

Full Length Research Paper

Investigations of antioxidant and antibacterial activity of leaf extracts of *Azadirachta indica*

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Active ingredients of medicinal plants have been used to cure several human diseases. *Azadirachta indica* is one of the most versatile medicinal plants having a wide spectrum of biological activity due to the presence of large number of bioactive compounds. The present study was conducted to evaluate antimicrobial and antioxidant efficiency and phytochemical screening of *A. indica* leaf extract using methanol as a solvent. A qualitative phytochemical screening was performed for the detection of various phytochemicals. Then, the quantitative determination of total phenols, flavonoids and proanthocyanidins was done and expressed in terms of gallic acid and rutin equivalent. Total phenolic, flavonoid and proanthocyanidin content were found to be 85.9 ± 4.0 , 104.9 ± 5.5 and 65.4 ± 13.9 mg/g of plant extract, respectively. Also, the antibacterial activity was performed using six different bacterial strains: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (ATCC 14028), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27853) and *Proteus vulgaris* (ATCC 35659). It was found that the maximum zone of inhibition of 22 ± 3 mm was shown against *S. aureus* using 700 μ g plant extract. Similarly, the antioxidant activity of the methanolic extract of plant was also determined and it was found that maximum inhibition obtained was 71.23% when 500 μ g plants extract was used.

Key words: *Azadirachta indica*, methanol, antibacterial, antioxidant.

INTRODUCTION

Azadirachta indica is one of the most versatile medicinal plant having a wide spectrum of biological activity and commonly known as 'neem'. It is naturalized in most tropical and subtropical countries. It is broad-leaved evergreen that grows up to 30 m tall and belongs to mahogany family, called Meliaceae. Frequently cultivated and naturalized throughout the drier regions of tropical

and subtropical countries like Nepal, India, Pakistan, Sri Lanka, Thailand and Indonesia, it is known for its therapeutic and ethnomedicinal values since prehistoric era (Chopra et al., 1952; Biswas et al., 2002).

A. indica has been extensively used in Ayurveda, Unani and homeopathic medicine as each part of either its leaves, bark, stem or root has some medicinal properties.

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A large number of biologically active compounds are present in *A. indica* including azadirone, promeliacin, limonoids, gedunin, vilasinin, C-secomeliacins, azadirachtin, nimbin, salanin and other non-iosprenoids, as well as protein/amino acids, polysaccharides, sulphurous compounds, polyphenolics such as flavonoids, their glycosides, dihydrochalcone, coumarins, tannins, aliphatic compounds, etc. (Biswas et al., 2002). The biologically most active compound is azadirachtin, which is actually a mixture of seven isomeric compounds labeled as azadirachtin A-G and azadirachtin E which is more effective (Verkerk and Wright, 1993). The plant extracts exhibit some antimicrobial, anti-inflammatory and antipyretic properties (Okpanyi and Ezeukwu, 1981). Also, the anti-fertility effects of the neem oil due to the potential spermicidal effect have been recorded (Lai et al., 1986; Riar et al., 1990). Neem oil also exhibited a spermicidal agent and inhibited sperm motility which highlighted its immuno-contraceptive properties (Upadhyay et al., 1990, 1992). Neem-oil has been shown to have anti-fertility activity and is also able to stimulate cell mediated immune response. Immuno-contraceptive properties, anti-implantational as well as abortifacient effects of neem are also well documented. (Upadhyay et al., 1992; Sinha et al., 1984).

Other various pharmacological actions of neem are also present such that they can be used as abortifacient, analgesic, anthelmintic, antibacterial, antiyeast, antiulcer, antifertility, antifilarial, antifungal, anti-hyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antinematodal, antipyretic, antispasmodic, insecticidal, antispermatogenic, antitumor, hypercholesteremic, hypoglycaemic, immunomodulator (Parotta, 2001; Ross, 2001). Similarly, neem is also useful in chickenpox, to increase the immunity of body, to reduce fever caused by malaria, for treating various fungi and is also a useful against termites and in curing neuromuscular pains. A wide range of personal care products are also derived from neem seed, oil and leaf which include skin care products- including eczema cream, antiseptic cream and nail care; hair care products - shampoo, and hair oils; oral hygiene- toothpaste and neem twigs; therapeutic- loose neem leaves – tea, vegetarian capsules, powders; household products- soaps, insect repellent (spray and lotion) and candles.

It is an established fact that polyphenolic compounds possess remarkable antioxidant and antimicrobial activities which are present quite commonly in the plant family Meliaceae. *A. indica* is well known in Nepal for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Therefore, taking into account these previous reports that the *A. indica* have huge medical importance, this study was conducted to evaluate the physicochemical and preliminary phytochemical studies, antimicrobial and antioxidant efficiency of *A. indica*, a medicinal plant (leaf extract) using methanol as a solvent.

MATERIALS AND METHODS

Specimen collection

The fresh leaves of *A. indica* were collected from Sunsari district, Nepal in the month of July 2014. After collection, the plant was sent for verification and was verified as *A. indica* by Rita Chhetri (1982) of National Herbarium and Plant Laboratory, Lalitpur Nepal. The healthy leaves of *A. indica* were then taken, washed with distilled water and shade dried for 4 weeks.

Preparation of extract

Dried leaves of plant were ground in a sterile blender to fine powder. Thirty grams of powder was soaked in 300 ml of methanol in conical flask and stored at room temperature for 72 h. The extract was filtered through Whatman No.1 filter paper and was evaporated to dryness using a rotary evaporator.

Preliminary phytochemical screening

Test of alkaloids was done to determine its presence or absence according to the method described by Kikuzaki and Nakatani (1993). Test of glycosides was done to determine its presence or absence according to the method described by Sharma and Singh (2012). Test of phenol and tannins was determined by the method described by Kikuzaki and Nakatani (1993). Test of steroids was done to determine its presence or absence as described by Sharma and Singh (2012). Test was done to determine the presence or absence of flavonoids according to the method described by Sharma and Singh (2012). Presence or absence of volatile oil was determined as described by Kikuzaki and Nakatani (1993). Presence or absence of carotenoids was determined according to the method described by Kikuzaki and Nakatani (1993). Test for fatty acids was done by the method described by Kikuzaki and Nakatani (1993). Test of saponins was done to determine its presence or absence according to the method described by Kikuzaki and Nakatani (1993). Presence or absence of polyphenols was determined by using the method described by Kikuzaki and Nakatani (1993).

Quantitative phytochemical screening

The total amount of phenolic content of plant extract was determined by Folin Ciocalteu method. The standard solutions were prepared by taking 10, 20, 30, 40, 50 µl sample from the stock of 10 mg/ml and total phenolic content was expressed as gallic acid equivalents (GAE) by following the method described by Sharma and Singh (2012). The presence of proanthocyanidins content was expressed as rutin equivalent by following the protocol described by Kikuzaki and Nakatani (1993). The total flavonols in the plant extract was expressed as rutin equivalent by following the method described by Kumaran and Karunakaran (2007).

Antimicrobial screening of the plant extract

The antimicrobial activity of plant extract was evaluated by well diffusion methods given by Perez et al. (1990). The microbial strains of six bacterial samples which were used for the test were obtained from National Public Health laboratory, Teku Kathmandu. The six different strains employed were Gram positive *Staphylococcus aureus* (ATCC 25923) and Gram negative *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 14028), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa*

(ATCC 27853) and *Proteus vulgaris* (ATCC 35659). They were taken and cultured on broth containing nutrient agar. A McFarland 0.5 standard was prepared and the bacterial suspension was compared with the 0.5 McFarland standards to adjust the turbidity of the inoculums for the susceptibility test. The inoculums of bacteria were transferred into Muller-Hinton agar plates using sterile cotton swab. Wells were prepared by punching the agar plate already inoculated with a pure culture of the test organism with the help of sterile glass pipe. Then 40, 50, 60, 70 μ l of samples were added in wells from the stock solution of 10 mg/ml.

In the two wells, 50 μ l of methanol (as negative control) and 50 μ l of reference antibiotic solution (as positive control) were added. Gentamycin (1%) and vancomycin (1%) were used as reference antibiotic for Gram-negative bacteria and Gram-positive bacteria, respectively. Diffusion of extracts, antibiotics and methanol were allowed at room temperature for 1 h. All the plates were then covered with lids and incubated at 37°C for 24 h and zone of inhibitions were measured.

1,1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity

1,1-Diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity was measured according to the method of Ilhami et al. (2005). The plant extract in methanol at various concentrations (10, 20, 30, 40 and 50 μ l) were mixed with an aliquot of 2 ml of 600 μ M DPPH solution in methanol and incubated at 25°C for 30 min and absorbance of the test mixture was read at 517 nm using a spectrophotometer against a DPPH control containing only 1 ml of methanol in place of the extract. All experiments were performed thrice and the results were averaged. Ascorbic acid was used as a standard. Percent inhibition was calculated using the following expression:

$$\text{Inhibition (\%)} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where, A_{control} and A_{sample} stand for absorbance of the control and absorbance of tested extract solution, respectively.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening of methanolic extract of *A. indica* leaves showed the presence of various secondary metabolites. Alkaloids, phenol, tannin, flavonoids, saponins were the most prominent and result of phytochemical test has been summarized in Table 1. Flavonoids, alkaloids and phenolic compounds are a major group of compounds that act as primary antioxidants or free radical scavenger. Subsequently, it may be used for the preparation of drug in a systematic way which may lead to the cure of many ailments in the future. So due to the presence of such secondary metabolites, *A. indica* may have higher medicinal value.

Antibacterial activity

The result for antibacterial activity of *A. indica* is shown in Table 2. The antibacterial potential may be due to various phytochemicals like phenols and flavonols. *A. indica* was the most effective against *S. aureus* (ATCC 25923) and

Table 1. Qualitative analysis of phytochemicals in methanolic extract of *A. indica* leaves.

Phytochemical	Plant extract
Alkaloids	+
Flavonoids	+
Steroids	+
Polyphenols	+
Glycosides	+
Phenol and tannins	+
Volatile oils	+
Carotenoids	+
Fatty acids	+
Saponins	+

+, Presence; -, absence.

S. typhi (ATCC 14028), whereas it was less effective against *E. coli* (ATCC 25922) and *K. pneumoniae* (ATCC 700603) and was moderately effective against *Proteus vulgaris* (ATCC 35659) and *Pseudomonas aeruginosa* (ATCC 27853). The diameter of zone of inhibition (ZOI) was measured and it was found that *A. indica* showed maximum zone of inhibition against Gram positive *S. aureus* and minimum zone of inhibition against Gram negative *E. coli*. Also, the zone of inhibition was increased on increasing concentration with maximum inhibition of 22 \pm 3 mm on *S. aureus* when 700 μ g extract was used.

DPPH radical scavenging activity

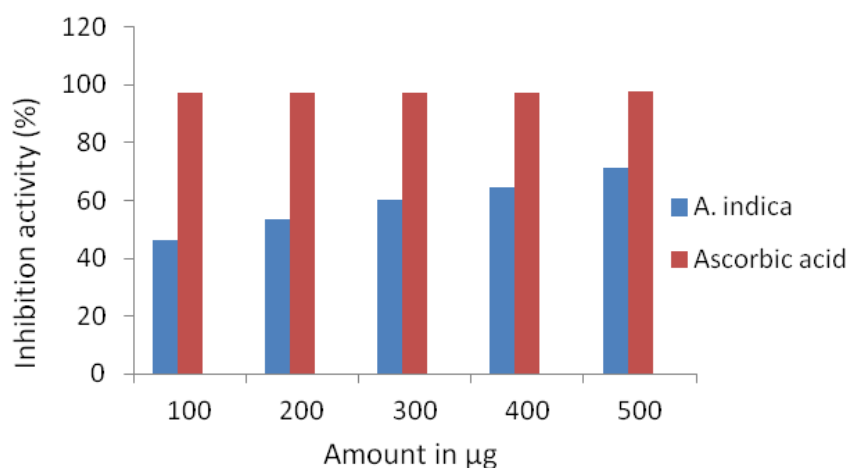
The DPPH radical scavenging activity of *A. indica* is shown in Figure 1. The extent of DPPH radical scavenging at different concentrations (10-50 μ l) from solution of 10 mg/ml of *A. indica* extract was measured, with ascorbic acid as the standard. The inhibition activity of the plant extract was comparatively lower than the ascorbic acid. The result revealed that 50 μ l (500 μ g) plant extract showed the highest inhibition activity of 71.23% followed by decreasing inhibition activity in lower concentrations.

Phenolics, flavonoids and proanthocyanidins

All the results in this study were computed from the calibration curve and expressed as gallic acid equivalent (GAE) in mg per gram (g) dry weight of plant extract. Total flavonoid content in the methanolic extract of *A. indica* leaves was expressed in rutin equivalent. The total proanthocyanidin content was calculated from the calibration curve and expressed as rutin equivalent in mg per gram (g) dry weight of plant extract. The results are shown in Table 3.

Table 2. Result of the antibacterial activity of *A. indica* plant extract.

Amount of sample (μg)	Diameter of zone of inhibition (mm)					
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. typhi</i>	<i>P. aeruginosa</i>	<i>Proteus vulgaris</i>	<i>S. aureus</i>
400	9 \pm 1	10 \pm 1	14 \pm 2	12 \pm 1	13 \pm 1	14 \pm 2
500	9 \pm 1	12 \pm 1	16 \pm 2	14 \pm 2	15 \pm 2	18 \pm 2
600	11 \pm 1	12 \pm 1	15 \pm 2	15 \pm 2	17 \pm 2	19 \pm 3
700	12 \pm 2	13 \pm 2	20 \pm 3	17 \pm 2	19 \pm 3	22 \pm 3
Gentamycin (1%)	18 \pm 3	26 \pm 2	20 \pm 1	18 \pm 2	20 \pm 1	-
Vancomycin	-	-	-	-	-	18 \pm 3

**Figure 1.** Antioxidant activity by DPPH assay.**Table 3.** Total amount of phenols, flavonoids and proanthocyanidins in methanolic extracts of *A. indica* leaves.

Plant	Total phenols (mg/g) of plant extract (GAE)	Total flavonoids mg/g of plant extract (rutin equivalent)	Total proanthocyanidins mg/g of plant extract (rutin equivalent)
<i>A. indica</i>	85.9 \pm 4.0	104.9 \pm 5.5	65.3 \pm 13.9

DISCUSSION

Till date, little work has been done on the biological activity and plausible medicinal applications of the phytochemical compounds and hence extensive investigation is needed to exploit the bioactive principles of *A. indica* for therapeutic utility. *A. indica* is used as medicine for various kinds of diseases and infection in Nepal and India. Some phytochemicals such as alkaloids, flavonoids, phenols, glycosides, proanthocyanidins, saponins and tannins were detected in the leaves extract of *A. indica*. Presence of these compound in the plant extract may be responsible for the investigated activities like antibacterial and antioxidant because it is well established that a wide range of bioactivities are dependent on these phytochemicals.

Medicinal plants constitute an effective and potent source of both traditional as well as modern medicines. With increasing number of multidrug resistant bacteria, there is need of potent and effective antimicrobial agents. Plant can be the best source for such bioactive compounds. Since the methanolic extract was used, this antibacterial activity was mainly the attribution of polar compounds present in leaf extract (Saha et al., 2013). The antimicrobial activities of methanol extracts appeared to be broad spectrum since both the Gram-positive and Gram negative bacteria were sensitive to their inhibitory effects.

In vitro antioxidant activity test showed that the investigated plant contains some potential antioxidant compounds. The antioxidant activity was measured in the term of percentage inhibition of DPPH free radical and it

was found that percentage inhibition was higher when higher concentration of plant extract was used. This may be due to the presence of high amount of antioxidant compounds at high concentration. Flavonoid presence indicated by the phytochemical investigation of plant extract may be accountable for this antioxidant activity (Karimi et al., 2010; Prochazkova et al., 2011). Since possible antioxidant and antibacterial activities were exhibited in the crude leaves extract of *A. indica*, this justifies its medicinal activities.

Conclusion

The present study has shown that crude extract of *A. indica* contains some medicinal active components. *A. indica* can be effective against multi drug resistant pathogens and can be used as a good source of antioxidant. Further research on active constituents of plant is expected to serve as lead in the development of novel bioactive antimicrobial and antioxidant compounds.

Conflict of interests

The author(s) did not declare any conflict of interest.

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