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RESEARCH ARTICLE

ANTI-MICROBIAL ACTIVITY IN *PHYLLANTHUS AMARUS* PLANT EXTRACTS USING POLAR AND NON-POLAR SOLVENTS AGAINST GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

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ABSTRACT

Anti-microbial activity of root, stem and leaf extracts of *Phyllanthus amarus* were studied with polar solvents such as ethyl acetate, dimethyl Formamide and non-polar solvents such as chloroform, dichloromethane and n-Hexane extracts against Gram positive bacteria *viz. Bacillus cereus ,Bacillus subtilis* and Gram negative bacteria *viz. Pseudomonas aeruginosa,, Escherichia coli, Klebsiella pneumoniae* using Agar well diffusion method. Root, stem and leaf extracts of *Phyllanthus amarus* were evaluated separately to determine the best explant source for potential antimicrobial effect. Amikacin and Nitrofurantoin were used as positive control. Leaf extracts in dimethyl formamide and dichloromethane exhibited better antimicrobial activity compared to root and stem extracts. The extracts were further subjected to GC-MS analysis for identification of compounds responsible for anti-microbial activity. MIC and MBC studies were carried out by macro-dilution method. The assessment of anti-microbial activity of this plant helps in correlating the ancient and modern way of disease treatment for large scale testing of novel pharmacopeia.

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INTRODUCTION

Plants play a major role in health care for sustainable development of the country. Plants are our saviors at all times and are nature's gift to mankind for their well-being. The usage of plants to various diseases is our ancient wisdom. Recently, the universal trend is shift from synthetic to herbal medicine. Ancient documents like Rig-Veda and Atharvana Veda tell us the use of medicinal plants in the treatment of man & animals. The knowledge of traditional medicine developed over generations before the era of modern medicine started. Man investigated nature with his intelligence and explored thousands of medicinal plants through trial and error method for his existence. For commercial exploitation of raw drugs, value addition of medicinal plants is very much essential. *Phyllanthus amarus* belonging to family Euphorbiaceae, is well recognized for its medicinal properties and plays a major role in Ayurvedic system of medicine for over 2,000 years. It is mostly found in tropical and sub tropical countries and the young shoots of the plant are used for the treatment of chronic dysentery (Nadkarmi, 1993; Babu, 2011).

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This herb primarily acts on liver and so it can be used for the treatment of jaundice. DNA polymerase enzyme, which is used for replication of Hepatitis B virus, can be blocked using *Phyllanthus* (Joseph and Raj, 2011). Plants of *Phyllanthus* help for treatment of a number of diseases including cold, diabetes, bladder infections, ulcers, urinary tract infections and Kidney disorders. Lignans, a type of phytoestrogens and anti-oxidant compounds and tannins which help in health enhancement are found in this plant (Thakur *et al*., 1989; Chatterjee and Prakash, 1991; Ross, 1999). Phyllanthin which is a lignan belonging to the type aryltetrahydronaphthalene, inhibit reverse transcriptase enzyme and display anti-viral activity against HIV (Sagar *et al*., 2004). Phyllanthin and hypophyllanthin have hepatoprotective and anti-genotoxic activities (Row *et al*., 1964), where as Phyltetralin displays anti-inflammatory activity by inhibiting neutrophil influx (Kassuya *et al*., 2005). Tannins such as Repandusinic acid repressed HIV type-1 reverse transcriptase (Ogata *et al*., 1992). Root, stem and leaf extracts in different solvents such as ethyl acetate, dimethyl formamide, chloroform, dichloromethane and n-Hexane were to evaluate anti-microbial properties. The leaf extracts of the plant in dimethyl formamide and dichloromethane displaying best antimicrobial activity were subjected to GC-MS analysis to evaluate the potential antimicrobial compounds.

MATERIALS AND METHODS

Plant material

The plants of *Phyllanthus amarus* were collected from fields of Andhra Pradesh, India .The plants were washed and dried under shade. The dried plant material was separated into root, stem and leaf .They were then ground to fine powder and extracts were prepared separately for root, stem and leaf using polar and non-polar solvents. About 10 grams of fine powder was added to 200 ml of solvent and kept on a rotary shaker at 190-220 rpm for 24 h. The filtrates were concentrated under reduced pressure using rota vapour (IKA,Germany).

Bacterial cultures

Root, stem and leaf extracts of *Phyllanthus amarus* were tested against two Gram positive pathogenic bacteria *viz.Bacillus cereus, Bacillus subtilis* and three Gram negative pathogenic bacteria *viz.Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*. The bacterial cultures were obtained from IMTECH,Chandigarh. Slants of Mueller-Hinton agar(Himedia,India) were used for maintenance of bacterial cultures with a storage temperature of 4^{o}c. Activation of bacterial cultures was done at 37^{0}c on Mueller-Hinton broth (Himedia,India) before antimicrobial assay.

Anti-microbial assay

Anti-microbial activity of plant extracts by Agar well diffusion method was evaluated based on the protocol of Perez *et al*. (1990). An inoculum size of 10^{6} colony forming units (c.f.u)/ ml of bacteria were used in Mueller-Hinton agar and poured on to petri plates.By using cork borer, four wells were made in the Mueller-Hinton agar plate with a diameter of 8mm. Each well was filled with 20µl of plant extract (root, stem and leaf) in respective polar and non-polar solvents .Broad spectrum antibiotics Amikacin and Nitrofurantoin were used in the form of discs on Mueller-Hinton Agar plate as control. The plates were then incubated in an incubator at 37 ºC for 24 hours. After 24 hours, the diameter of zones around the wells (including diameter of the well) were measured which reflect the sensitivity of bacteria to plant extracts in different solvents. All the results which were performed in triplicates were tabulated.

Evaluation of Minimum inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC) of *Phyllanthus amarus* plant extracts

Broth dilution method was used for determination of Minimum inhibitory concentration (MIC) (Chattopadhyay *et al*., 1998a).The crude extracts were prepared in Mueller-Hinton broth by two-fold serial dilution technique with antibiotic (Amikacin and Nitrofurantoin) as control (Chattopadhyay *et al*., 1998b). The concentration of each Amikacin and Nitrofurantoin discs were 30 µg and 300 µg respectively. 24-h-old suspension in Mueller–Hinton Broth was used for preparation of direct suspension of bacteria in 5 ml sterile distilled water. 0.5 McFarland standard was used for adjusting the turbidity of the suspension (McFarland, 1907). Each tube containing crude extracts was added with fifty microlitres of bacterial suspension for broth dilution tests at a concentration range of 0.005-5.120 mg/ml and incubated for 24 h at 37^{0}c. The tubes were evaluated macroscopically and the tube with lowest concentration which did not show any visible growth was used for determining MIC .The tubes not showing visible growth in serial dilutions were streaked on to Mueller-Hinton plates and incubated for 24h at 37^{0}c.They are then observed for visible growth. The tube with least concentration of extract which did not show visible growth after streaking on to the plate after 24 h was used for determining MBC. The data was tabulated after performing in triplicates.

Statistical analysis

The triplicate data were analyzed in mean $\pm$ standard deviation.

GC-MS analysis

In GC-MS analysis, Elite-5MS (30.0m length, 0.25mm ID, 250µm film thickness) column was used for sample injection. Perkin Elmer make, clarus 680: GC –MS model consisting of Clarus 600 (EI) Mass Spectrometer. The injector was set at 250°C.The Acquisition parameters used for injection was held at oven temperature of 60°C for 2 min, then from 60-300°C at the rate of 10°C/min,held for 6 min. The total run time was 32 min. The interface temperature used for GC-MS was 240°C, injection volume was 1 µl. The solvent was injected in a split ratio of 10:1 and the flow Rate was 1 ml/min and carrier gas is He. The mass condition (EI) involve a solvent delay of 2 min with a MS scan range of 50–6,000 Da. Compound identification was done with Turbo Mass ver 5.4.2 software for comparison of spectral data available in NIST-2008 library.

RESULTS AND DISCUSSION

The microbes tested in the present study cause an array of diseases *viz.* such as food borne diseases, allergic reactions, respiratory and urinary tract infections, skin infections, diarrheal illness etc. They may also cause chronic diseases like pneumonia which cause destruction to human lungs. The present study reveals that leaf extracts of *Phyllanthus amarus* work best towards all pathogenic bacteria compared to root and stem extracts in all the solvents tested. The antimicrobial activity of leaf extracts in dimethyl formamide and dichloromethane was more pronounced for all the gram positive and gram negative bacteria that were tested. The activity of plant extracts is more in polar solvents than non-polar solvents for inhibiting bacteria. Our results are in consonance with the reports of kiranmayee Rao *et al* (2010) using *Alpinia galangal* extracts and Bhuvaneswari (2012) using *Gymnema sylvestre* extracts. There were reports on anti-microbial activity of *Phyllanthus* leaf in various solvents *viz.* ethanol (Bhat *et al*., 2015), aqueous extract (Dhandapani *et al*., 2007), methanol (Saranraj and Sivasakthivelan, 2012), ethyl acetate (Sowparthani *et al*, 2011) and in different solvent systems (Bharathi *et al*, 2014) against pathogenic bacteria.

The antibiotics amikacin and nitrofurantoin were used as positive control and are active against all gram positive and gram negative bacteria that were used in the present study. The zones of inhibition displayed by plant extracts were more than the zones of standard antibiotic for some pathogens which indicate good potentiality of plant extracts towards pathogens. Leaf exhibited highest inhibition zone (25.4 ±0.24mm) in

dimethyl formamide extracts against *Bacillus subtilis* and lowest inhibition zone (5.72 ±0.64mm) in ethyl acetate extracts against *Escherichia coli* (Fig.1).

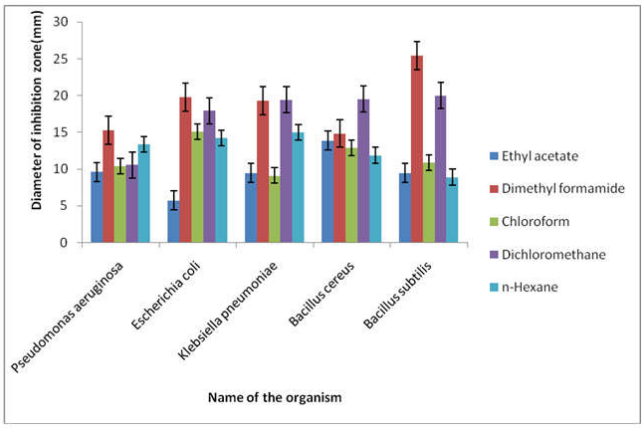

Fig.1. Comparative study of inhibitory zones of leaf extracts of *Phyllanthus amarus* against various bacteria in different solvents

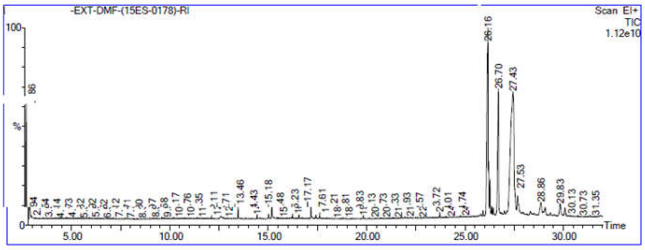

Fig.2. GC-MS Chromatogram of Dimethyl formamide leaf extract of *Phyllanthus amarus*

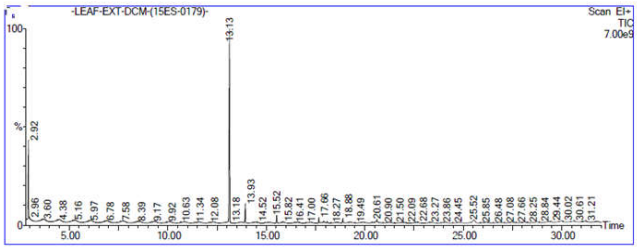

Fig.3. GC-MS Chromatogram of Dichloromethane leaf extract of *Phyllanthus amarus*

Stem exhibited maximum inhibition zone (19.44 ±0.22mm) in ethyl acetate extracts against *Bacillus cereus* and there were no inhibitory zones in dimethyl formamide and n-Hexane stem extracts against several pathogenic bacteria that were used. Root extracts in dimethyl formamide displayed maximum zone of inhibition (21.63 ±0.44mm) against *Escherichia coli* and minimum zone of inhibition (2.44 ±0.4mm) in chloroform against *Pseudomonas aeruginosa* From the above findings, maximum diameter of inhibitory zone (25.4 ±0.24mm) was displayed in dimethyl formamide leaf extract against *Bacillus subtilis* and minimum diameter of inhibitory zone (2.44±0.40mm) was displayed in chloroform root extract against *Pseudomonas aeruginosa*. *Bacillus subtilis* is found to be most sensitive as it has highest inhibitory zone (25.4 ±0.24mm) (Table 3). The MIC values of plant extracts ranged from 0.04 to 1.28 mg/ml and MBC values ranged from 0.08 to 2.56 mg/ml. After comparison of MIC and MBC values with standard antibiotics, it was found that the values of *B.Subtilis* and *Klebsiella pneumoniae* were lower than standard values which indicate a better choice for therapeutic applications.

Table 1. List of compounds detected by GC-MS analysis in *Phyllanthus amarus* Dimethylformamide leaf extracts

S. No.	Rt (min.)	Compound Name	CAS
1	2.863	FORMAMIDE, N,N-DIMETHYL-	68-12-2
2	26.163	CARISSANOL DIMETHYL ETHER	41328-80-7
3	26.203	No library	
4	26.273	No library	
5	26.703	No library	
6	27.434	SYLVATESMIN	487-39-8
7	27.674	FUMARIC ACID, 2-ISOPROPYLPHENYL PENTADECYL ESTER	900344-90-4
8	28.869	PHENETHYLAMINE, 2-METHOXY-.ALPHA.-METHYL-4,5-(METHYLENEDIOXY)-	23693-18-7
9	29.845	1-HEPTACOSANOL	2004-39-9

Rt-Retension time in minutes

Almost all other bacteria had similar MIC and MBC values as standard which shows good potentiality of *Phyllanthus amarus* plant extract for drug development. In the present study, the plant extracts exhibited high MIC and MBC values for *Pseudomonas aeruginosa* indicating highest resistance to plant extracts, even then these plant extracts in crude state had a challenging role in inhibiting such resistant bacteria (Table 4).

Table 2. List of compounds detected by GC-MS analysis in *Phyllanthus amarus* Dichloromethane leaf extracts

S.No	Rt (min.)	Compound Name	CAS
1	2.923	Formamide N-N Dimethyl	68-12-2
2	13.133	Di-Phenyl Methane	101-81-5
3	13.928	Phenol 2-4 BIS(1-1 Di-MethylEthyl)	96-76-4
4	15.523	Benzo Phenone	119-61-9
5	17.664	3,7,11,15 Tetra Methyl 2-HexaDecen-1-OL	102608-53-7
6	18.885	N-Hexa Decanoic Acid	57-10-3

Rt-Retension time in minute

Our study is in collaboration with the results of Saranraj *et al.* (2012) and Bharathi *et al.* (2014) where they mentioned that leaf extracts have shown best response towards antimicrobial activity against various pathogens. In our study also, leaf was found to display maximum antimicrobial activity compared to stem and root extracts.

GC-MS analysis of *Phyllanthus amarus*

GC-MS result of Dimethyl formamide leaf extract of *Phyllanthus amarus* detected 9 compounds *i.e* Formamide, N,N-dimethyl, Carissanol dimethyl ether, Sylvatesmin, Fumaric acid- 2-isopropylphenyl pentadecyl ester, Phenethylamine-2-methoxy-alpha-methyl-4,5-(methylenedioxy)- and1-Heptacosanol.Three compounds were not detected in the library. (Fig.2, Table 1). GC-MS result of Dichloromethane leaf extract of *Phyllanthus amarus* detected 6 compounds *i.e* Formamide N-N Dimethyl, Di-Phenyl Methane, Phenol 2-4 BIS(1-1 Di-MethylEthyl), Benzo Phenone, 3,7,11,15 Tetra Methyl 2-HexaDecen-1-OL and N-Hexa Decanoic Acid. In earlier reports on *Phyllanthus amarus* extracts, presence of Di-phenyl methane was not reported (Fig. 3, Table 2).

Table 3. Susceptibility patterns of Gram Negative and Gram Positive bacteria to plant parts of P*hyllanthus amarus* in different solvents

S.No	Name of the Organism	Gram +ve OR Gram –ve	Plant Part	Concentration (igms)	Diameter of Inhibition Zone(mm)					Antibiotic A-Amikacin N-Nitrofurantoin
					Solvent 1 Ethyl acetate	Solvent 2 Dimethyl formamide	Solvent 3 Chloroform	Solvent 4 DiChloro Methane	Solvent 5 n-Hexane	
					Polar	Polar	Non polar	Non polar	Non polar	
	Pseudomonas aeruginosa	Gram negative	Root		16.26±0.14	13.48±0.62	2.44±0.40	11.06±0.18	11.12±0.62	A – 5mm
			Stem	100µg	18.66±0.66	13.08±0.50	6.74±0.64	7.96±0.24	7.06 ±0.38	N - 6mm
1			Leaf		9.62±0.14	15.3 ± 0.48	10.42±0.34	10.56±0.20	13.34±0.72	
	Escherichia coli	Gram negative	Root		11.66 ±0.28	21.63±0.44	10.62±0.14	10.2 ±0.08	10.32±0.56	A – 14mm
2			Stem	100µg	19.4 ±0.32	16.38 ± 0	8.84±0.08	16.24 ±0.40	0	N – 10mm
			Leaf		5.72 ±0.64	19.74±0.66	15.1 ±0.20	17.94 ±0.52	14.22±0.76	
	Klebsiella pneumoniae	Gram negative	Root		9.62 ±0.62	9.08 ±0.20	12.72±0.08	10.6 ±0.24	7.06±0.38	A – 13mm
3			Stem	100µg	14.7 ±0.70	11.62±0.20	9.06 ±0.36	12.18 ±0.56	6.78 ±0.36	N – 14mm
			Leaf		9.5 ±0.90	19.32±0.22	9.12 ±0.16	19.42±0.32	14.96±0.84	
	Bacillus cereus	Gram Positive	Root		15.5 ±0.20	8.72±0.36	11.12±0.24	14.42 ±0.62	9.56 ±0.52	A – 6mm
4			Stem	100µg	19.44 ±0.22	11.9 ±0	6.88 ±1.66	16.34±0.32	0	N – no zone
			Leaf		13.86 ±0.20	14.84±0.1	12.92±0.14	19.5 ±0.26	11.86±0.66	
		Gram Positive	Root		11.74 ±0.66	10.86±0.18	11.2 ±0.42	9.22 ±0.60	10.7 ±0.60	A – 6mm
5	*Bacillus subtilis*		Stem	100µg	19.3 ±0.38	15.16 ±0	8.72 ±0.18	14.66 ±0.24	0	N – 7mm
			Leaf		9.46 ±0.54	25.4 ±0.24	10.9 ±0.42	20.00 ±0.40	8.92 ±0.50	

Statistical data is defined in terms of mean ± SD, n=3

Table 4. MIC and MBC values towards various pathogens using *Phyllanthus amarus* plant extracts

S.No.	Name of the Organism	Plant Parts	Concentration (mg / ml)										Antibiotic			
			Ethyl acetate		Dimethyl formamide		Chloroform		Dichloro methane		n-Hexane		Amikacin		Nitro-furantoin	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	Pseudomonas	Root	0.08	0.16	0.64	2.56	1.28	2.56	0.64	2.56	0.64	1.28	0.04	0.08	0.02	0.08
	aeruginosa	Stem	0.32	1.28	0.16	0.32	1.28	2.56	0.64	2.56	0.32	0.64				
		Leaf	0.16	0.64	0.04	0.08	0.64	2.56	1.28	2.56	0.64	1.28				
2	*Escherichia coli*	Root	0.32	0.64	0.08	0.16	0.16	0.32	0.16	0.32	0.64	2.56	0.005	0.01	0.04	0.08
		Stem	0.32	1.28	0.04	0.08	0.16	0.32	0.16	0.64	0.64	1.28				
		Leaf	0.16	0.32	0.08	0.32	0.16	0.64	0.08	0.32	0.64	1.28				
3	*Klebsiellaspp*	Root	0.32	0.64	0.08	0.16	0.64	1.28	0.32	0.64	0.16	0.32	0.08	0.32	0.08	0.16
		Stem	0.16	0.32	0.04	0.08	0.16	0.32	0.16	0.32	0.32	1.28				
		Leaf	0.08	0.16	0.08	0.16	0.16	0.64	0.64	2.56	0.32	1.28				
4	*Bacillus cereus*	Root	0.08	0.16	0.08	0.64	0.08	0.16	0.08	0.32	0.32	1.28	0.08	0.32	0.04	0.16
		Stem	0.08	0.32	0.04	0.32	0.16	0.64	0.32	1.28	0.64	2.56				
		Leaf	0.32	1.28	0.08	0.32	0.32	1.28	0.08	0.16	0.16	0.32				
5	*Bacillus subtilis*	Root	0.08	0.16	0.08	0.16	0.16	0.64	0.64	1.28	0.08	0.32	0.08	0.16	0.08	0.32
		Stem	0.04	0.08	0.04	0.16	0.32	1.28	0.32	0.64	0.32	1.28				
		Leaf	0.08	0.16	0.08	0.32	0.32	1.28	0.16	0.32	0.16	0.32				

Esters, alcohols, phenols and palmitic acid (Hexadecanic acid) present in the plant extracts may be responsible for its antimicrobial activity which is also reported in ethanolic leaf extracts of *Phyllanthus amarus* (Mamza *et al*., 2102). Berger *et al* (1953) reported that glycerol ethers and related compounds present in dimethyl ether extract may be responsible for the antimicrobial activity due to respiratory collapse (Berger *et al*., 1953). Compounds having methoxy group exhibit antimicrobial action by causing distortion to cell surface (Porosa *et al.,* 2013; Baluja *et al*., 2015). Benzophenone is a photoreactive group and is used for development of antimicrobial coatings as it interacts with phospholipid bilayer of bacterial cell membrane and alters its structure. This causes stress on the cell wall spilling cytoplasmic material and causing cell death (Porosa *et al.,* 2013). In *Aloe vera*, fumaric acid which is an organic acid has been reported as antibacterial component (He *et al*., 2011). A series of diphenyl methane compounds display antimicrobial action against various microorganisms (Florestano, 2006). In Malaysian mango kernel, Phenol, 2, 4-Bis (1, 1-Dimethyl ethyl) was identified and reported to have antibacterial activity (Abdullah 2001).

Conclusion

Secondary metabolites, because of their toxic effect, protect plants from herbivores and pathogens helping plants in defence mechanism. Since these compounds interfere with the biochemical pathway of pathogens, some of these compounds are useful to humans as medicines, flavorings or recreational drugs. *Phyllanthus amarus* is called as wonder drug plant as it cures many of diseases and as the plant is easily available, drugs from this plant can be manufactured at a cheaper rate which can be useful to the poor and needy. Constituents of *Phyllanthus amarus* such as alkaloids, flavonoids, lignans and a variety of other phytochemicals such as bioflavonoids, glycosides, ellagitannins, phenylpropanoids, Common lipids, sterols, flavonols are the main cause for good anti-microbial activity of this plant towards pathogens (Kumar, 2002; Bagalkotkar, 2006). As the leaf extract of *Phyllanthus amarus* in dimethyl formamide and dichloromethane solvents showed good microbiocidal activity towards pathogenic bacteria, this plant can be used for innovation of new phyto drugs.

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