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EVALUATION OF ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF THE LEAF AND BARK EXTRACTS OF *AZADIRACHTA INDICA* A. JUSS

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ABSTRACT

The antibacterial and DPPH radical scavenging activities of the leaf and bark extracts of *Azadirachta indica* A. Juss were investigated. The antibacterial potential of the petroleum ether, chloroform, ethyl acetate and methanol extracts of the leaves and bark of *Azadirachta indica* were studied against human pathogenic bacteria viz. *Bacillus cereus, Enterobacter faecalis, Salmonella paratyphi, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens* by 'agar well diffusion' method. Ethyl acetate, chloroform and methanol extracts of *Azadirachta indica* bark and leaves exhibited pronounced activity against Gram-positive and Gram-negative bacteria and their activity is quite comparable with the standard antibiotics such as tobramycin, gentamicin sulphate, ofloxacin and ciprofloxacin screened under similar conditions. Among the leaf and bark extracts of *A. indica* studied, methanol and ethyl acetate extracts of both leaf and bark showed potent scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The remarkable antibacterial and antioxidant activity exhibited by the plant extracts can be attributed to the synergic effect of the active compounds present in it. The results obtained showed that the leaf and bark methanol and ethyl acetate extracts of *A. indica* can be considered as good sources of natural antioxidants and antimicrobial compounds and can be incorporated into the drug formulations.

Key words: *Azadirachta indica*, antibacterial activity, agar well diffusion method, antioxidant activity, DPPH radical scavenging activity, drug formulations

INTRODUCTION

Medicinal plants are the source of great economic value all over the world. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. Herbal medicine is still the mainstay of about 75-80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents. The microorganisms have developed resistance against many antibiotics due to the indiscriminate use of antimicrobial drugs[1]. Antibiotics are sometimes associated with side effects[2] whereas there are some advantages of using antimicrobial compounds of medicinal plants, such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature[3].

The plant sources are rich of antioxidants, phyto-constituents are capable to terminate free radical reactions and prevent our body from oxidative damage. Phytoconstituents are conferring less side effect and compatible to body physiology. Therefore it is demand of modern era to use such phytoconstituents or phytomedicines[4].

Neem (*Azadirachta indica*), belongs to the family Meliaceae, is a hardy evergreen tree commonly found in South Asia and part of Africa. Its scientific name has been derived from the Persian word azadiracht-e-hind which means a freely growing tree of India[5]. Neem leaves are eaten as vegetable, and twigs are used as toothbrushes. Neem is a nature's pharmacy. Today, researchers are saying that neem could be called "a wonder tree" and eventually expect it to benefit everyone on the planet. The medicinal properties of neem have been known since time immemorial. The earliest ayurvedic literature refers to the benefits of all parts of this majestic tree - fruit, leaf, bark, flower and root[6]. The hypoglycemic actions of its leaves; stem bark and seeds have been reported[7].

Plants have provided a good source of anti-infective reagents; tannins, terpenoids, alkaloids and flavonoids continue to be highly efficient instruments in the fight against microbial infections[8]. Therefore, in the present study an attempt have been made to evaluate antibacterial and antioxidant potentials of the leaf and bark extracts of *Azadirachta indica*. The antibacterial activity of the plant extracts was tested against various multi-drug resistant Gram positive and Gram negative bacterial strains by 'agar well diffusion method'. The

antioxidant activity of the leaf and bark extracts were studied by DPPH radical scavenging assay. The results showed that methanolic extracts of bark and leaves of *A. indica* is a good source of active compounds and antioxidants.

MATERIALS AND METHODS

Plant Material

The leaves and bark of *Azadirachta indica* A. Juss were collected from Thrissur district of Kerala, South India and authenticated by Dr. Kochuthressia M.V., HOD, Department of Botany, Vimala College, Thrissur. Voucher specimen is deposited in the specially maintained herbarium, Department of Botany, Vimala College, Thrissur.

Preparation of Plant Extracts

Fifty grams of the powered plant material were extracted successively with 150mL of petroleum ether, chloroform, ethyl acetate and methanol as solvents for 24hours by Soxhlet equipment.

Test microorganisms

The microorganisms used for antibacterial activity evaluation were obtained from Microbial Type Culture Collection and gene bank (IMTECH, Chandigarh, India), which were maintained on Nutrient broth media. They were Gram-positive bacteria such as *Bacillus cereus* (MTCC-1305), *Staphylococcus aureus* (MTCC-96) and *Enterobacter faecalis* (MTCC-5112) and Gram-negative bacteria such as *Salmonella paratyphi* (MTCC-735), *Escherichia coli* (MTCC-729), *Klebsiella pneumoniae* (MTCC-109), *Pseudomonas aeruginosa* (MTCC-647), *Proteus vulgaris* (MTCC-426) and *Serratia marcescens* (MTCC-86).

Culture medium and inoculums

The stock cultures of microorganisms used in this study were maintained on Plate Count Agar slants at 4^0C. Inoculum was prepared by suspending a loop full of bacterial cultures into 10mL of nutrient broth and was incubated at 37^0C for 24hours. On the next day Muller-Hinton agar (MHA) (Merck) sterilized in a flask and cooled to 45-50^0C was distributed by pipette (20mL) into each sterile Petri dish and swirled to distribute the medium homogeneously. About 0.1mL of bacterial suspension was taken and poured into Petri plates containing 20mL nutrient agar medium. Using the L-shaped sterile glass spreader bacterial suspensions were spread to get a uniform lawn culture.

Antibacterial activity assay

The agar well diffusion method is used for the antimicrobial evaluations. Wells of 8mm (0.8cm) diameter were dug on the inoculated nutrient agar medium with sterile cork borer and 50µL of the petroleum ether, chloroform, ethyl acetate and methanol extracts of the leaves and bark of *Azadirachta indica* were added in each well. Wells introduced with 50µl of pure petroleum ether, chloroform, ethyl acetate and methanol served as negative controls. The plates were incubated at 37^0C over night and examined for the zone of inhibition. The diameter of the inhibition zone was measured in mm. The standard antibiotic drugs such as tobramycin, gentamicin sulphate, ofloxacin and ciprofloxacin were also screened under similar conditions for comparison. An extract was classified as active when the diameter of the inhibition was equal to or larger than 8mm[9]. All the assays were performed in triplicate and expressed as average values.

Preliminary Phytochemical analysis

The sample extracts were analysed for the presence of various phytoconstituents like flavonoids, alkaloids, glycosides, steroids, phenols, saponins and tannins according to standard methods[10].

DPPH free radical scavenging assay

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants[11]. Hydrogen or electron donation abilities of the compounds were measured from the bleaching of the purple-colored ethanol solution of 1, 1-diphenyl-2-picrylhydrazyl (DPPH). This spectrophotometer assay uses the stable radical DPPH as a reagent. The sample solution of material (50 µl) at four concentrations (1.0, 0.5, 0.25 and 0.125 mg/ml) was mixed with freshly prepared methanolic solution of DPPH (634 μM) and allowed to stand for 30 min at room temperature. The absorbance was then measured at 515nm using a spectrophotometer and the inhibition of free radical DPPH in percent (%) was calculated using the formula below:

The percent of inhibition of DPPH reduction (decolourization)

$$\% \text{ of inhibition} = \frac{A_0 - A_{sample}}{A_0} \times 100$$

where (A_0) is the absorbance of the control (blank) and (A_{sample}) is the absorbance of the test compound. The compound concentration demonstrating 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage against sample concentration. Tests were carried out in

triplicate. Samples and DPPH were dissolved in methanol. L-ascorbic acid was used as positive control.

RESULTS

Antibacterial screening

The leaf and bark extracts of *Azadirachta indica* showing the zone of inhibition in millimeters, for Gram positive and Gram negative bacteria are summarized in Table1. In addition, the inhibition zones formed by standard antibiotics and those of negative controls are listed in Table 2.

Phytochemical screening

Phytochemical evaluation was performed with methanol, ethyl acetate, chloroform and petroleum ether extracts of the leaves and bark of *A.indica* (Table 3).

Antioxidant activity

The antioxidant activity of *Azadirachta indica* leaves and bark extracts in solvents of varying polarity were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH. The method is based on the reduction of alcoholic DPPH· solutions in the presence of a hydrogen donating antioxidant. DPPH· solutions show a strong absorption band at 515 nm appearing as a deep violet color. The absorption vanishes and the resulting decolourization is stoichiometric with respect to degree of reduction. The remaining DPPH·, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant. The results of the free radical scavenging activity of leaf and bark extracts of *A.indica* assessed by DPPH assay was summarized in Table 4.

Azadirachta indica leaf methanol, ethyl acetate and chloroform extracts and bark methanol extract possess potent free radical-scavenging activity. The amount of the sample needed for 50% inhibition of free radical activity is expressed by IC_{50}. Lower IC_{50} value indicates higher antioxidant activity. IC_{50} values of *A.indica* leaf and bark extracts and the authentic antioxidant L-ascorbic acid are given in Table 5.

Table 1: Inhibition zones formed by *Azadirachta indica* leaf and bark extracts

	Diameter of inhibition zones(mm/50µL)							
	Azadirachta indica							
	Leaf extracts				Bark extracts			
Microorganisms	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
1. *Bacillus cereus*	23	20	15	13	20	18	16	13
2. *Enterobacter faecalis*	21	17	15	13	26	25	19	14
3. *Salmonella paratyphi*	18	17	15	12	20	17	16	14
4. *Staphylococcus aureus*	19	13	12	11	26	24	19	14
5. *Escherichia coli*	32	30	25	19	30	26	19	15
6. *Proteus vulgaris*	22	20	19	19	23	20	17	12
7. *Klebsiella pneumoniae*	29	20	17	14	26	20	16	11
8. *Pseudomonas aeruginosa*	20	17	15	15	25	23	22	20
9. *Serratia marcescens*	24	19	18	13	23	19	17	15

A: methanol; B: ethyl acetate; C: chloroform; D: petroleum ether

Used concentrations: 50µL of 10mg/mL of plant extracts

Table 2: Inhibition zones formed by the standard antibiotics and negative controls

	Diameter of inhibition zones (mm/50µl)				
Microorganisms	**Tob 10µg**	**Gen 10µg**	**Oflo 10µg**	**Cip 10µg**	**Control A, B, C, D**
1. *Bacillus cereus*	28	32	34	30	--
2. *Enterobacter faecalis*	26	32	32	26	--
3. *Salmonella paratyphi*	25	30	28	30	--
4. *Staphylococcus aureus*	26	28	24	24	--
5. *Escherichia coli*	30	36	32	34	--
6. *Proteus vulgaris*	26	30	24	32	--
7. *Klebsiella pneumoniae*	26	32	32	36	--
8. *Pseudomonas aeruginosa*	26	24	32	28	--
9.*Serratia marcescens*	24	32	30	30	--

Controls- A: methanol; B: ethyl acetate; C: chloroform; D: petroleum ether

Tob: tobramycin, Gen: gentamicin sulphate, Oflo: ofloxacin, Cip:ciprofloxacin

Table 3: Phytochemical screening of *Azadirachta indica* leaf and bark extracts

Phytoconstituents	*A. indica* leaf extracts				*A. indica* bark extracts			
	Methanol extract	Ethyl acetate extract	Chloroform extract	Pet.ether extract	Methanol extract	Ethyl acetate extract	Chloroform extract	Pet.ether extract
Flavonoids	+++	++	+	+	-	-	-	-
Alkaloids	-	-	-	-	-	-	-	-
Glycosides	+	-	-	-	+++	+	+	+-
Steroids	-	-	-	-	-	-	-	-
Phenols	++	-	-	-	-	-	-	-
Terpenoid	++	++	+	+	++	++	+	+
Saponins	-	-	-	-	-	-	-	-
Resins	-	-	-	-	-	-	-	-
Tannins	+	-	-	-	++	-	-	-

\+ *Present* ++ *Moderately present* +++ *Appreciable amount*

Table 4: The DPPH free radical scavenging activity of the leaf and bark extracts of *Azadirachta indica*

	Concentration(mg/ml)			
Samples	**1.0**	**0.5**	**0.25**	**0.125**
	Radical scavenging effect (%)			
A.indica leaf methanol extract	97.55	93.27	91.43	89.60
A.indica leaf ethyl acetate extract	96.64	92.66	89.90	88.38
A.indica leaf chloroform extract	94.49	90.21	88.37	86.23
A.indica leaf pet.ether extract	92.04	88.69	86.85	81.95
A.indica bark methanol extract	95.68	92.66	90.71	88.78
A.indica bark ethyl acetate extract	89.20	86.61	85.75	84.45
A.indica bark chloroform extract	80.99	80.13	78.83	73.00
A.indica bark pet.ether extract	38.80	30.88	25.26	21.80
L-ascorbic acid	98.90	94.51	93.29	90.85

Table 5: Antioxidant activities of the *Azadirachta indica* leaf extracts and positive control using the (DPPH) free radical-scavenging assay

Samples	IC_{50} (microgram/ml)
A.indica leaf methanol extract	72.9
A.indica leaf ethyl acetate extract	74.6
A.indica leaf chloroform extract	83.3
A.indica leaf petroleum ether extract	87.5
A.indica bark methanol extract	74.6
A.indica bark ethyl acetate extract	80.3
A.indica bark chloroform extract	86.5
L-ascorbic acid	58.3

DISCUSSION

Antibacterial screening of plant extracts

The leaf and bark extracts of *Azadirachta indica* showed pronounced antibacterial activity against all the microorganisms tested. The leaf methanol extract of *A.indica* exhibited higher activity than the other extracts and petroleum ether extract showed least activity. Methanol (18-32mm/50µl inhibition zone), ethyl acetate (13-30mm/50µl inhibition zone), chloroform (12-25mm/50µl inhibition zone) and petroleum ether (11-19mm/50µl inhibition zone) extracts of the leaf exhibited marked activity against all the tested organisms such as *Bacillus cereus, Enterobacter faecalis, Salmonella paratyphi, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens.*

Leaf methanol extract showed appreciable activity against *Serratia marcescens* (24mm/50µl inhibition zone) and *Escherichia coli* (32mm/50µl inhibition zone) which is similar to the activity of standard antibiotics tobramycin and ofloxacin (10µg each) respectively which are screened under similar conditions. The leaf ethyl acetate extract showed pronounced activity against *Escherichia coli* (30mm/50µl inhibition zone); leaf methanol extract exhibited remarkable activity against *Klebsiella pneumoniae* (29mm/50µl inhibition zone) which is similar to the activity of standard antibiotic tobramycin (10µg).

The bark methanol extract of *A.indica* exhibited higher activity than the other extracts and petroleum ether extract showed least activity. Methanol (20-30mm/50µl inhibition zone), ethyl acetate (17-26mm/50µl inhibition zone), chloroform (16-22mm/50µl inhibition zone) and petroleum ether (11-20mm/50µl inhibition zone) extracts of the bark exhibited marked activity against all the tested organisms such as *Bacillus cereus, Enterobacter faecalis, Salmonella paratyphi, Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens.*

Bark methanol extract of *A.indica* showed pronounced activity against *Klebsiella pneumonia* (26mm/50µl inhibition zone), *Escherichia coli* (30mm/50µl inhibition zone), *Enterobacter faecalis* (26mm/50µl inhibition zone) and *Staphylococcus aureus* (26mm/50µl inhibition zone) and the activity is comparable with that of the standard antibiotic namely tobramycin. Bark ethyl acetate extract showed pronounced activity against *Staphylococcus aureus* (24 mm/50µl inhibition zone), comparable with that of standard antibiotics ciprofloxacin and ofloxacin (10µg each) screened under similar conditions.

Phytochemical analysis

Phytochemical studies revealed the presence of various secondary metabolites in the leaf and bark extracts of *A.indica*. The various phytochemical compounds detected are known to have beneficial importance in medicinal sciences[12]. The leaf methanol extract of *A.indica* was found to be rich in flavonoids, phenols, terpenoids, glycosides and tannins and bark methanol extract was rich in terpenoids, glycosides and tannins. Ethyl acetate, chloroform and petroleum ether leaf extracts gave positive test for flavinoids and terpenoids whereas bark extracts gave positive test for terpenoids and glycosides. Antibacterial and antioxidant potential of leaf and bark extracts can be attributed to the presence of these phytochemicals.

DPPH free radical scavenging activity assay of plant extracts

DPPH free radical scavenging activity of the leaf and bark extracts of *A.indica* are sorted in descending order: Methanol extract > Ethyl acetate extract > Chloroform extract > Petroleum ether extract. Out of the four samples tested, *A.indica* leaf methanol extract showed the highest scavenging activity (% inhibition 97.55, 93.27, 91.43 and 89.60 at 1.0, 0.5, 0.25 and 0.125mg/ml respectively), followed by *A.indica* leaf ethyl acetate extract. Leaf petroleum ether extract exhibited least DPPH radical scavenging ability with percentage inhibition 92.04, 88.69, 86.85 and 81.95 at 1.0, 0.5, 0.25 and 0.125mg/ml respectively.

A.indica bark methanol extract showed the highest scavenging activity (% inhibition 95.68, 92.66, 90.71 and 88.78 at 1.0, 0.5, 0.25 and 0.125mg/ml respectively), followed by bark ethyl acetate extract. Bark petroleum ether extract exhibited least DPPH radical scavenging ability with percentage inhibition 38.80, 30.88, 25.26 and 21.80 at 1.0, 0.5, 0.25 and 0.125mg/ml respectively.

By comparing the IC_{50} value of the leaf and bark extracts of *A.indica* with that of the authentic antioxidant L-ascorbic acid, it was found that the antioxidant activity of *A.indica* leaf methanol extract (IC_{50}: 72.90 µg/ml) was quite comparable with that of L-ascorbic acid (IC_{50}: 58.3µg/ml). IC_{50} value of leaf ethyl acetate and bark methanol extracts (IC_{50}: 74.60 µg/ml) was not significantly different from that of L-ascorbic acid (IC_{50}: 58.3 µg/ml).

CONCLUSIONS

It is interesting to note that even crude extract of both leaf and bark of this plant showed prominent activity against various pathogenic bacteria where modern therapy has failed. The variation of the susceptibility of the tested microorganisms could be attributed to their intrinsic properties that are related to the permeability of their cell surface to the extracts. The activity of these extracts against the pathogenic microorganisms have been attributed to the presence of biochemicals such as flavonoids, polyphenols and tannins in the leaves[13]; terpenes, tannins and glycosides in the bark extract of *A.indica*[14].

From the above experiment it can be inferred that methanolic extract of bark and leaves of *A.indica* showed significant activity against Gram-positive and Gram-negative bacteria. The activity of leaf and bark methanol extracts was found to be quite comparable with the standard antibiotics screened under similar conditions. So they can be used as an external antiseptic in the prevention and treatment of bacterial infections caused by various pathogenic bacteria. The incorporation of these samples into the drug formulations is also recommended.

Among the leaf and bark extracts of *Azadirachta indica* studied, leaf and bark methanol extracts showed potent scavenging activity on DPPH free radical comparable with the standard antioxidant L-ascorbic acid. Antioxidant activities of the extracts from medicinal plants are mainly attributed to the active compounds present in them. This can be due to the high percentage of main constituents, but also to the presence of other constituents in small quantities or to synergy among them. The results obtained showed that the leaf and bark methanol extracts of *A.indica* can be considered as good sources of natural antioxidants. The

results of this study support the use of this plant for human diseases and reinforce the ethnobotanical importance of plant as a potential source of bioactive substances.

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