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ANTIMICROBIAL EFFICACY OF *AZADIRACHTA INDICA* LEAF EXTRACTS

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ABSTRACT
The antimicrobial activity of the hexane, ethyl acetate and methanol extracts of the leaves of *Azadirachta indica* were quantitatively assessed on the basis of zone of inhibition and minimum inhibitory concentration They were tested against 5 bacteria: Gram –positive bacteria *Staphylococcus aureus* and Gram – negative bacteria such as *Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and against 3 fungi such as *Aspergillus niger, Mucor spp.* and *Rhizopus spp*. The susceptibility of the microorganisms to the extracts of the plant was compared with selected antibiotics – ciproflaxin (bacteria) and nystatin (fungi). Ethyl acetate extracts showed maximum activity followed by hexane and methanol extract. Hexane and Ethyl acetate extracts of *Azadirachta indica* exhibited high antimicrobial activity with a zone of inhibition of more than 20mm against all the bacterial and fungal strains tested at the concentration of 100µg/ml. The methanol extract exhibited pronounced antifungal activity with 35mm zone of inhibition for *Aspergillus niger*. MIC of all the extracts was found to be less than 80µg/ml for all the tested strains. *E.coli* and *Staphylococcus aureus* were found to be highly susceptible and *Pseudomonas aeruginosa* the least susceptible (MIC: 100µg/ml). The present study emphasizes that the neem extracts can be used to produce drugs, which can effectively function as broad spectrum antimicrobial agent and combat multidrug resistant strains commonly reported in various infections.

KEY WORDS: Multidrug-resistant, *Azadirachta indica , Staphylococcus aureus, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumonia, Aspergillus niger, Mucor spp* and *Rhizopus spp*.

INTRODUCTION

Infectious diseases are the leading cause of death world – wide. Antibiotic resistance has become a global concern (Westh *et al*., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug – resistant pathogens (Bandow *et al*., 2003). Plants have been a source of herbal remedies throughout the history of mankind. Various medicinal plants have been used for years in daily life to treat diseases all over the world (Nisri *et al.,* 1999: Saxena, 1997). According to recent estimates by the World Health Organisation (WHO) more than 3.5 million people in the developing world rely on plants as source of medicine for various ailments. Over 20,000 plants have medical values and many plants are yet to be explored for their potentials. In addition, many of the existing synthetic drugs cause various side effects. Hence, drug development from plant based compounds could be useful in meeting this demand for newer drugs with minimal side effects (Srivastava *et al*., 2000). Ethno pharmacologists, Botanists, Microbiologist and natural – products chemists are combing the Earth for phytochemical and "leads" which could be developed for treatment of infectious diseases. While 25 to 50 % of current pharmaceuticals are derived from plants, they are used as antimicrobials. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial properties (Chattopadhyay *et al.,* 1993). Neem is an evergreen tree, cultivated in various parts of the subcontinent. Every part of the tree has been used as traditional medicines for house hold remedy against various human ailments, from antiquity (Kausik *et al*., 2002). Neem has been extensively used in ayurveda, unani and homeopathic medicine and has become a cynosure of modern medicine. The Sanskrit name of the neem tree is 'Arishtha' meaning 'reliever of sickness' and hence is considered as ' Sarbaroganibarini'. The tree is still regarded as 'village dispensary'in India. The importance of the neem tree has been recognized by the US National academy of sciences, which published a report in 1992 entiled 'Neem – a tree for solving global problem'(Schmutterer, 1995; Singh *et al*., 1996). The isolation of nimbin was reported as first bitter compounds isolated from neem oil, more than 135 compounds have been isolated from different parts of neem (Kausik *et al*., 2002). Extracts of neem leaf, neem oil and seed kernels are effective against certain human fungi including *Trichophyton, Epidermophytons, Microsporum, Trichosporum, Geotricum* and *Candida* (Khan *et al*., 1987). Oil from the leaves, seeds and bark possess a wide spectrum of antibacterial action against Gram-negative and Gram-positive microorganisms,including *Mycobacterium tuberculosis* and streptomycin resistant strains (Chopra *et al*., 1952). *In vitro,* it inhibits *Vibrio cholerae, Klebsiella pneumoniae, Mycobacterium tuberculosis* and *Mycobacterium pyogenes* (Satyavathi *et al*., 1976). The antibacterial activity of neem seed oil was assessed *in vitro* against 14 strains of pathogenic bacteria

including Gram-negative and Gram- positive microorganisms *M.tuberculosis* and streptomycin resistant strains.Though many reports are available on the antimicrobial activity of neem leaf extracts, only few studies have reported the comparative antimicrobial activity of various solvent extract of neem leaf. Therefore, this study was carried out to determine the comparative antimicrobial activity of various solvent extract *Azadirachta indica* (neem) leaf on Gram – positive and Gram – negative bacteria such as *Stapylococcus aureus (S.aureus*) and *Escherichia coli* (*E.coli*), *Pseudomonas aeruginosa* (*P.aeruginosa*), *Proteus vulgaris* (*P.vulgaris*), *Klebsiella pneumoniae* (*K.pneumoniae*), and fungi such as *Aspergillus niger* (*A.niger*), *Mucor and Rhizopus.*

MATERIALS & METHODS

Selection of plant

The plant neem (*Azadirachta indica*) was selected for study.

Preparation of plant extracts

The powdered plant materials obtained from authurticated Ayurvedic dealer were extracted with n-hexane, methanol and ethyl acetate to corresponding fractions (Dabur *et al*., 2004). 100g of dried powder of *Azadirachta indica* leaves were soaked separately in 300ml of methanol, ethyl acetate, hexane in a separating conical flask for 72 hours with intermittent shaking. The plant extract were then collected and filtered through whatman no.1 filter paper separately from the filtrates, the obtained liquid extracts were subjected to rotary evaporator and subsequently concentrated under reduced pressure (in vaccum at 40ºc). The dried powder of the plant extract was stored in air tight bottle.

Collection of test microorganism

The test microorganisms of *Escherichia coli* (NCIM 2065) , *Pseudomonas aeruginosa* (NCIM 2036) *Staphylococcus aureus* (NCIM 2079) *Proteus vulgaris* (NCIM 2027) *Klebsiella pneumonia* (NCIM 2098) *Aspergillus niger* (NCIM 105) *Mucor*(NCIM 108) *Rhizopus* were obtained from National Chemical Laboratory (NCL) Pune and maintained by periodical sub culturing on Nutrient agar and Sabouraud dextrose agar medium for bacteria and fungi respectively.

Antimicrobial screening

Medium

3.8g of Muller Hinton Agar is added to 100ml distilled water and sterilized at 121ºc for 15minutes and poured in sterile petriplates upto a uniform thickness of approximately 4mm and the agar is allowed to set at ambient temperature and used.

Constituents of Muller Hinton agar :

Beef extract	-	2.0g
Acid hydrolysate of casein	-	17.5g
Starch	-	1.5g
Agar	-	17.0g

Inoculums

The microorganisms were inoculated in peptone medium and incubated at 37ºc for 3 – 4 hours and this was used as inoculums.

METHOD

A sterile cotton swab was inserted into the bacterial suspension and then rotated and compressed against the wall of the test tube so to express the excess fluid. The surface of Muller Hinton Agar plate was inoculated with the swab. To ensure that the growth is uniform and confluent (or semi confluent) the swab is passed three times over the entire surface, by repeating the procedure, taking care the second and third time to turn the plate through 60º. Leaf extract and which were prepared using Dimethyl sulfoxide (DMSO) solvent to dissolve the plant extract and then placed on the inoculated agar surface using sterile forceps. Standard disc of antibiotic Ciprofloxacin (5µg/disc) for bacteria and Nystatin(100units /disc) for fungi were used as positive control and the solvent used for preparing extract was used as negative control. The plates were incubated overnight at 37º for 18-24 hours. Antimicrobial activity was evaluated by measuring zone of inhibition by using Hi-media zone scale.

Antimicrobial sensitivity testing using disc diffusion method

This method is suitable for organism that grows rapidly over night at 35 -37ºc. The antibiotic (specific concentration) impregnated disc absorbs moisture from the agar and antibiotic diffuses in to the agar medium. The rate of extraction of the antibiotic from the disc is greater than the rate of diffusion. As the distance from the disc increases, there is a logarithmic reduction in the antibiotic concentration. Zone of inhibition of microbial growth around each disc is measured. Circular disc of 6mm diameter were made from the whatman no.1 filter paper. Discs were impregnated with equal volume (1000µl) of the plant extract were aseptically placed over plates of Muller Hinton Agar seeded with each of test pathogens. The plates were incubated in an upright position at 37ºc for 24hours and the zone of inhibition was measured (in mm diameter). Inhibition zones with diameter less than 12mm were considered as having low antimicrobial activity, diameters between 12 and 16mm were considered moderately active and these with 16mm were considered highly active.

Determination of zone of inhibition

The freshly prepared inoculum was swabbed all over the surface of the muller hinton agar plate using sterile cotton swab. Sterile disc were placed in the medium with the help of sterile forceps and labelled properly and fifty microlitres of the working suspension/solution of different medicinal plant and same volume of extraction solvent control was filled in the wells with the help of micropipette. Plates were left for same time till the extract diffuse in the medium with the lid closed and incubated at 37ºc for 24 hour and measured using scale and mean were recorded after incubation. Plates were observed for zone of inhibition.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION:

Microdilution method

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound to inhibit the growth of microorganisms. The MIC values were determined by broth dilution assay of micro dilution assay. Varying concentrations of the extracts (1000µg/ml, 800µg/ml, 600µg/ml, 400µg/ml, and 200µg/ml) were

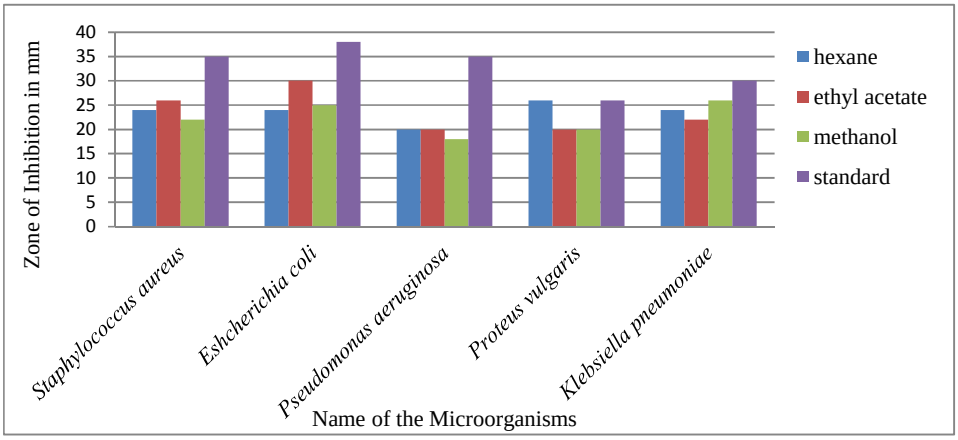
prepared. 0.1ml of standardized test organism of controls was equally set up by using solvents and test organisms without extract. The tube with least concentration of extract without growth after incubation was taken and recorded as the MIC.

RESULTS & DISCUSSIONS

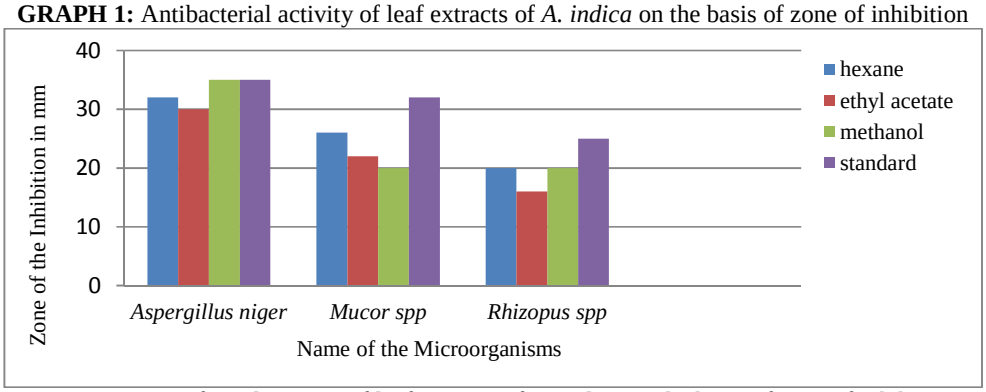
The leaf extracts of *Azadidirachta indica* showed in table 1. The antimicrobial activity of various neem extracts like methanol, hexane and ethyl acetate are evaluated and compared using disc diffusion method. All the test extracts of *Azadirachta indica* possess significant antimicrobial activity against the human pathogens.

TABLE 1: Extractive values of leaf extracts of *Azadirachta indica*

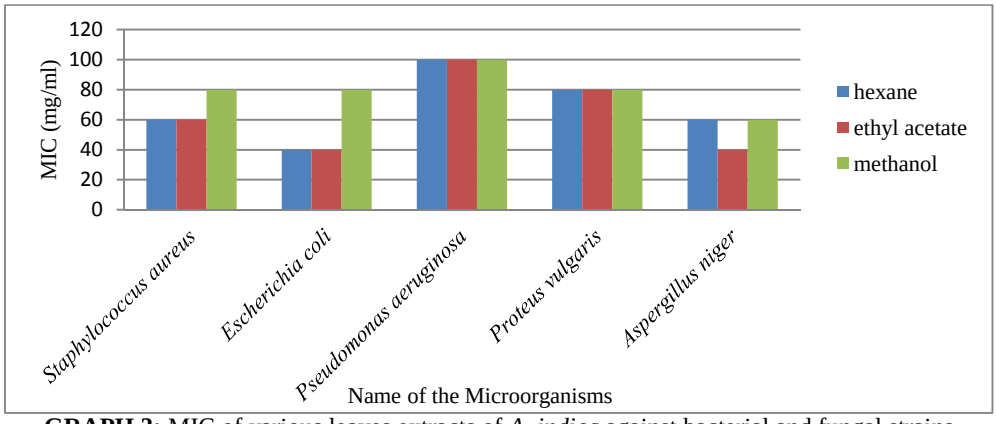
S.no	Type of extracts	Extractive value in gm
1	Methanol	6.5
2	Hexane	3.3
3	Ethyl acetate	3.0



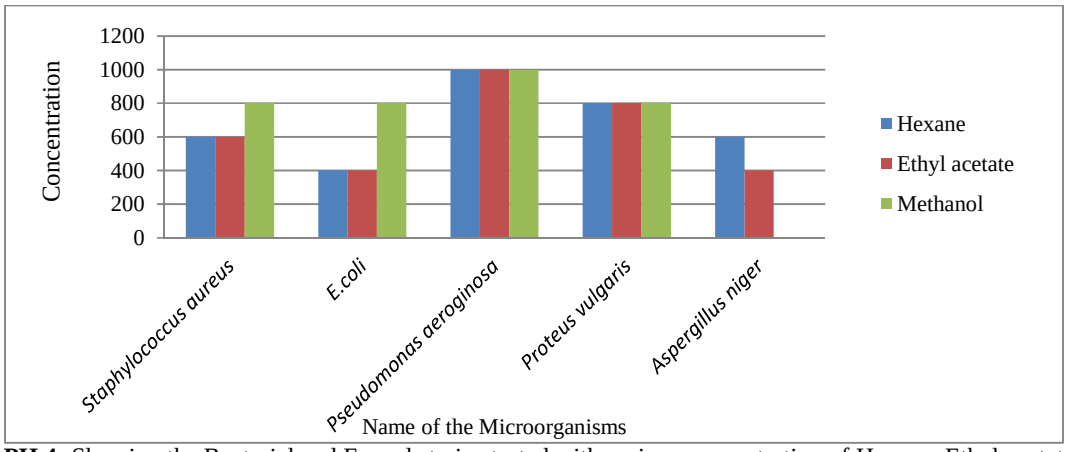
GRAPH 1: Antibacterial activity of leaf extracts of *A. indica* on the basis of zone of inhibition



GRAPH 2: Antifungal activity of leaf extracts of *A. indica* on the basis of zone of inhibition



GRAPH 3: MIC of various leaves extracts of *A. indica* against bacterial and fungal strains

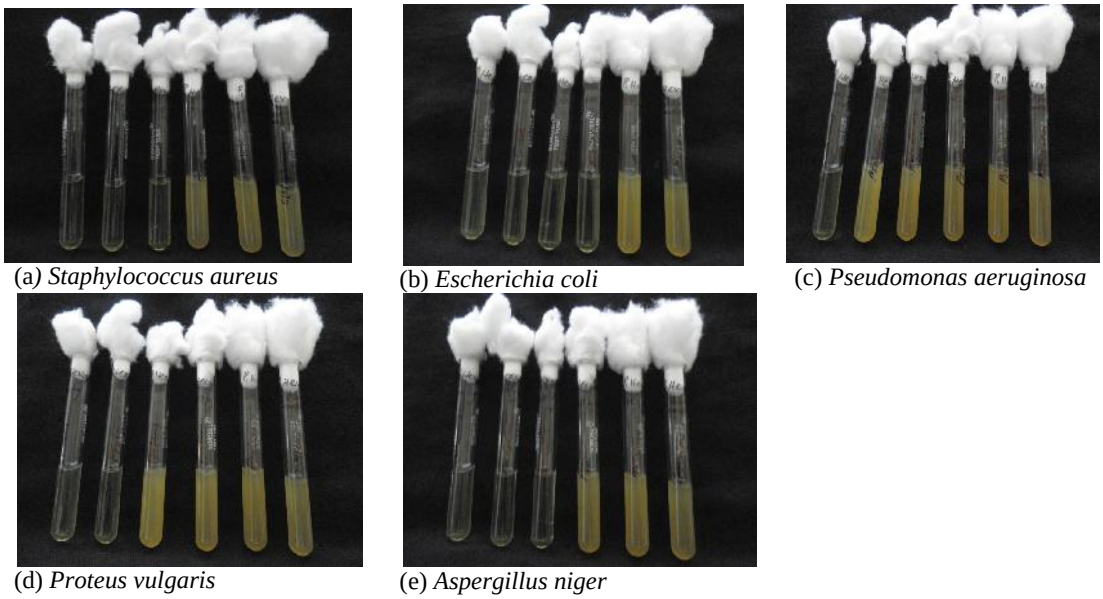


GRAPH 4: Showing the Bacterial and Fungal strains tested with various concentration of Hexane, Ethyl acetate and Methanol Extracts using MIC

Graph 1, 2 and 3 shows the microbial growth inhibition of Hexane, Methanol and Ethyl acetate extracts of the screened microorganisms. Graph 1 shows the antibacterial effect against the bacterial strains tested with hexane, ethyl acetate and methanol extracts of *A.indica* and standard. Hexane extract of *A.indica* showed maximum zone of inhibition (24mm) against *Staphylococcus aureus* followed by *Pseudomonas aeroginosa* (20mm), ethyl acetate extract of *A.indica* showed maximum zone of inhibition (26mm) against *Staphylococcus aureus* followed by *Pseudomonas aeroginosa* (20mm) and methanol extract of *A.indica* showed maximum zone of inhibition (22mm) against *Staphylococcus aureus* followed by *Pseudomonas aeroginosa* (18mm).

Graph 2 shows the antibacterial effect against the bacterial strains tested with hexane, ethyl acetate and methanol extracts of *A.indica* and standard. Hexane extract of *A.indica* showed maximum zone of inhibition (26mm) against *Proteus vulgaris* followed by *E.coli* (24mm) and *Klebsiella pneumoniae* (24mm), ethyl acetate extract of *A.indica* showed maximum zone of inhibition (30mm) against *E.coli* followed by *Klebsiella pneumoniae* (22mm) and *Proteus vulgaris* (20mm) and methanol extract of *A.indica* showed maximum zone of inhibition (26mm) against *Klebsiella pneumoniae* followed by *E. coli* (25mm) and *Proteus vulgaris* (20mm).

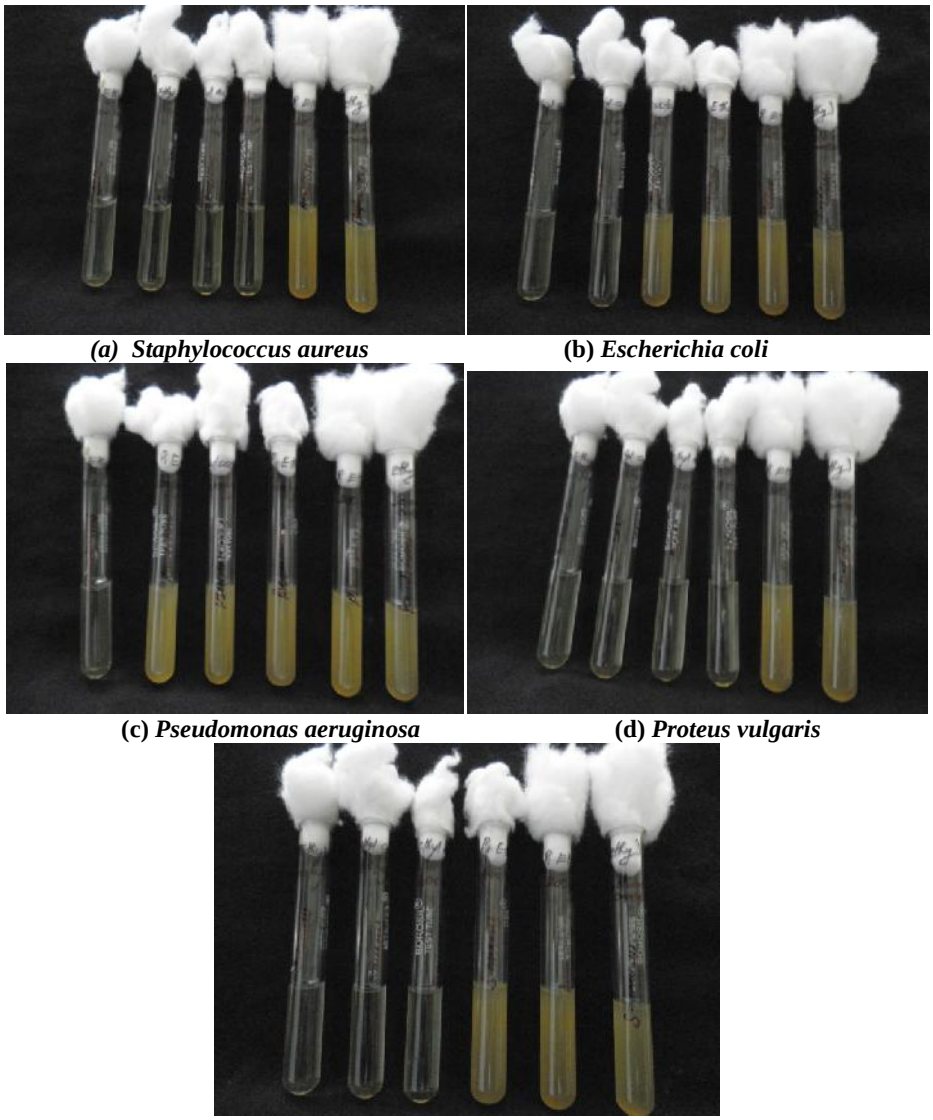
Graph 3 shows the antifungal effect against the fungal strains tested with hexane, ethyl acetate and methanol extracts of *A.indica* and standard. Hexane extract of *A.indica* showed maximum zone of inhibition (32mm) against *Aspergillus niger* followed by *Mucor* (26mm) and *Rhizopus* (20mm), ethyl acetate extract of *A.indica* showed maximum zone of inhibition (30mm) against *Aspergillus niger* followed by *Mucor* (22mm) and *Rhizopus* (16mm) and methanol extract of *A.indica* showed maximum zone of inhibition (35mm) against *Aspergillus niger* followed by *Mucor* (20mm) and *Rhizopus* (20mm).



(a) *Staphylococcus aureus* (b) *Escherichia coli* (c) *Pseudomonas aeruginosa*

(d) *Proteus vulgaris* (e) *Aspergillus niger*

FIGURE 1: MIC of hexane extract of *A.indica* leaves against bacterial and fungal strains



(a) Staphylococcus aureus **(b) *Escherichia coli***

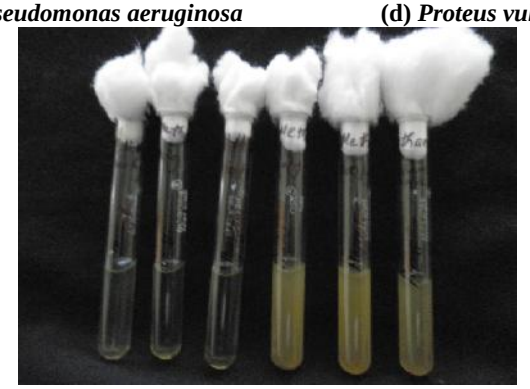
(c) *Pseudomonas aeruginosa* **(d) *Proteus vulgaris***

(e) *Aspergillus niger*

FIGURE 2: MIC of ethyl acetate extract of *A.indica* leaves against bacterial and fungal strains

(a) *Staphylococcus aureus* **(b) *Escherichia coli***

(c) *Pseudomonas aeruginosa* **(d) *Proteus vulgaris***



(e) *Aspergillus niger*

FIGURE 3: MIC of methanol extract of *A.indica* leaves against bacterial and fungal strains

TABLE 2: MIC of Hexane extract of *A.indica* leaves against bacterial and fungal strains

S.NO	Name of the microorgansim	Turbidity					
		Hexane extract of leaves of *A.indica*					
		100µg/ml	80µg/ml	60µg/ml	40µg/ml	20µg/ml	Solvent control
1.	*Staphylococcus aureus* (NCIM 2079)	_	_	MIC	++	++	+
2.	*Escherichia coli* (NCIM 2065)	_	_	_	MIC	+	+
3.	*Pseudomonas aeruginosa* (NCIM 2036)	MIC	+	+	++	+++	+
4.	*Proteus vulgaris* (NCIM 2027)	_	MIC	++	++	+++	+
5.	*Aspergillus niger* (NCIM 105)	_	_	MIC	+	++	+

MIC - Minium Inhibitory Concentration, _ - No Growth, + - Mild Turbiditory, ++ - Moderately Turbid, +++ - More Turbitory

TABLE 3: MIC of Ethyl acetate leaf extract of *A.indica* against bacterial and fungal strains

S.NO	Name of the microorgansim	Turbidity					
		Ethyl acetate extract of leaves of *A.indica*					
		100µg/ml	80µg/ml	60µg/ml	40µg/ml	20µg/ml	Solvent control
1.	*Staphylococcus aureus* (NCIM 2079)	_	_	MIC	+	++	+
2.	*Escherichia coli* (NCIM 2065)	_	_	_	MIC	+	+
3.	*Pseudomonas aeruginosa* (NCIM 2036)	MIC	+	+	++	+++	+
4.	*Proteus vulgaris* (NCIM 2027)	_	MIC	+	+	++	+
5.	*Aspergillus niger* (NCIM 105)	_	_	_	MIC	+	+

MIC - Minium Inhibitory Concentration, _ - No Growth, + - Mild Turbiditory, ++ - Moderately Turbid, +++ - More Turbitory

TABLE 4: MIC of Methanolic leaf extract of *A.indica* against bacterial and fungal strains

S.NO	Name of the microorgansim	Turbidity					
		Methanolic extract of leaves of *A.indica*					
		100µg/ml	80µg/ml	60µg/ml	40µg/ml	20µg/ml	Solvent control
1.	*Staphylococcus aureus* (NCIM 2079)	_	MIC	++	++	+++	+
2.	*Escherichia coli* (NCIM 2065)	_	MIC	+	+	+++	+
3.	*Pseudomonas aeruginosa* (NCIM 2036)	MIC	+	++	+++	+++	+
4.	*Proteus vulgaris* (NCIM 2027)	_	MIC	+	++	++	+
5.	*Aspergillus niger* (NCIM 105)	_	_	MIC	+	+	+

MIC - Minium Inhibitory Concentration, _ - No Growth, + - Mild Turbiditory, ++ - Moderately Turbid, +++ - More Turbitory

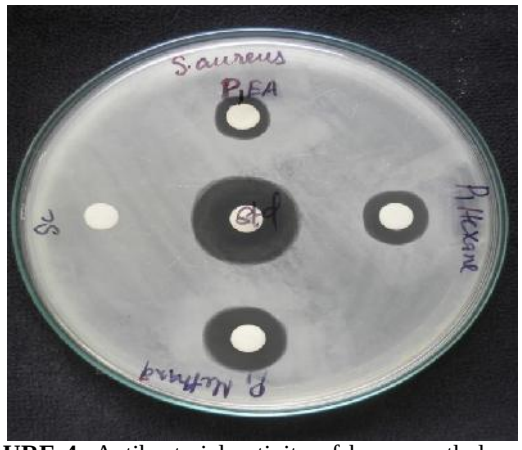


FIGURE 4: Antibacterial activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Staphylococcus aureus*

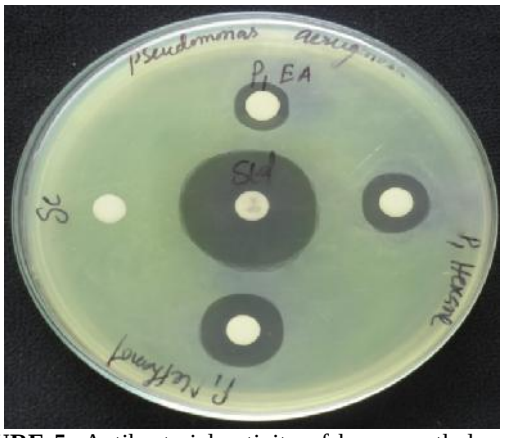


FIGURE 5: Antibacterial activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Pseudomonas aeroginosa*

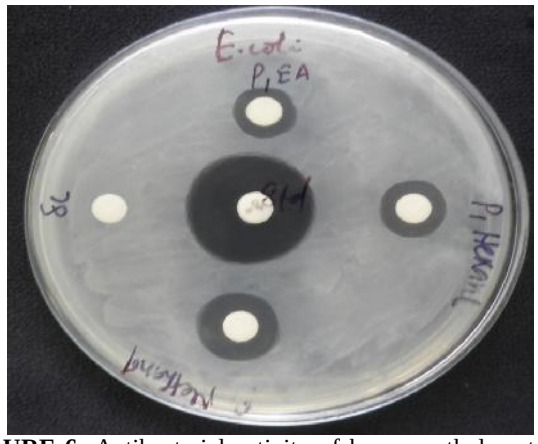


FIGURE 6: Antibacterial activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Escherichia coli.*

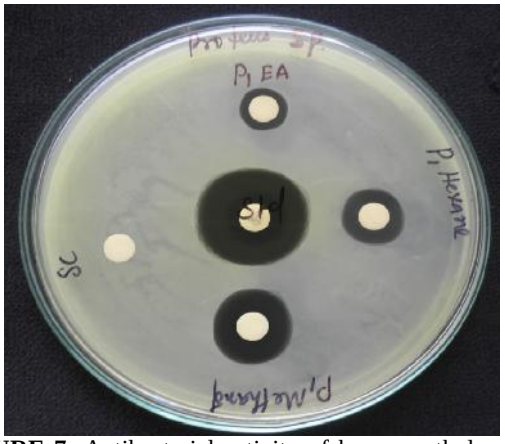


FIGURE 7: Antibacterial activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Proteus vulgaris*

Ciprofloxacin(5µg) was used as standard antibiotic reference for bacteria (positive control) values ranges from 26 – 38mm against all tested bacteria and Nystatin (100 units/disc) for fungi values ranges from 25 – 35mm against all tested fungi.

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 (Srivastava *et al.,* 2000). Neem leaf showed a broad spectrum of antimicrobial activity from the results obtained in this study as its extracts successfully inhibited the growth of the Gram - positive oganisms such as *Staphylococcus aureus* and Gram – negative organisms such as *E.coli, Proteus vulgaris, Klebsiella pneumonia* and *Pseudomonas aeruginosa* and fungi species such as *Aspergillus niger, Mucor and Rhizopus*.

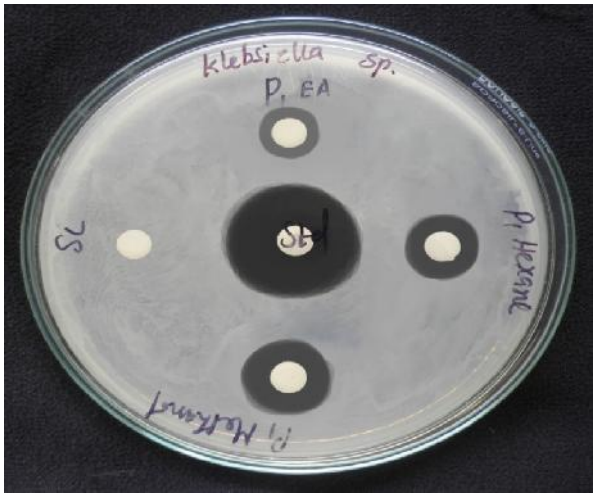


FIGURE 8: Antibacterial activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Klebsiella pneumoniae*

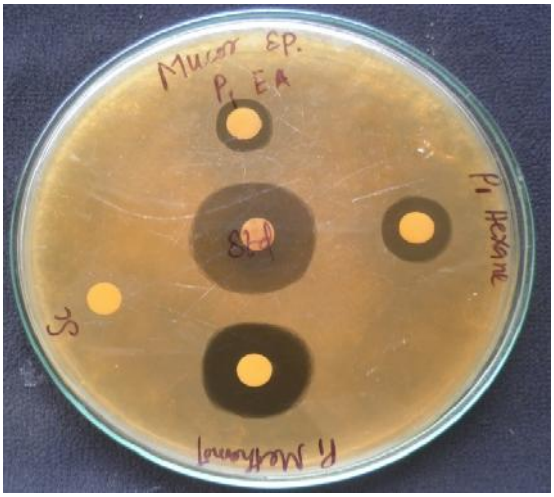


FIGURE 9: Antifungal activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Mucor spp*

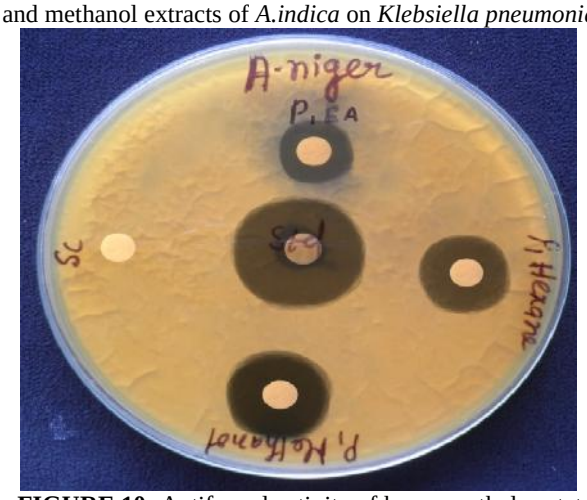


FIGURE 10: Antifungal activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Aspergillus niger*

FIGURE 11: Antifungal activity of hexane, ethyl acetate and methanol extracts of *A.indica* on *Rhizopus spp*

Proteus vulgaris belongs to Gram – negative bacteria and most susceptible bacteria to hexane and methanol extracts of *A.indica*. This selective toxicity could be linked to the difference in the composition of the lipid bilayer for these strains of bacteria. A greater degree of depolarization and hence increased permeability was expressed in the lipid bilayer of the Gram – negative bacteria for this cembranoid, because they contain more lipids in their cell walls (Stainer *et al*., 1986). This depolarization effect is suggested to be associated with hydrogen bonding on the hydroxyl group in the carboxylic functionally situated at the c – 19 positions in the diterpene (Jente *et al.*, 1990). Similarly in our study found that Gram – negative bacteria such as *E.coli, Pseudomonas aeroginosa, Proteus vulgaris* and *Klebsiella pneumonia* are susceptible to hexane and methanol extracts of *A.indica*.Infection caused by *Pseudomonas aeruginosa* are among the most difficult to treat with conventional antibiotics (Levison Jawetz *et al*., 1992). The growth of *Pseudomonas aeruginosa* was partially inhibited by chloroform, methanol and aqueous extracts. So the plant *A.indica* can be used as a source which could improve the treatment of infection caused by this organism. In the present study the methanolic leaf extract of *A.indica* moderately inhibit *Pseudomonas aeruginosa*. Antibacterial activity of the extracts of *A.indica* was effective on *E.coli* and *Klebsiella pneumoniae* (Jagannadh and Radhika, 2006). The present study report also shows high efficacy of hexane, ethyl acetate and methanol extracts on *E.coli* and *Klebsiella pneumoniae*. The hexane, ethyl acetate and methanol extracts of *A.indica* exhibited pronounced activity against Gram – positive bacteria *Staphylococcus aureus* and Gram – negative bacteria such as *E.coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa.* The antibacterial activity *A.indica* might be due to presence of triterpenoids, phenolic compounds, carotenoids, steroids, valavinoids, ketones and tetra – triterpenoids, azadirachtin (Sairam *et al.*, 2000). Himalpaudelchhetri *et al*., (2008) reported that the ethanolic extract of *A.indica* whole plant shoes presence of flavonoids and tannins only. Similarly in our study the methanolic extract of *A.indica* is active against *Klebsiella pneumoniae* followed by *E.coli, Staphylococcus aureus, Proteus vulgaris* and *Pseudomonas aeruginosa*. Srinivasan *et al.*, (2001)

reported that, the methanolic leaf extract of *A.indica* showed the antifungal and antibacterial activity. In our study also showed methanolic leaf extract of *A. indica* have activity against the bacterial strains such as *Staphylococcus aereus*, *E.coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Klebsiella pneumoniae* and fungal strains such as *Aspergillus niger*, *Mucor and Rhizopus sp.*

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