Int J Pharm Bio Sci 2016 April; 7(2): (P) 194 - 199

Original Research Article **Natural chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OIL OF LEAVES AND TWIGS OF *AEGLE MARMELOS*

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ABSTRACT

Aegle marmelos is used extensively in traditional medicine. The antibacterial and antifungal activity of essential oils from leaves and twigs was screened against 13 human pathogenic bacteria and 7 human pathogenic fungi. The oil obtained from twigs exhibited potent broad spectrum activity against *Bacillus subtilis*, *Streptococcus mutans*, *Staphylococcus epidermis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Yersinia enterocolitca*, *Mycobacterium smegmatis*, *Enterobacter aerogenes* and it also showed activity against two fungi *Sporothrix schenckii* and *Aspergillus flavus*.

KEY WORDS: **Aegle marmelos, Rutaceae, leaves and twigs essential oil; antibacterial activity and antifungal activity.**

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INTRODUCTION

Aegle marmelos Correa (Rutaceae) occurs wild in India. It occurs throughout the deciduous forests of India ascending to altitudes of 1200 m in the Western Himalayas and also occurs in Andaman Islands. It is extensively planted near Hindu temples because the leaves have been frequently used in religious offerings in temples through the ages and are valued in indigenous medicine.[1] Almost every part of the plant is used in traditional system of Indian medicine[1], as well as in Unani medicine for the treatment of various diseases. The leaves of bael (*Aegle marmelos*) are reported to have hypoglycemic effect while the root bark and stem bark infusions are used in the treatment of intermittent fever.[1] In Bangladesh; the leaves are used for fertility control. [2] The fruit is used in chronic diarrhea and the unripe or half-ripe fruits are regarded as astringent, digestive and effective against stomachache.[3] The essential oil from twigs is reported to have broad spectrum antifungal activity as compared to 0.5% hamacycin.[4] The antifungal activity of leaf oil was evaluated against the fungus *Fusarium udum* and it showed 80% inhibition at 400 ppm.[5] The seed oil of *A. marmelos* exhibited antibacterial activity[6] and is also used as purgative. The medicinal importance of this plant led us to undertake the present investigation of antimicrobial potential of essential oil obtained from leaves and twigs. The oils were tested against 13 human pathogenic bacteria and 7 pathogenic fungi. It was found that essential oil obtained from twigs possessed broad spectrum activity against bacteria. It also demonstrated significant activity against *Aspergillus falvus* and *Sporothrix schenkii*

MATERIALS AND METHODS

i) Plant material

Fresh leaves of *Aegle marmelos* were collected from Central Institute of Medicinal and Aromatic Plants Farm, Lucknow and Pantnagar, India while twigs were collected from Pantnagar. A voucher specimen has been deposited in Herbarium of the Institute with a voucher no. of CAM101

ii) ***Extraction of essential oil and identification of its constituents:***

The fresh plant material (100 g each of twigs and leaves) was hydro-distilled in the Clevenger's type apparatus for 4 hrs. The oil was dried over anhydrous sodium sulphate and stored in refrigerator. Gas chromatography was carried out on Perkin Elmer Auto System XL fitted with a PE-5(5% phenyl, 95% dimethyl polysiloxane), capillary column (50 m X 0.32 mm); film thickness 0.20 µm; carrier gas H_2. Oven temperature 100 ^{0}C for 2 min and then programmed from 100-280 ^{0}C at 3 ^{0}C/min. Injector and detector temperature 220 ^{0}C and 300 ^{0}C respectively. GC/MS analysis were carried out on a Perkin Elmer Turbomass coupled with GC-Auto XL, MS at 70 eV; column and temperature programme same as above using gas Helium Inlet pressure 10 psi. The constituents were identified by comparing their retention indices with those of authentic samples or identified in essential oils of known composition. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using NBS54K.L and WILEY.L built-in libraries and those reported in the literature. [7, 8]

iii) Microbial strains and media

The microorganisms used in the present study and their origin are presented in Table-1. The bacterial and fungal sensitivity testing was performed on the Muellar-Hinton Agar (Himedia) and Sabauraud dextrose agar (Himedia) culture media respectively. All the bacterial strains were grown on nutrient agar/broth for routine cultivation. Tetracycline was used as control antibiotic in the bioactivity assay with bacteria. The test samples (oils) were used either neat or diluted with DMSO (Merck) and stored at 4 ^{0}C before use.

Table 1
Growth conditions for microorganisms

S No.	Name of microorganism	Strain designation/source	Incubation temp. (^{0}C)	Medium used
1	*Bacillus subtilis* (BS)	MTCC[a] 121	30	MH[b]
2	*Streptococcus mutans* (SM)	MTCC 890	37	BHIA[d]
3	*Staphylococcus epidermis* (SE)	MTCC 435	37	MH
4	*Staphylococcus aureus* (SA)	MTCC 96	30	MH
5	*Enterococcus faealis* (EF)	MTCC 439	37	MH
6	*Escherichia coli* (EC)	MTCC 723	37	MH
7	*Pseudomonas aeruginosa* (PA)	MTCC 741	37	MH
8	*Salmonella typhi* (ST)	MTCC 733	37	MH
9	*Salmonella typhimurium* (STM)	MTCC 98	37	MH
10	*Klebsiella pneumoniae* (KP)	MTCC 109	37	MH
11	*Yersinia enterocolitica* (YE)	MTCC 861	30	MH
12	*Mycobacterium smegmatis* (MS)	UDSC[c]	37	MH
13	*Enterobacter aerogenes* (EA)	MTCC 111	30	MH
14	*Sporothrix schenkii* (SS)	AIIMS[e]	28	SDA
15	*Aspergillus falvus* (AF)	AIIMS	28	SDA
16	*Microsporum gypseum* (MG)	AIIMS	28	SDA
17	*Aspergillus niger* (AN)	AIIMS	28	SDA
18	*Trichophyton rubrum* (TR)	AIIMS	28	SDA
19	*Candida albicans* AI (CAI)	AIIMS	28	SDA
20	*Candida albicans* (CA)	AIIMS	28	SDA

[a]MTCC: Microbial type culture collection; [b]MH: Meuller-Hinton Agar
[c]UDSC: University of Delhi South Campus; [d]BHIA: Brain-Heart infusion Agar
[e]AIIMS: All India Institute of Medical Sciences; [f]SDA: Sabauraud dextrose Agar

iv) Disc diffusion assay

Antimicrobial activity testing was carried out according to the method reported by Bauer *et al.* [9] All the bacteria were subcultured from -80 ^{0}C stock cultures into 5 ml of Muellar-Hinton Agar and incubated for 24 hrs at desired temperatures. For use as an inoculum, the turbidity of the bacterial suspension was adjusted to the MacFarland standard 0.5 (2-4 X10^6 cfu/ml). About 100 µl of bacterial culture was spread, plated on solid medium and discs (5 mm diameter) impregnated with 5 µl of test oil were placed on the pre-inoculated agar surface. Observations were recorded after 24 hrs of incubation of plates at desired temperatures. The fungi were subcultured from -80 ^{0}C and grown on SDA at 28 ^{0}C for seven days except for *C. albicans*, which was incubated for 24 hrs suspension of each fungus was prepared in 0.85% normal saline containing 0.1% Tween 80.[10] For use as inoculum, the turbidity of the fungal suspensions was adjusted to the McFarland standard (0.5) in each assay. 20 µl of fungal culture (0.7 X 10^5 spores or 0.2X10^5 cfu/ml in case of yeast) was spread plated on soild medium and discs (5 mm diamater) impregnated with 8 µl of test oil was placed on the pre-inoculated agar surface. Observations were recorded after incubation of plates at 28 ^{0}C after seven days except for *C. albican,* which was incubated for 24 hrs.

v) Microbroth dilution assay

Two-fold serial dilution technique (Petersdorf *et al*) [11] was employed to assess the minimum inhibitory dilution (MID) of test essential oils against microbial strains. In a series of 8 tubes serial dilutions was made. In first tube, 2ml of nutrient broth was taken and in subsequent tube 1 ml of broth was taken after that, 10 µl of test oil was added in the first tube and mixed properly. From the first tube 1 ml of broth containing antibiotic was taken and added to the second tube and mixed properly. This was repeated until the seventh tube. 1 ml of mixture was expelled out from the last tube. Only broth culture was used as a control. To each of this tube, 10 µl of properly diluted log phase culture of test organism with a titre of 10^4 cfu /ml was added. The tubes were incubated at desired temperature and examined by turbidity measurement. The optical density of the culture was measured using Spectra Max 190 microplate reader (Molecular Devices Corp. USA). The MID was taken as the lowest dilution of the test oil, which inhibited the appearance of visible growth.

RESULTS AND DISCUSSION

In order to determine the antimicrobial activity, bioassays of these 3 essential oils were conducted against 13 human pathogenic bacteria and 7 pathogenic fungi. It was found that essential oil obtained from twigs possessed broad spectrum activity against bacteria (table-2). It also demonstrated significant activity against *Aspergillus falvus* and *Sporothrix schenkii* (table-4).

Table 2

Antibacterial activity of essential oil measured as diameter of the zone of growth inhibition (mm)

S No.	Bacterial strains	Zone of inhibition of essential oil (mm)		
		B1	B2	B3
1	SM	08±0.5	-	07±0.5
2	MS	-	08	07±0.5
3	EF	07±0.5	-	07±0.5
4	SA	-	-	08±0.5
5	SE	-	-	10±0.5
6	EA	06±0.5	-	08±0.5
7	EC	-	-	-
8	KP	-	-	-
9	SY	-	-	07±0.5
10	ST	-	-	08±0.5
11	BS	06±0.4	06±0.4	10±0.5
12	YE	-	-	09±0.5
13	PA	08±0.4	06±0.4	17±0.5

B1=Essential oil of leaves (CIMAP farm, Lucknow) of A. marmelos
B2=Essential oil of leaves (Pant Nagar) of A. marmelos
B3=Essential oil of twigs (Pant Nagar) of A. marmelos
= no inhibition

The essential oil obtained from leaves (CIMAP farm, Lucknow and Pant Nagar, India) exhibited significant activity against *B. subtilis* and *P aeruginosa* (table-2) while no activity was observed against any of the fungus that were tested (table-4). It was observed that *M. smegmatis* was particularly sensitive to the oil obtained from leaves (Pant Nagar) whereas *S. mutans*, *E. faecalis* and *E. aerogenes*) were sensitive to the essential oil obtained from leaves (CIMAP farm, Lucknow) (table-2).

Table 4

Antifungal activity of essential oil of Aegle marmelos measured as diameter of the zone of growth inhibition (mm) and minimum inhibitory dilution

SNo	Fungal strains	Zone of inhibition of oil (mm)			Minimum inhibitory dilution of oil		
		B1	B2	B3	B1	B2	B3
1	AF	-	-	11	-	-	1/1600
2	SS	-	-	05	-	-	1/800

B1=Essential oil of leaves (CIMAP farm, Lucknow) of A. marmelos
B2=Essential oil of leaves (Pant Nagar) of A. marmelos
B3=Essential oil of twigs (Pant Nagar) of A. marmelos
- = no inhibition

A comparison of zone of inhibition was done to find out the effectiveness of oil in controlling the microbial growth as depicted in chart 1.

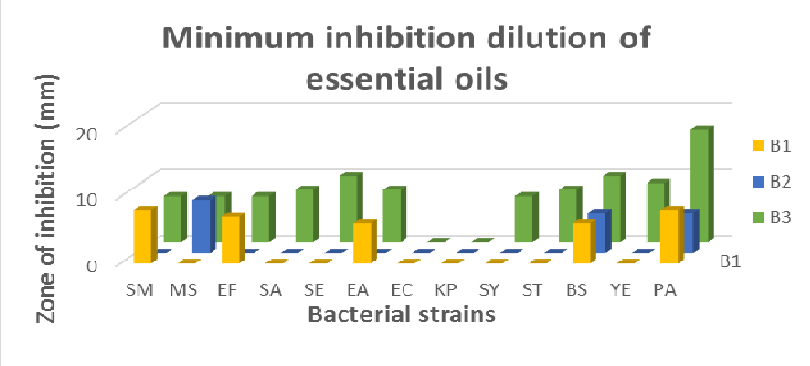

Chart 1

Comparative study of zone of inhibition of essential oils

The minimum inhibitory dilution was evaluated for all these three oil by broth dilution assay. The minimum inhibitory dilution for the essential oil obtained from twigs varied between 1/400 to 1/1600 (table-3 and 4) for all microorganism that were tested. The highest dilution (1/1600) for disappearance of visible microbial growth was observed against *P. aeruginosa* and fungus *A. falvus* for this oil. The minimum inhibitory dilution of the essential oil obtained from leaves (Pant nagar) (Table-3) was found to be 1/400 for MS, BS and PA while the value of minimum inhibitory dilution for essential oil obtained from leaves (CIMAP farm, Lucknow) varied between 1/400 to 1/800 for different strains of bacteria (table-3).

Table 3
The minimum inhibitory dilution of essential oil of Aegle marmelos

SNo.	Bacterial strains	Minimum inhibitory dilution of essential oil		
		B1	B2	B3
1	SM	1/800	-	1/800
2	MS	-	1/400	1/800
3	EF	1/800	-	1/800
4	SA	-	-	1/800
5	SE	-	-	1/800
6	EA	1/400	-	1/800
7	EC	-	-	-
8	KP	-	-	-
9	SY	-	-	1/400
10	ST	-	-	1/800
11	BS	1/400	1/400	1/800
12	YE	-	-	1/800
13	PA	1/800	1/400	1/1600

B1=Essential oil of leaves (CIMAP farm, Lucknow) of A. marmelos
B2=Essential oil of leaves (Pant Nagar) of A. marmelos
B3=Essential oil of twigs (Pant Nagar) of A. marmelos
- = no inhibition

It is quite evident from the above results that oil obtained from twigs is very active against most of the test microorganisms and minimum inhibitory dilution is also quite low which indicates that very small volume (1/400 to 1/1600) of the oil is needed to inhibit the microorganisms. A comparative study of minimum inhibitory dilution of all the oils was carried out and depicted in chart 2, the lower height shows more efficacy of essential oil.

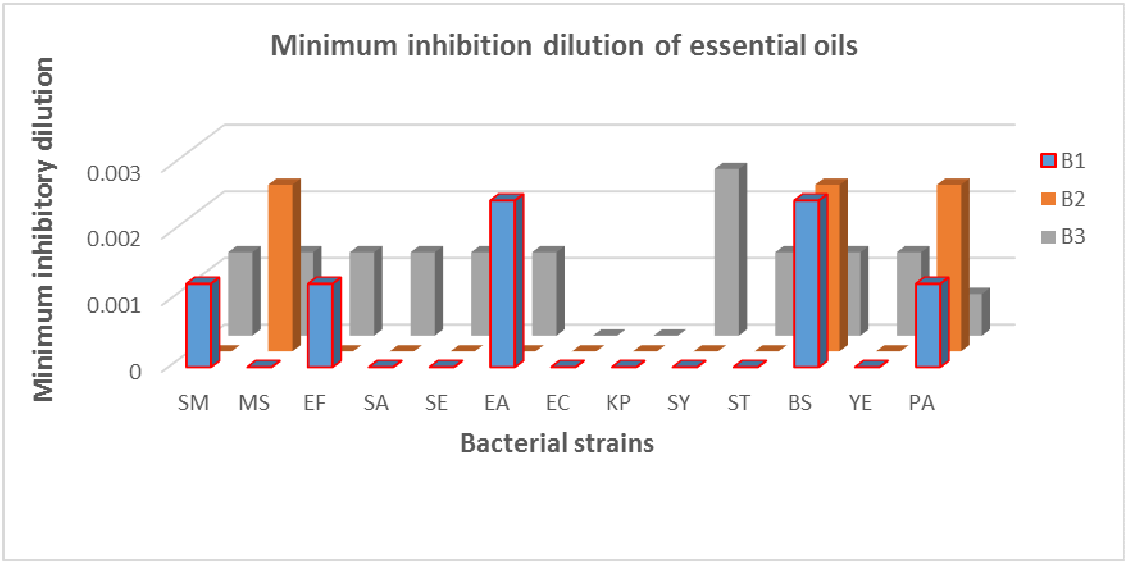

Chart 2
Comparison of the minimum inhibitory dilution of essential oil of Aegle marmelos

The percentage of various chemical constituents as studied by GC and GC/MS is summarized in table-5. The composition is similar as obtained earlier. [12] The composition of all the three oils was found to be more or less the same, differing in percentage of some constituents which might be responsible for the potency of essential oil of twigs against the tested pathogens for instance the % of (Z)- β-ocimene was found to be 39.80 while this compound was present in traces in leaves oil. The percentage of limonene was around 82% as reported earlier, this might make this oil more selective for inhibition of *Mycobacterium smegmatis.*

Table-5
Chemical composition (percentage) of essential oil of Aegle marmelos

Constituents	RI	B1	B2	B3	Method of identification
α-pinene	935	3.63	-	0.20	MS, RI, std
Camphene	950	0.15	-	0.10	MS, RI, std
sabinene	970	-	1.02	T	MS, RI, std
β-myrcene	987	2.02	2.10	0.10	MS, RI, std
α-phellendrene	1010	11.26	-	-	MS, RI, std
p-cymene	1021	1.95	-	0.80	MS, RI, std
limonene	1027	57.75	82.38	51.70	MS, RI, std
β- phellendrene	1032	14.57	-		MS, RI, std
(Z)- β-ocimene	1035	5.10	5.10	39.80	MS, RI, std
linalool	1094	0.15	0.18	2.10	MS, RI, std
Limonene oxide	1107	-	-	0.40	MS, RI, std
Terpinen-4-ol	1155	0.13	-	-	MS, RI, std
2,6-dimethyl-1,3,5,7-octatetraene	1173	-	-	0.10	MS, std
piperitone	1238	-	0.19	-	MS, RI, std
eugenol	1351	-	-	T	MS, RI, std
β-elemene	1380	0.22	0.95	0.30	MS, RI, std
β-caryophyllene	1398	4.15	0.37	1.20	MS, RI, std
α-humulene	1451	-	0.10	-	MS, RI, std
Y-curcumene	1460	-	0.12	-	MS, RI, std
germecrene	1465	-	-	0.50	MS, RI, std
β-selinene	1471	0.20	-	0.20	MS, RI, std
elemol	1550	-	0.11	-	MS, RI, std
Caryophyllene oxide	1575	0.15	0.22	0.20	MS, RI, std
Total percentage		96.33	92.84	97.70	

B1=Essential oil of leaves (CIMAP farm, Lucknow) of A. marmelos
B2=Essential oil of leaves (Pant Nagar) of A. marmelos
B3=Essential oil of twigs (Pant Nagar) of A. marmelos
- = no inhibition
T = traces

ACKNOWLEDGMENT

The authors are thankful to Instrumentation Division, CIMAP (Lucknow) for GC and GC/MS spectra and CSIR for financial support

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