Tropical Journal of Phytochemistry & Pharmaceutical Sciences

Available online at https://www.tjpps.org

Original Research Article

Polyphenol Profile and Antioxidant Properties of Various Solvent Fractions of *Phyllanthus amarus*

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ABSTRACT

The prevention of various diseases and the promotion of health are the major functions of natural antioxidants. In the current study, the polyphenols, phytochemicals and antioxidants contents of various fractions of *P. amarus* obtained through fractionation were examined. Liquid-liquid partition separation was used in separating the *P. amarus* crude extract into various fractions (methanol, diethyl ether, n-hexane, n-butanol, and aqueous fractions). The amount of polyphenols were assessed using high performance liquid chromatography (HPLC). Phytochemicals and antioxidants assay were performed with standard methods. The phytochemical screening of *P. amarus* crude extract and different fractions revealed the presence of saponins (4.00 ± 0.50 mg/dL), tannins (7.50± 1.20 mg/dL), alkaloids (6.40 ± 0.05 mg/dL), steroids (1.55 ± 0.15 mg/dL), and flavonoids (6.00 ± 1.02 mg/dL). There was a significant improvement in the DPPH scavenging activity, flavonoids, total phenol, FRAP, ABTS, TAC, and ascorbate oxidase when comparing the crude extract to various fractions. Nine polyphenols such as o-salicyclic acid, caffeic acid, p-anisic acid, protocatechuic acid, gallic acid, ferulic acid, vanillic acid, p-coumaric acid and sinapinic acid were targeted in crude extract and different fractions. The total polyphenol content of crude extract and various fractions were as follows; crude extract > methanol fraction > diethyl ether fraction > n-hexane fraction > n-butanol fraction > aqueous fraction. Overall, diethyl ether and methanol were found to be the best solvents for the extraction of antioxidant compounds from *P. amarus* leaves.

Keywords: *A*ntioxidant, Diethyl ether, Methanol, *Phyllanthus amarus*, Polyphenol, solvent

Received 05 June 2024
Revised 19 June 2024
Accepted 19 June 2024
Published online ****

Introduction

Antioxidants remove free radicals from the body's cells and stop or lessen oxidation-related damage.[1,2] The most effective way to eliminate reactive oxygen species (ROS) which cause the oxidative stress is with the help of antioxidants. [3-9] Antioxidants may be considered exogenous, endogenous, synthetic, or natural. Free radical generation can be decreased and prevented by using antioxidants.[10, 11] The understanding of therapeutic plants has grown over time in many centuries based on different medicinal systems.[12] Many plant foods include beneficial polyphenols, which can be divided into flavonoids, phenolic acid, polyphenolic amides, and other polyphenols. In addition to hydroxyl groups, polyphenols frequently have other functional groups. Both carboxylic acids and ether ester connections are frequently seen. [9] A functional group is a particular set of atoms or bonds inside a compound that are in charge of the chemical processes that are unique to that compound.[9,13] The dominance of polysaccharide, protein, aromatic linkages and hydrocarbon linked with medicinal plant has been revealed through spectroscopic analysis. [9,14]

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Citation: Ibobo , GO, Okpoghono, J, Onyesom, I. . Polyphenol Profile and Antioxidant Properties of Various Solvent Fractions of *Phyllanthus Amarus*. Trop J Phytochem Pharm. Sci. 2024; 3(3): 246-253
http://www.doi.org/10.26538/tjpps/v3i3.6

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Phyllanthus amarus (PA), a member of the *Phyllanthaceae* family, has been traditionally used to treat gonorrhea, renal issues, pain, chronic dysentery, diabetes, skin ulcers [15] and malaria.[16] Chronic intake of PA has potential adverse effect on kidney. [17] In both young and old mice, treatment with
s PA extract and phyllanthin enhanced impairment of memory and had anticholinesterase action. [15] *P. amarus* has been shown to ameliorate memory deficits brought on by scopolamine, chlorpyrifos or sodium nitrite in different animal models of cognitive behaviour, which further support a notion of their neuroprotective role. [18] Although it demonstrates a variety of pharmacological actions, the unifying features of all these actions are directed towards the plant's antioxidant and anti-inflammatory potentials. The presence of several components or active substances, each with a unique mode of action based on the polarity of the extraction solvents, accounts for the protective benefits and potential of plant products.[19,20] Innovative analytical methods may help identify active substances with biological characteristics. Spectrophotometry methods are primarily responsible for the quantification of a broad estimate of the content of phenolic chemicals.[19] Still, more precise research focuses on phenolic class identification with sensitive detectors such as gas chromatography (GC) or high performance liquid chromatography (HPLC).[19,20] A very contemporary method for assessing polyphenols both qualitatively and quantitatively is HPLC in conjunction with diode array detector (DAD).[20] For the examination, identification, and evaluation of natural chemicals such as polyphenols, the HPLC is a powerful technique. [21] Anticipated objectives of this study were to examine the phytochemicals, antioxidants and HPLC-DAD polyphenol profile of *P. amarus* fractions harvested from Umuosele community Amai, Delta State, Nigeria. The findings of the research will be useful in evaluating the potential of *P. amarus* polyphenols in respect to the ethnomedical

applications of the plant by the people of Delta State, Nigeria and other regions of the world where it is extensively grown.

Materials and Methods

Chemicals and reagents

The chemicals potassium persulphate, aluminium chloride and tripyridyltriazine were purchased from Sigma chemical company, London, England. The reagents n-hexane, methanol, diethyl ether, n-butanol, folin-Ciocalteau were all purchased from BDH chemical laboratory England. All other chemicals/reagents used were of analytical grade.

Plant harvest and authentication

Phyllanthus amarus is an erect annual herb of not more than one and half feet tall and has small leaves. The plant was harvested from loamy soil at 26–37 ℃ in Umuosele community Amai, Latitude: 5° North, Longitude: 6° East, Delta State, Nigeria. The plant was collected in September, 2021. The plant was identified with voucher specimen number FHI109728 at the National Institute of Forestry Research Ibadan.

Preparation of crude methanolic leaf extract

A portion of five hundred grams (500 g) of *P. amarus* powder sample was suspended in 2 L of methanol in 48 hours in large amber bottles with intermittent shaking. At the end of the extraction, filtration of crude methanol extract using muslin cloth was done and then concentrated in a water bath maintained at 45^0C. Using standard measures, phytochemical screening was done on ten grams of *P. amarus* crude extract. Fractionation was carried out on the crude sample. Both the fractions and crude methanol extract of *P. amarus* percentage yield were determined as a percent of the weight (g) of the extract/fractions to the original weight (g) of the dried sample.

Percentage yield of each extract/fractions were determined as shown in equation 1:

$$\frac{Dry\ weight(extract/fractions)}{Dry\ sample\ weight} \times \frac{100}{1}$$ ----------Equation 1

Fractionation of methanol P. amarus leaves crude extract

Liquid-liquid partition separation was used to separate the crude *P. amarus* extract into its various fractions. Then, 50 g of crude extract was reconstituted with 250 mL of methanol, n-hexane, diethyl ether, n-butanol solvents and 250 mL of water 1:1 (v/v) individually in a separator funnel. The sample was left to stand on the separator funnel for 30 minutes for each solvent until a fine separation line was clearly visible indicating the supernatant from the sediment before it was eluted sequentially. The process was repeated three times in order to get adequate quantity for each fraction. The methanol, n-hexane, diethyl ether, n-butanol and the aqueous residue fractions were evaporated to dryness in a water bath (WB-2R6H-25, Infiteck, China) to obtain five fractions respectively. Phytochemical screening, antioxidant and polyphenol analyses were conducted on the fractions.

Biochemical analysis

Qualitative phytochemical analyses

Qualitative phytochemical screening was done to determine the existence of possible phytochemicals, including alkaloids, saponins, tannins, flavonoids and phenols in the extracts of various fractions of *P. amarus* leaves with the aid of standard procedures outlined by Sofowora.[22] Quantitative phytochemical analysis was done following the method of Harborne.[23]

Antioxidant analysis

2,2-diphenyl-1-picrylhydrazyl (DPPH)

The sample capacity to scavenge free radicals against those of DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined by the method of Ursini et al. [24] The extracts (50 µg) was diluted with 3 ml ethanol and mixed with 3 ml DPPH solution. The mixture was shaken, and then incubated using incubator (Icb-160, Infiteck, China) in dark for 30 minutes. In comparison to a blank, the samples absorbance was measured using spectrophotometer (SP-UV5100I, Scitek Global Co.,Ltd, China) at 517 nm.

Total flavonoid assay

Total flavonoid was determined with colourimetric aluminium chloride methods as described by Ebrahimzadeh et al. [25] In a nutshell, 0.5 mL of sample was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water, and then kept at room temperature for 30 minutes. The absorbance was measured at 415 nm with spectrophotometer (SP-UV5100I, Scitek Global Co., Ltd, China) The amount of total flavonoid content in a gram of samples was quantified as mg quercetin equivalents (QE).

Total phenolic content

The procedure outlined by Dewanto et al. [26] was followed to determine the total phenolic content. Zero point five millilitres (0.5 ml) of sample were dissolve in 100 µl of Folin-Ciocalteau reagent and 6 ml of distilled water. It was vortexed for 1 minute, and 2 ml of 15% Na_2CO_3 was added and the mixture vortexed once again for thirty seconds (30 seconds). With distilled water, a solution of up to 10 ml was made. After 1 hour, 30 minutes, the absorbance of the samples was read at 750 nm with a UV spectrophotometer. Gallic acid solution was used for calibration curve preparation. Total phenolic contents of samples were expressed as the milligrams equivalent of gallic acid (mg GAE)/100 g of dry weight.

Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured by Benzie and Strain method. [27] The 300mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ (tripyridyltriazine) solution, and 20.0 mM $FeCl_3$.6H2O solution were combined to make the FRAP reagent in a ratio of 10:1:1 in volume. About 0.5 ml of samples at different concentrations (100 – 400µg/ml) was then added to 3 ml of FRAP reagent. The mixture was incubated at 37°C for 30 min. The absorbance at 593 nm was measured.

2,2'-azino-bis(3ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging activity

ABTS radical-scavenging activity was determined by Re et al. Method. [28] The cation of ABTS radical was formed by the effect of ABTS stock solution (5 mL) and 2.45 mM potassium persulphate ($K_2S_2O_8$) solution (5 mL), stored in the dark for 16 hours (room temperature). Exactly 0.5 ml of sample were added to 4.5 ml of cation solution of ABTS radical in test tubes, and allowed to incubate for 6 min at ambient temperature. Blanks were also done in each assay. The sample absorbance was recorded at 734 nm.

Total antioxidant capacity (TAC)

Total antioxidant capacity in the samples were estimated by Prieto et al. Method [29]. The sample (0.1 mL) were added to 1 mL of reagent solution (28 mmol/L Na_3PO_4, 4 mmol/L ammonium molybdate and 0.6 mol/L H_2SO_4) in test tubes. The tubes underwent a 90 minute incubation at 95°C in a thermal block. The mixture was allowed to cool at room temperature. At 695 nm, the absorbance was read against a blank. Equivalent of gallic acid in mg/g dry weight (mg GAE/g DW) was used to describe antioxidant ability.

Ascorbate Oxidase Activity

The assay of ascorbate oxidase was assessed by Vines and Oberbacher method. [30] The samples were mixed [1:5 (v/v)] with phosphate buffer (0.1 M/ pH 6.5) and centrifuged at 3000 g for 15 min at 5 ºC. The supernatant obtained functioned as enzyme source. The enzyme source (0.1 ml) was added to 3.0 ml of substrate solution (8.8 mg ascorbic acid in 300 ml phosphate buffer, pH 6.5) and the change in absorbance were determined at 265 nm for every 30 seconds for a period of 4 minutes. The enzyme activities were expressed as U/ml. One unit is the amounts that produce 1 µmole of dehydroascorbic acid per minute.

Polyphenol analysis

The polyphenols in the extract were determined using high performance liquid chromatography (HPLC). The analysis was done by Seal method. [31] Acetonitrile (Solvent B) and 1% aqueous acetic acid solution (Solvent A) were both present in the mobile phase and 2ml/min was adjusted as the flow rate. The column was controlled at 28^0C and the sample injection volume was 5 µL. A gradient elution was done by varying the proportion of solvent B to solvent A. The composition of the mobile phase back to initial condition of solvent B: solvent A: 10: 90 in 55 minutes and then allowed to stand for another 10 min., before another sample injection. Each sample underwent analysis for a total of 65 minutes. A photo diode array UV detector (DAD) was used to detect HPLC chromatograms at three distinct wavelengths: 272, 280 and 310 nm according to compounds analysed. Based on their withholding time and by spike with standards under the same circumstances, compounds were detected. Sample quantification was determined by the integrated peak area measurement and the content was calculated. Total polyphenols and individual polyphenols content were calculated using equation 2

$$\text{Concentration of polyphenols (mg/g)} = \frac{\text{Peak area}}{\text{Standard peak area}} \times \text{Standard concentration}$$

----------Equation 2

Total polyphenol standard profile
Standard conc. = 19.20 mg/g
Standard peak area= 4388.1550 mg/g
Individual polyphenol standard profile
Standard conc. = 1.745 mg/g
Standard peak area= 398.9231mg/g.

Statistical Analysis

The results were expressed in mean bars, mean ± SD. The results were analyzed using Analysis of variance (ANOVA). Significant difference between means were determined at $p< 0.05$ confidence level using least significant difference (LSD). The SPSS-PC programme package (version 21.0) was used for statistical analysis.

Results and discussion

Percentage yield and phytochemicals screening of P. amarus leaves

Table 1 presented the percentage yield of *P. amarus* leaves. Yield, which is typically given as a percentage, is a measurement of how many moles of a product are produced in a chemical reaction in comparison to the amount of reactant used. The percentage yields of *P. amarus* leaves were as follows; methanol crude extract (37.62%) > methanol fraction (28.40%) > diethyl ether fraction (27.60) > n-hexane fraction (23.00 %) > n-butanol fraction (21.00 %) > aqueous fraction (7.30 %) (Table 1). The percentage yields for the methanol fraction were the highest perhaps because of its polarity. El-mahmood et al. [32] reported that polarity of solvents is very important in extraction process. Factors like plant material type, age of plants and solvent polarity used for extraction could affect the extract percentage yield. [14]

The quantitative and qualitative phytochemical analysis of *P. amarus* leaves are shown in Table 2 and 3. *P. amarus* leaves crude extract and varying fractions of qualitative phytochemicals analysis indicated the occurrence of alkaloids, saponins, phenols, tannins, flavonoids, cardiac glycosides, steroids, and reducing sugar (Table 2). In quantitative analysis saponins had the highest concentration followed by flavonoids, alkaloids and tannins. Steroids had the lowest concentration (Table 3). *P. amarus* leaves have high concentration of saponins, flavonoids, and alkaloids chemicals, which may be a factor in their antioxidant capabilities. Antioxidants have a variety of effects on biological systems due to their abilities as metal ion chelators and protein precipitating agents. [33] They are crucial in the scavenging of free radicals. [34] Their notable antitumor effects and astringent action may be a result of these features.[34]

Antioxidant property of P. amarus leaves crude extract and fractions

Scavenging activity of DPPH radical, Flavonoids, total phenol, FRAP, ABTS, TAC and ascorbate oxidase are shown in Figure 1-7 respectively. The market for packaged foods places a high value on antioxidant activity since it lowers the risk of heart disease, guards against degenerative diseases, and extends the shelf life of the products by avoiding or delaying oxidation reactions. [35] Significant increase was observed in the DPPH, flavonoids, total phenol, FRAP, ABTS, TAC and ascorbate oxidase of crude extract when compared to methanol, diethyl ether, n-hexane, n-butanol, aqueous fraction. However, as the concentration of sample increases (100 - 400 µg/mL) the antioxidant properties increases. Trends of the antioxidants (flavonoids, total phenol, FRAP, ABTS, and ascorbate oxidase) were as follows; crude extract > methanol fraction > diethyl ether fraction > n-hexane fraction > n-butanol fraction > aqueous fraction. DPPH and TAC had the following trends: crude extract > methanol fraction > diethyl ether fraction > n-hexane fraction > n-butanol fraction > BHT > water fraction. Fruits and vegetables can be extracted of their phenolic components via solvent extraction. The various solvents employed in the current investigation may be crucial to the extraction process. The solvent's capacity to dissolve a special group of antioxidant compounds may influence the activity of antioxidant estimated. Interestingly, whenever species that are charged are present in extraction medium the reaction barrier is highly dependent on solvent's polarity. [14, 36] In order to boost the reactivity of the extraction process, solvent may aid in stabilizing the reactants. [36]

Crude extract and fractions of P. amarus leaves polyphenol content

Polyphenols content of *P. amarus* crude extract and the different fractions are shown in Figures 8-13 and Table 4-9. Nine polyphenols compounds were targeted in *P. amarus* crude extract and the different fractions. They were as follows: o-salicyclic acid, p-coumaric acid, caffeic acid, gallic acid, p-anisic acid, protocatechuic acid, ferulic acid, vanillic acid, and sinapinic acid. The total polyphenols content of *P. amarus* in varying fractions and crude extract trends as follows; crude extract > methanol fraction > diethyl ether fraction > n-hexane fraction > n-butanol fraction > aqueous fraction (Figure 14). The increased polyphenol compounds in methanol fraction and crude extract may be as a result of the polar hydroxyl group towards this solvent. Thus, polyphenol compounds such as o-salicyclic acid, p-coumaric acid, protocatechuic acid, p-coumaric acid in the crude extract and p-anisic acid, vanillic acid, caffeic acid and sinapinic acid in methanol fraction might mainly contribute to the overall antioxidant capacity. Furthermore, various monomeric polyphenol compounds showed certain property for different antioxidant systems.[36]According to studies, polarity increases extraction yield, antioxidant activity; and plant extracts ability to scavenge free radicals occurs because polyphenol compounds have a higher affinity for more polar solvents than non-polar ones. [37] Secondary metabolites known as plant polyphenols are identified by the association of one or more hydroxyl groups with one or more aromatic rings.[37,38]

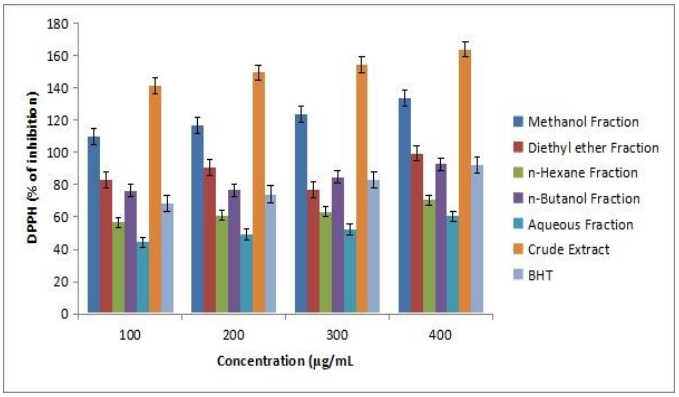

Figure 1: DPPH radical scavenging properties *P. amarus* leaves in fractions and crude extract. Bars represents means of triplicates values. Bars with unlike alphabets vary at $p<0.05$.

Table 1: Percentage yield of fractions and crude extract of *P. amarus* leaves

Extracting solvent	*P. amarus* dried leaves (g)	Extracted powder of *P. amarus* leaves (g)	% extract yield (%)
Methanol crude extract	400	150.50	37.62
Methanol fraction	50	14.20	28.40
n-hexane fraction	50	11.50	23.00
n-butanol fraction	50	10.50	21.00
Diethyl ether fraction	50	13.80	27.60
Aqueous fraction	-	29.20	14.60

Table 2: Qualitative phytochemical analysis of *P. amarus leaves* in crude extract and diverse fractions.

	Methanol Fraction	Diethyl ether fraction	n-Hexane Fraction	n-Butanol Fraction	Aqueous Fraction	Crude extract
Tannins	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	+
Cardiac Glycosides	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+

Key: + = Present

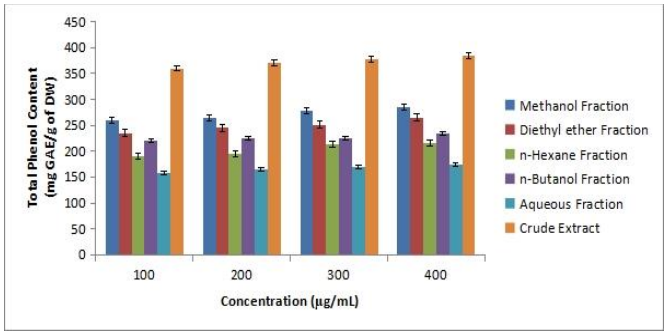

Figure 2: Total phenol content of *P. amarus* leaves in different fractions and crude extract.
Bars represents means of triplicates values. At $p<0.05$, bars with dissimilar alphabets differs significantly.

Table 3: Quantitative phytochemical screening of *P. amarus* leaves crude sample.

Parameters	**(mg/dL)**
Tannins	4.00 ± 0.50
Saponins	7.50± 1.20
Alkaloids	6.40 ± 0.05
Steriods	1.55 ± 0.15
Flavonoids	6.00 ± 1.02

Conclusion

The research revealed high concentration of flavonoid and polyphenol components with potent antioxidant properties in the various fractions and extract of *P. amarus*. Methanol and diethyl ether were found to be the most effective solvents for extracting polyphenols and antioxidant compounds from *P. amarus* leaves. However, the ability of the different fractions to act as a free radical scavenger and reducing agent raises the possibility that they may have therapeutic benefits for treatment of diseases brought on by free radicals.

Table 4: Polyphenol content of *P. amarus* leaves crude extract.

Target Compounds	**Concentration (mg/mL)**
O-Salicyclic Acid	11.02
p-Anisic Acid	7.93
Vanillic Acid	4.55
Protocatechuic Acid	9.37
p-Coumaric Acid	13.02
Gallic Acid	1.02
Ferulic Acid	1.05
Caffeic Acid	nd
Sinapinic Acid	4.33
Total	42.92

nd =not detected

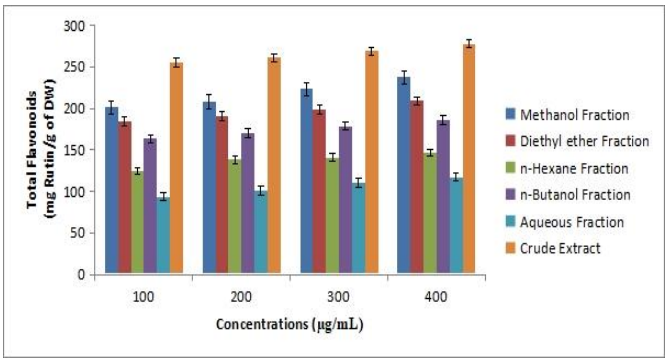

Figure 3: Total flavonoid content of *P. amarus* leaves crude extract and fractions. Bars represents means of triplicates values. Bars with dissimilar alphabets vary at $p<0.05$.

Table 5: Polyphenol content of *P. amarus* leaves methanol fraction

Target Compounds	Concentration (mg/mL)
O-Salicyclic Acid	0.20
p-Anisic Acid	3.74
Vanillic Acid	8.12
Protocatechuic Acid	0.25
p-Coumaric Acid	nd
Gallic Acid	0.47
Ferulic Acid	1.31
Caffeic Acid	1.32
Sinapinic Acid	2.65
Total	18.06

nd =not detected.

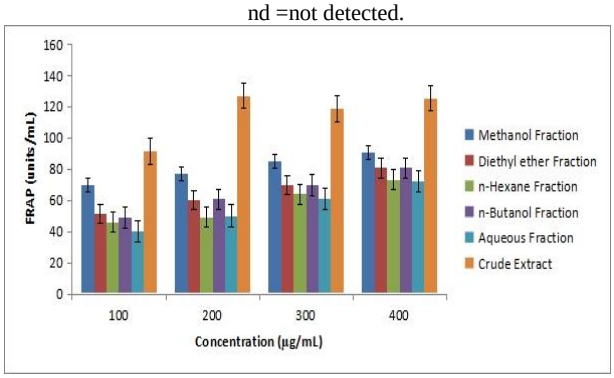

Figure 4: FRAP of *P. amarus* leaves in various fractions and crude extract. Bars indicate means of triplicates values. At $p<0.05$, bars with divergent alphabets differs significantly.

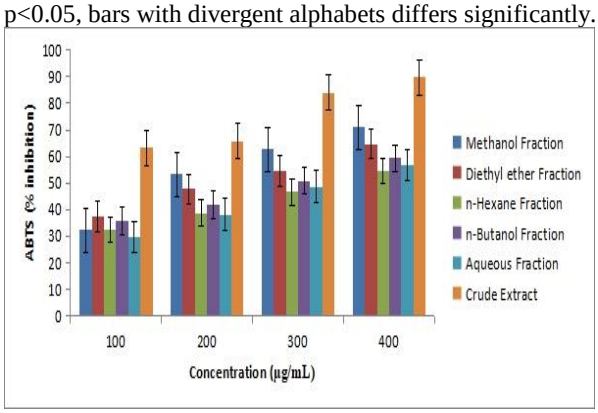

Figure 5: ABTS of *P. amarus* leaves in crude extract and varying fractions. Bars signify means of triplicates values. Bars with differing alphabets differs at $p<0.05$.

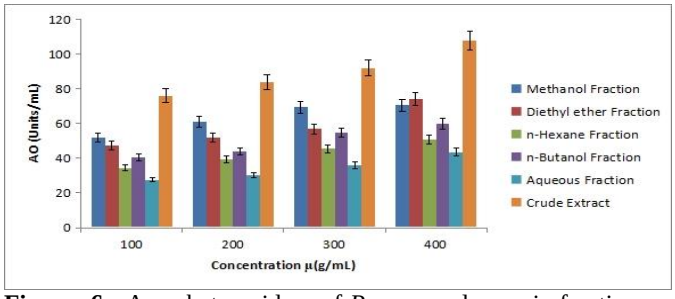

Figure 6: Ascorbate oxidase of *P. amarus* leaves in fractions and crude extract. Bars denote means of triplicates values. Bars with varying alphabets differs at $p<0.05$.

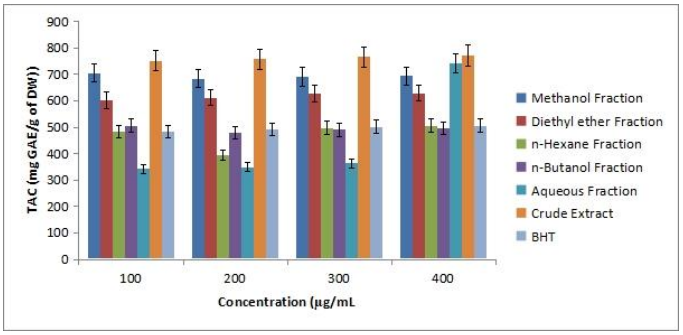

Figure 7: Total antioxidant capacity of *P. amarus* leaves in crude extract and fractions. Bars represents means of triplicates values. Bars with different alphabets vary at $p<0.05$.

Table 6: Polyphenol content of *P. amarus* leaves n-butanol fraction

Target Compounds	Concentration (mg/mL)
O-Salicyclic Acid	0.91
p-Anisic Acid	0.59
Vanillic Acid	1.13
Protocatechuic Acid	0.59
p-Coumaric Acid	nd
Gallic Acid	0.85
Ferulic Acid	1.29
Caffeic Acid	1.52
Sinapinic Acid	1.78
Total	8.66

nd =not detected.

Table 7: Polyphenol content of *P. amarus* leaves diethyl ether fraction

Target Compounds	Concentration (mg/mL)
O-Salicyclic Acid	0.25
p-Anisic Acid	2.04
Vanillic Acid	5.94
Protocatechuic Acid	nd
p-Coumaric Acid	nd
Gallic Acid	4.80
Ferulic Acid	0.76
Caffeic Acid	0.83
Sinapinic Acid	nd
Total	14.62

nd =not detected.

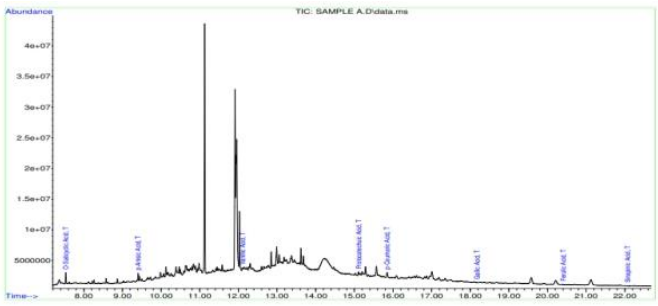

Figure 8: Chromatogram of *P. amarus* leaves crude extract polyphenol content.

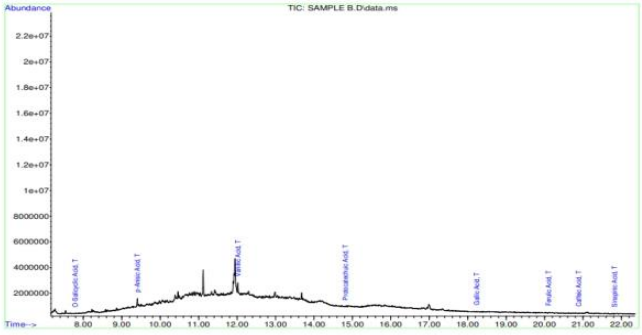

Figure 9: Chromatogram of *P. amarus* leaves methanol fraction polyphenol content.

Table 8: Polyphenol content of *P. amarus* leaves n-hexane fraction

Target Compounds	Concentration (mg/mL)
O-Salicyclic Acid	0.25
p-Anisic Acid	2.55
Vanillic Acid	7.45
Protocatechuic Acid	0.37
p-Coumaric Acid	0.41
Gallic Acid	0.43
Ferulic Acid	0.88
Caffeic Acid	1.32
Sinapinic Acid	nd
Total	13.66

nd= not detected

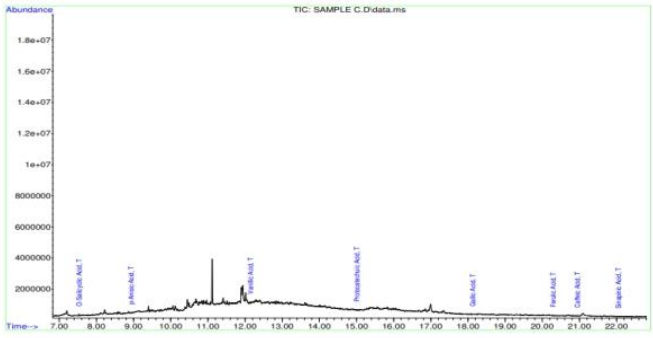

Figure 10: Chromatogram of *P. amarus* leaves n-butanol fraction polyphenol content.

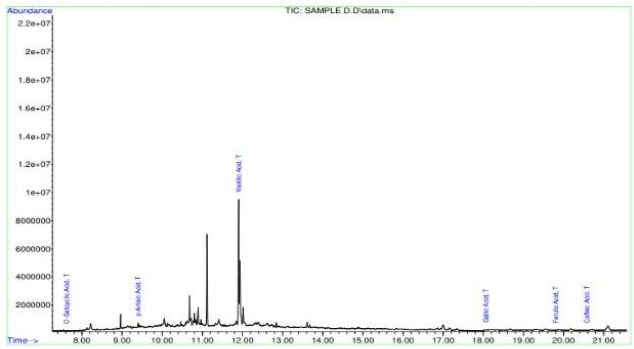

Figure 11: Chromatogram of *P. amarus* leaves diethylether fraction polyphenol content.

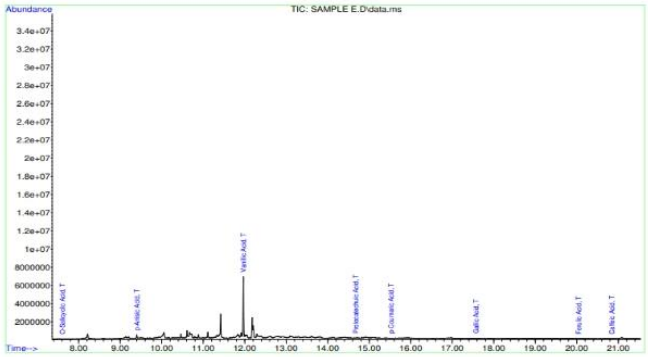

Figure 12: Chromatogram of *P. amarus* leaves n-hexane fraction polyphenol content

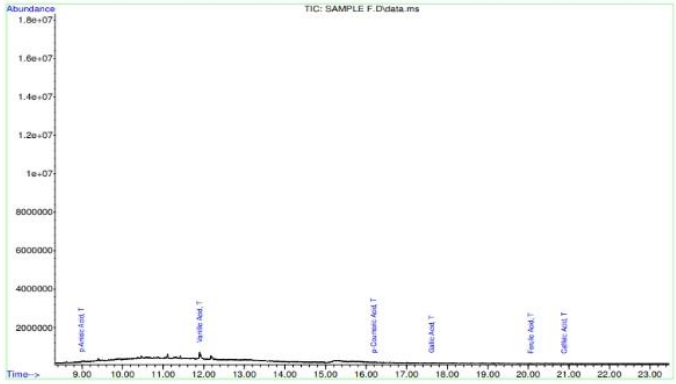

Figure 13: Chromatogram of *P. amarus* leaves aqueous fraction polyphenol content

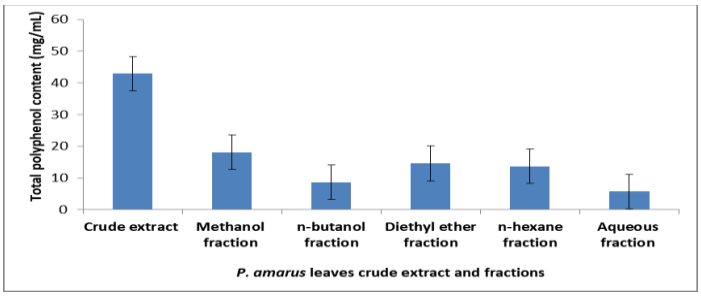

Figure 14: Total polyphenol content of fractions and crude extract of *P. amarus* leaves

Table 9: Polyphenol content of *P. amarus* leaves aqueous fraction

Target Compounds	Concentration (mg/mL)
O-Salicyclic Acid	nd
p-Anisic Acid	0.45
Vanillic Acid	2.36
Protocatechuic Acid	nd
p-Coumaric Acid	0.16
Gallic Acid	0.36
Ferulic Acid	1.17
Caffeic Acid	1.20
Sinapinic Acid	nd
Total	5.70

nd= not detected

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