

Phytochemical analysis and antimicrobial activity of aqueous, ethanol, and acetone extract of *Azadirachta indica* Juss in combination with streptomycin

Chilka N, Sarda P, Dr. Prashar K, Deshmukh P, * Warkhade B

Department of Biotechnology, Badrinarayan Barwale Mahavidyalaya, Jalna, Maharashtra, India

Abstract

Azadirachta indica A. Juss. Is a very useful traditional medicinal plant in the sub-continent and each part of the tree has some medicinal properties. The present study was carried out to screen and evaluate antimicrobial activity of leaf extract of *Azadirachta indica*. Ethanol, Acetone and aqueous extract leaves of *A. indica* were tested against *Escherichia coli* and *Staphylococcus aureus* which are known to be resistant to various antibiotics. These extracts were prepared from dry *Azadirachta indica* leaves and evaluated for their part in increasing antibacterial activity of streptomycin against *S. aureus* and *E. coli*. The antibacterial activity of streptomycin was enhanced against the test organism in the presence of these extracts. Streptomycin in combination with these extracts showed maximum inhibition against *S. aureus* and *E. coli*. Phytochemical analysis gave positive results for steroids, triterpenoids, alkaloids, glycosides, phenolic compounds, flavonoids, and tannins. Leaf extract of *Azadirachta indica* contains pharmacologically bioactive constituents that may be responsible for its activity against test organisms.

Keywords: *Azadirachta indica*, phytochemical analysis, bioactive constituents, Streptomycin.

1. Introduction

The Neem (*Azadirachta indica* A. Juss) is an evergreen robust tree, belongs to the family Meliaceae. It is mostly found in tropics and sub-tropical areas of the world (Africa and Asia). Occur in medium to large size and have brown to dark grey bark and a dense rounded pinnate leaves^[1]. It has an extensive deep root system which is responsible for their survival in arid and semi-arid area of the world. The Chemical constituents contain many biologically active compounds that can be extracted from neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, Carotenoids, steroids and ketones. Azadirachtin is actually a mixture of seven isomeric compounds labeled as azadirachtin A-G and azadirachtin E is more effective^[2]. Other compounds that have a biological activity are salannin, volatile oils, meliantriol and nimbin^[3]. Neem leaf is effective in treating eczema, ringworm, acne, anti-inflammatory, antiheperglycemic properties and it is used to heal chronic wounds, diabetic food and gangrene developing conditions. It is believed to remove toxins from the body, neutralize free radicals and purify the blood. It is used as anticancer agent and it has hepato-renal protective activity and hypolipidemic effects (Fitoterapia part I and part II). Medicinal plants have been found useful in the cure of a number of diseases including bacterial diseases.

Medicinal plants are a rich source of antimicrobial agents^[4]. Almost every part of the tree is bitter and finds application in indigenous medicine. Neem extract has been reported to have antidiabetic, antibacterial and antiviral activity. Almost every part of the tree has been in use since ancient times to treat a number of human ailments and also as a household pesticide. The extract from bark, leaves, fruits and root have been used to control leprosy, intestinal helminthiasis and respiratory disorders in children^[5]. Flavonoids, flavonoglycosides, dihydrochalcones, tannins and others are also important constituents of bark, leaves, fruits and flowers of neem. The biological activities and medicinal properties of neem have recently been reported^[7].

Now a day, antibiotic resistance in medically important bacteria is the major problem faced by the world. The indiscriminate use of commercial antimicrobial drugs has resulted in multiple drug resistance. Antibiotics may also cause adverse effects on the host including allergies, hypersensitivity and immune-suppression. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases.

In current study, screening the bioactive components and the antibacterial effects of the Ethanol, Acetone and Aqueous extract and the extracts were evaluated in combination with streptomycin to assess their antibacterial activity against pathogenic bacteria.

2. Material and Methods

Sample collection

The fresh leaves of *A. indica* were obtained from campus of Badrinarayan Barwale Mahavidyalaya, Jalna (MS). They were washed under running tap water to remove surface dirt and impurities, followed by distilled water. These leaves were air dried and kept for shade dry for 10-15 days. After drying the leaves were crushed to get fine coarse powder. This powder was used for preparation of different plant extracts.

Preparation of leaf extracts

For aqueous extract 10gm of fine powder of neem leaves was added to 100ml of distilled water and then boiled for 15min. after cooling this was filtered through Whatman's filter paper and filtrate was used as an aqueous extract. This extract was stored at 4 °C for further use.

For ethanolic extract absolute ethanol was taken 100ml and to this 10gm of fine coarse powder of neem leaves was added and kept overnight. After that this was filtered through Whatman's filter paper and filtrate was used as an Ethanolic extract. This extract was stored at 4 °C for further use.

For Acetone extract 100ml Acetone was taken and to this 10gm of fine coarse powder of neem leaves was added and

kept overnight. After that this was filtered through Whatman's filter paper and filtrate was used as an Acetone extract. This extract was stored at 4 °C for further use.

Test organism

Bacterial cultures were selected from American type culture collection (ATCC). The strain used for the study were *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). These were grown on their respective selective media and purity was determined by morphological and biochemical characterization.

Inoculum preparation

Loopful of pure culture from selective media was picked up and inoculated in Muller Hinton Broth (Himedia). It was incubated at 37 °C for 3-7 hrs. until moderate turbidity develops. Inoculum turbidity was compared with that of 0.5 McFarland standard.

Preparation of Disc

Whatman's filter paper no.1 was punched to get disc of 6mm diameter. These discs were sterilized under UV light. Each sterile disc was impregnated with ethanol extract, acetone extract, aqueous extract and excess of solvent was dried in controlled temperature.

Antimicrobial activity of extract

The antimicrobial activity of the extract was evaluated by standard disc diffusion method (Baur *et al.*, 1966). Plates of Muller Hinton agar (Himedia) medium having media up to 4 mm were prepared. After solidification lawn of inoculum was prepared on to agar plates for each organism. Inoculum was taken by socking the sterile swab (Himedia) in prepared inoculum of test organism i.e. *Staphylococcus aureus* and *Escherichia coli* and spread over the agar plates for respective organism. Ethanol extract disc, acetone extract disc and aqueous extract disc of *Azadirachta indica* was applied and incubated at 28-30 °C for 16-18 hours.

Disc diffusion assay to evaluate combined effects

Disc diffusion method was used to evaluate *in vitro* antibacterial activity of standard streptomycin disc (10mcg Himedia) against *Staphylococcus aureus* and *Escherichia coli* on Muller Hinton Agar (Himedia). To determine combined effect, each standard paper disc was further impregnated with 20µl of each single extract. Muller Hinton Agar plates were inoculated with *Staphylococcus aureus* and *Escherichia coli*. Standard antibacterial streptomycin disc were used as positive control and streptomycin disc impregnated with aqueous, ethanol, and acetone extract were place onto Muller Hinton Agar plate inoculated with test organisms. These plates were incubated 16-18 hours. After incubation, the zones of inhibition were measured. The assays were performed in triplicate.

Assessment of increase in fold area

The increase in fold area was assessed by calculating the mean surface area of the inhibition zone of each antibacterial agent (streptomycin) and streptomycin *plus* extract. The fold

increase area of different test organism for streptomycin and for streptomycin *plus* extract was calculated by equation $(B^2 - A^2)/A^2$, where A and B were zones of inhibition for streptomycin and streptomycin *plus* extract, respectively.

Phytochemical analysis

Phytochemical tests were done to find the presence of the active chemical constituents such as alkaloid, flavonoids, glycosides, triterpenoids, steroids, tannin and phenols, reducing sugar, carbohydrates and protein and amino acids by the following procedure. (C.K. Kokate; 2000, J.B. Harbone; 1999, Prashanth Tiwari *et.al.*; 2011)

Tests for Alkaloids

To the extract, dilute hydrochloric acid was added, shaken well and filtered. With the filtrate, the following tests were performed.

Mayer's reagent test

To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates the presence of alkaloids.

Tests for Carbohydrates

Molisch test

2 ml of aqueous extract was treated with 2 drops of alcoholic α -naphthol solution in a test tube and then 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates.

Tests for Reducing Sugars

Benedict's test

Equal volume of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

Tests for Flavonoids

Alkaline reagent test

The extract was treated with few drops of sodium hydroxide solution separately in a test tube. Formation of intense yellow color, which becomes colorless on addition of few drops of dilute acid indicates the presence of flavonoids.

Tests for Glycosides

Borntrager's test

To 3 ml of test solution, dilute sulphuric acid was added, boiled for 5 minutes and filtered. To the cold filtrate, equal volume of benzene or chloroform was added and it was shaken well. The organic solvent layer was separated and ammonia was added to it. Formation of pink to red color in ammoniacal layer indicates the presence of anthraquinone glycosides.

Tests for Tannin and Phenolic compounds

Ferric chloride test

A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added.

Formation of blue, green or violet color indicates presence of phenolic compounds.

Test for Saponin

Froth test

The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of saponins.

Tests for Protein and Amino acids

Ninhydrin test

3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution on a water bath for 10 minutes. Formation of blue color indicates the presence of amino acids.

Tests for Triterpenoids and Steroids:

Salkowski's test

The extract was treated with chloroform and filtered. The filtrate was added with few drops of concentrated sulphuric acid, shaken and allowed to stand. If the lower layer turns red, sterol is present. Presence of golden yellow layer at the bottom indicates the presence of triterpenes.

3. Result and Discussion

The antibacterial activity of Acetone, Ethanol and aqueous extract of *A. indica* along with Streptomycin against *E. coli* and *S. aureus* were shown in Table 1. Ethanol extract shows highest 19mm zone against *E. coli* and 18 mm zone against *S. aureus*, followed by acetone extract shows 17mm against *E. coli* and 15 mm zone against *S. aureus*, whereas aqueous extract shows 13mm zone diameter against *E. coli* and 12 mm zone against *S. aureus*. Standard antibiotic streptomycin shows 20 mm zone diameter against *E. coli* and 23 mm zone against *S. aureus* (Fig1).

Table 1: Antibacterial activity of Ethanol, Acetone and Aqueous extract of *A. indica*

Test Organism	Zone of inhibition of <i>A. indica</i> Extract in mm			Zone of inhibition of Streptomycin in mm
	Ethanol	Acetone	Aqueous	
<i>E. coli</i>	19	17	13	20
<i>S. aureus</i>	18	15	12	23

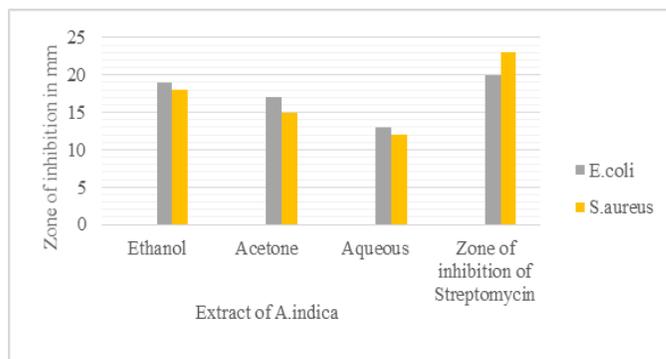


Fig 1: Antibacterial activity of Ethanol, Acetone and Aqueous extract of *A. indica* and Streptomycin

In the *in vitro* antibacterial activity of streptomycin an antibacterial agent that is widely used against many bacterial infection, was used as positive control for comparison with *A. indica* extracts. The diameter of zone of inhibition and increase in fold area for all the test organism was measured. The antibacterial activity of streptomycin increased significantly in presence of Ethanol, Acetone and Aqueous extract of *A. indica* and it was confirmed by calculating increase in fold area, result were indicated in Table 2.

Table 2: Zone of inhibition of streptomycin against test organism in absence and in presence of Neem extract at content 20µl per disc

Test Organism	<i>A. indica</i> Extract	Streptomycin + Extract (B)	Streptomycin (A)	Increase in Fold B^2-A^2/A^2 Area
<i>E. coli</i>	Ethanol	24mm	20mm	0.44
	Acetone	23mm	20mm	0.32
	Aqueous	21mm	20mm	0.1
<i>S. aureus</i>	Ethanol	26mm	23mm	0.27
	Acetone	26mm	23mm	0.27
	Aqueous	24mm	23mm	0.08

Phytochemical analysis

The phytochemical analysis of plant extracts using Acetone, Ethanol and Aqueous was showed in Table 3. From the phytochemical analysis, reducing sugar were found in *Azadirachta indica* in the solvents such as Acetone, Ethanol and Aqueous. The Ethanol extract of *Azadirachta indica* showed the presence of alkaloid, glycosides, flavonoids, saponins, tannin and phenolic compound, reducing sugar, triterpenoids and steroids. Alkaloid, glycosides, saponins, tannin and phenolic compound, reducing sugar, triterpenoids

and steroids were observed in Acetone extract of *Azadirachta indica* but there was absence of flavonoids. Alkaloid, glycosides, flavonoids, saponins, tannin and phenolic compound, reducing sugar, triterpenoids and steroids were found in presence of aqueous extract of *Azadirachta indica*. Ethanol, Acetone and Aqueous extract of *Azadirachta indica* shows absence of carbohydrates, proteins and amino acid by giving negative results for test of carbohydrate and test of protein and amino acid.

Table 3: Phytochemical analysis of plant extracts

Phytochemical test	Ethanol extract	Acetone extract	Aqueous extract
Tests for Alkaloids	+	+	+
Tests for Carbohydrates	-	-	-
Tests for Reducing Sugars	+	+	+
Tests for Flavonoids	+	-	+
Tests for Glycosides	+	+	+
Tests for Tannin and Phenolic compounds	+	+	+
Test for Saponins	+	+	+
Tests for Protein and Amino acids	-	-	-
Tests for Triterpenoids and Steroids	+	+	+

(+) indicates presence while (-) indicates the absence of the components

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 [7]. In this study, we have shown that streptomycin in combination with *A.indica* extract shows increase in bactericidal effect and can be used to treat the infection caused by these bacteria. The preliminary phytochemical tests performed were of qualitative type and from the phytochemical investigations it was observed that alkaloids, tannins and phenolic compounds, flavonoids, terpenoids and steroids, saponins, Glycoside and reducing were present in the extracts. *Azadirachta indica* leaves possessed good anti-bacterial activity confirms the presence of bioactive compounds and is useful for rationalizing the use of this plant in primary health care [8]. The presence of these secondary metabolites in plants, produce some biological activity in man and animals and it is responsible for their use as herbs. These compounds also serve to protect the plant against infection by microorganisms, predation by insects and herbivores, while some give plants their odors and or flavors and some still are responsible for their pigments [9].

In some cases, the activity has been associated with specific compounds or classes of compounds. These active constituents can be used to search for bioactive lead compounds that could be used in the partial synthesis of more useful drugs [9, 10].

4. Conclusion

Moreover, the combined effects of a standard antibacterial agent (streptomycin) with extracts against pathogenic bacteria is similarly a new finding. Further, it can be concluded that extract alone or their formulations (combination) can be used as effective agents against human bacterial pathogen.

5. References

- Ogbuewu IP. Physiological responses of rabbits fed graded levels of neem (*Azadirachta indica*) leaf meal. M.Sc. Thesis, Federal University of Technology, Owerri. 2008.
- Verkerk RHJ, Wright DJ. Biological activity of neem seed kernel extract and synthetic azadirachtin against

larvae of *Plutella xylostella*. Pesticide Science. 1993; 37:83-91.

- Jacobson M. Review of neem research in the United States. In: Locke JC, Lawson, RH (eds) proceedings of a workshop in neems potential in pest management program. USDA-ARS. Beltsville, MD.ARS. 1990; 86:4-14.
- Mahesh B, Satish S. Antimicrobial activity of some important medicinal plant against plant and human pathogens. World J Agric Sci. 2008; 4(S):839-843.
- Chattopadhyay RR, Chattopadhyay RN, Maitra SK. Possible mechanism of anti-inflammatory activity of *Azadirachta indica* leaf extract. Indian J. Pharm. 1993; 25:99-100.
- Venugopal PV, Venugopal TV. Antidermatophytic activity of neem (*Azadirachta indica*) leaves *in vitro*. Indian J. Pharmacol. 1994; 26:141-143.
- Srivastava A, Shukla Kumar YN. Recent development in plant derived antimicrobial constituents A Review. J Med Arom PI Sci 2000; 20:717-72.
- Saradha Jyothi Koon, Subbarao Budida. Antibacterial potential of the extracts of the leaves of *Azadirachta indica* Linn. Nat Sci Biol. 2011; 3(1):65-69.
- EL-Mahmood AM, Doughari JH, Ladan N. Antimicrobial screening of stem bark extracts of *Vitellaria paradoxa* against some enteric pathogenic microorganisms. Afr. J. Pharm. Pharmacol. 2008; 2(5):089-094.
- Ogbonnia SO, Enwuru NV, Onyemenen EU, Oyedele GA, Enwuru CA. Phytochemical evaluation and antibacterial profile of *Treulia Africana* Decne bark extract on gastrointestinal bacterial pathogens. Afr. J. Biotechnol. 2008; 7(10):1385-1389.