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Identification of Phytochemical Constituents of *Aegle marmelos* Responsible for Antimicrobial Activity against Selected Pathogenic Organisms

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Abstract

Antimicrobial activity and phytochemical constituents of an ethanolic extract of *Aegle marmelos* were investigated. The phytochemical screening of the crude extract revealed the presence of Alkaloids, Cardiac glycosides, Terpenoids, Saponins, Tannis, Flavonoids, and Steroids. The crude ethanolic extract was tested for antimicrobial activity against gram positive organisms of *Bacillus subtilis* (NCIM: 3471), *Staphylococcus aureus* (NCIM: 2079), gram negative *Escherichia coli* (NCIM: 2065) and *Pseudomonas aeruginosa* (NCIM: 2200) at different concentrations levels of 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml. At the 2.5 mg/ml concentration, gram negative *Escherichia coli* exhibits a zone of inhibition about 25.7mm; *Pseudomonas aeruginosa* 19.9mm; gram positive *Staphylococcus aureus* 29.0 mm; and *Bacillus subtilis*, a maximum zone of inhibition about 28.1 mm as compared to the control drug penicillin. *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* exhibit a maximum zone of inhibition, hence they were considered as susceptible to the plant extracts but *Staphylococcus aureus* doesn't exhibit such a zone of inhibition and is therefore considered as resistant.

Key Words: Phytochemical Screening, Antimicrobial activity, Gram positive organisms, Gram negative organisms, Ethanolic extract.

Introduction

Plants are known to be the source of many chemical compounds. Medicinal plants were used by people of ancient cultures without knowledge of their active ingredients. The common practice of taking crude extract orally is laden with hazards as the extracts may contain some toxic constituents. There is an ever increasing need to limit toxic clinical drugs (Lown, 1993).

In modern times, the active ingredients and curative actions of medicinal plants were first investigated through the use of European Scientific methods (Herborn, 1998). The most important ingredients present in plant communities turn out to be alkaloids, terpenoids, steriods, phenols glycosides and tannins (Abayomi, 1993).

The information obtained from extracts of medicinal plants makes pharmacological studies possible. The mode of action of plants producing therapeutic effects can also be better investigated if the active ingredients are characterized.

Infectious diseases are the leading cause of death worldwide. The clinical efficiency of many existing antibiotics is being threatened by the emergence of multidrug resistant pathogens (Bandow et al., 2003). Bacterial pathogens

have evolved numerous defense mechanisms against antimicrobial agents and resistance to old and newly produced drug is on the rise. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity (Colombo and Bosisio, 1996; Scazzocchio et al., 2001). There are several reports in the literature regarding the antimicrobial activity of crude extracts prepared from plants (El-seedi et al., 2002; Rojas et al., 2003; Duraipandiyan et al., 2006; Parekh and Chanda, 2007a).

Plants produce a diverse range of bioactive molecules making them a rich source of different types of medicines (Stuffness and Douros, 1982). Higher plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care since ancient times. Over 50% of all modern clinical drugs are of natural product origin and natural products play a vital role in modern drug development in the pharmaceutical industry (Baker et al., 1995).

Plants with possible antimicrobial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extract on bacteria have been studied by a very large number of researches in different parts of the world (Ates and Erdogrul, 2003). Much work has been done on ethnomedicinal plants in India (Negi et al., 1993). Interest in a large number of traditional natural products has increased (Taylor et al., 1996). It has been suggested that aqueous and Ethanolic extract from plants used in allopathic medicine are potential sources of antiviral, Anti tumural and antimicrobial agents. The selection of crude plant extracts for screening programmes has the potential of being more successful in initial steps than the screening of pure compounds isolated from natural products. The present results of our plant extract studies show that such extracts can be used by communities for curative purpose (Cordell, 1981; Micheal, 1990; Daiziel, 1955; Pamploma-Roger, 1999).

Materials and Methods

Collection of Plant materials and Identification

Plant material of *Aegle marmelos* was purchased from the local Ayurvedic medicinal shop Chennai, and they were identified and authenticated by the Chief Botanist, Tamil Nadu Aromatic Medicinal Plants Corporation Limited (TAMPCOL), Arignar Anna Siddha Medical College and Hospital Campus, Chennai, Tamil Nadu, India.

Ethanolic extraction

The plant materials were dried in the shade and powdered by a mechanical grinder. The powder of *Aegle marmelos* was initially defated with petroleum benzene (60 - 80˚C) followed by 1000 ml of ethanol, by using a Soxhlet extractor for 72 hours at a temperature not exceeding the boiling point of the solvent. The extract was filtered using whattman filter paper (No 1) and then concentrated in a vaccum and dried at 45˚C for ethanol elimination. The extracts were kept in a sterile bottle under refrigeration conditions of about 2-8˚C.

Test for Phytochemical Analysis

The extracts were analyzed for the presence of alkaloids, terpenoids, reducing sugars, saponins, tannins, carbonyls, flavonoids, phlobatannis and steriods (Adetuyi et al., 2001; Trease and Evans, 1989; Sofowora, 1982).

Test for Alkaloids

Weigh about 0.2 gm of plant extract in separate test tube and warmed with 2% Sulphuric acid for 2 minutes. And it was filtered in separate test tube and few drops of Dragencloffs reagent were added and observed for the presence

of orange red precipitates for the presence of alkaloids.

Test for Cardiac glycoside

Keller-Killani Test

Weigh about 0.5 gm of plant extract in a separate test tube with 2 ml of glacial acetic acid containing a drop of ferric chloride solution. This was under layered with 1 ml of concentrated tetra oxo sulphate (VI) acid. And observe for brown ring formation at the interface (Finar, 1983).

Test for Terpenoids

Weigh about 0.5 g plant extract in separate test tubes with 2 ml of chloroform. And add concentrated Sulphuric acid carefully to form a layer. And observe for presence of reddish brown color interface to show positive results for the presence of terpenoids.

Test for reducing sugars

Take a test tube and add 2 ml of crude plant extract and add 5 ml of Distill water and filter. The filtrate was boiled with 3-4 drops of fehlings solution A and B for 2 minutes. Observe for orange red precipitate which indicates the presence of reducing sugars.

Test for Saponins

Weigh about 0.2 gm of plant extract in the test tube and add 5 ml of distilled water and then heat to boil. Observe for the occurrence of frothing (appearance of creamy mass of small bubbles) which then indicates the presence of Saponin.

Test for Tannin

To small quantity of plant extract was mixed with water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. And observe for dark green solutions that indicate the presence of a tannin.

Test for Carbonyl

Take 2 ml of plant extract in separate test tubes and add few drops 2,4, di nitro phenyl hydrazine solution and shake. And observe for the presence of yellow crystals immediately for the presence of an aldehyde.

Test for Flavonoids

Weigh about 0.2 gm plant extract in separate test tubes and dissolved diluted Sodium hydroxide and add diluted Hydrochloride. And observe for yellow solutions that turn colorless. This indicates the presence of flavonoids

Test for Phlobatanin

Weigh about 0.5 gm of plant extract in a test tube and dissolve with distilled water and filter. The filtrate was boiled with 2% Hydrochloric acid solution. Observe for a red precipitate that shows the presence of Phlobatanin

Test for Steroids

To the plant extract add 2 ml of acetic anhydride and add 0.5 gm of ethanolic extract of each sample with 2 ml of Sulphuric acid .Observe for the color change from violet to blue or green in samples indicating the presence

of steriods

Antibacterial activity

Bacterial strains and Growth conditions

The following cultures were used: *Staphylococcus aureus* (NCIM 2079), *Bacillus subtilis* (NCIM 3471), *Escherichia coli* (NCIM 2065) and *Pseudomonas aeruginosa* (NCIM 2200). The cultures are obtained from National Collection of Industrial Microorganism (NCIM) Pune, India. Cultures of these bacteria were grown in nutrient broth at 37˚C and maintained nutrient agar slants <12˚C.

Reference antibiotic

Reference antibiotic penicillin was obtained from the authorized medical shop, "Chennai."

Preparation of Antibiotic and plant extract for the experiment:

The antibiotic and dried plant extract were weighed and dissolved in sterile distilled water to prepare appropriate dilution to get required concentrations of 0.5, 1.0, 1.5, 2.0, 2.5mg/ml.

Preparation of Inoculum:

Inoculum of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were prepared in nutrient broth medium and kept incubation at 35˚C for 8 hours

Preparation of Medium

The required amount of Mueller-Hinton plates (Hi media) is prepared as per manufacturer instruction.

Procedure for performing the Disc Diffusion test (Bayer et al., 1986)

A sterile cotton swab was dipped into the turbid culture suspension. The dried surface of Muller-Hinton agar plate were inoculated by streaking two more times rotating the plate approximately 60˚each time. The lid may be left aside for 3-5 minutes and allow to dry for the excess surface moisture content.

The previously prepared discs were poured with different concentrations of the above prepared antibiotic and plant extract solutions, the discs were placed on the medium and the plates were incubated at 5˚C for 1 hour to permit good diffusion, and then transferred to an incubator at 37˚C for 24 hours. The negative control was included without adding the cultures to know the sterile conditions. The antibacterial activity was recorded by measuring the width of the clear inhibition zone around the disc.

Results and Discussion

The ethanolic extracts of *Aegle marmelos* were subjected for phytochemical analysis and antimicrobial activity and the results were investigated. Phytochemical screening of the crude extract revealed the presence of alkaloids, cardiac glycosides, terpenoids, saponins, tannin, flavonoids, and steriods, but reducing sugars, carbonyl (aldehyde) and Phlobatanin show negative results (see explanation in Table -1).

Table-1: Phytochemical constituents of *Aegle marmelos*.

S. No	Test parameters									
	Alkaloids	Cardiac glycosides	Terpenoids	Reducing sugars	Saponins	Tannis	Carbonyl	Flavonoids	Phlo-batanin	Steriods
1	+	+	+	-	+	+	-	+	-	+

Key: + = Positive, - = Negative

The crude ethanolic extracts of *Aegle marmelos* and the control drug penicillin were subjected to antimicrobial activity. The results are tabulated and discussed below in Table -2.

In the case of *Escherichia coli*, the control drug penicillin showed less activity (about 22.0mm) when compared with the plant extract of *Aegle marmelos* (this 25.7mm). At a higher dilution of about 2.5 mg/ml, the plant extract is effective against gram negative *Escherichia coli*.

The same dilutions were tested on *Pseudomonas aeruginosa*. The plant extract showed a 19.9mm zone of inhibition, but the control drug Penicillin exhibited 18.9mm, and hence this plant extract is effective against gram negative *Pseudomonas aeruginosa*

In gram positive *Staphylococcus aureus*, *Aegle marmelos* and the control drug penicillin, the organisms exhibit a similar zone of inhibition (about 29.0mm), hence they are considered as resistant. The same dilutions were subjected to *Bacillus subtilis*, the zone of inhibition of which is about 28.1mm, but as the control drug penicillin exhibits 26.2mm, the plant extract is considered as susceptible.

In this study, the results of the investigation show that the plant extracts from *Aegle marmelos* have good antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* due to the presence of alkaloids, cardiac glycosides, terpenoids, saponins, tannin, flavonoids, and steriods. However, *Staphylococcus aureus* is considered resistant at different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/ml) against the control drug Penicillin.

TABLE-2 : Minimum inhibitory concentration of *Aegle marmelos* and the control drug Penicillin with the cultures of *Escherichia coli*, *Pseudomonas aeruginosa Staphylococcus aureus* and *Bacillus subtilis.*

S. No	Name of organism	Concentration (mg/ml)	Zone of Inhibition(mm)	
			Aegle marmelos	Penicillin
1	*Escherichia coli*	0.5	16.3	12.5
		1.0	18.2	14.0
		1.5	20.1	16.0
		2.0	22.7	18.0

		2.5	25.7	22.0
2	*Pseudomonas aeruginosa*	0.5	12.3	08.5
		1.0	13.7	10.3
		1.5	15.6	12.0
		2.0	17.7	16.7
		2.5	19.9	18.9
3	*Staphylococcus aureus*	0.5	17.4	17.2
		1.0	21.1	20.4
		1.5	23.4	23.5
		2.0	26.1	26.1
		2.5	29.0	29.0
4	*Bacillus subtilis*	0.5	17.0	16.3
		1.0	21.0	18.7
		1.5	23.6	21.8
		2.0	26.0	23.9
		2.5	28.1	26.2

Conclusion

This research work states that the presence of alkaloids, cardiac glycosides, terpenoids, saponins, tannin, flavonoids, and steriods in the ethanolic extract of *Aegle marmelos* were responsible for its antimicrobial activity. These compounds exhibit a maximum zone of inhibition against *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis, w*hen compared with the control drug penicillin. Such a zone of inhibition was not found in the case of *Staphylococcus aureus,* which is considered resistant. Hence, the present study suggests that pathogenic microorganisms may become resistant to existing drugs. Moreover, this study shows that some plants show much promise in the development of phytomedicines having antimicrobial properties. In this endeavour, traditional herbal medicines must perforce be granted the benefits of modern science and technology to serve further global needs. The drugs derived from herbs may have the possibility of use in medicine because of their antibacterial activity.

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