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Research Article

Antimicrobial activity of *Azadirachta Indica* (neem) leaf, bark and seed extracts

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ABSTRACT

Screening of medicinal plants for bioactive compounds leads to development of less expensive new antimicrobial agents with improved safety and efficacy. *Azadirachta Indica* (neem) is a multipurpose tree with multiple health benefits. Different parts of the plant are shown to exhibit antimicrobial effects against a wide variety of microorganisms. In the present study we compared the antimicrobial efficacy of aqueous extracts of leaf, bark and seeds of *A. Indica* against human pathogenic bacteria (S*taphylococcus aureus, Enterococcus feacalis, Proteus mirabilis* and *Pseudomonas aeuroginosa*) and fungi (*Aspergillus fumigatus* and *Candida albicans*). Agar well diffusion method and micro-broth dilution methods were used to determine the minimum inhibitory concentration (MIC). Results showed that leaf extract exhibited strong antimicrobial activity against bacteria and fungi at all the concentrations tested (500, 1000 and 2000µg/ml). Antimicrobial activity of bark extract was found to be moderate on bacteria and fungi (effective at 1000 and 2000µg/ml), whereas seed extract exhibited least antimicrobial activity. Minimum inhibitory concentration (MIC) of leaf and bark extract was found to be in the range of 500 to 2000µg/ml for all the tested microorganisms, where as the seed extract did not inhibit the microorganisms at all the concentrations tested except *Candida albicans* (1000µg /ml). Our results suggest that aqueous extracts of *Azadirachta Indica* leaf and bark exhibit high antimicrobial activity.

Keywords: *Azadirachta Indica*; agar well diffusion method; antimicrobial; MIC; pathogenic microorganisms

INTRODUCTION

Drug resistance is a serious global problem, and spread of resistance poses additional challenges for clinicians and the pharmaceutical industry. Use of herbal medicines in the developed world continue to rise because they are rich source of novel drugs and their bioactive principles form the basis in medicine, nutraceuticals, pharmaceutical intermediates and lead compounds in synthetic drugs (De N et al 2002 and Ncube N S et al 2008). Screening medicinal plants for biologically active compounds offers clues to develop newer antimicrobial agents. These compounds after possible chemical manipulation provide new and improved drugs to treat the infectious diseases (Natarajan et al 2003, Shah et al 2006). Plant based products/ extracts are cheaper alternatives to the development of synthetic drugs.

Azadirachta Indica (A. Indica) belongs to the family Meliaceae, commonly known as neem. It is used in traditional medicine as a source of many therapeutic agents. *A. indica* (leaf, bark and seed) are known to contain antibacterial, antifungal activities against different pathogenic microorganisms and antiviral activity against vaccinia, chikungunya, measles and coxsackie B viruses (Biswas K et al 2002). Different parts of neem (leaf, bark and seed oil) have been shown to exhibit wide pharmacological activities including; antioxidant, antimalarial, antimutagenic, anticarcinogenic, anti-inflammatory, antihyperglycaemic, antiulcer and anti-diabetic properties (Talwar et al 1997). The biological activities are attributed to the presence of many bioactive compounds in different parts.

Antimicrobial activity has been investigated for neem leaves, bark and seed, but there are no studies on the comparative evaluation of aqueous extract of leaves, bark and seed. Hence, the current study was designed to investigate the comparative antimicrobial activity of neem leaves, bark and seed aqueous extract against human pathogenic bacteria and fungi. A number of factors such as, thickness and uniformity of the gel, size of the inoculum, temperature and pH that affect the accuracy and reproducibility of the agar diffusion method were also taken into consideration to obtain reliable results.

MATERIALS AND METHODS

Collection of raw materials and preparation of extracts

The leaves, bark and fruits of *Azadirachta Indica* were collected locally and authenticated by a botanist, Ayurveda pharmacy, Tirupati, Andhra Pradesh. Fruits were manually separated into their seeds and seed hulls (kernels) were milled prior to extraction. Raw materials (leaves, bark and seed) were cleaned, shade dried for one week and pulverized to coarse powder. Approx-

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Table 1: Antimicrobial activity of aqueous extracts of *A. Indica* leaf, bark and seed

Extract /Drug	Diameter of zone of inhibition (mm)						
	Conc. (µg/ml)	*Staphylo-coccus aureus*	*Pseudomonas aeruginosa*	*Proteus mirabilis*	*Enterococcus faecalis*	*Aspergillus fumigatus*	*Candida albicans*
Leaf	500	10	10	13	12	-	-
	1000	12	15	14	15	-	11
	2000	15	19	15	18	15	12
Bark	500	-	9	9	10	-	-
	1000	10	12	12	11	12	11
	2000	13	17	14	14	13	13
Seed	500	-	-	-	-	-	-
	1000	-	-	-	-	-	12
	2000	-	-	-	-	-	13
Ciprofloxacin	5 µg	29	25	23	25	-	-
Amphotericin B	100 µg	-	-	-	-	16	14
NC (DMSO)	99.8 %	-	-	-	-	-	-

PC: Positive control, **NC:** Negative control, **mm:** millimeter, - indicates no zone of inhibition.

imately 200 grams of bark, leaves and seed powder was weighed and added to 600 ml of distilled water (1:3 ratio) in a conical flask separately and soaked for 48 hours with intermittent mixing. Mixture was then filtered through whatman No.1filter paper and evaporated in a rotavapour at 40^{o}C. Extracts of leaves, bark and seed were stored in air tight containers at 4^{o}C for further use.

Preparation of culture media

Dehydrated media and standard antimicrobial drugs (discs) were purchased from Hi-Media Laboratories Ltd, India. All the media were prepared in sterile glass petriplates (4 mm thickness) according to the manufacturer's instructions.

Microorganisms used

The bacterial cultures used in the present study include *Staphylococcus aureus*, *Enterococcus faecalis* (Gram positive) and *Pseudomonas aeruginosa, Proteus mirabilis* (Gram negative). *Candida albicans* and *Aspergillus fumigatus* fungi were included in the study. All the cultures were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial technology (IMTECH), Chandigarh, India. The bacterial strains were maintained in Muller Hinton Agar (MHA, pH 7.2) at 37±1^{o}C and fungi were maintained in Sabouraud dextrose agar (SDA, pH 5.4) at 25±1^{o}C. The stock culture slants were maintained at 4^{o}C.

Determination of antimicrobial activity

The aqueous extracts of leaf, bark and seed of *A. Indica* were screened for antimicrobial activity by agar well diffusion method. Agar surface was cut with the help of sterile cork borer having a diameter of 6.0 mm size. All bacterial and fungal strains were grown in nutrient broth (NB) and Sabouraud dextrose broth (SDB) for 4-6 hours at specified temperatures. The turbidity of the broth culture was adjusted to 0.5 McFarland units. This gives a suspension containing approximately 1-2 x 10^{6} colony forming units (CFU)/ml (Mackie & Mac Cartney 1996).

An aliquot (0.02 ml) of microbial culture was added to molten MHA at 45°C and poured into the petriplate. After solidification of the agar, appropriate wells were made on agar surface by using sterile cork borer (3 wells per 90 mm diameter plate). Different concentrations of the extracts were prepared using dimethyl sulfoxide (DMSO) and 50µl of each concentration was added to the wells. Bacterial cultures were incubated at 37°C for 24 hours and fungal cultures at 25^{o}C for 48 hours. Antimicrobial activity was determined by measuring the zone of inhibition surrounding the well. The assays were carried out under aseptic conditions. Ciprofloxacin (5µg/disc) and Amphotericin B (100µg/disc) were used as positive controls for bacteria and fungi respectively and DMSO as a negative control. Each concentration included duplicates and the results are average of two independent experiments.

Determination of Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the aqueous extracts was determined by micro broth dilution method (Andrews JM 2001). For MIC, two-fold serial dilutions of the extracts were prepared (500, 1000 and 2000µg/ml) in microtire wells. Incubation of the microtire plates was carried out at 37^{o} C for 18-24 hours for bacteria and at 25^{o}C for 48 hours for fungi. After incubation, micotire wells were observed for any visible growth. The bacterial suspensions were used as positive control and extracts in broth were used as negative control. The MIC was interpreted as the lowest concentration of the extract that did not show any visible growth when compared to control tubes.

RESULTS

All test strains of bacteria were found to be sensitive to Ciprofloxacin and fungal strains were sensitive to Amphotericin B. DMSO was used as the negative control

Table 2: MIC values of the *A. Indica* aqueous extracts of leaf, bark and seed

Name of the microorganism	Minimum inhibitory concentration (MIC) in µg/ml		
	Leaf	Bark	Seed
Staphylococcus aureus	500	1000	ND
Pseudomonas aeruginosa	500	500	ND
Proteus mirabilis	500	500	ND
Enterococcus faecalis	500	500	ND
Aspergillus fumigatus	2000	1000	ND
Candida albicans	1000	1000	1000

ND- not detected

which did not show any zone of inhibition against tested bacteria and fungi.

Results of the agar well diffusion method are shown in table-1. Leaf extract exhibited antibacterial activity against all the tested bacteria at all concentrations, where as antifungal activity was observed only at 2000µg/ml.

The bark extract exhibited significant antimicrobial activity on *Pseudomonas aeruginosa, Proteus mirabilis* and *Enterococcus faecalis* at all the concentrations tested, whereas its antimicrobial activity on *Staphylococcus aureus, Aspergillus fumigates* and *Candida albicans* was observed at higher concentrations (>500µg/ml).

Seed extract did not show any antibacterial activity, but antifungal activity was observed at 1000 and 2000 µg/ml against *Candida albicans.* No activity was observed against *Aspergillus fumigatus* at any of the concentrations tested.

Minimum inhibitory concentration (MIC) was tested for the aqueous extract of leaves, bark and seed. Results are shown in the table-2. MIC for leaf and bark extract against bacteria was found to be at 500µg/ml and for fungi at 1000µg/ml concentration. MIC of seed extract for fungi was found to be at 1000µg/ml concentration, but there was no inhibition of bacteria at the tested concentrations.

DISCUSSION

Antibiotic resistance is a major concern and development of new agents from plants could be useful in meeting the demand for new antimicrobial agents with improved safety and efficacy (Srivastava et al 2000). In this study, we have shown that the aqueous extracts of neem leaf exhibited highest antimicrobial activity compared with the bark and seed. The difference in the antimicrobial efficacy could be due to variable distribution of phytochemical compounds in different parts. Margolone, margolonone and isomargolonone are tricyclic diterpenoids isolated from stem bark are shown to exhibit antibacterial activity (Pennington et al 1981). Nimbidin and nimbolide from seed oil show antifungal, antimalarial and antibacterial activity including inhibition of *Mycobacterium tuberculosis* (Rojanpo et al 1985, Khalid et al 1989). However presence of high concentrations of azadirachtins, quercetin and β-sitosterol in *A. Indica* leaves might be responsible for strong antibacterial and antifungal activity compared with bark and seed (Subapriya R. Nagini S 2005).

Although crude extracts from various parts of neem have medicinal applications from time immemorial, very little work has been done on the biological activity and plausible medicinal applications of isolated compounds. Hence drug-development programmes could be undertaken to investigate the bioactivity, mechanism of action, pharmacokinetics and toxicity of compounds isolated from neem plant. Newer antimicrobials from plant extracts could also be useful in food, dairy and pharmaceutical industries to prevent contamination by limiting the microbial growth. The tests performed in the current study, compared the antimicrobial efficacy of aqueous extracts of neem leaf, bark and seed which showed high, moderate and low antimicrobial activities respectively.

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