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Medicinal potential of *Melodorum gracile and Mkilua fragrans* extracts

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ABSTRACT

Crude ethanol extracts of *Melodorum gracile* and *Mkilua fragrans* were screened for antimicrobial activity against *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum* and *Escherichia coli* of veterinary importance. Agar diffusion technique was used to determine the inhibition of microbial growth and broth dilution technique was used to determine MIC and MBC. The crude extracts exhibited predominantly antibacterial activity with the root extract showing the strongest inhibition against the test bacteria at a MIC of between 7 µg/ml and 500 µg/ml. The study has shown that *Mkilua fragrans* extract is very potent against *Streptococcus agalactiae* and *Staphylococcus aureus* but no effect against *Salmonella gallinarum* and *Escherichia coli*. The study also showed that *Melodonium gracile* extracts were potent against *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum* and *Escherichia coli.* Most of the plant extracts were significantly lethal towards brine shrimps. This is the first scientific evaluation of the veterinary medicinal potential of the *Melodorum gracile and Mkilua fragrans* indigenous to Tanzania, providing the baseline for further investigations on the plants towards new drug discovery.

INTRODUCTION

Melodorum gracile and Mkilua fragrans are species of plants in the family Annonaceae. The family consists of about 120 genera and over 2000 species among which 1000 species are practically confined to tropical and subtropical regions, particularly in lowland evergreen forests (Watt and Breyer-Brandwijk, 1962). All but one of the species is trees or shrubs and is usually evergreen; some of them are climbers (Leboeuf *et al*., 1982). Taxonomists consider Annonaceae among plant families, which constitute species that are still in their primitive evolutionary stages (Watt and Breyer-Brandwijk, 1962). Such plant may therefore be considered to possess chemical constituents with unknown and unusual chemical structures and bioactivities (Makangara, 1995). This has in fact been exemplified from already reported investigations of annonaceous plants, which yielded several natural products with hitherto unknown chemical structures. Some of the compounds that so far has been obtained only from this plant family include a unique class of bioactive acetogenins cyclohexane epoxides, benzopyranyl sesquiterpenes, alkalated indols and C-benzylated flavonoids, the compounds exhibited potent antitumour, antimalarial, insecticidal and other bioactivities (Watt and Breyer-Brandwijk, 1962, Makangara, 1995).

Phytochemical investigations done on *Isolona maitlandii, Piptostigma fugas* and *Monocyclanthus vignei* shown significant antibacterial and antifungal activities (Makangara, 1995), *Friesodielsia obovata* and *Hexalobus monopetalus* crude extracts have been reported to have mild antimalarial activity against the multidrug resistant K1 and chloroquine sensitive NF54 strains of *Plasmodium falcipalum*. The above extracts have been found to be mild cytotoxic in the brine shrimp test (Joseph, 1993). Also, the presence of various classes of compounds including polyphenols, essential oils, terpenes, acetogens and hexalobines, isoquinoline, aporphine and oxaporphine alkaloids has been reported. Acetogenins which are found only in the family Annonaceae have very potent bioactivities (antitumour, antimicrobial, pesticidal and anthelminthic) (Makangara, 1995). *Asteranthe asterias* ethanol extract from stem and root barks yielded two new diphenilated indols, 2',3'-epoxyasteranthine & 2',3'-dihydroxyasteranthine, both of the compounds showed a remarkable antimycotic activity against the fungi *Saprolegnia asterophora* and *Rhizoctonia soloni*, this activity being comparable

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with that of standard antimycotic agent Naftifine (Mdee, 1998). A study on *Isolona cauliflora* crude extracts showed high antimicrobial activity against some bacteria of veterinary importance (Magingo, 2000). Investigations of *Melodorum maccreae* (accepted name: *Xylopia maccreae*) yielded flavonoids with extended benzylation (Nkunya, 2002). Phytochemical investigation on methanol extract of stem barks of *M. fragrans* collected from East Usambara Mountains in Tanzania, yielded two classes of natural products aporphinoids and acids, the structure of which are still tentative. The crude extract was found to be inactive on brine shrimp test (Tesso, 1997).

However, no reports available on the antimicrobial activity of the Tanzanian *M. gracile and M. fragrans* extracts against pathogenic microorganisms of veterinary importance. This study aimed at investigating the antimicrobial activity of *M. gracile and M. fragrans* extracts against pathogenic microorganisms isolated from sick domesticated animals. The study showed that *M. fragrans* is very potent against *Streptococcus agalactiae* and *Staphylococcus aureus* but no effect against *Salmonella gallinarum* and *Escherichia coli*. The study also showed that *Melodonium gracile* extracts are potent against all four bacteria *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum* and *Escherichia coli.* The finding provides baseline information for further studies on the medicinal potentials of *M. gracile* and *M. fragrans* in search for new medicines against bacterial infections.

MATERIALS AND METHODS

Plant material

Mkilua fragrans rootbarks and stembarks and *Melodonium gracile* leaves and rootbarks were collected from Longuza area, Muheza district in Tanga region. The plant materials were air-dried in a well-ventilated room (25 to 28°C). The dried materials were ground into powder. Ethanol extraction was done by soaking in ethanol (1:3 ratios) 3-times, with magnetic steering at room temperature for 8 h, and then collecting the extract. The 3 extracts were pooled together and filtered by vacuum filtration and the filtrate was concentrated by using rotary evaporator at 40°C. The known concentration of the crude extract was tested for *in vitro* activity against microbial pathogens isolated from sick domesticated animals, then tested for toxicity against brine shrimps.

Isolation and culturing of the test microorganisms

Specimen collection from sick animals, isolation and identification of the microorganisms were done in the laboratory of the Department of Veterinary Microbiology and Parasitology of Sokoine University of Agriculture. *Streptococcus agalactiae, Staphylococcus aureus, Salmonella gallinarum* and *Escherichia coli* were isolates and pure cultures prepared: *S. gallinarum* and *E. coli* each onto MacConkey agar (MA) Petri-dishes, *S. aureus* and *S. agalactiae* each streaked onto sterile nutrient agar (NA) and blood agar (BA) petri-dishes. The culture media were prepared according to manufacture's specifications in slunts or culture plates. The plates were incubated, and observed for growth and purity after the 24 h at 37°C. The observation was recorded, and the positive result plates were stored at 4°C. Sub-culturing of pure strains was done at UDSM microbiology laboratory monthly, with the aim to maintain the strains.

Preparations of the pathogen inocula

Pure cultures of *S. aureus, S. gallinarum* and *E. coli* were sub cultured onto sterile NA slant, *S. agalactiae* was maintained on BA slant at 37°C for 24 h. A loopful from the aforementioned pure culture slant was inoculated into 100 ml of sterile broth medium in 200-volumetric flask, which was then incubated at 37°C in a shaking incubator. The broth culture was diluted to make an inoculum of approximately 10^5 C.F.U/ml. Determination of the number of cells in the broth cultures was done by the viable cell count method according to Grigorova and Norris (1990).

Antimicrobial activity assays

Both the agar diffusion and broth (or tube) dilution techniques were used to test for antimicrobial activity of extracts from leaf, stembark and rootbark according to Lyantagaye and Magingo (2012). The agar diffusion technique was used to determine zones of inhibition of the microbial growth whereas the broth dilution was used to determine minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC). Paired Two Sample *t-test* for means statistical test was used to determine if the data means of the activity of extracts from two different plant parts were different from each other. ANOVA was used for testing if there was significance difference in the means of *S. agalactiae* sensitivity among all the 4 plants extracts when using AWM.

C**ytotoxicity test**

The presence and levels of toxicity of the active plant extracts were determined by the Brine Shrimp Test (BST) method described by Meyer *et al*. (1982) and modified by Lyantagaye and Magingo (2012). From this method, an extract is considered to be cytotoxic if it kills at concentrations less or equal to 240 µg/ml.

RESULTS

Inhibition of the Microbial growth

Each of the plant extracts tested showed antimicrobial activity against at least one of the tested bacterial strains. The activity was quantified as a diameter (in mm) of the zones of growth inhibition surrounding the points of application of the extracts (Figure 1). Figure 1 shows the results from the AWM, from which the application of *M. fragrans* stembark and rootbark and *M. gracile* leaf and rootbark extracts into wells of the inoculated agar resulted in clear zones of growth inhibition of *S. agalactiae*. The minimum inhibition concentrations (MIC) and minimum bactericidal concentration (MBC) are summarized in Table 1. There was no bacterial growth inhibition surrounding the untreated control well as can be seen in Figure 1.

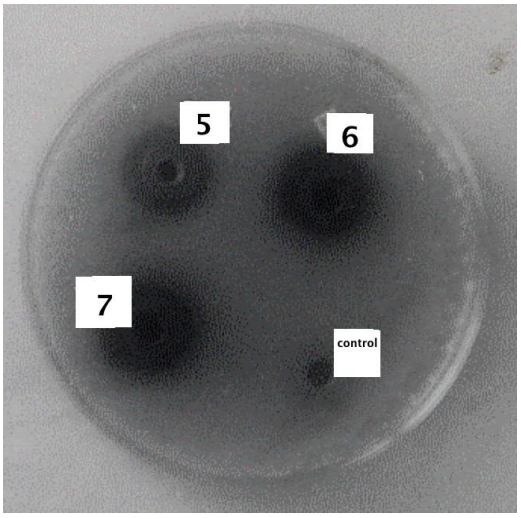

Fig 1. Agar well method (AWM) results showing *S. agalactiae* growth inhibition activity by *M. fragrans* stembark (5) and rootbark (6) and *M. gracile* leaf (7) extracts. A well was left without an extract as untreated control.

Table 1. Broth Dilution Method (BM) results showing the MIC and MBC values of some plant crude extracts, in µg/ml, against bacterial isolates of veterinary importance. Each assay was done ten times.

Plant species	Plant part	Sample code	*Staphylococcus aureus*		*Salmonella gallinarum*		*Escherichia coli*	
			MIC	MBC	MIC	MBC	MIC	MBC
Mkilua fragrans	stem	5'	105	125	-	-	-	-
Mkilua fragrans	root	6'	-	-	-	-	-	-
Melodorum gracile	leaf	7'	7.81	250	250	500	250	500
Melodorum gracile	root	9'	-	-	62.5	125	-	-

Table 2 shows all the results from AWM in which the *M. gracile* leaf extract showed the broadest spectrum antimicrobial activity (against all the tested strains) while *M. fragrans* stembark and rootbark showed narrowest spectrum only against *S. agalactiae.* There was a significant difference in activity between *M. gracile* leaf extracts and rootbark extract from different plant parts against *S. gallinarum* $p < 0.001$. *S. agalactiae* was sensitive to all the plant extract, but there was significant difference in the activity among different plant extracts, $p < 0.0001$, with *M. fragrans* exhibiting the highest and *M. fragrans* stembark the least. Similar scenario was observed with DM as shown in Table 3. The observed activity with the DM shows similar profile as with the AWM but with a slight decrease in the sizes of inhibition.

The cytotoxicity of the plant extracts

Table 4 shows that *M. fragrans* stembark and rootbark, and *M. gracile* leaf extracts killed 50 % of the *Artemia salina* larvae at concentrations below 240 µg/ml. A crude extract is considered to be active up to a concentration of 240 µg/ml (Meyer *et al*., 1982). *M. gracile* rootbark extract did not show any toxicity.

Table 4. The Brine Shrimp Test (BST) results showing the cytotoxicity of the plant crude extracts to *Artemia salina* larvae, and their IC_{50} at 24 h treatment.

Plant species	Plant part	Sample code	% Deaths at 24 h for different concentrations (µg/ml)						
			8	24	40	80	120	240	IC_{50}
Mkilua fragrans	Stem bark	5	0	0	20	70	90	100	34
Mkilua fragrans	Root bark	6	0	10	20	50	100	100	85
Melodorum gracile	leaf	7	0	20	30	50	70	90	69
Melodorum gracile	Root bark	9	0	0	0	0	10	20	NT

Note: NT = non toxic.

DISCUSSION AND CONCLUSIONS

This study aimed at examining if crude plant extracts of *M. fragrans* rootbark and stembark, and *M. gracile* rootbark and leaf have antimicrobial activity on some pathogenic bacterial isolated from sick domesticated animals. The results provids evidence of the efficacy of extracts from these plants to treat bacterial infections. The plants have never before been reported to have antimicrobial activity against the tested bacterial strains of veterinary importance, hence no reference literature is available.

The selectivity of *M. fragrans* towards Gram-positive bacteria may suggest that the active content of the extract could be a ligand to certain receptors found in the cell membrane of the Gram-positive bacteria tested (Boily and van Puyvelde, 1986).

The observed lower activity in DM as compared to AWM implies that sensitivity of a test method is also proportional to the amount of active agent(s) applied to the agar (Carter and Chengappa, 1991). Holding capacity of a 7mm diameter disc of Whatman filter paper could not equal the amount of extract 50 µl loaded in the agar well. The lower disc capacity the less the amount of the antimicrobials available to diffuse through inoculated agar media, hence smaller zones of inhibition (Aszalos, 1986).

Table 2. Agar Well Method (AWM) results showing the growth inhibition zones (mm) ± SD of plant crude extracts against four different bacterial strains of veterinary importance obtained by the Agar Well Method. n = 10.

Plant species	Plant part	Sample code	Zone of microbial growth inhibition (mm)			
			Streptococcus agalactiae	*Staphylococcus aureus*	*Salmonella gallinarum*	*Escherichia coli*
M. fragrans	Stembark	5	10.52 ± 0.80	10.40 ± 0.84	0	0
M. fragrans	rootbark	6	16.03 ± 0.87	0	0	0
M. gracile	leef	7	15.19 ± 0.36	20.00 ± 0.67	10.30 ± 0.67	10.60 ± 0.71
M. gracile	rootbark	9	15.22 ± 0.15	0	14.80 ± 0.79	0

Table 3. Disc Method (DM) results showing the growth inhibition zones (mm) ±SD of plant crude extracts and six standard test antibiotics against four different bacterial strains of veterinary importance obtained by the Disc Method. n = 10.

Plant species	Plant part	Sample code	Zone of microbial growth inhibition (mm)			
			Streptococcus agalactiae	*Staphylococcus aureus*	*Salmonella gallinarum*	*Escherichia coli*
Mkilua fragrans	Stembark	5	7.19 ± 0.13	7.20 ± 1.12	0	0
Mkilua fragrans	rootbark	6	11.54 ± 0.25	0	0	0
Melodorum gracile	leef	7	12.26 ± 0.17	16.50 ± 0.92	7.20 ± 0.90	7.30 ± 0.74
Melodorum gracile	rootbark	9	12.25 ± 0.16	0	11.70 ± 1.30	0

The observed difference in the activity between *M. gracile* leaf extract and rootbark extract against *S. gallinarum* ($p < 0.001$), the high sensitivity of *S. agalactiae* to all the plant extract, and the difference in the activity $p < 0.0001$ among the different extracts may not be explained easily at this stage. However, the activities of plant crude extracts vary according to the antimicrobial properties of the active ingredients, which in turn vary with extract and the plant part (Kalyoncu *et al*., 2006). Although almost all ethanol extracts in this study exhibited varying degrees of antibacterial activity, MICs varied between the different plants ethanol extracts as well as among extracts of different plant parts. Gram-negative bacteria including *S. garinallum* and *E. coli* seem to be more tolerant to the extracts. Al-Bakri and Afifi (2007) and Al-Hussaini1 and Mahasneh (2011) reported similar trends of antibacterial activity of some selected Jordanian plant extracts.

The BST procedures have proven the convenience of the method for toxicity assays as suggested by Meyer *et al*., (1982). The toxicity of the crude extracts on *A. salina* larvae does not necessarily mean they can be lethal to animals or humans. Toxicity of plant extracts towards *A. salina* could be encountered by the presence of certain compounds, which are in fact non-toxic to humans and animals (Mtolera, 1991). The toxicity towards *A. salina* of the crude extracts can simply be explained as the plants containing bioactive substances, which on modification can be used in a variety of ways as pesticides, or as a source of medicines.

The observed antimicrobial activity of these crude extracts could be attributed to multiple compounds interacting synergistically, antagonistically, and additively (Mtolera, 1991). The study findings provide promising leads for the isolation of antimicrobial compounds; and are therefore worth investigating further using bioassay guided fractionation.

In conclusion, ethanolic extracts of the tested plants exhibited varying degrees of antibacterial activity against Gram-positive and Gram-negative bacteria. The diameters of growth inhibition zones of some extract showed superior activities although the MIC values tended to be relatively high for some microorganisms. *M. fragrans* and *M. gracile* antimicrobial activity against pathogenic microorganisms of veterinary importance is reported for the first time. The study reveals a promising medicinal potential for possible therapeutic use of the *M. fragrans* and *M. gracile* extracts against pathogenic bacteria. However, further studies are needed to identify the active ingredients to ascertain their potential for their clinical application. Such potential plants should be protected in their natural environments, and if possible be propagated in botanical gardens to avoid their disappearances.

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