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Antibacterial Potential of the Extracts of the Leaves of *Azadirachta indica* Linn.

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Abstract

Azadirachta indica A. Juss (syn. *Melia azadirachta*) is well known in India and popularly known as Indian neem. To evaluate antibacterial potential, the agar well diffusion assay was used against Gram-negative and Gram-positive bacteria. Penicillin and Dimethyl sulfoxide were used as positive and negative controls, respectively. Methanol extract showed the highest and chloroform extract showed moderate to good antibacterial activity. *Proteus vulgaris* and *Micrococcus luteus* were the most susceptible bacteria while *Bacillus subtilis* was more resistant bacterium to the hexane, chloroform and methanol extracts of neem. The study recommended for the isolation and separation of bioactive compounds responsible for the antibacterial activity.

Keywords: agar-well diffusion, bacteria, medicinal plants, neem, solvent extracts

Introduction

Plants have been a source of herbal remedies throughout the history of mankind. Various medicinal plants have been used for years in daily life to treat diseases all over the world (Nimri *et al*., 1999; Saxena, 1997). The wide spread use of herbal remedies and healthcare preparations, such as those described in ancient texts like the Vedas and Bible, has been traced to the occurrence of natural products with medicinal properties. Existence and survival of mankind is impossible without plant kingdom, as plants are the primary procedures and play important role in sustaining the life forms on earth. Search of newer drugs from plants has been rise; since many of the microorganisms are posing series health related disorders. According to recent estimates by the WHO more than 3.5 billion people in the developing world rely on plants as source of medicine for various ailments. Over 20,000 plants have medicinal values and many plants are yet to be explored for their potentials. In addition, many of the existing synthetic drugs cause various side effects. Hence, drug development from plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects (Srivastava *et al.,* 2000).

Azadirachta indica A. Juss (syn. *Melia azadirachta*) is well known in India and its neighboring countries. It is popularly known as Indian neem (margosa tree) or Indian lilac. It is an evergreen tree, cultivated in various parts of the Indian subcontinent. Every part of the tree has been used as traditional medicine for household remedy against various human ailments. Neem has been extensively used in Ayurveda, Unani and homoeopathic medicine and has become a cynosure of modern medicine. The Sanskrit name of the neem tree is 'Arishtha' meaning reliever of 'sickness' and hence is considered as 'sarbarigabubarini'. The tree is still regarded as 'village dispensary' in India. Chemical investigation on the products of the neem tree extensively undertaken in the middle of the isolation of nimbin, the first bitter compound isolated from neem oil, more than 135 compounds have been isolated from different parts of neem (Ganguli, 2002). Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex (Subapriya and Nagini, 2005). Seeds contain a complex secondary metabolite azadirachtin. The medicinal properties of the plant *Azadirachta indica* were studied by several workers. They were Antipyretic (Khattak *et al.*, 1985; Okpanyi and Ezeukwk, 1981), anti-malarial effect (Rochankij *et al.*, 1985; Tella, 1977), anti tumour effect (Fujiwara *et al.*, 1982), anti ulcer effect (Pillai and Santhakumari, 1984), anti diabetic effect (Shukla *et al.,* 1973), antifertility effect (Sinha *et al.,* 1984), effect on central nervous system (Singh *et al.,* 1987) cardiovascular effect (Thompson and Anderson, 1978) and Antioxidant activity (Bandyopadhyay *et al.,* 2002; Sultana *et al.,* 2007). Boiled neem leaf water makes an excellent antiseptic to clean wounds, soothes, swellings and eases skin problems (Shahidi Bonjar *et al.,* 2004). Oil from the leaves, seeds and bark possesses a wide spectrum of antibacterial activity action against Gram-negative and Gram-positive microorganisms, including *M. tuberculosis* and Streptomycin resistant strains (Chopra *et al.,* 1956). *In vitro* it inhibits *Vibrio cholerae, Klebsiella pneumoniae, M. tuberculosis* and *M. pyrogenes* (Satyavati *et al.*, 1976). Rao *et al.* (1986) reported the antimicrobial activity of the seed oil against a variety of pathogens. The antifungal effect of leaf extract against *Alternaria alternata* (Bhomick and Choudhary, 1982). Antimicrobial effects of neem extract have been demonstrated against *Streptococcus* species (Almas, 1999).

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Recently, the antibacterial activity of neem seed oil was assessed *in vitro* against 14 strains of pathogenic bacteria (Baswa *et al.,* 2001). We planned the present study to find out the antibacterial activity of neem leaves against human pathogenic bacteria, including *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Micrococcus luteus, Bacillus subtilis, Enterococcus faecalis* and *Streptococcus faecalis*.

Materials and methods

Plant material and extracts preparation

The leaves of *Azadirachta indica* were collected from Kambalalkonda forest in Visakhapatnam district, Andhra Pradesh. The collected leaves were identified and authenticated by a Botanist, Department of Botany, Andhra University. The collected leaves were washed thoroughly with running tap water and finally with sterile distilled water. The material was chopped into small pieces and then air dried on a sterile blotter under shade for 20-30 days.

The completely shade dried material was coarsely powdered and allowed to Soxhlet for successive extraction with hexane, chloroform and methanol. The obtained liquid extracts were subjected to Rotary evaporator and subsequently concentrated under reduced pressure (in vaccum at 40°C). The residues obtained were designed as crude extracts were labeled and stored in refrigerator for further study (Nostro *et al.,* 2006). The dried plant extracted residues obtained were re dissolved in 0.1% Dimethyl sulfoxide (DMSO) to get 100mg/ml concentration and filtration through a 0.45 µm membrane filter and stored in sterile brown bottles in a freezer at 20°C until bioassay.

Test microorganisms

The microorganisms in the present study were including *Escherichia coli* (ATCC B9637), *Klebsiella pneumoniae* (MTCC B2405), *Proteus vulgaris* (MTCC B0426), *Micrococcus luteus* (MTCC B1538), *Bacillus subtilis* (MTCC B2274), *Enterococcus faecalis* (MTCC B0439) and *Streptococcus faecalis* (MTCC B0459). All the cultures were purchased from microbial type culture collection (MTCC), Institute of Microbial technology (IMTECH), Chandigarh, India. The strains were maintained and tested on nutrient agar (Himedia). Active cultures were generated by inoculating a loop full of culture in separate 100ml nutrient broths and incubating on a shaker at 37°C overnight. The cells were harvested by centrifuging at 4000 rpm for 5 min, washed with normal saline, spun at 4000 rpm for 5 min again and dilute in normal saline to obtain $5x10^5$ CFU/ml.

Determination of antibacterial activity

The hexane, chloroform and methanol extracts of *Azadirachta indica* were screened for antibacterial activity by agar well diffusion method (Perez and Paul Mand Bazeque, 1990) with cork borer of size 6.0 mm. For all bacterial strains, overnight cultures grown in nutrient broth were used for inoculation of the nutrient agar plates. An aliquot (0.02 ml) of inoculums was introduced to molten and cooled to 45°C nutrient agar and placed on petriplate by pour plate technique. The appropriate wells were made on respective agar plate by using cork borer. In agar well diffusion method 0.05 ml of hexane, chloroform and methanol extracts were introduced to their respective wells following an incubation period of 24 to 48 hours at 37°C. Antibacterial activity was evaluated by qualifying inhibition zones (IZ) of bacterial growth surrounding the plant extracts. The entire antibacterial assay was carried out under strict aseptic conditions. Penicillin (5 µg/disc) was used as positive control and DMSO as a negative control. Triplicates were carried out for each extract against each of the test bacterium. The results of screening are shown in the Tab. 1.

Results and discussion

The preliminary screening results of hexane, chloroform and methanol extracts are presented in Tab. 1. All the three extracts of leaves of *Azadirachta indica* were expressed antibacterial activity at least one bacterium. Methanol extract was the most effective against all the tested bacteria. The chloroform extract showed good to moderate where as hexane extract showed low antibacterial activity. The inhibition zone values were interpreted as sensitive (18 mm), intermediate (14-17 mm) and resistant (<14 mm) (Barry *et al.,* 1970).

Tab. 1. Inhibitory zones of extracts of *Azadirachta indica* against Gram-negative and Gram-positive bacteria

Extracts/ Antibiotic	Inhibition zones(mm)						
	EC	KP	PV	ML	BS	EF	SF
Hexane	13	11	20	19	--	15	12
Chloroform	18	14	31	21	10	23	20
Methanol	22	28	28	33	10	23	20
Penicillin	19	20	18	19	17	11	16
DMSO	--	--	--	--	--	--	--

--: no zone of inhibition; EC: *Escherichia coli*; KP: *Klebsiella pneumoniae*; PV: *Proteus vulgaris*; ML: *Micrococcus luteus*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis* ; SF: *Streptococcus faecalis*

Hexane extract of *Azadirachta indica* found effective against *Proteus vulgaris* and *Micrococcus luteus*, while the same bacteria were highly sensitive to chloroform and methanol extracts, respectively. *Escherichia coli, Proteus vulgaris, Micrococcus luteus, Enterococcus faecalis* and *Streptococcus faecalis* were showed fairly high degree of sensitivity (IZ=18-33 mm) to both chloroform and methanol extracts. *Enterococcus faecalis* and *Klebsiella pneumoniae* were found to be intermediate sensitivity (IZ=14-15 mm) to hexane and chloroform extracts, respectively. *Escherichia coli, Klebsiella pneumonia* and *Streptococcus faecalis* were most resistant (IZ=11-13 mm) to hexane extract. Metha-

nol extract of *Azadirachta indica* showed fairly high degree of sensitivity (IZ=20-33 mm) (Fig. 1) to all tested bacteria, except *Bacillus subtilis*, which was least susceptible to chloroform and methanol extracts, while did not showed any activity to hexane extract. Penicillin (5 µg) was used as standard antibiotic reference (positive control) exhibited zone of inhibition values range from 11-20 mm against all tested bacteria (Fig. 2). These values were lower than values observed by methanol extract of *Azadirachta indica*. Hence methanol extract was exhibited a broad spectrum of antibacterial activity. Negative control, DMSO had no effect on microbial growth.

Proteus vulgaris and *Micrococcus luteus* belongs to Gram-negative bacteria and the most susceptible bacteria to hexane, chloroform and methanol extracts of *Azadirachta indica*. This selective toxicity could be linked to the differences in the composition of the lipid bilayer for the two strains of bacteria. A greater degree of depolarization and hence increased permeability, was expressed in the lipid bilayer of the Gram-negative bacteria for this cembranoid, because they contain more lipids in their cell walls (Stainer *et al.,* 1986). This depolarization effect is suggested to be associated with hydrogen bonding on the hydroxyl group in the carboxylic functionally situated at the C-19 position in the diterpene (Jente *et al.,* 1990).

The antibacterial activity of *Azadirachta indica* might be due to presence of triterpenoids, phenolic compounds, carotenoids, steroids, valavinoids, ketones and tetra-triterpenoids azadirachtin. Earlier studies on *Azadirachata* claim that a spermicidal fraction of neem oil (NIM-76) is

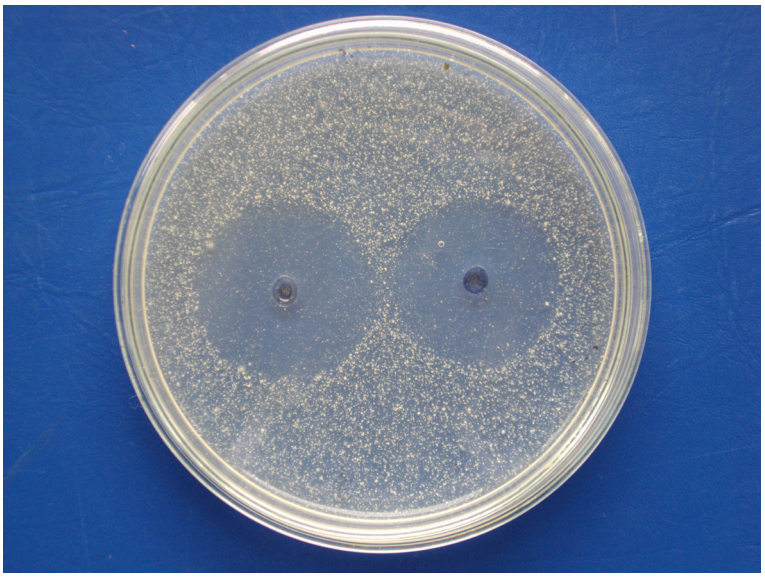

Fig. 1. Inhibition zones produced by methanol extracts

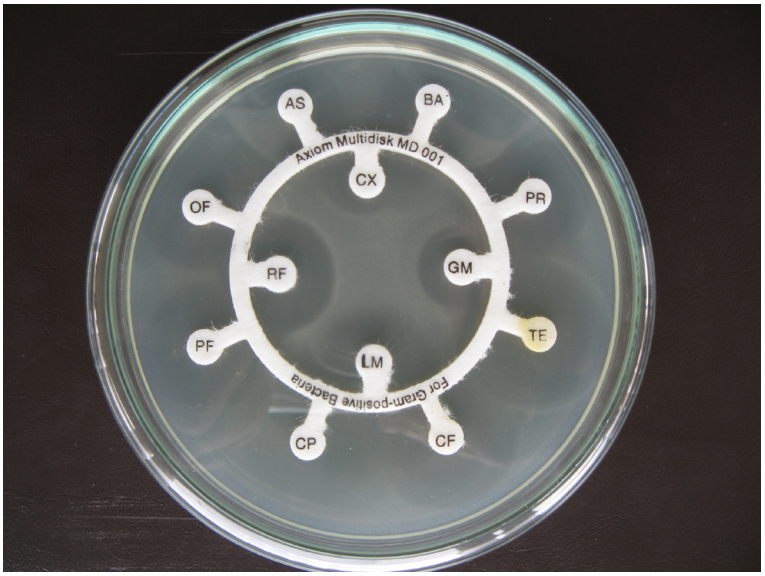

Fig. 2. Inhibition zones produced by standard antibiotic

more effective as an antimicrobial agent as compared to the neem oil itself especially its effect is less on *Escherichia coli* and *Klebsiella pneumonia* (Sairam *et al.,* 2000). Antibacterial activity of the extracts of *Azadirachta indica* was effective on *Escherichia coli, Klebsiella pneumoniae* and *Bacillus subtilis* etc. and needs further confirmation (Jagannadh and Radhika, 2006). Almas (1999) reported antimicrobial activity of neem oil on *Escherichia coli* and *Klebsiella pneumonia* with methanol but not with chloroform and hexane extracts as is influenced by pH of the final extract. The present study report also shows very high efficacy of chloroform and methanol extracts on *Proteus vulgaris* and *Micrococcus luteus* and to a lesser extent against *Escherichia coli, Klebsiella pneumonia, Enterococcus faecalis* and *Streptococcus faecalis*. This study is also needs further study to isolate and purification of bioactive compounds responsible for the antimicrobial activity.

Conclusions

Although this study investigating the *in vitro* antibacterial activity, the results showed that the extracts from *Azadirachta indica* leaves possessed good antibacterial activity, confirming the great potential of bioactive compounds and are useful for rationalizing the use of this plant in primary health care. *In vivo* data may be helpful in determining the real potential usefulness of this plant for the treatment of infectious diseases.

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