



Research Article

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Elucidation of bioactive compounds in hydroalcohol extract of *Phyllanthus amarus* Schum. and Thonn. leaf using GC-MS analysis

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Abstract

Phyllanthus amarus is one of the most important medicinal plant in tropical and subtropical nations which are used for stomach, liver, kidney and spleen diseases treatment. This work involves the elucidation of the phytochemical constituents in 70% ethanolic leaf extract of *P. amarus*. Phytochemical screening, quantitative and gas chromatography-mass spectrometry (GC-MS) analyses were carried out on the extract. The qualitative and quantitative analyses showed the presence of the following phytochemicals and their contents in mg/100g of extract as phenolic (34.31 ± 0.07), tannin (21.15 ± 0.16), phlobatanin (26.36 ± 0.19), terpenoid (14.71 ± 0.14), steroid (20.37 ± 0.13), cardiac glycoside (20.15 ± 0.19) and alkaloid (23.90 ± 0.05), and absence of flavonoids and saponins. 9,12,15-octadecatrienoic acid, ethyl ester (Z,Z,Z)- (22.47%), Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro- (12.68%), hexadecanoic acid, ethyl ester (12.63%), Beta Tocopherol (12.63%) and phytol (12.61%) were revealed by GC-MS as the major bioactive constituents. The results of this study validated the rich bioactive constituents in *P. amarus* which may be the reason the plant is being used for the treatment of various ailments.

Keywords: *Phyllanthus amarus*, Bioactive compounds, Gas chromatography, Mass spectrometry.

INTRODUCTION

Phyllanthus amarus is a branching annual glabrous herb which belong to a large plant family of Euphorbiaceae. The *Phyllanthus* genus consists of about 1000 species, spread over the tropical and subtropical regions of the world like Africa, America, Asia and Australia [1-2]. *P. amarus* is slender and 30 - 60 cm high, bearing leaf-branchlets and distichous leaves with sessile, elliptic-oblong, obtuse, rounded base [3]. It is commonly called Gale of wind, Hurricane weed, Stone breaker, carry me seed, Shatterstone or Gulf leaf flower [2] however, in Yoruba, Southwest Nigeria, it is known as eyin olobe or dobisowo while the Asaba people in South south Nigeria called it buchi oro [4]. Medicinal plants have been used for the treatment of diseases for as long as man came into existence and *P. amarus*, as a result of its richness in medicinal values, has been in use since ancient time. It plays important roles in green medicine as a result of its safety and dependability than the costly synthetic drugs [2]. It has been reported in folk medicine that the plant is useful in the treatment of jaundice, urinogenital disorders, wounds, scabies, dropsy, intermittent fevers as well as diabetes, gonorrhoea, pain, inflammation, chronic dysentery, appendix, kidney problems and urinary bladder disturbances [5-7].

Several pharmacological activities of *P. amarus* have been reported. The methanolic extract was reported to have antioxidant properties by *in vitro* inhibition of lipid peroxidation and scavenging of superoxide and hydroxyl radicals [8]. Some of the isolated phenolics from the plant like phyllanthusiin D, amariin and repansunic acid have shown higher degree of antioxidant activity among the ellagitannins which were comparable to the rutin, flavonoids and quercetin 3-O-glucoside [9]. It has also been reported to have antidiabetic [10], antimicrobial [11], anticancer [12], antiviral [13] and anti-inflammatory [14] activities. Anti-inflammatory property of extracts from the plant was showed to have iNOS and COX-2 expression reduced and NF- κ B activation inhibited but did not affect AP-1. In human whole blood, the extracts were also found to inhibit interferon- γ , interleukin (IL)-1 β and IL-10 induction and reduce TNF- α production [14].

Phytochemistry has been known to be the heart of herbal therapy, and phytochemical research has continued to play important roles in the development of new drugs. The major class of bioactive compounds reportedly present in *P. amarus* are phenols, flavonoids, alkaloids, lignans, terpenes, tannins and volatile oils. Many of these phytoconstituents which have been isolated from the plant include alkaloids (securinine, nor-securinine, isobubbialine, epibubbialine, dihydrosecurinine), flavonoids (kaempferol, astragalol, quercetin, quercetin-3-O-glucoside, quercitrin) and tannins (geraniin, amariin, furosin, amarulone, corilagin, melatonin, phyllanthusin D) [2,3]. Others are lignans (Hypo-phyllanthin, phyllanthin, 5-dimethoxy-niranthin, phyltetralin, hinokinin, nirtetralin, 4-(3,4-dimethoxy-phenyl)-1-(7-methoxy benzo[1,3]dioxol-5-yl)-2,3-bismethoxymethyl-but --an-1-ol), volatile oils (Phytol and Linalool), Sterols (Amarosterol A and amarosterol B), triterpenes (Phenazine and phenazine derivatives, 2Z, 6Z, 10Z, 14E, 18E, 22E-farnesyl farnesol) [2,3].

The evaluation of different classes of organic compounds of medicinal importance present in *P. amarus* are presently not exhaustive. Therefore, this study is designed to further investigate the bioactive compounds in hydroalcohol extract of *P. amarus* leaf using GC-MS analysis.

MATERIALS AND METHODS

Plant collection

Fresh aerial parts of *Phyllanthus amarus* were collected from Sango-Otta, Ogun State, Southwest Nigeria. Plant sample was identified by Mr. Adeleke of the Department of Pharmacognosy, Faculty of Pharmacy, College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria.

Preparation of plant extract

The plant was thoroughly cleaned with running tap water and air-dried on the laboratory bench under normal atmospheric temperature for 7 days. Dried leaves were separated from stalks and ground to fine powder using porcelain mortar and pestle. Alcoholic extract was prepared by weighing 100 g dried powder, soaked in 2 L 70% ethanol for 72 h and shook intermittently. This was filtered with a muslin cloth and then Whatman No.1 filter paper and filtrate was concentrated and evaporated at 40°C under reduced pressure using rotary evaporator to obtain the extract.

Phytochemical screening

Phytochemical tests were carried out on the ethanol extract of *P. amarus* leaf to identify the constituents using standard procedures [15,16].

Tannins test

0.5 g of extract was boiled in 20 ml of water in a test tube and filtered. Few drops of 0.1% ferric chloride was added to filtrate. Brownish green or a blue-black coloration shows that tannin is present.

Phlobatannins test

0.5g extract was boiled with 2mL of 1% aqueous hydrochloric acid for 10 minutes. Red precipitate deposit shows the presence of phlobatannin.

Saponin test

2 g extract in 20 ml of distilled water was boiled in a water bath and then filtered. 5 ml of distilled water was mixed 10 ml of filtrate and vigorously shaken to obtain a persistent froth. Then, 3 drops of olive oil was mixed with the froth and vigorously shaken again. Emulsion formation indicates the presence of saponin.

Flavonoids test

5 ml dilute ammonia solution was added to a small portion of plant extract and conc. H₂SO₄ was also gently added, brownish precipitate indicates that flavonoid is present.

Steroids test

0.5 g ethanol extract was added to 2 ml of acetic anhydride with 2 ml H₂SO₄. The changed of colour from violet to blue or green shows the presence of steroids.

Terpenoids test (Salkowski test)

5 ml ethanol extract was added to 2 ml of chloroform and mixed, then 3 ml concentrated H₂SO₄ was gently added to form a layer. Formation of reddish brown colouration at inter indicates the presence of terpenoids.

Cardiac Glycosides test (Keller-Killani test)

5ml extract was mixed with a solution of 2 ml of glacial acetic acid and a drop of ferric chloride. 1 ml of concentrated H₂SO₄ was carefully added. Brown ring at interface shows the deoxysugar characteristic of cardenolides.

Alkaloid test

5 mg of ethanol extract was dissolved in 3 ml of acidified ethanol and slightly warmed, then filtered. To 1 ml of filtrate was added 1 ml of Dragendroff's and few drops of Mayer's reagents. Any turbidity observed shows that alkaloid is present.

Quantitative analysis

The quantitative analysis of plant extract was carried out to quantify the contents of various phytochemicals using standard procedures as described previously.

Estimation of total phenol content

The extract sample (0.5 mL, 1:100 dissolved in distilled water, w/v) was added to Folin-Ciocalteu reagent (5 mL, 1:10 diluted with distilled water, v/v) and mixed for 5 min, then aqueous Na₂CO₃ (4 mL, 1 M) was added. This was allowed to stand for 15 min and then determined the phenols using spectrophotometer at 765 nm. A standard curve of gallic acid (0-250 mg/mL) in methanol: water (50:50, v/v) was prepared. The total phenol value was expressed as mg gallic acid equivalent (mg GAE/g of extract) [17,18]

Estimation of total alkaloids content

0.5 g extract was dissolved in 95% solution of acetic acid in ethanol with a ratio of 1:95 of the solution. The mixture was filtered after 24 hours and filtrate was concentrated by evaporation to one quarter of its volume. Concentrated aqueous NH₄OH was added drop wise until the alkaloid was precipitated. The precipitate was filtered with filter paper, washed with 1% ammonia solution and dried in the oven at 80°C. The alkaloid content was calculated as a percentage of the weight of original extract analyzed [15].

Estimation of total condensed tannins content

The total condensed tannins content was estimated by a modified version of a method reported previously [19,20]. 0.5 g extract sample was mixed with 5 mL vanillin-HCl (8% conc. aq. HCl and 4% vanillin in methanol). After 20 min, the absorbance was taken at 500 nm. Catechin was used as the standard. The condensed tannin content was expressed as mg catechin equivalent (mg CAE/g of extract).

Estimation of total terpenoids content

10 g of ethanol extract was soaked in alcohol for 24 hours. It was filtered and filtrate was extracted with petroleum ether. The extract was dried, weighed and regarded as total terpenoids [21].

GC-MS analysis

The GC-MS analysis was carried out on hydroalcohol extract of *P. amarus* according to the method described earlier by Ajayi *et al.* [22, 23].

The GC-MS used comprised of a Gas Chromatograph (Model 6890 series), a product of Hewlett Packard which was equipped with FID (flame ionization detector) and coupled with 7683 series injector (Hewlett Packard) possessing 250°C temperature MS transfer line. The column of the GC was a fused silica capillary column- HP-5MS (30 x 0.25 mm) with film thickness of 1.0 µm. Oven temperature was initially held at 50°C for 5 min and then raised to 250°C at a rate of 2°C/min and carrier gas used was helium gas (99.999%). 1.0 µ extract was injected at a split ratio of 1:30. Agilent Technology Network Mass Spectrometer (Model 5973 series) equipped with NIST08 Library software database was used for the identification of compounds by comparing the mass spectrum of unknown compounds with the known compounds stored in the software database Library.

Statistical analysis

All statistical values were expressed as mean ± standard error of mean (SEM). The analysis was carried out using GraphPad Prism version 5.04.

RESULTS

Qualitative phytochemical screening

The qualitative phytochemical screening of hydroalcohol extract of *P. amarus* leaf revealed the presence of tannins, phenol, phlobatanins, terpenoids, steroids, alkaloids, cardiac glycosides and reducing sugars whereas, saponins and flavonoids were absent (Table 1).

Qualitative phytochemical analysis

The total phenols and total tannins contents were quantified from the pre-established gallic acid and catechin standard curves while the total terpenoids and total alkaloids contents were also estimated. Results showed that the total phenols and tannins contents were 34.31±0.07 mg GAE/100 g of extract and 21.15±0.16 mg CAE/100 g of extract respectively (Table 2). Total terpenoids and total alkaloids contents were also showed to be 14.71±0.14 mg/100 g of extract and 23.90±0.05 mg/100 g of extract respectively (Table 2). This showed that the total phenols content value is the highest among the quantified phytochemical components.

GC-MS analysis

The chromatographic profile of the hydroalcoholic extract of *P. amarus* leaf is shown in Figure 2 while the list of phytochemical compounds is in Table 3. There are 12 bioactive compounds identified (Table 3) corresponding to the peaks shown in Figure 2. However, 5 major compounds were identified which include 9,12,15-Octadecatrienoic acid, 1-methyl ester, (Z,Z,Z)- (22.47%), Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro (12.68%), β-Tocopherol (12.63%), Hexadecanoic acid, ethyl ester (12.63%) and Phytol (12.61%) (Tables 3 and 4). The GC-MS mass spectrum and structures of these major phytochemicals are shown in Figures 3-8. Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro- (12.63%), C₂₀H₄₀O₂, 302 mol. wt is identified for the first time by GC-MS in hydroalcohol extract of *P. amarus* leaf.

DISCUSSION

Plants are sources of new drugs and their various species are still poorly harnessed for this important use. Secondary metabolites such as phenolics, flavonoids, tannins, saponins, alkaloids, phlobatanins, steroids, anthraquinones, cardiac glycosides and carbohydrates are known to be present in plants. These phytochemicals or secondary metabolites are also known to possess biological activities such as anticancer, antioxidant, anti-inflammatory, antidiabetic, antimicrobial, hepatoprotective, antianthritic, hypoglycemic and hypocholesterolemic

[23,25]. Several of these secondary metabolites and phytochemicals have been reportedly present in *P. amarus*, which include alkaloids, flavonoids, lignans, tannins, terpenoids, sterols and volatile oils [2]. In this study, tannins, phenol, phlobatanins, terpenoids, steroids, alkaloids, cardiac glycosides and reducing sugars were noted to be present in the hydroalcohol extract of *P. amarus* leaf. These compounds are responsible for many biological functions in human body, therefore possess great pharmaceutical applications.

Presence of phenolics which are noted to be good free radical scavengers, is an indication that the plant may have antioxidant properties, and its estimation in this extract provides a measure of antioxidant potential of the plant. This study also showed the presence of tannin, this secondary metabolites are linked to antibacterial activity and glycoside which are involved in several pharmacological effects including lowering of blood pressure [26,27]. Formation of tannins is by quinone units polymerization and also complex with protein through their molecular actions. Hence, tannins antimicrobial action may be due to its ability to produce microbial adhesions inactivation, and inhibit enzymes and cell membrane transport proteins [28]. Alkaloids are generally known to be toxic to organisms. However, they also have pharmacological activities and are commonly used as medications, as analgesic, antimicrobial, antipyretic, antihypertension agent, stimulant and local anesthetic, psychedelic, anticancer, antibacterial, cholinomimetic, vasodilator, anticholinergic, antiarrhythmia, antimalarial and antiasthma [28]. In this study, alkaloid is present, which further confirms the use of the plant as antipyretic, antimalarial and analgesic drug. The use of the plant as antimalarial, antiarrhythmia and analgesic agents may also be attributed to the presence of alkaloids and tannins [29]. Terpenes and essential oils are major bioactive constituents of plants, and *P. amarus* collected from our source in Nigeria was found to possess a good amount of terpenoids. Active biological components of plants tend to change with time, season and age, chemistry of soil and plant variety, and the secondary metabolites production in plants are influenced by these major factors [29]. These conditions could be attributed to the absence of flavonoids and saponins in this plant we studied.

Gas chromatography, mass spectrometry analysis has recently become a major technique in identification of bioactive compounds and elucidation of their chemical structures in plants and at the same time suggest their possible curative properties [23,30]. Phyllanthin, one of the important bioactive phytoconstituents lignan from *P. amarus* was characterized by using techniques like UV-visible spectrophotometry, mass spectrophotometry, elemental analysis, CNMR, FTIR, HNMR, and mass spectral analysis [2]. Several other bioactive compounds have been isolated and characterized from *P. amarus*. In this study, 12 phytochemicals were revealed to be present in hydroalcohol extract of plant leaf. The 5 major bioactive compounds are 9,12,15-Octadecatrienoic acid, 1-methyl ester, (Z,Z,Z)- (22.47%), Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro (12.68%), β-Tocopherol (12.63%), Hexadecanoic acid, ethyl ester (12.63%) and Phytol (12.61%). The oils from *P. amarus* were previously analyzed by GC-MS and 82 compounds were reportedly identified which were responsible for 87.6% of the oil content. These oils include phytol (13.0%) and linalool (36.4%). Others were hexahydrofarnesyl acetone (3.4%), nonacosane (2.1%), pentacosane (2.5%), (E)-b-ionone (2.3%), naphthalene (2.4%), tetracosane and octacosane (ca. 1.7%). Classes of compounds also reportedly present in *P. amarus* oil are oxygenated monoterpenoids (11.0%), monoterpene hydrocarbons (0.2%), sesquiterpene hydrocarbons (1.3%), diterpenoids (8.5%), oxygenated sesquiterpenoids (3.3%), fatty acids (3.9%), aliphatic alcohols (51.2%), ketones (0.5%), aldehydes (8.0%) and esters (0.3%) [31]. Two new lignans, 3-(3,4-dimethoxybenzyl)-4-(7-methoxy-benzo[1,3]dioxol-5-yl-methyl)-dihydrofuran-2-one and 4-(3,4-dimethoxy-phenyl)-1-(7-methoxy-benzo[1,3]dioxol-5-yl)-2,3-bis-methoxymethyl-butan-1-ol were also reportedly isolated and characterized from the leaves of *P. amarus* [32].



Figure 1: *Phyllanthus amarus* leaves

Table 1: Phytochemical screening results of hydroalcohol extract of *P. amarus* leaf

S/N	Parameters	Result
1	Saponin	Absent
2	Tannin	Present
3	Phenol	Present
4	Phlobatanin	Present
5	Reducing sugar	Present
6	Flavonoid	Absent
7	Terpernoid	Present
8	Steroid	Present
9	Cardiac glycoside	Present

Table 2: Quantitative analysis results of hydroalcohol extract of *P. amarus* leaf constituents

Phytochemical constituent	Mean \pm SEM(mg/100g)
Tannin	21.15 \pm 0.16
Phenol	34.31 \pm 0.07
Terpernoid	14.71 \pm 0.14
Alkaloid	23.90 \pm 0.05

Values were expressed as Mean \pm SEM of 3 readings

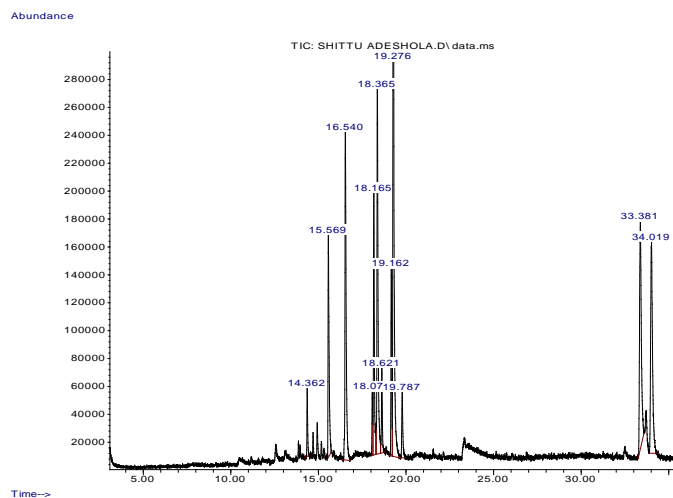


Figure 2: GC-MS chromatogram of hydroalcohol extract of *P. amarus* leaf showing different peaks of various phytochemical components present

Table 3: Phytochemical compounds identified by GC-MS in hydroalcohol extract of *P. amarus*

Peaks	Retention Time (min)	Identified compound	% of Total	Chemical Formula
1	14.362	Neophytadiene	1.49%	C ₂₀ H ₃₈ .
2	15.569	Hexadecanoic acid, methyl ester	7.27%	C ₁₇ H ₃₄ O ₂ .
3	16.54	Hexadecanoic acid, ethyl ester	12.63%	C ₁₈ H ₃₆ O ₂
4	18.079	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	1.45%	C ₁₈ H ₃₂ O ₂
5	18.165	9-Octadecenoic acid, methyl ester,	7.46%	C ₁₉ H ₃₆ O ₂
6	18.365	Phytol	12.61%	C ₂₀ H ₄₀ O
7	18.621	Methyl stearate	2.54%	C ₁₉ H ₃₈ O ₂
8	19.162	Linoleic acid ethyl ester	4.57%	C ₂₀ H ₃₆ O ₂
9	19.276	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	22.47%	C ₂₀ H ₃₄ O ₂
10	19.787	Octadecanoic acid, ethyl ester	2.21%	C ₂₀ H ₄₀ O ₂
11	33.381	Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro-	12.68%	C ₂₂ H ₂₄ N ₂ O ₄
12	34.019	Beta Tocopherol	12.63%	C ₂₈ H ₄₈ O ₂ .

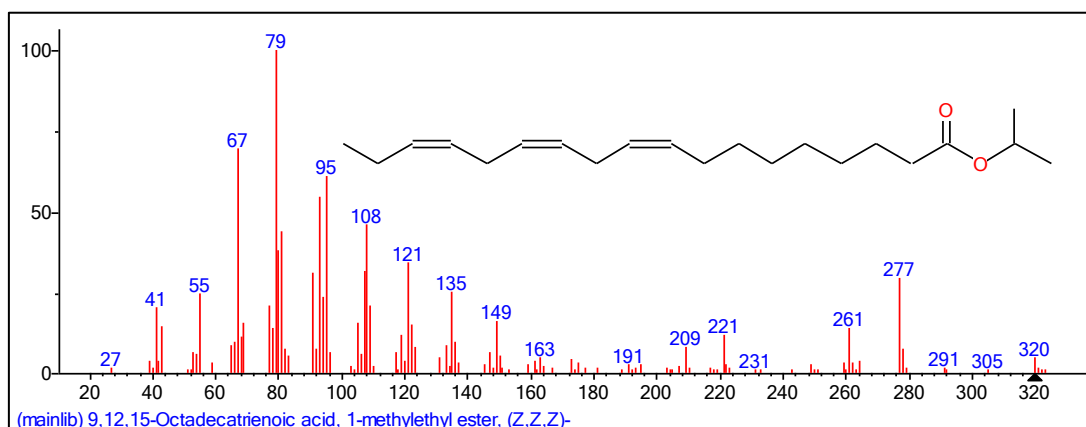


Figure 3: GC-MS mass spectrum and molecular structure of 9,12,15-Octadecatrienoic acid, 1-methyl ester, (Z,Z,Z)-

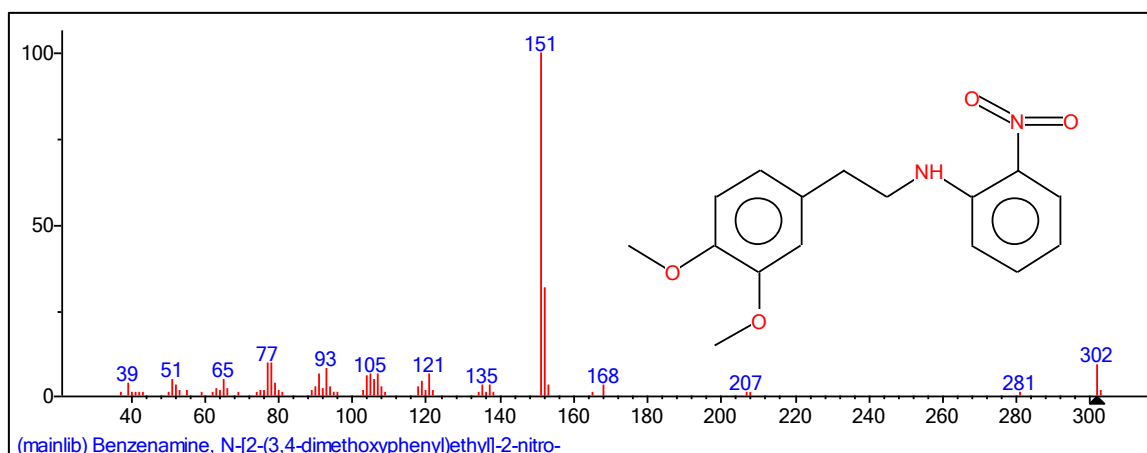


Figure 4: GC-MS mass spectrum and molecular structure of Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro-

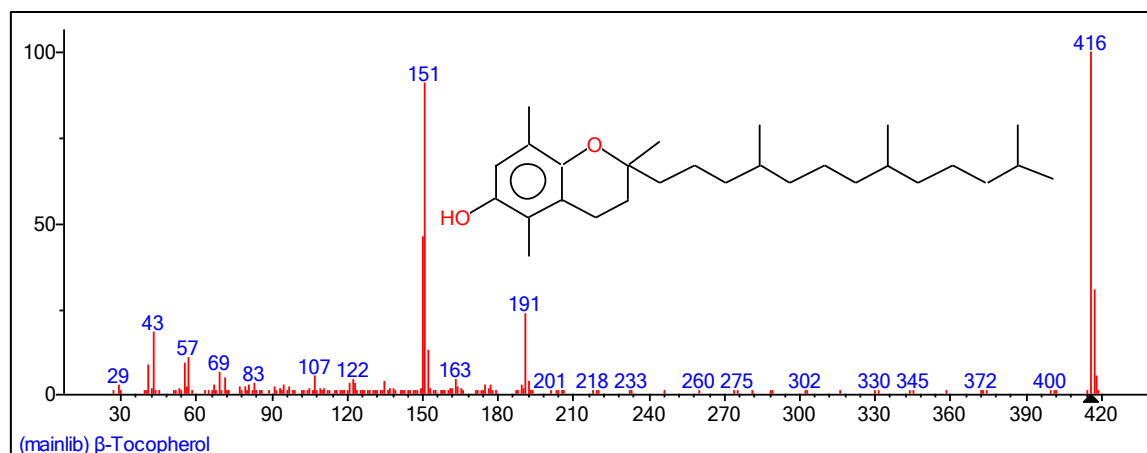


Figure 5: GC-MS mass spectrum and molecular structure of β -Tocopherol

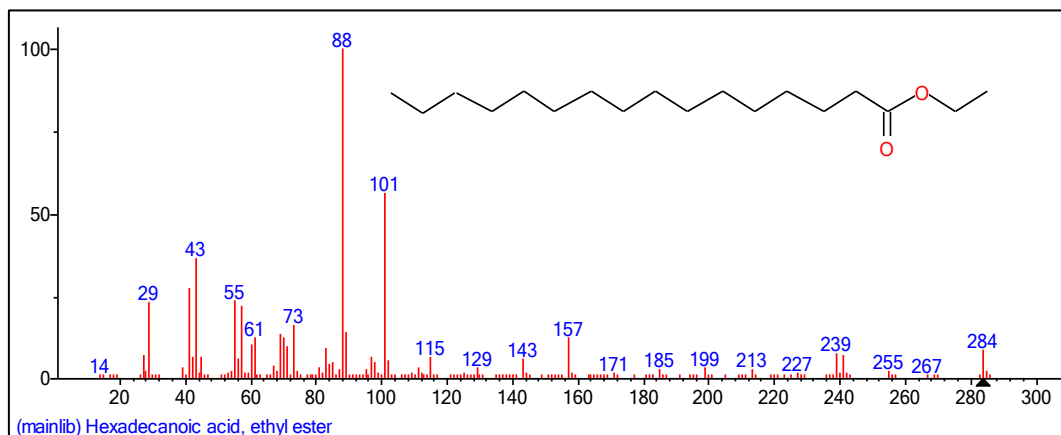


Figure 6: GC-MS mass spectrum and molecular structure of Hexadecanoic acid, ethyl ester

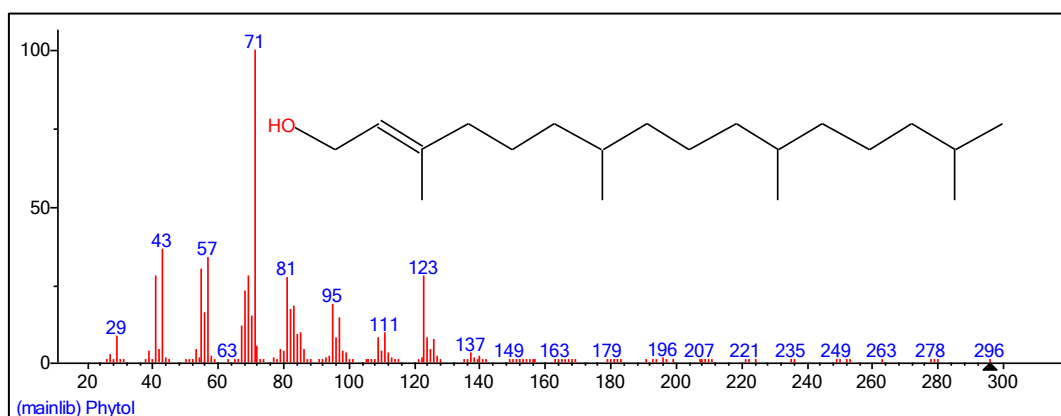


Figure 7: GC-MS mass spectrum and molecular structure of Phytol

Table 4: Molecular weight and biological activities of major phyto-compounds in *P. amarus* identified by GC-MS

S/N	Name of compound	Molecular weight	Biological activities
1.	9,12,15-Octadecatrienoic acid, 1-methyl ester, (Z,Z,Z)-	320	Anti-Inflammatory, Hypo-cholesterolemic, Cancer preventive, Hepato-protective
2.	Benzenamine,N-[2-(3,4-dimethoxyphenyl) thyl]-2-nitro-	302	Bactericidal
3.	β -Tocopherol	416	Antioxidant.
4.	Hexadecanoic acid, ethyl ester	284	Flavor and fragrance
5.	Phytol	296	Anticancer, antioxidant, anti-inflammatory, diuretic, antitumor, antimicrobial

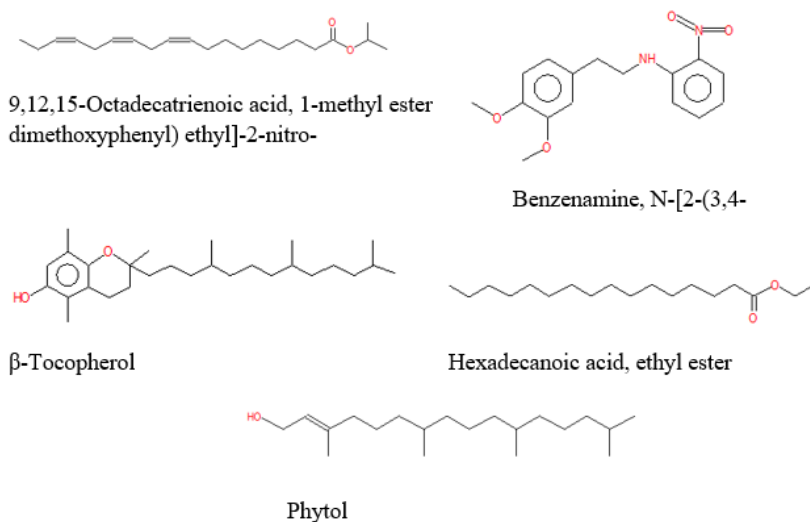


Figure 8: Structures of major bioactive components in hydroalcohol extract of *P. amarus* leaf

CONCLUSION

Today, there is an increased demand globally for the various products of medicinal plant like nutraceuticals, pharmaceuticals, phytochemicals, cosmetics and other products. Several bioactive compounds have been isolated and studied for phyto pharmacological activity. In the last few years, pharmaceutical industries have made massive investment in pharmacological and phytochemical researches in an effort to discover much more potent plant drugs and/or new drugs. Therefore, the present study has shown that hydroalcohol extract of *Phyllanthus amarus* leaf possess major phytochemicals that corroborate well with the claims of the traditional medicinal values of the plant. Likewise, Benzenamine, N-[2-(3,4-dimethoxyphenyl)ethyl]-2-nitro- (12.63%) was first identified by GC-MS in hydroalcohol extract of *P. amarus* leaf.

Conflict of interest

Authors declare no conflict of interest

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