ORIGINAL ARTICLE

Determination of antimicrobial potentialities of different solvent extracts of the medicinal plant: *Phyllanthus amarus* Schum. and Thonn.

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The antimicrobial efficiency of *Phyllanthus amarus* Schum. and Thonn., medicinal plants (leaf extracts), was examined using Methanol, Ethanol, Petroleum ether. and water, as solvents and tested against eight human pathogens like Bacteria: *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa,* Fungi: *Aspergillus niger, Aspergillus flavus, Fusarium oxisporum,* and *Rhizopus stolonifer,* using the agar well-diffusion method and minimum inhibitory concentration. All the plants showed significant activity against all pathogens, but the alcoholic extract of *P. amarus* showed the 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 the aqueous extract of *P. amarus* showed less antimicrobial activity against all the experimental strains. The alcoholic extracts of these plants could be a possible source of obtaining new and effective herbal medicines to treat infections, hence, it justified the ethnic use of *P. amarus* against various infectious diseases.

Key words: Agar well well-diffusion method, antimicrobial activity, medicinal plants, minimum bactericidal concentration, minimum fungicidal concentration, minimum inhibitory concentration, *Phyllanthus amarus*

INTRODUCTION

To survive on earth, there has to be a suitable relationship between people and the environment. As the human population grows and people strive for improved living standards, this relationship is prone to many stresses and strains.[1] Since the beginning of civilisation, survival of the human race was dependent on plants, not only as a source of food and oxygen, but also as a source of natural remedies.[2,3]

Plants with their complex chemical storehouse of biodynamic compounds, serve as plant defence mechanisms against invasion by microorganisms and insects, and can provide valuable sources of natural antibacterial agents.[4,5] The active principles isolated from plants appear to be one of the important alternatives, when compared to many sub-standard orthodox synthetic medicines, because of their less or no side effects and better bioavailability.[6]

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Antimicrobials of plant origin have an extremely large therapeutical potential. They are effective in the treatment of infectious diseases, while simultaneously alleviating many of the side effects that are often connected with synthetic antimicrobials.[7]

Phyllanthus amarus has a long history of usage by people, because of its rich medicinal values, It has been reported to possess potent anti-inflammatory,[8] antihepatotoxic,[9] antispasmodic, antiviral, antiamnesic,[10] antilithic, analgesic, hypotensive, diuretic, antimutagenic, and hypoglycemic properties.[11,12]

It is in view of this, that the present research was set up, to evaluate the antimicrobial activity of *P. amarus*, using different plant extractions against some pathogenic bacteria and fungi.

MATERIALS AND METHODS

Collection of Plant Material

The mature plants of *P. amarus* used for this study were collected from the University's Botanical Garden, Botany Department, University of Rajasthan, Jaipur. Different plant extractions (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

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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 sub-culturing regularly on the same medium and stored at 4°C, before use in experiments.

For the present study, Tables 1 and 2 pure bacterial and fungal cultures were taken.

Table 1: Bacterial culture

Name	Type	MTCC No.
Bacillus cereus	Gram positive	MTCC4317
Staphylocous aureus	Gram positive	MTCC3160
Escherichia coli	Gram negative	MTCC1652
Pseudomonas aeruginosa	Gram negative	MTCC4676

Table 2: Fungal cultures

Name	MTCC No.
Aspergillus niger	MTCC282
Aspergillus flavus	MTCC2456
Fusarium oxisporum	MTCC6659
Rhizopus stolonifer	MTCC2591

Preparation of Plant Extract

In vivo leaves of *P. amarus*, collected from the source plant, were washed twice or thrice with tap water and finally with distilled water, followed by an ethanol wash, and then allowed to dry at 50°C overnight, and finally milled to a coarse powder. One hundred grams of powdered material was soxhlet extracted with different solvents like, ethanol, methanol, petroleum ether, and aqueous (12 hours each). All the extracts were evaporated in vacuum under reduced pressure and 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[13,14] and minimum inhibitory concentration (MIC).[15]

Media Preparation and its Sterilization

For the agar well-diffusion method[13,14] antimicrobial susceptibility was tested on solid (Agar–agar) media in petri plates. For the bacterial assay, nutrient agar (NA) (40 gm/L), and for fungus, PDA (39 gm/L), were used for developing surface colony growth. The MIC, the minimum bactericidal concentration (MBC), and the minimum fungicidal concentration (MFC) values were determined by the serial micro-dilution assay.[15] The suspension culture, for bacterial cell growth was done by preparing 2% Lauria Broth (w/v), and for fungus cell growth, 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared were then sterilized by autoclaving the media at (121°C) for 20 minutes.

Agar Well-diffusion Method

The 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 eight-hour-old broth cultures of the respective bacteria and fungi. Wells (10 mm diameter and about 2 cm apart) were made in each of these plates by using a sterile cork borer. The stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts, namely, Methanol, Ethanol, Petroleum Ether, and Water. About 100 µl of different concentrations of the plant solvent extracts were added with a sterile syringe into the wells and allowed to diffuse at room temperature for two hours. Control experiments comprising inocula without plant extract were set up. The plates were incubated at 37°C for 18–24 hours for bacterial pathogens, and at 28°C for 48 hours for 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 replicate, and the readings were taken in three different fixed directions and the average values were recorded.

Minimum Inhibitory Concentration

Minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. This is determined from the readings on the culture plates after incubation. The most commonly employed methods are the tube dilution method and agar dilution method. Serial dilutions are made up of the products in the 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 the 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, 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 the 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 thrice.

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 the agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with the 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 till 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 Minimum Inhibitory Concentrations

The MIC, MBC, and MFCs were performed by a serial dilution technique using 96-well microtitre plates. The different plant extracts, namely, Methenol, Ethanol, Petroleum Ether, and Aqueous were taken (1 mg/ml) and serial dilution of the extracts with luria broth for bacterial culture and potato dextrose broth medium for fungus, with their respective inocula 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 the MICs.

Determination of Minimum Bactericidal Concentrations

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 a Microplate reader (Perlong, ENM8602) and compared with the standard Ampicillin for Bacteria (Himedia Laboratories, India) as the positive control. All experiments were performed in duplicate and repeated thrice.

Determination of Minimum Fungicidal Concentrations

The fungicidal concentrations (MFCs) were determined by serial sub-cultivation of a 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 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 and Flucanazole (Sigma) were used as the positive controls (1 – 3000 µg/ml) for fungi. All experiments were performed in duplicate and repeated thrice.

OBSERVATION AND RESULTS

In the present investigation, the inhibitory effect of different extracts (viz., Methanol, Ethanol, Petroleum ether, Aqueous) of the *in vivo* leaves from *P. amarus* were evaluated against both fungicidal and bacterial strains. The antimicrobial activity was determined using the agar well-diffusion method and the micro-dilution method summarized in Tables 3 and 4. The activity was quantitatively assessed on the basis of the inhibition zone, and their activity index was also calculated along with the 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, namely, Ampicillin (1.0 mg/disc) and Flucanazole (1.0 mg/disc). The results revealed that all the extracts were potent antimicrobials against all the microorganisms studied. Among the different solvent extracts studied methanol and ethanol showed a high degree of inhibition, followed by petroleum ether and aqueous extract.

In the present investigation, the ethanol and methanol extracts were found to have the highest antibacterial activity against *B. cereus, E. coli, S. aureus*, and *P. aeroginosa* respectively. In the presence of the ethanol extract, the maximum inhibition zone diameter was obtained, that is, *19.2±0.32* mm in *E. coli* and 16.4±0.82 mm in *B. cereus,* whereas, in the methanol extract, the maximum antibacterial activity was obtained in S *.aureus* and *P.aeruginosa,* with the inhibition zone diameter of 18.3±0.56 mm and 17.9±0.49 mm, respectively. Petroleum ether showed moderate antibacterial activity against all the tested microorganisms (12 – 15 mm). The aqueous extract showed minimum activity against all pathogenic bacteria (8 – 10 mm) [Table 3; Figure 1a-d].

The antifungal activity was also studied for different extracts using four different fungal strains, namely, *A. niger, A. flavus, R. stolonifer,* and *F. oxisporum*. In the present study, maximum antifungal activity was observed for the ethanol extract against *A. niger* (16.3±0.75mm) and *F.*

Table 3: Antimicrobial activity (zone of inhibition, mm) of various plant extracts *Phyllanthus amarus* against clinical pathogens

Microorganism		EtOAC	MeOH	Petroleum ether	Aqueous	Standard
Bacteria						
B. cereus	IZ	16.4±0.82	15.2±0.61	13.1±0.54	10.2±0.79	24.40
	AI	0.672	0.623	0.537	0.418	
E. coli	IZ	19.2±0.32	17.6±0.71	14.6±0.55	9.4±0.35	12.63
	AI	1.520	1.394	1.156	0.744	
S. aureus	IZ	16.3±0.91	18.3±0.56	12.5±0.65	8.4±0.52	19.07
	AI	0.855	0.960	0.655	0.440	
P. aeruginosa	IZ	15.8±0.45	17.9±0.49	13.2±0.32	10.7±0.45	21.52
	AI	0.734	0.832	0.613	0.497	
Fungi						
A. niger	IZ	16.3±0.75	15.5±0.52	14.3±0.63	10.7±0.72	17.63
	AI	0.925	0.879	0.811	0.607	
A. flavus	IZ	15.2±0.32	17.3±0.42	13.4±0.51	10.3±0.79	17.07
	AI	0.890	1.013	0.785	0.603	
F. oxisporum	IZ	14.4±0.51	13.2±0.51	12.3±0.75	9.3±0.65	21.64
	AI	0.665	0.610	0.568	0.430	
R. stolonifer	IZ	16.3±0.49	18.5±0.42	13.3±0.41	10.4±0.51	15.33
	AI	1.063	1.207	0.868	0.678	

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 4: MIC (µg/ml), MBC and MFC performance of different extracts of *Phyllanthus amarus* against pathogenic organisms

Microorganism		EtOAC	MeOH	Petroleum ether	Aqueous
Bacteria					
B. cereus	MIC	32.2	39.7	42.8	54.0
	MBC	68.8	75.6	88.7	109.0
E. coli	MIC	56.4	59.3	61.2	63.5
	MBC	114.8	119.7	122.5	124.5
S. aureus	MIC	49.5	42.0	53.7	58.0
	MBC	99.2	85.4	108.5	107.0
P. aeruginosa	MIC	42.5	38.7	45.7	49.5
	MBC	85.1	77.5	91.5	99.1
Fungi					
A. niger	MIC	32.0	38.5	42.5	47.8
	MBC	64.5	77.1	95.2	95.6
A. flavus	MIC	34.7	28.4	39.9	42.5
	MBC	79.5	57.9	79.9	85.1
F. oxisporum	MIC	43.2	47.2	51.5	54.7
	MBC	86.5	95.5	103.1	110.7
R. stolonifer	MIC	47.3	45.4	51.4	53.5
	MBC	85.9	95.7	103.9	108.7

MBC – Minimum bactericidal concentration; MFC – Minimum fungicidal concentration; MIC – Minimum inhibitory concentration

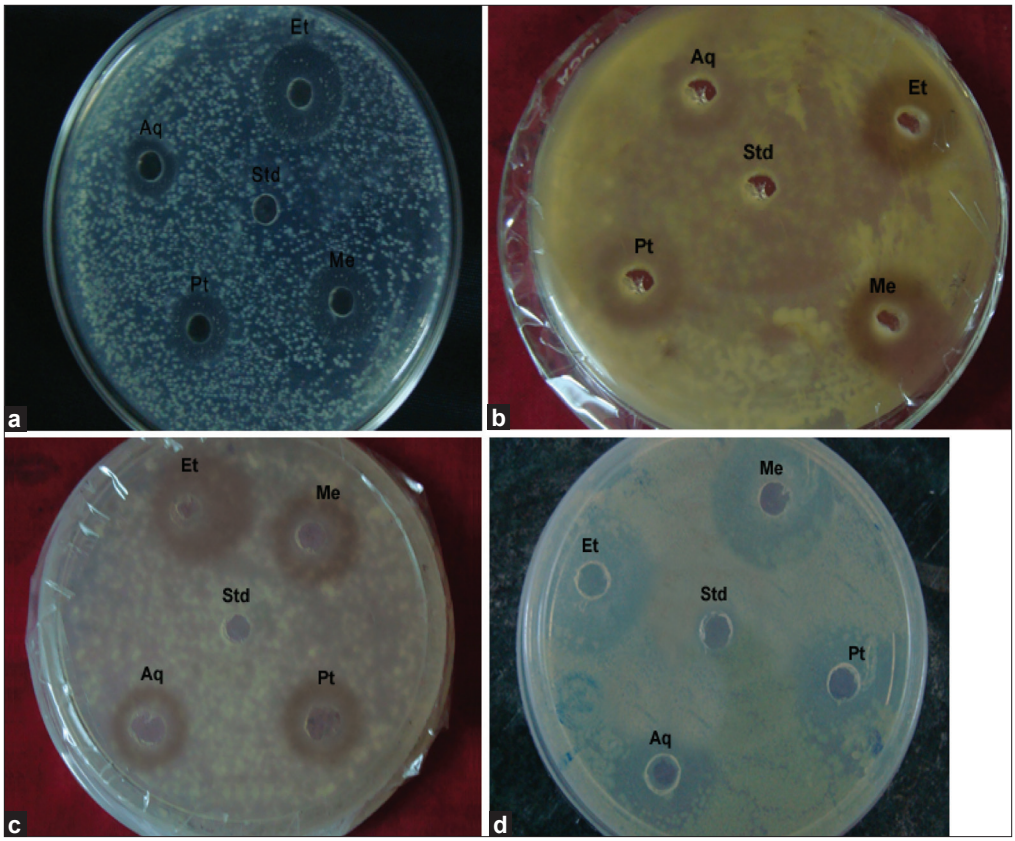
oxisporum (14.4±0.51mm), whereas, the methanol extract was found to be very effective against *A. flavus* (17.3±0.42 mm) and *R. stolonifer* (18.5±0.42 mm) as compared to the other tested microorganisms. Both petroleum ether (15–12 mm) and the aqueous extract (8–11 mm) showed negligible antifungal activity as compared to other alcoholic extracts [Table 3; Figure 2a-d].

Determination of Minimum Inhibitory Concentration, Minimum Bactericidal Concentration, and Minimum Fungicidal Concentration Values

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit the growth of organisms. Determination of MIC is important in diagnostic laboratories, as it helps to confirm the resistance of the micro-organism to an antimicrobial agent, and it monitors the activity of new antimicrobial agents. The MBC and MFC are determined by sub-culturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 hours. The concentration of the plant extract that completely killed the bacteria and fungi has been taken as MBC and MFC, respectively.

The ethanol extract of *Phyllanthus amarus* showed the least MIC value, that is, 32.2 µg/ml against *B. cereus,* while the methanol extract showed a value of 38.7 µg/ml against *P. aeroginosa*. *S. aureus* and *E. coli* showed effective MIC values of 42 µg/ml and 56.4 µg/ml in methanol and ethanol extracts, respectively. The aqueous extract showed the lowest MIC value, that is, 63.5 µg/ml against *E. coli,* while the ethanol extract showed the highest activity against *B. aureus* at a 32.2 µg/ml concentration [Table 4]. The petroleum ether extract of *P. amarus* showed moderate activity against all the tested microorganisms.

Fungi like *A. flavus and A. niger* proved to have the highest activity of 28.4 µg/ml and 32 µg/ml in the methanol and ethanol extracts, respectively. The same ethanol and methanol extracts were confirmed to have a high activity



Figures 1: Antimicrobial activity of different extracts *Phyllanthus amarus* Schum. and Thonn. against, (a) *Bacillus cereus;* (b) *Staphylococcus aureus;* (c) *Escherichia coli;* (d) *Pseudomonas aeruginosa* (Std – Standard, Et – Ethanol extract, Mt – Methanol extract, Pt – Petroleum ether extract)

at 43.2 µg/ml and 45.4 µg/ml against *F. oxisporum* and *R. stolonifer,* respectively. Petroleum ether and aqueous extract, both showed minimum antimicrobial activity, with comparatively the least MIC value. The least MBC and MFC values 68.8 µg/ml and 57.9 µg/ml were observed in ethanol and methnol extracts, against *B. cereus* and *A. flavus,* respectively [Table 4].

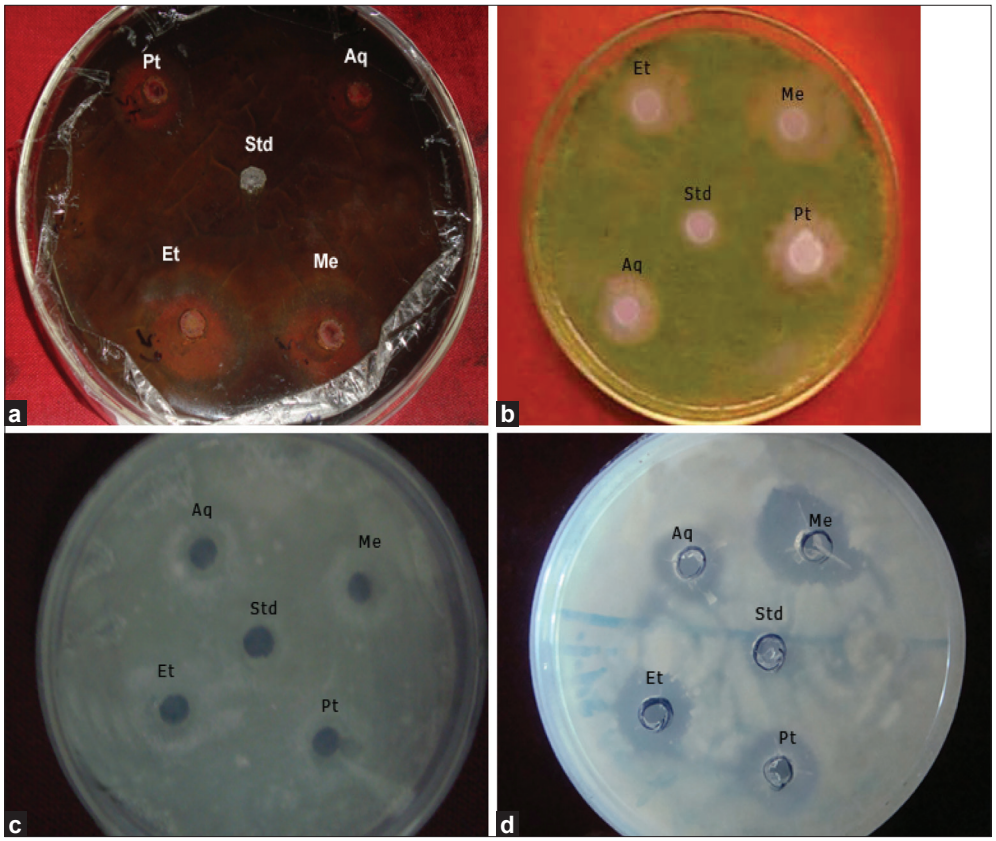
DISCUSSION

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 antimicrobial agent to replace synthetic ones. Phytochemicals derived from plant products serve as a prototype to develop less toxic and more effective medicines for controlling the growth of microorganisms.[16] These compounds have a significant therapeutic application against human pathogens, including bacteria, fungi, or virus. Numerous studies have been conducted with the extracts of various plants, screening of antimicrobial activity, as well as for the discovery of new antimicrobial compounds.[17] Therefore, medicinal plants are finding their way into pharmaceuticals, naturalceuticals, and food supplements.

In the present investigation, different extracts of *P.amarus* were evaluated for exploration of their antimicrobial activities against certain Gram-negative and -positive bacteria and fungus, which were regarded as human pathogenic microorganisms. The susceptibility of each plant extract was tested by the serial microdilution method (MIC) and was determined by the agar well-diffusion method.

Our preliminary investigation showed that all Ethanol, Methanol, Petroleum ether, and aqueous extracts of *P. amarus* were active against the locally isolated human pathogens like *Escherichia coli, Staphylococcus aureus*, *Bacillus cereus*, and *Pseudomonas aeruginosa*. This analysis of using several extracts to study the efficacy of plants for antimicrobial activity has also been realized by many scientists in many plant species like *Trianthema decandra* L,[18] *Argemone mexicana* L,[19] *Tinospora cordifolia,* and *Cassia fistula*.[20]

The alcoholic extracts from the plants of *P. amarus* showed significant antimicrobial activity against multi-drug resistant, clinically isolated microorganisms. Although the mechanism of 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 a more powerful antimicrobial



Figures 2: Antimicrobial activity of different extracts *Phyllanthus amarus* Schum. and Thonn. against, (a) *Aspergillus niger;* (b) *Aspergillus flavus;* (c) *Fusarium oxisporum;* (d) *Rhizopus stolonifer* (Std – Standard, Et – Ethanol extract, Mt–Methanol extract, Pt – Petroleum ether extract)

activity, as compared to the aqueous extracts.[21] Similar results showing that the alcoholic extract had the best antimicrobial activity was also reported by Jana and Shekhawat[22] in *Anethum graveolens*, and Preethi[23] in *Leucas aspera, Holarrhena antidysenterica.*

In the present study, the MIC value of the active plant extracts obtained in this study were lower than the MBC values Table 4 suggesting that the plant extracts were bacteriostatic at a lower concentration, but bactericidal at a higher concentration.[24]

In conclusion, the present investigation shows that *Phyllanthus amarus* contains the potential antimicrobial components that may be of great use to the development of pharmaceuticals in industries, as a therapy against various diseases. The ethanol, methanol, petroleum ether, and aqueous extracts of *Phyllanthus amarus* possess significant inhibitory effects 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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