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Research Article
Purification and GC-MS Spectroscopic Identification of Active Antioxidant Principles in Ethanol Extract of *Phyllanthus amarus* Leaves

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Abstract

Background and Objective: The presence of natural antioxidant activity in plants has been well acknowledged worldwide. There is increasing demand for a natural antioxidant to replace synthetic additives in the food and pharmacological industries. The main goal of this study was to fractionate and identify active antioxidant principles in *Phyllanthus amarus* leaves. **Materials and Methods:** Samples of matured and healthy looking leaves of *Phyllanthus amarus* were collected. Extraction of the plant was done using ethanol and fractionation with different solvents of varied polarity via column chromatography, starting from n-hexane, chloroform, ethyl acetate, ethanol, methanol and lastly, water. The fractions were screened for antioxidant activity by a 2, 2-Diphenylpicrylhydrazyl (DPPH) assay. Fraction 10b shows high antioxidant activity and was subjected to further analysis using the GC-MS and FTIR analysis and 32 compounds were identified. Total phenol and total flavonoid contents were analyzed by the Folin Ciocalteu method and aluminium chloride colorimetric method, respectively. **Results:** The result for total antioxidant activity shows that fraction 10b has the highest antioxidant activity (83 mg mL^{-1}) while fraction 2a shows the lowest antioxidant activity (13 mg mL^{-1}). The result for total phenol content showed that fraction 10b has the highest phenol content (430 mg mL^{-1}) and fraction 2a has the lowest phenol content (9 mg mL^{-1}). Fraction 3b shows the highest concentration of total flavonoid content (143 mg mL^{-1}) while fraction 2a shows the lowest concentration (23 mg mL^{-1}). The result shows a strong positive correlation between total antioxidant and total phenol content. The relationship between total antioxidant and total flavonoid content shows negative. **Conclusion:** This present study concluded that the high phenol content present in *Phyllanthus amarus* leaves is entirely responsible for the antioxidant activity of the plant.

Key words: Fractionation, antioxidant, GC-MS profile, *Phyllanthus amarus*, flavonoid content

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The use of herbal plants to treat various human diseases is global and universal, particularly in third world countries due to their easy access and low cost, compared with advanced Western medicines[1,2]. Plants with medicinal properties are efficiently used, mainly by the rural or tribal communities in remote areas, not only today but also in ancient communities in prehistoric times[1,3,4] and are highly respected in certain tribal communities due to belief such as being a tonic of life[5].

The medicinal value of plants lies in some chemical substances or group of compounds that produce a definite physiological action in the human body. These chemical substances are called phytochemicals[6]. These chemical substances can also be referred to as secondary metabolites. The most important of these bioactive groups are the alkaloids, terpenoids, steroids, flavonoids, tannins, saponins, glycosides and phenol compounds[7].

Phyllanthus amarus is a cosmopolitan herb that belongs to the family Euphorbiaceae. Its common names include the stone breaker (English), Eyin Olobe (Yoruba), Geron-Tsuntsaye (Hausa), Enyi Kwo Nwa (Igbo). The *P. amarus* is synonymous with *Phyllanthus niruri* L. and sometimes the are misidentified for each other. However, reorganization of the *Phyllanthus* genus has classified *P. amarus* as a type of *P. niruri*[8]. *Phyllanthus amarus* has shown a wide range of pharmacological effects which includes hepatoprotective, antiviral, antibacterial, antioxidant, anti-inflammatory, anticancer, hypolipidemic, antidiabetic, antispasmodic, nephroprotective and diuretic properties[9]. The use of *P. amarus* has gained considerable momentum because of its novel antiviral activity against the hepatitis B virus and for its several other biological activities[10]. Of all the *Phyllanthus* species, the phytochemistry of *P. amarus* is well studied and phyllanthin is the predominant compound responsible for many biological actions. This present study highlights the active antioxidant principles of the plant.

MATERIALS AND METHODS

Sample collection and preparation: Matured and healthy looking leaves of *Phyllanthus amarus* leaves were collected from the School Environment of Federal University, Wukari, Taraba State, Nigeria in May, 2019. The leaves were identified at the species level at the Herbarium Unit of the Department of Biological Sciences Federal University. The leaves were thoroughly washed with tap water to remove the dust and soil particles. The leaves were air-dried under the shade to prevent ultra-violet rays from inactivating the chemical constituents[11,12]. The dried leaves were pounded into a fine powder using mortar and pestle and then stored and labelled in dry containers until needed. Only healthy plants were used as the leaves were examined to be free from diseases. The experiment was carried out in 18 months.

Ethanolic extraction: Five hundred grams of the pulverized leaves were weighed and soaked in 1300 mL of ethanol for 48 hrs at room temperature. It was stirred continually after every 8 hrs, the extracts were filtered out first using a clean white sieving mesh and then using Whatman No.1 filter paper. The filtrate was allowed to dry by air under fan and extract was transferred to air-tight containers, corked and preserved in the refrigerator at 4°C until required.

Fractionation of ethanolic extract: The ethanol extract was assigned to column chromatography to separate the extract into its component fractions. Silica gel was used in packing the column while varying solvent combinations of increasing polarity were used as the mobile phase.

Packing of the column: The packing of the column was done according to the method of Yakubu *et al.*[13]. The lower part of the glass column was soaked with glass wool with the aid of a glass rod. The 235 g of silica gel (mesh size) was dissolved in 255 mL of absolute n-hexane to make the slurry. The chromatographic column (30 mm diameter by 40 cm height) was packed with silica gel and the free flow of the solvent was allowed into a conical flask below. The set-up was then seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked and the column was then allowed for 24 hrs to stabilize after which, the clear solvent at the top of the silica gel was allowed to drain down the silica gel meniscus.

Elution: The method of Yakubu *et al.*[13] was adopted for the elution. The extract (2 g) was dissolved in 15 mL absolute ethanol and the solution was applied to the chromatographic column (30 mm diameter by 40 cm height). Elution of the extract was done with the solvent system of gradually increasing polarity, beginning from n-hexane, ethyl acetate, ethanol, methanol and distilled water. The following ratio of solvent combinations was sequentially used in the elution process: N-hexane: Chloroform 100:0, 50:50, Chloroform: Ethyl acetate 100:0, 50:50, Ethyl acetate: Ethanol 100:0, 50:50,

Ethanol: Methanol 100:0, 50:50, Methanol: Distilled water 100:0, 50:50, Distilled water 100. A measured volume (400 mL) of each solvent combination was poured into the column each time using a separator funnel. Eluted fractions were then collected in aliquots of 200 mL using beakers.

Total antioxidant capacity (TAC): DPPH Assay for total antioxidant capacity was carried out as described by Kumar *et al.*[14]. The absorbance was measured at 517 nm. Measurements were done in duplicate for each fraction. About 0.02 g of DPPH was dissolved in 100 mL of methanol, 2 mL of DPPH solution was added into test tubes, 100 µL of each fraction were added and the solution was thoroughly mixed. The absorbance was read within 30 sec at 517 nm in a UV-Visible spectrometer. Total Antioxidant Capacity (TAC) was calculated as mg mL^{-1} of Trolox Equivalent (TE).

Total phenolic content (TPC): The concentration of phenolic content in the plant extract was determined colorimetrically at 415 nm, using Folin-Ciocalteu reagent and expressed as Gallic Acid Equivalent (GAE). Exactly 100 µL of the sample was added to the test tube containing 2.5 mL Folin reagent. Sodium carbonate solution (2 mL) was added and was allowed to stand for about 15 min at room temperature. Results were expressed as GAE.

Determination of total flavonoids content (TFC): Total Flavonoid Content was estimated using the aluminium chloride colorimetric method of Chang *et al.*[15]. Derivation of the calibration curve was done using quercetin. Exactly 100 µL of the sample was added into a test tube containing 15 mL of methanol. The 100 µL of 10% aluminium chloride ($AlCl_3$) and 100 µL of potassium acetate (CH_3COOK) were added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 765 nm and the concentration of flavonoids in the sample was estimated from the calibration curve. The amount of 10% aluminium chloride was substituted by the same amount of distilled in the water blank. Total flavonoids were expressed as mg mL^{-1} Quercetin Equivalent (QE).

RESULTS AND DISCUSSION

Total antioxidant capacity, phenolic, flavonoids contents of different fractions: Figure 1 showed the result for Total Antioxidant Capacity (TAC) where the ratio of methanol to water (fraction 10b) has the highest antioxidant activity (83 mg mL^{-1}) with fraction 7b (ethanol) showing the lowest antioxidant activity (13 mg mL^{-1}). The result for total phenolic content as presented in Fig. 2 showed that fraction 10b (430 mg mL^{-1}) has the highest phenolic content and fraction 2a shows the lowest phenolic content (9 mg mL^{-1}). Figure 3 showed that fraction 3b ((143 mg mL^{-1}) has the highest concentration of total flavonoid content while fraction 2a (23 mg mL^{-1}) has the lowest concentration of total flavonoid content.

Correlation between total antioxidant capacity and total phenolic content of different fractions: There is a weak positive correlation ($R^2 = 0.281$) between the total antioxidant

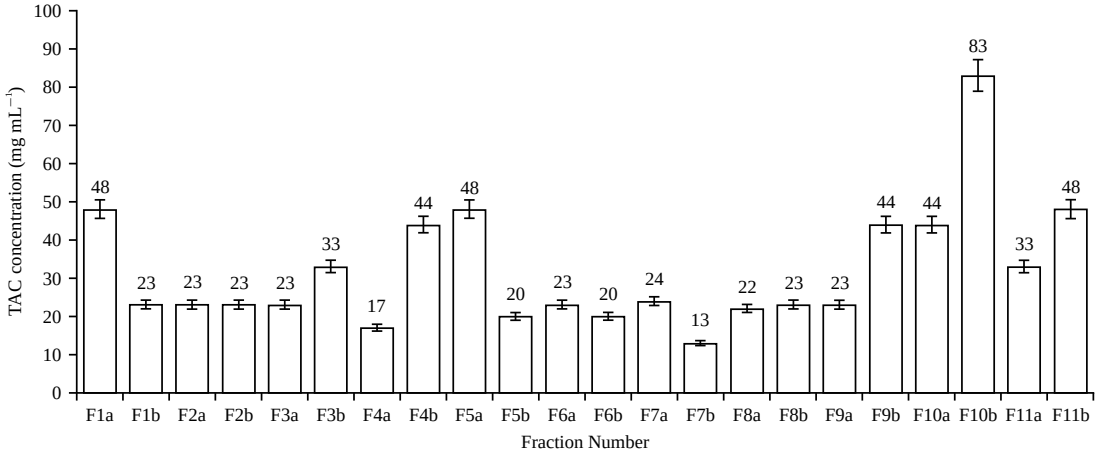


Fig. 1: Total antioxidant capacity

Fraction 1: N-hexane (100:0), F2: N-hexane+chloroform (50:50), F3: Chloroform (100:0), F4: Chloroform+ethyl acetate (50:50), F5: Ethyl acetate (100:0), F6: Ethyl acetate+ethanol (50:50), F7: Ethanol (100:0), F8: Ethanol+methanol (50:50), F9: Methanol (100:0), F10: Methanol+distilled H_2O (50:50) and F11: Distilled H_2O (100:0)

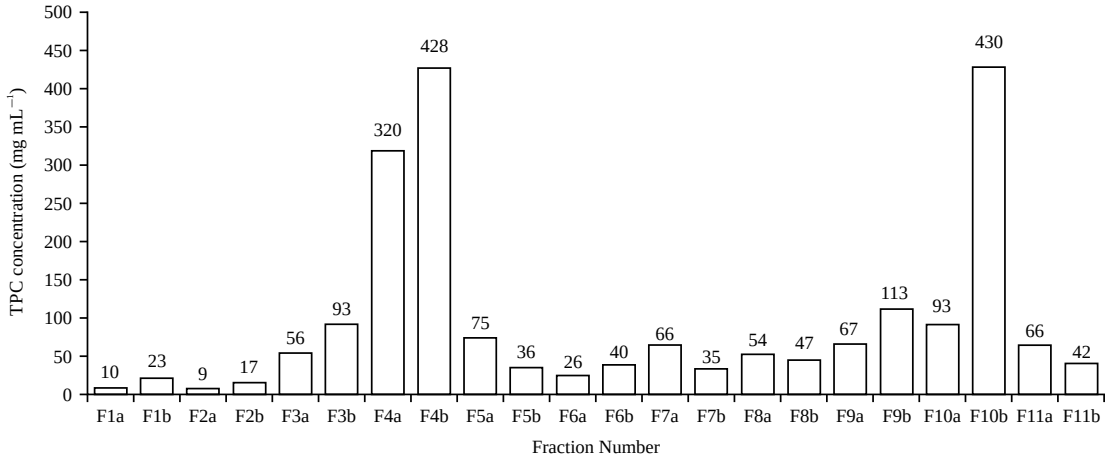


Fig. 2: Total phenolic content

Fraction 1: N-hexane (100:0), F2: N-hexane+chloroform (50:50), F3: Chloroform (100:0), F4: Chloroform+ethyl acetate (50:50), F5: Ethyl acetate (100:0), F6: Ethyl acetate+ethanol (50:50), F7: Ethanol (100:0), F8: Ethanol+methanol (50:50), F9: Methanol (100:0), F10: Methanol+distilled H_2O (50:50) and F11: Distilled H_2O (100:0)

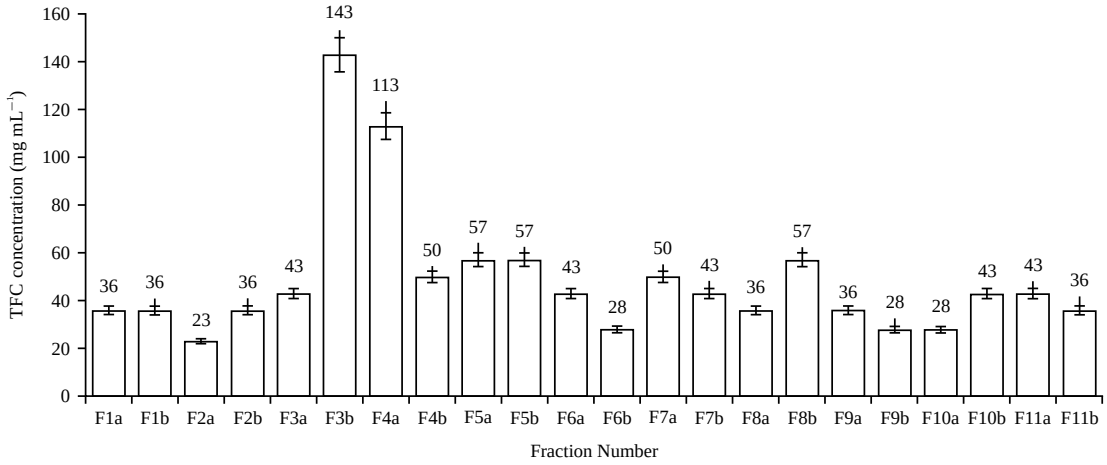


Fig. 3: Total flavonoid content

Fraction 1: N-hexane (100:0), F2: N-hexane+chloroform (50:50), F3: Chloroform (100:0), F4: Chloroform+ethyl acetate (50:50), F5: Ethyl acetate (100:0), F6: Ethyl acetate+ethanol (50:50), F7: Ethanol (100:0), F8: Ethanol+methanol (50:50), F9: Methanol (100:0), F10: Methanol+distilled H_2O (50:50) and F11: Distilled H_2O (100:0)

capacity and total phenolic content of the fractions obtained from the ethanolic extract of *Phyllanthus amarus* leaves, presented in Fig. 4.

Correlation between total antioxidant capacity and total flavonoids content of different fractions: There is a positive (R^2 = 0.010) correlation exhibited between the total antioxidant and total flavonoid contents of the fractions from the ethanolic extraction extract of *Phyllanthus amarus* leaves as presented in Fig. 5.

Correlation between total phenolic and total flavonoids contents of different fractions: A positive correlation (R^2 = 0.078) is shown between the total phenolic and total flavonoid contents of the fraction from the ethanolic extract of *Phyllanthus amarus* leaves as presented in Fig. 6.

Results of GC-MS and FTIR of fraction 10b of ethanolic extract: The compounds identified were from the fraction eluted with methanol and distilled H_2O (fraction 10b).

There were 32 compounds identified from the GC-MS profile of fraction 10b. Some of the compounds include pentadecanoic acid, hexadecanoic acid, 1-octadecene, heptadecanoic acid, tetrapentacontane and 9, 12, 15-Octadecatrienoic acid which were identified with antioxidant activity. Pentadecanoic acid ($C_{15}H_{30}O_2$) with a molecular weight of 242.39 g mol^{-1} and density of 842 kg m^{-3} is said to improve insulin sensitivity and also used as a marker

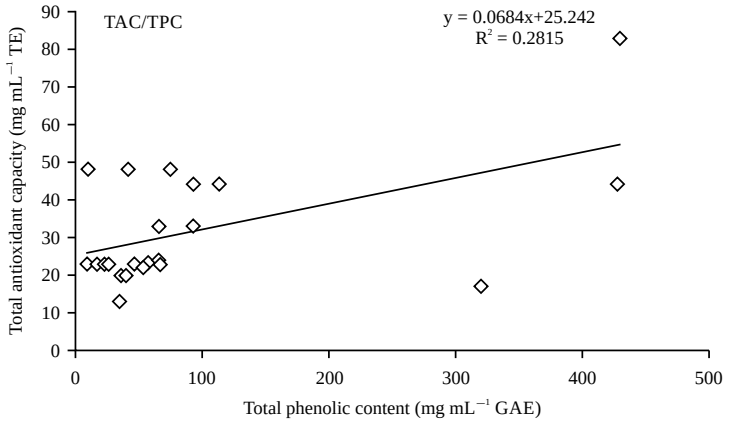


Fig. 4: Linear correlation between total antioxidant capacity and total phenol content of fractions obtained from the ethanolic extract of *Phyllanthus amarus* leaves

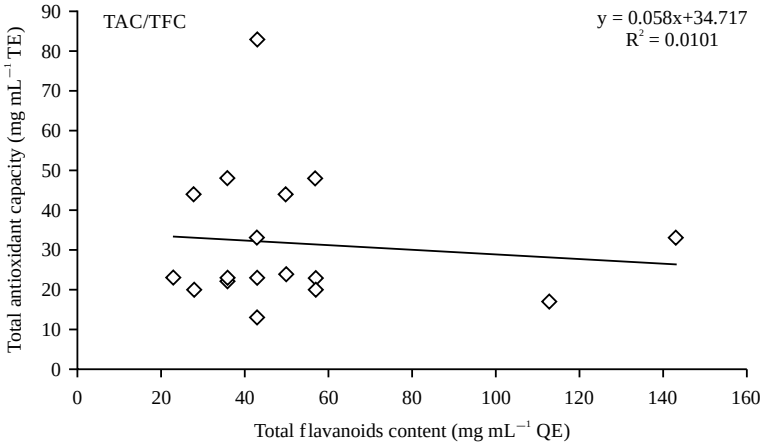


Fig. 5: Linear correlation between total antioxidant capacity and total flavonoid contents of the fractions from the ethanolic extract of *Phyllanthus amarus* leaves

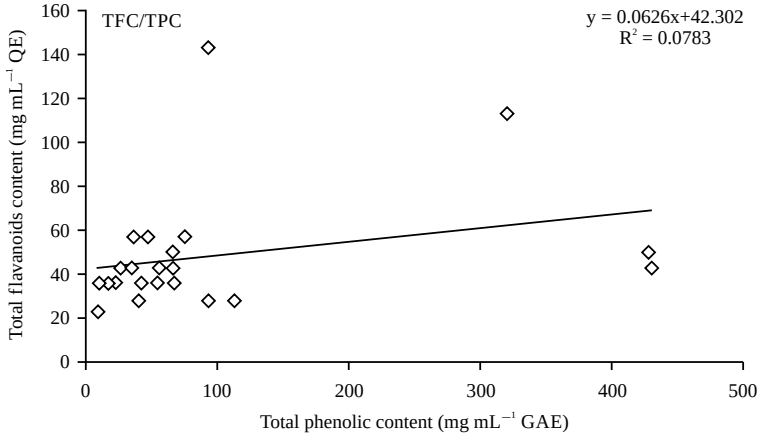


Fig. 6: Linear correlation between the total phenolic and total flavonoid contents of the fractions obtained from *Phyllanthus amarus* leaves

Table 1: GC-MS profile for fraction 10b (Methanol+distilled water)

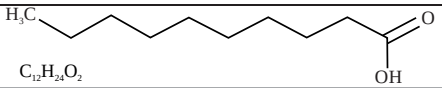
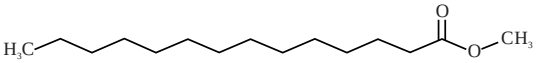
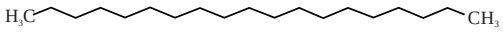
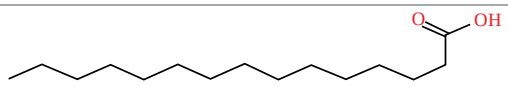
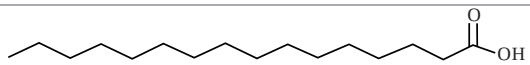
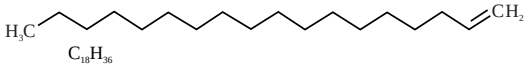
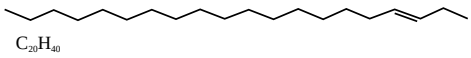
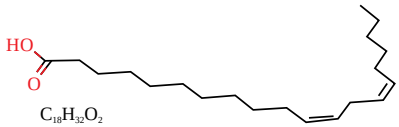
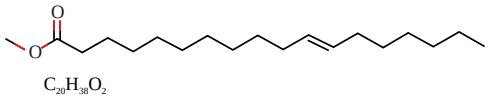
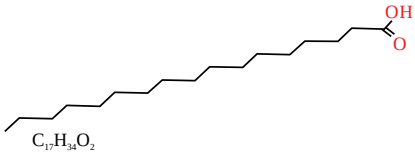
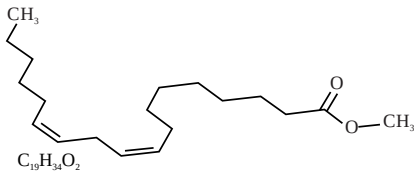
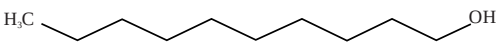
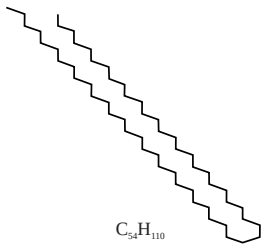
RT	Compounds	Area (%)	Chemical structures	Biological functions
7.255	Dodecanoic acid	0.44	 $C_{12}H_{24}O_2$	Antibacterial[16]
9.191	Methyl tetradecanoate	3.15	 $C_{15}H_{30}O_2$	Membrane stabilizer, energy storage[17]
12.219	Nonadecane	3.05	 $C_{19}H_4O$	It is used as a fragrance agent in cosmetics and perfumes
12.802	Pentadecanoic acid	7.34	 $C_{15}H_{30}O_2$	Antioxidant, antifungal and antibacterial[7]
13.508	Hexadecanoic acid	0.86	 $C_{16}H_{32}O_2$	Antioxidant, anti-inflammatory, anti-androgenic[18]
15.038	1-Octadecene	4.35	 $C_{18}H_{36}$	Anticancer and antioxidant[19]
15.263	3-Eicosene	3.14	 $C_{20}H_{40}$	Anti-nutritional[20]
15.746	9,12-Octadecadienoic acid	9.31	 $C_{18}H_{32}O_2$	Anticancer, anti inflammatory[21]
17.957	11-Octadecenoic acid, methyl ester	17.77	 $C_{20}H_{38}O_2$	Antihistaminic, antimicrobial
18.093	Heptadecanoic acid	1.45	 $C_{17}H_{34}O_2$	Antimicrobial, antioxidant
18.308	Methyl 10-Trans, 12-Cis-Octadecadienoate	3.14	 $C_{19}H_{34}O_2$	Cosmetic, flavour and fragrance agent
18.425	1-Decano	2.26	 $C_{10}H_{22}O$	It is used to manufacture plasticizers, lubricants, surfactants and solvents
18.598	Tetrapentacontane	0.97	 $C_{54}H_{110}$	Antioxidant, antimicrobial[22]

Table 1: Continue

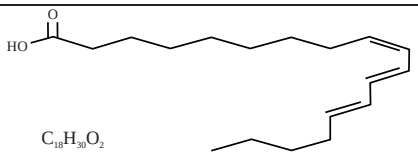
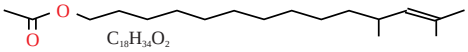
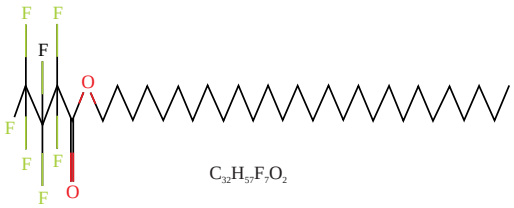
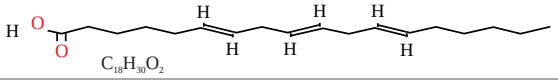
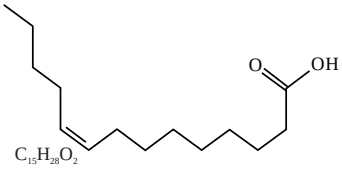
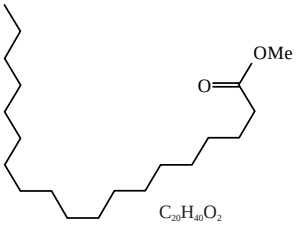
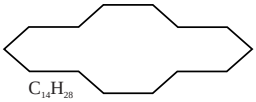
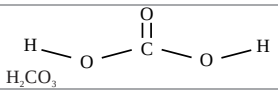
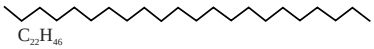
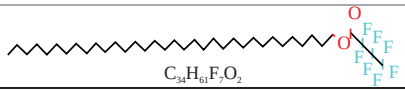
RT	Compounds	Area (%)	Chemical structures	Biological functions
18.684	9,12,15-Octadecatrienoic acid	1.17		Antioxidant, anticancer, antitumor[23]
18.764	11,13-Dimethyl-12-Tetradecen-1-Ol Acetate	2.10		No activity found
18.953	Octacosyl heptafluorobutyrate	1.16		Anticancer, anti-inflammatory[23]
19.163	Cycloeicosane 6, 9, 12-Octadecatrienoic acid	1.92		No activity found
19.291	Cis-9-Tetradecenoic acid	2.87		Apoptosis inducer[13]
19.582	Methyl 18-Methylnonadecanoate	2.62		Anti-tumor
19.902	1-Eicosene cyclotetradecane	0.62		Antimicrobial agent
20.017	1-Eicosene carbonic acid	0.36		No activity found
20.096	Carbonic acid	0.51		It maintains acid-base homeostasis
20.315	Tetracosane	0.61		Antibacterial
20.548	Docosane	0.17		Antimicrobial activity
20.656	1-Nonadecene oxalic acid	0.31		Cancer inhibitor and anti-obesity
20.974	1-Bromo-11-Iodoundecane	0.22		No activity found
21.214	Tetratriacontyl heptafluorobutyrate	0.28		No activity found

Table 1: Continue

RT	Compounds	Area (%)	Chemical structures	Biological functions
21.540	Pentacosane	0.32	$C_{25}H_{52}$	No activity found
21.968	Docosanoic acid	0.16	$C_{22}H_{44}O_2$	It is used as an anti-foaming agent in detergent[24]
22.317	Bis (2-Ethylhexyl) phthalate	0.41	$C_{24}H_{38}O_4$	It has a role as an apoptosis inhibitor, an androstane receptor agonist and a plasticiser[25]
22.850	Hexacosane	0.02	$C_{26}H_{54}$	Antifungal[26]
24.728	Tetracosanoic acid	0.02	$C_{24}H_{48}O_2$	Membrane stabilizer, energy source, energy storage and nutrient[27]

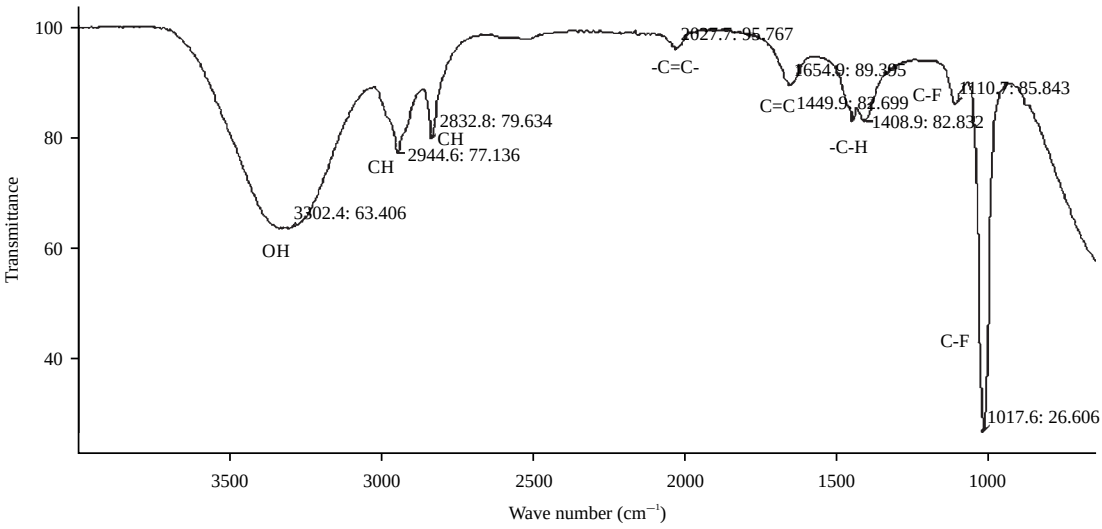


Fig. 7: FTIR spectra of ethanol extract of *Phyllanthus amarus* showing the various peaks and the functional groups

for butter and has antioxidant activity. Hexadecanoic acid ($C_{16}H_{32}O_2$) is a fatty acid known for its antibacterial and antifungal properties and heptadecanoic acid ($C_{17}H_{34}O_2$) can be used to detect and treat hyperferritinemia and metabolic syndrome in Dolphins. Tetrapentacontane ($C_{15}H_{110}$) is one of the prevailing compounds with a molecular weight of 759.4 g mol^{-1}.

The GC-MS profile for fraction 10b shown in Table 1 of the various compound. Hexadecanoic acid and methyl ester were found to have high biological functions as shown in Table 1. Other compounds such as pentadecanoic acid, hexadecanoic acid, 1-octadecene, heptadecanoic acid, tetrapentacontane, 9,12,15-octadecatrienoic acid, etc., were also identified as having different biological functions with antioxidant activity. Other compounds identified are shown in the Table 1 with their retention time, chemical structure and biological functions.

The FTIR spectra in Fig. 7 have one giant peak coming through at the last. There is a total of nine peaks with the two lowest peaks which could be the compound of most biological importance. The IR spectrum of the ethanol extract of *Phyllanthus amarus* was compared with the data of the

suggested GCMS spectrum, the spectra showed the presence of the OH group with strong and broad intensity at the absorption of 3303.4 cm^{-1}, C-H bond was observed at 2944.6 and 2832.8 cm^{-1}. The -C=C- stretch was observed at 20227.7, while C=C stretch was observed at 1449.9 and 1654.9 cm^{-1} and another functional group of C-F stretch were observed at 1110.7 and 1017.6 cm^{-1} with strong intensity. Thus the functional group obtained from the extract correlate with the chemical constituents obtained from the GCMS as shown in Table 1.

The result of this present study showed that the plant has a good antioxidant property due to the presence of the compounds identified.

The antioxidant activities of *Phyllanthus amarus* ethanol extract may be attributed to its phenolic and flavonoid contents, which are powerful *in vitro* antioxidant molecules[28] and predictable free radical scavengers due to their reducing properties as hydrogen or electron-donating agents[28].

The total phenolic content of the ethanolic extract of *Phyllanthus amarus* leaves ranges between 9-430 mg mL^{-1} as shown in Fig. 3 above with fraction 10b having the highest phenolic content and fraction 2a having the lowest phenolic content. This shows that the ratio of methanol to water was the best fraction for the extraction of phenols and another antioxidant.

Research has shown that phenolic compounds especially tannins are the major constituents of *Phyllanthus* plants. According to Yang *et al.*[29], more than 100 phenolic constituents with diverse biological activities were comprehensively identified in the fruits of *Phyllanthus emblica* Linn using HPLC-MS.

The total flavonoid content of the ethanolic extract of *Phyllanthus amarus* ranges between 23-143 mg mL^{-1} with fraction 3b showing the highest flavonoid content and fraction 2a showing the lowest concentration of total flavonoid. Different solvents with varied polarity were used for the elution of the ethanolic extract of *Phyllanthus amarus* leaves, this may be responsible for the variations in the concentration of the total flavonoid of the fractions. Flavonoids are commonly found in natural products and one of the most important natural phenolics[30].

The fractions obtained from *Phyllanthus amarus* leaves were analyzed for their constituents employing gas chromatography coupled with mass spectrometry (GC/MS) and Fourier-Transform Infrared Spectroscopy (FTIR). Results from GC-MS analysis of the fraction 10b of *P. amarus* revealed that 32 compounds were identified. Their biological functions, with their Retention Time (RT) are presented in Table 1. The prevailing compounds were pentadecanoic acid, hexadecanoic acid, tetrapentacontane and heptadecanoic acid identified with antioxidant activity.

Pentadecanoic acid ($C_{15}H_{30}O_2$) with a molecular weight of 242.39 g mol^{-1} and density of 842 kg m^{-3} has been said to improve insulin sensitivity[31] and also used as a marker for butter and has antioxidant activity. Hexadecanoic acid ($C_{16}H_{32}O_2$) is a fatty acid known for its antibacterial and antifungal properties[32] and heptadecanoic acid ($C_{17}H_{34}O_2$) can be used to detect and treat hyperferritinemia and metabolic syndrome in dolphins. Tetrapentacontane ($C_{15}H_{110}$) is one of the prevailing compounds with a molecular weight of 759.4 g mol^{-1}. More polar solvents can often extract antioxidant compounds in higher quantities. Overall high-polar solvent (ethanol) was very effective in extracting more antioxidant compounds when compared to an intermediate polar solvent, acetone and nonpolar solvent, hexane. The highest peak in Fig. 7 of FTIR spectra of ethanol extract of *Phyllanthus amarus* showing the highest peak of 2027.7, 95.767.

CONCLUSION

From the study carried out, there is a positive correlation shown between the total antioxidant and total flavonoids content of the fractions obtained from the ethanolic extract of *Phyllanthus amarus* leaves. However, a weak positive correlation is shown between the total antioxidant capacity and total phenolic content of the fractions obtained from the ethanolic extract of *Phyllanthus amarus* leaves implying that phenols are entirely responsible for the antioxidant activity of the plant.

SIGNIFICANCE STATEMENT

This study discovered the various antioxidants content of *Phyllanthus amarus* that can be beneficial for the development of human therapies and will help researchers to uncover the critical areas of plants antioxidants sources that many researchers were not able to explore. Thus a new theory on antioxidant properties may be arrived at shortly.

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