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Phytoconstituents and Antioxidant Activity of the Stem Bark of *Pentaclethra macrophylla* (Fabaceae)

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Abstract

Pentaclethra macrophylla is known for its nutritional and medicinal properties. Despite its wide usage, little information is known about the antioxidant potential of the stem bark extract and its fractions. This work reports the antioxidant activity of ethyl acetate and n-butanol soluble fractions, as well as chromatographic fractions, and the identification of probable antioxidant compounds using LC-MS. Vacuum liquid chromatography and gel filtration using a Sephadex LH-20 were used to isolate and identify the compounds. Antioxidant assays were carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothizoline-6-sulfonic acid (ABTS), and ferric reducing antioxidant power (FRAP) assays, and the scavenging activity was calculated as (IC_{50} ± SEM). The result revealed that in the ABTS assay; the n-butanol soluble fraction (Sample D) exhibited a dose-dependent scavenging activity with an IC_{50} of 43.76 ± 2.38 µg/ml followed by the ethyl acetate soluble fraction (Sample C) with IC_{50} of 49.90 ± 2.99 µg/ml in comparison to the standard drug ascorbic acid with IC_{50} of 20.20 ± 0.23 µg/ml. In the DPPH assay method, the n-butanol chromatography fraction (Sample E) showed the highest DPPH scavenging activity with IC_{50} of 16.53 ± 1.17 µg/ml followed by n-butanol soluble fraction which gave an IC_{50} of 18.14 ± 0.18 µg/ml which were both higher than the standard drug ascorbic acid with IC_{50} of 37.42 ± 1.67 µg/ml. In the reducing power assay, the n-butanol soluble fraction (Sample D) showed the highest activity with IC_{50} of 46.99 ± 2.10 µg/ml while the ethyl acetate fraction gave an IC_{50} value of 53.37 ± 1.53 µg/ml in comparison to ascorbic acid with an IC_{50} of 29.18 ± 0.66 µg/ml. Purification of the ethyl acetate and n-butanol fractions afforded 11-O-galloyl Bergenin, Myricetin-3-O-glucoside, and a mixture of bergenin and Methyl Bergenin. The compounds were identified using LC-MS. The presence of these compounds might explain the antioxidant and nutritional potential of this plant.

Keywords: Antioxidant activity, 11-galloylbergenin, Myricetin-3-O-glucoside, LC-MS

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Introduction

In developing nations, approximately 80% of the population derives their health needs from the use of medicinal plants or herbal remedies (Rates, 2001). Secondary metabolites from purified plant extracts possess numerous bioactivities and are considered safe and inexpensive (Lahlou, 2007). Plant parts such as fruits, vegetables, seeds, herbs, and cereals are known to contain a wide variety of antioxidants that protect against free radicals (Kaur and Kapoor, 2001), whereas phytoconstituents such as flavonoids, phenolic acids, and tannins are known to possess antioxidant activity (Syed *et al*., 2022). *Pentaclethra macrophylla* (Fabaceae) synonym, African oil bean tree also known commonly as 'Ugba' in South Eastern Nigeria is a legume tree found in both sub-tropical and tropical regions of the world. It is predominant in the southern and eastern regions, Nigeria, and other countries, such as Senegal, Cameroon, Gabon, and Angola (Butler, 2006).

The use of fruits, seeds, leaves, stem bark, and roots has been reported in herbal practices in different countries. In Nigeria, the stem bark is used for its anti-ulcer (Ojimelukwe, 2024), anti-diarrhea, antidiabetic, abortifacient (Nnamani *et al*., 2020), antinociceptive, and anti-inflammatory properties (Igbe and Osigwe, 2012; Okuronbo *et al*., 2009). The Seeds have been reported to possess anticancer activity (Ukoro, 2020), while in cameroon is used to treat infertility (Chidozie, 2006). The leaves have also been used to treat diarrhea, fever, and convulsions (Balogun, 2013; Akah *et al*., 1999; Asoegwu *et al*., 2006). We previously reported the isolation of methyl gallate, bergenin, and 11-galloyl bergenin from the stem bark extract (Chinaka *et al*., 2017), whereas a mixture of two new aromatic glycosides has been reported from the seed extract of this plant (Sinda *et al*., 2021). Despite the various ethnomedicinal uses and biological activities of this plant, there are no documented reports on the antioxidant activity of its stem bark. In this study, we report the antioxidant activity of ethyl acetate and n-butanol soluble fractions, as well as their chromatographic fractions, using DPPH (2,2-diphenyl-1-picrylhydrazyl) ABTS (2,2-Azinobis-3-Ethylbenzthiazolin-6-Sulfonic Acid) and Ferric Reducing Antioxidant Power (FRAP) assays, in addition to the identification of some of the chemical constituents present using liquid chromatography-mass spectrometry coupled with a photodiode array detector (LC-MS-PDAD).

Materials and Methods

Plant Collection

The stem bark was collected in Enugu South Eastern Nigeria in March 2024 and was authenticated by Dr A. Afieroho of the Department of Pharmacognosy and Phytotherapy, University of Port Harcourt, Nigeria where a voucher specimen which was compared with specimen voucher was deposited.

Extraction

The air-dried pulverized stem bark (450 g) was extracted to exhaustion at room temperature with 2 x 2 L of hydro alcohol. The combined extract was concentrated using a rotary evaporator to obtain a dark brown solid mass (21.7 g). A portion of the crude ethanol extract (15 g) was suspended in 300 ml of distilled water and partitioned exhaustively with 1.2 L of ethyl acetate and 1.5 L of n-butanol to give 3.5 g of the ethyl acetate-soluble fraction and 5.2 g of the n-butanol-soluble fraction.

Thin layer and Vacuum liquid separations

Thin layer chromatography (TLC) was carried out on silica gel G TLC plates (aluminum backed, 0.25 mm, Silicycle), Vacuum Liquid Chromatography (VLC) was carried out using a stationary phase made of silica gel 60 H 90 (0.2 mm), and gel filtration was performed using Sephadex LH-20 (Sigma Aldrich).

VLC Separation of the Ethyl acetate and n- butanol soluble fractions

A portion of the ethyl acetate fraction (Sample C) (2.5 g) was mixed with a small quantity of silica gel and subjected to vacuum liquid chromatography using a gradient elution technique starting with dichloromethane (100 %), followed by a mixture of dichloromethane and methanol (99:1, 98:2, 97:3, 96:4, 9 5 :5, 9 0 :10, 8 0 :20, 7 0 :30, 6 0 :40, 5 0 :50, 30:70:10:90), and methanol (100 %). Aliquots (100 ml of aliquots were collected. The progress of separation was checked by TLC using the solvent systems 1: ethyl acetate: dichloromethane (3:2), 2: ethyl acetate: dichloromethane: methanol: water (15:8:4;1), and 3:6:4:4:1), and those with similar TLC profiles were pooled together. Fractions eluted with dichloromethane (100%) and dichloromethane: methanol (1–4 %), which revealed similar TLC profiles, were pooled together, subjected to further purification over Sephadex LH-20, and eluted with pure methanol to give subfraction A. The fraction eluted with 10 % methanol in dichloromethane was purified on a Sephadex LH-20 column and eluted with methanol to obtain subfraction B. A portion of the n-butanol soluble fraction (Sample D), 4.4 g was subjected to Vacuum liquid chromatography using a gradient elution technique starting with dichloromethane (100 %) and dichloromethane: methanol mixtures as follows: dichloromethane: methanol (95:5; 90:10; 80:20; 70:30; 60:40; 50:50; 30:70; 10:90; methanol

(100 %) and 5 %water in methanol. Aliquots (100 ml) were collected and the progress of elution was checked by TLC using solvent systems 2 and 3. Fractions 5-9 eluted with 20 % and 30 % methanol in dichloromethane, which revealed a similar profile on TLC, were pooled and purified over Sephadex LH-20 by elution with methanol to give sub-fraction E. Subfractions A, B, and E were then subjected to LC-MS to identify the chemical constituents present.

LC-MS Separation

LC-MS was performed on a Waters 2695 separation module with W2998 PDA coupled to an ACQ-QDA MS. The samples were reconstituted in methanol, filtered through a membrane filter (0.45 µm). Separation was performed on a C-18 column (Sunfire C-18, 5.0 µm, 4.6 mm x 150 mm). The analysis was conducted at a flow rate of 1.0 ml/min using a mixture of the mobile phase with the following gradient: 0.1% formic acid in water as Solvent A 0.1% formic acid in Acetonitrile as Solvent B. A ratio of A/B (95:5) was used and maintained for 1 min, followed by A/B (5:95) for 13 -15 min, A/B (95:5) for 17 – 19 min, and finally 20 min. The PDA detector was set at 210–400 nm with a resolution of 1.2 nm and a sampling rate of 10 points/s. The mass spectra were acquired with a scan range from m/z 100 - 1250 Da after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8kv (positive) and 0.8kv (negative), probe temperature of 600 °C; flow rate of 10 ml/min; nebulizer gas 45 psi. The MS was set in the automatic mode by applying a fragmentation voltage of 125 V. The data were controlled and processed using the Empower 3 software (Garba *et al*., 2023).

Antioxidant Studies

The DPPH assay was performed according to the method described by Brand-Williams *et al.* (1995] with some modifications. 2, 2- diphenyl-1-picrylhydrazyl (DPPH) (8 mg) was dissolved in MeOH (100 mL) to give a solution concentration of 80 μg/mL. To determine scavenging activity, 100 μL DPPH reagent was mixed with 100 μL of the sample in a 96-well microplate and incubated at room temperature for 30 min. After incubation, absorbance was measured at 514 nm using a microplate reader, and 100 % methanol was used as a control. The DPPH scavenging activity was estimated using the following formula:
% DPPH scavenging = $(A_{blank} - A_{sample})/A_{blank}) * 100$.

Where $A_{blank\ represents}$ the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} represents the absorbance of the test sample/standard.

The 2,2-azabinos (3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) assay was performed as previously described (Arnao *et al.* 2019), with some modifications. 7mM 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2.45 mM potassium persulphate were mixed and dissolved in double-distilled water. The solution was diluted with distilled water to a 1:9 v/v ratio. A 190 µl volume of reagent was pipetted into a microtiter well with the addition of 10 µl of sample/standard (ascorbic acid). The absorbance was measured at 735 nm. A reagent blank reading was taken, and after the addition of the sample, the absorbance reading was taken 6 min after initial mixing. The antioxidant activity was estimated using the following formula:
% ABTS scavenging = $(A_{blank} - A_{sample})/A_{blank}) * 100$
For the FRAP assay, solutions were prepared as described by Benzie and Strain (1996). The FRAP working solution was prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of 2,4,6-tripyridyl-S-triazine (TPTZ) (40 mM dissolved in 40 mM HCl) and 1-volume of ferric chloride (20 mM in water). The microplate FRAP assay was performed according to previous reports with minor modifications.

Sample solutions (20 µL) were added directly to a 96-well microplate, followed by 280 µL of the working FRAP solution. The mixtures were shaken and incubated at 37 °C in the dark for 30 min, and absorbance was measured at 593 nm using a microplate reader. The values were estimated using micromolar equivalents of ferrous (II).

Statistical analysis

Data are presented as mean ±SEM. Graphs were plotted using GraphPad Prism version 8.0. The difference was considered statistically significant when $P < 0.05$

Results

Phytochemical constituents

Chromatographic separation of the ethyl acetate- and n-butanol-soluble fractions and subsequent purification over Sephadex LH-20 yielded sub-fractions A, B, and E based on their TLC profiles. Subfraction A obtained from the ethyl acetate soluble fraction was isolated as a colorless crystal (3.2 mg), and the LC-MS of this subfraction obtained from the ethyl acetate soluble fraction showed a major peak with a retention time of 17.848 mins in the chromatogram (Figure.1) and$[M-H]^+$ peak at 479.282 which points to a molecular weight m/z of 480 (Figure. 2). Sub-fraction B, also obtained from the ethyl acetate-soluble fraction as a brown solid (2.0 mg), showed a major peak in the chromatogram, with a retention time of 13.389 min

(Figure. 3) and a molecular $[M-H]^{+peak}$ at m/z 479.215, which translates to a molecular weight of 480.215 (Figure. 4), and molecular formula of $C_{21}H_{20}O_{13}$. Sub-fraction E was obtained from the n-butanol-soluble fraction as a colourless solid (3.5 mg), and LC-MS (Figure. 5) revealed two closely related major peaks with retention times of 5.961 and 6.166 min, respectively. The mass spectrum shows molecular $[M+H]^+$ peaks at m/z 329.182 and 343.215 (Figure. 6), with molecular weights of 328.182, 182, and 342.215.

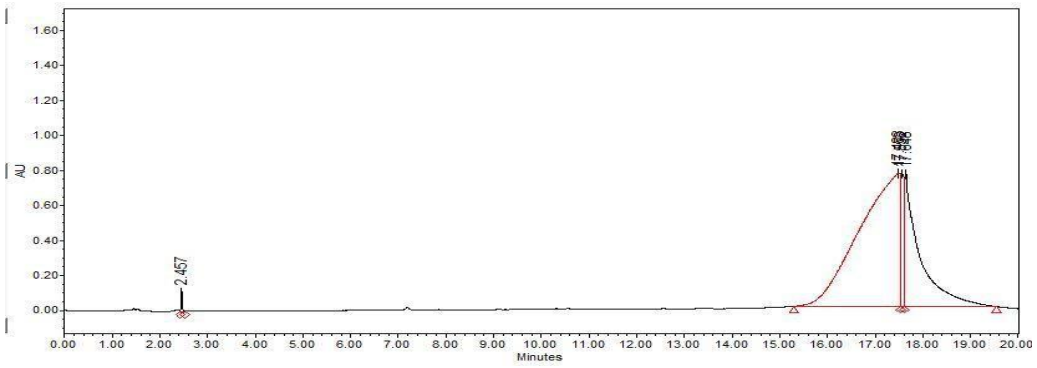

Figure. 1: Chromatogram of subfraction A showing separation on a C-18 column (Sunfire C-18, 5.0 µm, 4.6 mm x 150 mm).

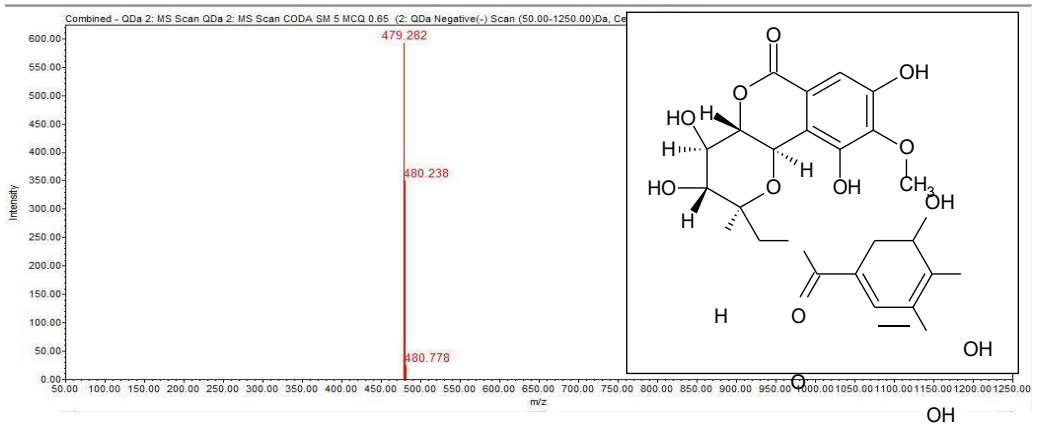

Figure. 2. Total Ion Chromatogram (TIC) and Mass Spectrum of Subfraction A

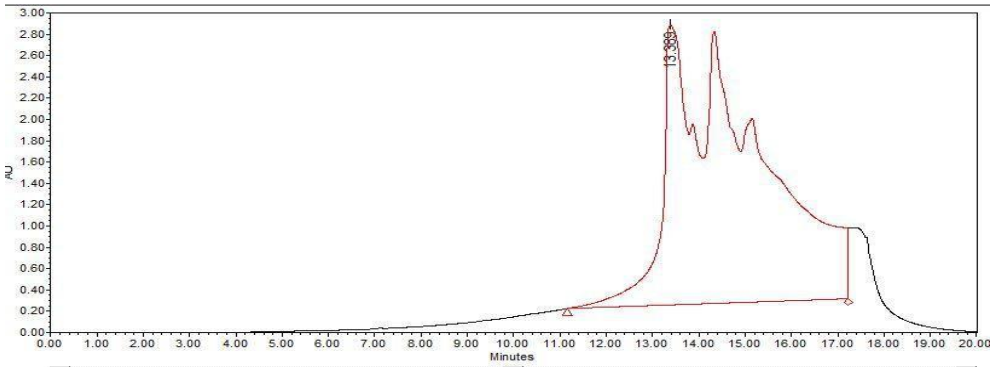

Figure. 3: Chromatogram of subfraction B showing separation on a C-18 column (Sunfire C-18, 5.0 µm, 4.6 mm x 150 mm).

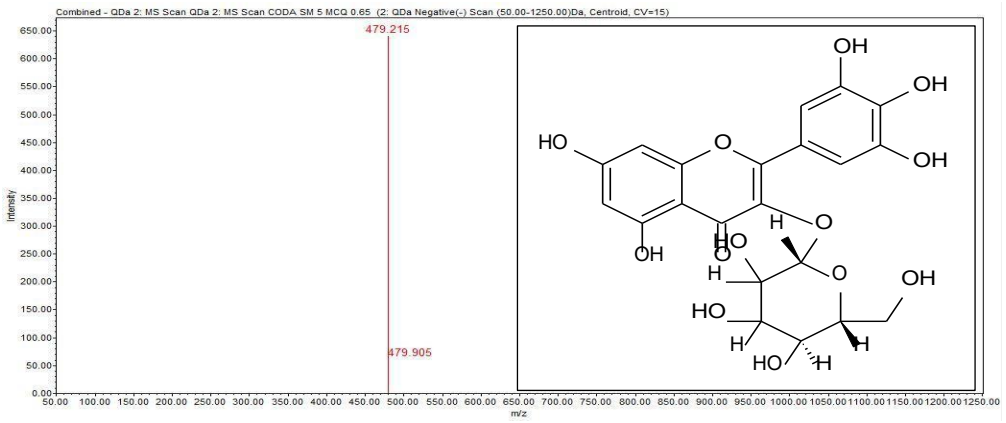

Figure. 4: Total Ion Chromatogram and mass spectrum of Subfraction B

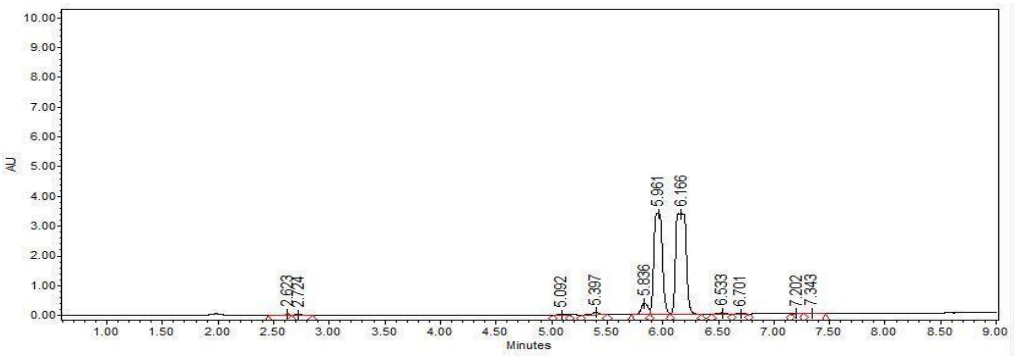

Figure. 5: Chromatogram of Sub Fraction E showing the separation on a C-18 column (Sunfire C-18, 5.0 µm, 4.6 mm x 150 mm).

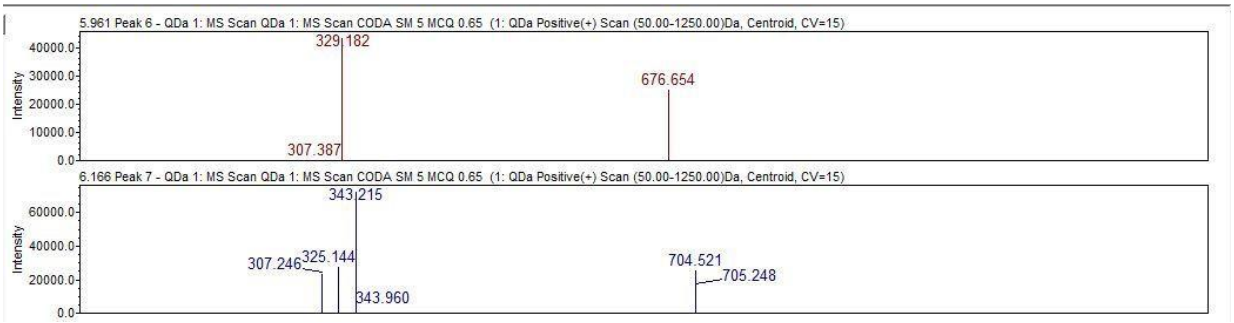

Figure. 6: TIC and Mass spectrum of Sub fraction E

Antioxidant capacity

The results of the antioxidant assays using ABTS, DPPH, and FRAP assays indicated that the ethyl acetate and n-butanol soluble fractions, as well as the chromatographic fractions, inhibited peroxidation induced by the ABTS, DPPH, and FRAP assays (Table 1). The n-butanol soluble fraction (Sample D) showed the highest ABTS radical scavenging ability with an IC_{50} of 43.76 µg/ml followed by the ethyl acetate soluble fraction with an IC_{50} of 49.90 µg/ml while ascorbic acid, the standard drug, showed an IC_{50} of 20.20 ± 0.23µg/ml (Table 2, Fig. 7). ABTS radical scavenging activity was in the order of D>C>E>B>A (Table 1). The chromatographic sub-fraction of the n-butanol

soluble fraction (Sample E) showed the highest DPPH radical scavenging activity, with an IC_{50} of 16.53 µg/ml followed by the n-butanol soluble fraction (Sample D), which gave an IC_{50} of 18.14 µg/ml (Table 1). The ethyl acetate soluble fraction (Sample C) had an IC_{50} of 24.19 ±3.16 µg/ml. The radical scavenging activities of n-butanol, the chromatography subfraction, and the ethyl acetate fraction were all higher than those of ascorbic acid, with IC50 values of. 37.42 ±1.67 µg/ml. The order of DPPH radical scavenging activity was E>D>C>A>B (Table 1). In the FRAP assay, the n-butanol soluble fraction exhibited the strongest reducing ability among the samples tested (Table 1) with IC_{50} 0f 46.99 µg/ml followed by the ethyl acetate with IC_{50} of 53.37 ±1.53 µg/ml in contrast to ascorbic standard which showed a reducing ability of IC_{50} 29.18 ±0.66 µg/ml (Table 1, Figures: 8 and 9).

Table 1: Antioxidant Activities of Samples A - E and Ascorbic acid[#]

	IC_{50} ± SEM (µg/mL)		
Sample	AB	DPPH	FRAP
A	68.58 ± 0.05	25.65 ± 1.46	78.13 ± 0.82
B	55.07 ± 4.82	42.00 ± 1.51	61.52 ± 0.11
C	49.90 ± 2.99	24.39 ± 3.16	53.37 ± 1.53
D	43.76 ± 2.38	18.14 ± 0.18	46.99 ± 2.10
E	52.08 ± 4.02	16.53 ± 1.17	74.62 ± 1.34
Ascorbic Acid (Standard)	20.20 ± 0.23	37..42 ± 1.67	29.18 ± 0.66

A, chromatographic subfraction of ethyl acetate; B, chromatographic subfraction of ethyl acetate; C= Ethyl acetate fraction; D, n-butanol fraction; E, chromatographic subfraction of n-butanol fraction. Results are expressed as Mean ± SEM where n = 3

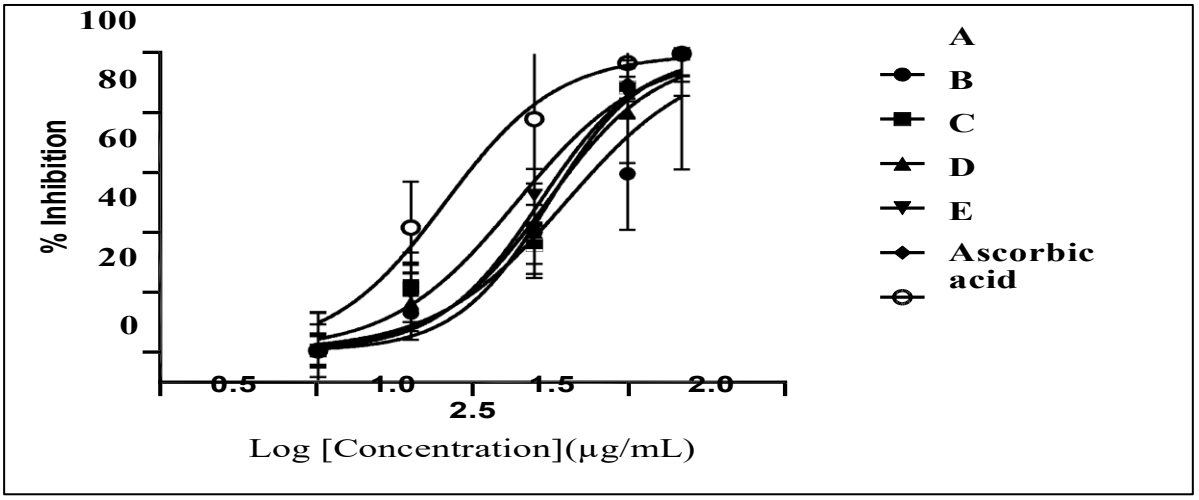

Figure. 7: Antioxidant activity of samples A-E in comparison to ascorbic acid using ABTS assay

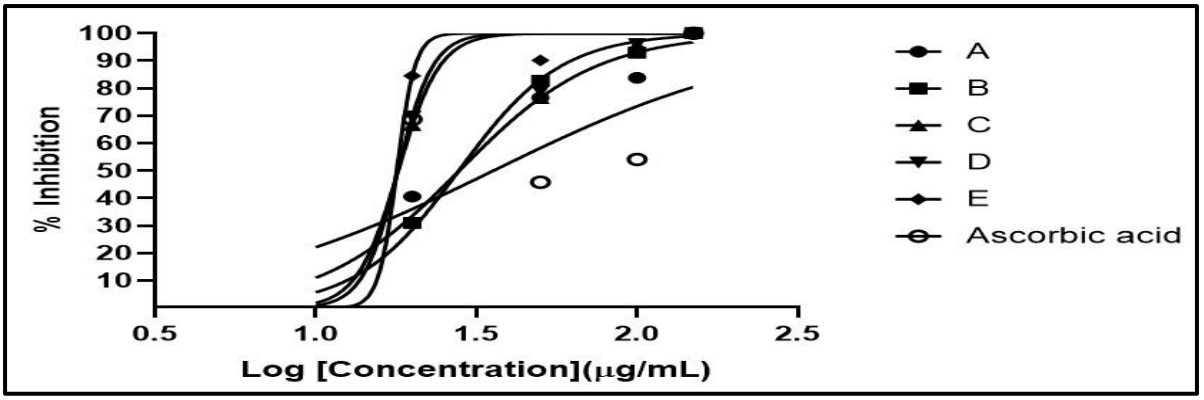

Figure. 8: Antioxidant activity of samples A-E in comparison to ascorbic acid using DPPH Assay

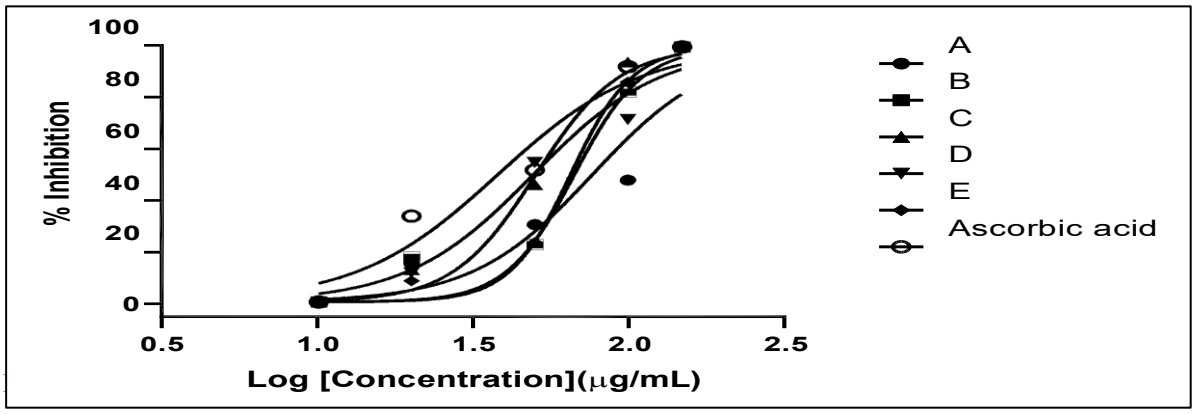

Figure. 9: Antioxidant activity of samples A-E in comparison to ascorbic acid using FRAP Assay

Discussion

Subfraction A was isolated as a colorless crystal, and LC-MS of this subfraction obtained from the ethyl acetate-soluble fraction showed a molecular $[M-H]^+$ peak at 479.282, which points to a molecular weight m/z of 480 and a molecular formula $C_{21}H_{20}O_{13}$, which was identified as a monogalloyl ester of bergenin, 11-galloyl bergenin (Khan *et al*.,2006; Wu et al., 2023). Sub-fraction B, also obtained from the ethyl acetate-soluble fraction, showed an $[M-H]^+$ peak at m/z 479.215, which translates to a molecular weight of 480.215 and molecular formula of $C_{21}H_{20}O_{13}$. Sub-fraction B, although with a molecular weight similar to that of sub-fraction A, was isolated from a more polar region than sub- fraction A; thus, sub-fraction B was identified as myricetin 3-O-glucoside (Khan *et al.,* 2016). Sub- fraction E was obtained from the n-butanol soluble fraction as a colourless solid, and the mass spectrum revealed two molecular $[M+H]^+$ peaks at m/z 329.182 and m/z 343.215, which point to the molecular weights of 328.182 and 342.215 and the molecular formula $C_{14}H_{16}O_9$ and $C_{15}H_{18}O_9$, respectively, consistent with that of bergenin (Subramanian *et al*., 2015), and proposed methyl bergenin. The two compounds differ by a methyl groups. Bergenin is a C- glycoside of 4-O-methyl gallic acid, a colorless crystalline-like polyphenol found in many medicinal plants, such as *Bergenia ciliata* and *Bergenia ligulata* (Khan *et al*., 2016). Bergenin is known to exhibit many pharmacological activities, including inhibitory activity against arrhythmias and anti-HIV, antifungal, anti-inflammatory, and anti-cancer activities (Chitte *et al.,* 2024). Yang *et al*, (2016) and Piacente *et al*, (1996) have reported the antidiabetic activity of bergenin and anti-HIV activity of bergenin and nor-bergenin respectively, while 11- galloyl bergenin was reported to exhibit higher antioxidant activity using DPPH and FRAP assay methods than bergenin (Brand-Wiliams ,2000). The same authors also reported the antiplasmodial activities of bergenin and 11-O-galloyl bergenin. Antioxidant activity is the ability of an active molecule to reduce free radical generation by scavenging reactive oxygen species (ROS) and inhibiting injuries to the degradation and oxidation

of biomolecules. The results of the antioxidant assays using ABTS, DPPH, and FRAP assays revealed that the ethyl acetate and n-butanol soluble fractions, as well as the chromatographic fractions, inhibited peroxidation induced by ABTS, DPPH, and FRAP reduction. In the presence of potassium persulfate, ABTS reacts to create a persistent green radical that is suppressed in the presence of an antioxidant. The n-butanol soluble fraction (Sample D) showed the highest ABTS radical scavenging ability with an IC_{50} of 43.76 µg/ml followed by the ethyl acetate soluble fraction with an IC_{50} of 49.90 µg/ml while ascorbic acid, the standard drug, showed an IC_{50} of 20.20 ± 0.23µg/ml. DPPH is a consistent free radical that can be used to evaluate the antioxidant properties of medicinal plant extracts or compounds that can be employed as food additives (Benzie, 1996). It is a free radical that is used to evaluate the antioxidant activity of compounds faster than any other method (Khan *et al*. 2006). The FRAP assay determines the overall reducing capacity by forming a blue complex in the presence of antioxidants, and the change in absorbance at 593 nm represents antioxidant capacity (Benzie, 1996). The soluble and chromatographic subfractions tested positive for phenols and flavonoids. From subfractions A, B, and E, 11-galloylbergenin, Myricetin -3-O-glucoside, bergenin, and methyl bergenin were identified using LC-MS. Bergenin has been reported to exhibit antioxidant activity against DDPH, and hydroxyl and nitric oxide scavenging activities at 100 µg/ml and less ferric-reducing power (Wu, 2023). Khan *et al* (2016) also reported the antioxidant and anti-plasmodial activities of bergenin and 11-O-galloyl bergenin isolated from *Mallotus phillippensis*. Bergenin has been reported to exhibit antioxidant potential by reducing free radical generation and scavenging ROS (Nazir *et al*., 2011). Flavonoids are known to exhibit radical scavenging activity, which has been attributed to the phenolic OH group, and the radical scavenging activity increases with an increase in the number of hydroxyl groups. Flavonoids are known antioxidants, and the 5,7 phenolic hydroxyl groups of ring A are necessary for their antioxidant activity (Sarian *et al*., 2017). Flavonoids containing the o-diphenolic hydroxyl group are known to exhibit better antioxidant activity than those containing the m-diphenolic group (Agraharam, 2022). Myricetin is a flavonoid that exhibits these characteristics and hence, its antioxidant potential. The presence of bergenin in the n-butanol-soluble fraction might be responsible for the higher antioxidant activity of this fraction than that of the ethyl acetate-soluble fraction. The observed antioxidant activity of the ethyl acetate, n-butanol, and chromatographic fractions was likely due to the presence of 11-galloyl bergenin, myricetin, bergenin, and methyl bergenin, which were identified using LC-MS.

Conclusion.

The antioxidant activity and the presence of bergenin, methyl bergenin, 11-O-galloyl bergenin and Myrecetin-3-O-glucoside reported in the stem bark of *Pentaclethra macrophyla* might be responsible for the nutritional and medicinal applications of this plant

Declaration of Interest

The authors declare that they have no conflicts of interest.

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