Investigation on Phytochemical and Biological analysis of Acetonic extracts of *Phyllanthus amarus* (Linn.)

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ABSTRACT

Medicinal plants play an important role in treatment of several human diseases since ancient times. One such species which emerged more than 3000 years had wide patronage of use in the history of medicines. *Phyllanthus amarus* belongs to the family of Euphorbiaceae has several experimental investigations that explored its phytochemical constituents and pharmacological uses. The analysis of chromatography techniques of this medicinal plant shows the potentiality of compounds which can be estimated into a natural drug. The compounds were purified using column chromatography and detected the purified samples applied to liquid chromatography and mass spectrometry (LCMS), Gas Chromatography and Mass Spectrometry (GC-MS) techniques to find purified components present in the medicinal plants.

Key Words: *Phyllanthus amarus*, TLC, GCMS, LCMS.

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INTRODUCTION

Medicinal plants constitute a source of novel chemical compounds which are of potential use in medicine and other pharmacological applications. These medicinal plants possess many active compounds which are deposited in various parts of the plants. The medicinal effects of plant material results from the combination of secondary products which are consider as very essential elements by World Health Organisation (WHO)[1].

Phyllanthus amarus belongs to the Euphorbiaceae family which has about 800 species and found abundantly in tropical and subtropical parts of the world. It is a annual branching herb roughly about 30-60

cm in height and has slender branchlets, distichously leaves, sub sessile elliptic-oblong, rounded or obtuse in base. Flowers of *Phyllanthus amarus* are of different colours which are yellowish, whitish or greenish, with auxillary attached while male flowers are present in groups of 1-3 and females are solitary i.e single flower in nature. Fruits are present underneath the branches with depressed-globose with smooth capsules and seeds are brown in color, trigonous and has parallel ribs. The plant is widely found in different parts of the world like Philippine, Cuba and Nigeria. In India, *Phyllanthus amarus* is distributed predominantly as a weed in cultivated and waste lands [2,3]

The studies on phytochemical analysis have the presence of various secondary metabolites such as flavonoids, alkaloids, lignans, fats, hydrolysable tannins (ellagitannins), polyphenols, triterpenes, sterols and oils. These medicinal plants are known to posses antioxidant, antibacterial, antifungal, antidiabetic, and anti-inflammatory due to their properties they are largely used for medicinal purpose. The development of drug resistance and the undesirable side effect of certain antibiotic have led to the search for new techniques are applied in to medicinal plants[3].

The molecules which are bioactive in nature will possess different compounds and its chemical structures and properties of physicochemical analysis shows the potential bioactive phytochemicals. To analyze these Column chromatographic techniques are performed and used for the purification of the bioactive compounds present in the samples. Developed instruments such as High Pressure Liquid Chromatography (HPLC) accelerate the process of purification of the bioactive molecule. The Gas chromatography portion separates volatile and semi-volatile compounds with great resolution, but it cannot identify them. Mass Spectrometry can provide detailed structural information on most compounds such that they can be exactly identified and quantified (Oregon State University, 2012). The analysis of different functional groups by means of Fourier Transform Infra-Red spectroscopy (FT-IR) helps in function group identification of chemically or naturally synthesized compounds[4]. The FTIR works by the principle of vibration and Rotation between the chemical bonds and provides the structure of molecules in analyzing organic and inorganic materials.

MATERIALS AND METHODS

Collection and Authentication of plant materials

The plant samples was collected from University of Madras, Guindy campus, Chennai and it was identified as *Phyllanthus amarus*, with the help of Flora of Presidency of Madras (Gamble, 1967) and the flora of the Tamil Nadu and Carnatic[6]. The specimens were authenticated by **Prof. P. Jayaraman**, PRAC, West Tambaram, Chennai.

Preparation of crude extract

The dried leaf sample of *Phyllanthus amarus* was powdered with the help of mechanical pulverizer. The powdered material was then soaked in 1000 mL of acetone. The extract was suction filtered using Whatmann filter paper. This was repeated for 2 to 3 times and similar extracts were pooled together and concentrated at 40 to 45˚C under reduced pressure using vacuum rotary evaporator. The concentrated crude acetonic extract was subjected to a preliminary analysis and biological activities[7, 8].

Thin Layer chromatography analysis

Thin Layer chromatographic technique is an important analytical tool for separation, identification and detection of different classes of natural products.

TLC Analysis

Silica gel G Chromatographic technique was performed. The crude acetonic leaf, extracts were spotted at 2 cm from the edge of the silica coated glass plate. The chromatogram was developed in a mixture of suitable solvent system and dried at room temperature. The developed plates were observed for visible spots. Then, the developed TLC plates were finally placed in iodine chamber[7, 8]. The R_f values of the colored spots were recorded.

$$R_f \text{ value} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Column chromatography

The stationary phase material is suitably moistened with mobile phase and packed with a cotton or asbestos pad at the bottom of the column. The plant extract is separated by placing above the stationary phase with a second cotton or asbestos pad in between them. The mobile phase is poured into the column over the sample and the solvent is eluted. As the mobile phase passes through the column, various components of the sample travel with different polarity rates through the stationary phase possessing silica gel which are eluted by the range of adsorption and molecules possessing affinity towards the silica gel (stationary phase) and the solvent (mobile phase). The sample eluted different fractions each possessing different components from the crude mixture. The greater affinity molecules pass to mobile phase faster and lesser affinity compounds moves slowly. Each color is an indicator of one particular set of compound in the sample mixture. The elution of components takes place drop by drop and approximately the elution of different components may

vary from few hours to days based on the sample size, length, polarity of mobile phase and the packing material used.[9]

Fourier transforms infra red spectroscopy (FT-IR)

The FAME samples were analyzed under infrared (Perkin Elmer model spectrum – I PC). The FT-IR spectra with the resolution of 4 cm^{-1}, Scan Number: 3 were performed after the evaporation of the lipid fraction on Thalium bromide tablets. The FT-IR spectrums of all the FAME samples were obtained as a percentage of transmission ranged from 600 cm^{-1} to 4000 cm^{-1}.

Gas chromatography and Mass spectrometry (GC-MS)

The column used was Perkin Elmer Elite - 5 capillary column measuring 30m × 0.25mm with a film thickness of 0.25mm composed of 95% Dimethyl polysiloxane. The carrier gas used was Helium at a flow rate of 0.5ml/min. 1µl sample injection volume was utilized. The inlet temperature was maintained as 250°C. The temperature of oven was maintained at 110°C for 4 min, and gradually increased to 240°C and then the sample was automatically increased to 280°C at a rate of 20°C ending with a 5 min. Therefore the Total time to carryout GCMS analysis for single sample is 36 min. The calculation was performed by means of relative percentage by comparing the total areas with the average peak area. Turbo mass software was used to identify the mass spectra and chromatogram with NIST 2009 library match.The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.[10]

Liquid Chromatography Mass Spectrometry (LC-MS)

An Agilent 1100 HPLC Series system (Agilent, USA) was used equipped with a degasser, binary gradient pump, column thermostat, auto sampler, and UV detector. For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18, 100 x 3.0 mm i.d., 3.5 µm particle); the work temperature was 48°C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using Chem Station and Data Analysis software from Agilent, USA. The MS spectra obtained from a standard solution of compounds were integrated in a mass spectra library. Later, the MS traces/spectra of the analyzed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral mach. The UV trace was used for quantification of identified compounds from MS detection. However, all

compounds can be selectively identified in MS detection (qualitative analysis) based on differences among their molecular mass and MS spectra. Quantitative determinations were performed using an external standard method.

Results:

Collection and extract preparation

The *Phyllanthus amarus* leaf samples were collected and powdered using mechanical pulvenizer and subjected to extraction using acetone as solvent and condensed the sample using rotary evaporator **(Fig.1).**

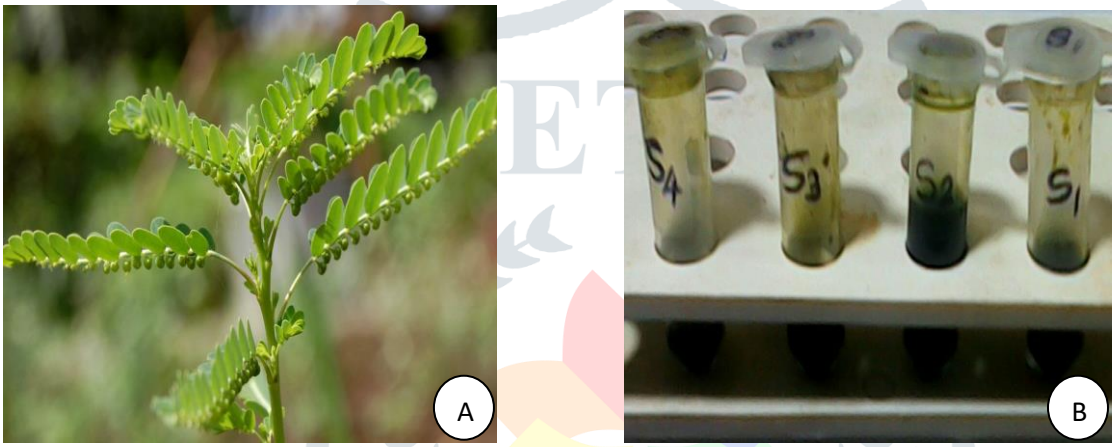


Fig. 1: A - Entire picture of *Phyllanthus amarus* B- Acetone extracted leaf samples

Thin Layer chromatography analysis

The TLC profile of acetonic extracts of leaf sample of *Phyllanthus amarus* were carried out. The chromatogram was developed in a mixture of suitable solvent system as hexane : acetone in the ratio of (8:2) and dried at room temperature. Different spots were identified under visible light. The R_f value was calculated for leaf samples of *Phyllanthus amarus.* The samples when spotted in TLC showed more number of bands in the leaf extracts **(Fig.2)**.

Fig 2: TLC profile of *Phyllanthus amarus* under visible light

Column Chromatography

The sample was passed through the silica gel 60. Hexane: Acetone in the ratio of 8:2 was used as the eluting solvent. The solvent fractions eluted were collected in pre 3ml/min. The fractions were collected and analyzed for its purity. The solvent fractions eluted were collected in pre-weighed container for further analysis (**Fig.3**).

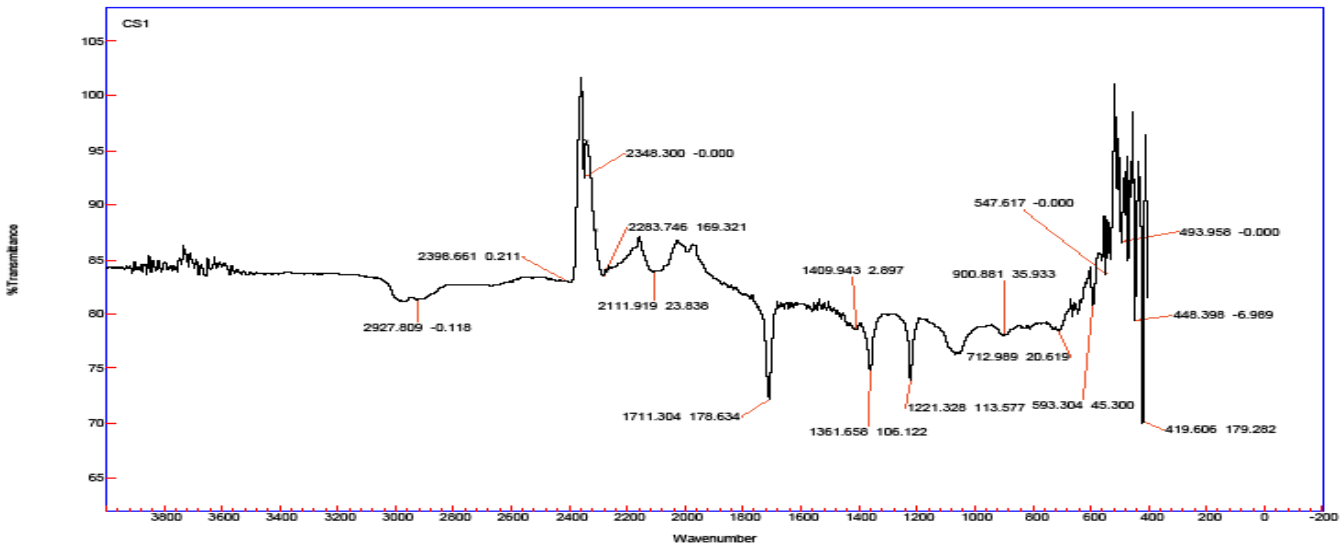
Fig. 3: Six different Fractions collected from the samples.

Fourier Transform Infra-Red spectroscopy (FT-IR)

The volatile products can be found in the spectrum of *Phyllanthus amarus* leaf extracts. The collected extractions give bands at 1409 to 2927cm^{-1}. There are totally seven peaks each representing one functional group as represented **(Table 1 and Graph 1).**

Table 1: FT-IR Spectrum of *Phyllanthus amarus*

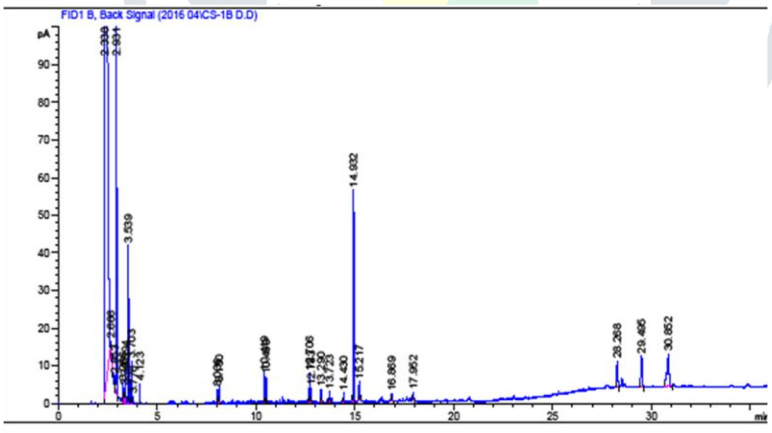
Frequency	Bond	Functional group
2927.809	C-H Stretch	Alkyl Group
2396.661	C-H Stretch	Nitrile Group
2348.300	C-H Stretch	Alkyl Group
2283.746	C-H Stretch	Nitrile Group
2111.919	C=H Stretch	Alkyl Group
1711.304	O-H Stretch	Carboxylic Acids
1409.943	C-C Bond	Aromatic Group



Graph 1: FT-IR Spectrum of *Phyllanthus amarus*

Gas Chromatography and Mass spectrometry (GC-MS)

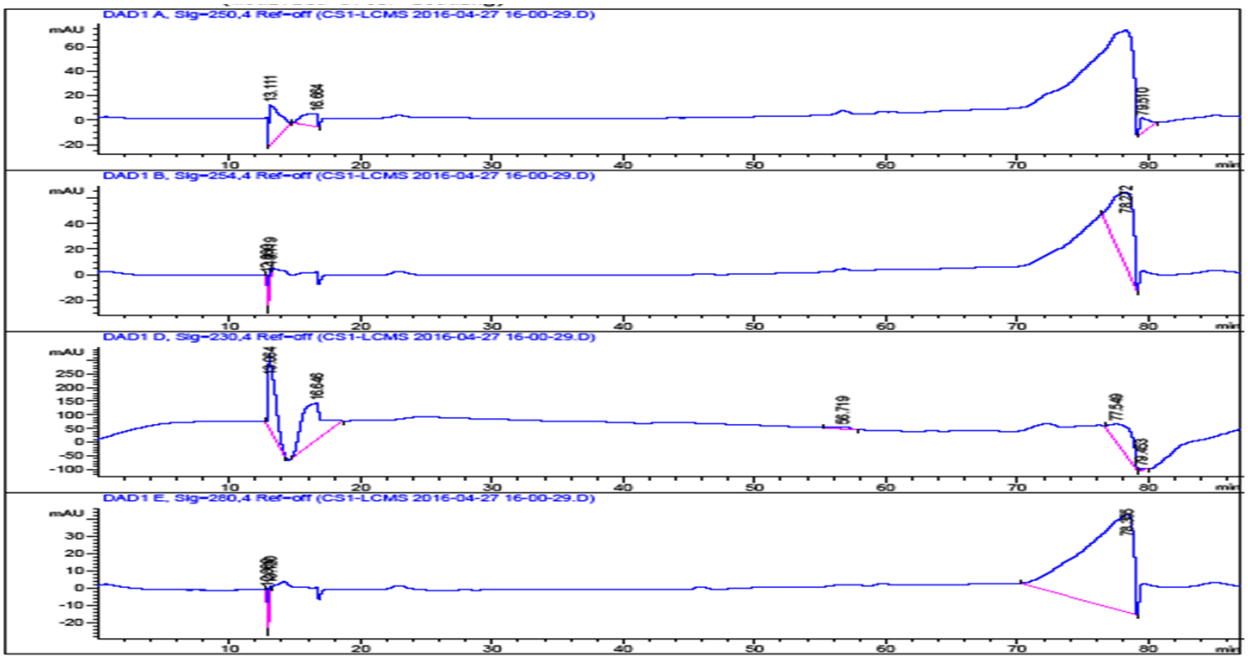
The GC-MS of *Phyllanthus amarus* leaf extract showed the presence of various compounds as represented in Graph 2. The predominant compounds present in acetone extract of *P. amarus* are 3, 4-Dimethoxy-di-phenylanine, Phenethylamine, 2-methoxy-alpha-methyl-4, 5-(Methlenedioxy) and 5, 8, 11, 14- Eicosatetraynoic acid. The remaining compounds are at least level and they are Benzhydrazide,4-methoxy-N2-(2-bromo-5-(2-prophnyloxy) benzylideno), 2(3H)-Cyclopent(e)-1,3-oxaxin-2-one, hexahydro, 2H-Pyan-2,6(3H)-dione, dihydro-4,4-dimethyl, Hex-5-encylamine, Cycloheptylamine and Cycloctanamine.



Graph 2 : GC-MS of *Phyllanthus amarus*

Liquid Chromatography and Mass spectrometry (LC-MS)

The LC-MS of *Phyllanthus amarus* leaf extract showed the presence of various compounds as represented in Graph 3. When these peaks were subjected to LCMS analysis, the molecular weight of the compounds observed was 795.70, 782.72, 775.40 and 77.62On comparing with the available databases of *P. amarus*, these compounds were identified as derivatives of Phyllanthine.



Graph 3: LC-MS of *Phyllanthus amarus*

DISCUSSIONS

The results of the study showed that *Phyllanthus amarus* has adverse effect on phytopharmacological studies. *Phyllanthus amarus* (Euphorbiaceae) were collected in Dhanbad district of Jharkhand, India[11]. In the present study, *Phyllanthus amarus* was collected from University of Madras, Guindy campus, Chennai – 600 025. The phyllanthin and hypophyllanthin obtained by TLC method shows poor resolution of compounds from other closely related compounds Deb and Mandal (1996). Whilst the mobile phase reported by Sane *et al.* (1997) gave resolved peaks of the content of the latter (0.858%) was reportedly higher than that of the former (0.709%). Therefore, a number of mobile phases of different compositions were tried for TLC. Depending on the suitable solvent phases, hexane : acetone : ethyl acetate (7.4:1.2:0.8, v/v/v) showed different compounds. The extract thus obtained was concentrated and column chromatography was carried out to ensure the separation of phyllanthin and other unwanted compounds. The solvent system was optimized to Hexane : Toluene : Ethyl acetate (2 : 2 : 1 %v/v) which gave good separation of phyllanthin[9].Similarly Hexane : Acetone in the ratio of 8:2 was used for the elution of crude samples and six different fractions were collected. Better efficacy of acetone and ethanol solvents were also supported by GC-MS analysis which has revealed the presence of bioactive compounds in the acetone extract and ethanolic extract. This plant can be exploited for the development of drugs which can reduce the side effects of commonly used synthetic drugs. Further studies are needed with this plant on its isolation, structural elucidation of bioactive compounds and about its toxicological effects for new drug formulation[12]. LCMS analysis of ethanolic extracts of *P. amarus*, showed the presence of active components like Phyllanthine and Nirphyllin or its derivatives. The obtained component by means of LCMS shows the component belongs to Lignan group of and has good Anti-oxidation property.[13]

CONCLUSION

Phyllanthus amarus having bio active phyto-constituents present in the plant were the potential source for drugs and therapeutic leads. The results of this study revealed that acetone extract of *Phyllanthus amarus* contains pharmacologically active substances which are proven by analytical techniques. Therefore the crude extracts of *Phyllanthus amarus* leaf could be new source of drug development to cure several diseases.

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