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Antimicrobial Activity of Neem (*Azadirachta Indica)* Leaf Extracts

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***Abstract* – Neem ethanol and water extracts were prepared by using soxhlet apparatus and Hydro distillation respectively. Then these extracts were screened for the presence of different phytochemicals. . Tannins, Flavonoids, Terpenoids, Saponins and Steroids have shown positive result in neem ethanol extracts. Extracts were then tested for the antimicrobial activity against M.roseus, E.coli, B.licheniformis, M.luteus, and S.aureus by MIC test. After the MIC test Ethanol extract of *Azadirachtaindica* found highly effective against Micrococcus roseus and Escherichia coli compare to other organisms. The zone of inhibition found to be 9.5mm for *E.coli*and 8.5mm for *M.roseus*.**

***Keywords* – Neem Extracts, Ethanol, Phytochemical Screening, M.Roseus, E.Coli, B.Licheniformis, M.Luteus, S.Aureus, MIC Test.**

I. INTRODUCTION

Medicinal plants have gained lot of importance in the present world. Throughout the history of mankind plants have been used for several ayurvedic remedies. Various medicinal plants have been used to treat diseases since ancient days all over the world. These medicinal plants are known to treat the disease completely with minimal side effects [1]. These medicinal plants are used as antimicrobial, antifungal, antibacterial and antiviral agents all over the world.

Neem is a tree in the mahogony family Meliacea. The scientific name of neem is *AzadirachtaIndica*.It grows mostly in tropical and sub-tropical regions .It is a fast growing tree that can reach a height of 15-20 metres. It is evergreen, but in severe drought it may shed most or nearly all its leaves. The branches are wide and spreading [2].

Neem is used for several biological and medicinal activities. Different parts of neem (leaf, bark and seed) have shown to fight against several diseases. Azadirachtaindica is one among the medicinal plants and is used in ayurveda, unani and homeopathic medicine. Neem twigs are used as toothbrushes in some tropics, but this can cause illness, neem twigs are often contaminated with fungi within two weeks of their harvest and should be avoided [3].

Neem's antiseptic properties are widely recognized now. "Neem preparations are reportedly efficacious against a variety of skin diseases, septic sores, and infected burns. The leaves, applied in the form of poultices or decoctions, are also recommended for boils, ulcers, and eczema. The oil is used for skin diseases such as scrofula, indolent ulcers and ringworm. Renowned for its antiseptic and disinfection properties, the tree is thought to be particularly protective of women and children. Delivery chambers are fumigated with its burning bark (Margosa seed oil has been chemically tested as an external contraceptive, used by women as a spermicide). Dried margosa leaves are burned as mosquito repellent. Fresh leaves, notorious for their bitterness, are cooked and eaten to gain immunity from malaria.

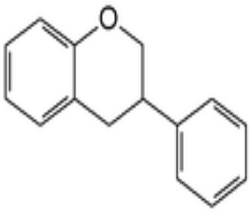
Each and every part of the plant is used for various purposes. The extract from bark, leaves, fruits, root and seeds is being used to treat many diseases in humans and also to control pests. Neem extract is used to treat many dentary and skin related problems. It is also used to balance blood sugar level and it has been used as a house hold pesticide. *A.indica* extracts are found to be antimicrobial, antifungal, antiviral, antibacterial, and antidiabetic. It has been regarded as a natural drug in curing many diseases.

Neem leaves are used for leprosy, eye disorders, bloody nose, intestinal worms, stomach upset, loss of appetite, gum diseases, antibacterial agent [4], stomach and intestinal ulcers, skin diseases, pain and fever, antifungal agent [5]. The leaves are often mixed with rice and consumed as a cure to all prophylactic against bacterial and helminthic infections. Neem leaf pastes are used to repair scarred skins arising from the effects of chicken pox. Not surprisingly, many believe that the Neem tree itself can ward off demons.

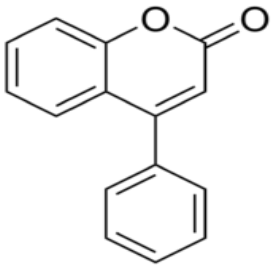
Secondary metabolites are organic compounds that are not directly involved in the normal growth, development and reproduction of organisms. Absence of secondary metabolites doesn't result in immediate death, but long-term impairment of organisms. They play an important role in plant defence. These secondary metabolites are used for medicines, flavorings and recreational drugs.

The chemical constituents and phytoconstituents of neem are biologically active. Compounds may include secondary metabolites like flavonoids, steroids, tannins, terpenoids, saponins in varying concentrations. Azadirachtin, terpenoid is a low toxic compound and found in neem seeds.

Flavonoids or bioflavonoid: Flavonoids were referred to as Vitamin P. It is classified into iso flavonoid and neo flavonoid.



Iso-flavon structure



Neo Flavonoids structure

These are ketone-containing compounds and as such are anthoxanthins (flavones and flavonols). It is a most important plant pigment [6]. Acts as chemical messengers, physiological regulators and cell cycle inhibitors. Some clinical studies have suggested that Flavonoids have an role in cancer prevention. It is said to have a direct antimicrobial activity.

Terpenoids also called as isoprenoids are naturally occurring organic chemicals similar to terpenes. Plant terpenoids are used for their aromatic qualities. They also have a role in herbal remedies. Azadirachtin is the terpenoid present in neem plant [7].

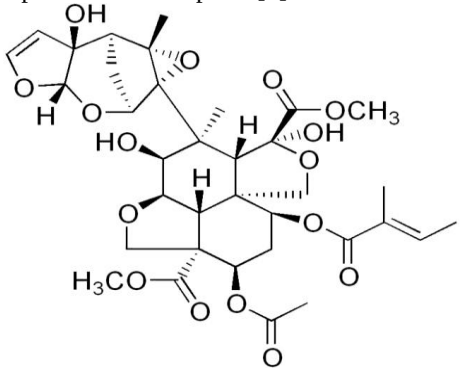


Fig. Azadirachtin

Saponins are amphipathic glycosides. When shaken in aqueous solutions it forms foams. In plants saponins serve as anti – feedants and also protect the plant against microbes and fungi. It may also enhance nutrient absorbtion and aid in animal digestion. Saponins are found to be toxic in cold blooded organisms and in insects at particular concentrations [8]. Saponins are often bitter to taste and are easily dissolved in water.

Tannin is an astringent, bitter plant polyphenolic compounds which binds to and precipitate proteins and compounds like amino acids and alkaloids [9]. It has a role in plant growth regulation, protection from predation and as pesticides. Tannins are found in leaf, seed, root and stem tissues. Depending on the chemical structure and dosage tannins are considered as anti-nutritional [10]. The best human dietary source is tea, wine and also fruit juices but not citrus.

Microorganisms are microscopic organism, which may be a single cell or multicellular organism. The study of microorganism is called microbiology. Microorganism are of several types of which some are useful for mankind like purification of water, digestion, production of enzymes, etc, while some are pathogenic/harmful leading to harmful diseases.

An antimicrobial is an agent, which kills or inhibits the growth of microorganisms. Neem shows antimicrobial activity against some microorganisms like the organisms causing dental caries and plague. In this study we are using Minimum inhibitory concentration (MIC) test to show the antimicrobial activity of neem extracts.

MIC (minimum inhibitory concentration) TEST:

In microbiology, MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC testis important in diagnostic lab to confirm resistance of microorganism to an antimicrobial agent and also to monitor the activity of new antimicrobial agents [11].

Summary of the Minimum Inhibitory Concentration Test (MIC Test):

A pure culture of a single microorganism is grown in Mueller-Hinton broth, or other broth as appropriate.

The culture is standardized using standard microbiological techniques to have a concentration of very near 1 million cells per milliliter. The more the standard of the microbial culture, the more will be the reproducible test results.

The antimicrobial agent is diluted a number of times, usually 1:1, through a sterile diluent (typically Mueller-Hinton broth).

After the antimicrobial agent has been diluted, a volume of the standardized inoculum equal to the volume of the diluted antimicrobial agent is added to each dilution vessel, bringing the microbial concentration to approximately 500,000 cells per milliliter.

The inoculated, serially diluted antimicrobial agent is incubated at an appropriate temperature for the test organism for a pre-set period, usually 18 hours. The more consistent the incubation period, the more reproducible the test results.

After incubation, the series of dilution vessels is observed for microbial growth, usually indicated by turbidity and/or a pellet of microorganisms in the bottom of the vessel. The last tube in the dilution series that does not demonstrate growth corresponds with the minimum inhibitory concentration (MIC) of the antimicrobial agent.

Strengths of the Minimum Inhibitory Concentration Test (MIC Test):

The MIC test is relatively straightforward and easy to prepare for and execute, which naturally enhances reproducibility.

The MIC test can be done on a very small scale without using too much antimicrobial agent. This is important for experimental antimicrobials such as biologically synthesized antimicrobial peptides [12].

The MIC test is an easy way to test the antimicrobial attributes of a formulation across many different parameters, such as across microbial species or surfactant blends.

Because little preparation is required for the minimum inhibitory concentration testing, test turnaround times can be kept low.

Weaknesses of the Minimum Inhibitory Concentration Test (MIC Test):

Minor variations in the way MIC test parameters can have major impacts on the apparent MIC. For example, extended incubation will make the MIC appear to be higher, and lower inoculum concentrations will make the MIC appear to be lower.

Results from MIC studies must be evaluated in the appropriate context. In the tube corresponding to the MIC, microorganisms were merely prevented from growing and not necessarily killed - there could still be 500,000 viable cells in that dilution vessel just waiting to grow should the antimicrobial agent become chemically neutralized [13, 14]

MIC tests are an important and unique part of Antimicrobial Test Laboratories' portfolio of **testing services**. MIC tests are especially appropriate if liquid antimicrobial agents will be used to inhibit the growth of microorganisms in some context [15].

Antimicrobial activity has been investigated for neem leaves, bark and seeds but using different solvents and different bacteria. The present study involves the antimicrobial activity of neem leaf extracts against *Escherichia coli, staphylococcus aureus, Micrococcus roseus, Micrococcus luteus, Bacilluslicheniformis*.

II. MATERIALS AND METHODS

Selection of plant:

The neemplant was selected for the study from 23-14/1, Shanti niketan, Lingampally, Hyderabad, Andhrapradesh. Its healthy leaves were collected for the research.

Leaf Sample Preparation:

The collected leaves were completely shade dried and were finely powdered with mortar and pestle. Then the powder was filtered which was approximately 12 grams. Fresh leaves were also collected and cleaned for neem water extract preparation.

Ethanol extract

12g of dried neem leaf powder was taken in a soxhlet apparatus along with 100ml ethanol. The two were allowed to soxhlet at a temperature of around 50°-70°C. Within 1-2 hours ethanol extract was prepared and taken in a container.

Water extract

Fresh leaves were taken and distilled water was added and allowed for hydro- distillation.

Screening for the secondary metabolites:

Qualitative analysis for secondary metabolites in neem extracts were carried out in the following ways.

Test for Flavonoids:

Shinoda test:

Reagents: Neem-ethanol extract, Neem-water extract, Ethanol, Magnesium turnings, Conc.HCl

2ml of neem ethanol extract and neem water extract were taken in two different test tubes.95% of ethanol and few magnesium turnings were added to both the tubes and few drops of conc.HCl was added and boiled. Pink or red color shows the presence of flavanoids.

NaOH test:

Reagents: Neem powder, Neem-water extract, 10% aqueous NaOH, dil.HCl

2ml of neem ethanol extract and neem water extract were taken in two different test tubes and 10% aqueous NaOH is added to the solution. This produces yellow coloration and then dil.HCl is added, the yellow color slowly disappears which indicates the presence of flavanoids. The same procedure was done with 5mg of neem powder. The powder was dissolved in water, warmed and filtered. Then 10% aqueous NaOH was added to the filtrate, and then later dil.HCl.

Lead acetate test:

Reagents: Neem powder, Distilled water, Lead acetate

5g of neem powder was dissolved in water, warmed and filtered. To the filtrate lead acetate solution was added. Yellow precipitate indicates the presence of flavonoids.

Test for Steroids:

Reagents: Neem-ethanol extract, Neem-water extract, Chloroform, Conc. H_2SO_4

1ml of each Neem-ethanol and Neem-water extracts were taken in two test tubes.10ml chloroform and equal volume of conc. H2SO4 was added to the side of the test tubes. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicates the presence of steroids

Test for Alkaloids:

Reagents: Neem-ethanol extract, Picric acid solution

2ml of Neem-ethanol extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids.

Test for Tannins:

Reagents: Neem-ethanol extract, Neem-water extract, 1% lead acetate

2ml of neem ethanol and neem water extract was added to few drops of 1% lead acetate. A yellowish precipitate indicated the presence of tannins.

Test for Saponins:

Reagents: Neem powder, Distilled water

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 stoppered and shaken vigorously for about 30 seconds. Honeycomb froth indicated the presence of saponins.

Test for reducing sugars:

Reagents: Neem-ethanol extract, Neem-water extract, Fehlings solution

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.

Test for Terpenoids:

Reagents: Acetic anhydride, Chloroform, Conc. H_2SO_4

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 color was observed for terpenoid.

Microorganisms:

The bacterial cultures used in the present study for the antimicrobial activity include *Escherichia coli, Staphylococcus aureus, Bacillus licheniformis, Micrococcus luteus and Micrococcus roseus*.

Preparation of culture media:

The media used for the present study is nutrient agar media.

Nutrient Agar media:

Composition: Peptone -5g/lit, Beef extract-3g/lit, NaCl-5g/lit, Agar-18g/lit, Distilled water-100ml

100ml of nutrient agar media was prepared and autoclaved at 121 degree centigrade for 15 minutes at 15lbs and poured in five sterile Petri plates, 20 ml each. The media was allowed for solidification under laminar airflow hood.

Suspension culture preparation:

1ml sterile water was put to five eppendorfs each. A loop full of bacterial cultures (used for the MIC test) was inoculated to each eppendorf.

Determination of antimicrobial activity:

The aqueous extracts of leaf of *A.Indica* were screened for antimicrobial activity by agar well diffusion method.100µl of each bacterial suspension (*Escherichia coli, Staphylococcus aureus, Bacillus licheniformis, Micrococcus luteus, Micrococcus roseus*) was inoculated into 5 agar plates respectively through spread plate technique under laminar air flow hood. Two wells of 1cm diameter was made on the surface of agar plates. Then 50µl of Neem - ethanol extract was added to one well and neem water extract was added to another well. After 1 hour all the agar plates were incubated at 37°C for 24 hours in the incubator and the results were observed. Antimicrobial activity was determined by measuring the zone of inhibition surrounding the well. The assays were carried out under aseptic conditions.

III. RESULTS

Neem extract Preparation:

Neem ethanol extract and neem water extract was prepared by soxhlet and hydro distillation method respectively.

Neem Powder

Neem water: Hydrodistillation

Neem Ethanol Extract: Soxhlet

Phytochemical screening:

NaOH test with neem powder showed a colour change from yellow to colourless which indicates the presence of Flavonoids, where as the characteristic result was not shown by Neem-ethanol and Neem-water extract.

Lead acetate test with Neem powder showed a yellow precipitate indicating the presence of Flavonoids, where as Neem-ethanol and Neem-water extracts did not show any colour change.

Neem ethanol extract when treated with chloroform and Conc.H_2SO_4 the upper layer turns red and sulphuric acid

layer showed yellow with green fluorescence indicating the presence of steroids.Neem-water extract did not show any colour change.

Neem ethanol extract when treated with 1% lead acetate solution a yellowish precipitate was observed indicating the presence of tannins. Neem-water extract did not show any colour change.

Neem powder dissolved in distilled water when shaken vigorously formed ahoney comb froth which indicates that saponins are present.

Extracts	Flavonoids			Steroids	Alkaloids	Tannins	Saponins	Reducing sugars	Terpenoids
	Shinoda test	NaOH test	Lead acetate test						
Neem-ethanol	-	-	-	+	-	+	-	-	+
Neem-water	-	-	-	-	-	-	-	-	-
Neem powder	-	+	+	-	-	-	+	-	+


Fig. Tannins: +ve


Fig. Tannins: -ve


Fig. Saponins: +ve

MIC (Minimum Inhibitory concentration) test:

Micrococcus roseus: After incubating the Petri plate for 24Hrs at 37°C we observed a zone of inhibition of about 8.5mm around the Neem ethanol well which indicates that the Neem ethanol extract shows an antimicrobial activity against *M.roseus*.

Escherichia coli: After incubation for 24hrs at 37°C, there was a zone of clearance of about 9.5mm around the Neem ethanol well showing its antimicrobial activity.

Bacillus licheniformis: The plates observed after incubation at 37°C for 24hrs, zone of inhibition of about 8mm was observed.

Micrococcus luteus: After incubating the Petri dishes for 24hrs at 37°C, zone of inhibiton of about 7.5mm was observed.

Staphylococcus aureus: After incubating the Petri plate for 24hrs at 37°C, a zone of inhibition of about 7.3mm around the neem ethanol extract which indicated the antimicrobial activity of neem ethanol against *S.aureus*.

Extract	Inhibition zones(mm)				
	M.roseus	E.coli	B.licheniformis	M.luteus	S.aureus
Neem water	-	-	-	-	-
Neem ethanol	8.5mm	9.5mm	8mm	7.5mm	7.3mm

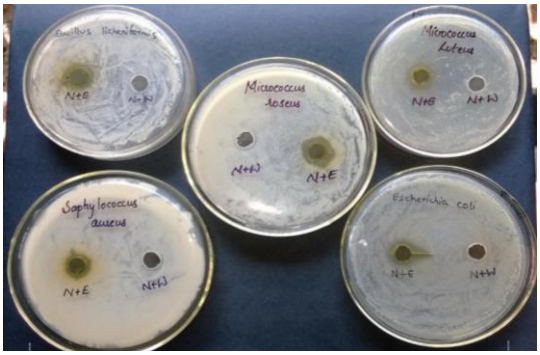


Fig: MIC test result

IV. DISCUSSION

The qualitative analysis of secondary metabolites and antimicrobial activity of neem-extracts were depicted in the respective tables.By performing qualitative analysis we found that tannins, Flavonoids, Terpenoids, saponins and steroids are present in the extracts.

Ethanol extract of *Azadirachtaindica* found highly effective against micrococcus roseus and Escherichia coli compare to other organisms. But we see that neem-water extract didnot show any inhibition activity against these microorganisms.

The presence of phytochemicals in the ethanol extract is mainly found to be responsible for the inhibition activity, because we see that neem-water extract did not show the presence of any phytochemicals during qualitative analysis. *Azadirachtaindica* leaves possessed good antimicrobial activity, confirming the great potential of bioactive compounds.

V. CONCLUSION

Neem extracts were prepared by using water and ethanol separately. These extracts were screened for the presence of secondary metabolites, which showed positive results for tannins, saponins, flavonoids, Terpenoidsand steroids. In MIC test Ethanol extract of *Azadirachta indica* found highly effective against micrococcus roseus and Escherichia coli compare to other organisms.

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