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RESEARCH ARTICLE

Antibacterial, antifungal, antioxidant, brine shrimp lethality and polyphenolic content of *Holarrhena mitis* (Vahl) R.Br. ex Roem. & Schult.

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Abstract: *Holarrhena mitis* (Vahl) R.Br. ex Roem. & Schult. which is an endemic plant growing mainly in the dry regions of the low-country has been used in the treatment of dysentery in Ayurvedic medicine. During the present study, we tested antibacterial, antifungal, antioxidant activities and brine shrimp lethality, as well as the total phenolic content of the dichloromethane, ethyl acetate and methanol extracts of the bark and leaves of *H. mitis.* The methanol extract of bark produced a measurable zone of inhibition against two *Candida* species, namely *Candida albicans*, *Candida krusei* among all five tested species and against both dermatophytes, *Microsporum gypseum* and *Tricophyton mentagrophytes*. In addition, the methanol extract of bark showed very strong antifungal activity against *C. krusei* (20 mm), which is very close to that of the positive control ketoconazole (22 mm). Both dicholoromethane extracts of bark and leaves and methanol extracts of leaves showed an activity against both tested strains of *Staphylococcus aureus* but were negative against *Escherichia coli* and *Salmonella enteritidis*. However, the rest of the extracts exerted an active against all the tested bacterial strains. In the brine shrimp cytotoxicity assay, the dichloromethane extracts of both leaves and bark showed lower LC_{50} values (27.13 ppm and 9.38 ppm, respectively) than that of positive control, $K_2Cr_2O_7$, (35.74 ppm) indicating cell toxicity. Compared to the positive control, DL-α-tocopherol (IC_{50}: 12.2 ppm), the antioxidant activity of the ethyl acetate and methanol extracts of leaves exhibited comparable activity (IC_{50}:16.9 ppm and 29.8 ppm, respectively). Antioxidant activity correlated well with the polyphenol content of methanol and ethyl acetate extracts (473.25 and 138.74 mg (GAE) /g, respectively) of leaves, with respect to gallic acid. These empirical results revealed that methanol extract of bark of *H. mitis* showed strong antifungal activity as well as very low brine shrimp lethality indicating that it would be a potential nontoxic anti-fungal natural product. The dichloromethane extracts exhibited strong brine shrimp lethality and may contain potential natural anticancer lead compounds. Ethyl acetate extracts of both leaves and bark having significant antibacterial activity, would be source of potential antibacterial lead compounds.

Keywords: Apocynaceae, endemic plant, *Holarrhena mitis,* antioxidant, antidysenteric, antifungal, antibacterial activities and low cytotoxicity.

INTRODUCTION

Natural products from plants and animals have been considered as a rich source of compounds for drug discovery and have continued to be the leading compounds in clinical trials particularly as anticancer and antimicrobial agents (Dias *et al*., 2012; Harvey, 2008; Harvey *et al*., 2015; Rishton, 2008). According to the Food and Drug Administration (FDA) United States, 34 % of the new medicines that have been approved during 1981-2010 are natural products or direct derivatives of natural products such as statins, tubulin binding anticancer drugs and immuno suppressants (Newman and Cragg, 2012). Unlike synthetics, natural products provide an infinite pool of chemical structures with an immensely high structural diversity and high degree of stereochemistry, which would be expected to contribute to more difficult screening targets such as protein-protein interactions (Drewry and Macarron, 2010).

The variety, richness and abundance of flora in Sri Lanka in general and the high percentage of endemic plants found in the is landmakes it a fertile testing ground for pharmaceutical discovery. The World Conservation Monitoring Center has designated Sri Lanka as a "hotspot" in terms of biodiversity (Caldecott *et al.,* 1994). Antimicrobial and insecticidal activities of Sri Lankan endemic plants have shown some potent findings (Hewage *et al*., 1997). In addition, biological activity of Sri Lankan lower, non-flowering plants such as lichens have also exhibited a great potential as sources for drug leads (Karunaratne *et al.*, 2005; Thadhani *et al*., 2012). Importantly, in Sri Lanka, so far it has not been a large-scale systematic search for bioactive potential of its plants. The urgency for such an activity is highlighted because out of 3,154 indigenous species evaluated in the Red List (MOE, 2012), 1,386 (44%) are threatened (critically endangered, endangered or vulnerable). The non-endemics on the other hand, are more accessible. Thus, investigating new drug leads from such medicinally important endemic natural sources from Sri Lanka is critically important. *Holarrhena mitis* (Vahl) R.Br. ex Roem. & Schult. is one

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such endemic plant growing mainly in the dry regions of the low-country. In the present study, we evaluated the preliminary pharmaceutical potential of endemic and vulnerable *H. mitis* which has been used in the treatment of dysentery in Ayurvedic medicine.

Genus *Holarrhena* belongs to the family Apocynaceae and there are five species, namely *H. congolensis* Stapf., *H. curtisii* King and Gamble., *H. floribunda* (G.Don) T. Durand & Schinz, *H. antidysenterica* Wall, and *H. mitis* which have been distributed in various geographical areas. However, according to the Red List of Fauna and Flora, *H. mitis* is a vulnerable species (there is a high risk of the plant becoming extinct in the future) (Weerakoon, 2012).

H. mitis is known **as** *Kalinda*/*Kiri-mawara*/*Kiri-walla* in Sinhala and *Kuluppalai* in Tamil has been used in Ayurvedic medicine. *H. mitis* grows mostly in the dry regions of the low-country up to 460 m asl (Mirigama, Habarana, Sigiriya, Negombo, Ratnapura etc) **(***Hooker, 1882)*. It is a tall slender tree with a whitish, rather smooth bark and slender drooping branchlets which bear a smooth, purplish bark and glabrous young parts. Leaves are simple and opposite. They are 3.5-8.7 cm long and 1.2-2.5 cm broad, on short petioles, thin and usually curved. Flowers are regular and bisexual. They are white and sweet scented. Seeds are narrow **(***Trimen, 1895)*.

In terms of the chemistry and the biological activity studies in genus *Holarrhena*, *H. antidysenterica* has been thoroughly investigated. These studies revealed that its bark, leaves and seeds showed anti-diabetic, antioxidant (Ali *et al*., 2009; Bhusal *et al*., 2014; Ganapathy *et al*., 2011; Hegde and Jaisal, 2014; Korpenwar, 2011; Preethi *et al*., 2010; Ray, 2014; Sabira *et al*., 2014;), antibacterial and antifungal activities (Ballal *et al*., 2001; Preethi *et al*., 2010; Rath and Padhy, 2014). In addition, seeds have acetylcholinesterase inhibitory activity which is useful for treating neurological disorders (Yang *et al*., 2012) and has diabetic defense as well as the ability to reduce triglycerides which affect the total cholesterol level (Pathak *et al*., 2015). Further *H. floribunda* acts against *Mycobacterium ulcerans* (MIC 125 µg/ml) (Loukaci *et al*., 2000) and it has anticancer activity and weak anti-leishmanial activity (Loukaci *et al*., 2000) The chemistry and the biological significance of *H. curtisii* have also been investigated and anthelmintic, appetizing, astringent and anti-diarrheal properties have been identified (Bhutani *et al*., 1988). However, there are no reports relating to the bio activity studies of *H. congolensis* which is distributed in Zaire and only few study reports about the alkaloids of *H. mitis* (Arseculeratne *et al*., 1981; Bhavanandan and Wannigama,1960; Gunatilaka, 1978; Gunatilaka, 1999; Leboeuf*et al*., 1972; Wannigama and Cave, 1972).

During this study, we focused on the antibacterial, antifungal, antioxidant activities and brine shrimp lethality assay, as well as the total phenolic content of the dichloromethane, ethyl acetate and methanol extracts of the bark and leaves of *H. mitis.*

MATERIALS AND METHODS

Collection and identification of the plant

The bark and the leaves of *H. mitis* were collected from the National Botanical Garden, Peradeniya and the identity of the plant was confirmed by the National Herbarium, Department of National Botanical Garden, Peradeniya, where a voucher specimen 6/01/H/03 was deposited on 12/12/2013.

Preparation of extracts

Fresh leaves and bark of the *H. mitis* were washed with tap water followed by distilled water, and each part was air dried and homogenized to a coarse powder separately. They were percolated with distilled dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) sequentially at room temperature (3 days for each solvent) and concentrated using the rotary vacuum evaporator below 40 ^{0}C to obtain DCM (0.81 %), EtOAc (0.16 %) and MeOH (1.96 %) extracts of bark and DCM (2.11 %), EtOAc (3.25 %) and MeOH (15.25 %)extracts of leaves separately.

Antimicrobial activity

Antifungal activity

Antifungal screening of the plant extracts of *H. mitis* was performed using the cut-well diffusion method (Kumar *et al*., 2009). Microbial suspensions of test organisms were adjusted to McFarland turbidity of 0.5 (approximately 1.5 x 10^{7-8} cfu/ml) and inoculated onto Mueller Hinton agar plates (MHA, Oxoid, Hampshire, England). The inoculated plates were swirled evenly to distribute the organisms and excess broth was removed using a sterile pipette. The plates were left at room temperature for 30 min after which 12 mm diameter wells were bored on the agar and the bottom sealed with molten MHA. The dissolution of organic extract was aided by 10% (V/V) dimethylsulfoxide (DMSO). Using a prepared template, aliquots of each reconstituted extract (10 mg/ml) was pipetted into the wells and the plates incubated at 35 °C for 24 h. The diameter of the zone of inhibition (ZOI) around the well was measured. Each screening was carried out in triplicate and the mean diameter of the ZOI was recorded.

Antifungal activity of plant extracts was screened in triplicate against the clinically isolated dermatophytes (*Trichophyton mentagrophytes*, *Microsporum gypsium* and standard cultures of *Candida*sp. (*C. tropicalis*-ATCC 750, *C. albicans*- ATCC 90028, *C. parapsilosis*-ATCC 23019, *C. Krusei*-ATCC 6258, *C. glabrata*-ATCC 90030) using the agar well diffusion assay. For each fungal strain, negative controls were maintained using pure solvents instead of extract. Econazole was used as a positive control for the dermatophytes and ketoconazole for the *candida* sp. These cultures were taken from the culture collection maintained at the Department of Mycology, Medical Research Institute, Sri Lanka.

Antibacterial activity

Antibacterial activity of *H. mitis* plant extracts was screened in triplicate for the selected cultures of *Staphylococcus aureus* (ATCC 25923 and 29213), *Escherichia coli* (ATCC 25922), and *Salmonella enteritidis* (clinically isolated) which were taken from the confirmed culture collection maintained at the Department of Microbiology, Faculty of Medicine, University of Peradeniya using agar dilution method (Hussain *et al.*, 2011). All the petri dishes were labeled with the pathogen tested and the dilution series (20 mg/ml, 10 mg/ml and 5 mg/ml). Each test solution (2 ml) was added to the sterile MHA (18 ml) and mixed well by inverting the universal bottles 2-3 times. Each solution was poured into the appropriately labeled petri dish to a depth of approximately 4 mm and allowed to set on a level surface. Ten-fold dilution of 0.5 McFarland suspension of each test organism was inoculated on to the appropriately labeled plate using a micropipette (10 µl). The plates were incubated (37 ^{o}C for 24 h).

Brine Shrimp Lethality Assay (BSLA)

Brine shrimp lethalities of the crude extracts were determined by using the brine shrimp (*Artemia salina*) lethality assay (Carvalho-silva *et al*., 2012) which is considered as a useful tool for preliminary assessment of toxicity as a positive correlation exists between brine shrimp lethality and human carcinoma. Shrimp eggs (50 mg), were incubated at 37 ºC in brine solution (38 g sea salt in 1 L of distilled water, pH 7.4), for 48-72 hours to provide large numbers of larvae. After 2 days of hatching and maturation, 10 larvae were placed (using pasture pipette) in each of the vials, containing 1, 10, 25, 50, 100, 250, 500, 750, 1000 and 2000 ppm test samples (10 mg/mL in DMSO). The total volume was brought up to 5 mL with seawater and incubated at 28 ± 1 ^{o}C for 24 h under illumination. The vials supplemented with DMSO and potassium dichromate also served as negative and positive controls, respectively. The lethal concentration at 50 % mortality after 24 h of exposure and the chronic LC_{50} with 95 % confidence level were determined by Probit analysis method using the statistical software "Minitab® 16.1.0".

Antioxidant activity [DPPH (1, 1-Diphenyl-picrylhydrazyl) free radical scavenging assay]

The radical scavenging activity of plant extracts against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined spectrophotometrically according to the method described by Budzianowski and Budzianowska (Budzianowski and Budzianowska, 2006). A methanol solution of DPPH radicals of 1.5×10^{-3} moldm^{-3}was prepared freshly and 0.2 mL of that was added to each test sample prepared in MeOH (10, 20, 40, 60, 80, and 100 ppm). Absorbance measurements were recorded immediately with a UV-Visible spectrophotometer (UV-160, SHIMADZU) for each concentration. The change of absorbance at 517 nm was recorded after 30 minutes. As the positive reference control, the antioxidant activity of DL-α-tocopherol was determined following the above procedure. The percent antioxidant activities (AA%) of extracts and DL-α-tocopherol were calculated at each concentration level according to the formula,

$$AA\ \% = 100-[(Abs_{Sample}- Abs_{Blank}) \times 100/Abs_{Control}]$$

and $Abs_{Control}$ is the absorbance of 1.0×10^{-4} mol dm^{-3} DPPH solution without antioxidant, Abs_{Blank} is the absorbance of the extract at different concentrations (10, 20, 40, 60, 80 and 100 ppm) without adding DPPH radical and Abs_{Sample} is the absorbance of test solution after 30 minutes.

Total Phenolic Content

The total polyphenol content of methanol extracts was determined by the Folin-Ciocalteu method (ISO-14502-C). Absorbance of the test sample, mixture of different concentrations (10, 20, 40, 60, 80, 100 ppm) of gallic acid (0.4 mL), sodium carbonate (7.5% W/W) (1.6 mL) and Folin-Ciocalteu (10 times diluted) (2 mL), were determined at 765 nm and the calibration curve was plotted for gallic acid (gallic acid concentration vs. absorbance). The total polyphenol content of each extracted was obtained using the plotted curve and expressed as mg of gallic acid equivalent of dry material (mg GAE/dry weight g).

RESULTS AND DISCUSSION

Crude extracts of leaves and barks of *H. mitis* exhibit antifungal and antibacterial activities as indicated by the inhibition zones formed around the well with a transparent ruler in millimeters at the end of incubation. The MeOH extract of bark produces a measurable zone of inhibition against two *Candida* species, namely *C. albicans*, *C. krusei* among five tested species and both dermatophytes, *M. gypseum* and *T. mentagrophytes*. Interestingly, the MeOH extract of bark shows a very strong antifungal activity against *C. krusei* (20 mm), which is very close to that of the positive control ketoconazole (22 mm). However, all the DCM and EtOAc extracts of leaves and of bark show no activity against all the tested fungal strains. Both DCM extracts of bark and leaves and MeOH extracts of leaves, display positive results against both tested strains of *S. aureus* but is negative against *E. coli* and *S. enteritidis* (Table 1). However, the rest of the extracts are active against all the tested bacterial strains (MIC 10 mg/mL).

Brine shrimp lethality of the plant extracts is determined using brine shrimp (*Artemia salina*) lethality assay. The DCM extracts of both leaves and bark show lower LC_{50} values (27.13 ppm and 9.38 ppm, respectively) compared to $K_2Cr_2O_7$ (35.74 ppm) indicating cell toxicity. The EtOAc extracts of leaves and bark (LC_{50}: 283.17 ppm and 173.63 ppm, respectively) and both MeOH extracts of leaves and bark (LC_{50}: 199.05 ppm and 1223.36 ppm, respectively) demonstrate lower cytotoxicity. According to the above data, the DCM extracts of bark and leaves are more cytotoxic than the MeOH or EtOAc extracts of bark and leaves.

Compared to the positive control, pure DL-α-tocopherol (IC_{50}: 12.2 ppm), the antioxidant activity of both the EtOAc and MeOH extracts of leaves are promising and comparatively high (IC_{50}:16.9 ppm and 29.8 ppm, respectively). The MeOH extract of bark exhibits moderate antioxidant property (IC_{50} value of 85.4 ppm) and both DCM extracts of leaves and bark and EtOAc extract

Table 1: Microbial activities, brine shrimp lethality, antioxidant activity and total phenolic content of DCM, EtOAc and MeOH extracts of leaves and bark of *H. mitis.*

	Extracts	Antifungal Activity	Antibacterial Activity	Cyto-toxicity	Antioxidant Activity	Total Phenolic Content
				LC_{50} (ppm)	IC_{50} (ppm)	mg (GAE)g^{-1}
Leaves	DCM	Negative	Positive[2]	27.13	183.7	1.05
	EtOAc	Negative	Positive[3]	283.17	16.9	138.74
	MeOH	Negative	Positive[2]	199.05	29.8	473.25
Bark	DCM	Negative	Positive[2]	9.38	473.4	5.61
	EtOAc	Negative	Positive[3]	173.63	169.0	1.86
	MeOH	Positive[1]	Positive[3]	1223.36	85.4	12.72
Positive Control	$K_2Cr_2O_7$	-	-	35.74	-	-
	α-tocopherol	-	-	-	12.2	

[1]Positive for *C. albicans*, *C. krusei, M. gypseum* and *T. mentagrophytes*

[2]Positive only for *S. aureus*

[3]Positive for all tested strains

possess even less activity (IC_{50}: 183.7 ppm, 473.4 ppm, 169.0 ppm, respectively) .

The polyphenol contents in 1 g of both MeOH and EtOAc extracts of leaves, with respect to gallic acid, are 473.25 and 138.74 mg (GAE) /g, respectively while all the other extracts show very low values with respect to gallic acid. Both MeOH and EtOAc extracts of the leaves display strong antioxidant activity and high yield of polyphenols. Therefore, it is assumed that the antioxidant activity of *H. mitis* have a relationship with phenol content of the plant.

Other authors have shown that, phosphatidylethanolamine is essential to the promotion of carboxyfluoresce in leakage from bacterial model membranes by antioxidant galloylated catechins, indicating their bactericidal activities, at least at the membrane level may be due to antioxidant catechins (Caturla *et al*., 2003). Interestingly, in agreement with the literature, there appears to be a positive correlation between antimicrobial activity of MeOH and EtOAc extracts of *H. mitis* and their total polyphenol content and antioxidant activity.

CONCLUSION

The MeOH extract of bark of *H. mitis* showed strong antifungal activity in antifungal assay studies and showed very low brine shrimp lethality (LC_{50}:1223.36 ppm) indicating that it would be a potential nontoxic anti-fungal natural product. The DCM extracts exhibited strong brine shrimp lethality and may contain potential natural anticancer lead compounds. EtOAc and MeOH extracts of leaves showed the highest antioxidant activity and the highest phenolic content. EtOAc extract of both leaves and bark showed antibacterial activity. Future studies are needed to isolate active antifungal compounds from the MeOH extract of bark, antibacterial compounds from EtOAc extracts and cytotoxic compounds from both DCM extracts of bark and leaves.

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