ions observed at m/z 269.02 and 259.03 were almost identical those in previous reported [14]. Then, they were preliminary identified as engeletin and its isomer. The same method was used for the peak with retention time at 20.3min and observed $[M-H]^{-}$ at m/z 451.1062 was originally identified as cinchonain.

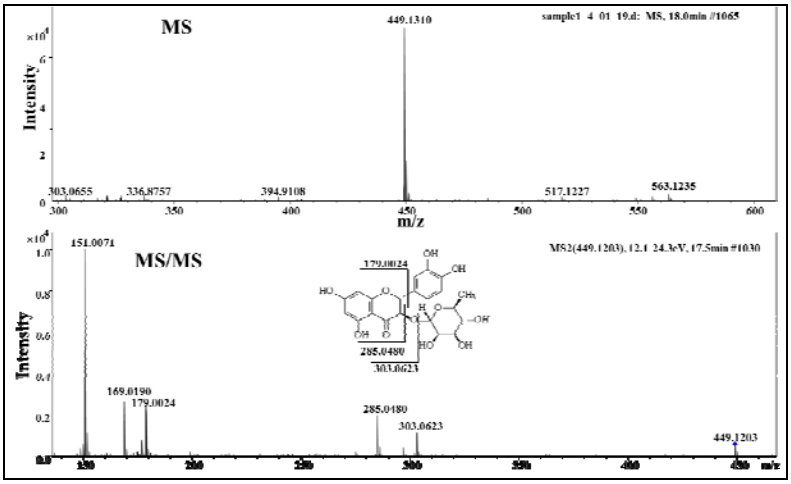

Figure 2. MS and MS/MS of peak 2, 3, 4 and 5 and their molecular formula were elucidated as $C_{21}H_{22}O_{11}$ and astilbin as well as its stereoisomers.

Results of SPE-NMR

The ^{1}H NMR and ^{1}H-^{1}H COSY spectra of peak 2 to peak 8 were analyzed using SPE-NMR with CryoFit, comparison with standard of astilbin, the peak 3 was identified as astilbin firstly (Figure 3), since peak 2, 4 and 5 were preliminary elucidated as stereoisomers of astilbin, comparing with literature [15], the peak 2, 4 and 5 were further confirmed as neoastilbin, neoisoastilbin, isoastilbin. Using same methods, peak 6, 7 and 8 were identified as engeletin, isoengeletin and cinchonain, respectively.

The quantitative analyses of these major flavanonols were performed by quantitative NMR, in briefly, using tetramethylsilane as internal standard, chosen H-2 and/or H-3 (chemical shift from 4.0 to 5.5ppm) as quantitative peaks, using same NMR parameters, comparing with the peak area of tetramethylsilane, the amounts of the major flavanonols were estimated.

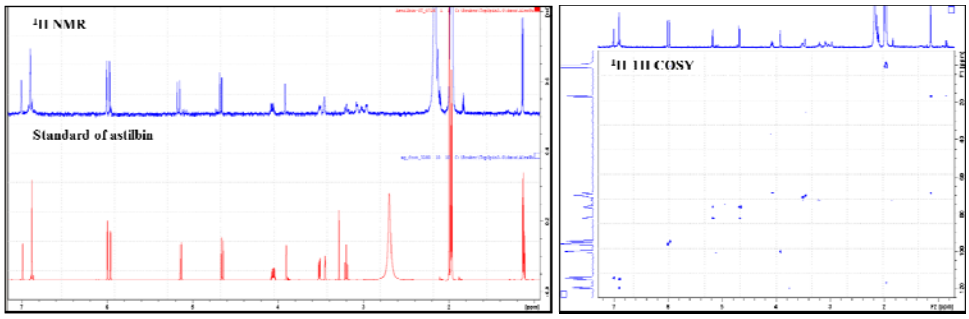

Figure 3. ^{1}H NMR spectra of peak 3 and standard of astilbin (left), ^{1}H-^{1}H COSY of peak 3 (right).

4. CONCLUSIONS

Using the approach of on line integrated UHPLC-Q-TOF-MS/SPE-NMR, the major flavanonols in *S. glabrae* were separated by UHPLC, and identified using UV, mass spectrometry, and NMR simultaneously, and the major flavanonols were fast identified and quantified without standards.

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Effect of solvents on total phenolic compounds and antibacterial activity of *Ceriops tagal* extracts

Luksamee Vittaya*[a], Chakhriya Chalad[a], Thunwadee Ritthiwigrom[b]
[a]Faculty of Science and Fisheries Technology, Rajamangala University of Technology Srivijaya, Trang, Thailand 92150
[b]Chemistry Department, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200

*nokluksamee@hotmail.com phone +66-74204064 fax +66-74204064

ABSTRACT

Ceriops tagal is a mangrove plant in RHIZOPHORACEAE family which plays an importance role in coastal ecological balance and exhibit versatile pharmacological area. In this study, leaves and barks were collected from Rajamangala beach, Rajamangala University of Technology Srivijaya, Trang province and were dried to successively extract with hexane, ethyl acetate and methanol. The total phenolic content was evaluated by Folin-ciocaltue method. The results showed that methanolic barks extracts exhibited the highest total phenolic content values 6.063 ± 0.205 mg GAE/g extract. The antimicrobial activities against *Bacillus cereus* TISTR 687, *Escherichia coli* TISTR 780, *Staphylococcus aureus* TISTR 1466, and *Samolnella typhi* TISTR 292 of the leaf and bark extracts were determined using paper disk diffusion method. The methanol extracts from the bark exhibited the best activity. None of the extracts showed antibacterial activity against *E*.coli.

Keywords: Total phenolic, Antibacterial, Mangrove, Extracts, Disk diffusion, *Ceriops tagal*

1. INTRODUCTION

Thailand has several kinds of medicinal plants which have been used to treat many diseases for a long time. Among these potential Thai plants, *Ceriops tagal* are medicinal mangrove plants which widely use as Thai traditional medicine for therapy. It is traditional Thai plants from Rhizophorceae family and generally dominant interface ecosystems between the land and the sea in the tropical forest. It is important in the economy of those regions in terms of mangrove-linked fisheries and forestry. Almost parts of both plants can be used in traditional medicine for treatment of various ailments, for example, anti-inflammability [1], anticancer activity [2-3], induced antidiabetes in rats from leaves extract [4]. However, there are quite a few data on antimicrobial activity of *C. tagal* [5]. Therefore, we have investigated the antimicrobial activity of hexane, ethyl acetate and methanolic extracts from leaf and barks of *C. tagal* against *Bacillus cereus* TISTR 687, *Escherichia coli* TISTR 780 *Staphylococcus aureus* TISTR 1466 and *Samolnella typhi* TISTR 292. The correlation between antimicrobial and total phenolic compounds was also study.

2. MATERIALS AND METHODS

Plant identification

C. tagal were collected from Rajamangala beach, Rajamangala University of Technology Srivijaya, Trang province which located in the southern part of Thailand in September, 2012.

Plant material and extraction procedures

Both dried parts of *C. tagal* was collected and were successively extracted in solvents by increasing polarity organic solvents as hexane, ethyl acetate, and methanol over a period of seven days each at room temperature. Crude extracts were acquired by concentrating the extract under reduced pressure.

Determination of total phenolic content

The content of total phenolic compounds of leaf and barks crude extracts were determined using the Follin-Ciocalteau method which is modified by Miliauskas *et al* [6]. A standard curve was plotted using gallic acid as standard. Ten milligram of each samples and standard was diluted with methanol to the final volume of 0.5 mL. Then, 0.25 mL of the Follin-Ciocalteau reagent and 1.25 mL of Na_2CO_3 solution (20%) were added in each tube, respectively. The tubes were vortexed and the absorbance of all samples and standard were measured at 725 nm using a UV-vis spectrophotometer after 40 min. Total phenolic content were expressed as gallic acid equivalent (GAE) calculated from the calibration curve.

Antimicrobial screening

Antimicrobial activity of leaf and barks of both plants were performed using a paper disk diffusion method which modified from National Committee for Clinical Laboratory Standards (NCCLS) [7]. *Bacillus cereus* TISTR 687 *Escherichia coli* TISTR 780 *Staphylococcus aureus* TISTR 1466 and *Samolnella typhi* TISTR 292 obtained from the National Center for Genetic Engineering and Biotechnology, Thailand, were used for antibacterial study. The bacterial culture media (Nutrient agar) was autoclaved for 20 min at 121°C and at 15 lb pressure before inoculation and then it was poured into a plate and allowed to solidify. The bacteria were incubated in Nutrient broth (NB) at 36°C for 24 h. Turbidity was adjusted at 0.5 McFarland standard (10^8 CFU/ml)and was swabbed over the surface of media using a sterile cotton swab to ensure even growth of the organisms. The tested crude extracts were dissolved in dimethylformamide (DMSO) to give stock solutions of 100 mg mL^{-1}. Sterile filter paper discs were measured as 6 mm in diameter (Whatman No. 1 filter paper), before placed on the solidified nutrient agar medium and 10 μL of tested crude extracts were then placed on sterile filter paper discs. After overnight incubation for 24 h at 37°C, the zones of inhibition were measured in mm and compared with standard antibiotics (gentamicin and penicillin). All experiments were repeated three times and the average values are presented.

3. RESULTS

Sample of leaf and bark of this plant were died and successively extract with hexane, ethyl acetate and methanol to obtain six extracts. Total phenolic content of extracts was determined by Folin-Ciocalteu method and the data was shown in Table 1. Total phenols of *C. tagal* extracts were calculated according to the equation $y = 0.005x + 0.008$ ($R^2 = 0.992$) as gallic acid equivalent (GAE, mg/g dry material).

Table 1. Tatal phenolic content of crude extracts of *C. tagal*.

Parts	*C. tagal* extracts (mg of GAE/g)		
	Hexane extract	Ethy acetate extract	Methanol extract
Leaf	0.227±0.033	0.385±0.031	1.130±0.039
Bark	0.301±0.034	4.681±0.051	6.063±0.205

Total phenolic content show value between 0.227 to 6.063 mg GAE/g of the dried material in various extracts. In regard to amount of phenolic in leaf and bark of *C. tagal* crude extract, methanolic extract contained the higher content of phenolics (1.130 and 6.063 mg QE/g of the dried material), compared to ethyl acetate extracts (0.385 and 4.681 mg QE/g of the dried material) and hexane extract (0.227 and 0.301 mg QE/g of the dried material). It is hypothesized that tannin present in leaf and bark of *C. tagal* play an important role in the biological activity [8]. Moreover, the difference in the antibacterial activity of extracts may be attributed to the difference in the total phenolic content. Antibacterial activities in various solvents of leaf and barks of *C. tagal* were carried out (Table 2) and represented in Figure 1.

Table 2. The antimicrobial activities of crude extracts of *C. tagal*.

Part used	solvents	Zone of inhibition diameter (mm)			
		B. cereus	*E. coli*	*S. aureus*	*Salmonella*
leaf	H	8.66 ± 0.14	-	8.76 ± 0.85	6.27 ± 0.13
	E	8.33 ± 0.43	-	8.73 ± 0.69	7.78 ± 0.36
	M	9.78 ± 0.32	-	9.25 ± 0.84	9.47 ± 0.07
bark	H	10.61 ± 0.95	-	10.50 ± 0.38	10.58 ± 0.80
	E	11.89 ± 0.99	-	12.80 ± 0.68	11.70 ± 0.27
	M	12.39 ± 0.55	-	13.37 ± 0.97	12.14 ± 0.63

Values are mean ± SD; n = 3
H = hexane, E = ethyl acetate, M = methanol

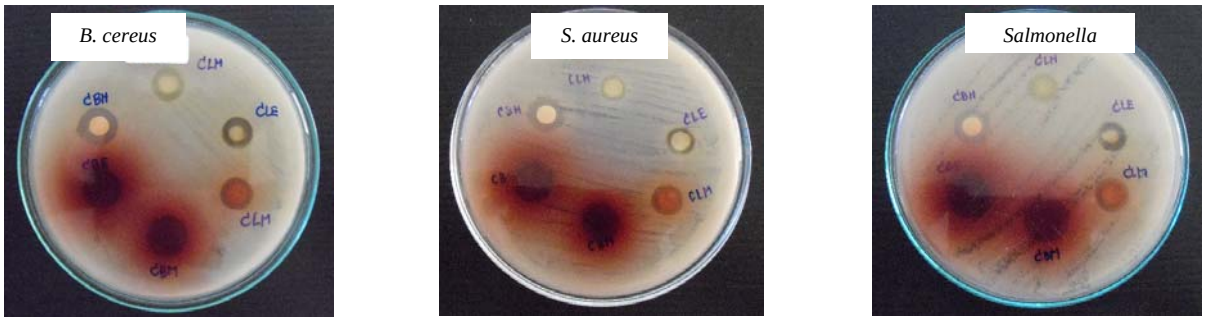

Figure 1. Antimicrobial activity of crude extracts to *B. cereus* (left) *S. aureus* (middle) and *Salmonella* (Right). CBH, CBE and CBM are bark of *C. tagal* extracted by hexane, ethyl acetate and methanol, respective. CLH, CLE, CLM are leaves of *C. tagal* extracted by hexane, ethyl acetate and methanol, respective. The technique were performed by disk diffusion method

The results revealed that the extracts were active against *B. cereus, S. aureus, Samonella typhi* exceptionally *E. coli* with their inhibition zones ranged 6.27-13.37 mm. In addition, the results indicated that barks of hexane, ethyl acetate, and methanolic extracts have greater antibacterial activities against the tested microorganisms compared to all leaf extracts. Moreover, the crude extracts of *C. tagal* leaves and bark active against only *B. cereus, S. aureus* and *Samonella typhi* and did not show any active for *E. coli*. This is explained that *E. coli* has outer membrance and periplasmic space having lopopolysaccharide which put up extracts across permeable membrance [9].

4. CONCLUSIONS

The results obtained from this study showed that the leaf and barks crude extracts of *C. tagal* inhibitory effect against *B. cereus* TISTR 687, *S. aureus* TISTR 1466 and *Salmonella* except *E. coli* TISTR 780. Therefore, this mangrove plant may has potential as antibacterial activity. In addition, the determination of total phenolic content related to antimicrobial activity of *C. tagal* leaf and barks in hexane, ethyl acetate, and methanol solvents.

ACKNOWLEDGEMENTS

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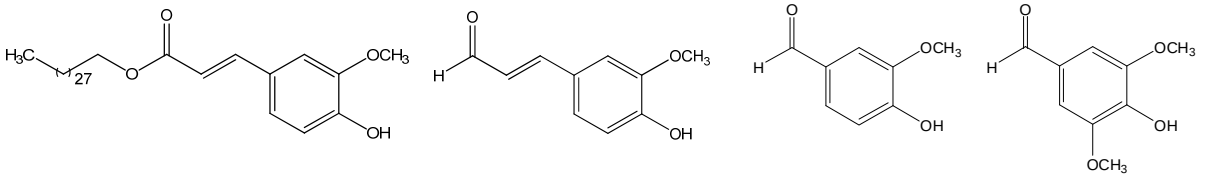
Chemical Constituents from the Stem Bark of *Fagraea fragrans* Roxb.

Suwaibah Madmanang[a], Suda Chakthong*[a]
[a]Department of Chemistry, Faculty of Science and Natural Product Research Center of Excellence, Prince of Songkla University, Hat Yai, Songkhla, Thailand 90112

*suda.ch@psu.ac.th phone +66-74288425

ABSTRACT

Fagraea fragrans Roxb. is a plant in the Gentainaceae family. This plant grows abundantly in Southeast Asia. It is known locally as Tammusu or Temmusu. F. *fragrans* has been used in the folk medicine. The isolated compounds from this plant were reported to show antimalarial, anti-HSV-1 and mild antimycobacterial activities. In the present work we investigated the chemical constituents of the crude methylene chloride extract from stem bark of F. *fragrans*. Four aromatic derivatives, (2*E*)-nonacosyl ester (1), cinnamaldehyde (2), vanillin (3) and syringaldehyde (4), were isolated. Their structures were elucidated by analysis of spectroscopic data, including comparison of data with those previously reported.



(1) (2) (3) (4)

Keywords: *Fagraea fragrans* Roxb., (2*E*)-Nonacosyl ester, Cinnamaldehyde, Vanillin, Syringaldehyde

1. INTRODUCTION

Fagraea fragrans Roxb. is a tall tree, 8 - 30 meter in height. This plant grows sparsely in southern and northeastern parts of Thailand, where it is called Kankrao (central), Tamsao or Thamsao (southern) and Man Pla (northern and northeastern). The medicinal uses of this plant have been reported in various regions where it grows. In Malay folk medicine, a decoction of the leaves and twigs of *F. fragrans* is used for the treatment of dysentery. The bark is believed to have medicinal value for malaria. The decoction of the bark is used to treat malaria in India, Cambodia, and Malaysia and as a febrifuge in Philippines. In Thai traditional medicine, it is believed that leaves contain antimalarial, element tonic, and antiasthmatic agents, and are externally used for mild infectious skin diseases, while an aqueous extract of the stems is used as a remedy for coughs [1-2]. In this report, we investigated the constituents of *F. fragrans* Roxb. and described the isolation and characterization of four aromatic derivatives.

2. MATERIALS AND METHODS

Plant material

The stem bark of *F. fragrans* Roxb. was collected from Satun province, in the southern of Thailand, in May 2012.

General Experimental Procedure

Quick column chromatography and column chromatography were carried out on silica gel 60 (230-400 Mesh ASTM, Merck) and silica gel 100 (70-230 Mesh ASTM, Merck), respectively. Aluminum Sheets of silica gel 60 F_{254} (Merck) were used for thin layer chromatography (TLC) and preparative thin-layer chromatography (PTLC), glass plates of silica gel 60 F_{254} (20×20 cm, 0.2 mm, Merck) were used for analytical purposes and the compounds were visualized under the ultraviolet light. Solvents for extraction and chromatography were distilled at their boiling ranges prior to use. The IR spectra were measured with a Perkin-Elmer FT-IR spectrophotometer. The ^{1}H- and ^{13}C-Nuclear magnetic resonance spectra were recorded on a FT-NMR Bruker Ultra Shield^{TM} 300 MHz spectrometer. Spectra were recorded in deuterochloroform ($CDCl_3$) and recorded as δ values in ppm downfield from TMS (internal standard δ 0.00). The Ultraviolet spectrums were measured with a Hewlett-Packard 8453. Melting point was recorded in °C on a digital Electrothermal 9100 Melting point apparatus. The EIMS mass spectra was recorded using Mass Spectrometer, MAT 95 XL, Thermo Finnigan, Germany at the Scientific Equipment Center, Prince of Songkla University.

Extraction and Isolation

The dried stem bark of *F. fragrans* (4.10 kg) was extracted successively three times with methylene chloride over a period of 3 days at room temperature. After evaporation, a dark brown gum of the methylene chloride extract (49.0 g) was separated by quick column chromatography using gradient solvent systems of hexane, hexane-methylene chloride, methylene chloride-methanol and finally with pure methanol. On the basis of their TLC characteristics, the fractions which contained the same major components were combined to give thirteen fractions (T1-T13). Fraction T3 (3.32 g) was separated by column chromatography on silica gel 100 and eluted with methylene chloride:hexane (4:6 v/v) to obtain fractions T3A-T3I. Fraction T3E was further purified by column chromatography on silica gel 100 and eluted with ethyl acetate:hexane (1:9 v/v) as an eluent to give a white solid of **1** (57.1 mg). Fraction T4 (2.81 g) was separated by column chromatography on silica gel 100 and eluted with ethyl acetate:hexane (1:9 v/v) to obtain fractions T4A-T4K. Fraction T4I (80.6 mg) was further purified by column chromatography on silica gel 100 and eluted with methylene chloride:hexane (9:1 v/v) as an eluent to give a yellow crystal of **3** (3.0 mg). Fraction T4J (102.5 mg) was further purified by column chromatography on silica gel 100 and eluted with acetone:methylene chloride (1:9 v/v) to obtain fractions T4J1-T4J5. Fraction T4J3 (21.1 mg) was further purified on preparative TLC and eluted with acetone:methylene chloride:hexane (1:4:5 v/v) to give a yellow powder of **2** (2.5 mg). Fraction T5 (4.90 g) was separated by column chromatography on silica gel 100 and eluted with methylene chloride:hexane (8:2 v/v) to obtain fractions T5A-T5L. Fraction T5F (275.6 mg) was further purified by column chromatography on silica gel 100 and eluted with acetone:hexane (1:9 v/v) as an eluent to give a yellow crystal of **4** (54.6 mg).

Compound 1 a white solid; m.p. 76.0-78.0ºC; EIMS (*m/z*) = 600; UV λ_{max} (MeOH) (log ε): 218 (3.16), 227 (3.10) and 324 (2.94) nm; IR (neat): 3518, 1701 and 1630 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 7.61 (1H, *d*, *J* = 15.9 Hz, H-1′), 7.08 (1H, *dd*, *J* = 8.1, 1.9 Hz, H-6), 7.04 (1H, *d, J* = 1.9 Hz, H-2), 6.91 (1H, *d, J* = 8.1 Hz, H-5), 6.29 (1H, *d, J* = 15.9 Hz, H-2′), 6.01 (H, *brs*, 4-OH), 4.19 (2H, *t*, *J* = 6.7 Hz, H-1″), 3.91 (3H, *s*, 3-OCH_3), 1.69 (2H, *t*, *J* = 6.7 Hz, H-2″), 1.25-1.47 (52H, *m*, H-3″-H-28″), 0.90 (3H, *t*, *J* = 6.7 Hz, H-29″); ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 14.1

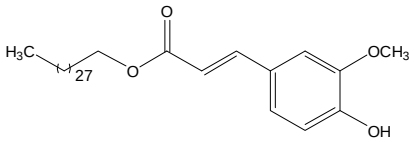
(C-29″), 22.7-32.8 (C-3″-C-28″), 26.0 (C-2″), 55.9 (3-OCH_3), 64.6 (C-1″), 109.3 (C-2), 114.7 (C-5), 115.6 (C-2′), 123.0 (C-6), 127.0 (C-1), 144.7 (C-1′), 146.8 (C-3), 147.9 (C-4), 167.4 (C-3′).

Compound 2 a yellow powder; m.p. 65.0-66.0ºC; UV-Vis (MeOH) λ_{max} (nm) (log ε) 201 (3.02), 221 (2.98) and 339 (3.09) nm; IR (neat): 3431, 1653 and 1628 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 9.65 (1H, *d*, *J* = 7.7 Hz, H-3′), 7.41 (1H, *d*, *J* = 15.8 Hz, H-1′), 7.13 (1H, *dd*, *J* = 8.2, 1.9 Hz, H-6), 7.07 (1H, *d*, *J* = 1.9 Hz, H-2), 6.96 (1H, *d*, *J* = 8.2 Hz, H-5), 6.60 (1H, *dd*, *J* = 15.8, 7.7 Hz, H-2′), 6.00 (1H, *brs*, 4-OH), 3.95 (3H, *s*, 3-OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 55.9 (3-OCH_3), 109.5 (C-2), 114.9 (C-5), 124.0 (C-6), 126.5 (C-2′), 126.7 (C-1), 147.0 (C-3), 149.0 (C-4), 152.9 (C-1′), 193.5 (C-3′).

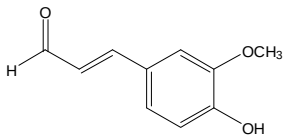
Compound 3 a yellow crystal; m.p. 78.0-79.0ºC; UV λ_{max} (MeOH) (log ε): 206 (3.38), 230 (3.39) and 307 (3.20) nm; IR (neat) : 3420, 1652 and 1540 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 9.83 (1H, *s*, H-7), 7.44 (1H, *d*, *J* = 1.8 Hz, H-2), 7.42 (1H, *dd*, *J* = 8.5, 1.8 Hz, H-6), 7.04 (1H, *d*, *J* = 8.5 Hz, H-5), 3.97 (3H, *s*, 3-OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 56.1 (3-OCH_3), 108.8 (C-2), 114.4 (C-5), 127.6 (C-6), 129.9 (C-1), 147.2 (C-3), 151.7 (C-4), 191.0 (C-7).

Compound 4 a yellow crystal; m.p. 117.0-118.0 ºC; UV-Vis (MeOH) λ_{max} (nm) (log ε) 215 (3.36), 230 (3.31) and 307 (3.21) nm; IR (neat): 3425, 1689 and 1538 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ_H 9.82 (1H, *s*, H-7), 7.16 (2H, *s*, H-2, H-6), 3.98 (6H, *s*, 3, 5-OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ_C 56.5 (3, 5-OCH_3), 106.7 (C-2, C-6), 128.4 (C-1), 140.9 (C-4), 147.4 (C-3, C-5), 190.7 (C-7).

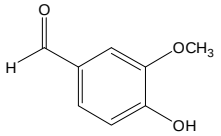
3. RESULTS



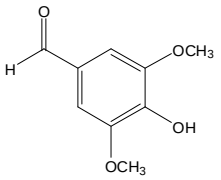
Compound 1 was obtained as a white solid. The UV spectrum exhibited the absorption bands characteristic of aromatic and carbonyl at 218, 227 and 324 nm. The IR spectrum showed absorption bands at 3518 (O-H stretching), 1701 (C=O stretching), and 1630 (C=C stretching) cm^{-1}. The EIMS mass spectrum afforded a signal for at *m/z* 600, consistent with the molecular formula $C_{39}H_{68}O_4$. In the ^{1}H NMR spectrum of compound **1** exhibited the signal of aromatic protons of a ferulic acid moiety at δ_H 7.08 (1H, *dd*, *J* = 8.1, 1.9 Hz, H-6), 7.04 (1H, *d*, *J* = 1.9 Hz, H-2) and 6.91 (1H, *d*, *J* = 8.1 Hz, H-5) which indicated the presence of a 1,3,4-trisubstituted benzene ring. A methoxyl singlet signal at δ_H 3.91 (3-OCH_3) and a hydroxy proton signal at δ_H 6.01 (*brs*, 4-OH) were proposed to be substituted at C-3 and C-4 position, respectively. The spectrum further showed the two *trans*-olefinic protons at δ_H 7.61 (1H, *d*, *J* = 15.9 Hz, H-1′) and 6.29 (1H, *d*, *J* = 15.9 Hz, H-2′). The large coupling constant of 15.9 Hz indicated *E*-configuration of H-1′ and H-2′, together with 29 carbon side-chain signals seen as signal of oxygenated methylene proton at δ_H 4.19 (2H, *t*, *J* = 6.7 Hz, H_2-1″), methylene proton at δ_H 1.69 (2H, *m*, H_2-2″) and 1.25-1.47 (52H, *m*, H_2-3″-H_2-28″) and one methyl proton at δ_H 0.90 (3H, *t*, *J* = 6.7 Hz, H_3-29″). These data corresponded to the feruloyl moiety with long chain hydrocarbon of 29 carbons. whose HMBC correlations of H-2 at δ_H 7.04 with the carbons at δ_C 147.9 (C-4), 123.0 (C-6) and 144.7 (C-1′). The proton signal at δ_H 6.91 (H-5) showed correlation with the carbons at δ_C 127.0 (C-1), 109.3 (C-2), 146.8 (C-3), and 147.9 (C-4) while the proton signal at δ_H 7.08 (H-6) showed correlation with the carbons at δ_C 147.9 (C-4), 144.7 (C-1′) and 109.3 (C-2). A methoxy proton signal at δ_H 3.91 showed correlation with the carbon at δ_C 146.8 (C-3) suggested this methoxyl group was located at C-3, and the hydroxyl group at δ_H 6.01 showed correlation with the carbons at δ_C 147.9 (C-4) and 114.7 (C-5) suggested this hydroxyl group at C-4 of benzene ring. The olefinic proton at δ_H 7.61 (H-1′) showed correlation with the carbons at δ_C 127.0 (C-1), 109.3 (C-2), 123.0 (C-6), 115.6 (C-2′) and 167.4 (C-3′). The olefinic proton at δ_H 6.29 (H-2′) showed correlation with the carbons at δ_C 127.0 (C-1), 144.7 (C-1′) and 167.4 (C-3′). The ester linkage was confirmed by the HMBC correlations of H_2-1″ (δ_H 4.19) and H-2′(δ_H 6.29) to the ester carbonyl carbon at δ_C 167.4 (C-3′). Thus, the structure of compound **1** was identified as (2*E*)-nonacosyl ester [3].



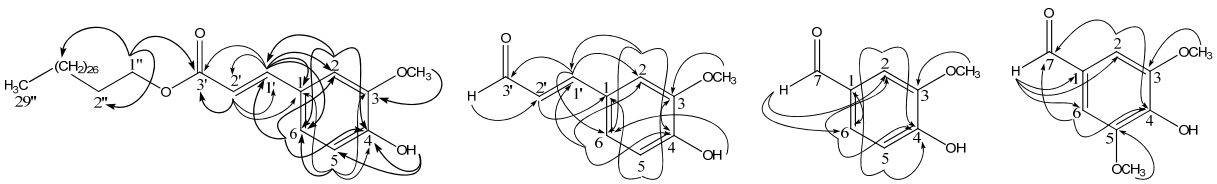
Compound 2 was obtained as a yellow powder. The UV spectrum exhibited the absorption bands characteristic of aromatic and carbonyl at 201, 221 and 339 nm. The IR spectrum showed absorption band at 3431 (O-H stretching) and 1653 (C=O stretching) and 1628 (C=C stretching) cm^{-1}. The ^{1}H NMR spectrum of compound **2** showed a signal pattern similar to those of compound **1**. The differences were shown as the absence of signals (*E*) nonacosyl as in compound **1** but the presence of a singlet signal of aldehyde group at δ_H 9.65. In the HMBC correlation spectrum, the olefinic proton at δ_H 6.60 (H-2′) showed correlation with the carbon at δ_C 126.7 (C-1) and another olefinic proton at δ_H 7.41 (H-1′) showed correlation with the carbons at δ_C 109.5 (C-2), 124.0 (C-6) and aldehyde carbon at δ_C 193.5 (C-3′), indicating the attachment of a propenal group at C-1. Thus on the basis of its spectroscopic data and comparison with the previously reported therefore compound **2** was identified as cinnamaldehyde [4].



Compound 3 was obtained as a yellow crystal. The UV spectrum exhibited the absorption bands characteristic of aromatic and carbonyl at 206, 230 and 307 nm. The IR spectrum showed absorption band at 3420 (O-H stretching), 1652 (C=O stretching) and 1540 (C=C stretching) cm^{-1}. The ^{1}H NMR spectrum of compound **3** showed a signal pattern similar to those of compound **2**. The differences were the absence of propenal signals but the presence of the aldehydic proton at δ_H 9.83, δ_C 191.0. The formyl group (δ_H 9.83) was assigned to be at C-1 (δ_C 129.9) due to HMBC correlations between the carbons at δ_C 108.8 (C-2) and 127.6 (C-6). Therefore compound **3** was identified as vanillin [4].



Compound 4 was obtained as a yellow crystal. The UV spectrum exhibited the absorption bands characteristic of aromatic and carbonyl at 215, 230 and 307 nm. The IR spectrum showed absorption band at 3425 (O-H stretching), 1689 (C=O stretching) and 1538 (C=C stretching) cm^{-1}. The ^{1}H and ^{13}C NMR spectral data of compound **4** were similar to those of compound **3**. The differences were the absence of signals for aromatic ABX system but the presence of a singlet signal of two aromatic protons at δ_H 7.16, suggesting the presence of a symmetrical 3,4,5-trisubstituted benzaldehyde. The formyl group at δ_H 9.82 was assigned to be at C-1 due to HMBC correlations between the carbons at δ_C 106.7 (C-2, C-6) and 128.4 (C-1). The proton signal at δ_H 7.16 (H-2, H-6) showed correlations with the carbons at δ_C 128.4 (C-1), 147.4 (C-3, C-5), 140.9 (C-4) and 190.7 (C-7). The two methoxyl groups at δ_H 3.98 (6H) showed HMBC correlations with the carbon at δ_C 147.4 (C-3 and C-5), indicating the location of these two methoxyl groups at C-3 and C-5, respectively, leaving the hydroxyl group at C-4. Therefore compound **4** was identified as syringaldehyde [5].



1 **2** **3** **4**

Figure 2. Major HMBC correlations of compounds **1-4** (H➔C).

4. CONCLUSIONS

The methylene chloride extract from the dried stem bark of *Fagraea fragrans* Roxb. was purified by chromatographic technique led to the isolation of four aromatic derivatives: (2*E*)-nonacosyl ester (**1**), cinnamaldehyde (**2**), vanillin (**3**) and syringaldehyde (**4**). Their structural elucidations were determined by spectroscopic data.

ACKNOWLEDGEMENTS

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Quantitative analysis of ligustilide in roots and stems of *Angelica sinensis* (Oliv.) Diels.

Pattra Ahmadi Pirshahid*[a], Thongchai Hemthanon[a], Yaowaluk Khamphan[a], Phatsuda Chueboonmee[a], Chuleratana Banchonglikitkul[a]

[a] Pharmaceutical and Natural Products Department,Thailand Institute of Scientific and Technological Research, 35 Moo 3, Klong 5, Klong Luang, Pathum Thani, Thailand 12120

*pattra_a@tistr.or.th phone +66-5779107 fax +66-5779107

ABSTRACT

Medicinal herb, *Angelica sinensis* (Oliv.) Diels. belongs to Umbelliferae family, is indigenous plant in the eastern country such as China, Korea and Japan. Northern part of Thailand, people always use *A. sinensis* as for cookong. In Asia, the plants have been cultivated for medicinal purpose and was recorded for treating a wide variety of female disorders. Currently, the popular use of *A. sinensis* is the treatment of menopausal symptoms, hot flashes, promote a healthy menstrual cycle and painful during menstruation. Ligustilide is the most abundant active component in *A. sinensis* and was reported that the therapeutic pharmacological activity is most often attributed to the Ligustilide content. In this paper, the quantitative analysis of Ligustilide in the ethanol extract of separated whole root (RE) and aerial stems including leaves (SE) of *A. sinensis* are investigated. The result of Ligustilide analysis will be used as a marker compounds and could be served as a fingerprint for quality control of *A. sinensis*. The content of Ligustilide can lead us to utilize the *A. sinensis* extract as a dietary supplement products for hormone replacement from both whole root and aerial stems including leaves. This analysis had performed by High Performance Liquid Chromatography, method validation; specificity, linearity, range, Limit of Detection (LOD). Limit of Quantitation (LOQ), precision as well as accuracy (%recovery) are reported.

Keywords: *Angelica sinensis* (Oliv.) Diels, Ligustilide, Menopause

1. INTRODUCTION

The deficiency of estrogen hormone is one of the main causes of women menopause such as sweating, mood swing, hot flashes [1] and contributes to adverse health problems such as osteoporosis or the loss of bone density [2]. Hormone replacement therapy (HRT) has been shown as an effective method in relieving menopause symptoms [3]. However, HRT is generally not recommended due to the risk of breast cancer [4, 5]. Medicinal plants are a natural alternative to conventional HRT to treat menopause [6]. In traditional Chinese medicine, the hot water extract of the herb mixture of *Radix Astragali* and *Radix Angelica sinensis* (Oliv.) Diels. has been used to treat blood circulation and immune system [7]. Health benefits of the herbal extract are attributed to active compounds found in *A. sinensis*, mainly ligustilide that is largely responsible for the pharmacological activity [8]. Ligustilide is a bioactive marker for the authentication and standardization of *A. sinensis*. The objective of the present study was to develop a reversed-phase high performance liquid chromatography (RP-HPLC) method for quantitative determination of Ligustilide from the ethanol extracts of separated whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*. Validation of the analytical method was performed in termed of specificity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ).

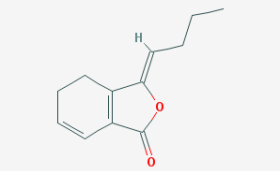

Figure 1. Structure of ligustilide ((3Z)-3-butylidene-4,5-dihydro-2-benzofuran-1-one)

2. MATERIALS AND METHOD

Materials

About six month old *Angelica sinensis* (Oliv.) Diels. plants were obtained in June 2013 from The Agricultural, Forest, Environmental Demonstration and Transfer Station Project at Baan Lee Sor, Paek Sam, Chiang Mai province. Commercial grade ethanol 95% was provided by The Liquor Distillery Organization. HPLC grade methanol and phosphoric acid were obtained from RCI Labscan Ltd. Ligustilide reference standard was purchased from Standlord Chemicals, Co., Ltd.

Preparation of plant material

The whole roots and aerial stems including leaves were separately rinsed with water, sliced into small pieces, and then oven-dried at 45°C for 15-24 h. The dried roots and stems were ground and stored in a plastic bag at room temperature for subsequent extraction.

Preparation of extracts

1 gm of the dried ground sample of each part (roots and stems) of *A.sinensis* was extracted with 95% ethanol under stirring at ambient temperature for 20 min. The solid was separated from the liquor by filtration, followed by re-extraction for 4 times. The filtered solution was combined together, and then evaporated under vacuum at 40°C to yield the crude extracts of roots (RE) and stems (SE) of *A. sinensis.*

HPLC analysis

Chromatographic analysis was performed with a Waters 600 controller equipped with a Waters 486 tunable absorbance detector. Data were analyzed using Clarity chromatography software (Data Apex, Czech Republic). Chromatographic analysis was performed with a Waters Xterra reversed-phase C_{18} column (5 µm, 3.9 x 150 mm) with a mobile phase gradient consisting of acetronitrile and 0.05% phosphoric acid (pH 5.0) at a flow rate of 1 ml/min and a detection wavelength at 288 nm.

Preparation of standard solution

An accurate weight of 5 mg of ligustilide reference standard was transferred into a 25 ml volumetric flask, sonicated for 15 min and adjusted to the final volume of 50 ml with methanol. The stock solution was then diluted to concentrations of 10, 20, 30, 40, 50 and 60 µg/ml. These serial different dilutions were filtered through 0.2 µm cellulose membrane before use.

Preparation of sample solutions

An accurate weight of 0.1 g each of RE and SE of *A.sinensis* was dissolved with 20 ml methanol in a 50 ml volumetric flask, and sonicated for 15 min. The mixture was then adjusted to the final volume of 50 ml with methanol. The solution was filtered through a 0.45 µm cellulose membrane prior injects to HPLC.

Validation of HPLC method

The validation of HPLC method was examined in terms of specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

Specificity

The standard Ligustilide solution and sample solutions of RE and SE were prepared in acetronitrile. A volume of 10 µl was injected into the HPLC column individually.

Linearity

The linearity was validated by preparing the standard ligustilide solutions at least 5 concentrations. A volume of 10 µl of each concentration was injected to the HPLC column. Triplicate determinations were performed. The standard curve was analyzed using the linear least-squares regression equation derived from the peak area.

Precision and sample preparation

The precision of the analytical method was validated by determining the content of ligustilide in RE and SE of *A. sinensis*. Concentrations of the standard ligustilide were calculated with the linear equation of standard curve. Triplicate determinations were performed. Coefficient of variation (CV) was calculated as a standard deviation (SD) to the mean value from the results obtained and not more than 2%.

Accurately weighted 0.1 g of RE in a volume flask 50 ml for 6 samples sonicated for 15 min then adjusted to final volume with methanol and filter through cellulose membrane 0.2 µm prior to HPLC.

Accuracy and sample preparation

The standard ligustilide with the determined amount was spiked to the RE sample solution. Triplicate injections for each concentration were performed. Recovery (%) of the standard ligustilide was calculated.

Accurately weighted 0.1 g of RE for 9 samples sonicate for 15 min, added standard ligustilide at concentration 20, 40 and 60 ppm to each 3 sample of RE respectively, then adjust to final volume of 50 ml by methanol , filter through 0.2 µm cellulose membrane prior to HPLC injection for triplicate.

LOD and LOQ

For the limit of detection (LOD) and limit of quantification (LOQ), serial dilutions of ligustilide were prepared and analyzed using the HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

Figure 2. *Angelica sinensis* (Oliv.) Diels.

3. RESULTS

Specificity validation

HPLC chromatograms for specificity validation are shown in Figure 3. Ligustilide was identified as the main compound in the ethanol extracts of the whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*, with retention time of 32.2 min.

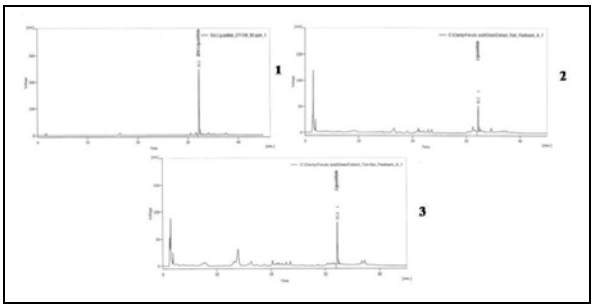

Figure 3. HPLC chromatograms for specificity validation: (1) ligustilide reference standard; (2) RE and (3) SE.

Linearity and Range

The standard curve of ligustilide was examined from the results of Table 1. The curve is linear at the concentrations range of 10-60 µg/ml. The linear equation of $Y = 28.34X + 21.04$ and correlation coefficient (R^2) of 0.999 were obtained as shown in Figure 4.

Table 1. Peak areas of standard ligustilide at the concentrations range of 10 to 60 µg/ml for the linearity

Concentration (µg/ml)	Peak area 1	Peak area 2	Peak area 3	Mean peak area	SD	% CV (%RSD)
10	299.84	299.702	295.545	298.362	2.441	0.818
20	588.83	589.408	585.109	587.782	2.333	0.397
30	880.792	879.079	878.300	879.390	1.275	0.145
40	1159.515	1157.976	1159.993	1159.161	1.054	0.091
50	1437.161	1434.986	1436.18	1436.109	1.089	0.076
60	1711.782	1717.532	1722.824	1717.379	5.523	0.322

Coefficient of deviation (CV) = SD/mean x100%

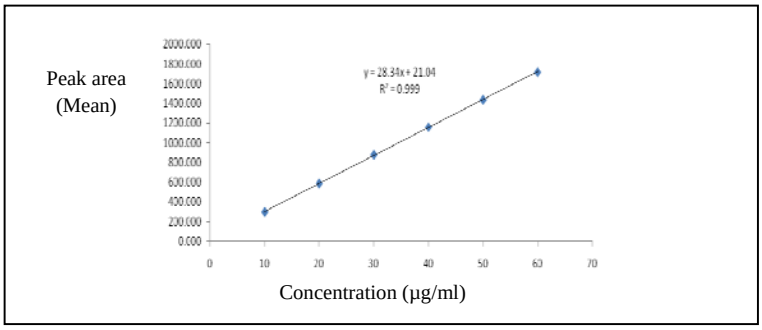

Figure 4. The standard linear curve of ligustilide: $Y = 28.34X + 21.04$, $R^2 = 0.999$, Y is peak area, X is the concentration of the analyzed sample.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ for ligustilide are 0.63 and 2.12 µg/ml, respectively. The LOD and LOQ values are low, indicating the good sensitivity of the HPLC method.

Precision

Comparing the measured concentration with the standard concentration, the coefficient variations for ligustilide in RE and SE of *A.sinensis* were 0.257. All the coefficient variations were less than 2%, indicating that the HPLC method for quantitative determination of ligustilide from the ethanol extracts of *A.sinensis* has good precision.

Table 2. Precision validation of the HPLC method for determination of ligustilide from RE of *A. sinensis* (Ang khang area)

Samples	Standard concentration (µg/ml)	Measured concentration (µg/ml)	CV (%)
RE	46-53	2.269 ± 0.006	0.257

Values are mean ± SEM as obtained by triplicate determinations, Coefficient of deviation (CV) = SD/mean x100%

Accuracy

As presented in Table 3, the percentage recovery of ligustilide ranged from 99.52 to 101.17%, with 0.54 to 1.40% of coefficient variations. The results demonstrate that the HPLC method has good accuracy.

Table 3. Accuracy validation of the HPLC method for ligustilide

Spiked level (µg/ml)	Nominal (%)	Recovery (%) (n = 3)	CV (%)
20	50	101.17	0.54
40	100	99.52	1.34
60	150	99.94	1.40

Coefficient of deviation (CV) = SD/mean x100%

Determination of ligustilide contents in ethanol extracts of *A. sinensis*

The amounts of ligustilide found in ethanol extracts of the whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis* were 0.562 and 0.887 mg/g as shown in Table 4.

Table 4. Ligustilide contents (mg/g) in ethanol extracts of *A. sinensis*

RE	SE
0.562 ± 0.001	0.887 ± 0.001

Values are mean ± SEM as obtained by triplicate determinations

4. CONCLUSION

A reversed-phase high performance liquid chromatography (HPLC) method was used for analysis of Ligustilide from the ethanol extracts of separated whole roots (RE) and aerial stems including leaves (SE) of *A. sinensis*. The analytical method was validated and showed good linearity, precision, accuracy and low LOD and LOQ. This method is suitable for quality control of ligustilide from the ethanol extracts of *A. sinensis*, which will be further developed to dietary supplement product.

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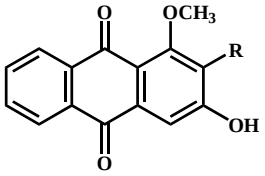
Anthraquinone derivatives from the roots of *Morinda elliptica*

Asadhawut Hiranrat*[a], Wanrudee Hiranrat [a], Chalita Tukwun [a]
Natural Products Research Laboratory, Faculty of Science, Thaksin University, Phatthalung Thailand 93110

*hasadhawut@tsu.ac.th phone +66 74693992 fax +66-74693992

ABSTRACT

This work was aimed to investigate the chemical constituents from the methanol extract of *Morinda elliptica* roots. Three anthraquinones (**1**-**3**) were isolated from this extract by preparative HPLC using gradient solvent manner of H_2O and MeOH. The structures of all isolated compounds were elucidated by the analyses of 1D and 2D NMR spectroscopic data.



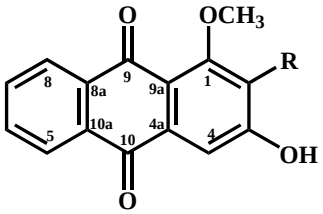
1 : R = CH_2OH
2 : R = CH_3
3 : R = CHO

Keywords: Rubiaceae, *Morinda elliptica*, anthraquinones, spectroscopic data, HPLC

1. INTRODUCTION

The genus *Morinda* belongs to the family Rubiaceae. It consists of about thirteen species in Thailand [1]. Phytochemical investigation of this genus has been reported to obtain several compounds. The structures were identified as anthraquinones [1-5], iridoids [1, 6-11], flavonoids [8-10, 12], coumarins [12] and benzophenones [5].

Morinda elliptica Ridl. is a shrub or small tree up to 25 meters in high. The young is greenish and smooth bark while shallow fissure in older. Leaves are elliptic shape with 4-6.5 x 10-19.5 cm. It is widely distributed throughout South-east Asia [6]. This plant have been used as a traditional medicine for loss of appetite, headaches, diarrhea, fever, heamorrhoids, antipyretic and anti-inflammatory agents [2, 6]. In this work we have reported on the isolation and structure determination of the anthraquinone derivatives obtained from the methanol extract of the roots of *M. elliptica*. The preparative HPLC separation resulted in the isolation of three anthraquinones. The spectroscopic data of these compounds associated with comparison to the previous reports were elucidated as damnacanthol (**1**), rubiadin-1-methyl ether (**2**) and damnacanthal (**3**).



2. MATERIALS AND METHODS

General procedures

Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected. UV spectra were recorded by a UV-1700 PharmaSpec spectrophotometer (SHIMADZU). The IR spectra were measured with a Cary 630 ATR FT-IR spectrophotometer (Agilent Technologies). The ^{1}H and ^{13}C NMR spectra were recorded by a FT-NMR Bruker Avance 300 MHz spectrometer using TMS as the internal standard. The Agilent 1100 HPLC system was used for separation purposes with Lichrospher®100 RP-18 endcapped column (⌀ 25 x 250 mm, particle size 5 μm, Merck). Column chromatography (CC) was performed on silica gel 100 (70–230 Mesh ASTM, Merck). For thin-layer chromatography (TLC), aluminium sheets of silica gel 60 F_{254} (20 × 20 cm, layer thickness 0.2 mm, Merck) was used for analytical purposes. The compounds were visualized under ultraviolet light. All solvents for extraction and chromatography were distilled at their boiling ranges prior to use except for HPLC separation (MeOH) was analytical grade (Merck).

Plant material

The roots of *M. elliptica* were obtained from Pa Phayom District, Phatthalung Province in southern part of Thailand during October 2013. The identification was made by Associate Prof. Dr. Kitichate Sridith and the voucher specimen has been deposited at the Prince of Songkla University Herbarium, Department of Biology, Faculty of Science, Prince of Songkla University, Thailand.

Extraction and isolation

The air-dried roots of *M. elliptica* (336.8 g) were chopped and successively macerated with MeOH at room temperature for twice (each for 4 days) to give a dark brown gum (4.2 g). The extract (2.0 g) was subjected to HPLC separation using gradient solvent of MeOH-H_2O. The elution was conducted at a flow rate of 3.0 mL/min under linear gradient conditions of 20% MeOH up to 100% MeOH in 180 min, then 100% MeOH for a further 60 min to afford 80 fractions (3 min each). Fractions 49 (17.6 mg), 58 (19.9 mg) and 62 (13.6 mg) were washed with CH_2Cl_2 to yield brownish powder of compound **1** (9.5 mg, 0.0059% dry wt), yellowish powder of compound **2** (8.3 mg, 0.0052% dry wt) and compound **3** as yellow powder (4.3 mg, 0.0027% dry wt).

Structure identification

Compound 1: brownish powder, mp. 290-291ºC; UV (MeOH) λ_{max} nm (log ε) 230 (4.45), 250 (4.10), 273 (4.50), 368 (3.52); FT-IR (ATR) ν_{max} (cm^{-1}) 3282 (OH stretching), 1672 (C=O stretching); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.18 (*dd*, J = 8.0 and 1.2 Hz, H-8), 8.14 (*dd*, J = 8.0 and 1.2 Hz, H-5), 7.88 and 7.83 (*m*, H-6 and H-7), 7.52 (*s*, H-4), 4.60 (*s*, H-11), 3.90 (*s*, 1-OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 185.4 (C-10), 182.9 (C-9), 165.2

and 165.1 (C-1 and C-3), 140.0 (C-4a), 137.8 (C-7), 136.7 (C-6), 136.5 (C-8a), 135.4 (C-10a), 131.8 (C-2), 130.0 (C-8), 129.7 (C-5), 120.8 (C-9a), 113.0 (C-4), 65.2 (1-OCH_3), 56.0 (C-11)

Compound 2: yellowish powder, mp. 289-290ºC; UV (MeOH) λ_{max} nm (log ε) 234 (4.40), 248 (4.25), 279 (4.62), 370 (3.70); FT-IR (ATR) ν_{max} (cm^{-1}) 3296 (OH stretching), 1671 (C=O stretching); ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.16 (*dd*, J = 7.9 and 1.0 Hz, H-8), 8.11 (*dd*, J = 7.9 and 1.0 Hz, H-5), 7.90 and 7.84 (*m*, H-6 and H-7), 7.51 (*s*, H-4), 3.60 (*s*, 1-OCH_3), 2.20 (*s*, H-11); ^{13}C NMR (75 MHz, DMSO-d_6) δ 183.0 (C-10), 180.9 (C-9), 162.0 (C-3), 161.1 (C-1), 135.1 (C-7), 134.0 (C-4a), 139.8 (C-10a), 133.6 (C-6), 132.5 (C-8a), 127.0 (C-8), 126.8 (C-2), 126.5 (C-5), 118.8 (C-9a), 110.0 (C-4), 61.1 (1-OCH_3), 9.6 (C-11)

Compound 3: yellow powder, mp. 210-211ºC; UV (MeOH) λ_{max} nm (log ε) 248 (4.20), 279 (4.32), 312 (4.00), 373 (3.50); FT-IR (ATR) ν_{max} (cm^{-1}) 3300 (OH stretching), 1670 (C=O stretching); ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.28 (*s*, 3-OH), 10.48 (*s*, H-11), 8.30 (*dd*, J = 7.8 and 1.4 Hz, H-8), 8.25 (*dd*, J = 7.8 and 1.4 Hz, H-5), 7.84 and 7.78 (*m*, H-6 and H-7), 7.69 (*s*, H-4), 4.13 (*s*, 1-OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.6 (C-11), 182.0 (C-10), 180.1 (C-9), 166.8 and 166.7 (C-1 and C-3), 141.7 (C-4a), 134.8 (C-7), 133.7 (C-6), 133.6 (C-8a), 132.4 (C-10a), 127.4 (C-8), 127.0 (C-5), 118.0 (C-2), 117.8 (C-9a), 113.0 (C-4), 64.8 (1-OCH_3)

3. RESULTS

Chemical studies of the constituents from the MeOH extract of *M. elliptica* roots had led to the isolation of three anthraquinones, damnacanthol (**1**), rubiadin-1-methyl ether (**2**) and damnacanthal (**3**). The structures of all isolated compounds were elucidated using the analyses of spectroscopic data especially 1D and 2D NMR techniques.

Compound **1** was isolated as brownish powder. The UV spectrum showed absorption bands maxima at 230, 250, 273 and 368 nm which expected for an anthraquinone skeleton [2]. The FT-IR spectrum exhibited the absorption bands at 3282 and 1672 cm^{-1} assignable for hydroxyl and conjugated carbonyl groups stretching, respectively. The ^{1}H NMR spectrum revealed the resonances of four adjacent aromatic protons at δ 8.18 (*dd*, J = 8.0 and 1.2 Hz), 8.14 (*dd*, J = 8.0 and 1.2 Hz), 7.88 (*m*) and 7.83 (*m*) indicated that one of the aromatic rings was an unsubstituted anthraquinone [3]. These coupled protons were clearly confirmed by the ^{1}H-^{1}H COSY experiment. The spectrum showed further a singlet aromatic proton at δ 7.52, an oxygenated methylene group at δ 4.60 (*s*) and a methoxyl group at δ 3.90 (*s*). These data suggested the other anthraquinone ring was a trisubstituted moiety. The presence of the oxygenated methylene and methoxyl groups corresponded to the signals at δ 56.0 and 65.2 in the ^{13}C NMR spectrum, respectively. Among these two carbons, the spectrum exhibited additional signals due to the resonances of two carbonyl carbons at δ 185.4 and 182.9, seven quaternary carbons at δ 140.0, 136.5, 135.4, 131.8, 120.8 including the oxygenated carbons at δ 165.2 and 165.1 and five aromatic methine carbons at δ 137.8, 136.7, 130.0, 129.7 and 113.0. In the HMBC experiment, the signals resonated for the oxygenated methylene protons (δ 4.60) showed the correlation peaks with the two oxygenated quaternary carbons at δ 165.2 and 165.1 indicating that the hydroxymethyl group was located at the C-2 position (δ 131.8). The aromatic proton H-4 (δ 7.52) displayed the ^3J HMBC cross peaks to the carbon signals at δ 185.4 (C-10), 131.8 (C-2) and 120.8 (C-9a) and the ^2J correlations with the signals at δ 165.2 (C-3) and 140.0 (C-4a) confirmed the location of this proton at the C-4 position and suggestively oriented the same side with the carbonyl carbons C-10. In addition, the ^{1}H NMR spectrum showed the methoxyl group at δ 3.90 and disappeared a chelated hydroxyl signal at the down field region indicated that the C-1 position was replaced by the methoxyl group. Consequently the free hydroxyl group was positioned at the carbon C-3. These concluded the structure of compound **1** as 3-hydroxy-2-hydroxymethyl-1-methoxyanthraquinone or known as damnacanthol [3, 13]. The HMBC correlations were fully supported this assigned anthraquinone structure.

Compound **2** was obtained as yellowish powder. The UV spectrum displayed absorption bands maxima at 234, 248, 279 and 370 nm indicating the same chromophore as for compound **1**. The FT-IR spectrum showed the absorption bands for hydroxyl group at 3296 cm^{-1} and for conjugated carbonyl group at 1671 cm^{-1}. The ^{1}H NMR spectrum of anthraquinone **2** exhibited the similar signals as those of compound 1 cosisting of the unsubstituted aromatic ring [δ 8.16 (*dd*, J = 7.9 and 1.0 Hz, H-8), 8.11 (*dd*, J = 7.9 and 1.0 Hz, H-5), 7.90 and 7.84 (*m*, H-6 and H-7)] and the trisubstituted aromatic ring [δ 7.51 (*s*, H-4), 3.60 (*s*, 1-OCH_3), 2.20 (*s*, H-11)]. The only difference data was the presence of a singlet methyl group at δ 2.20 in compound **2** in stead of the hydroxymethyl group in compound **1**. The C-2 of compound 2 was then placed by the methyl group. The ^{13}C NMR and HMBC spectra were obviously confirmed this conclusion. Therefor compound **2** was identified to be 3-hydroxy-1-methoxy-2-methylanthraquinone or rubiadin-1-methyl ether [13, 14].

Compound **3** was isolated as yellow powder. The UV and FT-IR spectra showed the similar absorption bands to those of compound **1** indicating the same anthraquinone structure. The ^{1}H NMR spectrum also demonstrated the resonance signals closely to compound **1** except for the signals of the chelated hydroxyl and aldehydic protons at δ 12.28 (*s*) and 10.48 (*s*), respectively. The ^{13}C NMR signal resonated at δ 195.6 supported the existence of the formyl group. The ^{3}J HMBC correlations of the formyl proton (δ 10.48) with the oxygenated quaternary carbons at δ 166.8 and 166.7 (C-1 and C-3) suggested the placement of this formyl group at the C-2 position. The HMBC correlations of the chelated hydroxyl group (δ 12.28) to the carbons resonated at δ 118.0 (C-2) and 113.0 (C-4) indicated the location of this hydroxyl group at the C-3 position. Consequently the methoxyl group (δ 4.13) was clearly substituted at the C-1. Comparison the spectroscopic data with the previous works concluded that compound **3** was closely corresponded to 2-formyl-3-hydroxy-1-methoxyanthraquinone or widely known as damnacanthal [13,14].

4. CONCLUSIONS

Investigation of chemical constituents from the methanol extract of *M. elliptica* roots performed by reversed phase preparative HPLC separation using MeOH-H_2O gradient solvents resulted in the isolation of three known anthraquinones, damnacanthol (**1**), rubiadin-1-methyl ether (**2**) and damnacanthal (**3**).

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Chemical compounds and toxicity of crude extracts from *Gynura divaricata* DC. reduced in blood sugar level in diabetes rats

Sasamol Phasuk*[a], Prasert Meeratana[b],
[a]Department of Science Education, Faculty of Science and Technology, Valaya Alongkorn Rajabhat University under the Royal Patronage, Pathumthanni, Thailand 13180
[b]Faculty of Allied Health Science, Burapa University, Chonburi, Thailand 20000

*phasuk_ss@yahoo.co.th phone +02-9093042 fax +66-29093041

ABSTRACT

The aims of this study were 1) to study acute and subchronic toxicity of the ethanol crude extracts of *Gynura divaricata* DC. on the blood sugar in diabetes rats and 2) to analyze the chemical compounds of *Gynura divaricata* DC. ethanol crude extract. Fresh *Gynura divaricata* DC. leaves (21.6 kg) were macerated in ethanol. 200-350 grams of Spraque-Dawley's rats, both male and female were randomly sampled. The rats were divided into 3 control and 3 experimental groups, each groups were contained 9 rats. Subchronic toxicity was focused on rats while acute toxicity was emphasized on mices. The experimental groups were orally daily fed with 200 400 and 800 mg/70 kg bw of crude extract for 4 weeks. The control groups were fed with water and control solvent. Crude extract was separated by thin-layer chromatography and column chromatography. In addition, the separated crude extract was then analyzed using spectrometry technique. The results were shown as follow:

1. The diabetes rats receiving 200 mg/70 kg.bw were exhibited the lowering of blood sugar. The blood sugar level displayed the non-significance different comparing with the control group on the first and third weeks (118.0 ± 75.1 and 155.9 ± 81.8 mg% respectively). In addition, both 400 and 800 mg/70 kg.bw rats were shown the high blood sugar level with the significance different comparing with the control groups ($p < 0.05$).

2. The subchronic toxicity on gross appearance of the organs of the circulatory system, urinary system and biliary system was not apparently observed in the sacrificed rats at the end of experiment. However, blood chemistry of the experimental groups showed the significant increasing in liver function parameters including ALP, AST, ALT and direct bilirubin comparing with the control groups. Among these groups, the animals of 200 mg/70 kg.bw group showed less increasing in blood chemistry of liver function parameters.

3. The acute toxicity experiment exhibited the LD_{50} of the oral ethanol crude extract of *Gynura divaricata* DC. The LD_{50} of ethanol crude extract was higher than 8 gm/kg that was 2,800 times of the 200 mg/70 kg bw dosage.

4. The chemical compounds of *Gynura divaricata* DC. ethanol crude extract were separated and analyzed by spectrometry technique. The major chemical compound was triterpenoidal saponin; bellericagenin B 3-*O*-[β-D-glucopyranosyl-(1-2)-α-D-glucopyranoside.

Keywords: Acute and subacute toxicity, *Gynura divaricata* DC., Ethanol extract, Hypoglycemic effect, iabetes rats.

1. INTRODUCTION

The number of patients suffering from diabetes mellitus has been increasing [2]. Although drugs possessing potent efficacy are apparently important for the treatment of diabetes mellitus. Nowadays, patients are interesting in alternative medicine concern natural product such as *Gynura divaricata* DC. It is a medicinal plant, traditionally used to treat diabetes mellitus, for health promotion and longevity [1]. The toxicity of *Gynura divaricata* DC. ethanol extract is not study by scientific process. Thus, this study aimed to study acute and subacute toxicity and analyze the chemical compounds of the ethanol crude extract of *Gynura divaricata* DC. reduced on the blood sugar in diabetes rats.

2. MATERIALS AND METHODS

The fresh leaves were pulverized into powder. The powder was extracted with ethanol by maceration. The animals were purchase from National Animal Research Center, Salaya, Thailand. They were rare in standard polyethylene cage at Animal Husbandry and Research Unit, Faculty of Allied Health Sciences, Burapha University, Chonburi, Thailand. Animals are acclimatized for two weeks in 24-25 degree celcius with 60 percent humidity room under 12:12 h. light dark ratio. 200–350 g of Spraque-Dawley rats, both male and female were randomly sampled. The rats were divided into 3 control and 3 experimental groups, each groups were contained 9 rats. Subacute toxicity was focused on rats while acute toxicity was emphasized on mices. The experimental groups were orally daily fed with 200, 400 and 800 mg/70 kg bw of crude extract for 4 weeks. The control groups were fed with water and control solvent. Checked blood biochemistry and study subacute toxicity in rats and acute toxicity in mices. Crude extract was separated by thin-layer chromatography and column chromatography. In addition, the separated crude extract was then analyzed using spectrometry technique.

3. RESULTS

Results of subacute toxicity

Blood chemistry of rats in forth weeks as shown in Table 1. The subacute toxicity on gross appearance of the organs of the circulatory system, urinary system and biliary system was not apparently observed in the sacrificed rats at the end of experiment. However, blood chemistry of the experimental groups showed the significant increasing in liver function parameters including ALP, AST, ALT and direct bilirubin comparing with the control groups. Among these groups, the animals of 200 mg/70 kg BW group showed less increasing in blood chemistry of liver function parameters.

Table 1. Blood chemistry of rats

Group	HCT. %	BUN mg/dl	Creati-nine mg/dl	Total protein mg/dl	Albu-min g/dl	Globu lin g/dl	Total billiru-bin g/dl	Direct billiru-bin mg/dl	AST U/L	ALT U/L	ALP U/L
Control gr. (normal)	55.8± 3.6	20.3±2. 5	0.4± 0.3	6.6± 0.3	3.8± 0.1	2.8± 0.2	0.2± .0	0.02± 0.03	89.5± 34.1	70.9± 61.8	80.8± 13.4
Control gr. (compare)	55.8± 4.5	21.7± 1.9	0.5± 0.2	6.6± 0.2	3.8± 0.1	2.8± 0.2	0.1± 0.1	0.0	88.3± 29.7	64.8± 31.0	77.9± 5.1
Control gr. (diabetes)	55.3± 3.6	52.6± 9.9*	0.6± 0.2*	5.9± 0.2*	3.6± 0.1	2.3± 0.1*	0.2± .0	0.1± 0.1*	129.9± 35.3*	105.1± 13.6	283± 107.6*
Experimental gr. 1 (200mg/70kg BW)	53.67± 2.8	21.1± 9.8	0.6± 0.6	5.5± 0.6*	3.7± 0.3	1.8± 0.5*	0.3± 0.1	0.1± 0.1*	112.4± 49.2	99± 44.4	362.4± 187.1*
Experimental gr. 2 (400mg/70 kg BW)	53.9± 2.6	20± 25.8	0.6± 0.4*	5.4± 0.4*	4.2± 0.4	2.0± 0.4*	0.4± 0.2	0.1± 0.1*	189.7± 216.2*	205.9± 159.1	245.8± 86.6*
Experimental gr. 3 (800mg/70 kg BW)	53.33± 2.2	24.6± 22.4	0.7± 0.2*	5.8± 0.2*	3.6± 0.3	2.2± 0.3*	0.2± 0.1	0.1± 0.1*	130.9± 74.9*	125.3± 81.9	368.3± 175.9*

Mean ± SD, * = $p < 0.05$

Results of acute toxicity

The acute toxicity experiment exhibited the LD_{50} of the oral ethanol crude extract of *Gynura divaricata* DC. The LD_{50} of ethanol crude extract was higher than 8 g/kg that was 2,800 times of the 200 mg/70 kg bw dosage.

Result of chemical compound

The major chemical compound analyzed by spectrometry technique such as ^{13}C NMR and ^{1}H NMR as shown in Table 2

The major chemical compound was triterpenoidal saponin; bellericagenin B 3-*O*-[β-D-glucopyranosyl-(1-2)-α-D-glucopyranoside. The chemical structure as shown in Figure 1.

Table 2. Chemical shift (δ) ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR (in C_5D_5N**)** of major chemical compound in *Gynura divaricata* DC. ethanol crude extract

Position	δ_C	C-type	δ_H (mult, J in Hz)	Position	δ_C	C-type	δ_H (mult, J in Hz)
1	47.8	CH_2	1.59 (1H,dd,,J=14.1 ,4.4) 1.97 (1H, dd, J=9.5, 4.4)	23	64.3	CH_2	4.02, 4.62 (AB d, J=11.4)
2	69.7	CH	4.41 (ddd, J=14.1, 9.5, 4.4)	24	62.8	CH_2	4.27, 4.81 (AB d, J=11.4)
3	81.4	CH	4.32 (d, 9.5)	25	17.1	CH_2	1.12 s
4	47.9	C	-	26	17.4	CH_2	1.13 s
5	48.6	CH	1.51m	27	24.8	CH_2	1.54 s
6	19.4	CH_2	1.55 (1 H, m) 1.48 (1 H, m)	28	180.2	C=O	-
7	33.3	CH_2	2.10, 1.65 m	29	30.3	CH_2	1.31 s
8	40.2	C	-	30	26.3	CH_2	1.10 s
9	48.2	CH	1.87 (1H, dd, J=11.3,4.9)	“Glc-1”	104.5	CH	6.32 (d, J=2.9)
10	38.3	C	-	“2”	82.3	CH	4.06 m
11	24.4	CH_2	2.03, 1.30	“3”	77.9	CH	4.16 m
12	123.2	CH	5.52 (t, J=3.2)	“4”	71.3	CH	4.11 m
13	144.3	C	-	“5”	78.1	CH	3.77 m
14	42.1	C	-	“6”	62.6	CH_2	4.28 (dd, J=11.5, 5.0); 4.43 (dd, J=11.5, 2.5)
15	28.9	CH_2	1.78 m	“Glc-1”	102.8	CH	5.91 (d, J=7.7)
16	27.9	CH_2	0.91 m	“2”	76.9	CH	4.06 m
17	46.4	C	-	“3”	78.3	CH	4.16 m
18	44.8	CH	3.43 (br s)	“4”	71.2	CH	4.24 m
19	80.1	CH	3.52 (d, J=4.4)	“5”	78.2	CH	3.87 m
20	35.5	C	-	“6”	62.2	CH_2	4.37 (dd, J=11.5, 4.0); 4.45 (dd, J=11.5, 3.1)
21	28.8	CH_2	1.82, 2.01 m				
22	33.2	CH_2	0.88, 2.76 m				

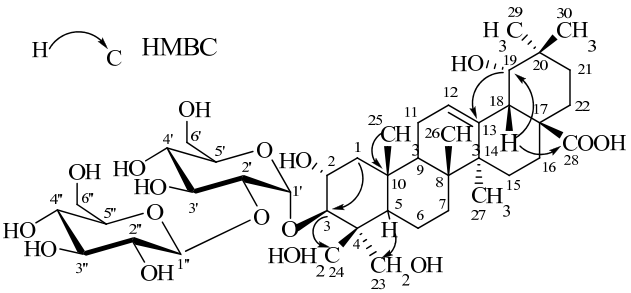

Figure 1. HMBC correlation of triterpenoidal saponin; bellericagenin B 3-*O*-[β-D-glucopyranosyl-(1-2)-α-D-glucopyranoside.

4. CONCLUSIONS

The subacute effects of the ethanol crude on physical, behavioral, circulatory and urinary system could not be observed in all rats, except rising in blood chemistry of the liver function test of both control and experimental rats. The possible mechanism of this phenomena would be the side effect of natural alkaloids and terpenoids [3], which present in all plants extract. The acute side effect of the extract could not be observed up to 2,800 times of the effective dose 200 mg/70 kg BW.

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Biological activities of stem extracts from *Luvunga scandens*

Prangchanok Sirinut[a], Awanwee Petkongkeaw[b], Panumart Thongyoo*[a]
[a]Department of Chemistry, [b]Department of Food Science, Faculty of Science and Technology, Rangsit Campus Thammasat University, Pathumthani, Thailand 12120

*tpanumas@tu.ac.th phone +66-59410465 fax +66-25644483

ABSTRACT

Natural products have long been known as one of the most excellent sources of lead drug candidate in drug discovery. Nowadays, a large number of Thai medicinal plants have been utilized for the treatment of a variety of diseases. *Luvunga scandens* is commonly known as one of Thai folk medicinal plants applied for the treatment of small pox, kidney diseases, wasting disease and urinal disorders. Stem bark of *Luvunga scandens* was extracted in dichloromethane, ethyl acetate and methanol to evaluate anti-oxidative, anti-inflammatory and anti-bacterial activities. The methanolic extract was found to reduce the level of cytokine (Tumor Necrosis Factor type-alpha (TNF-α) and interleukin-10 (IL-10) of monocyte), both of which are known as mediators for inflammation responses. Interestingly, all crude extracts were also found to have very promising anti-bacterial activities against *Staphylococus aureus, Bacillus cereus, Enterococus faecalis, Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA) with MIC ranging from 3.125 to 100 mg/ml. Additionally, dichloromethane, methanolic and ethyl acetate extracts were found to be moderately active as anti-oxidants in the DPPH radical scavenging assay with IC_{50} = 94, 445 and 480 μg/ml, respectively.

Keywords: *Luvungascandens*, TNF-α, Anti-oxidant, Antibacterial activity

1. INTRODUCTION

Inflammatory response is an important element in the pathogenesis of chronic inflammation-related disorders, involving the sequential activation of various signaling pathways, including cyclooxygenase, nitric oxide synthase, some proteases (such as tryptase) and cytokines [tumor necrosis factor-α (TNF-α) and interleukins (IL-6 and IL-1β)]. The inappropriate production of pro-inflammatory mediators; such as interleukins (IL-6 and IL-1β) and tumor necrosis factor-α (TNF-α) is implicated in several inflammation diseases; such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, psoriasis, endotoxemia and etc. Therefore, the inhibition of the over-production of pro-inflammatory mediators IL-6, IL-1β and TNF-α should be an effective approach for treating the diseases. *Luvunga scandens*is commonly known as South East Asian traditional herb, belonging to Rutaceae family, and distributed widely in Eastern Bengal, Assam, Khasia hills, Burma and Thailand. The family Rutaceae has been known to produce many compounds, ranging from triterpene, oxiranecarbamide to sesquiterpene, some of which displayed anti-insecticidal activity, anti-tumor activity, antipyretic activity using for the treatment of gonorrhea. In Thailand, *L. scandens*has been used widely as folk medicine for the treatment of many diseases, such as abscess, wasting disease, adrenocortical insufficiency, neurogenic bladder[1]. Presently, the roots and berries of *L. scandens* have been prescribed for the treatment of snake-bite or scorpion sting (http://www.mpbd.info/plants). To this report we focused on the evaluation ofthe inhibitory effect of the extract of *L. scandens* on lipopolysaccharide (LPS) induced tumor necrosis factor-alpha (TNF-α) secretion from isolated human peripheral blood mononuclear cells, and anti-oxidative activity was also investigated *via* DPPH scavenging radical assay as well as an anti-bacterial activity against a wide range of bacterial strains such as *S. aureus, B. cereus, E. faecalis*, Methicillin-resistant *S. aureus* (MRSA) and *E. coli*.

2. MATERIALS AND METHODS

General experimental procedure

The NMR spectra were recorded in $CDCl_3$ using BRUKER-NMR 400MHz spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS as internal standard, and chemical shifts are expressed in δ values. Analytical and preparative TLC was carried out on pre-coated silica gel 60F254 and RP-18F254 plates (Merck, 0.25 or 0.50 mm thickness).

Plant material

The stems and roots of *L. scandens* used in this experiment were collected at Hoey Sai National Park, Prachuabkirikhun Province, Thailand, in May 2010. A voucher specimen (LS 150655) was deposited at Department of Chemistry, Thammasat University, Thailand.

Plant preparation

The stems and roots of *L. scandens* (1 kg) were sliced into small pieces, and consecutively extracted with dichloromethane (1000 ml), ethyl acetate (1000 ml), and methanol (1000 ml) for 2 weeks each. The decanted crude CH_2Cl_2 extract was filtered by Whatman filter paper 1 and subsequently concentrated *in vacuo* to afford the crude dichloromethane extract (1.920 g), ethyl acetate extract (2.578 g) and methanolic extract (4.695 g), respectively [2].

Anti-oxidative property

The antioxidant activity of plant extracts was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay[3, 4]. The stock solutions (1000 µg/mL) of crude extract were prepared, and subsequently diluted to final concentrations of 500, 250, 125, 50, 25, 10 and 5 µg/mL in methanol, respectively. To this solutions were added by 0.3 mM DPPH (1 mL) dissolved in methanol and kept in the dark for 30 minutes. Absorbance was measured at 520nm. Methanol (1 mL) was used as blank and DPPH solution (0.3 mM, 1 mL) with methanol (2.5 mL) was used as control. Ascorbic acid was used as a standard control. The absorbance values were record and the percent of inhibition was calculated.

Anti-bacterial activity

The antibacterial activity of crude extracts was determined by resazur in microplate assay (REMA) by modified method [5]. 50 µL of 0.85% normal saline was added into column 2-11 of sterile 96 wells plate**.** Then, 100 µL of crude extracts (100 mg/mL) was added into the first column serial dilution was performed and was trashed 50 µL of solution in column 11. 3.3x strength isosensitised nutrient broth (30 µL) was added to all wells to confirm the final volume. 10 µL of bacteria (5×10^{6}cfu/mL) was added to each well. The plate was incubated at 37°C for 24 h. After 24 h 10 µL of 0.1% resazurin was added to each well and incubated at 37°C for 3 h. After 3 h, the color of resazurin wasn't changed from blue to pink were recorded as positive. On the other hand, the color of resazurin was

changed from blue to pink were recorded as negative. Cephalexin (1.5 mg/mL) was used as positive control and 0.85% normal saline was used as negative control.MBC values were determined by streak on nutrient agar plate.

Isolation of Human PBMC and Culture

PBMC (Polymorphonuclear cells) from healthy donors were isolated from EDTA blood by Ficoll-Hypaque gradient centrifugation. Peripheral blood from the donors was diluted with sterile phosphate buffer saline and overlaid on the Ficoll-Hypaque solution, and centrifugation was performed at 350 × *g* for 10 min at room temperature. The recovered PBMC were rested in RPMI-1640 and incubated at 37°C 95% O_2 + 5% CO_2 for 30 min before performing the experiments. Cell viability was determined by a Trypan blue dye exclusion assay. The percentages of cell viability were calculated by the ratio of Trypan blue excluding cells to total cell number

LPS Stimulation and Incubation of the *Luvunga scandens* Extracts with PBMC

After the PBMC isolation and pre-incubation period, 200 l of 1×10^5 cells/ml PBMC were cultured in a 96-well polypropylene plate in serum-free RPMI-1640 medium with LPS at a final concentration of 10 ng/ml and various concentrations of the methanol extract of *Luvunga scandens* (0.0-3.0 mg/ml, final concentration) in DMSO. In the control wells, cells were incubated with LPS and 0.01% DMSO vehicle. Cells in all conditions were incubated at 37°C, 95% O_2 + 5% CO_2 for 6 h. Cell viability was determined by Trypan blue dye exclusion and culture medium was collected and stored at -20°C until analysis for TNF-α production

Determination of TNF-α Production

Supernatants were collected after an optimum incubation period and stored at -20°C until used. TNF-α ELISA was performed according to the manufacturer's instructions. Briefly, 100 µL of the collection medium and 50 µL of the detection antibody, conjugated with horseradish peroxidase, were added to 96-well plates pre-coated with capture antibody for 2 h at room temperature. TMB substrate was added to the reaction for 30 min at room temperature. The reactions were terminated by the addition of the stop solution before measuring the absorbance at 450 nm. The concentration of TNF-α can be calculated from the standard curve produced by the serial-diluted standard TNF-α.

3. RESULTS

Anti-bacterial activity and anti-oxidative activity *via* DPPH assay of *Luvunga scandens* extracts

Ethyl acetate, dichloromethane and methanol extracts of *L. scandens* exhibited moderate anti-oxidative activities as radical scavenger in the DPPH assays with IC_{50} values of 94, 445 and 480 µg/mL respectively, in comparison with ascorbic acid (IC_{50} < 5 µg/mL) as a reference standard. All five bacterial strains were sensitive to extracts from *Lunvunga scandens.*All of extracts, Ethyl acetate extract demonstrated anti-bacterial activity against all bacterial strains used for this study with MIC value ranging from 3.125 to 25 mg/mL, and also methanol extract displayed a moderate anti-bacterial activity against *E. coli* (TISTR 780), *S. aureus* (TISTR 1466), *B. cereus* (TISTR 687) and *E. faecalis* (TISTR 379) with MIC value of 25 mg/mL. Dichloromethane extract displayed an anti-bacterial activity against S. *aureus* (TISTR 1466) and Methicillin-resistant *S. aureus* (MRSA) (ATCC 43300) with MIC value of 6.25 and 12.5 mg/mL, respectively.

Table 1. Minimum inhibitory concentration (MIC) of stem extracts from *Luvunga scandens*

Bacterial strains	Gram stain	MIC and MBC values are in mg/mL					
		CH_2Cl_2 extract		EtOAc extract		MeOH extract	
		MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli(TISTR 780)	-	100	100	12.5	25	25	50
Bacillus cereus(TISTR 687)	+	100	>100	25	50	25	100
Enterococusfaecalis(TISTR 379)	+	>100	>100	25	100	25	100
Staphylococusaureus (TISTR 1466)	+	6.25	50	3.125	12.5	25	50
Methicillin-resistant *S. aureus* (MRSA)(ATCC 43300)	+	12.5	100	3.125	6.25	50	50

Effect of methanolic extract of *Luvunga scandens* on LPS-induced TNF-α production

For activating TNF-α-production, isolated human PBMCs were exposed to 10 ng/ml LPS for 6, 12 and 24 h. The optimum condition for LPS-induced TNF-α production was 6 h. Subsequently, the effect of the methanolic extract of *L. scandens* on anti-inflammation was evaluated by the addition of various concentrations of the *L. scandens* extracts. Exposure of the *L. scandens* extracts reduced the TNF-α level in a dose-dependent manner (Figure 1b). It was observed that at the final concentration of the methanolic extract at 5 mg/ml significantly reduced the TNF-α level. Furthermore, we tested whether the reduction of TNF-α as treated with the extracts, as seen in the previous results, was not due to the toxicity of the extract. Therefore, cell viability was performed by a Trypan blue dye exclusion assay. Exposure of human PBMCs to the *L. scandens* extracts did not reduce the percentages of cell viability at all concentrations as shown in Figure 1a.

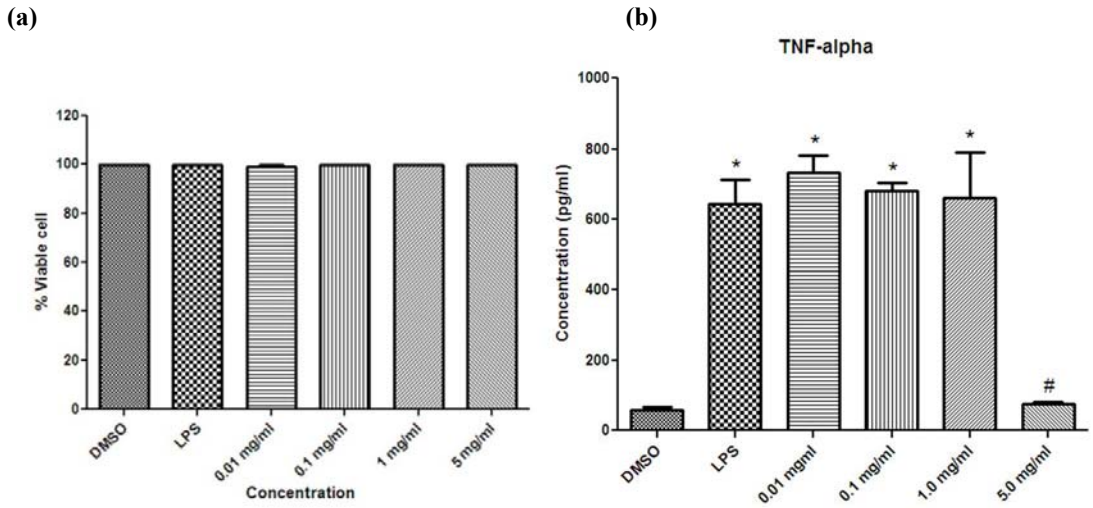

Figure 1. (a) Effect of *Luvunga scandens* extract on cell viability, (b) Inhibitory effect of the methanolic extract from *L. scandens* on the production of TNF-α in LPS-activated monocytes. White blood cells were stimulated LPS in the presence of the methanolic extract (0.01 mg/ml-5.0 mg/ml) for 6 h. Results are expressed as means ± SEM of three experiments made in triplicate. (DMSO or blank = cells without treatment; LPS = cells previously treated with LPS). *$p < 0.05$ vs control and #$p < 0.05$ vs LPS, LPS + 0.01 and LPS + 0.1.

4. CONCLUSIONS

To this research, we reported the investigation of anti-inflammatory anti-bacterial and anti-oxidative activities of *L. scandens* extracts. It was found that the methanolic extract of displayed a markedly anti-inflammatory inhibitory activity *via* reducing the level of TNF-αsecreted by monocytes, and also showed anti-oxidative and anti-bacterial activities against a variety of bacterial strains. This finding has paved the way of the identification of active components from *L. scandens* with therapeutic applications.

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Anti-oxidant and anti-bacterial properties of leaf extract of *Pithecellobium dulce*

Watsika Vichaidit[a], Panumart Thongyoo[*a]
[a]Department of Chemistry, Faculty of Science and Technology, Rangsit Campus Thammasat University, Pathumthani, Thailand 12120

*tpanumas@tu.ac.th phone +66-59410465 fax +66-25644483

ABSTRACT

Pithecellobium dulce is one of Thai medicinal plants belonging to the *Fabaceae* family and applied extensively as a folk medicine for the treatment of wounds, dental caries, and diarrhea. This research highlighted the anti-oxidative and anti-bacterial activities from the methanolic extract of *Pithecellobium dulce*. This methanolic extract was assessed an anti-bacterial activity against *Stapphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Escherichia coli* and Methicillin-resistant *Stapphylococcus aureus* (MRSA) by using disk diffusion and minimum inhibitory concentration (MIC) methods. Interestingly, the methanolic extract demonstrated a promising anti-bacterial activity against *Stapphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Escherichia coli* and Methicillin-resistant *Stapphylococcus aureus* (MRSA) with MIC values ranging from a concentration of 6.25 mg/ml to 50 mg/ml. Additionally, the methanolic extract also exhibited a very potent anti-oxidative property *via* DPPH assay. According to their anti-bacterial and anti-oxidative activities, the leaf extract from *Pithecellobium dulce* might possibly be applied as natural preservative ingredients in food and pharmaceutical industries.

Keywords: *Pithecellobium dulce*, Antibacterial, Antioxidant, Anti-inflammatory

1. INTRODUCTION

For the last century, a variety of medicinal plants have been applied for the treatment of human diseases, especially infectious diseases from microorganism. The increasing number of bacterial resistance to antibiotics has become a great concern worldwide. For this reason, the exploration of novel alternative medicines for the treatment of infectious diseases is urgently required. In Thailand, biodiversity is relatively high according to their plant and animal types distributed in different regions. To date, a vast number of medicinal plants have been studied to explore novel natural antimicrobial compounds. Much interest has been focused so far on the exploration of the new active compounds from natural product especially from plants for the treatment of human diseases. *Pithecellobium dulce* [1,2] is one of Thai medicinal plants belonging to the Fabaceae family. *P. dulce* is an evergreen tree widely distributed throughout India and also found in Southeast Asia, South Africa and Australia. This plant has been used for hedges, street trees and used extensively as a folk medicine. Leaves of *P. dulce* are used as for the treatment of wounds, dental caries, and diarrhea. The bark of the plant is used as for the treatment of dysentery, febrifuge and eye inflammation. In addition, the fruits of *P. dulce* have been consumed as a dietary supplement. The fruits are used as for the treatment of gastric ailments. To this report, we focused on the evaluation the inhibitory effect of the extract of *P. dulce* against a panel of bacterial strains ranging from *S. aureus*, *B. cereus*, *E. faecalis,* MRSA–to *E. coli*, including the evaluation of anti-oxidative activity of the methanolic extract by DPPH.

2. MATERIALS AND METHODS

General experimental procedure

The NMR spectra were recorded in $CDCl_3$ using BRUKER-NMR 400MHz spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS as internal standard, and chemical shifts are expressed in δ values. Analytical and preparative TLC was carried out on pre-coated silica gel 60F254 and RP-18F254 plates (Merck, 0.25 or 0.50 mm thickness).

Plant material

The leaves of *P. dulce* used in this experiment were collected at Prachuabkirikhun Province, Thailand, in April 2010. A voucher specimen (LS 150650) was deposited at Department of Chemistry, Thammasat University, Thailand.

Plant preparation

The leaves of *P. dulce* (2 kg) were grinded with a mortar, and subsequently extracted with methanol (1500 ml) for 2 weeks. The decanted crude methanolic extract was filtered by Whatmann filter paper 1 and subsequently concentrated *in vacuo* to afford the crude methanolic extract (200 mg).

Bacterial species

Staphylococcus aureus (TISTR 1466), *Bacillus cereus* (TISTR 687), *Enterococcus faecalis* (TISTR 379), *Escherichia coli* (TISTR 780), which were obtained from Thailand Institute of Scientific and Technological Research, and Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300) from the American Type Culture Collection (ATCC).

Determination of anti-bacterial activity

Four concentrations of the extract (0.05 g/mL, 0.10 g/mL, 0.20 g/mL, and 0.40 g/mL) were prepared and assessed an anti-bacterial activity by using a disc diffusion method as a screening test [3]. Stock culture of test bacteria were grown in nutrient broth medium at 37°C for 22 h. The culture suspensions were prepared and adjusted to approximately 1.5 x 10^8 cfu of bacteria/mL (0.5 McFarland turbidimetry). One hundred microliters of the inoculum were spread over plates containing sterile nutrient agar. Circular discs of 6 mm diameter impregnated with 50 microliters of each concentrations of extract were placed on the surface of the media. The plates were incubated at 37°C for 24 h. Each concentration of extracts were carried out in triplicate. After incubation, the diameter of the zone of bacterial growth inhibition around each disc were measured and recorded in millimeter. Cephalexin (50 mg) was used as a positive control and distilled water was used as a blank.

Determination of minimum inhibitory concentration

0.85% normal saline was added in sterile plate. A stock concentration of 100 mg/mL for crude extracts in methanol was pipetted into the first row of the plate and prepared a serial dilution. To each well 30 µL of 3.3x strength isosensitised broth and 10µL 5x10^5 cfu/mL of bacteria were added. Cephalexin (1.5 mg) was used as positive control and methanol was used as solvent control. The plates were incubated at 37°C for 24 h. After that, 10 µL of resazurin indicator was added in each well. The plates were incubated at 37°C for 3 h. and recorded for the result. From result, blue color was recorded as positive. The lowest concentration at which color did not change

from blue to pink was recorded as the MIC value. The average of three from four values was calculated to MIC value [4].

Determination of minimum bactericidal concentration

A nutrient broth from well recorded in the MIC assay was cultured on nutrient agar. The plates were incubated at 37°C for 24 h. After incubation, the highest dilution (least concentration) that inhibited the colony formation on nutrient agar was recorded as MBC value.

Determination of anti-oxidant activity by thin layer chromatography (TLC) analysis

The methanol extract of *P. dulce* was loaded on TLC plates. The plates were developed in methanol: ethyl acetate (0.5:9.5, v/v) to separate compound from crude extract. The plates were developed, subsequently dried by air, and sprayed with 0.05% of DPPH solution in methanol to evaluate an anti-oxidative activity. The appearance of yellow color was recorded as anti-oxidant activity [5].

3. RESULTS

The evaluation of an anti-bacterial activity against a panel of bacterial strains was assessed by disc diffusion method as displayed in Table 1. It was found that the methanolic extract of *P. dulce* demonstrated a promising anti-bacterial inhibition with the highest activity at 0.4 g/mL against *E. coli*, *S. aureus*, MRSA, *B. cereus* and *E. faecalis*. At the concentration of 0.2 g/mL, this extract exhibited an anti-bacterial activity only against *E. coli* and MRSA. Additionally, at the lowest concentration studied in this experiment (0.1 g/mL) the methanolic extract showed an inhibition only against *E. coli* as shown in Table 1.

Table 1. The anti-bacterial activity of leaves of *Pithecellobium dulce* methanolic extract assessed by disc diffusion method**.**

Bacterial strains	Gram stain	Diameter of inhibition zone (mm) against various concentration of extract (g/mL)			
		0.05	0.10	0.20	0.40
Escherichia coli	-	N	7.0	7.0	7.0
Bacillus cereus	+	N	N	N	7.0
Enterococus faecalis	+	N	N	N	7.0
Staphylococus aureus	+	N	N	N	12.2
Methicillin-resistant *S. aureus* (MRSA)	+	N	N	7.0	8.0

N = no inhibition zone, diameter disc (6 mm)

The determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. dulce* methanolic extract was assessed and the result was shown in Table 2. Interestingly, all five bacterial strains used in this experiment were sensitive to the methanolic extract from *P. dulce* with different MIC values, ranging from 12.5 mg/mL to 100 mg/mL. It is very important to note that the methanolic extract from *P. dulce* demonstrated a promising inhibion against medicinally important bacterial strain named as MRSA with that MIC value of 25 mg/mL, and against *S. aureus* with the MIC value of 50 mg/mL.The MBC of each bacterial strains was displayed in Table 2. Additionally, the methanolic extract of *P. dulce* also exhibited an anti-oxidative activity as confirmed by DPPH.

Table 2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of *Pithecellobium dulce*

Bacterial strains	Gram stain	MIC and MBC values are in mg/mL	
		MIC	MBC
Escherichia coli	-	100	100
Bacillus cereus	+	50	100
Enterococus faecalis	+	100	100
Staphylococus aureus	+	12.5	100
Methicillin-resistant *S. aureus* (MRSA)	+	25	100

4. CONCLUSIONS

P. dulce is one of Thai medicinal plants and applied extensively as a folk medicine for the treatment of wounds, dental caries, and diarrhea. It was observed that methanolic extract of *P. dulce* demonstrated anti-bacterial and anti-oxidative properties against a panel of bacterial strains, ranging from *E. coli, S. aureus,* MRSA, *B. cereus* to *E.faecalis* with impressive MIC and MBC values. Additionally, the methanolic extract of *P. dulce* also showed an anti-oxidative activty as confirmed by DPPH. This finding has paved the way of the exploration of pure active compounds from *P. dulce* with therapeutic applications.

ACKNOWLEDGEMENTS

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Phytochemistry and lethality effect to brine shrimp of selected *Callicarpa* species

Sarinthip Muensaen*[a, b], Siripen Jarikasem[a], Nongluck Ruangwises[b], Pongtip Sithisarn[c], Sawanya Buranapalin[b]

[a]Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand 12120

[b]Department of Pharmaceutical Chemistry Faculty of Pharmacy, Mahidol University, Bangkok, Thailand 10400

[c]Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand 10400

*sarinthip@tistr.or.th phone +66-25779116 fax +66-25779110

ABSTRACT

Four *Callicarpa* species which includes *C. candicans*; CC, *C. arborea*; CA, *C. longifolia*; CL and *C. rubella*; CR were phytochemically investigated for a potential insecticidal component, callicarpone using TLC, HPLC-DAD and HPLC-ESI-MS techniques. Toxicity against *Artemia salina* (brine shrimp) was simultaneously tested for the water extract of the four plant species. HPLC fingerprint analysis was conducted using XBridge Shield RP18 column (150 mm ×4.6 mm i.d., 5 µm). The gradient mode of mobile phases with 0.005% trifluoroacetic acid in water and 0.005% trifluoroacetic acid in acetonitrile was used. The results showed that each plant species has typical terpenoid fingerprint. By comparing the UV absorbance spectral obtained from HPLC-DAD together with HPLC-ESI-MS with the literature data, the component at t_R of 21.76 min corresponding to callicarpone i.e. λ_{max} at 266 nm and m/z at 332 was only found in *C. candicans*. For toxicity assessment, percentage of brine shrimp lethality tested at the extract concentration of 1000 µg /ml from CC, CA, CL and CR were found to be 86.67, 16.67, 20 and 3.33, respectively.

Keywords: *Callicarpa* species, Callicarpone, HPLC-DAD, HPLC-EIMS, Brine shrimp

1. INTRODUCTION

The genus *Callicarpa* comprising about 140 plant species has been regarded as a significant member of Lamiaceae family [1,2]. Ethnomedical reports of several *Callicarpa* species indicated their uses for the treatment of hepatitis, rheumatism, fever, headache, indigestion, cancer and other ailments [3]. Moreover, some *Callicarpa* species have been traditionally used as fish poisoning agent due to the presence of a bioactive diterpenoid constituent named callicarpone. The pesticidal activity of callicarpone has been reported to be 10 times more potent than a natural insecticide rotenone [4,5]. Thus callicarpone containing *Callicarpa* species are believed to have potential for using as a natural insecticide. In this study we have investigated on chemical constituents of 4 *Callicarpa* species focusing on di- and triterpenoids using TLC, HPLC-DAD and HPLC-ESI-MS techniques. Preliminary evaluation on cytotoxic potential of extracts from these plant species also has been performed using brine shrimp lethality test.

2. MATERIALS AND METHODS

Plant materials

Leaf samples of 4 different *Callicarpa* species were collected from different locations in Thailand including *C. arborea* (CA, from Prachuapkhirikhan), *C. candicans* (CC, from Krabi), *C. longifolia* (CL, from Songkhla) and *C. rubella* (CR, from Chiang Mai) during December 2010 to February 2011. Plant samples were identified by Dr. Charan Leeratiwong, a botanist in Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. Plant specimens were deposited at Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. All plant samples were dried at 40^0 C for 6 h in hot air oven and pulverized.

Preparation of the extracts

Extract for chemical analysis

Plant samples (5 g) were separately extracted with 50 ml of methanol under sonication for 30 min and filtered. The process was repeated for 3 times. The combined filtrates were concentrated under reduced pressure and the final volume was adjusted to 5 ml with methanol, then filtered through a 0.45-µm PTFE syringe filter before applied to the TLC and HPLC system.

Extract for brine shrimp lethality test

Plant samples (15 g) were refluxed (80°C) with distilled water for 3 h (plant/solvent ratio 1:10 w/v), then filtered. The filtrate was taken to dryness by lyophilization.

Phytochemical analysis

Thin layer chromatography analysis [4, 5]

Thin layer chromatography of all extracts was performed on TLC silica gel 60 GF_{254} aluminium sheet, using chloroform-ether (4:1) as the solvent system. TLC plates were detected under UV at white R and 366 nm, vanillin sulfuric acid spray reagent.

High performance liquid chromatography analysis [6, 7]

HPLC-DAD: Shimadzu (Kyoto, Japan) LC-10ADvp series liquid chromatography with binary pump, a model 7725i manual injector valve with a 5 µL sample loop, thermostated column compartment and diode array detector (DAD), XBridge™ Shield RP18 column (150 mm x 4.6 mm i.d., particle size 5 µm) were used for the analysis. HPLC column was maintained at room temperature. HPLC analysis of the extracts was carried out using the conditions applied from the methods for terpenoid analysis previously reported. The gradient mode of mobile phases with acetonitrile containing 0.005% trifluoroacetic acid (solvent A) and Milli-Q water containing 0.005% trifluoroacetic acid (solvent B) was used. The flow rate was 0.6 mL/min, the injection volume was 5 µL and the chromatograms were monitored at 266 nm.

HPLC-ESI-MS: HPLC conditions were the same as described above. The MS analysis was performed using the Shimadzu (Kyoto, Japan) 2100 series mass selective detector (MSD) equipped with an electrospray ionization (ESI) source operated in the positive ion mode. Instrument control and data acquisition were performed using Shimadzu LC-MS solution software. The ion source temperature was 25°C, and the needle voltage was set at 1.5 kV. Nitrogen was used as the drying and nebulizer gases at a flow rate of 1.5 L/min and a backpressure of 27.6 MPa. The scanning range was starting *m/z* 100 and ending *m/z* 800.

Brine shrimp lethality test [8, 9]

Eggs of *Artemia salina* were hatched by incubating in saline water for 48 h at room temperature under light source. Stock solution of each plant extract was prepared by dissolving in saline water at the concentration of 10

mg/ml. An aliquot of 500 µL was added to well containing 4.5 mL of saline water and mixed thoroughly. Ten larvae were then added to the well and maintained at room temperature under light source for 24 h. A control treatment contained only saline water. The experiment was done in triplicate. Survivors were counted and calculated for percentage of lethality.

3. RESULTS

Phytochemical analysis

TLC analysis

TLC analysis of methanolic leaf extracts from *Callicarpa* species showed specific terpenoid pattern detected by vanillin sulfuric acid spray reagent. Based on the R_f value and color reaction, spots corresponding to triterpene oleanolic acid, *β*-sitosterol and diterpene abietic acid were observed in all samples, the most prominent one belonged to oleanolic acid (Figure 1).

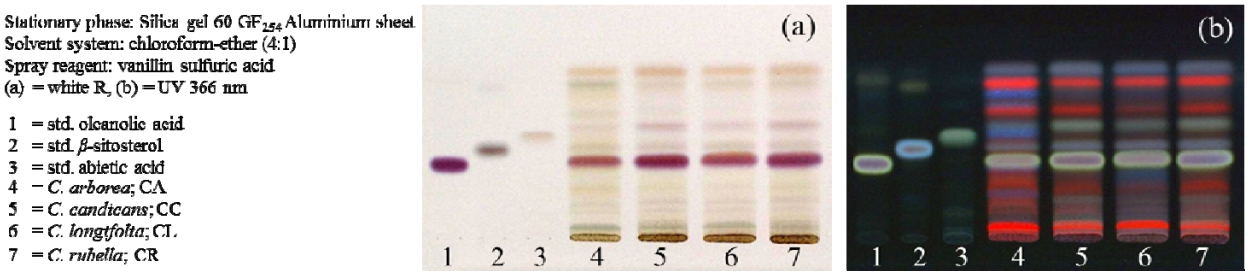

Figure 1. Thin-layer chromatography of leaf extracts from the 4 investigated *Callicarpa* species.

HPLC analysis

From HPLC-DAD analysis (Figure 2), typical terpenoid fingerprints of 4 test samples were observed at 266 nm and six common characteristic peaks (peaks 3, 4, 6, 8, 17 and 18) were found in all samples. By comparing their retention times (t_R) and UV spectra with the reference standards, three out of those six peaks were structurally confirmed as *β*-sitosterol (peak 3), abietic acid (peak 17), oleanolic acid and/or ursolic acid (peak 18) with the t_R of 12.68, 34.03 and 36.83 min, respectively. It is noteworthy that two main peaks at t_R 16.57 and 21.76 min (peaks 7and 9, respectively), were only presented in CC HPLC fingerprint. Positive mode ESI-MS spectrum of compound from peak 9 showed *m/z* at 332 (Fig. 3A) and DAD absorbance spectrum (Fig. 3B) showed λ_{max} at 266 nm corresponding to chemical character of callicarpone previously reported [4, 5]. ESI MS result has suggested a presence of the pesticidal active component, callicarpone in *C. candicans*.

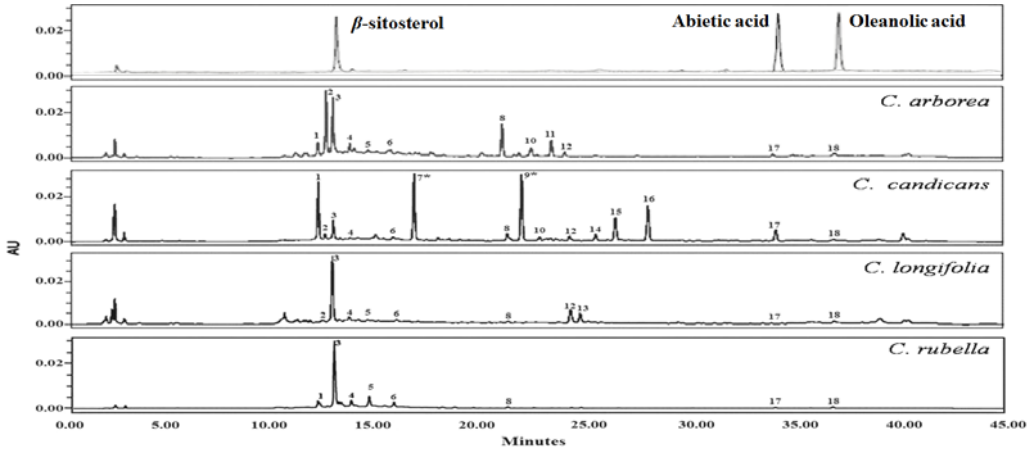

Figure 2. HPLC-DAD chromatograms of leaf extracts from the 4 investigated *Callicarpa* species at 266 nm.
* Only found in *C. candicans*

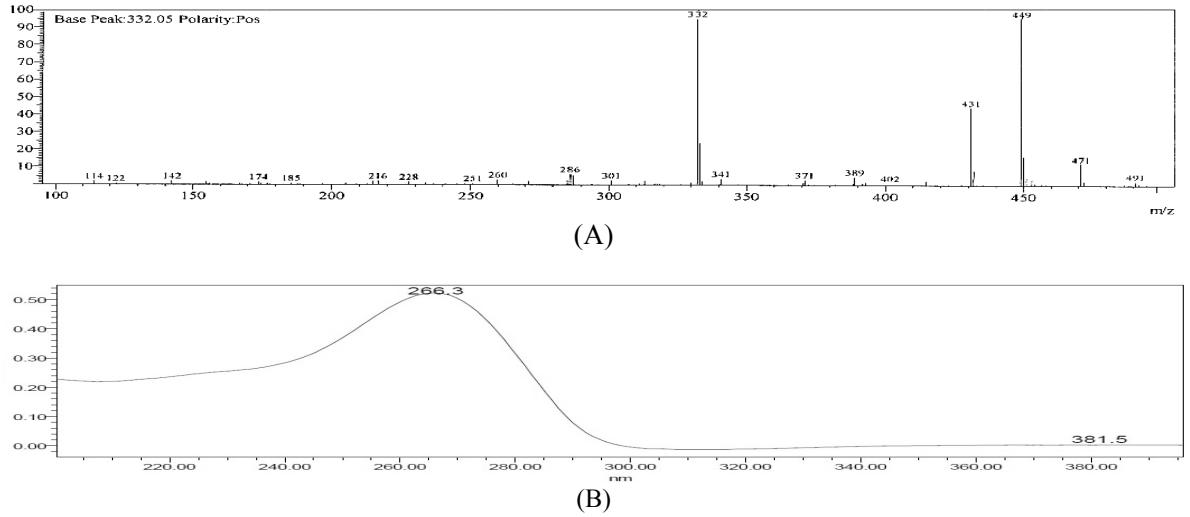

Figure 3. ESI-MS fragmentation pattern (A) and UV spectra (B) of compound from peaks 9.

Brine Shrimp Lethality Test

The results of toxicity to brine shrimp of aqueous leaf extract from *Callicarpa* species were shown in Table 1. Extract from CC showed the highest cytotoxic effect (86.67 % lethality) while that from CR was the lowest activity (3.33 % lethality).

Table 1. Toxicity to brine shrimp of leaf extracts from the 4 investigated *Callicarpa* species.

Test sample	% Lethality
CA	16. 67
CC	86.67
CL	20.00
CR	3.33
Control (saline water)	0.00

4. CONCLUSIONS

Our studies demonstrate HPLC-DAD fingerprints of di- and triterpenoid components of leaf extracts from the 4 investigated *Callicarpa* species. The fingerprints of all samples showed six similar chromatographic peaks. By comparing of retention time together with UV absorbance spectral data, three out of the six peaks were structurally confirmed as *β*-sitosterol, abietic acid, oleanolic acid and/or ursolic acid. Compound corresponding to callicarpone was observed only in *C. candicans* fingerprint. Preliminary toxicity evaluation of aqueous extract of 4 plant species showed a strongest effect in *C. candicans* which corresponded to its chemical profile.

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A glucose/mannose-specific lectin with alpha-glucosidase inhibitory activity from *Sterculia monosperma* Vent seeds

Aphichart Karnchanatat*[a], Polkit Sangvanich[b]
[a]The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330
[b]Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330
*i_am_top@hotmail.com phone +66-22188078 fax +66-22533543

ABSTRACT

Alpha-glucosidase inhibitors (GIs), delay digestion and thus impair absorption of glucose in the intestines by inhibiting the enzyme responsible for breaking down maltose. It can thus be used to control human non-insulin dependent diabetes mellitus and obesity. The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to the suppression of glucose production from dietary carbohydrates in the gut or glucose absorption from the intestine. GI from the seeds of *Sterculia monosperma* were purified by using a combination of 80% ammonium sulfate precipitation, ion exchange chromatography with DEAE cellulose, and gel filtration chromatography with Superdex 200, respectively. The enriched preparation had a specific activity of 355.76 GI U/mg proteins for a yield of 13.88% total protein. The molecular weight of this GI was estimated by SDS-15% (w/v) PAGE to be about 30.8 kDa, of which 15.8% was carbohydrate. The pH stability of this GI was between pH 6.0-8.0, and it is stable up to 40^{o}C but is totally inactivated after exposure to 60 ^{o}C for 120 min, and a high activity with some divalent cations such as Ca^{2+} and Mn^{2+} at less than 25 mM but not by Mg^{2+}, Fe^{2+}, Hg^{2+}, Co^{2+}, and EDTA, and appeared as a non-competitive inhibitor of alpha-glucosidase (K_i of 0.58 mg protein/ml). The amino acid sequence of an internal fragment of this purified GI had a similarity to the sequence from plant glucose/mannose-specific lectin family.

Keywords: Alpha-glucosidase inhibitory activity, Glucose/mannose-specific lectin, *Sterculia monosperma*

1. INTRODUCTION

Diabetes mellitus is a common disorder associated with increased morbidity, mortality rate. At the present time it is estimated that 150 million people worldwide have diabetes and that the member will increase to 220 million by 2010 and 300 million by 2025. Globally, the percentage of type 2 diabetes (non insulin dependent diabetes mellitus) is grater than 90% [1]. Patients with diabetes have an increased risk of cardiovascular disease (CVD). Recently, much attention has been paid to evidence that abnormalities of the postprandial state are an important contributing factor to the development of arthrosclerosis, even in diabetes mellitus. Postprandial hyperglycemia is more important in a development of macrovascular disease [2]. Therefore, effect control of postprandial hyperglycemia is more strongly warranted than previous thought. To control postprandial hyperglycemia, α-glucosidase inhibitors are widely used, as monotherapy as well as combination therapy with antidiabetes agents [3].

In Thailand, the use of herbal medicine by the suffers of chronic disease is encouraged because there is concern about the adverse effects of chemical drugs and treatment using medicines of natural origin appears to offer more gentle means of managing such disease [4-7]. Herbal drugs are prescribed widely because of their effectiveness, fewer side effect and relatively low cost. To this end, research has begun to embrace traditional medicines from various cultures, as scientists search for clues to discover new therapeutic drugs for diabetes [8]. Therefore, investigation on such agents from traditional medicinal plants has become more important and researches are competing to find the new effective and safe therapeutic agent for treatment of diabetes.

α-glucosidase is a very important enzyme responsible for the hydrolysis of dietary disaccharides into absorbable monosaccharide in microbial system and in small intestine of animal digestive system. Glucosidases are not only essential for carbohydrate digestion but it is also very important for processing of glycoproteins and glycolipids and are also involved in a variety of metabolic disorders and other diseases such as diabetes [9]. Inhibition of α-glucosidase activity in animal guts decreases the blood glucose levels via delaying digestion of poly- and oligo-saccharides to the absorbable monosaccharides [10]. Thus, α-glucosidase inhibitors (GIs) testing is useful for screening plants that could be used for blood glucose treatment. Previous studies have revealed that the GI activity of cyanidin-3-galactoside, a natural anthocyanin, can be used in combination with acarbose for the treatment of diabetes [11]. Some GI active substances have been developed from bacterial sources to pharmaceutical applications, such as acarbose (glucobay®) from *Actinoplane* sp. 5 [12], voglibose (basen®) from *Streptomyces hygroscopicus* var. *limoneus* [13] and miglitol (glyset®) from *S. roseochromogenus* [14]. Thus, considerable effort has been made to search for more effective and safe GIs from natural materials to develop physiologically functional foods to treat diabetes mellitus.

The small evergreen china chestnut tree, *Sterculia monosperma* Ventenat (Sterculiaceae), also commonly known as Seven Sister's fruit, Ping pong and Pheng Phoh, is a member of the tropical chestnut trees and is found throughout the tropical and subtropical areas. In Thailand, this plant is known as "Kao-lat Thai" because these trees are found abundantly in the northern part of Thailand, especially in the Nan province. Seeds of *S. monosperma* have long been used in cooking and one of its distinctive characteristics is that when cooked, they have the color of an egg yolk. Vegetarians lavish them in their cooking. Chinese gourmets use them to enrich the taste and flavors of dumplings and in brewing pots of herbal chicken, duck or pork. Then the aim of this research was to study the GI activity of *S. monosperma* seeds in relation to their proteinaceous content.

2. MATERIALS AND METHODS

Material

The fresh seeds of *S. monosperma* were obtained from Nan Province in the northern part of Thailand. Thus, the exact cultivar, geographical location and season of cultivation are not known and so the effect of such variations within the species in enzyme isoforms or levels is not addressed here. Methyl-α-D-glucopyranoside was purchased from Fluka (Germany). Ammonium sulfate, acrylamide, bis-acrylamide, hydrochloric acid, mercaptoethanol, sodium acetate, TEMED (Tetramethylethylenediamine), Tris (hydroxymethyl) aminomethane, the divalent metal salts and (Ethylenediaminetetraacetic acid) EDTA was purchased from Merck group, Germany. Ammonium persulfate, coomassie Blue G-250, glacial acetic acid, methanol, sodium chloride (NaCl), sodium hydroxide and sodium dodecylsulfate (SDS) were from BDH and purchased from VWR international, USA. α-Glucosidase from *Saccharomyces cerevisiae* Type I, as a lyophilized powder, ≥10 units/mg protein, diethylaminoethyl-cellulose (DEAE-cellulose) and Superdex 200 were purchased from Sigma-Aldrich Co. Ltd, USA. All chemicals were analytical grade.

Extraction of GI from seeds of *S. monosperma*

One kilogram of *S. monosperma* seeds was homogenized in and defatted in acetone at 4°C (200 mL aqueous acetone per 10 g seed). *T*he insoluble material was then removed by vacuum filtration and extracted overnight at 4°C with 20 volumes of TBS (20 mM Tris-HCl buffer, pH 7.2, plus 150 mM NaCl). The suspension was then clarified by filtration through double-layered cheesecloth followed by centrifugation at 15,000 × *g* for 30 min. The clarified supernatant was harvested and ammonium sulfate added, with stirring, to 90% saturation and left with stirring overnight at 4°C. The precipitate was harvested by centrifugation at 15,000 × g for 30 min, discarding of the supernatant, and dissolved in TBS prior to being dialyzed against 3 changes of 5 L of water and then freeze-dried.

GI activity

The assay method was modified from that reported previously [15]. GI activity was evaluated at every step of the enrichment procedure. Twenty µL of α-glucosidase (1 U/mL) in TB (20 mM Tris-HCl buffer, pH 7.2) was mixed with 10 µL of the test protein sample and 60 µL of TB and then incubated at 37°C for 10 min before 10 µL of 1 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) in TBS as substrate was added. After incubation at 37°C for 30 min, the reaction was stopped by the addition of 100 µL of 0.5 M Na_2CO_3. The GI activity was determined by measuring the release of the yellow *p*-nitrophenol at 400 nm, and calculated as follows;

$$\% \text{ Inhibition} = \frac{(OD_{control}-OD_{blank})-(OD_{sample}-OD_{blank})}{(OD_{control}-OD_{blank})} \times 100$$

IC_{50} determination

The half maximal inhibition concentration (IC_{50}), as the concentration of the protein sample that inhibited 50% of the maximal α-glucosidase enzyme activity, was evaluated by using two-fold dilutions of each test protein sample and then proceeding as described in above.

Enrichment of the GI protein

DEAE-cellulose ion-exchange chromatography

Following the 80% saturation ammonium sulfate cut off the next purification step was performed using DEAE-cellulose ion-exchange chromatography on an ÄKTA Prime system (Amersham Biosciences, Uppsala, Sweden). The ammonium sulfate cut off fraction, resolved at 50 mg/mL was applied (5 mL) to the TB pre-equilibrated column (1.6 cm × 20 cm) and then eluted with the same buffer at a flow rate of 1.0 mL/min collecting 10 mL fractions. After 280 mL a linear gradient of 0-1.0 M NaCl in the same buffer was then applied over the next 750 mL. Fractions were assayed for α-glucosidase inhibitory activity (assay for α-glucosidase inhibition activity section), and those found to contain α-glucosidase inhibitory activity were pooled, dialyzed (3.5 kDa cut-off tubing) against TB, and concentrated by freeze dry to 50 mg/mL ready for further purification by gel filtration chromatography and analysis, and is subsequently referred to as the "post-DEAE-cellulose GI fraction".

Superdex-200 gel filtration chromatography

The post-DEAE-cellulose GI fraction (pooled fractions from DEAEcellulose ion exchange chromatography that displayed α-glucosidase inhibitory activity) was applied (2 mL at 50 mg/mL) to a pre-equilibrated (TB/100 mM NaCl) Superdex-200 column (1.6 cm × 60 cm) and then eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 5.0 mL were collected and assayed for α-glucosidase inhibitory activity (as per assay for α-glucosidase inhibition activity section), and contiguous GI positive fractions (from the same peak) were pooled and dialyzed (3.5 kDa cut-off tubing) against an excess of same buffer prior to further analysis. This final preparation is referred to as the "enriched GI protein fraction".

Determination of the protein content

The protein concentration was determined following the standard Bradford assay [16], with dilutions of a known concentration of bovine serum albumin as the standard. The absorbance at 595 nm was monitored with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). During the column chromatographic separations, the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Carbohydrate assay

Total carbohydrate concentration was estimated using the phenol sulfuric acid method [17]. This method involved adding phenol solution (80% w/v, 50 µL) and concentrated sulfuric acid (2.0 mL) to the sample (100 µL) and allowing the mixture to stand at room temperature for 10 min. The solution was vortexed and allowed to cool before reading the absorbance at 490 nm. Absorbance values obtained were translated into glucose equivalent using a glucose standard curve.

Molecular weight determination by SDS-PAGE

Discontinuous reducing SDS-PAGE gels were prepared with 0.1% (w/v) SDS in 15% and 5% (w/v) acrylamide separating and stacking gels, respectively, with Tris-glycine buffer pH 8.3 containing 0.1% (w/v) SDS as

the electrode buffer, according to the procedure of Laemmli [18]. Samples to be analyzed were treated with reducing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Molecular weight standards were co-resolved in each gel alongside the samples to determine the subunit molecular weight of the purified protein(s). After electrophoresis, proteins in the gel were visualized by standard Coomassie Blue R-250 staining as detailed in determination of the protein pattern by native-PAGE section.

Effect of temperature on the GI activity

The effect of temperature on the GI activity was determined by incubating the enriched GI protein fraction samples in TB at various temperatures (4-90°C at 10°C intervals) for 30 min, and then assaying the residual GI activity with 100% and 0% activity controls, as described in assay for α-glucosidase inhibition activity section.

pH-dependence of the GI activity

Incubating the enriched GI protein fraction samples in buffers of broadly similar salinity levels, but varying in pH from 2 to 14 was used to assess the pH stability and the pH optima of the GI. The buffers used were 20 mM glycine-HCl (pH 2-4), 20 mM sodium acetate (pH 4-6), 20 mM potassium phosphate (pH 6-8), 20 mM Tris-HCl (pH 8-10), and 20 mM glycine-NaOH (pH 10-12). The enriched GI protein fraction was mixed in each of the different buffer pH compositions, or TB for the control, and then left for 30 min at room temperature. Next, the samples were adjusted back to pH 7.2 and assayed for GI activity (assay for α-glucosidase inhibition activity section), and the activities attained were compared with the control which was set as 100% activity.

Effect of metal ions on the GI activity

The effect of pre-incubation of the enriched GI protein fraction with six different divalent metal cations and the chelating agent EDTA on the resultant GI activity was evaluated as follows. The enriched GI protein fraction (1 mg/mL) was incubated for 10 h with one of Ca^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, or EDTA at one of the indicated concentrations (12.5, 25, 50, and 100 mM) in TB with continuous shaking and was then tested for GI activity as described in assay for α-glucosidase inhibition activity section, using at least three replicates for each assay.

Mechanism of the inhibition

To evaluate the inhibition mode of the enriched GI protein against α-glucosidase, the PNPG solution at one of 0.025-0.2 mM, as the substrate, was added to the α-glucosidase (1 U/mL) in TB in the presence of 0, 0.05 and 0.075 mg/mL of the enriched GI protein fraction sample. The remaining α-glucosidase activity, and thus the GI activity, was determined as outlined in above. The inhibition type was determined by Lineweaver-Burk plots, where v is the initial velocity and [S] is the substrate concentration used.

Internal amino acid sequence of GI by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS)

A selected protein band from SDS-PAGE was excised, cut into small pieces (ca. 1 mm^3) and washed with 100 µL deionized water. The sample preparation process then followed the published method of Mortz [19], with the trypsinization using 100 ng of proteomics grade trypsin (Sigma) in 40 µL of 50 mM NH_4HCO_3 at 37 ^oC overnight. The supernatant was then harvested following centrifugation at 15,000 × g for 1 min. The remaining peptides in the gel were extracted with a solution of 50% (v/v) acetonitrile containing 5% (v/v) formic acid for 10 min with shaking, and subsequently pooled with the supernatant and taken to dryness. The likely amino acid sequence of each internal fragment of the trypsinized peptide was then analyzed by LC/MS/MS mass spectrometry. The extracted tryptic peptides were then subjected to LC-nano ESI/MS/MS. All collected LC/MS/MS data were processed and submitted to a MASCOT (http://www.matrixscience.com) search of the NCBI database (http://blast.ncbi.nlm.nih.gov). The following criteria were used in the Mascot search: (i) trypsin cleavage specificity with up to three missed cleavage sites, (ii) cysteine carbamidomethyl fixed modification, (iii) methionine oxidation variable modifications, (iv) ± 0.2 Da peptide tolerance and MS/MS tolerance and (v) ESI-TRAP fragmentation scoring [19].

3. RESULTS

GI was extracted from the seeds of *S. monosperma* using method by Boonmee [15]. In this study, the purified GI from crude extract was precipitated with 80% ammonium sulfate and dialyzed in TB. The dialysate was further purified by DEAE-cellulose column, Superdex 200 and each fraction was tested for its inhibitory activity. Figure 1A showed the elution chromatographic profile from DEAE-cellulose column. The GI activity remained in the bound fraction and eluted from the column in the main peak at ~150-175 mM NaCl. Fractions showing inhibitory activity against α-glucosidase were pooled, concentrated and applied on to the Superdex 200 column. The eluted chromatogram of proteins was showed in Figure 1B. The enriched GI protein fraction had a specific activity of 171.44 GI U/mg proteins for a yield of 43.35% total protein (Table 1). Concentrate fraction from Superdex 200

column of the purified GI showed a single protein band on 15% SDS-PAGE with ~7.5 mg loading protein after coomassie blue staining (Figure 2). This support that the obtained protein was pure unless contamination of other proteins. In the denature from and under the breaking of disulfide bonds this protein have 1 subunit of 30.8 kDa, as in this fraction, was sub sequentially found to contain 17.2% sugar by Dubois's method.

No significant changes in the inhibition activity of the enriched GI fraction was seen when pretreated for 30 min within the temperature range of 4 - 40°C, but at 60°C the observed GI activity was essentially abrogated (Figure 3). This is a very broad temperature range for GI activity. Pre-incubation of the inhibitor at 37°C for 30 min at various pH from 2 - 12. After incubation the solution was adjusted the pH back to pH 7.2, substrate 1 mM PNPG were added for the assay of α-glucosidase activity. Figure 3B showed α-glucosidase inhibitory activity had increased from pH 5.0 to 6.0, maximal inhibitory activity at pH 6.0 - 8.0 and reduced ability of α-glucosidase inhibitory at pH 8.0-9.0. Incubating the enriched GI protein fraction preparation (1 mg/mL) with each of the six different divalent metal cation salt solutions, plus EDTA, at five different concentrations for 10 h prior to assaying for GI activity, revealed a requirement for Ca^{2+}, Mg^{2+}, and Mn^{2+} at less than 25 mM to be effective for GI activity, suggesting they are essential for the stability of the AI protein structure and activity (Table 2). In contrast, Fe^{2+}, Hg^{2+}, Co^{2+}, and EDTA did not support any GI activity.

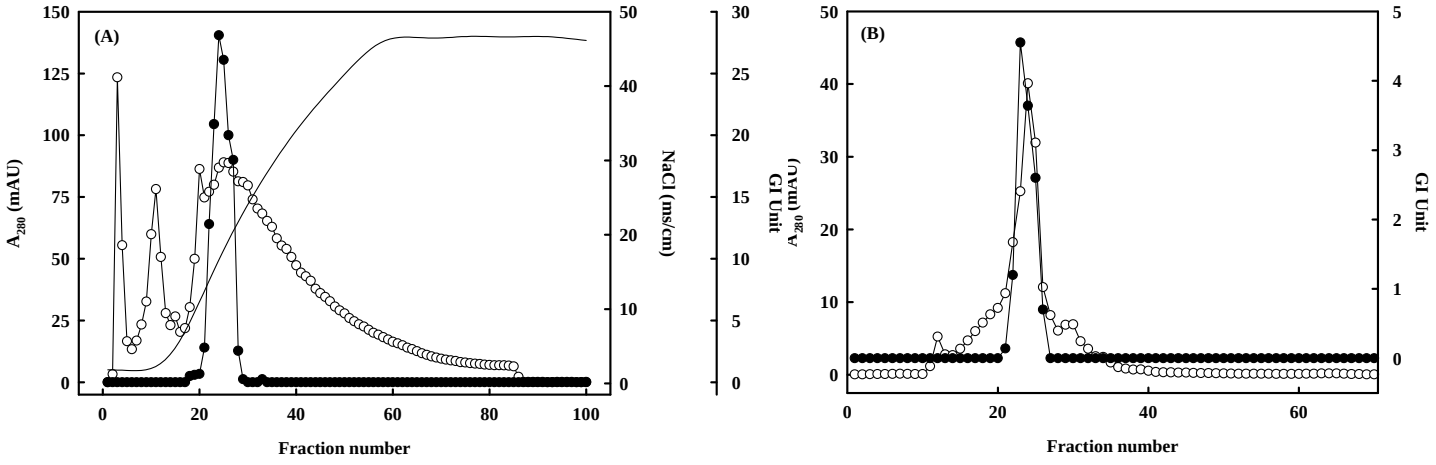

Figure 1. (A) DEAE-cellulose ion exchange chromatography of the ammonium sulfate cut fraction solubilized in TB, and eluted in the same buffer but with a linear gradient of 0-1.0 M NaCl at a flow rate of 1 mL/min. (○) Absorbance at 280 nm, (●) α-glucosidase inhibitory activity. (B) Superdex-200 gel chromatography of the post-DEAE-cellulose GI fraction. Fractions (5 mL) were eluted with TB/100 mM NaCl at a flow rate of 0.5 mL/min. (○) Absorbance at 280 nm, (●) α-glucosidase inhibitory activity. Profile shown is representative of three independent trials.

Table 2. Summary of the enrichment of the α-glucosidase inhibitor (GI)[a]

Purification step	Total protein (mg)	Total inhibitory activity (GI U)	Specific GI activity (GI U / mg)	Yield (%)	Purification (fold)
Crude extract	523.53	2,086.47	3.96	100.00	1.00
80% $(NH_4)_2SO_4$ cut	122.60	1,725.44	14.07	82.70	3.56
DEAE-cellulose	17.22	960.84	55.80	46.05	14.11
Superdex-200	1.45	248.60	171.44	11.91	43.35

[a]Data shown is representative of three independent trials.

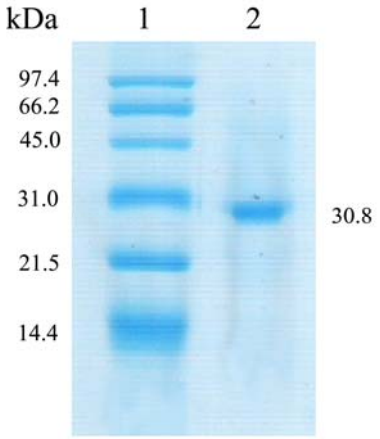

Figure 2. Lane 1, molecular weight standards; Lane 2, enriched GI protein fraction from Superdex 200 fraction (7.5 µg of protein).

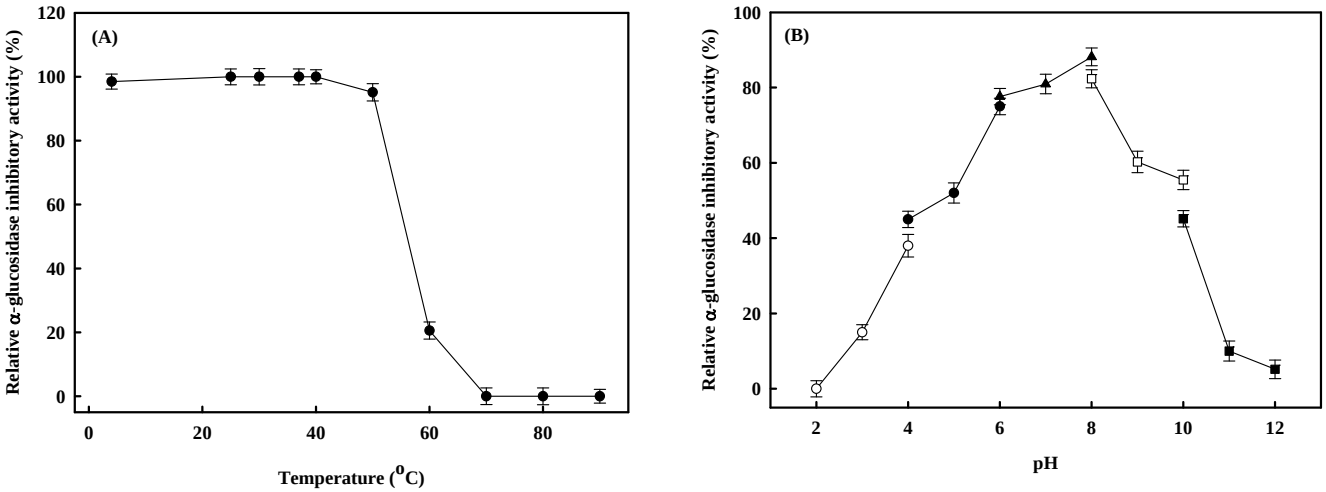

Figure 3. (A) Effect of pretreatment temperature on the GI activity of the enriched GI protein fraction towards α-glucosidase. (B) The effect of pH pretreatment on the AI activity of the enriched GI protein fraction against α-glucosidase. The data are shown as the mean ± 1 SEM and are derived from three repeats. The following buffer systems were used: (○) 20 mM glycine-HCl (pH 2.0 - 4.0), (●) 20 mM sodium acetate (pH 4.0 - 6.0), (▲) 20 mM potassium phosphate (pH 6.0 - 8.0), (□) 20 mM Tris-HCl (pH 8.0 - 1 0.0) and (■) 20 mM glycine-NaOH (pH 10.0 - 12.0). For both panels the data are shown as the mean ±1 SEM and are derived from three repeats.

The GI protein band from SDS-PAGE was excised for in gel trypsin digestion and anaysis by LC-MS/MS, revealed two peptide fragments with the likely sequence TSFIVSDTVDLK, and GNVETNDVLSWSFASK. Sequences comparison showed that amino acid sequence of peptide fragments from *S. monosperma* GI was similar to those from *Phaseolus vulgaris* and *P. coccineus*. This suggested that *S. monosperma* GI was related to plant glucose/mannose-specific lectin family. The catalytic kinetic studies for α-glucosidase activity, with different substrate and enriched GI lectin fraction concentrations were initially analyzed using Lineweaver-Burk plots (Figure 5) Both the maximal velocity (V_{max}, y-intercept) and the Michaelis-Menten constant (K_m, slope of the trend lines) decreased with increasing concentrations of the enriched GI fraction, and so this GI acted as a non-competitive inhibitor of α-glucosidase. K_i value for the GI of 1.39 µg/mL was obtained via non-linear regression using the least squares difference method.

Table 2. The effect of divalent metal cations and EDTA on the α-glucosidase inhibitory activity [a]

Metal salt	Concentration (mM)			
	12.5	25	50	100
Ca^{2+}	-	0.78 ± 0.003	0.63 ± 0.001	0.24 ± 0.004
Mn^{2+}	-	0.77 ± 0.015	0.35 ± 0.001	0.32 + 0.002
Mg^{2+}	-	0.48 ± 0.001	-	-
Fe^{3+}	-	-	-	-
Hg^{2+}	-	-	-	-
Co^{2+}	-	-	-	-
EDTA	-	-	-	-

[a]Data are shown as the mean ± 1 SEM, and are derived from triplicate assays
[*]α-glucosidase from *S. cerevisiae* (1.0 U/ml)
[*]Control contained no salt in TB

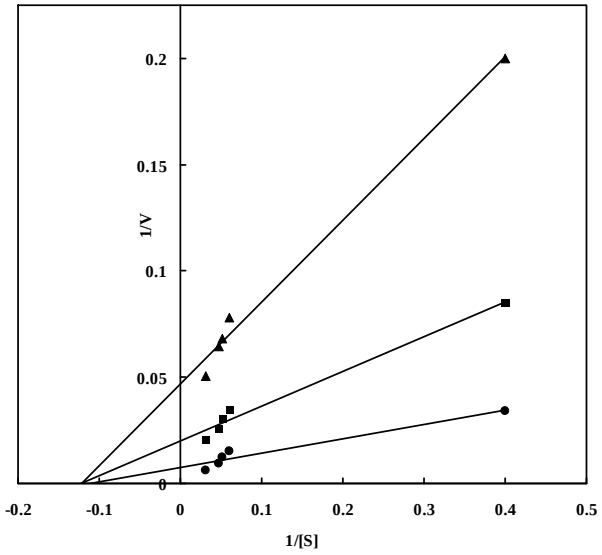

Figure 5. Line weaver-Burk plots derived from the inhibition of α-glucosidase by the enriched GI lectin fraction from *S. monosperma* seeds. α-glucosidase was treated with each indicated concentration of PNPG solution (one of 0.025-0.2 mM) in presence of the enriched GI lectin fraction at (●) 0, (■) 0.05 and (▲) 0.075 mg protein/mL. Data are shown as the mean ± 1 SD, derived from three repeats.

4. CONCLUSIONS

A GI of ~30 kDa and a potential member of the plant glucose/mannose-specific lectin family, was purified from the seeds of *S. monosperma*, by ammonium sulfate precipitation followed by DEAE-cellulose ion exchange and Superdex-200 gel chromatography, respectively. The GI activity was optimal at a pretreatment pH range of 6.0-8.0 and relatively stabile, but was markedly reduced by more basic pH values above 9.0 or acidic pH values below 4.0. The GI was heat stable below 40°C for 30 min. Divalent cations appeared to be essential for the GI activity, with this requirement being met by some (Ca^{2+}, Mg^{2+}, and Mn^{2+}) but not other (Fe^{2+}, Hg^{2+} and Co^{2+}). The inhibitor inhibited α-glucosidase activity through the Vmax decreased indicating that the type of inhibition is non-competitive.

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Comparative study on antioxidative activity of the seeds of hoary basil (*Ocimum basilicum*) protein hydrolysates produced by papain, pepsin and Protease G6 (alcalase)

Aphichart Karnchanatat*[a], Karittha Semanit[b], Sajee Noitang[a], Jittra Piapukiew[c]
[a]The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330
[b]Program in Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330
[c]Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330

*i_am_top@hotmail.com phone +66-22188078 fax +66-22533543

ABSTRACT

Excessive free radical production have known to be the cause of various degenerative disorder of human such as cardiovascular diseases, aging, cancer, and neurodegenerative disease like Alzheimer's disease. By-products form the chemical extraction of essential oil and fiber of the seeds of hoary basil (*Ocimum basilicum*) are abundant and underutilized resources that can be used as a unique protein source to make protein hydrolysates. Antioxidative activity of protein hydrolysates prepared from hoary basil seed protein hydrolysates (HBPs) using papain, pepsin and Protease G6 (alcalase) were determined. In addition, all HBPs exhibited antioxidative activity in a concentration-dependent manner. Protease G6-HBPs generally showed greater antioxidative activity (0.0196 ± 0.064 mg/ml) than papain-HBPs (0.2033 ± 0.219 mg/ml), and pepsin-HBPs (0.0813 ± 0.00813 mg/ml) protein hydrolysates as indicated by the higher 1,1-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity. Protease G6-HBPs were fractionated into three fractions, HBPs-I (MW> 10 kDa), HBPs-II (MW = 5-10 kDa), and HBPs-III (MW < 5 kDa) and the *in vitro* antioxidant activities of all fractions were determined. HBPs-III showed the lowest IC_{50} value at 0.02523 ± 0.14 mg/mL. In addition, the protein of hoary basil seed have a high percentage of essential amino acids, Moreover, protein showed the presence of phenlyalanin, tyrosine, and histidine, these amino acids have also been reported to show antioxidant activity. HBPs had a high nutritional value and could be used as supplement to poorly balanced dietary proteins.

Keywords: Antioxidative activity, Protein hydrolysates, Hoary basil, *Ocimum basilicum*

1. INTRODUCTION

Oxidation is one of the main causes for diseases and pathogenesis in human. For example, free radical attack on proteins, lipids, and nucleic acids, results in cell damage, apoptosis and plays an important role in atherosclerosis, Alzheimer's disease, inflammatory bowel disease, and certain cancers [1]. Essentially, all the cellular components and the specific constituents are susceptible to reactive oxygen species (ROS) and reactive nitrogen species (RNS), for example, hydroxyl radicals ($^{\cdot}OH$), peroxyl radicals ($^{\cdot}OOR$), superoxide anion ($O_2^{-\cdot}$), and peroxyl nitrite ($ONOO^-$). The accumulation of protein carbonyl compounds, a result of oxidation, is believed to be a main mechanism of aging process in human [2]. Antioxidants play a vital role in human body, reducing oxidative processes. In the human body, endogenous antioxidants, including enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and various nonenzymatic compounds such as selenium, α-tocopherol, and vitamin C help to protect tissues and organs from oxidative damage caused by ROS and RNS [3]. Apart of these, amino acids, peptides and proteins also contribute to the overall anti-oxidative capacity of cells and towards maintaining the health of biological tissues. In recent years, a considerable amount of research has also focused on the liberation of antioxidant peptides encrypted within food proteins, with a view to utilizing such peptides as functional food in gradients aimed at health maintenance. Interestingly, with the parent protein sequence, the peptides are inactive and thus must be released to exert an effect. These bioactive peptides are 2-20 amino acid residues in length, although some have been reported to have more than amino acid residues [4].

Protein hydrolysate is a product of hydrolysis reaction from protein. Mixtures of free amino acid are prepared by splitting a protein with chemical or enzyme. Chemical protein degradation is the low cost, but limited in its use in food product or pharmaceutical, make it difficult to control product quality [5]. The enzymatic hydrolysis by controlling condition such as hydrolysis time, temperature, pH, unit enzyme is a method that is more effective to provide the maximum among of peptides. Where with the enzyme has a specific substrate [6]. Enzymatic hydrolysis, which is also in a mild condition, can control degradation by choosing type of enzymes and optimized condition. The obtained protein hydrolysate was with desired properties. The source of protein hydrolysate is mostly waste or cheap materials from agricultural derived from plants or animals [7]. Protein hydrolysate from plants is the product of the hydrolyzed material that is high in protein as amino acids, peptides and other compound which can have aromatic ring types, such as phenylalanine, tyrosine, tryptophan, histedine and cysteine [8]. These amino acids can donate proton to the free radicals resulting this hydrolysate has an antioxidant activity. Reported low molecular polypeptide components with histedine, tryptophan and tyrosine showed highly antioxidant activity [9].

Hoary basil (*Ocimum basilicum*) is a biennial plant, one of the major oil producing species, commonly used in many kind of food. The seed are eaten as a dessert and used as a mild laxative. It can also decrease blood sugar levels in diabetes's patients [10]. The initial study found that essential oil of Hoary basil composed of highly essential fatty acid [11]. Hoary basil seed is a by-product form the chemical extraction of essential oil and fiber. It is interested material in producing active protein hydrolysate. It increase value of waste and also, also it is reported that defatted Hoary basil seed was analyzed composed of 17.16% protein [11]. Although protein content of this seed less than the other, however we have trialed for the amount of active free amino acid before, other than hoary basil seed is a new source of protein hydrolysate. The hoary basil seed protein hydrolysates have not been study for bioactivity elsewhere. The goal of this study was to evaluate of antioxidant activity of bioactive protein hydrolysate by Protease G6 form hoary basil seed.

2. MATERIALS AND METHODS

Biological and chemical materials

Hoary basil seed were purchased from Yoawarat market Bangkok Thailand. The samples were quickly taken to laboratory and kept in dark 4°C room until used. Ascorbic acid, bovine serum albumin (BSA), 2, 2-Diphenyl-1-picryhydrazyl (DPPH), and trichloroaceticacid (TCA) were purchased from Sigma Chemicals Co. (USA). Protease G6 was purchased from Siam Victory Chemicals Co., Ltd (Thailand). All other unlabeled chemicals and reagents were of analytical grade.

Defatted hoary basil seed preparation

Raw hoary basil seed was selected and cleaned to remove contaminated, crushed with a blender and it was defatted by petroleum ether using soxhlet for 10-12 hour at 65°C. Hot air oven with 60°C then the defatted hoary basil seed was sieved using laboratory hammer mill through 90 meshes and was kept in the dedicator until used.

Total amino acid analysis

Acid hydrolysis

Five milliliters of HCl 6N was added (5 mg protein/ml HCl) and mixed. The tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110°C for 22 h. The internal standard (10 ml of 2.5 mM L-α-amino-*n*-butyric acid in HCl 0.1 M) was added and diluted with water to 250 ml. The solution was filtered with 0.20 µm filter and was then derivatized with 6 aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Flour reagent). It was then heated in a heating block at 55°C, for 10 min. Heating converts a minor side product of tyrosine to a major mono-derivatized compound. Total amino acid content was determined by high performance liquid chromatography.

Chromatographic conditions

Chromatographic separation was carried out in a Waters Alliance 2695 with heater amino acid analysis Hypersil Gold column C18. The column was thermostatted at 35 ± 1°C and the flow rate was 1.0 ml/min. The injection volume was 5 µl. Mobile phase A consisted of sodium acetate buffer pH 4.90 and 60% acetonitrile.

Preparation of hoary basil seed protein hydrolysate (HBPs)

The sieved hoary basil seed less than 90 meshes was hydrolyzed using papain, pepsin, and ProteaseG6, and using a hydrolyze ratio of substrate: enzyme was 0.5:10 (w/v). The hydrolysate was conducted for 4 hours at 50°C with shaking 150 rpm. The reaction was stopped by heating at 90°C for 10 min. After that, centrifugation was used to keep supernatant at 15,000 × *g*, 15 min, 4°C. The protein content was determined by Bradford's procedure.

Determine of degree of hydrolysis (DH)

The degree of hydrolysis was estimated by determination of free amino acid groups. Nitrogen content was analyzed by Kjeldahl method [12]. Samples were treated with 10% trichloroaceticacid (TCA) and centrifuge 15,000 x *g* for 30 min at 4°C. The supernatant was considered and %DH was calculated by formula below:

$$\text{DH\%} = \frac{\text{Soluble Nitrogen in TCA 10\%}}{\text{Total Nitrogen in the sample}} \times 100$$

Ultrafiltration

The protein hydrolysates were fractionated through ultrafiltration membranes using a bioreactor system (Amersham Biosciences, Sweden). From research showed that peptide which is a small molecules had antioxidant properties better than bigger, more over short peptides can be transform to use in pharmaceutical and industrial. HBPs peptide solution was pumped through a range of nominal molecular weight cutoff (MWCO) membranes of 10, and 5 kDa, respectively, in the order of decreasing pore size. MW > 10 kDa; retentate from 10 kDa membrane, MW = 5-10 kDa; retentate from 5 kDa membrane, and MW < 5 kDa; permeate from 5 kDa membrane were collected.

DPPH radical scavenging activity

The DPPH radical scavenging activity was modulated the previous method [13], sample with various concentration were mixed with 0.1 M DPPH in ethanol for 30 min prior measured by using spectrophotometer at 517 nm. The IC_{50} value (the concentration that causes a decrease in initial DPPH concentration 50%) was determined from the linear regression of the DPPH inhibition against the concentration of protein. The negative control (blank) was water instead of the protein / protein hydrolysate sample while the positive control was ascorbic acid (0 - 50 µg/mL). The percentage of radical scavenging was calculated as follows:

$$\text{\% radical scavenging} = \frac{(Ac - Acb) - (As - Asb)}{(Ac - Acb)} \times 100$$

where *Ac* is the absorbance of water plus DPPH (in methanol), *Acb* is the absorbance of the blank (water plus methanol without DPPH), *As* is the absorbance of the sample plus DPPH (in methanol) and *Asb* is the absorbance of the sample plus methanol without DPPH. Dilution of sample was used in order to obtain calibration curves and to calculate the IC_{50} values (IC_{50}: concentration required to obtain a 50% radical scavenging activity).

Protein concentration

The protein content was determined [14]. Bovine serum albumin (BSA) was used as the standard with four different concentrations between 5-20 µg/ml to construct the calibration curve.

Statistical analysis

All investigations were done in triplicate. The results were indicated as the mean values ± stand deviation. GraphPad Prism (Version 6.00, GraphPad Software Inc, La Jolla, CA, USA) for windows was used to calculated

IC_{50} values. Statistical tests of significant difference were performed by ANOVA, with $p < 0.05$ being accepted as significant.

3. RESULTS

Amino acid content of hoary basil seed

Intact proteins, protein hydrolysates, individual peptides and amino acids have been reported to have significant antioxidant properties. Usually protein hydrolysates or peptide fractions showed greater antioxidant activity than intact proteins and amino acids. The antioxidant activities of protein digests vary depending on their peptide structure *ie.,* size of the peptides and their amino acid sequences, which are influenced by the source of protein and conditions of hydrolysis involved [15]. The relationship between hydrophobic amino acid and the reducing power of hydrolysates have been previously reported [16]. The reducing power assay is used to evaluate the ability of compound to donate electron or hydrogen to free radical thereby converting the radical to stable substances [17]. Data concerning qualitative and quantitative amino acids composition is presented in Table 1. Amino acid composition indicates the nutritional quality of protein. Glutamic acid and aspartic acid were found to be the major non-essential amino acids in the samples tested. Results indicated that all essential amino acids, except S-containing types and tryptophan, are present in high amounts in this specie. Moreover, protein showed the presence of phenlyalanin, tyrosine, and histidine, these amino acids have also been reported to show antioxidant activity. Therefore, protein hydrolysates have antioxidant activity as well as high nutritive value because of the presence of important amino acids [15]. Results are comparable to those of earlier worker [18] while there is no previous report on amino acid composition of hoary basil seeds.

Table 1. Total amino acid profile of hoary basil seed protein

Amino acids	mg/100mg
Aspartic acid	4.61
Serine	3.58
Glutamic acid	10.55
Glycine	3.12
Histidine	1.70
Arginine	8.48
Threonine	2.16
Alanine	2.65
Proline	2.25
Tyrosine	2.08
Valine	2.63
Lysine	1.56
Isoleucine	1.91
Leucine	4.02
Phenylalanine	3.49

Degree of hydrolysis

The maximum DH exhibited by hoary basil seed protein hydrolysate (HBPs) in the current study (55.134 ± 0.1 %) for 270 min as show in Figure 1a. It was greater than the DH values reported for protease hydrolyzed proteins from other oilseeds such as soy, which had DH of 39.5 % upon 8 h of hydrolysis [19] and DH of 5.46 to 17.86 % upon 1 to 8 h of hydrolysis [20] and sunflower that had DH of 42.2 % upon 3 h of hydrolysis [21]. These findings are in agreement with the results of enzymatic hydrolysis of other proteins reported by other workers. The DH for rice endosperm protein [22] and wheat germ protein hydrolysates [23] was reported to be 11.7 % and 25 % after 6 h of hydrolysis respectively; the % DH increased with increase in time of hydrolysis. The maximum DH value of HBPs obtained by Protease G6 hydrolysis alone was also lower than the recently reported values for Alcalase-Flavourzyme hydrolysates of yellow pea protein hydrolysate (DH: 58.89 %), Kabuli (DH: 77.58 %) and Desi (DH: 77.53 %) chickpea protein hydrolysates prepared by the protease enzymes [24]. Hydrolyzed protein was separated into soluble supernatants, and this fraction was subjected to antioxidant assays. It was found DPPH radical scavenging ability of HBPs increased, with an increase in the degree of hydrolysis (Figure 1b).

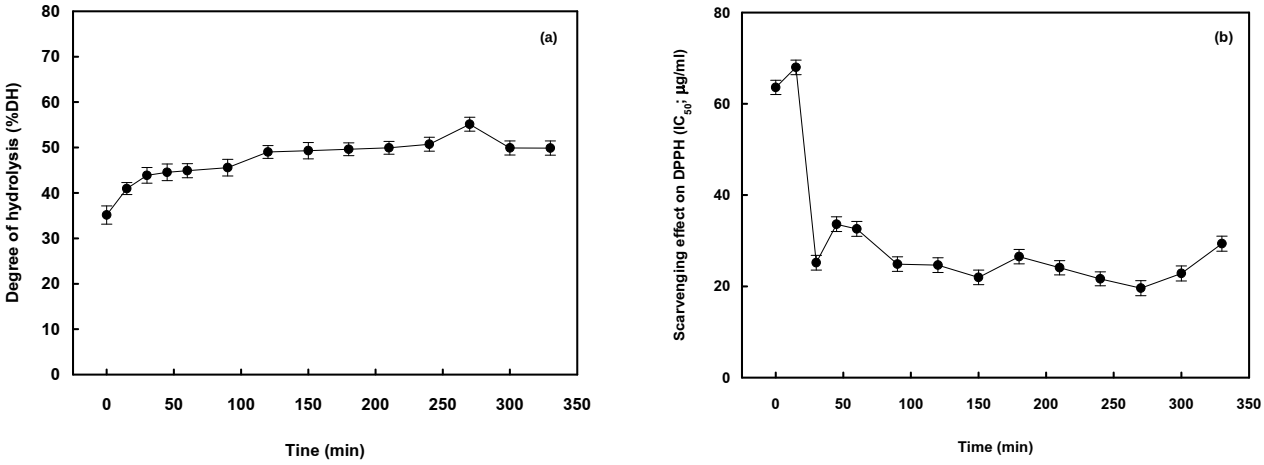

Figure 1 (a) Hydrolysis curve of hoary basil seed protein hydrolysate (HBPs) treated with Protease G6. Reaction conditions: pH, 8.0; 50°C with shaking 150 rpm, substrate: enzyme was 0.5:10 (w/v). (b) Antioxidant activity curve for HBPs.

Antioxidant assays

Ultrafiltration membranes were used to separate the HBPs into three fractions, HBPs-I (MW> 10 kDa), HBPs-II (MW = 5-10 kDa), and HBPs–III (MW < 5 kDa). As shown in Table 2, HBPs–III (MW < 5 kDa) showed the lowest IC_{50} value, which indicated the highest free radical scavenging activity. However, after ultrafiltration the *in vitro* antioxidant activity of the protein hydrolysate from HBPs was dramatically reduced. Previous studies support the presence as well as absence of free radical scavenging capacity activity of protein hydrolysates obtained by microbial protease catalyzed hydrolysis. Flavourzyme hydrolyzed soy concentrate upon passing through a 50 kDa membrane previously showed an activity of 21.81 % (concentration used: 0.1 mg protein/mL) at a DH of 28.8 % and this value was found to be similar to the free radical scavenging capacity activity shown by flaxseed protein hydrolysate at similar DH (25). Udenigwe et al., 2009 (26) digested flaxseed protein with pancreatin and reported that < 1 kDa ultrafiltered fraction of the resulting hydrolysate had an $OH^{\bullet}$ scavenging activity with an IC_{50} of 0.06 mg protein/mL. Since the protein hydrolysate from hoary basil seed showed by far the strongest antioxidation activity it was selected for further investigation, involving bioassay guided fractionation, in order to isolate the antioxidation activity constituent(s) responsible. These activities showed that this plant may have potential to be one of new effective antioxidant.

Table 2 Ultrafiltration separation of HBPs prepared by Protease G6 digestion and their free radical scavenging capacity by DPPH assay

Sample	Molecular weight (kDa)	IC_{50} (µg/ml)[a]
HBPs		14.65 ± 0.14
HBPs-I	> 10 kDa	41.17 ± 0.14
HBPs-II	5-10 kDa	37.00 ± 0.13
HBPs-III	< 5 kDa	25.23 ± 0.14
ascorbic acid (vitamin C)		142.80 ± 0.03

[a]All data are shown as the average mean ± 1 standard error of mean and are obtained from 3 replicated determination.

4. CONCLUSIONS

A potential HBPs-III that displays antioxidant was enriched from the hoary basil seed. by an ultrafiltration procedure. HBPs-III has a high dose-dependent antioxidant activity in DPPH assays. However, determination of the antioxidant assay that plays the important roles in various diseases and aging. The mechanism(s) of such activity of HBPs-III, along with conformation of its multimeric state and role of such, await further research.

ACKNOWLEDGEMENTS

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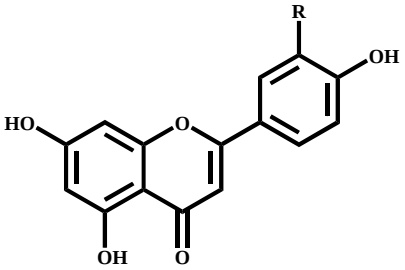
Flavones from the twigs of *Cynometra cauliflora*

Wanrudee Hiranrat*[a], Asadhawut Hiranrat [a], Weerapat Jaijong [a]
[a]Natural Products Research Laboratory, Faculty of Science, Thaksin University, Phatthalung Thailand 93110

*kaewnok@hotmail.com phone +66-74693992 fax +66-74693992

ABSTRACT

Phytochemical study of the methanol extract from the twigs of *Cynometra cauliflora*, resulted in the isolation of two known flavones apigenin (1) and luteolin (2). The chemical constituents were isolated and purified by preparative high performance liquid chromatography (prep-HPLC) using gradient solvent system of H_2O and MeOH. The structures of the isolated compounds were elucidated using the basis of their NMR spectroscopic data and comparison with those reported in the literature. These flavones were isolated from this plant for the first time.



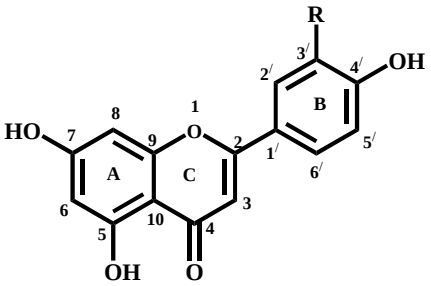
1 : R = H
2 : R = OH

Keywords: *Cynometra cauliflora,* Flavones, Spectroscopic data, HPLC

1. INTRODUCTION

Cynometra cauliflora is a native plant of Malaysia, commonly known as Nam-nam, grown mainly in northern Peninsular Malaysia and can be more widely cultivated in other states, mainly in kampongs and villages. This tree belongs to the bean family. It is a small tree with a thick much branched stem. The flowers are rather small about 1.2 cm across. They appear on the stem in clusters. The fruits are kidney-shaped, 5-10 cm long and 5 cm wide. The pod does not split open readily, but a line is visible along the fruit and divides it into two. The texture of the fruit surface is rough and wrinkled, pale greenish/yellow and dull looking. The flesh is juicy and yellow in colour. It produces a smell and tastes sourish. The seeds are large [1]. The fruit of *C. cauliflora* was reported to have low antioxidant capacity and moderately in total phenolic constents [2]. The methanolic extract of *C. cauliflora* whole fruit was cytotoxic towards HL-60 cells and induced the cell death mode, but less cytotoxic towards NIH/3T3 cells [3]. To the best of our knowledge, no phytochemical work has been done on the constituents of this species. The investigation carried out on some species of genus Cynometra led mainly to the isolation of imidazole alkaloids [4, 5].

We report herein the isolation and the structure elucidation of two new flavones in this species (**1**-**2**) from the twigs of *C. Cauliflora.* The structure of compounds were established using their NMR, UV and IR data.



1 : R = H
2 : R = OH

2. MATERIALS AND METHODS

General experimental procedures

IR spectra were recorded on a ATR FT-IR spectrometer (Agilent Technologies Cary 630). UV spectra were recorded on UV-VIS Spectrophotometer (UV-1700 PharmaSpec SHIMADZU). The ^{1}H and ^{13}C NMR spectra were recorded with Bruker spectrometer at 300 MHz. Chemical shifts (δ) are quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS). The Agilent 1100 HPLC system was used for separation purposes with Lichrospher®100 RP-18 endcapped column (Ø 25x250 mm, particle size 5 µm, Merck). For thin-layer chromatography (TLC), aluminum sheets of silica gel 60 F_{254} (20 x 20 cm, layer thickness 0.2 mm, Merck) was used for analytical purposes. The compounds were visualized under ultraviolet light. All solvents for extraction and chromatography were distilled at their boiling ranges prior to use except for HPLC separation (MeOH) was analytical grade (Merck).

Plant materials

The twigs of *C. cauliflora* were collected from Ranong province, Thailand. They were then dried by a hot air oven at 60^{o}C, and then ground into fine powder with a blender (500 g).

Extraction and Isolation

The dried powder of samples was extracted with methanol for 7 days at room temperature. The filtrate was collected through a filter paper and the plant material was re-soaked for twice. The combined filtrate was evaporated to dryness under vacuum to obtain a dark brown gum (11.94 g).

The methanol extract was separated by preparative reversed-phase HPLC with gradient solvent of CH_3OH-H_2O in the presence of 0.1% TFA to afford 90 fractions each which were combined based on TLC characteristics into 12 fractions.

Fraction 9 was subjected to purification by preparative reversed-phase HPLC with gradient solvent of CH_3OH-H_2O in the presence of 0.1% TFA to afford 50 subfractions each which were combined using the TLC into 7 subfractions. Subfraction 3 gave **2** by crystallization. (1.5 mg)

Fraction 10 was further purified by crystallization to afford **1** (5.0 mg)

Structural Identification

Compound 1; Yellow needle, mp 276-277ºC; UV (CH_3OH) λ_{max} (logε) 343 (2.15), 300 (2.12), 245 (1.52) nm; FT-IR (ATR) ν_{max} : 3268, 1649, 1603, 1553, 1350 1153 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 12.92 (1H, s, 5-OH), 10.74 (1H, s, 7-OH), 10.24 (1H, s, 4′-OH), 7.91 (2H, d, *J* = 8.80 Hz, H-2′, H-6′), 6.93 (2H, d, *J* = 8.80 Hz, H-3′, H-5′), 6.78 (1H, s. H-3), 6.49 (1H, d, *J* = 2.04 Hz, H-8), 6.20 (1H, d, *J* = 2.02 Hz, H-6); ^{13}C NMR (125 MHz, DMSO-d_6): δ163.6 (C-2), 102.2 (C-3), 181.1 (C-4), 161.4 (C-5), 98.3 (C-6), 163.3 (C-7), 93.3 (C-8), 156.9 (C-9), 103.0 (C-10), 121.4 (C-1′), 127.9 (C-2′), 117.4 (C-3′), 160.5 (C-4′), 116.5 (C-5′), 130.1 (C-6′).

Compound 2; Yellow powder, mp 327-330ºC; UV (CH_3OH) λ_{max} (logε) 342 (2.27), 314 (1.51), 263 (0.93) nm; FT-IR (ATR) ν_{max} : 3411, 1649, 1612, 1509, 1428, 1352, 1256, 1164, 1120, 1031, 838 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ: 13.01 (1H, s, 5-OH), 7.47 (1H, d, *J* = 8.0 Hz, H-6′), 6.98 (1H, d, *J* = 8.0 Hz, H-5′), 6.67 (1H, s, H-3), 6.56 (1H, s, H-2′)), 6.51 (1H, s, H-8), 6.20 (1H, s. H-6); ^{13}C NMR (125 MHz, DMSO-d_6): δ163.0 (C-2), 101.9 (C-3), 180.7 (C-4), 156.4 (C-5), 97.3 (C-6), 163.2 (C-7), 92.9 (C-8), 160.9 (C-9), 102.8 (C-10), 118.4 (C-1′), 114.9 (C-2′), 142.48 (C-3′), 150.5 (C-4′), 115.1 (C-5′), 122.7 (C-6′).

3. RESULTS

The methanol crude extract of the twigs of C. *cauliflora* was separated by preparative reversed-phase HPLC to afford two flavones apigenin (**1**) and luteolin (**2**). The isolation of **1** and **2** from the genus Cynometra is reported here for the first time. The compounds were identified by comparison of their spectroscopic data with those reported in the literature.

Compound (**1**) was obtained as yellow needles. The bands exhibited at ν_{max} 3268 (OH stretching), 1649 (C=O stretching) and 1153 (C-O stretching) cm^{-1} and the maximum absorption bands observed at λ_{max} 343, 300 and 245 nm on its IR and UV spectra, respectively, were suggestive for a flavone skeleton [6]. The ^1H NMR displayed signals of two aromatic doublets at δ6.49 (1H, d, *J* = 2.04 Hz) and 6.20 (1H, d, *J* = 2.02 Hz) assignable, respectively, to proton H-8 and H-6, and characteristic for A-ring of flavones with the oxygenation at positions 5 at δ12.92 (1H, s) and 7 at δ10.74 (1H, s). Moreover, the ^1H NMR spectrum exhibited signals of one olefinic proton at δ6.78 (1H, s) for H-3 of ring-C. And also showed signals of two doublets at δ7.91 (2H, d, *J* = 8.80 Hz) and 6.93 (2H, d, *J* = 8.80 Hz) corresponding, respectively, to the aromatic proton H-2′, H-6 and H-3′, H-5′ of B-ring, and signal at δ10.24 (1H, s) of 4′-OH. The ^{13}C NMR spectrum displayed characteristic signals of one disubstituted aromatic ring (ring B) at δ_c 121.4 (C-1′), 127.9 (C-2′), 117.4 (C-3′), 160.5 (C-4′), 116.5 (C-5′), 130.1 (C-6′), one tetrasubstituted aromatic ring (ring A) at δ_c 161.4 (C-5), 98.3 (C-6), 163.3 (C-7), 93.3 (C-8), 156.9 (C-9), 103.0 (C-10), two olefinic carbon at δ_c 163.6 (C-2) and 102.2 (C-3) and one carbonyl carbon at δ_c 181.1 (C-4). Compound **1** was thus assigned as apigenin by comparison of the physical and spectral data with the literature [7].

Compound (2) was obtained as yellow needles**.** It showed similar IR (ν_{max} 3411 (OH stretching), 1649 (C=O stretching) and 1164 (C-O stretching) cm^{-1} and UV (λ_{max} 342, 314 and 263 nm) data compared with **apigenin** (**1**), and characteristic of flavones skeleton [6]. The ^1H NMR data had signals of two aromatics at 6.51 (1H, s) and 6.20 (1H, s) assignable, respectively, to proton H-8 and H-6, and characteristic for A-ring of flavones with the oxygenation at positions 5 at δ13.01 (1H, s) and exhibited signals of one olefinic proton at δ6.67 (1H, s) for H-3 of ring-C similar to **1**. Another two hydrogen atoms exhibited a doublet at δ6.98 with coupling constant (*J*) of 8.0 Hz and δ7.47 with *J* of 8.0 Hz, to the aromatic H-5′, H-6′ and one hydrogen atom singlet signal were observed at δ6.56 , to the aromatic H-2′ (ring B). Compound **2** was thus assigned as luteolin by comparison of the physical and spectral data with the literature [8].

4. CONCLUSIONS

Two known flavones were isolated from the methanol extracts of twigs of C. *cauliflora.* Flavone **1** and **2** were assigned as apigenin and luteolin, respectively by comparison of the physical and spectral data with the literature. This is the first report of secondary metabolites isolated from C. *cauliflora.*

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Potential *halal* food colorants and active pharmaceutical ingredients in 24 Malaysian traditional vegetables (*ulam*)

Rashidi Othman*[a], Fatimah AzzahraMohd Zaifuddin[b], NorazianMohd Hassan[b]
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia
[b]Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Malaysia

**rashidi@iium.edu.my* phone +60-126644772 fax +60-361964864

ABSTRACT

Synergistic combination of two National Key Economic Areas (NKEAs) in healthcare and agricultural sectors counts for promoting extensive exploitation of Malaysia's mega-biodiversity to develop pharmaceuticals and related products from natural resources. Additionally, it is noted that nowadays *halal* products are gaining wider recognition as a new benchmark for safety measure and quality assurance especially among the Muslim consumers. In this study, we established quantification of a group of plant pigments which are potentially to be introduced as *halal* active pharmaceutical ingredients (API) and natural food colorants from 24 species of local traditional vegetables (*ulam*), identified as neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene. These targeted biocolorant and phytopharmaceutical ingredients were found to be comparable in concentrations after being developed through series of phytochemical extraction, purification and finally analysed through HPLC. Results obtained shown that *Sauropus androgynus* contained highest amount of neoxanthin, violaxanthin and β-cryptoxanthin at 142.40 ± 3.57, 28.06 ± 0.65 and 0.07 ± 0.00 mg/g DW, respectively. Highest accumulation of lutein and α-carotene were observed in *Centella asiatica* at 16.53 ± 0.97 and 2.14 ± 0.12 mg/g DW accordingly. *Piper sarmentosum* accumulated high zeaxanthin content (123.45 ± 12.3 mg/g DW) whereas *Oenanthe javanica* has the largest amount of β-carotene (3.09 ± 0.06 mg/g DW). The extracted yellow-to-red pigments can be further commercialised especially in the *halal* market for pharmaceutical and coloring products. The significant outcome of this research will be new dicoveries of natural resources for food colorant and API which covers not only the *shariah* requirement, but also the hygiene, sanitation and safety aspects.

Keywords: *Halal*, Food colorant, Active pharmaceutical ingredients, Traditional vegetables, Ulam

1. INTRODUCTION

Halal is an Islamic term which is defined as permissible, allowed or lawful in accordance to *shariah* laws for the Muslims [1]. Recently, the *halal* related industries are growing worldwide since *halal* products are gaining wider recognition not only among the Muslims but also consumers of other religions. The announcement of Malaysia as a global *halal* hub in combination with National Key Economic Areas (NKEAs) in healthcare and agriculture seems to promote extensive exploration and exploitation of local mega-biodiversity to develop various food additives, pharmaceutical, nutraceutical and cosmeceutical products from natural resources [2]. These natural resources could include the traditional vegetables (locally known as *ulam*) seem to be potential candidates for massive exploitation to compensate the high demand for *halal* pharmaceutical ingredients and natural food colorants.

There are more than 120 plant species which are consumed as traditional vegetables or *ulam* [3].Those *ulam* species were reported to be nutritious, full of health benefits and important sources for aroma and flavors [4-6]. In this study, 24 of them were selected to be extracted and saponified to separate their carotenoid compounds. These carotenoids were sources for yellow, orange and red colored pigments [7]. Carotenoids were already used for industrial colorants and food ingredients [8-9] but yet the extracted compounds need to be further improvised in order to comply with the *shariah* requirement for better quality of food and pharmaceutical products.

2. MATERIALS AND METHODS

Sample preparation

All edible parts of *ulam* samples (Table 1) were collected in 2011 and freeze-dried for 72 h, after which the samples were ground into fine powder and kept at -20°C until further analysis.

Extraction of carotenoids

The extraction procedure essentially follows the methods described by Fatimah [10], with some modifications. 0.1 g of each powdered sample was rehydrated with distilled water and extracted with a mixture of acetone and methanol (7:3, v/v) at room temperature until colorless. The crude extracted was then centrifuged for 5 min at 10, 000 g and stored at 4ºC in the dark prior to analysis. To extract carotenoids an equal volume of hexane and distilled water was added to the combined supernatants. The solution was then allowed to separate and the upper layer containing the carotenoids was collected. The combined upper phase was then dried to completion under a gentle stream of oxygen-free nitrogen.

Saponification

Samples were saponified with a mixture of acetonitrile and water (9:1, v/v) and methanolic potassium hydroxide solution (10% w/v). Base carotenoids were then extracted by addition of 2 mL hexane with 0.1% butylated hydroxytoluene (BHT), followed by addition of 10% NaCl until phase separation was achieved. The extracts were washed with distilled water, dried under a gentle stream of oxygen-free nitrogen and re-suspended in ethyl acetate for HPLC analysis as described detail in Othman [11].

HPLC analysis

The HPLC analysis of saponified carotenoids were performed on an Agilent model 1200 series comprised of a quaternary pump with auto-sampler injector, micro-degassers, column compartment equipped with thermostat and a diode array detector. The column used was a ZORBAX Eclipse XDB-C_{18} end capped 5 µm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile: water (9:1, v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min^{-1}. The column would be allowed to re-equilibrate in 100% A for 10 min prior to the next injection. The temperature of the column was maintained at 20°C. The injection volume is 10 µL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β-carotene (454 nm), β-cryptoxanthin (450 nm) and α-carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (mg/g DW).

3. RESULTS

In this study, a group of 24 plant species which are commonly consumed as *ulam* by local folks were subjected to evaluation for their carotenoid profiles. The evaluated *ulam* species were: *Sauropus androgynus*, *Allium tuberosum*, *Centella asiatica*, *Oroxylum indicum*, *Ocimum basilicum*, *Piper sarmentosum*, *Oenanthe javanica*, *Ocimum americanum*, *Zea mays*, *Brassica chinensis*, *Lactuca sativa*, *Morinda citrifolia*, *Murray akoenigii*, *Piper betle*, *Apium graveolens*, *Cosmos caudatus*, *Pluchea indica*, *Polygonum minus*, *Anacardium occidentale*, *Euodia redlevi*, *Allium cepa*, *Ipomea batatas* and *Daucus carota*.

There were seven types of carotenoids identified and quantified in this study particularly neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene. These individual carotenoids were found in comparable amount and can be further categorized into several group based on number of carotenoids accumulated in them, as tabulated in Table 1. The results established that *S. androgynus* has the substantially highest total carotenoid concentration at 190.30±3.43 mg/g DW while *D. carota*has the least carotenoids level at 1.31±0.01 mg/g DW. Again, *S. androgynus*contained the highest concentration of neoxanthin, violaxanthin and β-cryptoxanthin at 142.40 ± 3.57, 28.06 ± 0.65 and 0.07 ± 0.00 mg/g DW, respectively. Lutein and α-carotene were observed the most in *Centella asiatica* at 16.53±0.97 and 2.14±0.12 mg/g DW accordingly. *Piper sarmentosum* accumulated high zeaxanthin content (123.45±12.3 mg/g DW) whereas *Oenanthe javanica* has the largest amount of β-carotene (3.09 ± 0.06 mg/g DW)

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Table 1: Distribution of total and individual carotenoids in 24 species of *ulam*.

Species	Total Carotenoid (mg/g DW)	Neoxanthin (mg/g DW)	Violaxanthin (mg/g DW)	Lutein (mg/g DW)	Zeaxanthin (mg/g DW)	β-cryptoxanthin (mg/g DW)	α-carotene (mg/g DW)	β-carotene (mg/g DW)
Species with 6 types of individual carotenoids								
S. androgynus	190.30±3.43	142.40±3.57	28.06±0.65	15.57±0.32	nd	0.07±0.00	1.36±0.42	2.84±0.37
A. tuberosum	24.61±1.00	13.95±0.75	2.98±0.24	5.00±0.38	nd	0.06±0.00	0.75±0.08	1.86±0.27
Species with 5 types of individual carotenoids								
C. asiatica	130.61±15.03	96.10±11.4	13.45±2.68	16.53±0.97	nd	nd	2.14±0.12	2.39±0.06
O. indicum	100.78±2.45	81.79±2.70	4.36±0.12	13.12±0.31	nd	nd	0.38±0.03	1.12±0.03
O. basilicum	95.28±3.25	65.16±3.22	17.97±0.50	9.66±0.96	nd	nd	0.53±0.11	1.95±0.24
Species with 4 types of individual carotenoids								
P. sarmentosum	161.36±12.72	24.06±4.63	nd	12.58±1.28	123.45±12.3	nd	nd	1.27±0.29
O. javanica	144.48±4.93	115.55±4.09	11.06±0.70	14.80±0.44	nd	nd	nd	3.09±0.06
O. americanum	108.79±6.35	74.62±3.30	24.12±2.63	9.27±0.35	nd	nd	nd	0.78±0.16
Z. mays	61.53±5.55	nd	nd	1.54±0.03	58.87±5.38	0.05±0.00	1.06±0.14	nd
B. chinensis	27.00±2.73	18.75±1.84	nd	6.51±0.63	nd	0.04±0.00	nd	1.68±0.26
L. sativa	15.14±0.65	10.15±0.69	1.87±0.09	2.58±0.07	nd	nd	nd	0.54±0.02
Species with 3 types of individual carotenoids								
M. citrifolia	57.11±1.94	nd	nd	5.20±0.00	52.08±0.25	nd	nd	1.31±0.01
M. koenigii	51.48±2.60	nd	nd	4.04±0.14	45.74±2.32	nd	nd	1.69±0.28
P. betle	18.30±0.08	nd	nd	15.49±0.10	nd	0.07±0.00	nd	2.74±0.10
A. graveolens	14.35±0.14	nd	nd	11.53±0.09	nd	0.06±0.00	nd	2.76±0.09
C. caudatus	12.59±0.27	nd	nd	9.60±0.32	nd	nd	1.56±0.16	1.43±0.05
P. indica	10.66±0.39	nd	nd	5.76±0.17	nd	nd	1.92±0.16	2.98±0.31
P. minus	7.40±0.38	nd	nd	4.16±0.11	nd	nd	0.71±0.08	2.53±0.25
Species with 2 types of individual carotenoids								
A. occidentale	14.20±0.29	nd	nd	12.46±0.55	nd	nd	nd	1.74±0.28
E. redlevi	11.65±0.14	nd	nd	10.39±0.15	nd	nd	nd	1.30±0.03
A. cepa	6.90±0.04	nd	nd	4.83±0.02	nd	nd	nd	2.07±0.02
I. batatas	1.34±0.06	nd	nd	nd	nd	nd	0.38±0.09	0.97±0.06
D. carota	1.31±0.01	nd	nd	0.72±0.00	nd	nd	nd	0.60±0.01

nd – non-detectable, results are significant at $p < 0.0001$

4. CONCLUSIONS

In this study, we established the production of *halal* active pharmaceutical ingredients and food colorants from 24 *ulam* species namely neoxanthin, violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene. These targeted phytochemicalcompoundscan be introduced as individual ingredients or as a mixture of few carotenoids for the production of pharmaceutical and food colorant products. The phytochemical extraction, purification and manipulation of postharvest storage condition which are *shariah* compliance can be applied prior to commercialisation in *halal* market. The significant outcome of this research will be new findings for new *halal*natural sources of API and food colorants.

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Constitutive, instuitive and up-regulation of carotenogenesis regulatory mechanism via *in vitro* culture model system and elicitors

Rashidi Othman*[a], Fatimah Azzahra Mohd Zaifuddin[b], Norazian Mohd Hassan[b]
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia
[b]Department of Pharmaceutical Chemistry, Kulliyyah of Pharmacy, International Islamic University Malaysia, 25200 Kuantan, Malaysia

*rashidi@iium.edu.my phone +60-126644772 fax +60-361964864

ABSTRACT

Every single step in the pathway of carotenogenesis in the leaves or any part of the plants is regulated and controlled by specific genes. It was inferred that these genetic components would be inherited by either undifferentiated (callus) or differentiated (*in vitro* shoot) cells since they were initiated from their respective intact plants. Thus, the *in vitro* cultures are most likely to develop and respond similarly upon various internal and external stimuli like the intact plants. Therefore, this study was aimed at establishing *in vitro* cultures as potential research tool to study the regulatory mechanism of the carotenoid biosynthesis in medicinal species through a controlled environment. This establishment would be beneficial as researchers in this field could overcome problems encountered in researches using intact plants. Via HPLC analysis, it was found that different types of cultures accumulated different types of carotenoid as compared to their intact plants, accordingly. The results obtained in this study suggested that the key factors which involved in regulatory mechanisms of individual carotenoid biosynthesis in a particular biology system of plants can be assessed and predicted. On top of that, further exploration and manipulation of the environmental conditions is made possible for enhancement or enrichment of certain carotenoid of interest in food crops without altering the genes and thus issues regarding the introduction of genetically modified (GM) crops into natural environment and acceptability of consumers towards GM crops could be minimized.

Keywords: *In vitro* cultures, Carotenogenesis, Elicitors, Regulatory mechanisms

1. INTRODUCTION

The carotenogenesis often commences with the formation of phytoene from condensation of two blocks of geranylgeranyl diphosphate (GGPP) molecules by phytoene synthase [1]. Desaturation of phytoene by phytoene desaturase resulted in formation of phytofluene, ζ-carotene, neurosporene and finally red coloured lycopene [2]. Then, cyclization of lycopene with lycopene cyclases (β-cyclase and ε-cyclase) is a significant branching point in carotenoid biosynthesis which resulted in the formation of α- and β-carotene. Hydroxylation of α- and β-carotene will produce the well-known xanthophyll pigments lutein and zeaxanthin, respectively. Violaxanthin is formed from zeaxanthin through epoxidation. This reaction sequence is reversible and de-epoxydation can convert violaxanthin back to zeaxanthin. Neoxanthin is synthesized and derived from violaxanthin. Cleavage products of this last plant carotenoid serve as precursors in the production of important plant hormones such as abscisic acid and strigolactones [3].

Over the past decade several approaches of genetic manipulation or modification have been performed to increase carotenoid content and composition in different plant species and tissues [4-6]. Collectively, the results suggested that different plant species will react differently towards the stability of individual carotenoids accumulated in transgenic plants. The overexpression of genes and modification of sink capacity in the carotenoid biosynthetic pathway have resulted in increased levels of carotenoids and proven as a new strategy to enhance carotenoids in food crops quantitatively and qualitatively but yet these techniques still need to become more precise to avoid the inadvertent introduction or expression of undesirable genes causing allergenicity, weediness or endanger natural ecosystems [7]. Hence, more detailed knowledge of the diversity of carotenoid pigments in plants and environmental factors influencing their accumulation urgently required for better understanding of plant carotenogenesis regulation. The question remains is which mechanism will stimulate carotenogenesis more effectively. In order for this question to be answered, it is important to identify and to understand the key control factors for carotenoid accumulation in plant. In this study carotenoid biogenesis is investigated in selected plant *in vitro* cultures as a potential model system for rapid initiation, extraction and analysis of carotenoids by providing stringent control of genetic, developmental and environmental factors. The value of this experimental system for investigating variables controlling carotenoid accumulation is then tested by assessing the effects of environmental variables, such as drought stress, light intensity and hormone strength on carotenoid accumulation.

2. MATERIALS AND METHODS

Sample preparation

Intact leaves and *in vitro* cultures of *Morinda citrifolia*, *Ocimum basilicum* and *Ruta angustifolia* were freeze-dried for 72 h, ground into fine powder and kept at -20ºC until further analysis.

Extraction of carotenoid and saponification

0.1 g of all samples were rehydrated, extracted, partitioned to hexane and dried under gentle stream of nitrogen free oxygen gas as detailed in [8]. Each of the samples was prepared in triplicate. The extracts were then re-suspended in ethyl acetate (HPLC grade) prior to subsequent HPLC analysis.

HPLC analysis

The HPLC analysis of saponified carotenoids were performed on an Agilent model 1200 series comprised of a quaternary pump, auto-sampler injector, micro-degassers, column compartment equipped with thermostat and photo-diode array detector. The column used was ZORBAX Eclipse XDB-C18 end capped 5 μm, 4.6x150 mm reverse phase column (Agilent Technologies, USA). The eluents used were (A) acetonitrile:water (9:1, v/v) and (B) ethyl acetate. The column separation was allowed via a series of gradient such as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 mL min-1. The temperature of the column was maintained at 20 °C. The injection volume is 10 μL each. Detection of individual carotenoids was made at the wavelengths of maximum absorption of each carotenoid in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm), β-carotene (454 nm), β-cryptoxanthin (450 nm) and α-carotene (456 nm). Compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks was in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometry. The total and individual carotenoid concentration would be expressed in terms of milligram per 1.0 g dry weight of freeze-dried matter (mg/g DW).

Tissue culture, callus and shoot initiation

Virus-free *in vitro* callus and shoots of *M. citrifolia, O. basilicum* and *R. angustifolia* were induced as detailed in [9-11], accordingly. The cultures were incubated in a growth room at 24ºC day and night temperature. Every 4 weeks, the *in vitro* callus and shoots were subcultured on fresh MS medium supplemented with 30 g/L sucrose, 10 g/L agar and plant growth regulators. Media were adjusted to pH 5.7 and sterilized by autoclaving (15 min, 121ºC) and 50 mL aliquots poured into pre-sterilised 250 mL polycarbonate culture vessels (7 cm diameter x 8 cm high).

Effect of environmental factors on carotenoid biosynthesis

In three independent experiments the influence of light, water-stress and PGRs availability on carotenoid biosynthesis were tested in *M. citrifolia*, *O. basilicum* and *R. angustifolia*. Callus harvested after 4 weeks from two culture vessels were pooled for each of three replicates established under the following conditions: 1) Light incubation under cool-white, fluorescent lamps (80-85 μmol m^{-2} s^{-1}; 16 h photoperiod); 2) Incubation under same light condition, in three concentrations of PGR strength (1.0, 1.5 and 2.0 mg/L); and, 3) Incubation under same light condition, with and without 50 mg and 100 mg salicylic acid to impose water-stress.

3. RESULTS

Carotenoid accumulation in intact leaves and callus cultures of different colour strains of *M. citrifolia* in response to light.

When developing in natural environment, the intact leaves of *M. citrifolia* accumulated predominantly zeaxanthin followed by lutein and β-carotene. The callus cultures of *M. citrifolia* possessed lesser carotenoids as compared to the leaves as shown in Table 1.

Table 1. Carotenoid accumulation in intact leaves and callus cultures of *M. citrifolia*.

	Carotenoid content (mg/g DW)				
	Total carotenoid	Lutein	Zeaxanthin	β-cryptoxanthin	β-carotene
Intact leaves	58.59 ± 0.26	5.20 ± 0.00	52.08 ± 0.25	nd	1.31 ± 0.00
Green callus	2.17 ± 0.01	2.00 ± 0.00	nd	nd	0.16 ± 0.00
Yellow callus	13.00 ± 0.08	1.29 ± 0.00	11.53 ± 0.09	0.05 ± 0.00	0.14 ± 0.00

nd - non-detectable, not significantly different at $p < 0.0001$

Carotenoid accumulation in intact leaves and callus cultures of *O. basilicum* of different sources of explants and PGRs supplementation in response to light

Intact leaves of *O. basilicum* accumulated five individual carotenoids particularly neoxanthin, violaxanthin, lutein, α-carotene and β-carotene when grown in nature as shown in Table 2. Different source of explants and PGR supplementation in this experiment resulted in more or less the same level carotenoids.

Table 2. Carotenoid accumulation in intact leaves and callus cultures of *O. basilicum*.

Source of explants	Samples	Carotenoid content (mg/g DW)					
		Total carotenoid	Neoxanthin	Violaxanthin	Lutein	α-carotene	β-carotene
-	Intact leaves	95.28 ± 3.25	65.16 ± 3.22	17.97 ± 0.50	9.66 ± 0.00	0.53 ± 0.11	1.95 ± 0.00
Shoot	S1	1.81 ± 0.01	nd	nd	1.62 ± 0.01	nd	0.19 ± 0.00
tips	S2	1.65 ± 0.03	nd	nd	1.52 ± 0.02	nd	0.14 ± 0.00
	S3	1.66 ± 0.01	nd	nd	1.51 ± 0.01	nd	0.15 ± 0.00
Leaves	L1	1.71 ± 0.00	nd	nd	1.57 ± 0.00	nd	0.14 ± 0.00
	L2	1.70 ± 0.01	nd	nd	1.55 ± 0.00	nd	0.15 ± 0.01
	L3	1.68 ± 0.00	nd	nd	1.54 ± 0.03	nd	0.16 ± 0.00

nd - non-detectable, significantly different at $p < 0.0001$

Carotenoid accumulation in intact leaves and *in vitro* cultures of *R. angustifolia* in response to light

The leaves of *R. angustifolia* accumulated three major carotenoids where lutein was found in highest level. The shoot cultures derived from stems contained the highest level of total carotenoids while callus cultures have the least carotenoid accumulation as tabulated in Table 3.

Table 3. Carotenoid accumulation in intact leaves and *in vitro* cultures of *R. angustifolia*.

	Carotenoid content (mg/g DW)				
	Total carotenoid	Neoxanthin	Lutein	β-cryptoxanthin	β-carotene
Intact leaves	6.45 ± 0.56	nd	6.00 ± 0.11	0.04 ± 0.00	1.10 ± 0.01
Shoot cultures	14.70 ± 0.84	10.27 ± 0.62	2.75 ± 0.12	nd	1.68 ± 0.11
Callus cultures	1.76 ± 0.01	nd	1.54 ± 0.01	nd	0.22 ± 0.00

nd - non-detectable, significantly different at $p < 0.0001$

Carotenoid accumulation in shoot cultures of *R. angustifolia* in response to light and elicitor

In this experiment, treating the shoot cultures of *R. angustifolia* has resulted in interesting findings. The concentration of 50 mg salicylic acid (SA) used in this preliminary study has significantly enhanced the accumulation of neoxanthin in the cultures (see Table 4).

Table 4. Carotenoid accumulation in shoot cultures of *R. angustifolia* in response to light and elicitor (salicylic acid).

	Carotenoid content (mg/g DW)				
	Total carotenoid	Neoxanthin	Violaxanthin	Lutein	β-carotene
Control (0 mg SA/L media)	12.49 ± 0.40	7.47 ± 0.35	1.30 ± 0.04	2.94 ± 0.08	0.79 ± 0.02
Treatment 1 (50 mg SA/L media)	16.51 ± 4.24	15.88 ± 2.16	0.75 ± 0.00	1.98 ± 0.01	0.18 ± 0.00
Treatment 2 (100 mg SA/L media)	1.50 ± 0.01	nd	nd	1.42 ± 0.00	0.07 ± 0.00

nd - non-detectable, significantly different at $p < 0.0001$

4. CONCLUSIONS

The results suggested that zeaxanthin appears to be a key factor and indicator for the presence of environmental stress. Due to the presence and time of occurrence of environmental stress, some genotypes accumulated merely violaxanthin and neoxanthin in order to generate xanthoxin or precursors of the abscisic acid biosynthesis pathway. Not surprisingly, the response to such environments appeared to be highly genotype dependent and time duration exposed to stress. Another factor is the activity of functional enzymes and candidate enzymes that regulate carotenogenesis which will determine type and quantity of individual carotenoids. By understanding the environmental factors that affected carotenogenesis, it should be possible to enhance the amount and type of carotenoid that accumulates in food crops. In conclusion, resistance or sensitivity of plants to stress depends on the species, type of stress and development age.

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Total phenolic contents, total flavonoids and antioxidant activity of Thai basil (*Ocimum basilicum* L.)

Ratchadaporn Oonsivilai*[a], Panita Prasongdee[a]
[a]School of Food Technology, Suranaree University of Technology, 111 University Avenue, Muang, Nakhon Ratchasima, Thailand 30000

*roonsivi@sut.ac.th phone +66-44224233 fax +66-44224250

ABSTRACT

Two species of Thai basils was studied for their total phenolic and flavonoid contents including antioxidant activity. Thai basils in species of *thyrsiflorum (Ocimum basilicum* var. *thyrsiflorum*) and Jumbo 4320 (*Ocimum basilicum* cv. Jumbo 4320) were extracted by 3 solvents such as water, ethanol and ethyl acetate. The total phenolic contents of Thai basils extracts were evaluated by Folin-Ciocalteu method. The results showed that water extraction of both Thai basils species gave the highest yield percentage of the extracts. Moreover, the Jumbo 4320 Thai basils water extract showed the highest total phenolic content at the value of 459.62 ± 3.07 mg gallic acid equivalent/100 g follow by ethanol and ethyl acetate extracts at 179.80 ± 0.55 and 56.95 ± 0.99 mg GAE/100 g, respectively. Likewise, *thyrsiflorum* Thai basils water, ethanol, and ethyl acetate extracts showed total phenolic contents at 353.93 ± 1.65, 179.80 ± 0.55, and 56.95 ± 0.99 mg GAE/100 g respectively. In addition, total flavonoid contents of Thai basils extracts were determined by aluminum chloride colorimetric method. It was found that Jumbo 4320 Thai basils ethanol extract showed the highest total flavonoid content at 557.12 ± 14.27 mg quercetin equivalent/100 g follow by ethanol and ethyl acetate at 557.12 ± 14.27 and 209.07 ± 23.31 mg quercetin equivalent/100 g. In addition, the antioxidant activities were carried out by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power assay (FRAP). The results showed that Thai basil species Jumbo 4320 were extract showed the highest antioxidant activity by DPPH assay at IC_{50} 48.52 ± 1.15 mg/ml follow by ethanol and ethyl acetate extracts at IC_{50} at 60.54 ± 0.52, 82.09 ± 3.18 mg/ml where as BHT and ascorbic acid showed IC_{50} at 0.18 and 0.06 mg/ml. Otherwise, *Thyrsiflorum* Thai basils ethanol extract showed the highest antioxidant activity by FRAP assay at the value of 0.0186 ± 0.00 mmol Fe^{2+}/g follow by ethanol and ethyl acetate extracts at the value of 0.0012 ± 0.00 and 2.6159 ± 0.02 mmol Fe^{2+}/g respectively. In conclusion, Thai basils species and solvent extraction affect the total phenolic content and antioxidant activities of three Thai basils extracts. More than that, the antioxidant activity also affected by Thai basils species and solvent used for extraction. Finally, Thai basils extracts showed the potential to be applied as food ingredients for health benefit.

Keywords: Total phenolic, Total flavonoid, Antioxidant activity, Thai basil

1. INTRODUCTION

Ocimum basilicum Linn., commonly known as basil or sweet basil (family Lamiaceae), is an important medicinal plant and culinary herb. Pharmacological reports revealed that various *Ocimum basilicum* extracts have anticandidal, antibacterial, antifungal effects, anti-giardial, antiviral and antioxidant activities. Basil has a high level of antioxidants, which are the result of large groups of polyphenols and flavonoids such as quercetin, kaempferol, and myricetin; Tannins such as catechin and pigments such as found anthocyanins in purple basil and essential oils such as eugenol and methyl chavicol. The plant has also shown hypolipidaemic, anti-inflammatory, anti-platelet aggregation, antithrombotic, bronchodilatory, antiulcerogenic and anticarcinogenic [1, 2]. The purpose of this research is evaluating total phenolic, total flavonoid and antioxidant activity of *Ocimum basilicum* extracted by different solvent such as water, ethanol and ethyl acetate.

2. MATERIALS AND METHODS

Plant Material

Thai basil leaves prepared from *thyrsiflorum* (*Ocimum basilicum* var. *Thyrsiflorum*) species and Jumbo 4320 (*Ocimum basilicum* cv. Jumbo 4320) species collected in Nakhon Ratchasima province, Thailand during the months of September to January 2013. Thai basil leaves were rinsed, dried at 50°C for 24 h, after which they were ground by Ultra Centrifugal Mill Model ZM-1000 (Retch, Germany), sieved by mesh size 0.2 mm and stored in vacuum package at -20°C until use.

Chemical and standards

All chemicals of analytical grade including: 1,1-diphenyl-2-picryhydrazyl free radical (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ferric, chloride-6-hydrate, ferrous, sulphate 7-hydrate, acetate buffer pH 4.6, gallic acid, Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate (Na_2CO_3), catechin, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$) and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich Co. (St. Louis, USA). Solvents including ethanol and ethyl acetate were purchased from Mallinckrodt-Baker (Phillipsburg, NJ, USA).

Proximate analysis

Dry two species of Thai basil powder were analyzed for chemical composition. Moisture analysis was done by AOAC method 925.10. Ash and sand quantification was done by AOAC method 900.02A and 900.02D respectively. Protein and fat quantification was carried out with AOAC method 928.08 and 963.15 respectively. Crude fiber determination was done according to AOAC method 978.10 [3].

Preparation of Thai basil extracts

Thai basil powder of about 5.0 g was extracted with 50 ml portions of water, ethanol and ethyl acetate by shaking for 18 hours and then centrifuged at 2800 rpm for 15 min, filtrated and the volumes were adjusted to 50 ml with same solvent. Aliquots of 2 ml were added in culture test tubes (VWR, Wilmington, NC). The solvent was removed on vacuum dryer (Rapid Vap® Vacuum N2 and N2/48 Evaporation Systems, Labconco corporation, Kansas city, MO) for ethanol and ethyl acetate, on freeze dryer for water, the extract dried. The samples were kept at -20°C until use [1].

Total phenolic

Total soluble phenolic constituents of the extracts were determined by Folin-Ciocalteu reagent and gallic acid was used as standard [3]. 20 µl of the crude extract solution, standard or blank was added into a 1.5 ml cuvette, after that 1.58 ml DI water was added followed by 100 ml Folin-Ciocalteu reagent then the mixture was thoroughly mixed and incubated for 5 min at room temperature. Following incubation, 300 µl of the Na_2CO_3 (2% w/v) solution was added and the mixture was allowed to stand at room temperature for 2 h. Absorbance was measured at 765 nm. Results were expressed as gallic acid equivalents.

Total flavonoid

Total flavonoid contents of the extracts were determined by aluminum chloride colorimetric method [4]. 250 µl of crude extract, standard or blank were added into a 1.5 ml cuvette, after that 1.25 ml DI water was added followed by $NaNO_2$ 75 µl then mixture was incubated for 6 min. Following incubation, 150 µl of the 10% $AlCl_3$ solution was added followed by 1 M NaOH 0.5 ml, DI water 275 µl, respectively following with incubation for 5 min. Absorbance was measured at 510 nm. Results were expressed as catechin equivalents.

DPPH radical scavenging activity

The DPPH free radical scavenging activity of Thai basil extracts (water, ethanol and ethyl acetate), BHT and ascorbic acid were determined using DU 800 Spectrophotometer (Beckman Coulter, CA) according to method described by Oonsivilai et al. [5]. An aliquot (100 µl) of an extract at the vary concentration was mixed with 1.9 ml

of MeOH DPPH solution. The BHT and ascorbic acid in MeOH solution were used as positive controls. The IC_{50} of extracts was calculated using nonlinear regression of Sigma Plot 9.1 (Systat Software Inc, Illinois).

Ferric reducing antioxidant power assay

The method of Oonsivilai et al. [5] was utilized. Briefly, FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol ferrous chloride solution in the proportion of 10:1:1 (v/v), respectively. The extract of 50 µl was added to 1.5 ml of the FRAP reagent. The absorbance of mixture was recorded at 593 nm after 4 min incubation. The standard curve was constructed using ferric sulphate solution (100-2000 µm) and the results were expressed as mmol equivalents of ferric per g dry weight of plant materials.

Statistical Analysis

The analyses were carried out in triplicate and results were expressed as mean ± standard deviation. Analyses of variance (ANOVA) were conducted and differences among samples means were analyzed by Duncan's multiple range test ($p<0.05$) by using SPSS (version 16.0, SPSS Inc., USA).

3. RESULTS

Proximate analysis

The main chemical compositions of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 are carbohydrate (39.73 ± 0.32%, 38.38 ± 0.30%) and protein (27.31 ± 0.13%, 29.94 ± 0.16%), respectively. It was found that *Ocimum basilicum* var. *thyrsiflorum* powder showed significantly ($P<0.05$) higher moisture and carbohydrate than *Ocimum basilicum* cv. Jumbo 4320 powder. While as *Ocimum basilicum* cv. Jumbo 4320 powder showed significantly ($p<0.05$) higher ash and protein than *Ocimum basilicum* var. *thyrsiflorum* powder.

Thai basil was extracted by three solvents such as water, ethanol and ethyl acetate. The yield percentage of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts are investigated. It was found that yield percentage of all solvents are significantly different ($p < 0.05$). The *Ocimum basilicum* var. *thyrsiflorum* crude extract gave higher yield percentage than *Ocimum basilicum* cv. Jumbo 4320. *Ocimum basilicum* var. *thyrsiflorum* extracted by water gave the highest yield percentage at 15.12 ± 0.08%.

Total phenolic and flavonoid contents

Total phenolic contents of *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 extracted by water, ethanol and ethyl acetate solvent was investigated by Folin-Ciocalteu reagent. The total phenolic contents in each extract are presented in Table 3. The total phenolic contents in all solvents extracts are significantly different ($p < 0.05$). Also, the total phenolic contents of water extract showed the highest value followed by ethanol and ethyl acetate extracts, respectively. Furthermore, total phenolic content of both species are also significantly different ($p < 0.05$). *Ocimum basilicum* cv. Jumbo 4320 extracted by water showed highest total phenolic content at 459.62 ± 3.07 mg gallic acid equivalent/100 g raw material.

Table 3. Total phenolic contents and total flavonoid contents of Thai basil crude extracts.

Solvents	Total phenolic (mg gallic acid equivalent /100 g of raw material)		Total flavonoid (mg quercetin equivalent /100 g of raw material)	
	Ocimum basilicum var. *thyrsiflorum*	*Ocimum basilicum* cv. Jumbo 4320	*Ocimum basilicum* var. *thyrsiflorum*	*Ocimum basilicum* cv. Jumbo 4320
Water	$353.93\pm1.65^{a,e}$	$459.62\pm3.07^{b,e}$	$115.80\pm0.21^{a,c}$	$131.41\pm0.37^{b,c}$
Ethanol	$179.80\pm0.55^{b,d}$	$171.20\pm1.10^{a,d}$	$497.38\pm3.30^{a,e}$	$557.12\pm14.27^{b,e}$
Ethyl acetate	$56.95\pm0.99^{a,c}$	$66.02\pm0.99^{b,c}$	$156.77\pm28.97^{ns,d}$	$209.07\pm23.31^{ns,d}$

Note: Each value is mean ± SD

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$).

^{c,d,e} Data within the same column with different superscripts are significantly different ($p<0.05$).

^{ns} means not significant.

From Table 3, when comparison among solvents, the total flavonoid contents of all solvents extracts are significantly different ($p < 0.05$) including ethanol extracts showed the highest value followed by ethyl acetate and water extracts, respectively. When compared between two species studied, the *Ocimum basilicum* cv. Jumbo 4320 extracted by water and ethanol showed higher value than *Ocimum basilicum* var. *thyrsiflorum* with significantly

different ($p < 0.05$). Both species of Thai basils extracted by ethyl acetate is not significant different ($p < 0.05$). *Ocimum basilicum* cv. Jumbo 4320 extracted by ethanol showed the highest total flavonoid content at 557.12±14.27 mg quercetin equivalent /100 g raw material.

Antioxidant activity

DPPH radical scavenging activity and ferric reducing antioxidant power assay

The studied extracts exhibited the scavenging activity of various strengths and were dose dependent in all extracts. In addition, positive control with BHT and ascorbic acid were tested for their DPPH radical scavenging. The calculated IC_{50} for 15 min incubation time are report in Table 4. The comparison between the solvents, the antioxidant activities by DPPH assay are significantly different ($p < 0.05$). When compared between two species studied, the antioxidant activities of Thai basils extracted by water and ethanol are significantly different ($p < 0.05$). Two species of Thai basils extracted by ethyl acetate is not significant different ($p < 0.05$). *Ocimum basilicum* cv. Jumbo 4320 extracted by water showed the highest antioxidant activity by DPPH assay at IC_{50} value of 48.52 ± 1.15 mg /ml.

Table 4. Antioxidant activities of Thai basil crude extracts on DPPH assay and FRAP assay.

Solvents	DPPH IC_{50} (mg /ml)		FRAP (mmol Fe^{2+}/ g)	
	Ocimum basilicum var. *thyrsiflorum*	*Ocimum basilicum* cv. Jumbo 4320	*Ocimum basilicum* var. *thyrsiflorum*	*Ocimum basilicum* cv. Jumbo 4320
Water	$105.62 \pm 3.77^{b,e}$	$48.52 \pm 1.15^{a,c}$	$0.0139 \pm 0.00^{b,d}$	$0.0090 \pm 0.00^{a,d}$
Ethanol	$53.88 \pm 0.74^{a,c}$	$60.54 \pm 0.52^{b,d}$	$0.0186 \pm 0.00^{b,e}$	$0.0153 \pm 0.00^{a,e}$
Ethyl acetate	$72.48 \pm 5.57^{ns,d}$	$82.09 \pm 3.18^{ns,e}$	$0.0012 \pm 0.00^{a,c}$	$0.0016 \pm 0.00^{b,c}$
BHT	0.18 ± 0.00	0.18 ± 0.00	2.6159 ± 0.02	2.6159 ± 0.02
Ascorbic acid	0.06 ±0.00	0.06 ± 0.00		

Note: Each value is mean ± SD

^{a,b} Data within the same row with different superscripts are significantly different ($p<0.05$).

^{c,d,e} Data within the same column with different superscripts are significantly different ($p<0.05$).

^{ns} Means not significant.

It was found that the antioxidant activity investigated by FRAP assay are significantly different ($p < 0.05$) which ethanol extract showed the highest antioxidant activity followed by water and ethyl acetate extracts, respectively. When compared between two species, the antioxidant activities of both species are significantly different ($p < 0.05$). Wong et al. [6] classified categories of medicinal plants based on their antioxidant activities: extremely high (>500 µmol Fe(II)/g, high (100-500 µmol Fe(II)/g, medium (10-100 µmol Fe(II)/g), and low (<10 µmol Fe(II)/g). Under this classification, the *Ocimum basilicum* var. *thyrsiflorum* extracted by water and ethanol and *Ocimum basilicum* cv. Jumbo 4320 extracted by ethanol exhibited medium antioxidant activity. The other are classified as exhibited low antioxidant activity. *Ocimum basilicum* var. *thyrsiflorum* extracted by ethanol showed highest antioxidant activity by FRAP assay at the value is 0.0186±0.00 mmol Fe^{2+}/ g.

4. CONCLUSIONS

Total phenolic contents and antioxidant activities (DPPH and FRAP assay) of two species of Thai basils as *Ocimum basilicum* var. *thyrsiflorum* and *Ocimum basilicum* cv. Jumbo 4320 crude extracts were assessed. Total phenolic contents and antioxidant activities of *Ocimum basilicum* different between species and solvent that applied for extraction. Unfortunately, total phenolic contents of the extracts showed relationship with antioxidant activities. At high total phenolic contents of *Ocimum basilicum* crude extracts showed strong antioxidant activities.

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Screening *Lagerstroemia speciosa* flower for anti-infective activity against human pathogens using *Caenorhabditis elegans* as *in vivo* model

Arumugam Kamaladevi[a], Krishnaswamy Balamurugan*[a]
[a]Department of Biotechnology, Science campus, Alagappa University, Karaikudi, Tamilnadu, 630 003, India

ABSTRACT

The objective of present study was to evaluate anti-infective activity of methanol extract from *Lagerstroemia speciosa* flower against human pathogens using *Caenorhabditis elegans* as an *in vivo* screening tool. Crude methanol extract was tested for its ability to rescue *C. elegans* against human pathogens *Pseudomonas aeruginosa*, *Serratia marcescens Staphylococcus aureus* and *Vibrio alginolyticus* in liquid medium. Extract rescuing 50% of *C. elegans* against infection was selected for further studies. EC_{50} value of the active fraction was calculated using dose response assays. Data revealed that the methanol extract from *L. speciosa* flower promoted survival of *C. elegans* (50%) against clinical and drug resistant *S. aureus* infections. The active fraction reduced pathogen burden by reducing the *in vivo* colonization of *S. aureus* in *C. elegans* gut. *In vitro* assay indicated the ability of flower extract to inhibit *S. aureus* growth in plate assay. MIC was evaluated using broth dilution method and compared with *in vivo* EC_{50} values. GC-MS analysis of active fraction confirmed presence of fatty acids like hexadecanoic acid, Octadecanoic acid methyl ester and 1,2-benzenedicarboxylic acid as constituents. *In vivo* assays using pure compound of one of the major constituents (palmitic acid) confirmed its efficiency to promote survival of *C. elegans* against *S. aureus*. To our knowledge this is the first study to report the use of model organism *C. elegans* to screen plant extract for its bioactivity against clinical and drug resistant *S. aureus* strains.

Keywords: *Caenorhabditis elegans*, *Lagerstroemia speciosa*, *In vivo* Screening, Antibacterial

1. INTRODUCTION

Continuous emergence of highly virulent bacterial strains and their increased resistance to available antibiotics has become a challenge for clinicians and researchers in the field of infectious disease. Traditional methods for screening anti-infectives fail to deliver a potent lead for drug discovery due to various disadvantages in the *in vitro* plate assays [1]. Recent studies on the use of model organism in the field of drug discovery proved the ability to identify structurally different active compounds with efficient *in vivo* activity [2]. The role of model organism in the process of drug discovery at the stage of preclinical screening is well exploited due to their simplicity and feasibility [3]. *Caenorhabditis elegans* is a simple model host that can be infected and killed by a remarkably large number of human pathogens including Gram-negative *Pseudomonas aeruginosa*, *Vibrio alginolyticus* etc., and Gram-positive *Enterococcus faecalis*, *Staphylococcus aureus etc.*, and the fungal pathogen *Cryptococcus neoformans*. This simple feeding-based pathogenicity assay facilitates high-throughput genetic analysis to understand the role of innate immune system against bacterial infections [4]. *C. elegans* has been proved as an efficient model for screening anti-infectives from both microbial and plant resources [5]. It has been reported that there is a broad overlap between the bacterial virulence factors required for pathogenesis in mammals and for *C. elegans* killing. Owing to their popular use in traditional medicine for the treatment of various ailments including infectious diseases, interest in medicinal plants as a source of novel antimicrobial compounds has been growing [6]. Historically, pharmacological screening of medicinal plants has been a key to innumerable therapeutic agents and antibiotics. Nearly 130 drugs used at present are products or semi-synthetic products availed from chemical entities of plant materials [7]. *Lagerstroemia speciosa* is a tropical plant found in many countries including India. Research in *L. speciosa* was initiated as early as 1940 [8] and researchers around the globe are interested in this tropical plant for its anti-diabetic and anti-obesity activities [9]. Dried leaves of *L. speciosa* were used for treating diabetes patients in Philippines. The antibacterial activity of *L. speciosa* seed extract was reported earlier against few pathogens. The ability of *L. speciosa* fruit extract to inhibit bacterial cell to cell communication has been reported recently [10]. With the knowledge from our previous studies in establishing *C. elegans* as model for bacterial infections [11,12], the present study was designed to evaluate the anti-infective activity of methanol extract from *L. speciosa* flower against human pathogens using *C. elegans* as an *in vivo* screening model.

2. MATERIALS AND METHODS

Plant material and microorganisms

Fresh plant materials of *L. speciosa* flower were collected from gardens in and around Hosur, Tamilnadu, India. 100 g dried powder was incubated in 1000 ml of methanol, chloroform, ethyl acetate and hexane and the concentrated crude extracts were dissolved in DMSO and stored at 4°C until use. The test organisms *S. aureus* (ATCC 11632), *V. alginolyticus* (ATCC 17749) and Methicillin Resistant *Staphylococcus aureus* (ATCC 33591) were obtained from American Type Culture Collection (ATCC). Clinical isolates of *S. aureus* were collected from Rajaji government hospital Madurai, India during 2009-2010 [13]. Other reference strains include *P. aeruginosa* (PAO1) and *S. marcescens* (GenBank accession No: FJ584421).

Preliminary screening

C. elegans wild-type strain N2 was routinely maintained at 20°C on nematode growth medium (NGM) agar plate seeded with *E. coli* OP50 by the standard method. Preliminary screening was carried out in a liquid medium comprising M9 buffer (80%) and culture medium (20%) along with the pathogen (0.3 $O.D_{600\ nm}$). The experiment was carried out in 48 well culture plates in triplicates. Test experiment wells included flower extract and wells without flower extract served as negative control, wells with *E. coli* OP50 served as food control. The plates were incubated at 20°C for 96 h and observed every 4 h, animals were considered to be dead when they showed no response or movement to external stimuli like a gentle tap or touch with platinum loop.

Silica gel column fractionation and GC/MS

The crude extract showing antibacterial activity was fractionated using silica gel column (SRL, India) with using the Methanol: Chloroform: Hexane solvent system in the ratio of (0.5:2.5:7). The fractions showing antibacterial activity were once again purified by the same column with the solvent system as described earlier and the purity was confirmed by TLC (Merck, USA) analysis. GC-MS analysis of the column purified active fraction from the methanol extract was performed using a Shimadzu GCMS-QP 2010 PLUS using standard protocol [13].

Microscopic observations, colony count assay and pharyngeal pumping assay

C. elegans fed with *E. coli* OP50 or *S. aureus* in control and treatment plates were washed thrice with M9 buffer and placed on a 2% agarose pad containing 1 mM sodium azide for microscopic observations using an inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan). *In vitro* CFU assay was performed according to standardized protocol [11].

***In vitro* antibacterial assay and Minimum inhibitory concentration (MIC) evaluation**

The sensitivity of different bacterial strains to solvent extracts was determined using standard agar diffusion assay. The MIC of the plant extract was evaluated as per Clinical and Laboratory Standards Institute guidelines. Assay was also performed using standard antibiotic ciprofloxacin.

Statistical analysis

Experiments were conducted in triplicates conducted in triplicates and the statistical analysis was performed by one way ANOVA using SPSS software 10.0. Significant difference between the groups was calculated by Dunnet's Multiple range test ($p < 0.05$).

3. RESULTS AND DISCUSSION

Preliminary screening identified the ability of methanol extract from *L. speciosa* flower to rescue 50% *C. elegans* in comparison to the control against *S. aureus* infection at the concentration of 2.5 mg/ml (Figure 1). Since no phytochemical data was available on *L. speciosa* flower, prior to proceeding with further assays, the crude extract was fractionated in silica column and fractions were checked for their activity *in vivo* and purity of the extract was assessed using precoated TLC plates. GC-MS analysis of the column purified active fraction of extract confirmed the presence of fatty acids and hexadecanoic acid (Palmitic acid as major constituent). The ability of column purified active fraction was tested against clinical and drug resistant *S. aureus* isolates at different concentrations. The dose response assays proved that the column purified active fraction displayed significant rescue activity with EC_{50} value of 0.2 mg/ml against the reference and few clinical strains and varied up to 1 mg/ml against the drug resistant clinical isolate. Interaction of *C. elegans* with *S. aureus* and its immune responses during infection has been previously reported from our laboratory [12]. *C. elegans* is considered as one of the suitable models for studying infection and host response against *S. aureus*. The *C. elegans* based survival assay was previously used for screening bioactive compounds from the chemical and natural libraries and the assay proved to be more efficient than the *in vitro* screening for indentifying bioactive compounds with unique structure and interesting mode of action [2]. Plant extracts inhibiting the Quorum sensing phenomenon in *P. aeruginosa* was also tested using *C. elegans* bio assay [5].

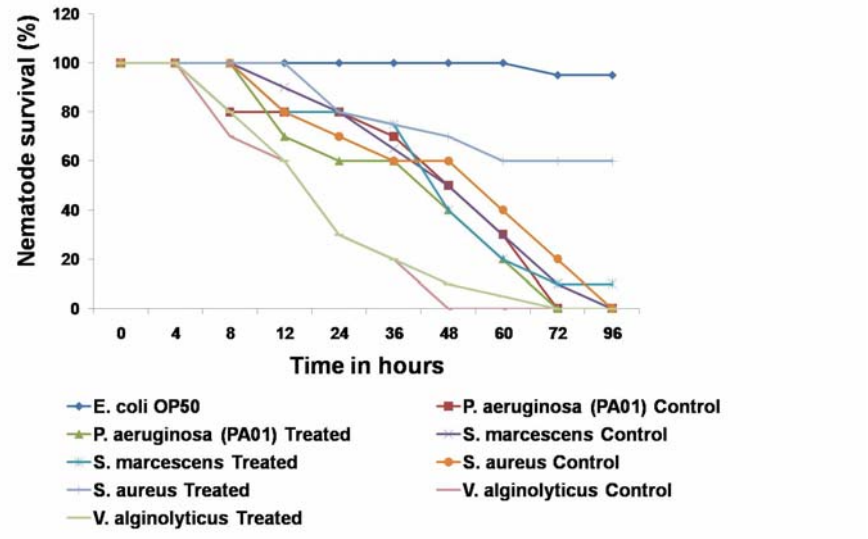

Figure 1. Survival graph of *Caenorhabditis elegans* against human pathogens in presence and absence of *Lagerstroemia speciosa* flower extract.

The advantage of *C. elegans* based screening system is that apart from screening for bio-actives it also checks the toxicity during the screen process. In the present study, no toxicity was observed with the active fraction in all the tested concentrations; however, above 5 mg/ml *C. elegans* displayed aversive response leading to dauer formation. It is also noted that beyond 2.5 mg/ml of active fraction, no significant increase in rescue was observed (data not shown). The killing of *C. elegans* by *S. aureus* is correlated with the accumulation of bacteria in the digestive tract of the nematode. Animals fed on *S. aureus* in the presence of plant extract showed a reduced colonization. Time course CFU assays from the control and treated samples proved reduction in colony count (Figure 2).

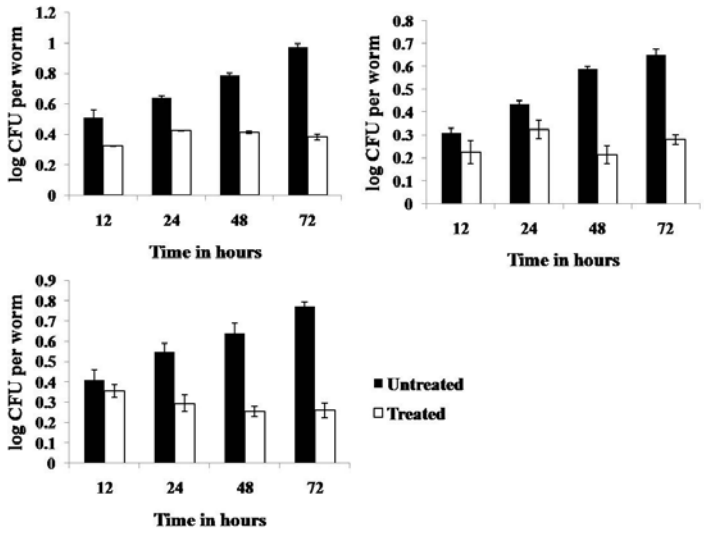

Figure 2. CFU assay: Bacterial colonization of *S. aureus* strain (A) ATCC 11632; (B) ATCC 33591 and (C) GSA 410) in presence and absence of active fraction (0.25 mg/ml).

The presence of column purified fraction promoted other activities of *C. elegans* including pharyngeal pumping, and locomotion during infection. In contrast, the animals fed on *S. aureus* in the absence of the flower extract at same time period showed retarded growth, delayed movement and pharyngeal pumping arrest leading to paralysis and death of the nematode**.** Infection with *S. aureus* also caused immature vulval development leading to egg laying defective worms resulting in the internal hatching. Microscopic observation of control and treated *C. elegans* displayed no such abnormality in the presence of flower extract. Several studies previously reported the ability of fatty acids to inhibit bacterial growth and antibacterial activity of fatty acids from essential oils has been documented [14]. Since Hexadecanoic acid methyl ester also called as Palmitic acid constituted the major portion of the fraction with major peak area of (36.49%) ability of palmitic acid pure compound to rescue *C. elegans* was again assessed by liquid assays. The results confirmed the increase in survival of *C. elegans* against *S. aureus* in the presence of pure palmitic acid at the concentration of 0.05 mg/ml (P<0.005**)**. Role of hexadecanoic acid against *S. aureus* colonization was well studied in the clinical context. Confirmation using commercial compound in the present study further supported the findings with the activity of column purified fraction against *S. aureus*. Several studies previously reported the ability of fatty acids present in the skin and mucosal region to protect humans from bacterial infections and colonization of *S. aureus* in the skin was found to be lower in regions rich in fatty acids like hexadeconoic acid and sphingosine[14]. The presence of fatty acids in the active fraction of flower extract supports the bioactivity of the extract against clinical and drug resistant *S. aureus. In vitro* MIC varied among the isolates with a minimum for *S. aureus* ATCC at concentration of 0.312 mg/ml and maximum of 1.25 mg/ml (P < 0.05) against clinical isolates. By comparing the *in vitro* with *in vivo* results, it is observed that the concentration of active fraction required for *in vivo* rescue was significantly less that *in vitro* active concentration in many cases. Results of present study clearly suggest that the *in vivo* efficacy of *L. speciosa* flower extract against *S. aureus* infection. *L. speciosa* flower enriched with fatty acids could be potent source of anti-infective drug against *S. aureus* infection. Further studies on the biomedical importance of flower extract against various clinical aspects using *C. elegans* model will further enrich value of present study.

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Hepato-protective effect of *Azadirachta indica* leaf aqueous extract against *Plasmodium berghei* infected mice

Voravuth Somsak*[a], Ubonwan Jaihan[a], Somdet Srichairatanakool[b], Chairat Uthaipibull[c]
[a]Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Kanchanaburi, Thailand 71170
[b]Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand 50200
[c]National Center for Genetic Engineering and Biotechnology (BIOTEC), National of Science and Technology Development Agency (NSTDA), Pathumthani, Thailand 12120

*voravuthsomsak@gmail.com phone +66-898009939

ABSTRACT

Malaria is caused by protozoa parasite in genus *Plasmodium* and transmitted by *Anopheles* mosquito. It is estimated 1 million deaths annually, most of them are children less than 5 years of age in sub-Saharan Africa. Causes of death in malaria are variable including severe anemia, cerebral malaria, and liver damage and failure. Especially in liver damage during malaria infection is a major focus of this study. The objective of this study was to determine protective effect of *Azadirachta indica* leaf extract against *Plasmodium berghei*-induced liver damage by using aspartate and alanine aminotransferase (AST and ALT, respectively) as biological markers. Aqueous leaf extract of *A. indica* was prepared. For *in vivo* test, ICR mice were inoculated with $1x10^7$ infected red blood cells of *P. berghei* ANKA. The extract (1,000 mg/kg) was orally given twice a day for 6 consecutive days, and AST and ALT were then measured using commercial kits. It was found that AST and ALT levels in plasma were significantly ($p<0.05$) increased on day 6 post infection resulting to parasite development *in vivo*. Interestingly, infected mice treated with this extract, AST and ALT levels were normalized significantly and no difference to normal mice. It can be concluded that *A. indica* leaf extract exerted protective effect on liver damage during malaria infection. However, active components and mechanism of action should be studied in more detail for validating this extract as alternative malaria treatment.

Keywords: Hepato-protective effect, *Azadirachta indica*, *Plasmodium berghei*

1. INTRODUCTION

Malaria is an infectious disease with ravaging effects in the world. It is estimated that half the world's population is at risk of malaria, and that 1-2 million annual deaths can be attributed to malaria alone [1, 2]. This disease is caused by protozoa parasite *Plasmodium* and transmitted by female *Anopheles* mosquito. The causes of death in malaria involve severe anemia, cerebral malaria, and organ damage and failure [3-5]. In organ damage, liver is a critical organ and one of most targets for malaria. Malaria-associated liver damage and failure occurs between 2-5% of hospitalized patients with a mortality that can reach up to 45% [6-8]. This has prompted research towards the discovery and development of compounds to treatment and protects liver damage during malaria infection. In this respect, plant resources are potential targets for research and development of alternative drugs.

Azadirachta indica, commonly known as Neem, is found throughout Southeast Asia including Laos, Myanmar, Cambodia and Thailand. This plant is used for treatment of some pathological conditions related to oxidative disorders such as inflammation and skin diseases, rheumatic, arthritis disorders and treatment of fever and diabetes [9, 10]. Moreover, extract of *A. indica* has been used traditionally to treat malaria in several endemic countries. For *in vitro* study, *A. indica* leaf extract possess inhibitory activity on *P. falciparum* asexual stage [11-13]. However, there is no publication concerning the biological activities of *A. indica* in protection of liver damage during malaria infection. The aim of this study was to investigate protective effect of aqueous leaf extract of *A. indica* against *P. berghei*-induced liver damage.

2. MATERIALS AND METHODS

Plant material and preparation of extract

Fresh leaves of *Azadirachta indica* were collected in Kanchanaburi, Thailand. The sample was identified by Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand. The Voucher specimen was deposited at Department of Clinical Chemistry, Faculty of Medical Technology, Western University, Thailand. Sample was cleaned, dried in a hot air oven (55^{o}C) for 6 h then powdered. Dried powder of leaf sample was separately boiled with distilled water (DW) for overnight at room temperature (plant: water = 1: 20, w/v). The suspension was centrifuged at 5,000 g for 20 min and filtered through Whatman no. 1 filter paper [14]. The filtrate was then lyophilized to yield 11.9% (w/w) and stored at -20^{o}C. The extract was dissolved in DW at a suitable concentration prior to experiment.

Experimental animal

Free-pathogen 4-week female ICR mice with 25-30 g purchased from National Laboratory Animal Center, Mahidol University, Thailand were used. They were kept at 25^{o}C with 12 h day/night cycle, and fed with standard pellet diet and clean water *ad libitum*. All experimental animals were ratified by the Animal Ethical Committee from Western University, Thailand.

Rodent malaria parasite

Chloroquine sensitive strain of *Plasmodium berghei* ANKA (PbANKA) gifted from Dr. Chris Janse at Leiden University was used in this study. Parasite was grown in animal by intraperitoneal (IP) injection of 1x10^{7}infected red blood cells (iRBC). Parasitemia was monitored daily by Giemsa stained thin blood smear under light microscope with x100 oil immersion lens. When parasitemia reached to 15-20%, mechanical passage was performed into new ICR mice. Moreover, assessment of liver function during malaria infection was also determined.

Assessment of liver function

Enzyme markers, aspartate and alanine aminotransferase (AST and ALT) were measured using commercial kit (BioSystems, Spain), according to manufacturer's instruction. Blood was collected by cardiac puncture and centrifugation was then performed to collect serum. The serum was used as subject for measurement.

Efficacy test *in vivo*

The *in vivo* test was based on standard Peters' test [15]. Groups of ICR mice (5 mice of each) were inoculated with 1x10^{7} iRBC of PbANKA by IP injection. The extract (1,000 mg/kg) was given orally twice a day for 6 consecutive days. Blood was collected then AST and ALT were measured. Normal and untreated mice were used as controls.

Statistics

Statistical analysis was performed using GraphPad Prism software. Data was expressed as mean $\pm$ standard error of mean (SEM). One-way ANOVA was used to compare the data, and significance was considered at 95% confident, $p < 0.05$.

3. RESULTS

In order to examine blood stage propagation of PbANKA infection in mice, parasitemia was daily monitored. Figure 1A showed that parasitemia was first detectable on day 2 post infection with a parasitemia of <1%. Parasite was growing and parasitemia reached to 60% on day 10 post infection. Infected mice died on day 11 post infection. During *Plasmodium* propagation in RBC, ring form is developed into trophozoite and schizont containing thousand merozoites. Then, merozoites are released into bloodstream and re-invade new RBC. At this phenomenon, RBC was destroyed and hemolysis is occurred resulting to severe anemia [16, 17]. Therefore, infected mice could die from severe anemia. In addition, levels of AST and ALT in plasma were increased during parasite development, and significance ($p < 0.05$) was firstly observed on day 6 post infection (Figure 1B). It was suggested that during hemolysis, free radical is increased followed by oxidative stress and inflammation is developed. Hemolysis is the important factor in the onset and progress of severe anemia mainly by producing oxidative stress [18, 19]. It has been reported that oxidative stress could damage several organs, especially liver [20, 21]. The mechanism suggested involvement of cytoadherence of iRBC, pro-inflammatory response as well as hepatitis due to oxidative stress [6]. So, biological markers for liver function, AST and ALT were then increased.

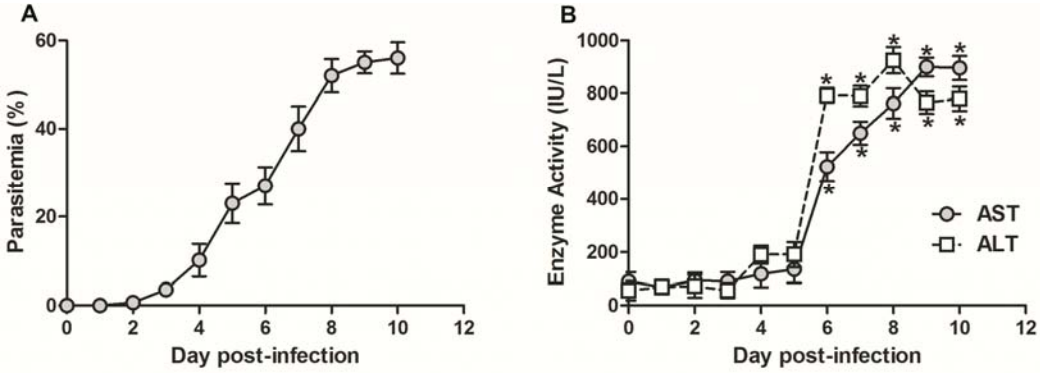

Figure 1. Assessment of liver damage during *Plasmodium berghei* infection in mice. ICR mice (5 mice) were inoculated with $1x10^7$ iRBC of PbANKA by IP injection, and (A) parasitemia and (B) AST and ALT were then measured as previously described. Results were expressed as mean ± SEM. * $p < 0.05$, compared to day 0 post-infection.

For efficacy test *in vivo*, aqueous leaf extract of *A. indica* was given orally twice a day for 6 consecutive days in PbANKA infected mice. The results showed that AST and ALT were decreased and normalized into normal levels in infected mice treated with this extract, and there were no significant difference when compared to normal mice (Figure 2A and B). Moreover, no toxicity was observed in normal mice treated with the extract as indicated by levels of AST and ALT were normal (data not shown). The extract with a dose of 1,000 mg/kg has been descried to have no any toxicity to mice [22]. Moreover, it has been reported that aqueous leaf extract of *A. indica* exhibited antioxidant activity as well as anti-inflammation with potent free radical scavenging [14, 23]. Hence, these properties of the extract might exert protective effect on liver damage during malaria infection in order to inhibit free radical and oxidative stress followed by protection of liver. Moreover, it has been reported that the efficacy of this extract is attributed to limonoid, a class of highly oxygenated terpenoids, endowed with a range of biological properties including insecticidal, anti-microbial, anti-inflammation, and immune-modulatory activities [24].

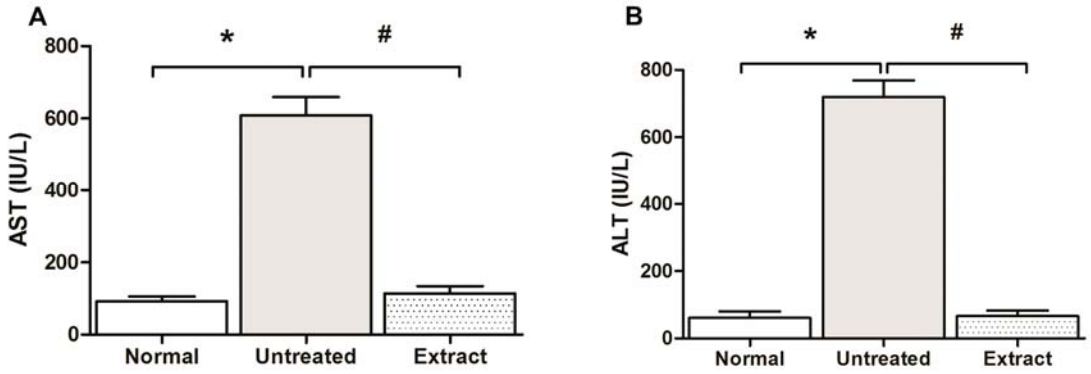

Figure 2. Efficacy of aqueous leaf extract of *Azadirachta indica* on liver damage during *Plasmodium berghei* infection. Groups of ICR mice (5 mice of each) were inoculated with $1x10^7$ iRBC of PbANKA by IP injection. The extract (1,000 mg/kg) was given orally twice a day for 6 consecutive days, and (A) AST and (B) ALT were consequently measured as previously described. Results were expressed as mean $\pm$ SEM. * $p < 0.05$, compared to normal group. # $p < 0.05$, compared to untreated group.

4. CONCLUSIONS

The aqueous leaf extract of *A. indica* showed the protective effect on liver damage during *P. berghei* infection in mice as indicated by normalizing of AST and ALT levels. Further work should include the separation and identification of active components and mechanism of action.

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Functional analysis of 70% ethanolic extract of pearl grass (*Hedyotis corymbosa* (L.) Lamk.) on rheumatoid arthritis rat

Anton Bahtiar*[a], Margaretha Silvana Mawar Utami[a], Indana Ayu Soraya[a], Dadang Kusmana[a]
[a]Faculty of Pharmacy University of Indonesia, Indonesia

*anton.bahtiar@gmail.com phone +62-217864049 fax +62-217863433

ABSTRACT

This study aims to analyze the anti-inflammatory effect of 70% ethanolic extract of pearl grass (*Hedyotis corymbosa* (L.) Lamk). In term of reduction in edema induced by Complete Freud's Adjuvant (CFA) and its effect on bone density, serum calcium levels of RA Rats. A total of 36 Sprague Dawley-strain rats were divided into 6 groups, whole rats was induced with 0.1 mL of CFA in subplantar on day-1 rats except normal control groups were injected by saline 0,9% NaCl. On day 2nd to day 28th, the rat were administered as follow: negative control groups were given 0.5% CMC, positive control groups were given sodium diclofenac, and 3 groups of pearl grass with varying doses 28 mg/200g; 63 mg/200g and 142 mg/200g, respectively. Edema volume was evaluated on day 1, 7, 14, 21 and day 28. The results showed that 70% ethanolic extract of pearl grass could decrease edema volume, osteoclast number on calcaneus bone and also could increase calcium serum of RA rat. This result indicated that extract could be promising drug for inflammation condition.

Keywords: Calcium serum, Osteoclast, Rheumatoid arthritis, *Hedyotis corymbosa* (L.) Lamk

1. INTRODUCTION

RA is the prototype of a destructive arthritis. The disease directly leads to joint damage, with only a few signs of repair. Traditionally, structural damage in RA has been identified using conventional radiography to detect cortical bone erosions, joint space narrowing, and periarticular osteoporosis. Imaging has shown unequivocally that there is a net loss of cartilage and bone in patients with RA. In particular, the presence of bone erosions has emerged as an indicator of irreversible damage resulting from a continuous inflammatory attack of the synovial membrane on bone [1].

Bone-resorbing osteoclasts are important effector cells in inflammation-induced bone loss. The RANK/RANKL pathway was shown to be essential for osteoclast differentiation in inflammatory bone destruction. In addition, in vitro and in vivo studies have demonstrated that many cytokines elaborated by inflammation, including the proinflammatory cytokines TNF-a and interlukin-1 (IL-1), may contribute to osteoclast differentiation and activation [2].

2. MATERIAL AND METHODS

Plant and Chemical Materials

Hedotys corymbosa (L.) Lamk used in the present study were collected from "Kampoeng Djamoe Organic" PT Martha Tilaar. Ethanol 96%, methanol p.a, ethyl acetate p.a and petroleum ether p.a. were obtained from Sigma (St. Louis, MO, USA). Estradiol was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

Preparation of 70 % *Hedotys corymbosa* extracts.

The dried powders of pearl grass herb were extracted by maceration using ethanol. 250 g of pearl grass herb was mix with ethanol (1:5) for 6 h in shaker. Then the extract was filtered and dried using evaporator. The extractive value of ethanol from dried powders was calculated as % w/w yield and was found to be 3.71%.

Animals

Female Sprague–Dawley rats, aged 36 days, were purchased from Litbangkes. The animals were grouped and housed in polyacrylic cages with one animal per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (12/12 h) and allowed free access to commercial pellet diet and water ad libitum.

Rats were acclimatized to laboratory condition for 1 week before commencement of experiment. All procedures described conducted in accordance with Guideline for Care and Use of Faculty of Medicine, University of Indonesia. Animals were assigned to experimental groups, Normal control groups were injected by saline 0.9% NaCl. On day 2nd to day 28th, the rat were administered as follow: Negative control groups were given 0.5% CMC, Positive control groups were given sodium diclofenac, and 3 groups of Pearl Grass with varying doses 28 mg/200g; 63 mg/200g and 142 mg/200g, respectively. On the day after the last dose, the rats were blood collected from orbital plexus after and sacrificed under light anesthesia. The heels were removed immediately for histology analysis.

Calcium content of the blood

The blood collected from orbital plexus after and sacrificed under light anesthesia for calcium analysis, dried for 12 h at 110°C and reweighed to obtain the dry blood weights. Calcium content in ash blood was determined by atomic absorption spectrophotometry (AAS).

Histological Analysis

The Heels bones were fixed in 10% buffered formalin for 48 h. All samples were embedded in paraffin and 3-μm thick sections were cut, mounted, and stained with hematoxylin and eosin (H&E) for microscopic analysis

Statistical Analysis

Data from the animal experiments were expressed as the mean ± S.E.M. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni or LSD post-hoc test analysis using software SPSS. p values of less than 0.05 were considered to indicate significant differences.

3. RESULTS

Effect of pearl grass extract on inflammation

Whole rats was induced with 0.1 mL of CFA in subplantar on day-1 rats except normal control groups were injected by saline 0,9% NaCl. On day 2 to day 28, the rat were administered as follow: Negative control groups were given 0.5% CMC, Positive control groups were given sodium diclofenac, and 3 groups of Pearl Grass with varying doses 28 mg/200g; 63 mg/200g and 142 mg/200g, respectively. Edema volume was evaluated on day 1, 7, 14, 21 and 28.

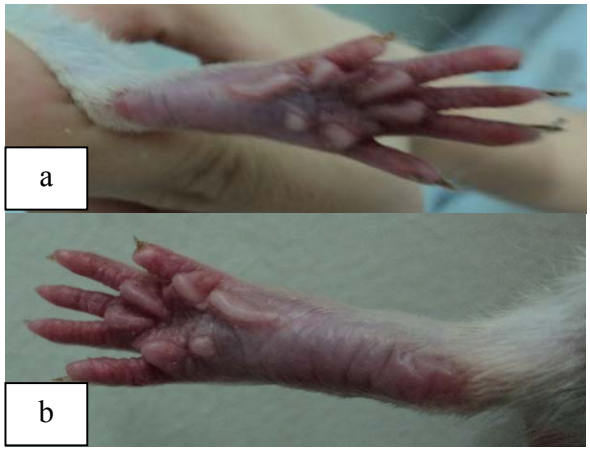

Figure 1. Rat injected by 0.9% NaCl as normal control (a) and rat injected by CFA (b).

Tabel 1 Percentage of edema inhibition

Groups		Percentage of edema inhibition			
		Day 7	Day 14	Day 21	Day 28
Normal Rat		0	0	0	0
Negative control		0	0	0	0
Diclofenac sodium		33, 33	17, 48	8, 33	4, 76
Pearl grass extract	Dose 1	11, 43	33, 98	16, 67	7, 62
	Dose 2	25, 71	19, 42	20, 83	21, 90
	Dose 3	5, 71	11, 65	21, 87	12, 38

In diclofenac sodium-treated rat, the inhibition of inflammation was start from the beginning of CFA induction but at the end of experiment the inhibition was decreased. In Pearl grass-treated rat shows that the inhibition of inflammation was greatest at the end of experiment, Dose 2 showed better effect compared groups dose 1 and 3. This result indicated that between diclofenac sodium and pearl grass extract have different mechanism in inhibited inflammation and dose 2 of extract was optimum dose for RA inhibition.

Analysis of Calcium in the blood

Blood calcium concentration was decreased in RA rat, and either diclofenac sodium treated rat or pearl grass treated rat could increase blood calcium concentration to normal condition.

Tabel 2. Analysis of Calcium content of blood by Atomic Absorption Spectrophotometer

Groups		Blood Calcium concentration (mg/dL)
Normal rat		48.23 ± 6.8
RA rat		28.27 ± 8.3*
Diclofenac sodium		53.84 ± 9.6#
Pearl grass extract	Dose 1	50.08 ± 4.1#
	Dose 2	45.77 ± 7.9#
	Dose 3	43.5 ± 9.5#

Note : * significantly different compare with normal rat
significantly different compare with RA rat

Analysis of osteoclast number on calcaneous bone

On histology analysis on calcaneous bone, osteoclast number were increased in RA rat and administration of Diclofenac sodium was decreased osteoclast number and same effect was found in Pearl grass treated rat.

Tabel 3. Jumlah osteoklas per daerah pengamatan setiap kelompok perlakuan

Groups		No. of Osteoclast/ mm^2
Normal rat		122,83 ± 37,98
RA rat		167,17 ± 7,33*
Diclofenac sodium		97,00 ± 10,49#
Pearl grass treated Rat	Dose 1	132,33 ± 10,78#
	Dose 2	111,67 ± 2,24#
	Dose 3	96,33 ± 13,02#

Note : * significantly different compare with normal rat
significantly different compare with RA rat

4. CONCLUSION

1. 70% ethanolic extract of Hedyotis corymbosa Lamk could decrease inflammation on *Complete Freund's Adjuvant* (CFA) induced Rheumatoid arthritis
2. 70% ethanolic extract of Hedyotis corymbosa Lamk could increase blood calcium level of RA rat.
3. There is a relationship between anti-inflammatory effect with improved levels of calcium, so that the blood serum calcium levels may be can be use for diagnosis of rheumatoid arthritis disease
4. 70% ethanolic extract of Hedyotis corymbosa Lamk could decrease osteoclast number on Calcaneous bone

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Antibacterial activity of *Stephania suberosa* extract against methicillin-resistant *Staphylococcus aureus*

Nongluk Autarkool[*a], Yothin Teethaisong[a], Sajeera Kupittayanant[b], Griangsak Eumkeb[a]
[a]School of Pharmacology, [b]School of Physiology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand 30000

*Autarkool@hotmail.com phone+66-812621056 fax +66-44224650

ABSTRACT

Bacterial resistance to β-lactam antibiotics is a global problem. *Staphylococcus aureus* (*S. aureus*) strains are resistant to penicillin, methicillin around the world and in most of the Asian countries nowadays. Strain of methicillin-resistant *S. aureus* (MRSA) is now pose serious problem to hospitalized patients, and their care providers. Antibiotics available for the treatment of MRSA infection are fairly toxic and their use is frequently associated with unwanted side-effects. Novel antibiotics and/or new generation of phytopharmaceuticals approaches that can reverse the resistance to well tried agents which have lost their original effectiveness are research objectives of far reaching importance. The aim of this investigation was to examine antibacterial and synergistic activities of *Stephania suberosa* extract (SSE) against MRSA when used singly and in combination with ampicillin. The minimum inhibitory concentrations (MICs) determination of ampicillin and SSE against MRSA were 455 μg/ml and 4,000 μg/ml, respectively. Synergistic activity was observed in the combination of ampicillin and SSE with fractional inhibitory concentration index (FICI) 0.5. The viability of MRSA was determined using time-killing assay showed dramatically reduced from $5x10^5$ CFU/ml to 10^3 CFU/ml within 6 h and throughout 24 h. Electronmicroscopic study revealed that 0.125 μg/ml ampicillin in combination with 2,000 μg/ml SSE caused severe damage to MRSA envelopes. This study establishes evidence that SSE may be used combination with ampicillin, as a new generation of phytopharmaceuticals, to treat MRSA that currently almost untreatable microorganism. These *in vitro* results have to be confirmed in an animal test or in humans.

Keywords: β-lactam antibiotics, MRSA, *Stephania suberosa* Forman, Synergistic activity, Ampicillin

1. INTRODUCTION

Plant-derived antibacterials are an interesting source of novel therapeutics. *Stephania suberosa* Forman belongs to the family Menispermaceae, has been traditionally used in folk Medicine as a tonic, carminative, and expectorant [1]. Drug-resistant bacteria has emerged to be one of the greatest test to human health worldwide. MRSA is a major cause of community and health care associated infections. Recently, this strain has acquired resistant to practically antibiotics, its primary antibiotics cannot be effectively used against this strain. Thus, the development of a novel antibacterial agent MRSA strain is urgently needed. There is no a wealth of evidence regarding on antibacterial activity of *S. suberosa* against MRSA. Therefore, the purpose of this study was to determine antibacterial and synergistic activities of *S. suberosa* against MRSA when used alone and in combination with ampicillin.

2. MATERIALS AND METHODS

Preparation of plant extracts

Root of *S. suberosa* was dried and powdered. 50 g *S. suberosa* powdered was extracted with ethanol at 75°C for 8 hours and each extract was concentrated using a rotary evaporator. Then, freeze dryer was performed to yield a brown powder and a dark brown sticky oil of ethanol extract [1, 2].

Bacterial strains and antibiotics

Clinical isolates of MRSA DMST 20651 were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. *S. aureus* ATCC 29213 obtained from the American Type Culture Collection (ATCC) was used as reference strain. Ampicillin was obtained from Sigma.

Bacterial suspension standard curve

In order to select bacterial suspensions with a known viable count [3] the method was followed.

Minimum inhibitory concentration determination (MIC)

To determine MIC of crude extract and antibiotic against these strains, the broth macrodilution method was performed. Briefly, bacterial suspension was adjusted to approximately 1 x 10^8 CFU/ml. Inoculum (0.1 ml) of each stain was added to 0.9 ml MHB, plus serial dilutions of the antibacterial agents, to give final concentration approximately 1x10^5 CFU/ml. The lowest concentration that showed no visible growth was recorded as the MIC [3, 4].

Checkerboard determination

Checkerboard determinations of antimicrobial combinations were performed in accordance with Odds's method [5]. The interactions between antibacterial agents and crude extracts were determined by the FICI was calculated and interpreted by previously described: FICI $\leq$ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism [5].

Killing curve determinations (viable counts)

Viable count of MRSA was examined using determination of killing curve in according to previously described [6]. Inocula (5×10^5 CFU/ml) were exposed to the antibacterials either singly or in combination with crude extract after contact time of 0, 0.5, 1, 2, 4, 6 and 24 h incubation at 37°C for 18 h were allowed counting of growing colonies. The lowest detectable limit for counting is 10^3 CFU/ml.

Transmission electron microscopy (TEM)

To assess the effect of *S. suberosa* induced MRSA envelope damage when either used alone or in combination with ampicillin, the TEM study was performed in according to previously described [6, 7].

3. RESULTS

The present study reported the antibacterial activity of *S. suberosa* extract when used alone and in combination with conventional antibiotic. The MICs of the extract and ampicilln against MRSA were 4 mg/ml and 455 µg/ml, respectively, while against susceptible *S. aureus* strain were 16 mg/ml and 2 µg/ml, respectively (Table 1). These results indicated MRSA used in this study revealed high resistant level to ampicillin. Interestingly, synergistic effect was observed in crude extract and ampicillin combination (FICI = 0.5) (Table 1). These finding indicated that the ability of *S. subersa* can reverse the resistance strains to be susceptible to primary antibiotics. The

alkaloids have been found to be the major bioactive compound of *S. suberosa* [8], thus alkaloids could probably be the bioactive compound of this extract against drug resistant bacteria.

Table 1. MICs of crude extract from *S. suberosa* when used singly and in combination with ampicillin against drug resistant bacteria

Microorganisms	Agents	MIC	Combination	FICI	Outcome
S. aureus DMST 20651 (MRSA)	Ampicillin	455 µg/ml	0.125 µg/ml	0.5	Synergism
	SSE	4 mg/ml	2 mg/ml		
S. aureus ATCC 29213	Ampicillin	2 µg/ml	NT	NT	NT
	SSE	16 mg/ml	NT		

FICI ≤ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism; *S. aureus* ATCC 29213 were used as control strains. NT = not test.

The killing curve assay is shown in Figure 1. The cell grown in absence of antibacterial agents (control) revealed no reduction in viable counts. No significant change was observed in cells treated with the SSE and ampicillin alone. Interestingly, the combination of the SSE and ampicillin exhibited steady reduction of 5×10^5 CFU/ml to 10^3 CFU/ml within 6 h and did not recover within 24 h. These results establish evidence that the combination of the SSE and ampicillin have synergistic activity against MRSA. These findings seem consistent with previously reported that ceftazidime in combination with flavonoids caused markedly reduction in viable counts of MRSA [7].

Electron microscopic investigation clearly exhibited that the cytoplasmic membrane and cell wall of MRSA treated without antibacterial agents (control) can be undoubtedly distinguished (2A). MRSA treated with ampicillin alone showed minimal peptidoglycan damage to a minority of these cells (2B). Cells treated with the SSE alone caused quite rather cell wall damage to several of these cells (2C). These findings suggest that the SSE alone cause higher peptidoglycan damage than ampicillin alone. Interestingly, the combination of ampicillin plus SSE inhibited definitely damage to peptidoglycan and cytoplasmic membrane, cell shape distortion, cell wall and cytoplasmic membrane of these cells cannot be distinguished in a majority of these cells (Figure 2D). These results provide evidence that SSE exerts synergy effect with ampicillin by reversing ampicillin resistance to be susceptible to its primary antibiotic. These results are in substantial agreement with previously reported that TEM clearly showed damage to the ultrastructures of MRSA strain after exposure to the combination [6, 7].

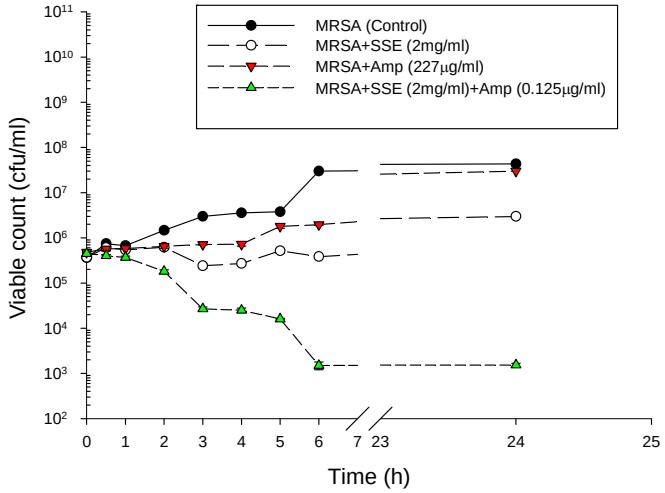

Figure 1. The effect of ampicillin either alone or in combination with *S. suberosa* extract (SSE) on clinical isolates of Methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA). Symbol represents: (●) control (antibacterial free); (○) SSE (2 mg/ml); (▼) ampicillin (227 µg/ml); (▲) SSE (2 mg/ml) + ampicillin (0.125 µg/ml). The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

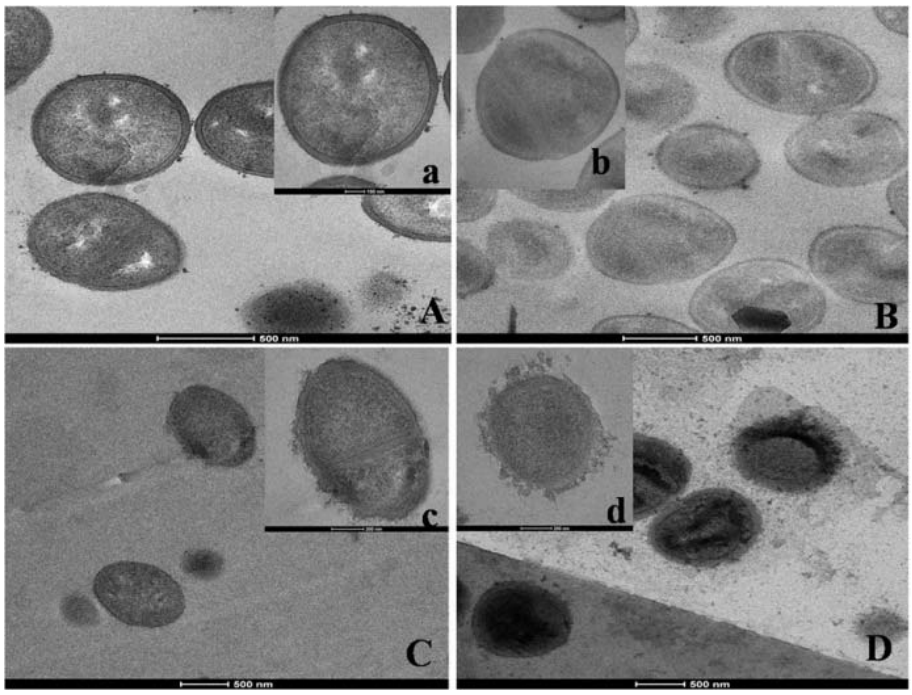

Figure 2. Ultrathin sections of log phase clinical isolates of Methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA) grow in MHB: Control (A: bar=500 nm, x19500; a: bar=100 nm, x43000); 227 µg/ml ampicillin (B: bar=500 nm, x15000; b: bar=200 nm, x38000); 2 mg/ml SSE (C: bar=500 nm, x9900; c: bar=200nm, x38000); 2 mg/ml SSE plus 0.125 µg/ml ampicillin (D: bar=500 nm, x9900; d: bar=200 nm, x29000).

4. CONCLUSIONS

The results of present study support the traditional use of *S. suberosa* and also suggest that this plant possess antibacterial properties. Our results also demonstrate the ability of *S. suberosa* extract also have the ability to reverse the resistance of such bacterial strain to the activity of the primary antibiotic. Moreover, the combination of SSE plus ampicillin could be offered for the development of a valuable adjunct to ampicillin, as a new generation of phytopharmaceuticals, treatments against, MRSA, otherwise resistant strain of currently almost untreatable microorganism.

ACKNOWLEDGEMENTS

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Antiproliferative effect on cancer cells and mutagenic activity of *Pseuderanthemum palatiferum* (Nees) Radlk.

Benjawan Dunkhunthod[a], Benjamart Chitsomboon*[a]
[a]School of Pharmacology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand 30000

*benja@sut.ac.th phone +66-897180683 fax +66-44224185

ABSTRACT

Pseuderanthemum palatiferum (Nees) Radlk. is one of the most commonly used medicinal plants in Thailand. It has been reported to have antioxidant, anti-inflammatory, antidiabetic, and antimicrobial activities. This study aimed to investigate the *in vitro* antiproliferation of 95% ethanol extract of *P. palatiferum* (EEP) against different human cancer cell lines (Jurkat, HepG2, MCF-7, and PC-3) using the MTT assay. The mutagenic effect of EEP as a possible health risk from its long term use was also evaluated by the Ames test. The results showed that various types of cancer cells exhibited different susceptibilities to EEP in a dose dependent manner. Jurkat cells was the most sensitive to the lethal effect of EEP. The LC_{50} of EEP in Jurkat and HepG2 were 476.35 ± 31.51 and 927.01 ± 90.84 µg/ml, respectively, and the LC_{50} in MCF-7 was higher than 1,500 µg/ml. No growth inhibition of EEP on PC-3 cells was observed. The cytotoxicity of EEP was mediated through apoptotic mechanism as evidenced by the nuclear condensation and DNA laddering fragmentation profile of Jurkat cells exposed to 300 µg/ml EEP for 12 and 24 h and 600 µg/ml for 24 h. Apoptosis induction in Jurkat cells was further confirmed by Hoechst 33258 and Annexine-V/PI staining using flow cytometry. There was no mutagenic effect of EEP on the *Salmonella typhimurium* strains TA98 and TA100, regardless of the absence or presence of S9 mix. Overall, this study suggested that EEP exhibits antiproliferative effect on Jurkat cells by apoptosis induction, and the extract possesses no mutagenic activity

Keywords: Antiproliferation activity, Mutagenic activity, Apoptosis induction, *Pseuderanthemum palatiferum* (Nees) Radlk.

1. INTRODUCTION

Pseuderanthemum palatiferum (Nees) Radlk. or Hoan-ngoc has long been used as medicinal plant by Vietnamese people. A few years ago, it has become popular among Thai people for alleviating or curing various diseases, including cancer. Hoan-ngoc leaves have been reported to have antioxidant and antidiabetic [1], anti-inflammatory [2], and antimicrobial activities [3]. Moreover, the major bioactive compounds in Hoan-ngoc leaves are revealed to be flavonoid, stigmasterol, β-sitosterol, and apigenin-7-0-β-glucoside [4]. Flavonoid and β-sitosterol were reported to induce apoptosis in cancer cells [5, 6]. However, up to present, the pharmacologic studies of Hoan-ngoc's claimed properties are still very limited, and the long-term effects of its use are still largely unknown. Therefore, the aim of this study was to investigate the *in vitro* antiproliferative activity against different human cancer cell lines (Jurkat, HepG2, MCF-7, and PC-3) and evaluated apoptosis induction on Jurkat cells of 95% ethanol extract of *P. palatiferum* (EEP). Besides, the mutagenic effect activity of the extract was assessed.

2. MATERIALS AND METHODS

Preparation of *Pseuderanthemum palatiferum* (Nees) Radlk leave extracts

Fresh leaves of Hoan-ngoc were blended in 95% ethanol and filtered through gauze. The extract was centrifuged and then the supernatant was filtered through a Whatman No.1 filter paper. After that, the ethanolic filtrate was concentrated using a vacuum rotary evaporator and lyophilized into powder of ethanol crude extract.

Cell culture

HepG2 human hepatocyte carcinoma cell line, MCF-7 human breast adenocarcinoma cell line, and PC-3 human prostate adenocarcinoma cell line were obtained from American Type Culture Collection (ATCC). Jurkat leukemic cell line was obtained from Cell Line Services (CLS), Germany. HepG2 and MCF-7 cells were cultured in DMEM with high glucose supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin. Jurkat cells and PC-3 cells were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37ºC in 5% CO_2 and 95% humidity.

Cytotoxic assay

The cytotoxic effect of EEP on cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [7].

DNA fragmentation

Jurkat cells were treated with 100, 300, and 600 µg/ml of EEP for 24 h and 300 µg/ml for 6, 12, and 24 h. After treatment, cells were collected, and then DNA was extracted using QIAamp® DNA Mini Kit (QIAGEN, Germany). Five micrograms of DNA sample were loaded on a 1.5% agarose gel. The gel was run at 70 volts for 1.50 h and then stained with 0.5 µg/ml ethidium bromide. The DNA fragment was visualized under ultraviolet light.

Hoechst 33258 staining

After Jurkat cells were treated with EEP, the cells were fixed with ρ-formaldehyde (4%, v/v) for 20 min and further stained with 10 µg/ml of Hoechst 33258 for 30 min at room temperature in the dark. The stained cells were washed with PBS and visualized under the inverted fluorescence microscope.

Annexin V-PI staining

After EEP treatment, the Jurkat cells were collected and stained for 15 min with Annexin V-FITC and PI using the Annexin V-FITC Apoptosis Detection Kit (EXBIO, Czech Republic). The stained cells were analyzed by flow cytometry.

Ames test

The mutagenicity of EEP was evaluated by the Ames test using *Salmonella typhimurium* strains TA98 and TA100. The assay was performed by pre-incubation method [8] and conducted under both absence and presence of S9 mix. The extract was considered as mutagenic if the number of revertant per plate was at least double over the spontaneous revertant frequency.

3. RESULTS

The *in vitro* cytotoxic effect of EEP against various human cancer cell lines, namely Jurkat, HepG2, MCF-7 and PC-3 after 24 h of exposure are shown in Figure 1A. The results showed that various types of cancer cells exhibited different susceptibilities to EEP in a dose dependent manner. The LC_{50} was calculated from a dose response curve using linear regression analysis. EEP exerted antiproliferation only in Jurkat and HepG2 cell lines with the LC_{50} values of 476.35±31.51 and 927.01 ± 90.84 µg/ml, respectively. EEP at the concentration up to 1,500 µg/ml had no cytotoxicity towards PC-3. The breast cancer MCF-7 cells showed less susceptible to EEP treatment (LC_{50} > 1,500 µg/ml). Being the most sensitive target of EEP, the Jurkat cells were selected for further investigation whether the cytotoxic effect of EEP was mediated through the apoptotic mechanism.

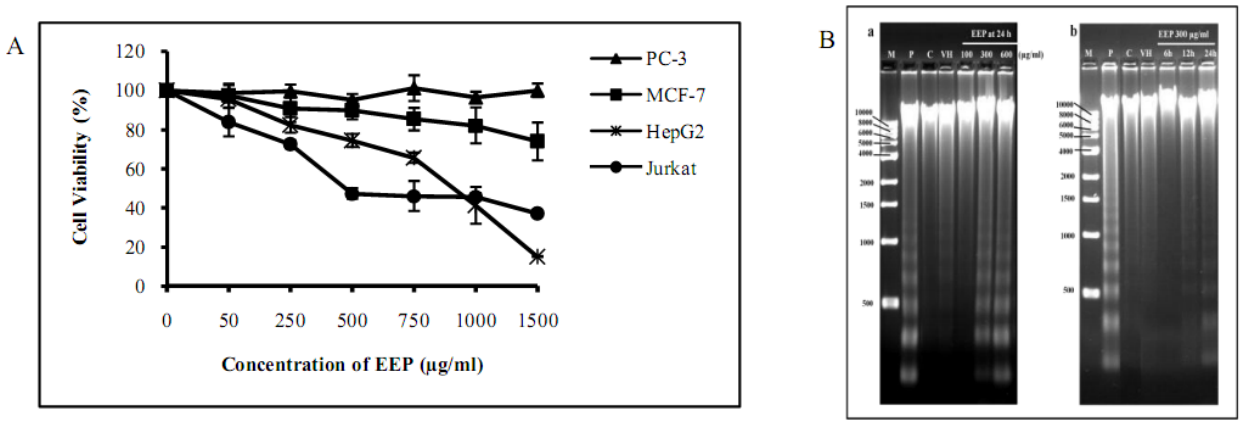

Figure 1. (A) Cytotoxic effect of EEP against different human cancer cell lines, PC-3, MCF-7, HepG2 and Jurkat cells. The cells were exposed to various concentrations of EEP for 24 h and were assessed for cell viability by MTT assay. Reported means ± SD values (n=4) are from a representative of three independent experiments. (B) DNA fragmentation in Jurkat cells exposed to EEP for 24h. Lane M, 1 kb DNA marker; lane P, 40 µg/ml of etoposide (positive control); lane C, media alone (negative control); lane VH, 0.1% DMSO (vehicle control).

The DNA fragmentation analysis (Figure 1B) showed that EEP induced the apoptotic cell death in both concentration- and time-dependent manners. The fragmented DNA was clearly observed in Jurkat cells after exposure to EEP at 300 and 600 µg/ml for 24 h and at 300 µg/ml for 12 and 24 h, respectively. No DNA fragmentation was observed in both negative and vehicle control cells, whereas the DNA ladder formation was clearly observed in the positive control group (40 µg/ml etoposide) at 24 h of exposure.

The nuclear morphological changes of Jurkat cells after EEP treatment were observed by Hoechst 33258 staining. The extent of apoptotic cell death induced by EEP was dose- and time- dependent (Figure 2A). At 300 and 600 µg/ml, EEP induced apoptotic cell death in Jurkat cells, but had no effect at 100 µg/ml. The time course study revealed that Jukat cells exposed to 300 µg/ml of EEP showed nuclear condensation and DNA fragmentation at 12 and 24 h post exposure, but no alteration of nuclear morphological changes was observed at the earlier time point (6 h). As expected, non-treated cells showed normal nuclear morphology.

The Annexin V-PI assay was further performed to confirm the apoptotic cell death induced by EEP. AnnexinV-PI staining was used to evaluate early and late apoptotic cell death. The percentage of EEP-induced apoptosis in Jurkat cells were increased in both dose- and time-dependent manners (Figure 2B). The percentages of early apoptotic in EEP-treated Jurkat cells were 5.21%, 7.93%, and 14.24% upon treatment with 100, 300, and 600 µg/ml of EEP for 24 h, respectively. Likewise, the percentages of early apoptotic in Jurkat cells after treatment with 300µg/ml of EEP for 6, 12, and 24h were 6.93%, 8.44%, and 11.42%, respectively.

The mutagenicity of EEP was evaluated by the Ames test using *S. typhimurium* strains TA98 and TA100. The assays were performed in both absence and presence of S9 mix. A compound tested with a mutagenic index of 2.0 or more is regarded as a potent mutagen. EEP ranging from 150 µg/plates up to 600 µg/plates had the mutagenic index of less than 2.0 on both tested strains, regardless the presence or absence of S9 mix (Table 1). Therefore, EEP in the range of 150 - 600 µg/plates had no mutagenic activity, whereas all positive controls always induced a clear mutagenic response with high values of mutagenic indexes (13.6-50.6).

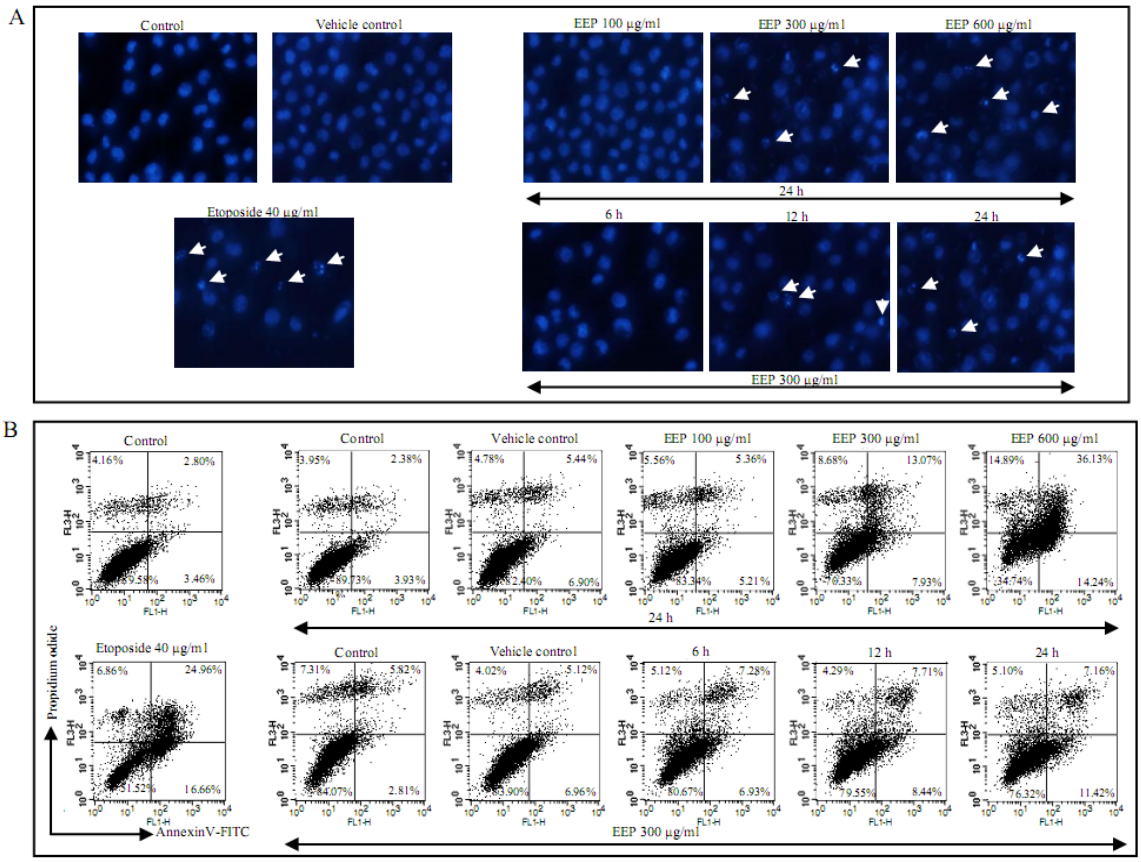

Figure 2. (A) Effect of EEP on nuclear morphological changes in Jurkat cells. The nuclear morphological changes were evaluated by staining with Hoechst 33258 and visualized under fluorescence microscopy at 400 ×magnification. The fragmented or condensed nuclei are indicated as white arrows. (B) Flow cytometric analysis of apoptosis in Jurkat cells exposed to various concentrations of EEP for 24 h and kinetics of apoptosis induction in Jurkat cells exposed to 300 µg/ml of EEP. The apoptosis of Jurkat cells was detected by flow cytometry using AnnexinV-PI staining method. Data are a representative of two independent experiments.

Table 1. Mutagenic effect of EEP on the *Salmonella typhimurium* strains TA98 and TA100 in the absence and presence of S9 mix

Sample	Number of revertants/plate (Mean±SD), (Mutagenic Index)			
	TA98		TA100	
	(-) S9 mix	(+) S9 mix	(-) S9 mix	(+) S9 mix
Control	22±2	24±5	117±6	111±5
Vehicle control(1.4%DMSO)	20±5	26±8	121±10	112±9
2-NF(10µg/plate)^{PC}	1,012±225,(50.6)	-	-	-
Sodium azide(10µg/plate)^{PC}	-	-	1,587±7,(13.56)	-
2-AA(2.5µg/plate)^{PC}	-	903±28,(34.74)	-	4,691±473,(41.88)
EEP (150µg/plate)	24±2, (1.20)	25±5, (0.96)	131±29, (1.08)	115±11, (1.03)
EEP (300µg/plate)	20±4, (1.00)	26±4, (1.00)	105±10, (0.87)	92±7, (0.82)
EEP (600µg/plate)	20±3, (1.00)	22±1, (0.85)	108±23, (0.89)	82±20, (0.73)

PC, positive control. Data were expressed as means ± SD of two independent experiments (n=3). Mutagenic Index (MI) = Number of revertant colonies of the extract/Number of revertant colonies of the vehicle control (spontaneous revertant). Values in brackets (MI) ≥2 indicate mutagenicity.

4. CONCLUSIONS

The current study demonstrated that the ethanolic extract of *Pseuderanthemum palatiferum* exerts the most potent antiproliferative effect on human T cell leukemia Jurkat cells, and its cytotoxicity is mediated through apoptosis pathway. The extract in the range of 150 to 600 µg/plate has no mutagenicity in the Ames assay.

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Evaluation of analgesic activity of the methanolic extract from the galls of *Quercus infectoria* (Olivier) in rats

Sook-Ha Fan*[a], Dayang Fredalina Basri[b], Noraisah Akbar Ali[b]
[a]Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman (UTAR), Perak, Malaysia
[b]School of Diagnostic and Applied Health Sciences, Faculty of Health Sciences, National University of Malaysia (UKM), Kuala Lumpur, Malaysia.

*fansh@utar.edu.my phone: +60-54688888 ext: 4454 fax: +60-54661676

ABSTRACT

The present study was to investigate the analgesic activity of the methanolic extract of the galls of *Quercus infectoria.* Analgesic effects were evaluated in rats using hot plate and tail-flick methods. The extract was administered intraperitoneally at a dose of 20 mg/kg while morphine sulphate and sodium salicylate (10 mg/kg each) served as standards. The methanolic extract exhibited significant analgesic activity in the tail-flick model ($p < 0.05$) by increasing the reaction time of the rats to 8.0 s at 30 min post-treatment in comparison to control (4.4 s). Morphine sulphate produced a reaction time of 11.9 s in the same test. At the peak of activity (30 min), the extract produced a maximum possible analgesia (MPA) of 34.2%, whilst morphine sulphate achieved a peak MPA of 70.9% at the same interval. No analgesic effects have been observed using sodium salicylate except for 30 min post-treatment in the tail-flick model. In the same model, the extract demonstrated stronger analgesic activity than sodium salicylate at all time intervals. Tail-flick is a better method to evaluate analgesic activity compared to hot plate as no significant results were observed for all treatments using hot plate with the exception of morphine sulphate, which showed significant results only at 45 and 60 min post-treatment. In conclusion, the methanolic extract of the galls of *Quercus infectoria* displayed analgesic activity and warrants further study to elucidate the mechanisms involved.

Keywords: *Quercus infectoria,* Analgesic activity, Tail-flick, Hot plate.

1. INTRODUCTION

According to International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage" [1]. Drugs used for pain relief such as aspirin and morphine have been widely used in the recent decades. In most instances, these analgesic drugs particularly opioids and non-steroidal anti-inflammatory drugs (NSAIDs) can only relieve 50% of the pain in about 30% of patients [2]. In addition, many of these drugs cause serious side effects. Studies have shown that opiates cause physical dependency, tolerance and addiction while NSAIDs usually cause gastrointestinal disorders [3].

As such, research to discover other alternatives to treat pain is crucial. Medicinal herbs have been used for centuries for therapeutic purposes. The galls of *Quercus infectoria* Olivier (Fagaceae) are locally known as "biji manjakani" in Malaysia. It has been used in traditional Malay medicine to restore postpartum uterine elasticity [4]. Pharmacologically, it has been documented to possess activities such as antioxidant, anti-inflammatory, antimicrobial and anti-diabetic [5]. The present study aimed to evaluate the analgesic activity of methanolic extract of galls of *Quercus infectoria* in animal models.

2. MATERIALS AND METHODS

Plant materials

The galls of *Quercus infectoria* used in this study were purchased from local market in Kuala Lumpur. The galls were identified and deposited at the Forest Research Institute Malaysia (FRIM) with the voucher number EZ186/93. The dried galls were crushed to small pieces using pestle and mortar and pulverised using an electric grinder.

Preparation of extract

The methanol extract was prepared by cold extraction technique [6]. One hundred g of the dried gall powder was immersed in 500 ml methanol for 24 h at room temperature. The mixture was then filtered and the process was repeated using the remaining residue with 300 ml methanol. Both filtrates were then combined and concentrated under reduced pressure using rotary evaporator. The resulting semi-solid residue was pounded to dryness under hot-air dryer to obtain a powdery crude methanol extract.

Animals

Mature albino Wistar rats (*Rattus norvegicus*) (150-200 g) of both sexes obtained from the laboratory animal units of the Faculty of Medicine, Universiti Kebangsaan Malaysia were used for the experiment. The animals were housed under standard laboratory conditions at room temperature with relative humidity of 70-80%. They were fed with standard commercial diet and water *ad libitum*. Prior to the experiment, the animals were fasted for 12 h with water given *ad libitum* and weighed. All procedures described were reviewed and approved by the Universiti Kebangsaan Malaysia Animal Ethics Commitee (UKMAEC) (UKM 1.5.24/111/61/2).

Study design

The rats were randomly assigned to four groups of six animals each for the two different experimental models. The first group served as negative control received normal saline (10 ml/kg). The second and third group served as positive control and were given standard drugs morphine sulphate and sodium salicylate, respectively (10 mg/kg each). The methanolic extract of the galls of *Quercus infectoria* was given at a dose of 20 mg/kg to the last group. All treatments were administered intraperitoneally.

Tail-flick test

Antinociceptive (analgesic) activity of the extract was evaluated by the hot tail-flick method described by Sewell and Spencer (1976) [7]. About 5 cm from the distal end of the tail of each rat was immersed in warm water maintained at 50°C. The reaction time (in second) was the time taken by the rat to flick its tail due to pain. The first reading was omitted and reaction time was taken as the average of the next two readings. The reaction time was recorded before (0 min) and at 15, 30, 45 and 60 min after the administration of the treatments. The maximum reaction time was fixed at 15 s to prevent any tail tissue injury. If the reading exceeds 15 s, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$\text{MPA} = \frac{\text{Reaction time for treatment - reaction time for saline}}{\text{15 - reaction time for saline}} \times 100$$

Hot plate test

Evaluation of analgesic activity of the extract was also carried out using hot plate method [8]. The rats were placed on a hot plate maintained at 55°C within the restrainer. The reaction time (in second) or latency period was

determined as the time taken for the rats to react to the thermal pain by licking its paws or jumping. The reaction time was recorded before (0 min) and at 15, 30, 45 and 60 min after the administration of the treatments. The maximum reaction time was fixed at 45 s to prevent any injury to the tissues of the paws. If the reading exceeds 45 s, it would be considered as maximum analgesia. The maximum possible analgesia (MPA) was calculated as follows:

$$MPA = \frac{\text{Reaction time for treatment - reaction time for saline}}{\text{45 - reaction time for saline}} \times 100$$

Statistical analysis

Data were presented as mean ± SEM. The results were analysed using Statistical Package for the Social Sciences (SPSS) version 16. Statistical significance was determined by student t-test and p value less than 0.05 was considered as significant.

3. RESULTS

Tail-flick test

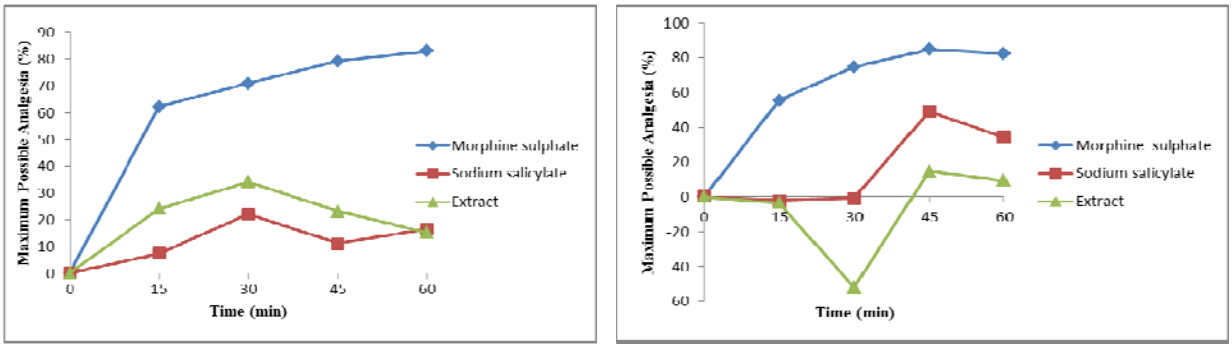
The results of the analgesic activity of the methanolic extract of the galls of *Quercus infectoria* are shown in Table 1. Rats treated with normal saline (negative control) did not show any significant difference in the reaction time on tail-flick throughout the 60 min observation. In comparison with the baseline values within the same treatment groups, the increase in reaction time at different time points significantly differed ($p < 0.05$) for morphine sulphate only. Duration of the reaction time in morphine sulphate and extract treated animals were significantly higher compared to saline treated animals, except for the extract group at 60 min. The highest reaction time for the extract treated group was 8.0 seconds at 30 min, while saline and morphine sulphate was 4.4 s and 11.9 s, respectively. At all time points, the tail-flick latency time differed significantly between the extract and morphine sulphate groups, being greater for the latter group. No significant difference in reaction time was observed between the extract and sodium salicylate. Observation in rats treated with sodium salicylate did not give any significant analgesic effect in comparison with baseline values, saline or extract (except for 30 min post-treatment).

Table 1: Analgesic effect of methanolic extract from the galls of *Quercus infectoria* by tail-flick method in rats

	Reaction time in s (mean ± SEM)				
Treatments	0 min	15 min	30 min	45 min	60 min
Control (normal saline)	4.25 ± 0.57	4.50 ± 0.34	4.42 ± 0.45	4.58 ± 0.44	5.17± 0.80
Morphine sulphate	6.50 ± 1.22	11.04 ± 0.73*[a b]	11.92 ± 0.84*[a b]	12.83± 0.35*[a b]	13.33 ± 0.83*[a b]
Sodium salicylate	4.13 ± 0.54	5.29 ± 0.57	6.75 ± 0.62*[a]	5.75 ± 0.56	6.79 ± 1.24
Quercus infectoria (galls) extract	6.67± 0.85[ac]	7.04± 0.67[a]	8.04 ±0.73[a]	7.00 ±0.92[a]	6.67 ± 0.86

All values by student t test, significant at p<0.05, SEM = Standard error mean.*$p < 0.05$ vs baseline of the respective treatment, [a]$p < 0.05$ treatment vs control, [b]$p < 0.05$ extract vs morphine sulphate, [c]$p < 0.05$ extract vs sodium salicylate.

The analgesic effects of morphine sulphate, sodium salicylate and extract could be seen from the maximum possible analgesia (MPA) graph (Figure 1a). The analgesic effect of morphine sulphate was evident within 15 min following intraperitoneal administration. The MPA remained elevated during the observation period, reaching its peak at 60 min (83.0%). Likewise, the extract also showed analgesic activity beginning at 15 min, with highest MPA at 30 min and gradually decreased towards 60 min (34.2%). For sodium salicylate, the MPA exhibited similar trend, producing a peak at the same time point (22.0%). With reference to MPA value, the extract demonstrated stronger analgesic activity than sodium salicylate at all time points.



(a) (b)

Figure 1: Maximum possible analgesia, MPA (%) representing the effect of the methanol extract of the galls of *Quercus infectoria* compared to morphine sulphate and sodium salicylate (positive control) administered into rats, evaluated by (a) tail-flick method and (b) hot plate method

Hot plate test

The results of the analgesic effect of the methanolic extract of the galls of *Quercus infectoria* using hot plate method are presented in Table 2. The results showed that there was no significant difference on the thermal stimulus in rats treated with normal saline (negative control) throughout the 60 min observation. There was no increase in reaction time at all time points compared to baseline values (0 min) within the same treatment groups. In comparison to the saline treated animals, the significant increase in the reaction time to thermal pain was not detectable in both sodium salicylate and extract with the exception of morphine sulphate. However, the observation in morphine sulphate treated animals is only noted at 45 and 60 min. The reaction time was significantly different between the extract and morphine sulphate, being greater for morphine sulphate at 30, 45 and 60 min post-treatment. No significant difference was observed between the extract and sodium salicylate.

Table 2: Analgesic effect of methanolic extract from the galls of *Quercus infectoria* by hot plate method in rats

	Reaction time in s (mean ± SEM)				
Treatments	0 min	15 min	30 min	45 min	60 min
Control (normal saline)	30.67± 5.15	28.25± 4.87	31.50± 4.57	24.08± 6.37	24.83±6.02
Morphine sulphate	30.75± 5.64	37.54± 5.55	41.58±3.22 [b]	41.79±2.87[a b]	41.46±2.55[a b]
Sodium salicylate	32.04± 4.59	27.92± 4.04	31.42± 2.61	34.38± 2.08	31.73± 1.46
Quercus infectoria (galls) extract	30.88±4.08	27.67± 2.17	24.50± 3.08	27.17± 3.92	26.71±4.06

All values by student t test, significant at p<0.05, SEM = Standard error mean. *$p < 0.05$ vs baseline of the respective treatment, [a]$p < 0.05$ treatment vs control, [b]$p < 0.05$ extract vs morphine sulphate, [c]$p < 0.05$ extract vs sodium salicylate.

Figure 1b illustrates the analgesic effect of morphine, sodium salicylate and extract using MPA. Morphine sulphate elicited significant analgesic activity within 15 min following administration as evidenced by the gradual increase throughout the observation period. At the peak of activity (45 min), morphine sulphate showed a MPA of 84.7%. Rats treated with sodium salicylate exhibited analgesic activity at a slower interval, which began at 45 min (49.2%) and then declined. The MPA value for the extract did not show any analgesic effect in the first 30 min post-treatment, but increased at 45 min (14.8%) and declined thereafter.

On the basis of these findings, tail-flick is a better method to evaluate analgesic activity compared to hot plate as no significant results were observed for all treatments using hot plate with the exception of morphine sulphate.

Tail-flick and hot plate are two of the several methods available for evaluating central analgesic activity [9]. Although both methods employed thermal stimuli, the tail-flick response indicates spinally mediated reflex while the paw- licking hot plate response is due to complex supraspinally intergrated behaviour [10]. Findings from this study demonstrated that the methanolic extract prolonged the reaction time in the tail-flick method but showed an apparent

lack of effect in the hot plate method. This might indicate higher sensitivity of the spinally mediated reflex response in the tail-flick method. However, intra-animal variation may also contribute to the lack of effect in hot plate method. Unlike the typical tail-withdrawal reflex in rats, problem arises in the hot plate method as the rats have to learn what nociceptive response they need to show in order to stop the thermal stimulus [11]. Taken together, the differences in sensitivity of the both methods as well as the mechanism involved may explain the analgesic effects observed in this study.

4. CONCLUSIONS

In conclusion, the methanolic extract of the galls of *Quercus infectoria* displayed analgesic activity and support the traditional use of this plant in pain relief. Further study is warranted to identify the active compounds present in this extract and to elucidate the mechanisms involved in its analgesic properties.

ACKNOWLEDGEMENTS

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Antibacterial activity of plasma fractions from Siamese crocodile (*Crocodylus siamensis*) on Ceftazidime-resistant *Enterobacter cloacae*

Nittaya Rojtinnakorn*[a], Sajeera Kupittayanant[b], Yosapong Temsiripong[c], Griangsak Eumkeb[a]

[a]School of Pharmacology, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang District Nakhon Ratchasima, Thailand 30000

[c]Sriracha Moda Co., Ltd, 383 Moo 4, Nongkham

[b]School of Physiology, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang District Nakhon Ratchasima, Thailand 30000

[c]Sriracha Moda Co., Ltd, 383 Moo 4, Nongkham, Sriracha, Chonburi, Thailand 20110

*erkorat03@gmail.com phone +66-44224273 Fax +6644224650

ABSTRACT

The resistance of bacteria, is a major problem in the world including Thailand, lead to increasing the mortality and cost of medical care. Thus, the objective of this study was to investigate the activity of plasma fractions from Siamese crocodile *(Crocodylus siamensis)* against drug resistant bacteria, when use alone and in combination with *β*-lactams. The crocodile plasma was purified to give five fractions (P1, P2, P3, P4 and P5) using column chromatography. The MICs of P1, P2, P3, P4 and P5 against ceftazidime-resistant *Enterobacter cloacae* DMST 21394 (CREnC) revealed 1024, >1024, >1024, >1024 and 1024 mg/ml, respectively, while MICs for methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA) displayed 1024 mg/ml for all fractions. The FICs index of either P1 or P5 plus ceftazidime against CREnC revealed both equal at 0.062, also these fractions plus cloxacillin demonstrated both equal 0.375 against MRSA. The killing curves confirmed that both P1 and P5 in combination with either ceftazidime or cloxacillin caused markedly decrease of CREnC or MRSA cells from 6 throughout 24 h. CREnC treated with the ceftazidime plus either P1 or P5 combination revealed a dramatically significant smaller cell size than the control cells ($p < 0.01$), cell distortion, cell envelope damage, increased the OM and CM permeability, β-lactamase inhibition ($p < 0.01$) and OMPG associated protein synthesis disruption. In conclusion, the combination of either P1 or P 5 and ceftazidime showed strong synergistic activity against CREnC strain. These findings provide evidence that these combinations can reverse resistance strain to be susceptible to its primary antibiotic.

Keywords: *Enterobacter cloacae, Staphylococcus aureus,* Ceftazidime, Cloxacillin, *Crocodylus siamensis,* Synergistic activity

1. INTRODUCTION

The resistance of bacteria is a major problem in the world lead to increasing the use of newer, higher generation and expensive antibacterial agents. Thus, the main purpose of current research is emphasized on naturally-derived substances, which have antibacterial activity against drug resistant bacteria or enhance the effectiveness of existing antibiotics. The crocodiles encounter high rates of injury, however, these animals rarely exhibit outward bacterial infection wound. The bacterial activities of Siamese crocodile (*Crocodylus siamensis*) blood were reported in many recent studies [1,2]. However, no work has been investigated on the effect of antibacterial activity of plasma from the Siamese crocodile on *β*-lactam resistant bacteria. Thus, the objective of this study was to investigate the activity of separated fractions from Siamese crocodile plasma against drug resistant bacteria, when use alone and in combination with *β*-lactams antibiotic.

2. MATERIALS AND METHODS

Separated fractions from Crocodile Plasma

Crocodile blood was collected by drawing blood from supravertebral vein from anterior dorsal sinus (40 mL) and was transferred to EDTA tube, kept at 4°C overnight, then centrifuged at 4000 rpm for 10 minutes to obtain the plasma and kept at -70°C until tested. Separation of plasma was performed by ion exchange chromatography over Q Sepharose fast flow column. The protein fractions from ion exchange chromatography were further separated to select molecular weight 0-250 kDa by gel filtration chromatography with Sephadex G-50 and 60% acetronitile in 0.1% TFA [2].

Bacterial suspension standard curve

To select bacterial suspensions with a known viable count, the method of Eumkeb [3] and Liu et al. [4] was followed.

Minimum inhibitory concentration (MIC) and checkerboard determinations

MIC and checkerboard determinations of selected β-lactam drugs against CREnC DMST 21394 were performed following Liu et al. [4] and CLSI [5] methods. MICs were determined using broth microdilution method. The MICs of separated fractions were recorded as the lowest concentration where no visible growth was observed in the 96-microwell plates after incubation for 18-24 h [5]. Checkerboard determinations in antimicrobial combinations were performed [6] with slight modification [3].

Killing curve determinations (viable counts)

Viable counts for the determination of killing-curves were performed as previously described [7] with slight modification [3]. Positive controls with free plasma fractions or drugs were used.

Transmission electronmicroscopy (TEM)

Ceftazidime plus P1 either P5 that dramatically decreased the MICs against CREnC DMST 21394 were chosen for electronmicroscopy study when used singly and in combination. Subculture of this strain was prepared to examine by TEM [8].

Outer and cytoplasmic membrane permeability

The separated fractions of P1, P5 either alone or in combination with antibacterials induced permeabilization of the OM of CREnC 21394 was carried out. NCF was used a substrate of *β*-lactamase. The concentration of half-maximal membrane permeabilization (EC50) was determined from dose–response curves of peptide-exposed cells and peptide-free wells at 500 nm (OM permeabilization) [9,10].

Cytoplasmic membrane permeability was determined by the ability of the peptides to unmask cytoplasmic *β*-galactosidase activity in bacteria by using ortho-nitrophenylgalactoside (ONPG) as the substrate. The method of sample preparation was prepared the same as for the OM permeability determinations. To assay CM permeabilization, the wells contained 50 μL selected separated fraction P1, P5 either alone or in combination with antibiotic drugs and 50 μL ONPG solution. Complete permeabilization was induced in the presence of 5 μM. PMX was used as a positive control and wells lacking separated fraction and drugs served as negative control. The concentration of half-maximal membrane permeabilization (EC50) was determined from dose–response curves of peptide-exposed cells and peptide-free wells at 420 nm after 40 mins.

Electrophoresis

To examine the effect of antibacterial characteristic from separated fractions of P1, P5 either alone or in combination with antibiotics drugs on the outer membrane and peptidoglycan associated protein (OMPG) were performed [3]. Bovine serum albumin (Sigma) was used as the protein standard. The extract was stored at -70°C and

re-diluted in sample buffer before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation. The extract was shown to be stable for over 2 months under these conditions [3].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used a gel system having a 4% stacking gel and a 15% separating gel. A volume of the OMPG extract was mixed with a volume of sample buffer containing 0.125 M Tris-HCl buffer, pH 6.8, and boiled for 5 min. The electrophoresis was performed at 8 mA per gel for stacking gel and 15 mA per gel for separating gel to maximise the resolution at the important subtyping areas of the gel. After electrophoresis the separating gel was stained with a Coomassie Brilliant Blue stain for 2 h at room temperature with gentle mixing. The gel was then destained. The following standard proteins (BDH) was used as molecular mass markers: myoglobin (17,200), carbonic anhydrase (30,000), ovalbumin (42,700), albumin (66,250) and ovotransferrin (76,000-78,000) [3].

Enzyme assays

The β-lactamase type IV of *E. cloacae* was obtained from Sigma (Poole, England). Enzymes activities of Richards et al. [8] method were followed.

Statistic analysis

OM and CM permeability and enzyme assay were carried out in triplicate. At least ten cells in each treated cells from TEM study were measured. The data were expressed as mean ± SEM. Significant differences between these groups were examined using one-way ANOVA. $p < 0.05$ and $p < 0.01$ of Tukey's HSD post hoc test were considered as a statistically significant difference.

3. RESULTS

The crocodile plasma was separated to give five fractions (P1, P2, P3, P4 and P5) using column chromatography. The MICs of P1, P2, P3, P4 and P5 against clinical isolates of Ceftazidime-resistant *Enterobacter cloacae* DMST 21394 (CREnC 21394) revealed 1024, >1024, >1024, >1024 and 1024 mg/mL, respectively, while MICs for methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA 20651) displayed 1024 mg/mL for all fractions. These MICs show high resistant of both strains to these fractions. Furthermore, both CREnC 21394 and MRSA 20651 were also high resistant to ceftazidime and cloxacillin (both MICs >1024 µg/mL). The checkerboard results displayed that the FICs index of either P1 or P5 plus ceftazidime against CREnC 21394 revealed synergistic effects both equal value at 0.062, besides either P1 or P5 plus cloxacillin demonstrated synergistic effects both equal value at 0.375 against MRSA strain. The killing curves confirmed that both P1 and P5 in combination with either ceftazidime or cloxacillin caused markedly decrease of CREnC or MRSA cells, respectively, within 6 h and throughout 24 h period (Figure 1-2).

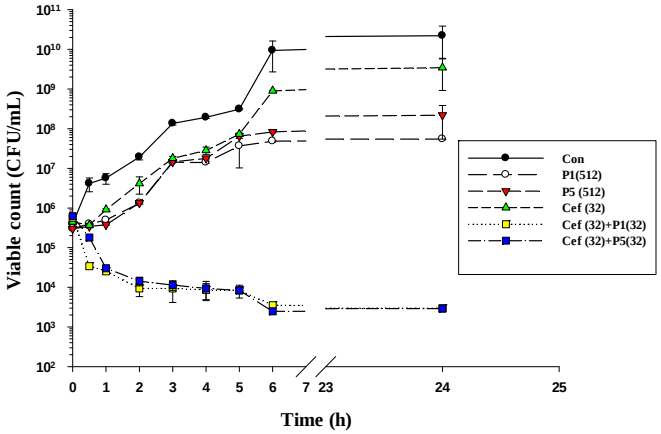

Figure 1. The effect of separated fraction either P1, P5 and ceftazidime alone or in combination on the clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394. The values plotted are the means of three observations, and the vertical bars indicate the standard errors of the means. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(32) = ceftazidime at 32 µg/mL, P1(32)+Cef(32) = P1 at 32 mg/mL + ceftazidime at 32 µg/mL, P5(32)+Cef(32) = P5 at 32 mg/mL + ceftazidime at 32 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

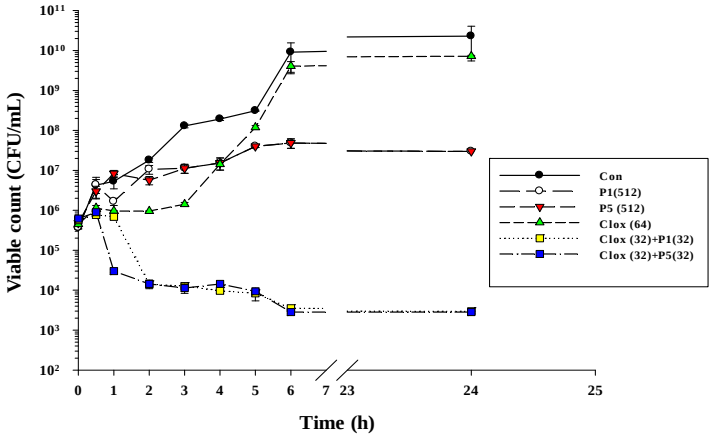

Figure 2. The effect of cloxacillin combined with separated fraction either P1, P5 and cloxacillin alone or in combination on the clinical isolates of methicillin- resistant *S. aureus* DMST 20651. The values plotted are the means of three observations, and the vertical bars indicate the standard errors of the means. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Clox(64) = cloxacillin at 64 µg/mL, P1(32)+Clox(32) = P1 at 32 mg/mL + cloxacillin at 32 µg/mL, P5(32)+Clox(32) = P5 at 32 mg/mL + cloxacillin at 32 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

The TEM study exhibited that the effect of the combination of ceftazidime plus either P1 or P5 on CREnC revealed a dramatically significant smaller cell size than the control cells ($p < 0.01$) (Figure 4), cell shape distortion and cell envelope damage in most of these cells (Figure 3). In addition, the OM and CM permeabilization results demonstratedthat P1 and P5 either alone or in combination with ceftazidime steadily increased the OM and CM permeability of this strain ($p < 0.01$). The SDS-PAGE results revealed that the outer membrane and peptidoglycan (OMPG) associated protein band at MW 25 kDa of either ceftazidime alone or in combination with P1 appeared slightly paler than others. As well as, the 35 and 45 kDa OMPG associated protein bands of ceftazidime plus either P1 or P5 were slightly paler than control. The results of enzyme assay indicated that the combination of ceftazidime plus either P1 or P5 exhibited β-lactamase type IV inhibition activity compared to others ($p < 0.01$).

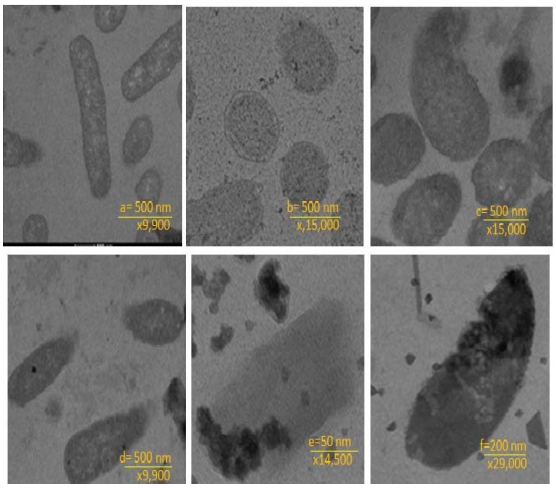

Figure 3. Ultrathin sections of log phase ceftazidime resistant *E. cloacae* DMST 21394 (CREnC) grown in cation-adjusted Mueller–Hinton (MH) broth containing: (a) drug-free (control); (b) 512 mg/mL P1; (c) 512 mg/mL P5; (d) 16 µg/mL ceftazidime; (e) 16µg/mL ceftazidime plus 32 mg/mL P1; (f) 16µg/mL ceftazidime plus 32 mg/mL P5 (a, b, c, d, e, f original magnification, ×9,900, ×15,000, ×15,000, ×9,900, ×14,500, ×29,000; bar, 500, 500, 500, 500, 50, 200 µm, respectively).

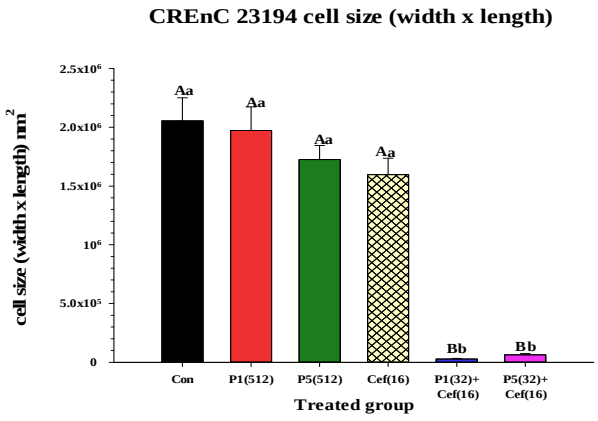

Figure 4. The comparison of cell size for CREnC 23194 cells grown in the presence of separated fraction P1, P5, ceftazidime either alone or in combination. The mean ± SEM for ten treated cells in each group are presented. The graph shows area of cell determined by cell width x cell length (nm^2). The different superscript alphabet are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.05$ (small alphabet) and $p<0.01$ (capital alphabet) are presented. Con=control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(16) = ceftazidime at 16 µg/mL, P1(32)+Cef(16) = P1 at 32 mg/mL + ceftazidime at 16 µg/mL, P5(32)+Cef(16) = P5 at 32 mg/mL + ceftazidime at 16 µg/mL.

4. CONCLUSIONS

These results can be concluded that the combination of either P1 or P5 plus ceftazidime showed strong synergistic activity against CREnC strain. These findings provide evidence that these combinations can reverse resistance strain to be susceptible to its primary antibiotic. In conclusion, antibacterial and synergistic activities of the fractions when used alone and in combination with ceftazidime may involve three mechanism of actions. Firstly, these plasma fractions show synergistic effect with ceftazidime and may inhibit peptidoglycan synthesis leads to cell shape distortion and cell envelope damage. Secondly, increase in OM and CM permeability in this strain. Thirdly, *β*-lactamase inhibition. In addition, the OMPG associated protein synthesis may be interfered and leads to slightly paler bands at 35 and 45 kDa of this strain. So, the fractions from Siamese crocodile serum would be offered as a good candidate for the development of a novel valuable adjunct to ceftazidime against CREnC, which currently almost resistant to practically antibiotics. However, toxicity test in animal and human are required. If possible, blood and tissue levels would be achievable to work synergistically.

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Pharmacognostic standardization and wound healing perspectives of *Aegle marmelos* (Linn.) *and Mucuna pruriens* (Linn.)

Fedelic Ashish Toppo*[a], Rajesh Singh Pawar[a]
[a]Pharmacognosy and Phytochemistry Division, Faculty of Pharmacy, VNS Group of Institutions, Vidya Vihar, Neelbud, Bhopal-462044, Madhya Pradesh, India
*ashish_two@yahoo.co.in phone +91-9907027908 fax +91-7552696748

ABSTRACT

Plants are a great source of medicines, especially in traditional medicine, which are useful in the treatment of various diseases. *Aegle marmelos* Linn. (AM) and *Mucuna pruriens* Linn. (MP) have been used traditionally for cuts and wounds. In order to ensure the use of only genuine and uniform material for isolation and characterization to produce more effective novel phytoconstituents for wound healing potential, work on standardization was carried out. The shape of leaves of AM is lanceolate which are green in colour and the shape of seeds of MP is reniform with black and grayish colour. The loss on drying of the plant material after drying, the moisture content was found to be 1.36% w/w and 12.10% w/w and total ash was found to be 5.38% w/w and 4.18% w/w and light green and dark violet colour was observed when powder was subjected to fluorescence analysis for AM and MP respectively. Pharmacognostic parameters such as organoleptic, physio-chemical and fluorescence analysis etc. are useful for detecting low grade products. The result of the pharmacognostical standardization of the candidate plant will serve as a reference and will help in future for identification and authentification of this plant specimen.

Keywords: Wound healing, *Aegle marmelos*, *Mucuna pruriens,* Pharmacognostical standardization

1. INTRODUCTION

The prevalence of chronic wounds in the community was reported as 4.5 per 1000 population, whereas an acute wound was about 10.5 per 1000 populations [1]. It is estimated that about 6 million people are suffering from chronic wounds worldwide [2]. It is estimated that 1 - 1.5% of the population is affected by a wound at any point time [3].

World Health Organization (WHO) encourages, recommends and promotes traditional/herbal remedies in natural health care programmes because these drugs are easily available at low cost, safe and people have faith in them. The WHO assembly in number of resolutions has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and applying suitable standards [4].

The assessment of the plants for their therapeutic activity is done on the basis of either their chemotaxonomic examination or ethnobotanical information for a particular disease [5]. Plants are utilized extensively as raw drugs for many formulations in traditional systems of medicine. To check the genuineness of the raw drugs and to detect adulteration of these materials, an authentic pharmacognostic study for standardization is needed for each raw drug. Pharmacognosy is a simple and reliable tool, by which complete information of the crude drug can be obtained [6-9]. The process of standardization can be achieved by stepwise pharmacognostic studies.

Herbs have affinities to certain phases of wound healing and different types of wounds. Therefore, they can accelerate the process of healing. A large number of plant extracts and their pastes are equally used by tribes and folklore traditions in India for treatment of cuts, wounds, and burns. Many herbal plants have a very important role in wound healing process because they promote their repair mechanism in the natural way. Agents that accelerate the process of wound healing are active terpenes [10] flavonol glycosides [11,12] and β- sitosterol. Tannins [13], flavonoids [14] triterpenoids [15] and sesquiterpenes [16] are known to promote wound healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelisation.

Aegle marmelos (Linn) correa, commonly known as bael (or bel), belonging to the family Rutaceae, is a moderate-sized, slender and aromatic tree. It is indigenous to India and is abundantly found in the Himalayan tract, Bengal, Central and South India [17]. Leaves are also use in Abscess, backache, abdominal disorders, vomiting, cut and wounds [18,19]. The leaves are having astringent, laxative, and expectorant [20]. Antimicrobial activity [21] and antioxidant activity [22] have been reported. *Mucuna pruriens* Linn. belongs to the family fabaceae, commonly known as cowhage plant or kapikacho or kevach in Hindi, is the most popular drug in Ayurvedic system of medicine [23]. Leaves paste is also applied on cuts and wounds [24]. Antioxidant activity [25] and antimicrobial activity [26] have been reported. These plants have been used in the Indian traditional medicines from time immemorial. They form the easily available source purposes in rural and tribal areas. It is associated with various important medicinal properties. Therefore pharmacognostic standardization was carried out to explore the physiochemical and phytochemical profile of *Aegle marmelos* Linn. (AM) and *Mucuna pruriens* Linn. (MP).

2. MATERIALS AND METHODS

Identification and collection of plant material

The seeds of *Mucana pruriens* were collected / purchased in the month of November from Bhopal local market and leaves of *Aegle marmelos* were collected in the month of September from the medicinal garden of VNS group of Institutions, Bhopal (M.P.). These were identified and authenticated by Dr. S. N. Dwivedi (HOD) and voucher specimens were deposited in the herbarium of the Department of Botany, Janata PG College, A.P.S. University, Rewa (M.P.). The seeds and leaves were shade dried and powdered moderately and stored in well closed container.

Extraction of crude drugs

The powdered seeds (200 g) of *Mucana pruriens* and leaves of *Aegle marmelos* (400 g) were extracted in soxhlet assembly for 36 hours with petroleum ether for defatting. The defatted plant materials were dried and then exhaustively extracted with ethyl acetate, methanol and water in soxhlet apparatus, separately. The completion of extraction was confirmed by evaporating a few drops of the extract on the watch glass and ensuring that no residue remained after evaporating the solvent. The extracts were concentrated under reduced pressure at a bath temperature below 50°C to yield semisolid mass and stored in well-closed container for further studies.

Pharmacognostic study

The seeds of *Mucana pruriens* (MP) and leaves of *Aegle marmelos* (AM) were subjected to macroscopic studies, fluorescence analysis and physio-chemical analysis and extract were subjected to phytochemical analysis to detect the presence of plant constituents [27-30].

3. RESULTS AND DISCUSSION

Macroscopic characteristics

The shape of leaves of AM is lanceolate which are green in colour and the shape of seeds of MP is reniform with black and grayish colour. Leaves of AM have aromatic odour, taste is peculiar astringent and are 7 - 13 cm in length, 3-6cm in width. Seeds of MP are odourless, taste is slightly bitter and are 15 – 20 mm in length, 7 – 12 mm in width. (Figure 1 and Table 1)

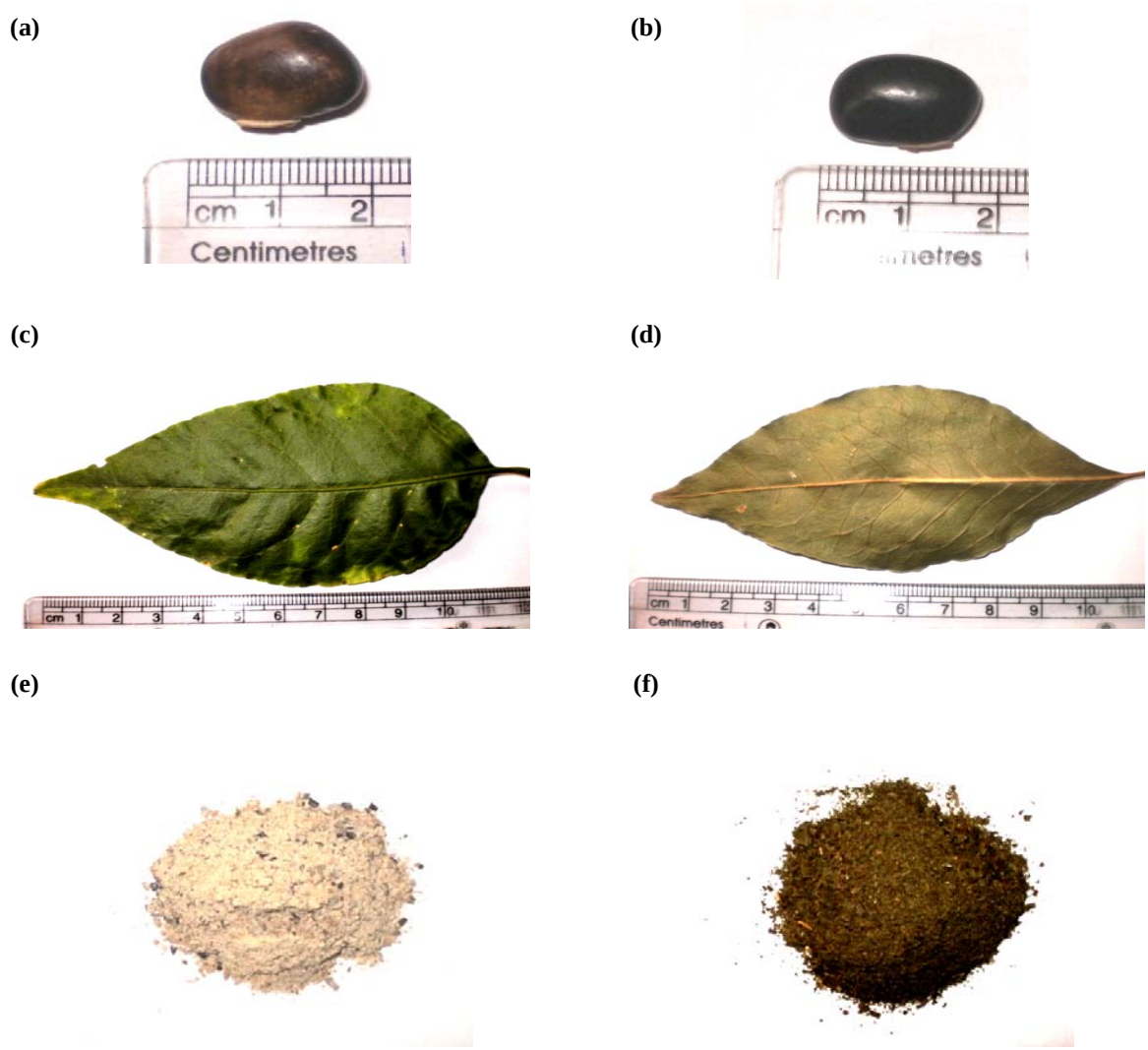

Figure 1. Macroscopic observations of *Mucana pruriens* and *Aegle marmelos*. (a) Fresh seed of *Mucana pruriens*, (b) Dried seed of *Mucana pruriens*, (c) Fresh leaf of *Aegle marmelos*, (d) Dried leaf of *Aegle marmelos*, (e) *Mucana pruriens* seeds powder, (f) *Aegle marmelos* leaves powder

Table 1. Macroscopic characterization of *Mucana pruriens* and *Aegle marmelos*

Parameters	Observations	
	Mucana pruriens	*Aegle marmelos*
Shape	Reniform	Lanceolate
Colour	Black and grayish	Green
Odour	Odourless	Aromatic
Taste	Slightly bitter	Peculiar astringent
Length	15-20 mm	7-13cm
Width	7-12 mm	3-6 cm

Fluorescence analysis

Fluorescence characteristic of powdered seeds of MP and leaves of AM were observed for resolution of doubtful specimen. Powder seeds of MP were observed under visible light and ultra violet light and they were found to be in light violet and dark violet in colour. Powdered leaves were observed dark green and light green under visible light and ultra violet light. (Table 2)

Table 2. Fluorescence analysis of *Mucana pruriens* and *Aegle marmelos*

Mucana pruriens	*Aegle marmelos*
Visible light	Ultra violet light
Wheat colour	Green
Dark violet fluorescence	Dark green

Physicochemical parameters

Physicochemical parameters of any drug give an idea of the earthy matter and/or inorganic composition and/or other impurities present along with a drug. The moisture content of the powdered seeds of MP was evaluated using loss on drying method and valve observed was 12.10% w/w. The total ash value, acid insoluble ash value and water soluble ash value of MP were found to be 4.18% w/w, 0.44% w/w and 0.61% w/w respectively. The moisture content of the powdered leaves of AM, total ash value, acid insoluble ash value and water soluble ash was found to be 1.36%w/w, 5.38%w/w, 1.02%w/w and 0.84%w/w. (Table 3).

Table 3. Physiocochemical observations of *Mucana pruriens* and *Aegle marmelos*

Parameters	Observations	
	Mucana pruriens	*Aegle marmelos*
Loss on drying	12.10% w/w	1.36% w/w
Total Ash	4.18% w/w	5.38% w/w
Acid insoluble ash	0.44% w/w	1.02% w/w
Water soluble ash	0.61%w/w	0.84%w/w

The percentage yield of powdered seeds of MP extracts viz. petroleum ether, ethyl acetate, methanol and aqueous extract were found to be 7.62%w/w, 2.80%w/w, 4.90%w/w and 23.08% w/w. The percentage yield of powdered leaves of AM extracts viz. petroleum ether, ethyl acetate, methanol and aqueous extract were found to be 8.92%w/w, 3.54%w/w, 12.44%w/w and 6.10% w/w. (Table 4)

Table 4. Percentage yield of *Mucana pruriens* and *Aegle marmelos*

Extracts	Yield (%)	
	Mucana pruriens	*Aegle marmelos*
Petroleum ether	7.62% w/w	8.92% w/w
Ethyl acetate	2.80% w/w	3.54% w/w
Methanol	4.90% w/w	12.44% w/w
Water	23.08% w/w	6.10% w/w

Phytochemical analysis

The dried and coarse powdered seeds of MP extracts and powdered leaves of AM were tested for the presence of phytoconstituents using reported methods mentioned in the standards and results are given in Table 5 & 6. The preliminary phytochemical screening of various extracts of AM was carried out and it was found that petroleum ether extract showed the presence of steroids, fats and oil, flavonoids and saponin glycosides, ethyl acetate extract showed the presence of carbohydrates, alkaloids, flavonoids, tannin and phenolic compounds and anthraquinone glycosides, methanol extract showed the presence of steroids, carbohydrates, alkaloids, flavonoids, tannin and phenolic compounds, protein and amino acids and saponin glycosides, aqueous extract showed the presence of carbohydrates, alkaloids, flavonoids, tannin and phenolic compounds, protein and amino acids and coumarin glycosides.

The preliminary phytochemical screening of various extracts of MP was carried out and it was found that petroleum ether extract showed the presence of steroids, fats and oil, flavonoids, saponin glycosides and terpenoids, ethyl acetate extract showed the presence of steroids, fats and oils, alkaloids, flavonoids, tannin and phenolic compounds, protein and amino acids and saponin glycosides, methanolic extract showed the presence of steroids, fats and oils, carbohydrates, alkaloids, flavonoids, tannin and phenolic compounds, protein and amino acids, cardiac glycosides, anthraquinone glycosides, saponin glycosides and terpenoids, aqueous extract showed the presence of alkaloids, flavonoids, tannin and phenolic compounds and protein and amino acids.

It has been reported that only 1–3% of drugs listed in Western pharmacopoeia for the use of skin and wounds; and at least one third of herbal remedies are for such use [31]. The herbal medicines for wound healing are very cheap and affordable and are safe as hypersensitive reactions [32]. It has been known that the process of wound healing can be promoted by several plant extracts rich in active compounds, such as flavonoids, triterpenes, alkaloids, tannins and other biomolecules [33]. Plants exhibit strong antioxidant activities, which might be helpful in preventing the oxidative stress by scavenging the highly reactive free radicals, involved in delaying the wound healing process. Polyphenolic compounds play an important role in wound healing due to their antimicrobial and antioxidant activity, metal chelating ability and enzyme inhibition properties [34]. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. However, there is a need for scientific validation and safety evaluation of plants before these could be recommended for healing of the wounds.

Table 5. Phytochemical analysis of aqueous, methanol, ethyl acetate and petroleum ether extracts of *Aegle marmelos*

S. No.	Tests	Petroleum ether	Ethyl acetate	Methanol	Aqueous
1.	**Test for steroids**				
	Salkowaski test	+	-	+	-
	Liebermann-Burchard test	+	-	+	-
	Liebermann's reaction	+	-	+	-
2.	**Tests for fats & oils**				
	Solubility test	-	-	-	-
	Filter paper stain test	-	-	-	-
3.	**Test for carbohydrates**				
	Molisch's test	-	+	+	+
	Fehling test	-	+	+	+
	Tollen's test	-	+	+	+
4.	**Test for alkaloids**				
	Mayer's test	-	+	+	+
	Wagner's test	-	+	+	+

S. No.	Tests	Petroleum ether	Ethyl acetate	Methanol	Aqueous
	Dragondroff's test	-	+	+	+
	Hagger's test	-	+	+	+
5.	**Test for flavonoid's**				
	Alkaline reagent test	+	+	+	+
	Shinoda test	+	+	+	+
6.	**Test for tannins and phenolic compounds**				
	Ferric chloride test	-	+	+	+
	Lead acetate test	-	+	+	+
7.	**Test for protein and amino acids**				
	Millon's test	-	-	+	+
	Xanthoproteic test	-	-	+	+
	Ninhydrin test	-	-	+	+
8.	**Test for glycosides**				
	A. Cardiac glycosides				
	Keller-killani test	-	-	-	-
	Legal's test	-	-	-	-
	Liebermann's test	-	-	-	-
	B. Anthraquinone glycosides				
	Borntrager's test	-	+	-	-
	Modified Borntrager's test	-	+	-	-
	C. Saponin glycosides				
	Foam test	+	-	+	-
	D. Coumarin glycosides	-	-	-	+
9.	**Test for Terpenoids**	-	-	-	-

+, - indicates presence and absence of chemical constituents, respectively.

4. CONCLUSION

The purpose of pharmacognostic standardization of crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material. Morphological studies and fluorescence analysis of the plant material will enable to identify the crude drug. Ash values, extractive values can be used as reliable aid for detecting adulteration. Phytochemical screening gives a quick answer to the various types of phytochemicals and is a important tool in bioactive compound analyses which may give a significant effect against various diseases and disorders. The information obtained from pharmacognostic standardization will be useful in finding out the genuinely of the drug.

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Table 6. Phytochemical analysis of aqueous, methanol, ethyl acetate and petroleum ether extracts of *Mucana pruriens*

S. No.	Tests	Petroleum ether	Ethyl acetate	Methanol	Aqueous
1.	**Test for steroids**				
	Salkowaski test	+	+	+	-
	Liebermann-Burchard test	+	+	+	-
	Liebermann's reaction	+	+	+	-
2.	**Tests for fats & oils**				
	Solubility test	+	+	+	_
	Filter paper stain test	+	+	+	_
3.	**Test for carbohydrates**				
	Molisch's test	-	-	+	-
	Fehling test	-	-	+	-
	Tollen's test	-	-	+	-
4.	**Test for alkaloids**				
	Mayer's test	-	+	+	+
	Wagner's test	-	+	+	+
	Dragondroff's test	-	+	+	+
	Hagger's test	-	+	+	+
5.	**Test for flavonoid's**				
	Alkaline reagent test	+	+	+	+
	Shinoda test	+	+	+	+
6.	**Test for tannins and phenolic compounds**				
	Ferric chloride test	-	+	+	+
	Lead acetate test	+	+	+	+
7.	**Test for protein and amino acids**				
	Millon's test	-	+	+	+
	Xanthoproteic test	-	+	+	+
	Ninhydrin test	-	+	+	-
8.	**Test for glycosides**				
A. Cardiac glycosides					
	Keller-killani test	-	-	-	-
	Legal's test	-	-	-	-
	Liebermann's test	-	-	+	-
B. Anthraquinone glycosides					
	Borntrager's test	-	-	+	-
	Modified Borntrager's test	-	-	+	-

C. Saponin glycosides					
	Foam test	+	+	+	-
D. Coumarin glycosides		-	-	_	-
9.	**Test for Terpenoids**	+	-	+	-

+, - indicates presence and absence of chemical constituents, respectively.

Feasibility of Hemp (*Cannabis sativa* L.) utilization for pharmaceutical purposes in Thailand

Prapatsorn Tipparat[*a], Natthachai Duangnil[a,b], Jeerawan Klangjorhor[b], Sarita Pinmanee[c], Peeraphan Potachareon[b], Prachya Kongtaweelert[b]
[a]Regional Medical Sciences Center 1 Chiang Mai, Chiang Mai, Thailand 50180
[b]Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand 50200
[c]Highland Research and Development Institute (Public Organization), Chiang Mai, Thailand 50200

[*]ptipparat@yahoo.com phone +66-53112188 fax +66-53112191

ABSTRACT

Presently, more than 30 countries in the world grow industrial hemp (*Cannabis sativa* L.) as potential economic crop for fiber and seed usages. In Thailand, hemp is still enforced as schedule V controlled substance. However, Thai government recognizes that hemp may make useful contribution to the economy as an alternative crop. This work was hence conducted to give information about the feasibility of hemp utilizing for pharmaceutical purposes in Thailand. Four varieties of hemp grown at Pang Da Royal Agricultural Station, Chiang Mai including HH, V50, MSM and PU were examined. The results revealed that the proportions of CBD to Δ^9THC, the major cannabinoids, were 7.61, 8.38, 5.51 and 1.79, respectively. The anti-oxidant abilities among four varieties of hemp examined by ABTS, DPPH and FRAP assay were explicit in the same manner and the relevant results given between them were positively correlation ($0.88 \leq r \leq 0.97$, $p<0.05$). PU possessed the highest anti-oxidant activities following by MSM, HH and V50, respectively. The cytotoxicity studies by MTT assay showed that the hemp extracts may be toxic to HAC cells at concentrations greater than 10 µg/ml. Thus, the concentrations of the extracts at 0.1, 1, 5, and 10 µg/ml were then studied for their anti-inflammation activity. The results indicated that all kind of hemp extracts could significantly reduce the pro-inflammatory genes, including IL-1β, COX-1 and iNOS after inducing the HAC cells by lipopolysaccharide in likely dose dependent manner. PU was the one having the most effectiveness. Suggesting that anti-oxidant and anti-inflammation activity existing in hemp extracts could be a useful target in the search for possible treatments for arthritis.

Keyword: Hemp, *Cannabis sativa* L., Anti-oxidant activities**,** Anti-inflammation activity**,** Arthritis

1. INTRODUCTION

Cannabis sativa L. has evolved into two races; hemp or industrial hemp for fiber and seed usage and marijuana for using as intoxicant. Presently, more than 30 countries in the world grow industrial hemp as potential economic crop. Hemp fiber has been used in textiles, plastic composites for automobile and building construction products. Hemp seed and hempseed oil are currently used for health foods, dietary supplements, cosmetics and for medicinal purposes [1-3]. Cannabis contains unique class of chemicals called cannabinoids as well as terpenoids and flavonoids secreted as resin component by glandular trichomes found mostly in flowering tops and bracts [4]. The major psychoactive constituent, THC has been used for reducing the effects on nausea/vomiting due to cancer chemotherapy, as appetite stimulant, on some painful conditions and on symptoms of multiple sclerosis [5]. CBD has been reported to provide neuroprotective action, possibly based on its anti-oxidative and anti-inflammatory activities, in acute and chronic neurodegeneration in Parkinson's disease [6]. Cannabis-based medicine extracts containing multiple cannabinoids have been offered significant advantages over single cannabinoid and trended to supplement and potentially replace medicines currently used in relieving neuropathic pain, symptoms of multiple sclerosis such as muscle spasms as well as sleep disorders [7]. In Thailand, hemp is still enforced as schedule V controlled substance. However, Thai government recognizes that hemp may make useful contribution to the economy as an alternative crop, many researches for hemp cultivation and its utilizing have been continuing supported. Nevertheless, the uses of discarded parts after harvesting are approached for hemp added values. This work was hence conducted to give information about the feasibility of hemp utilizing for pharmaceutical purposes in Thailand.

2. MATERIALS AND METHODS

Plant materials

Four varieties of hemp including HH, V50, MSM and PU were cultivated during March-May, 2012 at Pang Da Royal Agricultural Station in Samoeng District Chiang Mai; a trial field authorized by the Food and Drug Administration of Thailand. Each of them was grown in 10 m. X 10 m. trial field with plastic shelter. All cultivated areas were treated in the same conditions with distance between rows of 75 cm, between pots of 25 cm and sowing rate of 3-4 plants per pot. No pesticides were supplied to these crops.

Sample preparation and extraction

At harvest time (90-days after sowing), leaves on the upper third of the main stem of hemp samples were collected separately and dried at 40°c for 48 hours. Dried leaves were ground and sieved through 32-mesh sieving. For crude extraction, 100 g of 500 µm dried powder of each hemp varieties was percolated with 400 ml methanol for 24 hours. The extracts was filtrated through Whatman® filter paper No.1 and collected. The extraction was repeated until the percolated extract was clear. The collected extract was then evaporated and measured % yield of crude extract (%w/w by dry weight).

Cannabinoids analysis by GC-FID

1 g. of each sample was extracted with 25 ml methanol by shaking for 1 h, 10 ml of the extract was centrifuged at 4,000 rpm for 5 min and then 1 ml of supernatant was diluted to 1:1 with 0.2 mg/ml 2,2,2 triphenylacetophenone as internal standard. 1.0- µl aliquot of the extract was injected and quantitatively analyzed by using Chrompack® 9002 GC-FID on DB-1 capillary column (30 m x 0.32 mm I.D. film thickness 0.25) with following conditions: nitrogen as carrier gas with flow rate 2 ml min^{-1}; split ratio 1:20; injector and detector temperature, 260 and 270°C respectively; oven temperature programmed, 7 min. at 230 °C, increase to 260 °C at 10 °C/min, hold at 260 °C for 2 min. THC, CBD and CBN contents were thus quantified upon the peak area ratio of each cannabinoids to internal standard in comparing sample with the peak area ratio of each standard to internal standard [8].

Anti-oxidant activity assay

100 µl of each hemp extract (0.8-1.2 µg/µl of crude extract) was performed with 3 different colorimetric methods including ABTS (9), DPPH (10) and FRAP (11) assay. The range of Trolox concentration (0.1 – 1.0 mM) was used as positive control and generated standard curve. The anti-oxidant ability from each method was presented in Trolox equivalent anti-oxidant capability (TEAC) per gram of dried weight powder.

Cytotoxicity examination

Appropriated dose of hemp extracts was screened using MTT assay. Human articular cartilage (HAC) cells were treated with various concentrations of hemp extracts for 24 hours then cultured supernatants were drained.

Cells were washed with 1x PBS for 2 times, added with MTT reagent, incubated for 4 hours and then MTT containing media was removed. DMSO was added and absorbance was measured at 540 nm.

Anti-inflammatory activity assay

Anti-inflammatory activity of each hemp extracts was studies on its ability to reduce the proinflammatory genes, including IL-1β, COX-1 and iNOS after inducing the HAC cells by Lipopolysaccharide (LPS). By using Reverse Transcription – Polymerase Chain Reaction (RT-PCR) assay, total cellular RNA was isolated using RNA isolation reagent (NeucleoSpin® RNA II) according to manufacturer's instructions. 1.0 μg/ml of total RNA was required for reverse transcription to produced cDNA using RevertAid^{TM} First Strand cDNA synthesis kit. The transcribed cDNAs were mixed with set of primer, including IL-1β, COX-1 and iNOS genes, along with DreamTaq^{TM} Green PCR Master Mix then amplified as previous described. The PCR products were analyzed on 1.2% agarose gel and visualized by ethidium bromide staining. The data were normalized with constitutive gene GAPDH and analyzed quantitatively using TotalLab® TL120 software [12].

3. RESULTS

The crude methanol extract of HH, V50, MSM and PU were 25.33, 16.17, 23.75 and 26.12% w/w by dry weight, respectively. PU and HH extract tended to have more oily paste comparing to the others. The amount of Δ^9THC in HH, V50, MSM and PU were 0.302, 0.091, 0.341 and 1.259% w/w and the amount of CBD were 2.297, 0.763, 1.879 and 2.256% w/w by dried weight, respectively. The ratio of CBD content to Δ^9THC, of HH, V50, MSM and PU were 7.61, 8.38, 5.51 and 1.79, consequently. V50 and HH were found to be predominant in CBD.

The hemp extracts of each varieties was tested for anti-oxidant properties using 3 different methods including ABTS, DPPH and FRAP. Each method examined in triplicate and the average value was reported as TEAC unit as shown in Table 1. The anti-oxidant abilities among four varieties of hemp examined by 3 methods showed positively correlation ($0.88 \leq r \leq 0.97$, $p < 0.05$). PU has possessed the highest anti-oxidant activities following by MSM, HH and V50, respectively.

Table 1 Anti-oxidant activity of various varieties of hemp measured with 3 methods

Hemp variants	Anti-oxidant activity (μ mol TE/g of d.w.); n=3		
	ABTS	DPPH	FRAP
HH (RPF1)	163.41 ± 5.02	139.66 ± 2.09	36.27 ± 2.05
V50 (RPF2)	84.61 ± 2.83	74.02 ± 1.53	24.93 ± 4.01
MSM (RPF3)	172.20 ± 15.68	149.17 ± 4.81	57.26 ± 5.26
PU (RPF4)	209.93 ± 1.92	206.87 ± 3.77	69.16 ± 1.35

After the HAC cells were treated with various concentration of hemp extracts (1–1000 μg/ml), cell viability was measured using MTT dye by colorimetric method at 540 nm. The cytotoxicity studies showed that the hemp extracts may be toxic to HAC cells at concentrations greater than 10 μg/ml. Thus, the concentrations of the extracts at 0.1, 1, 5, and 10 μg/ml were then studied for their anti-inflammation activity.

Interleukin-1-beta (IL-1β), cyclo oxygenase-1 (COX-1) and inducible nitric oxide synthase (iNOS) which were the genes playing important role in inflammatory process, were measured by RT-PCR after treating HAC cells with various kind of hemp extracts in different concentration (0.1-10 μg/ml) along with 0.1 μg/ml LPS as the trigger for cellular inflammation. The results revealed that the extracts from all hemp varieties could affect the expression of iNOS, IL-1β and COX-1 which were gradually reduced in dose dependent manner as shown in Fig.1. PU was the one having the most effectiveness.

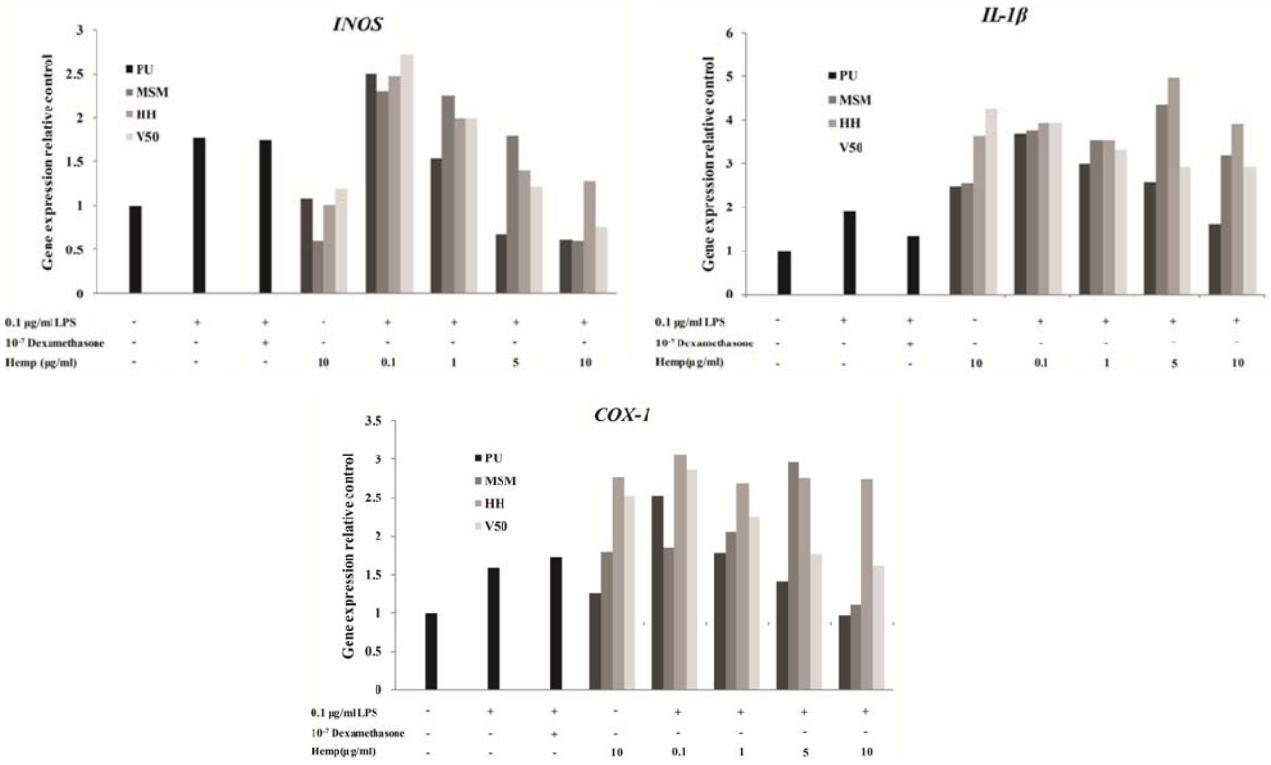

Figure 1. An effect of hemp extracts on iNOS (A), IL-1β (B) and COX-1 (C) expression after stimulation of LPS. 10^{-7} Dexamethasone was used as the positive control for inflammatory inhibition**.**

4. CONCLUSIONS

Suggesting that anti-oxidant and anti-inflammation activity existing in hemp extracts could be a useful target as anti-inflammatory agent which may be possible for the treatments of arthritis. Anti-inflammatory activity *in vivo* as well as acute and chronic toxicity of those extracts would be conducted in further study.

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Ageratum conyzoides leaf extract inhibit inflammatory response via suppression of NF-κB and MAPKs pathway in LPS-induced macrophages

Sawinee Seemakhan[a,b], Klaokwan Srisook[*b,c]
[a]Biological Science Program, Faculty of Science, Burapha University, Saensook, Muang, Chonburi, Thailand 20131
[b]Center of Excellence for Innovation in Chemistry, Burapha University, Saensook, Muang, Chonburi, Thailand 20131
[c]Department of Biochemistry, Faculty of Science, Burapha University, Saensook, Muang, Chonburi, Thailand 20131

*klaokwan@buu.ac.th phone +66-3810-3058 fax +66-3839-3495

ABSTRACT

Ageratum conyzoides (Asteraceae) has been widely used in traditional medicine in several countries for the treatment of skin diseases, ulcer wound, diarrhea, fever, pain and inflammation. Leaf extracts from *Ageratum conyzoides* have been shown anti-inflammatory activity in several *in vivo* models. However, the mechanism of its action has not been described yet. In this study, we determined the anti-inflammatory activity and the molecular mechanism of the ethanol extract of *Ageratum conyzoides* leaves (ACE) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage model. ACE exhibited an inhibitory effect on inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E_2 (PGE_2) production with IC_{50} values of 23.4 and 18.5 μg/ml, respectively. ACE showed no significant cytotoxic effect determined by MTT assay. ACE attenuated the expression of iNOS and COX-2 at mRNA as well as protein levels in a concentration-dependent manner. Additionally, ACE suppressed the level of nuclear factor-B (NF-B) translocation and phosphorylation of p38 kinase, extracellular receptor kinase (ERK) and c-jun NH2 terminal kinase (JNK) of mitogen-activated protein kinases (MAPKs). These results indicate that ACE inhibits inflammatory response, at least in part, by inhibition of NO and PGE_2 production through suppression of iNOS and COX-2 expression via a signaling pathway that involves NF-B nuclear translocation and MAPKs phosphorylation. These findings provide the scientific evidence to justify the anti-inflammatory therapeutic use of *Ageratum conyzoides* leaves in traditional medicine.

Keywords: *Ageratum conyzoides,* Nitric oxide, Prostaglandin E_2, iNOS, COX-2, Macrophage

1. INTRODUCTION

Inflammation is a critically important aspect of host responses to infection and injury. In response to inflammatory stimuli such as LPS, macrophages secrete various pro-inflammatory mediators including inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E_2 (PGE_2) which are involved in eradication of infection and injury. However, excessive or prolonged secretion of these mediators is implicated to the pathogenesis of a variety of diseases, including asthma, atherosclerosis, cancer, diabetes, inflammatory bowel diseases, and rheumatoid arthritis [1, 2]. Therefore, inhibition of NO and PGE_2 production is a promising target in the development of anti-inflammatory agents. Expression of iNOS and COX-2 in LPS-stimulated macrophages is mainly regulated by nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPKs) pathways [3-5].

Ageratum conyzoides L. (Asteraceae) is annual herb used in traditional medicine in many countries in the world. The leaves of *A. conyzoides* have been used in the treatment of chronic pain and various inflammatory diseases [6]. *A. conyzoides* leaf extracts have been shown to exert anti-inflammatory activity in several *in vivo* models [7-9]. However, the molecular mechanisms of its action have not been described. The purpose of this study was to investigate underlying anti-inflammatory mechanism of *A. conyzoides* leaf extract in LPS-induced RAW 264.7 macrophages.

2. MATERIALS AND METHODS

Preparation of extract

Leaves of *A. conyzoides* were collected from Ban Ang-Ed official community forest, Chantaburi Province. The plants were cleaned with tap water, dried and finally powdered. The powdered were soaked in 95% ethanol in a ratio 1:10 for 5 days with occasional shaking, after that ethanol extract was filtered through filter paper. Plant residues were re-extracted with 95% ethanol 2 times. The filtrate were pooled and evaporated by rotary evaporator until dryness before storage at -20°C.

Cell viability assay

Cell viability of RAW 264.7 macrophage were assessed by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as determined by Buapool et al, 2013 [5].

Measurement of nitric oxide production

Nitrite, an oxidation product of NO, in the cultured media is an index of NO production determined by Griess reaction according to the method reported by Buapool et al, 2013 [5].

Determination of PGE_2

PGE_2 produced from LPS-activated RAW 264.7 cells was quantified using PGE_2 competitive enzyme immunoassay kit (R&D Systems, USA.).

Western blotting analysis

Levels of iNOS, COX-2 and β-actin proteins in macrophages were determined by Western blotting analysis as described by Buapool et al, 2013 [5].

Real time-reverse transcription-polymerase chain reaction (Real time RT-PCR) analysis

Total RNA was reverse transcribed into cDNA using 5x iScript™ Reverse Transcription Supermix. The cDNA was used for real-time quantitative PCR which conducted in a CFX96™ Real-Time System (Bio-Rad) using 2x iTaq™ Universal SYBR® Green Supermix. The fold increase or decrease of iNOS and COX-2 were determined relative to a control after normalized to a housekeeping gene (EF-2) using formula $2^{-\Delta\Delta CT}$.

Nuclear protein extraction

For analyses of NF-κB p65subunit level, nuclear protein extracts were isolated according to the method reported by Srisook et al., 2011 [10].

3. RESULTS AND DISCUSSION

This study was performed to investigate the mechanism of anti-inflammatory effect of *A. conyzoides* leaves in LPS-induced RAW 264.7 macrophages. The ethanol leaf extract of *A. conyzoides* (ACE) inhibited the production of NO and PGE_2 in a dose-dependent manner with IC_{50} values of 22.69 ± 0.14 and 25.92 ± 5.72 µg/mL, respectively (Figure 1). ACE at concentrations 3.125 to 50 µg/mL did not significantly affect cell viability when compared to unstimulated control cell, indicating that the reduction of NO and PGE_2 production was not attributed to cell cytotoxicity. As shown in Figure 2, iNOS and COX-2 proteins were increased dramatically in cells treated with LPS and ACE attenuated the expression of protein iNOS and COX-2 in a dose-dependent manner. In accord, we found that ACE attenuated the level mRNA of iNOS and COX-2 in a dose-dependent manner (Figure 3). The results show that inhibition of NO and PGE_2 by ACE as a result of the suppression of iNOS and COX-2 mRNA and protein syntheses.

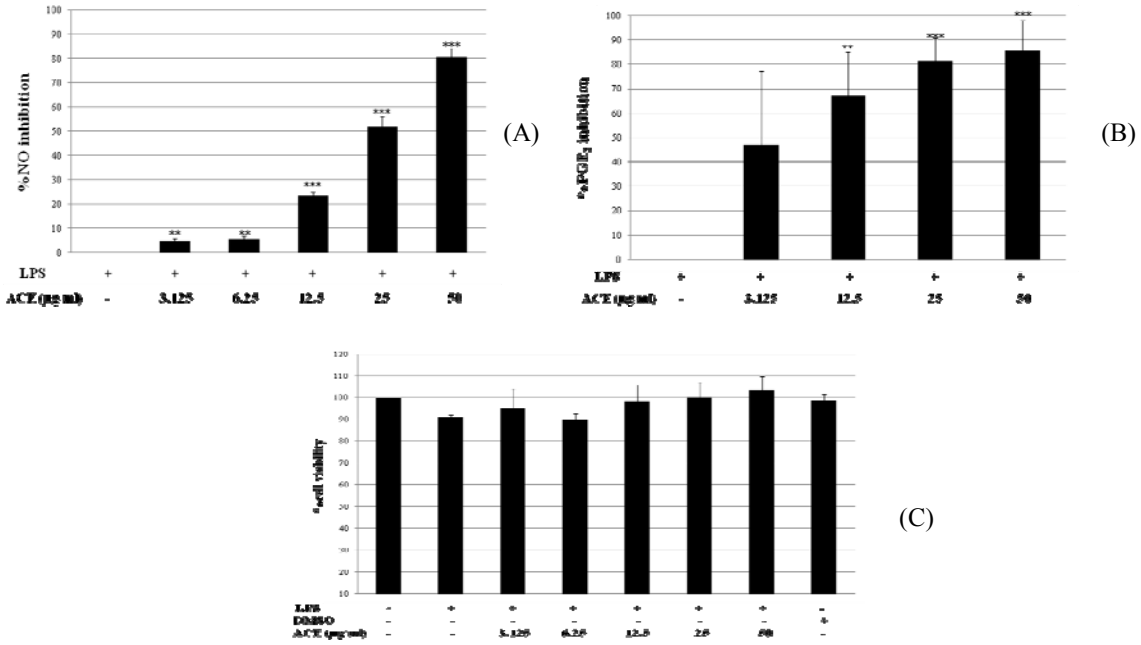


Figure 1. Inhibitory effects of ACE on NO (A) and PGE_2 (B) production in LPS-induced RAW 264.7 macrophages. Cells were incubated with ACE and LPS for 24 h. Accumulated nitrite and PGE_2 concentrations present in the medium were determined. (C) Viability of cells was determined using the MTT assay. Each column represents the mean ± SD of at least three independent experiments with triplicate samples. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS alone.

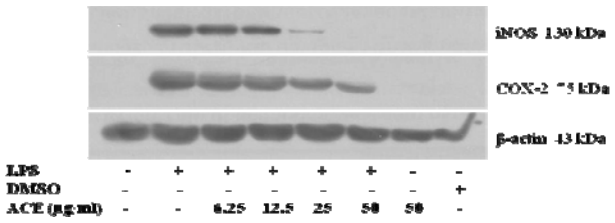
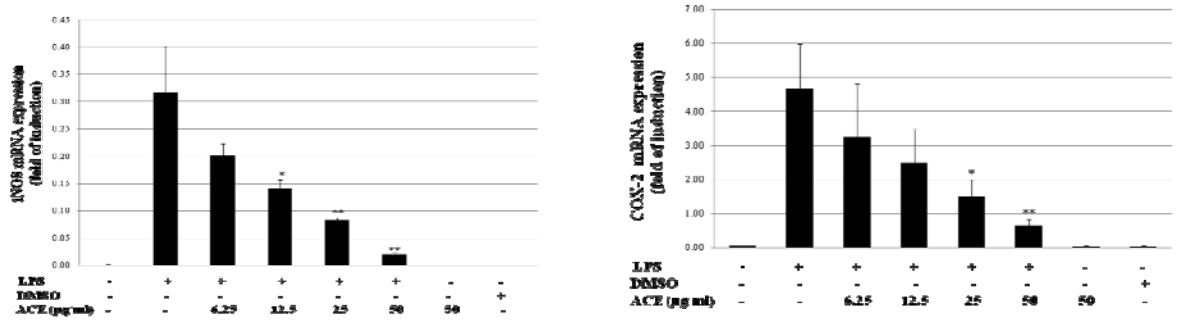


Figure 2. Effect of ACE on iNOS and COX-2 expression in LPS-induced RAW 264.7 macrophages. Cells were stimulated with ACE and LPS for 24 h. iNOS and COX-2 proteins were detected by Western blot analysis.

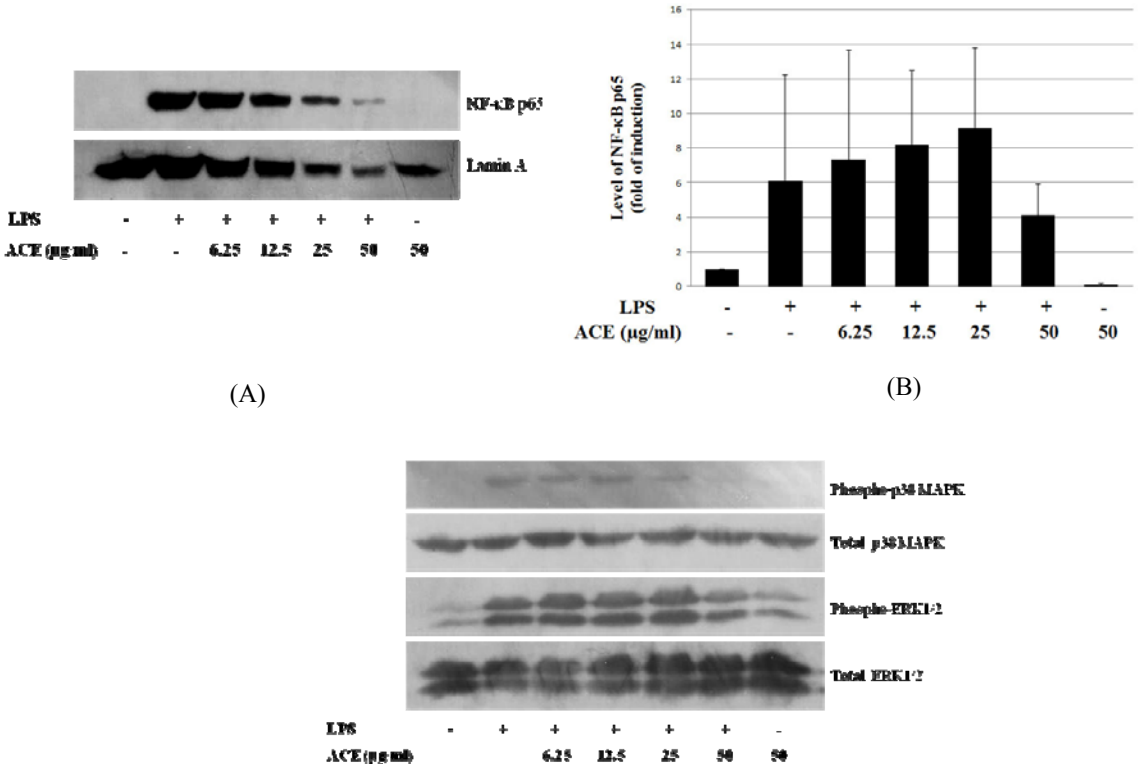


(A) (B)

Figure 3. Effect of ACE on iNOS (A) and COX-2 (B) mRNA expression in LPS-induced RAW 264.7 cells. Cells were treated with LPS and ACE for 9 h. Total RNA were isolated, iNOS and COX-2 mRNA levels were determined by real-time RT-PCR. Each column represents the mean ± SD of at least three independent experiments. *$p < 0.05$, **$p < 0.01$ vs. LPS alone.

In unstimulated cells, transcription factor NF-κB is bound to inhibitor of κB (IκB) in cytoplasm. Upon activation, NF-κB is rapidly translocated to the nucleus, subsequently facilitates the transcription of target genes, involved in inflammation, such as pro-inflammatory cytokines, chemokines, and inducible enzymes such as iNOS and COX-2 [3, 11]. We next determined the effect of ACE on the level of NF-κB p65 subunit since p65 protein is a subunit of the most common heterodimer NF-κB. LPS induced NF-κB p65 subunit translocation in nucleus, but ACE suppressed the level of NF-κB p65 subunit (Figure 4A). The other major signal pathway of iNOS and COX-2 expression stimulated by LPS is the mitogen-activated protein kinase (MAPKs) pathway composed of p38 kinase, extracellular receptor kinase (ERK), and c-jun NH_2 terminal kinase (JNK) [4, 5]. As shown in Western blot analysis data in Figure 4B, LPS induced the phosphorylation of ERK and p38 MAPK while ACE suppressed the phosphorylated ERK and p38 MAPK in a dose-dependent manner. These results indicate that ACE inhibited NO and PGE_2 production through suppression of iNOS and COX-2 expression, at least in part, via a reduction of NF-κB p65 subunit nuclear translocation and ERK and p38 MAPK phosphorylation.

The extracts from *A. conyzoides* have been shown anti-inflammatory effects in inflammation models of animal which associated with the secretion of inflammatory mediators including PGE_2 and NO [7-9]. The obtained results from LPS-induced PGE_2 and NO release in RAW 264.7 macrophages indicate that the mechanism of anti-inflammatory effect of *A. conyzoides* on several *in vivo* models [7-9] might be attributed to the inhibition of PGE_2 and NO. Literature review reports indicate the presence of kaempferol and quercetin in *A. conyzoides* [6] which have been shown to possess anti-inflammatory activities [12]. Thus these compounds might be attributed to the potent anti-inflammatory activity of ACE in LPS-induced macrophages. However, the bioactive compounds which responsible for anti-inflammatory property of ACE should be identified.



(A) (B)

(C)

Figure 4. Effect of ACE on the nuclear translocation of subunit p65 of NF-κB and phosphorylation of MAPKs in RAW 264.7 macrophages. Cells were pre-treated with the indicated concentrations of ACE for 30 min, and stimulated with LPS for 1 h for NF-κB and 30 min for MAPKs. The nuclear extracts were analyzed the levels of NF-κB p65 subunit (A). Graph shows the mean ± SD of results of densitometric analyses of NF-κB p65 subunit which were normalized to Lamin A densitometric values (n = 3) (B). Whole cell extracts were determined the phosphorylated ERK1/2 and p38 MAPK level by Western blot analysis (C).

4. CONCLUSIONS

These obtained results demonstrate that the mechanism of anti-inflammatory activity of ACE is, at least in part, the inhibition of NO and PGE_2 production by inhibition of iNOS and COX-2 expression, via NF-κB and MAPKs signaling pathway. These findings support the uses of *Ageratum conyzoides* leaves in traditional medicine.

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Comparative fibrinolytic activities of Nattokinases from *Bacillus subtilis* var. *natto*

Amira Abdel Motaal*[a], Inas Fahmy[b], Ali El-Halawany[a], Nahed Ibrahim[b]
[a]Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Kasr-El-Ainy St. Cairo11562, Egypt
[b]Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center, Giza, Egypt.

*a_motaal@hotmail.com phone +201221770823

ABSTRACT

Two Nattokinase genes were cloned using chromosomal DNA from *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872. Recombinant *Natto* (GenBank accession number KF734090) and *Natto-19* were expressed using a pQE-30 expression vector, and their fibrinolytic activities were studied. *Natto* nucleotide sequence contained an open reading frame of 1146 base pairs encoding 106 amino acids for signal peptide and 275 amino acids for mature subtilisin. It showed 100, 99.74 and 98.69 % identities with subtilisin NAT, subtilisin E and subtilisin J from *B. subtilis*, respectively. While the new *Natto-19* sequence contained 1088 base pairs encoding only 87 amino acids for signal peptide and 275 amino acids for mature subtilisin. Nattokinase was also produced by *B. subtilis* at pH 7.0 in a fermentation medium (g%): 1.0 glucose, 5.5 peptone, 0.5 $CaCl_2$, 0.2 $MgSO_4$. Quantitative analysis of the fibrinolytic activity was conducted by the fibrin plate method using urokinase (20000 U/mg) as the reference standard. The fibrinolytic activities of Natto and Natto-19 were 1000 ± 101 and 1187.5 ± 134 U/mg, respectively, compared to 400 ± 97 U/mg of the supernatant of the fermented culture broth of *B. subtilis*.

Keywords: Nattokinase, *Bacillus subtilis*, Fermentation, Fibrinolytic activity

1. INTRODUCTION

Atherosclerosis, hypertension and several cardiovascular diseases are ever prevailing in Egypt and the Middle East due to bad eating habits and lack of exercise. This requires a continuous search for safe, effective and cheap thrombolytic agents. One known potent fibrinolytic enzyme is nattokinase (NK) from *Bacillus subtilis* var. *natto* which was first found in the traditional Japanese soybean food natto [1]. NK possesses several advantages over several other conventional clot dissolving drugs. This includes safety, efficacy, preventive and prolonged effects, low cost, convenience in oral administration, as well as stability in the gastrointestinal tract [2, 3]. These properties make from NK a promising thrombolytic therapy especially in developing countries.

The three-dimensional structural model of NK from *Bacillus natto* was previously constructed by homology modeling [4]. Weng et al., 2009 [5] compared between the specific activity of wild type subtilisin NAT and two of its mutants (T220S and M222A). The protein coding regions of subtilisins NAT, E and J were identified to code for a 29-residue signal peptide for protein secretion from the cell membrane, a 77-residue propeptide which functions as an intramolecular chaperone that organizes the *in vivo* folding to achieve the active conformation and a 275-mature subtilisin [6, 7].

The fermented soybeans produced using *B. subtilis* bacteria, however, may have a preventive effect on not only thrombosis but also cancer. Previous reports proved that using the fermentation medium components glucose, peptone, calcium chloride and magnesium sulphate resulted in a maximum NK activity of 3194.25 U/ml [8]. Therefore, with the increase in yield and activity and simultaneous cost reduction, the industrial NK production by *B. subtilis* fermentation can be regarded as economically attractive.

The aim of our study was to produce a lyophilized fermentation product using *B. subtilis* culture broth and compare its fibrinolytic activity to two new cloned and expressed NK enzymes (*Natto* and *Natto-19*).

2. MATERIALS AND METHODS

Microorganism and inoculum preparation

Bacillus subtilis (Ehrenberg 1835) Cohn 1872 was purchased from RIKEN BioResource Center, Ibaraki, Japan (Catalogue#20036). The bacterial strain was grown overnight on LB agar plates. The culture was maintained at 4°C and the LB medium consisted of trypton 10 g/L, yeast 5 g/L and sodium chloride 5 g/L.

Cloning and expression of nattokinases

The nucleotide sequence database of National Center for Biotechnology Information was searched for a NK homologue and the NK NAT from *B. subtilis* (P35835) was found. Chromosomal DNA from *B. subtilis* (Catalogue#20036) was prepared by the standard method [9], and was used as the template for PCR. *Natto* and *Natto-19* genes were amplified by PCR using BamHI-linked sense primers (5'-CGCGGATCCGTATGAAAATAGTTATTTCG - 3' for *Natto* and CGCGGATCCATGGCGTTCAGCAACATGTCTGCG for *Natto-19*) and PstI-linked antisense primer (5'-AAAACTGCAGTTATT- GTGCAGCTGCTTGTACGTTG - 3'). PCR amplification was performed under the following conditions: 35 cycles of 95°C for 2 min, 95°C for 30 s, 50°C for 30 s, 72°C for 3 min and 72°C for 10 min. The PCR-amplified 1146 bp (*Natto*) and 1088 bp (*Natto-19*) DNA fragments were extracted from agarose gel and then ligated into pBluescript II SK (-) plasmid (Stratagene). After digestion with BamHI and PstI, the *Natto* and *Natto-19* fragments were inserted into the bacterial expression vector pQE-30 (Qiagen). *Escherichia coli* M15[pREP4] was the host bacterial strain for the expression vector. Transformed cells were then grown at 37°C in LB-medium, supplemented with kanamycin (25µg/ml) and ampicillin (100 µg/ml) until an OD_{600} of 0.6 was reached. Protein expression was induced by 1.0 mM IPTG (Sigma) and incubation was continued for 4-5 h at 37°C. The cells were harvested by centrifugation (20 min, 4000 x g, 4°C). Protein purification was carried out through the N- terminal hexa histidine residue (coded in the pQE-30 vector, 6xHis-tag). The purification was done according to QIA expressionist protocol (Qiagen, Fifth Edition, 2001) using Ni^{2+}-NTA agarose. The purified protein was stored at -20°C, then lyophilized to be used for the activity assays.

Determination of the concentration of the purified protein

After purification of the protein to homogenity, 2 µl of the protein in 800 µl water were mixed with 200 µl Biorad reagent and mixed. Samples were measured at 595 nm and the protein concentration was determined by means of a standard calibration curve established using 4, 6, 8 and 12 µg of bovine serum albumin [10].

Production of nattokinase by fermentation

The bacterial strain *B. subtilis* var. *natto* grown overnight on an LB plate, was washed in 5ml LB medium and transferred into a 1L flask containing 500 ml LB medium. The culture was incubated with shaking at 300 rpm at 30°C for 6 h. A 70 L fermentor (BIO F10 5000, New Brunswick Scientific, New Brunswick, NG, USA), containing 50 L of the fermentation medium (g%): 1.0 glucose, 5.5 peptone, 0.5 $CaCl_2$, 0.2 $MgSO_4$ and pH was adjusted to 7.0 [8], was inoculated with 1 L of the seed culture. Cells were grown at 30°C with an air flow rate of 1.5 slpm and an agitation speed between 200-300 rpm under an overhead pressure of 1 psi. The cell culture was harvested by centrifugation at 4000g, 4°C for 10 min, where the clear supernatant (F) was lyophilized and assayed for the NK activity.

Fibrinolytic activity assay

Quantitative analysis of the fibrinolytic activity was conducted by the fibrin plate method [11-13], using urokinase as the reference standard. In brief, 15 ml of 0.8 mg/ml fibrinogen solution (in 0.1M sodium phosphate buffer, pH 7.4) were mixed with 20 ml of 2% agarose solution and warmed up in a 45°C water bath for 15 min, followed by the addition of 1 ml of thrombin solution (7.5 U/ml) in a 90 mm Petri dish. The mixture was left for 1 h at room temperature to form a fibrin clot layer. The enzyme protein or fermentation product (F) were lyophilized into a powder form and dissolved in 50 µl of phosphate buffer, pH 7.4. Each 10 µl of the enzyme sample solutions were placed on a filter disc (5 mm in diameter) and incubated at 37°C for 18 h. After measuring the diameter of the clear zone, the units (U) of the enzyme activities were determined according to the urokinase established standard calibration curve. The specific activity was denoted by urokinase units of fibrinolytic activity in each milligram of the enzyme or F. Urokinase from human urine, fibrinogen from human plasma and thrombin from bovine plasma were purchased from Sigma Aldrich.

3. RESULTS

Cloning of nattokinase

The subtilisin *Natto* gene of *Bacillus subtilis* (*natto*) was cloned and its nucleotide sequence was determined (deposited in GenBank under accession number KF734090). The sequence revealed only one open reading frame composed of 1146 base pairs and 381 amino acid residues. The amino acid sequence of NK *Natto* was compared with the published sequences of other subtilisins (Figure 1) and showed 100, 99.74 and 98.69 % identities with subtilisin NAT, subtilisin E and subtilisin J from *Bacillus subtilis*, respectively. On the other hand, subtilisin *Natto-19* gene was cloned and its sequence revealed an open reading frame composed of 1088 base pairs and 362 amino acid residues. It had 19 amino acid residues missing at the N-terminal of the signal peptide compared to other subtilisins. The catalytic domain of 275 amino acids as well as the catalytic triad (Asp-32, His-64, and Ser-221) were well conserved in both genes as other *Bacillus* subtilisins [4].

Expression and purification of nattokinase

Cultures of transformed *E. coli* M15[pREP4] were induced with 1.0 mM IPTG at 37°C, which led to the production of major proteins of 40 and 42 kDa. Protein purification was carried out through the N- terminal hexa histidine residue using Ni^{2+}-NTA agarose. The concentrations of the purified proteins were calculated to be 500 and 400 µg/ml for *Natto-19* and *Natto*, respectively, using the established standard calibration curve [10].

Comparative fibrinolytic activity

The fibrinolytic activities of the cloned *Natto-19* and *Natto* were compared to that of the fermentation product using urokinase as a reference standard. Serial dilutions of urokinase (100 to 0.001 U) were added to the fibrin plate and the clear zones resulting from hydrolysis of the fibrin clot were recorded and a standard calibration curve was established (Figure 2). Ten µl of each of the expressed NK samples (*Natto-19* and *Natto*) and the fermentation product (F) containing 8, 10 and 75 mg/ml protein, respectively, were added to the fibrin clot. All samples revealed fibrinolytic effects as indicated by the clear zones of 1.6 and 1.7 and 1.9 cm corresponding to 95, 100 and 300 U, respectively. As shown in Table 1, the specific activity of *Natto-19* (1187.5 ± 134 U/mg) was slightly higher than *Natto* but was 3-fold that of the fermentation product. Previously, Weng et al., 2009 [5] expressed a wild type subtilisin NAT and two of its mutants T220S and M222A possessing specific fibrinolytic activities of 1760 ± 154, 1230 ± 90 and 933 ± 97 U/mg, respectively, at pH 7.75.

```
Natto MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
  NAT MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
  E   MRSKKLWISLLFALTLIFTMAFSNMSAQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
  J   MRSKKLWISLLFALTLIFTMAFSNMSVQAAGKSSTEKKYIVGFKQTMSAMSSAKKKDVIS
      *************************.**********************************

Natto EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
  NAT EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
  E   EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
  J  EKGGKVQKQFKYVNAAAATLDEKAVKELKKDPSVAYVEEDHIAHEYAQSVPYGISQIKAP
      ************************************************************

Natto ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
  NAT ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
  E   ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
  J   ALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQDGSSHGTHVAGTIAA
      ************************************************************

Natto LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
  NAT LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
  E   LNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPTGSTA
  J   LNNSIGVLGVSPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLGGPSGSTA
      **********:*************************************************:****

Natto LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGS
  NAT LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSVGS
  E   LKTVVDKAVSSGIVVAAAAGNEGSSGSTSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGS
  J   LKTVVDKAVSSGIVVAAAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGS
      ****************************:****************************.**

Natto  ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
  NAT ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
  E   ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
  J   ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTA
      ************************************************************

Natto TYLGNSFYYGKGLINVQAAAQ
  NAT TYLGNSFYYGKGLINVQAAAQ
  E   TYLGNSFYYGKGLINVQAAAQ
  J   TYLGNSFYYGKGLINVQAAAQ
      ********************
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Figure 1. Comparison between the amino acid sequence of *Natto* (KF7340090) and other subtilisin proteases; Subtilisin NAT (P35835), Subtilisin E (P04189), Subtilisin J (P29142).

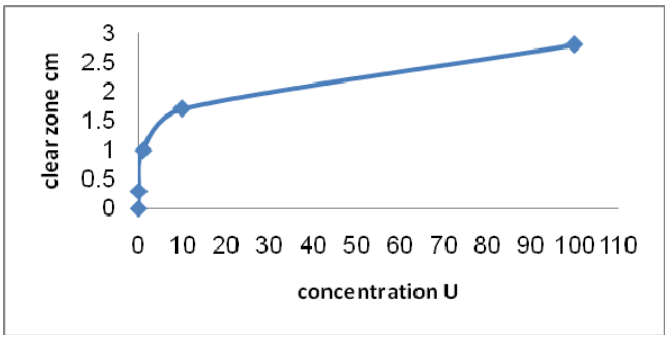

Figure 2. Standard calibration curve for urokinase showing its serial dilutions and the corresponding clear zones on a fibrin plate.

Table 1. Comparison between the fibrinolytic activities

Tested sample	Specific activity (U/mg)*
Natto-19	1187.5 ± 134
Natto	1000 ± 101
Fermentation product (F)	400 ± 97

*****The data shown are expressed as a mean ± SD, based on three independent experiments.

4. CONCLUSIONS

The fibrinolytic activities of the two cloned Nattokinases, *Natto-19* and *Natto*, were more or less the same although 19 amino acid residues were missing at the N-terminal of *Natto-19*. The lyophilized fermentation product of *Bacillus subtilis* (natto) showed quite a good activity compared to the two lyophilized pure expressed Nattokinases. Production of such a fermentation product on a larger scale and optimizing the fermentation conditions in order to achieve higher activities to incorporate the product into pharmaceutical preparations are our next goals.

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Antiproliferative effect of *Moringa oleifera* extract on cancer cells

Suphachai Charoensin*[a]
[a]Division of Biochemistry and Nutrition, School of Medical Sciences, University of Phayao, Phayao, Thailand 56000

*suphachai.ch@up.ac.th phone +6654466666 fax +66-54466663

ABSTRACT

In present study, free radical scavenging and antiproliferative activities of *Moringa oleifera* (drumstick) extract have been investigated *in vitro*. Leaves were extracted with hot water to obtain aqueous extract. The 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was used to determine free radical scavenging activity. Antiproliferative assays on cancer cell lines, hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2) and breast adenocarcinoma (MCF-7), were carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Concentrations causing fifty percent of cell death or radical scavenging were calculated as IC_{50}. The result showed that the extract had radical scavenging activity (IC_{50} = 16.15±1.04 µg/ml). According to MTT assay, HepG2 was most sensitive to the extract (IC_{50} = 146.40±2.31 µg/ml). Caco-2 and MCF-7 had relatively low sensitivity than HepG2. Both had IC_{50} of extract more than 170 µg/ml. No inhibitory effect was observed on human dermal fibroblast treated with 0-400 µg/ml of extract. These results suggest that aqueous extract of *M. oleifera* leaves has antioxidant activity against free radicals and inhibits cancer cell proliferation. This chemotherapeutic effect could be promising for further work on cancer chemotherapy *in vivo*.

Keywords: *Moringa oleifera*, Antiproliferation, Cytotoxicity, Antioxidant activity

1. INTRODUCTION

According to the World Health Organization (WHO) report, cancer is one of the major causes of death around the world [1]. The effective strategy to overcome various kinds of cancers is application of natural products in terms of inhibiting cell proliferation and inducing cell death. Due to this strategy, research interests in cancer chemotherapy and phytochemicals have been focused [2].

Moringa oleifera (*M. oleifera*) or drumstick is a member of Moringaceae. It is widely grown in Thailand and other Asian countries [3]. The leaves contain essential amino acids, vitamins, minerals and *β*-carotene [4-5]. Recent reports on disease prevention by *M. oleifera* have been reported. The leaf extract was capable of reducing hyperglycemia and dyslipidemia [6]. Budda et al. reported that tender pods demonstrated inhibitory potential against azoxymethane-induced colon carcinogenesis [7]. Moreover, it has been reported that polyphenols in *M. oleifera* leaves were potent antioxidant with cytotoxicity on some cancer cell lines [8, 9], and enhanced chemotherapy of drug on pancreatic cancer cells [10]. Due to limited reports on cancer chemotherapy of *M. oleifera* leaves, the aim of present study was to investigate the *in vitro* antiproliferative activity of *M. oleifera* extract on HepG2, Caco-2 and MCF-7, along with free radical scavenging activity.

2. MATERIALS AND METHODS

Plant material and extraction

The leaves of *Moringa oleifera* were collected during November-December 2012 from Lampang Herb Conservation, Lampang, Thailand. This plant was identified and confirmed by comparing it with voucher specimens of known identity (ID: WP2614) deposited at the Queen Sirikit Botanical Garden, Chiang Mai, Thailand. The air-dried leaves of *M. oleifera* were ground into powder and stored at 4°C until extraction. Fifteen grams of leaf powder were extracted with 350 ml of hot water (80°C). Then, the liquid extract was filtered through Whatman no. 1 filter paper, and was lyophilized. The aqueous extract was kept in amber glass at -20°C until use.

ABTS radical cation decolorization assay

The protocol of Re et al. was used with slight modification [11]. Briefly, the ABTS radical was prepared in 2.45 mM potassium persulfate. The solution was then left for 15 min in a dark place to obtain an ABTS radical solution. This solution was subsequently diluted with ethanol before use. To the diluted solution, various concentrations of extract (300 µl) were added. Trolox was used as positive control. After incubating for 8 hours in the absence of light, the absorbance was measured at 731 nm. The percentage of ABTS radical scavenging activity was calculated using the following equation:

$$\%\ Inhibition = \frac{(OD_{control} - OD_{extract})}{OD_{control}} \times 100$$

The inhibitory percentage was calculated, and the IC_{50} was determined.

Cell culture

Human hepatocellular carcinoma (HepG2) (ATCC: 77400), colorectal adenocarcinoma (Caco-2) (ATCC: HBT-37) and breast adenocarcinoma (MCF-7) (ATCC: HTB-22) cell lines, and human dermal fibroblast (ATCC: PCS201012) were used in an antiproliferative assay. The cancer cell lines were cultured in 25 cm^2 culture flask using Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The human dermal fibroblast was cultured in fibroblast basal medium supplemented with FBS, glutamine, ascorbic acid, hydrocortisone and hemisuccinate. These cells were maintained in a humidified incubator with an atmosphere comprising 5% CO_2 and 95% air at 37°C. Subcultures were made when cell proliferation reached 80% confluence.

Cytotoxicity test

The cytotoxic effect of *M. oleifera* extracts on HepG2, Caco-2, MCF-7 and human fibroblast was evaluated by the MTT assay [12]. The cells were plated at 1.0×10^3 cells per well in 96-well plates. Twenty four hours after plating, the cells were incubated with extract (0 – 250 µg/ml) or cisplatin (anticancer drug) for 48 h at 37°C. The final dimethyl sulfoxide (DMSO) concentration did not exceed 0.2%. Then, 20 µl of MTT solution (5 mg/ml) were added to each well. The insoluble purple formazan crystal was dissolved in 100 µl of DMSO, and the absorbance was determined at 540 nm and 630 nm using a microplate reader. The percentage of cell viability was calculated. The concentration of the extract causing 50% inhibition of cancer cell growth was considered as IC_{50}.

Statistical analysis

Data were expressed as mean ± standard error of mean (SEM) of three-independent experiments. The analysis of variance was performed using ANOVA. The Bonferroni test with $p < 0.05$ was considered to test for a significant difference between control and treated groups.

3. RESULTS AND DISCUSSION

ABTS radical cation decolorization was carried out to assess free radical scavenging activity of *M. oleifera* extract. The radical scavenging potential was compared with trolox (standard antioxidant). It was found that the extract scavenged ABTS radicals with IC_{50} = 16.15 ± 1.04 µg/ml. The present work confirms the reports on antioxidant activity of *M. oleifera*, which were previously described in other papers [13-15]. *M. oleifera* leaves are rich in antioxidant polyphenols and flavonoids [14-15]. Moreover, polyphenols such as crypto-chlorogenic acid, isoquercetin and astragalin were recently reported by Vongsak et al. [16]. Quercetin and kaempferol were also found in the leaves [8, 14]. According to accumulating research on cancer chemoprevention and chemotherapy, the current trend is directed towards naturally-occurring antioxidants. Due to advantage as safe substances, polyphenols with antioxidant activity have been evaluated antiproliferation on cancer cell lines. For example, *M. oleifera* leaf extract inhibited SW480, HCT18 and Panc-1 cancer cell proliferation [9-10]. In this study, following MTT assay, *M. oleifera* extract showed, for the first time, antiproliferative activity against HepG2, Caco-2 and MCF-7, with different potentials. It significantly inhibited HepG2 cell growth (IC_{50} = 146.40 ± 2.31 µg/ml) compared with control (DMSO). Caco-2 and MCF-7, however, showed less sensitivity to the extract. Fifty percent growth inhibitions of both cancer cells occurred above 170 µg/ml. Human dermal fibroblast was not affected by the extract (0 - 400 µg/ml). The mechanistic studies regarding cell death (apoptosis) and cell proliferation are needed to be clarified.

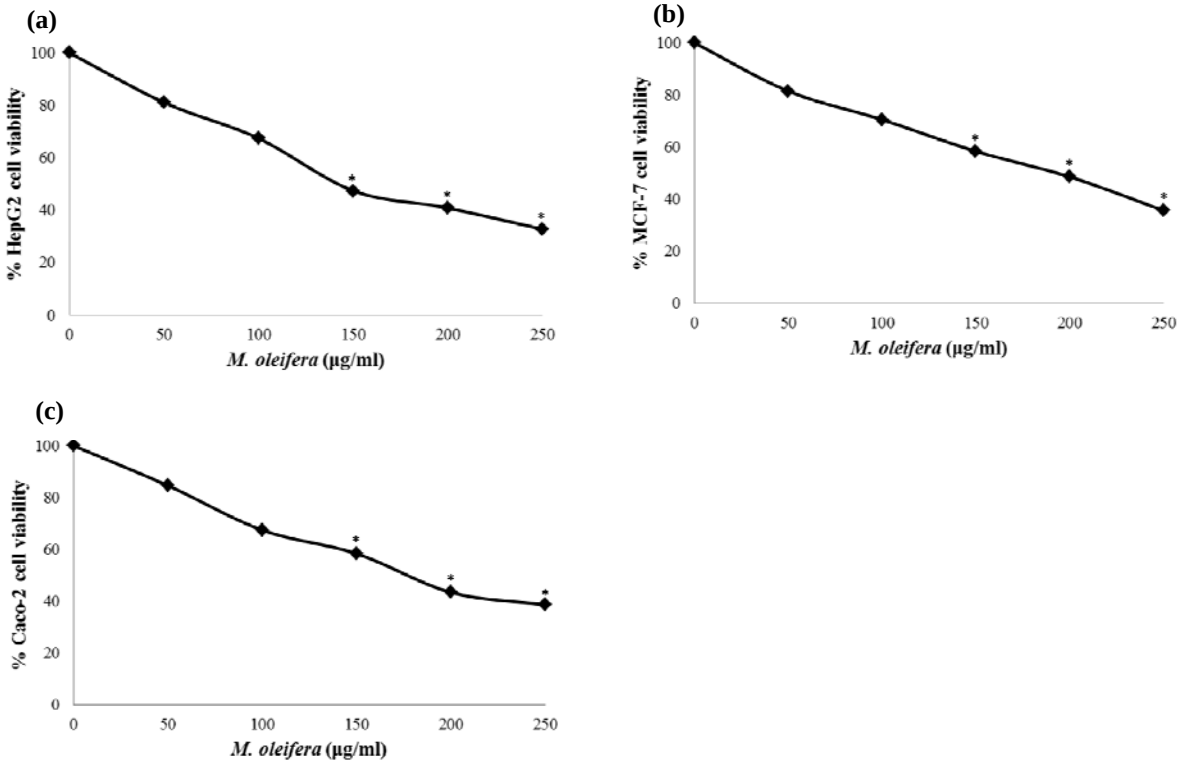

Figure 1. Antiproliferation of *M. oleifera* extracts on (a) HepG2, (b) MCF-7 and (c) Caco-2. Each cancer cell type was incubated with various concentrations of extracts (0 – 250 µg/ml) for 48 h. Data are obtained from three independent experiments, and are shown as mean ± standard error of mean. An asterisk (*) indicates significant difference ($p < 0.05$) between negative control and treated groups.

4. CONCLUSION

M. oleifera aqueous extract exhibits antiproliferative effect on HepG2, Caco-2 and MCF-7, as well as antioxidant activity. This could be beneficial and applicable for further phytochemical-cancer research in animal model.

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Anti-mutagenic and anti-oxidative DNA damage effects of *Moringa oleifera* Lam. leaves using micronucleus test and comet assay

Ratsada Praphasawat*[a], Maitree Suttajit[b], Prapaipat Klungsupya[d], Suphachai Charoensin[b], Rungthip Thongbuntho[b], Paween Kunsorn[c],Kwanchaya Jeerawut[c], Thanchanok Muangman[d] and Sarunya Laovitthayanggoon[d]

[a]Department of Pathology, School of Medicine, University of Phayao, Thailand 56000

[b]Department of Biochemistry, School of Medical Sciences, University of Phayao, Thailand 56000

[c]Department of Applied Thai Traditional Medicine, School of Medicine, University of Phayao, Thailand 56000

[d]Department of Pharmceuticals and Natural products, Thailand Institute of Scientific and Technological Research (TISTR), Thailand 10210

*ratsada.pr@gmail.com phone +66-878315522 fax +66-54466298

ABSTRACT

Moringa oleifera Lam., a leguminous plant, is mostly cultivated in Thailand. The leaves of *M. oleifera* have long been used by many people as food because of their rich source of nutrients and essential minerals. A number of publications have been supported that *M. oleifera* possesses anti-oxidative potential due to antioxidant capacity and free radical scavenging activity detected by various antioxidant assays. However, there is limited information available regarding to their anti-DNA damage effects. The aim of this study was to investigate the anti-DNA damage property of *M. oleifera* leaves (extracted by distilled water) using micronucleus test and comet assay in TK6 cells. The micronucleus results show that the leaf extract of *M. oleifera* do not induce the micronucleus frequency up to a concentration of 150 µg/ml. Interestingly, *M. oleifera* leaves have dramatically reduced micronucleus induction by mitomycin C (a well-known mutagen) at the concentration ranging from 25-100 µg/ml. For the comet assay, TK6 cells were incubated with various concentrations of *M. oleifera* leaf extracts before being exposured to H_2O_2 for DNA damage induction. We found that pretreatment with the leaf extracts of *M. olerifera* possess an anti-DNA- damage potential by significant reduction of the tail length of DNA and tail moment values. These findings could be useful for future development of leaf extracts from *M. oleifera* as dietary supplement. However, *in vivo* study of leaf extracts from *M. oleifera* is required for a practical use in the future.

Keywords: *Moringa oleifera*, Micronucleus test, Comet assay

1. INTRODUCTION

Moringa oleifera Lam., commonly known as drumstick tree, horse-radish tree, and ma-rum tree belongs to the Moringaceae Family. It has been cultivated in many tropical and sub-tropical countries such as India, Pakistan, Philippines, Thailand and Africa. The leaves of these trees have long been used as traditional medicines and dietary consumptions because of its rich sources of nutritional and therapeutic compounds. Many nutritional compounds have been reported such as a number of proteins (albumin, globulin, prolamin, and insoluble proteins), carbohydrate, ashes, some mineral (calcium, iron) [1]. Moreover, phenolic compounds and flavonoids were found as the major phytochemical constituents in leaves [2]. These compounds are responsible for various properties such as anti-oxidant, anti-inflammation, and anti-hypertension activities [3-5]. Some publication show low toxicity of leaf extracts from *M. oleifera* [6] and their significant function as anti-oxidative properties possibly inhibitory effect of DNA damage as well as reduced risk of mutations and cancers. The aim of this study was to investigate the mutagenic and anti-oxidative DNA damage effects of *M. oleifera* from leaf extracts. In this research, micronucleus test and single cell gel electrophoresis or comet assay were performed in TK6 human lymphoblastoid cell culture [7-11]

2. MATERIALS AND METHODS

Extraction of *M. oleifera* leaves

Fresh leaves of *M. oleifera* were harvested and collected from Phayao Province, North of Thailand. The leaves were cleaned by tab water and air-dried. Two hundred-fifty grams of fresh leaves were blended with 2 L distilled water and heated for 1 h at 80°C in a water bath. Following, filtration through Whatman no.1 filter paper using a suction apparatus, the extract was lyophilized giving a reddish brown color. The dry extracted was weight and kept at -20°C. The yield of freeze-dried powder from fresh leaves was about 3.14%W/V [12].

Cell culturing and maintenance

The stock TK6 human lynphoblastoid (TK6) cell line (CRL-8015) was purchased from American Type Culture Collection (ATCC) in Maryland, US. Cells were grow in suspension and maintained as exponentially phase in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and incubated at 37°C in an incubator containing 5% CO_2. Cells were subculture every 48 h.

Determination of mutagenicity and anti-mutagenicity using micronucleus test

Prior to the anti-mutagenicity assay, the mutagenicity of *M. oleifera* leaves were evaluated at selected doses based on viability greater than 70%. In this study the extract at doses of 10, 50, 100 and 150 µg/ml were incubated in TK6 cells for 24 h at 37°C. The anti-mutagenicity experiment was performed at doses (>70% cell viability) of 25, 50, 75 and 100 µg/ml RPMI in combination with a known mutagen, mitomycin C (MMC at 0.8 µg/ml) for 24 h. To treated-cell, a cytochalasin B solution (Cyt B 3 µg/ml) was added during 6 h to collect the cells at a binucleated stage. Following washing and harvesting steps, treated cells were prepared as monolayer on glass slides using cytospin equipment (Shadon, UK). Slides were left to dry at room temperature and then fixed in cold methanol for 30 mins. Cells on slides were stained using 10% Giemsa solution. Micronuclei (MN) formations were scored in 1000 binucleated (BNC) cells under light microscope (40×) (Figure 1)

Determination of anti-oxidative DNA damage property using comet assay

After seeding the TK6 cells (2×10^5 cells/ ml) into a 6-well plate, cells were treated for 18 h with *M. oleifera* leaf extract at doses of 25, 50, 100, 200 µg/ml RPMI. By the end of the treatment time, cells were harvested by centrifugation at 3000 rpm for 2 mins to remove extract-containing medium. Cells were then treated with 50 µM H_2O_2 for 5 mins at 4°C. Following treatment, cells were washed twice with cold HBSS and re-suspended in RPMI medium to be ready for comet assay.

The comet assay was performed following the method described by Tice RR. 2003. In brief, 20 µl of cell suspension was mixed with 75 µl of 0.5% low melting point (LMP) agarose at 37°C, layered onto a pre-coated slide with 0.75% normal melting point (NMP) agarose and covered with a coverslip allowing gel-solidification on a flat surface ice box. The coverslip was gently removed and 95 µl of LMP agarose was layered and covered with the coverslip. After coverslip removal, the slides immersed into a lysis solution (2.5M NaCl, 10 mM $Na_2EDTA{\cdot}2H_2O$, 10 mM Trisma base, pH 10 with 1% triton X-100, 10% DMSO) for 2 h at 4°C. After lysis, slides were exposed to freshly made alkaline electrophoresis buffer (200 mM EDTA, 10 N NaOH, pH 13) for 20 min to allow DNA unwinding. The slides were then placed on a electrophoresis tank filled with sufficient electrophoresis solution and kept in an ice bath (4°C). Electrophoresis was carried out for 20 mins at constant 25V and a current of 300mA using a powerpact supply. Then, the slides were neutralized in 0.4 M Trizma base buffer (pH 7.5) and stained with 20 µg/ml ethidium bromide. At least 50 cells per slide and per treatment were randomly analyzed for comet images using the fluorescence microscope (at 40x magnification) connected to a computer equipped with an automated

image analysis system (Comet assay III, Perceptive Instrument, UK). Two parameters, tail length of DNA (TL) and tail moment (TM) were considered as indicator of DNA damage.

Statistical analysis

In the micronucleus test, the presented data were the mean ± standard derivation (SD.) of at least two experiments and were statistically analyzed by one way ANOVA with Scheffe test applied to compare groups. A $p < 0.05$ was considered to be significant different. For the comet assay, the mean of 50 comet cells from two independent experiments were compared with those in the negative control group by Tamhane test after being tested by one way ANOVA. The difference between the means at the level of $p < 0.05$ was considered as significant.

3. RESULTS AND DISCUSSION

Mutagenic and anti-mutagenic effects of *M. oleifera* leaves

In this study, we performed the micronucleus test to investigate the mutagenic and anti-mutagenic properties of the *M. oleifera* leaf extracts. This assay is technically simple and rapid test to indicate chromosome damage, such as chromosome breaks which were investigated by use of the *in vitro* cytokinesis-blocked micronucleus assay (CBMN). Addition of cytochalasin-B led to the accumulation of the cell at binucleated stage. Then, the cytokinesis block proliferation index (CBPI) values were concurrently calculated in order to assure that the cell have undergone divided once throughout the experiment. More so this method is frequently used in genotoxicity testing and human biomornitoring [13-16]. As our result, the survival rates of TK6 cells of all treatment were greater than 60%. Moreover, the CBPI values revealed no significant altered for all concentration tested.

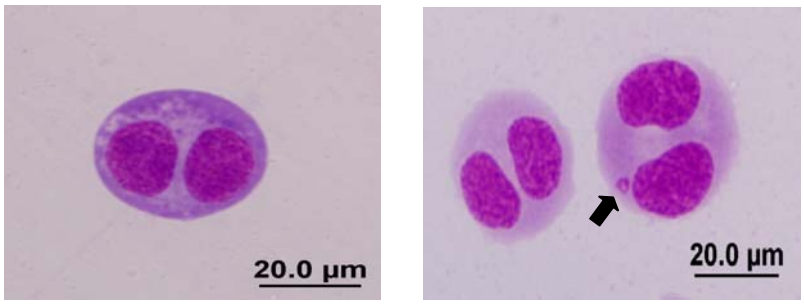

Figure 1. Photomicrographs illustrate binucleated (BNC) cells (left) and BNC with one micronucleus (arrow point), of TK6 cells (right).

Results of the mutagenic study (Figure 2) showed that of *M. oleifera* leaf extracts in all concentrations (10, 50, 100, 150 µg/ml) revealed no significant increase in micronuclei frequencies to TK6 cells compared with untreated cells. By contrast, for positive control, TK6 cells treated with MMC revealed significant a number of MN frequencies. For the anti-mutagenic assay (Figure 3) positive control or MMC-treated cell without *M. oleifera* leaf extracts showed the amount of MN frequencies as 25.05. Whereas in cell treated with *M. oleifera* leaf extracts and MMC combination groups showed a concentration dependent decreased in the MN frequencies by 21.36, 19.03, 14.44, and 9.33 according to the doses of *M. oleifera* leaf extracts 25, 50, 75, 100 µg/ml, respectively. These results agree well with earlier findings where the water extracts of the leaves of this plant have no mutagenicity but showed anti-mutagenicity against the standard mutagens tested in *Salmonella typhimurium* TA100 strain [17]. In addition, the previous study of Sathya N.T. and colleagues has shown that the mouse micronucleus test of *M. oleifera* extracted from leaves could prevent the genotoxic effect of cyclophosphamide-induce DNA damage in mice [18].

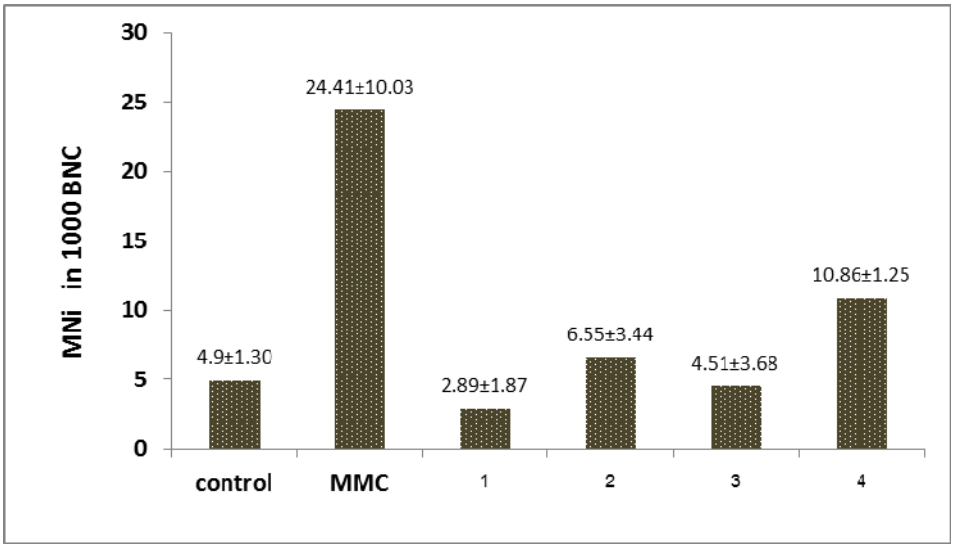

Figure 2. Bar plot illustrates the MN frequency in TK6 cells following treatment of vehicle (control group), MMC (0.8 µg/ml) and *M. oleifera* leaf extract (defined as 1=10, 2=50, 3=100, and 4=150 µg/ml). Results are expressed as mean values of three separate experiments. *Significant different from other groups at $p < 0.05$ (ANOVA).

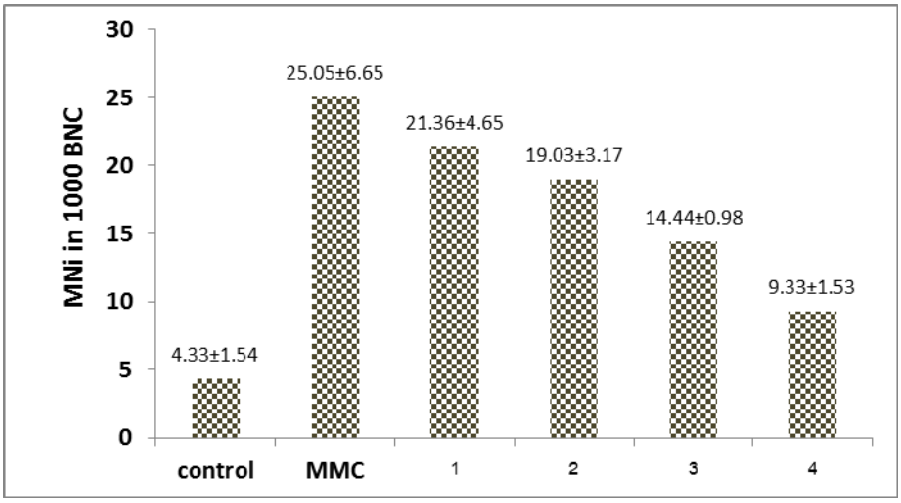

Figure 3. Bar plot illustrates the MN frequency in TK6 cells following treatment with vehicle control, MMC (0.8 µg/ml) and combination treatment of MMC (0.8 µg/ml) and *M. oleifera* leaf extract (defined as 1=25, 2=50, 3=75 and 4=100 µg/ml). Results are expressed as mean value of three separated experiments.

Anti-oxidative DNA damage effect of *M. oleifera* leaves

We assessed the anti-DNA damage induced by H_2O_2 using the single cell gel electrophoresis or comet assay. In the present study, alkaline condition (pH > 13) was used in the lysing and electrophoresis steps which produce DNA denature expressed as single strand breaks, double strand break, base damage or alkaline labile sites to TK6 cells [19, 20]. Results used two parameters as indicator of DNA damage like tail length (TL= a distance of damage-DNA migration) and tail moment (TM; a DNA damage intensity) values.

Our comet assay results demonstrated that DNA damage in terms of TM values was significantly increased in case of H_2O_2 exposure compared to the vehicle or negative control up to 9-10 fold (from 2.17 to 20.91) whereas TL values increased 1-2 fold (from 30.02 to 66.84). In ascorbic treated-TK6 cells, both the TM and TL values showed a

strong decrease down to 11.21 and 57.13, respectively. Interestingly results of our study showed that pre-incubated TK6 cells with *M. oleifera* leaf extracts revealed a clear decrease in both TM and TL values. DNA damage was reduced by TM values to 16.83, 16.15, 12.67, and 16.05 at doses of 25, 50, 100, 200 µg/ml, respectively (Figure 4). In fact, the TM values were more accurate than TL values. Therefore, our experiment used TM values as the main DNA damage parameter [21]. We calculated the percentage of inhibitory DNA damage based on TM values. Our results clearly demonstrated that pre-treatment of TK6 cells with 25, 50, 100, 200 µg/ml of *M. oleifera* leaf extracts suppressed H_2O_2-induced DNA damage effects by 19.51%, 22.76%, 39.41%, 23.24% respectively. The anti-oxidative DNA damage of *M. oleifera* observed in this study corroborated with the previous finding [22]. They exhibited that these extracts could have oxidative-DNA damage protective activity by significantly inhibit the hydroxyl radical (OH◦)-dependent damage of pUC18 plasmid DNA. Similar observation was demonstrated by others [23] who found that *M. oleifera* leaf extracts mediated concentration dependent protection of oxidative DNA damage induced by OH◦.

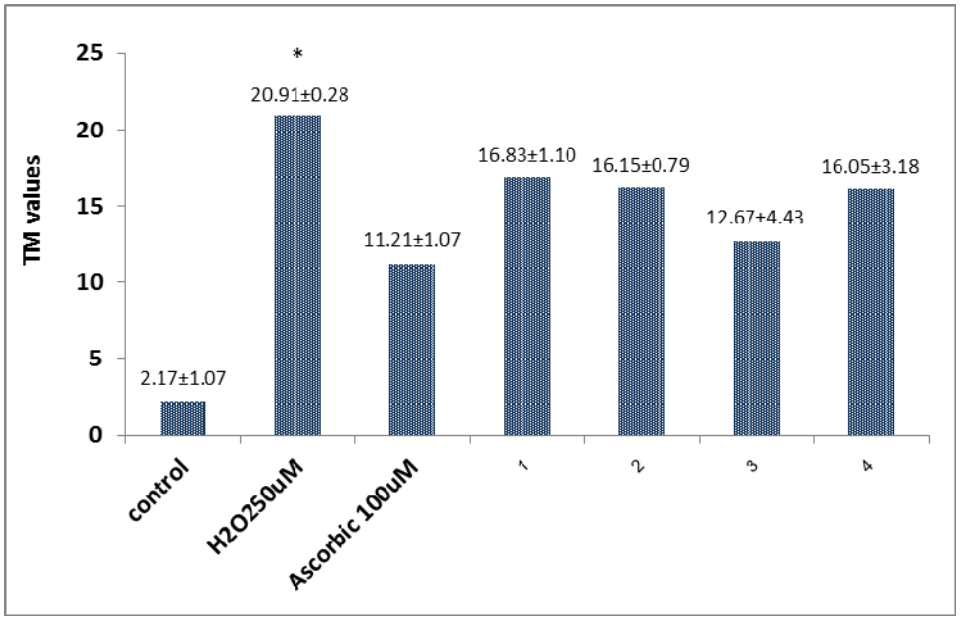

Figure 4. The result of the comet assay represented by tail moment (%) value of TK6 cells after treatment with difference concentrations of *M. oleifera* leaf extract (defined as 1=25, 2=50, 3=100, and 4=200 µg/ml) for 18 h before H_2O_2 (5 mins) exposure. Results are expressed as mean values of two separated experiments and each quantified in 50 comet cells. *Significant different from other groups at $p < 0.05$ (ANOVA).

4. CONCLUSIONS

The present study demonstrated that *M. oleifera* extracted from leaf not only had no mutagenic effects but also had effectiveness in reduction of chromosome and DNA damage induced by a mutagen (MMC) and H_2O_2 in TK6 cells. These findings were supported by the results of the micronucleus test and the comet assay in TK6 cells. Therefore, *M. oleifera leaves* might be a new candidate or an alternative compound used for anti-mutagenic and anti-oxidative DNA damage activities. However, *in vivo* studies are needed to better assess the use of this plant.

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Antioxidant capacity of indigenous plant extracts from Ban Ang-Ed Official Community Forest Project (The Chaipattana Foundation) at Chantaburi Province

Chatchawin Petchlert[*a], Sureechai Wongla[a], Jareewan Phumphinicha[a]
[a]Department of Biochemistry, Faculty of Science, Burapha University, Chon Buri, Thailand 20131

[*]chatchaw@buu.ac.th phone +66-38103151 fax +66-38393495

ABSTRACT

In this study, the antioxidant activities of water extracts from indigenous plants including *Barrington augusta* Kurz. (Jig) leaves, *Syzygium gratum* (Wight) S.N. Mitra var. gratum (Sa-med-dang) leaves, *Cissus hastate* Miq. (San-dan-ban) leaves, *Lepisanthes fruticosa* (Roxb) Leenh. (Cham-ma-leang) leaves, *Sauropus amabilis* Airy Shaw (Pak-wan) leaves and *Brucea javanica* (L.) Merr. (Pya-dab-hak) seeds were determined the ferrous-ion chelating, ABTS radicals decolorization assay and ferric ion reducing antioxidant power (FRAP). The results showed that Jig leaves expressed the highest iron chelating effect followed by Pya-dab-hak seeds, San-dan-ban leaves and Sa-med-dang leaves (99.18%, 96.92%, 95.37% and 91.85%, respectively) with IC_{50} of Jig, Pya-dab-hak, San-dan-ban and Sa-med-dang by 0.08, 0.34, 1.80, and 0.34 mg/ml, respectively. Moreover, Jig leaves at 5 mg/ml also expressed the highest TEAC value (72.41±0.001 µM trolox equivalent/mg extract). In addition, San-dan-ban leaves showed the highest FRAP value by 16.70 ± 0.02 mM/mg extract. In conclusion, we observe that extracts of indigenous plants from Ban Ang-Ed Official Community Forest Project at Chantaburi province possess good antioxidant capacities that probably promote the better health.

Keywords: ABTS, ferrous-ion chelating, FRAP, Indigenous plants

1. INTRODUCTION

The oxidation of macromolecules is a major cause of deterioration in the food quality and the reduction in nutritional value [1]. Moreover, this condition elicits cellular toxicity of various targets such as DNA, proteins and lipids. In human, reactive oxygen species-induced cellular damage has been implicated in the development of the aging process, several chronic degenerative diseases including the inflammation, cardiovascular disease and cancers [2]. Indigenous plants usually contain several hundred different types of antioxidants that may directly react with reactive oxygen or nitrogen species forming products with much lower reactivity. Alternatively, compounds in a plant-based diet may increase the capacity of the endogenous antioxidant defenses by modulating genes related to antioxidant and cytoprotective enzymes [3]. However, knowledge of the potential antioxidant compounds present in medicinal plants still confounds in many aspects. In Thailand, various researchers attempt to investigate such activities in medicinal plants as well. In this study, the antioxidant capacities of water extracts from indigenous plants including *Barrington augusta* Kurz. (Jig) leaves, *Syzygium gratum* (Wight) S.N. Mitra var. gratum (Sa-med-dang) leaves, *Cissus hastate* Miq. (San-dan-ban) leaves, *Lepisanthes fruticosa* (Roxb) Leenh. (Cham-ma-leang) leaves, *Sauropus amabilis* Airy Shaw (Pak-wan) leaves and *Brucea javanica* (L.) Merr. (Pya-dab-hak) seeds were investigated.

2. MATERIALS AND METHODS

Sample preparation and extraction

Indigenous plants from Ban Ang-Ed Official Community Forest Project including *Barrington augusta* Kurz. (Jig), *Syzygium gratum* (Wight) S.N. Mitra var. gratum (Sa-med-dang), *Cissus hastate* Miq. (San-dan-ban), *Lepisanthes fruticosa* (Roxb) Leenh. (Cham-ma-leang), *Sauropus amabilis* Airy Shaw (Pak-wan) and *Brucea javanica* (L.) Merr. (Pya-dab-hak) were cleaned and chopped in small pieces, then they were dried in hot air oven at 40^0C. They were ground well. Distilled water were added in 1:10 (w/v) and boiled for 30 min. After that, each extract was filtered. Repeated this step 3 times, then the filtrate was centrifuged at 3,000 rpm for 5 min. the supernatant was collected and filtered with Buchner funnel. The extract was concentrated by the rotary evaporator, and then kept at -80°C before lyophilization. After that, all extracts were kept at -20°C until use.

Ferrous-ion chelating activity

The chelation of ferrous ions by the extracts was estimated by the method of Dinis et al. [4]. Briefly, standard EDTA was dissolved in water to prepare in different concentrations. 200µL EDTA or extract was mixed with distilled water, and then 2mM $FeCl_2$ 10 µL was added. 5mM Ferrozine was administered for 20 µL. The mixture was allowed to stand at room temperature for 5 min. Absorbance at 562 nm was conducted by the microplate reader. The results were calculated as %chelating from [A-(B-C)]/A x 100 where A = the absorbance of control, B = the absorbance of EDTA and C = the absorbance of extract. IC_{50} was also expressed for this activity.

ABTS assay

An improved ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) radical cation decolorization assay was used. It involves the direct production of the blue/green $ABTS^{+\bullet}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of antioxidants to the preformed radical cation reduces it to ABTS, to an extent and on a timescale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction [5]. ABTS was diluted with phosphate buffer, pH 7.4 to read $A_{734} = 0.70 \pm 0.02$. The extracts were dissolved with 70% ethanol at different concentrations. 1 ml of prepared ABTS was added. The reaction was incubated for 6 min. The absorbance at 734 nm was measured. The results were shown as Trolox equivalent antioxidant capacity (TEAC).

Ferric reducing antioxidant power (FRAP assay)

The ability to reduce ferric ion was determined using a modified method described by Benzie and Strain [6]. 1mg extract in 1mL water was mixed with 1 mg/mL FRAP reagent. Mixed well and incubated in the dark for 30 min. The absorbance at 596 nm was recorded, calculated and then expressed as FRAP value.

Statistical analysis

All assays were realized in triplicate. The difference of each group was statistically analyzed by one-way ANOVA at *P* value < 0.05 by SPSS version 13.0 for windows.

3. RESULTS

% Yields of each extract were shown in Table 1. *Sauropus amabilis* Airy Shaw or Pak-wan leave extract possessed the greatest yield (35.44%) compared to other extracts.

Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radical and hydroperoxide decomposition reactions via Fenton chemistry [6]. These processes can be delayed by iron chelation and deactivation. Therefore, the ability of the extracts to chelate Fe (II) ion was evaluated and expressed as % chelation capacity. Figure 1 displays the metal chelating power of different indigenous plant extracts. It is clear that this activity of Jig leaves, Pya-dab-hak seeds and Sa-med-dang leaves was higher as compared to the other three samples. From IC_{50} values in Table 2, Jig leave extract markedly exhibits the iron chelating ability close to EDTA, the standard chelating agent.

Determination of the antioxidant capacity by ABTS assay of various extracts was carried out the next approach in this study. The trolox equivalent antioxidant capacity (TEAC) values for the extracts are shown in Table 3. All extracts were able to reduce the $ABTS^{+\bullet}$ radical, particularly Jig, Sa-med-dang, San-dan-ban and Cham-ma-leang leaves were found to be apparently most active than Pya-dab-hak seeds and Pak-wan leave extracts. The result indicated that H atoms of hydroxyl groups in these indigenous plants were readily abstracted by N-centered radical. Zhao and Liu [7] previously described that H atoms in *ortho*-hydroxyl groups of many phytochemicals could form an intramolecular hydrogen bond that hindered the H atom in hydroxyl group to be abstracted by radicals.

The ferric reducing antioxidant potential (FRAP) assay is based on the reducing power of a compound. It measures the reduction of Fe^{3+} to Fe^{2+}, the values in the FRAP assay express the corresponding concentration of electron donating antioxidants in test samples. As shown in Table 3, the highest reducing ability was found in San-dan-ban leave extract followed by Sa-med-dang leaves, Pak-wan leaves, Jig leaves, Cham-ma-leang leaves and Pya-dab-hak seeds, respectively.

Table 1. % Yield of indigenous plant extracts used in this study

Sample	% yield
Sauropus amabilis Airy Shaw leaves (Pak-wan)	35.44
Barrington augusta Kurz. leaves (Jig)	27.85
Brucea javanica (L.) Merr. seeds (Pya-dab-hak)	16.10
Syzygium gratum (Wight) S.N. Mitra var. gratum leaves (Sa-med-dang or Mek)	12.17
Lepisanthes fruticosa (Roxb) Leenh. leaves (Cham-ma-leang)	15.55
Cissus hastate Miq. leaves (San-dan-ban)	18.50

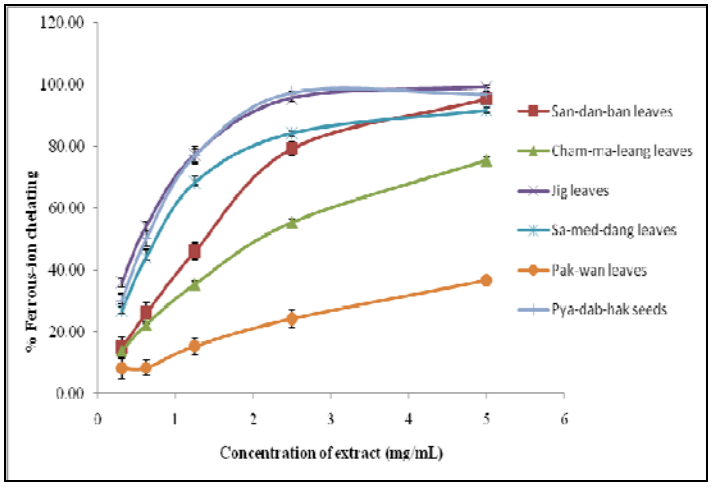

Figure 1. Ferrous-ion chelating activity of different indigenous plant extracts from Ban Ang-Ed Official Community Forest Project (The Chaipattana Foundation) at Chantaburi province.

Table 2. Antioxidant activity of various plant extracts observed with IC_{50} of ferrous-ion chelation.

Sample	IC_{50} (mg/mL)
Cham-ma-leang leaves	2.68
San-dan-ban leaves	1.80
Pya-dab-hak seeds	0.34
Jig leaves	0.08
Sa-med-dang leaves	0.88
Pak-wan leaves	6.98
EDTA	0.03

Table 3. ABTS scavenging activity and FRAP values of various plants extracts.

Sample	TEAC (μM Trolox equivalent/mg extract)	FRAP value (mM/mg extract)
Pya-dab-hak seeds	63.75 ± 0.001	1.22 ± 0.054
Sa-med-dang leaves	72.90 ± 0.002	15.61 ± 0.026
San-dan-ban leaves	72.59 ± 0.001	16.70 ± 0.016
Jig leaves	74.21 ± 0.001	8.003 ± 0.022
Pak-wan leaves	63.55 ± 0.002	15.38 ± 0.051
Cham-ma-leang leaves	72.41 ± 0.001	2.42 ± 0.003

4. CONCLUSIONS

The results show that *Barrington augusta* Kurz. or Jig leave extract is the most effective either great chelating ability or radical scavenging activity. In addition, *Cissus hastate* Miq. (San-dan-ban) leave extract expresses the maximum FRAP value; it corresponds to be a good antioxidant for electron donation to the other molecules. Conclusively, the extracts of indigenous plants from Ban Ang-Ed Official Community Forest Project (The Chaipattana Foundation) at Chantaburi province possess good antioxidant capacities that probably promote the better health.

ACKNOWLEDGEMENTS

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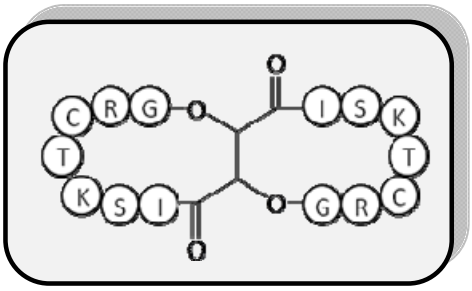
Anti-Asthmatic property of derived bivalent SFTI-1 inhibitor

Kittinat Saiphruk[a], Puthon Kraisuriyawong[a], Yuttana Senpradit[a], Panumart Thongyoo*[a]
[a]Department of Chemistry, Faculty of Science and Technology, Rangsit Campus Thammasat University, Pathumthani, Thailand 12120

*tpanumas@tu.ac.th phone +66-59410465 fax +66-25644483

ABSTRACT

Asthma is one of the most serious allergic and common chronic diseases caused by many factors ranging from exhaust gas, pollen grain to chemical irritation. Tryptase is the most abundant secretory granule-derived serine proteinase found in mast cells whereas its structure is composed of four equivalent monomers arranged in a square ring. Tryptase is released once mast cell is activated by certain allergic and inflammatory disorders. Presently, a various bivalent inhibitors have been applied for targeting tryptase. SFTI-I (sunflower trypsin inhibitor-1), composed of 14 amino acids together with one disulfide bond, is an example of tryptase inhibitor. The aim of this project is to explore the synthetic approach of bivalent SFTI-I inhibitor in which its structure was comprised of cyclic moiety and two inhibitory loops, resulting in the increase of both stability and inhibitory activity against tryptase. To our bivalent derived SFTI-I synthetic strategy, tartaric acid has been applied as the core of bivalent SFTI-I inhibitor. According to SFTI-I inhibitory activity against tryptase, bivalent derived SFTI-I inhibitor could become an important candidate of targeting tryptase. Importantly this synthetic approach could be one of the most challenging synthetic strategies for the synthesis of human-β-tryptase inhibitor.



Keywords: Bowman-Birk protease inhibitor, Sunflower trypsin inhibitor-1 (SFTI-1), Bivalent inhibitor

1. INTRODUCTION

Protease inhibitors are important molecules capable of regulating the proteolytic activity of their target proteases, and widely found in plants, microorganisms and animals. A large number of protease inhibitors have been isolated, and identified to date. Notably, newly discovered protease inhibitors have been increasingly isolated, ranging across cysteine, serine, aspartyl and metallo protease inhibitors. Serine protease inhibitors have been the great majority of protein-based inhibitors known and characterized to date. They are distinguished according to the sequence similarity, mechanism of binding, the topological similarity, disulfide pattern and the three dimensional structure. Presently, at least eighteen different families have been categorized. These serine protease inhibitors usually interact with enzymes according to the substrate-like standard mechanism. Of particular interest to the work in this project, the Bowman-Birk inhibitor, namely sunflower trypsin inhibitors-1 (SFTI-I) [1] in which two inhibitory loops were introduced, the so-called bivalent SFTI-I inhibitor was synthesized and this bivalent SFTI-Is could potentially be applied as an anti-asthmatic agent [2, 3].

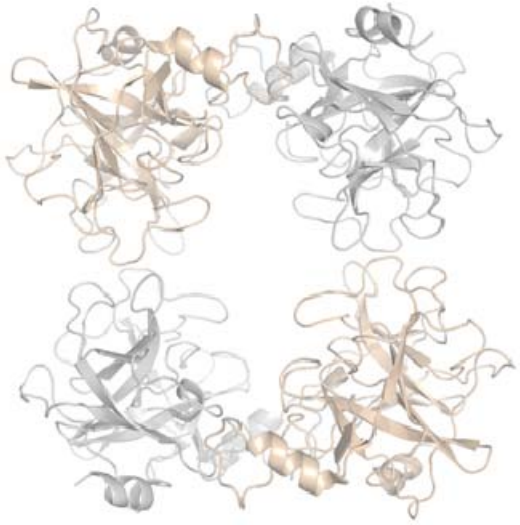


Figure 1**.** The structure of human beta-tryptase tetramer.

2. MATERIALS AND METHODS

General Experimental Procedure

The NMR spectra were recorded in $CDCl_3$ using BRUKER-NMR 400MHz spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR using TMS as internal standard, and chemical shifts were expressed in δ values. Analytical and preparative TLCs were carried out on pre-coated silica gel 60F254 and RP-18F254 plates (Merck, 0.25 or 0.50 mm thickness).

The Attachment of first amino acid on 2-chlorotrityl chloride resin

Fmoc-Thr-OH (1.4 mmol, 2 eq.) was dissolved in dichlorimethane (DCM, 5 mL), followed by the addition of DIPEA (2.8 mmol, 4 eq.). To this solution was added by 2-Chlorotrityl resins (0.7 mmol) under nitrogen atmosphere and was stirred the reaction mixture for 120 min. The resin was washed with dimethylformamide (DMF, 3 × 1 mL), DCM (3 × 1 mL) and diethyl ether (3 × 1 mL), respectively. The resin was dried *in vacuo* and the loading of resin was estimated by Fmoc test. The % loading was found to be approximately 35%.

The synthesis of linear peptide chain *via* solid phase peptide synthesis (SPPS)

After the first amino acid loading, the resin was swelled in DMF for 60 min and then drained off. Acetic anhydride, DIPEA and DMF were added to the resin. The mixture was shaken at room temperature for 60 min. The solution was filtered off. The capped resin was washed with DMF (3 × 1 mL) and DCM (3 × 1 mL). The Fmoc deprotection was carried out by 20% piperidine in DMF for 30 min. The resin was drained off and washed with

DMF (3 × 1 mL) and DCM (3 × 1 mL), following by the coupling of Fmoc-Lys(Boc)-OH to the resin. 1-Hydroxybenzotriazole hydrate (HOBT), Fmoc-Lys(Boc)-OH, 4-Dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were added to the resin and the reaction was shaken for 120 min, followed by washing with DMF (3 × 1 mL) and DCM (3 × 1 mL), respectively. The resin was deprotected by 20% piperidine in DMF (deprotection step) and coupled with Fmoc-Ser(^{t}Bu)-OH in the presence of HOBT, DMAP and DCC and the reaction was shaken for 120 min, following by washing with DMF (3 × 1 mL) and DCM (3 × 1 mL). The resin was deprotected by 20% piperidine in DMF and coupled with Fmoc-Ile-OH in the presence of HOBT, DMAP and DCC. The reaction was shaken for 120 min, followed by washing with DMF (3 × 1 mL) and DCM (3 × 1 mL). The resin was dried and kept at 0°C. Fully protected peptide was cleaved by treating with 1% TFA in DCM for 2 min, followed by draining off and rinsing with a small amount of 1% TFA in DCM. The filtrate was evaporated to dryness under the reduced pressure.

The synthesis of tartaric acid derivatives

The synthesis of dimethyl tartrate

Tartaric acid, methanol and a small amount of p-toluenesulfonic acid (*p*-TsOH) were added to round bottom flask. The reaction was refluxed overnight and subsequently purified by silica gel column chromatography to afford dimethyl tartrate (5.23g, 89 %). ^{1}H-NMR (400MHz, $CDCl_3$) δ 3.69 (s, 3H), 4.54 (s,1H).

The synthesis of Fmoc-Gly-Dimethyl tartrate

Fmoc-Gly-OH was activated in the form of HOBt ester by mixing Fmoc-Gly-OH (5 eq., 125 μmol) with the mixture of HOBt/HBTU (125 μmol), and allowed the reaction for 45 min. To the reaction mixture was added by tartaric acid (62.5 μmol), and allowed the reaction for 3 hrs. The reaction mixture was quenched by the addition of water, and separated by column chromatography to yield a peptidyl tartaric acid (Fmoc-Gly-OH). Fmoc-Gly-tartaric acid was deprotected by the addition of 20% piperidine in DMF, and the reaction mixture was allowed for 3 hrs, worked up and separated by column chromatography to yield Fmoc-Gly-dimethyl tartrate (73.6 mg, 17%).^{1}H-NMR (400MHz, $CDCl_3$) δ 3.82 (s, 3H) 4.21 (t, 2H) 4.35 (t, 1H) 4.42 (d, 2H) 5.81 (s, 1H) 7.35-7.49 (m, 4H) 7.62 (d, 2H) 7.79 (d, 2H).

The synthesis of Fmoc-Arg(Pbf)-Gly-Dimethyl tartrate

The Fmoc deprotection was carried out with 20% piperidine in DMF for 30 min. Fmoc-Arg(Pbf)-OH was activated in the form of HOBt ester by the addition of Fmoc-Arg(Pbf)-OH (5 eq., 125 μM) to the mixture of HOBt/HBTU (125 μmol) and DIPEA (125 μmol), and the reaction was allowed for 45 min. To the reaction mixture was added by NH_2-Gly-tartaric acid (62.5 μmol) which was dissolved in DMF, and the reaction was allowed for 3 hrs. The reaction mixture was quenched by the addition of water, and separated by column chromatography to afford Fmoc-Arg(Pbf)-Gly-Dimethyl tartrate (86.5 mg, 55%). ^{1}H-NMR (400MHz, $CDCl_3$) δ 1.0-1.7 (m, 22H), 1.9 (s, 2H), 2.1 (d, 3H), 2.55 (d, 6H), 3.0 (s, 2H), 3.4 (t,1H), 4.15 (q, 1H), 4.22 (t, 2H), 4.44 (s, 2H), 4.6 (d, 1H), 5.5 (s, 1H), 7.15-7.45 (m, 4H), 7.59 (d, 2H) , 7.77 (d, 2H) , 7.9 (d, 1H).

The synthesis of Fmoc-Cys(trt)-Arg(Pbf)-Gly-Dimethyl tartrate

The Fmoc deprotection was carried out by 20% piperidine in DMF for 30 min. Fmoc-Cys(trt)-OH was activated in the form of HOBt ester by the addition of Fmoc-Cys(trt)-OH (5 eq., 125 μM) to the mixture of HOBt/HBTU (125 μmol) in the presence of DIPEA (125 μmol), and the reaction was allowed for 45 min. To the reaction mixture was added by NH_2-Gly-tartaric acid (62.5 μmol) which was dissolved in DMF, and the reaction was allowed for 3 hrs. The reaction mixture was quenched by the addition of water, and separated by column chromatography to afford Fmoc-Cys(trt)-Arg(Pbf)-Gly-Dimethyl tartrate (22.7 mg, 31%). ^{1}H-NMR (400MHz, $CDCl_3$) δ 1.90-2.25 (m, 4H), 2.30-2.40 (m, 1H), 2.41-2.65 (m, 2H), 2.95-3.20 (m, 3H), 3.40-3.70 (m, 3H), 4.15 (q,1H), 4.22 (t, 2H), 4.35-4.45 (m, 2H), 5.15 (t, 1H), 5.7 (d, 2H), 7.10-7.50 (m, 26H), 7.63 (d, 2H), 7.79 (d, 2H).

The ligation of Fmoc-Cys(trt)-Arg(Pbf)-Gly-Dimethyl tartrate with Fmoc-Ile-Ser(tBu)-Lys(Boc)-Thr(tBu)-OH

Fmoc-Cys(trt)-Arg(pbf)-Gly-dimethyl tartrate was deprotected by 20% piperidine in DMF, stirred for 30 min and dried under reduced pressure. Fmoc-Ile-Ser(tBu)-Lys(Boc)-Thr(tBu)-OH was activated with a solution of HOBT, HBTU and DIPEA in DMF and the reaction was stirred at room temperature for 60 min. To this solution was added by H-Cys(trt)-Arg(Pbf)-Gly-dimethyl tartrate in DMF and the reaction was allowed at room temperature overnight. The reaction was dried *in vacuo* and purified by sephadex LH-20 column chromatography to afford Fmoc-Ile-Ser(tBu)-Lys(Boc)-Thr(tBu)-Cys(trt)-Arg(Pbf)-Gly-Dimethyl tartrate (3.2 mg, 11.8%). HPLC analysis: Retention time 2.289.

The cyclization of bivalent peptide inhibitor

The ligated peptide was deprotected by 20% piperidine in DMF for 30 min. The ligated peptide was dried *in vacuo* and subsequently dissolved in DMF. To this solution was added by DIPEA. A solution of 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU) in DMF

was added by syringe pump for 12 hrs. The reaction mixture was stirred overnight at room temperature. The reaction was dried *in vacuo* and purified by sephadex LH-20 column chlomatography to afford bivalent SFTI-I inhibitor (0.1 mg, 1%). HPLC analysis: Retention time 2.551. Molecular weight (M.W.): Calcd $[M + H]^+$ 1608.1. Found: 1608.3.

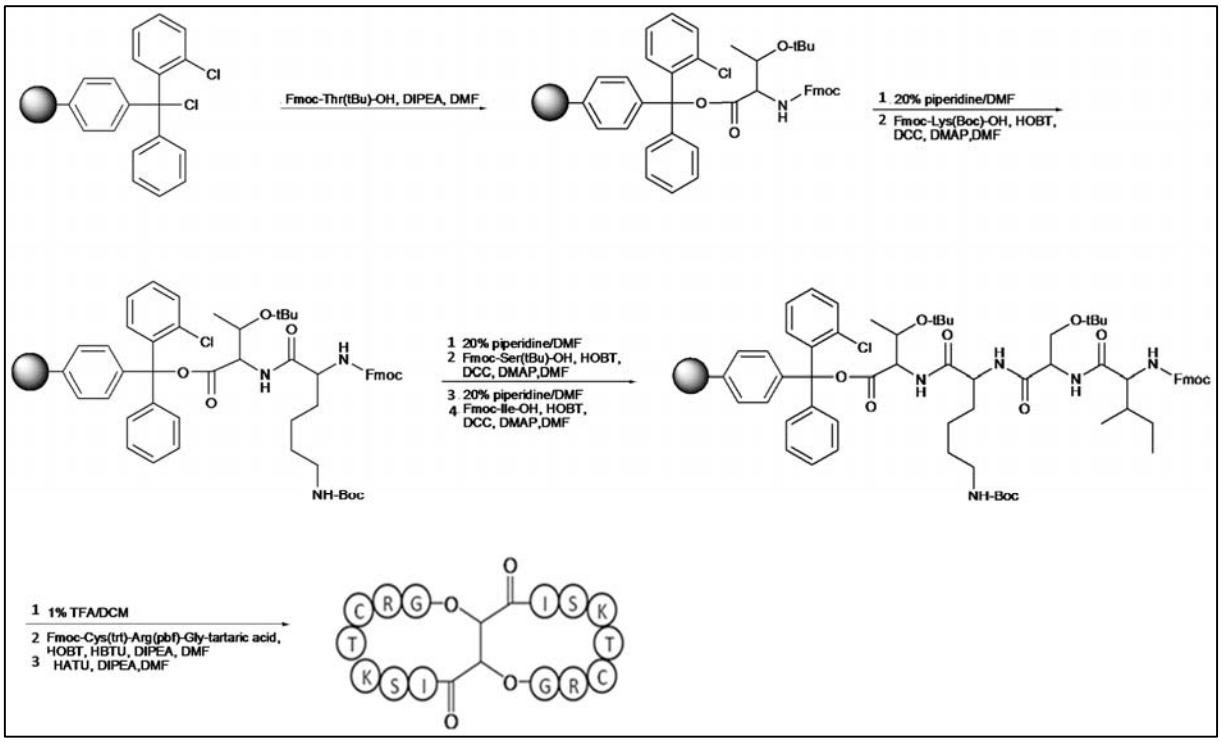


Figure 2. The synthesis of bivalent SFTI-1 inhibitor.

3. RESULTS

The bivalent SFTI-1 inhibitor was successfully synthesized by using tartaric acid applied as a core to generate a bivalent motif. To our synthetic strategy, both solution and solid phase peptide synthesis were employed. For the solution phase, dimethyl tartrate was firstly synthesized by esterification reaction in order to protect both carboxyl groups which can then be cleaved under mild basic condition. The resulting dimethyl tartrate was subsequently coupled with Fmoc-Gly-OH by using DCC as a coupling agent to afford Fmoc-Gly-dimethyl tartrate. Interestingly, monomer of Fmoc-Gly-dimethyl tartrate was also observed as a minor product. Furthermore, Fmoc-Gly-dimethyl tartrate was coupled with Fmoc-Arg(Pbf)-OH, Fmoc-Cys(trt)-OH, respectively to yield Fmoc-Cys(trt)-Arg(Pbf)-Gly dimethyl tartrate with overall approximately 12%. It was found that the coupling efficacy was greatly depended on the steric hindrance from both amino acid side chain and the size of Fmoc protecting group which in turn affected the observed yield. To the solid phase peptide synthesis part, Fmoc-Thr(tBu)-OH was firstly loaded to Cl-trityl resin and further coupled with other amino acids as shown in Figure 2 by using standard Fmoc-base SPPS and HOBT and HBTU used as coupling agent. The peptide was subsequently cleaved by using 1% TFA/DCM to afford the resulting peptide product with relatively good yield (80%), which was coupled with Fmoc-Cys(trt)-Arg(Pbf)-Gly tartaric acid, followed by Fmoc deprotection with 20% piperidine to give the unprotected linear precursor cyclization which can then be cyclized by using HATU as a coupling agent in very high dilution to avoid intramolecular coupling between two ligated peptides and following by side chain deprotection (80%TFA) to afford the cyclized product with overall yield approximately 1%. The mass analysis confirmed the molecular mass of bivalent SFTI-I inhibitor. This bivalent SFTI-I inhibitor will be further assessed the biological activity against tryptase, which is protease related to asthma.

4. CONCLUSIONS

We achieved the synthesis of bivalent SFTI-1 inhibitor in which tartaric acid was applied as a core to generate a bivalent motif. To this approach, both solution and solid phase peptide synthesis were used. This bivalent SFTI-I will be further examined its biological activity against human-beta tryptase.

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Subchronic toxicity test of quercetin and cloxacillin in mice

Griangsak Eumkeb[a], Aphai Duangkhamb[b], Tanaporn Hengpratom*[a]
[a]School of Pharmacology, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang District Nakhon Ratchasima, Thailand 30000
[b]School of Biology, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang District Nakhon Ratchasima, Thailand 30000

*Minneymouse_1010@windowslive.com phone +66-44224273 Fax +6644224650

ABSTRACT

Quercetin is a naturally occuring flavonol, which is classified in flavonoid family. It occurs ubiquitously in the normal human diet and also exhibits numerous pharmacological properties. However, in vivo toxicity test of quercetin has only few reports. Thus, the purpose of this study is to investigate the subchronic toxicity of quercetin alone and in combination with cloxacillin antibiotic in vivo. The mice were administered intraperitoneally (i.p.) with quercetin either alone at 20 and 40 mg/kg BW/day (group 1 and group 2 respectively) or in combination with cloxacillin at 20 plus 150 (group 3) and 40 plus 300 mg/kg BW/day (group 4) of quercetin plus cloxacillin, respectively twice daily for 90 days. At the end of the experiments, blood and the selected main organs were collected for haematological and histological analysis. There was no significant difference in either the growth rate measured by living body weight or the relative weight of the selected main body organs of mice treated with all doses of either quercetin alone or in combination with cloxacillin, when compared to control. The histology of the liver, spleen, heart, kidney and stomach all exhibited a normal appearance in comparison with the control. Results of blood haematological and chemistry marker indicated that there was no significant changes of RBC, Hb, Hct WBC, MCV AST, BUN, FBS, and Uric acid levels between pre- and post-treatment and compared to control in all groups ($p<0.05$). However, cholesterol level exhibited a significant reduction in the group treated with 20 plus 150 mg/kg BW/day of quercetin plus cloxacillin ($p<0.05$).

Keywords: Quercetin, Cloxacillin, Subchronic toxicity, Haematological, Histological, Mice

1. INTRODUCTION

Bacterial resistance to antibiotics is a serious global problem [1]. New approaches to resolve this problem is needed. Active compounds from medicinal plants have long been isolated to use for multipurpose, including antibiotic purpose.

Flavonoids are polyphenolic compounds generally found in fruit, vegetables, gains, bark, stems, roots, wine, and tea. They are considered as integral components in diets. More than 4,000 varieties of flavonoids have been identified. It is widely accepted that the high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of coronary heart disease and also inhibited the proliferation of various tumor growth. Furthermore, epidemiologic studies suggested a protective role of dietary flavonoids against cancer induction in several human tissues, including colon, lung, prostate and urinary bladder [2]. In addition, the combination of β-lactams (ampicillin, cloxacillin and ceftazidime) with flavonoids (baicalein, galangin and quercetin) showed potential activity against drug-resistant bacterial strains [3].

It is very well established that quercetin is chemically related to a class of flavonoids called (pro) anthocyanins. Quercetin has been indicated from pharmacological studies to possess antihistamine, anti-inflammatory, antiallergic, antiviral properties. Quercetin was also active against *Bacillus cereus* when present in the amount of 2.5 μg/ml [4]. Furthermore, quercertin has shown to cause chromosomal mutations in certain bacteria in *in vitro* study. It was reported that quercetin at the dose of 0.33 mg/kg can protect liver cells and mitochondria from oxidative stress by maintaining normal levels of serum transaminases and preventing lipid peroxidation [5].

Although antibiotics in combination with active compounds isolated from medicinal plants are considered as the new approach for overcoming various resistant bacteria, *in vivo* toxicity test of the some flavonoids, including quercetin, have not been investigated. Therefore, the aim of this study was to investigate subchronic toxicity tests of quercetin alone and in combination with antibiotic, cloxacillin *in vivo*.

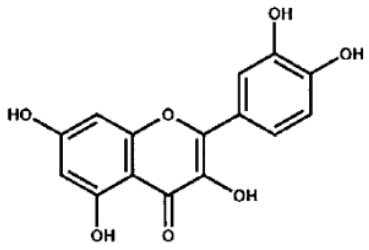

Figure1. Chemical structure of quercetin

2. MATERIALS AND METHODS

Chemicals

Quercetin and Cloxacillin were purchased from Indofine Chemical Company (New Jersy, USA) and dissolved in Normal Saline Solution (NSS) for toxicity test.

Animals preparation

Male and female mice (ICR mouse), 7 ± 1 weeks of age, were purchased from National Laboratory Animal Centre, Mahidol University, Thailand. The animals were housed in stainless cages, in a light- and temperature-controlled room (light on 06.00-20.00 h) temperature 25 ± 0.5°C at the Animal Care Building at Suranaree University of Technology, Nakhon Ratchasima, Thailand. Mouse chow diet (Pokaphan Animal Feed Co., Ltd, Bangkok, Thailand) and water were provided ad libitum. The experimental protocol was approved in accordance with guideline for the care and use of laboratory animal by Animal Care and Use Committee (ACUC), Suranaree University of Technology.

Subchronic toxicity study in mice

Fifty adult mice of body weight 28-40 g were divided into five groups. Ten mice per group (five males and five females) were classified into group one to four including control. The control group was injected with 0.9 % NaCl intraperitoneally (i.p.) twice a day. The tested one to four groups were administered intraperitoneally (i.p.) with quercetin alone at 20 (group 1) and 40 mg/kg BW/day (group 2), quercetin plus cloxacillin at 20 plus 150 (group 3) and 40 plus 300 mg/kg BW/day (group 4), respectively twice a day for three months. These mice were analyzed the blood composition before and after sample injection. Their body weights were recorded weekly.

Haematology and blood chemistry

At the end of the experiments, blood samples were collected by tail under thiopental sodium anesthesia from 9.00 to 10.00 a.m. and were partly used for haematology. From the remainder blood serum was prepared by centrifugation at 1000 x g for 30 min and kept at -20°C for blood chemistry analysis, including fasting blood sugar (FBS), cholesterol, aspartate aminotransferase (AST), blood urea nitrogen (BUN) and uric acid. The assays were employed with automated analytical systems at the centre for medical and public health service, Suranaree University of Technology. The replications of serum from each of the treated groups and control group were run at the same analysis.

Necropsy

After the blood sampling, the mice were sacrificed under thiopental sodium anesthesia and subjected to necropsy. The heart, liver, spleen, lung, kidney, and stomach were removed and weighed. Body weight measured on the day of necropsy was used to calculate the relative organ weight. All organs were preserved in 10% (w/v) neutral phosphate buffer formaldehyde. Heart, liver, spleen, lung, kidney, and stomach fixed-tissue were embedded in paraffin and prepared for microtome sectioning at 5 µm. Haematoxylin and eosin were used for staining. The pathohistology of the organ tissue slides were examined under light microscope.

Statistical analysis

All data are presented as the mean ± S.E.M. Significant differences between the relative selected organ weight or body weight of control and treatment groups were analyzed by ANOVA. The difference of haematology, blood chemistry, growth rate analysis between pre- and post- treatment groups were calculated by *paired student's t-test*. Then, significant difference between each group was compared using ANCOVA. The Tukey HSD post hoc test at $p < 0.05$ and $p < 0.01$ were also considered statistically significant difference between each group.

3. RESULTS

Animal growth and histology analysis

There was no significant difference in either the growth rate measured by living body weight or the relative weight of the selected main body organs of all groups of treated mice for 90 consecutive days, when compared to the control (Figure 2). The pathohistology of all organs showed normal appearance compared to the control organs (data not shown).

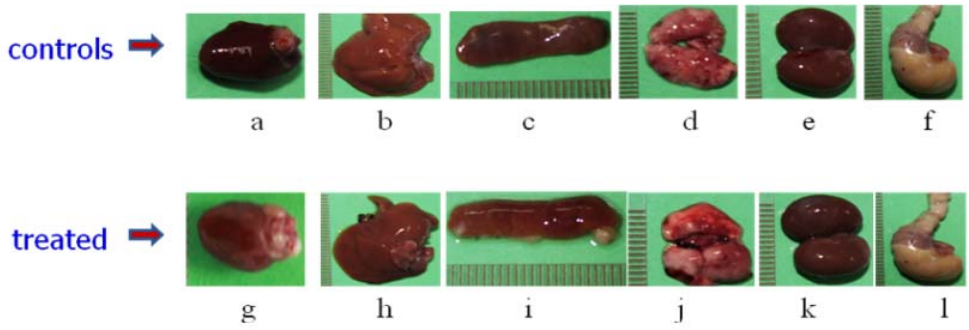

Figure2. Morphology of main body organs of mice treated with quercetin alone and in combination with cloxacillin for 90 consecutive days in comparison with the negative control. Control group; a = heart; b = liver; c = spleen; d = lung; e = kidney; f = stomach. Treated group; g = heart; h = liver; i = spleen; j = lung; k = kidney; l = stomach

Haematology and blood chemistry

Subchronic toxicity study indicated that there was no significant changes of AST, BUN, FBS, Uric acid, WBC, RBC, Hb, Hct, MCV levels between either pre and post treatment in all groups or between all treated groups compared to control ($p < 0.05$) (data not shown). Cholesterol level exhibited a significant reduction in the post-treated group with 20 mg/kg BW/day quercetin plus 150 mg/kg BW/day cloxacillin compared to pre-treated ($p < 0.05$). Also, other post-treated groups displayed cholesterol level lower than pre-treated groups. However, these levels were not significant difference compared to control ($p < 0.05$) (Figure 3).

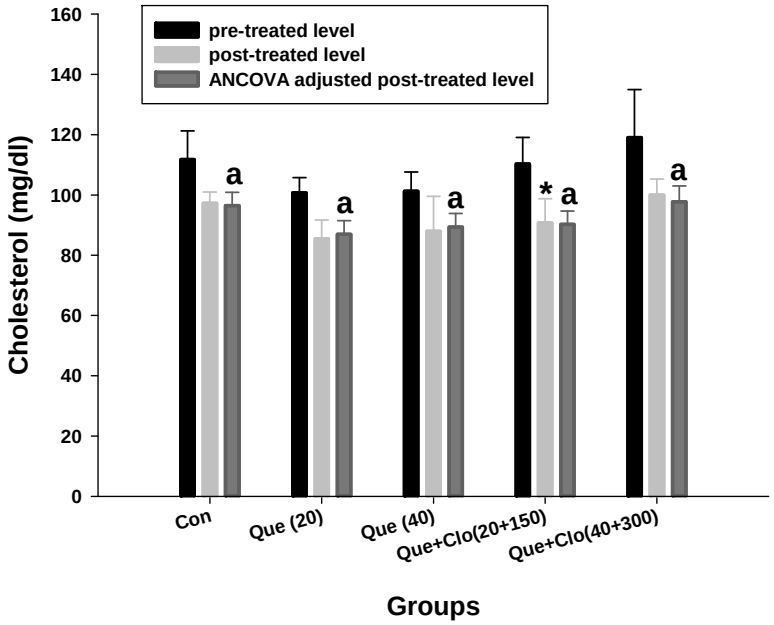

Figure 3. Effects of quercetin, cloxacillin either alone or in combination of mice. Con = control, Que (20) = Quercetin 20 mg/kg BW/day, Que (40) = Quercetin 40 mg/kg BW/day, Que+Clo (20+150) = Quercetin 20 mg/kg BW/day plus Cloxacillin 150 mg/kg BW/day, Que+Clo (40+300) = Quercetin 40 mg/kg BW/day plus Cloxacillin 300 mg/kg BW/day. Significant difference between pre- and post-test in each group was compared using paired student t-test at * $p<0.05$. Significant difference between ANCOVA adjusted post-treated level in each group was compared using ANCOVA and Tukey HSD post hoc test at ^{a} $p < 0.05$.

4. CONCLUSIONS

In conclusion, these findings lead us to believe that quercetin alone and in combination with cloxacilin at these concentrations show no toxicity with blood chemistry, hematology and main organs in mice. Interestingly, these flavonoids provide evidence that its can reduce cholesterol in mice blood when these are taken at high dose for long duration. This study provides essential information for further investigation in other higher mammals, including human.

ACKNOWLEDGEMENTS

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Stimulation of dermal fibroblast collagen synthesis *in vitro* by saponin enriched extract from soybeans

Sarunya Laovitthayanggoon*[a], Siripen Jarikasem [a], Sarinthip Muensaen [a]
[a]Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand 12120

*sarunya@tistr.or.th phone +66-25779094 fax +66-25779110

ABSTRACT

Soybean (*Glycine max* L.) is a species of legume native to East Asia, widely grown for its edible bean which has numerous uses. It has been used as a source of human food of high quality protein and other nutrients for hundreds of years. Recently, soyasaponin, one of the significant bioactive constituents from soybeans, has received more attention to be used in skin care cosmetic products due to its relevant beneficial properties including antioxidation, anti-inflammation and water retention. Previous works have also reported anti-wrinkle property of certain saponins of which effect on collagen synthesis is of interest. In this study, saponin enriched extract from soybeans prepared by use of Diaion HP-20 macroporous resin was investigated for *in vitro* stimulating effect on type 1 collagen synthesis. The human dermal fibroblasts cell line (ATCC CRL-1744) was treated with three different concentrations (50, 100, 200 µg/ml) of the extract for 24 h and then the amount of type 1 collagen was measured using ELISA test kit. The results revealed that the percentage of stimulating effect on collagen synthesis after saponin enriched extract treatment were 8.08 ± 1.23 and 21.12 ± 1.54 at concentrations 100 and 200 µg/ml, respectively while that of a positive control ascorbic acid at 50 µg/ml was 31.08 ± 0.28. The present study demonstrates the collagen synthesis stimulating potential of soysaponins and provides a possibility to develop as anti skin aging agent in cosmetic products.

Keywords: Soybean, Saponin, Human dermal fibroblast, Collagen synthesis

1. INTRODUCTION

Skin aging has been suggested to be associated with a decrease in dermis collagen content which clinically manifested on wrinkle and laxity. Collagen deficiency is partly arisen from its reduced synthesis [1]. Presently, substances which possess stimulatory effect on collagen synthesis have received more attention to be used for skin anti-aging cosmetic preparations. Currently, several plant-derived saponins have been reported to possess collagen synthetic property including ginsenoside [2], asiaticoside [3] and astragaloside [4]. *Glycine max* L. (Legumonosae) or soybean is a well known edible bean crop which has been used as a source of human food of high quality protein and other nutrients for hundreds of years. Moreover soybeans have been reported to contain about 0.17-6.16% of saponins [5]. Interestingly, beneficial effects for skin care of soysaponins have been demonstrated including antioxidation [6] and anti-inflammation [7]. In order to obtain scientific evidence to support a use of soyasaponins for aging skin treatment targeting on collagen alteration, we investigated on its collagenesis enhancing effect in dermal fibroblast monolayer. Fractionation of saponins was performed by column chromatographic technique using Diaion HP-20 adsorptive resin.

2. MATERIALS AND METHODS

Plant material

Soy beans were purchased from health food shop (Lemon green) in Bangkhen District, Bangkok, in October 2011. They were pulverized into coarse powder, then exhaustedly soxhletted with hexane to give the defatted powder.

Extraction and fractionation

Defatted powder 1.1 kg was macerated with 11 L of 70% ethanol for 72 h with occasional stirring, then filtered. After removal of ethanol under reduced pressure, 300 ml portion of aqueous solution equivalent to 360 g of powder was passed through Diaion HP-20 column (450 ml) and sequentially eluted with 0, 25, 50 and 95% aqueous ethanol. The fractions were concentrated under reduced pressure and freeze-dried. Saponin enriched extract (1.20 g) was obtained from 50% ethanolic eluate as monitored by TLC.

Determination of cytotoxicity and collagen stimulation

Cell culture

Human dermal fibroblast cells (ATCC CRL-1474) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated in a humidified 5% CO_2 atmosphere at 37^0 C for 72 h.

MTT cytotoxicity test [8, 9]

The cells were seeded in a 96-well plate at a density of 10^5cells/ml, and incubated for 24 h. Various concentrations of test samples dissolved in DMSO (200 µl, 500- 4000 µg/ml) were added to the cells and incubated for 24 h, and were then washed out. MTT (50 µl, 5 mg/ml) and the medium (150 µl) were added into each well. The cells were then incubated at 37^0 C 5% CO_2 for additional 4 h. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 150 µl of DMSO under 15 min agitation. The absorbance was read at 540 nm. The toxicity of extract was indicated by 50% inhibitory concentration (IC_{50}).

Determination of collagen synthesis using ELISA [10-12]

The cells were seeded in a 6-well plate at a density of 2×10^5 cells/ml, and incubated for 24 h. After that the media were removed and added with different concentrations of extract (50, 100 and 200 µg/ml), incubated continually for 24 h and the extracts were then removed. Attached cells were transferred to a microcentrifuge tube using cell scraper and digested for collagen using pepsin solution in acetic acid (0.1mg/ml 50 mM acetic acid). Finally, the supernatant was detected for human collagen type I using ELISA test kit (Cosmo Bio Co.LTD). Collagen determination was performed by measuring absorbance at 412 nm using microplate reader. Then collagen concentration was calculated by interpolating sample OD value to the standard curve. After that the percentage of collagen stimulation was generated with the following equation:

$$\% \text{ Collagen stimulation} = [(A_C - A_T)/ A_C] \times 100$$

A_C = the collagen concentration of control

A_T = the collagen concentration of test sample

3. RESULTS

The cytotoxicity results showed the %survival of dermal fibroblast cell line, at each concentration compared to control and IC_{50} value over the test concentrations of 500-4000 µg/ml. The results showed that the IC_{50} value of soyasaponin enriched extract was 2,445.99 ± 0.00 µg/ml, as shown in Figure 1. According to classification of the cytotoxicity for natural ingredients [13], our saponin enriched extract could be classified as potentially non toxic substance.

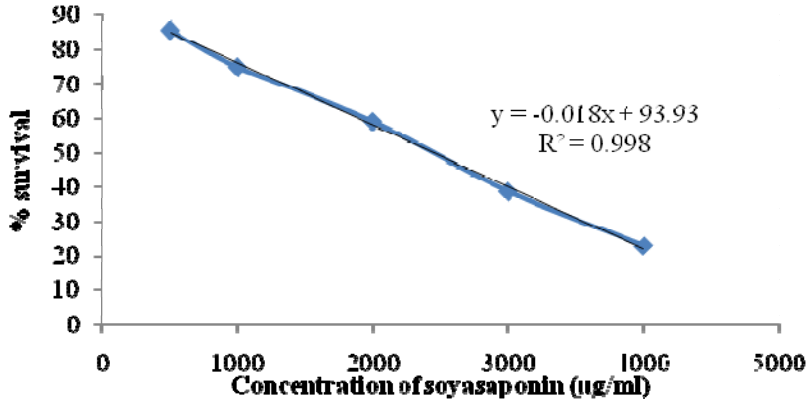

Figure 1. The survival of dermal fibroblast cell line following exposure to soyasaponin enriched extract.

The collagen synthesis stimulating potential of soyasaponin enriched extract was investigated by using ELISA assay compared with the positive control ascorbic acid. The results as shown in Figure 2 revealed that the percentage of stimulating effect on collagen synthesis after saponin enriched extract treatment were 0, 8.08 ± 1.23 and 21.12 ± 1.54 at concentrations 50, 100 and 200 µg/ml, respectively. The effect was in a dose dependent manner. Ascorbic acid at 50 µg/ml showed 31.08 ± 0.28% of collagen synthesis stimulating effect.

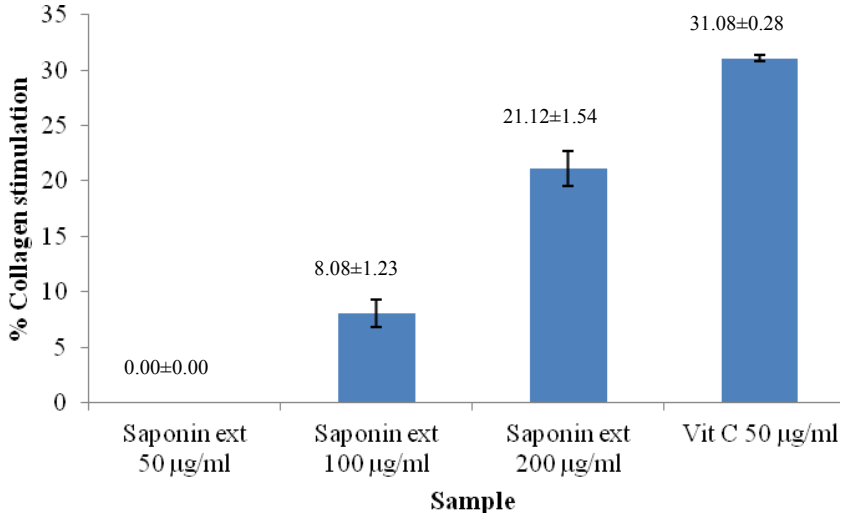

Figure 2. The percentage of collagen stimulation of dermal fibroblast following exposure to saponin extract.

4. CONCLUSIONS

Saponin enriched extract from soybeans is nontoxic to normal human dermal fibroblast cell line (IC_{50} 2,445.99 ± 0.00 µg/ml). The extract at the concentration of 100 and 200 µg/ml showed the percentage of collagen stimulatory effect of 8.08 ± 1.23 and 21.12 ± 1.54, respectively. The results demonstrate a collagen synthesis stimulating potential of soyasaponin extract and provide a possibility to develop as skin anti- aging agent in cosmetic products.

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Anti-oxidant and cytotoxic activity of *Cajanus cajan* (L) Millsp alkaline extracts

Ubon Rerk-am*[a], Sarunya Laovitthayanggoon[a], Bantika Kongsombat[a], Sinn Tangsatirapakdee[a], Chuleratana Banchonglikitkul[a]
[a]Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research. 35 Moo 3, Technopolis, Tambon Klong 5, Amphoe Klong Luang, Pathum Thani Thailand 12120

*ubon@tistr.or.th phone +66 25779114 fax +66 25779110

ABSTRACT

Pigeon pea (*Cajanus cajan* (L) Millsp) is among the dry leguminous seed. Pigeon pea has high levels of proteins and important amino acids such as methionine, lysine and tryptophan. The present study was performed to evaluate antioxidant and cytotoxic activity of the alkaline extracts from Pigeon pea. The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazin (DPPH) radical scavenging assay and lipid peroxidation (β-carotene bleaching model). The EC_{50} value was 61.39 µg/ml and 45.37 µg/ml, respectively. The cytotoxicity (IC_{50}) was determined using MTT assay. The ATCC CRL-1474, dermal human fibroblast was treated with alkaline extracts at 1000, 500, 250, 125, 62.50, 31.25, 15.63, and 7.81 µg/ml. for 24 h. The IC_{50} value was more than 1000 µg/ml. The results showed that the alkaline extracted have high potential in antioxidant activity and non toxic to human skin. It would be interesting to do further study as anti-wrinkle agents.

Keywords: *Cajanus cajan* (L) Millsp., Pigeon pea, Anti-oxidative activity, Cytotoxicity

1. INTRODUCTION

Pigeon pea (*Cajanus cajan* (L) Millsp) is among the dry leguminous seed which important source of proteins in human diet food, especially in the vegetarian population. Pigeon pea has high levels of proteins and important amino acids such as methionine, lysine and tryptophan. The Extraction of seed and leaves are widely used in traditional folk medicine [1]. The rich content of phenolic and flavonoids contained in leaves extracts of pigeon pea were beneficial compounds for their bioactivity such as antipasmodic, anti-inflammatory, antimicrobial and antioxidant activities [2]. The ethanolic extract of leaves are contain pinostrobin, cajanin, longstylin C, longistylin A and cajaninstilbene, it have potent to antioxidant, antiplasmodical, anti-inflammatory and hypocholesterolemic activities. While, the crude ethanolic extracts of roots are contain genistein, genistin, longstylin C, longistylin A and cajanol, which have antioxidant and anticancer activities [3]. The fractionated proteins of pigeon pea seed using water-solubility properties, salts, alcohol and acid/alkali are albumin, globulin, prolamins and glutilins, respectively [4]. The globulin fraction from seed has a hypolipidaemic activity in rats fed a high-fat-high-cholesterol diet [5]. The present study was taken up to evaluate antioxidant and cytotoxic activities of pigeon pea alkaline extracted. The antioxidants are claimed to be biologically active in protecting the body, the skin collagen and elastic tissue against damaging by reactive oxygen species. MTT assay is a tetrazolium-dye based colorimetric microtitration assay. Metabolism competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability.

2. MATERIALS AND METHODS

Preparation of alkaline protein extraction

The dried seed of pigeon pea were ground into powder, extracted with acetone 3 times before and then extracted with hexane using soxhlet extraction technique for defat. The alkaline protein of pigeon pea was extracted using the isoelectric point precipitation (IP) technique correlation to pH of NaOH solution. The meal of pigeon pea extracted by 1% NaOH (1:10, meal:solvent, w/v) and adjusted pH to 12. The suspension was homogenized at 1500 rpm for 1 h followed by centrifugation at 7,000 *g* for 15 min. The supernatant was filtered and protein precipitated by adjusting the pH to 4.5 using 0.1 M HCl. The precipitate protein was recovered by centrifuging at 10,000 *g*, followed by washing three times in excess water and centrifuging. The precipitate was dried using lyophilizer and stored in freezer.

Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals assay

The free radical scavenging activity of pigeon pea alkaline extracts was analyzed by the DPPH assay [6]. The amount of 100 µl of various concentrations sample were reacted with 100 µl of $6x10^{-3}$ M DPPH ethanolic solution in a 96-well plate, incubated at 37°C for 30 min. The absorbance was measured at 517 nm using a UV–VIS microplate reader. All experiments were carried out in triplicates.

Lipid peroxidation (β-carotene bleaching model)

The antioxidant activity of pigeon pea alkaline extract was measured by β-carotene bleaching model system with slight modification [7]. Emulsion I was prepared by dissolving 10 mg of β-carotene in 10 ml of chloroform. Four milliliters of β-carotene solution, 40 mg of linoleic acid and 400 mg of Tween 40 were mixed and removed chloroform at 50°C under vacuum by rotary evaporator. The emulsion was further made up to 100 ml with MillQ water. Emulsion II was prepared same as emulsion I with out β-carotene. Test sample, 50 µl of varied concentration sample were mixed with emulsion I. Blank sample, 50 µl of varied concentration sample were mixed with emulsion II. Absorbance was measured at 450 nm after incubated reaction mixture in oven at 50°C and the reading take from zero time (t=0) till 60 min.

Cell culture

The human dermal fibroblast (ATCC CRL-1474:NHFF) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated for 72 h. at 37°C in a fully humidified, 5% CO_2: air atmosphere.

MTT cytotoxicity test [8]

The cells were seeded in a 96-well plate at a density of 10,000 cells/well, and incubated for 24 hours. The sample at various concentrations were added to the cells and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested with MTT assay. Briefly, 50 µl of MTT in PBS at 5 mg/ml was added to the medium in each well and the cells were

incubated for 4 hours. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 µL of DMSO and 25 µl of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (Inhibitory concentration 50; IC_{50}) of the cells compared to controls.

3. RESULTS

The biological activity of pigeon pea alkaline extracts was shown in Table 1. The DPPH radical was used to evaluate the free radical scavenging capacity of antioxidants extensively. The concentration of pigeon pea alkaline extract to quench DPPH radical (EC_{50}) was 61.39 µg/ml. Lipid peroxidation was estimated of capacity of pigeon pea alkaline extract to protect β-carotene color change from yellow to colorless. The activity (EC_{50}) was 45.37 µg/ml. The results shown that the antioxidant activity of pigeon pea alkaline extracts was lower activity than Trolox, which know compound had high antioxidant activity.

Table1. Antioxidant activity (EC_{50}) of pigeon pea alkaline extracts, compared with standard.

Sample	DPPH assay (µg/ml)	Lipid peroxidation (µg/ml)
Pigeon pea alkaline extract	61.39	45.37
Trolox	1.90	1.20

The cytotoxicity test was shown in Table 2 and Figure 1. The treatment of NHFF cells for 24 h with various concentrations of pigeon pea alkaline extracts at 500, 400, 300, 200, 100, 50, 25 and 12.5µg/ml indicated that the IC_{50} value was more than 1000 µg/ml for treatment times.

Table 2. Viability of NHFF following exposure to pigeon pea alkaline extracts at various concentrations (µg/ml).

Concentration (µg/ml)	% viability	IC_{50} (µg/ml)
1000.00	93.25 ± 0.02	> 1000
500.00	99.56 ± 0.05	
250.00	105.20 ± 0.13	
125.00	103.04 ± 0.15	
62.50	101.00 ± 0.02	
31.25	102.00 ± 0.06	
15.63	101.00 ± 0.05	
7.81	102.00 ± 0.07	

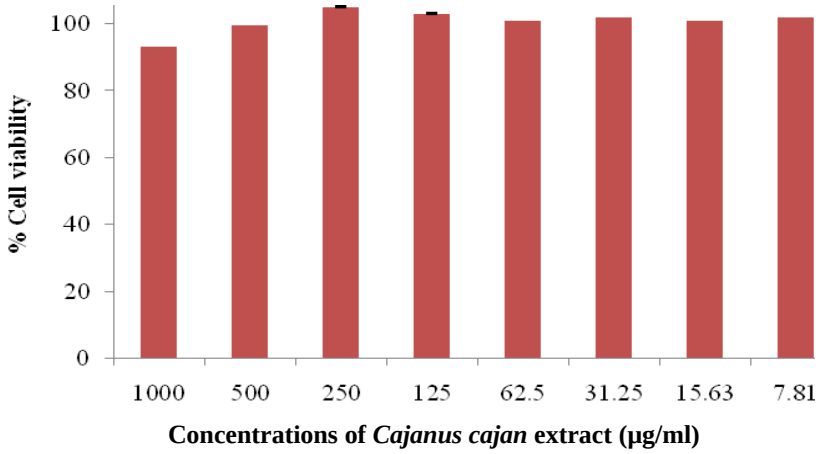

Figure 1. The Viability of NHFF following exposure to pigeon pea alkaline extracts (µg/ml)

4. CONCLUSIONS

The results reveal that Pigeon pea alkaline extracts had high antioxidant activity, although less activity than Trolox. These crude extracts could be potential sources of antioxidant and non toxic to human dermal fibroblast. It would be promising to do further studies as anti-wrinkle agents in cosmetic application.

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Salacinol and related analogs, new leads for type 2 diabetes therapeutic candidates from Thai traditional natural medicine *Salacia chinensis*

Toshio Morikawa*[a], Junji Akaki[a,b], Kiyofumi Ninomiya[a], Eri Kinouchi[a], Genzoh Tanabe[b], Masayuki Yoshikawa[a], Osamu Muraoka[a,b]

[a]Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan

[b]School of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan

*morikawa@kindai.ac.jp phone +81-667212332 fax +81-667293577

ABSTRACT

Genus *Salacia* (Hippocrateaceae), climbing shrubs widely distributed in Thailand, Sri Lanka, India, and Southeast Asian countries, have traditionally been used for treatment of diabetes in traditional medicine. A hot water extract from the stems of *S. chinensis* was found to significantly suppress the increase of postprandial blood glucose levels in α-starch loading rats in a dose-dependent manner (30–300 mg/kg, *p.o.*). Administrations of 0.25 and/or 0.50% of this extract containing dietary feeds for 3 weeks to KK-A^y mice were found to significantly suppress both blood glucose and HbA1c levels without significant changes of the body weight and food intake. The mechanism of action was revealed to be α-glucosidase inhibition, and through the bioassay-guided separation by monitoring the inhibitory activity against rat small intestinal α-glucosidase, we isolated several novel thiosugar sulfonium inner salts and their desulfonated analogs, *e.g.* salacinol (1), kotalanol (2), neosalacinol (3), and neokotalanol (4). These potent enzymatic inhibitory activities were also observed in human α-glucosidase (IC_{50} = 3.9-9.0 μM for maltase). These sulfoniums were found highly stable in an artificial gastric juice (residual rate (%) of **1**: 92.5 ± 6.1; 2: 91.4 ± 4.6; 3: 93.2 ± 6.2; 4: 96.5 ± 4.7, treated at 37°C for 180 min). In addition, the sulfoniums were scarcely absorbed from the intestine in the experiment using the *in situ* rat ligated intestinal loop model (residual rate (%) of 1: 97.6 ± 3.9; 2: 99.7 ± 6.0; 3: 94.5 ± 4.1; 4: 96.6 ± 3.7, treated for 120 min). These results indicate that sulfoniums (1-4) are promising leads for the new type of antidiabetes agents.

Keywords: Type 2 diabetes therapeutic candidate, *Salacia chinensis*, α-Glucosidase inhibitor

1. INTRODUCTION

The Hippocrateaceae plant, *Salacia chinensis* is a woody climbing plant found in Thailand and Laos *etc*. [1–4]. From a methanol and/or water extract of *S. chinensis*, we previously isolated a novel thiosugar sulfonium sulfate inner salt, salacinol (**1**) [2], as a bioactive constituent responsible for the postprandial anti-hyperglycemic activity. We also isolated its analogs, neosalacinol (**2**) [5], kotalanol (**3**) [6], neokotalanol (**4**) [5], ponkoranol (**5**) [7], neoponkoranol (**6**) [8], salaprinol (**7**) [7], and neosalaprinol (**8**) [8], and these analogs were found to have the same level of postprandial anti-hyperglycemic activity as **1**. The mechanism of the antidiabetogenic action of these sulfoniums were shown to be mediated by the inhibition of α-glucosidases, and their activities against maltase and sucrase were found as potent as those of acarbose or voglibose, the clinical inhibitors (Table 1). Clinical trials of the extract demonstrated the efficacy [9] with no side effects [10]. Based on these findings, interests in *S. chinensis* as a possible nutraceutical product for diabetic patients is increasing, and there has been a strong demand for efficient quality control measurement to ensure the authenticity and the active contents of these products, as well as to verify the claims on product labels. In this presentation, following *in vivo* examination of the hot water extract (SCE) and/or its sulfonium constituents (**1**–**4**) were examined; i) hypoglycemic activity on postprandial blood glucose level in α-starch loading rats, ii) improvement effects on blood glucose and HbA1c levels after 3 weeks administration to KK-A^y mice. In addition, the stability test was carried out using an artificial gastric juice. An intestinal absorption study was also performed using an *in situ* rat ligated intestinal loop model.

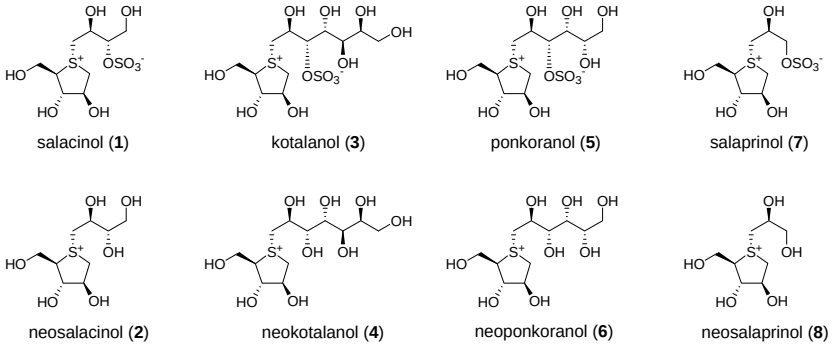

Figure 1. Structures of sulfonium constituents from *Salacia chinensis*

2. MATERIALS AND METHODS

Hypoglycemic activity of SCE, 1, 3, and 4 on postprandial blood glucose level in α-starch loading rats

Test sample suspended in 5%(w/v) α-starch solution (1g/kg) was administered orally to 20 h fasted male Sprague Dawley (SD) rats (6 w). Blood samples were collected from the tail vein at 0.5, 1, 2, and 3 h after the oral administration. Blood glucose level was immediately measured by glucose oxidase method.

Improvement effects of SCE on blood glucose and HbA1c levels after 3 weeks administration in KK-A^y mice

Male KK-A^y mice (6 w, CLEA Japan, Inc., Tokyo, Japan) were housed under the following conditions: room temperature (24 ± 2°C), 50 ± 10% humidity, 12 h illumination per day and *ad libitum* access to food and water. Mice caged individually were divided into four groups of six mice each and fed for 21 days on control (CE-2, CLEA Japan, Inc.) or (0.10, 0.25, and 0.50% (w/w) *S. chinensis* stem hot water extract containing CE-2). Food and water consumption and the body weight of each mouse were measured every day. At 15th day and the end of the treatment period, blood samples were collected from the tail vein. The HbA1c was measured by DCA2000 (Siemens, Eschborn, Germany).

Table 1. IC_{50} (μM) values of sulfoniums (**1**—**8**) for α glucosidases

Enzyme origin:	Human	Rat		
	maltase	maltase	sucrase	isomaltase
salacinol (1)	4.9	6.0	1.3	1.3
kotalanol (3)	3.9	2.0	0.43	1.8
ponkoranol (5)	5.0	5.6	0.41	4.6
salaprinol (7)	—[a]	>329	>329	14
neosalacinol (2)	9.0	22.2	2.5	0.68
neokotalanol (4)	3.9	1.6	1.5	0.46
neoponkoranol (6)	4.0	5.1	1.0	1.4
neosalaprinol (8)	—[a]	>444	90	6.5
voglibose	1.3	1.3	0.22	2.2
acarbose	15.2	1.7	1.5	645
miglitol	3.7	8.2	0.43	4.6
1-deoxynojirimycin	—[a]	0.67	0.12	0.26

[a]: not tested

Stability of 1–4 in artificial gastric juice

A solution of the extract (50.0 mg) in artificial gastric juice (50 mL, components: 0.2% (w/v) NaCl and 0.32% (w/v) pepsin from porcine, pH was adjusted to 1.2 with HCl) was incubated at 37°C at 60 or 180 min. Each reaction mixture was neutralized with 0.1 M NaOH, then filtered by ultrafiltration using Amicon Ultra (MWCO 3000 Da, Millipore, Massachusetts, USA). The sulfonium contents (**1**—**4**) of each filtrate were measured by LCMS as described previously [5, 6].

***In situ* intestinal absorption study of 1–4 using rat ligated intestinal loop model.**

Male SD rats (10 w, fasted 18–24 h) were anesthetized with thiobutabarbital (80 mg/kg, *i.p.*), and then prepared ligated jejunal loop (*ca.* 20 cm). The extract (300 mg/body/2 mL H_2O) was injected and indwelled into the loop for 30 or 120 min). The indwelling solution was collected using saline and the sulfonium contents (**1**–**4**) were measured by LCMS as described previously [5, 6].

3. RESULTS

As shown in Figure 2, SCE was found to significantly suppress the increase of postprandial blood glucose levels in *α*-starch loading rats in a dose-dependent manner (30–300 mg/kg, *p.o.*). The principal sulfoniums (**1**, **3**, and **4**) were also found to show the activity (EC_{50} = >2.06, 0.62, and 0.54 mg/kg, respectively). SCE also significantly inhibited blood glucose and HbA1c levels in 21 days administration at doses of 0.25 and/or 0.50% (w/w) containing CE-2 fed KK-A^y mice as shown in Figure 3, without producing significant changes in body weight and food intake (data not shown).

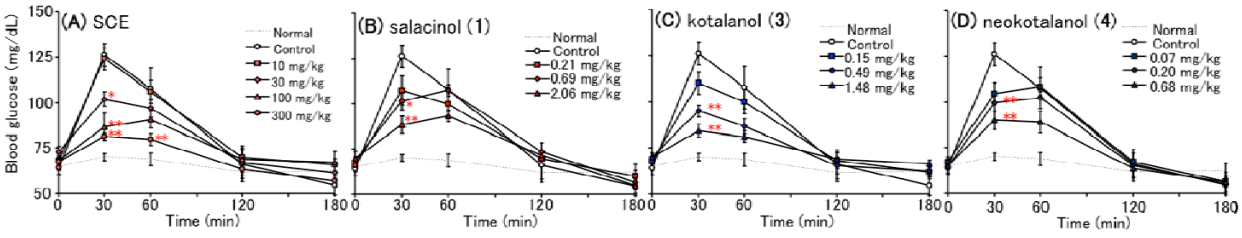

Figure 2. Effects of single dose of SCE and its principal sulfonium constituents (1, 2, and 4) on blood glucose levels in *α*-starch loading rats

Next, the stability test was carried out using an artificial gastric juice, and the intestinal absorption study was performed using an *in situ* rat ligated intestinal loop model. As the results, high stability of the principal sulfoniums (**1**—**4**) in the artificial gastric juice were observed (residual rate (%) of **1**: 92.5 ± 6.1; **2**: 91.4 ± 4.6; **3**: 93.2 ± 6.2; **4**: 96.5 ± 4.7, treated at 37°C for 180 min). In addition, these sulfoniums were scarcely absorbed from the intestine (residual rate (%) of **1**: 97.6 ± 3.9; **2**: 99.7 ± 6.0; **3**: 94.5 ± 4.1; **4**: 96.6 ± 3.7, treated for 120 min) as shown in Figure 4.

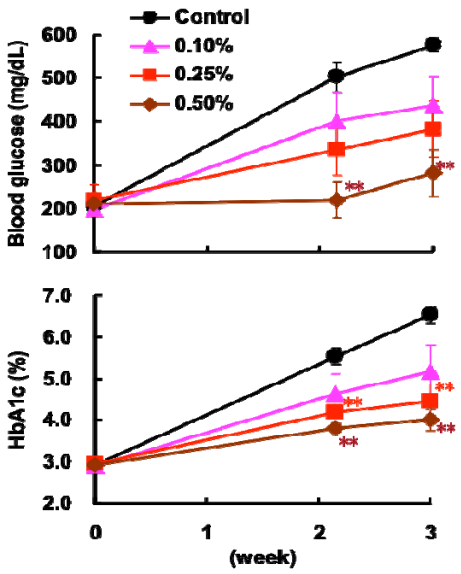

Figure 3. Effects of 21 days administration of SCE on blood glucose and HbA1c levels in KK-A^{y} mice.

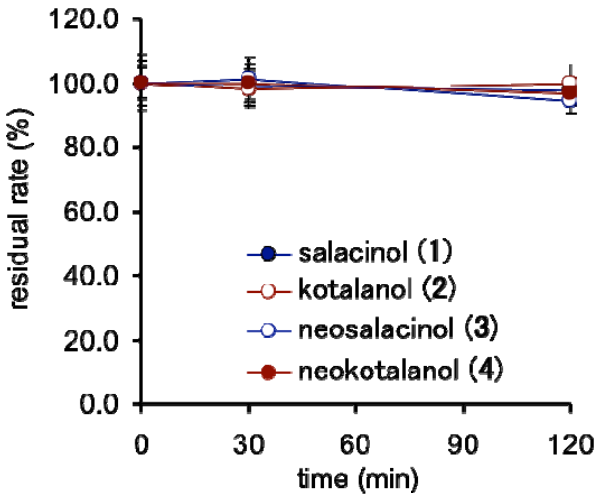

Figure 4. Effects of intestinal absorption of sulfoniums (1—4) in *in situ* rat ligated loop.

4. CONCLUSIONS

These findings indicate that sulfoniums (**1**—**4**) are promising leads as candidates for a new type of antidiabetics.

ACKNOWLEDGEMENTS

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Lipidemic, glycemic and organ protective actions of Tea seed oil in rats fed with high fat and high carbohydrate diet

Thamolwan Suanarunsawat*[a], Warinna Pinthong[a], Wacharaporn Devakul Na Ayutthaya[b]
[a]Physiology Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Pathumtani, Thailand 12000
[b]Pharmacology and Toxicology Unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Pathumtani, Thailand 12000

*tmw706@gmail.com phone +66-29972200 fax +66-29972200

ABSTRACT

The present study was conducted to investigate the lipidemic, glycemic and organ protective effects of tea seed oil (TSO) in rats fed with high fat and high carbohydrate (HFHC) diet. Three groups of male Wistar rats were used including normal control group, group fed with HFHC diet (cholesterol + lard + fructose) for three months, and HFHC group treated with TSO. At the end of the experiment, serum lipid profile, blood glucose, oral glucose tolerance test (OGTT), serum AST, ALT, LDH, CK-MB, creatinine and BUN were determined. Individual fatty acids containing in TSO was assayed by Gas chromatography. The results showed that oleic acid was the primary fatty acid in TSO (83.36%). HFHC diet increased serum lipid profile and atherogenic index (AI). The high serum levels of lipid profile and AI were decreased in HFHC rats treated with TSO. The elevation of area under the curve of OGTT was alleviated by TSO. TSO also normalized the high serum levels of AST, ALT, LDH, CK-MB, creatinine and BUN. It can be concluded that TSO was able to decrease the high serum levels of lipid profile, and high blood glucose level of OGTT in rats fed with HFHC, indicating its therapeutic potency to prevent atherosclerosis and hyperglycemia. It also protects liver, heart and kidney in HFHC. Oleic acid containing in TSO might be responsible for these activities.

Keywords*:* Tea seed oil, High fat diet, Liver function, Cardiac function, Fructose

1. INTRODUCTION

Hyperlipidemia and hyperglycemia are widely known to be the major risk factor for the development of atherosclerosis, ultimately causes coronary artery disease [1, 2]. The modern life style with high fat and high carbohydrate diet and less physical activity are the significant causes of hyperlipidemia and hyperglycemia. Hyperlipidemia and hyperglycemia have been found to induce oxidative stress in various organs such as liver, heart and kidney [3, 4]. Since synthetic anti-hyperlipidemic and anti-hyperglycemic drugs cause several adverse effects especially liver damage, searching for natural products that have less or no side effects has been increasing. Among many kinds of medicinal plants available in eastern countries, *Camellia oleifera* Abel. oil (tea seed oil, TSO) is promising since it has several outstanding properties such as high smoking point, stable, and high nutritional contents, especially Vitamin E, and high unsaturated fat which is useful for human foods [5]. Tea seed oil has been shown to lower serum lipid profile [6, 7] and protected vital organs in normal and several stress conditions [6, 8-10]. However, no experimental study to elucidate its effects on serum lipid profile, blood glucose and organ protection in subjects fed with high fat and high carbohydrate diet has been reported. Therefore, the present study was conducted to investigate its lipidemic, glycemic and organ protective effects in rats fed with high fat and high carbohydrate diet

2. MATERIALS AND METHODS

Identification of fatty acids in tea seed oil using Gas Chromatography-Mass Spectrometry (GC-MS)

The tea seed oil was extracted from Lamsoon, Ltd, Thailand. Fatty acids containing in TSO were transformed into methyl ester (FAMEs). The FAMEs were analyzed by gas chromatography mass spectrometry (GC-2010+AOC20i, SHIMADZU), equipped with a flame ionization detector (Electron-Impact mass spectrometry). The temperature of the detector was set at 300°C. The carrier gas was helium at split ratio 50:1, flow rate was 62.9 mL/min. The content of fatty acids was expressed as a percentage of the total fatty acids

Animal preparation

Male Wistar rats weighing between 180-220 g from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornprathom, Thailand were used in the study. All animals were cared for in accordance with the principles and guidelines of the Institutional Animal Ethics Committee of Rangsit University which is under The National Council of Thailand for Animal Care. High fat and high carbohydrate (HFHC) diet was prepared by adding 2 g% cholesterol powder **(**Sigma-Aldrich Co., Ltd**,** USA**)** along with 10% lard in normal diet, and 60% fructose powder (Vidhyasom Co., Ltd., Thailand) mixed in drinking water. Three groups of seven rats each were fed on the following diet for three month: group I, normal diet for a control; group II, a high fat and high carbohydrate (HFHC) diet; and group III, HFHC group treated with TSO orally once a day at the dose of 5.69 g/kg BW/day for three months. This dose has been chosen because of its anti-oxidative effect to protect liver against CCl_4 [9]. In addition, it is the average dose that showed blood lipid lowering in previous reports [11].

Experimental protocol

Effect of tea seed oil on blood glucose and oral glucose tolerance tests in rats fed with HFHC diet

After 3 months of normal or HFHC diet feeding, the rats were fasted overnight. Blood was collected from rats' tail to determine blood glucose using automatic glucometer (Medisense UK Co., Ltd, Abbott Lab, UK**).** Oral glucose tolerance test (OGTT) was evaluated by feeding 2 g/kg BW of 50% glucose solution. After glucose loading, blood was collected from the tail to determine blood glucose every 30 min for 120 min. Area under the curve (AUC) was calculated from blood glucose through 120 min.

Effect of tea seed oil on serum lipid profile and organ protection in rats fed with HFHC diet

At the end of the experiment, the rats were fasted overnight, and were anesthetized by intraperitoneal injection with sodium pentobarbiturate (60 mg/kg BW). Blood was collected from abdominal vein. Serum was separated by refrigerated centrifuge at 3000 rpm, 4°C for 5 min for determination of serum lipid profile including total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C). The atherogenic index (AI) was then calculated as the ratio of TC-(HDL-C)/(HDL-C). Liver damage was evaluated by determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST). Cardiac damage was also assessed by determination of serum lactate dehydrogenase (LDH) and creatine kinase MB subunit (CK-MB). Renal function was evaluated by determination of serum creatinine and blood urea

nitrogen (BUN). All serum enzymes were determined using diagnostic kit (Gesellschaft Für Biochemica und Diagnostica GmbH Co.,Ltd, Germany, and Randox Laboratories Co.,Ltd, UK).

Statistical Analysis

All values were presented as means ± SEM. The results were analyzed by ANOVA. Duncan multiple rank test was performed to determine statistical significance among groups by using SPSS software version 11.5. Significant difference was accepted at $p < 0.05$.

3. RESULTS

Both saturated and unsaturated fatty acids were found in OS fixed oils as shown in Fig. 1 and Table 1. The major fatty acid contained in tea seed oil was Oleic acid (83.36%). The high intake of fructose and lipid have been linked with the induction of insulin resistance commonly observed with high fructose and/or high fat feeding in both humans and animal models and then finally results hyperglycemia [12-13]. The present study shows that basal blood glucose in HFHC rats was slightly but not significantly different from normal rats whereas AUC of OGTT in HFHC rats was significantly increased (Figure 2-3). TSO slightly decreased basal blood glucose but the level was not significantly different from HFHC rats. In contrast, the high AUC of OGTT in HFHC rats treated with TSO was significantly decreased (Figure 3). These implies that TSO might be able to stimulate glucose-induce insulin secretion or improves insulin action which then eventually prevents the occurrence of hyperglycemia in rats fed with HFHC diet. The serum levels of TC, TG, HDL-C, LDL-C and AI were shown in the Table 2. Three months of HFHC diet feeding significantly increased serum TC, TG, LDL-C and AI whereas HDL-C was slightly decreased. TSO treatment significantly decreased the serum lipid profile and AI in HFHC rats, indicating that TSO could be effective to alleviate atherosclerosis which eventually prevents the occurrence of coronary artery disease. It has been known that the liver, heart and kidney are the primary risk organs in hyperlipidemia and hyperglycemia conditions. Three months of HFHC diet feeding impaired the liver, heart and kidney functions as shown by the significantly increased serum levels of AST, ALT, LDH, CK-MB, creatinine, and BUN in HFHC rats. The high levels of AST, ALT, LDH, CK-MB, and BUN were significantly decreased in HFHC rats treated with TSO (Table 3). These results reflect that TSO not only shows anti-hyperglycemic effect but also anti-hyperlipidemic and organ protective activities against hyperlipidemia.

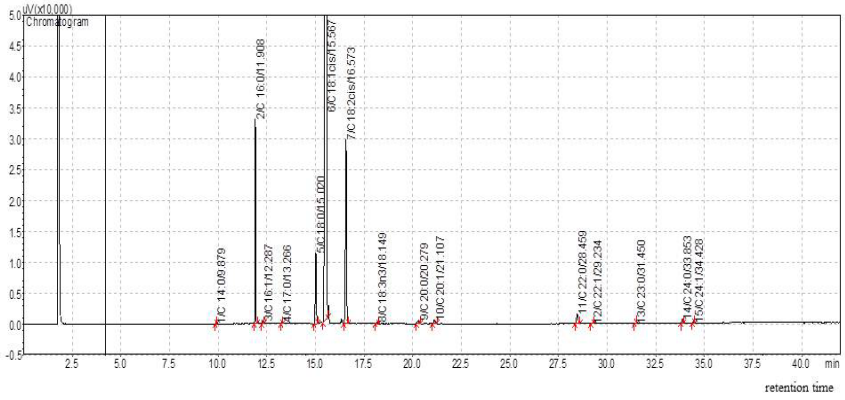

Figure 1. GC chromatogram of fatty acids composition in tea seed oil.

Table 1. Fatty acid compositions in tea seed oil.

Fatty acid	Molecular formula	%
Palmitic acid	C 16:0	4.74
Palmitoleic acid	C 16:1	0.07
Margaric acid	C 17:0	0.03
Stearic acid	C 18:0	2.84
Oleic acid	C 18:1 n-9 cis	83.36
Linoleic acid	C 18:2 n-6 cis	7.68
Linolenic acid	C 18:3 n-3	0.06
Arachdic acid	C 20:0	0.18
Behenic acid	C 22:0	0.55
Erucic acid	C 22:1 n-9	0.04
Lignoceric acid	C 24:0	0.17
Nervonic acid	C 24:1	0.03

Table 2. Serum total cholesterol, triglyceride, HDL-C, LDL-C, atherogenic index (AI) in normal rats, HFHC rat and HFHC rats treated with TSO

Group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)	Atherogenic Index
control	54 ± 5.67^{a}	29 ± 3.57^{a}	21.19 ± 1.98^{a}	26.97 ± 3.49^{a}	1.54 ± 0.11^{a}
HFHC	157 ± 8.64^{b}	50 ± 5.27^{b}	17.49 ± 1.57^{a}	130.04 ± 8.92^{b}	8.49 ± 1.01^{b}
HFHC+TSO	120 ± 9.16^{c}	21 ± 2.12^{a}	18.92 ± 1.74^{a}	96.86 ± 10.11^{c}	5.86 ± 1.07^{c}

Values are expressed as mean ± SEM of seven rats per group. Values with different superscripts in each colume are significantly different at $p < 0.05$.

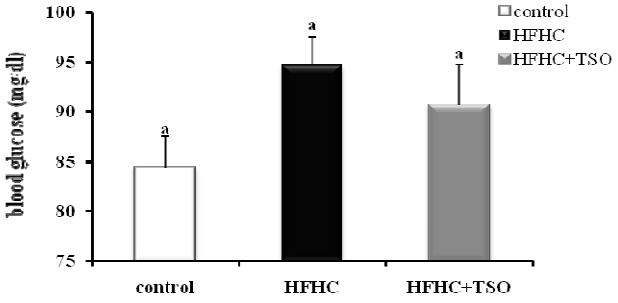

Figure 2. Basal blood glucose in normal rats, HFHC rat and HFHC rats treated with TSO. Values are expressed as mean ± SEM of seven rats per group. Values with the same superscripts on graph are not statistically significant difference. HFHC: high fat and high carbohydrate.

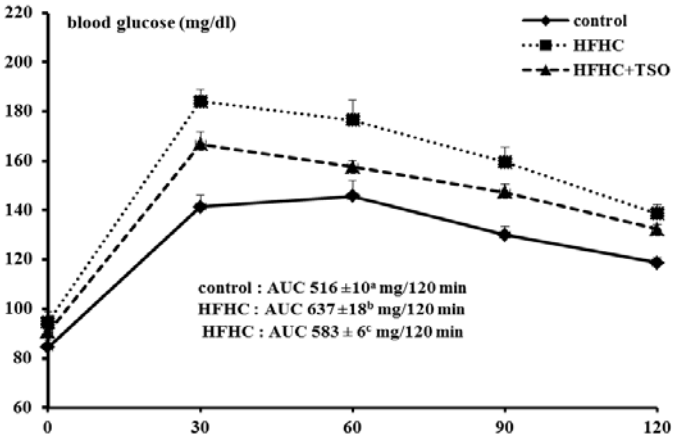

Figure 3. Area under the curve (AUC) of oral glucose tolerance test (OGTT) in normal rats, HFHC rat and HFHC rats treated with TSO.Values are expressed as mean ± SEM of seven rats per group. Values with different superscripts are significantly different at $p < 0.05$. HFHC: high fat and high carbohydrate

Table 3. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase MB subunit (CK-MB), lactate dehydrogenase (LDH), creatinine and BUN in normal rat, HFHC rat and HFHC rat treated of TSO

Group	Serum AST (U/L)	Serum ALT (U/L)	CK-MB (U/L)	LDH (U/L)	Creatinine (mg/dl)	BUN (mg/dl)
control	83 ± 9^{a}	33 ± 3^{a}	$581 \pm 40^{a,b}$	454 ± 43^{a}	1.14 ± 0.03^{a}	15.24 ± 0.55^{a}
HFHC	181 ± 12^{b}	142 ± 14^{b}	757 ± 58^{b}	579 ± 23^{b}	1.28 ± 0.04^{b}	17.79 ± 0.74^{b}
HFHC+TSO	119 ± 11^{c}	54 ± 6^{a}	536 ± 79^{a}	415 ± 43^{a}	$1.22 \pm 0.04^{a,b}$	13.51 ± 0.76^{a}

Values are expressed as mean ± SEM of seven rats per group. Values with different superscripts in each column are significantly different at $p < 0.05$.

4. CONCLUSIONS

The present study shows that three months of HFHC diet feeding slightly raised the basal blood glucose whereas it significantly raised AUC of OGTT. TSO treatment slightly decreased basal blood glucose but it significantly decreased the high level of AUC in HFHC diet. HFHC diet also raised serum lipid profile, atherogenic index (AI) and serum levels of AST, ALT, LDH, CK-MB, creatinine, and BUN. TSO treatment attenuated the high serum lipid profile, AI and all these serum marker enzymes. Qualitative analysis of the fatty acid composition in TSO shows that oleic acid was the primary fatty acid in TSO (83.36%). Oleic acid containing in TSO might be responsible for these effects.

ACKNOWLEDGEMENTS

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Chemical fingerprints and anti-inflammatory activity of polar fraction from *Cajanus cajan*

Siripen Jarikasem*[a], Amonrat Khayankarnnawee[a], Sarinthip Muensaen[a]
[a]Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research, Pathum Thani, Thailand 12120

*siripen@tistr.or.th phone +66-25779116 fax +66-25779110

ABSTRACT

Cajanus cajan (L.) Millsp., Leguminosae or pigeon pea is traditional edible pea crop widely distributed in the northern part of Thailand. Besides it is also served as food due to its high protein content, the seed has been used traditionally for the treatment of stomatitis, gingivitis and as energy stimulant. Previous studies have demonstrated its interesting biological activities including *in vitro* Anti-oxidation, suppression of inflammatory cytokine production in macrophage which was relevant to the presence of certain phenolic components. In this study, pigeon pea polar components i.e. *n*-butanol fraction from the aqueous ethanolic seed extract was investigated for chemical constituents and *in vivo* anti-inflammatory activity. HPLC-DAD-ELSD chromatographic analysis of the fraction was performed using X-Terra RP18 column with 0.1% trifluoroacetic acid and acetonitrile as mobile phase. Confirmation of components was done by comparision of R_t and absorbance spectral with reference standards. Four compounds were identified as genistin, genistein, soyasaponin I and soyasaponin II. Anti-inflammatory test was performed by carrageenan induced rat paw oedema model. The effect was observed at time 1, 2, 3 h after oedema induction. The polar fraction given orally to rats at 100 and 200 mg/kg, at time 3 h showed 35% and 61 % oedema inhibiting activity, respectively while that of diclofenac at 50 mg/kg was 68 %. The results indicate therapeutic potential of pigeon pea's polar components which confirm its traditional uses as anti-inflammatory remedy.

Keywords: *Cajanus cajan*, anti-inflammation**,** Rat paw oedema, HPLC fingerprint

1. INTRODUCTION

Pigeon pea (*Cajanus cajan*) is a perennial plant widely cultivated in tropical and subtropical regions of many countries as well as in the north of Thailand. Besides serving as food due to its high protein content, the seeds have been used traditionally as a tea for the treatment of inflammation and blood disorders and as diuretic and energy stimulant [1, 2]. Previous studies have demonstrated its *in vitro* antioxidation and suppression of inflammatory cytokine production in macrophage [3] and hypocholesterolemic effects [4] of which polar phenolic components take the responsibilities. In order to provide scientific evidences to confirm the traditional uses of pigeon pea, we investigated on its chemical components and anti-inflammatory activity in animal model focusing on the polar constituents.

2. MATERIALS AND METHODS

Plant material:

Mature seeds of pigeon peas collected from Kanchanaburi province in October 2011 were dried in hot air oven at 50°C for 6 h then pulverized into coarse powder and used for extraction**.**

Extraction and fractionation: Plant powder 1.5 kg was macerated with 15 L of 70% ethanol for 72 h with occasional stirring, then filtered, the method was repeated twice. The combined filtrate, after removal of ethanol under reduced pressure afforded 206.85 g crude extract (13.79% yield). A 30 g portion of the extract was suspended in 200 mL of 10% aqueous methanol and partitioned with 3 x 200 mL of *n*-butanol. The combined *n*-butanol solution was concentrated under reduced pressure to give 4.39 g of dried extract (Bu Fr).

High performance liquid chromatography analysis

BuFr was dissolved in methanol (1 mg/mL) and PTFE 0.45 µm filtered, then subjected to HPLC analysis. Confirmation of components was done by comparision of R_t and absorbance spectral with reference standards i.e. soy isoflavones and soy saponins from Chromadex, USA.

HPLC condition: Waters Pump 600, thermostated column compartment and diode array detector (DAD), X-Terra RP18 column (150 mm x 3.9 mm i.d., particle size 4 µm) were used for the analysis. HPLC column was maintained at room temperature. HPLC analysis of the extract was carried out using the conditions applied from the methods for saponins analysis previously reported [5]. The gradient mode of mobile phases with water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile (solvent B) : 100A for 3 min, 90A/ 10B for 17 min, 80A/ 20B for 10 min, 60A/ 40B for 20 min to 10A/ 90B isocratic for 10 min was used. The flow rate was 1.0 mL/min, the injection volume was 20 µL and the chromatograms were DAD detected at 205 nm. For evaporative light scattering detector (ELSD), a probe temperature 50°C, a gain of 6.0 and nebulizer nitrogen gas of 3.3 ± 0.1 bar were used.

Anti-inflammatory test

Animals:

Male Wistar rats purchased from Laboratory Animal Centre, Mahidol University, Salaya, Nakornprathom, Thailand were housed in the animal facility of Thailand Institute of Scientific and Technological Research (TISTR) under standard conditions (25 ± 2°C), 50-60% of humidity and 12 h/ 12 h light/dark cycles. Food and water were allowed *ad libitum*. The animals were kept under laboratory conditions for one week prior to the start of the experiment. The Animal Ethics Committee of TISTR approved all experimental protocols.

Carrageenan induced paw oedema [6]

Rats weighing 80-100 g were divided in groups of six: vehicle control (distilled water), positive control (diclofenac 50 mg/kg, test sample I (BuFr 100 mg/kg) and test sample II (BuFr 200 mg/kg). At the beginning of the experiment, initial paw volumes were determined using a water plethysmometer (Ugo Basil, Italy). Then, individual animal group was orally received sample accordingly. One hour after sample administration, paw oedema was induced by injection of 0.1 mL of carrageenan (λ-carrageenan, type IV, Sigma) diluted in saline in the right hind foot pad. Paw volumes were determined at time 1, 2, and 3 h after oedema induction. The percentage of oedema was calculated as follows:

$$\% \text{ Oedema} = (T_t - T_0) \times 100 / T_0$$

where T_0 = initial paw volume and T_t = paw volume after sample application and induced oedema.

The percentage of oedema inhibition was calculated with reference to vehicle control group.

Analysis of Data

The results are expressed as means ± S.E.M. Differences in mean values between groups were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. *P* values less than 0.05 ($P < 0.05$) is considered significant.

3. RESULTS

HPLC analysis

HPLC chromatogram of BuFr detected under DAD at 205 nm (Fig. 1D) showed the presence of isoflavones genistin and genistein at R_t of 25.90 and 36.74 respectively as confirmed by R_t of standard isoflavones (Fig. 1A). Peaks corresponding to soyasaponin I and soyasaponin II were also observed at R_t 38.94 and 39.55 as confirmed by ELSD (Fig. 1B, Fig. 1C). Structurally, there have been no UV active chromophores in saponin molecule. Thus, DAD detector at 205 nm gave a low intensity peak of soyasaponin I and soyasaponin II while the saponin selective ELSD detector gave higher intensity peaks (Fig. 1C, Fig. 1D).

Anti-inflammatory activity

BuFr at 100 and 200 mg/kg orally given to rats exhibited significant oedema inhibitory effect at time 2 and 3 h similar to that of diclofenac 50 mg/kg (Table 1). Maximum inhibitory effects of 54% (100 mg/kg) and 64% (200 mg/kg) were observed at 2 h after oedema induction while that of diclofenac appeared to be more than 64% at time not less than 3 h. The oedema inhibitory effect of BuFr was in a dose dependent manner. The result is in accordance with its chemical profile obtained from this study as well as a previously reported on *in vitro* anti-inflammatory activity [3].

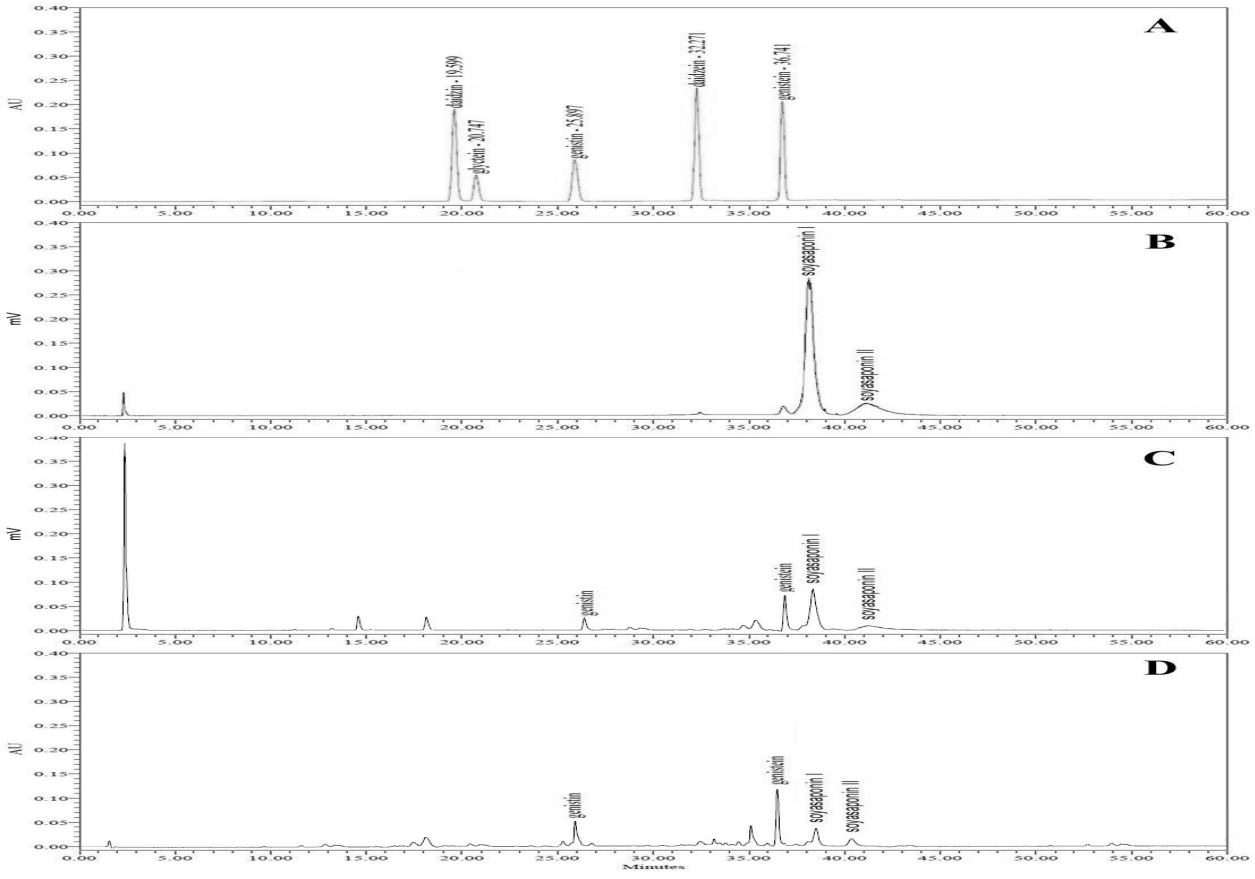

Figure 1. HPLC Chromatograms of polar fraction from pigeon peas (BuFr) and reference standards.
(A) standard isoflavones, DAD 205 nm, (B) soyasaponins, ELSD, (C) BuFr, ELSD, (D) BuFr, DAD 205 nm

Table 1. Oedema inhibitory effect of polar fraction, BuFr from pigeon pea tested by carrageenan induced paw oedema in rat**.**

Samples	% Oedema			Oedema inhibition (%)		
	1 h	2 h	3 h	1 h	2 h	3 h
Control	31.06 ± 2.27	63.40 ± 7.84	76.17 ± 7.24	-	-	-
Diclofenac 50 mg/kg	23.44 ± 1.93	20.92 ± 3.60*	19.53 ± 3.36*	24.00	64.00	68.00
BuFr 100 mg/kg	29.36 ± 3.18	28.59 ± 3.51*	48.95 ± 4.99*	5.00	54.00	35.00
BuFr 200 mg/kg	22.44 ± 3.41	22.45. ± 5.24*	37.17 ± 3.41*	27.00	64.00	61.00

* $p < 0.05$, compared with control using Tukey's test

4. CONCLUSIONS

n-Butanol fraction from the 70% aqueous ethanolic extract of pigeon peas contained both isoflavones and saponins detected by HPLC-DAD-ELSD. Four components could be characterized from HPLC-DAD profile at 205 nm including genistin, genistein, soyasaponin I and soyasaponin II. The fraction given orally at 100 and 200 mg/kg significantly showed *in vivo* anti-inflammatory effect tested by carrageenan induced rat paw oedema. Our study demonstrates both chemical and pharmacological evidences to support the traditional use of pigeon peas as anti-inflammation remedies.

ACKNOWLEDGEMENTS

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Antioxidant activities of *Pluchea indica* Less tea after *in vitro* digestion

Krisadaporn Polsiri[a], Chatchawin Petchlert*[b]
[a]Biological Science Graduate Program, Faculty of Science, Burapha University, Chon Buri, Thailand 20131
[b]Department of Biochemistry, Faculty of Science, Burapha University, Chon Buri, Thailand 20131

*chatchaw@buu.ac.th phone +66-805930482 fax +66-38393495

ABSTRACT

In this study, antioxidant activities of *Pluchea indica* Less. tea both before and after *in vitro* digestion were investigated. Such activities were performed using DPPH, reducing power assay, total phenolic and total flavonoid contents. Before the digestion, the result showed the greatest inhibition on DPPH with IC_{50} = 0.05 ± 0.005 mg/ml. However, after digestions, the remarkable decrease of DPPH scavenging activity was observed in all stages (IC_{50} = 0.34 ± 0.05 (oral), 0.44 ± 0.01 (gastric) and 0.42 ± 0.02 mg/ml (intestinal stage), respectively). A little difference of reducing power was found between pre-digestion (143.46 ± 0.04 mg quercetin equivalent (QE)/g sample) and post-digestion (154.67 ± 0.01 mg QE/g sample for oral stage, 240.08 ± 0.008 mg QE/g sample for gastric stage and 146.04 ± 0.001 mg QE/g sample for intestinal stage, respectively) at the highest concentration of the experiment. In addition, total phenolic and total flavonoid contents of samples in post-digestion have significantly increased (p<0.05) when compared to the pre-digestion condition. Conclusively, the digestive process in GI tract possibly influenced to the antioxidant activity and bioactive compounds of *Pluchea indica* Less. tea.

Keywords: *Pluchea indica* Less. tea; *in vitro* digestion, DPPH, Reducing power, Total phenolic content, Total flavonoid content

1. INTRODUCTION

Pluchea indica Less. (Asteraceae) or locally known as Khlu is a widespread medicinal plant of Asia, especially India, Thailand, Malaysia and Philippines. It is a perennial shrub plant with medicinal properties and antioxidant activities which have many beneficial effects [1]. A decoction of the leaves has been used to combat fever. The sap expressed from leaves is used to treat dysentery. A poultice of leaves is applied externally to treat ulcers and soothe sores [2]. However, its antioxidant activity after digestion has not been studied. The aim of this study is to investigate the antioxidant activity of *P. indica* Less. tea both before and after digestion that simulate the human gastrointestinal (GI) system. Such activities were performed using DPPH assay, reducing power, total phenolic and total flavonoid contents. *P. indica* Less. tea may provide a potential natural source of bioactive compounds and may be beneficial to the human health.

2. MATERIALS AND METHODS

Preparation of extract

P. indica Less. leaves were obtained from Chantaburi province, Thailand. Samples were cleaned, washed with water, cut into small pieces, dried overnight in a hot air oven at 60°C and rendered to smaller size particles using a grinder. *P. indica* Less. (dried leaves) was ground into a fine powder. Powder (100g) was added into boiling distilled water (10L) and further heated for 30 min. Water extract was filtered by vacuum pump (GAST, USA) via Whatman filter paper No.1 (Whatman, UK) at room temperature. The remaining residues were re-extracted with boiling water as described above. Then, extract was centrifuged at 3,000 rpm for 5 minutes (K240R, Centurion Scientific, UK). The collected filtrate was evaporated by rotary evaporator and dried in a freeze-dryer (GAST, U.S.A). All extracts were weighed and stored at -20°C in an airtight container until use.

DPPH assay

The scavenging activity of *P. indica* Less. tea was evaluated the on the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals according to the previous procedure described [3]. Stock solution of *P. indica* Less. tea was prepared as sample in methanol. For each concentration, 50 µl of the test fraction was mixed with 100 µl of 0.2 mM DPPH in methanol in a 96-wellplate, incubated at room temperature for 30 min in the dark, and then the absorbance was measured at 517 nm by a microplate reader. Percentage of inhibition was calculated using the following formula: $OD_{(DPPH)} - OD_{(DPPH + sample)}/OD_{(DPPH)} \times 100$ [4]. The IC_{50} value denotes the concentration of the sample that inhibited DPPH by 50%. All tests were employed in triplicate. Quercetin was used as positive control while methanol was the negative control [5].

Reducing power assay

1 mL of *P. indica* Less. tea was mixed with 0.2mM phosphate buffer, pH 6.6 (2.5 ml) and 1% potassium ferricyanide (2.5 ml) and incubated at 50°C for 30 min. Aliquots of 10% trichloroacetic acid (2.5 ml) were added to the mixture, which was then centrifuged at 3,000 rpm for 10 min whenever necessary [6]. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared 0.01% ferric chloride solution (0.5 ml). The absorbance was measured at 700 nm. Quercetin at various concentrations was used for standard curve [7].

Total phenolic content

The total phenolic content of each sample was determined using Folin-Ciocalteu method [8] with slight modification. In this method, quercetin was used as a standard. Briefly, 125 µl of sample or the standard were mixed with 500 µl distilled water. 125 µl Folin-Ciocalteu reagent was added and then incubated for 6 minutes 1.25 ml of 7%(w/v) sodium bicarbonate was added then adjusted volume to 3 ml with distilled water and the incubation was allowed for 90 min. At the end, the absorption of each concentration was measured at 765 nm.

Total flavonoid content

The total flavonoid content was determined using a colorimetric method [9]. The sample was diluted with distilled water or methanol. The sample 0.5 ml was mixed with distilled water 2 ml. The next step adding 150 µl 5% $NaNO_2$ and mixed. Then, 150 µl of 10% $AlCl_3$ was added, the volume was adjusted to5 ml with distilled water. Mixed well and the absorbance was measured at 510 nm. Total flavonoid content was expressed as mg quercetin equivalents/g extract.

***In vitro* digestion**

An understanding of the basic physicochemical and physiological processes that occur as a food passes through the human gastrointestinal (GI) tract is required to develop effective *in vitro* models that accurately simulate digestion. After ingestion, food experience a complex series of physical and chemical changes as they pass through the mouth, stomach, small intestine, and large intestine, which affect their ability to be digested and/or absorbed.

However, this method was adapted from previous study [10-12]. Briefly, *P. indica* Less. tea were digested with a mixture ofα-amylase (pH 6.8) for 1h for oral digestion, followed by 1h gastric digestion with pepsin (pH 3-4) and then 2h intestinal digestion with pancreatin and bile salts (pH 7-8). All steps of the digestion were incubated in shaking water bath at 37°C (55 oscillation/min) [13]. All digestive products were collected and stored at -80°C in an airtight container until analysis.

3. RESULTS

The antioxidant activity was investigated. The results of the assay were expressed as % inhibition of DPPH radical both pre and post-digestion in oral, gastric and in intestinal phases (Figures 1 and 2). Pre-digestion showed the great inhibition of DPPH by 84.77% at 0.1 mg/ml. After digestion, % inhibition of DPPH radical markedly decreased nearly tenfold. When considered the IC_{50} to indicate the potential of this activity, the value in pre-digestion had lower than post-digestion of *P. indica* Less. The structural modification of bioactive compounds in sample due to the pH change during the GI system may influence to this scavenging activity [14].

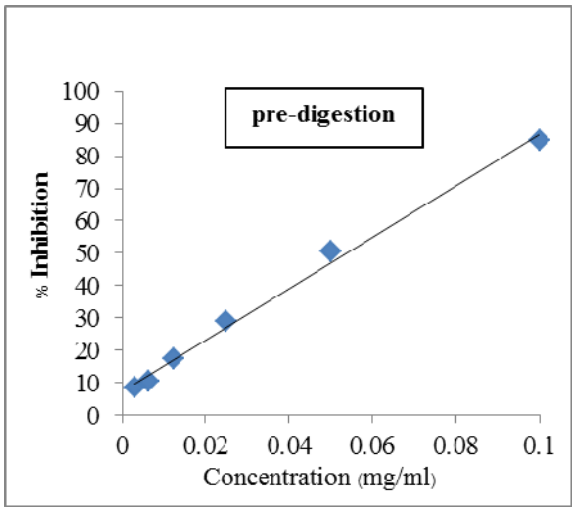

Figure 1. DPPH scavenging activity of *Pluchea indica* Less. tea prior to digestion.

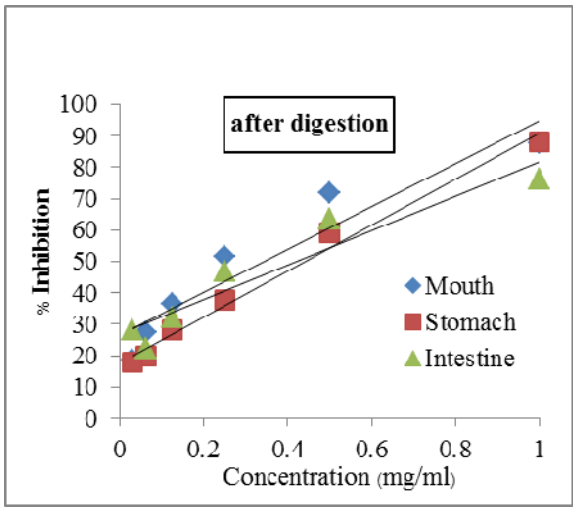

Figure 2. DPPH scavenging activity of *Pluchea indica* Less. tea after digestion.

The results of the reducing power, total phenolic contents and the total flavonoid content are shown in Table 1. All experiments were tested at a concentration of 1 mg/ml, and used quercetin as a reference for standard curve. No difference of recducing ability was observed both pre-digestion (143.46 ± 0.04 mg QE/g sample) and after the intestinal digestion (146.04 ± 0.001 mg QE/g sample). However, *P. indica* Less. tea was still effective for this activity. Total phenolic and total flavonoid contents of *P. indica* Less. tea displayed with high amounts at pre-digestion (196.70 ± 0.01mg QE/g sample) while the post-digestion (230.41 ± 0.01mg QE/g sample) significantly increased their phenolic and flavonoid contents. Especially, total flavonoid contents had the increasing trend in the intestinal stage. It may be due to some enzymes in the digestive system react with any bioactive compounds that present in *P. indica* Less. tea and thus free aglycone flavonoids were released [14-16].

Table 1. Antioxidant activities of *Pluchea indica* Less. tea at 1 mg/ml, which was used quercetin as a standard antioxidant.

Method	mg of quercetin equivalent / g of extract			
	Pre-digestion	Mouth	Stomach	Intestine
Reducing power assay	143.46±0.04	154.67±0.01	240.08±0.008	146.04±0.001
Total phenolic content	196.70±0.01	158.54±0.01	312.64±0.005	219.02±0.004
Total flavonoid content	230.41±0.01	551.22±0.002	643.22±0.001	454.00±0.003

Each value is expressed as mean ± standard deviation (n = 3).

4. CONCLUSIONS

P. indica Less. tea demonstrates the great antioxidant activity before digestion. Although some antioxidant effect will reduce after digestion. However, *P. indica* Less. tea was still effective activity. The digestive process in GI tract possibly influenced to the antioxidant activity and bioactive compounds of *P. indica* Less. tea. This tea may be considered as an alternative healthcare to consumer. But the study of the human digestive system is difficult because there are many factors involved, such as pH, digestive enzymes and the stability of chemical structure of bioactive compounds in this herbal tea.

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Neonothopanus nambi Speg., a new source of antibiotic and anti-inflammatory agents

Ampornrut Prapaiwong[*a], Sukanlaya Leejae[a], Teeratat Sudsai[a], Somporn Phonkrathok[a], Acharee Suksuwan[b,c], Prasan Tangyuenyongwatana[a], Krisana Kraisintu[a,c]
[a]Faculty of Oriental Medicine, Rangsit University, Pathum Thani, Thailand 12000
[b]Faculty of Pharmacy, Rangsit University, Pathum Thani, Thailand 12000
[c]Sino-Thai Traditional Medicine Research Center, Rangsit University, Pathum Thani, Thailand 12000

*sarcina_sine789@hotmail.com phone +66-29972200 fax +66-29972200

ABSTRACT

Neonothopanus nambi Speg. is a poisonous basidiomycete luminescent mushroom. The information on antibacterial and anti-inflammatory activities of the fungus has not been established. Study on antibacterial and anti-inflammatory potencies of *N. nambi* extracts using *in vitro* model were assessed. The results indicated that the mycelia of the luminescent mushroom extracted with $CHCl_3$, EtOAc, and culture filtrate extracted with EtOAc demonstrated excellent antibacterial activity against *Staphylococcus aureus* ATCC 25923 with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranged from 2-4 µg/ml, which is very closed to that of vancomycin (0.5-1 µg/ml). Moreover, hexane fraction of the collected mycelia exhibited obvious antibacterial potency against the pathogen with MIC and MBC values of 16 µg/ml. In contrast, all of the fractions were inactive against *Escherichia coli* ATCC 25922, a Gram-negative bacterium. For anti-inflammatory assay, $CHCl_3$ fraction of *N. nambi* mycelia showed the most potent inhibitory effect on nitric oxide-released macrophage cells with an IC_{50} of 10.9 µg/ml followed by EtOAc and hexane fractions with IC_{50} values of 19.7 and 25.6 µg/ml, respectively. Both of the culture filtrate and the collected mycelia extracted with $CHCl_3$ exhibited comparable effect to the positive controls, L-NA (NO synthase inhibitor, IC_{50} = 10.2 µg/ml), indomethacin (IC_{50} = 16.6 µg/ml), the standard used non-steroidal anti-inflammatory drugs. Purification, antibacterial mechanism of action, and anti-inflammatory activity of the extracts are still under investigations.

Keywords: *Neonothopanus nambi*, Luminescent mushroom, Antibacterial activity, Anti-inflammation

1. INTRODUCTION

Neonothopanus nambi Speg. is a poisonous basidiomycete luminescent mushroom which is found in Australia, South America, Central America and Malaysia. Pharmacological effects of sesquiterpenes isolated from the fungus exhibited pronounced cytotoxicity against cancer cell lines as well as antimalarial and antimycobacterial activities [1]. Moreover, the effect of bioactive compounds from *N. nambi* was investigated on infectious larvae of root-knot nematode (*Meloidogyne incognita*) *in vitro*. The results demonstrated that the compounds caused mortality of the nematode without adverse effects on beneficial organisms [2, 3]. However, antibacterial and anti-inflammatory activities of this luminescent mushroom have not been evaluated. Therefore, the objectives of this research were to study on antibacterial and anti-inflammatory activities of *N. nambi* extracts using *in vitro* model.

2. MATERIALS AND METHODS

Preparation of the luminescent mushroom extracts

Cultivation of *N. nambi* was carried out following a method from Kanokmedhakul et al. [1]. Briefly, the mushroom isolate No. 3 was collected from Ratchaburi province and the mushroom hyphae were grown in potato dextrose agar. The mycelia were transferred to potato dextrose broth and incubated in a dark room without shaking with 2 h of light per day at 25°C for 18 days.

Crude extracts from broth culture and mycelia were prepared as follows: liquid culture of *N. nambi* was filtered using Whatman filter paper No. 1 to collect culture filtrate. The filtrate was thrice partitioned and then evaporated to yield *n*-hexane-, $CHCl_3$-, EtOAc-, and H_2O-extracts (295, 25, 385 and 6,035 mg/l, respectively). The collected mycelia were macerated with MeOH (3 x 500 ml) for 3 days, under constant stirring at 250 rpm at room temperature. The mycelium mixture was filtered and the combined filtrate, once dried, was then added with 10% MeOH/H_2O mixture. The mixture solution was extracted three times with equal volume of *n*-hexane, $CHCl_3$, and EtOAc solvents. The water-crude fraction was freeze-dried and the organic crude extracts were dried over anhydrous Na_2SO_4 and then evaporated to obtain *n*-hexane-, $CHCl_3$-, EtOAc-, and H_2O-extracts with yield 56, 196, 114, and 366 mg/l, respectively. All of the mushroom extracts were kept at -20°C for future antimicrobial and anti-inflammatory assays.

Antibacterial assay

A modified broth microdilution method outlined by Clinical and Laboratory Standards Institute (CLSI) [4] was performed. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used in this study. The mushroom extracts were dissolved in 10% dimethyl sulphoxide and diluted two-fold to give final concentrations ranged from 1,024-0.5 µg/ml. One hundred microliters of the bacterial suspensions, containing approximately 10^6 colony forming unit/ml of the microorganism, was inoculated in 80 µl of Mueller-Hinton broth supplemented with 20 µl of the compound. The microtiter plates were incubated at 37°C for 16-18 h. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were recorded.

Nitric oxide measurement

Nitric oxide (NO) production by RAW264.7 cells was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent as previously described [5]. The % inhibition was calculated based on the following equation and IC_{50} values were determined graphically (n = 4):

$$\text{Inhibition (\%)} = [(A-B)/(A-C)] \times 100$$

A-C: NO_2^- concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)].

Assay of cell viability

Viability of RAW264.7 cells was assayed using a MTT colorimetric method [5].

3. RESULTS

The luminescent mushroom extracts were tested in broth microdilution assays to determine their MICs. The activities of the extracts against human pathogens are indicated in Table 1. The results demonstrated that the collected mycelia extracted with hexane (M1), $CHCl_3$ (M2), EtOAc (M3) and its culture filtrate extracted with EtOAc (F3) elucidated pronounced antibacterial activity against *S. aureus* ATCC 25923. The MIC and MBC values of the extracts against the microorganisms ranged from 2-16 µg/ml. The results suggested that the main bioactive compounds may obtain from the mycelia and EtOAc fraction of the culture filtrate. Purification and antibacterial mechanisms of the extracts are still under investigation. In contrast, the culture filtrate extracted with hexane (F1),

$CHCl_3$ (F2), and H_2O (F4) showed no antibacterial activity against the tested Gram-positive bacterium. All of the mushroom extracts were inactive against *E. coli* ATCC 25922, a Gram-negative bacterium.

Lipopolysaccharide (LPS) induced RAW264.7 macrophage cells are widely used as screening platform for anti-NO production by induction the transcription and protein synthesis of iNOS, and increased NO production [6]. The result demonstrated that the inhibitory effects of the mushroom extracts on NO production in a concentration-dependent manner were observed. $CHCl_3$ fraction of *N. nambi* mycelia exhibited the most potent inhibitory effect with an IC_{50} of 10.9 µg/ml followed by EtOAc and hexane fractions with IC_{50} values of 19.7 and 25.6 µg/ml, respectively. In addition, the hexane, $CHCl_3$, EtOAc, and H_2O extracts of the culture filtrates elucidated anti-inflammatory activity against NO-released macrophage with IC_{50} values of >100, 64.6, 27.4, and >100 µg/ml, respectively. However, the cytotoxic effect of the culture filtrate and the mycelium extracts on RAW264.7 cells was observed at a dosage of 10-100 µg/ml (Table 1). This aspect has to be considered in the application of the extracts for therapeutic purposes. Both of the culture filtrate and the collected mycelium extracted with $CHCl_3$ exhibited comparable effect with positive controls, L-NA (NO synthase inhibitor, IC_{50} = 10.2 µg/ml), indomethacin (IC_{50} = 16.6 µg/ml), the standard used non-steroidal anti-inflammatory drugs. Therefore, it also suggested that the major active compounds of *N. nambi* may obtain from $CHCl_3$ fraction. $CHCl_3$ fractions might show potent inhibitory effect on NO production in LPS stimulated RAW264.7 cells through the suppression of iNOS and COX-2 genes.

Table 1. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *Neonothopanus nambi* extracts against pathogenic bacteria and its anti-inflammatory activity on inhibition of nitric oxide production in RAW264.7 cells

Sample	Solvent extraction	Yield (mg/l)	MIC/MBC (µg/ml)		Inhibition of NO production IC_{50}[1] (µg/ml)
			Staphylococcus aureus ATCC 25923	*Escherichia coli* ATCC 25922	
F1	Hexane	295	1,024/>1,024	1,024/>1,024	>100
F2	$CHCl_3$	25	512/>1,024	1,024/>1,024	64.6
F3	EtOAc	385	4/4	256/512	27.4
F4	H_2O	6,035	>1,024/NA[2]	>1,024/NA	>100
M1	Hexane	56	16/16	>1,024/NA	25.6
M2	$CHCl_3$	196	2/2	512/512	10.9
M3	EtOAc	114	4/4	512/>1,024	19.7
M4	H_2O	366	1,024/>1,024	>1,024/NA	-
Vancomycin	-	-	0.5/1	-	-
Indomethacin	-	-	-	-	16.6
L-NA	-	-	-	-	10.2

[1]Each value represents mean ± S.E.M. of four determinations.

[2]Not applicable.

4. CONCLUSIONS

The mycelia of *N. nambi* extracted with hexane, $CHCl_3$, EtOAc, and culture filtrate extracted with EtOAc elucidated pronounced antibacterial activity against *S. aureus* ATCC 25923 with MIC and MBC values ranged from 2-16 µg/ml. Moreover, the fractions exhibited anti-inflammatory activity against NO-released macrophage with IC_{50} values ranged from 10.9-64.6 µg/ml. Purification, antibacterial mechanism, and *in vivo* anti-inflammatory activity of the extracts are still under investigations.

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Synergistic antibacterial activity of *Boesenbergia rotunda* extract and β-lactam antibiotic combination against multidrug-resistant bacteria

Yothin Teethaisong*[a], Nongluk Autarkool[a], Griangsak Eumkeb[a]
[a]School of Pharmacology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand 30000

*teethaisong.y@gmail.com phone +66-44224260 fax +66-44224650

ABSTRACT

An increase of drug resistance in pathogenic bacteria has been continuously documented, affecting patients and health care providers worldwide. Practically-prescribed antibiotics to treat these strains are not successful. Thus, searching for novel antibacterial compounds or new strategies are urgently necessary. To this aim, the present study investigated antibacterial activity of *B. rotunda* extract (BRE) and synergism with antibiotics against multidrug-resistant bacteria. Dried powdered roots of BR were extracted with 95% ethanol. MIC and checkerboard assays were performed to determine antibacterial and synergistic activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis*. The antibacterial activity was confirmed using killing curve assay. BRE revealed antibacterial activity at MIC 2 mg/ml against both MRSA and *S. epidermidis*, while weak activity was found in *Enterobacter cloacae* and *Escherichia coli* at equal MICs >1024 mg/ml. In checkerboard assay, the strongest synergistic activity was observed in BRE and amoxicillin combination against *S. epidermidis* at fractional inhibitory concentration index (FICI) 0.313. Other synergisms were also found in either BRE plus ampicillin or cloxacillin counter this strain. Similarly, BRE in combination with amoxicillin also revealed synergistic activity against MRSA. Killing curve assay was confirmed dramatic reduction in viable count in *S. epidermidis* treated with BRE and amoxicillin combination compared to control. In conclusion, our results suggest that BRE plus these penicillins can inhibit multidrug resistant bacteria. Interestingly, these combinations are an attractive application to develop as the novel anti-drug resistant bacteria, which almost resistant to practically prescribed antibiotics.

Keywords: *Boesenbergia rotunda*, Synergistic activity, β-lactam antibiotic, Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*

1. INTRODUCTION

Antibiotics resistance in pathogenic bacteria, in particular β-lactams, has emerged as a global health problem. Staphylococci are one of the most frequent resistances to many new and commonly prescribed antibiotics in current year as a consequence of the selective pressure produced by therapeutic misuse of antibiotics and abuse [1, 2]. Increased emerging multidrug resistances in extended-spectrum Enterobacteriaceae, such as *E. coli* and *E. cloacae,* in both hospitals and in the community have also been continuously reported during the past decade [3]. Consequently, practically-prescribed antibiotics in treatment of these strains are not effective. Thus, searching and development for novel antibacterial compounds and new strategies to combat drug resistant bacteria are urgently required. Plant-derived antibacterials are one of the most interesting sources of new therapeutics. *B. rotunda* (BR), or locally known in Thai as Krachai, is widely cultivated in Asian countries. This plant has traditionally been used as food ingredients and in treatments of several diseases such as antimicrobial, parasitic infections, antiulcer effect, anticariogenic, antimutagenic and anticancer/antitumor, as well as obesity treatment [4]. In addition, a drug combination approach is an attractive avenue to treat drug resistant bacteria by achieving synergistic multidrug targets, interacting with resistant mechanisms of bacteria and neutralizing or eliminating adverse effects [5]. To develop a novel agent against multidrug resistant bacteria, the present study investigated antibacterial activity of BRE when used alone and in combination with antibiotics against multidrug-resistant bacteria.

2. MATERIALS AND METHODS

Plant extraction

Fresh rhizomes of BR were purchased locally in Nakhon Ratchasima province, Thailand. Dried powdered of BR rhizomes were extracted with 95% ethanol for 8 h using Soxhlet extractor. Then, the extracts were evaporated and lyophilized to remove the solvent and to yield a brown crystal.

Bacterial strains and antibiotics

Clinical isolates of Methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA 20651), *Enterobacter cloacae* DMST 21394 (*E. cloacae* 21394) and *Escherichia coli* DMST 20662 (*E. coli* 20662) were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. *Staphylococcus epidermidis* ATCC 12228 (*S. epidermidis* 12228), *Staphylococcus aureus* ATCC 29213 (*S. aureus* 29213) and *Escherichia coli* ATCC 25922 (*E. coli* 25922) were obtained from the American Type Culture Collection (ATCC). Amoxicillin (AMX), cloxacillin (CLX), ampicillin (AMP) and ceftazidime (CTZ) were obtained from Sigma.

Bacterial suspension standard curve

To select a known viable count of bacterial suspension, the method of Liu et al. (2000), as previously described was followed [6].

Minimum inhibitory concentration (MIC) determinations

Antibacterial activities of BRE and selected antibiotics were performed using standard agar dilution in accordance with previously described by Clinical Laboratory and Standard Institute (CLSI) [7]. Briefly, bacterial suspension was adjusted spectrophotometrically to approximately 1 x 10^8 CFU mL^{-1}. Aliquot 0.1 mL of each adjusted strain to 0.9 mL MHB plus vary concentration of serial dilution antibacterial agents to give bacterial suspension approximately 1 x 10^7 CFU mL^{-1}. Antibacterial-free tubes were use as control. Then, Aliquots 2µL from each tube were spotted on agar surface (the final concentration of each spot was approximately 1 x 10^4 CFU mL^{-1}). The MICs of each antibacterial agent were read after incubated at 37ºC for 24 h. The MIC was defined as the lowest concentration of the drugs at which no visible growths in the triplicate spots were observed.

Checkerboard determinations

The interaction between BRE and antibiotics were investigated using checkerboard determinations in according to the method of Odds (2003) description with slight modifications [8]. The cultured and antibacterial agents were prepared and performed similarly with MIC determination. In different, the combinations of antibiotics plus BRE were mixed before dropping on agar plate in each spot. After incubated at 37ºC for 24 h, the FIC index (FICI) were calculated to determine drug interaction, and interpreted as previously described: FICI ≤ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism [8].

Killing curve determinations (viable counts)

In order to confirm antibacterial and synergistic activities of BRE when used singly and in combination with antibiotic, the combination that exhibited the lowest FICI was selected and carried out the killing curve assay. The viabilities of drug resistant bacteria after exposure to these agents alone and in combination at eight distinct times (0, 0.5, 1, 2, 4, 6, 8 and 24 h) were counted. The assay was followed the previously described by Mun et al. (2013) [9].

Briefly, Inocula (5×10^5 CFU mL^{-1}) were exposed to BRE either singly or in combination with penicillins. Aliquots (0.1 mL) of each exposed time was removed and diluted in normal saline as needed to enumerate 30-300 colonies. The diluted cultures were platted and spread on plate containing MHA. The growing colonies were counted after incubated at 37°C for 24 h. The lowest detectable limit for counting is 10^3 CFU mL^{-1}. The experiment was done in triplicate; data are expressed as mean ± SEM.

3. RESULTS

This study reported the synergistic antibacterial activity of BRE plus β-lactams antibiotic against multidrug-resistant bacteria was exhibited. The MICs of the BRE against clinical isolates of MRSA 20651, *S. epidermidis* 12228, *E. cloacae* 21394 and *E. coli* 20662 were 2, 2, >1024 and >1024 mg mL^{-1}, respectively, while against reference strain *S. aureus* 29213 was 4 mg mL^{-1} (Table 1). These results suggest that BRE alone has a high potency to inhibit staphylococci strains, which are gram positive bacteria, while show weak activities against gram negative *E. coli* 20662 and *E. cloacae* 21394. These may due to gram negative bacteria have more multi-layered and a high complex structure that its outer membrane can act as a barrier to antibiotics [10]. Furthermore, MIC results for selected β-lactams; AMX, CLX, AMP, and CTZ ranged from 16 - 1024 μg mL^{-1} against those selected drug-resistant bacteria (Table 1). These results reveal that those resistant bacteria are high resistant to all selected antibiotics. The reference strains used in the present study exhibited susceptible to antibiotics [7]. As previously reported, Panduratin A, a major compound isolated from BR, had been reported to possess antibacterial against clinical *Staphylococcus* strains and *Enterococci* clinical isolates [1, 11]. Therefore, the major active compounds of BRE against multidrug-resistant bacteria could probably be Panduratin A. These findings provide evidence that BRE may be developed to treat multidrug-resistant staphylococci. In checkerboard assay, BRE plus β-lactams combinations against MRSA 20651 and *S. epidermidis*12228 showed that there were strong synergistic activities among BRE plus AMX, CLX or AMP at FICI between 0.3-0.5, with exception for BRE plus AMP against MRSA at FICI 1 (Table 2).

The lowest FICI was observed in the combination of BRE plus AMX against *S. epidermidis*12228 at 0.313. The findings from checkerboard assay suggest that BRE can reverse multidrug-resistant Staphylococci to its susceptible to primary antibiotics. Moreover, the concentrations of these penicillins were dramatically reduced when they were combined with BRE. Probably, the mechanisms in inhibiting those bacteria may be due to interaction with several resistant factors such as inhibition of β-lactamase, increase in membrane permeability, and inhibition of bacterial cell wall synthesis [5, 12].

Table 1. MICs of *B. rotunda* extract and antibiotics against multidrug resistant bacteria

Antibacterial agents	Minimum inhibitory concentration (MIC)				
	AMX (μg mL^{-1})	CLX (μg mL^{-1})	AMP (μg mL^{-1})	CTZ (μg mL^{-1})	*B. rotunda* (mg mL^{-1})
MRSA DMST 20651	512 [R]	>1024 [R]	512 [R]	NT	2 [ND]
S. epidermidis ATCC 12228	128 [R]	16 [R]	128 [R]	NT	2 [ND]
E. cloacae DMST 21394	>1024 [R]	>1024 [R]	>1024 [R]	>1024	>1024 [ND]
E. coli DMST 20662	>1024 [R]	>1024 [R]	>1024 [R]	>1024	>1024 [ND]
**S. aureus* ATCC 29213	1 [S]	8 [S]	0.25 [S]	NT	4 [ND]
**E. coli* ATCC 25922	8 [S]	NT	NT	2 [S]	NT

AMX= amoxicillin; CLX=cloxacillin; AMP= ampicillin; CTZ= ceftazidime; NT= Not test. [S] = susceptible; [R]=resistant; [ND] = No data in CLSI; **S. aureus* ATCC 29213 and **E. coli* ATCC 25922 were used as reference strains.

Table 2. Results of *B. rotunda* extract in combination with amoxicillin, ampicillin and cloxacillin against MRSA DMST 20651 and *S. epidermidis* ATCC 12228

Compounds		MRSA DMST 20651					*S. epidermidis* ATCC 12228				
A	b	MIC_a	MIC_b	FIC_a	FIC_b	FICI	MIC_a	MIC_b	FIC_a	FIC_b	FICI
BR	AMX	0.5	64	0.25	0.125	0.375*	0.5	8	0.25	0.063	0.313*
BR	AMP	2	2	1	0.004	1.00	0.5	16	0.25	0.125	0.375*
BR	CLX	1	4	0.5	0.004	0.504*	0.5	2	0.25	0.125	0.375*

AMX= amoxicillin; CLX=cloxacillin; AMP= ampicillin; MIC_a= MIC of compound a in combination; MIC_b = MIC of compound b in combination. FIC_a = MIC of a in combination /MIC of a alone. FIC_b= MIC of b in combination /MIC of b alone; FICI = FIC_a+FIC_b. FICI ≤ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism. * Synergistic activity against multidrug-resistant bacteria.

Killing curve assay demonstrated that the control cells revealed steady increase in log phase viable counts. Whereas, BRE at 1/4 MIC and AMX at 1/2 MIC can inhibit growth at only first 4 h and slow increase growths were observed after 4 h. Interesting, BRE alone at 1/3 MIC, BRE + AMX at 1/8 + 1/32 and 1/5 + 1/21 MICs showed steep reduction in CFU throughout 24 h. While no CFU was observed after exposure to BRE alone at 1/2 MIC (data not showed). These results indicate that BRE antistaphylococal potency is depend on its concentration manner. The present findings establish evidence that the combination of BRE and AMX has synergistic activity against *S. epidermidis*.

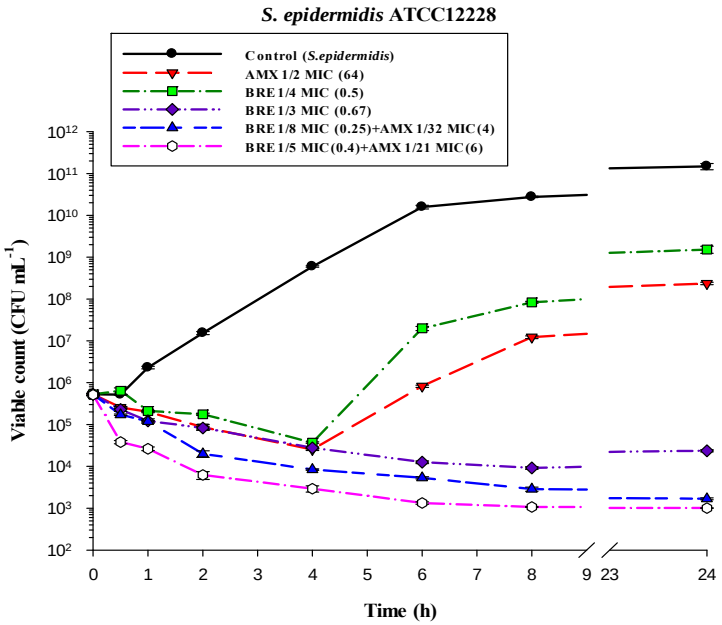

Figure 1. Time killing-curves of *S. epidermidis* ATCC 12228 after exposure BRE either alone or in combination with amoxicillin (AMX). Symbol represents: (●) control (antibacterial free); (▼) AMX alone at 1/2 MIC (64 µg mL^{-1}); (■) BRE alone at 1/4 MIC (0.5 mg mL^{-1}); (♦) BRE alone at 1/3 MIC (0.67 mg mL^{-1}); (▲) BRE at 1/8 MIC (0.25 mg/ml) combined with AMX at 1/32 MIC (4 µg mL^{-1}). (○) BRE at 1/5 MIC (0.4 mg mL^{-1}) combined with AMX at 1/21 MIC (6 µg mL^{-1}). The experiment was performed in triplicate. The plotted values are expressed as means ± SEM.

4. CONCLUSIONS

The results of this investigation suggest that BRE possesses antimultidrug-resistant bacteria, especially Staphylococci. Interestingly, this extract also has strong synergistic antibacterial activity with AMX, CLX and AMP against these Staphylococcal strains. These findings provide evidence that BRE plus these penicillins may develop to a novel phytopharmaceutical approach for combating multidrug-resistant bacteria, which almost resistant to practically prescribed antibiotics. However, the mechanism of action of these combinations should be further investigated. Of course, the animals and humans tests are still necessary to confirm the efficacy and toxicity of these combinations.

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Dillenia suffruticosa extracts inhibit proliferation of cervical cancer cells via G_2/M arrest and apoptosis through mitochondrial dysregulation and endoplasmic reticulum stress-induced apoptosis pathway

Wan Nor Hafiza Wan Abd Ghani[a,b], Latifah Saiful Yazan*[a,c], Jhi Biau Foo[c], Yin Sim Tor[c], Nurdin Armania[a,c], Heshu Sulaiman Rahman[d,e]

[a]Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[b]College of Medical Laboratory Technology, Institute for Medical Research, Ministry of Health, Jalan Pahang 50588 Kuala Lumpur, Malaysia

[c]Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[d]Faculty of Veterinary Medicine, Department of Microbiology and Pathology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

[e]UPM MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

*latifahsy@upm.edu.my phone +60-389472308 fax +60-389472336

ABSTRACT

The anticancer properties of ethyl acetate and dichloromethane extract of *Dillenia suffruticosa* (EADS and DCMDS) were studied. The cytotoxic effects were determined by MTT assay and flow cytometric technique. The effects on apoptotic-related genes and proteins were evaluated by qRT-PCR, Western blot and ELISA, respectively. EADS and DCMDS were found to significantly ($p<0.05$) inhibit the growth of HeLa and SiHa in a time- and dose-dependent manner. The expression of cyclin B1 was downregulated leading to G2/M arrest in HeLa and SiHa cells treated with DCMDS. Further, the extrinsic apoptotic pathway via caspase-8 and NF-κB pathways was activated. Meanwhile, the intrinsic signaling cascades were triggered upon activation of PARP1, NF-κB, p53 and JNK. Increased accumulation of Bax but decreased expression of Bcl-2 further enhanced the release of cytochrome c and increase in caspase-3 and -9 activities which ultimately led to apoptosis. DCMDS treatment also activated the endoplasmic reticulum (ER) stress genes chaperones *GRP78* and calreticulin (*CRT*); and ER transcription factors *CHOP/GADD153*, *XBP1* and *ATF4.* GRP58 is believed to play a role in DCMDS-induced ER stress-induced apoptosis whereby increased rate of apoptosis was noted with reduced level of the protein. On the other hand, expression of other ER stress genes was up regulated. The data indicate the prominent anti cervical cancer properties of DCMDS.

Keywords: *Dillenia suffruticosa*, Cervical cancer, Cell cycle arrest, Apoptosis, ER stress

1. INTRODUCTION

Dillenia suffruticosa (Griffith ex Hook. F. & Thomson) Martelli has a potential as a new anticancer agent [1-4]. In this study, the mechanisms underlying their cytotoxicity towards cervical cancer cells have been the major focus. The objective was to evaluate the expression of apoptotic-related genes and proteins in the human cervical cancer cell lines following treatment with dichloromethane extract of *D. suffruticosa* DCMDS.

2. MATERIALS AND METHODS

Preparation of EADS and DCMDS extract

The extracts used in this study were obtained by using a sequential cold solvent extraction method and tested as described elsewhere [2].

Cell culture

The human cervical adenocarcinoma (HeLa) cell line was purchased from the American Type Culture Collection (ATCC), Rockville MD, USA, and was grown in RPMI-1640. The culture medium was supplemented with 10% (v/v) of FBS and 1% of penicillin-streptomycin (100 IU/mL of penicillin and 100 µg/mL of streptomycin). The cells were allowed to grow at 37°C with 5% CO_2 atmosphere.

Determination of cytotoxicity of EADS and DCMDS

Cytotoxicity was assessed by using MTT assay [5]. The cells (0.7 x 10^5 cells/mL) were treated with different concentration of EADS and DCMDS (3.0 -200 µg/mL) in a 96-well plate for 24, 48 and 72 h. Control without the treatment was also included. Following incubation, 20 µL of MTT (5 mg/mL) was added to each well and placed at 37°C for 3 h. Next, 100 µL of DMSO was added to each well for solubilizing the blue crystal precipitate (formazan product). The absorbance at 570 nm and the reference wavelength of 630 nm was measured with an ELISA reader (Opsys MR, USA). Further analysis was performed on the DCMDS as the results showed that DCMDS was more cytotoxic than EADS. Determination of mode of cell death was carried out by using Annexin V–FITC Apoptosis Detection Kit I (BD Biosciences Pharmingen, Franklin Lakes, NJ, USA). The sample was analyzed by using the FACScan flow cytometer (Becton Dickinson, USA) equipped with FACStation running Cell Quest software.

Quantitative real-time Polymerase Chain Reaction (RT-qPCR)

HeLa cells were treated with DCMDS (12.5 µg/mL and 25 µg/mL) for 24 and 48 h. RNA was extracted using Total RNA Extraction Kit (Mini) Cells according to the manufacturer's instructions (Real Biotech Corporation, USA). Highly purified salt-free primers were designed by Next Gene Scientific Sdn. Bhd. Malaysia and synthesized by AIT Biotech, Singapore. The descriptions of the primers used for amplification of genes are shown in table below:

Primers for amplification of selected genes

Name		Primer sequence	Accession number
GRP78	F	5'-AGTTCTTCAATGGCAAGGAA-3'	NM_005347.4
	R	5'-CAGTTCAATACCAAGTGTAAGG-3'	
GRP58	F	5'-CATGTACGTTGCTATCCAGGC-3'	NM_005313.4
	R	5′-TGCTAAAGGAGAGAAGTTTG-3'	
CHOP	F	5′-ATGGCAGCTGAGTCATTGCCTTTC-3′	[6]
	R	5′-AGAAGCAGGGTCAAGAGTGGTGAA-3′	
XBP1	F	5'-GGAGTTAAGACAGCGCTTGGGGA-3'	[7]
	R	5'-TGTTCTGGAGGGGTGACAACTGGG-3'	
ATF4	F	5′-AACCGACAAAGACACCTTCG-3′	[8]
	R	5′-ACCCATGAGGTTTGAAGTGC-3′	
Calreticulin	F	5'-ACGAGCCAAGATTGATGACC-3;	[9]
JNK	R	5'-CAGAAGCTCCACCACCAAAGAT-3'	NM_139046
	F	5'-GCCATTGATCACTGCTGCAC-3'	
NF-κB	R	5'-GCGGGCGTCTAAAATTCTG-3'	NM_001077493
		5'-TTCCACGATCACCAGGTAGG-3'	
PARP-1	F	5'-TATCGAGTCGAGTACGCCAA-3'	NM_001618
	R	5'-GTGTGGGACTTTTCCATCAAA-3'	
Cytochrome c	F	5'-CCAGTGCCACACCGTTGAA-3'	[10]

	R	5’-TCCCCAGATGCCTTTGTT-3’	
	F	5’-TTCCATTCGCCCTTGTATTC-3’	
HPRT	R	5′-CTGCTACCACTACCTTCA-3′	NM_001101.3
	F	5'-CTCCTTAATGTCACGCACGAT-3'	
β-actin	R	5'-CGAGATGTGATGAAGGAGATG-3'	NM_000194.2
		5'-CCTGTTGACTGGTCATTACAA-3’	

F: Forward; R: Reverse

PCR products were synthesized using IQ™ SYBR® Green Supermix (Bio-Rad, USA). Real-time PCR reactions were performed in a total volume of 25 µL using the CFX 96 Real-Time PCR Detection System (Bio-Rad, USA). Thermal cycling programs were performed using the three-step cycling protocol in low-profile 0.2 mL tube strips (Bio-Rad, Hercules, CA, USA) in the dark. Conditions for all PCRs were optimized in a gradient cycler (Mastercycler Gradient, Bio-Rad, USA) with regard to various annealing temperatures (50–60°C). The gene expression was analyzed by CFX Manager Software Version 2.0.

Western Blot analysis of apoptotic related proteins

A HeLa cell was treated with DCMDS (12.5 µg/mL and 25 µg/mL) for 24 and 48 h. Western blot procedures were followed as described previously [11] with minor modifications. The membrane was incubated overnight at 4°C with the primary antibody, mouse anti-GRP58 (Abcam Corporation, USA) and anti-β-actin (Santa Cruz, USA) at a dilution of 1:2000. These steps were followed by incubation with secondary antibody, rabbit anti mouse IgG (Santa Cruz, USA) for 60 minutes at 25°C on rocker (dilution 1:4000). All immunoblots were visualized by enhanced chemiluminescence (ECL) plus Western blotting detection reagents (Abcam Corporation, USA). Densitometrical quantification of autoradiograms was analyzed by ImageJ software (version 1.41, National Institutes of Health, Bethesda, MD). GRP58 protein levels in cells treated with cisplatin and TQ were normalized against the intensity of β-actin.

Enzyme-linked immunosorbent assay (ELISA) of apoptotic related proteins

Untreated and treated HeLa cells (12.5 µg/mL and 25 µg/mL) were scraped, washed twice with ice-cold PBS and lysed for 60 minutes at 27°C in Lysis Buffer (1X) (Invitrogen, USA). The p53 and Bcl-2 protein concentration in the extract was estimated using the Human p53 Platinum ELISA kit and the Human Bcl-2 Platinum ELISA kit, respectively (Bender MedSystems GmbH, Vienna, Austria). The protease activity of caspases-3, caspase-8 and caspase-9 was evaluated spectrophotometrically using colorimetric assay kit (Gene script, USA) and caspase 12 using the Human Caspase-12 ELISA kit (Cusabio Biotech, China) in DCMDS-treated HeLa cells. Experiments were performed in triplicate. The samples extinction values were determined using ELISA plate reader (Biotek, USA) at 450 nm.

Statistical analysis

Statistical analysis was performed with General Linear Model (Univariate), Duncan’s multiples range test (DMRT) and Dunnett’s test using Statistical Package of Social Sciences (SPSS) for Window version 21.0 (SPSS Inc., Chicago, IL, USA). All the data are presented as mean ± standard deviation (SD). A mean difference is considered significant when $P<0.05$.

3. RESULTS

DCMDS promoted cell death machinery activation of the extrinsic pathway via caspase 8. Meanwhile, intrinsic signaling cascades were activated by activation of PARP1, NF-κB, p53and JNK molecules. Increased accumulation of Bax, but decreased expression of Bcl-2 further enhanced the release of cytochrome *c* and increase in caspase-9 and caspase-3 activities, and ultimately leading to apoptosis. DCMDS also activated the ER stress genes chaperones *GRP78* and *CRT*; and ER transcription factors *CHOP/GADD135*, *XBP1* and *ATF4.* GRP58 is believed to play a role in DCMDS-induced apoptosis as increased rates of apoptosis were noted with reduced levels of the protein. At the same time, increased levels of cyclin B1 led to G2/M arrest in HeLa.

4. CONCLUSIONS

DCMDS induced cellular stress in HeLa cells by dysregulation of mitochondrial pathways and ER-stress induced apoptosis causing morphological changes, cell cycle arrest and ultimately apoptosis. This study demonstrates the significant anti cervical cancer properties of DCMDS and its potential to be developed into an agent for the treatment of cervical cancer.

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Antibacterial activity of *Cyperus rotundus* extract against methicillin-resistant *Staphylococcus aureus*

Prairadda Cheypratub[a]*, Wilairat Leeanansaksiri[b], Griangsak Eumkeb[a]
[a]School of Pharmacology, [b]School of Microbiology, Institute of Science, Suranaree University of Technology, 111 University Avenue, Muang District Nakhon Ratchasima, Thailand 30000

*pcheypratub@gmail.com phone +66-44224273 Fax +66-44224650

ABSTRACT

Antibiotic resistance is the resistance of bacteria to antibiotics which originally sensitive. Therefore, medical standard treatments become ineffective and increase the risk of spread of bacteria leading to a worldwide problem. The *Cyperus rotundus (C. rotundus)*, a turf grass known as *Motha*, has been widely used in several fields of pharmacological applications including antibiotic resistance. The objective of this study was to investigate the effect of *C. rotundus* extract (CRE) on antibiotic resistant bacteria when use either alone or in combination with antibiotics. *C. rotundus* was extracted by soxhlet extraction with 95% ethanol for 8 h to get crude extract. The antibacterial susceptibility test of CRE against methicillin-resistant *Staphylococcus aureus* **(**MRSA**)** was primary tested by agar disc diffusion and agar dilution technique. The results showed that clear zone was 15 mm and the minimum inhibitory concentration (MIC) was displayed at 128 mg/ml. Checkerboard assay of CRE plus ampicillin exhibited synergistic activity against this strain at fractional inhibitory concentration index (FICI) at 0.25. Killing curve assay was confirmed dramatic reduction in viable count of MRSA after treated with the sub-MIC of this combination compared to control. In conclusion, the combination of CRE plus ampicillin demonstrated strong synergistic activity against MRSA. Therefore, this combination can be employed for the potential treatment of MRSA, which almost resistant to practically β-lactam antibiotics.

Keywords: Methicillin-resistant *Staphylococcus aureus, Cyperus rotundus,* Ampicillin, Synergistic activity

1. INTRODUCTION

Staphylococcus aureus is a bacterial pathogen for various infectious diseases such as, skin infection, wound infection, myositis, osteomyelitis, sepsis and bacteremia [9] including antibiotic resistances especially to methicillin. The methicillin-resistant *Staphylococcus aureus* (MRSA) causes many medical-therapeutic complications since *S. aureus* can pass nearly all the barriers of the host defenses [7, 9, 18]. Therefore, the medical standard treatments become ineffective and increase the risk of spread of bacteria leading to a worldwide problem. Nowadays, the developments of new therapeutic approaches are required as well as the alternative therapies such as medicinal plants. The *Cyperus rotundus* (*C. rotundus*), a turf grass known as *Motha*, has been widely used in several fields of pharmacological applications including antidiarrhoeal, anti-inflammatory, antidiabetic, anticancer activities and antibiotic resistance [8,12,13,20,21,22]. According to phytochemical reports, *C. rotundus* elicits the presence of some active ingredients e.g. alkaloids, flavonoids, monoterpenes, sesquiterpenes, sitosterols, tannins and essential oil [2, 22]. *C. rotundus* has a dramatic effect on *Proteus vulgaris* (*P. vulgaris*) while *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) can resist to the herb [22, 24]. However, *C. rotundus* has strong inhibitory effect on *Salmonella enteritidis* (*S. enteritidis*), *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*) [8, 22]. In contrast, the essential oil extract is reported to be antibacterial activity against several bacteria as follows *E. coli, S. aureus, P. aeruginosa* and *Bacillus subtilis* (*B. subtilis*) [2]. The essential oil of *C. rotundus* revealed significant antibacterial activity against gram-positive bacteria compared to that of gram–negative bacteria [25]. So, the objective of this study was to investigate the effect of CRE on antibiotic resistant bacteria when use either alone or in combination with antibiotics.

2. MATERIALS AND METHODS

Plant extraction

The ethanolic extractions of medicinal plants were the most active against several bacterial strains comparing to the other solvents [16, 19]. Therefore, dried tubers of *C. rotundus* was extracted by 95% ethanolic soxhlet extraction for 8 h [3, 10] to obtain crude extract followed by filtration through Whatman No 1. [15], evaporation at 110 mbar to expel the solvent and freeze-drying for 4-5 days. The CRE was stored in refrigerator at -20°C. The percent yield of CRE was calculated as:

$$\% \text{ Yield of CRE} = \frac{\text{Weight of freeze-dried CRE} \times 100}{\text{Weight of dried } \textit{C. rotundus}}$$

Bacterial suspension standard curve

Both *S. aureus* sensitive strain and MRSA were cultured in 50 ml of Mueller-Hinton Broth (MHB) for 18 h followed by harvesting and washing the bacterial cells three times with 0.85% NaCl. The bacterial cells were re-suspended in 10 ml of 0.85% NaCl then diluted to **o**ptical density (O.D.) of 0.05, 0.10, 0.15, 0.20 and 0.25 at 500 nm wavelength using 0.85% NaCl as a blank. Spread plate technique was employed for triplicate viable plate count on overdried Mueller-Hinton Agar (MHA). Standard curves of both bacterial strains mentioned above were plotted to obtain 10^8 cfu/ml [5, 14].

Agar disc diffusion

The 18 h cultures of *S. aureus* sensitive strain (ATCC 29213) and MRSA (DMST 20651) in MHB were prepared to achieve 10^8 cfu/ml by using bacterial suspension standard curve. Each bacteria with 10^8 cfu/ml was thoroughly swabbed on MHA plates. Each 10 µl of *Cyperus rotundus*, *Capsicum annuum* and *Curcuma longa* crude extracts at 250 mg/ml in 10% DMSO was subjected to agar disc diffusion for primary sensitivity screening test. The 10 µg/ml of ampicillin in distilled water and 100% DMSO were also subjected for screening test as positive and negative controls, respectively. Each sample was separately dropped onto each paper disc. All discs were put on the overdried surface of Mueller-Hinton agar plates and were incubated at 37°C for 18 h. Finally, diameters of inhibition zone were measured and compared to positive and negative controls [4, 14].

Minimum inhibitory concentration (MIC) by agar dilution technique

MIC is defined as the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a microorganism after overnight incubation. The 18 h of bacterial culture was tested in MHB containing various concentrations of *C. rotundus* crude extract (CRE) and ampicillin by two-fold serial dilutions. The two-fold serial dilutions were started from 0.125 to 256 mg/ml for CRE and 2 to 1,024 µg/ml for ampicillin. The 18 h of MHB cultures for *S. aureus* sensitive strain and MRSA were prepared to reach 10^8 cfu/ml according to bacterial

suspension standard curve. After 5 mins incubation in MHB with various concentrations of CRE and ampicillin, the 2 µl containing 10^4 cfu/ml of each test samples were separately transferred onto MHA surface and incubated at 37°C for 18 h. 10% DMSO without CRE and ampicillin were used as control. The lowest concentration of CRE at the first clear spot of no bacterial growth is defined to MIC [4, 14].

Checkerboard assay

Checkerboard assay is the technique that has been used to assess the activity of antibacterial combinations *in vitro*. The 18 h of MRSA culture in MHB was harvested, washed and diluted to 10^8 cfu/ml using bacterial suspension standard curve as a reference. The MRSA was incubated for 5 mins in MHB with various concentrations of CRE and ampicillin starting from 0-128 mg/ml and 0-1,024 µg/ml, respectively. The 2 µl containing 10^4 cfu/ml of each test samples was transferred separately onto MHA surface and incubated at 37°C for 18 h. MICs of CRE (128 mg/ml) and ampicillin (> 1024 µg/ml) were determined for antibacterial combination and the isobologram was plotted: Y axis was CRE concentration in mg/ml, while X axis was ampicillin concentration in µg/ml [5].

Fraction inhibitory concentration (FIC) index

FIC index for antibacterial combination was calculated by following formula to determine synergistic activity [4].

$$FIC_{index} = \frac{\text{Concentration of A in MIC of A+B}}{\text{MIC of A alone}} + \frac{\text{Concentration of B in MIC of A + B}}{\text{MIC of B alone}}$$

FIC index $\leq$ 0.5 Synergism
FIC index $>$ 0.5-4.0 No interaction
FIC index $>$ 4.0 Antagonism

Time killing assay

Time killing assay can be employed to evaluate the antibacterial activity of CRE either alone or in combination with antibiotics. Furthermore, the assay can also be used to confirm checkerboard assay. The MRSA was cultured in 50 ml of MHB for 18 h followed by re-culturing again in 49 ml of MHB for 4 h to get the mid-log phase culture. The mid-log phase MRSA in MHB was prepared to obtain the final concentration of 5 x 10^5 cfu/ml by using bacterial suspension standard curve. Then, the MRSA test samples were subjected to three different conditions with their half MIC treatments as follows: 512 µg/ml of ampicillin, 64 mg/ml of CRE and the combination of 32 mg/ml of CRE mixing with 1 µg/ml of ampicillin. The MRSA in 10% DMSO without antibacterial substance was used as a control. All test samples and control were incubated at 37°C and taken every 1 h interval for triplicate viable plate count on overdried MHA ranging from 0 to 24 h [5, 6].

3. RESULTS

The 95% ethanolic extraction of CRE gave the percent yield of 7.23% w/w after evaporation and freeze-drying. The dried CRE was kept at -20°C for study of a whole research.

Antibacterial susceptibility of medicinal plants by agar disc diffusion was recorded in Table 1. CRE gave the highest inhibition zone for both *S. aureus* sensitive strain and MRSA at 12 mm and 15 mm, respectively. Furthermore, 10 mm of inhibition zone was shown for *S. aureus* sensitive strain (Figure 1A) whereas there is no inhibition zone for MRSA treated with ampicillin (Figure 1B).

Table 1. Antibacterial susceptibility of medicinal plants by agar disc diffusion method.

Pathogenic bacteria	Inhibition zone of medicinal plants (mm.)				
	Capsicum annuum (250 mg/ml.)	*Cyperus rotundus* (250 mg/ml.)	*Curcuma longa* (250 mg/ml.)	Ampicillin 10 µg/ml (positive control)	100% DMSO (negative control)
S. aureus ATCC 29213	0 mm.	12 mm.	8 mm.	10 mm.	0 mm.
S. aureus DMST 20651	0 mm.	15 mm.	9 mm.	0 mm.	0 mm.

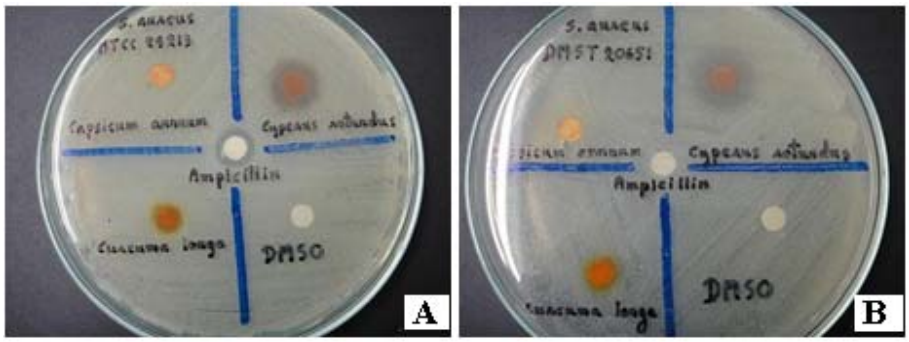

Figure 1. Antibacterial susceptibility of medicinal plants by agar disc diffusion method (A) *S. aureus* sensitive strain, (B) MRSA.

According to bacterial suspension standard curve, it indicated that in order to obtain 10^8 cfu/ml of both *S. aureus* sensitive strain and MRSA, the bacterial suspension with O.D 0.08 must be taken (data not shown).

MICs for both *S. aureus* sensitive strain and MRSA treated with CRE and ampicillin were demonstrated in Table 2. MRSA treated with CRE displayed the results at 128 mg/ml which is higher than that of *S. aureus* sensitive strain at 64 mg/ml (Figure 2A and Figure 2B). The bacteria treated with ampicillin also showed the results at 64 µg/ml for sensitive strain and >1,024 µg/ml for MRSA (Figure 3A and Figure 3B).

Table 2. Minimum inhibitory concentration (MIC) of CRE by agar dilution method.

Pathogenic bacteria	MIC	
	Cyperus rotundus (mg/ml)	Ampicillin (µg/ml)
S. aureus ATCC 29213	64	64
S. aureus DMST 20651	128	>1,024

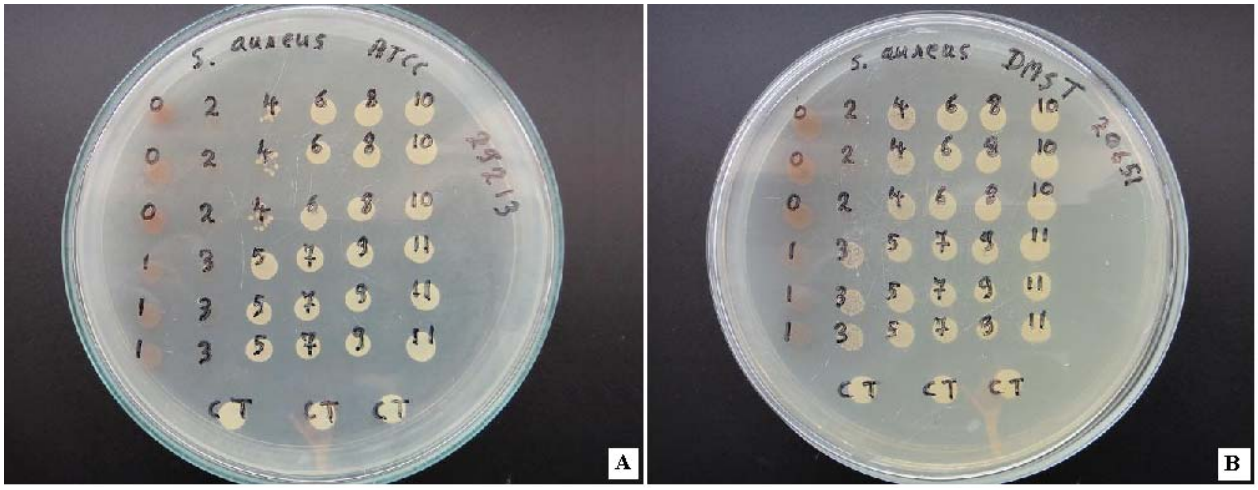

Figure 2. Minimum inhibitory concentration (MIC) of CRE by agar dilution method (A) *S. aureus* sensitive strain, (B) MRSA: where 0 = 256 mg/ml, 1 = 128 mg/ml, 2 = 64 mg/ml, 3 = 32 mg/ml, 4 = 16 mg/ml, 5 = 8 mg/ml, 6 = 4 mg/ml, 7 = 2 mg/ml, 8 = 1 mg/ml, 9 = 0.5 mg/ml, 10 = 0.25 mg/ml, 11 = 0.125 mg/ml, CT = control.

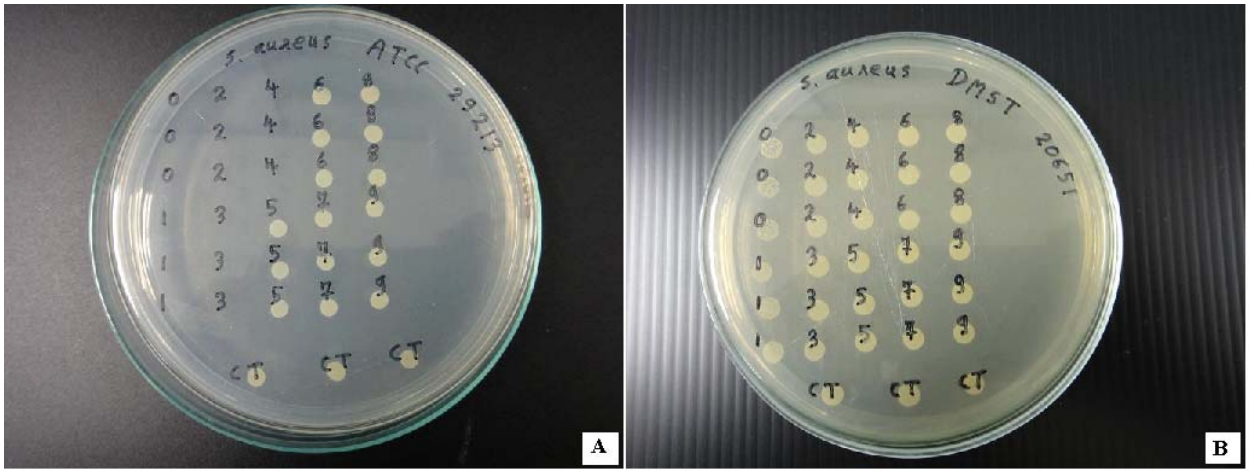

Figure 3. Minimum inhibitory concentration (MIC) of amicillin by agar dilution method (A) *S. aureus* sensitive strain, (B) MRSA: where 0 = 1,024 µg/ml, 1 = 512 µg/ml, 2 = 256 µg/ml, 3 = 128 µg/ml, 4 = 64 µg/ml, 5 = 32 µg/ml, 6 = 16 µg/ml, 7 = 8 µg/ml, 8 = 4 µg/ml, 9 = 2 µg/ml, CT = control.

Moreover, checkerboard assay and FIC $_{index}$ at 0.25 indicated synergistic activity for combination of CRE and ampicillin against MRSA at 32 mg/ml CRE and 1µg/ml ampicillin (Figure 4).

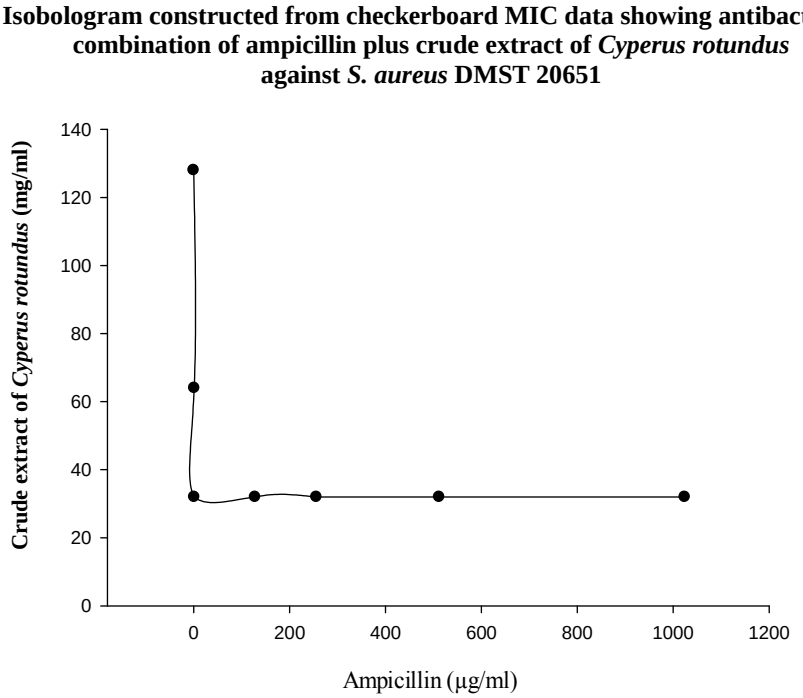

Figure 4. Isobologram constructed from checkerboard assay showing antibacterial combination of CRE and ampicillin against MRSA.

Synergistic activity was defined as a decrease of equal to or more than $2\log_{10}$ cfu/ml in comparison to the initial inoculum while the interaction was considered for bactericidal agent if there was a decrease of equal to or more than $3\log_{10}$ cfu/ml [1, 11, 23]. Bacteriostatic activity was defined as a reduction of less than $3\log_{10}$ cfu/ml [17]. In this study, time killing assay showed synergistic activity for the combination of CRE at 32 mg/ml and ampicillin at 1 µg/ml against MRSA (Figure 5). The mid-log phase viable cell count of the CRE-ampicillin treated MRSA was

dramatically reduced to 133 cfu/ml or $4.263\log_{10}$ cfu/ml which is more than $2\log_{10}$ cfu/ml comparing to initial inoculum at 567,000 cfu/ml. The data not only indicated dramatically synergistic activity but also indicated bactericidal activity due to the reduction of bacterial cfu/ml more than $3\log_{10}$. Moreover, the number of MRSA was not able to recover within 24 h. According to the time killing curve, MRSA treated with 512 µg/ml of ampicillin gave the high survival rate because of its highly resistant capacity whereas 64 mg/ml of CRE alone revealed the ability to inhibit MRSA as a bacteriostatic agent with the viable cell count (14,330,000 cfu/ml or $1.628\log_{10}$ cfu/ml) less than $3\log_{10}$ cfu/ml when compare to control.

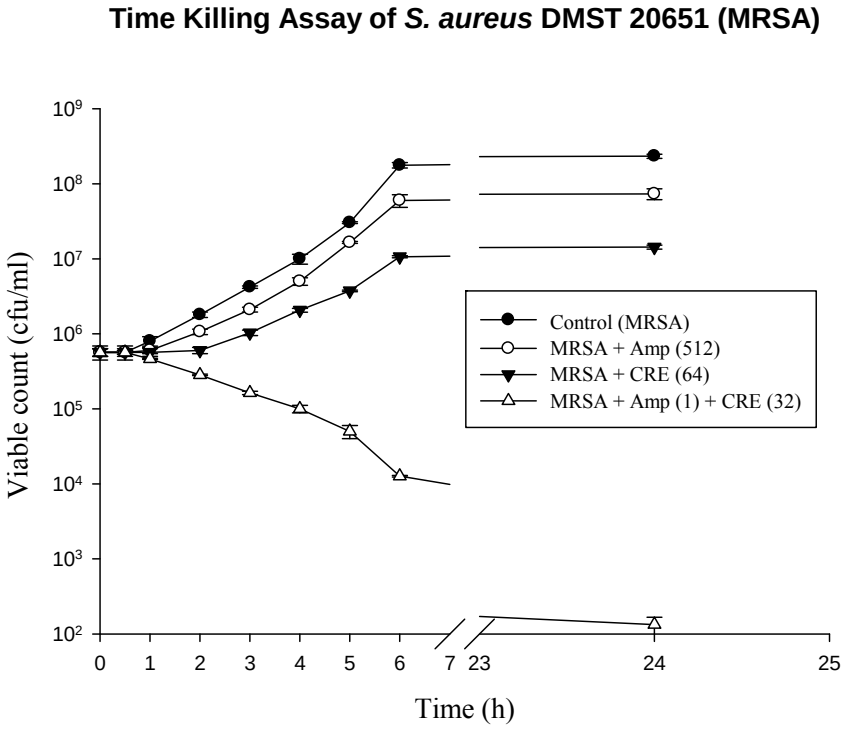

Figure 5. The medicinal effect against MRSA by ampicillin and *Cyperus rotundus* crude extract alone including the combination of ampicillin and *Cyperus rotundus* crude extract: Control = MRSA; Amp = Ampicillin 512 µg/ml; CRE = *Cyperus rotundus* crude extract 64 mg/ml; Amp + CRE = Combination of Ampicillin 1 µl/ml and *Cyperus rotundus* 32 mg/ml.

4. Conclusions

In conclusion, the combination of CRE plus ampicillin demonstrated strong synergistic activity against MRSA whereas CRE alone revealed the ability to inhibit MRSA as a bacteriostatic agent. The results obtained in this study indicate that CRE possess the active compound with antibacterial property. Nowadays the available antibiotics are becoming ineffective against several bacteria either Gram-positive or Gram-negative as they are developing resistance to its. Therefore, CRE offers for the development of a valuable adjunct to ampicillin against MRSA, which almost resistant to practically β-lactam antibiotics.

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The intrinsic values of mangrove plant species among Malay community: case study at Tanjung Dawai, Kedah

Rashidi Othman*[a], Razanah Ramya[a], Zainul Mukrim Baharuddin[a],
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit,
Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design
International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia

*rashidi@iium.edu.my phone +60-126644772 fax +60-361964864

ABSTRACT

Presently, mangrove forest is in serious declination and about 1% area per year has been loss in Malaysia due to over exploitation for timber, aquaculture activities, urbanization and increasing number of sea level. The community in coastal area who attached directly to this resource may have reflected the knowledge about the botany and ecology of this forest. The aim of this study is to explore the intrinsic values particularly ethno-botany of mangrove species that influenced Malay community in their daily life who inhabit near mangrove forest. Fieldwork was conducted in Tanjung Dawai at Kedah, Matang Mangrove Forest at Perak and Sungei Buloh Wetland Reserve and Kranji Nature Trail at Singapore using the methods of semi-structured interviews, participant observation and informal discussion with resident and plant specialist to collect plant material specimens. The results revealed that, there were almost 35 mangrove plant species had been identified and from 50 respondents from Malay community in selected villages, 19% of mangrove species were used for utility, 14% were used for food and 9% were used for medicine. This research finding showed the soil conditions and environmental factors influenced the species growth meanwhile community, locality, cultural factors and environmental condition may influence people to adopt their resources in daily life. This study emphasizes on the local people perspectives in manipulating mangrove species as their halal natural resources which later can be turned into plant materials selection for landscape ethno-botany planting design in creating genus loci or identity for that particular location or space.

Keywords: Mangrove, Landscape ethno-botany, Malay culture, Halal natural resources

1. INTRODUCTION

The increasing temperature about 0.7ºC will give an impact towards mangrove species by changing in species composition, phonological patterns (flowering and fruiting) and mangrove productivity. Temperature is vital factor effect on types of forest and geography especially distribution of mangrove species [1-3]. Malaysia experiences the increasing of sea level two times from the previous average about 1 meter and currently increase about 2 meter with current average climate changes is in limitation of temperature (0.7°C - 2°C). If the temperature increase beyond that level about 5°C- 6°C may destroy the world [4]. In Malaysia, mangrove ecosystem located in silt-rich and brackish water environment along tropical and nearly to river estuarine as a good indicator of coastline area [5]. Mangrove botanist has divided mangrove species into three categories which are exclusive (mangrove habitat in tidal area), non-exclusive (species that is not restricted by mangrove community) and associate (transitional mangrove species between land and sea [6]. Ethno-botany defined as knowledge on how people of particular regions and culture use the plants in their local environment in term of food, medicine, utilities, religious, aphrodisiacs, poisonous and tannin [1-8]. This research aimed to explore intrinsic ethno-botanical values of mangrove species in Malay community as well to preserve this knowledge from disappear.

2. MATERIALS AND METHODS

Sample and study location

Tanjung Dawai (5°41'5"N 100°21'50"E) is located in the largest mainland mangrove forest of Kedah at Sungai Merbok Mangrove Forest Reserve that considered as one of the most diverse mangrove species in the world with more than 30 species of true mangrove plants species [9-11]. Two villages had been chosen for this study which is Kampung Nelayan and Kampung Huma. The mangrove forest is resourceful to the coastal community that depends on mangrove forest in their daily life for variety uses [12]. 50 respondents from Kampung Nelayan and Kampung Huma had been interviewed based on how mangrove plants species associate in their daily life as natural resources in term of food, medicine, utilities and others with focus on Malay community. To facilitate cross-checking of plant species, the specimens were identified through various floristic records or secondary data such as sources from books, internet, University of Malaya herbarium and Forest Research Institute of Malaysia (FRIM) herbarium, Kepong; and also from previous research studies and journals to ascertain the nomenclature [13].

3. RESULTS

50 respondents from Malay communities have been interviewed from two villages which are Kampung Nelayan and Kampung Huma at Tanjung Dawai near to Sungai Merbok. 19% of the Malay community used the mangrove species for utility, 14% used for food and only 9% used for medicine as detailed in table 1. This study established that, the Malay community use mangrove species in their daily life in term of utility, food and medicine. Six types of mangrove species were observed to be used for utilities such as charcoal, pole and fence. Seven types of mangrove species are commonly used for food which mainly from fruit such as *Acrostichum aureum, Bruguiera cylindrical, B. parviflora, Excoecaria agallocha, Nypa fruticans, Sonneratia caseolaris* and *S. ovata*. Meanwhile, five types of mangrove species were found to be used as medicine such as *Acanthus ebracteatus, Morinda citrifolia, Nypa fruticans, Rhizophora* sp. and *Sonneratia ovata.* The most useful part is leaves and normally used to stop bleeding and control blood pressure. Interestingly, the results showed that the Malay community used mangrove species in their daily life in term of food, utility and medicine throughout the year. There are some limiting factors that contribute them to use or select specific mangrove species such as the followings:

1. Location
   The location of the village is surrounded by mangrove forest which forced them to explore functions of mangrove species to be adopted in their daily life.
2. Economy factor
   The poverty factor of the Malay community makes them to utilize mangrove species in term of food, utility and medicine for their benefit in daily life.
3. Environment
   It refers to the types of mangrove forest ecosystem such as clayey or sandy type as a part of their living environment. Different type of soil will have different type of mangrove species
4. Culture

The role of shaman (*bomoh*) and Malay folk also influenced the selection of mangrove species by Malay community s to support their daily life.

4. CONCLUSIONS

In conclusion, the result established that mangrove species can be considered as one of the most important plant in Malay ethnic and in their daily life. In general, mangrove species have a very strong relationship with Malay. Throughout the history, Malay has held mangrove species in great esteem, not only as their source of food, utility, medicine but also as a symbol of their belief.

Table 1. List of mangrove species used by the Malay community in their daily life at Kampung Huma and Kampung Nelayan.

Ethnobotany	Scientific Name	Botanical Name	Part used	Remarks
Utility	*Avicennia* sp.	Api-api	Pole	Piling, firewood, roof, *wakaf*, charcoal
	Bruguiera cylindrica	Berus-berus	Pole	Fence
	Bruguiera parviflora	Lenggadai	Pole	Fence, charcoal
	Nypa fruticans	Nipah	Leaves, shoot	Roof, cigarettes
	Rhizophora sp.	Bakau	Pole	Piling, firewood, fish trap, fence, charcoal, furniture
	Xylorcarpus sp.	Nyireh	Pole	Fish trip
Food	*Acrostichum aureum*	Piai raya	Leaves	Vegetable
	Bruguiera cylindrica	Berus-berus	Seed, fruit	Porridge, cake (*bingka*), eat raw
	Bruguiera parviflora	Lenggadai	Seed, fruit	Porridge, eat raw
	Nypa fruticans	Nipah	Fruit, flower	Drink, eat raw
	Sonneratia caseolaris	Berembang	Fruit	Eat raw
	Sonneratia ovata	Gedabu	Fruit	Eat raw, cake (*bingka*)
Medicine	*Acanthus ebracteatus*	Jeruju	Leaves	Abscess, control high blood pressure, stop bleeding
	Morinda citrifolia	Mengkudu	Leaves	Control blood pressure
	Nypa fruticans	Nipah	Flower, fruit	Diabetes mellitus, control high blood pressure, cough
	Rhizophora sp.	Bakau	Bark, leaves	Skin parasite, stop bleeding
	Sonneratia ovata	Gedabu	Seed	Headache

Avicennia sp.	*Rhizophora sp.*	*Xylorcarpus sp.*	*Nypa fruticans*

S. caseolaris	*Acanthus ebracteatus*	*A. aureum*	*S. ovata*

Figure 1. Mangrove species used by Malay community at Kampung Huma and Kampung Nelayan.

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Traditional Malay midwifery practices for body treatment (*Param*) and forehead treatment (Pilis) in Kelantan, Terengganu, Pahang and Johor of Malaysia

Rashidi Othman*[a], Razanah Ramya[a], Nooriszai Ishak[a]
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environmental Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia

*rashidi@iium.edu.my phone +60-126644772 fax +60-361964864

ABSTRACT

Midwifery is the practice of assisting a woman through childbirth using natural procedures with limited access to biomedicine. However, today in Malay community, midwifery continues to play an important role in both traditional and modern cultural settings that involved with providing health care during pregnancy, childbirth and postnatal care to both mother and newborn. Param or outer body treatment and Pilis known as forehead treatment are two famous traditional Malay midwifery practices among Malay culture and exhibit a wide variation in plant materials selection. This study established that plant materials selection differs with Malay midwifery practices, locality, environmental factors and culture. A total of 19 Malay midwives were observed in four different states of Malaysia in Kelantan, Terengganu, Pahang and Johor. There were at least 8 most common plant materials used at all 4 localities during Berparam which are lime, Lengkuas, Kunyit, Cekur, Halia, Halia bara, garlic and Serai wangi. Surprisingly none were similar in plant composition selection during Berpilisat all localities. At Kelantan and Terengganu 2 species were used whereas in Pahang 3 species and Johor 9 species were used during Berpilis. All plant materials composition for Berpilis at 4 different states was not similar. Paradoxically, for Berparam, many similarities in term of species used from Kelantan, Pahang and Terengganu whereas from Johor the selection of plant materials were also quite similar. The study highlighted on traditional Malay midwifery practices (Param and Pilis) in creating ethnic's identity and sense of place for landscape ethno-botany planting design in particular locality.

Keywords: Traditional Malay midwifery, Postnatal treatment, Landscape ethno-botany, Param, Pilis.

1. INTRODUCTION

Introduction of western medicine and medical practices in Malaya (Malaysia) in the last century has become widespread however many rural communities are still heavily dependent on traditional or indigenous plant medicine as their primary health care [1]. Due to rapid changes in socio-economic, environmental and cultural belief in Malaysia, the use of ethnobotanical plant species as herbal medicine is in transformation. The impacts towards the society in traditional medicine and modern medical system are as follows: traditional knowledge of herbal medicine is disappearing; traditional healers are becoming rare and less respected and medicinal plants are over-harvested [2-3]. Ethno-botany can make a positive contribution to alternative treatment in modern medical practices by identifying locally available plant resources, indigenous knowledge and traditional healers [4].

Midwifery is the practice of assisting a woman through childbirth using natural procedures. In both traditional and modern cultural settings, midwifery is involved with providing health care, during pregnancy, childbirth and postnatal care to both mother and newborn. The concept or stages in traditional Malay midwifery practices are swinging the tummy or coconut belly rubs (*lenggang perut*), traditional confinement (*pantang*), point massage (*bertungku*), body treatment (*param*), forehead treatment (*pilis*), vaginal heat or herbal treatments (*tangas*), body girdle (*bengkung*), massage (*urut*), traditional bath (*mandi serom*), herbal decoction (*ubat periuk/jamu/makjun*), heat treatment (*bersalai*), diet during pregnancy, confinement and miscarriage [5]. *Param* or body treatment is a form of herbal concoction made into a body spread for applying to the whole body especially after or before traditional bath. The purpose is to smooth the skin, tone the muscles and burn the body fat and at the same time remove body odour, avoid unwanted cool wind and occurrence of varicose veins [5-6]. The ingredients of *param* used among traditional Malay midwives are quite similar which are uncooked rice, glutinous rice, tumeric, *cekur, jerangau, lempoyang* and *rambai* with mixture of floral water. The purpose of *pilis* is to avoid newborn mother from stress or postpartum depression (meroyan) during pregnancy. *Pilis* ingredients made up of herbal concoctions which are rice flour, cinnamon stick, garlic and mixture of floral water and will be placed onto forehead in between the eyebrows and below hair lines after every baths [6-7]. Therefore this study aimed to determine plant materials selection by Malay midwifery practices of *param* and *pilis* from different locality, environmental factors and culture in order to prevent this knowledge from become history and disappear.

2. MATERIALS AND METHODS

Sample and study location

This semi structure interview and observation were conducted at Kelantan, Terengganu, Pahang and Johor and 19 Malay midwives were selected for *param* and *pilis* treatments. Ethnobotanical data were collected according to the methodology [8]. A semi-structured questionnaire was used to extract information on the ethno-botanical uses of plants. To facilitate cross-checking of plant species, the specimens were identified through various floristic records or secondary data such as sources from books, internet, University of Malaya herbarium and Forest Research Institute of Malaysia (FRIM) herbarium, Kepong; and also from previous research studies and journals to ascertain the nomenclature [9].

3. RESULTS

There were at least 8 most common plant materials used at all 4 localities during *berparam* which are lime, *lengkuas, kunyit, cekur, halia, halia bara,* garlic and *serai wangi*. All these species were quite popular as kitchen garden or ingredient in Malay culture. Surprisingly none were similar in plant composition selection during *berpilis* at all localities. At Kelantan and Terengganu 2 species were used during *berpilis* whereas in Pahang 3 species were used and Johor 9 species were used. All plant materials composition selection for *berpilis* at 4 different states was not similar. Paradoxically for *berparam*, many similarities in term of species used were observed especially localities from east coast (Kelantan, Pahang and Terengganu) whereas from west coast (Johor) the selection of plant materials were not much different.

Considerable research interest has recently focused on the Malay traditional midwifery practices in Malaysia [5-7]. Unfortunately little information is available on the influence of the environment on plant materials composition selection in Malay garden identity, especially locations and culture. Culture and environment interactions have been reported to account for variation in plant species selections towards midwifery practices [5-7]. Table 1 and Table 2 demonstrated that different localities can exert some influences on the composition of plant materials selection in traditional Malay midwifery practices of *berparam* and *berpilis*. The correlation between plant

species and environment can be indicative of the particular culture and locality. For example during *berpilis*, 2 species were used in Kelantan (rambai and kunyit) and Terengganu (asam jawa and halia) whereas in Pahang 3 species were used (chenderai, leban and kelapa) and Johor 9 species were used (cucur atap, gelam, turi, cengkih, halia bara, jintan manis, sireh, ekor lada and lada hitam). A notable similarity between 4 localities was the most common plant materials composition used in *berparam.* Lime, *lengkuas, kunyit, cekur, halia, halia bara*, garlic and *serai wangi* were used quite frequently at all sites. It is therefore important to make further studies in soil and forest type of the location. Trees and palms are common and abundance in clay soil of dipterocarp forest. Climbers, gingers and herbaceous plant will occur under low light intensity whereas macrophytes and ferns will occur under saturated soil like freshwater bodies

4. CONCLUSIONS

Overall this study clearly demonstrated that culture and environment can strongly influence plant materials composition selection in traditional Malay midwifery practices which can be significantly affecting landscape character or identity of Malay landscape design. Therefore in addition to culture from different locality, environmental factors also play an important role in selecting plant materials for traditional Malay midwifery practices, especially in Kelantan, Terengganu, Pahang and Johor. These results clearly indicate that selection of plant materials cannot be made on the basis of a single locality results. However, valid comparisons can be made between data from different localities and under different environmental conditions. This study suggests that environmental factors such as soil type and ecosystem variation may influence the selection of plant materials composition of different stages in Malay midwifery practices. Clearly further studies are required to confirm this hypothesis. As an alternative treatment to modern medical practices, ethno-botany plays an important role in classifying types of plants used and knowledge of the traditional practitioners. This traditional knowledge should be preserved for the next generation as a significant part of Malay culture especially for postnatal treatment. Therefore, future studies are needed to discover the valuable history of Malay culture by establishing information database of medicinal plants towards Malay heritage conservation and appreciation.

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Table 1. List of plant materials used in traditional Malay midwifery in Pahang (1), Johor (2), Terengganu (3) and Kelantan (4) during *berparam*.

Scientific name	Local name	Types of plant	Locality
Curcuma longa	Kunyit	Zingiber	1, 2, 3, 4
Kaempferia galanga	Cekur	Zingiber	1, 2, 3, 4
Zingiber officinale	Halia putih	Zingiber	1, 2, 3, 4
Cymbopogon citratus	Serai merah	Herbaceous	1, 2, 3
Cymbopogon nardus	Serai wangi	Herbaceous	1, 2, 3
Allium sativum	Bawang putih	Herbaceous	2, 3, 4
Zingiber officinale var *rubrum*	Halia bara	Zingiber	2, 3, 4
Citrus aurantifolia	Limau	Shrub	1, 3, 4
Alpinia galangal	Lengkuas	Zingiber	1, 3, 4
Lawsonia inermis	Inai	Tree	1, 3
Curcuma xanthorrhiza	Temu lawak	Zingiber	1, 3
Cinnamomum verum	Kulit kayu manis	Tree	2, 3
Acorus calamus	Jerangau	Macrophyte	2, 3
Curcuma zedoaria	Kunyit mas	Zingiber	3, 4
Garcinia atroviridis	Asam gelugur	Tree	3, 4
Allium cepa	Bawang merah	Herbaceous	3, 4
Piper nigrum	Lada hitam	Climber	3, 4
Baccaurea motleyana	Rambai	Tree	1
Fagraea fragrans	Tembusu	Tree	1
Garcinia hombroniana	Beruas	Tree	1
Mangifera indica	Mempelam	Tree	1
Micromelum pubescens	Cemumar	Tree	1
Cocos nucifera	Kelapa	Palm	1
Flemingia strobilifera	Seringan	Shrub	1
Melastoma malabathricum	Senduduk	Shrub	1
Zingiber cassumunar	Bonglai	Zingiber	1
Entada rheedii	Beluru/Gandu	Climber	1
Smilax myosotiflora	Ubi jaga	Climber	1
Melicope lunu-ankenda	Tenggek burung	Shrub	2
Ficus aurantiaca	Tengkuk biawak	Climber	2
Parameria polyneura	Serapat	Climber	2
Piper longum	Cabai pintal	Climber	2
Myristica fragrans	Pala	Tree	3
Oroxylum indicum	Bonglai/beko	Tree	3
Tamarindus indica	Asam jawa	Tree	3
Carmona microphylla	kesinai	Shrub	3
Cassia alata	Gelenggang	Shrub	3
Justicia gendarussa	Gandarusa	Shrub	3
Zingiber zerumbet	Lempoyang	Zingiber	3
Nigella sativa	Jintan hitam	Herbaceous	3
Piper betle	Sireh	Climber	3
Sida rhombifolia	Getam guri	Shrub	4
Curcuma alba	Kunyit putih	Zingiber	4
Eupatorium odoratum	Daun Kapal Terbang	Herbaceous	4
Papaver somniferum	Kaskas	Herbaceous	4

Table 2. List of plant materials used in traditional Malay midwifery in Pahang (1), Johor (2), Terengganu (3) and Kelantan (4) during *berpilis*.

Scientific name	Local name	Types of plant	Locality
Cocos nucifera	Kelapa	Palm	1
Vitex pinnata	Pokok leban	Tree	1
Grewia paniculata	Chenderai	Tree	1
Baeckea frutescens	Cucur atap	Tree	2
Melaleuca cajuputi	Kulit kayu seputih	Tree	2
Sesbania grandiflora	Turi	Tree	2
Syzygium aromaticum	Bunga cengkih	Tree	2
Zingiber officinale var *rubrum*	Halia bara/ halia merah	Zingiber	2
Pimpinella anisum	Jintan manis	Herbaceous	2
Piper betle	Sireh	Climber	2
Piper cubeba	Ekor lada	Climber	2
Piper nigrum	Lada hitam	Climber	2
Zingiber officinale	Halia	Zingiber	3
Tamarindus indica	Asam jawa	Tree	3
Curcuma longa	Kunyit	Zingiber	4
Baccaurea motleyana	Kulit pokok rambai	Tree	4

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Traditional Malay midwifery practices of point massage (bertungku) and vaginal heat (bertangas) treatment for postnatal treatment among Malay culture at east coast of Malaysia

Rashidi Othman*[a], Nur Illani Abdul Razak[a], Nooriszai Ishak[a]
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environment Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia

*rashidi@iium.edu.my phone +60-126644772 fax +60-361964864

ABSTRACT

This paper examines the role of point massage (bertungku) and vaginal heat (bertangas) in postpartum recovery treatment among traditional midwives (bidan kampong) in East Coast Malaysia. The data were derived from three different localities namely Kelantan, Terengganu and Pahang where 23 types of plant material used during bertungku and 18 species during bertangas. Bertungku, a point massage using heated stones wrapped with few layers of rugs lined with leaves of selected herbs, is applied to all parts of body especially on the front, back, abdomen, thigh and uterus. It is important to enhance the healing process of the uterus, to break up fats, toning up the abdomen and refresh the body. While bertangas is an herbal vapour therapy used to dry and tighten up the vagina. It can reduce uterus swelling, eliminate foul odour, enhance the healing of cuts and wounds and tighten the pelvic floor muscle which later on will improve sexual response while preventing gynecological problems such as prolapsed. There are three types of bertangas; dry tangas, oily tangas, and wet tangas where the process is done after the thirtieth day of giving birth. Even though there are different types of bertangas, yet the benefits are the same.

Keywords: Malay midwifery, Landscape ethnobotany, Point massage, Vaginal heat, Malay culture

1. INTRODUCTION

In African and Afro-Surinamese cultures, the practice where plants and other intravaginal desiccants are used to minimize vaginal secretions is known as dry sex. The purpose of dry sex or vaginal heat is to create a vagina that is dry, tight and heated, which is supposed to generate an increased sensation for the man during intercourse. Although this is uncomfortable and painful, African and Afro-Surinamese women express the need to please their husbands with dry sex in order to keep them from leaving and to minimize the number of girlfriends [1-5]. On the other hand, women in Asia and Latin America especially share several postpartum precautions, including physical confinement; restrictions on bathing, the prescription of hot and proscription of cold foods; for many women these precautions are supplemented with the direct application of heat, including by 'mother roasting', steaming, or smoking [6]. In Malay culture it was strongly believed that the same practice are also imposed for the same purposes but are differ in plant materials used. The practice is known as *bertungku* and *bertangas*.

Bertungku is a form of point massage using heated stones wrapped with a few layers of rugs lined with leaves of some selected herbs. Normally this process applied at all parts of the body especially front and back, abdomen and thigh and special care need to be taken when performing point massage around the uterus. Point massage are important to enhance the healing process of the uterus, to break up and release blood clots, to cleanse up the newborn mother's uterus, to break up fats, to tone the stomach, to improve blood flow and rejuvenate the body healing [7].

Tangas refers to the use of plants to dry and tighten the vagina. The purposes of tangas in Malay cultures are to reduce uterus swelling, eliminate foul odour, enhance healing of cuts and wounds and tighten the pelvic floor muscles. Pelvic floor tightening is a very important postpartum treatment. The pelvic floor muscles are considerably stretched and often left sagging after birth. Toning of the muscles will improve sexual response and help prevent gynaecological problems such as uterine prolapsed [7-8].

There are three types of *tangas* practices which are dry *tangas*, oily *tangas* and wet *tangas*. During dry *tangas* vaginal area will be exposed to the aromatic heat where a small fire or embers will be put under the chair with holes and herbs ground. Then, the newborn mother will seat on it for several times in order to shrink off the mother's uterus. Meanwhile oily *tangas* will be using herbal oil where the oil will be placed into a container, fixed with some form of wicks, burnt and placed one foot below the chair for herbal aromatic. The wet *tangas* also applying the same methods to the previous *tangas* techniques and process, which is, seat on the special chairs with holes where vaginal area exposed to heat and herbal vapor. The selected plants materials and herbs used will be boiled in water. *Tangas* normally being practiced 3 to 7 days continuously before traditional body massage or right after thirtieth day of giving birth and usually before the main process of *tangas* being held, the newborn mother need to drink a glass of water to avoid dehydration [9].

2. MATERIALS AND METHODS

Sample and study area

This semi structure interview and observation were conducted at 3 different states in the east coast of Malaysia namely Kelantan, Terengganu and Pahang and 16 Malay midwives were selected. Ethnobotanical data were collected according to the methodology described by [8]. A semi-structured questionnaire was used to extract information on the ethno-botanical uses of plants. To facilitate cross-checking of plant species, the specimens were identified through various floristic records or secondary data such as sources from books, internet, University of Malaya herbarium and Forest Research Institute of Malaysia (FRIM) herbarium, Kepong; and also from previous research studies and journals to ascertain the nomenclature as further detailed by [10].

3. RESULTS

This survey recorded 23 types of plant species used by 16 *bidan kampung* (traditional Malay midwives) during *bertungku* and 18 species during *bertangas* at three different localities which are Kelantan, Terengganu and Pahang. The plant species can be divided into trees, palms, shrubs, zingibers, aroids, climbers, ferns, groundcovers and herbaceous. Interestingly plant materials used for *bertungku* and *bertangas* at these three localities were totally different. *Bertungku* in Kelantan involved 9 types of plant species, Terengganu 13 species whereas in Pahang only 5 species. Only one species was similar in all the three localities which are *Morinda citrifolia* (mengkudu) as further detailed in Table 1. Meanwhile for *bertangas*, 10 types of plant species were discovered in Kelantan, 7 types in

Terengganu and 3 types in Pahang. Only *Pandanus amaryllifolius* (pandan) and *Cocos nucifera* (kelapa) were found similar in all three localities as detailed in Table 2.

Table 1: List of plant species used during *bertungku* by 16 traditional Malay midwives at 3 different localities in the east coast of Malaysia (Kelantan, Terengganu and Pahang)

Scientific name	Family	Local name	Type of plant	Locality
Alstonia angustiloba	Apocynaceae	Daun Pulai	Tree	Kelantan
Cinnamomum verum	Lauraceae	Kulit kayu manis	Tree	Terengganu
Morinda citrifolia	Rubiaceae	Mengkudu	Tree	Kelantan Terengganu Pahang
Oroxylum indicum	Bignoniaceae	Bonglai/beko	Tree	Pahang
Pisonia alba	Nyctaginaceae	Mengkudu siam	Tree	Terengganu
Syzygium polyanthum	Myrtaceae	Serai kayu	Tree	Terengganu
Vitex trifolia	Lamiaceae	Daun Lemuni	Tree	Kelantan
Justicia gendarussa	Acanthaceae	Gandarusa	Shrub	Terengganu
Lantana camara	Verbenaceae	Bunga tahi ayam	Shrub	Terengganu
Melastoma malabathricum	Melastomataceae	Senduduk	Shrub	Terengganu
Alpinia galanga	Zingiberaceae	Lengkuas	Zingiber	Kelantan Terengganu
Curcuma longa	Zingiberaceae	Kunyit	Zingiber	Kelantan Pahang
Kaempferia galanga	Zingiberaceae	Cekur	Zingiber	Terengganu
Musa paradisiaca	Musaceae	Daun Pisang	Zingiber	Kelantan Pahang
Zingiber officinale	Zingiberaceae	Halia putih	Zingiber	Terengganu
Cymbopogon citratus	Gramineae	Serai merah	Herbaceous	Terengganu
Eupatorium odoratum	Asteraceae	Daun Kapal Terbang	Herbaceous	Kelantan
Pandanus amaryllifolius	Pandanaceae	Pandan	Herbaceous	Kelantan
Alocasia macrorrhiza	Araceae	Keladi birah	Aroid	Terengganu Pahang
Homalomena pendula	Araceae	Kelemoyang	Aroid	Kelantan
Parameria polyneura	Apocynaceae	Serapat	Climber	Pahang
Piper betle	Piperaceae	Sireh	Climber	Kelantan
Asplenium nidus	Aspleniaceae	Tanduk rusa	Fern	Terengganu

Table 2: List of plant species used during *bertangas* by 16 traditional Malay midwives at 3 different localities in the east coast of Malaysia (Kelantan, Terengganu and Pahang)

Scientific name	Family	Local name	Type of plant	Locality
Grewia paniculata	Tiliaceae	Chenderai	Tree	Pahang
Morinda citrifolia	Rubiaceae	Mengkudu	Tree	Kelantan
Nephelium lappaceum	Sapindaceae	Rambutan	Tree	Kelantan
Parsonsia helicandra	Apocynaceae	Pokok lemak ketam	Tree	Kelantan
Syzygium polyanthum	Myrtaceae	Serai kayu	Tree	Kelantan
Vitex pinnata	Verbenaceae	Pokok leban	Tree	Pahang
Cocos nucifera	Arecaceae	Kelapa	Palm	Kelantan Pahang
Citrus aurantifolia	Rutaceae	Limau	Shrub	Terengganu
Jasminum sambac	Oleaceae	Melor	Shrub	Terengganu
Melastoma malabathricum	Melastomataceae	Senduduk	Shrub	Kelantan
Rosa chinensis	Rosaceae	Mawar	Shrub	Terengganu
Curcuma longa	Zingiberaceae	Kunyit	Zingiber	Terengganu
Cymbopogon nardus	Poaceae	Serai wangi	Herbaceous	Kelantan
Elephantopus scaber	Asteraceae	Tapak Sulaiman	Herbaceous	Kelantan
Nigella sativa	Ranunculaceae	Jintan hitam	Herbaceous	Terengganu
Pandanus amaryllifolius	Pandanaceae	Pandan	Herbaceous	Kelantan Terengganu
Trigonella foenum-graecum	Leguminosae	Halba	Herbaceous	Kelantan
Mimosa pudica	Leguminosae	Semalu	Groundcover	Terengganu

4. CONCLUSIONS

The documentation of plant species used in *bertungku* and *bertangas* practices in Kelantan, Terengganu, and Pahang has proved to be an effective tool for investigating the environmental and cultural factors in herbal traditional bath practices among Malay community. This shows that the total number of *bertungku* and *bertangas* plant species is vary based on its locality depended on the availability of the plants in certain places and culture influence itself. Moreover, medicinal plants are seldom recorded in a written form by the villagers but are still mainly based on oral history and cultural traditions passed down through the generations. Ethno-botany can make a positive contribution to alternative treatment in modern medical practices by identifying locally available plant resources, indigenous knowledge and traditional healers**.** Development activities which put indigenous knowledge into the context of natural resource management are particularly important. Therefore, future studies are needed in order to establish a medicinal plant information database in order to educate young people especially and Malaysian about the importance of ethno-botany functions and uses and to prevent this knowledge to become history.

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Mandi serom or herbal traditional bath practices among traditional Malay midwives at east coast of Malaysia

Rashidi Othman*[a], Nur Illani Abdul Razak[a], Nooriszai Ishak[a]
[a]International Institute for Halal Research and Training (INHART), Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture and Environment Design, International Islamic University Malaysia, 53100 Kuala Lumpur, Malaysia

*rashidi@iium.edu.my phone +60-126644772 fax +60-361964864

ABSTRACT

Mandi serom, a herbal traditional bath is a conventional method used among the Malay midwives for postpartum recovery and rejuvenation therapy. Interviews were conducted in Kelantan, Terengganu and Pahang in order to document the preparation and plants used for the mandi serom. In Kelantan, mandi serom practice accumulated 32 different plant species whereas Terengganu accumulated only 19 species and Pahang 17 species. The most frequently plants composition used for mandi serom are *Morinda citrifolia*, *Justicia gendarussa*, *Alpinia galangal*, *Curcuma longa*, *Cymbopogon nardus* and *Pandanus amaryllifolius* in all the three localities. Specializing for postpartum mothers, mandi serom serves four significant syndromes particularly regarding body odour, spiritual cleansing, hygienic purposes and mystical forces known as makhluk halus in Malay culture. The act of bathing mandi serom should be made continuously within 7 days after giving birth as the mother is known to be in vulnerable state and easy to draw mystical forces (makhluk halus) intervention which will cause baby blues (merian or meroyan), insomnia, nightmare, moody, solitariness, forgetful and unconscious reflection (melatah). Mandi serom is normally conducted by the midwife (bidan kampung) who usually recites prayer (doa) or spell (jampi) during the bath. Other than magical spell, position of the mother, time and how the plants were plucked also been considered for the effectiveness of the bath. Consequently, identifying plant species helps to distinguish medicinal values in mandi serom and meanwhile protecting traditional knowledge from disappearing.

Keywords: Landscape ethnobotany, Malay midwifery, Traditional bath, Malay culture

1. INTRODUCTION

Traditional bathing in Malay culture means a total body bath, from head to toe. It is designated for specific purpose and occasion, mainly conducted by traditional Malay midwives, known as *bidan kampung*. *Bidan kampung* in Malay culture often an old respected skillful woman who holds the highest place in the community and seen as specialist doctor in attending childbirth, assisting woman and advising young lady. They normally get knowledge through matrineally transmission, along the female line, from either mother to daughter or grandmother to granddaughter. This knowledge is regarded as sacred and esoteric [1]. Midwifery practice was primarily among traditional peoples with limited access to biomedicine. However, today it is also practiced in Western societies as an alternative to biomedicine and continues to play an important role in providing health care to women and children [2-12].

As in postpartum practice, Malay traditional bath *(mandi serom)* is the essential part in afterbirth recovery that involved selected plant species chose by the midwives together with the taboos *(pantang-larang)* that must be complied for the bathing ceremony [13]. Malay culture believes in the concept that illnesses are the consequence of physical as well as supernatural causes. Supernatural causes include a wide variety of hantu (malevolent spirits), witchcraft and badi (supernatural aura that emanates from slain animals and men) and reflects the Malay cultural concept of the "universe" [14-15].

Most midwifery plant species are wild, but many important species are native to certain location or area. The documentation of traditional medicinal plants used by the midwives in Malaysia is very limited and traditional knowledge is disappearing due to reliance of modern medical care. Therefore Malay midwives are becoming rare and less respected. The purpose of this study is to assess and document the knowledge of traditional medicinal plant pertaining herbal traditional bath *(mandi serom)* in Malay culture, which is a part of an initiative systematic study baseline data for future ethnopharmacology studies and reference in Malaysia.

2. MATERIALS AND METHODS

Sample and study area

This semi structure interview and observation were conducted at 3 different states in the east coast of Malaysia namely Kelantan, Terengganu and Pahang and 16 Malay midwives were selected. Ethnobotanical data were collected according to the methodology described by [13]. A semi-structured questionnaire was used to extract information on the ethno-botanical uses of plants. To facilitate cross-checking of plant species, the specimens were identified through various floristic records or secondary data such as sources from books, internet, University of Malaya herbarium and Forest Research Institute of Malaysia (FRIM) herbarium, Kepong; and also from previous research studies and journals to ascertain the nomenclature as further detailed by [16].

3. RESULTS

Table 1 showed that there was marked difference in plant species composition in *mandi serom* from Kelantan, Terengganu and Pahang. In Kelantan, *mandi serom* practice accumulated 32 species which predominated by trees, zingibers and herbaceous. However, *mandi serom* in Terengganu accumulated only 19 species and Pahang 17 species. Analysis of plant materials composition demonstrated that trees, zingibers and herbaceous are the most common group of plants being used by Malay midwives in all localities. *Morinda citrifolia* (mengkudu), *Justicia gendarussa* (gandarusa), *Alpinia galanga* (lengkuas), *Curcuma longa* (kunyit), *Cymbopogon nardus* (serai wangi) and *Pandanus amaryllifolius* (pandan) were observed being used in all localities by midwives.

This study revealed that there were significant differences between locality, culture, environment and all combinations of interactions. The importance of the interaction components emphasizes that the changes in plant materials composition in traditional Malay midwifery practices are complex and the selection to every stages or processes are not consistent across Kelantan, Terengganu and Pahang.

Table 1: List of plant species used during *mandi serom* by 16 traditional Malay midwives at 3 different localities in the east coast of Malaysia (Kelantan, Terengganu and Pahang)

Scientific name	Family	Local name	Type of plant	Locality
Citrus grandis	Rutaceae	Limau bali	Tree	Kelantan
Barringtonia racemosa	Lecythidaceae	Putat sungai	Tree	Kelantan
Fagraea fragrans	Loganiaceae	Tembusu	Tree	Pahang
Fagraea racemosa	Loganiaceae	Tembusu/Sepulih	Tree	Terengganu
Glycosmis pentaphylla	Rutaceae	Nerapi/Merapi	Tree	Kelantan
Lawsonia inermis	Lythraceae	Inai	Tree	Kelantan
Mallotus barbatus	Euphorbiaceae	Balik angin	Tree	Kelantan
Micromelum pubescens	Rutaceae	Cemumar/Semuru	Tree	Kelantan
Morinda citrifolia	Rubiaceae	Mengkudu	Tree	Kelantan Terengganu Pahang
Pittosporum ferrugineum	Pittosporaceae	Belalang puak	Tree	Terengganu
Psidium guajava	Myrtaceae	Jambu batu	Tree	Kelantan Terengganu
Syzygium polyanthum	Myrtaceae	Serai kayu	Tree	Kelantan
Vitex pinnata	Verbenaceae	Pokok leban	Tree	Pahang
Vitex trifolia	Lamiaceae	Daun Lemuni	Tree	Kelantan Terengganu
Cocos nucifera	Arecaceae	Kelapa	Palm	Pahang
Citrus aurantifolia	Rutaceae	Limau nipis	Shrub	Kelantan
Citrus hystrix	Rutaceae	Limau purut	Shrub	Kelantan
Claoxylon longifolium	Euphorbiaceae	Salang/chapa batu	Shrub	Pahang
Jasminum sambac	Oleaceae	Bunga Melor	Shrub	Kelantan
Justicia gendarussa	Acanthaceae	Gandarusa	Shrub	Terengganu Pahang
Melastoma malabathricum	Melastomataceae	Senduduk	Shrub	Kelantan
Rhodomyrtus tomentosa	Myrtaceae	Kemunting	Shrub	Kelantan
Strobilanthes crispus	Acanthaceae	Pecah kaca	Shrub	Terengganu
Alpinia galanga	Zingiberaceae	Lengkuas	Zingiber	Kelantan Terengganu Pahang
Curcuma aeruginosa	Zingiberaceae	Kunyit hitam	Zingiber	Kelantan Pahang
Curcuma longa	Zingiberaceae	Kunyit	Zingiber	Kelantan Terengganu Pahang
Curcuma xanthorrhiza	Zingiberaceae	Temu lawak	Zingiber	Kelantan Pahang
Curcuma zedoaria	Zingiberaceae	Kunyit mas	Zingiber	Terengganu
Etlingera coccinea	Zingiberaceae	Kesing	Zingiber	Pahang
Kaempferia galanga	Zingiberaceae	Cekur	Zingiber	Kelantan
Musa paradisiaca	Musaceae	Pisang kelat	Zingiber	Pahang
Zingiber cassumunar	Zingiberaceae	Bonglai	Zingiber	Kelantan Pahang
Zingiber officinale	Zingiberaceae	Halia putih	Zingiber	Kelantan Terengganu
Zingiber officinale var *rubrum*	Zingiberaceae	Halia bara/halia merah	Zingiber	Kelantan
Andrographis paniculata	Acanthaceae	Hempedu bumi	Herbaceous	Kelantan

Scientific name	Family	Local name	Type of plant	Locality
Bidens pilosa	Asteraceae	Kancing baju	Herbaceous	Kelantan
Blumea balsamifera	Asteraceae	Sembong/Capor	Herbaceous	Kelantan Terengganu
Coleus amboinicus	Labiatae	Bangun-bangun	Herbaceous	Johor
Cymbopogon nardus	Gramineae	Serai wangi	Herbaceous	Kelantan Terengganu Pahang
Elephantopus scaber	Asteraceae	Tapak Sulaiman	Herbaceous	Terengganu
Eupatorium odoratum	Asteraceae	Daun Kapal Terbang	Herbaceous	Terengganu
Euphorbia hirta	Euphorbiaceae	Pokok susu nabi	Herbaceous	Pahang
Henckelia platypus	Gesneriaceae	Semboyen/susuh nabi	Herbaceous	Terengganu
Leucas lavandulifolia	Labiatae	Ketumbit	Herbaceous	Terengganu
Pandanus amaryllifolius	Pandanaceae	Pandan	Herbaceous	Kelantan Terengganu Pahang
Piper sarmentosum	Piperaceae	Kaduk	Herbaceous	Kelantan
Trigonella foenum-graecum	Leguminosae	Halba	Herbaceous	Kelantan Pahang
Phyllanthus amarus	Phyllanthaceae	Dukung anak	Herbaceous	Kelantan
Piper betle	Piperaceae	Sireh	Climber	Kelantan
Asplenium nidus	Aspleniaceae	Tanduk rusa	Fern	Terengganu

4. CONCLUSIONS

The use of plants as ethno-botanical uses such as in *mandi serom* has a long tradition amongst Malay community in Malaysia. It involves a diversity of indigenous knowledge and cultural beliefs and constitutes an important basis for the development of Malay society. Due to rapid changes in socio-economic, environmental and cultural beliefs in Malaysia, the use of ethno-botanical plant species as herbal medicine is in transformation. The impact on Malay society of traditional Malay midwifery practices as well as modern medical systems has varied, but the facts are these traditional Malay midwifery practice knowledge of *mandi serom* is disappearing, traditional Malay midwives are becoming rare and less respected and knowledge regarding medicinal plants used during *mandi serom* are becoming history.

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Anticancer PSP and phenolic compounds in *Lentinus squarrosulus* and *Lentinus polychrous*

JongrakAttarat*[a], Rapee Thamisak[a]
[a]Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand 65000

*rakae19@hotmail.com phone +66-55964639 fax +66-55964770

ABSTRACT

At present, free radicals were originated from environment, stress and food. The free radicals are major issues in aging, diabetes, hypercholesterolemia and especially in tumor promotion. Many bioactive compounds in mushrooms such as peptides, proteins, vitamins and phenolic compound were served as antioxidant and anticancer activity for disposal free radicals.An anticancer PSP (Polysaccharide peptide) was detected in protein extracts from *Lentinus squarrosulus* and *Lentinus polychrous* at 9.87 ± 0.03, 0.41 ± 0.03 μg/g protein, respectively. Anti-oxidant activity of ethanolic mushroom extracts was identified by DPPH and FRAP. Percentage of inhibition onto scavenging activity by DPPH assay was higher than 70% in ethanolic extract of both *Lentinus squarrosulus* and *Lentinus polychrous*. In FRAP assay, micromolar trolox equivalent of *Lentinus squarrosulus* showed higher than and *Lentinus polychrous*. Catechin and tannic acid were major group of phenolic compounds in *Lentinus polychrous* via LC-MS. In summary, PSP, catechin and tannic acid from *Lentinus squarrosulus* and *Lentinus polychrous* may be effective natural product for clinical trial in patients because this edible mushrooms are nontoxic and also as functional food for human health.

Keywords: Anticancer, Mushrooms, *Lentinus squarrosulus*, *Lentinus polychrous*, Anti-oxidant assay, Catechin, PSP, polyphenol compounds

1. INTRODUCTION

Bioactive proteins isolated from more than 30 mushroom species have shown antitumor, antiviral, antimicrobial, antioxidant and immunomodulatory action such as lectins, fungal immunomodulatory proteins (FIP), laccases, protease, ribonuclease, proteoglycans, PSP (polysaccharopeptide) and other proteins [1-2]. PSP extracted fromTurkey tail (known as *Coriolus versicolor* or Yun-zhi) has a molecular mass of approximately 100 kDa [3]. PSP contains α-1,4 and *β*-1,3 glucosidic linkages in their polysaccharide moieties. Of the *C. versicolor*-derived therapeutics extracts, polysaccharopeptides are commercially the best established. The polysaccharopeptides obtained from *C. versicolor*, known as *C. versicolor* polysaccharides, is a complicated protein-bound polysaccharide extracted from its mycelium, or fruiting body. PSP significantly improved the quality of life, provided substantial pain relief, and enhanced immune status in 70-97% of patients with cancers of the stomach, esophagus, liver, lung, ovary, and cervix [4]. Presently, PSP is considered as a potential candidate for drug development in treatment and prevention of human cancer because of its immunological properties as well as its ability to distinguish cancerous cells from normal cells. Besides bioactive proteins, mushrooms have become rich sources of natural antioxidant compounds such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids. There are many phenolic compounds found in mushrooms such as benzoic acid, gallic acid, catechin, tannic acid, caffeic acid, and resveratrol [5]. Many phenolic compounds have been reported to process potent antioxidant activity and to have anticancer or anticarcinogenic/antimutagenic, antiatherosclerotic, antibacterial, antiviral, and anti-inflammatory activities to a greater or lesser extent. The aim of this study is to quantitate PSP and phenolic compounds in local cultivated edible mushrooms and then verify the antioxidative activity *in vitro*. *L. squarrosulus* was outstanding mushroom here in antioxidant and anticancer properties.

2. MATERIALS AND METHODS

Chemicals

All chemicals are from Sigma (Sigma Chemical Co., St. Louis, MO) unless otherwise noted.

Preparation of protein extract in mushrooms

Fruiting bodies of edible mushrooms, *Lentinus polychrous* and *Lentinus squarrosulus* were collected from farm in Chiang Mai province. Those fruiting bodies were dried at 50°C and ground into powder and stored at -50°C until using for protein preparation and the phenolic compounds assay. Approximately 10 g of the mushroom powder was prepared by homogenizing in hot water and 0.15 N NaCl. The homogenized samples were then precipitated with 40-80% $(NH_4)_2SO_4$ saturation. Fractions extracted were resuspended in 50 mM Tris-HCl, pH 7.5, and dialyzed overnight against the same buffer. The resulting solution was added with protease inhibitor cocktail (AMRESCO LLC, OH 44139, USA) and collected at -20°C in order to quantitate protein concentration, PSP levels, and treat Cholangiocarcinoma cell lines. Protein concentration of the extract was determined by Bradford assay with BSA (Bovine serum albumin) as standard.

Development of ELISA to quantify PSP concentration in edible mushrooms

To quantify PSP in fruiting bodies, ELISA was performed following the procedure described previously in quantitation of vitellin and vitellogenin in ovary and hemolymph of prawns. Briefly, PSP and proteins extracted from fruiting bodies were coated on 96 well plates at room temperature. Anti-PSP antibody specific to commercial PSP (JHS Natural Products, OR 97402, USA) was used as a primary antibody at a dilution of 1:500. The enzyme reaction was carried out by *o*-phenylenediamine after an addition of anti-rabbit IgG-HRP (dilution 1:5,000). PSP concentrations in the samples were calculated on the basis of PSP concentrations according to a standard curve. The sensitivity of the assay for immunoreactive PSP was from 1-100 ng per assay.

Quantitation of phenolic compounds

Ten grams of dried mushrooms were incubated in 100 ml solvent [absolute ethanol and HCl acid media (1% v/v)] at room temperature on a shaker at 150 rpm for 24h and then centrifuged at 5,000 rpm for 10 minutes. The solution was filtered through a 0.45 μm membrane filter and then the residue was re-extracted. An Agilent 1100 HPLC system equipped with a quaternary pump, refrigerated autosampler, column heater and withDAD, FLD and MS ion trap detectors was used. Forphenolic analysis, a 150 x 4.6 mm Zorbax SB C18 column wasused at a flow rate of 1 ml/min and gradient elution of 0–5 min B 100% constant, 5–10 min A 0–20%, 10–20 min A 20% constant, 20–60 min A 20–40% (buffer A: 10 mM formic acid pH 3.5 withNH_4OH; Buffer B: 100 % methanol with 5 mM ammonium formate). Column temperature was 40°C and UV detectionwas at 270, 330, 350 and 370 nm. Compound identification was confirmed by injection of authentic standard. Compounds were quantitated by the external

standard method using authentic standards, gallic, tannic acid, catechin, rutin, isoquercetin, eriodictyol, quercetin, hydroguinin, kaempferol, and apiginin.

Preparation of ethanolic mushroom extract

Five grams of dried mushrooms were extracted by stirring with 100 ml of ethanol at 25 °C for 24 hours and filtering through Whatman No.4 filter paper. The residue was then extracted with addition of 100 ml of ethanol. The combined ethanolic extracts were then rotary evaporated at 40°C to dryness. The dried extract was resuspended in ethanol and stored at 4°°C for further use in DPPH, FRAP and total phenolic compound assays.

DPPH assay

The DPPH (2,2-diphenylpicrylhydrazyl)assay was used to determine free radical scavenging activity of mushroom extracts. Each mushroom extract in ethanol was mixed with 1 ml of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm.

FRAP assay

The FRAP (Ferric reducing-antioxidant power assay) was done according to Benzie and Strain with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM$FeCl_3$.$6H_2O$ solution. The fresh working solution wasprepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. Mushroom extracts were allowed to react with FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm absorbance. The standard curve was linear between 25 and 800 mM trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E). Results are expressed in µM TE (trolox equivalent antioxidant capacity) per gram of dried mushroom. Additional dilutions were needed if the FRAPvalue measured was over the linear range of the standard curve.

3. RESULTS

In Figure 1, PSP level was quantitated in different edible lentinus mushrooms using indirect ELISA. In one gram of protein concentration in three mushrooms, PSP level in *L. squarrosulus* was significantly higher than *L. polychrous*. As previously reported, PSP was produced from *C. versicolor* mushrooms harvested in the wild or cultivated commercially, or from mycelial growth of *C. versicolor* in submerge fermentation [3]. PSP also enhanced the cytotoxicity of certain S-phase targeted-drugs, such as doxorubicin, etoposide, camptothecin and cyclophosphamide, on human cancer [6]. In addition, PSP has shown a chemopreventive effect on prostate cancer via the targeting of prostate cancer stem cell-like populations. Immunoreactive protein of anti-commercial PSP antibody using ELISA may be PSP as reported in *C. versicolor.*

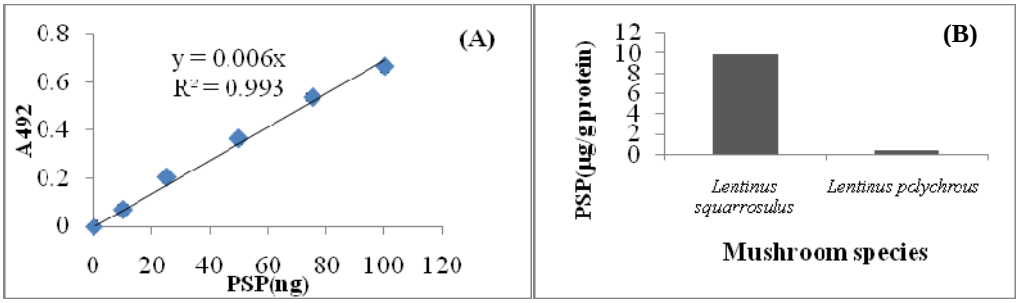

Figure 1. Detection of PSP concentration with indirect ELISA in three mushrooms species, *L. squarrosulus* and *L. polychrous*. (A) Calibration curve of PSP standard. (B) Concentration of PSP (mg/g of protein) two mushroom species. Error bars indicate mean±standard deviation (n = 3).

Table 1. Antioxidant activity of ethanolic extract from mushrooms

Mushrooms	DPPH assay (%Inhibition)	FRAP assay (Trolox equivalent)
L. squarrosulus	80.13 ± 0.002	117.27 ± 0.009
L. polychrous	73.13 ± 0.008	20.91 ± 0.013

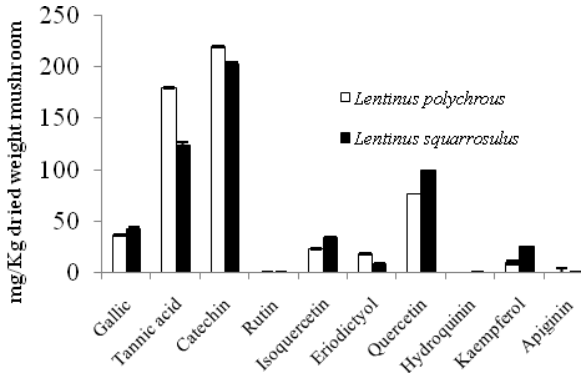

Figure 2. Concentration of phenolic compounds in three mushrooms, *L. squarrosulus* and *L. polychrous*. Catechin and tannic were the major phenolic compound in three lentinus mushrooms. Error bars indicate mean ± standard deviation (n=3).

Catechin was the major phenolic compound in both *L. polychrous* and *L. squarrosulus*; the higher concentration was existent in *L. polychrous*. From results in Figure 2, the amount of catechin comprised over 200 mg/kg dried weight of the mushroom. The second and third amount of phenolic compound in ethanol extract of mushrooms were tannic and quercetin, respectively. Gallic, isoquercetin, kaempferol and eriodictyol level were less than 50 mg/kg dried weight of the mushroom and these phenolic contents were detected at the highest amount in *L. squarrosulus*. Rutin and hydroquinin were not detected in any ethanolic extracts of mushrooms in this study. Catechin is a group polyphenol of condensed tannins. Normally, cathechins are the main compounds in green tea [7]. Catechin served as powerful antioxidant against lipid peroxidation when phospholipid bilayers are exposed to aqueous oxygen radicals. Catechin prevented cancer of the liver, lung, breast and colon, and also suppresses cancer promotion. Besides anticancer activity, antibacterial, antiviral agent and anti-hyperglycemic action of catechin were also reported. A systematic review revealed that tannic acid or commercial product of tannin have also been reported to exert many physiological effects, such as accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immunoresponses [8]. The antioxidant activities of mushroom extracts were estimated by means of trolox equivalent antioxidant capacity (TEAC) calculated from the DPPH and FRAP assay. At concentration of 50 mg/ml, scavenging effects of *L. squarrosulus*, *L. polychrous* and *L. edodes* were about 80, 73, and 30%, respectively, while scavenging activity of trolox by DPPH assay at 0.15 mg/ml was 88%. The scavenging effect of 50 mg/ml *L. squarrosulus* extract showed its highest value at 500 µM TE/g dried mushroom in FRAP assay; this data was correlated with the TPC and DPPH assays.

4. CONCLUSIONS

Some advantages of using mushrooms over plants as sources of bioactive compounds are that often the fruiting body can be produced in much less time and can be manipulated to produce optimal quantities of active compounds. In this study, it is the first time to detect PSP in protein extracts from fruiting body of *L. squarrosulus* and *L. polychrous* by ELISA. In addition, other bioactive proteins except PSP may also exist in *L. squarrosulus*. However, further studies are required to confirm that this is the same PSP as characterized in *C. versicolor*. Purification and characterization of the PSP of *L. squarrosulus* has been studied and this data will be reported soon in a different manuscript. A good antioxidant activity of *L. squarrosulus* extract and high phenolic content in *L. polycrous* will challenge us to prove its mechanism in cancer cell lines.

ACKNOWLEDGEMENTS

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Stability evaluation of liposomal formulation comprising of *Pueraria Mirifica* extract

Thanachod Thammachat*[a], Supanee Fahrungsang[a], Sitthiphong Soradech[b]

[a]Thai Herbal Medical Plant, The Office of Kamphaengsaen Campus, Kasetsart University, Kamphaengsaen, Nakhon Pathom, Thailand 73140
[b]Pharmaceutical and Natural products Department, Thailand Institute of Scientific and Technological Research, Pathumthani, Thailand 12120

*thanachod@ku.ac.th phone +66-867330184 fax +66-034351907

ABSTRACT

Encapsulation of active ingredients derived from natural products into the liposome vesicles is utilized for cosmetic products. Owing to their efficacy on the skin permeation enhancement, prolong stability and decrease the toxicity of active ingredients was also reported. The main aim of this study was to investigate the stability of liposome containing *Pueraria mirifica* extract. The samples of liposomes were prepared by a thin film stability parameters, such as percentage of encapsulation efficiency (%EE), color, pH, particle sizes and aggregation were investigated. The results showed that the crude extracts obtained from *Pueraria mirifica* at 1 and 2% could be loaded into the liposomal formation and exhibiting the good stability during 6 cycles of study. The particle sizes were increased with the increase in the cycles of study. The %EE 1-2% crude extract into the liposome vesicles slightly decreased after 6 cycles of heating and cooling. Therefore, these results showed the innovative technology for using in herbal cosmetic delivery systems and hence the high efficacy of cosmetic product was occurred.

Keywords: Phospholipids, Liposome, *Pueraria mirifica* extract, Encapsulation efficiency

1. INTRODUTION

Liposomes have been used for encapsulating bioactive substance, due to their efficacy on the permeation enhancement of skin, prolong stability of active ingredients and decrease the toxicity of active ingredients. Liposomes are a lipid vesicles that contains both of hydrophilic and hydrophilic structure, are widely used as cosmetic delivery systems. Liposomes are biodegradable and non-toxic, are also able to encapsulate both hydrophobic and hydrophilic materials. Varying methods for preparation of liposomes have been developed which are dependent on the vesicle diameter and aqueous volume. Liposomes could be obtained from several approaches which include the use of organic solvents, mechanical procedure and by removal of detergent from phospholipids/detergent micelle mixture. The effect on liposome formation were depend on various factors i.e., composition and concentration of phospholipids, liposomal size and surface charge [5-8].

Pueraria mirifica (PM) also known as Kwao Krua Kao (*Pueraria candollei* Graham var. *mirifica* (Airy Shaw & Suvat.) Niyomdham), is the plant found in northern and north eastern of Thailand. PM has a history of use in folk medicine. Although the name "Kwao Krua" had been applied to several species of plants having tuberous roots, it was definitively identified as PM in 1952. The PM was promoted to be champion herbal products of Thailand in 2013, is a unique herbal hormone supplement that contains various phytoestrogens including miroestrol, deoxymiroestrol, daidzein, genistin, genistein, β-sitosterol, stigmasterol, coumestrol, puerarin, campesterol, mirificoumestan, kwakhurin and mirificine. An unusual estrogenic phenol, miroestrol, was isolated eight years later from this plant. Some cosmetic products and herbal supplements claim various health benefits of the extracts of PM including increasing appetite, enlarging breasts, improving hair growth and other rejuvenating effects. [1-4].

Thus, the purpose of this research was to evaluate the stability of liposome containing PM. A thin film hydration method was used for preparation of the liposome formations. The both of 1% and 2% crude extract of PM concentrations were chosen to this study. The stability study was assessed using heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles). The stability parameter, such as percentage of encapsulation efficiency (% EE), color, pH, particle sizes and aggregation were also investigated.

2. MATERIALS AND METHODS

Materials

Phosphatidylcholine from soy bean (SPC) was purchase from Fluka, (USA) while cholesterol was obtained from sigma Aldrich. All other solvents and chemicals used are of analytical grade. The PM SARDI 190 extract were collect to Kasetsart University.

Preparation of crude extract loaded liposomes

The suitable ratio between phosphatidylcholine and cholesterol were weight at various molar ratios and added into round bottom flask. Phosphatidylcholines were dissolved in appropriate amount of chloroform. phosphatidylcholine were dried by a rotary evaporator with a suitable condition of pressure and temperature at 150 bars and 40°C to produce a thin lipid film for 10 min. After thin film from phosphatidylcholine and cholesterol was obtained, 5 ml of PM crude extract (1% and 2%) in phosphate buffer solution pH 7.4 was then added and the mixture was vortexes for 5 min and then sonicated with ultrasonicator for 10 min. The suspensions of liposomes were annealed with the rotary evaporator for 20 min (150 bars and 40°C). The blank liposome was prepared with the similar condition but it used phosphate buffer solution pH 7.4 without PM crude extract.

Stability evaluations

The stability studies of liposomal formulation containing PM was assessed using heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles). The stability parameter, such as percentage of encapsulation efficiency (%EE), color, pH, morphology, particle sizes and aggregation were also investigated (Figure 1).

Particle size measurement

The diameter of the liposome was detected by the method of light diffraction (Horiba L 950, Japan). All analyses were performed in triplicate.

Determination of pH:

The pH of the samples was determined by pH meter (pH 700, German). The samples were determined in triplicate.

Morphology

The morphology and surface property of liposomes were investigated by using inverts microscope (Eclipse TE2000-s, Japan).

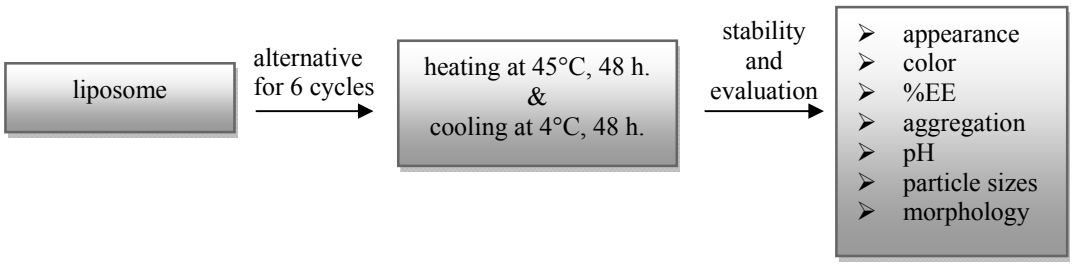

Figure 1. Diagram of stability testing by heating and cooling method

Determination of Color

The colors of liposome were investigated by color measurement (Miniscan EZ, USA). The color of the product was investigated based on three parameters including: L*, a* and b*.

Encapsulation efficiency (%EE)

The %EE of liposomes containing crude extracts at 0.25 and 0.5% were assessed. Initially, the unencapsulated was separated from the liposome dispersion by centrifugation. The liposome was centrifuged at 6,000 rpm, 4ºC for 1 h in a centrifugation in order to separating the incorporation of the active ingredient from the free form. The supernatant was analyzed by using total phenolic content to determine the amount of active ingredients for determination of percentage of encapsulation efficiency of crude extract within liposome vesicles.

3. RESULTS

Liposome containing PM extract could be developed, predicting by the morphology and %EE of liposomes. The morphology was shown in figure 1 that presented the droplet shape. However, the stability of liposome formulation was concerned a critical rule. Therefore, the stability of liposomes loaded PM extracts at 1 and 2% were also study as displayed in figure 2-3 and table 1-2.

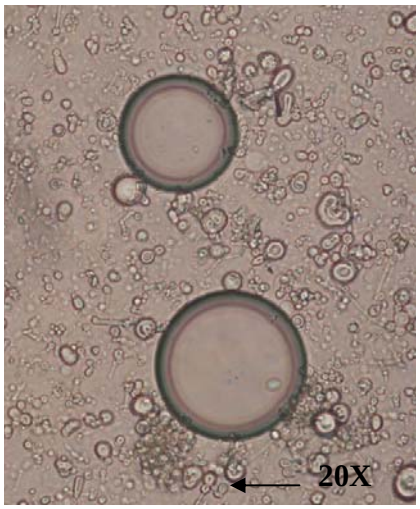

Figure 2. Morphology of liposome containing *Pueraria mirifica* extract

Stability assessment of the liposome with and without herbal extract were carried out using heating and cooling test at 45°C 24 h and 4°C 24 h for 6 cycles. The results revealed that the appearance, odor and texture of all formulation did not change. The pH, were slightly changed after 6 cycles of storage in accelerated conditions. The pH value slightly changed from 7.01, 6.99 and 7.9 to 7.06, 6.9 and 7.1 for blank liposome (Blank_LP), liposome containing 1% crude extract (LP_1% crude extract) and liposome containing 2% crude extract (LP_2% crude extract), respectively during 6 cycles of evaluation. The color of the product was investigated based on three

parameters including: L*, a*, b* which showed slight change for Blank_LP, LP_1% crude and LP_2% crude extract after 6 cycles.

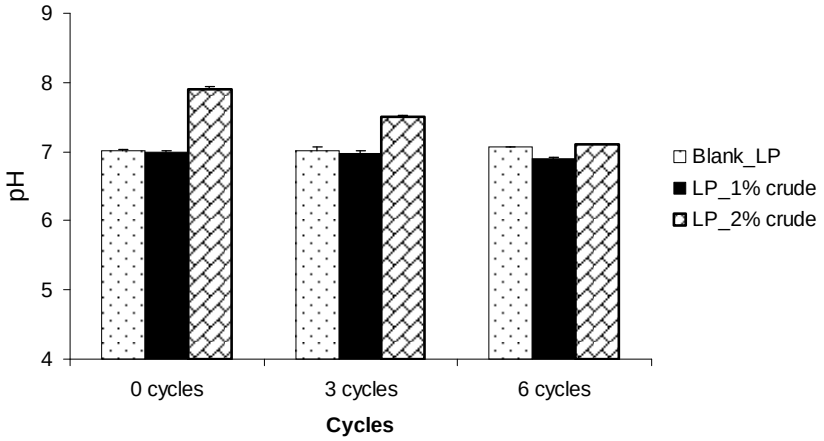

Figure 2. pH of all liposome formulations after heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles)

The result of color was in agreement with the appearance and pH of all formulations. The particle sizes were increased as increasing the cycles of heating and cooling. The particle sizes increased from 6.10, 6.70 and 6.87 µm to 7.25, 7.60, and 7.80 for blank _LP, LP_ 1% crude and LP_2% crude extract, respectively after 6 cycles of storage. This result was due to the combination between particles of liposomes. The change in sizes of liposome resulted in the change in % EE of *PM* extract loading. The %EE of LP_ 1% crude and LP_2% crude extract slightly decreased from 51.48% and 31.28% to 45.02% and 28.76% after 6 cycles of storage. Therefore, this result showed the innovative technology for using in herbal cosmetic delivery systems.

Table 1. Color of all liposome formulation after heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles)

Classification	0 cycles	3 cycles	6 cycles
Blank _LP	L* = 65.76 ± 0.22	L* = 64.12 ± 0.35	L* = 65.43± 0.10
	a* = -0.55 ± 0.03	a* = -0.56 ± 0.08	a* = -0.54 ± 0.02
	b* = 0.66 ± 0.04	b* = 0.67± 0.15	b* = 0.68 ± 0.05
LP_ 1% crude	L*=61.91 ± 0.63	L*=60.43 ± 0.45	L*=59.23 ± 0.34
extract	a*=-0.84 ± 0.03	a*=-0.87 ± 0.12	a*=-0.90 ± 0.16
	b*=9.26 ± 0.09	b*=9.78± 0.24	b*=9.98 ± 0.15
LP_2% crude	L*=61.93 ± 0.24	L*=59.67 ± 0.17	L*=58.43± 0.56
extract	a*=-0.35 ± 0.03	a*=-0.37± 0.05	a*=-0.39 ± 0.10
	b*=13.93 ± 0.15	b*=14.06 ± 0.12	b*=14.56 ± 0.07

Table 2. Encapsulation efficiency of all liposome formulation after heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles)

Classification	Times		
	0 cycles	3 cycles	6 cycles
LP_1% crude extract	51.48 ± 3.41	45.19 ± 4.63	45.02 ± 1.09
LP_2% crude extract	31.28 ± 4.89	30.54 ± 2.54	28.76 ± 2.45

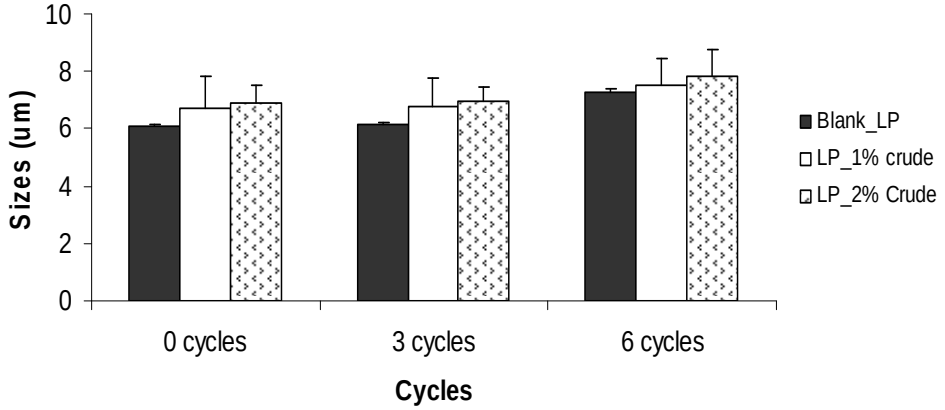

Figure 3. Particle sizes of all liposome formulations after heating and cooling method (45°C, 48 h and 4°C, 48 h for 6 cycles)

4. CONCLUSIONS

The formulation of liposome consisting of a PM extract was successfully prepared from the thin film hydration method. The formulation of liposomes containing PM of both concentrations showed good formulation and good stability for applying to cosmetic delivery and adding to cosmetic products.

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The authors are gratefully acknowledge the Thai Herbal Medical Plant, The Office of Kamphaengsaen Campus, Kasetsart University, Kamphaengsaen, Nakhon Pathom 73140, Thailand

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Formulation and encapsulation efficiency of crude extract derived from PM loaded liposomal formation

Thanachod Thammachat[*a] Supanee Fahrungsang[a], Sitthiphong Soradech[b]
[a] Thai Herbal Medical Plant, The Office of Kamphaengsaen Campus, Kasetsart University, Kamphaengsaen, Nakhon Pathom, Thailand 73140
[b] Pharmaceutical and Natural products Department, Thailand Institute of Scientific and Technological Research, Pathumthani, Thailand 12120

[*]thanachod@ku.ac.th phone +66-867330184 fax +66-034351907

ABSTRACT

Liposomes are lipid vesicles that contained both part of hydrophilic and hydrophilic structure. It is widely used as cosmetic delivery. Their efficacy on the permeation enhancement of skin, prolong stability of active ingredients and decrease the toxicity of active ingredients were also reported. Therefore, the purpose of this study was to prepare *Pueraria Mirifica* (PM) extract loaded liposome formulation. Liposomes were prepared by hydration of dried lipid films. The crude extract of both concentrations (1% and 2%) were performed to loading within the liposome vesicles. The physicochemical properties of liposomes were characterized on the morphology, sizes, color, pH and percentage of encapsulation efficiency (%EE). The %EE of liposomes containing crude extracts at 1 and 2 % were assessed using total phenolic content. The results indicated that the crude extracts obtained from PM could be loaded into the liposomal formation. The %EE of crude extract loaded liposomes was in the range of 30.0-50.0%. The particle sizes of liposomes was in the ranges of micrometer about 6.0–7.0 μm. Hence, crude extract derived from PM loaded liposome showed a good formulation, giving a high efficacy for applying to cosmetic delivery system.

Keywords: Phospholipids, Liposome, PM extract, Encapsulation efficiency

1. INTRODUTION

Pueraria mirifica (PM) is the herb as origin of Thailand. It has also been called Kwao Krua Kao and Thai Kudzu, *PM* is a plant which belongs to the family Papilionaceae (Leguminosae) same family as soy. It has a history of usage in Thai herbal medicine pharmacopia which have been used for almost 100 years and well known in benefit of PM. The tuberous of PM were found hormones from natural components; including phytoestrogens contains similar estrogen-like compounds which are more potent than those found in soy, such as miroestrol and deoxymiroestrol.

Thus PM shows a valuable role in helping to maintain a healthy hormone balance in menopausal women, when estrogen levels drop and women experience changes in mood, hot flashes, lower libido, sleep interruption and other health issues. PM has earned a reputation for its supportive effects for breast health, and has been featured in products that help support breast firmness, as well as protecting breast tissue. In addition to their hormone supporting effects, these substances have a high level of antioxidant activity, probably due to their ability to increase the cell protective substance called superoxide dismutase [1-4].

In recent years, liposomes have been widely applied as a carrier system which can improve the activity and safety of many active ingredients. Liposomes are widely used as cosmetic delivery systems and drug delivery systems. Due to their efficacy on the permeation enhancement of skin, prolong stability of active ingredients and decrease the toxicity of active ingredients. Liposomes, which are biodegradable and non-toxic, are also able to encapsulate both hydrophobic and hydrophilic materials. Varying methods for preparation of liposomes have been developed which are dependent on the vesicle diameter and aqueous volume. Liposomes could be obtained from several approaches which include the use of organic solvents, mechanical procedure and by removal of detergent from phospholipids/detergent micelle mixture. The structure formation of liposomes was depend on many factors i.e., composition and concentration of phospholipids, liposomal size and surface charge [5-8].

Therefore, the purpose of this research was to formulate and evaluate the percentage of encapsulation efficiency of crude extract derived from PM loaded liposomes. A thin film hydration method was used for preparation of the liposome formations. The physicochemical properties were characterized on size, pH, color, morphology and encapsulation efficiency (EE %).

2. MATERIALS AND METHODS

Materials

Phosphatidylcholine from soy bean (SPC) was purchase from Fluka, (USA) while cholesterol was obtained from sigma Aldrich. All other solvents and chemicals used are of analytical grade.

Preparation of crude extract derived from *Pueraria mirifica*

Specific extracts of PM SARDI 190 was separately prepared by macerating the powdered plant with ethanol-water and filtering through Whatman paper No. 41. The solvent was removed under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision) at 45°C. An appropriate mixing ratio was used to prepare the mixed extract.

Preparation of crude extract loaded liposome

The suitable ratio between phosphatidylcholine and cholesterol were weight at various molar ratios and added into round bottom flask. phosphatidylcholines were dissolved in appropriate amount of chloroform. Phosphatidylcholine were dried by a rotary evaporator with a suitable condition of pressure and temperature at 150 bars and 40°C to produce a thin lipid film for 10 min. After thin film from phosphatidylcholine and cholesterol was obtained, 5 ml of crude extract (1% and 2%) in phosphate buffer solution pH 7.4 was then added and the mixture was vortexes for 5 min and then sonicated with ultrasonicator for 10 min. The suspensions of liposomes were annealed with the rotary evaporator for 20 min (150 bars and 40°C). The blank liposome was prepared with the similar condition but it used phosphate buffer solution pH 7.4 without crude extract.

Particle size measurement

The diameter of the liposome was detected by the method of light diffraction (Horiba L 950, Japan). All analyses were performed in triplicate.

Determination of pH

The pH of the samples was determined by pH meter (pH 700, German). The samples were determined in triplicate.

Morphology

The morphology and surface property of liposomes were investigated by using inverts microscope.

Determination of color

The colors of liposome were investigated by color measurement (Miniscan EZ, USA). The color of the product was investigated based on three parameters including: L*, a* and b*.

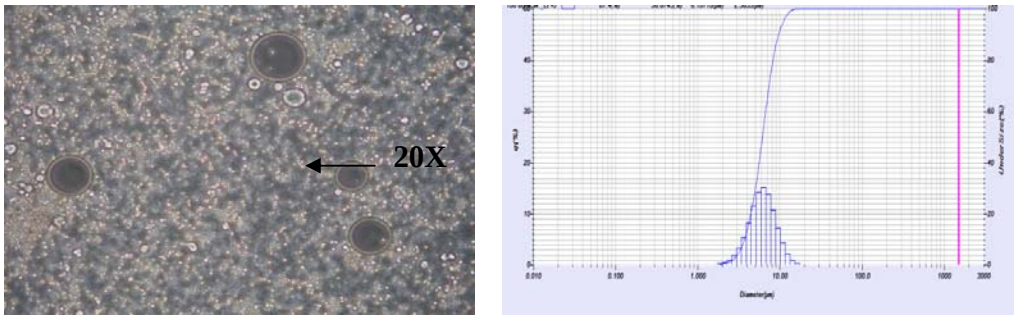
Encapsulation efficiency (%EE)

The %EE of liposomes containing crude extracts at 0.25 and 0.5% were assessed. Initially, the unencapsulated was separated from the liposome dispersion by centrifugation. The liposome was centrifuged at 6,000 rpm, 4ºC for 1 hrs in a centrifugation in order to separating the incorporation of the active ingredient from the free form. The supernatant was analyzed by using total phenolic content to determine the amount of active ingredients for determination of percentage of encapsulation efficiency of crude extract within liposome vesicles.

3. RESULTS

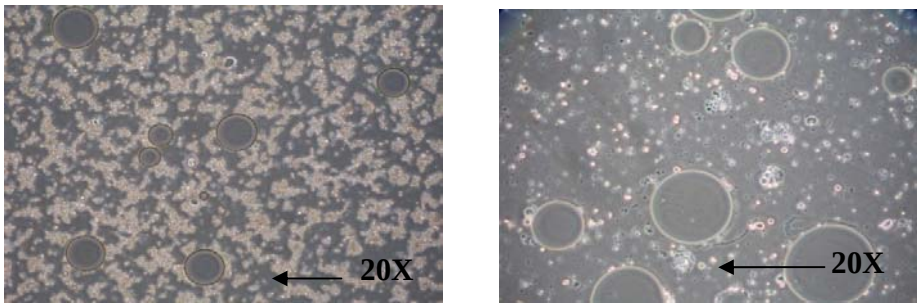
PM extract loaded liposomes were formulated through thin film hydration method varying with the composition of the phosphatidylcholine and cholesterol. The suitable ratio between phosphatidylcholine (Phospholipids) and cholesterol was 10:1, due to it was easily prepared liposomes as shown in figure 1a. The results indicated that the liposomes showed the spherical shape (1a) and white suspension of colloids. The particle sizes of blank liposomes were about 6 µm as displayed in figure 1b.

This condition was to formulate *PM* extract loaded liposomes formation. The concentrations crude extract of PM at 1% and 2% was used in this study. The results demonstrated that the crude extract of both concentrations could be added into the structure of liposomes indicating by the % EE of both concentrations. The % EE of crude extract loaded in liposomes at 1% and 2% concentrations were 51.48% and 31.28% respectively. The addition of crude extract into liposomal vesicles did not affect the change in pH of liposome while the color of liposomes was extremely changed. The higher concentrations of crude extract, the higher yellow color (b*) of liposomes were observed. The b* value increased ($p < 0.05$) from 0.66 to 13.93. The change in color was due to the influence of crude extract.



(1a) **(1b)**

Figure 1. Shows the invest micrograph (1a) and particle sizes (1b) of blank liposomes at 10:1 (lecithin and cholesterol)



(2a) **(2b)**

Figure 2. Shows the invest micrograph of 1.0% crude extract of *Pueraria mirifica* loaded liposomes (2a) and 2.0% crude extract loaded liposomes (2b)

Table1. Physicochemical properties and encapsulation efficiency of *Pueraria mirifica* extract loaded liposomes

Formulas	pH	Shape	Sizes	Color	% EE
Blank liposome	7.01 ± 0.02	Spherical	6.10 ± 0.05	L* = 65.76 ± 0.22 a* = -0.55 ± 0.03 b* = 0.66 ± 0.04	none
1% crude extract loaded liposomes	6.99 ± 0.01	Spherical	6.16 ± 0.03	L* = 61.91 ± 0.63 a* = -0.84 ± 0.03 b* = 9.26 ± 0.09	51.48 ± 3.41
2% crude extract loaded liposomes	7.90 ± 0.01	Spherical	7.25 ± 0.17	L* = 61.93 ± 0.24 a* = -0.35 ± 0.03 b* = 13.93 ± 0.15	31.28 ± 4.89

4. CONCLUSIONS

The formulation of liposomes comprising of a PM extract was successfully prepared from thin film hydration method. The formations of liposome with and without crude extract were spherical shape, indicating the suitable condition for fabrication of liposome formulation. The particle sizes of liposomes with and without crude extract were in the range of 6.0 - 7.0 µm while % encapsulations were in the range of 30.0 – 50.0% which depended on the concentrations of crude extracts. This research contributed to a new formulation of PM extract for applying to cosmetic formulation.

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Development of antioxidant soluble drinking powder from mamao (*Antidesma ghaesembilla*) fruit extract

Pongtip Sithisarn*[a], Wandee Gritsanapan[a]
[a]Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand 10400

*pongtip.sit@mahidol.ac.th phone +66-26448677 ext. 5521 fax +66-26448701

ABSTRACT

Antidesma ghaesembilla or mamao is a plant in Euphorbiaceae family. This plant has reddish purple fruits which are edible and become more popular in the recent years as beverages and health products. This experiment was set up in order to investigate free radical scavenging activities of extracts from the fruits of mamao prepared by different methods of extraction and drying including decoction, fresh squeezing and maceration using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Folin-Ciocalteu and pH differential methods were also conducted to quantitatively analyze of total phenolic and total anthocyanin contents of the extracts. Finally, the extract from the most suitable extraction and drying methods was selected for development of health supplement product. Decoction and drying by evaporation on a water bath promoted extract of mamao ripe fruits with the strongest free radical scavenging activity (IC_{50} = 72.42 ± 3.52 µg/ml) with high amount of total phenolic and total anthocyanin contents of 1.22 ± 0.36 g gallic acid equivalent in 100 g extract (g%GAE) and 7.09 ± 0.24 g cyanidin-3-glucoside equivalent in 100 g extract (g%C-3-GE), respectively. This extract was developed as soluble drinking powder by wet granulation method and qualitatively controlled by evaluations of loss on drying, thin layer chromatographic (TLC) and infrared spectroscopic (IR) fingerprints. The obtained product is pinkish red powder that contained 3.08 ± 0.81 g%C-3-GE in 1 sachet (14 g) and exhibited free radical scavenging activity equivalent to 0.004 g of vitamin C. The information from this study could be used as guideline for the developments of antioxidant products from mamao fruit extracts in the future.

Keywords: *Antidesma ghaesembilla*, Mamao, Free radical scavenging activity, Total phenolic, Total anthocyanin, Soluble drinking powder

1. INTRODUCTION

Many degenerative pathologies and diseases such as cardiovascular disease, Alzheimer's, atherosclerosis, inflammation and cancer are considered to relate to the excess production of free radicals [1]. To balance the obtaining and eliminating of free radicals in body system, consumption of antioxidant diets including fruits and vegetables could be the solutions to control oxidative stress in human being. *Antidesma ghaesembilla* which is called in Thai as mamao is a plant in Euphobiaceae family. This plant is a tree up to 20 m tall with simple alternate leaves and multiple fruits [2]. The leaves and the fruits have been traditionally used to treat anemia and promote blood circulation while the barks have been used as astringent and tonic [2]. Nowadays, the ripe fruits of mamao which have red to dark purple color have been developed as various nutritional products such as wine, jam, and fruit juice [3]. Since there are various nutritional applications from mamao fruits, however, there has been no report concerning the biological activities and phytochemicals in this plant. Therefore, this study was set up in order to investigate for antioxidant activity of extracts from the fruits of mamao using DPPH scavenging assay and quantitatively analyze for active components including phenolics and anthocyanins contents by Folin-Ciocalteu and pH differential methods, respectively. Moreover, investigation of suitable fruit extract preparations such as extraction and drying methods and product development were also conducted.

2. MATERIALS AND METHODS

Plant materials

Purple and red fruits of mamao (*A. ghaesembilla*) were collected from Nong Khai province, Thailand in January, 2012. Plant materials were identified by Assoc. Prof. Dr. Wandee Gritsanapan, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The samples were cleaned and air dried then the seeds were removed and the flesh was cut into small pieces. The flesh of purple and red fruits of mamao was separately extracted using the extraction procedures below.

Decoction

The flesh of purple fruits of mamao was boiled (80°C) with distilled water (plant/water ratio 1:10 w/v) for 8 h, then filtered. The filtrate was dried using spray drying (130°C), freeze drying (-45°C) and evaporation on a water bath method (60°C) to obtain dried purple fruit decoction extracts of spray drying, freeze drying and evaporation (PDS, PDF and PDE, respectively). The red fruits of mamao were also extracted by decoction using the same decoction protocol then freeze drying to obtain red fruit decoction freeze drying extract (RDF).

Maceration

The flesh of purple fruits of mamao was separately macerated with 95% ethanol, methanol and acetone (plant/water ratio 1:10 w/v) for 48 h with occasional shaking then filtered. The filtrate was evaporated under reduced pressure to dryness. These dried purple fruit ethanol, methanol and acetone extracts were labeled as PME, PMM and PMA, respectively.

Squeezing

The flesh of purple fruits of mamao was minced with distilled water (plant/water ratio 1:10 w/v) using electronic blender then filtered. The filtrate was dried using freeze drying method to yield purple fruit squeezing extracts (PS). All extracts were analyzed for antioxidant activities, total phenolic and total anthocyanin contents using DPPH scavenging assay, Folin-Ciocalteu method and pH differential method, respectively. The extract that promoted the best quality was selected for further product development.

Determination of free radical scavenging activity using DPPH scavenging assay

The free radical scavenging effect of all mamao fruit extracts as well of standard trolox corresponding to the quenching ability to DPPH radical was carried out as described by Yamasaki et al. [4]. DPPH solution was added to the sample solution in the same volume. The absorbance at 517 nm was determined after 15 minutes of the reaction and percentage of scavenging and IC_{50} were calculated. Each sample was assayed in triplicate and the average of IC_{50} value was calculated.

Determination of total phenolic content using Folin-Ciocalteu method

Using the method applied from Naithani et al. [5], plant extract solutions were oxidized with Folin-Ciocalteu reagent and the reactions were neutralized by sodium carbonate solution. The absorbance of the resulting blue colored solution was measured at 765 nm after 120 min. Total phenolic content was expressed as g gall acid equivalent in 100 g extract (g% GAE). Each sample was done in triplicate and the average result was calculated.

Determination of total anthocyanin content using pH differential method

Applied the methods from Sutharut and Sudarat [6], plant extract solution were separately prepared in potassium chloride buffer, pH 1.0 and sodium acetate buffer, pH 4.5 and stayed in room temperature for 15 minutes. Then the absorbance of each solution was measured at 510 and 700 nm. Total anthocyanin content was calculated and expressed as g cyanindin-3-glucoside equivalent in 100 g extract (g% C-3-GE). Each sample was done in triplicate and the average result was calculated.

Product development

Extract from ripe fruits of mamao prepared by decoction and evaporation on a water bath (PDE) which promoted the best antioxidant activity and high phenolic and anthocyanin contents was developed as a soluble drinking powder using wet granulation method. The product was determined for physical characteristics, loss on drying, TLC and IR fingerprint and evaluated for antioxidant activity using DPPH scavenging assay compared with ascorbic acid (vitamin C) standard curve. Antioxidant effect of soluble drinking powder from mamao fruit extract was expressed as g vitamin C equivalent per 1 sachet (14 g) of soluble drinking powder.

Loss on drying

Loss on drying of soluble drinking powder of mamao fruits was investigated using method according to USP 36 [7]. Soluble drinking powder (2 g) was dried in the hot air oven at 105 º C for 3 hours and then every 1 hour until constant weight is obtained and calculated for percent loss on drying. Loss on drying of soluble drinking powder was done in triplicate and the average result was calculated.

Thin layer chromatographic (TLC) fingerprint

Decoction extract from the ripe fruits of *A. ghaesembilla* (PDE) was extracted with 0.5 % HCl in methanol (1:10 w/v). The obtained hydrochloric acid methanol fraction was analyzed by thin layer chromatography on TLC pre-coated silica gel 60 GF_{254} plate using n-butanol-acetic acid-water (4:1:5) as solvent system. TLC plates were detected under UV 254 and 366 nm and DPPH spray reagent.

Infrared spectrometric (IR) fingerprint

The infrared spectra of soluble drinking powder of mamao fruits was identified by FTIR (KBr disc) techniques.

Statistical analysis

All data are reported as means ± standard deviation of triplicates. Least significant difference was used to compare means ($p < 0.05$).

3. RESULTS

Free radical scavenging activity by DPPH scavenging assay

Comparing between purple fruit extracts of mamao from various methods of extraction, extract from decoction promoted higher free radical scavenging activity than maceration and squeezing extracts. There is no significant difference in free radical scavenging effect among decoction extracts obtained from various drying methods. The red fruit extract also exhibited high free radical scavenging effect (Table 1).

Determination of total phenolic content by Folin-Ciocalteu method

As shown in Table 1, RDF exhibited the highest total phenolic content of 2.81 ± 0.88 g% GAE. Total phenolic contents of extracts from the purple fruits of mamao prepared from different methods of extraction ranged from 0.87 ± 1.00 to 2.63 ± 1.02 g% GAE.

Determination of total anthocyanin content by pH differential method

Squeezing extract from the purple fruits of mamao contained the highest amount of total anthocyanin content of 61.51±0.95 g%C-3-GE (Table 1). The decoction extracts from purple fruits of mamao obtained by freeze drying and evaporation on a water bath significantly contained higher amount of total anthocyanin contents than extract from spray drying. The results suggested that the high temperature during the process of spray drying had a significant decreasing effect to anthocyanin content.

From the results, the ripe purple fruit extract prepared from decoction and evaporation on a water bath method (PDE) which exhibited high DPPH scavenging activity, high amount total phenolic and total anthocyanin contents was selected for development of soluble drinking powder. Moreover, this extract contained the high percentage of yield and the extraction and drying procedures were convenient and simple which suitable for up-scaling in manufacturing process.

Table 1. Free radical scavenging activity, total phenolic and total anthocyanin contents of extracts from the fruits of mamao prepared by different extraction and drying methods

Sample	Yield (%w/w)	DPPH assay* (IC_{50}, µg/ml)	Total phenolic* (g% GAE)	Total anthocyanin* (g% C-3-GE)
PDF	32.56	70.14 ± 3.90^{a}	1.18 ± 0.44^{a}	6.03 ± 0.65^{a}
PDS	6.96	62.59 ± 6.63^{a}	1.68 ± 0.46^{a}	4.70 ± 0.23^{b}
PDE	28.48	71.26 ± 1.57^{a}	1.22 ± 0.36^{a}	7.09 ± 0.24^{a}
RDF	3.38	72.42 ± 3.52^{a}	2.81 ± 0.88^{b}	15.79 ± 2.09^{c}
PME	15.76	575.18 ± 17.14^{b}	0.87 ± 1.00^{c}	6.21 ± 0.57^{a}
PMM	10.95	3552.23 ± 393.03^{c}	1.72 ± 1.56^{a}	6.55 ± 1.04^{a}
PMA	6.91	541.70 ± 51.79^{b}	1.49 ± 0.86^{a}	6.56 ± 0.84^{a}
PS	7.06	120.73 ± 5.75^{d}	2.63 ± 1.02^{d}	61.51 ± 0.95^{d}
Trolox	-	5.49 ± 0.34^{e}	-	-

PDF = purple fruit decoction freeze drying extract, PDS = purple fruit decoction spray drying extract, PDE = purple fruit decoction water bath evaporating extract, RDF = red fruit decoction freeze drying extract, PME = purple fruit maceration ethanol extract, PMM = purple fruit maceration methanol extract, PMA = purple fruit maceration acetone extract, PS = purple fruit squeezing extract. Different letter in the same column indicated significant differences ($p < 0.05$).

Product development

Soluble drinking powder from decoction extract of ripe mamao fruit was developed as a pinkish red powder. In 1 sachet of 14 g preparation contained 3.08 ± 0.81 g%C-3-GE and exhibited free radical scavenging activity equivalent to 0.004 g of vitamin C determined by pH differential method and DPPH scavenging assay, respectively. The soluble drinking powder of mamao fruit exhibited loss on drying of 0.22 ± 0.03%. After applying 1 sachet of soluble drinking powder in 1 glass of water (240 ml), the solution appeared as brownish red solution with sour-sweet flavor. The physical appearances of soluble drinking powder and the drinking solution were shown in Figure 1.

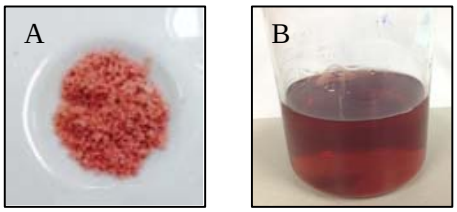

Figure 1**.** Soluble drinking powder from decoction extract of mamao fruits (A) and the drinking solution (B)

TLC fingerprints

Thin layer chromatogram of decoction extract from mamao fruits (PDE) showed specific fingerprints with some dark quenching and fluorescence bands by the detection under UV 254 and 366 nm, respectively. The results suggested the presences of phytochemicals with the chromophores such as conjugated double bonds of phenolics and flavonoids. Moreover, these compounds also showed positive results to DPPH spray reagent appeared as pale yellow bands on purple background suggesting the antioxidant effects (Figure 2). Compound appeared at Rf value of 0.74 was the major band which also promoted antioxidant activity and could be used as an antioxidant marker for quality control of mamao fruit extract in a future.

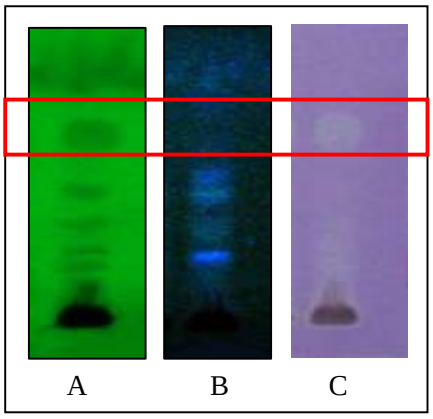

Figure 2. TLC fingerprint of decoction extract from mamao fruits (DE) stationary phase: silica gel GF_{245}
Solvent system: *n*-butanol-acetic acid-water (4:1:5)
Detection: A= UV 254 nm, B= UV 366 nm, C= DPPH spray reagent

IR fingerprint

Using IR spectroscopic technique (KBr disc), decoction extract from mamao fruits (PDE) promoted the major IR spectra of some functional groups including hydroxyl group (OH), C-H group, keto group (C=O), C=C group, C-O group and fingerprint of benzene ring corresponded to the functional groups of phenolic and flavonoid compounds. The results from IR analysis supported the results from TLC and spectrophotometric assays. The functional groups from IR spectra of soluble drinking powder from mamao fruit extract was described in Table 2 [8].

Table 2. IR spectra of soluble drinking powder from mamao fruit extract

Peak	Frequency (cm-1)	Functional group
1	3403	OH- stretching
2	2970	methyl C-H stretching
3	2901	methylene C-H stretching
4	1732	C=O
5	1622	C=C stretching
6	1418	CH_2- bending
7	1281	C-H bending
8	1080	C-O stretching
9	600 – 882	fingerprint of benzene ring

Product specification sheet

The physical, chemical and biological properties of soluble drinking powder from mamao fruit extract was described as product specification sheet in Table 3

Table 3. Product specification sheet of soluble drinking powder from mamao fruit extract

Property	Description
Raw material	Ripe purple fruit of mamao (*Antidesma ghaesembilla*)
Method of extraction	Decoction
Method of drying	Evaporation on a water bath
Physical characteristic	Fined powder
Color	Pinkish red
Odor	Odorless
Taste of the solution	Sour-sweet
Weight / sachet (g)	14
How to use	1 sachet soluble in 1 glass of water (240 ml)
Total anthocyanin content* of 1 sachet (g% GAE) (*pH differential method)	Not less than 3
Antioxidant activity of 1 sachet* (*DPPH scavenging assay)	Not less than 4 mg ascorbic acid equivalent
Loss on drying (%)	Less than 1
TLC fingerprint* (*condition as mentioned above)	Specific fingerprint as shown in figure 2 with chromatographic band at Rf = 0.74 which quenching under UV 254 nm and positive to DPPH spray reagent
IR fingerprint (KBr disc)	Specific IR spectra with peaks at wavenumbers of 3403, 2970, 2901, 1732, 1622, 1418, 1281, 1080, fingerprint around 700-800 corresponded to the functional groups as mentioned in Table 2

4. CONCLUSIONS

Extract from the ripe purple fruits of mamao (*Antidesma ghaesembilla*) prepared by decoction and drying by evaporation on water bath (PDE) was found to promoted high antioxidant activity tested by DPPH scavenging assay. This extract contained high amount of phenolic and anthocyanin contents. Soluble drinking powder from PDE was developed by wet granulation method. The finished product appeared as pinkish red powder that contained 3.08 ± 0.81 g%C-3-GE in 1 sachet (14 g) and exhibited free radical scavenging activity equivalent to 0.004 g of vitamin C. Thin layer chromatographic and infrared spectroscopic fingerprints and loss on drying were conducted to qualitatively control the specification of the product. Investigation for the stability, toxicity and other related biological activities of the soluble drinking powder should be performed in the future. The results of this study could be the guideline for further developments of antioxidant products from mamao fruit extracts in industrial enterprises.

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Anti-oxidant contents and activities from natural *Phellinus linteus* and *Phellinus igniarius* extracts

Wanwisa Moon-ai*[a], Poompong Chuchouisuwan[a], Panee Sirisa-ard[b], Frankie Chan[a]
[a]Thai-Korea Natural Phellinus Mushroom Research Center, Amazing Grace Health Products Limited Partnership, 203/41-42 Moo 1, Lakhok, Muang, Pathumthani, Thailand 12000
[b]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Thailand 50200

*bio_t@windowslive.com phone +66-29786481 fax +66-29786350

ABSTRACT

The natural *Phellinus* mushroom has been widely used as a medicinal plant for the treatment of various diseases. There are many commercial products derived from the mushroom available on the market today; however, the biochemical substances involving antioxidants and the associated activities are still unknown or have yet to be reported. In this study, we investigated the antioxidant contents and activities from four commercial products containing natural *Phellinus linteus* (PL-65 and PL-38) and *Phellinus igniarius* (PI-65 and PI-16). The evaluation of anti-oxidant contents in this study included total phenolic compound, flavonoids, tocopherol, ascorbic acid and glutathione. The highest antioxidant contents were found in the *Phellinus igniarius* product, under the commercial name PI-65, followed by PL-65, PL-38 and PI-16 respectively. The total antioxidant activity was assessed by using DPPH and ABTS assays. The results were reported as trolox equivalent antioxidant concentrations (TEAC). The PI-65 and PL-65 showed the highest TEAC values in both DPPH (1.00 and 1.00 g/ml) and ABTS (1.88 and 1.79 g/ml) assays. Based upon these results, all these products may have potential anti-oxidants to prevent oxidative stress which can cause pathogenesis and ultimately induced cell death in the human body.

Keywords: Anti-oxidants, Natural mushroom, *Phellinus linteus*, *Phellinus igniarius*

1. INTRODUCTION

Free radicals are normally generated by being metabolized in cells. However, exogenous factors, such as sunlight, ultraviolet light, and chemicals can cause free radicals as well [1]. The overproduction of free radicals results in an imbalance between factors that generate it and factors that protect the cells, this can cause oxidative stress [2]. Oxidative stress is associated with biological molecules like proteins, lipids and DNA and creates lipid peroxidation, protein, and DNA damage. These damages lead to ageing, inflammatory disease, atherosclerosis, cardiovascular disease, diabetes, neurological disease and cancer [3-4]. Although the human body possesses an antioxidant system to protect cells from free radicals, these systems are insufficient to provide complete protection. Essential dietary foods are abundant sources of antioxidants, such as phenols, flavonoid, tocopherols, ascorbic acid, carotenoids and glutathione for supporting the antioxidant system and reducing oxidative damages [5]. *Phellinus linteus* and *Phellinus igniarius* have been popularly used as a medicinal mushroom since ancient times. Their medicinal properties have various positive effects, such as being antitumor, anticancer, immunomodulatory activities and can treat various diseases such as gastro-enteric disorder, lymphatic disorder and cancer [6]. The antioxidant contents and activities in these mushrooms have been reported in many research studies. Quantitation of properties was significantly found for being used as a source of anti-free radicals [6-8]. The present study aimed to evaluate the antioxidant contents and properties of hot water extracts from natural *Phellinus linteus* and *Phellinus igniarius* using *in vitro* assays. The scavenging abilities for antioxidant activity are on DPPH and ABTS. Potential antioxidant components in these extracts were determined as well.

2. METERIALS AND METHODS

Materials

Hot water extracts of natural *Phellinus linteus* and *Phellinus igniarius* were used from commercial products of Amazing Grace Health Products. The products of *Phellinus linteus* were PL-65 and PL-38 and the products of *Phellinus igniarius* were PI-65 and PI-16.

Determination of Antioxidant contents

Total phenols were measured by The Folin-Ciocalteau method [9] with some modification. 0 µl of sample was mixed with 50 µl of Folin-Ciocateau reagent, which made up a volume of 400 µl. With added water, it was incubated at room temperature for 5 minutes then 500 µl of 7% Na_2CO_3 was added into the solution, after which it was kept at room temperature for 90 minutes. The mixture measured the absorbance at 750 nm by a spectrophotometer; Gallic acid was used as a standard.

Total flavonoid was determined with a slightly modified method of Kurkina (2011) by using a complex-forming reaction with an aluminum chloride solution [10]. The reaction mixture contained 100 µl of sample mixed with 200 µl of 3% lCl_3ethanolic solution and made up a volume of 1 ml with 95% ethanol. The mixture was mixed and incubated for 30 minutes at room temperature. The absorbance was measured at 403 nm with a spectrophotometer; Rutin was used as a standard.

Tocopherol was estimated by using the method previously described [11]. 100 µl of sample was mixed with 350 µl of 0.07% (/v) 2,2´bipyridine, 50 µl of 0.2% (w/v) $FeCl_3$ then made up a volume of 1 ml with 95% ethanol. The mixture was incubated at room temperature for 1 minute and measured absorbance at 520 nm by using spectrophotometer; α-Tocopherol was used as a standard.

Ascorbic acid was then measured by following the method of Marschner and Cakmak (1992) [12]. The reaction mixture contained 10 µl of the sample, which was then added 250 µl; 0.15M sodium phosphate buffer (pH 7.4) and 100 µl of water. The color was developed by adding the followed reagents; 200 µl of 10% trichloroacetic acid (TCA), 200 µl of 42.5% ortho phosphoric acid, 200 µl of 4% 2,2´bipyridine and 100 µl 3% $FeCl_3$. The mixture was mixed and incubated at room temperature for 10 minutes. The absorbance was read at 525 nm with a spectrophotometer; L-ascorbic acid was used as a standard.

Glutathione (reduced form) was determined by using Ellman's reagent according to method of Greppin and Castillo (1988) [13]. The mixture solution contained with 50 µl of sample mixed with 950 µl of 60 mM potassium phosphate 2.5 mM EDTA buffer (pH 7.5), 2 mM 5,5´-dithiobis-2-nitrobenzoic acid (DTNB). The mixture was incubated at room temperature for 10 min. The absorbance was measured at 412 nm by using spectrophotometer. L-glutathione (reduced form) was used as a standard.

Antioxidant scavenging capacity

DPPH scavenging activity was determined by standard method [2] with modifications. 50 µl of sample was mixed with 350 µl of absolute methanol and then combined with 400 µl of 0.15 mM methanolic DPPH. The mixture

was incubated at room temperature for 30 minutes in the dark. The absorbance was read at 517 nm with a spectrophotometer. The results were expressed in grams; trolox equivalent antioxidant capacity (TEAC)/ml.

ATBS scavenging activity was estimated by following Jung et al. (2008) [8] with some modifications. The stock solution was prepared by mixing 7 mM ATBS in water and 2.45 mM potassium persulfate in equal volume then left in the dark for 12 hours. The working solution was diluted by mixing stock solution with absolute methanol to obtain an absorbance 1.0 ± 0.1 at 734 nm. $ATBS^{+}$ solution was freshly prepared for each assay. Twenty µl of sample was added into 980 µl of $ATBS^{\cdot+}$ solution for 5 minutes in the dark. The absorbance was measured at 734 nm by using spectrophotometer. The scavenging activity was expressed in gram TEAC/ml.

Statistical analysis

Data was shown as mean ± standard error (SE). All analyses were replicated three times. The results were analyzed by using one-way ANOVA and Duncan's multiple range test (DMRT) to test any significant differences.

3. RESULTS

The five antioxidants from four hot water extracts of *Phellinus linteus* and *Phellinus igniarius* in table 1 included total phenols, flavonoid, tocopherol, ascorbic acid and glutathione. The highest contents of all antioxidants was found in *Phellinus igniarius* product, PI-65. There were 16.50 ± 0.84, 0.71 ± 0.04, 37.76 ± 0.75, 1.21 ± 0.01 and 1.57 ± 0.07 mg/ml in total phenols, flavonoid, tocopherol, ascorbic acid and glutathione respectively. The lowest contents were found in *Phellinus igniarius* product, PI-16 as well. Its total phenols, flavonoid, tocopherol, ascorbic acid and glutathione contents were 6.01 ± 0.64, 0.26 ± 0.03, 14.67 ± 1.66, 0.36 ± 0.03 and 0.72 ± 0.01 mg/ml, respectively. This difference should be due to the concentration in both formulas being different as well as from the *Phellinus linteus* products. The PL-65 product was found to have lower antioxidant than PI-65 and higher than PL-38. However, there is no significant difference between PL-65 and PL-38 in flavonoid content.

Antioxidant activities were evaluated in hot water extracts using ABTS and DPPH assays. The DPPH and ABTS activity results were expressed in the term trolox equivalent antioxidant capacity (TEAC, g/ml). For DPPH assay, the synthetic DPPH radical can be reduced by an electron or hydrogen donation from extracts. The results showed that there were no significant differences of DPPH scavenging activity in both PL-65 and PI-65. The ability of extracts capable of scavenging DPPH free radicals follows this order: PI-65 = PL-68 > PL-38 > PI-16 (table 1). The ABTS assay was based on generating free radicals from the ABTS salt in the dark. As the results in table 1 revealed, all the hot water extract products were able to neutralize the ABTS radicals. The maximum capacity was found in PI-65 and PL-65 products. While activity in PL-38 was higher than PI-16, it was lower than PI-65 and PL-65.

Table1. Contents and activities of antioxidant components from hot water extract of natural *Phellinus linteus* and *Phellinus igniarius*.

Products	Antioxidant content (mg/ml)					Antioxidant scavenging activity (TEAC, g/ml)	
	Total phenols	Flavonoids	Total tocopherols	Ascorbic acid	Glutathione	DPPH	ABTS
PL-65	14.17±0.84 b	0.57±0.01 b	29.78±1.92 b	0.85±0.02 b	0.90±0.03 b	1.00±0.01 a	1.79±0.05 a
PL-38	9.03±0.84 c	0.48±0.03 b	18.78±1.61 c	0.37±0.03 c	0.78±0.00 c	0.87±0.02 b	1.23±0.06 b
PI-65	16.50±0.32 a	0.71±0.04 a	37.76±0.75 a	1.21±0.01 a	1.57±0.07 a	1.00±0.01 a	1.88±0.06 a
PI-16	6.01±0.64 d	0.26±0.03 c	14.67±1.66 c	0.36±0.03 c	0.72±0.01 c	0.72±0.03 c	0.88±0.02 c

Each value is expressed as mean ± standard error (n = 3)

Values indicated by different letters in a column are significantly different ($p < 0.05$)

4. CONCLUSION

Hot water extracts from *Phellinus linteus* and *Phellinus igniarius* in commercial products were evaluated in antioxidant contents and activities. According to the results, PI-65 was found the highest in antioxidant contents and PI-16 was found the lowest in antioxidant contents. In antioxidant activities, all the products had ability to reduced free radicals in DPPH and ABTS assays. The highest activities of DPPH and ABTS scavenging capacities were found in PI-65 and PL-65 products. These results showed that hot water extract products by *Phellinus linteus* and

Phellinus igniarius may contain a good source of antioxidants, which prevent oxidative damages from free radicals that cause human diseases.

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Biological efficacy of bacterial cellulose/alginate composite film plasticized with glycerol containing sappanwood ethanolic extract

Saranyou Oontawee[a], Chanan Phonprapai*[a], Arunporn Itharat[b],
[a]Department of Biotechnology, Faculty of Science and Technology, Thammasat University Rangsit Center, Pathum Thani, Thailand 12121
[b]Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University Rangsit Center, Pathum Thani, Thailand 12121

*chanan@tu.ac.th phone +66-871025380 fax +66-25644500

ABSTRACT

Infected wound can be caused by improperly wound care, which wound dressing can be used to prevent the wound from physical damages and bacterial invasions. Recently, commercialized dressing is usually produced from biopolymer due to their biocompatibility and biodegradability. Thus, this research had focused on the characterization of the bacterial cellulose (BC) /alginate composite film plasticized with glycerol that contained the sappanwood ethanolic extract (SE)which was invented to be the antibacterial dressing. In the preparations, the films were varied BC content (40% to 60% ($w_{SE}/w_{BC/ALfilm}$)), and SE content (0% to 60% ($w_{SE}/w_{BC/ALfilm}$)). Subsequently, the antibacterial activities were determined by using the agar disc diffusion method, and the releasing profiles were derived by using the Franz diffusion cells. From the biological characterization of the extract, the minimum inhibitory concentration (MIC) against *Staphylococcus aureus* TISTR1466 (0.078 mg/ml) was lower than *Pseudomonas aeruginosa* TISTR781 (1.250 mg/ml), and the half maximal inhibitory concentration (IC_{50}) against human dermal fibroblast was 0.056 mg/ml. Additionally, the film characterizations revealed that the film with 60% SE and 40% BC gave the largest zone of inhibition against *S. aureus* TISTR1466 (1.35 mm), whereas the average cumulative concentration at 360 min of 60% SE films were 0.026 mg/ml. Finally, the simulation of bacterial inhibiting periods will be added in the future works.

Keywords: Bacterial cellulose, alginate, Sappanwood ethanolic extract, Antibacterial activities, Releasing profiles

1. INTRODUCTION

The wound is an injury that damages the deep layer of the skin called dermis. To protect the wound properly, the wound dressing will be applied.Ithas been used to prevent the wound from physical damages and microbial invasions since ancient time [1]. In the last few decades, bio-based wound dressing mostly developed due to their biocompatibility and biodegradability. It is usually produced from alginate (AL), the brown algae extract. Normally, sodium alginate could be converted to calcium alginate when the calcium ions were presented [2]. When the dressing has been addedwith active compounds to provide the antimicrobial activity, it should obtain enough concentration of the active compound passed on the wound. Bacterial cellulose (BC) is a fine fiber that produced by acetic acid producing bacteria namely *Gluconacetobecter xylinus* [3]. It could obstruct the releasing due to it possesses fine fiber networks. In this study, the bacterial cellulose/alginate composite film plasticized with glycerol and containing the Thai herbal extract had practically prepared and biologically characterized. The sappan wood ethanolic extract (SE) was selected for introducing the antibacterial properties to the dressing. According to the pharmacological characterizations, the sappan wood extract was found to hold the activitiesthat promote the wound healing, such as anti-inflammatory activity, anti-oxidation activity, anti-hyperglycemic activity, and anti-microbial activity [4].

2. MATERIALS AND METHODS

Determination of minimum inhibitory concentration of SE against wound infecting bacteria

Firstly, the powdered sappan wood was extracted by using Soxhlet apparatus with 95% ethanol at 80ºC until appear a clear solvent.To determine the minimum inhibitory concentration (MIC) of sappan wood ethanolic extract, the microdilution broth method, modified from the research of Sarker et al. [5] was performed against the wound infecting bacteria, such as *Staphylococcus aureus* TISTR1466 and *Pseudomonas aeruginosa* TISTR781. Then, the microbes were activated and dilutedto the desired bacterial suspension ($0.5x10^6$ CFU/mL) by using Mueller-Hinton broth (MHB).After that, the extract, dissolved in dimethylsulfoxide (DMSO), was prepared to reach the concentration of 5 mg/mL by using MHB. The 50 µL of the prepared extract was filled into the first well and the second well of the 96-well plates and then performed the 2-fold serial dilutionsfromthe second well to the eleventh well. Each well was filled with the 50 µL of$5.0x10^5$CFU/mL bacterial suspensions. The concentration of the extract was then varied from 0.002 to 2.5mg/mL with the bacterial concentration of $2.5x10^5$CFU/mL, while the controlled well was only filled with a sterile MHB (without bacterial inoculation). This made the total volume of each condition was 100µL, which was done in triplicate. In addition, the 2-fold dilution of 0.05mg/mL gentamicin was also performedin the second well to the eleventh well (provided 0.00002 to 0.025 mg/mL gentamicin). Finally, the plates were then loosely wrapped and incubated at 37ºC on 100 rpm incubator shaker for 18 h. To determine the bacterial viability, the 10 µL of 1 mg/mL resazurin reagent was added to the wells, which were then incubated for 2 h. The MIC value was selected from the lowest extract concentrationwhichgavea dark blue color developed in the well.

Determination of cytotoxicity of SE against human dermal skin fibroblast

This experiment was performed by Animal Cell Cytotoxicity Testing Service (Bioassay Laboratory, BIOTEC, NSTDA, Thailand). Firstly, the extract was dissolved in DMSO to prepare a stock concentration of 100mg/mL. The sample was then carried on 2-fold serial dilution in the culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4mM L-glutamine, 1.5g/L sodium bicarbonate, 100 U/mL penicillin and 100µg/mL streptomycin)to prepare 7 concentrations of the extract (500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL).The cells were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. To determine the viability of the cells, the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used. When the cell survives, it is able to metabolize the tetrazolium (yellow) to formazan (blue) [6]. This method was modified from the conventional standard methods (BS-EN30993-5:1994 and ISO10993-5:1992). Then, 50 µl of MTT, 5 mg/mLin phosphate buffer saline pH 7.4 (PBS pH 7.4), was added into each well, which already contained the medium and cells. These wells were incubated for 4 h before aspirating the medium from the wells. The formazan was then solubilized with 200 µL of DMSO and 25 µL of Sorensen's glycine buffer pH10.5. Finally, the optical density of the solubilized formazan was detected using a microplate reader at 570 nm and then analyzed through the SoftMax software todetermine the $IC_{50}$of the extract.These experiments were donein three replicates, whicheach replicate was averagedfrom 4 wells to get themean of each point. To obtain the mean and the standarddeviation of each concentration, each replicate was summarized, averaged, and calculated the

standarddeviation. The dose-responsive curve was done by plotting the mean of each concentration, and the concentration that inhibited the cell viability for 50% was selected as IC_{50}.

Preparation of BC/AL composite films plasticized with GLY containing SE

The compositions of the films, which included sodium alginate (AL), shredded bacterial cellulose (BC), glycerol (GLY), and SE, were prepared before mixing. The sodium alginate solution was obtained fromdissolving sodium alginate powder in the water to get the concentration of 3.0 g/L aqueous solution. Likewise, shredded bacterial cellulose was obtained from crushing the purified bacterial cellulose in blender and diluting it to 3.0 g/L of aqueous suspension, andglycerol was diluted to be 50% (v/v) aqueous solution. In preparingthe SE solution, the crude extract was dissolved in 95% ethanol to obtain 45 g/L solution.During the preparation, the BC content was varied from 40 to 60% (wBC/wBC/AL film), and the SE content was varied from 0 to 60% (wSE/wBC/AL film), while the GLY content was fixed at 3% (vGLY/vBC/ALmixture).The compositions were then gently mixed by usinga magnetic stirrer for 10 min, where SE was gentlyadded during themixture process. The mixtures werethen casted onthe glass plates to get the 30 g/$m^2$of the films in dry basis, where the mixture was slowly evaporated in 45°Chot air oven at under atmosphere pressure for 48 h to obtain the films without entrappedair bubbles. When the mixtures were completely dried, it was carried on the cross-linking process for 15 min by pouring the 5% (w/v) calcium chloride solution into the platesand then soaked with distilled water for 15 min to eliminate the excessive salt. The film was re-dried for 24 h under the same condition and kept in silica gel desiccator to avoid the moisture absorption.

Preliminary determination of antimicrobial efficiency of BC/AL composite films plasticized with GLY containing SEagainst wound infecting bacteria

The agar disc diffusion method was used to determine the antimicrobial efficiency of the films against *S. aureus* TISTR1466 and *P. aeruginosa* TISTR781. Firstly, the activated microbes ($0.5x10^6$ CFU/mL in MHB) were fully smeared onMueller-Hinton agar (MHA), then the circular films (6 mm diameter), which was irradiated by the UVC light for 20 minto sterile the films, weregently placed on the smeared plate, which the plain filter papersand the filled filter paper, with 20 µL of 0.1 mg/mL gentamicin, were used as a negative control and a positive control, respectively. Finally, the plates were then incubated at 37°C for 18 h before observing and measuring the inhibition zone.

Releasing investigation of BC/AL composite films plasticized with GLY containing SE

Before determined the releasing, the films were cut into a round shape with 3 cm diameter and then kept intoa silica gel desiccator at room temperature. This study has used the modified Franz diffusion cells to investigate the releasing rate of the extract from the filmsby maintaining the temperatureat 37°C.The extracts were permitted to release through thecellophane membrane, while phosphate buffer saline pH 7.4 (PBS pH7.4) was used as a receptor medium, to simulate the human skin condition. The releasing was investigated for 360 min, while the phosphate buffer with SE was withdrawn for 500 µL every 30 min for 180 min and then every 60 min for 180 min. Every withdrawn sample was replaced by a fresh PBS pH7.4 with the equivalent volume. The SE extract concentration in the sample was determinedbyusingthe spectrophotometer with the highest absorption wave length (λ_{max}) of the extract. The concentrations were calculated from thecrude extract calibration curve.

3. RESULTS

The results of MIC of SE and gentamicin revealed that the MIC values of SE and gentamicin against *S. aureus* TISTR1466 and *P. aeruginosa* TISTR781 were 0.078 and 0.0002 mg/mL, for SE, respectively and 1.250 and 0.0008 mg/mL, for gentamicin, respectively. The cytotoxicity value which was obtained from Animal Cell Cytotoxicity Testing Service was exhibited as IC_{50} against human dermal skin fibroblast, was 0.056±0.013 mg/mL. The antibacterial efficiency was tested and the results showed that the zone of inhibition was found around 60% SE and 40% BC samples, which was tested against *S. aureus* TISTR1466, with the size of 0.967±0.28 mm in average. While the inhibition zone of positive controls against *S. aureus* TISTR1466 and *P. aeruginosa* TISTR781 was found at 6.78±1.14 and 4.30±0.50 mm, respectively. As a results, the releasing investigation was carried out only the 60% SE films with various BC contents, where the results show that the extract contained in the films were continuously released at different rates until it reached the constant concentration around 300 min (Figure 1). At the final releasing time (360 min), the releasing concentration of 40% BC film, 50% BC film, and 60% BC film were found to be 0.026±0.010, 0.017±0.008, and 0.035±0.007 mg/mL, respectively.

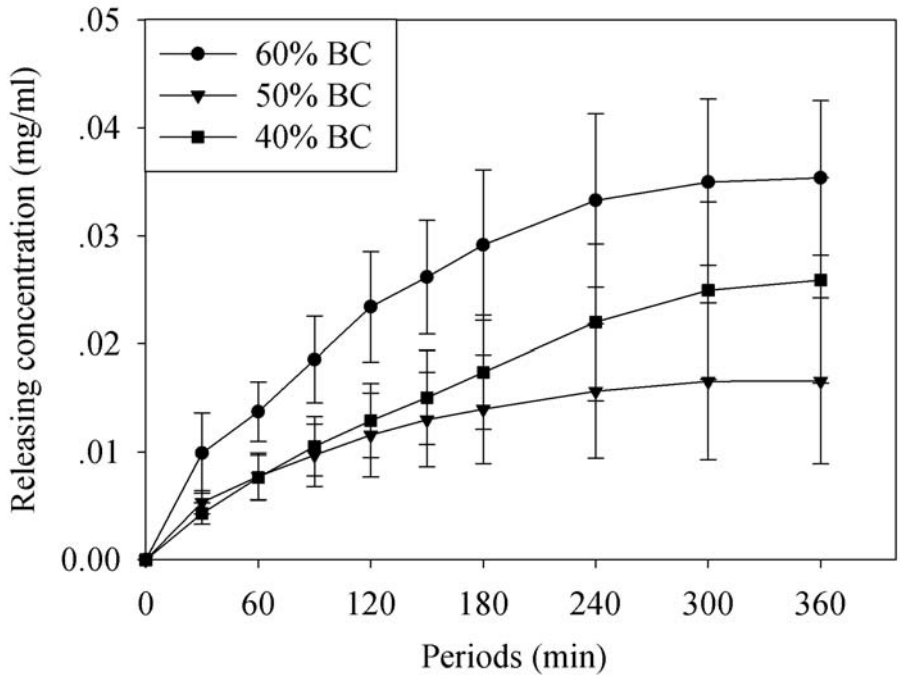

Figure 1. The releasing profiles of BC/AL composite films plasticized with GLY containing 60% SE that varied the BC contents.

4. CONCLUSIONS

Though the releasing concentration of the 60%SE-40%BC film (0.026 ± 0.010 mg/mL) is lower than the MIC value (0.078 mg/mL) for 3 times, it could inhibit the proliferation of *S. aureus* TISTR1466. These may be due to the released extract was continuously absorbed and accumulated by the bacteria during the determination of antimicrobial efficiency of the films using agar disc diffusion method that incubated for 18 h. According to the results, it could be concluded that the 60%SE-40%BC film could be used as the antibacterial wound dressing but it should be investigated on the bacterial inhibiting periods in the future works. Moreover, applying the film to the wound or the skin for 360 min should be safe from the irritation.This could be confirmed by the IC_{50} value of the extract against human dermal skin fibroblast (0.056 ± 0.013 mg/mL) which was two times higher than the releasing concentration at 360 min (0.026 ± 0.010 mg/mL). Nevertheless, the animal skin irritation test should be done in the future works also.

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Acute toxicity study of *Phaleria macrocarpa* (Scheff.) Boerl

Azad AK*[a], Wan Azizi WS[a], Taher M[b]

[a]Department of Basic Medical Sciences, Faculty of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200 Indera Mahkota, Kuantan, Pahang, Malaysia
[b]Department of Pharmaceutical Technology, Faculty of Pharmacy, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200 InderaMahkota, Kuantan, Pahang, Malaysia

ABSTRACT

Acute toxicity of a single oral dose 5000 mg/kg/b.w (max. dose) of *Phaleria macrocarpa* (Scheff.) Boerl. (PM) fruits *(*mesocarp and pericarp) ethanolic extract by *in vivo* and MTT-assay by cell line study with different concentration was evaluated. In the in *vivo* study, a total of twelve healthy adult male rats (Sprague–Dawley) 8–12 weeks weighing from 180 to 200 gm were obtained from the Laboratory Animal Centre, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. Ethanolic extract of the (PM*)* fruits was obtained based on the procedure described by the revised UP and Down method (OECD, 425). Group-I (Control- 10% normal saline) and Group-II (treated+ PM) consist of six rats of each group. The rats were fasted (16 h) overnight and the body weight (gm) of each rat was recorded prior to the test. A fixed dose of PM fruits extract (5000 mg/kg/b.w) was administered orally to each rat and observed closely at 4 h initially, then every 6 h intervals for changes. MCF-7 cells were used in the MTT-assay in cell lines study. *In-vivo* biochemical aspects for the treated animals' serum values were similar to the control animals. Results on histological studies of liver showed that there was no periportal necrosis of the hepatocytes and no inflammation of lymphocytes and macrophages in both control and treated groups. No difference was observed in glomeruli or any other segment of kidney tubules when compared with their respective normal rats. Similarly, *in vitro* cytotoxicity study showed that the percentage of cell viability was 56.65% (5.00 µl) and 95.62 (0.31 µl) but the percentage of cell viability was 106.23% at concentration of 1.25 µl. Findings of the present study suggest that the ethanolic extract of (PM*)* fruits at the dose of 5000 mg/kg/b.w (maximum) non-toxic and it could be used in the next step research for animal or cell lines study.

Keywords: *Phaleria macrocarpa*, Mesocarp, Acute toxicity, MCF7, Histology, Liver, Kidney

1. INTRODUCTION

There are general and herb-specific concerns regarding medicinal plants and their ability to produce toxicity and adverse effects. Accidental herb toxicity occurs not only as a result of a lack of pharmaceutical quality control in harvesting and preparation but also because herbal remedies are believed to be harmless. Reports suggested that spices and their chemical constituents may have diverse pharmacological activities and toxicity [1-2].

Studies on the management of diabetes mellitus have postulated the benefits of *Phaleria macrocarpa* (Scheff.) Boerl. (PM) in both hypoglycemic and antihyperglycemic effects that were conducted in animal models using the streptozotocin-induced diabetic sprague-Dawley rats in *in-vivo* and *in vitro* α-glucosidase and α-amylase inhibition study [3-5].

For centuries, the native Indonesians have been using the fruit of the *Mahkota dewa* tree, PM to cure, treat and prevent diabetes [6], liver diseases, vascular problems, cancer and high blood pressure.It'smost commonly used in Indonesian traditional medicine for the treatment of cancer, diabetes mellitus, and hypertension [7]. From an ancient era, the native Boerl traditionally used to combat diabetes, liver diseases, vascular problems, cancer and high blood pressure [8].

Previous studies have shown that PM contains some secondary metabolites that could combat not only cancers or infectious disease such as malaria [9] but also the so-called lifestyle diseases including diabetes, hypertension and atherosclerosis [10]. Ethyl acetate extracts of PM showed anti-diabetic activity against alloxan – induced diabetic rats which was assumed to be mediated either by preventing the decline of hepatic diabetes or hypertension [3,11]. Natural phytochemicals have been reported to possess a wide range of biological activities including antioxidant, antimicrobial and anti-inflammatory properties [12-13]. Itcontained kaempferol, myrecetin, naringin and rutin and these flavonoids showed very potent antibacterial and antifungal activities [7].

The leaves extract of PM showed antibacterial, radical-scavenging activities and cytotoxicity [14]. Empirically, PM has proved capable of controlling cancer, impotency, haemorrhoids, diabetes mellitus, allergies, liver and heart diseases, kidney disorders, blood diseases, rheumantism, high blood pressure, stroke, migraine, various skin diseases, acne and so forth [15].

Studies on the fruits of PM resulted in the isolation and characterization oficariside C_3 and mangiferin [16]. Hartati et al., [17] reported the identification of phalerin, a new benzophenoic glucoside (3,4,5-trihydroxy-4′-methoxy-benzophenone-3-0-β-D-glucoside) from the methanolic extract of PM leaves.

A new phenolic glycoside mahkoside A, together with six known compounds including mangiferin, kaempferol-3-0-β-D-glucoside, dodecanoic acid, palmitic acid, ethyl stearate and sucrose were isolated from the pit of PM [9]. Lignanspinoresinol, lariciresinol and matairesinol were isolated from different parts of PM [18]. Recent result showed that PM had potently inhibiting carbohydrate hydrolyzing enzymes [5].

The seeds of PM are very toxic and unpleasant taste due to the present of 29-norcucurbitacin, desacetylfevicordin A and other 29-norcucurbetacin derivatives,fevicordinA, fevicordin A glucoside and fevicordin D glucoside [19]. Seed of PM also contained flavonoid known as vizquercetin [9]. Traditionally seeds are only used for the treatment of skin conditions and for ornamental cultivation purposes or as a traditional biopesticide [18]. Based on previous toxicity studies on PM concentration of 250 mg/kg/b.w. [12], and 1000 mg/kg/b.w. [3] did not produce any toxicity effect. In the present study concentration of 5000 mg/kg/b.w. of PM extractfollowing revised UP and Down method (OECD, 425) was applied with the objective to identify acute liver and kidney toxicity following treatment with PM extract.

2. MATERIALS AND METHODS

Chemicals

Ethanol used for the extraction was analytical grade.

Collection of plant material

The ripen fruit (835 g) of PM was collected from area in Kuantan, Pahang, Malaysia in September 2012. Taxonomic identification was made by IIUM botanist and deposited in the Herbarium, Kulliyyah of Pharmacy, IIUM, Malaysia (Voucher specimen no.: PIIUM 0230). The mesocarp & pericarp of the fruits were sliced and dried in a protech laboratory dryer (FDD-720) at 37°C in the dark for 10 days, pulverized to powder using Fritsch Universal Cutting Mill-PULVERISETTE 19, Germany, and stored at 4°C until further use [20].

Preparation of ethanolic extract

PM fruits powder (710 g) was subjected to cold maceration using ethanol for 48 h at room temperature, filtered into a clean round bottom flask using adsorbent cotton wool and filter paper (Whatman No. A-1). The extraction using similar solvent was repeated for eleven times to ensure maximum yield of ethanol soluble

compounds. The ethanol extract was concentrated in vacuo using a rotary evaporator (BUCHI R-205) to a final adjusted volume of 500 mL. Theextract was frozen at −70°C and transferred immediately to two weeks continuous freeze drying at -50°C using bench top freeze dryer (ALPHA 1-4LD-2), to give a final yield of 213 g.

Experimental animals

The experimental procedures in the present study were approved by the local ethics committee (Institutional Animals Research Ethical Committee, IAEC, KOM, IIUM) and conformed to International guidelines for the use and care of laboratory animals (OECD). All efforts were made to minimize the number of animals used and their discomfort [21]. Twelve healthy adult male rats (Sprague–Dawley strain) (8–12 weeks) weighing from 180 to 200 g, were obtained from the Laboratory Animal Centre, Universiti Putra Malaysia (UPM), Serdang, Sealngor, Malaysia Animals were housed (in pairs) in standard propylene cages, acclimatized for 7 days to animal house conditions in air conditioned room (temperature: 22 ± 3°C; relative humidity: 46-70%; 12:12 h light: dark cycle [22, 23, 24,], adequate cross ventilation) prior to the test. Experimental animals were maintained on standard commercial dry pellet diet containing 22% crude protein, 46% fat, 4% fibre, 7.6% ash, 12.0% moisture, 1.2% calcium, 0.73% phosphorus (Gold Coin Feed mills Sdn. Bhd., Kuala Lumpur, Malaysia), and water *ad libitum*. Animals described as fasting were deprived of food for at least 16 h but had free access to drinking water (OECD) [25].

Safety profile estimation (acute oral toxicity test)

The safety profile study of the ethanolic extract of the PM fruits was determined according to the procedure described by the revised UP and Down method. Animal's weight had not fall in an interval within ± 20 % of the mean initial weight of previously dosed animals. The test substance was administered in a single dose by oral gavage using a stomach tube and a suitable intubation cannula. Following the period of fasting, the animals were weighed prior to administration of the test substance. The fasted body weight of each animal was determined and the dose was calculated according to the body weight. After the substance administered, food may be withheld for a further 3-4 hours in rats (OECD) [26], Briefly, group-I (control, 10% normal saline) a total of six male Sprague–Dawley rats were used for the control and group- II (treated, extract, one rat on daily basis) a six male Sprague-Dawley treated rats. The rats were fasted (16 h) overnight and the body weight (g) of each rat was recorded prior to the test. A fixed dose of PM fruits extract (5 g/kg) was administered orally to each rat and observed closely at 4 h initially, then every 6 h intervals for changes in skin and fur, eyes and mucous membranes, behavioral (alertness, restlessness, irritability, recumbence, vomiting, fearfulness), neurological (spontaneous activity, convulsion, gait, bleeding orifices, touch/pain response), autonomic (defecation, micturition) profiles, and/or mortality [27-30], and any significant morbidity or mortality within 24–168 h was recorded [31].

Biochemical estimation

Blood collection and preparation

At the end of 7 days period of ethanolic extract of PM oral administration, the rats were humanely sacrificed through the anesthetic agent i.e., diethyl either. Before sacrificed, about 3 to 5 mL of blood was collected directly from the heart of each rat via intra cardiac puncture using 23 G syringe. The blood was collected in EDTA tubes. Plasma and red blood cells were separated using refrigerated ultra-centrifuge (Allegra X-12R), 4°C at 2000 × *g* for 10 min. The plasma was stored in working aliquots at -80°C for subsequent biochemical analysis.

Serum biochemistry

The following parameters were determined calorimetrically by employing the standard ready-to-use kits and methods of Human (Human GesellschaftfürBiochemica and Diagnostica MBH,

Germany), Glutamate oxaloacetate transaminase (GOT, AST), glutamate pyruvate transaminase (GPT, ALT), alkaline phosphatase (ALP), total proteins, albumin, total bilirubin, glucose, urea, blood urea nitrogen (BUN), uric acid, total cholesterol and triglycerides (Cobas Mira, The automated Thermo Scientific Konelab 20 and 20XT analyzers). The manufacturer's instructions for each biochemical parameter were strictly followed in the course of the investigations.

Histological test (microscopy)

Kidney and Liver histology

Renal and liver samples from all the rats were harvested and immediately fixed in 10% formalin for tissue processing and paraffin embedding. Sections of 7µm thickness were cut from each paraffin embedded blocks- and stained with haematoxylin-eosin (H&E) followed by Periodic Acid Schiff (PAS) for histological examination. Prepared slides were studied by light microscopy for messangial matrix expansion, as well as glomerular and tubular structural changes [32-33].

Safety profile estimation by MTT-assay

The MCF-7 cell are known to response to physiological level of insulin and thus used in diabetic studies [34-35] MCF-7 cells were cultured in 25 t-flask in medium containing Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5%CO_2, 95% air and complete humidity. Once reached ~90% confluency, they were detached using 0.05% trypsin/EDTA and counted by means of trypan blue and hemocytometer. These cells were then resuspended at a concentration of 4×10^4 cells/cm^2 and added onto 96-well plate (i.e., 250 µL/well) by an 8-channel pipette. For background absorption, some wells were remained cell-free, i.e. as blank control [36-42]

Statistical analysis

One way analysis of variance (ANOVA) and Bonferroni post hoc test were used for data analysis. All the results were expressed as mean ± SEM, (P □ 0.05) were considered statistically significant.

3. RESULTS AND DISCUSSIONS

The individual weights of animals increased from the day of dosing to before sacrifice but it's a normal growth (Table 1). During two-weeks dosing period, all the animals were observed daily for clinical signs and mortality patterns once before dosing, immediately after dosing and up to 6 h after dosing. At the dose levels tested, there was no evidence of toxic symptoms or significant behavioral changes in all animals at a single large dose of 5000 mg/kg/b.w (Table 2) untoward clinical signs were observed in the surviving rats. Careful observation were made every 4h interval/day for any changes effecting physical behavior, CNS and ANS, motor activity, salivation, skin coloration and other general signs of toxicity were also observed and recorded. No any toxicity was observed at 5000mg/kg/b.w of the PM extracts to the group of animals and no mortality was observed in the rats that received 5000 mg/kg/body weight of PM fruits extract orally after 72 h and later (Table 2).

Table 1. Results showing the individual weights of animals at the day of dosing, after treatment day 3 and at the time of sacrifice in rats after 7 days treatment with ethanolic extract of the fruits of PM 5000 mg/kg/b.w

Rat No	Individual body wt. during dosing (g)		Individual body wt. after treatment(g)				Individual body wt. before sacrificed (g)	
			Control		Test		Control	Test
	Control	Test	6 h	3 day	6 h	3 day	7 day	7 day
1	195.62	190	196.68	199.39	190.98	198.3	231.18	224.3
2	190.12	187	190.23	194.50	187.97	194.59	227.23	222.67
3	187.35	188	187.98	190.30	188.94	196.34	223.54	224
4	189.63	189.63	190.	194.3	190.10	197	224.87	224.98
5	183.88	192.53	183.88	188.20	192.96	198.8	228.89	227.57
6	192	192	192.97	195.2	193	200.5	229.67	228.59
Mean ± SD	189.77 ± 4.00	189.77 ± 4.01	189.77 ± 4.02	189.77 ± 4.03	189.77 ± 4.04	189.77 ± 4.05	189.77 ± 4.06	189.77 ± 4.07

*P □ 0.05 No significantly different from control; n = 6

There are no significant differences between control and treatment group. It could be infer there is no effect on body weight after oral administration of 5000mg/kg fruits extract of PM.

Table 2. Results showing time course of onset of signs of toxicity on the safety profile estimation in rats after 7 days treatment with ethanolic extract of the fruits of PM at 5000 mg/kg/b.w.

Observations	Control group		Test group		Mortality	
	6 h	7 day	6 h	7 day	Control	Test
Physical activity	Normal	Normal	Normal	Normal		
CNS activity	Normal	Normal	Normal	Normal		
ANS profile	Normal	Normal	Normal	Normal		
Skin and fur	Normal	Normal	Normal	Normal		
Eyes	Normal	Normal	Normal	Normal		
Mucous Membrane	Normal	Normal	Normal	Normal	Nil	Nil
Salivation	Normal	Normal	Normal	Normal		
Lethargy	Normal	Normal	Normal	Normal		
Sleep	Normal	Normal	Normal	Normal		
Diarrhea	Normal	Normal	Normal	Normal		
Coma	N.O*	N.O*	N.O*	N.O*		
Tremors	N.O*	N.O*	N.O*	N.O*		

Serum Biochemistry

Effect of PM fruit extract on hepatic, renal and lipid function

Table 3. Effects of PM fruits extract on hepatic, renal and lipid function indices in male rats.

Test	Analyte Parameter	Control Values						Mean ± SD	Treated Values (5000 mg/kg/b.w)						Mean ± SD	Ref. values [43-45]
Renal Profile	Creatinine mg/dL	0.4	0.5	0.5	0.5	0.4	0.4	0.450 ± 0.022	0.4	0.5	0.4	0.6	0.4	0.5	0.46 ± 0.033	0.2-0.8
	Phosphorous mg/dL	6.4	6.2	5.4	6.7	5.8	5.5	6.00 ± 0.21	6.53	5.3	6.2	6	6.5	5.8	.05 ± 0.46	3.11-11
Lipid Profile	HDL Cholesterol mg/dL	30.6	39	46	32	38	31	36.10 ± 2.47	29.6	28	31	30.4	30.5	38.4	31.31 ± 1.48	□ 27
	LDL Cholesterol mg/dL	35	30.1	30.5	31.7	32.9	34.5	32.45 ± 0.83	30	29.7	30	30.9	32	31.4	30.66 ± 0.37	□ 46.8
	Cholesterol mg/dL	65.3	72	69.3	78	73,2	68.5	71.05 ± 1.78	67.1	64	73	69	70	66.3	68.23 ± 1.28	40-130
	Triglyceride mg/dL	78	78	69.9	75.3	80	74.2	75.90 ± 1.47	78	70	76.4	79	77.3	78.4	76.51 ± 3.31	60-90
Liver Profile	AST U/L	52.8	60	65.3	67	69	61.3	62.56 ± 2.39	52	60	65.3	70.2	68	63	63.08 ± 2.65	45.7 - 80.8
	ALT U/L	21.9	20	24.3	23	24.1	22.9	22.70 ± 0.64	21.8	19.3	23	22.7	24.2	21.3	22.05 ± 0.68	17.5 - 30.2
	ALP U/L	76	78	73.4	80.3	83.3	79.5	78.41 ± 1.41	77.6	80.4	77	78.9	82.5	78.6	79.16 ± 0.82	56.8 - 128
	T. BIL mg/dL	0.29	0.42	0.39	0.40	0.35	0.32	0.36 ± 0.02	0.32	0.40	0.37	0.39	0.41	0.34	0.37 ± 0.01	0.20 - 0.55
	ALB g/dL	3.9	3.8	3.9	4.0	3.8	4.2	3.93 ± 0.06	4.3	4.0	3.8	3.8	3.9	4.1	3.98 ± 0.07	3.8 - 4.8
	Glum g/dL	84.67	89.4	90	88.5	89	79.7	86.86 ± 1.63	82.3	79.4	80	86	85.4	78.9	82.00 ± 1.26	50 - 135
	T. Prot g/dL	5.9	6.1	6.0	6.7	6.8	6.3	6.30 ± 0.15	6.0	5.7	6.0	6.4	6.2	6.2	6.08 ± 0.09	5.6 - 7.6

AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, ALP = Alkaline Phosphatase, T.BIL = Total Bilirubin, ALB = Albumin, Glu = Glucose, T.PROT = Total Proteins;

*P □ 0.05 No significantly different from control; n = 6

In biochemical observations, treated animals serum glucose level was slightly decrease compare than control group. However, the normal treated rats showed minimal variations in few biochemical parameters in comparison to normal control. From statistical analysis showed that there are no significant differencesbetween the treatments animalscompare with the control (P □ 0.05).

Histopathological findings for control and treated animals

The liver tissue of the control rats were examined for structural changes under the light microscope using hemotoxylin and eosin staining. The liver of control and treated groups rats appeared to be divided into the classical hepatic lobules; each was formed of cords of hepatocytes radiating from the central vein to the periphery of the lobule. The cell cords were separated by narrow blood Sinusoids. Hepatocytes were in concentric arrangement around the central vein. The cells are large in size with more or less centrally placed prominent nucleus. The hepatic cells are hexagonal in nature (Figure 1). The current study showed that there was no periportal necrosis of the hepatocytes and no inflammation of lymphocytes and macrophages in both control and treated groups

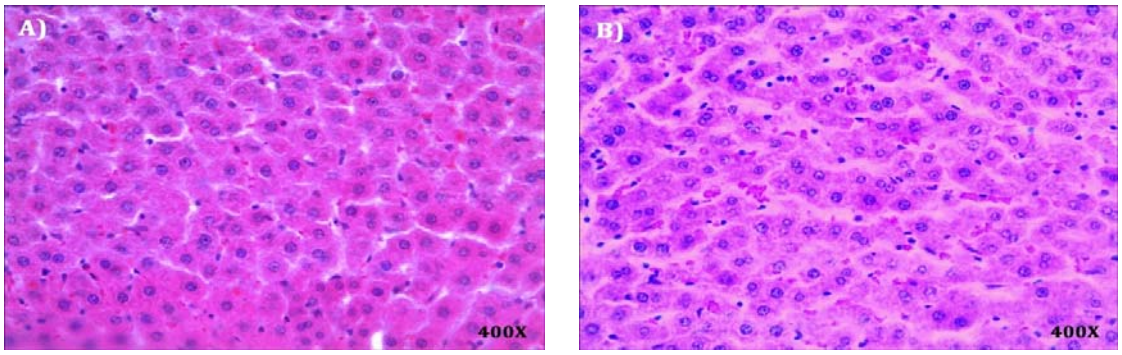

Figure 1. Light micrographs of rat liver tissue in control (A) and treated with (PM5000mg/kg/b.w) extracts (B)
(A) Normal control: Normal liver showing normal hepatic cells and architecture.
(B) Treated group: section of liver showing normal hepatocellular architecture with normal nucleus and cytoplasm. No significant damage detected in treatment group.

The histological preparation of kidney from control rats showed that the various segments of kidney tubules were well preserved. Abundant glomeruli portion of nephron segment with interspersed blood capillaries were also clearly seen (Figure 2).

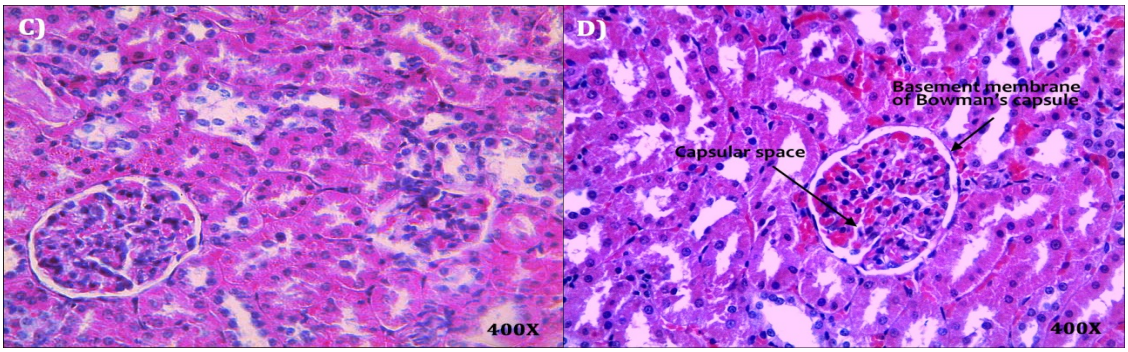

Figure 2. Light micrographs of rat kidney tissue in control (C) and treated with (PM5000mg/kg/b.w) extracts (D)
(C) Normal control: Normal kidney depicted normal renal corpuscle with glomerulus.
(D) Treated group: kidneys of treated animals revealed absence of matrix expansion and glomerular basement membrane thickening, suggesting normal histoarchitecture of renal.

In the treated group (PM ethanolic extract 5000 mg/kg/b.w) there was no significant histological alteration in the glomeruli or any other segment of kidney tubule following extracts treatment under the present experimental

protocol. Alltheconstituent structures of the kidney tubule in PM extracts treated group appeared to be well maintained. Various regions of the kidney tubules appeared to be normal without any changes in messangial matrix.

MTT-assay

The cytotoxicity study was done by MTT-assay on MCF-7 breast cancer cell. The MTT-assay results showed that the highest percentages of cell viability were 106.23% atconcentration of 1.25 µL and the lowest percentages were 13.04% at concentration of 10 µL. However, rest of the concentration showed a very well percentage of cell viability except for concentration of 5 µL which showed 56.66% cell viability. The overall results for in *vitro* MCF-7 cell line showed that the ethanolic extracts of PM fruits is non-toxic, except for the concentration of 10 µL (Figure 3).

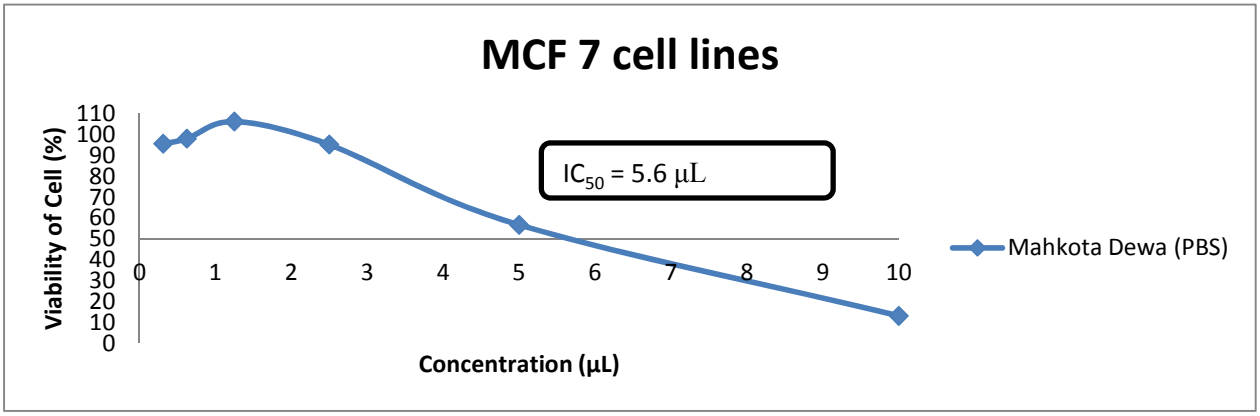

Figure 3 Percentage of cell viability at different concentration of PM fruits extracts using MTT-assay.

4. CONCLUSION

From the toxicity studies *in vivo* data revealed that the PM fruits extract proved to be non-toxic at tested dose levels and well tolerated by the experimental animals. *In vitro* toxicity study done using MTT-assay on MCF 7 cancer cell lines and the percentage of cell viability showed that the PM fruits extract is non-toxic. It's the first time acute oral toxicity study on PM. PM fruits extract could be applied for new line investigation such as antidiabetic screening in future study.

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Efficacy of tamarind seed polysaccharide in products development.

Porntip Chaimanee*[a], Supanee Chayabot[a,b], Pornchai Rachatanan[c], Suchada Piriyaprasarth[d], Somlak Kongmuang[d], Tanasait Ngawhirunpat[d]

[a]Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand

[b]Material Research Center for Art and Design, Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand

[c]Division of Packaging Technology, Faculty of Agro-Industry,Chaingmai University, Chaingmai, Thailand

[d]Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, NakhonPathom, Thailand

*porntip@ su.ac.th phone +66-034255797 fax +66-034271356

ABSTRACT

Tamarind seed is an underutilized by product of tamarind pulp industry. In Thailand, only small fraction of the seeds, in the form of tamarind kernel powder (TKP), is produced and exported. Though there are some uses for decorticated seeds as a binding material in traditional Thai mural painting, there have been hardly any other uses in food and non-food Thai industries. Extraction and purification of xyloglucan, a non toxic polysaccharide from TKP, was obtained in order to be use as a texture modifier. Since the aqueous solution of xyloglucan have high viscosity and stability against acids and heat with film forming properties. We now have examined the potential usage of xyloglucan as an excipient in pharmaceutical dosage forms such as, nanoemulsion, dry emulsion and transdermal films. The xyloglucan was used as viscosifiers in preparation of acrylic resin and water color paint for art. Furthermore, we have shown that pretreatment of cotton and silk surface with xyloglucan can enhance the quality of inkjet printing on the fabric.

Keywords: Tamarind seed polysaccharide, Xyloglucan

1. INTRODUCTION

Tamarind seed is a by-product of the commercial utilization of the fruit, the pulp is usually removed from the pod and used to prepare juice, jam, syrup and candy. The seed comprises the seed coat or testa (20-30%) and the kernel or endosperm (70-75%) [1]. Tamarind seed is the raw material used in the manufacture of tamarind seed kernel powder (TKP). In India, TKP is used as a source of carbohydrate for the adhesive or binding agent in paper and textile sizing, weaving and jute products as well as textile printing [2].

Xyloglucan is a non-ionic polysaccharide extracted from TKP and it is composed of a [1, 4] β- D-glucan backbone which has [1, 6] α-D- xylose branches that are partially substituted by [1, 2] β- galactoxylose (Figure 1).The glucose, xylose and galactose units are present in the ratio of 2.8:2.25:1.0 [3]. The average molecular weight of xyloglucan is 52,000-56,000 or around 115,000 depending on the method of measurement.

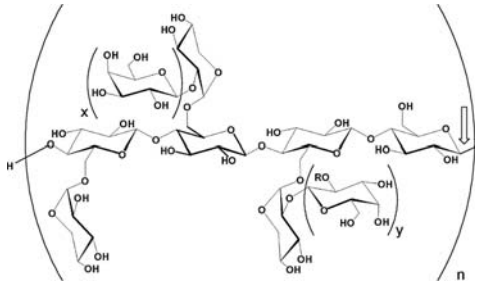

Figure 1. The structure of the repeating unit of tamarind xyloglucan.

Xyloglucan is insoluble in organic solvent and dispersible in hot water to form a highly viscous solution with a broad pH tolerance and high thermal stability [4]. This property led to its application as stabilizer, thickener, gelling agent, binder in food industry, particularly in Japan where it is a permitted food additive [5].

Due to its unique rheological properties with mucoadhesivity, biocompatibility and high drug holding capacity xyloglucan is a suitable carriers for the development of novel pharmaceutical formulations and for drug delivery system [6-7].

The general objective of the present study is therefore to evaluate the usage of xyloglucan in various products. The products included, (i) use as an excipient in pharmaceutical dosage forms such as, nanoemulsion, dry emulsion and transdermal films, (ii) use as viscosifiers in preparation of acrylic resin and water color paint for art and finally (iii) use xyloglucan to treated surface of cotton and silk fabrics before inkjet printing.

2. MATERIALS AND METHODS

Preparation of xyloglucan

Method 1 (X_1)

20 g of tamarind seed kernel powder was added to 200 ml of cold distilled water to prepare slurry. The slurry obtains is then poured into 800 ml of boiling water and are maintained at 80°C for 30 min. and kept overnight at room temperature . A clear solution was separated and poured into ethanol with continuous stirring to obtain a gelatinous precipitate. Then the precipitate was drained on cheesecloth and oven dried. The obtained polymer was stored in a desiccator.

Method 2 (X_2)

20 g of tamarind seed kernel powder was added to 1000 ml of cold distilled water to prepare slurry. The slurry obtains was left to stand for 4 hours so most of the large particle size TKP portion was settled down. Then the precipitate was resuspended in water, heat to 80°C for 30 min. and kept overnight at room temperature. A clear solution was separated and poured into ethanol with continuous stirring to obtain a gelatinous precipitate. The precipitate was drained on cheesecloth and oven dried. The obtained polymer was stored in a desiccator.

Xyloglucan use as pharmaceutical excipient.

Preparation of nanoemulsion

Stable nanoemulsion formulation was prepared from Tween80 castar oil/xyloglucan ($\mathbf{X_2}$) using high energy homogenization (Ultra-Turrax® T50 Basic, IKA, Germany) at 20,000 rpm for 15 min.

Preparation of dry emulsion

The liquid o/w-emulsions were prepared with 50% dry powder mass. The aqueous solution containing dissolved HPMC or HPMC with Xyloglucan($\mathbf{X_2}$) and coconut oil were homogenized in a high speed colloid mill (Ultra-Turrax® T25 basic IKA, Germany) for 3 min at 24,000 rpm. The liquid o/w-emulsions were dried at 70°C.

Preparation of film

Solution of 1% xyloglucan($\mathbf{X_2}$) was prepared by dissolving in 10% w/v propylene/glycerol solution. The above solution (20 g) was poured into a petridish and kept in an oven at 60°C for complete drying. The dried films were removed from the petridish and stored in a desiccator until use.

Xyloglucan use as viscosifier for paint

Xyloglucan ($\mathbf{X_1}$) solution was used to substitute gum Arabic or maltodextrin in water color base formulation. In acrylic paint formulation, xyloglucan (method1) solution was used to replace some of acrylic emulsions.

Xyloglucan use as textile surface coated

Cotton and silk fabrics were coated with xyloglucan ($\mathbf{X_1}$) solution using padding machine. Coated fabric was printed with inkjet printer using program Greatag Macbeth@2004 and follow with heating at 100°C for 5 min.

3. RESULTS

Aqueous extraction of TKP yield 50% and 30% of xyloglucan (w/w) by method 1 and 2 respectively. The absence of the contaminating proteins in xyloglucan (method2) is verified by sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (Figure 1). This purified xyloglucan was further use as a carrier in various drug delivery systems. It was possible to formulate nanoemulsion (100-500 nm) which composed of 30% castor oil, 20% Tween80 and 3% xyloglucan. The prepared nanoemulsion were stable for 14 day at 8°C. (Figure 2). Dry emulsion containing HPMC or HPMC/xyloglucan as emulsifier were prepared with 50% coconut oil. The reconstitution properties of xyloglucan containing dry emulsion can reformed the o/w-emulsion comparable to HPMC containing dry emulsion. (Figure 3). Xyloglucan with 10% plasticizer (PG/glycerol) showed excellent film-forming characteristics. The cast films were transparent and uniformly smooth and flat (Figure 4).

Xyloglucan prepared by method1 was used as a rhelogy control additive in both water base and acrylic base color emulsion. It is possible to prepared both water and acrylic color paint with good quality when compare to standard. (Figure 5).

Furthermore, the xyloglucan was used as pretreatment for coated cotton and silk fabrics before inkjet printing. The quality of the coated printing products were improved in printing sharpness and wider color area (Figure 6).

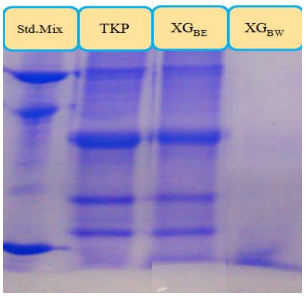

Figure 1. SDS-PAGE of (Std. Mix) Standard proteins, (TKP) tamarind kernel powder, (XG$_{BE}$) Xyloglucan ($\mathbf{X_1}$) and (XG$_{BW}$) Xyloglucan ($\mathbf{X_2}$)

Figure 2. Appearance of nanoemulsion compose of 30% castor oil, 20% Tween 80 and 3% xyloglucan formulated in distilled water

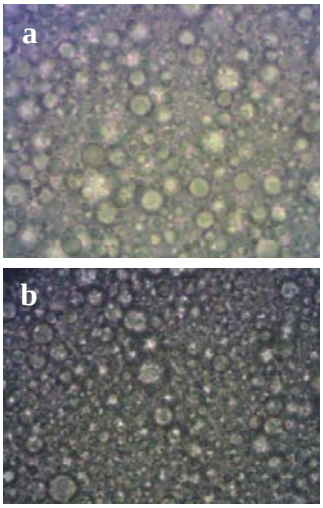

Figure 3. Reconstituted of dry emulsion in water of (a) HPMC (b) HPMC/xyloglucan from inverted microscopy.

Figure 4. Transparent xyloglucan film prepared from 1% (w/w) xyloglucan with 10% plasticizer (PG/glycerol)

a

b

Figure5 Twelve color of water base paint (a) Water color and (b) Acrylic color.

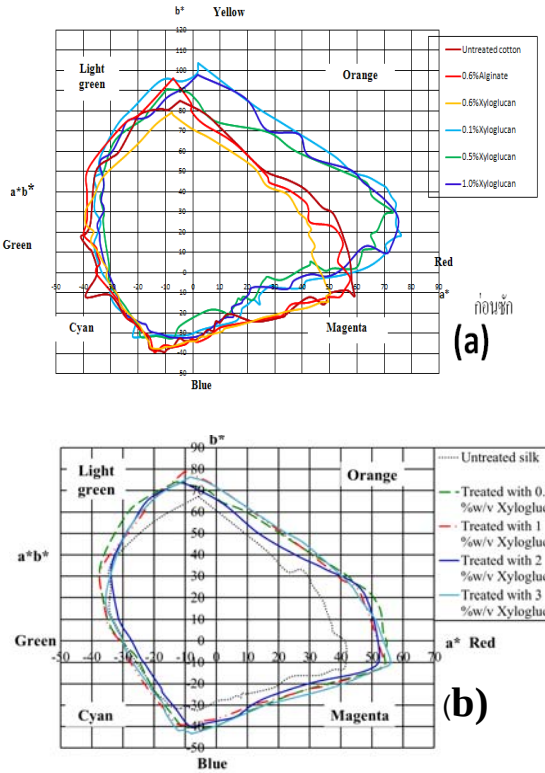

Figure 6. Color area of the treated and untreated fabrics is shown in a*b* diagram of the untreated and treated cotton (a) and silk (b).

4. CONCLUSIONS

Tamarind seed is a typical underutilized material in Thailand. In this study, xyloglucan was extracted and purified from tamarind seed kernel and was explored to find an application in various products ranging from pharmaceutical excipient to emulsifier in color paint and as the surface coated on fabrics to improve printing quality. It is desirable that more works has to be done to increase the use of this unexploited tamarind seed polysaccharide.

ACKNOWLEDGEMENTS

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Plant species used for the treatment of skin related problems in the Muzaffarabad, Kashmir Region, Western Himalaya

Tariq Habib[a], Afia Iqbal[a], Muhammad Ejaz Ul Islam Dar[*a], Muhammad Alatf Hussain[b]
[a]Deparment of Botany, University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan 13100
[b] Mirpur University of Science and Technology, Azad Jammu and Kashmir

* ejazdar1@gmail.com phone +92-3009888172 fax +92-5822960431

ABSTRACT

A total of 46 medicinal plant species belonging to 43 genera and 32 families, used against Skin Diseases were recorded from District Muzaffarabad and Kotli of Azad Jammu and Kashmir. Among the 32 families, Rutaceae, Solanaceae, Pinnaceae and Lamiaceae dominated by contributing 03 species each. Moreover, the plants used against maximum diseases were *Lawsonia inermis* L., *Juglans regia* L., *Curcuma longa* L., and *Azadirachta indica* Juss. Major skin problem recorded in Muzaffarabad and Kotli was Eczema being treated by *Achyranthus aspera, Cassia fistula* L. *Azadirachta indica* Juss., *Solanum nigrum* L., *Lawsonia inermis* L., *Dodonaea viscosa* Jacq., and *Curcuma longa* L., followed by injuries and wounds which were found to be treated by 06 different plants and Ringworm by 05 plants. These results emphesize the continuous needs for monitoring dermal problems in the people of rural areas.

Keywords: Natural products, Skin disease treatment, Ethnobotany, Kashmir region, Westren Himalaya

1. INTRODUCTION

Skin diseases have been of major concern recently due to their association with the Human Immunodeficiency Virus. Skin diseases occur worldwide and amount to approximately 34% of all occupational diseases encountered [1]. They affect people of all ages from neonates to the elderly and constitute one of the five reasons for medical consultation [2]. Skin ailments present a major health burden in both developed and undeveloped countries. Socio-economic environments such as household overcrowding play an enormous part in the spread of skin infections [3]. Furthermore, hot and humid climatic conditions exacerbate skin infections [4]. These factors are particularly problematic, although mortality rates for skin diseases are relatively low, they impact significantly on the quality of life and are often persistent and are difficult to treat.

Traditional medicinal resources, especially plants have been found to play a major role in managing skin disorders [5]. They have been employed in the treatment of skin ailments in many countries around the world where they contribute significantly in the primary health care of the population [5, 6]. The aim of the present study was to collect ethnomedicinal knowledge from lay people in Azad Jammu and Kashmir for the application of medicinal plants as a treatment for skin disorders. This is the first survey done in this region which specifically focuses on dermatological ailments.

2. MATERIALS AND METHODS

The study was performed in both an urban as well as a rural area adjoining to the Muzaffarabad city, which is the capital of Azad Jammu and Kashmir and has an area of 1642 km^2. According to 2011 estimate of the planning and development department of AJK, projected population of this metropolitan district was 0.650 million. Triangulation [7] was the principle method used in collecting data for the present study. Interviews, semi-structured questionnaires and personal observations were the techniques used in present investigation. Interviews and questionnaire were administrated in Kashmiri, Hindko Gojri and Urdu language to communicate respondents.

3. RESULTS

During this survey 85 respondents were interviewed across Muzaffarabad and the neighboring villages of Jhelum Valley. Among 85 respondents 56.47% (n=48) were female and 43.53% (n=37) were male. 30.59% of the key informants spoke Hindko, 24.70% were Kashmiri, and 20% were Gojri while Urdu speaking informants were 15.29%. The results of present findings showed that 19 skin diseases were treated by 46 plants among them. Eczema, injuries and wounds, ringworm and pimples were found to be treated by more than 05 plant species each (fig 1A).

Present findings showed that the major disease was eczema treated by 07 plants namely *Achyranthus aspera, Cassia fistula* L. *Azadirachta indica* Juss. *Solanum nigrum* L. *Lawsonia inermis* L. *Dodonaea viscosa* Jacq. and *Curcuma longa* L. Injuries and wounds were ranked second from the study area being treated by 6 different plants as *Brassica oleraccae* L. *Cynadon dactylon* (L) Pers. *Hedra helix* L. *Euphorbia hirta* L. *Rumax haustatus and Dodonaea vicosa* Jacq.

Ringworm was treated by 5 plants which were *Adhatoda vasica* L, *Allium sativum* L., *Ocimum bacillium* L., *Curcuma longa* L., *Cynadon dactylon* (L.). Pimples were found to be treated by (5 plants) *Anethum graveolnse* L., *Mentha longifolia* (L.) Huds, *Ficus religiosa* L., *Citrus sinensis* L. and *Daucus carota* L. whereas Psoriasis was treated by 4 species viz; *Lawsonia inermis* L., *Ocimum bascillium* L., *Curcuma longa* L. and *Ranunculus muricatus* L. Dryness was treated by *Cucumus sativus* L., *Momordica charantia* Descout. *Arachis hypogea* L. and *Citrus recticula* Blanco. Skin burn was found to be treated by four plants namely, *Pinus wallichiana* A.B. Jack, *Citrus medica, Lycopersicum esculentum* L. and *Luffa cylindrica* L.

Dandruff was cured with 3 plants, namely, *Brassica campestris* L., *Cuscuta reflexa* L. and, *Ficus religiosa* L. Rashes were also treated by 3 plants, namely, *Mangifera indica* L., *Zea mays* L. and .*Impatiens edgeworthii* L. Boils and Blisters were found to be treated by *Azadirachta indica* Juss and *Sambucus wightiana* Wall, *Adhatoda vasica* L. and *Aloe vera* Burn F were found useful against acne.

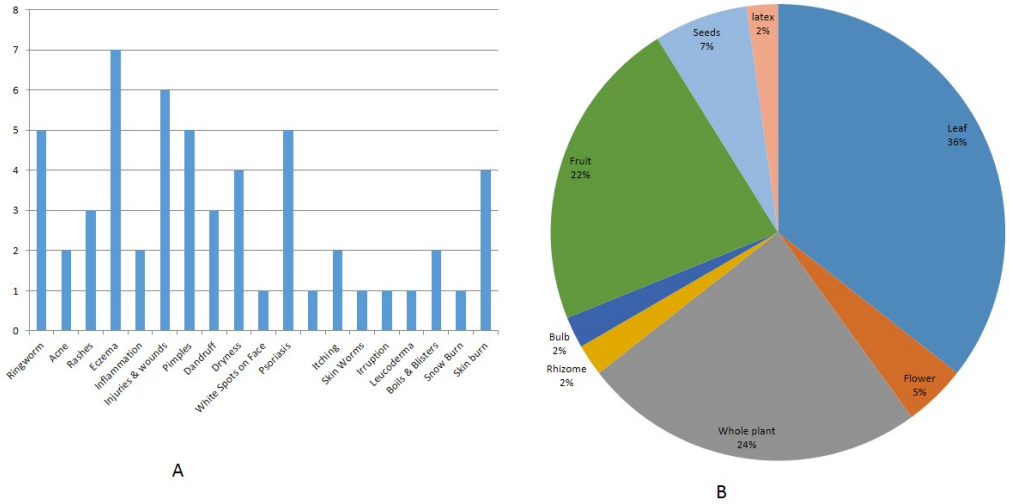

Figure 1. (A) Number of plants used against specific skin diseases from investigated area, (B) Graphical representation of plant parts used against skin diseases

Inflammation was being treated by *Adiantumn Venustum* L. and *Quercus incana*, while itching was found to be treated by *Ricinus communis* L. and *Solanum nigrum* L. Burn F. *Cedrus deodara* (Roxb.) G. Don, was recorded useful against snow burn.

Irruptions were treated by *Lawsonia inermis* L., skin worms by *Juglans regia* L., athlete's foot by *Allium satum* L. and white spots on face were being treated by *Cuscuta reflexa* L. The plant part used most of the times against maximum skin ailments was leave constituting 36%. Leaves were followed by whole plant i.e. 24% and fruit usage was 22%. Seeds and flowers were used 7% and 5% respectively. Rhizome, bulb and latex contributed 2% each to the treatment of skin problems (fig 1B).

4. CONCLUSIONS

The results obtained from the survey indicate that 46 plant species of 32 families are sought for medicinal curative properties in Muzaffarabad. The dominating families of medicinal plants used in skin ailments in the investigated area were Rutaceae, Solanaceae, Pinaceae, and Lamiaceae. These families are major contributors to the ethno botanical flora of Pakistan.

Based on the survey, there seem to be eight predominant treatments sought by informants in investigated area viz; eczema treated by 7 different plants followed by injuries and wounds being treated by 06 plants, ringworm, pimples and psoriasis treated by 05 plants each. Dryness and skin burn were found to be treated by 04 plants each, rashes were treated by 03 plants each, boils and blisters, acne, inflammation and itching were found to be treated by 02 plants each, and skin burn, irruptions, skin worms, athlete's foot and white spots on face were being treated by 01 plant each. This study has revealed the dynamic title role that medicinal plants play in the primary healthcare of the people of Muzaffarabad, Lesser Himalaya. Based on present findings, it is expected that the results of this study will lead to phytochemical and pharmacological investigations of the plants showing reasonable antimicrobial activity.

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Fatty acid composition and acute oral toxicity of rambutan (*Nephelium lappaceum*) seed fat and oil extracted with SC-CO_2

Jirawat Eiamwat*[a], Paramee Pengprecha[b], Benjaporn Tiensong[b], Tuanta Sematong[a], Sareeya Reungpatthanaphong[a], Patthanant Natpinit[a], Sukit Kampruengdet[a], Nantaprecha Hankhunthod[a]

[a]Thailand Institute of Scientific and Technological Research(TISTR), Head office, 35 Moo 3, Technopolis, Klong 5, Klong Luang, Pathum Thani, Thailand 12120

[b]Industrial Metrology and Testing Service Center, Samut Prakan, Thailand 10280

*jirawat@tistr.or.th phone +66-025779151 fax +66-025779141

ABSTRACT

Seeds of rambutan (*Nephelium lappaceum*), after soaking in hot water and dry grinding, were extracted with supercritical carbon dioxide (SC-CO_2) at 35 MPa and 45°C to obtain the fat and oil. The extractable fat and oil yield ranged from 16.0-23.0% and 5.0-7.6% respectively. The fatty acid composition of SC-CO_2 extracted rambutan seed fat and oil was broadly similar, with about 45% of saturated fatty acids (SFA), 51-53% of monounsaturated fatty acids (MUFA) and 2-3% of polyunsaturated fatty acids (PUFA) respectively. Oleic (C18:1) was a major fatty acid as MUFA and present 50-52% of the total fatty acid content. By comparing the SFA amounts of SC-CO_2 extracted rambutan seed fat and oil, it was observed differences of 2-5%, mainly due to variation on arachidic acid (C20:1), behenic acid (C22:0), stearic acid (C18:0) and palmitic acid (C16:0) contents. A 14-day acute oral toxicity test revealed that SC-CO_2 extracted rambutan seed fat and oil at the limit dose of 5000 mg/kg bodyweight to rats did not cause signs of intoxication, death or gross pathological lesions. Based on these results, rambutan seeds are promising as a potential fat and oil resource for edible use. Further investigation on physical and chemical characteristics of the rambutan seed fat and oil extracted with SC-CO_2 is in process.

Keywords: Acute oral toxicity, Fatty acids, Rambutan seeds, fat and oil, Supercritical carbon dioxide (SC-CO_2)

1. INTRODUCTION

Rambutan (*Nephelium lappaceum*) is widely consumed fresh and industrially processed to canned fruit products. Rambutan seeds are usually discarded as waste. The seeds, constituting 4-9% by weight of the fruit and 14-41% fat, are an attractive source of potential natural fat and oil for human consumption and industrial applications [1]. Recovery of rambutan seed fat and oil has performed by solvent extraction using hexane [1-2] and supercritical carbon dioxide (SC-CO_2) extraction [3]. SC-CO_2 is a viable alternative to the usual hexane for extraction of rambutan seed fat and oil due to the low critical point (P_c = 7.3 MPa, T_c = 31°C) and characteristics of CO_2 that allow for efficient extraction and replacement for toxic solvents, resulting in an eco-environmental friendly process [3]. The optimum condition of rambutan seed oil extracted by SC-CO_2 was obtained at 34.8 MPa and 56.7ºC using response surface methodology [3].

Fatty acid composition is quantitatively the important indication of fatty acids in fat and oil. In general, oil has much greater proportions of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) than fat which is at high contents in saturated fatty acids (SFA). The main fatty acids in the rambutan seed fat extracted with hexane were palmitic (C16:0), stearic (C18:0) and arachidic acid (C20:1) as SFA, oleic (C18:1) as MUFA, and linolenic acid (C18:3) as PUFA [1-2].

The objective of the present study, therefore, was to examine the fatty acid composition of rambutan seed fat and oil obtained by SC-CO_2 extraction at 35 MPa and 45°C, which is at lower temperature level than in the work of Yoswathana [3] and evaluate the toxicological value of the rambutan seed fat and oil by acute oral toxicity limit test.

2. MATERIAL AND METHODS

Preparation of rambutan seeds sample

Rambutan (*N. lappaceum*) seeds were obtained from a fruit processing company in Nakorn Pathom province, Thailand. The seeds were washed, soaked in hot water at 90°C for 1 h and oven-dried at 55°C for 10-12 h. The dried seeds were ground using a miller and then sieved using the sieve screens between 10 and 100 mesh. The ground seed sample of particle size ranging from 0.15 to 2.0 mm was stored in a sealed plastic bag at room temperature until used for extraction experiments.

Proximal chemical analysis

The proximate analysis of rambutan seeds sample was determined according to AOAC (2000). The analysis included moisture, crude protein, crude fat and ash. The moisture content was determined by drying in oven at 105°C to a constant weight. Total crude protein content was determined using the Kjeldahl method. The total fat content was determined by Soxhlet extraction with petroleum ether (40 to 60°C) for 3 h, and then oven-dried to dryness at 105°C for 1 h. Ash was determined by weighing the incinerated residue obtained at 550ºC for 30 min. Total available carbohydrate was calculated as 100% minus the sum of moisture, protein, fat and ash.

SC-CO_2 extraction

The SC-CO_2 extraction of fat and oil from rambutan seeds sample was performed using the Speed SFE instrument with a 300 ml extraction vessel (Applied Separations Inc., Allenton, PA, USA). The vessel was heated with an oven controlled by a thermostat (±1ºC). Liquid CO_2 was delivered into the vessel and pressurized to the operating value (±10 bar) with a high pressure pump (Applied Separations Inc., Allenton, PA, USA). For each extraction, about 100 g of ground rambutan seeds were loaded into the vessel, and packed with propylene wool. SC-CO_2 was left in contact with the sample for 30 min of static extraction. After that, dynamic extraction was performed with a CO_2 flow rate ranging about 2 L/min. The SC-CO_2 with dissolved oil passed through a heated micrometering valve at 110ºC, while the SC-CO_2 was expanded to atmospheric pressure. At time intervals of about 4 h, the fatty oil was collected in a pre-weighed glass vial immersed in a water bath at about 5°C, where the oil was cooled and fat was precipitated. The extraction was set to end when the fatty oil measured was less than 0.005 g/ g dry seeds. The oil and fat were subsequently separated from each other by filtration.

Fatty acid composition

$SC\text{-}CO_2$ extracted fat and oil were converted into fatty acid methyl esters (FAME) according to AOAC (2000). In brief, 0.2 g of the extracted sample was dissolved in 10 mL of 1M methanolic sodium hydroxide, refluxed at 100°C for 15 min, and then 12 mL of 12% BF_3 and 5 mL of *n*-heptane was added. After cooling, 30 mL of saturated sodium chloride was added to the mixture. The upper *n*-heptane phase was transferred into a vial and injected into a GCMS-QP2010 Ultra gas chromatrograph mass spectrometer (Shimadzu, Columbia, MD, USA) equipped with a capillary column Cp-Sil 88 (100 m long, 0.25 mm i.d., 0.2 µm film thickness). The initial oven temperature was from 100°C heated to 240°C (3°C/min). Injector and detector temperatures were set at 225°C. The mass spectrometer operated at ionization energy of 70 eV with a scan range of 30-320 amu. Identification of components was carried out based on retention time and mass spectra by matching with the NIST library.

Acute oral toxicity limit test

A total of 50 male and female Wistar rats were randomly divided into five groups of ten (five male and female each). The rats were around 6-7 weeks old and their body weight range for males 261-312 g and females 182-221 g. The animals were allowed to acclimatize under controlled conditions in a ventilated room at 24±1°C with relative humidity between 50% and 70% and a constant day/night cycle for a week. Feed and water *ad libitum* were provided throughout the study period. The animals were starved for 16 h overnight prior to the experiments. Rats (5/sex/group) were treated orally with $SC\text{-}CO_2$ extracted rambutan seed fat and oil at dose levels of 2,000 and 5,000 mg/kg body weight, while the control group received only distilled water. In all experiments, the animals were observed daily for 14 days for any signs of toxic symptoms or mortality. Body weight of the animals was noted before administration and weekly. At completion on the 14 day, all animals were euthanized and gross pathological examinations were performed.

Statistical analysis

Values were reported as means with standard error of means (SEM). Analysis of variance (ANOVA) was used to analyze the data, where $p < 0.05$ the means were considered statistically significant difference.

3. RESULTS

The yields of fat and oil extracted with $SC\text{-}CO_2$ ranged from 16.0-23.0% and 5.0-7.6% respectively (data not shown), compared to the mean fat yield of 31.2% recorded by Sirisompong et al [1] and about 30.4% reported by Yoswathana [3].

The proximate composition of rambutan (*N. lappaceum*) seeds is presented in Table 1. The results are in good agreement with findings of Solís-Fuentes et al [2] who reported rambutan seeds had high contents of carbohydrate (57.60%), fat (33.40%) and protein (7.80%) but a low ash content (1.22%) on a dry basis.

Table 1. Proximate composition of rambutan (*N. lappaceum*) seeds (g/100g dry weight)

	Proximate composition				
	Moisture	Protein	Fat	Ash	Carbohydrate
Rambutan seeds	6.30±0.05	7.90±0.09	28.18±0.82	1.49±0.05	62.43±0.70

Values are means of triplicate determinations.

The fatty acid composition of rambutan seed fat and oil extracted with $SC\text{-}CO_2$ at 35 MPa and 45°C is shown in Table 2. Statistically significant ($p < 0.05$) differences in several fatty acids were detected between the $SC\text{-}CO_2$ extracted fat and oil. In general, both the fat and oil displayed high compositions of 16:0, 18:0, 20:0 and 22:0 as SFA, 18:1 *cis*-9 as MUFA and low composition of 18:3 as PUFA. A comparison of the compositions of 16:0, 18:0, 20:0, 22:0 and 18:1 *cis*-9 fatty acids between the fat and oil were not large, about 2 to 5%.

Table 2. Fatty acid composition of rambutan seed fat and oil extracted with SC-CO_2 at 35 MPa and 45°C

	Percentage by weight of total fatty acids	
	Fat	Oil
Saturated fatty acid		
Myristic acid (C14:0)	0.01^{a}±0.01	0.04^{a}±0.01
Palmitic acid (C16:0)	7.39^{b}±0.20	10.33^{a}±0.15
Stearic acid (C18:0)	16.58^{a}±0.20	12.21^{b}±0.14
Arachidic acid (C20:0)	12.34^{b}±0.03	16.22^{a}±0.06
Behenic acid (C22:0)	8.91^{a}±0.03	6.53^{b}±0.05
Monounsaturated fatty acid		
Palmitoleic acid (C16:1)	0.49^{b}±0.03	1.35^{a}±0.05
Elaidic acid (C18:1 *trans*-9)	0.02^{a}±0.01	0.03^{a}±0.01
Oleic acid (C18:1 *cis*-9)	52.18^{a}±0.44	50.17^{b}±0.38
Polyunsaturated fatty acid		
Linolenic acid (C18:3)	2.02^{b}±0.01	3.04^{a}±0.05

Data are expressed as mean values of triplicate determinations. ^{a,b}Means with different subscripts are significantly different (I < 0.05)

The SC-CO_2 extracted fat obtained in our study had higher ($p < 0.05$) proportions of 16:0, 18:0, 22:0 and 18:1 *cis*-9 fatty acids, and lower ($p < 0.05$) proportions of 20:0 and 18:3 fatty acids, when the fatty acid composition of hexane extracted fat previously reported by Sirisompong et al. [1] and Solís-Fuentes et al. [2] was compared. The differences found with these results may be due to the solubility of fatty acids in SC-CO_2, which mainly affects the proportion of individual fatty acids in the SC-CO_2 extracted fat compared with the hexane extracted fat.

In the acute toxicity study, the 14-day observation period revealed that there was no mortality in all male and female animals. The oral LD_{50} of SC-CO_2 extracted rambutan seed fat and oil in Wistar rats was greater than 5,000 mg/kg body weight. There were no any gross pathological lesions observed on necropsy at the end of the study in male and female rats.

Data referring to the body weights of male and female rats receiving SC-CO_2 extracted fat and oil from rambutan seeds at dose levels up to 5,000 mg/kg bodyweight are presented in Table 3. There were no significant differences ($p > 0.05$) in the body weights of male and female rats when the control and treatment groups were compared. These results suggest that the extracted fat and oil were non-toxic and feeding at dose level of 5,000 mg/kg body weight to rats had no significant effect on body weights.

Table 3. Comparison of mean body weights for male and female rats during a 14-day trail toxicity study with SC-CO_2 extracted fat and oil from rambutan seeds

Day	Sex	Control	Fat (mg/kg bw)		Oil (mg/kg bw)	
			2,000	5,000	2,000	5,000
1	M	282.40±14.59	291.80±11.92	296.60±7.77	291.20±15.80	298.80±15.09
	F	192.60±4.98	194.20±12.13	203.00±10.17	194.20±6.98	201.80±9.58
8	M	308.40±21.20	316.00±18.81	328.80±13.33	316.20±16.84	324.60±13.60
	F	222.20±1.16	217.00±13.45	223.20±5.60	220.40±13.39	229.60±12.78
15	M	328.00±26.91	327.00±14.75	340.80±10.87	325.80±19.18	339.80±15.85
	F	235.60±11.33	231.80±13.70	241.40±13.54	226.20±14.48	243.80±15.12

Values are mean ± SEM for 5 rats in each group.

4. CONCLUSIONS

Rambutan seed fat and oil obtained by SC-CO_2 at 35 MPa and 45°C contained mainly palmitic (7.39 vs 10.33%), stearic (16.58 vs 12.21%), arachidic (12.34 vs 16.22%) and behenic (8.91 vs 6.53%) as SFA, oleic (52.18 vs 50.17%) as MUFA, and linolenic (2.02 vs 3.04%) as PUFA. The SC-CO_2 extracted rambutan fat and oil are safe to use and may be considered for edible purposes.

ACKNOWLEDGEMENTS

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Effect of tamarind (*Tamarindus indica* L.*)* seed polysaccharide on physical properties of itraconazole-loaded nanoemulsions

Suchada Piriyaprasarth*[a,b], Pornsak Sriamornsak[a,b], Porntip Chaimanee[c], Chatruedee Jiaranai[a], Sirunya Chuchan[a], Siwaporn Sakunpakdi[a], Suwannee Panomsuk[a], Julaluck Chaichiangkong[a], Vipaluk Patomchaiviwat[a,b]

[a]Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand 73000

[b]Pharmaceutical Biopolymer Group (PBiG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand 73000

[c]Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand 73000

*wsuchada@su.ac.th phone +66-34255800 fax +66-34255801

ABSTRACT

Recent trend toward the use of plant based and natural products demands the replacement of synthetic additives with natural ones. The objective of this study was to investigate the effect of tamarind (*Tamarindus indica* L.) seed polysaccharide on physical properties of itraconazole-loaded nanoemulsions prepared by simple homogenization (20,000 rpm, 15 min). Itraconazole, a low aqueous solubility substance, was used as a model drug. Castor oil, oleic acid and caprylic capric triglyceride at the concentration of 20-50% w/w were used as an oil phase. Polysorbate 80 and sorbitan sesquioleate 83 were used as emulsifier whereas tamarind seed polysaccharide was used as co-emulsifier. The formulations containing 30% oil and 20-30% w/w polysorbate 80 provided nano-sized emulsions (100-500 nm) while the use of tamarind seed polysaccharide alone up to 3% as emulsifier could not produce the nanoemulsions. The incorporation of 1-3% tamarind seed polysaccharide in the formulations containing 20% polysorbate 80 decreased the size of emulsion droplets. For the formulations containing 3% tamarind seed polysaccharide, the droplet size of nanoemulsions decreased with the increase of concentration of polysorbate 80. The addition of itraconazole did not affect droplet size of the nanoemulsions. The zeta potentials ranged from -16.97 to -16.41 mV. The viscosity was in the range of 1,067-1,595 cPs. The nanoemulsions were stable for at least 14 days at 8°C. The results pointed that tamarind seed polysaccharide, a biodegradable, edible and harmless biopolymer, could be applied as co-emulsifier in nanoemulsions.

Keywords: Tamarind seed, *Tamarindus indica* L., Co-emulsifier, Physical stability, Itraconazole, Nanoemulsions

1. INTRODUCTION

The use of plant-based and natural products demands the replacement of synthetic additives with natural ones becomes interesting due to their safety, biodegradability, biocompatibility and non-toxicity. Tamarind (*Tamarindus indica L.*), belongs to the family Leguminosae and grows naturally in many tropical and sub-tropical regions. Tamarind is an important food resource for Thai population. Flower and leaf are consumed as vegetables while the germ obtained from the seed is used for manufacturing Tamarind gum [1]. Tamarind fruit is a pulpy mass of a light reddish-brown color, changing with age to a dark brown, containing some branching fibers and numerous reddish brown, smooth, oblong or quadrangular, compressed seeds, each enclosed in a tough membrane. Tamarind seeds contain tamarind seed polysaccharide which was reported to use as binding agent, emulsifier, suspending agent, sustained release agent, hydrogels, mucoadhesive agent, rectal drug delivery and nasal drug delivery [2-3]. Itraconazole (ITZ), a triazole antifungal agent, is poorly water-soluble drug and its absorption in gastrointestinal tract is low. The various methods that could increase the solubility of ITZ included complexation with β-cyclodextrin [4], use of mesoporous silica [5], solid dispersion [6] and nanoemulsions [7]. Nanoemulsions are often referred to emulsions with droplet sizes in the nanometric scale, generally 100-500 nm [8]. It has been found that the use of nanoemulsions for oral administration to increase the bioavailability of poorly water-soluble drugs due to an enhancement of the intestinal absorption of the drug is well documented. The previous study of nanoemulsions could contain 140 µg/mL of ITZ in caprylic/capric triglyceride as internal phase [7,9]. Therefore, the aim of this study was to investigate effect of tamarind seed polysaccharide as co-emulsifier on physical properties of itraconazole-loaded nanoemulsions prepared by simple homogenization and evaluate the changes in physical properties, such as droplet size, viscosity, zeta potential as well as physical stability, to explore their potential in pharmaceutical industry.

2. MATERIALS AND METHODS

Materials

Tamarind fruits were procured from local market (Nakhon Pathom, Thailand). Itraconazole raw material used in this study was purchased from Nosch Labs Private (India). All other chemicals used were of analytical grade and used as received. Deionized water was used as an aqueous phase in all preparations.

Extraction of tamarind seed polysaccharide

Tamarind seed kernel (TKP) powder (20 g) was added to 1000 mL of cold distilled water to prepare slurry. The slurry obtained was allowed to stand for 4 hours so most of the large particle size (TKP portion) was settled down. The precipitate was resuspended in water, heated to 80°C for 30 min and kept overnight at room temperature. A clear solution was separated and poured into ethanol with continuous stirring. The precipitate obtained was dried and stored in a desiccator.

Preparation of nanoemulsions

Castor oil, oleic acid and caprylic capric triglyceride at the concentration of 20-50% w/w were used as an oil phase. Polysorbate 80 and sorbitan sesquioleate 83 were used as emulsifier while tamarind seed polysaccharide was used as co-emulsifier. Itraconazole, a low aqueous solubility substance, was used as a model drug. The nanoemulsions were prepared by using homogenizer (Ultra-Turrax® T50 Basic, IKA, Germany) at a speed of 20,000 rpm for 15 min in an ice-bath to avoid over-heating.

Determination of physical properties

Droplet size measurement

The prepared nanoemulsions were dispersed or diluted in deionized water with gentle stirring. The droplet size of nanoemulsions was investigated by static light scattering method (Laser scattering particle size distribution analyzer LA-950, Horiba, Japan). The median particle size was measured under continuous stirring. The measurements were repeated at least three batches of nanoemulsions.

Zeta potential measurement

The zeta potential analyzer (ZetaPlus, Brookhaven, USA) was used to measure zeta potential of the prepared nanoemulsions. Nanoemulsions were dispersed in deionized water at the ratio of 1:50 (v/v) and the electric field applied was 1 V. The measurement were performed three times and reported as the average and standard deviation.

Stability of nanoemulsions

All nanoemulsions, kept in the glass vials, were separated into two groups; the first group was stored in a refrigerator at 8°C while the other group was subjected to temperature cycling test by keeping at 4°C for 24 h and at 40°C for 24 h. The stability of nanoemulsions was examined by calculation of percent creaming [7, 9]. A greater

value of the percent creaming is an indication of a more stable emulsion. The size of the nanoemulsion droplets after stability test was also measured by a static light scattering method as described above.

3. RESULTS AND DISCUSSION

The effect of emulsifier types in the concentration of 20% w/w on droplet size of emulsions in various oils (30% w/w) is shown in Figure 1A. Castor oil, oleic acid and caprylic/capric triglyceride were used in this study. In all types of oil, polysorbate 80 alone provided nano-sized emulsions (100-500 nm) while the use of sorbitan monooleate 83 alone and the combination of polysorbate 80 and sorbitan monooleate 83 in the ratio of 1:1 could not produce the nanoemulsions. In addition, tamarind seed polysaccharide (TSP) was investigated for emulsifying activity. It was found that TSP alone up to 3% w/w could not produce nano-sized emulsions by the simple homogenizer (data not shown). The effect of oil concentration was investigated when polysorbate 80 was used as emulsifier. It was found that the increase in oil concentration tended to increase the size of the emulsion (Figure 1B). Due to high solubility of itraconazole and stability of nanoemulsion, 30% w/w castor oil was selected to prepare itraconazole-loaded nanoemulsions. The effect of co-emulsifying system between polysorbate 80 and TSP on droplet size of nanoemulsions is summarized in Figure 2. When 3% w/w TSP was incorporated in the formulation as co-emulsifier, the droplet size of nanoemulsions decreased with the increased concentration of polysorbate 80 (Figure 2A). The effect of TSP concentration as co-emulsifier is summarized in Figure 2B. When polysorbate 80 used was 20% w/w, the increasing of TSP concentration up to 3% w/w resulted in a decrease in droplet size of nanoemulsions (Figure 2B). Using excess biopolymer, however, may lead to the flocculation of emulsion droplets via depletion and bridging mechanism [10]. Therefore, it is suggested that an appropriate amount of polymer should be considered in nanoemulsion formulations.

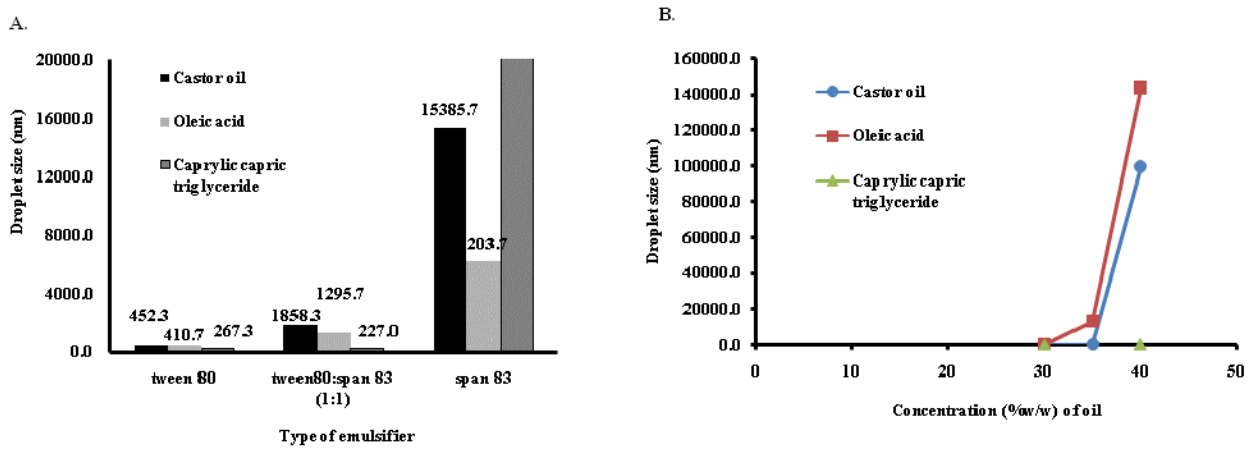

Figure 1. Effect of (A) emulsifier types and (B) oil concentration on droplet size of emulsions. The various oils used were 30% w/w (A) and the total emulsifier used was 20% w/w (B).

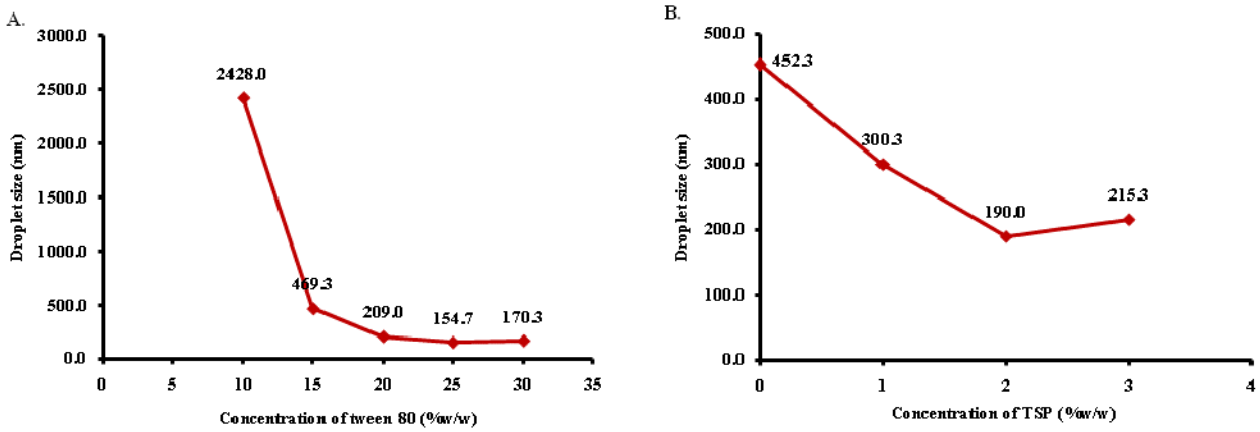

Figure 2. Effect of co-emulsifying system between polysorbate 80 and TSP on droplet size of nanoemulsions. (A) polysorbate 80 in various concentrations and 3% w/w TSP and (B) TSP in various concentrations and 20% w/w polysorbate 80. Castor oil was used in the concentration of 30% w/w.

The addition of itraconazole slightly decreased droplet size of the nanoemulsions. The zeta potentials ranged from -15.39 to -29.38 mV. The viscosity was in the range of 248.5-4,909 cPs. The percent creaming was 100%. The prepared nanoemulsions were subjected to stability test and found that the nanoemulsions were stable for at least 14 days at 8°C. After the temperature cycling test by storage at 4°C for 24 h and at 40°C for 24 h was performed, it was found that the formulation containing 30% w/w castor oil, 20% w/w polysorbate 80 and 1-3% w/w TSP was stable for 3 cycles.

4. CONCLUSIONS

Type and concentration of oils and emulsifiers affected stability of nanoemulsions prepared by simple homogenization. The decrease in the concentration of emulsifier and an increase in the oil concentration affected the stability of nanoemulsions. For the co-emulsifying system between polysorbate 80 and TSP, the increase in TSP concentration up to 3% w/w tended to decrease the droplet size of nanoemulsions. The total concentration of emulsifier also affected the size of the nanoemulsions. The formulation containing castor oil of 30% w/w, polysorbate 80 of 20% w/w and TSP of 1-3% w/w provided stable nanoemulsions. The results pointed that tamarind seed polysaccharide, a biodegradable, edible and harmless biopolymer, could be applied as co-emulsifier in nanoemulsions.

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Characterization of rice starch/carboxymethyl chitosan blend films

Rungsiri Suriyatem[a], Pornchai Rachtanapun*[b,c]
[a]Division of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand 50100
[b]Division of Packaging Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand 50100
[c]Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200

*p.rachta@chiangmai.ac.th phone +66-897903926 fax +66-53948224

ABSTRACT

Chitosan from shrimp was converted to carboxymethyl chitosan (CMCh) *via* carboxymethylation under alkaline condition. Rice starch/carboxymethyl chitosan (RS/CMCh) blend film was prepared by casting on an acrylic plate. The effect of different ratios between RS and CMCh (100:0, 88:12, 65:33, 50:50, 33:67, 12:88, and 0:100) on the properties of the RS/CMCh blend films was studied. Each RS/CMCh blend film was transparent. A higher amount of CMCh affected the transmittance at 550 nm of the blend films increased and the absorbance decreased. Scanning electron microscopy (SEM) revealed homogenious cross-sectional surface for the RS/CMCh blend films. Water vapor permeability (WVP) of the pure RS film and the pure CMCh film were 4.8 and 9.1 g.mm/m^2.mHg.day, respectively while the WVP of the RS/CMCh blend films (5.0-9.1 g.mm/m^2.mHg.day).Tensile strength (TS) of the pure RS and the pure CMCh were 5.5 and 17.7 MPa, respectively. The RS/CMCh blend films' TS (6.5-20.6 MPa) increased when the CMCh content increased. Elongation at break (EB) of the RS/CMCh blend films was separated into two groups; 1) higher EB (64.5-65.0%) was found from the RS/CMCh blend films contained 0-12% CMCh, 2) lower EB (47.9-52.3 %) was achieved from the blend films contained 37-100% CMCh.

Keywords: Blend film, Carboxymethyl chitosan, Property, Rice starch, Synthesis

1. INTRODUCTION

Starch is one of the most studied and promising natural polymers that are used to develop biodegradable films because it has the capability of forming into a film and it is a relative inexpensive, renewable and abundant resource. Rice starch (RS) is made from rice by-product after rice processing. Major components of rice starch, amylose and amylopectin, are biopolymers, which are attractive raw materials for use as barriers in packaging materials [1]. Nevertheless, the application of starch film was limited because starch cannot form film with adequate mechanical properties (high tensile strength and high elongation) [2]. Plasticizers are normally added to the film to overcome these films deficiencies.However, plasticizers createhigher elongation but lower tensile strength values and generally increase hydrophilicity of the film which in term promotes water vapor permeability [3]. In order to overcome the shortcomings, starch is often blended with other biopolymers to produce biocomposite films [1, 3-5].

Chitosan is a linear polysaccharide containing β-(1-4)-linked N-acetyl-D-glucosamine and D-glucosamine units [6]. It is nontoxic and biodegradable. Chitosan is extracted from chitin, which is abundantly available in nature. Chitosan exhibits excellent biocompatibility, biodegradability, non-toxicity, adsorption properties, etc[6]. So it has a wide range of applications, including utilization in food processing, agriculture, biomaterials, biotechnology, biomedicine, and pharmaceuticals [6]. However, its limitation of only dissolving in acidic water affected the finished product by causing a bad odor [7]. Carboxymethyl chitosan (CMCh) is an etherified chitosan which could be used in various industries including food and non-food. It is an interesting choice for use in polymer blending because of its dissolvability in cold water.

To date, no research has presented the RS/CMCh blend film preparation and its properties. In this study, the effects of CMCh proportions were evaluated on appearance, transparency, water vapor permeability, mechanical propertiesand morphologyof RS/CMCh blend films.

2. MATERIALS AND METHODS

Synthesis of carboxymethyl chitosan (CMCh)

Chitosan (deacetylation degree=98%) was purchased from Taming Enterprises (Thailand) and synthesized to be CMCh according to the method propounded by Rachtanapun and Suriyatem [8] with slight modifications. Monochloroacetic acid and isopropanol/distilled-water were employed as an etherifying agent and a medium, respectively. The reaction was carried out under alkaline condition. The final product, CMCh, was stored in a sealed polyethylene bag until use.

Film preparation and transparency

Rice starch was purchased from Thai Flour Industry Company Ltd.(Thailand). Film forming solutions (5% w/v) with different rice starch (RS) and CMCh ratios were prepared to yield different RS/CMCh blended films (Table 1). RS was dispersed in distilled water and constantly stirred at 90°C for 5 min. CMCh was dissolved in distilled water at 80°C for 10 min. The RS and CMC solution were mixed and glycerol (25%wt of solid content) was added as a plasticizer. The mixture was degasified, cooled to 25°C, cast on silicone plate, and then dried at 25°C for 36 h. The films were peeled and kept in a polyethylene bag until use. Film transparency was determined by measuring the transmittance (%) at 550 nm using a spectrophotometer (Labomed, USA).

Morphology observation

Morphological investigation of film was performed using a scanning electron microscope (JEOL, JSM-5910LV, Japan). A cross-sectional sample was prepared by fracturing films in liquid nitrogen. The sample was mounted on a specimen stub and sputter-coated with a thin layer of gold. The sample observed using an accelerating voltage of 10 kV with 3000x.

Water vapor permeability (WVP)

WVP was investigated using the ASTM E96-93 [9]. The cups containing dried silica gel werecovered with the circular specimens and sealed with paraffin wax. Sealed cups were weighed and kept in a desiccator with saturated solution of $NaNO_2$ to provide 65%RH, 25°C. The cups were re-weighed daily for 7 days. WVP was calculated byequation described elsewhere [10]. All measurements were performed in triplicate.

Mechanical properties

Mechanical properties, included tensile strength (TS) and elongation at break (EB), were determined using an Instron Model 1000 (H1K-S, UK) with the procedure according to ASTM D882-91 [11]. The samples were cut into specimens of 1.5 cm × 14 cm rectangular to be used as a test specimen. Initial grip separation and cross-head speed was set at 50 mm and 50 mm/min, respectively.There were ten sample measurements.

All data was analyzed using a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p \leq 0.05$) using SPSS version 11.

3. RESULTS

Blend films of RS and CMCh prepared were found to be easily peeled from the acrylic plate. The RS/CMCh-01 (pure RS) film was transparent and light white while the RS/CMCh-07 (pure CMCh) film was transparent and light yellow (Figure 1). Other blend films were transparent and showed the shade of very light yellow from the color of CMCh (Figure 1). The transparency of the films determined by transmittance at 550 nm is summarized in Table 1. Percent transmittance (T) of the pure CMCh film was about 2 times higher than that of the pure RS film. T value of the RS/CMCh blend film significantly increased when increasing the proportion of CMCh. The clarity of film depended on the content of linear structure. In this case, the linear molecules belonging to amylose tended to favor compact H-bonds between thehydroxyl groups of adjacent chainsto form an opaque polymer matrix, while CMCh structure is more bulky with the carboxymethyl groups' substitute on the cellulose backbone compared to starch. In similar, Mohanty et al. [12] reported that carboxymethyl cellulose can produce clearer films than starch.

The WVPs of the blend films with different ratios of RS to CMCh were measured at a vapor pressure difference of 0/65% across film and are listed in Table 1. The results presented that the pure CMCh and the film containing 88% CMCh provided the higher WVP than the pure RS film (about 2 times). This result could be explained by a higher hydrophilicity of the CMCh material (NH_3+ and COO- groups) than the RS (OH- groups) [1]. The other blend films, which compose 12-67% CMCh, showed no significant different WVP but all lower than that of CMCh film. Wu et al. [13] and Bourtoom and Chinnan [1] found similar observations for pullulan-CMCh blend film and RS-chitosan blend film, respectively. Moreover, the WVP of blend films increased with an increase of chitosan [1] or CMCh content [13]. This tendency is due to a higher hydrophillicity of NH_3+ groups by chitosan in the films [1] or the bulkier side groups of chitosan and CMCh that caused the increased free-volume of the composite matrix [13].

Table 1. Properties of rice starch/carboxymethyl chitosan blend films

Type of films	RS/CMCh ratio	Thickness (mm)	T (%)	WVP ($g.mm/m^2.mHg.day$)
RS/CMCh-01	100/0	0.128 ± 0.005 a	48.5 ± 2.3 a	4.800 ± 0.342 a
RS/CMCh-02	88/12	0.129 ± 0.001 a	52.8 ± 2.4 b	5.459 ± 0.492 a
RS/CMCh-03	67/33	0.136 ± 0.019 ab	57.8 ± 2.1 c	5.059 ± 0.426 a
RS/CMCh-04	50/50	0.143 ± 0.013 ab	62.3 ± 0.9 d	5.368 ± 0.628 a
RS/CMCh-05	33/67	0.138 ± 0.020 ab	65.0 ± 0.7 e	5.985 ± 0.767 a
RS/CMCh-06	12/88	0.144 ± 0.013 ab	73.6 ± 0.6 f	9.078 ± 1.909 b
RS/CMCh-07	0/100	0.159 ± 0.004 b	87.7 ± 2.0 g	9.068 ± 0.669 b

Different letters in the same column indicate significantly different ($p \leq 0.05$).

Figure 1. An appearance of the various RS/CMCh blend films.

The TS was an indicator of the film strength, and the EB determined the film flexibility [13]. The effect of CMCh proportion on the TS and EB of the RS/CMCh blend films is shown in Figure 2. It was found that the pure CMCh film had 3.2x higher film TS value than the pure RS film. The EB of the pure RS film had 1.2x higher than that of the pure CMCh film. This phenomenon suggested that the CMCh film was tougher than the RS film. The

amounts of CMCh (33-88%wt of solid content) decreased the EB of the RS film, except sample composing 12% CMCh which contained the highest value of the EB (64.5%) among other blends. The TS of the RS/CMCh blend films increased when CMCh proportion increased, and the maximum value was observed at 88% CMCh content which achieved 20.6 Mpa. The observed increase in TS value following the increase of CMCh portion could have been caused by the formation of inter-molecular hydrogen bonds between the COO- group of the CMCh backbone and OH- group of the RS [13]. Wu et al. [13] also proved that for the pullulan-CMCh blend film, the TS of the films increased when the CMCh to pullulan ratio increased. In addition, Xu et al. [14] found that at the higher starch:chitosan ratios, a phase separation between the two main components has been observed which cause the diminish of the TS value.

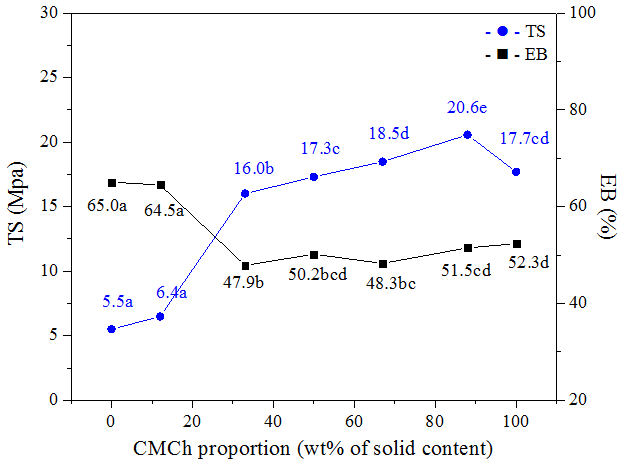

Figure 2. Mechanical properties of RS/CMCh blend films

The morphology of composite materials is a very important characteristic as it ultimately defines many properties of the biodegradable materials. The cross-sectional surface of the RS/CMCh blend films was examined by scanning electron microscopy (Figure 3). All films were found to be homogeneous. The surface of pure RS and pure CMCh as well as RS/CMCh-02 and RS/CMCh-03 were smooth. The smooth and homogeneous morphology suggests high miscibility and blend homogeneity between polymers [13]. For the increasing CMCh content (33-100%), the morphology of the blend film fractures changed from smooth to slightly rough. The roughness cross-sectional surface indicates the toughness of the material [15].The SEM images of the film composing 33-100%CMCh showed the presence of some fractures, especially for those of pure CMCh and RS/CMCH-06.The observed fracture on microstructures seems related to the result of WVP described above. However, more experiments will be further carry out to investigate this effect.

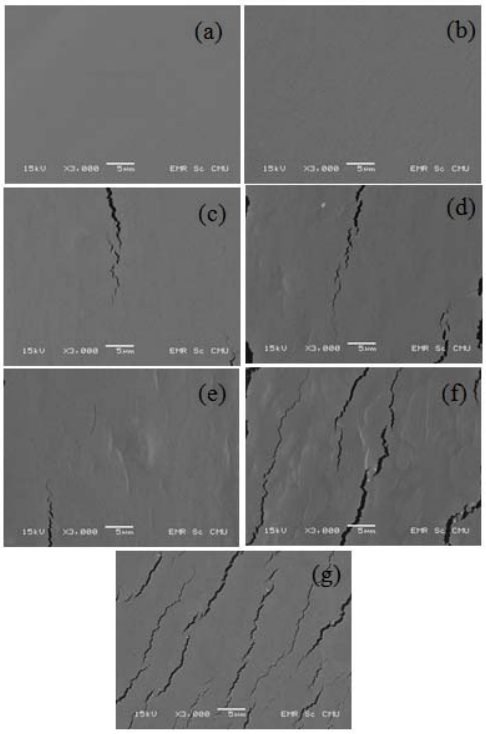

Figure 3. SEM images of (a) RS/CMCh-01,(b) RS/CMCh-02, (c) RS/CMCh-03, (d) RS/CMCh-04, (e) RS/CMCh-05, (f) RS/CMCh-06, and (g) RS/CMCh-07 films.

4. CONCLUSIONS

The addition of CMCh to RS films yield improvements in optical properties andtoughness, but do not improve the water resistance of the RS/CMCh blend films. The RS/CMCh blend films show potential to be used as edible film.

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Study on properties of xyloglucan/carboxymethyl cellulose blend film

Pornchai Rachtanapun*[a,b], Rungsiri Suriyatem[c], Porntip Chaimanee[d]
[a]Division of Packaging Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand 50100
[b]Materials Science Research Center, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200
[c]Division of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai, Thailand 50100
[d]Department of Chemistry, Faculty of Science, Silpakorn University, Nakorn Pathom, Thailand 73000

*p.rachta@chiangmai.ac.th phone +66-897903926 fax +66-53948224

ABSTRACT

Xyloglucan (XG), a neutral polysaccharide, was extracted from tamarind seed kernel powder using hot water procedure. Xyloglucan/carboxymethyl cellulose (XG/CMC) blend films with different XG/CMC ratios (100:0, 75:25, 50:50, 25:75, and 0:100) were prepared by solution casting. Water vapor permeability (WVP), sorption isotherm, and mechanical properties of the blend films were investigated. The WVP of the pure XG film and the pure CMC film were 1.63 and 1.79 g.mm/m^2.mHg.day respectively. The XG/CMC blend films provided lower WVP than both pure films (0.99-1.25 g.mm/m^2.mHg.day). The sorption isotherm of the XG/CMC blend films presented the characteristic of sigmoid-shaped type II. The equilibrium moisture content increased when the CMC content increased. Tensile strength (TS) of the pure XG and the pure CMC were 17.6 and 15.4 MPa respectively. Each XG/CMC blend film present higher TS (19.8-23.8 MPa) than both XG and CMC pure film. Elongation at break (EB) of the XG/CMC blend films had the similar trend with TS and it was in range between 45.4 and 72.4%. The highest TS and EB were provided by the film which contained 5:5 XG/CMC and this film was selected to be incorporated with various amounts of citric acid in the future study.

Keywords: Blend film, Carboxymethyl cellulose, Extract, Property, Xyloglucan

1. INTRODUCTION

Bio-based polymers have become increasingly important due to environmental awareness and demand for green technology. Polysaccharide such as starch and cellulose are example of bio-based polymers. These are known for their film-forming properties and have been used intensively for food and non-food applications. All these polysaccharides are by nature hydrophilic, causing performance problems, especially in relation to packaging of moist products. On the other hand these polysaccharides make film with excellent gas barriers. Addition of biodegradable plasticizer for hydrophilic polymer, such as starch helps to overcome the brittleness of the film [1].

Xyloglucan (XG) is a storage polysaccharide present in the tamarind seed waste which is obtained from the tamarind industry. XG from the tamarind seed is low cost and an abundantly available as a raw material in Thailand as well. It is a soluble hemicelluloses with a backbone composed of a β(1→4)-linked glucan backbone (as in cellulose) substituted with α(1→6)-linked xylose, which is partially substituted by β(1→2)-linked galactosyl residues. A representative partial structure of XG is illustrated in Figure 1 [2]. XG is soluble in water yielding highly viscous solution. Commercial interest in XG centers on its ability to thicken or gel in the presence of sugar or alcohol [3] and this can be utilized to make pectin-like gels. Carboxymethyl cellulose (CMC) is cellulose ether that is water soluble, edible and nontoxic. In literatures, the CMC could improve the mechanical properties of starches [4-6] and chitosan [7] and CMC could be well blended with corn flour and gelatin film to improve their permeability properties [8].

To date, there are a few researches that study preparation of XG film and its properties [9, 10]. No researchers have reported the preparation and characteristic of XG films blended with CMC. Thus the objective of this work was to investigate the influence of XG and CMC ratios on transparency, water vapor permeability (WVP), sorption isotherm, and mechanical properties (tensile strength and elongation at break) of the XG/CMC blend films.

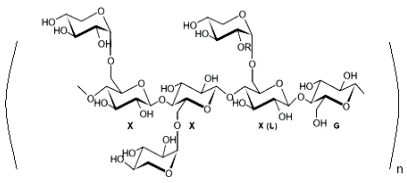

Figure 1. Representative partial structure of tamarind seed xyloglucan.

2. MATERIALS AND METHODS

Extraction of XG

XG was extracted from tamarind seeds kernel powder (GMICHIHARA Co., Ltd., Thailand) by hot water extraction method. The solution of tamarind seeds kernel powder (2wt %) was prepared in distilled water and mechanically stirred at 80°C for 30 min. Most of the water-insoluble fraction was removed by centrifugation at 6000 rpm for 10 min. The supernatant solution was precipitated with 95% ethanol in 2:1 ratio and stand for 60 min, which was followed by filtration with a Buchner funnel. The xyloglucan gel was oven dried at 50°C and ground to a fine powder.

Preparation of blend film and film clarity

Five different ratios of XG/CMC blend film forming solution (2wt% of solid content) were prepared (Table 1). First, XG powder was dissolved in 100 ml of distilled-water at 60°C for 2 h. CMC (Sigma-aldrich, Germany) was added and the solution was stirred at 80°C for 10 min. Sorbitol (30wt% of solid content) and butylated hydroxyl anisole (BHA, 10wt% of solid content) were used as a plasticizer and an antifungal respectively. The solution was degased, cooled to 25°C and cast onto an acrylic plate. The sample was dried at room temperature for 36 h. The film was peeled and kept in a polyetylene bag. Film clarity was determined by measuring the transmittance (%) at 550 nm using a spectrophotometer (Labomed, USA).

Water vapor permeability (WVP)

WVP was investigated using the ASTM E96-93 [11] as previously described by Rachtanapun [6] and Tongdeesoontorn et al. [8] with slight modification. A saturated solution of $NaNO_2$ was placed in a bottom of the

desiccator to provide 65%RH. The test was carried out at 25°C for 7 days. All measurements were performed in triplicate.

Sorption isotherm

Dried square specimens (2.0 cm × 2.0 cm) were placed in various desiccators containing saturated salt solutions (LiCl, $MgCl_2$, $Mg(NO_3)_2$, $NaNO_3$, NaCl and KCl) to create the specific RH (20, 39, 58, 63, 79, and 88%). Weight of film specimens was determined daily and when the difference between two consecutive weightings was approximately equal (not greater than 0.1% of the sample weight) it was assumed that an equilibrium condition had been reached. The percent equilibrium moisture content of film (%EMC) was determined as described in detail elsewhere [8, 12].

Mechanical properties

Tensile strength (TS) and elongation at break (EB) were determined using ASTM D882-91 [13] with Instron Model 1000 (H1K-S, UK). Rectangular specimens (1.5 cm × 14 cm) were required. Initial grip separation and cross-head speed was set at 50 mm and 50 mm/min respectively. There were ten sample measurements.

All data was analyzed by the one-way analysis of variance (ANOVA) and Duncan's multiple range test ($P \leq 0.05$) using SPSS version 11**.**

3. RESULTS

The different types of blend films from XG and CMC were prepared. The XG/CMC-01 (pure XG) film was transparent and light yellow while the XG/CMC-05 (pure CMC) film was transparent and colorless (Figure 2). The other films were transparent and showed the shade of very light yellow from the color of the XG (Figure 2). The clarity of the XG/CMC blend films determined by transmittance at 550 nm is shown in Table 1. The percent transmittance (T) of the pure CMC film was 3x of the pure XG film. The T increased significantly with increase of the CMC content.

Figure 2. An image of the XG/CMC blend films.

An evaluation of the water vapor permeability (WVP) of the film is one of the basic properties of the packaging material and is an indicator of the hydrophilicity of the material. Higher WVP is usually founded from a more hydrophilic material. The WVP value is the normalized transfer rate of the thickness and partial pressure gradient. It can then be compared even between films having different thicknesses according to the different ratios of XG and CMC. Table 1 shows that WVP of the pure XG film and the pure CMC film were not significantly different. WVP of the XG/CMC blend films ranged between 0.99-1.25 g.mm/m^2.mHg.day (Table 1), which is all lower than that of the pure CMC films. The addition of 25%wt CMC into the XG film provided the lowest WVP. However, when the CMC proportion reached to 50%wt, films maintained their WVP. Decrease of WVP after blending XG with CMC results in an improvement of the functional properties of the blends, considering the hydrophilic characteristics of the matrix. The reduction of WVP by incorporation with CMC is in agreement with Ghanbarzadeh et al. [4], who studied the properties of corn starch/CMC films. They reported that, at a low content of filler, CMC probably disperse well in the starch matrix and blocks the water vapor transmission. Nevertheless, additional amounts of CMC might congregate which diminished the effective contents of the CMC and facilitates the water vapor permeation through the film matrix.

The isotherms of various XG/CMC blend films showed the slow initial increase in moisture content with a_w increase of up to 0.58 [14]. T hen the moisture content rapidly increased with the increase of a further a_w (Figure 3a). A higher amount of water than usual could be absorbed by the films when water activity was increased [15]. The sorption isotherm of all films gave the characteristic sigmoid-shaped type II isotherm. The pure CMC film gave more EMC value than the pure XG film which can be clearly seen at an environmental relative humidity of 88% (Figure 3a). The EMC of blend films increased with the increase of CMC content. The related observations have been reported previously for cassava starch-CMC blend films [8] and rice starch/CMC from durian rind blend films [15].

Table 1. Compositions, transparency, and WVP of the blend films of XG and CMC

Type of films	XG/CMC ratio	Thickness (mm)	T (%)	WVP (g.mm/m^2.mHg.day)
XG/CMC-01	100/0	0.090 ± 0.025 ab	28.2 ± 1.3 a	1.629 ± 0.370 ab
XG/CMC-02	75/25	0.078 ± 0.014 a	31.6 ± 0.1 b	0.986 ± 0.160 c
XG/CMC-03	50/50	0.101 ± 0.003 ab	41.7 ± 1.3 c	1.251 ± 0.230 ac
XG/CMC-04	25/75	0.092 ± 0.007 ab	57.3 ± 0.4 d	1.241 ± 0.037 ac
XG/CMC-05	0/100	0.106 ± 0.009 b	88.0 ± 0.4 e	1.790 ± 0.130 b

Values followed with different letters in the same column are significant differences at the 5% level, according to Duncan ($p \leq 0.05$).

The effect of ratio between XG and CMC on the mechanical properties (TS and EB) of the XG/CMC blend films was investigated. The TS of the pure XG and the pure CMC film were as 17.6 and 15.4 Mpa respectively (Figure 3b). The TS of the films increased as the XG and the CMC were blended when comparing with that of the pure one. The blend film composing 50%wt CMC film (XG/CMC-03) was found to give the highest TS. The increasing TS of the blend films is up to 35% and 54% compared with that of the pure XG and the pure CMC film respectively. This result was similar to the case of corn starch/CMC films [4], CMC from papaya peel/corn starch blend films [6] and chitosan-starch composite film [7] as reported previously. Xu et al. [7] explained that the increasing TS of the chitosan-starch composite films are attributable to the formation of inter-molecular hydrogen bonds between NH_3+ of the chitosan backbone and OH^- of the starch. In addition, Ghanbarzadeh et al. [4] reported that the increasing TS of starch/CMC films are due to the interfacial interaction between the matrix and filler which is due to the chemical similarity (polysaccharide structure) of starch film and CMC. The EB of the blend films were affected by the XG and CMC ratios (Figure 3b). The EB values of the blend films behaved similar to the TS values, increasing EB of the blend film up to 39% and 59% compared with that of the pure XG and the pure CMC film respectively. The maximum occurred at XG/CMC ratio of 50:50. This was in agreement with others [4,6,7].

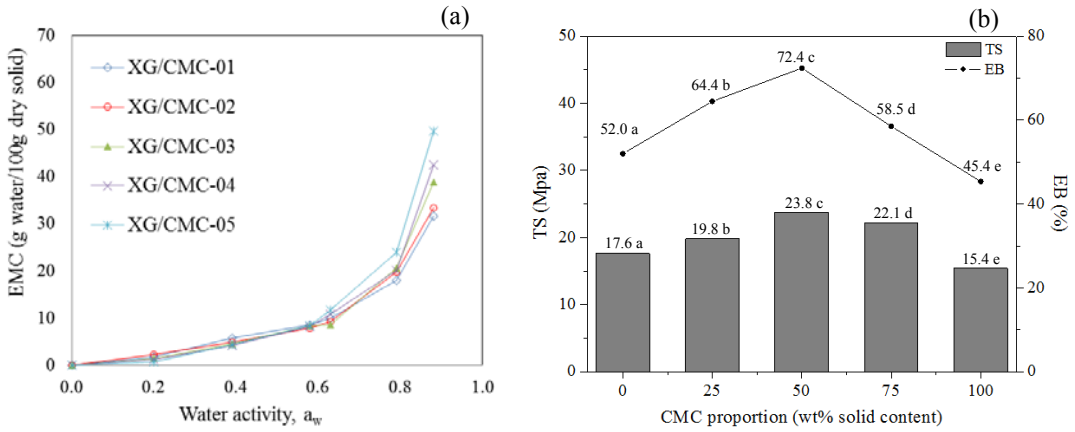

Figure 3. Moisture sorption isotherm (a) and mechanical properties (b) of the XG/CMC blend films with different of XG and CMC ratios.

4. CONCLUSIONS

XG could be blended with CMC and provide the transparent films with very light yellow shade. The transparency of the blend films increased with increases of CMC proportion. The XG/CMC blend films had increasing tensile strengths and elongation at breaks, and decreasing water vapor permeability when comparing with those of the pure ones.

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Determination of the content of hazardous heavy metals on *Curcuma longa* grown around a contaminated area

Napattaorn Buachoon*[a]
[a]Department of Science Education , Faculty of Science and Technology, Valaya Alongkorn Rajabhat University under Royal Patronage, Klongluang District, Pathumtani, Thailand 13180

* send2duang@hotmail.com phone +66-29093042 fax +66-29093041

ABSTRACT

The content of copper, lead, cadmium, and nickel on tissues of *Curcuma longa* grown around a contaminated area was determined by Inductively Coupled Plasma-Optical Emission Spectrometry. The area was divided into six sections, and each section was studied. Analyses were performed on sample rhizomes, stems, leaves, as well as the soil where the plant grew. Rhizomes showed a high content of the metals, followed by the leaves, and finally the stems, which had the lowest content of the metals. Lead concentrations in rhizomes, leaves, and stems were 550 mg/kg, 200 mg/kg and 150 mg/kg, respectively, while copper concentrations were 920 mg/kg, 593 mg/kg, and 270 mg/kg, respectively. In contrast, cadmium and nickel concentrations were lower and varied from 30 mg/kg on rhizomes, 108 mg/kg on leaves, and 56 mg/kg on stems for cadmium, and the content of nickel found ranged from 35 mg/kg on rhizomes, 43 mg/kg on leaves, and 40 mg/kg on stems. Soil concentrations were high in site 4 for lead and copper, 5,631 mg/kg and 5,033 mg/kg, respectively; lower concentrations were found for cadmium and nickel, 247 mg/kg and 67 mg/kg, respectively. The heavy metal content of the soils indicates the degree of pollution in the area. As expected, those sections which contained higher levels of heavy metals in the soil also showed to have higher heavy metal uptake by various parts of *Curcuma longa*. These data demonstrate *Curcuma longa's* ability to uptake copper and lead, and to some extent cadmium and nickel, from heavy metal contaminated soils. Analyses of other heavy metals will also be examined.

Keywords: *Curcuma longa***,** Hazardous heavy metals, Inductively Coupled Plasma-Optical Emission Spectrometry

1. INTRODUCTION

Elements are essential micro-nutrients and have a variety of biochemical functions in all living organisms. Some of them form an integral part of several enzymes. Although they are essential, they can be toxic when taken in excess; both toxicity and necessity vary from element to element and from species to species. Thus, information on the intake of heavy metals through food chain is important in assessing risk to human health.

Increasing environmental pollution has given rise to concern on the intake of heavy metals in humans. These metals enter the human body mainly by two routes namely: inhalation and ingestion. The intake of heavy metals through ingestion depends on the food habit.

For example, excess concentrations of lead, cadmium, copper and zinc significantly affected the plant water status of sunflowers, causing water deficit and subsequent changes in the plant. [9] Although the uptake of heavy metals is antagonistic to a myriad number of plant systems, other studies have shown that some plants are able to absorb heavy metals, adapt to them, and thrive. Currently, those sites that contain the highest concentrations of heavy metals are situated near industrial sources such as smelters and steel refineries. [7] Even in locations such as these, certain plant species have been able to adapt to heavy metal ions. [8] Both the mechanisms that the plants use for adaptation and the specific effects of the metals on the plants' bio-systems, however, remain unclear. Presently, a number of researchers are investigating the manner in which plants absorb heavy metal ions and are conducting experiments with these plants to see if they can be used to alleviate the metal contamination problem.

The industrial activity in the area has resulted in the accumulation of many heavy metals in the soil including copper, lead, cadmium, and zinc.[1] The accumulation of these heavy metals in the soils, however, may have lessened over time as a result of the metals being up taken by creosote bushes.

Curcuma longu, its main active constituent, is as powerful and antioxidant as vitamins C and E, making turmeric usage a consumer choice for cancer prevention, liver protection and premature aging. *Curcuma longu* can bind with heavy metals such as cadmium and lead, thereby reducing the toxicity of these heavy metals.

The aim of the present study is to determined of the content of hazardous heavy metals present in the individual tissues including the rhizomes, stems, leaves and the soils from which the plants grew. Three replicate samples were collected from various locations on the Pathumtani Province. The samples were oven dried and then the tissues of rhizomes, stems, and leaves were separated. The soil and tissue samples were acid digested and analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) for the determination of the content of copper, lead, cadmium, and zinc.

2. MATERIALS AND METHODS

Collection of *Curcuma longu*

For the present study, five locations were arbitrarily chosen to represent creosote bushes in various locations around the copper smelting industry. A six site, located 30 miles from sites 1-5, was chosen for control samples of the bushes. During the summer season of 2013, three replicate samples of creosote bushes, approximately 3 ft. high and of the same stage of maturity, were collected using a shovel. A diameter of approximately 2 ft. was dug around each bush. The bushes were pulled up by the rhizomes and the residual soils from the rhizomes were shaken off the bushes and collected in plastic containers for later analysis. All three bushes were combined for representative sampling purposes.

Preparation of *Curcuma longu* and soils

Samples of creosote bushes from each site were oven dried at a temperature of 90°C for four days. This mild temperature was chosen to avoid vapor loss of the metals or of their salts. After drying, the rhizomes, stems, leaves were separated and ground.

Soils were also sieved to -100 Tyler mesh to remove unwanted rock and sediment materials from the matrix. After the tissues were obtained, three replicate one gram samples of each tissue were acid digested according to EPA method 3050. A fourth replicate sample was also prepared and digested but was spiked with either 1.0 or 5.0 ppm each of copper, lead, cadmium, and zinc, which were the metals of interest. A blank was also prepared to ensure the integrity of the analytical procedure. The EPA protocol that was adopted for the acid digestion of the soil and tissue samples here can be described as follows: A 1.00-2.00 gram homogenous representative sample was obtained and placed in conical beakers. Sample slurries were prepared by adding 10 ml of 1:1 nitric acid (HNO_3). The slurries were then covered with watch glasses, heated to near boiling, and refluxed for 15 min. After refluxing, the slurries were cooled and then 5 ml of concentrated HNO_3 were added and the solution was again allowed to reflux for an additional 30 min. This last step was repeated to ensure complete oxidation of the metals. After the third refluxing

period, the sample was cooled to room temperature and 2 ml of deionized water and up to 10 ml of 30% hydrogen peroxide were added. The samples were then filtered to remove any particulates which might interfere with ICP-OES analysis. The filtrates were collected in 100 ml volumetric flasks and were diluted with deionized water to volume. The samples, which were approximately 5.0% (v/v) nitric acid, were now ready for ICP-OES analysis.

Data analysis

The experiments were performed in triplicate, and the samples were analyzed in triplicate. For each set of given data, standard statistical methods were used to determine the mean values and standard deviations. Confidence intervals of 95% were calculated for each set of samples to determine the error margin.

3. RESULTS

The range of linearity of concentration vs. absorbance curve is of great importance in determining of the Content of Hazardous Heavy Metals on *Curcuma longu*. The calibration curves for Zinc (Zn) Cadmium (Cd) Copper (Cu) and Lead (Pb) are shown in Figure 1.

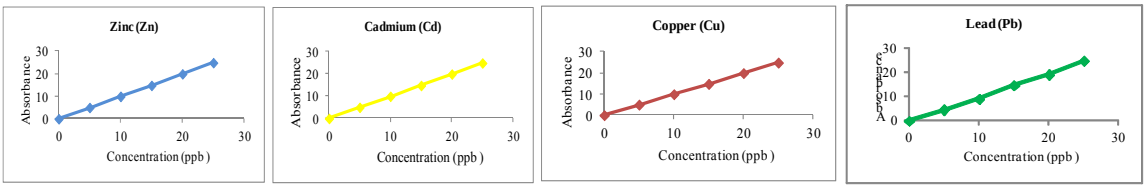

Figure 1. Concentration versus absorbance curves for Zinc (Zn) Cadmium (Cd) Copper (Cu) and Lead (Pb)

The highest concentrations of heavy metals were found in samples from site 3. Site 3 lead concentrations in rhizomes, stems, leaves and soil were 550 mg/kg, 200 mg/kg, 150 mg/kg, and 5,631 mg/kg, respectively, while copper concentrations were 920 mg/kg, 593 mg/kg, 270 mg/kg, and 5,033 mg/kg respectively. In contrast, cadmium concentrations from site 3 were lower and varied from 30 mg/kg in the rhizomes, 58 mg/kg in the stems, 56 mg/kg in the leaves, and 247 mg/kg in the soil. The content of Zinc determined in creosote from site 3 ranged from 35 mg/kg in the rhizomes, 43 mg/kg in the leaves, 40 mg/kg in the stems, and 67 mg/kg in the soil. In all of the samples, copper concentrations were the highest, followed by lead. Cadmium and Zinc concentrations were extremely low.

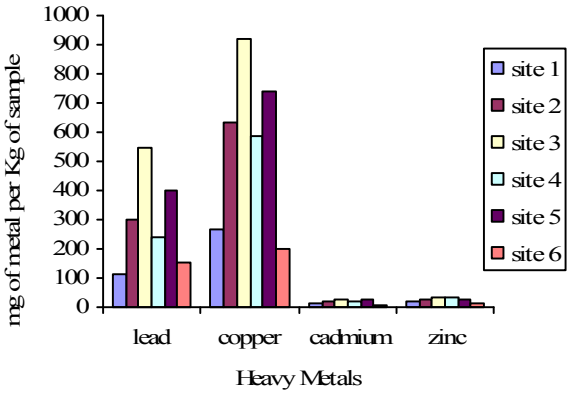

Figure 2. lead, copper, cadmium and zinc contamination in rhizomes where *Curcuma longu* (every data point represents the mean value of three replicate samples)

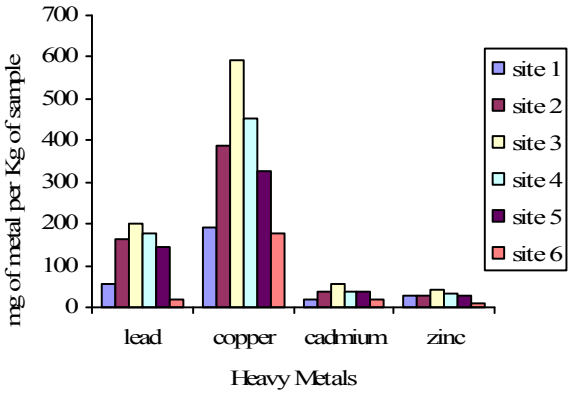

Figure 3. lead, copper, cadmium and zinc contamination in stems where *Curcuma longu* (every data point represents the mean value of three replicate samples)

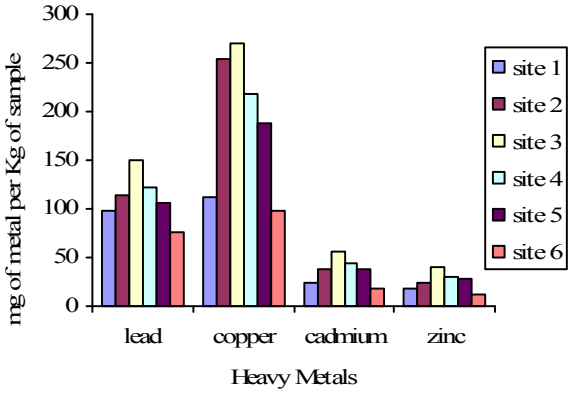

Figure 4. lead, copper, cadmium and zinc contamination in leaves where *Curcuma longu* (every data point represents the mean value of three replicate samples)

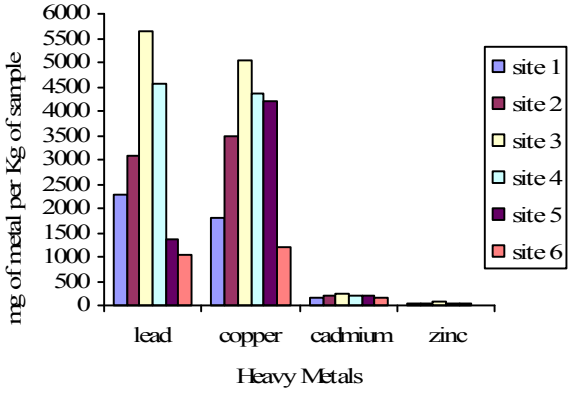

Figure 5. lead, copper, cadmium and zinc contamination in soil where *Curcuma longu* (every data point represents the mean value of three replicate samples)

Heavy metal concentrations for the rhizomes from which *Curcuma longu* grew are shown in Figure 2. Plant tissue metal contents for stems, leaves and soil are reported in Figures 3, 4, and 5, respectively. The total metal concentrations in soils and plants from each site were all nearly 100-fold higher than in the control samples (site 6).

As expected, those sections which contained higher levels of heavy metals in the soil also showed to have higher heavy metal uptake by the various parts of *Curcuma longu.* The highest levels of heavy metals found in various tissues of creosote were in conflict with the expected values. Particularly, because site 1 was located within the closest proximity to the suspected contamination source, the anticipated data should have been much higher at that site.

Clearly, *Curcuma longu* takes up heavy metals from the soil. It is unclear though whether the plant has evolved to adapt to the high concentrations of heavy metals or if the bushes already possess the capability of being planted under these contaminated conditions and still grow to maturity. Plants are known to have at least two defense mechanisms whereby they are able to incorporate metals into their tissues and continue to survive. In response to the toxic elements, plants can synthesize metalchelating proteins called metallothioneins. Another mechanism which may account for the accumulation of heavy metals in plants is the synthesis of phytochelatins, as suggested by Rauser.

Various other mechanisms have also been suggested regarding where the heavy metals are compartmentalized. Plants can also tolerate heavy metal contaminates by excluding the metals from sensitive sites, changing the metabolic pathways to prevent damage, or by synthesizing enzymes that would detoxify the heavy metals. Detrimental effects of heavy metals on plants have also been found to prevent the uptake of valuable nutrients such as potassium and phosphorus. All of these parameters regarding the effects of heavy metals in relation to *Curcuma longu* will require further investigation.

4. CONCLUSIONS

Creosote bush plants have succeeded in demonstrating the ability to uptake heavy metals such as copper and lead from contaminated soils. The metal concentrations in the plant are dispersed throughout the rhizomes, leaves, and stems with the highest concentration found in the rhizomes. As expected, the higher the contaminant soil concentration, the higher the metal uptake by the various parts of *Curcuma longu.* These data demonstrate the potential of phytoremediation via creosote bush as a low-cost, effective means of removing heavy metals from contaminated soils. The nature of the chemical groups that are responsible for the binding of the metals, however, is not fully understood and will be the purpose of further investigation.

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Inhibition of the tobacco-specific nitrosamine metabolizing cytochrome P450 2A13 by some plants from Eastern Thailand

Athitaya Insee[a,b], Pornpimol Rongnoparut [c], Panida Duangkaew[d], Songklod Sarapusit*[a]
[a]Biological Science Graduate Program, Faculty of Science, Burapha University, Thailand 20131
[b]Department of Biochemistry, Faculty of Science, Burapha University, Thailand 20131
[c]Department of Biochemistry, Faculty of Science, Mahidol University, Thailand 10400
[d]Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Petchaburi IT Campus, Petchaburi 76120

*songklod@buu.ac.th phone +66-38103058 fax +66-38393495

ABSTRACT

The lung-specific Cytochrome P450 2A13 (CYP2A13) is a heme containing enzyme that belongs to cytochrome P450 superfamily. This enzyme plays an important role in metabolism of tobacco-specific nicotine-derived nitrosamine ketone (NNK), or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone nitrosamine NNK and associate with development of NNK-induced lung cancer in various smokers. The 8-methoxypsoralaen (8-MOP) had reported to specifically inhibit the CYP2A13 mediated NNK-metabolism and coumarin 7-hydroxylation activity, resulting in decrease lung cancer formation in mice. As administration of 8-MOP caused various side-effects in smokers, the CYP2A13 inhibitor from natural-derived compounds is a safety strategy for lung cancer prevention. This research aims to investigate the inhibitory activity of some plants from Eastern Thailand on CYP2A13 enzyme *in vitro*. The *Escherichia coli* strain XL-1 blue containing the CYP 2A13 cDNA (pKK-Δ-23-2A13) was induced for protein expression by 0.2 mM IPTG and 0.005 μg/ml δ-ALA and the CYP 2A13 protein was purified by Ni^{2+}-Affinity chromatography and determined purity by SDS-PAGE. The purified CYP 2A13 had a molecular mass of 53.2 kDa. The inhibitory activities of some plant extracts from Eastern of Thailand at concentration of 10% μg/ml, 50% μg/ml and 100% μg/ml were investigated. The resulted indicated that the ethanolic extract of *Pluchea indica* Less extract showed the highest inhibitory activity with an IC_{50} value of 45.1 μg/ml compared to other plant extracts (p-value < 0.05). Further investigation suggested that *P indica* extract could inhibit CYP2A6 in time-, concentration- and NADPH-dependent manner, an indication of time dependent inhibition (TDI) pattern.

Keywords: Cytochrome P450 2A13 (CYP2A13), 8-methoxypsoralaen (8-MOP), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *Pluchea indica,* Time dependent inhibition (TDI)

1. INTRODUCTION

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent lung carcinogen in animals and human [1]. Metabolic activation (a-hydroxylation) of NNK, both the methyl and methylene carbons, to the formation of 4-oxo-4-(3-pyridyl)-1-butanone (keto alcohol) and 4-oxo-1-(3-pyridyl)-1-butanone (keto aldehyde), is required for its carcinogenicity. Several cytochrome P450 (P450) enzymes have been reported to be involved in the metabolic activation of NNK. However, CYP2A13 has the highest activity and the lowest *K*m value for NNK a -hydroxylation compared to CYP2A6 and other P450 enzymes [2,3]. Both CYP2A13 and CYP2A6 proteins share as high as a 93.5% identity in the amino acid sequence. CYP2A6 is mainly expressed in the liver and plays an important role in nicotine metabolism while CYP2A13 is predominantly expressed in the respiratory tract [3]. Since lung is the major target organ in NNK-induced carcinogenesis in laboratory animals and in smoking-related human cancers [1], the high activity of CYP2A13 in NNK α -hydroxylation and its predominant expression in human respiratory tissues suggest that CYP2A13 plays an important role in NNK induced carcinogenesis [4]. Therefore, the identification of a potent and specific inhibitor/mechanism-based inactivator of CYP2A13 could lead to lowering the metabolic activation of NNK in the target tissue, and, as a result, potentially lower the risk of lung cancer in smokers.

It has been demonstrated that several CYP2A6-mediated nicotine metabolism inhibitor compounds could also inhibit NNK-activation by CYP2A13. Methoxsalen (8-methoxypsoralen; 8-MOP) has been shown as both a competitive and a mechanism-based inhibitor of CYP2A6- and CYP2A13-mediated coumarin metabolisms [5]. Co-administration 8-MOP can completely inhibit *in vitro* nicotine metabolism in mice, and inhibits the NNK-induced lung adenoma in mouse [5, 6]. Recently, the *Rhinacanthus nasutus* extract and its constituents (Rhinacanthin A, B, and C) have shown efficiently inhibitory activity against both CYP2A6 and CYP2A13 enzymes *in vitro* [7].

This study aimed to preliminary screen for Thai herbal extracts in Eastern part of Thailand that previously shown to inhibit CYP2A6 enzymatic activity *in vitro* [8]. The results indicated the *Plucea indica* could highly inhibited CYP2A13-mediated coumarin metabolism in time-, concentration- and NADPH-dependent manner, an indication of mechanism-based inhibition (MBI) pattern.

2. MATERIALS AND METHODS

Preparation of herb extract and trial purification procedure

Medicinal folk plants were collected from Chachoengsao, Chanthaburi, Chon Buri, Rayong, and Trat provinces in Eastern Thailand. The plants (1kg) were extracted with 95% ethanol (1L × 3) for 5 days, filtered and solvent removed by vacuum rotary evaporator. *P indica* (leaves) was dry, macerated, extracted and fractionated in to Hexane, Ethyl acetate and water, respectively.

Cytochrome P450 CYP2A13 activity and inhibition assay

The human CYP2A13 and rat CPR, a P450s redox partner, proteins were expressed and purified as previously described [8,9], the purified enzymes were then used for P450-reconstitution enzymatic assay. Enzymatic activity of CYP2A13 to metabolize fluorescence coumarin substrate was determined as previously described [9], with some modification. The purified human CYP2A13 was pre-incubated with rat CPR in 50 mM Tris-Cl buffer for 10 min at room temperature, followed by incubation with coumarin substrate. To determine inhibition activity, various concentrations of extract or fraction and coumarin substrate were incubated with enzyme mixture for 5 min before starting reaction by addition of NADPH. Production of 7-hydroxycoumarin metabolite will be measured in real time at excitation 355 nm and emission 460 nm. The P450 enzymatic activity in the presence of extract was compared with the control incubations in which DMSO solvent was added instead of extract or fraction. The actual IC_{50} values (concentration causing 50% reduction of control activity) for inhibition were calculated using Graph-Pad Prism software, version 5 (La Jolla, CA). Since methoxsalen (8-MOP) is the known inhibitor of CYP2A13, it was used as control in the inhibition assays.

Inhibitory effect of *P. indica* extract and fractions at each concentration was performed. To characterize time-, NADPH- and concentration-dependent inhibition of CYP2A13 by *P. indica* extract and fractions, reactions with various concentrations of *P. indica* in the presence or absence of NADPH were carried out by pre-incubation with *P. indica* extract or fractions at various times (15 and 30 min) before addition of coumarin substrate. A natural logarithm of the percentage of the remaining activity was plotted against pre-incubation time by linear regression analysis using GraphPad Prism 5.

3. RESULTS AND DISCUSSION

The human CYP2A13 and rat CPR enzymes were successfully expressed and purified from *E. coli* expression system into homogeneity, as determined by SDS-PAGE. The cytochrome c reduction activity of the purified rat CPR is 58.69 ± 1.58 μmol of cytochrome c reduction/minute/mg protein. Using reconstitution enzymatic assay, the purified CYP2A13 protein could convert mediated coumarin metabolism (coumarin 7-hydroxylase activity) with the specific activity of 0.054 ± 0.001 μmol coumarin/minute/mg protein, comparable to those previously described [8, 9].

The present study attempted to screen for a candidate plant that possessed inhibition activity against lung specific CYP2A13 enzyme and widely distributed in Eastern Thailand. Initial inhibition screening with different plant extracts at various concentrations demonstrated that the extract of *P indica,* a commonly used mangrove plant for health tea product in Chantaburi province, shown strongest inhibitory activity against CYP2A13-mediated coumarin hydroxylation with an IC_{50} value of 30.60 ± 1.20 μg/ml follow by the turmeric extract from *Allium sativum* (Table 1). We had previously determined that the *P. indica* extract has no inhibitory activity against the rat CPR, a CYP2A13 redox partner [8]. Therefore, inhibitory activity of *P. indica* extracts may possibly caused by inhibitory activity against CYP2A13. As CPR plays role in electrons transfer for P450-mediated metabolism *in vitro*, and the effect of plant extract on CPR activity could impair metabolic function of various P450 isoforms, resulting in diverse un-predictable side-effects of herb-drug interaction. Thus, *P. Indica* extract could probably suitable as a safe natural product for safety smoking cessation therapy in near future.

Table 1. Inhibition of CYP2A13 by some medicinal folk plants in Eastern part of Thailand

Sample	IC_{50} (μg/ml)[a]	Sample	IC_{50} (μg/ml)[a]
A. indica	252.6 ± 1.79	*P. indica*	30.60 ± 1.20
A. sativum	123.6 ± 1.95	*P. nigrum*	255.9 ± 2.22
D. trifolia	213.9 ± 2.09	*S. aromaticum*	177.9 ± 2.06
C. longa (rhizome)	160.9 ± 1.73	*S. tuberosa*	ND
C. longa (leaf)	223.8 ± 1.85	*A. calamus*	167.1 ± 1.99

[a]each value represents mean ± SD of douplicate experiments
[b]could not determine as the extract activating enzymatic activity

The inhibition of cytochrome P450 enzymes has been frequently occurred through mechanism-based inhibition (MBI) causing irreversible loss of enzyme activity. To primarily determine whether inhibition by *P. indica* followed a mechanism-based inhibition pattern, the CYP2A13 inhibitory activity was measured by pre-incubation *P. indica* extract with purified CYP2A13 enzyme in the absence and presences of NADPH. The inhibition of CYP2A13-mediated coumarin 7-hydroxylase activity by the *P. indica* extract significantly increase from IC_{50} value of 30.60 ± 1.20 μg/ml to 18.24 ± 0.16 μg/ml when pre-incubated extract with purified CYP2A13 for 30 min in the absence and presence of NADPH, respectively.

Table 2. Inhibition of CYP2A6 by *P. indica* extract and fractions

Sample	IC_{50} (μg/ml) [a]	
	Co-incubation	Pre-incubation
Ethanolic extract	30.60 ± 1.20	18.24 ± 0.16
Hexane fraction	58.25 ± 0.08	31.24 ± 0.14
Ethyl acetate fraction	21.54 ± 0.07	3.00 ± 0.11
Aqueous fraction	90.9 ± 0.08	85.15 ± 0.11

[a]each value represents mean ± SD of triplicate experiments

In an initial attempt to obtain inhibitory compound(s) from *P. indica* extract, fractionation with hexane and ethyl acetate as solvents yielded hexane, ethyl acetate and aqueous fractions, respectively. As indicated in Table 2, ethyl acetate fraction (EtOAc fraction) showed the strongest inhibition followed by hexane fraction. Interestingly, the EtOAc fraction exhibited highly potent CYP2A13 inhibiting activity potency than the ethanolic extract, indicating that the majority of the candidate compound may possibly fractionate into this fraction. To determine whether inhibition by EtoAc fraction followed a mechanism-based inhibition pattern, inhibition assay by means of pre-incubation of enzyme mixture with EtOAc fraction in presence and absence of NADPH was performed. The IC_{50} values obtained from pre-incubation assayed in presence and absence of NADPH were 21.54 ± 0.07 and 3.00 ± 0.11 μg/ml, respectively (Figure 2A). Figure 2B shows the significant increase in inhibition of CYP2A13 by EtOAc fraction when pre-incubation time was increased These data primarily suggested that a candidate compounds in EtOAc fraction of *P. indica* could inhibit human CYP2A13 in a time-, concentration-**,** and NADPH-dependent mechanism-based inhibition manner. However further work is required to purified EtOAc fraction to obtained inhibitory compound(s) in sufficient amount for its chemical structure determined, together with its mechanism-based inhibition mode be proven.

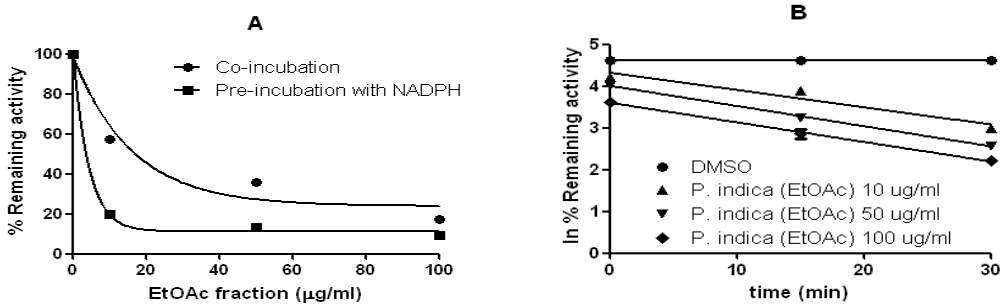

Figure 1. Inhibition of human CYP2A13 by EtoAc-fraction. (A) Inhibitory effect of EtoAc fraction on CYP2A13 by pre-incubation in the presence and absence of NADPH for 30 minutes. (B) Concentration- and time dependent inhibition of human CYP2A13 activity by EtOAc fraction. Each data point represents the mean of duplicate determination

4. CONCLUSIONS

In conclusion, the present study indicated high inhibitory activity and potency of *P. indica* (leaves) extract and the ethyl acetate (EtOAc) fraction against CYP2A13 mediated coumarin 7-hydroxylase activity *in vitro*. Although the chemical structure and detail inhibition mechanism of candidate compounds are remain to be investigating, the irreversible inhibition of CYP2A13 activity by *P. indica* extracts is very useful information for an application of *P. indica* extract as a safety smoking cessation therapy in Thailand.

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Inhibition studies of Cytochrome P450 2A6 by *Vernonia cinerea* Less and *Carthamas tinctorius* L. extracts

Tunyaporn Wongsri[a,b], Sarinya Thongjam[b], Pornpimol Rongnoparut[c], Panida Duangkaew[d], Songklod Sarapusit*[b]
[a]Department of Biochemistry, Faculty of Science, Burapha University, Thailand 20131
[b]Regional Medical Sciences center 6, Chon Buri, Thailand 20000
[c]Department of Biochemistry, Faculty of Science, Mahidol University, Thailand 10400
[d]Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Petchaburi IT Campus, Petchaburi Thailand 76120

*ssongklod@gmail.com phone +66-38103058 fax +66-38393495

ABSTRACT

The liver-specific cytochrome P450 2A6 (CYP2A6) enzyme play an essential role in metabolism of various endogenous and exogenous compound especially the tobacco-additive substance, nicotine, and coumarin probe substrate. The nicotine metabolism by liver-specific human CYP2A6 enzyme has been reported as an important route in nicotine detoxification and associate with smoking addiction behavior. In addition, genetic polymorphism of CYP2A6 enzyme is associated with smoking behavior, low CYP2A6 activity smoke fewer cigarettes per day. Thus, compounds that could inhibit the CYP2A6 mediated nicotine metabolism might be a good candidate for smoking cessation therapy. We had previously reported that the whole plant extract of *Vernonia cinerea* L. and. the flower extract of *Carthamus tinctorius* L are strongly inhibit the CYP2A6 enzymatic activity *in vitro*. Therefore, characterization of *V cinerea* and *C tinctorius* extracts on CYP2A6 enzyme were further investigated in this study. The *V cinerea* and *C tinctorius* were collected, macerated, extracted and fractionated into Hexane, Ethyl Acetate and Water fractions. The CYP2A6 enzyme was bacterially expressed and purified. The CYP2A6-mediated coumarin 7-hydroxylase activity was measured in the presence and absence of tested fractions. Interestingly, the F5 fractionation of Hexane fraction of *V cinerea* and the F4 and F5 fractionation of Hexane fraction (Hexane: Ethyl Acetate; 70:30) at 50 µg/ml could potently inhibit the CYP2A6 activity by 77.71%, 100% and 96.01% respectively. The inhibitory activities of this fractionation were comparable to the specific CYP2A6 inhibitor (8-methoxypsolaren: 8-MOP). These natural–derived compounds from *V cinerea* and *C tinctorius* could possibly useful for safety smoking cessation treatment.

Keywords: Cytochrome P450 2A6, Nicotine metabolism, 7-Hydroxylase activity, Inhibition, *Vernonia cinerea* L, *Carthamus tinctorius* L

1. INTRODUCTION

Cigarette smoking is widely prevalent due to the highly addictive properties caused by nicotine, a major constituent of tobacco. Because of addiction, continuing tobacco smoking leads to exposure to a diverse array of carcinogens in tobacco, causing tobacco-related diseases. Nicotine is metabolized mainly by the cytochromes P450 2A6 (CYP2A6) enzyme to cotinine, followed by conjugation with glucuronic acid and excreted in urine. However, addictive properties of nicotine lead to an exposure to various carcinogens, and as a result could contribute to the cause of lung diseases and cancers. In humans, inter-individual differences in nicotine metabolism have been shown in association with genetic polymorphisms of CYP2A6. Individuals with gene deletion variant have impaired nicotine metabolism, accordingly have reduced smoking behavior and are likely to stop smoking. Thus inhibition of CYP2A6 activity, to mimic genetic defect, could decrease nicotine metabolism and so maintain plasma nicotine level for longer periods of time and as a result may affect smoking behavior by smoking fewer cigarettes per day. Moreover reducing nicotine intake in the smokers concomitantly reduces the exposure to tobacco smoke contaminants and carcinogenic metabolites, thus decreases adverse health effects of tobacco smoking. In addition, the 8-methoxypsolaren (8-MOP), a specific inhibitor of CYP2A6 enzyme, shown to reduce number of cigarette smoke per day. However, 8-MOP causes various side effects and has been removed for smoking cessation [1-3].

Recently, commonly used Thai herbs were selected and screened for their CYP2A6 inhibitory activity by using fluorescence-based method. Interestingly, at 10% v/v, *Vernonia cinerea* tea (*VC* tea) and *Carthamas tinctorius* tea (*CT* tea) could inhibit CYP2A6-mediated coumarin 7-hydroxylase activity by 72% and 50%, respectively [4]. In this study, we aim to further investigate the inhibition activity of the *V. cinerea* and *C. tinctorius* extracts on bacterially expressed and purified CYP2A6 enzyme *in vitro*. The candidate compounds that could potential inhibited CYP2A6 activity could beneficial as an alternative safety-smoking cessation in Thailand.

2. MATERIALS AND METHODS

Preparation of herb extract and trial purification procedure

V. cinerea (whole plant) was collected in Bangsean and *C. tinctorius* (flower) was purchased from local traditional Chinese pharmacy in Chonburi province. Both plants were dry, macerate, extract and fractionate in to hexane, ethyl acetate and water as shown in Figure 1. The activity-guide assay was performed and the hexane fractions were further purified by column chromatography (CC) at various ratio of hexane:ethyl acetate and preliminary determined the purity by thin layer chromatography (TLC).

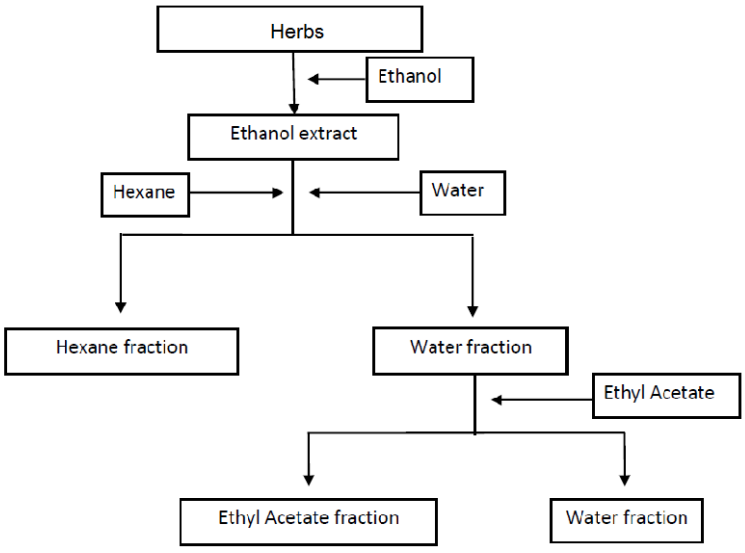

Figure 1. Extraction and fractionation flowchart of *Vernonia cinerea* less and *Carthamas tinctorius* less

Cytochrome P450 CYP2A6 activity assay

The human CYP2A6 and rat CPR, a P450s redox partner, proteins were expressed and purified as previously described [5-7], the purified enzymes were then used for P450-reconstitution enzymatic assay. Enzymatic activity of CYP2A6 to metabolize fluorescence coumarin substrate was determined as previously described [5, 7-8], with some modification. The purified human CYP2A6 was pre-incubated with rat CPR in 50 mM Tris-Cl buffer for 10 min at room temperature, followed by incubation with coumarin substrate. To determine inhibition activity, the various concentrations of extract or fraction and coumarin substrate (~ the K_m value) were incubated with enzyme mixture for 5 min before starting reaction by addition of NADPH. Production of 7-hydroxycoumarin metabolite will be measured in real time at excitation 355 nm and emission 460 nm. The P450 enzymatic activity in the presence of extract or fraction was compared with the control incubations in which DMSO solvent was added instead of extract or fraction. The actual IC_{50} values (concentration causing 50% reduction of control activity) for inhibition were calculated using GraphPad Prism5 software, version 5 (La Jolla, CA). Since 8-methoxypsolaren (8-MOP) is the known inhibitor of CYP2A6, it was used as control in the inhibition assays.

Further study of the inhibitory activity of extract and fraction on electron transfer of rat CPR was determined. The purified rat CPR was incubated with cytochrome c in 50 mM Tris-CL buffer pH 7.5, 50 µM NADPH was add to start reaction, increasing of cytochrome c (reduced form) was detected at 550 nm [6]. Specific activity was analyzed by SPSS and GraphPad Prism5 to verify remaining activity of enzyme compared between with or without *V. cinerea* and *C. tinctorius* extracts and fractions.

3. RESULTS AND DISCUSSIONS

The human CYP2A6 and rat CPR enzymes were successfully expressed and purified from bacteria-expression system into homogeneity, as determined by SDS-PAGE. The molecular mass of CYP2A6 and rat CPR were approximately 57.99 and 78.7 kDa, respectively. The cytochrome c reduction activity of the purified rat CPR is 58.69 ± 1.58 µmol of cytochrome c reduction/minute/mg protein which is comparable to previously report [6]. The *in vitro* reconstitution of rat CPR and human CYP2A6 enzyme was performed and the CYP2A6-mediated coumarin 7-hydroxylase activity was determined. The specific activity of the purified CYP2A6 enzyme is 0. 2951 ± 0.0007 µmol coumarin/minute/mg protein with a coumarin substrate binding affinity (coumarin K_m) about 3.25 ± 0.13 µM which is also comparable to previously report by our group [4].

Inhibitory herbs extract, hexane fraction, ethyl acetate fraction and water fraction on CYP2A6 enzyme was measured by fluorescence spectrometry assay, and analyzed by GraphPad Prism5 (ANOVA, p-value < 0.05). Interestingly, At 100 µg/ml of *V. cinerea,* hexane, ethyl acetate, and water fraction could inhibit CYP2A6 by 88.51% 93.43% and 45% respectively. Interestingly, at 50 µg/ml of *C. tinctorius*, ethanolic extract and all fractions could potently inhibit CYP2A6 enzymatic activity by 100%. Due to very low amount of ethyl acetate fractions, thus we aim to preliminary purify candidate compounds in hexane fractions by column chromatography. Various solvent composition were test, however, the composition of hexane:ethyl acetate at 70:30 gave high resolution of chemical compounds on TLC plate. At this separating condition, the *V. cinerea* was separated into 6 fractions while *C. tinctorius* was separated into 8 fractions. The inhibitory activities of each fraction at 50 µg/ml were determined. Interestingly, the F5 fractionation of hexane fraction of *V. cinerea* and the F4 and F5 fractionation of Hexane fraction (hexane: ethyl acetate; 70:30) at 50 µg/ml could potently inhibit the CYP2A6 activity by 77.71%, 100% and 96.01% respectively (Figure 2A and 2B). The inhibitory activities of this fractionation were comparable to the specific CYP2A6 inhibitor (8-methoxypsolaren: 8-MOP)

As CPR plays role in electrons transfer for P450-mediated metabolism *in vitro*, and the effect of plant extract on CPR activity could impair metabolic function of various P450 isoforms, resulting in diverse un-predictable side-effects of herb-drug interaction. We found that both F5 fractionation of *V. cinerea* and F4 and F5 of *C. tinctorius* (50 µg/ml) have no inhibitory activity against rat CPR enzyme (data not show), implicated that the candidate compound in Hexane fractionation could not be affected other P450 enzyme activity through inactivation of CPR enzyme. The specificity of inhibition against other P450 enzymes is waiting to determine in near future.

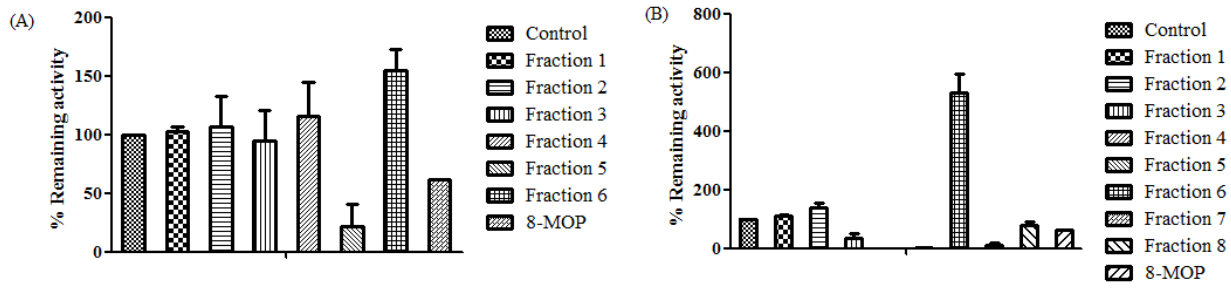

Figure 2. CYP2A6 remaining activity compared of 50 µg/ml of *V. cinerea* fractions (A), 50 µg/ml of *C. tinctorius* fractions (B) compared with 2 µM 8-MOP (specific CYP2A6 inhibitor).

Although, *V. cinerea* tea (whole plant) is widely available and well known in Thailand as an effective alternative smoking cessation strategy in Thailand [9], little is known about how *V. cinerea* tea could helps to quit smoking. Thus, according to the results, useful information that *V. cinerea* tea could strongly inhibit CYP2A6 activity may be a value added and strongly support the use of *VC* tea in smoking cessation therapy. In addition, the *C. tinctorius* (flower) is also widely available for healthcare treatment, not smoking cessation. This potent inhibition activity of *C. tinctorius* extract is value information for being used of *C. tinctorius* extract for smoking cessation. Thus, these natural-derived compounds from *V. cinerea* and *C. tinctorius* could probably suitable as a safe natural product for decreasing nicotine metabolism, an alternative safety smoking cessation therapy.

4. CONCLUSIONS

The liver-specific CYP2A6 is an important enzyme that plays an important role in nicotine metabolism in human. The CYP2A6 enzymatic activity has been reported to associate smoking behavior and number of cigarette smoked per day. Smoker who possess an impair CYP2A6 activity have reduced smoking behavior and are likely to stop smoking [1-3]. Thus inhibition of CYP2A6 activity could decrease nicotine metabolism and so maintain plasma nicotine level for longer periods of time and may affect smoking behavior by smoking fewer cigarettes per day [2, 3]. The results from this study indicated that the F5 fractionation (Hexane fraction) of *V. cinerea* and the F4 and F5 fractionation (Hexane fraction) of *C. tinctorius* (50 µg/ml) could effectively inhibit the CYP2A6 activity compared with CYP2A6 inhibitor (8-MOP). These natural-derived compounds from *V. cinerea* and *C. tinctorius* could possibly useful for safety smoking cessation treatment.

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Inhibitory effects of medicinal folk plants from Ban-Ang-Ed Official Community Forest Project (The Chaipattana Foundation) on drug-metabolizing cytochrome P450 3A4 and 2C9 enzymes

Janthip Anantakul[a], Tunyaporn Wongsri[a], Pornpimol Rongnoparut[b], Songklod Sarapusit[a*]
[a]Department of Biochemistry, Faculty of Science, Burapha University,Thailand 20131
[b]Department of Biochemistry, Faculty of Science, Mahidol University, Thailand 10400

*songklod@buu.ac.th phone +66-38103058 fax +66-38393495

ABSTRACT

The liver-specific cytochrome P450 3A4 (CYP3A4) and 2C9 (CYP2C9) enzymes are heme containing enzyme that belongs to cytochrome P450 superfamily. Both CYP3A4 and CYP2C9 enzymes are responsible for metabolizing various pharmaceutical drugs (~40-45% of prescribed drugs), suggesting an important role of CYP3A4 and CYP2C9 enzymes in health care treatment. In Thailand, healthcare treatment by co-administration of herbal products along with clinical drugs is becoming popular; however, this situation could lead to various side effects because of herb-drug interaction. In this study we investigate the inhibitory effect of commonly used medicinal folk plants from Ban-Ang-Ed official community forest project (The Chaipattana Foundation) on CYP3A4 and CYP2C9 enzyme. The plants were collected based on local intellectual suggestion of commonly used medicinal folk plants for healthcare treatment. The harvested plants were identified, macerated, and ethanolic extracted. The CYP3A4 and CYP2C9 enzymes were bacterially expressed and purified separately. The inhibitory activity of plant extracts from Ban-Ang-Ed at concentration of 50 μg/ml on CYP3A4 and CYP2C9 enzymatic activities were determined. The results showed that plant extracts differently affected CYP3A4 mediated testosterone 6-β hydroxylation activity and CYP2C9 mediated tolbutamide 4-hydroxylation activity *in vitro*. Among tested plant extracts, *Aglaonema nitidum* extract (commonly use for treatment of hemorrhoid) could potently inhibited CYP3A4 and CYP2C9 activities, followed by *Picrasma javanica* (commonly use for anti-anemia and treatment of inflammation) and *Tacca chantrieri* (commonly use as elixir and aphrodisiac). According to the results, care must be taken for co-treatment of diseases by using medicinal folk plants in Ban-Ang-Ed with CYP3A4 and CYP2C9 metabolizing pharmaceutical drugs.

Keywords: Cytochrome P450 3A4, Cytochrome P450 2C9, Ban-Ang-Ed Official Community Forest Project, Testosterone 6-β hydroxylation activity, Tolbutamide 4-hydroxylation activity

1. INTRODUCTION

There is a trend nowadays to employ natural products for treatment of diseases. The use of medicinal herbs, either pre-treatment or co-administration with drugs, has particularly increased over the past few years. However, this situation could lead to various side effects because of herb-drug interaction of natural derived compounds with the liver-specific cytochromes P450 3A4 (CYP3A4) and 2C9 (CYP2C9) enzymes that have been reported to metabolized various clinical drugs (~ 40-45% of prescribed drugs). Several reports have demonstrated that natural compounds and herbal products may cause pharmacokinetic interaction with clinically used drugs when co-administrated [1-6]. The Herb–drug interactions (HDIs) due to cytochrome P450 (CYP) induction, inhibition, and/or inactivation can lead to treatment failure, attenuation of the efficacy of concomitant medications and even death in some cases [7-9]. The traditional approach for assessing potential CYP induction or inhibition of test compounds has been to measure the change in enzyme activities of CYP3A4 and CYP2C9 using known CYP-specific probe substrates. In Thailand, many medicinal plants are used as traditionally to cure many diseases and almost unregulated. In addition, many patients do not inform their physician about the traditional medicines they consume. Therefore, interactions between traditional medicines and drugs prescribed clinically are becoming a concern. In this study, some of the selected medicinal plants which are widely used from Ban-Ang-Ed official community forest project (The Chaipattana Foundation) based on local intellectual suggestion were selected and evaluated for their action on CYP3A4 (testosterone 6-β-hydroxylation) and CYP2C9 (tolbutamide 4-hydroxylation) enzymes. The obtained information of HDIs will be beneficial for safety used of these selected plants.

2. MATERIALS AND METHODS

Preparation of herb extracts

The plants were collected based on local intellectual suggestion of commonly used medicinal folk plants for healthcare treatment. The harvested plants have been currently identified by Dr. Benchawon Chewpreecha, Department of Biology, Burapha University and collected in herbarium. All harvested plants were dry, macerate and extract with 95% ethanol for 5 days, filtered and solvent removed by vacuum rotary evaporator.

Cytochrome P450 CYP3A4 and CYP2C9 activity assay

The human CYP3A4, CYP2C9 and rat CPR, a P450s redox partner, proteins were expressed and purified as previously described [10-12], the purified enzymes were then used for P450-reconstitution enzymatic assay. Enzymatic activity of CYP3A4 to metabolize testosterone substrate to 6-β-hydroxytestosterone product (testosterone 6β-hydroxylation activity) and CYP2C9 to metabolize tolbutamide to 4-hydroxytolbutamide (tolbutamide 4-hydroxylation activity) were determined as previously described [10, 11], with some modification. The purified human CYP3A4 or CYP2C9 enzyme was pre-incubated with rat CPR in 50 mM Tris-Cl buffer for 10 min at room temperature, followed by incubation with 100 μM testosterone substrate for CYP3A4 enzyme and 100 μM tolbutamide substrate for CYP2C9 enzyme. The reaction was further incubated at room temperature for 5 min before starting reaction by addition of NADPH. The reaction was incubated at 37 C for 20 min before addition of 2M HCl (T20 min), addition of androsterdione internal standard and centrifuged for 10 min. The equal volume of Ethyl acetate was added to the supernatant to extract the products followed by centrifugation at 10,000 rpm for 10 min. The Ethyl acetate was removed and re- dissolved in methanol. The products formation was analyzed using a Novapak C_{18}-Reverse Phase High Performance Liquid Chromatography (C_{18}-RP-HPLC) with the mobile phase of methanol: water (80:20, v/v), a flow rate of 0.4 ml/min, and monitoring at 240 nm for 6-β-hydroxytestosterone and 229 nm for 4-hydroxytolbutamide. Total formation of either 6-β-hydroxytestosterone product or 4-hydroxytolbutamide were compared with the initial reaction (T 0 min), addition of 2M HCl right after NADPH addition.

Inhibition of CYP3A4 and CYP2C9 by medicinal folk plants

Inhibition screening assay was carried out by pre-incubation of either purified human CYP3A4 or CYP2C9 enzyme with rat CPR in 50 mM Tris-Cl buffer for 10 min at room temperature, followed by incubation with 100 μM testosterone substrate for CYP3A4 enzyme and 100 μM tolbutamide substrate for CYP2C9 enzyme in the presence of 50 μg/ml of plant extracts for 5 min before starting reaction by addition of NADPH. Products determination was performed as described. The remaining activity of CYP3A4 or CYP2C9 enzymes in the presence of plant extracts were compared with the control reaction in which the DMSO solvent was added instead of tested plant extracts using SPSS and Graph-Pad Prism software, version 5 (La Jolla, CA).

Further study of the inhibitory activity of extract on electron transfer of rat CPR was determined. The purified rat CPR was incubated with cytochrome c in 50 mM Tris-HCL buffer pH 7.5, 50 µM NADPH was add to start reaction, increasing of cytochrome c (reduced form) was detected at 550 nm [12]. Specific activity was analyzed by SPSS and GraphPad Prism5 to verified remaining activity of enzyme compared between with or without extracts.

3. RESULTS

The human CYP3A4, CYP2C9 and rat CPR enzymes were successfully expressed and purified from *E. coli* expression system into homogeneity, as determined by SDS-PAGE (data not shown). The cytochrome c reduction activity of the purified rat CPR is 58.69 ± 1.58 µmol of cytochrome c reduction/minute/mg protein which is comparable to previously report [10]. Using the *in vitro* reconstitution enzymatic assay, the specific activity of purified CYP3A4-mediated testosterone 6β-hydroxylation activity is 0.2887 ± 0.0008 µmol 6β - hydroxytestosterone/minute/mg protein while the specific activity of purified CYP2C9-mediated tolbutamide 4-hydroxylation is 0.2980 ± 0.0016 µmol 4-hydroxytolbutamide/minute/mg protein which are comparable to previously report [8,9].

The present study attempted to screen medicinal folk plant in Ban-Ang-Ed official community forest project (The Chaipattana Foundation) that possessed inhibitory activity against human hepatic CYP3A4 and CYP2C9 enzymes. These selected plants were commonly used for anti-inflammation, anti-flu and antibiotic based on local intellectual suggestion. We found that plant extracts at 50 µg/ml concentrations differently affected both CYP3A4 and CYP2C9 activity *in vitro* (Anova, p <0.05). Interestingly, the enzymatic activity of CYP3A4 was more affected by HDIs compared to the CYP2C9 activity (Figure 1 and 2). The CYP3A4 activity was modulated to the highest activity, almost 3-folds, in the presence of Chongko (leave) and Madiahom (leave) extracts followed by Mahunk (root), Hengmaina (trunk), and Klongkleng (leave) extracts. On the other hand, Klongkleng (root), Denguton (leave), and Huadiew (leave) are very potent inhibitory activity against CYP3A4 enzyme followed by kangkaodum (rhizome and leave), Rayomnoi (leave and root) compared to other extracts (Figure 1). In contrast, most of the selected plants merely affected the CYP2C9-mediated tolbutamide 4-hydroxylation activity in vitro (Figure 2). There were only Phanomsawan (root) and Uengmaina (rhizome) showed slightly activated CYP2C9 activity while Huadiew (leave), Denguton (leave) showed highest inhibitory activity among other plant extracts.

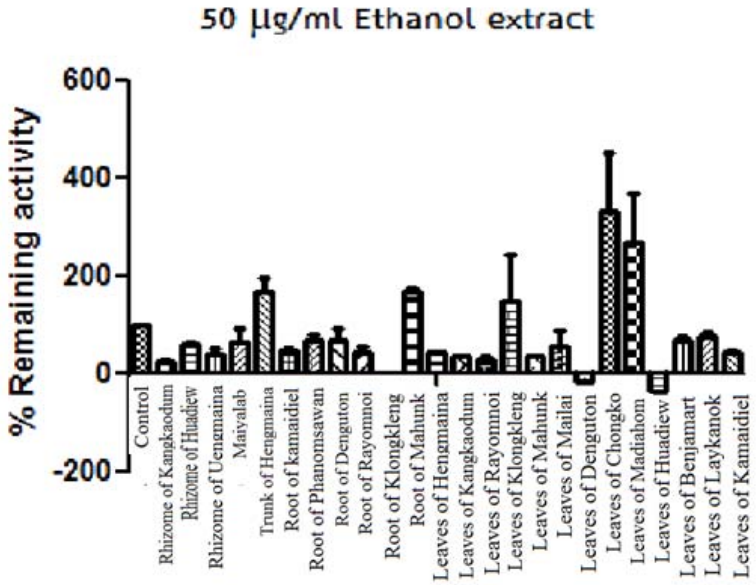

Figure 1. Effect of selected plant extract on *in vitro* CYP3A4 mediated Testosterone 6β-hydroxylation activity.

As CPR plays role in electrons transfer for P450-mediated metabolism *in vitro*, and the effect of plant extract on CPR activity could impair metabolic function of various P450 isoforms, resulting in diverse un-predictable side-effects of HDIs. Surprisingly, we found that none of selected extracts (50 µg/ml) affected *in vitro* cytochrome c reduction activity (data not show), implicated that the modulation in enzymatic activity of CYP3A4 or CYP2C9 enzyme did not through activation or inactivation of CPR enzyme.

According to these results, either pre-treatment or co-administration of medicinal plants should be in caution especially when supplement in patents who under healthcare treatment with CYP3A4 metabolizing drugs such as antibiotic (erythromycin), anti-arrhythmics (quinidine), benzodiazepines (diazepam, midazolam), immune modulators (cyclosporine), HIV antivirals (indinavir, saquinavir), antihistamines (astemizole, chlorpheniramine), calcium channel blockers (amlodipine, felodipine, verapamil), HMG CoA reductase inhibitors (atorvastatin, lovastatin), steroid 6 beta-OH (estradiol, progesterone, testosterone), miscellaneous (caffeine, cocaine, docetaxel, methadone, tamoxifen). For CYP2C9 enzyme, care must be taken in patient who co-administrated Phanomsawan (root), Uengmaina (rhizome), Huadiew (leave) and Denguton (leave) with NSAIDs **(**diclofenac, ibuprofen, meloxicam) Oral Hypoglycemic Agents **(**tolbutamide), Angiotensin II Blockers **(**losartan, irbesartan), Sulfonylureas **(**glyburide, tolbutamide), Others (amitriptyline, fluvastatin, tamoxifen S-warfarin) As the inhibition or inactivation could lead to high dose of drugs remaining, so it has side effect for body and failure of drug treatment. In contrast, activation by herbal constituents could lead to rapid elimination of drugs and failure of drug treatment [2, 3, 7]. These HDIs due to cytochrome P450 induction or inhibition, could lead to treatment failure, attenuation of the efficacy of concomitant medications and even death in some cases.

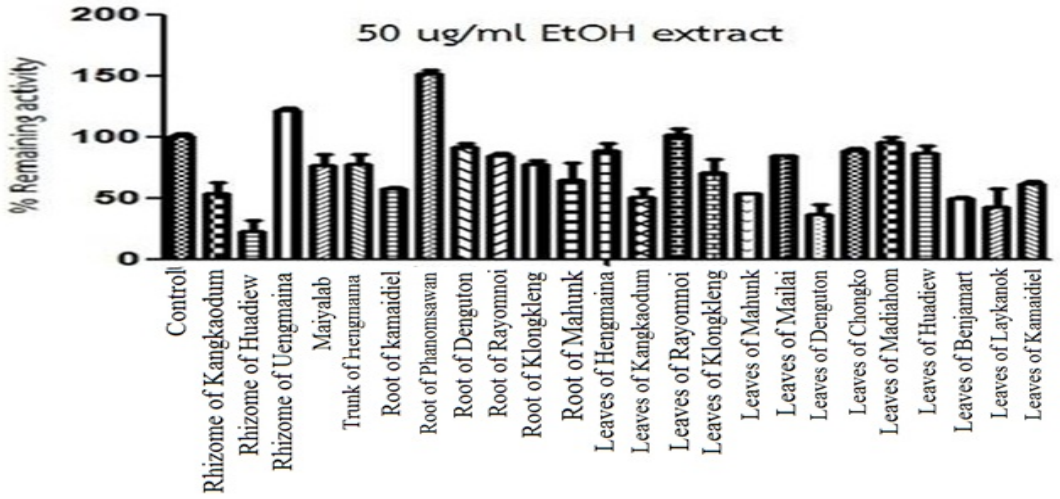

Figure 2. Inhibitory effect of herbs extract on tolbutamide 4-hydroxylation of CYP2C9.

4. CONCLUSIONS

In this study we investigate the inhibitory effect of commonly used medicinal folk plants from Ban-Ang-Ed official community forest project (The Chaipattana Foundation) on CYP3A4 and CYP2C9 enzyme. The results showed that plant extracts differently affected CYP3A4 mediated testosterone 6-β hydroxylation activity and CYP2C9 mediated tolbutamide 4-hydroxylation activity *in vitro*. Among tested plant extracts, *Aglaonema nitidum* extract (commonly use for treatment of hemorrhoid) could potently inhibited CYP3A4 and CYP2C9 activities, followed by *Picrasma javanica* (commonly used for antianemia and treatment of inflammation) and *Tacca chantrieri* (commonly use as elixir and aphrodisiac). According to the results, care must be taken for co-treatment of diseases by using medicinal folk plants in Ban-Ang-Ed with CYP3A4 and CYP2C9 metabolizing pharmaceutical drugs.

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Recombinant anti-oxidative peptides from algae protein waste hydrolysate expressed in *Escherichia coli* MG1655

Apapun Kongcharoen*[a], Ruethairat Boonsombat[b]
[a]Program in Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330
[b]Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, Thailand 10330

*felisamz@live.com phone +66-22188078 fax +66-22533543

ABSTRACT

Free radical is an electron missing atom which is unstable and has high energy. It generates as a byproduct from metabolism or in unusual conditions such as inflection, chemical and radioactive contamination. It can damage biomolecules including DNA and protein potentially resulting in several diseases. With pollution and current life style, antioxidant in the body may not be enough to eliminate free radical, so uptaking of alternative anti-oxidant will provide additional protection against free radical invaders. The amino acid peptide with the sequence VECYGPNRPQF, extracted from algae protein waste hydrolysate was found to be a potential antioxidant. Due to natural product extraction poses some problems, this research aims to construct recombinant *Escherichia coli* MG1655 to express this peptide. *E. coli* is widely used as an expression host because it easy to manipulate, grows rapidly in simple growth medium and many kinds of peptides have been successfully expressed in *E. coli.* Furthermore, this recombinant *E. coli* can be used for alternative source for anti-oxidative peptides production with similar activities to the native ones leading to more efficiency in large scale production. The 234 base pair DNA fragment containing 6 copies of the interested peptide linked by the codons of lysine was inserted into the *E. coli* expression vector, pQE-30Xa. Ampicillin resistant transformants are selected. DNA fragment encoding for the target peptide will be verified by restriction enzyme digestion and DNA sequencing. The expression and the anti-oxidative characterization of this recombinant peptide will be further study.

Keywords: Recombinant, Anti-oxidant, *Escherichia coli*

1. INTRODUCTION

Free radical is generated as a byproduct from aerobic metabolism, many endogenous systems, or in unusual conditions such as inflection, chemical and radioactive contamination. It is an unpaired electron atom which is unstable, sensitive, and has high energy. Free radical is considered as a potential cause of several diseases due to the ability to damage biomolecules including DNA and protein [1]. Unbalance of antioxidants and free radicals, called oxidative stress state, leads to oxidative damage of biomolecules. Mechanisms for free radical scavenging in the body may not be enough due to the accumulation of free radicals from pollution and current life style, so uptaking of antioxidants from other sources will provide additional protection. Many studies supported that receiving external antioxidant could protect and reduce the risk of some diseases [2]. Antioxidants can be synthetic or natural; however, the synthetic ones have been reported to be dangerous for human health because it is more durable which potentially remains in body for a longer period of time than the natural ones [3]. Therefore, antioxidants from natural sources, found in many organisms such as fungi, plants and algae, seems to be more attracted and widely applied including in pharmaceutical and food industries.

Macro and microalgae are widely applied as food supplement, animal feed and cosmetic products due to their several functional chemical ingredients and also some highly valuable molecules such as polyunsaturated fatty acid oil, supplements and pigments. It was reported that the peptide fraction extracted from algae protein waste, a byproduct from algae essence production of *Chlorella vulgaris*, had anticancer and antioxidant activities [4]. Moreover, the previous investigation by Sheih and co-workers also revealed a potential antioxidative peptide with the sequence VECYGPNRPQF which was extracted from this algae waste protein, generally discarded as animal feed, after being digested by pepsin [5].

Generally, natural product extraction encounters some problems such as large amount of raw material requirement, difficulty to control the quality of raw materials, high cost and time consuming. Recombinant DNA technology is an alternative to reduce these problems. The principle of this technique is to create recombinant DNA from two or more sources in an expression host. With the application of this technique, the production of recombinant peptide is expected to produce higher yield of production with less time and lower cost, and also relatively easy to be manipulated [6].

This research aims to construct a recombinant *E. coli* strain to express the potential antioxidative peptide originally found in algae waste hydrolysate. *E. coli* is widely used as an expression host because it is easy to be genetically manipulated, grows rapidly in simple growth medium and many kinds of peptides have been successfully expressed in this organism [7]. Furthermore, this recombinant *E. coli* is expected to be used as an alternative source for antioxidative peptides production with similar activities to the native ones. Due to small peptide, DNA fragment encoding 6 copies of this antioxidative peptide was synthesized. Lysine residue, chosen as a linker, for each copy could be removed by trypsin and carboxypeptidase B, and then six copies of small peptide of VECYGPNRPQF could be obtained from one recombinant peptide. The 234 base pair of this DNA fragment containing 6 copies was inserted into the *E. coli* expression vector, pQE-30Xa and then, transformed into *E. coli* to generate the expression strain. The expression and the antioxidative characterization of this recombinant peptide will be further studied.

2. MATERIALS AND METHODS

Strains and plasmids

All plasmid derivatives were transformed into HIT-DH5α *E. coli* (RBC Bioscience, Taiwan). The plasmid pCR®2.1-TOPO® (Invitrogen, USA) was used to maintain and amplify the synthesized DNA fragment. A pQE-30Xa plasmid (Invitrogen, USA) was used as an expression vector. *E. coli* was cultured in LB media. Ampicillin with final concentration of 100 µg/ml was added to maintain plasmids

Construction of *E. coli* strain for target antioxidative peptide expression

The 234 base pair DNA fragment encoding 6 copies of the antioxidative peptide with the sequence of 11 amino acids: VECYGPNRPQF with Lysine linker and *Xho*I restriction size at 5' and 3' terminal was synthesized as white powder of gBlocks Gene Fragments (Intergrated DNA Technologies, Inc). Then, the powder was resuspended in 20 µl TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). After that 15 µl reaction of 5 µl of diluted gBlocks® Gene Fragments, 1 µl of Taq polymerase, 1.5 µl of 0.05 mM dNTP, 1.5 µl of 1X *Taq* polymerase Buffer with 1.5 mM MgCl was performed at 70ºC for 15 to 30 minutes to amplify DNA fragment. The products from this reaction were ligated with pCR®2.1-TOPO® and then, transformed into HIT-DH5α *E. coli* according to TOPO® TA Cloning® Kit manual. White colonies of ampicillin resistant transformants were selected according to the blue/white screening

assay. By using restriction enzyme digestion, the verified plasmid was named as pWP2304. After that, the plasmids pWP2304 and pPICZαA were digested by *Xho*I, then ligated and transformed into HIT-DH5α *E. coli* competent cells. Single colonies of zeocin resistant transformants were selected by using restriction enzyme digestion and DNA sequencing (Macrogen, Korea). The verified plasmid was named as pWPTAαO.

The plasmids pWPTAαO and pQE-30Xa were digested by *Xho*I and *Sal*I restriction enzymes, respectively. Then, the approximately 230 base pair DNA band from pWPTAαO was ligated to 3500 base pair DNA band from pQE-30Xa by T4 ligase enzyme. The ligation reaction was transformed into *E. coli* MG1655. Plasmids from ampicillin resistant transformants were extracted, verified by *Xho*I and *Pme*I restriction enzyme digestion and DNA sequencing (Macrogen, Korea).

3. **RESULTS**

Synthesized 234 base pairs DNA encoding 6 copies of antioxidative peptide from algae waste protein of which separated by Lysine codon (Fig.1) was in the form of gBlock® Gene Fragments. The product was cloned into pCR®2.1-TOPO® plasmid and then, pPICZαA. The target gene fragment was screened by the blue/white screening assay and confirmed by *Xho*I digestion and DNA sequencing.

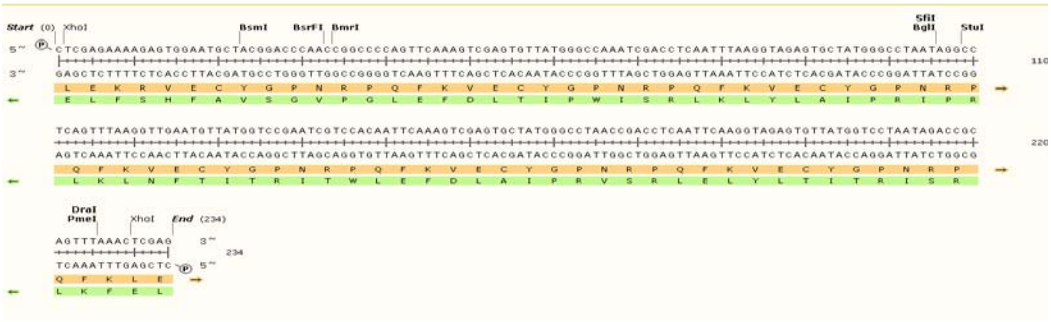

Figure 1. The sequence of DNA fragment encoding 6 copies of the antioxidative peptide with the sequence of 11 amino acids: VECYGPNRPQF. Each copy is linked by Lysine codon. The fragment contains *Xho*I restriction site at 5' and 3' terminal for further cloning.

The expression vector, pQE-30Xa was used as the vector for expressing VECYGPNRPQF antioxidative peptide in *E. coli*. The 234 base pair DNA fragment from pWPTAαO digested by *Xho*I (Fig 2B) was ligated to *Sal*I restriction site of pQE-30Xa (Fig.2A). This ligation reaction was transformed into *E. coli* MG1655. The selected Ampicillin resistant colonies are in the process of verifying by restriction enzymes and DNA sequencing.

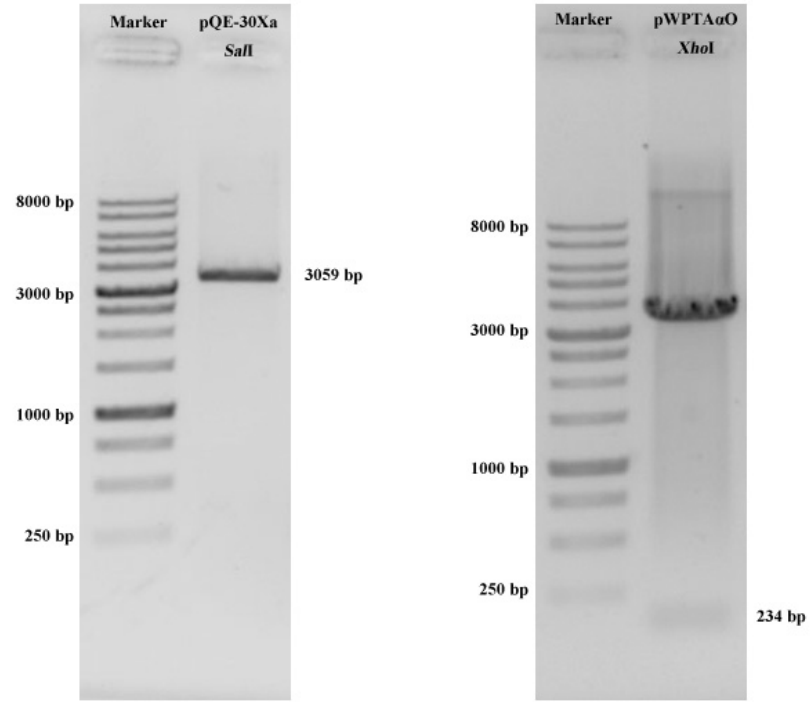

Figure 2. (A) The approximately 3509 base pair from pQE-30Xa after were digested by *Sal*I (B) The approximately 234 base pair of target DNA from pWPTAαO after were digested by *Xho*I. Both fragments were extracted from these 1% agarose gels for future ligation.

4. CONCLUSIONS

DNA fragment encoding 6 copies of antioxidative peptide originally from algae waste hydrolysate were cloned into pQE-30 Xa expression vector and transform into *E. coli* MG1655. The study is currently in the process of verification of recombinant expression vector. The *E. coli* strain containing verified plasmid will be further used for studying the target antioxidative peptide.

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Effect of domestic cooking methods on total anti-oxidant capacities, polyphenols, chlorophyll derivatives and carotenoids contents of selected local Thai green vegetables

Atinuch Nartnampong[a,b], Supatra Porasuphatana*[c], Worawit Kittiwongsunthorn[b]
[a]Graduate School Khon Kaen University, Khon Kaen, Thailand 40002
[b]Regional Medical Sciences Center 10 Ubon Ratchatani, Ubon Ratchatani, Thailand 34000
[c]Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand 40002

* psupatra@kku.ac.th phone +66-815452115 fax +66-43202379

ABSTRACT

Effects of domestic blanching and streaming on total anti-oxidant capacities (TAC), polyphenols, carotenoids, chlorophylls and pheophytins contents of selected local Thai green vegetables (Thorny tree, Dill, Chinese chive, Peninsular, Asiatic pennywort, Betel vine, Garden parsley, Vietnamese coriander, Swamp morning glory and Sweet basil) were investigated by FRAP and DPPH assay, Folin-Ciocalteu method and HPLC, respectively. Among all fresh vegetables, Vietnamese coriander contained the highest phenolic content and TAC. Dill had the highest chlorophylls and β-carotene contents while the highest content of lutein was found in Betel vine. Good correlation between chlorophylls and lutein contents was also observed. After cooking, phenolic content and TAC showed similar patterns in almost vegetables which were higher in some cooked vegetables compared to fresh samples. β-carotene and lutein contents increased in almost vegetables while chlorophylls content trended to decrease after cooking except Vietnamese coriander, Peninsular and Asiatic pennywort which increased after blanching. Chlorophylls significantly decreased in all streamed vegetables except Vietnamese coriander. Pheophytins content increased in all vegetables after cooking and most of pheophytins formation occurred in streaming vegetables. The overall results of this study demonstrated that changing of TAC, polyphenols, carotenoids, chlorophylls and pheophytins contents were dependent on vegetable type and cooking method which, long time of heat treatment such as streaming reduced chlorophylls content while, short time treatment such as blanching either increased or decreased TAC, polyphenols, carotenoids and chlorophylls contents. Results are expected to be beneficial for the use of these phytochemical contents from local Thai vegetables in various aspects.

Keywords: Green vegetable, Chlorophyll, Pheophytin, Carotenoids, Polyphenols, Anti-oxidant capacities, Cooking

1. INTRODUCTION

Phytochemical in plants have been increasing interest because of epidemiological evidences support that high consumption of fruits and vegetables reduce the risk of chronic disease such as cancer and cardiovascular [1,2]. Green leafy vegetables are rich source of phytochemical such as, chlorophylls and carotenoids. Polyphenols and antioxidant are also found [1-3]. Major source of chlorophylls and carotenoids for human consumption are from green vegetables with many types of preparations such as, fresh and cooked. Previous studies reported that vegetables with many types of cooking, boiling, streaming and microwaving resulted in different changing of phytochemical contents and antioxidant capacities. The results indicate that some cooking enhance nutritional levels of vegetables [4,5]. For Thailand, blanching and streaming are domestic and common cooking methods of vegetables, that usually found in Thai cuisine. However, few information were available for local Thai green vegetables cooked by different methods. Therefore, the aim of this study was to investigate the effects of domestic blanching and streaming on TAC, polyphenols, carotenoids, chlorophylls and pheophytin contents in 10 selected local Thai green vegetables, Thorny tree, Dill, Chinese chive, Peninsular, Asiatic pennywort, Betel vine, Garden parsley, Vietnamese coriander, Swamp morning glory and Sweet basil.

2. MATERIALS AND METHODS

Plants and cooking treatments

Ten species of freshly harvested local Thai green vegetables (Table 1) were purchased from local markets in Khon Kaen, Thailand. To obtain more homogeneous samples, each vegetable was prepared in batches of 150 g. Each batch was then divided into four equal portions. One portion was retained raw, and others were cooked. All cooking treatments were performed in triplicates. For blanching, vegetables were added to boiling tap water in a covered pot and cooked for 30 second. For streaming, a single layer of fresh vegetables was steamed into a domestic closed vessel using stream basket suspended above a small amount of boiling water. Cooking time was 5 min. After cooking, samples were freeze-dried then kept in seal bag and stored at -20°C.

Table 1 Thai local vegetable used in this study

Botanical names	Common names/Thai names	Plant part
Acacia pennata subsp. *Insuavia*	Thorny tree/ Cha-Om	Leaves
Anethum graveolens Linn.	Dill/ Phak-Chee-Laao	Leaves
Allium tuberosum Roxb.	Chinese chive/ Kui-chai	Leaves
Mentha cordifolia Opiz ex Fresen	Peninsular/ Saranae	Leaves
Centella asiatica (Linn.) Urban	Asiatic pennywort/ Boa-Bok	Leaves
Piper betel Linn.	Betel vine/ Cha-Poo	Leaves
Eryngium foetidum Linn.	Garden parsley/ Phak-Chee-Farang	Leaves
Polygonum odoratum Lour.	Vietnamese coriander/ Phak-Paew	Leaves
Ipomoea aquatica Forssk.	Swamp morning glory/ Phak-bung	Leaves
Ocimum basillicum L.	Sweet basil/ Horapha	Leaves

Preparation of vegetables extracts for carotenoids and chlorophylls contents

Chlorophylls and carotenoids were extracted according to the standard method [6] with minor modification. All preparations were extracted at 20°C. The residue was dissolved in 5 mL of acetone and filter before HPLC analysis. All experiments were done under dim light to prevent pigments degradation.

Determination of chlorophylls and carotenoids contents

The extraction of pigments was analyzed as described previously [7]. The HPLC mobile phase consisted of 90% acetonitrile and 100% ethyl acetate. The linear gradient system was used for the separation. The data was detected at 440 nm for carotenoids and 660 nm for chlorophylls and their derivatives.

Preparation of vegetables extracts for total polyphenols content and total antioxidant capacity (TAC)

Vegetables extracts for phenolic content and TAC were prepared using the method previously describe [8] with some modifications. Grounded sample 0.1 g was weighted, then 4 ml of acidic methanol/water (50:50, v/v; pH2) was added and the tube was shaken at room temperature for 1 h. The tube was centrifuged for 10 min and the supernatant is recovered. Four milliliters of acetone/water (70:30, v/v) is added to the residue, and shaking and centrifugation are repeated. Methanolic and acetonic extracts were combined and stored at -20°C until analysis.

Determination of total polyphenols content

The procedure followed the method of Herald et al.[9]. The absorbance of samples and standard were measured at 765 nm by a microplate reader. Gallic acid was used as a standard.

Determination of antioxidant capacity

Ferric reducing antioxidant power (FRAP) assay

The procedure followed the method previously described with minor modification [10]. Vegetable extract (25 µl) was incubated with 175 µl of FRAP solution in the dark at 37°C for 30 min and the absorbance at 595 nm was determined by a microplate reader. Trolox was used as a standard.

2,2-Diphenyl-1picryhydrazyl (DPPH) radical scavenging capacity assay

DPPH assay was measured by the method previously described [9] with minor modification. Vegetable extract (25 µl) was incubated with 175 µl of DPPH solution in the dark for 30 min and the absorbance at 517 nm was determined by a microplate reader. Trolox was used as a standard.

Statistical analysis

All data were calculated as mean ± SE. One-way analysis of variance (ANOVA), Duncan's multiple rang test and correlation were performed by SPSS (version 17.0, Chicago, IL, USA).

3. RESULTS

Effect of domestic cooking methods on chlorophyll derivatives and carotenoids contents

Fresh vegetables were found to vary in chlorophylls content. Dill had the highest chlorophylls content followed by Betel vine**,** Garden parsley, Chinese chive, and Asiatic pennywort (Table 2). Whereas, Vietnamese coriander had the lowest chlorophylls content. When blanched, Thorny tree, Dill, Chinese chive, Garden parsley, and Sweet basil, chlorophylls retained their chlorophylls content. Chlorophylls of all others (Asiatic pennywort, Peninsular, and Vietnamese coriander) were dramatically increased following blanching process. Chlorophylls content of almost analyzed vegetables changes significantly after long heat treatment, streaming. Pheophytins content increased in all vegetables after cooking and most of pheophytins formation occurred during streaming process.

Among all fresh vegetables, top three of vegetables which contained the highest β-carotene content were Dill, Asiatic pennywort, and Chinese chive. The highest content of lutein was found in Betel vine, followed by Dill, Garden parsley, and Asiatic pennywort. Good correlation between chlorophylls and lutein contents in fresh vegetables was also observed (r^2= 0.88). β-carotene and lutein contents when compared between fresh and cooked vegetables were found to be statistically significant ($p < 0.05$) in almost vegetables except Chinese chive that blanching and streaming did not affect carotenoids contents. β-carotene and lutein contents increased in almost cooked vegetables and only those of Betel leave decreased significantly.

Effect of domestic cooking methods on total antioxidant capacities, and polyphenols.

Among all fresh vegetables, Vietnamese coriander had the highest phenolic content and TAC. After cooking, phenolic content and TAC showed similar patterns in almost vegetables which were higher in some cooked vegetables compared to fresh samples. In four of ten cooked vegetables, Peninsular, Asiatic pennywort, Garden parsley, and Sweet basil, polyphenols significantly increased compared to fresh samples. While polyphenols and TAC in Thorny tree, Dill, and Betel vine significantly decreased after blanching. However, two cooking methods did not affect the phenolic content of Chinese chive.

Table 2 Effect of cooking methods on phytochemical contents of local Thai green vegetables (mg/g dry weight; mean ± SE). Mean in same column with different letter is statistically significant ($p < 0.05$)

Vegetables/ cooking method	Chlorophylls	Pheophytins	Lutein	β-carotene
Thorny tree				
Fresh	5.33 ± 0.05[a]	0.36 ± 0.01[a]	0.60 ± 0.01[a]	0.37 ± 0.01[a]
Blanching	4.73 ± 0.25[a]	0.32 ± 0.01[b]	0.60 ± 0.05[a]	0.34 ± 0.02[a]
Streaming	3.55 ± 0.24[b]	1.91 ± 0.02[c]	0.76 ± 0.04[b]	0.36 ± 0.01[a]
Dill				
Fresh	15.79 ± 0.28[a]	0.18 ± 0.00[a]	1.11 ± 0.02[a]	0.80 ± 0.02[b]
Blanching	15.83 ± 0.67[a]	0.41 ± 0.01[b]	1.44 ± 0.07[b]	1.03 ± 0.03[a]
Streaming	11.02 ± 0.55[b]	2.08 ± 0.01[c]	1.29 ± 0.06[a]	0.95 ± 0.04[a]
Chinese chive				
Fresh	11.51 ± 0.23[a]	0.15 ± 0.00[a]	0.80 ± 0.03[a]	0.62 ± 0.01[a]
Blanching	10.99 ± 0.10[a]	0.26 ± 0.00[b]	0.83 ± 0.01[a]	0.65 ± 0.02[a]
Streaming	8.61 ± 0.21[b]	1.60 ± 0.01[c]	0.85 ± 0.01[a]	0.65 ± 0.01[a]
Peninsular				
Fresh	11.33 ± 0.02[a]	0.16 ± 0.01[a]	0.85 ± 0.01[a]	0.58 ± 0.00[a]
Blanching	18.71 ± 0.02[b]	0.36 ± 0.00[b]	1.54 ± 0.01[b]	0.97 ± 0.01[b]
Streaming	10.62 ± 0.01[c]	2.53 ± 0.01[c]	1.22 ± 0.01[c]	0.90 ± 0.01[c]
Asiatic pennywort				
Fresh	11.44 ± 0.22[a]	0.30 ± 0.03[a]	0.90 ± 0.01[b]	0.64 ± 0.01[b]
Blanching	14.20 ± 0.46[b]	0.45 ± 0.01[b]	1.11 ± 0.03[a]	0.77 ± 0.02[a]
Streaming	8.82 ± 0.06[c]	2.06 ± 0.04[c]	1.05 ± 0.02[a]	0.76 ± 0.01[a]
Betel vine				
Fresh	14.65 ± 0.16[a]	0.13 ± 0.00[a]	1.37 ± 0.02[a]	0.46 ± 0.01[b]
Blanching	4.64 ± 0.55[b]	0.22 ± 0.01[a]	0.85 ± 0.03[b]	0.42 ± 0.01[a]
Streaming	7.28 ± 0.26[c]	1.68 ± 0.08[b]	1.04 ± 0.01[c]	0.45 ± 0.01[a]
Garden parsley				
Fresh	11.99 ± 0.20[a]	0.43 ± 0.00[a]	1.03 ± 0.02[a]	0.59 ± 0.01[a]
Blanching	12.45 ± 0.23[a]	0.38 ± 0.01[a]	1.20 ± 0.02[b]	0.73 ± 0.01[b]
Streaming	8.37 ± 0.47[b]	1.97 ± 0.05[b]	1.03 ± 0.00[a]	0.60 ± 0.01[a]
Vietnamese coriander				
Fresh	4.07 ± 0.05[a]	0.77 ± 0.04[a]	0.42 ± 0.00[b]	0.48 ± 0.00[b]
Blanching	10.94 ± 0.60[b]	0.63 ± 0.03[a]	0.71 ± 0.03[a]	0.81 ± 0.03[a]
Streaming	4.15 ± 0.44[a]	3.67 ± 0.08[b]	0.65 ± 0.04[a]	0.74 ± 0.04[a]
Swamp morning glory				
Fresh	10.09 ± 0.05[a]	0.25 ± 0.00[a]	0.68 ± 0.01[a]	0.55 ± 0.00[b]
Blanching	7.00 ± 0.13[b]	0.22 ± 0.01[b]	0.88 ± 0.01[b]	0.73 ± 0.02[a]
Streaming	7.65 ± 0.12[c]	1.93 ± 0.01[c]	1.08 ± 0.00[c]	0.75 ± 0.01[a]
Sweet basil				
Fresh	10.58 ± 0.10[a]	0.17 ± 0.00[a]	0.66 ± 0.01[b]	0.46 ± 0.01[b]
Blanching	10.85 ± 0.32[a]	0.30 ± 0.01[b]	0.89 ± 0.04[a]	0.68 ± 0.02[a]
Streaming	7.86 ± 0.29[b]	1.87 ± 0.01[c]	0.89 ± 0.03[a]	0.68 ± 0.02[a]

4. CONCLUSIONS

The overall results of this study demonstrated that changing of TAC, polyphenols, carotenoids, chlorophylls and pheophytins contents in vegetables were dependent on vegetable type and cooking method which, long time of heat treatment such as streaming reduced chlorophylls content while, short time treatment such as blanching either increased or decreased TAC, polyphenols, carotenoids and chlorophylls contents. Results suggest that some cooking methods enhance nutritional levels by increasing antioxidant capacities and phytochemical contents. These effects are expected to be beneficial for the use of Thai vegetables especially Asiatic pennyworth and Peninsular

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Stability of encapsulated Yanang leaves (*Tiliacora triandra*) extract with calcium alginate hydrogel beads

Jittra Singthong*[a], Jirawan Oonmetta-aree[b]
[a]Department of Agro-Industry, Faculty of Agriculture, Ubon Ratchathani University, Warinchamrap, Ubon Ratchathani, Thailand 34190
[b]Food Science and Technology Program, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Muang, Nakhon Ratchasima, Thailand 30000

*jittrawara@gmail.com phone +66-45353500 fax +66-45288373

ABSTRACT

Yanang (*Tiliacora triandra*) is used as a vegetable and herb in northeast Thailand. This work investigated the stability of bioactive compounds in encapsulated Yanang leaves extract which was evaluated during storage at different temperatures. In addition, the *in vitro* releasing the bioactive compounds in gastrointestinal tract (GI tract), stomach (pH 1.2) and small intestine (pH 7.4) at 37ºC was studied. Finally, encapsulated Yanang leaves extract was added to a food model system (soft drink model system) to find the effect of pH (pH 2, 3, 4, 5, 6, and 7) and temperature/time (65 ºC for 30 min and 77 ºC for 1 min) treatments on the release of bioactive compounds. The study of bioactive compounds in encapsulated Yanang leaves extract showed only a gradually reduction during storage at 4-10 ºC for 3 months (> 50% residual). Moreover, encapsulated Yanang leaves extract was released in small intestine (pH 7.4) more than in stomach (pH 1.2) at 24 hr and in the soft drink model system encapsulated Yanang leaf extract was released at pH 4 and 77 ºC for 1 min. In conclusion, this study demonstrates the potential of using hydrogel beads process in optimizing the encapsulation of herbal extract and it represents an interesting food additive option for incorporation into functional foods.

Keywords: Encapsulation, Yanang, *Tiliacora triandra*, Hydrogel, Calcium alginate, Bioactive compounds

1. INTRODUCTION

The herbal-related products used as food or food additives, food supplements, tradition medicines, etc. The world Bank has projected the global market for herbal products to grow from USD 200 billion in 2008 to USD 5 trillion in 2050 [1]. A key factor for the rapid growth of the market is the growing knowledge and confidence of consumers in natural product or medicines. In addition, bioactive compounds of herbal plants such as antioxidant, phenolic compounds, carotenoids and chlorophyll have shown to have multiple functional and remedial properties [2]. In contrast, the stability of bioactive compounds was unstable causing pH, light, oxygen, temperature and enzymatic activities [3]. Consequently, the stabilization of bioactive compounds could be improved using encapsulation technologies.

Yanang (*Tiliacora triandra*) is herbal in the northeast of Thailand. The extract from the root of Yanang is used as a medicinal herb for treating fever in local people causing it is rich in alkaloids [4]. Besides, some profile reports of this plant have shown to high antioxidant, high vitamin and moderate levels of calcium and iron [5].

Hydrogel is a commonly used material for encapsulation due to its capability to absorb large amount of water or biological fluids. Among many materials, calcium-alginate hydrogel is the most widely used due to several advantageous features such as non-toxicity, biocompatible, easily produced; thermally and chemically stable [6]. Solutions of alginate can be cross-linked by a mild gelling reaction with calcium ions to form a hydrogel. Alginate gel structure is relatively stable at acidic pH, but it is easily swollen and disintegrated under mild alkali conditions [7].

The goal of our research was to develop an effective bioactive compounds delivery system using an encapsulation technique for achieving the controlled release and improving bioavailability of a model nutrient.

2. MATERIALS AND METHODS

Materials

Yanang leaves, procured from a local market in the northeast of Thailand (Ubon Ratchathani Province) were cleaned with water to remove dust and infected leaves were separated. The dried leaves were prepared using the method of Singthong [8]. Encapsulation matrix polymer was calcium alginate and calcium chloride ($CaCl_2$). Sodium alginate was obtained from ISP Technologies Inc., Thailand.

Encapsulation

Yanang leaves extract was prepared using the method of Oonsivilai [9] with slightly modification. The encapsulation process was conducted using the method of Chan [1] with slightly modification. Optimized condition for the encapsulation was at the ratio of core to wall 1.60:8.40 in alginate concentration at 1% (w/v) and 1% (w/v) of $CaCl_2$ solution [10].

Stability of encapsulation

The stability of encapsulation was conducted using the method of Nori [11] with slightly modification. Encapsulation of Yanang extract was storage at cold temperature (4-10°C) and room temperature (25-30°C) for 3 months. Bioactive compounds were analyzed every week for 3 months.

***In vitro* releasing property of bioactive compounds**

In vitro bioactive compounds releasing property were evaluated using the encapsulated produced under the optimal condition of Yanang extract encapsulation with calcium-alginate hydrogel. To mimic the digestive tract in human body, 0.05 M HCl (pH 1.2) with 0.2% NaCl was used as a stimulated gastric fluid and 0.05 M phosphate buffer (pH7.4) as a stimulated intestinal fluid, but this simulation system did not contained digestive enzymes [7].

Application of encapsulation in soft drink-related model system

Effect of pH

The study the effect of pH changes on bioactive compounds release was performed following the method of Hansson [12] with slightly modification.

Effect of temperature/time

The study the effect of temperature and time changes (Low temperature long time (LTLT) as 65°C for 30 min and high temperature short time (HTST) as 77°C for 1 min) on bioactive compounds release was studied following the method of Pernice [13] with slightly modification.

Analysis of bioactive compounds

Total soluble phenolic constituents of the encapsultion of Yanang leaves extract using the Folin-Ciocalteu reagent with gallic acid as standard. [9]. Antioxidant activity was evaluated in accordance with the DPPH method

[14]. Total carotenoids and chlorophyll were carried out using the method of Oliveira [15] with slightly modification.

3. RESULTS AND DISCUSSION

The optimised Yanang leaves extract encapsulation was identified as the ratio of core material to wall material 1.60:8.40, alginate concentration 1% and concentrate of $CaCl_2$ solution 1%. Stability of bioactive compounds in encapsulated Yanang leaves extract was evaluated under various conditions of storage temperature. The total antioxidant contents of Yanang leaves extract encapsulations were decreased by 62% at the end of three months storage period at 25°C. At 4-10°C storage temperature, loss of total antioxidant content was determined as 34% (Figure 1). An increase in storage temperature led to decrease in bioactive compounds. Storage at 4-10°C increased stability of bioactive compounds 2 times according to 25°C storage temperature.

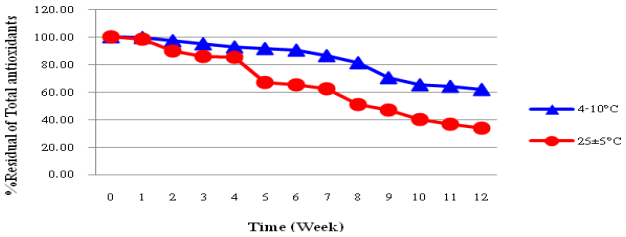

Figure 1. %Residual of total antioxidant contents of Yanang leaves extract encapsulations at storage temperature 25 ± 5°C and 4-10°C for 3 months.

The desirable encapsule for the nutrient delivery system should protect core material without acid-damaged destruction under strong acidic environment, which means that the coating material should be resistant against acid. Once the encapsule reach small intestine, it should initially release the core material rapidly, and keep the releasing rate to maintain the effective concentration of core material as the absorption rate decreases [7]. The Yanang extract encapsule produced under the optimal yield and bioactive compounds condition was applied for *in vitro* bioactive compounds releasing experiment in the simulated gastric fluid (pH1.2) and simulated intestine fluid (pH7.4) for 24 h period (Figure 2). Encapsulated Yanang leaves extract was released in small intestine (pH 7.4) more than in stomach (pH 1.2) at 37°C for 24 h.

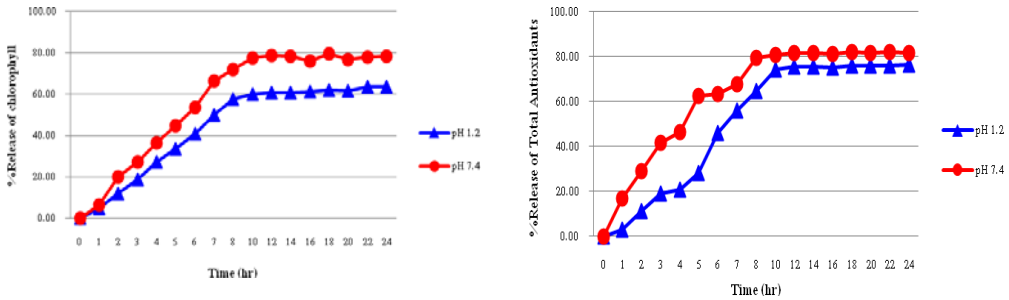

Figure 2. %Release of bioactive compounds of Yanang leaves extract encapsulations pH 1.2 and pH 7.4 at 37°C for 24 h.

Citric acid is the acid most often used in the soft drink industry. To study their effect on bioactive compounds release they were added in variable concentrations to a soft drink model system containing water and Yanag leaves extract encapsules. These results indicate that pH4 showed the highest amount of bioactive compounds (Table 1). Bioactive compounds capacity is shown in Table 2. Using high temperature short time (HTST), bioactive compounds were activity about 2-6 times greater than low temperature long time (LTLT).

Table 1. Effect of pH on bioactive compounds of Yanang leaves extract encapsulations.

pH	Total carotenoids (mg/g)	Total chlorophyll (mg/g)	Total phenolics (mg GAE/g)	Total antioxidant (AEAC mg/g)	IC_{50} (mg/g)
2	$51.27 \pm 0.47^{c/1}$	15.99 ± 0.20^{d}	57.03 ± 1.39^{d}	191.99 ± 1.55^{c}	8.13 ± 0.01^{c}
3	56.20 ± 0.66^{b}	17.49 ± 0.27^{c}	72.76 ± 1.39^{b}	224.77 ± 1.03^{b}	8.00 ± 0.00^{d}
4	61.00 ± 0.09^{a}	28.07 ± 0.48^{a}	90.46 ± 5.56^{a}	263.39 ± 5.16^{a}	7.89 ± 0.02^{e}
5	50.53 ± 1.51^{cd}	19.42 ± 0.41^{b}	79.64 ± 2.73^{b}	225.50 ± 7.73^{b}	8.08 ± 0.03^{c}
6	49.87 ± 3.39^{cd}	15.99 ± 0.48^{d}	64.90 ± 1.39^{c}	180.70 ± 7.73^{c}	8.86 ± 0.03^{b}
7	47.07 ± 0.94^{d}	9.66 ± 0.55^{e}	27.04 ± 2.09^{e}	103.10 ± 2.58^{d}	9.66 ± 0.01^{a}

[1/] Values with different letters in the same column are significantly different ($p \leq 0.05$).

Table 2. Effect of temperature and time on bioactive compounds of Yanang leaves extract encapsulations.

Temp/time	Total carotenoids (mg/g)	Total chlorophyll (mg/g)	Total phenolics (mg GAE/g)	Total antioxidant (AEAC mg/g)	IC_{50} (mg/g)
65°C:30 min	11.56 ± 0.56	11.18 ± 1.33	24.58 ± 1.97	159.32 ± 28.50	8.58 ± 0.06
77°C:1 min	$68.67 \pm 2.92^{*/1}$	$48.63 \pm 5.49^{*}$	$77.68 \pm 8.73^{*}$	$341.59 \pm 41.49^{*}$	$7.57 \pm 0.17^{*}$

[1/] Values with different letters in the same column are significantly different ($p \leq 0.05$).

4. CONCLUSION

The hydrogel beads method can give stabilize bioactive compounds. Storage at 4-10°C increased stability of bioactive compounds 2 times according to 25°C storage temperature. In the simulated intestine fluid (pH 7.4), the amount of released bioactive compounds was greater than in the simulated gastric fluid (pH 1.2). Encapsulated Yanang leaves extract was released at pH 4 and 77°C for 1 min in the soft drink model system. From this study, encapsulation of Yanang leaves extract was an interesting in food addition for incorporation into functional foods.

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Extraction of coconut oil by using Yan-Pang-Hom (*Paederia linearis*) extract

Jirawan Oonmetta-aree*[a], Tanida Phatisena[b], Jittra Singthong[c]
[a]Food Science and Technology Program, Faculty of Science and Technology,
Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima, Thailand 30000
[b]Faculty of Public Health, Nakhon Ratchasima Rajabhat University,
Nakhon Ratchasima, Thailand 30000
[c]Department of Agro-Industry, Faculty of Agriculture, Ubon Ratchathani University,
Warinchamrap, Ubon Ratchathani, Thailand 34190

*jirawan_o@yahoo.com phone+66-44009009 ext.1325 fax+66-44272939

ABSTRACT

Coconut oil contains the amount of medium chain fatty acids (MCFA) and nearly 48-53% lauric acid, which is benefit to various functional properties including digestibility. Yan-Pang-Hom (*Paederia linearis*: YPH) is a food plant and herb in Thailand. This research was to study the various YPH extracts concentration and different temperature for coconut oil extraction and determine this coconut oil qualities. Coconut milk and YPH extracts were mixed in various ratios (100:0, 100:5, 100:10, 100:15 and 100:20 (w/w)) and allowed to separate for 7-27 h. YPH extracts were able to separate the coconut oil from coconut milk within 7 h, which were shorter time than the fermentation (100:0) at 27 h. Oil yield (about 108-110 ml) were not difference ($p>0.05$) in various YPH extracts concentration. The coconut milk samples from ratio 100:5 were incubated at 20, 30 and 40ºC and detected the extraction times of coconut oil. It showed that extraction times were 98, 7 and 4 h, respectively. After that, the extracted oil (40 ºC, 4 h) was steamed at 80ºC for 5 min. The qualities of oil with YPH extract (ratio 100:5, 40ºC for 4 h) was moisture content (0.14 ± 0.03), total acidity (0.43 ± 0.01), iodine value (4.65 ± 0.29), saponification value (257.80 ± 1.49), peroxide value (2.59 ± 0.12), color (L* = 65.25 ± 0.38, a* = 2.52 ± 0.06, b* = -11.06 ± 0.30) and total viable counts were less than 10 CFU/ml, which was met the quality of the proposed International Standard by the Asian and Pacific Coconut Community (APCC). In conclusion, coconut oil from coconut milk with YPH extract concentration at the ratio of 100:5 (w/w), at 40ºC for 4 h was a method that reduced extraction time when compared to traditional fermentation.

Keywords: Coconut oil, Yan-Pang-Hom, *Paederia linearis*, Coconut milk, Extraction, Fermentation

1. INTRODUCTION

Coconut oil contains the amount of medium chain fatty acids (MCFA) and nearly 48-53% lauric acid, which is benefit to various functional properties including digestibility [1]. It is extracted by various methods such as dry or wet processing. Dry processing is widely used for coconut oil extraction by pressing copra follow by refining, bleaching and deodorizing [1-3]. Coconut oil from wet processing is obtained by the extraction of the coconut milk and destabilizing emulsion without thermal treatment, is known as virgin coconut oil (VCO). Several methods for VCO extraction are chemical solvent extraction, fermentation and enzymatic extraction etc. These methods, which are wet processing, separate the coconut milk into two layers: upper coconut oil layer and lower aqueous layer since coconut milk is broken emulsion. VCO from these methods is higher quality than dry processing [3-4].

Yan-Pang-Hom (*Paederia linearis*: YPH) is a food plant and herb in Thailand. Its root is rich in amylase enzyme which is used to be an ingredient in traditional glutinous rice cracker in northeast of Thailand for increasing sweet. Some reports were indicated various enzymes (amylase, protease etc.) were able to separate the coconut oil from coconut milk [3]. Therefore, YPH is utilized as enzyme in order to degrade components of the structural cell wall of coconut milk for extracting coconut oil or VCO. YPH extracts are investigated optimum concentration and temperature for coconut oil extraction and the obtained coconut oil is determined the chemical, physical and microbiological properties.

2. MATERIALS AND METHODS

Preparation of Materials

Coconut milk was prepared from squeezing the grated coconut meat mixed with water (1:1 w/w) by using coconut milk squeezer machine. Coconut milk was used to extract the coconut oil.

Roots of Yan-Pang-Hom (*Paederia linearis*: YPH) were washed in filtered water and soaked in 70% ethanol for 5 min before peeling, and then were grinded in a mortar. Grounded YPH root was mixed with filtered water (1:1 w/w), extracted, and squeezed through a layer of cheeseclothes. The filtrate was YPH extract which was used to extract coconut oil from coconut milk.

Extraction of coconut oil

Optimum concentration of YPH extracts

Coconut milk and YPH extracts were mixed in various ratios (100:0, 100:5, 100:10, 100:15 and 100:20 (w/w)) at room temperature (30°C) and allowed to settle and separate into two layers: upper coconut oil layer and lower aqueous layer. The amount of all treatment of extracted coconut oil was observed every 30 min until steady. The obtained oils were centrifuged (5,000 rpm, 15 min) and oil layer was separated and then steamed at 80°C for 5 min.

Optimum temperature for extraction

Coconut milk and optimum condition of YPH extract was mixed and incubated at 20, 30 and 40°C and observed the yield of separated oil every 30 min until steady. The obtained oils were centrifuged (5,000 rpm, 15 min) and oil layer was separated and then steamed at 80°C for 5 min.

Analysis of coconut oil properties

Extracted coconut oil from the YPH extract treatment, which showed the shortest extraction time, was selected for determination and comparison of chemical and microbiological properties with coconut oil from traditional fermentation, heat treatment and commercial coconut oil according to the proposed International Standard by the Asian and Pacific Coconut Community (APCC).

Determination of chemical and physical properties

The moisture content, acid value and peroxide value of extracted coconut oil were determined by AOAC (2000) method. The iodine value was determined according to AOCS Official Method 1d – 92 (1997). The saponification value was determined according to Paquot (1979). Color was measured by Hunter color value (L*, a*, b*).

Determination of microbiological properties

Total viable count of extracted coconut oil was performed using a standard plate count agar (Hi-media, India) and incubated at 35°C for 24 h.

Statistical analysis

The statistical analysis was carried out using SPSS statistic program (Version 17) for Window (SPSS Inc. Chicago, IL). The condition of coconut oil extraction and chemical of coconut oil properties were evaluated using one-way analysis of variance (ANOVA) and mean comparison was performed by Duncan's Multiple Range Test significant difference at a 5% level.

3. RESULTS

Optimum concentration of YPH extracts

YPH extracts were mixed into the coconut milk and stand to settle at room temperature. After that, coconut milk was separated to two layers: top coconut oil layer and lower aqueous layer. After 7 h extraction, the coconut oil was separated from the milk with all concentration of YPH extracts (100:5, 100:10, 100:15 and 100:20 w/w). They were shorter time than the fermentation (100:0) at 27 h. Oil contents (about 108-110 ml) were not difference ($p>0.05$) in all treatments (Table 1).

Table 1. Volume and extraction times of coconut oil from coconut milk with various concentration of Yan-Pang-Hom extract

Ratio of coconut milk : YPH extracts	Extraction times (h)	Volume of extracted coconut oil^{ns} (ml)
100:0	27	108.33 ± 5.77
100:5	7	110.00 ± 1.15
100:10	7	109.33 ± 5.00
100:15	7	109.33 ± 1.15
100:20	7	109.33 ± 1.15

^{ns} in the same column indicate no significant differences between volume of extracted coconut oil ($p>0.05$)

Means ± SD (n=3)

Extraction time of all concentration of YPH extracts were not difference therefore ratio of coconut milk:YPH extract at 100:5 was selected to study the suitable incubation temperature.

Optimum temperature for extraction

YPH extract (100:5) was mixed into coconut milk and incubated at 20, 30 and 40°C. At 40°C, YPH extract showed high efficacy in oil extraction (Table 2), it may be due to this condition was suitable temperature for activities of enzymes in YPH extract. YPH extract contained the protease and amylase for hydrolyzing the starch and protein in the cell wall structure of the coconut milk [5]. Protease hydrolyzed peptide bonds in the interior of the polypeptide chain therefore coconut milk emulsion was destabilized and caused oil droplets aggregation. Protein fragments moved towards the aqueous phase leading to phase separation [3].

Table 2. Volume and extraction times of coconut oil from coconut milk with Yan-Pang-Hom extract (100:5) at various incubation temperature

Incubation temperature (ºC)	Extraction times (h)	Volume of extracted coconut oil^{ns} (ml)
20	98	120±0.00
30	7	120±0.00
40	4	120±0.00

^{ns} in the same column indicate no significant differences between volume of extracted coconut oil ($p>0.05$)

Means ± SD (n=3)

Analysis of coconut oil properties

Extracted coconut oil from the YPH extract treatment (100:5), which showed the shortest extraction time, was selected for determination and comparison of chemical and microbiological properties with coconut oil from traditional fermentation, heat treatment and commercial coconut oil according to the proposed International Standard by the Asian and Pacific Coconut Community (APCC) (Table 3-4).

Coconut oil from heat treatment had lower L* value (darker yellow) than other treatments because the oil was oxidized by heat [6]. Coconut oil from YPH extract had color values (L*, a*, b*) similar to commercial coconut oil (oil from dry processing) (Table 3).

Table 3. The L*, a*, b* values of coconut oil from various extractions

Coconut oil from various methods	L*	a*	b*
YPH extraction	67.05±0.49[a]	3.06±0.06[b]	-13.72±0.07[a]
Traditional fermentation	66.48±0.25[b]	1.91±0.06[d]	-9.43±0.09[c]
Heat treatment	65.25±0.38[c]	2.52±0.06[c]	-11.06±0.30[b]
Commercial coconut oil	66.38±0.09[b]	3.21±0.05[a]	-13.86±0.10[a]

Different superscripts in the same column indicate significant differences between samples ($p \leq 0.05$), Means ± SD (n=9)
L* = Lightness (0 = black, 100 = white), a* = (+ red, - green), b*(+ yellow, - blue)

Table 4. Chemical properties of coconut oil from various extractions

Coconut oil from various methods	Moisture content (%)	Acidity (%)	Iodine value (iodine/100 oil)	Saponification value[ns] (mg KOH/g oil)	Peroxide value (meq peroxide oxygen/kg oil)
YPH extraction	0.17±0.10[b]	0.46±0.01[a]	7.34±2.35[a]	255.05±4.19	2.21±0.41[b]
Traditional fermentation	0.34±0.04[a]	0.42±0.01[b]	5.33±0.28[b]	254.60±2.50	2.22±0.05[b]
Heat treatment	0.14±0.03[b]	0.43±0.00[b]	4.71±0.33[b]	257.49±1.66	2.61±0.13[a]
Commercial coconut oil	0.21±0.05[b]	0.32±0.01[c]	4.29±0.13[b]	254.13±4.34	1.55±0.02[c]

[ns] in the same column indicate no significant differences between volume of extracted coconut oil ($p > 0.05$)
Different superscripts in the same column indicate significant differences between samples ($p \leq 0.05$), Means ± SD (n=9)

The chemical properties of extracted coconut oil were showed in Table 4. The extracted coconut oil was heat at 80°C for reducing the microbial contamination therefore total viable count was less than 10 CFU/ml. Peroxide value of oil from heat treatment was higher than other treatments, indicating this oil had low stability against oxidation [3]. Iodine value indicates a degree of saturation and unsaturated lipid content. The extracted oil (YPH extraction) showed high value, which indicated high unsaturated lipid content leading to a high sensitive to oxidative rancidity [3, 7]. Chemical, physical and microbiological properties of the extracted coconut oil from YPH extract indicated that had values within the limits of Asian and Pacific Coconut Community (APCC) standards for VCO.

4. CONCLUSIONS

Coconut oil from coconut milk with YPH extract concentration at the ratio of 100:5 (w/w), at 40°C for 4 h was a method that reduced extraction time when compared to traditional fermentation. The obtained coconut oil from YPH extract had % moisture content, % acidity, iodine value, saponification value and peroxide value within the limits of International Standard by the APCC. YPH extract was able to one of choices for coconut oil extraction method.

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Heavy Metal Determination of Ceramics Wares Imported from China into Thailand

Pannraphat Takolpuckdee[a], Napattaorn Buachoon[a], Sekporn Tansripraparsiri*[b]
[a]Department of Science Education, Faculty of Science and Technology, Valaya Alongkorn Rajabhat University under the Royal Patronage, Pathumthani, Thailand 13180,
[b]Department of Ceramic Technology, Faculty of Industrial Technology, Valaya Alongkorn Rajabhat University under the Royal Patronage, Pathumthani, Thailand 13180

*sekporn@yahoo.com phone +66-29093042 fax +66-29093041

ABSTRACT

Traditional pottery imported from China or other countries may be improperly glazed, and the glaze used to make the pottery may contain large amounts of lead and other heavy metals. Chineseceramic wares were randomly selected from products available in the shpos at Phatumtani Province, Sa Kaeo Province, and Nakhon Nayok Province in Thailand, and analyzed to determine the levels of heavy metals (Pb, Cd, Cu, Co, Cr, Zn, and Mn) in the products. All the ceramic wares contain heavy metals in varying concentrations. The results ranged from 356.21 - 961.32 μg/ml, 51.63 – 81.21 μg/ml, 155.00 – 778.00 μg/ml, 163.33 – 548.33 μg/ml,245.81 – 639.42 μg/ml, 4184.83 – 7833.30 μg/ml, and 4122.12 – 6901.43 μg/ml for Pb, Cd, Cu, Co, Cr, Zn, and Mn respectively. About 70% of the ceramic ware shows Pb concentration higher than 500 μg/ml whereas cadmium levels are generally low being less than 90 μg/ml. With the observed high levels of heavy metals in the ceramics used in this study, it is possible that acidic substances can easily induce leaching of these metals.

Keywords: Heavy metals, Ceramic, Leaching

1. INTRODUCTION

Ceramic wares were currently popular used in both rural and suburb areas due to the low price with varieties of products. Therefore, local convenience shops generally sold the ceramic products that imported from China. Mostly, ceramics products were made from clay. In order to increase the ceramic value, the coating technique was normally applied especially using glazed technique. The general chemical that used in the classical glazing technique was lead. The lead glaze was used to protect the surface of the ceramics ware as well as the beauty and durability of the products. The luster of the ceramic wares was also obtained from the glazed technique. In addition, some ceramic properties were adjusted by the addition of heavy metals. However, in the case of improper fire, lead glazed products could become toxic. Damage ceramic products were also harmful. Also, Lead and other heavy metals might be contaminated into the food or liquid by leaching. Frequently, the heating process could also increase the leaching and caused acidic food or drink.

Normally, the compositions of ceramics were contained with clay, glaze and color. In ceramic process, after body shape, the clay body was heated via biscuit firing prior to decoration painting on the body. The glost firing was then followed to protect the glazing and prevent the damage derived from acid or salts. The duration and colorful of ceramic products were obtained. However, the composition of glaze was sometimes contained lead and the glazing color was normally metal oxides. It could be concluded that the ceramic products the overglaze color could be released. were gained the heavy metals from both underglazing and overglazing processes. By rubbing and cleaning the ceramic products, the heavy metals might be released out.

Therefore, the aim of this work was to determine the heavy metals in Chinese ceramic products in suburb area, Pathumthani, Nakhon Nayok and Sa Kaeo provinces.

2. MATERIALS AND METHODS

Sample groups

The Chinese ceramic products, especially table wares such as saucer, cup, plate, pot, tiles or mortal, were sampling from the from the local shops in the area of Pathumthani, Nakhon Nayok and Sa Kaeo provinces, 35 shops with 120 samples. The products were prepared into fine powder.

Sample digestion

The ceramic digestion was followed and modified from BS 6748:1986 and ASTM 1466.00. The accurate weight of ground ceramic product (around 1 gram) was prepared. The mixture acids of nitric and hydrofluoric acids were used in an open vessel. The digest solution was then filtered to eliminate the insoluble particles.

Preparation of standard solutions

The standard solutions of lead, cadmium, copper, cobalt, chromium, zinc and manganese were prepared in the range of 0 to 50 ppb. The dilution solvent of the standard was deionized water. Both standard and sample solutions were quantitatively characterized via ICP-OES; Inductively Coupled Plasma: Model No.JY238Ultrace/ultimaz.

3. RESULTS

The results in ceramic samples of heavy metal concentrations were shown in Table 1.

Table 1. List of shops and samples

Province	Number of shops	Number of samples
Pathumthani	18	84
Nakhon Nayok	9	27
Sa Kaeo	8	24

From Table 1, it was displayed that the total of Chinese ceramic samples collected from 18 local shops in Pathumthani was 84 samples whereas the Chinese ceramic samples from 9 local shops in Nakhon Nayok and 8 local shops in Sa Kaeo were 27 and 24, respectively.

Table 2. Average of heavy metal concentration

Province	Average of heavy metal concentration (ppb)						
	Pb	Cd	Cu	Co	Pb	Zn	Mn
Pathumthani	2.307	0.108	0.315	0.602	0.226	7.011	1.993
Nakhon Nayok	1.661	0.203	0.718	0.247	0.636	2.474	8.313
Sa Kaeo	1.914	0.058	0.682	1.143	0.491	2.198	3.240

Table 2 was exhibited that the average amount of lead from Pathumthani province showed the highest value at 2.307 ppb while the lowest amount of lead was showed from the local shops at Nakhon Nayok province, 1.914 ppb. From the USFDA, it can be conclude that the average amounts of lead in three provinces were higher than the standard limit (not more than 0.5 ppb). Next, in the case of the amount of cadmium, the Chinese ceramic from local shops at Nakhon Nayok province was 0.203 ppb whereas the amounts of cadmium from the Chinese ceramic from local shops at Pathumthani and Sa Kaeo provinces were 0.108 and 0.058 ppb, respectively. In addition, the amount of copper was also observed in all three provinces with the range of 0.315 to 0.718 ppb. Then, the concentration of cobalt was also determined. The products from Sa Kaeo were recorded with the highest amount of cobalt with 1.143 ppb and then followed by the products from Pathumthani (0.602 ppb) and Nakhon Nayok (0.247 ppb). Chromium amounts were found in the highest amount at Nakhon Nayok (0.636 ppb). In Sa Kaeo and Pathumthani provinces, the amounts of chromium were 0.491 and 0.226 ppb, respectively. Both zinc and manganese were not of toxicological significance. The results were revealed that the amounts of zinc were in the range of 2.198 to 7.011 ppb. Manganese amounts were in the range of 1.993 to 8.313 ppb.

4. CONCLUSIONS

The toxic heavy metal of Chinese ceramic products was found the amount of lead above the USFDA allowance. It can be summarized that the imported Chinese ceramic products in the three provinces was rather high possible to leaching those heavy metals into human body.

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