Research Article

Screening of Leaf Extracts of *Azadirachta Indica* (Neem), *Aegle Marmelos* (Bael) and *Trigonella Foenum Graecum* (Methi) for their Inhibitory Activity on the Strains of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida Spp.*

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ABSTRACT

Leaf extracts of three plants *Azadirachta indica* (Neem), *Aegle marmelos* (Bael) and *Trigonella foenum graecum* (Methi) were evaluated for their inhibitory activity *in vitro* on five clinical isolates of *Candida spp.* (one strain each of *C. tropicalis, C. Parapsilosis, C. cruezi*) and two strains of *C. albicans*. Also this activity was further evaluated against three clinical isolates each of *S. aureus and P.aeruginosa*. Aqueous, methanolic and ethanolic extracts were prepared by cold maceration of leaves of the plants which were collected from Solan and Chandigarh regions (India). For comparison of the inhibitory activity standard preparations were used such as Amphotericin B against *Candida spp.,* gentamicin and ampicillin against *Staphylococcus aureus,* and ciprofloxacin against *P.aeruginosa spp*. Significant zone of inhibition was exhibited by ethanolic and methanolic extracts against all the isolates whereas most isolates were resistant to the standard antibiotic/antifungal discs. However, we did not observe any significant antimicrobial activity in the aqueous extracts. These findings are important in light of the fact that the extracts showed significant activity against the drug resistant clinical isolates. Phytochemical analysis of the plant extracts revealed the presence of tannins, saponins, quinones, coumerin and gum in all the plants studied.

Keywords: Zone of inhibition, *Azadirachta indica* (Neem), *Aegle marmelos* (Bael) *and Trigonella foenum graecum* (Methi), Clinical isolates, Phytochemicals.

INTRODUCTION

Candida species are opportunistic fungal pathogens of humans responsible for superficial and systemic infections. Among these species, *Candida albicans* is responsible for majority of the infections[1]. *Staphylococcus aureus* is another leading organism causing diverse infections, particularly the methecillin resistant *Staphylococcus aureus* (MRSA) which are multidrug resistant and pose a challenge in treating these infections. *P. aeruginosa* is the second most frequent gram negative nosocomial pathogen. Keeping the rising prevalence of drug resistant microorganisms, there is an urgent need to search for new effective drugs having natural or synthetic origin[2]. Being rich in antimicrobial compounds, the medicinal and aromatic plants could offer a better option to combat bacterial diseases[3, 4]. Since phytochemicals from medicinal plants have been considered the lead compounds in drug discovery and design[5, 6]. Keeping this in view, we attempted to evaluate the extracts of leaves of *Azadirachta indica* (Neem), *Aegle marmelos* (Bael) and *Trigonella foenum graecum* (Methi) plants for their inhibitory activity against clinical isolates of *Candida spp.,* strains of *S. aureus and P. aeruginosa*.

MATERIAL AND METHODS

Preparation of Plant extracts

Azadirachta indica (Neem), *Aegle marmelos* (Bael), *Trigonella foenum graecum* (Methi) leaves collected from Solan and Chandigarh regions (India) were washed and soaked in sterilized distilled water for 10 minutes, and air dried. Leaves thus dried were powdered in a grinder. Using cold maceration method, aqueous, ethanolic and methanolic extracts of 10g powder of each plant were prepared for phytochemical analysis and evaluation of antimicrobial activity[7].

Screening for Phytochemicals

Phytochemicals are non-nutritive, chemically active substances present in plants that produce a definite physiological action on the human body and play a significant role in the treatment of serious diseases. Since ancient times, the plants have been used in prevention and treatment of various diseases. In the present study the leaf powder of plants under study were analyzed for alkaloids, tannins saponins, quinones, coumerins, sugar and gums as per standard protocols given below[8].

1. Detection of alkaloids: The Mayer's test was followed for the detection of alkaloids. Precisely, 2-3 drops of 2N HCl were mixed with one ml of respective aqueous, ethanolic and methanolic leaf extracts of each plant. The aqueous layer was separated and 1-2 drops of Mayer's reagent were added to it. Formation of white turbidity or precipitates indicated the presence of alkaloids.

2. Detection of tannins: Few drops of basic lead acetate solution were added to one ml. of leaf extract (ethanolic, methanolic and aqueous) of all the selected plants. Formation of white precipitates indicated the presence of tannins.

3. Detection of saponins: A few drops of distilled water were added to one ml. of ethanolic, methanolic and

aqueous leaf extracts of all the selected plants. Foamy leather formation indicated the presence of saponins.

4. Detection of quinones: A few drops of sodium hydroxide were added to one ml. of ethanolic, methanolic and aqueous leaf extracts of all the selected plants. Change of color to blue green or red indicated the presence of quinones.

5. Detection of coumerin: Two to three drops of 10% of sodium hydroxide and chloroform each were added to the ethanolic, methanolic and aqueous leaf extracts of all the selected plants. Formation of yellow color indicated the presence of coumerin.

6. Detection of sugars: Fehling's test was followed for the detection of sugars. Equal volume of ethanolic, methanolic and aqueous extracts of each plant was added to the mixture of Fehling A and Fehling B solutions and heated for 5-10 min. in boiling water and allowed to cool thereafter. Depending upon the presence of amount of reducing sugar the color changed from green yellow to red, otherwise no color change would occur.

7. Detection of gum: A few drops of distilled water were added to one ml of each plant extract and shaken vigorously. Formation of swells or adhesives indicated the presence of gum.

Screening for Antimicrobial activity

Microorganisms: *Candida sp.* from clinical cases were procured from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh and isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa* recovered from clinical cases were obtained from Indira Gandhi Medical College (IGMC), Shimla (H.P).

Antifungal assay: Antifungal activity of the plant extracts was assayed by disc diffusion method on Sabouraud's Dextrose Agar (SDA) plates. The autoclaved (at 15 lb for 15 minutes) media was poured into plates aseptically, allowed to solidify at room temperature. Each inoculum in a volume of 0.2ml was placed on SDA plates and spread uniformly over the surface of the agar, allowed to dry for 5 minutes. Each plant extract in a volume of 10ul was loaded onto sterile discs. The discs were then placed in the plates with the help of sterile forceps, incubated at 28°C for 24-48 hours. The plates were observed for the zones of inhibition around the discs at 24 and 48 hours. Amphoterecin B (25mcg) discs were used to see the antifungal activity against each strain of *Candida species* and for comparing the efficacy of each plant in this regard. Solvent controls were also kept in the assay in order to rule out the inhibitory effect if any due to the solvent alone. The diameter of zone of inhibition (in millimeters) of fungal growth around the discs were measured for determine antifungal activity.

Antibacterial assay: Antibacterial activity of the plant extracts was assayed by disc diffusion method on Muller Hinton agar. Sterile plates were prepared by pouring autoclaved (at 15 Lb for 15 minutes) media under aseptic conditions. After solidification of the medium, 0.2ml inoculum of each clinical isolate was inoculated and spread uniformly over the surface of agar, allowed to dry for 5 minutes. Each plant extract in a volume of 10ul was loaded onto sterile discs which were then placed on the surface of inoculated plates, incubated at 37°C for 24-48 hours. The plates were observed for the zone of inhibition around the plant extract discs. In the assay, ampicillin (10mcg), gentamicin (10mcg) and ciprofloxacin (5mcg) discs were used as positive controls against the strains of *S.aureus* and *P.aeruginosa* and for comparing the efficacy of plant extract. Solvent controls were also kept in these experiments in order to rule out the inhibitory effect if any due to the solvent alone. The diameter of zone of inhibition (in millimeters) of bacterial growth around the discs were measured for determining antibacterial activities.

RESULTS

Identification of plants and their phytochemical analysis

Three plants, namely, *Azadirachta indica* (Neem), *Aegle marmelos* (Bael), *Trigonella foenum graecum* (Methi) were authenticated at the Department of Botany, Shoolini Institute of Life Sciences and Business Management (SILB), Solan Himachal Pradesh. Their dried leaves which were used for the preparation of plant extracts and phytochemical analysis are presented through Fig.1 (a,b,c). Using standard protocols for phytochemical analysis, tannins, saponins, quinones, coumerins, gum were demonstrable in the ethanolic and methanolic extracts of these plants. Gum was also demonstrable in the aqueous extracts. However alkaloids and sugars could not be demonstrated (Table-1).

Screening of antimicrobial activity

Antimicrobial activity of the extracts of all the three plants used in the study was assessed by *in vitro* culture sensitivity assay by disc diffusion method in which five clinical isolates of *Candida species* and three isolates each of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used. Amphotericin-B (25mcg), ampicillin (10 mcg), ciprofloxacin (5 mcg) respectively were used as positive control for comparison with the zones of inhibition produced by the 10µl of each plant extract.

As given in table-2, all the strains of *Candida spp.* were resistant to the standard antifungal agent, Amphotericin-B, as zones of inhibition were not observed. Interestingly, both ethanolic and methanolic extracts of all the three plants produced fairly large zones of inhibition, particularly the ethanolic extract of *Azadirachta indica* (Neem) and *Trigonella foenum graecum* (Methi) produced the zones of inhibition of 19mm each.

The extracts of neem produced zones of inhibition comparable to those produced by the ampicillin against different strains of *Staphylococcus aureus.* All the strains, however, were ampicillin resistant. It is particularly interesting to observe that the extracts of other two plants produced larger zones of inhibition whereas no

zones of inhibition were recorded with ampicillin (Table-3).

In case of P*seudomonas aeruginosa* no zones of inhibition were observed against ciprofloxacin (5 mcg) but both methanolic and ethanolic extracts of all the three plants produced fairly large zones of inhibition up to 19mm (Table-3).

DISCUSSION

The present study has been undertaken to determine the antimicrobial activity of plant extracts of leaves of three medicinal plants namely, *Azadirachta indica* (Neem), *Aegle marmelos* (Bael), *Trigonella foenum graecum* (Methi) in different solvents (10% w/v, aqueous, methanolic and ethanolic). The significance of such study is particularly important keeping in view the growing resistance of the bacterial and fungal species to commercially available antibiotics. Because of the emergence of many resistant strains against commonly used antibiotics, the researchers are showing their enthusiasm in new antibacterial and antifungal agents of natural origin.

The phytochemical analysis of the plants under study showed that the ethanolic and methanolic extracts of *Azadirachta indica* (Neem), *Aegel marmelos* (Bael) and *Trigonella foenum graecum* (Methi) had tannins, saponins, quinones, coumerin, sugar and gum whereas the aqueous extract of all the three plants contained gum only. These plant extracts need to be correlated to the antimicrobial activity.

All the five strains of *Candida spp.* namely, *C. albicans* (2), *C. tropicalis* (1), *C.cruzei* (1), *C. parapsilosis* (1) were resistant to the standard antifungal agent, Amphotericin-B, zones of inhibition were not observed with different extracts of all the three plants used. Interestingly, both ethanolic and methanolic extracts of these plants produced fairly larger zones of inhibition, particularly the ethanolic extract of *Azadirachta indica* and *Trigonella foenum graecum* produced the zones of inhibition of 19mm each.

Table 1: Phytochemical analyses of aqueous, methanolic and ethanolic extracts of *Azadirachta indica* (Neem), *Aegle marmelos* (Bael), *Trigonella foenum graecum* (Methi).

Phytochemical Tests	*Azadirachta indica* (Neem)			*Aegle marmelos* (Bael)			*Trigonella foenum graecum* (Methi)		
	Aqueous extract	Methanolic extract	Ethanolic extract	Aqueous extract	Methanolic extract	Ethanolic extract	Aqueous extract	Methanolic extract	Ethanolic extract
Alkaloids	–	–	–	–	–	–	–	–	–
Tannins	–	–	–	–	–	–	–	–	–
Saponins	–	+	+	–	+	+	–	+	+
Quinone	–	+	+	–	+	+	–	+	+
Coumarin	–	+	+	–	+	+	–	+	+
Sugar	–	–	–	–	–	–	–	–	–
Gum	+	+	+	+	+	+	+	+	+

(+ Presence, - Absence)

Table 2: Susceptibility of *Candida Species* to different plant extracts and standard antifungal drug Amphoterecin-B

Test strains used	Diameter of zone of inhibition (mm) Ethanolic Extract 10µl disc					Diameter of zone of inhibition (mm) Methanolic Extract 10µl disc				
	Azadirachta indica (Neem)	*Aegle marmelos* (Bael)	*Trigonella foenum graecum* (Methi)	Positive control	Susceptibility to positive control	*Azadirachta indica* (Neem)	*Aegle marmelos* (Bael)	*Trigonella foenum graecum* (Methi)	Positive control	Susceptibility to positive control
C.tropicalis (B-1389/09)	15	12	13	nil	R	16	12	13	Nil	R
C.albicans (CAGMC6)	16	15	15	nil	R	17	15	16	Nil	R
C.albicans (B-1622/09)	19	13	19	nil	R	14	13	14	Nil	R
C.parapsilosis (B-1597/09)	15	11	14	nil	R	15	14	19	Nil	R
C.cruzei (ATCC-6258)	17	14	15	nil	R	14	12	15	Nil	R

(R- Resistant, S-Sensitive)

Table 3: Susceptibility of *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains to different plant extracts and standard antibiotics.

Plant Used	Ethanolic Extract				Methanolic Extract		
	Test strain used	zone of inhibition 10µl disc (Dia in mm)	Ampicillin (+ve control) 10mcg disc	Susceptibility to Ampicillin	zone of inhibition 10µl disc (Dia in mm)	Ampicillin (+ve control) 10mcg disc	Susceptibility to Ampicillin
Azadirachta indica (Neem)	SPMIC-29	14	11	R	12	11	R
	SPMIC-130	15	10	R	13	10	R
	SPMIC-132	14	10	R	14	10	R
Aegle marmelos (Bael)	SPMIC-29	15	Nil	R	16	Nil	R
	SPMIC-130	15	Nil	R	19	Nil	R
	SPMIC-132	16	Nil	R	18	Nil	R
Trigonella foenum graecum (Methi)	SPMIC-29	12	Nil	R	16	Nil	R
	SPMIC-130	13	Nil	R	20	Nil	R
	SPMIC-132	15	Nil	R	17	Nil	R
			Ciproflox. (5mcg disc)			**Ciproflox. (5mcg disc)**	
Azadirachta indica (Neem)	PA-37	15	Nil	R	12	Nil	R
	PA-38	19	Nil	R	13	Nil	R
	PA-39	12	Nil	R	12	Nil	R
Aegle marmelos (Bael)	PA-37	15	Nil	R	15	Nil	R
	PA-38	12	Nil	R	13	Nil	R
	PA-39	11	Nil	R	14	Nil	R
Trigonella foenum graecum (Methi)	PA-37	14	Nil	R	13	Nil	R
	PA-38	15	Nil	R	11	Nil	R
	PA-39	13	Nil	R	12	Nil	R

(R- Resistant, S-Sensitive)

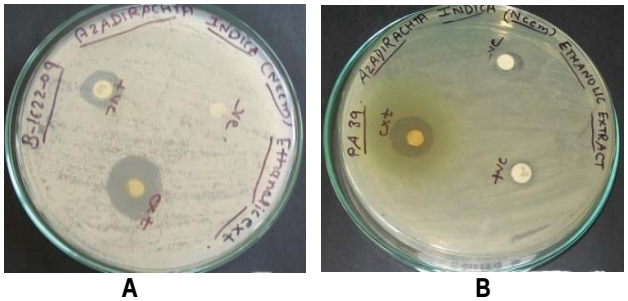
This finding is valuable as the extracts could provide an alternative to Amphotericin-B as well as other antifungal agents. The lead compound of such plant extracts need to be identified and considered for designing therapy. Although the phytochemical analysis of these compound has been done but further studies are required to pin point the active compounds.

The extracts of neem produced zones of inhibition comparable to those produced by the ampicillin against different strains of *Staphylococcus aureus.* All the strains, however, were ampicillin resistant. It is particularly interesting to observe that the extracts of other two plants produced larger zones of inhibition as compared to ampicillin (Table-3). Also, all the three strains of *Pseudomonas aeruginosa sp.* were resistant to ciprofloxacin while the extracts produced fairly larger zones of inhibition to the extent of 19mm in case of ethanolic extract of neem plant against PA-38 strain. It is worthwhile to mention here that zone of inhibition was not observed with the solvent alone which thus excludes such apprehension. Other workers have observed higher zones of inhibition with ethanolic extract of *Azadirachta indica* which supports our observations[9]. In the present study the extracts of *Aegle marmelos* produced bigger zones of inhibition against *Candida sp*. as compared to *Staphylococcus and Pseudomonas*. This finding is consistent with similar observations made by Umadevi *et al.*, 2011[10]. We, however, did not observe antimicrobial activity of aqueous extracts of all the three plants. This could be due to the absence of suitable phytochemicals responsible for such activity in the aqueous extracts as we could demonstrate only gum only in these extracts or this may be due to the better solubility of the active components in organic solvents than in water[11,12]. Finally, the amount of the antimicrobial compounds extracted in the experiments in the alcoholic solvents could be present in these extracts only and not in the aqueous extracts. All the five strains of *Candida sp*. tested were susceptible to the alcoholic extracts of *Trigonella foenum graecum* but were not sensitive to standard antifungal agent Amphotericin B (25mcg) and to alcoholic solvents alone. Further, all the three strains of *Staphylococcus aureus* had shown sensitivity to the ethanolic, methanolic extracts but were not susceptible to aqueous plant extract and Ampicillin (10mcg) and to alcoholic solvent alone. However, all the three strains of *Pseudomonas aeruginosa* were not inhibited by any extract of this plant (Table-3).

It can be concluded from the present study that, the alcoholic extracts of different plants exhibited, antifungal and antibacterial activity. It is worthwhile to mention that the strains of these organisms which were resistant to standard antibacterial agents such as ampicillin, ciprofloxacin and standard antifungal agents amphotericin-B, could be inhibited by the plant extracts which suggests that these extracts have potential for offering alternative to these drugs. Further studies are required for evaluating their clinical efficacy.

A B C

Figure 1: Leaves of (a) *Azadirachta indica* (Neem), (b) *Aegle marmelos* (Bael) and (c) *Trigonella foenum graecum* (Methi) used for preparation of extracts.

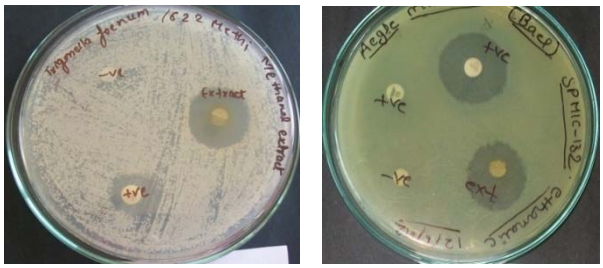


A B

Figure 2: (A) & (B) Diameter of zone of Inhibition in (mm) by ethanolic and methanolic extract of *Azadirachta indica indica* (Neem) leaves against (B-1622-09) *Candida* strain and (PA-39) *Pseudomonas aeruginosa* strain respectively.

C D

Figure 2: (C) & (D) Diameter of zone of Inhibition in (mm) by ethanolic and methanolic extract of *Aegle marmelos* (Bael) leaves against (CA-GMC-6) *Candida* strain and (PA-38) *Pseudomonas aeruginosa* strain respectively.



E F

Figure 2: (e) & (f) Diameter of zone of Inhibition in (mm) by ethanolic and methanolic extract of *Trigonella foenum graecum* (Methi) leaves against (B-1622-09) *Candida* strain and (SP-MIC-132) *Staphylococcus aureus* strain respectively.

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