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NATURAL DRUGS

ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF SERIAL EXTRACTS FROM LEAVES OF *AEGLE MARMELOS* (LINN.)

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Abstract: The *in vitro* antimicrobial activity of serial petroleum ether, chloroform and methanol extracts from leaves of *Aegle marmelos* were investigated against bacterial and fungal species. All the extracts exhibited broad spectrum antimicrobial activity with zones of inhibition ranging from 10 to 22 mm against bacteria: *Staphylococcus aureus, β Streptococcus haemolyticus group A, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi,* fungi: *Candida albicans, Candida tropicalis and Aspergillus flavus*. The minimal inhibitory concentrations (MIC) and the minimal microbicidal concentrations (MMC) of the extracts ranged from 1.25 to 10 mg/mL and 2.5 to 20 mg/mL respectively. Assessment of antibacterial efficacy of different extract revealed that *Staphylococcus aureus, β Streptococcus haemolyticus group A, Pseudomonas aeruginosa* and *Escherichia coli* showed high susceptibility to petroleum ether extract. *Proteus mirabilis, Klebsiella pneumoniae* showed high susceptibility to chloroform extract and *Salmonella typhi* showed high susceptibility to methanol extract. Petroleum ether extract exhibited the highest antifungal efficacy against all tested fungal species. Phytochemical screening revealed the presence of phenols, sterols in petroleum ether and chloroform extracts, whereas tannins, flavonoids, coumarins, saponins and triterpenoids in methanol extract. The ability of the leaf extracts of *Aegle marmelos* to inhibit growth of bacteria and fungi is an indication of its broad spectrum antimicrobial activity which could be a potential source for development of novel bioactive antimicrobial agents.

Keywords: *Aegle marmelos*, antimicrobial activity, serial extracts, phytochemicals

The continued emergence or persistence of drug resistant organisms and the increasing evolutionary adaptations by pathogenic organisms to commonly used antimicrobials have reduced the efficacy of antimicrobial agents currently in use. In addition to this, antibiotics are associated with adverse effects, therefore, the search for new drugs from novel sources, such as plants, is necessary. It has been pointed out that more than 80% of world's population depends on plants to meet their primary health care needs (1). Plants continue to be a major source of commercially consumed drugs. Even many synthetic drugs have their origin from natural plant products. The trend of using natural products has increased in recent years and the active plant extracts are frequently screened for new drug discoveries (2).

Aegle marmelos (Linn.) belongs to family *Rutaceae*, commonly known as bael (Hindi) and golden apple (English). It is found throughout India and is known from pre-historic time. *Aegle marmelos* has been used from time immemorial in traditional systems of medicine for relieving constipation, diarrhoea, dysentery, peptic ulcer and respiratory infections (3). Several studies on different parts of *Aegle marmelos* showed that the plant possesses antidiarrhoeal (4), antidiabetic (5), anti-inflammatory, antipyretic, analgesic (6), anticancer (7), radioprotective (8) and antimicrobial activities (9, 10). Limited information is available regarding antimicrobial activity of *Aegle marmelos* leaves; therefore, present study is carried out to investigate antimicrobial activity of serial extracts from leaves of *Aegle marmelos* against various bacterial and fungal species. Preliminary phytochemical studies of these extracts are also undertaken to find out bioactive compounds having antimicrobial activity.

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MATERIALS AND METHODS

Plant material

The leaves of *Aegle marmelos* were collected from their natural habitat from Gwalior city, Madhya Pradesh, India in the month of April 2008. The plant leaves were identified by Dr. K.K. Koul, Professor and Head of the Department, School of Studies in Botany, Jiwaji University, Gwalior and a voucher specimen (skam/08) has been retained in our laboratory for further reference.

Preparation of extract

The shade dried leaves were powdered using a mechanical grinder and passed through 40 mesh sieve. Powder (300 g) was successively extracted with 1.5 L of petroleum ether, chloroform and methanol, in a Soxhlet apparatus at 60–70°C each for 10–12 h consecutively. Solvents used were of analytical grade and removed from all the three extracts under vacuum and a semisolid mass was obtained. Yield of petroleum ether, chloroform and methanol extracts were 2.10, 1.58 and 11.5% w/w, respectively. Extracts were stored in sterile amber colored storage vials in refrigerator until used for experiment.

Formulation of extract

Each extract was dissolved in 20% dimethyl sulfoxide (DMSO) treated water and sterilized by passing through membrane filter of 0.2 µm pore size before antimicrobial testing.

Test microorganisms

Bacterial and fungal isolates used in the present study (bacteria*: Staphylococcus aureus* (ATCC 25923)*, β Streptococcus haemolyticus group A, Proteus mirabilis, Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella typhi,* fungi*:Candida albicans, Candida tropicalis and Aspergillus flavus*) were obtained from HiMedia Laboratories Pvt. Ltd. Navi Mumbai, culture collections of microbiology departments of All India Institute of Medical Sciences, New Delhi and Gajara Raja Medical College, Gwalior, India. The bacterial isolates were first subcultured in a nutrient broth and incubated at 37°C for 18 h while the fungal isolates were subcultured on a Sabouraud dextrose agar for 72 h at 25°C.

Phytochemical screening

The extracts were subjected to preliminary phytochemical screening for possible presence of bioactive antimicrobial compounds by the methods of Trease and Evans (11) and Harborne (12).

1. Test for tannins – (1 mL of extract + few drops of 10% lead acetate), appearance of precipitate indicated the presence of tannins.

2. Test for flavonoids – (2 mL of extract + conc. hydrochloric acid + magnesium ribbon), appearance of pink-red color indicated the presence of flavonoids.

3. Test for saponins – (1 mL of extract + 9 ml distilled water, shaken vigorously), appearance of stable froth indicated the presence of saponins.

4. Test for phenols – (1 mL of extract + 5 mL distilled water + few drops of neutral ferric chloride) appearance of dark green color indicated the presence of phenol.

5. Test for coumarins – (1 mL of extract + 1 mL of ethanol KOH solution) appearance of precipitate indicated the presence of coumarins.

. Test for sterols – (25 mg of extract + 1 mL chloroform + few drops of acetic anhydride + 2 drops of conc. sulfuric acid) appearance of green solution indicated the presence of sterols.

7. Test for triterpenoids – (Liebermann-Burchard) (2 mL of extract + 1 mL of chloroform + few drops of acetic anhydride + conc. sulfuric acid added along the side of test tube) appearance of red-brown color indicated the presence of triterpenoids.

Antimicrobial activity

The antimicrobial sensitivity patterns for the extracts were studied by disc diffusion method (13). Sterile discs (6 mm) prepared from Whatman's filter paper no. 1 were made to absorb (500 µg) of the test samples. Discs were left to dry under laminar flow cabinet overnight. Standard reference antimicrobial discs with cefuroxime (30 µg) for bacteria and fluconazole (10 µg) for fungal species were used as positive control and solvent discs were used as negative control. The microbial isolates were first grown in a nutrient broth for 18 h before use and standardized to 0.5 Mc Farland standards (1.5×10^8 cfu/mL). Mueller-Hinton agar was prepared on the plates as the medium for the test organism. The microbial inoculum was spread evenly onto the surface of agar plate using the sterile cotton bud and then the extracts discs, 20% DMSO impregnated discs and standard antimicrobial discs were positioned on the inoculums agar surface. The antimicrobial activity was interpreted from the size of diameter of zone of inhibition measured to the nearest mm as observed from clear zone surrounding the disc. Each extract was assayed in triplicate and the mean of the three values was taken.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentrations of different extracts were determined by twofold serial micro dilution method using sterile 96 well microtitre plates (14). Hundred microliters of the test extracts at a final concentration ranging from 10 to 0.0049 mg/mL were introduced into the wells before 100 µL of standardized cell suspensions were added in each well. Microbial suspensions were used as a positive control and extract in broth was used as negative control. The MIC was taken as the lowest concentration of the extract in the well of microtitre plate that showed no turbidity after 24 h of incubation at 37°C. The turbidity of the wells was interpreted as the visible growth of microorganism.

Determination of minimal microbicidal concentration (MMC)

The MMC of the extracts was determined by a modification of the method of Spencer and Spencer (15). Samples were taken from plates with no visible growth in the MIC assay and subcultured on freshly prepared nutrient agar plates and SDA plates, later incubated at 37°C for 48 h and 25°C for 72 h for bacteria and fungi, respectively. MMC was taken as the concentration of the extract that did not show any visible growth on new set of agar plates.

RESULTS

Antibacterial activity

All the three extracts showed varying degree of antibacterial activity against the test organisms (Table 1).

Disc diffusion assay revealed maximum inhibition zones against Gram positive organisms *Staphylococcus aureus, β Streptococcus haemolyticus group A* and Gram negative organism *Pseudomonas aeruginosa and Escherichia coli* with petroleum ether extract suggesting the highest antibacterial efficacy of petroleum ether extract against these organisms. Further, it compared favorably with standard antibacterial drug cefuroxime. Antibacterial activity of petroleum ether extract was moderate against *Proteus mirabilis* and mild against *Klebsiella* and *Salmonella typhi*. *Proteus mirabilis* and *Klebsiella pneumoniae* showed maximal zone of inhibition with chloroform extract suggesting high antibacterial efficacy of chloroform extract against these organisms. Further, it compared favorably with cefuroxime. The antibacterial activities of chloroform extract were moderate against *Pseudomonas aeruginosa* and *Escherichia coli* and were mild against *Salmonella typhi*. Methanol extract showed maximum zone of inhibition against *Salmonella typhi* suggesting highest efficacy against this organism. Further, it compared favorably with cefuroxime. The antibacterial activities of methanol extract were mild against the rest of the tested microorganisms.

The MIC of different extracts ranged from 1.25 to 10 mg/mL and are shown in Table 2.The MIC for Gram positive organisms *Staphylococcus aureus* and *β Streptococcus haemolyticus group A* organisms were the lowest with petroleum ether extract suggesting that the smallest amount of this extract was required and was most potent. Also the MIC for control cefuroxime ranged from 0.0195 to 0.0391 mg/mL. The MIC for Gram negative organisms *Proteus mirabilis* and *Klebsiella pneumoniae* were the lowest with chloroform extract suggesting that the smallest amount of chloroform extract was required and was most potent. Also the MIC for control cefuroxime ranged from 0.0391 to 0.078 mg/mL. The MIC for *Salmonella typhi* was the lowest with methanol extract suggesting that the smallest amount of this extract was required and was most potent. The MIC of the standard drug cefuroxime was 0.078 mg/mL. The MMC of the petroleum ether, chloroform and methanol extracts for different bacteria ranged from 2.5 to 20 mg/mL.

Antifungal activity

The extracts showed broad spectrum varying degree of antifungal activity against the tested fungal isolates (Table 1).The antifungal activity against *Candida albicans, Candida tropicalis* and *Aspergillus flavus* showed the maximum zone of inhibition with petroleum ether extract, suggesting the highest efficacy of this extract. Further, it compared favorably with standard antifungal drug – fluconazole. The antifungal efficacy of chloroform extract was mild and methanol extract was moderate against test fungal species. The MIC (Table 2) ranged from 1.25 to 10 mg/mL of petroleum ether, chloroform and methanol extracts. The lowest concentration of ether extract was required against all test fungal species and was most potent. The MIC for control fluconazole ranged from 0.0049 to 0.0098 mg/mL. The MMC (Table 2) against different fungi ranged from 2.5 to 20 mg/mL of the tested extracts.

Phytochemical screening

Phytochemical screening of the extracts revealed the presence of phenols and sterols in petroleum ether and chloroform extracts, whereas

Table 1. Antimicrobial activity of serial extracts from leaves of *Aegle marmelos*.

Test microorganism	Zone of inhibition (mm) (the mean ± SD)				
	Pet. ether (500 µg/mL)	Chloroform (500 µg/mL)	Methanol (500 µg/mL)	Cefuroxime (30 µg/mL)	Fluconazole (10 µg/mL)
Staphylococcus aureus	16 ± 0.4	10 ± 0.2	10 ± 0.3	22 ± 0.2	–
β Streptococcus haemolyticus group A	18 ± 0.8	14 ± 1	12 ± 0.4	23 ± 0.5	–
Proteus mirabilis	14 ± 1.2	20 ± 0.1	12 ± 1.3	24 ± 0.5	–
Pseudomonas aeruginosa	16 ± 0.8	16 ± 1.5	10 ± 0.6	22 ± 1.1	–
Escherichia coli	18 ± 0.4	14 ± 0.6	10 ± 0.2	24 ± 1.4	–
Klebsiella pneumonia	12 ± 1.2	18 ± 0.4	10 ± 1.1	24 ± 1.2	–
Salmonella typhi	12 ± 0.6	14 ± 0.8	22 ± 0.6	20 ± 1.5	–
Candida albicans	22 ± 0.2	12 ± 0.6	16 ± 0.4	–	24 ± 0.5
Candida tropicalis	22 ± 0.3	14 ± 0.2	16 ± 0.8	–	26 ± 0.1
Aspergillus flavus	20 ± 1.1	16 ± 0.8	14 ± 0.3	–	24 ± 0.6

Pet. = petroleum, – = not done

Table 2. MIC and MMC values of extracts from leaves of *Aegle marmelos* and standard drugs in mg/mL.

Test microorganism	Petroleum ether		Chloroform		Methanol		CF	FL
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MIC
Staphylococcus aureus	2.5	5	10	20	10	10	0.0391	–
β Streptococcus haemolyticus group A	1.25	2.5	10	20	5	10	0.0195	–
Proteus mirabilis	10	20	1.25	2.5	5	10	0.0195	–
Pseudomonas aeruginosa	5	20	5	10	10	10	0.0391	–
Escherichia coli	5	10	5	10	10	10	0.0391	–
Klebsiella pneumonia	10	20	2.5	5	10	20	0.0391	–
Salmonella typhi	10	10	5	10	1.25	2.5	0.078	–
Candida albicans	1.25	2.5	10	20	2.5	5	–	0.0098
Candida tropicalis	1.25	2.5	5	10	2.5	5	–	0.0049
Aspergillus flavus	1.25	2.5	10	20	2.5	5	–	0.0098

Pet = Petroleum; CF = Cefuroxime; FL = Fluconazole MIC = minimum inhibitory concentration; MMC = minimum microbicidal concentration.

Table 3. Phytochemical screening of serial extracts from leaves of *Aegle marmelos*.

Phytochemicals	Ether extract	Chloroform extract	Methanol extract
Tannins	–	–	+
Flavonoids	–	–	+
Saponins	–	–	+
Phenols	+	+	+
Coumarins	–	–	+
Sterols	+	+	+
Triterpenoids	–	–	+

(+) present; (–) absent

phenols, sterols, tannins, flavonoids, saponins, coumarins and triterpenoids were present in methanol extract (Table 3).

DISCUSSION

Aegle marmelos leaf extracts showed varying degree of broad spectrum antimicrobial activities against tested bacterial and fungal species. Antimicrobial activities of petroleum ether and chloroform extracts could be attributed to the presence of phenols and sterols as such activities with these compounds are reported (16, 17). The antimicrobial activities of methanol extract may be due to the presence of tannins, triterpenoids and flavonoids. Tannins have been known to form irreversible complexes with prolene rich protein resulting in the inhibition of cell wall synthesis (18). Triterpenoids are known to weaken the membranous tissue, which results in dissolving cell wall of microorganism (19). Flavonoids, another constituent of methanol extract, have exhibited a large number of biological activities like anti-inflammatory, antioxidant and antimicrobial properties (20). Antifungal activity exhibited by methanol extract of *Aegle marmelos* leaves against all tested fungi may additionally be contributed due to the presence of coumarins. This is supported by earlier work (21) showing antifungal activity of herbal plants containing coumarins.

It is concluded that serial petroleum ether, chloroform and methanol extracts from leaves of *Aegle marmelos* showed variable broad spectrum antimicrobial activities. Although the exact active components of these extracts that showed these effects were not identified, yet the positive presence of antimicrobial active principles such as phenols, sterols, flavonoids, tannins, triterpenoids and coumarins seems to cause these activities. The ability of the leaf extracts of *Aegle marmelos* to inhibit growth of bacteria and fungi is an indication of its broad spectrum antimicrobial activity, which may be employed as a source to develop new antimicrobial agents.

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