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Antimicrobial Potential of *Memecylon* L. species from Western Ghats against clinical isolates of pathogenic bacteria.

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ABSTRACT

To study the antibacterial efficiency of *Memecylon* species *viz., M. umbellatum*, *M. malabaricum*, *M. wightii* and *M. edule*. Antibacterial efficiency of *Memecylon* species were examined using hexane, ethyl acetate, methanol and water extracts major groups of phyto constituents were determined by chemical assays. The extracts were tested against five human pathogens *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Escherichia coli,* and *Pseudomonas aeruginosa* using agar well diffusion method and its minimum inhibitory concentration (MIC). The extracts of different plant species showed significant activity against gram-negative bacteria compared to gram-positive bacteria, however, highest zone of inhibition and lowest inhibitory concentration was observed in the methanol extract of *M. umbellatum* and *M. malabaricum* against all the target microorganisms. The MIC for methanol extracts for different bacterial strains ranged from 3.4 to 22 mg/ml in *M. umbellatum* and 3.4 to 28 mg/ml in *M. malabaricum*, 3.2 to 28 mg/ml in *M. edule* and 3.4 to 28 mg/ml in *M. wightii* for different clinical isolates. The ethyl acetate and aqueous extracts of *Memecylon* species showed less antibacterial activity compared to methanol extracts whereas, no activity was observed for hexane extracts.

Keywords: *Memecylon* species, Minimum inhibitory concentration (MIC), Phyto constituents, Antibacterial activity

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INTRODUCTION

Over 80% of world population depends on medicinal plants for their fundamental needs mainly for the treatment of ailments caused by bacteria, viruses, parasites and fungi [1]. Plant metabolites showed a broad spectrum of antimicrobial activity [2]. Numerous reports are available for the use of phytochemicals as promising alternatives to antimicrobial synthetic drugs, to which many infectious microorganisms have become resistant. In Indian traditional medicine a number of medicinal plants have been screened for their antimicrobial activity that include *Datura metel*, *Curcuma longa*, *Eucalyptus globules*, *Zingiber officinale* [3], and also as a source for natural compounds that act as anti-infection agents. Biologically active compounds from the natural source have been of enormous interest to scientists working on infectious diseases. Many useful drugs from higher plants have been revealed by following up ethnomedical uses. The diversity of plants growing in India, along with their known ethnopharmacological uses, offers an enormous possibility of finding new structures with antimicrobial properties.

The genus *Memecylon* L. (*Melastomataceae*) comprises about 300 species and is distributed in Asia, tropical Africa, Australia, Madagascar and Pacific Islands. The genus is represented by about 30 species in India [4-5], of which 15 are found endemic to peninsular India [6]. *Memecylon umbellatum*, is a small tree or shrub. The leaves of the plant are extensively used to treat eye troubles, leucorrhoea, gonorrhea, conjunctivitis, snake bite and skin diseases [7]. Several pharmacological studies have shown its promising antidiabetic, anti-inflammatory, antimicrobial and antioxidant activity [8-9]. This plant also possesses several phyto constituents such as umbelactone, β-amyrine, oleanolic acid, ursolic acid, sitosterol and tannins [10]. The young shoot tip paste of *Memecylon malabaricum*, along with cumin seeds is applied externally on the skin for the treatment of herpes which is mainly used in traditional medicinal system [11], and this plant is reported to have antimicrobial properties against both Gram-positive and Gram-negative bacteria, and fungi [12]. *Memecylon wightii,* mainly used as a folk medicine is distributed in Western Ghats [13]. *Memecylon edule,* is mainly used in the treatment of bruises, B-chronic lymphocytic leukemia and astringent and several pharmacological studies have shown that it has potential for anti-inflammatory and antimicrobial properties [14-15]. The phytochemical analysis of the genus *Memecylon* has shown the presence of 13 fatty acids, 12 methyltetradeconate, glucose, amino acids, carotenoids, a phenolic glycoside and possibly undefined saponins [16].

The present research is designed to determine the antibacterial activity along with its phyto constituents of leaf extracts of four *Memecylon* species *viz., M. umbellatum*, *M. malabaricum*, *M. wightii*, and *M. edule*, available in Western Ghats against certain human pathogenic bacteria namely *Staphylococcus aureus*, *Bacillus subtilis, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Plant sample collection

Different *Memecylon* species *viz*., *M. umbellatum*, *M. malabaricum*, *M. wightii* , and *M. edule*, were collected from Sringeri region in Western Ghats of Karnataka State, India during April 2012. Leaves of the collected plant samples were carefully compared with the deposited voucher specimans of *M. umbellatum* Burm, *M. malabaricum* Clarke, *M. wightii* Thwaites, and *M. edule* Roxb at the herbarium (#IOE LP0002, #IOE LP0003, #IOE LP0004, #IOE LP0005) at the Department of Studies in Biotechnology, University of Mysore and also authenticated by plant taxonomist. The leaves were separated, washed to remove adhering dust particles under running tap water, followed by slow drying at a regulated temperature of 30^{o}C. The dried leaves were ground to a coarse powder using the mechanical grinder and stored at 4^{o}C.

Preparation of extracts

Leaf powder (250 g) was extracted sequentially using 500 ml of non-polar, moderately polar and polar solvents (E. Merck, Bangalore, India) in increasing polarity (hexane < ethyl acetate < methanol < water) using Soxhlet apparatus by continuous hot percolation (boiling point, 52 to 62^{o}C) until the solvent became colorless. The resultant solvent extracts were concentrated in a rotary evaporator (Speed Vac, Savant SPD 2010, Thermo Scientific, Germany) under controlled pressure. Required amount of extracts were weighed and

solubilized in minute quantities of dimethyl sulphoxide (DMSO) and were further diluted as indicated in the sections below.

Phytochemical screening

The screening for alkaloid, flavonoid, carbohydrates, glycosides, proteins and amino acids, steroids, fat and fixed oil was carried out according to the method described by Trease and Evans (1989) [17].

Determination of Total Phenol and Flavonoid Content

The total phenolic content of the extracts of *Memecylon* species was determined using Folin-Ciocalteu reagent [18]. To each extract 50 µl of Folin-Ciocalteu reagent (1/10 diluted) and 7.5 % of Na_2CO_3 (2 ml, w/v) were added and mixed well. The mixture was incubated at 45^{o}C for 15 min. The absorbance of the resulting blue color solution was measured at 765 nm using UV-Vis spectrophotometer (Beckman Coulter, DU 730 Life Sciences) with Na_2CO_3 solution as blank. Quantification was done using Gallic acid as an internal standard, and the results were expressed as mg of Gallic acid equivalents (GAE) per 100 g of dry weight of the sample (mg GAE / 100g DW).

The total flavonoid content was measured by aluminium chloride ($AlCl_3$) method [19]. The reaction mixture consisted of leaves extract (1.0 ml), 1.2 % $AlCl_3$ (0.5 ml) and 120 mM potassium acetate (0.5 ml). The mixture was allowed to stand for 30 min at room temperature and the absorbance was measured at 415 nm. The flavonoid content of the extract is presented as mg quercetin equivalence / mg dry weight extract.

Microorganisms used for the study

Microbial strains used in the study were human pathogens procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. These microbes include the gram-negative bacteria such as *Escherichia coli* (MTCC 724), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (MTCC 733), and Gram-positive bacteria such as *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441). All the aforementioned bacterial strains were cultivated in Luria Bertani (LB) medium from Difco, Becton Dickinson, at 37°C and were maintained at 4°C till further use.

Antibacterial assay

Antibacterial activities of the extracts were determined by Agar well-diffusion method by Perez et al. (1990) [20]. Individual colonies of the bacterial strains of 18h old culture were maintained on TSA and MHB medium and incubated for 16 h at 37 ^{o}C. Bacterial suspensions were prepared and adjusted to approximately 2.0 X 10^8 CFU / ml (optical density at 600 nm = 0.2). They were further swabbed evenly on the surface of MHB agar plates. Four wells (10mm diameter) were made in each of these plates using sterile cork borer. About 0.2 ml of different concentrations of plant solvent extracts and standard antibiotic, ampicillin (5 Mg/ml) (20 ?l) were added to the wells and allowed to diffuse at room temperature for 2h and the plates were incubated for 24 h at 37^{o}C. DMSO, solvent controls/ distilled water and inoculums without plant extract were used as controls. The zone of inhibition was measured, and the antibacterial activity was recorded as average diameter of the zone of inhibition in millimeters was recorded. The experiment was repeated thrice, and triplicates were maintained and the average values were recorded.

MIC was tested using broth dilution method as determined by Wiegand *et al.,* (2008) using 96-well micro titer plate [21]. The bacteria were inoculated into a liquid growth medium in the presence of different concentrations of an antimicrobial agent viz., Ten µl of each concentration was added to each corresponding well of a 96-well micro titer plate and 90 µl of bacteria ($1x10^5$) in MH medium added. Growth is assessed after incubation for a defined period of time (16 – 20 h) at 37^{0} C and the MIC value is read at 595 nm**.** MIC was taken as the lowest concentration where no visual growth of bacteria was detected.

Statistical analysis

All experiment / measurements were made in triplicate, and all the values are expressed as the mean ± SE of three replicates. Data was subjected to ANOVA, and the significant differences were tested by post hoc comparison test (Student-Newman-Keuls) at $p < 0.05$.

RESULTS AND DISCUSSION

The dried leaves of *Memecylon* species were used to prepare the extracts as reported earlier; dried components have higher concentrations of active chemical compounds than crude plant components [22]. Four different types of solvent extracts were prepared including hexane, ethyl acetate, methanol and water for *Memecylon* species namely *M. umbellatum*, *M. malabaricum*, *M. wightii* and *M. edule*. Preliminary phytochemical screening of various extracts of the leaves were tabulated in Table 1. Total phenol and flavonoid content of *Memecylon* species were given in Table 2. The results from the current investigation exhibited that methanol and ethyl acetate extracts of *M. umbellatum* and *M. malabaricum* has significant antibacterial activity whereas hexane and water extract has no activity against tested bacterial pathogens. Further the bacterial pathogens showed differential susceptibility to each extract (Table 3) and the antibacterial activity was not observed in controls (ethyl acetate, methanol and water). We found that Gram-negative bacterial pathogens are more sensitive to the solvent extracts when compared to Gram-positive bacterial pathogens. The studies of Basha *et al.,* [7] in *M. umbellatum*, Elavazhagan *et al.,* [15] in *M. edule*, Hullati *et al.,* [12] in *M. malabaricum* showed potent antibacterial activity for Gram-negative bacteria compared to Gram-positive bacterial pathogens which supports our findings.

The MIC values for tested bacterial pathogens are shown in Table 4. The tested bacterial pathogens showed lower MIC for methanol and ethyl acetate extracts. The MIC value for *S. aureaus* (12 mg/ml), *B. subtillis* (3 mg/ml), *S. typhi* (3 mg/ml), *P. aerguniosa* (15 mg/ml) and *E. coli* (11 mg/ml) was observed in methanol extracts of *Memecylon* species. The MIC values reported by our study were supported by Hullati *et al.,* [12] for some bacterial pathogens such as *B. subtillis* and *S. typhi*. The MIC values were in the range of 3.5 - 15 mg/ml for methanol extract of *M. umbellatum* against *S. aureaus, B. subtillis, S. typhi, E. coli* and *P. aeruginosa*, in the range of 3.4-22 mg/ml for *M. malabaricum*, the MIC values in the range of 3.2-28 mg/ml for *M. edule* and the MIC values in the range of 3.9-28 mg/ml for *M. wightii*. Methanol extract of *M. umbellatum* and *M. malabaricum* was most promising antibacterial agent for *S. aureaus*, *B. subtillis*, *S. typhi* and *E. coli* whereas *P. aeruginosa* was observed to be less sensitive compared to other bacterial pathogens. The ethyl acetate and water extract of *M. umbellatum* and *M. malabaricum* was less sensitive compared to methanol extracts against *S. aureaus* and *B. subtillis*. (Table 4).

The antibacterial activity in plants is mainly due to secondary metabolites [23] (Lamothe *et al.,* 2009) the active constituents of these secondary metabolites includes phenolic compounds and tannins [24] In order to identify the metabolites present in different extracts of *Memecylon* species phytochemical analysis was performed. The data in Table 1 depicted that hexane, ethyl acetate, methanol and water extracts had glycosides, terpenoids, flavonoids and phenolics mainly in the methanol extract followed by ethyl acetate and water extracts. However the highest concentration of these constituents was found mainly in *M. malabaricum*, *M. edule*, *M. umbellatum* and the lowest concentration was found in *M. wightii*. The results obtained were supported by the previous studies reported by Murugesan *et al.,* [25] for which antimicrobial activity and phytochemical analysis were carried out in *M. umbellatum.* Elavazhagan *et al.,* [15] reported the antibacterial activity and detailed evaluation of phytochemical constituents in seed extracts of *M. edule*. The total phenolic and flavonoid content expressed as mg Gallic acid and Querectin equivalents per gram of different *Memecylon* species of various solvent extracts are presented in Table 2. The highest total phenol concentration 0.42 to 1.73 mg/ml was found in *M. umbellatum* and total flavonoid varied from 0.54 to 2.32 mg/ml where as in *M. malabaricum* total phenol was varied from 0.47 to 1.43 mg/ml and total flavonoid varied from 0.85 to 1.85 mg/ml in *M. edule* total phenol was varied from 0.24 to1.59 mg/ml and total flavonoid varied from 0.43 to 1.65 mg/ml and for *M. wightii* total phenol varied from 0.14 to 1.17 mg/ml and total flavonoid varied from 0.42 to 0.75 mg/ml in 5 mg of extracts. These results were in accordance with other previous reports of *Memecylon* species [26].

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Table 1: Analysis of phytoconstituents from various solvent extracts of the leaves of *Memecylon* species.

Sl.no	Phytoconstituents	*M. umbellatum* Leaves extract				*M. malabaricum* Leaves extract				*M. edule* Leaves extract				*M. wightii* Leaves extract			
		Hexane	Ethyl acetate	Methanol	Water	Hexane	Ethyl acetate	Methanol	Water	Hexane	Ethyl acetate	Methanol	Water	Hexane	Ethyl acetate	Methanol	Water
1	Alkaloids	+	-	-	-	-	-	-	-	+++*	+	-	+	+++*	+	-	-
2	Glycosides	-	-	+	+	+	+	++	+	+	-	+	-	-	-	-	-
3	Terpenoids	-	+	+++*	+	-	+	+++*	-	+	++	++	+	+	++	+++*	+
4	Carbohydrates	-	+	+	++	-	-	+	+	+++*	++	+	+	+	++	+	+
5	Proteins	-	+	-	-	-	-	+	-	+	+	+	+	+	+	+	-
6	Phenols	-	+	++	+	-	+	++	-	+	+	-	-	+	+	-	-
7	Saponins	-	+	-	++	-	-	+	-	+++*	+	-	-	+++*	+	-	+
8	Aminoacids	-	+			-	-	-	-	+++*	+	+	+	+++*	++	-	-
9	Flavonoids	-	+	+++*	++	-	-	+++*	-	+	-	+	-	-	+	+	-
10	Steroids	+	+	+	+	-	-	+	-	+++*	+	-	+	+++*	+	-	-

Not detected (-) the particular phytochemical was not detected. The rating (+++) Present in High concentration; (++) Present in moderate amount; (+) Present in trace amounts are subjective estimates based upon relative quantities.

Table 2: Total phenol and flavonoid content of *Memecylon* species

Sl no.	Sample name	Total Phenol Content Gallic acid equivalence				Total Flavonoid Content Quercetin equivalence			
		Methanol	Ethyl acetate	Hexane	water	Methanol	Ethyl acetate	Hexane	Water
1	*M. umbellatum*	1.73±0.2	0.60±0.11	0.42±0.03	0.83±0.02	2.32±0.31	1.89±0.12	0.82±0.03	0.54±0.1
2	*M. malabaricum*	1.43±0.4	0.89±0.01	0.47±0.01	0.91±0.04	2.13±0.23	1.85±0.11	0.10±0.02	0.85±0.2
3	*M. wightii*	1.17±0.2	0.64±0.53	0.148±0.2	0.514±0.3	0.72±0.1	0.75±0.3	0.10±0.2	0.42±0.2
4	*M. edule*	1.59±0.3	0.87±0.2	0.24±0.4	0.15±0.8	1.65±0.3	1.62±0.1	0.84±0.2	0.23±0.2

*Data expressed as mean ± S.E.M. of triplicate experiments

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Table 3: Antibacterial activity of *Memecylon* species (Zone of inhibition in mm) using agar well diffusion method

Medicinal Plant extracts	Gram-positive bacteria						Gram-negative bacteria								
Bacterial pathogens	*Staphylococcus aureus*			*Bacillus subtilis*			*Salmonella typhi*			*Escherichia coli*			*Pseudomonas aerigunosa*		
Control antibiotic ampicillin 5 mg/ml	20 mm			22 mm			16 mm			18 mm			22 mm		
Concentration mg/ml	**1**	**3**	**5**	**1**	**3**	**5**	**1**	**3**	**5**	**1**	**3**	**5**	**1**	**3**	**5**
***M. wightii* Methanol**	-	5	7	-	2	3	-	8	9	6	8	8	2	5	8
Ethyl acetate	-	5	6	2	3	5	-	4	6	2	4	6	5	6	6
Water	1	3	3	-	3	4	1	4	4	-	3	3	-	4	5
***M. edule* Methanol**	-	4	6	2	3	3	2	6	9	3	4	4	-	6	9
Ethylacetate	-	2	3	-	5	5	4	4	4	-	4	5	3	4	5
Water	-	3	3	-	4	5	2	5	6	2	3	3	-	-	3
***M. umbellatum* Methanol**	10	14	14	5	3	3	4	3	4	5	6	12	4	4	6
Ethyl acetate	-	1	8	3	3	3	2	2	5	5	5	10	4	6	6
Water	2	2	2	1	1	1	3	2	2	3	2	2	3	4	4
***M. malabaricum* Methanol**	10	13	13	3	3	5	3	6	9	4	6	8	3	8	8
Ethylacetate	1	3	3	2	3	3	3	3	6	3	4	5	2	4	6
Water	-	2	4	-	3	6	3	4	4	2	2	2	1	2	4

Different solvent leaf extracts of *Memecylon* species 100 µg/disc, Zone of inhibition in mm, (-) indicates no activity, standard antimicrobial compound ampicillin – 5 µg/disc. data expressed as mean ± S.E.M.

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Table 4: Minimum inhibitory concentration (MIC) mg/ml of extracts on the test pathogens as detected by spectrophotometric method at 595 nm.

Sl. No	Plant name	Plant extracts	Gram-positive bacteria		Gram-negative bacteria		
			Staphylococcus aureus	*Bacillus subtilis*	*Salmonella typhi*	*Escherichia coli*	*Pseudomonas aeruginosa*
		Control ampicillin	14.5^{a}	11^{a}	20^{b}	15^{a}	18^{b}
1	*M. umbellatum*	Methanol	13^{a}	3.5^{a}	3.6^{a}	15^{a}	15^{a}
		Ethylacetate	13^{a}	35^{d}	24^{c}	25^{c}	22^{c}
		Water	25^{c}	23^{c}	32^{d}	33^{d}	35^{d}
2	*M. malabaricum*	Methanol	13^{a}	3.4^{a}	3.2^{a}	11^{a}	22^{c}
		Ethylacetate	15^{a}	18^{b}	20^{c}	28^{d}	24^{c}
		Water	28^{d}	26^{d}	12^{a}	21^{c}	22^{c}
3	*M. edule*	Methanol	12^{a}	3.4^{a}	3.2^{a}	12^{a}	28^{d}
		Ethylacetate	23^{c}	26^{d}	40^{d}	44^{d}	44^{d}
		Water	38^{d}	39^{d}	-	-	-
4	*M. wightii*	Methanol	12^{a}	3.9^{a}	3.9^{a}	16^{b}	28^{d}
		Ethylacetate	28^{d}	30^{d}	49^{d}	42^{d}	48^{d}
		Water	40^{d}	34^{d}	-	-	-

MICs were determined as described under materials and methods. The bacterial growth inhibition was read at 540 nm. Data expressed as mean ± S.E.M. of n = 3 experiments. Results with different alphabets in superscript in columns are significantly different ($p < 0.05$).

$3\text{-}15^{a}$ - Potent antimicrobial activity
$16\text{-}20^{b}$ - Moderate antimicrobial activity
$21\text{-}25^{c}$ - Slight antimicrobial activity
Above 26^{d} - No activity

CONCLUSION

The methanol extracts of all the *Memecylon* species have inhibited the *in vitro* growth of target bacterial human pathogens. The extracts of *M. umbellatum* and *M. malabaricum* are more promising whereas *M. edule* was moderately potent and *M. wightii* were less active against all the tested human pathogens the antibacterial potential of these plants was confirmed by evaluating its phyto constituents. Hence, the present study provides the scientific rationale for medicinal use of *Memecylon* species. The use of *Memecylon* extracts is of great significance as substitute antimicrobial agent in medicine.

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