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METABOLITE PROFILING AND PHARMACOLOGICAL EVALUATION OF *PHYLLANTHUS AMARUS* STEM EXTRACT

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ABSTRACT

The plant *Phyllanthus amarus* contains a wide range of pharmacologically active compounds and is used for the treatment of several ailments in traditional folklore medicine in Bangladesh. The present study was done on *P. amarus* stem extract in order to investigate the metabolite profiling as well as evaluation of its pharmacological (antibacterial, antioxidant, cytotoxicity bioassay) profiles. The metabolite profiling was done by color test and nuclear magnetic resonance spectroscopy. The antibacterial activities were determined by measuring the diameter of the zones of inhibition in millimeter. The cytotoxic potential of the test samples was determined by using brine shrimp lethality bioassay. The metabolite profiling techniques mentioned above revealed the presence of flavonoids, anthraquinones, terpenoids, steroids, coumarins, tannins, saponin, and reducing sugars in the crude extract of *P. amarus* stems. The stem extract displayed a remarkable antibacterial activity against all the tested bacteria. The extract also showed good scavenging activity having IC_{50} values 12.87 µg/ml. This study gives some concrete evidence to support the pharmacological use of *P. amarus* stems extract as antimicrobial, antitumor or pesticidal activity.

KEYWORDS: Metabolite profiling, *Phyllanthus amarus*, antibacterial activity, antioxidant activity, cytotoxicity.

INTRODUCTION

Plants have been utilized as medicines for thousands of years. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations.[1] The specific plants to be used and the methods of application for particular ailments were passed down through oral history. Eventually information regarding medicinal plants was recorded in herbals. In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from opium in the early 19th century.[1,2] Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use.[3,4] Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds. On the other hand, the R & D thrust in the pharmaceutical sector is focused on development of new drugs, innovative/indigenous processes for known drugs and development of plant-based drugs through investigation of leads from the traditional systems of medicine. Recently, there has been a renewed interest in natural product research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas such as immunosuppression, anti-infectives and metabolic diseases. Metabolite profiling is an analytical method for relative quantitation of a number of metabolites from biological samples. Commonly, these samples have been garnered from a specific tissue or a part of a tissue of interest, but, depending on the biological question, also either from a larger mixture of different organs (such as whole-shoots) or conversely on a micro scale from single cells or purified organelles. *Phyllanthus amarus* is a plant of the family Euphorbiaceae and has about approximately 800 species which are found in tropical and subtropical countries of the world.[5,6] It has found its traditional usefulness in several health problem such a diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds. Further, these are used in the treatment of kidney problems, urinary bladder disturbances, pain, gonorrhea, diabetes and chronic dysentery. Topically, it is used for several skin problems ranging from skin ulcers, sores, swelling and itchiness, wounds, bruises, scabies, ulcers and sores, edematous swellings, tubercular ulcers, ringworm, scabby and crusty lesions.[7-9] The herb has found use in several female problems such as in leucorrhoea, menorrhagia and mammary abscess and can act as galactagogue. The young shoots of plant are administered in the form of an infusion for the treatment of chronic dysentery. The stem juice is also used as wound healers. The whole plant extract is used in

urinary problems and swelling of liver.[10-13] Bangladesh is a good repository of medicinal plants belonging to various families, including Euphorbiace. No work on Metabolite profiling of *P. amarus* grown in Bangladesh was done. Therefore, an attempt has been taken to study the metabolite profiling of *P. amarus* stems extract and its pharmacological evaluation.

MATERIALS AND METHODS

Collection and preparation of the plant sample

The stems of *P. amarus* were collected from an authentic source and were identified by a taxonomist Mst. Nadira Begum, Scientific Officer, BCSIR Laboratories, Dhaka. A voucher specimen (DACB 35212) that contains the identification characteristics of the plant was submitted to the herbarium for future reference. At first the sample was sun-dried and then, dried in an oven at reduced temperature (not more than 50°C) to make it suitable for grinding purpose. The coarse powder was then stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

Preparation of extracts

The powdered plant materials is submerged with dichloromethane: methanol (1:1) solvent in an air-tight flat bottomed container for several days, with occasional shaking and stirring. The major portion of the extractable compounds of the plant material will be dissolved in the solvent during this time and hence extracted as solution. The individual extractives are then filtered through several means, e.g., cotton, cloth, filter paper etc. All the extractives are concentrated with a rotary evaporator at low temperature (40 ^{0}C - 50 ^{0}C) and reduced pressure. The concentrated extract thus obtained is termed as crude extract.

Phytochemicals screening of the crude extract

Phytochemicals screening was performed using standard procedures.[14]

Test for Reducing sugars (Fehling's test)

The extract (0.5 g in 5 ml of water) was added to boil Fehling's solution (A and B) in a test tube. The formation of brick red color indicates the presence reducing sugars.

Test for Anthraquinones

0.5 g of the extract was boiled with 10 ml of sulfuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another

test tube and 1 ml of dilute ammonia was added. A brown coloration indicates the presence of anthraquinones.

Test for Terpenoids (Salkowski test)

2 ml of chloroform was added to 0.5 g each of the extract. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

Test for Flavonoids

Three methods were used to test for flavonoids. First, dilute ammonia (5ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulfuric acid (1 ml) was added. Second, a few drops of 1% aluminum solution were added to a portion of the filtrate. Third, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. In all the cases, a yellow coloration indicating the presence of flavonoids was observed.

Test for Saponins

0.5 g of extract was added to 5 ml of distilled water in a test tube. The solution was shaken vigorously and the mixture was observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for Tannins

About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration.

Test for Cardiac glycosides (KellerKilliani test)

0.5 g of extract diluted with 5 ml water was added to 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlaid with 1 ml of concentrated sulfuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a ring may form just above the brown ring and gradually spread throughout this layer.

Metabolite Profiling by NMR Spectroscopy

The ^{1}H-NMR spectrum of the crude extracts was recorded by using Ultra Shield Bruker DPX 400 NMR instrument, (Bruker BioSpin AG, Switzerland) to identify the presence of following compounds such as flavonoids, anthraquinones, coumarins, sterol/steroidal, saponins etc.

Antibacterial activity assay

The measured amount of each test sample was dissolved in specific volume of solvent to obtain the desired concentrations in an aseptic condition. The antimicrobial activities were then carried out by the disc diffusion method.[15] The diluted samples were applied onto sterile filter paper discs at different concentration (µg/disc) for the antibacterial assays.

Assay of free radical scavenging activity

The hydrogen atoms or electrons donation ability of the corresponding extracts was measured from the bleaching of purple colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as a reagent.[16] 2.0 ml of a methanol solution of the samples (extractives/control) at different concentration (200 µg/ml to 0.78125 µg/ml) were mixed with 2.0 ml of a DPPH methanol solution (20 µg/ml). After 30 min reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spetrophotometer.

Inhibition of free radical DPPH in percent *(I%)* was calculated as follows:

$(I\%) = (1 - A_{sample}/A_{control}) \; X \; 100$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test material).

Extract concentration providing 50% inhibition (IC_{50})was calculated from the graph plotted inhibition percentage against extract concentration.

Cytotoxicity bioassays

The cytotoxic activity was performed as described previously.[17,18] The test samples for crude extracts were dissolved in DMSO and serial dilution were made as 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781µg/ml. Then each of these test solutions was added to test tubes containing 10 shrimps in simulated brine water (5 ml) and incubated at room temperature for 24 h. After 24 h, the median lethal concentration (LC_{50}) of the test samples

was obtained by a plot of percentage the shrimps killed against the logarithm of the sample concentration.

RESULTS AND DISCUSSION

Phytochemical study

Thousands of diverse natural products are produced by plants and many of these are involved in plant defense. The phytochemical diversity of antimicrobial compounds include terpenoids, saponins, phenolics and phenyl propanoids, pterocarpans, stilbenes, alkaloids, glucosinolates, hydrogen cyanide, indole and also elemental sulphur, the sole inorganic compound.[19] In this study, the phytochemical analysis of the crude extract of the stem of *P. amarus* showed the presence of different groups of secondary metabolites such as terpenoids, flavonoids, anthraquinones, saponin, tannins and reducinig sugar which are of medicinal importance (Table 1).

Table 1: Phytochemicals screening of the crude extracts of *P. amarus* stem.

Class of compounds	Stem extract
Reducing sugars	+
Anthraquinones	+
Terpenoids	+
Flavonoids	+
Saponin	+
Tannins	+
Cardiac glycosides	-

Metabolic profiling by NMR

The ^{1}H-NMR spectrum (Fig. 1) of the crude extract obtained from the stem exhibited a range of chemical shifts. The chemical shifts and splitting pattern in the region of approximately 5.0 ppm to 8.30 ppm could be attributed for the aromatic/olefinic protons belonging to different sub classes of flavonoids, anthraquinones and coumarins. Moreover the chemical shifts and splitting pattern of the aliphatic region such as approximately 2.0 ppm to 5.0 ppm may be attributable for protons of flavonoids (flavonone, flvanonoletc). The sharp singlets in the region of approximately 3.0 ppm to 4.0 ppm may be indicated the presence o-methyl protons of any flavonoids or in coumarins. The presence of multiplets near about at 3.50 ppm and 5.30 ppm typical for H-3 and H-6 of a steroidal nucleus. The presence of multiplet near about at 4.30 ppm typical for the anomeric proton of steroidal saponin and also existence of many peaks in the region of approximately 3.0 ppm to 4.0 ppm indicated the presence of glycosidic compounds.

It is evident that the identification of some metabolites in the ^{1}H-NMR spectrum are also supportive to the previous experiments of chemical group test (color test).

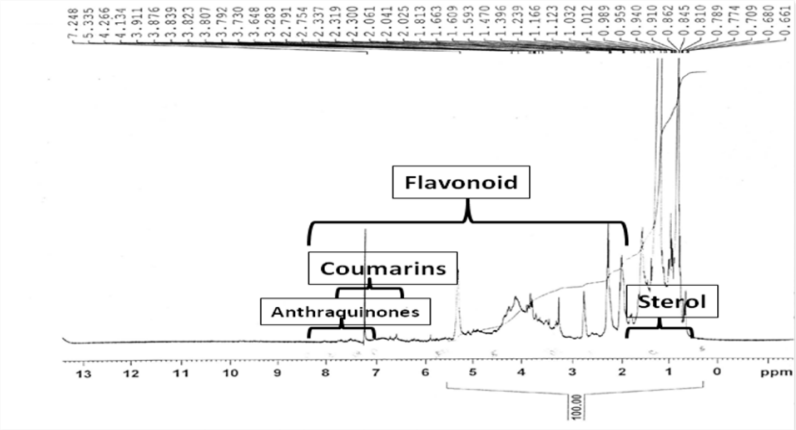

Fig. 1: ^{1}H-NMR spectrum of crude extract from stem.

***In vitro* antibacterial activity**

There has been an increasing effect on microbial growth inhibition with increasing concentration of the extracts. However, the effects observed were less than those produced by the standard agents. The results on antimicrobial activity of the *P. amarus* extracts against four bacterial strains are presented in Table 2. Our results showed that *P. amarus* stem exhibited strong activity against *Staphylococcus aureus* (18 mm) and *Bacillus megaterium* (14 mm). On the other hand, the extract showed moderate activity against *Escherichia coli* (10 mm) and *Pseudomonas aeruginosa* (12 mm).

Table 2: Antimicrobial activity of stem extract of *P. amarus*.

Bacterial strain	Diameter of zone of inhibition (mm)	
	Stem extract	Azithromycin
Staphylococcus aureus (ATCC 25923)	18	28
Bacillus megaterium	14	22
Escherichia coli (ATCC 8739)	10	21
Pseudomonas aeruginosa (ATCC 27833)	12	22

Scavenging activity of DPPH radical

The DPPH free radical is a stable free radical, which has been widely used as tool to estimate free radical-scavenging activity of antioxidants. Antioxidants, on interaction with DPPH, either transfer electrons or hydrogen atoms to DPPH, thus neutralizing the free radical character.[17] The color of the reaction mixture changes from purple to yellow, and a decrease in absorbance. The DPPH-radical-scavenging activity of the extracts is shown in Table 3.

Lower IC_{50} value indicates higher antioxidant activity. DPPH-radical scavenging capacity of extracts of *P. amarus* was compared to butylated hydroxyanisole (BHA) and trolox (Table 3). The stem extract had the remarkable free radical-scavenging activity with an IC_{50} value of 12.68 µg/ml. The antioxidant activity may be due to the presence of phenolic hydroxyl or methoxyl groups, flavones hydroxyl, keto groups, free carboxylic groups and other structure features.[20]

Table 3: IC_{50} values of the standard and crude extract of *P. amarus* stem.

Test sample	IC_{50}
Butylated hydroxyanisole	1.21
Trolox (water soluble form of vit-E)	1.51
P. amarus stem extract	12.68

Cytotoxic activity

The cytotoxic activity of the extracts were determined by using brine shrimp lethality bioassay. The LC_{50} for stem extract was found 2.45 µg/ml. Fig. 2 shows the percent mortality of brine shrimp nauplii with different concentration of plant extracts. This positive results suggested that the stem may contain antitumor or pesticidal activity.

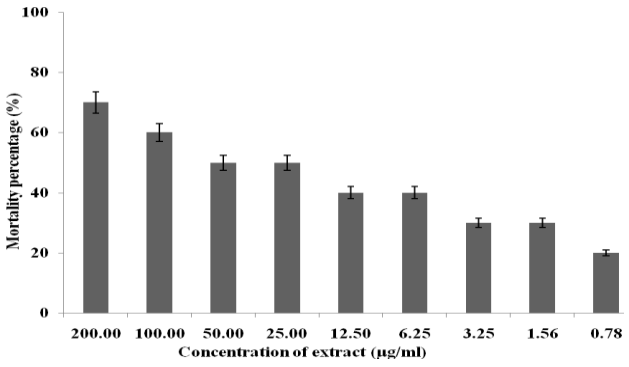

Fig. 2: Cytotoxicity of the stem extracts of *P. amarus* at different concentrations.

CONCLUSION

These investigations may provide some interesting information on *P. amarus*, which may be pharmacologically important and significant for extensive phytochemicals work (target-oriented isolation), drug development/drug discovery, to avoid dereplication, quality control and standarization of herbal medicine.

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