

EVALUATION OF ANTIMICROBIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF MEDICINAL PLANT: *MELIA AZEDARACH* L.

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ABSTRACT

Antimicrobial efficiency of *Melia azedarach* L. a medicinal plants (leaf extracts) were examined using Methanol, Ethanol, Petroleum ether and water, as solvents and tested against eight human pathogens like Bacteria: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, Fungi: *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxisporum*, *Rhizopus stolonifer* using agar well diffusion method and Minimum inhibitory concentration. All the plants showed significant activity against all pathogens, but the alcoholic extract of *M. azedarach* showed maximum zone of inhibition and minimum inhibitory concentration against all the microorganisms. The minimum zone of inhibition and comparatively greater inhibitory concentration were determined in petroleum ether and aqueous extract of *M. azedarach* showing less antimicrobial activity against all the experimental strains. The Spectrum of activity observed in the present study may be indicative of the present study alcoholic extracts of these plants could be a possible source to obtain new and effective herbal medicines to treat infections, hence justified the ethnic uses of *M. azedarach* against various infectious diseases.

Keywords: Antimicrobial activity, *Melia azedarach* L. medicinal plants, Agar well diffusion method, MIC, MBC, MFC

INTRODUCTION

The use of plant and its products has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine¹. Since antiquity, many plants species reported to have pharmacological properties as they are known to possess various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes which is therefore, should be utilized to combat the disease causing pathogens^{2,3,4}.

With the advancement in Science and Technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drugs⁵. Antibiotics are undeniably one of the most important therapeutic discoveries of the 20th century that had effectiveness against serious bacterial infections. However, only one third of the infectious diseases known have been treated from these synthetic products⁶. This is because of the emergence of resistant pathogens that is beyond doubt the consequence of years of widespread indiscriminate use, incessant and misuse of antibiotics^{7,8}. Antibiotic resistance has increased substantially in the recent years and is posing an ever increasing therapeutic problem. One of the methods to reduce the resistance to antibiotics is by using antibiotic resistance inhibitors from plants^{9,10}. Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens¹¹. Medicinal plants have been used as traditional treatments for numerous human diseases for thousands of years and in many parts of the world. Hence, researchers have recently paid attention to safer phytomedicines and biologically active compounds isolated from plant species used

in herbal medicines with acceptable therapeutic index for the development of novel drugs^{12,14}.

Melia azedarach L., is traditionally been used as anthelmintic, antilithic diuretic, astringent and stomachic¹⁴. Various scientific studies reported the anticancer¹⁵, antimalarial activity, analgesic and anti-inflammatory activity¹⁶.

After scrutiny of published literature showing its medicinal importance, the present protocol has been outlined regarding the antimicrobial activity on these selected plant using different extracts. It is in view of this, that the present research was set up to evaluate the antimicrobial activity of *M. azedarach*, using different plant extractions against some pathogenic bacteria and fungi.

MATERIAL AND METHODS

Collection of plant material

Mature plants of *M. azedarach* were used for this study was collected from University Botanical Garden, Botany Department, University of Rajasthan, Jaipur. Different plant Extraction (Methanol, Ethanol, Petroleum Ether and Water) were used for further studies.

Culture and Maintenance of microorganisms

Pure cultures of all experimental bacteria and fungi were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The pure bacterial cultures were maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium. Each bacterial and fungal culture was further maintained by subculturing regularly on the same medium and stored at 4°C before use in experiments.

Table: For the present study following pure bacterial and fungal cultures were taken: Bacterial culture

S. No.	Name	Type	MTCC No.
1.	<i>Bacillus cereus</i>	Gram positive	MTCC4317
2.	<i>Staphylococcus aureus</i>	Gram positive	MTCC3160
3.	<i>Escherichia coli</i>	Gram negative	MTCC1652
4.	<i>Pseudomonas aeruginosa</i>	Gram negative	MTCC4676

Table: Fungal cultures

S. No.	Name	MTCC No.
1.	<i>Aspergillus niger</i>	MTCC282
2.	<i>Aspergillus flavus</i>	MTCC2456
3.	<i>Fusarium oxisporum</i>	MTCC6659
4.	<i>Rhizopus stolonifer</i>	MTCC2591

Preparation of plant extract

In vivo leaves of *M. azedarach* collected from source plant were washed for 2-3 times with tap water and finally with distilled water, followed by ethanol wash and then allowed to dry at 50°C for overnight and finally milled to a coarse powder. 100 gm of powdered material was soxhlet extracted with different solvents like, Ethanol, methanol, petroleum ether and aqueous (12 hour each). All the extracts were evaporated in vacuum under reduced pressure. All extracts were stored in sterile glass bottles at room temperature until screened.

Microbiological screening

Antimicrobial activities of different extracts were evaluated by the agar well diffusion method (Murray *et al.*)¹⁷ modified by (Olurinola,¹⁸) and Minimum inhibitory concentration (MIC)¹⁹.

Media Preparation and Its Sterilization

For agar well diffusion method (Murray *et al.*, 1995 later modified by Olurinola, 1996) antimicrobial susceptibility was tested on solid (Agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungus PDA (39 gm/L) was used for developing surface colony growth. The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) values were determined by serial micro dilution assay. The suspension culture, for bacterial cells was done by preparing 2% Lauria Broth (w/v), and for fungus cells growth, 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old -broth culture of respective bacteria and fungi. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts viz. Methanol, Ethanol, Petroleum Ether, Water. About 100 µl of different concentrations of plant solvent extracts were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 h for bacterial pathogens and 28°C for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Minimum Inhibitory concentration

The minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. This was determined from readings on the culture plates after incubation. The most commonly employed methods are the tube dilution method and agar dilution methods. Serial dilutions are made of the products in bacterial and fungal growth media. The test organisms are then added to the dilutions of the products, incubated, and scored for growth. This procedure is a standard assay for antimicrobials.

Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism.

Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents.

Preparation of Inoculum

Test for antibacterial activity

The antibacterial assay was carried out by microdilution method in order to determine the antibacterial activity of compounds tested against the pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^7 CFU/ml. The inocula were prepared and stored at 4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were performed in duplicate and repeated three times.

Test for antifungal activity

In order to investigate the antifungal activity of the extracts, a modified micro dilution technique was used. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately $1.0 - 10^7$ in a final volume of 100 µl per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid potato dextrose agar to verify the absence of contamination and to check the validity of the inoculum.

Determination of MIC

The minimum inhibitory concentrations (MIC), MBC and MFCs were performed by a serial dilution technique using 96-well microtiter plates. The different plant extracts viz. Methanol, Ethanol, Petroleum Ether, Aqueous were taken (1 mg/ml) and serial dilution of the extract with luria broth for bacterial culture and for fungus, potato dextrose broth medium with respective inoculum were used. The microplates were incubated for 72 hours at 28°C, respectively. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs.

Determination of MBC

The MBCs were determined by serial sub-cultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 72 hours. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by Microplate reader (Perlong, ENM8602) and compared with the standards Ampicillin for Bacteria (Hi-media lab, India) as the positive control. All experiments were performed in duplicate and repeated three times.

Determination of MFC

The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of a 2 µl into microtiter plates containing 100 µl of broth per well and further incubation 72 hours at 28°C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. Commercial standards, Flucanazole (Sigma), was used as positive controls (1-3000 µg/ml) for fungi. All experiments were performed in duplicate and repeated three times.

Observation and Result

In the present investigation, the inhibitory effect of different extracts (viz. Methanol, Ethanol, Petroleum Ether, Aqueous) of *in vivo* leaves from *M. azedarach* were evaluated against both fungicidal and bacterial strains. The antimicrobial activity was determined using agar well diffusion method and micro dilution method summarized in Table 1-2. The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated along with minimum inhibitory concentration (MIC).

Measurement of antimicrobial activity using Agar well diffusion Method

The antimicrobial potential of both the experimental plants was evaluated according to their zone of inhibition against various pathogens and the results (zone of inhibition) were compared with the activity of the standards, viz., Ampicillin (1.0 mg/disc), Flucanazole (1.0 mg/disc). The results revealed that all the extracts are potent antimicrobials against all the microorganisms studied. Among the different solvents extracts studied methanol and ethanol

showed high degree of inhibition followed by petroleum ether and aqueous extract.

For all the tested microorganisms Methanol and Ethanol showed maximum antibacterial activity in *M. azedarach*. In Ethanol extract maximum inhibition zone diameter was obtained in *P. aeruginosa* and in *S. aureus* with diameter 22.3±0.42 mm 19.5±0.52 mm, respectively. Similarly, Methanol extract showed maximum inhibition zone with diameter of 21.5±0.86 mm in *E. coli* and 17.6±0.43 mm *B. cereus*. The Petroleum Ether (12-15 mm) and aqueous extract (8-11 mm) showed restrained and minimum activity, respectively. More specifically, aqueous extract represented higher susceptibility to all bacterial strains (Table 1; Fig.1 (A-D)).

For the antifungal activity, *A. flavus* (21.5±0.32 mm) and *R. stolonifer* (20.1±0.62 mm) showed efficient antifungal activity for ethanol plant extract and for methanolic extract. *A. niger* (20.1±0.62mm) and *F. oxisporum* (22.4±0.86mm) showed proficient antifungal activity. Petroleum Ether and aqueous extract showed lowest inhibition zone with diameter ranging between 15-18 mm and 9-12 mm against all pathogenic fungal strains, respectively (Table 1 Fig.2 (A-D)).

Determination of MIC, MBC and MFC values

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit growth of

organisms. Determination of the MIC is important in diagnostic laboratories because it helps in confirming resistance of micro-organism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The MBC and MFC was determined by subculturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 h. The concentration of plant extract that completely killed the Bacteria and fungi was taken as MBC and MFC, respectively. Moreover, it was noted that most of the antimicrobial properties in different plant part extractions shows, MBC value that is almost two fold higher than there corresponding MICs²⁰.

Methanol extract of *Melia azedarach* L. showed least MIC value 22.4 µg/ml against *B. cereus* while ethanol extract 37.6 µg/ml against *P. aeruginosa*. *S. aureus* and *E. coli* showed comparatively efficient MIC value 38.7 µg/ml and 39.6 µg/ml in methanol and ethanol extract respectively (Table 2).

R. Stolonifer and *A. flavus* was proved to have highest activity 39.5 µg/ml and 47.3 µg/ml in ethanol and methanol extract respectively. Relatively high activity at 48.3 µg/ml and 49.6 µg/ml of *F. oxisporum* and *A. niger* was observed in methanol and ethanol extract, respectively. The least MBC and MFC value 43.8µg/ml and 79 µg/ml was observed in ethanol extracts against *B. cereus* and *R. stolonifer* respectively (Table 2)

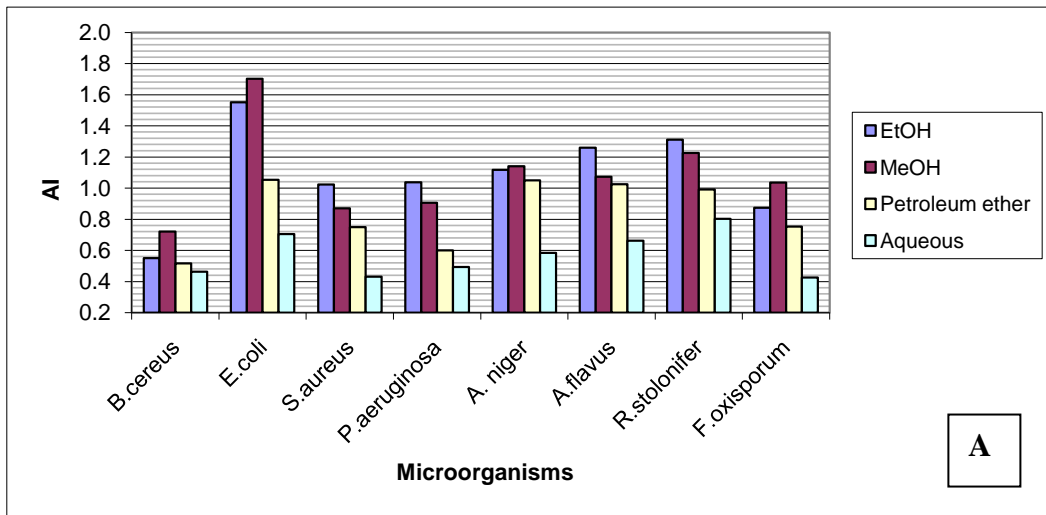
Table 1: Antimicrobial activity (zone of inhibition, mm) of various plant extracts *Melia azedarach* against clinical pathogens.

Microorganism		EtOAC	MeOH	Petroleum ether	Aqueous	Standard
Bacteria						
<i>B.cereus</i>	IZ	13.4±0.56	17.6±0.43	12.6±0.52	11.3±0.31	24.4
	AI	0.549	0.721	0.516	0.463	
<i>E.coli</i>	IZ	19.6±0.65	21.5±0.86	13.3±0.52	8.9±0.41	12.63
	AI	1.552	1.702	1.053	0.705	
<i>S.aureus</i>	IZ	19.5±0.52	16.6±0.13	14.3±0.40	8.2±0.51	19.07
	AI	1.023	0.870	0.750	0.430	
<i>P.aeruginosa</i>	IZ	22.3±0.42	19.5±0.52	12.9±0.31	10.6±0.25	21.52
	AI	1.036	0.906	0.599	0.493	
Fungi						
<i>A. niger</i>	IZ	19.7±0.11	20.1±0.62	18.5±0.54	10.3±0.35	17.63
	AI	1.117	1.140	1.049	0.584	
<i>A.flavus</i>	IZ	21.5±0.32	18.3±0.54	17.5±0.56	11.3±0.41	17.07
	AI	1.260	1.072	1.025	0.662	
<i>R.stolonifer</i>	IZ	20.1±0.62	18.8±0.52	15.2±0.91	12.3±0.51	15.33
	AI	1.311	1.226	0.992	0.802	
<i>F.oxisporum</i>	IZ	18.9±0.11	22.4±0.86	16.3±0.51	9.2±0.35	21.64
	AI	0.873	1.035	0.753	0.425	

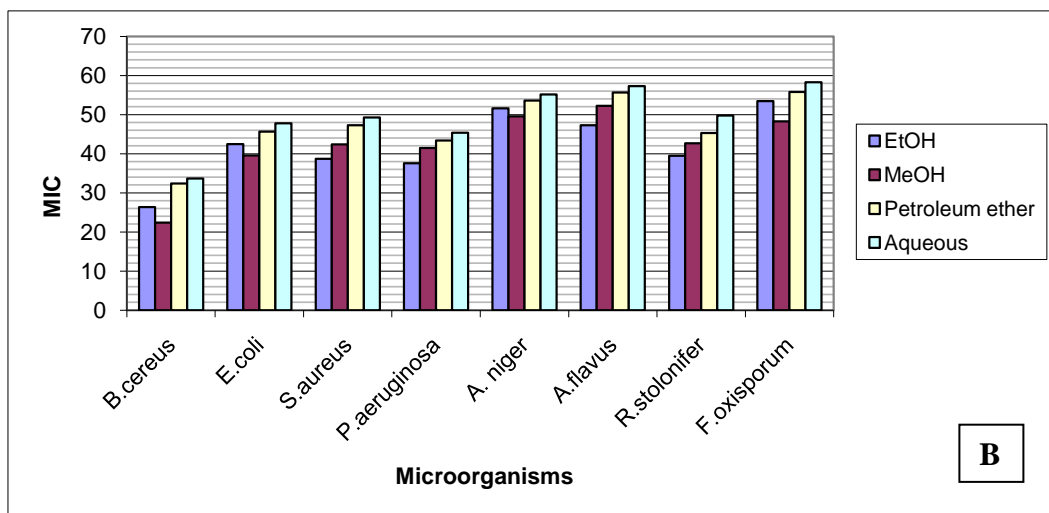
IZ= Inhibition zone (in mm) includes the diameter of disc (6 mm); Standards: Ampicillin (1.0 mg/disc), Flucanazole (1.0 mg/disc); AI- activity index = IZ of test sample / IZ of standard. Values are mean of triplicate readings (mean ± S.D).

Table 2: MIC (µg / ml), MBC and MFC performance of different extracts of *Melia azedarach* L. against pathogenic organisms

Microorganism		EtOAC	MeOH	Petroleum ether	Aqueous
Bacteria					
<i>B.cereus</i>	MIC	26.4	22.4	32.4	33.7
	MBC	43.8	45.8	65.7	67.5
<i>E.coli</i>	MIC	42.5	39.6	45.7	47.8
	MBC	84.3	78.3	93.5	95.6
<i>S.aureus</i>	MIC	38.7	42.4	47.3	49.3
	MBC	76.5	85.9	86.7	97.8
<i>P.aeruginosa</i>	MIC	37.6	41.5	43.4	45.4
	MBC	75.3	93.1	97.9	99.8
Fungi					
<i>A. niger</i>	MIC	51.6	49.6	53.6	55.2
	MFC	103.2	98.4	110.8	113.4
<i>A.flavus</i>	MIC	47.3	52.3	55.7	57.3
	MFC	94.6	104.6	111.4	114.6
<i>R.stolonifer</i>	MIC	39.5	42.7	45.3	49.8
	MFC	79	95.4	90.7	99.6
<i>F.oxisporum</i>	MIC	53.5	48.3	55.8	58.3
	MFC	107.1	96.6	110.6	116.6



Graph A: Activation index against various microorganisms.



Graph B: MIC against various microorganisms.

Graph 1: Graph showing comparative antimicrobial activity of different extract of *M. azedarach* against pathogenic bacteria

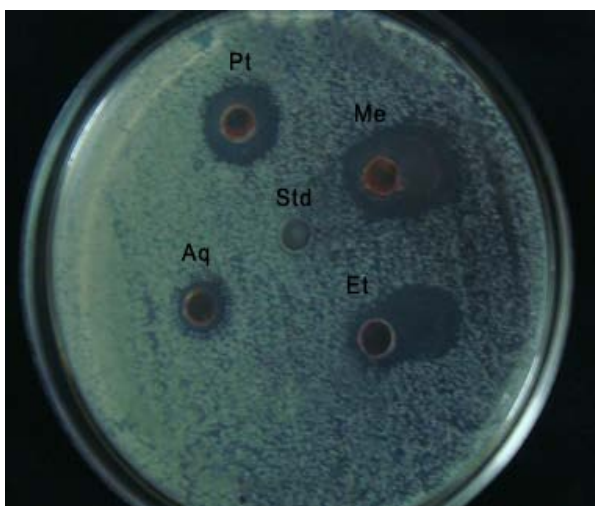


Fig. A: *Bacillus cereus*

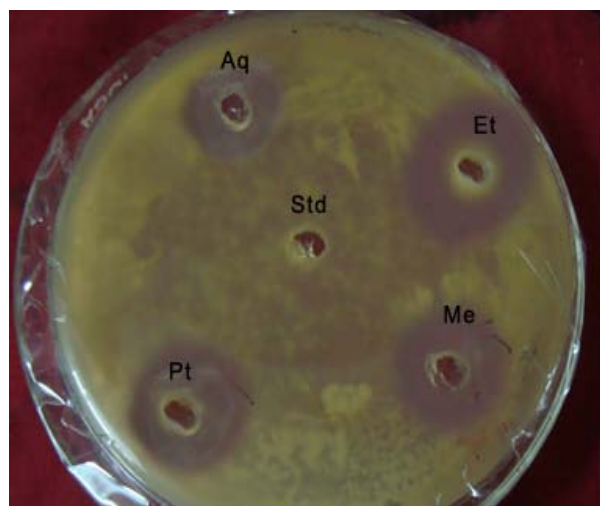


Fig. B: *Staphylococcus aureus*

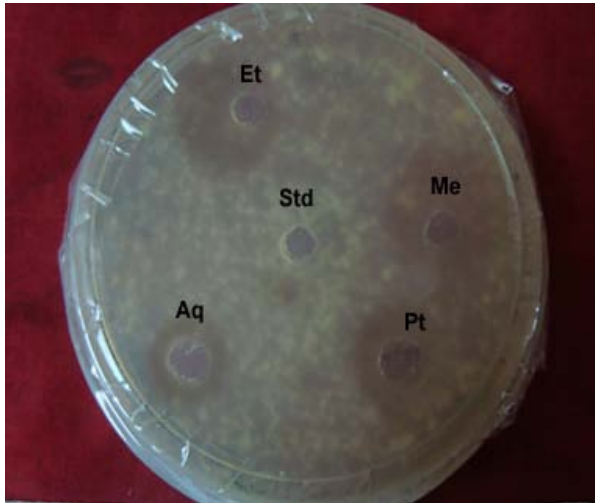


Fig. C: *Escherichia coli*

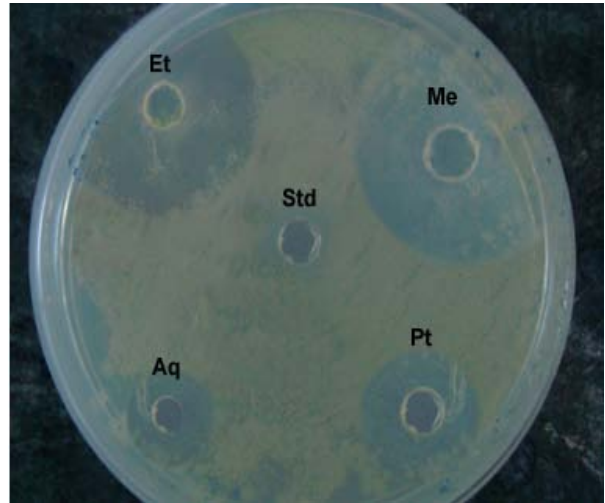


Fig. D: *Pseudomonas aeruginosa*

Fig. 1: Antimicrobial activity of different extracts *Melia azedarach* L. against:

Abbervation: Std.- Standard, Me- Methanol, Et- Ethanol, Pt- Petroleum ether, Aq- Aqueous

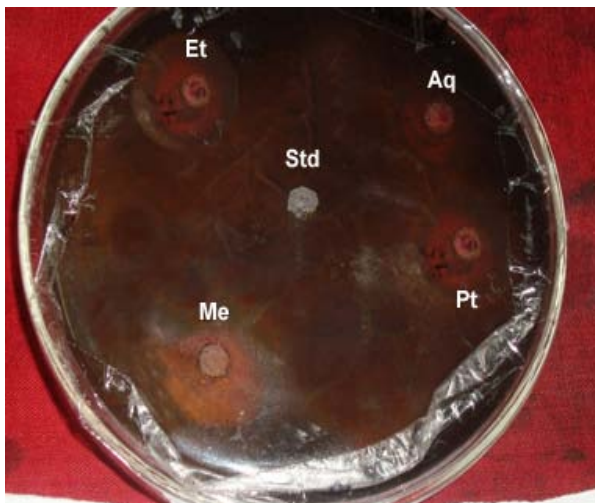


Fig. A: *Aspergillus niger*

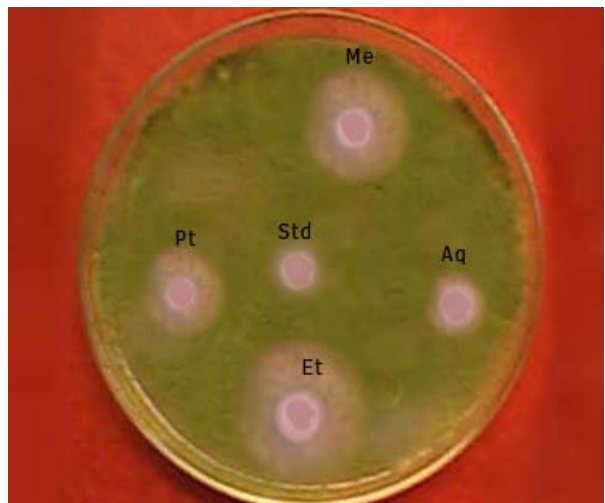


Fig. B: *Aspergillus flavus*

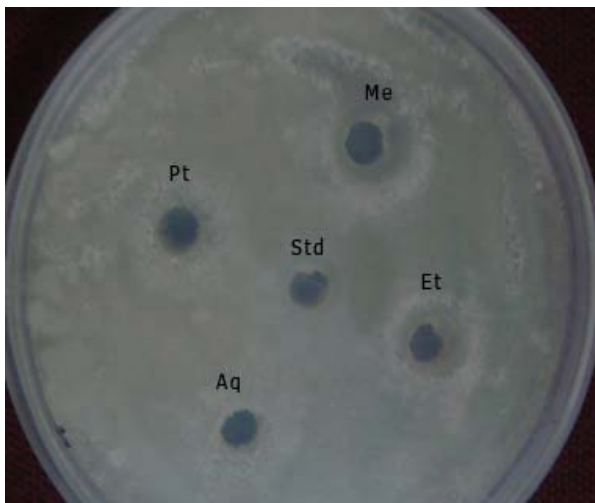


Fig. C: *Fusarium oxysporum*

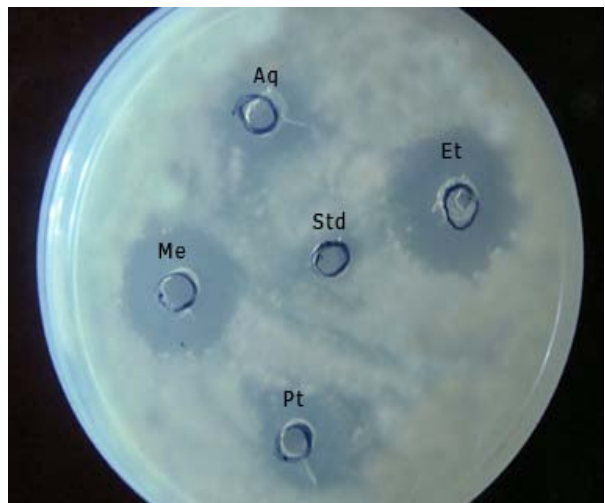


Fig. D: *Rhizopus stolonifer*

Fig. 2: Antimicrobial activity of different extracts *M. azedarach* L. against:

Abbervation: Std.- Standard, Me- Methanol, Et- Ethanol, Pt- Petroleum ether, Aq- Aqueous

DISCUSSION

The search for antimicrobials from natural sources has received much attention and efforts have been put in to identify compounds that can act as suitable antimicrobials agent to replace synthetic ones. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines in controlling the growth of microorganism^{21,22}. These compounds have significant therapeutic application against human pathogens including bacteria, fungi or virus. Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds^{23,24}. Therefore, medicinal plants are finding their way into pharmaceuticals, nutraceuticals and food Supplements.

In the present investigation, different extracts of *M. azedarach* was evaluated for exploration of their antimicrobial activity against certain Gram negative and Gram positive bacteria, fungus which was regarded as human pathogenic microorganism. Susceptibility of each plant extract was tested by serial microdilution method (MIC) and agar well diffusion method was determined.

Our preliminary investigation showed that all Ethanol, Methanol, Petroleum ether and aqueous extracts of *M. azedarach* were active against the locally isolated human pathogens like *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. This analysis of using several extracts so as to study the efficacy of plant for antimicrobial activity has also been realized by many scientist in many plant species like *Adhatoda zeylanica*, *Medic25*, *Trianthema decandra* L.²⁶, *Argemone mexicana* L.²⁷, *Tinospora cordifolia* and *Cassia fistula* ²⁸.

The alcoholic extracts of *M. azerarach* showed significant antimicrobial activity against multi-drug resistant clinically isolated microorganisms (Graph 1(A-B)). Though, the mechanism of the action of these plant constituents is not yet fully known it is clear that the effectiveness of the extracts largely depends on the type of solvent used. The organic extracts provided more powerful antimicrobial activity as compared to aqueous extracts. This observation clearly indicates that the existence of non-polar residues in the extracts which have higher both bactericidal and bacteriostatic abilities. Cowan ²⁹ mentioned that most of the antibiotic compounds already identified in plants are reportedly aromatic or saturated organic molecules which can easily solubilized in organic solvents. Similar results showing that the alcoholic extract having the best antimicrobial activity is also reported by Preethi ³⁰ in *Leucas aspera*, *Holarrhena antidysenterica*. Seyyidnejad ³¹ also studied the effect of different alcoholic viz. ethanol and methanol for antimicrobial activity and observed that this difference in the activity between different alcoholic extract is due to the difference between extract compounds in this two extract.

The study also revealed that Petroleum ether extract shows moderated and aqueous extract shows minimum antimicrobial activity. However, Murugesan³² showed that petroleum ether extract of plant *Memecylon umbellatum* Burm. f. shows significant antimicrobial activity. Furthermore, water extract from leaves of *P. acerifolium* had been reported to have prominent antimicrobial activity against several gram positive and gram negative human pathogenic bacteria³³.

The antimicrobial analysis using the agar well diffusion method and MIC value is been used by many researchers^{34,35,36}. In the present study the MIC value of the active plant extracts obtained in this study were lower than the MBC values (Table 1-2, Graph 1 (A-B)) suggesting that the plant extracts were bacteriostatic at lower concentration but bactericidal at higher concentration³⁷.

In conclusion, of the present investigation *Melia azedarach* contain potential antimicrobial components that may be of great use for the development of pharmaceutical industries as a therapy against various diseases. The ethanol, methanol, petroleum ether and aqueous extracts of *Melia azedarach* possess significant inhibitory effect against tested pathogens. The results of the study support the folklore claim along with the development of new antimicrobial drugs from both the plants.

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