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Research Article

FORMULATION AND EVALUATION OF ANTIBACTERIAL ACTIVITY OF A HERBAL OINTMENT PREPARED FROM CRUDE EXTRACTS OF *AEGLE MARMELOS*, (BAEL)

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ABSTRACT

Objective: The main objective of the work was to analyse the phytochemical constituents from different parts of *Aegle marmelos* to determine its antibacterial potential against *Staphylococcus aureus* and to apply this antibacterial potential in the preparation of ointment.

Methods: Various phytochemicals were extracted from leaves, ripe fruit, unripe fruit and stem of *Aegle marmelos* by solvent extraction using Soxhlet apparatus and aqueous extraction and these phytochemicals were qualitatively analysed. The antibacterial activity and minimum inhibitory concentration of different extracts of *Aegle marmelos* was investigated against *Staphylococcus aureus.* Ointment was formulated and evaluated using selective extracts.

Results: The qualitative analysis showed presence of maximum phytochemicals in leaves, ripe and unripe fruit and very less phytochemicals were present in stem of *Aegle Marmelos*. The antibacterial activity and MIC tests showed that ethanol and methanol extracts of unripe fruit have greater antibacterial potential compared to other extracts. The physicochemical parameters of the formulated ointments were identified. The antibacterial activity of ointments was tested and ethanol and methanol extract of unripe fruit using Soxhlet apparatus showed good antibacterial potential.

Conclusion: Unripe fruit showed maximum antibacterial activity against *S. aureus* compared to leaves, ripe fruit and stem. Also ethanol extract showed maximum zone of inhibition compared to methanol and aqueous extract. The antibacterial activity was retained after the formulation of ointment. Stability studies of the ointment showed that the ointment was stable after two months.

Keywords: *Aegle marmelos*, phytochemicals, Antibacterial activity, Minimum inhibitory concentration, Ointment.

INTRODUCTION

Use of medicinal plants as traditional medicine is one of the common practices in India due to their wide pharmacological activities. Traditional medicines are being used at the primary health care level by many developed and developing countries. More than 80% of world's population depends on plants for primary health care needs. Many currently used drugs are expensive or not readily available and a major setback to their continued usage is the development of resistance. Some drugs may also have side effects. This situation urgently forced scientists for searching drugs which are inexpensive, safe, biodegradable, have less side effects and which will be able to act for longer periods before resistance sets in.

Since ancient time in India, herbal medicines have been the basis of treatment and cure for various diseases physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Herbal medicines have various therapeutic uses such as healing wounds, treating inflammations due to infection, skin lesions, leprosy, diarrhoea, scabies, venereal diseases, snake bite and ulcers etc.

Many infectious agents such as virus, fungi, and parasites may harm the plants. All plants synthesize a variety of secondary metabolites capable of providing them protection against such infectious agents. The secondary metabolites obtained from medicinal plants are known as phytochemicals which serve as lead compounds in drug discovery and design.

Aegle marmelos (L) Corr. belongs to the family Rutaceae and is popularly known as Bael tree. Hindu physicians regard the unripe or half ripe fruit as astringent, digestive and stomachic and prescribe it for diarrhoea and dysentery. The fresh juice of the leaves is taken with honey as a laxative and febrifuge; it is used in asthmatic complaints. The fruit is used as a remedy for diarrhoea and also used for dryness of eyes. Leaf juice mixed with honey is a folk remedy for fever [1].

Several studies on different parts of *Aegle marmelos* showed that the plant possesses anti-diarrhoeal, anti-diabetic, anti-inflammatory, antipyretic, analgesic, anticancer, antiviral, radio protective and antimicrobial activities ([2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13]). Limited information is available regarding antimicrobial activity of *Aegle marmelos* plant; therefore, present study was carried out to investigate antimicrobial activity of serial extracts from leaves, ripe fruit and unripe fruit of *Aegle marmelos* against gram positive bacteria *S. aureus* (NCIM 2079) and fungus *A. niger*. Preliminary phytochemical studies of these extracts were also undertaken to find out bioactive compounds having antimicrobial activity. Since *Aegle marmelos* has anti-inflammatory potential, the study will also be focused on its application in treating skin infections.

MATERIALS AND METHODS

Collection of Plant material

The *Aegle marmelos* leaves, ripe fruit, unripe fruit and stem were collected from local area of Pune, Maharashtra in the month of July 2012. The samples were washed with distilled water to clean the adhering dust particles. Then the leaves and bark were dried in shade for 3 weeks and fruits were freeze dried. The dried plant samples were grinded using mechanical grinder to fine powder and passed through 40 micron sieve the samples were stored in airtight container.

Preparation of plant extracts

An extract is a mixture of phytochemicals from any plant which is obtained by extraction of specific parts of the plant. The crude extracts of different parts of *Aegle marmelos* were prepared using different solvents such as ethanol (70%), methanol (80%) and distilled water. The different methods used for extraction were as follows:

1) Solvent extraction: Plant material was dissolved in 70% ethanol and 80% methanol, (1:10); 1 g sample should be dissolved in 10 ml of solvent. Mixtures were kept in the dark for 3 days at room temperature in sterilized beakers wrapped with aluminium foil to avoid evaporation and exposure to sunlight. After 3 days, mixtures were filtered through Whatman no.1 filter paper and kept in water bath at 40°C till all the solvent had completely evaporated from mixtures [1].

2) Solvent extraction using Soxhlet apparatus: Plant powder (10 g) was successively extracted with 250 ml of ethanol (70%) and

methanol (80%), in a Soxhlet apparatus at 75°C and 63°C respectively each for 10 to 12 h. Extracts were filtered through Whatman no.1 filter paper. The extracts were concentrated by keeping in water bath at 40°C till all the solvent had completely evaporated from mixtures [4].

3) Aqueous extraction: The powdered plant material was mixed with distilled water (1:5) and magnetically stirred in a separate container for overnight at room temperature. The residue was removed by filtration through Whatman no.1 filter paper and the aqueous extracts were lyophilized and stored in airtight containers [2].

All extracts were stored in sterilized containers at -20°C until used for testing. At the time of testing, the extracts were reconstituted in Dimethyl Sulphoxide (DMSO).

Qualitative Phytochemical Analysis

The extracts were subjected to preliminary phytochemical screening for possible presence of bioactive antimicrobial compounds

1. Test for tannins: Take 2ml of extract in a test tube and add 2 drops of 5% ferric chloride, brown colour or dark green colour gives positive result [1].

2. Test for phlobatannins: Take 2ml plant sample in a test tube and add 10ml de-ionized water and boil at 100ºC with few drops of 1%HCl. Deposition of red precipitation gives positive result [1].

3. Test for saponins: The extract was diluted with 20 ml of distilled water and it was agitated in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam showed the presence of saponins [14].

4. Test for terpenoids: Take 5ml of aqueous extract add 2 ml chloroform followed by addition of 3 ml conc. sulphuric acid, observe the reddish brown interface for presence of terpenoids ([1], [15], [16]).

5. Test for reducing sugar: Take 1 ml of plant sample in a test tube and add 10 ml de-ionized water then add few drops of Fehling solution (Fehling A and B) and heat at 100ºC in water bath. Brick red precipitate shows a positive result ([1], [16]).

6. Test for alkaloids: Take 1 ml of aqueous extract in test tubes and add 2- 3 drops of Wagner's reagent it gives orange red precipitation [1].

7. Test for flavonoids: To one ml of the extract, a few drops of dilute sodium hydroxide were added. An intense yellow colour was produced in the plant extract, which become colourless on addition of a few drops of dilute acid indicates the presence of flavonoids ([17], [16]).

Antibacterial activity

The antibacterial activity of all the extracts was tested by well-diffusion using pour plate method against *Staphylococcus aureus* (NCIM 2079) which was purchased from NCL, Pune.

Well-diffusion using pour plate method: Mueller-Hinton agar was prepared and autoclaved. 500 µl of inoculum was added in 250 ml of the media under aseptic conditions and the media was poured in petriplates. After the medium was solidified wells were bored with the help of sterile borer. 50 µl of extract (200mg/ml), control (DMSO 5%) and standard (Cefadroxil 20 µg/ml) was loaded in the wells and kept in the incubator at 37˚C for overnight [18].

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is lowest concentration of antimicrobial that will inhibit the visible growth of microorganism after overnight incubation. It is also a confirmatory test of antibacterial activity. A lower MIC is indication of better antimicrobial agent. It was carried only for the extracts that showed best antibacterial potential using serial dilution method. 1 ml of nutrient broth was added into 8 test tubes. Stock of 400 mg/ml of the extracts was prepared. 1 ml of this was added in the first tube as to obtain concentration of 200 mg/ml. The extracts were then serially diluted to obtain the concentration from 200 mg/ml to 6.25 mg/ml. Loop full of microbial suspension (*S. aureus*) was added to each of the tube. Microbial suspension was used as a positive control and extract in broth was used as negative control. The tubes were then incubated at 37˚ C for 24 hours. The lowest concentration of the extract in the test tube that showed no turbidity was taken as MIC ([19], [20], [21]).

Ointment preparation

Table 1: Formulation of ointment

Emulsifying wax (6gm)	
Component	**Amount**
Cetosteryl alcohol	5.4 gm
Sodium Lauryl Sulphate	0.6 gm
Distilled water	6 ml
Emulsifying ointment (20gm)	
Component	**Amount**
Emulsifying wax	6 gm
Soft paraffin	10gm
Liquid paraffin	4 ml
***Aegle marmelos* ointment**	
Sample	**Amount**
1. Unripe fruit ethanol extract	
2. Ripe fruit methanol extract using Soxhlet apparatus	
3. Unripe fruit ethanol extract using Soxhlet apparatus	0.4 gm
4. Unripe fruit methanol extracts using Soxhlet apparatus	
5. Mixture of all four extracts	
Emulsifying ointment	20 gm

The constituents of base shown in table 1 were weighed and melted in a beaker at 70˚C using heating mantle. The ingredients were stirred gently maintaining the temperature at 70˚C for 5 min. This was cooled with continuous stirring. To this adequate quantity of extract was added and stirred well until a homogeneous mass was obtained ([22], [23], [24], [25]).

Evaluation of ointment

Preliminary evaluation of formulations was carried out as follows [25]:

1) Colour and odour: Colour and odour of the prepared ointments were visually examined.

2) pH: The pH of various formulations was determined by using digital pH meter. One gram of ointment was dissolved in 100 ml of distilled water and stored for 2 hours. The measurement of pH of each formulation was done after 2 hours.

3) Antibacterial activity of ointment: The antibacterial activity of ointment was evaluated using well diffusion pour plate method. The ointment was dissolved in DMSO (200mg/ml) and added into the agar wells and Mupirocin as a standard was also added into the well. The plates were incubated at 37˚C for 24 hours and the antibacterial activity was checked.

4) Stability studies: The stability studies were carried out for the prepared formulations at 37˚ C for 2 months.

RESULTS AND DISCUSSION

Qualitative phytochemical analysis

Aegle marmelos have beneficial therapeutic effects in traditional Indian system of medicine. Various phytochemicals that are present in different parts of *Aegle marmelos* are responsible for this therapeutic effect. Presence of these phytochemicals were analysed by the qualitative test which showed the following results.

Table 2: Phytochemical analysis of leaves

Tests	Aqueous Extraction	Solvent extraction		Solvent extraction using Soxhlet apparatus	
		Methanol	Ethanol	Methanol	Ethanol
Tannins	+	+	+	+	+
Phlobatannins	+	-	-	-	-
Saponins	+	+	+	+	+
Terpenoids	+	+	+	+	+
Reducing sugar	+	+	+	-	+
Alkaloids	+	-	-	-	-
Flavonoids	+	+	+	+	+

Table 3: Phytochemical analysis of unripe fruit

Tests	Aqueous Extraction	Solvent extraction		Solvent extraction using Soxhlet apparatus	
		Methanol	Ethanol	Methanol	Ethanol
Tannins	+	+	+	+	+
Phlobatannins	-	-	-	-	-
Saponins	+	+	+	+	+
Terpenoids	+	+	+	+	+
Reducing sugar	+	+	+	+	-
Alkaloids	-	-	-	-	-
Flavonoids	+	+	+	+	+

Table 4: Phytochemical analysis of ripe fruit

Tests	Aqueous Extraction	Solvent extraction		Solvent extraction using Soxhlet apparatus	
		Methanol	Ethanol	Methanol	Ethanol
Tannins	+	+	+	+	+
Phlobatannins	-	-	-	-	-
Saponins	+	+	+	+	+
Terpenoids	+	+	+	+	+
Reducing sugar	+	+	+	+	+
Alkaloids	-	-	-	-	-
Flavonoids	+	+	+	+	+

Table 5: Phytochemical analysis of stem

Tests	Solvent extraction		Solvent extraction using Soxhlet apparatus	
	Methanol	Ethanol	Methanol	Ethanol
Tannins	-	-	-	+
Phlobatannins	-	-	-	-
Saponins	-	-	-	-
Terpenoids	+	+	+	+
Reducing sugar	-	-	+	-
Alkaloids	-	-	-	-
Flavonoids	-	-	-	+

Table 6: Antibacterial activity

	Zone of inhibition (mm)					
	Aqueous extraction	Solvent extraction		Slovent extraction using Soxhlet apparatus		Standard (Cefadroxil)
		Methanol	Ethanol	Methanol	Ethanol	
Leaves	11	-	13	-	-	16
Unripe fruit	12	-	11.5	13	15	16
Ripe fruit	-	-	12.5	11.75	-	16
Stem	-	-	-	-	-	16

It was observed that the phytochemical analysis of leaves (table 2), unripe fruit (table 3) and ripe fruit (table 4) showed presence of tannins, saponins, terpenoids and flavonoids in all the extracts; whereas phlobatannins, reducing sugar and alkaloids were present only in some extracts. In case of stem (table 5) very less phytochemicals were found to be present.

The absence of few phytochemicals might be due to different parts of *Aegle marmelos* used, different solvents used for extraction and different extraction methods used.

This is supported by various researchers who have also elucidated that some phytochemicals were absent in the extracts ([26], [14], [4], [1], [17]).

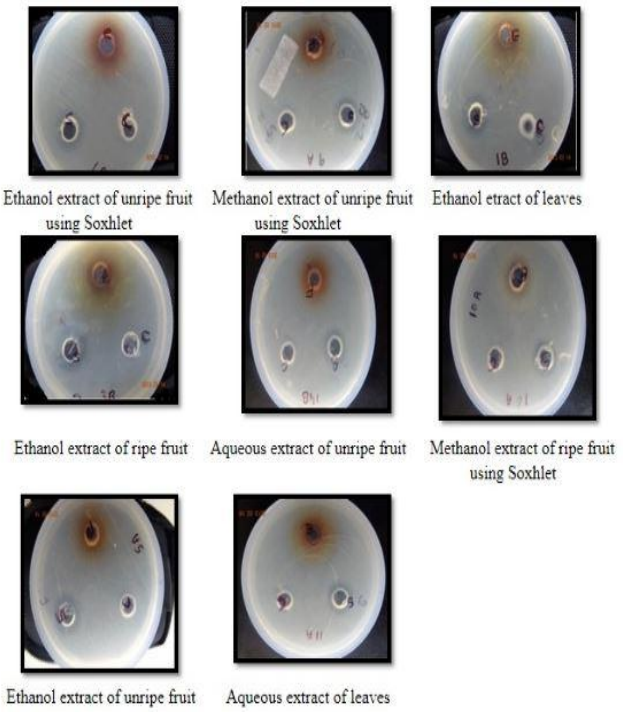

Fig. 1: Antibacterial activity

Antibacterial activity

The antibacterial potential of leaves, ripe fruit, unripe fruit and stem of *Aegle marmelos* against *S. aureus* was determined by using well diffusion pour plate method. The zone of inhibition for all the extracts is shown in table 6. It was observed that the zone of inhibition for different extracts were obtained in the range 11 – 15 mm; maximum being for the ethanol extract of unripe fruit using Soxhlet apparatus and minimum for the aqueous extract of leaves.

The antibacterial activity of these extracts is shown in figure 1

In general, the unripe fruit of *Aegle marmelos* showed maximum antibacterial activity. The antibacterial activity of these extracts may be due to presence of tannins, terpenoids and flavanoids as tannins have been known to form irreversible complexes with prolene rich protein resulting in the inhibition of cell wall synthesis Terpenoids are known to weaken the membranous tissue which results in dissolving cell wall of microorganism. Flavanoids exhibit large number of biological activities antioxidant, antimicrobial and anti-inflammatory properties. This is supported by various researchers who have also got the zone of inhibition for *S. aureus* in the range of 10 – 19 mm ([4], [1], [27])

Minimum Inhibitory Concentration

The antibacterial activity of *Aegle marmelos* was confirmed by Minimum Inhibitory Concentration test. MIC test was carried only for the extracts that showed best antibacterial potential. This test was carried in the test tubes and the concentration range used for MIC was 200 mg/ml to 6.25 mg/ml. Following are the results obtained for MIC test

Table 7: Minimum Inhibitory Concentration

MIC of extracts against *S. aureus*									
Tube no.	**Extracts concentration (mg/ml)**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	200	-	-	-	-	-	-	+	+
2	100	-	-	-	-	-	-	+	+
3	50	-	-	-	+	+	+	+	+
4	25	+	+	+	+	+	+	+	+
5	12.5	+	+	+	+	+	+	+	+
6	6.25	+	+	+	+	+	+	+	+

A – Ethanol extract of unripe fruit, B – Ethanol extract of unripe fruit using Soxhlet apparatus, C – Methanol extract of unripe fruit using Soxhlet apparatus, D – Ethanol extract of leaves, E – Methanol extract of ripe fruit using Soxhlet apparatus, F – Aqueous extract of unripe fruit, G – Ethanol extract of ripe fruit, H – Aqueous extract of leaves.

From table 7, the best MIC 50 mg/ml was shown by ethanol extract of unripe fruit, ethanol extract of unripe fruit using Soxhlet apparatus and methanol extract of unripe fruit using Soxhlet apparatus compared to other extracts. The ability of *Aegle marmelos* to inhibit the growth of bacteria may be attributed to various secondary metabolites.

Evaluation of formulated ointment

Few extracts that showed good MIC were used as active agent in the preparation of ointment and various physiochemical parameters and the antibacterial potential of the formulated ointments were studied. Ointments were stored in the bottles.

Five different ointments were formulated and the physicochemical parameters such as colour, odour and pH were evaluated. Also the antibacterial potential of the ointments was determined as shown in table 8. All ointments had different colours and characteristic odour. The pH of the five ointments was in the range of 6 – 6.5. The antibacterial test showed that 12.5 mm zone of inhibition was obtained for ointment prepared by methanol extract of unripe fruit using Soxhlet apparatus and 12 mm for ointment prepared by ethanol extract of unripe fruit using Soxhlet apparatus. The other three ointments did not show any antibacterial activity. This work is supported by researchers ([22], [25]).

Stability studies from table 9 showed that there was change in the colour of ointment prepared using ethanol extract of unripe fruit (indicated by bold text). Also slight change in pH was observed for all the ointments which were acceptable ([22], [1]).

CONCLUSION

The antibacterial activity of *Aegle marmelos* was successfully evaluated. This activity was retained when the extracts were incorporated into the ointment base. The ointments were stable after two months. Further the extracts can be used for the commercial production of *Aegle marmelos* ointment. Similarly the antifungal, anti-diabetic, activity can be studied.

Table 8: Evaluation of ointment

Formulations	**Colour**	**Odour**	**pH**	**Zone of Inhibition (mm)**
Ethanol extract of unripe fruit	Yellowish	Characteristic	6.27	-
Methanol extract of ripe fruit using Soxhlet	White	Characteristic	6.21	-
Ethanol extract of unripe fruit using Soxhlet	Cream	Characteristic	6.13	12
Methanol extract of unripe fruit using Soxhlet	Off-white	Characteristic	6.47	12.5
Mixture of all four extracts	CreamishWhite	Characteristic	6.35	-
Standard (Mupirocin)	White	Characteristic	6.12	15

Stability studies

Table 9: Stability studies of ointment

Formulations	Colour	Odour	pH
Ethanol extract of unripe fruit	**Change (dark)**	Characteristic	**6.30**
Methanol extract of ripe fruit using Soxhlet	White	Characteristic	**6.25**
Ethanol extract of unripe fruit using Soxhlet	Cream	Characteristic	**6.10**
Methanol extract of unripe fruit using Soxhlet	Off-white	Characteristic	**6.45**
Mixture of all four extracts	CreamishWhite	Characteristic	**6.32**
Standard (Mupirocin)	White	Characteristic	6.12

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