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Phytochemical Screening and Antimicrobial Potential of Phyllanthus amarus Linn Extracts

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ABSTRACT

The aim of the present study is the phytochemical screening of various extracts of Phyllanthus amarus Schum & Thorn leaves and study the antibacterial potential against human pathogens. Three different extracts such as, petroleum ether, ethanol and chloroform were subjected for phytochemical analysis which revealed the presence of various bioactive phytochemical components such as glycosides, steroids, alkaloids, tannins and saponnis. The extracts were also subjected for antibacterial activity against Staphylococus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa. The ethanolic extracts showed better activity against the tested pathogens among the other extracts tested. The MIC values of the ethanolic extracts were found to be in the range of 10 to 20 mg/ml against the tested pathogens.

Key words: *Phyllanthus amarus, Staphylococus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa.*

INTRODUCTION

The natural resources of bioactive materials such as plant materials imply that there is a possibility of exploitation in the field of medicinal research. Most of the medicinal plants are significantly useful and essential which contain various active elements that are used in the treatment of various diseases pertaining to humans. Despite using different approaches for the discovery of various therapeuticals, the natural products still remain as best source for traditional and/or orthodox medicine [1, 2].

The species of the Genus *Phyllanthus* (Euphorbiaceae) commonly called as 'Bhuiamlki' in India have been used for the treatment of liver, kidney, bladder problems, intestinal parasites and diabetes from earlier times. Various parts of the *Phyllanthus* sp. are ethnobotanically considered to have enormous therapeutic activities such as leaves used as expectorant and also useful in strangury and sweats; the seeds were used as diuretic, laxative, diaphoretic, carminative, astringent to the bowels and also used for earache, griping, opthalmia, ascites and bronchitis [3, 4].

The genus *Phyllanthus* contains over 600 species worldwide, distributed throughout the tropical and subtropical areas [5]. Most of the *Phyllanthus* extracts were used by traditional healers [6], especially in the case of treatment of kidney stones, gallstones, inflammation in appendix and also in the case of prostate problems [7]. In many countries around the world various species in the genus *Phyllanthus* are used in folk remedies. Therefore, this genus is of great importance in traditional medicine.

Plant based therapy plays a vital role in the modern drug development involving traditional medicinal plants. World plant biodiversity is the immense source of various herbal drugs and still huge population rely on plant based therapies. India is one of the countries which has great potential in the exploration of medicinal plants where a large amount of plants have been screened for various properties. Various scientific techniques have been used for the evaluation in herbal medicine industry and the main focus of interest is on the active principles (bioactive molecule). However, more research is required to develop a drug formulation from the natural sources, an important field to be addressed. Several infectious diseases are still a major problem to health issues all over the country [8].

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The adverse effects of antibiotics against various bacterial infections have further created problem of increase in antibiotic resistance among various pathogens [9]. The need for the development of potential antimicrobials has become a daunting task. The ethnobotanical importance of the tested medicinal plants has been highlighted and it is used for various diseases [10]. Based on the above background, in this study, the plant *Phyllanthus amarus Schum & Thorn* (leaf) was screened for the presence of photochemical substances and also tested for their potential antibacterial activity against different human pathogens.

MATERIAL AND METHODS

Chemicals and reagents:

All the chemicals used in the present study were of high analytical grade, purchased from SRL Chemicals Ltd., Mumbai and readymade bacteriological media were obtained from HiMedia Laboratories, Mumbai, India. All the reagents and solutions were prepared using freshly prepared double distilled water.

Collection and processing of plant material:

The leaves of mature plants of known *P. amarus Schum & Thorn* used for the present study were collected from the CAS in Botany, University of Madras, Guindy Campus, Chennai. The plant leaf materials were then transported to the laboratory and washed thoroughly using tap water followed by metal free distilled water. The washed leaves were shadow dried at room temperature for 3-5 days till they became fully crispy while retaining the green coloration because volatile constituents such as glycosides and amino acids, important in medicinal use may be lost due to evaporation or degradation. The samples were ground by using mixer - grinder mechanically and the powder was stored in air tight vessels for further study.

Preparation of different plant extracts:

Fifty grams of powdered plant sample of *P. amarus* was defatted using petroleum ether, followed by extraction using 400 ml of different solvents such as ethanol and chloroform. All the extracts were evaporated in vacuum under low pressure and the obtained crude extracts were stored in sterile amber bottles at 4°C for further experiments.

Preliminary phytochemical analysis:

All the three extracts, petroleum ether, ethanol and chloroform of *P. amarus* were subjected for qualitative phytochemical analysis for the presence or absence of different phytochemicals [11].

Test for alkaloids:

Dragendroff's test:

Equal amount of (100 µl) diluted hydrochloric acid and Dragendroff's reagent were added to 2 ml of different extracts in sterile test tubes. The formation of orange - brown coloured precipitate indicates the presence of alkaloids.

Mayer's test:

Two milliliter of different extracts were taken separately in a test tube, then 0.2 ml of diluted hydrochloric acid and 0.1 ml of Mayer's reagent were added and observed for yellowish buff colored precipitate that indicates the positive results for the presence of alkaloids.

Test for steroids:

Salkowski test:

One milliliter of concentrated sulphuric acid was mixed to 10 mg of crude extracts, dissolved in 1 ml of chloroform. The preparation was then observed for reddish brown colour in chloroform layer and green fluorescence in the acid layer to identify the presence of steroids.

Liebermann-Burchard test:

Ten micrograms of crude extracts was mixed in 1 ml of chloroform, followed by addition of 1 ml of acetic anhydride and then 2 ml of concentrated sulphuric acid on the sides of the test tube. The preparation was observed for the formation of reddish violet colour for confirming the presence of steroids, triterpenoids and cardiac glycoside.

Test for saponins:

Foam formation test:

To one milliliter of the plant extracts, distilled water was added separately up to 20 ml and mixed in a graduated cylinder for 15 min. The formation of stable foam indicates the presence of saponins in the plant extracts.

Test for tannins:

Ferric chloride test:

To 5 ml of each crude extract, 1 ml of 5% ferric chloride solution was added and observed for the presence of greenish black color for the presence of tannins.

Potassium dichromate test:

To 5 ml of each crude extract, 1 ml of 10% aqueous potassium dichromate solution was added and observed for the formation of yellowish brown precipitate which confirms the presence of tannins.

Test for flavonoids:

Lead acetate test:

One milliliter of 10 % aqueous lead acetate solution was reacted with 5 ml of each extract and observed for the development of yellow colour precipitate for the presence of flavonoids.

Test for sugars:

Molisch's test:

Two milliliter of each extract and a few drops of 15 % of ethanolic alpha-naphthol solution was mixed in a separate test tube. To this mixture, 2 ml of concentrated sulphuric acid was added drop by drop along the sides of the test tubes and observed for the formation of a reddish violet ring at the junction of two layers to confirm the presence of carbohydrates.

Fehling's test:

To five milliliter of each extract, 5 ml of Fehling's solution (equal volume of Fehling's solution A and B mixture) was added and boiled in the water bath and observed for the formation of brick - red precipitate which shows the presence of reducing sugars.

Estimation of total flavonoids:

The total flavonoid content was estimated using Chang *et al.* (2002) method [12] in which 0.5 ml of each crude extract (1mg ml^{-1}) in methanol was separately mixed with 1.5 ml of methanol, 0.1ml of 1M potassium acetate, 0.1ml of 10% aluminium chloride and 2.8 ml of distilled water and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was read at 415 nm using Visible Spectrophotometer. The total flavanoid content of each crude extract was expressed in equivalent of β-carotene as mg/g of dried leaf powder.

Estimation of total phenolic compound:

The total phenolic content of the three different plant extracts was analyzed Spectrophotometrically by modified method of Kahkonen *et al.* (1999) [13] using Folin-Ciocalteau's reagent. To the 300 µl of each extract (in triplicates), 1.5 ml of Folin-Ciocalteau reagent (diluted 10 times) and 1.2 ml of sodium carbonate (7.5 %) were added. The contents in the tubes were mixed and kept in the dark condition for 30 minutes and then absorbance was measured at 765 nm using Visible Spectrophotometer. Total phenolic content was expressed in equivalent of β –carotene as mg/g dry weight.

Antimicrobial Studies of solvent extracts:

The *in vitro* antimicrobial activity of different extracts was studied using standard agar method [14]. The pure test strains for the present study were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh. The crude extracts of three different solvents were tested for their activity against the four different test pathogens such as, *Escherichia coli* MTCC 1652, *Staphylococus aureus* MTCC 3160, *Pseudomonas aeruginosa* MTCC 424 and *Bacillus subtilis* MTCC 121.

Briefly, 24 hours old Nutrient broth (HiMedia, Mumbai) cultures of test bacteria were swabbed uniformly on solidified sterile Nutrient agar (HiMedia, Mumbai) plates using sterile cotton swab. Then, wells of 4 mm diameter were cut into the inoculated plates using sterile cork borer and each extract were added separately into respectively labeled wells. The plates were then incubated at 37°C for 24 hours in upright position and the zone of inhibition formed around the well was measured and the experiments were performed in triplicates under sterile experimental conditions. The solvent extracts which show better activity against the test strains were further evaluated for Minimum Inhibitory Concentration (MIC) determination.

MIC determination of solvent extracts:

The Minimum Inhibitory Concentration of different extracts against the test strains was determined in accordance with Clinical and Laboratory Standard Institute (CLSI) methodology [15]. The method used was the tube dilution method and dilutions of the extracts were incorporated in Muller - Hinton broth. The individual extracts were dissolved in Muller–Hinton broth (MHB) at different concentrations of 10µg/ml to 100µg/ml in separate sterile test tubes with a final volume of 1ml in each test tube. Then each organism was separately suspended in 5ml of Muller - Hinton broth and incubated overnight. Thereafter, 0.1 ml was added to all the test tubes and the preparations were incubated at 37°C for 18 hours. After incubation, a loop full from each one of test tube was sub-cultured on nutrient agar and observed for visible growth. The lowest concentration of extract which shows no growth was recorded as minimum inhibitory concentration (MIC).

RESULTS AND DISCUSSION

Development of multi-drug resistant phenomenon against pathogenic microbes has created a necessity for the search for new antimicrobial substances from other natural sources, especially plants. Most of the traditionally used medicinal plants synthesize a variety of compounds which are known to be therapeutic value [16, 17]. The substances that can inhibit pathogens and have little toxicity to host cells are considered candidates for developing new antimicrobial drugs. Most of the infectious diseases are known to be treated with various herbal remedies throughout the history of mankind. Even today, plant components play an important role in primary health care as therapeutic remedies in many developing countries [18]. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases [19]. Therefore, various scientific workers are increasingly moving their attention to folk medicines looking for new leads to develop better drugs against microbial infections [20, 21].

The phytochemical analysis of the three different extracts was performed and tabulated (**Table 1**). The results reveal that presence of various proteins, carbohydrates, phenols and tannins, flavonoids and saponins were observed in three different extracts.

Through phytochemical analysis of the extracts was necessary to determine the presence of various classes of secondary metabolites which play a major biological activities such as antioxidant [22], antimicrobial [23], antitumor and antiophidic [24].

Tannin possesses spasmolytic activity in smooth muscle cells, free radical scavenger and antioxidant properties [25]. Tannins bind to proline-rich proteins and interfere with the protein synthesis. Flavonoids are hydroxylated phenolic substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Possible mechanism of antimicrobial activity may be due to their ability to complex with extracellular and soluble proteins and to complex with cell walls of bacteria.

Several researchers have reported that natural and synthetic derivates of alkaloids possess medicinal value which include antisplasmodic, analgesic and antibacterial activities, antioxidant and are useful in renal disorder [26, 27]. Similar phytochemicals were reported during characterization of medicinal plants [28].

Antimicrobial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell. Steroids have been reported to have antibacterial properties which may interfere with the membrane lipids and exert their action by causing leakages from liposomes [29].

The estimation of total flavonoid content was observed to be significantly higher in ethanolic extracts (35 mg/g) of *P. amarus* when compared to petroleum ether and chloroform which were observed as 27 and 21 mg/g dry weight respectively (**Table 2**). Over the years, the study on medicinal plants to reveal the mechanism of action and to justify the claims of traditional healers has been increased. The total phenolic content was determined for all the three different extracts (**Table 3**), ethanolic extracts show higher amount of total phenolics of 142 mg/g compared to the other extracts.

Alkaloids are secondary metabolites originating generally from amino acids and therefore, contain nitrogen in their molecular structure. Their physiological activity on other plants and animals and especially on humans renders them as important drugs. Their role in the plants is not absolutely clear but they are involved in plant defense mechanisms, biochemical or physiological, waste products and storage reservoirs of nitrogen [30]. The presence of these phenolic and flavonoid compounds, contribute different biological activities such as anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic. These properties might be attributed to their free radical scavenging and antioxidant activities [31]. Similar results were reported by various studies that methanolic fraction possesses highest amount of phenolic and poly phenolic compounds [32, 33]. Flavonoids are naturally occurring substances in plants that are thought to have positive effects on human health [34]. The most important function of flavonoids is the antioxidant properties. Flavonoids have been reported to have highly effective scavengers of most of the oxidizing molecules, which include singlet oxygen and other free radicals [35]. Phenolic and flavonoid substances were associated with antioxidant property which plays a major role in stabilization of lipid peroxidation [36].

The antibacterial assay was carried out using agar well diffusion tests and broth dilution techniques for all the extracts. Antibacterial activity was determined from the zone of inhibition around the wells. The results reveal that ethanolic extracts of *P. amarus* show better antibacterial activity against the tested bacterial strains (**Table 4**), followed by chloroform and petroleum ether. The MIC values were also determined for the two different extracts such as chloroform and ethanol on *P. amarus* which gave significant results in the well diffusion method for antibacterial effect. The MIC results revealed that ethanolic extracts having better activity compared to chloroform, showing a value of 10 µg/ml for both *B. subtilis* and *P. aeruginosa;* 20 µg/ml for *S. aureus* and *E. coli*. Similarly, chloroform extract shows MIC value in the range of 20 µg/ml for *B. subtilis* and 30 µg/ml for *P. aeruginosa*, *E. coli* and *S. aureus* respectively.

The present study shows that the application of organic solvents for the preparation of plant extracts provide more consistent antimicrobial activity. Similar findings were reported by several workers [37, 38] who have used different solvents for the antimicrobial property of various plant extracts. Eloff (1998) [39] in his study revealed that methanol and ethanol were considered as most efficient solvent for the plant extraction process. The present study clearly indicates that the ethanolic solvent shows significant results which show that under experimental conditions ethanol is ideal solvent to extract antimicrobial compounds from the leaves.

Due to emergence of antibiotic resistant strains as well as side effects of synthetic drugs, investigation of potent antimicrobial drugs obtained from natural resources has been an objective of researchers and investigators. The findings of our results showed that methanolic, ethanolic and butanolic fractions had significant antibacterial activity against all pathogenic strains. Our results agreed with results reported by Ndhlala *et al.* (2009) [40] during antimicrobial characterization of the South African plant aloe (*Aloe barberae*) due the presence of bioactive polyphenolic constituents. Narod and coworkers (2004) [41] reported the antimicrobial effects of *Toddalia asiatica* extracts against Gram negative and Gram positive bacteria which were similar to our findings.

Table No. 1: Qualitative Phytochemical analysis of various extracts

Phytochemical tests		Extracts		
		Petroleum ether	Ethanol	chloroform
Test for alkaloids	Dragendroff's test	++	+	+
	Mayer's test	++	++	++
Test for steroids	Salkowski test	++	--	+
	Liebermann-Burchard test	++	+	++
Test for saponins	Foam formation test	++	++	+
Test for tannins	Ferric chloride test	++	++	++
	Potassium dichromate test	++	++	++
Test for flavonoids	Lead acetate test	++	++	++
Test for sugars	Molisch's test	+	+	--
	Fehling's test	--	++	--

++ = highly positive; -- = Negative

Table No. 2: Determination of total flavonoids of various extracts

Determination of total flavonoids		
Plant name	**Extracts**	**Total Flavonoids (β –carotene as mg/g)**
Phyllanthus amarus	Petroleum ether	27
	Ethanol	35
	Chloroform	21

Table No. 3: Determination of total phenolic compounds of various extracts

Determination of total phenolic compounds		
Plant name	**Extracts**	**Total phenolic (β –carotene as mg/g)**
Phyllanthus amarus	Petroleum ether	126
	Ethanol	142
	Chloroform	114

Table No. 4: Antibacterial evaluation of *P. amarus* extracts against test strains

Test Strains	Zone of inhibition (mm)		
	Plant extract		
	Petroleum ether	Chloroform	Ethanol
B. subtilis	6	11	12
S.aureus	6	13	15
E. coli	4	10	12
P. aeruginosa	6	12	14

Table No. 5: MIC value of *P. amarus* extract on bacterial pathogens

Strains	MIC (mg/ml)		
	Petroleum ether	Chloroform	Ethanol
B. subtilis	50	20	10
S.aureus	70	30	20
E. coli	70	30	20
P. aeruginosa	60	30	10

CONCLUSIONS

The study showed that ethanolic extract of *P. amarus* leaves shows an effective antibacterial property against the tested pathogens such as *Staphylococcus aureus*, B*acillus subtilis*, *Pseudomonas aeruginosa* and *Escheichia coli*, which are considered as potential pathogens to cause health hazards to humans. The study also showed the presence of various phytochemical in all the three extracts which may found to be useful in pharmaceutical products. However, detailed investigation is necessary to study the active principles responsible for the antibacterial property which may provide a complete evidence of bioactive mechanism of medicinal plants.

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