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Research Article

PHYTOCHEMICAL ANALYSIS AND ANTI-BACTERIAL EFFECT OF CRUDE EXTRACT OF *AZADIRACHTA INDICA* BY USING *ESCHERICHIA COLI, BACILLUS SUBTILIS AND STAPHYLOCOCCUS AUREUS*

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ABSTRACT
With the widespread development of resistance of microbial pathogens against currently available modern antibiotics, medical science is now making efforts to discover novel antibiotics. *Azadirachta indica* (Meliaceae) commonly known as Neem is native of India and naturalized in most of tropical and subtropical countries is of great medicinal value and distributed wide spread in the world. The Chemical constituents contain many biologically active compounds that can be extracted from Neem, including alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoid, steroids and ketones. Leaf extract has a good therapeutic potential as anti hyperglycemic agent. Anti-inflammatory effect of Neem extract is less than that produced by dexamethasone. Neem leaves has antibacterial properties and could be used for controlling airborne bacterial contamination in the residential premise. Neem seeds are used in traditional medicine to treat infections conditions especially those involving the eye and ear. 20-30 grams of fresh leaves were boiled with 200 ml of solvent for 1 hour. The extract was filtered using Whatmann filter paper No. 1 and then concentrated in vacuum at 40°-50°C using a rotary evaporator. Evaporation of solvent in the rotary evaporator affords a crude extract of the soluble components and these extracts were subjected to the qualitative phytochemical analysis and antibacterial studies. The literature survey reveals that Neem(*Azadirachta indica*) possessed various biological activities such as anti cancer, anti inflammatory, anti bacterial activity, antiviral activity, anti oxidant effect, skin diseases, digestive disorders more important biological activities present in Neem. The aim of the present investigation is to achieve phytochemical analysis and anti-bacterial activity of crude extract of *Azadirachta indica*.

Keywords: *Azadirachta indica, E.coli, Bacillus subtilis and Staphylococcus aureus, Antibacterial activity, Cup Plate Method*

INTRODUCTION
Natural herbs and their varied extracts have been used globally in therapeutic since antiquity (Summar 9 WHO guidelines 1993). In developing countries majority of the population still exploits traditional folk medicine derived from plant resources (Farnsworth, 1994; Saxena, 1997). Medicinal use of herbal preparations is first mentioned in ancient Hindu texts like Vedas and these herbs are an important part of 'medicinal science of Indian culture – Ayurveda (Rastogi and Mehrotra, 2002).
In current epoch quick results are expected; hence haphazard use of synthetic antimicrobial drugs is copious these days which is now resulting in multiple drug resistance (Davis, 1994) and evidences of serious adverse effects are noted in various studies (Ahmad *et al.,*). Therefore natural herbs are gaining importance in overcoming this problem (Cordell, 2000) as the traditional herbs are found to be more economical and having lesser side effects than synthetic drugs (Nair *et al.,* 2005; Menon, 2003). Neem (*Azadirachta indica*) belonging to family Meliaceae used for medicinal purpose since centuries in India (Jayraman *et al.,* 1995). Twig of its stem is widely used for chewing (brushing) in rural areas since time immemorial and it is now proved to have antiplaque and related antibacterial properties (Wolinsky *et al.,* 1996).

Research Article

Plant Profile

❖ ***Neem Leaves***

❖ **BIOLOGICAL SOURCE:** *Azadirachta indica*
❖ **FAMILY:** Meliaceae
❖ **KINGDOM:** Plantae
❖ **DIVISION:** Magnollophyta
❖ **ORDER:** Sapindales
❖ **GENUS:** *Azadirachta*
❖ **SPECIES:** *Indica*

MATERIALS AND METHODS

❖ ***Collection of Plant Materials:*** The experiment was conducted in the year 2014 in the college laboratory. Leaves were collected from the *Azadirachta indica* tree in the college campus. It was ensured that the plant was healthy and uninfected. The leaves were washed under running tap water to eliminate dust and other foreign particles and to clean the leaves thoroughly and dried.

❖ ***Preparation of Leaf Extracts:*** 20-30 grams of fresh leaves were boiled with 200 ml of solvent for 1 hour. The extract was filtered using Whatmann filter paper No. 1 and then concentrated in vacuum at 40°-50°C using a rotary evaporator. Evaporation of solvent in the rotary evaporator affords a crude extract of the soluble components and these extracts were subjected to the qualitative phytochemical analysis and antibacterial studies.

❖ ***Phytochemical Analysis:*** The extracts were analyzed by the following procedures to test for the presence of the alkaloids, saponins, tannins, Terpenoids, flavonoids, glycosides, volatile oils and reducing sugars

❖ ***Saponins:*** Saponins were detected using the froth test. 1g of the sample was weighed into a conical flask in which 10ml of sterile distilled water was added and boiled for 5 minutes. The mixture was filtered and 2.5 ml of the filtrate was added to 10ml of sterile distilled water in a test tube. The test tube was stopper and shaken vigorously for about 30 seconds. It was then allowed to stand for half an hour. Honeycomb froth indicated the presence of saponins.

❖ ***Tannins:*** To a portion of the extract diluted with water, 3-4 drops of 10% ferric chloride solution is added. A blue colour is observed for gallic tannins and green colour indicates for catecholic tannins.

❖ ***Reducing Sugars****:* To 0.5ml of plant extracts, 1ml of water and 5-8 drops of Fehling's solution was added and heated over water bath. Brick red precipitate indicates the presence of reducing sugars.

❖ ***Glycosides:*** 25ml of dilute sulphuric acid was added to 5ml extract in a test tube and boiled for 15 minutes, cooled and neutralized with 10%NaOH, then 5ml of Fehling solution added. Glycosides are indicated by a brick red precipitate.

❖ ***Alkaloids:*** 2ml of extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids.
❖ ***Flavonoids:*** 4ml of extract solution was treated with 1.5 ml of 50% methanol solution. The solution was warmed and metal magnesium was added. To this solution, 5-6 drops of concentrated hydrochloric acid was added and red colour was observed for flavonoids and orange colour for flavones.
❖ ***Volatile Oils:*** 2ml of extract was shaken with 0.1ml dilute NaOH and a small quantity of dilute HCl. A white precipitate is formed if volatile oils are present.
❖ ***Terpenoids:*** Four milligrams of extract was treated with 0.5 ml of acetic anhydride and 0.5 ml of chloroform. Then concentrated solution of sulphuric acid was added slowly and red violet colour was observed for terpenoid.
❖ ***Ethanol Extract:*** *Azadirachta indica* leaves (100 g) were ground into fine powder using a stainless-steel grinder, and deep in100% ethanol (200 ml) for overnight. The ethanol fraction was separated using sterile muslin cloth and filter through sterile Whatmann filter paper (no. 02). The filtered extract was concentrated by rotary film evaporator.
❖ ***Methanol Extract:*** Ten grams of dried plant material was extracted with 100 ml of methanol kept on a rotary shaker for 24 hours. Thereafter, it was filtered and centrifuged at 5000 g for 15 minutes. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume (17). It was stored at 4°C in airtight bottles for further studies.
❖ ***Source of Microorganisms:*** The organisms used were *Escherichia Coli, Pseudomonas aeroginosa, Staphylococcus aureus* and *Basillus substilis.*

Sterilization of Nutrient Media

❖ Nutrient media
❖ Peptone: 0.3%
❖ Beef Extract: 0.3%
❖ Nacl: 0.5%
❖ Agar: 2%
❖ pH: 7±0.2
❖ Distilled water: 100ml.
❖ All the required components that are 0.3gm of peptone, 0.3gm of beef extract, 0.5gm of Nacl were weighed accurately and were taken in a conical flask and distilled water was added up to 95ml to it.
❖ All the components were dissolved with the aid of stirring.
❖ Then the pH was adjusted to 7±0.2.
❖ Volume was adjusted to 100ml.
❖ The conical flask was plugged with non-absorbent cotton and it was sterilized by using autoclave at 121°C temperature, 15 lb pressure and 20 minutes time.
❖ After sterilization the nutrient media was transferred to culture tubes.

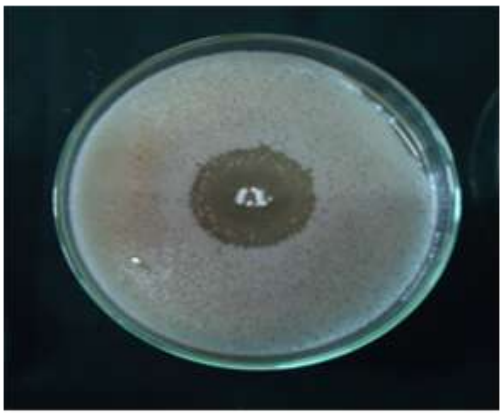
Determination of Antibacterial Activity by Cup Plate Method

The antibacterial activity of the extracts was determined by using the agar well diffusion technique. Mueller Hinton agar plates (Himedia, Mumbai) were seeded with 0.1 ml of overnight culture, allowed to incubate for 24hrs. Cups were made in Petri plates using sterile cork borer (0.85 cm) and 50 µl of each extract was added into each well. Then bacterial plates were incubated at 37 C 24 hrs. Antibacterial activity was determined by measurement of zone of inhibition around each well in plate using zone reader. Measured inhibition zones were recorded as mean diameter in mm.

Antibiotic Assay Medium

❖ Peptic digest of animal tissue – 6gm
❖ Yeast extract-3gm
❖ Beef extract-1.5gm
❖ Agar-1.5gm.
❖ Distilled water-1000ml.

❖ The agar tube was taken and medium was liquefied and pour and cooled to 45 C aseptically loopful of required micro-organism from pure culture was inoculated and then poured into a sterile Petridis and allowed to solidify added to it. In cup plate method four cups were made on the solidified medium and to this different antibiotics were added. These Petridis were inoculated at 37 °C for 24hours.



S.aureus

Rifampicin

B.subtilis

E.coli

1. Antibacterial activity of Ethanol, Methanol extracts of Azadirachta indica medicinal plants against human pathogen:

Sl.No.	Culture	Extract of crude product	Diameter	Radius	Area
1.	*Bacillus subtilis*	Methanol extract	20mm	10mm	0.031cm
2.	*Bacillus subtilis*	Ethanol extract	25mm	12.5mm	0.020cm

Sl.No.	Culture	Extract of crude product	Diameter	Radius	Area
1.	*E.coli*	Methanol extract	18mm	12mm	0.021cm
2.	*E.coli*	Ethanol extract	23mm	14mm	0.016cm

Sl.No.	Culture	Extract of crude product	Diameter	Radius	Area
1.	*Staphylococcus aureus*	Methanol extract	11mm	10.5mm	0.028cm
2.	*Staphylococcus aureus*	Ethanol extract	19mm	16.5mm	0.0115cm

2. Qualitative Photochemical Analysis of Ethanol and Methanol extracts of *Azadirachta Indica* leaf.

Solvents used for extraction	Alkaloid	Reducing sugar	Flavonoid	Saponin	Tannin	Volatile oil	Glycoside	Terpenoids
Methanol	-	+	-	-	-	-	+	+
Ethanol	-	+	+	+	+	-	-	-

RESULT AND DISCUSSION

Results

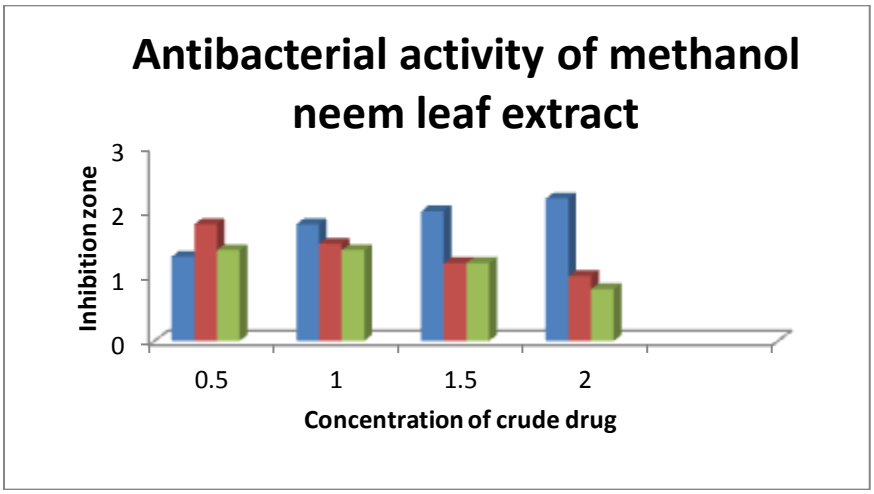
We designed several formulations and prepared them using standard methods and compare with a commercially antibiotic i.e. Rifampicin. The methanol extract of *A.Indica* against *E.coli*, *S.aureus and B. subtillus* bacteria show varied zone of inhibition. Results obtained from this study, indicate that the Neem plant leaf extracts showed the strongest antibacterial activity than a commercially (Rifampicin) available antibiotics.

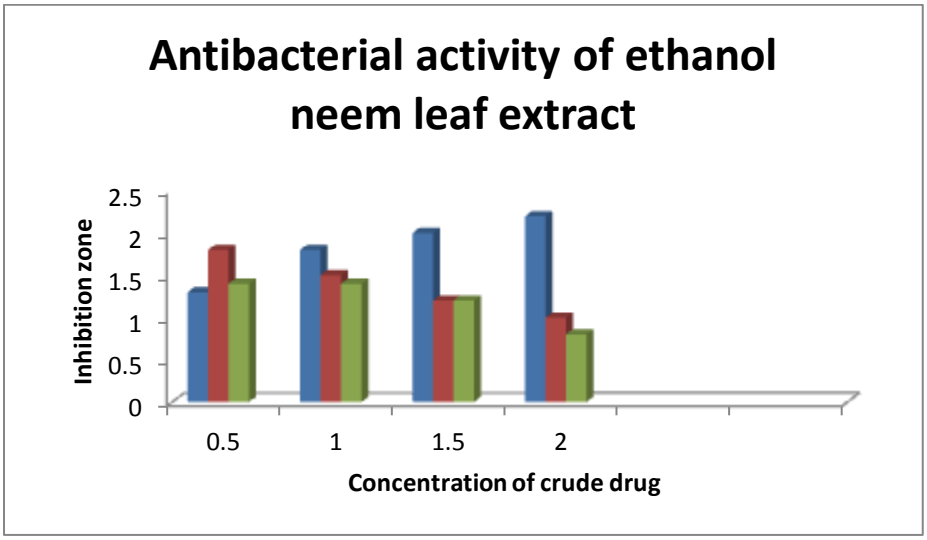
Antibacterial activity of ethanol Neem leaf extract:

Concentration	Bacterial Strains (Zone of inhibition in mm)		
	E.coli	***S.aureus***	***B.subtilis***
0.5%	2.1	2.2	2.0
1.0%	2.3	2.4	2.3
1.5%	2.4	2.2	2.1
2.0%	2.0	2.5	2.0

Antibacterial activity of methanol Neem leaf extract:

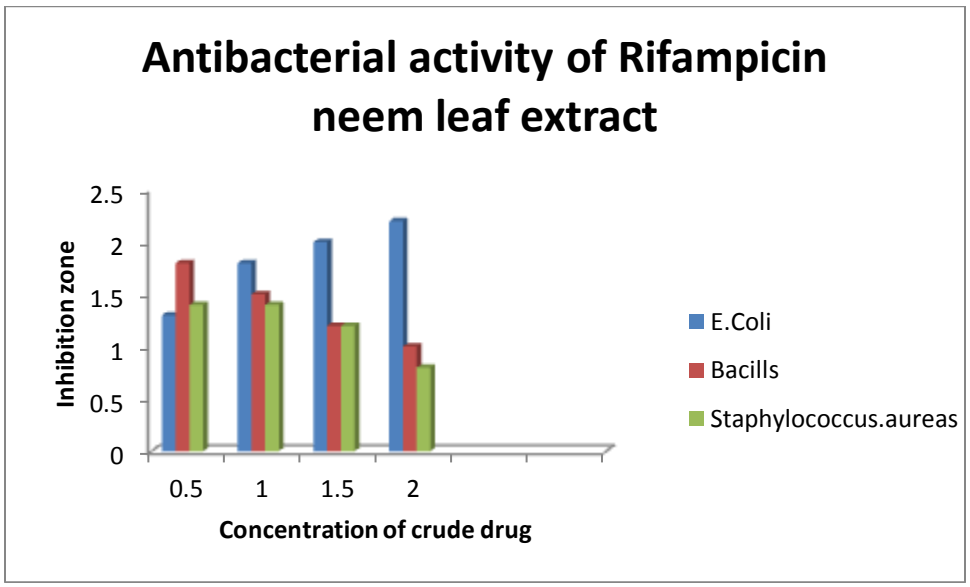
Concentration	Bacterial Strains (Zone of inhibition in mm)		
	E.coli	***S.aureus***	***B.subtilis***
0.5%	2.0	2.1	2.0
1.0%	2.2	2.5	2.4
1.5%	2.3	2.2	2.2
2.0%	2.0	2.5	2.3





Antibacterial activity of Rifampicin Neem leaf extract

Concentration	Bacterial Strains (Zone of inhibition in mm)		
	E.coli	***S.aureus***	***B.subtilis***
0.5%	1.3	1.8	1.4
1.0%	1.8	1.5	1.4
1.5%	2.0	1.2	1.2
2.0%	2.2	1.0	0.8



The phytochemical analysis showed the presence of tannins, glycosides, flavonoids, reducing sugars and saponin, were present in some of the Neem plant leaf extracts. Plants are known to have beneficial therapeutic effects documented in Traditional Indian System of Medicine. Though bioactive products of Neem have been used in treatment of various aliments since time immemorial, role of phytochemical in inhibition of growth of microorganisms has gained less prominence. Phytochemical extracts from Neem leaf are potential sources of antiviral, antitumor and antimicrobial agents. Several workers have evaluated

antibacterial, antisecretory, antihemorrhagic, insecticidal activity of *A. indica* based drugs to meet the health care needs.

Discussion

The presence of these photochemical components may be responsible for the observed antimicrobial activity of the plant leaf extract. This finding conforms to the antiprotozoal and antibacterial activities. Flavonoid has also been reported to have greater potential benefit to human Health. Many of the existing synthetic drugs cause various side effects. Hence, drug development plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects. *A.indica* leaves possessed good antibacterial activity confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary healthcare. The extract of *A.indica* when used as medicinal plant, could be useful or the growth inhibition of the carcinogenic bacterium, Bacillus subtilis. The alkaloids, glycosides, flavanoids and saponins are antibiotic principles of plants. These antibiotic principles are actually the defensive mechanisms of the plants against pathogens.

Conclusion

This study strongly suggest that the ethanol and methanol extract from Neem leaves having more potential antibacterial activity against *Staphylococcus aureus*, *E.coli*, *Bacillus subtilis* than rifampicin.

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