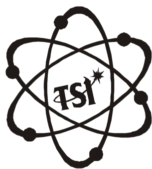
September 2009 Volume 5 Issue 3



Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 5(3), 2009 [120-124]

Extraction, isolation and characterization of phyllanthin from *Phyllanthus amarus* with preliminary phytochemical evaluation of the crude extract

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Received: 5th July, 2009 ; Accepted: 15th July, 2009

ABSTRACT

Phyllanthin is a characterizing compound present in the plant *Phyllanthus amarus,* used as hepatoprotective. The crude extract of phyllanthin was obtained from *P. amarus* using solvents of varied polarity. Preliminary phytochemical evaluation was performed on the crude extract obtained by successive extraction. The crude extract confirmed the presence of alkaloids, glycosides, carbohydrates, terpenes, flavonoids, from the tests conducted. The result of bioburden studies performed also was within limits. A detailed extraction, isolation and characterization method was also optimized for phyllanthin. Soxhlet extraction; followed by Preparative Thin Layer chromatography (PTLC) and characterization by spectral studies was conducted on the methanolic extract. The spectral studies conducted on the extract after isolation confirmed the presence of phyllanthin.

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KEYWORDS

Phyllanthus amarus;
Phyllanthin;
Isolation;
Phytochemical;
Characterization.

INTRODUCTION

The annual herb *Phyllanthus amarus* Schum and Thonn, belonging to family Euphorbiaceae is grown throughout India. The plant is erect around 10-60 cm long, with a simple or branched stem, and 1.5 - 14 m long stipulated, deltoid acuminate, deciduous branches. The leaves are distichously oblong with minutely apiculate at the apex and obtuse slightly inequilaterally base[1]. The genus Phyllanthus has been used historically in the treatment of diabetes, intestinal parasites and liver, kidney and bladder problems[2-4]. Commonly known as Bhuiamla, the plant *P. amarus* is highly valued in the treatment of liver ailments and kidney stones and has been shown to posses anti-hepatitis B virus surface antigen activity in both *in vivo* and *in vitro* studies[5,6]. The major lignan of the genus, namely phyllanthin, has shown to be hepatoprotective against carbon tetrachloride- and galactosamine-induced hepatotoxicity in primary cultured rat hepatocytes[7]. Thus, phyllanthin (Figure 1) major lignan of the plant serves as a characterizing compound for the analytical studies.

Literature survey reveals that some chromatographic methods[8-10] have been described. But all the reported methods describe only the chromatographic techniques in detail. However none of the method describes the extraction, isolation and characterization of phyllanthin from phyllanthus amarus in detail. Thus an

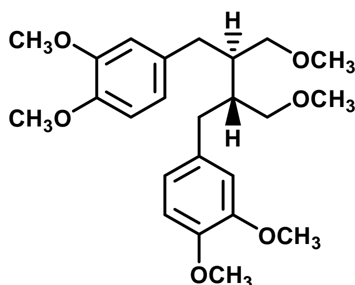

Figure 1 : Chemical structure Phyllanthin

attempt has been made develop and optimize a method for detailed extraction, isolation and characterization of phyllanthin from *P. amarus*. Also preliminary phytochemical evaluation of the crude extract of phyllanthin was conducted using successive solvent extraction. Extraction and isolation forms an important tool for the analytical studies and hence considering the importance the following study was undertaken.

EXPERIMENTAL

Chemicals and Reagents

HPLC grade Hexane, Toluene and Ethyl acetate was purchased from Merck (Darmstadt, Germany). And Petroleum ether, Chloroform, Acetone, Methanol AR (Analytical Reagent) grade was procured form Qualigens Fine Chemicals, Mumbai, India. Deionised and ultra pure water used in all experiment was obtained from Milli-Q system (Millipore, USA).

Plant material

The whole plant of *P. amarus* Schum and Thonn was procured from Mumbai, and authenticated at Agharkar Research Institute, Pune, India. (Certificate dated 12/02/07)

Collection and Preparation

The authenticated whole plant of *P. amarus* was dried for 6 hours at 30-40 ^{0}C in shade. The dried samples were then crushed into powder using an electronic mixer. The powder sample was stored in bottle at room temperature prior to analysis.

Preparation of Extracts

(Extract A): Successive Extraction for Crude Extract

50 gm of powdered stems, roots and raw samples of *P. amarus,* were packed in a white muslin cotton bags and were then extracted successively using the following solvents in a Soxhlet apparatus. The solvents used for extraction were petroleum ether, chloroform, acetone, methanol, and water. The polarity was increased with time. Each time before extracting with the next solvent, the powdered material was dried in an air oven below 50°C. Finally the marc was macerated with water for 24 hours to obtain aqueous extract. The extracts for further study were labeled as petroleum ether (E1), chloroform (E2), acetone (E3), methanol (E4), and water (E5). The extractive values and preliminary phytochemical studies were carried out on these successive extracts.

(Extract B): Methanol Extraction for Isolation

The powder of 100 gm of *Phyllanthus amarus* was exhaustively extracted with methanol at room temperature using Soxhlet apparatus. The plant extract was then collected and filtered through Whatman No.1 filter paper. The extract was further concentrated and subjected to silica gel column chromatography.

Phytochemical Evaluation

The phytochemical evaluation of the crude extract obtained from successive extraction was carried out as per procedure defined in the Indian Pharmacopoeia[11].

Extract A, obtained after successive extraction was used for the phytochemical studies.

Successive Extractive Values

3-5 ml of crude extract was taken in a previously tarred evaporating dish. The solvent was completely evaporated and the residue was weighed. From this residue, the percentage extractive value was calculated for each extract (E1, E2, E3, E4, E5) separately.

Qualitative Screening

The qualitative chemical tests were performed on different successive extracts (E1, E2, E3, E4, E5) of the roots, stems and raw sample extracts for the phytoconstituents such as alkaloids, glycosides, carbohydrate, steroids and terpenes, saponins, tannins, and phenols, fixed oils and fats, flavonoids, gums and mucilages.

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Quantitative Screening

The various parameters like extractive value, ash value, moisture content, and foreign organic matter were studied, in accordance with the Indian Pharmacopoeial procedure.

Bioburden studies

The microbial load on the crude extract was checked by bioburden studies. The testing was for pathogenic fungi, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *and Escherichia coli, Salmonella species* were done as per standard Pharmacopoeial procedure.

Elemental Analysis

Elemental analysis was carried out to detect the traces of elements quantitatively using inductively coupled plasma atomic emission spectroscopy.

Isolation

Extract B, was used for further isolation of phyllanthin from *P. amarus*.

Chromatographic conditions

The chromatographic conditions for isolation of phyllanthin were optimized. The silica gel was used as an adsorbent for column chromatography. The column used was 40 cm in height with an internal diameter of 1 cm. the solvent system used for isolation was optimized to Hexane: Ethyl acetate (97:3) with a rate of elution of 30 drops per minute. The sample was loaded in the ratio of sample size to the amount of adsorbent (1:30). Sequentially 85 fractions of 4 ml each were collected throughout the isolation process. The solvent system eluted with the fractions was completely removed by evaporation at 50 ^{0}C under a stream and the residue was reconstituted with 0.5 ml methanol. After reconstitution, TLC of these fractions was carried out to find out in which fraction phyllanthin was present. The 64-76th fraction contained phyllanthin but none of the fraction contained exclusively phyllanthin. It was not possible to isolate exclusively phyllanthin by column chromatography and hence for the further purification step preparative thin layer chromatography was employed.

Preparative Thin Layer chromatography (PTLC)

Final purification of Phyllanthin was performed by Preparative Thin Layer Chromatography. Silica gel G F_{254} plates were used as the stationary phase. A band of the fraction collected from the column was applied using narrow tip glass capillary. The solvent system was optimized to Hexane: Toluene: Ethyl acetate (2:2:1) which gave the best separation on silica gel plates. For detection of phyllanthin, the developed plates were observed for the blue fluorescence under the UV (254 nm) as phyllanthin gives blue fluorescence spot under UV (254 nm) light. After the detection the silica gel covering the area on which phyllanthin was present was carefully scraped out and phyllanthin was recovered by eluting it in methanol. The adsorbent material and the solvent were homogenized on a vortex mixer, to ensure complete elution, centrifuged and supernatants were collected and evaporated to obtain phyllanthin.

Identification and Characterization of Phyllanthin

Spectroscopic techniques were used for the identification and characterization of phyllanthin.

Sample preparation

For UV identification the 1 mg/ml sample of isolated phyllanthin in methanol was prepared with suitable dilution to record the spectra. For NMR identification isolated phyllanthin was dissolved in $CDCl_3$ and the H^1 Nuclear Magnetic spectrum was recorded. And the MS was recorded with 1 μg/ml solution of isolated phyllanthin in methanol.

RESULTS AND DISCUSSION

The results after evaluation of successive extractive values were as follows: 1.82 % - Petroleum ether, 0.42 % – Chloroform, 2.60 % – Acetone, 13.56 % – Methanol, 9.70 % – Water. The total extractive value was thus found to be 28.10 %. Quantitative estimation of the physicochemical properties expressed as (% w/w) of the dry *P. amarus* plant were: moisture content 4.21, water soluble extractives 16.38, alcohol soluble extractives 14.42, total ash 5.37, acid insoluble ash 0.42, water soluble ash 0.92, foreign organic matter 0.56. From all the obtained results it was observed that water soluble extractive value was higher indicating that the amount of polar constituents are more in water extract than alcohol extract. The bioburden studies carried out on *Sta-*

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phylococcus aureus, *Pseudomonas aeruginosa*, *and Escherichia coli, Salmonella* indicated complete absence of all these organisms, thus it was free from any microbial load.

The qualitative screening of extract of *P. amarus* revealed the presence of phytoconstituents alkaloids, glycoside, and tannins which are indicated in TABLE 1.

TABLE 1 : Qualitative Screening of Phyllanthus amarus Extract

Test	E1	E2	E3	E4	E5
Alkaloids	-	+	-	+	-
Glycosides	-	-	+	+	-
Carbohydrates	-	-	-	+	+
Steroids and terpenes	+	+	+	-	-
Saponins	-	-	-	-	+
Tannins and phenols	-	-	-	+	+
Fixed oils and fats	+	+	-	-	-
Flavonoids	-	+	+	+	+
Gums and mucilages	-	-	-	-	+

+ = present, - = absent

The quantitative screening for the presence of element's in the crude extract revealed the presence of iron 0.513 ppm, silver 0.002 ppm and zinc 0.019 ppm with absence of other elements.

The solvent chosen for extraction must take into account the chemical nature and polarity of the sample constituents. Because the objective was quantitation of the semi polar compound phyllanthin, which is soluble in methanol, hence methanol was chosen for extraction of phyllanthin using classical method. The extract thus obtained was concentrated and column chromatography was carried out to ensure the separation of phyllanthin and other unwanted compounds. For further purification of phyllanthin PTLC method was employed which gave purified phyllanthin. The solvent system was optimized to Hexane: Toluene: Ethyl acetate (2:2:1 %v/v) which gave good separation of phyllanthin.

The identification of phyllanthin was done under UV (254 nm) as phyllanthin gives blue fluorescence spot under UV (254 nm) light. The spectroscopic characterization revealed the following details:

UV: Isolated Phyllanthin showed absorption at 206 nm, 230 nm and 279 nm in UV region, which corresponds to the lambda max. of phyllanthin.

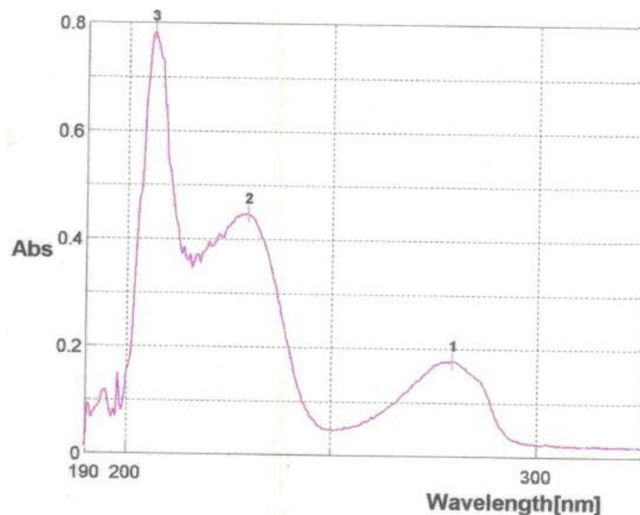

Figure 2 : UV Spectra of Isolated Phyllanthin

NMR: The peaks observed: multiplet (-CH=) at 7.26 and 6.61, a singlet ($-OCH_3$) at 3.81 and 3.25, doublet ($-CH_2$) 2.65 and 2.02, singlet (-CH=) 1.55 corresponds to the structure of phyllanthin.

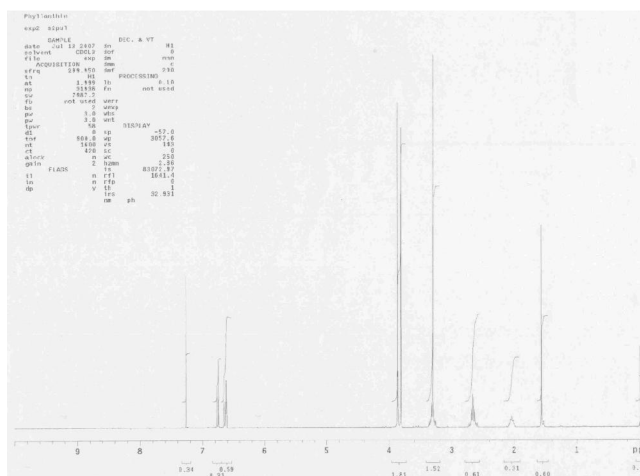

Figure 3 : NMR Spectra of Isolated Phyllanthin

MS: A molecular ion peak can be seen at m/z 418, corresponds to the molecular weight of the phyllanthin.

Phyllanthin the major lignan of *P. amarus* was successfully detected by above spectroscopic techniques. Thus the compound was successfully isolated with the above method.

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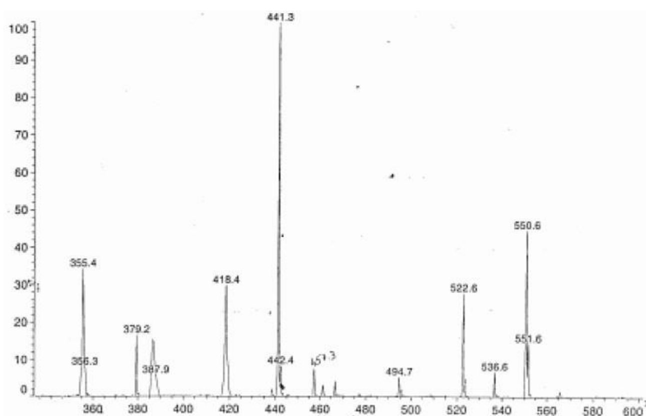

Figure 4 : MS Spectra of Isolated Phyllanthin

CONCLUSION

Extraction and isolation are of major importance in herbal industry. The developed method for extraction and isolation is simple and rapid and thus can be extended to large scale extraction and isolation of phyllanthin from the plant *P. amarus*. Thus the present study provides a valuable tool to the herbal industry.

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