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Original Research Article

Estimation of Total Phenolics, Total Flavonoids Content and *In Vitro* Antioxidant Activities of Extract and Fractions of *Asplenium platyneuron* (Carl Linnaeus)

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ABSTRACT

Phenolic compounds possess the potential to function as antioxidants, hence the growing interest in ethnomedicine to use them for treatment or prevention of diseases associated with oxidative stress. This study measured the total phenolics and flavonoid contents (TPC and TFC) and *in vitro* antioxidant activity of *Asplenium platyneuron* extract and its fractions. The methanol extract was partitioned successively in *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol. Thereafter, the EtOAc soluble was fractionated using vacuum liquid chromatography (VLC) in gradients of *n*-hexane/ EtOAc and dichloromethane/methanol. The free radical scavenging activity was determined by using diphenyl-picrylhydrazyl (DPPH), and the reducing power by ferric reducing antioxidant power (FRAP) and the total antioxidant capacity assays. The TPC of EtOAc fraction was 9.31 ± 0.76 mg GAE/g extract compared with 1.33 ± 0.61 mg GAE/g extract of *n*-hexane fraction. The TFC for EtOAc was 139.73 ± 8.03 mg QE/g extract higher than 35.73 ± 15.01 mg QE/g extract of *n*-butanol fraction. The ethylacetate fraction exhibited the highest DPPH free radical scavenging capacity of 80.50%, and FRAP of 413.00 ± 1.28 mg GAE/g extract with IC_{50} of 1.31 ± 0.68mg/mL. There was a strong correlation between the antioxidant activity and the TFC/TPC. The VLC fractions, AP_2 and AP_7 (IC_{50} 1.43 ± 1.34 and 1.41± 1.11 mg/mL respectively) elicited antioxidant activity higher than other fractions. Dereplication analysis identified three phenolics; apigenin 6-C-hexoside-8-C-pentoside, kaempferol 3-*O*-caffeoylsophoroside and schaftoside from *A. platyneuron*. The high TPC/TFC and its strong antioxidant properties makes *A. platyneuron* a promising source of novel lead antioxidants.

***Keywords*:** Phenolics, flavonoids, *Asplenium platyneuron*, gallic acid, quercetin, antioxidant activities

Introduction

With the advancement of civilization and technology, people are now continually and progressively exposed to environmental pollution, smoking, alcoholism, poor nutrition, stress-inducing behaviours, and unhygienic lifestyles. These factors present a significant health risk. Oxidative stress, which is caused by an imbalance between reactive oxygen species (ROS) and the biological system's capacity to detoxify the reactive oxygen intermediates, is thought to be the root cause of several illnesses, including Parkinson's disease, ulcers and premature ageing of the skin [1,2]. Many compounds found in plants used in traditional medicine can be employed as medications to treat serious chronic non-communicable diseases as well as infectious disorders [3]. To reduce oxidative cell damage, there is a lot of interest right now in discovering antioxidants from natural sources.

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Increased levels of reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide, hydroxyl radicals (OH^-), and peroxyl radicals (ROO^-) can cause oxidative stress in the body, which can lead to pathogenic and degenerative effects such DNA and cell damage. Cancer, ageing, atherosclerosis, ischemia injury, inflammation, and neurological diseases have all been linked to oxidative stress.[4] By contributing electrons to stabilize and counteract the negative effects of the free radicals, antioxidants protect DNA, proteins, and cells from oxidative damage caused by the free radicals. Being radical scavengers, plant-derived antioxidants have drawn more attention. Effective antioxidants that occur naturally are phenolic chemicals.[5]

Many therapeutic plants include chemical constituents with antioxidant effects. The hypothesis that plant elements with antioxidant activity are capable of exerting protective effects against oxidative stress in biological systems is strongly supported by several studies on medicinal plants.[6] Plant phenolics may have health advantages in part because of their antioxidant capabilities, which include metal chelation, electrophile scavenging, and the suppression of reactive oxygen species (ROS).[7] Additionally, pharmacological features like anticancer, antiviral, antibacterial, anti-inflammatory, anti-hypertensive, and antioxidant activity have been found for natural antioxidants like phenols and flavonoids.[8-9] *Asplenium platyneuron* (Carl Linnaeus), is a fern in the Aspleniaceae family and it is called Okpáká in the Igbo language. The ethnomedicinal uses of *A. platyneuron* have been harnessed for the treatment and prevention of stroke and can also be traditionally used for the treatment of spleen enlargement, kidney, anti-inflammatory, bronchitis, fever, and bowel diseases.[10,11] However,

some of these ethnomedicinal claims, including antioxidant activity, have not been well documented. This study aims to search for alternative and natural remedies for the treatment of diseases caused by oxidative stress determining the total phenolic/flavonoid content (TPC/TFC) of the extract and fractions. The study fractionated the ethyl acetate (EtOAc) soluble of the methanol extract by vacuum liquid chromatography, determined the total antioxidant capacity (TAC) of the extracts and fractions, the qualitative phytochemical analysis of the extracts and fractions, and the *in vitro* antioxidant assay of the extracts and fractions. The HPLC analysis of the VLC sub-fractions was undertaken to determine the distribution of secondary metabolites and antioxidants.

Materials and Methods

Reagents and Chemicals

Folin-Ciocalteau reagent from Lobal Chemie in India, *n*-hexane, methanol, *n*-butanol, ethyl acetate, and other chemicals from Sigma-Aldrich in Germany. BDH (England) supplied the ascorbic acid, quercetin, tetraoxosulphate (VI) acid, and sodium hydroxide. Gallic acid and sodium nitrite were purchased from Qualikems in India. The following items were purchased from JHD (China): sodium trioxocarbonate (IV), aluminium chloride hexahydrate ($AlCl_3.6H_2O$), sodium phosphate (NaH_2PO_4), and ammonium molybdate. Sigma-Aldrich in Germany was used to obtain 1,1-diphenyl-2-picrylhydrazyl (DPPH). The following items were purchased from Sigma-Aldrich (Germany): potassium ferric cyanide ($K_3F(CN)_6$), phosphate buffer, ferric chloride hexahydrate ($FeCl_3.6H_2O$), trichloroacetic acid, and silica gel. Analytical-grade chemicals were employed throughout.

Preparation of plant material and extract

Fresh leaves of *A. platyneuron* (Carl Linnaeus) were procured from the University of Nigeria Secondary School in Nsukka, Nigeria (6.8429°N, 7.3733 °E) in December 2015 and were identified and authenticated by Mr. Alfred O. Ozioko, a taxonomist at the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria. The voucher number is UNN/11231. The plant was air dried for 14 days, powdered, and 500 g was macerated in 1.25 L of methanol at room temperature for 48 h with agitation. To obtain the extracts, the filtrate was concentrated *in vacuo* under reduced pressure and at 40°C.

Solvent partitioning of extract

The crude methanol extract was partitioned successively in *n*-hexane, ethyl acetate, and *n*-butanol.[12] Methanol extract (12.5 g) was dissolved in 10% methanol (400 mL) and partitioned using *n*-hexane (250 × 10 mL), ethyl acetate (250 × 10 mL), and *n*-butanol (250 × 4 mL) to obtain four solvent fractions: *n*-hexane (0.453 g) (HF), ethyl acetate (4.20 g) (EF), *n*-butanol (3.90 g) (BF) and residual aqueous fractions respectively. After drying *in vacuo*, the percentage yields for the methanol extract and each of these fractions were recorded to be 2.45, 0.09, 0.84, and 0.78%w/w of the powdered sample respectively.

Vacuum liquid chromatographic fractionation

The ethyl acetate (4.20 g) fraction was further separated by vacuum liquid chromatography (VLC) using silica gel (60 – 200 mesh) as the stationary phase in a glass column (150×3.0 cm) and eluted with a gradient of *n-hexane* in ethyl acetate (10:0, 8:2, 6:4, 4:6, 2:8, 0:10, each 500 mL) followed by dichloromethane in methanol (9:1, 7:3, .5:5, 3:7,1:9, each 500 mL) to afford 11 sub-fractions (AP_1-EF_{11}). Sub-fractions AP_1 (0.024 g, 0.0048%), AP_2 (0.029 g, 0.0.0058%), AP_3 (0.048 g, 0.0096%), AP_4 (0.069 g, 0.0138%), AP_5 (0.046 g, 0.0092%), AP_6 (0.076 g, 0.0152%), AP_7 (0.016 g, 0.0032%) per weight of powdered sample were obtained.

Phytochemical analysis

The methanol extract and its fractions were subjected to a qualitative phytochemical analysis using standard methods with slight modifications.[13]

Determination of the total phenolic content (TPC)

Gallic acid was employed as an internal standard with a small modification, as previously reported.[14] In a volumetric flask (20 mL), the extract (1.0 mg/mL) was combined with distilled water (9.0 mL). A 2.5 mL addition of 10-fold diluted Folin-Ciocalteau phenol reagent (FCPR, 1:10) was made. After waiting for five minutes, 10 mL of 7.5% Na_2CO_3 solution was added to the mixture and adjusted with distilled water to the proper concentration. The mixture was incubated at room temperature for 90 minutes in the dark. The same method used to prepare the extracts was used to prepare a set of standard solutions of gallic acid (20, 40, 60, 80, and 100 mg/L). Using a UV/Visible spectrophotometer (UV-1800, Shimadzu, Japan), the absorbance of the extract and standard solutions were measured against the reagent blank at 760 nm. The total phenolic content of the extracts was calculated in triplicate. The calibration curve was used to calculate the total phenolic content, which was then reported as milligrams of gallic acid equivalent (GAE) per gram of the extract.[15]

Determination of total flavonoid content (TFC)

The total flavonoids included in the extract were assayed using an aluminium-chloride colourimetric test, as previously reported [14,15]. In a 20 mL volumetric flask, the extract (1.0 mg/mL) was combined with distilled water (4.0 mL). The flask received 0.30 mL of 5% sodium nitrite. 10% $AlCl_3.6H_2O$ solution (0.30 mL) was added to the mixture after 5 minutes, and after another 5 minutes, 1.0 M NaOH (2.0 mL) was added. The mixture was then diluted to the desired strength using distilled water. The same procedure used to make the extracts was used to create a set of standard solutions of quercetin (20, 40, 60, 80, and 100 mg/L). Using a UV/Visible spectrophotometer Model 721G from Yoke Instruments Co., China. The absorbance of the extract and standard solutions was measured against the reagent blank at 510 nm. In triplicates, the total flavonoids in the extract and standards were determined.

In-vitro antioxidant assays

Three assay models, the phosphomolybdate method, 1,1-diphenyl-2-picrylhydrazyl DPPH method, and ferric reducing antioxidant power (FRAP) were used in this study [16,17]. The extract was dissolved in distilled water (100 mL) to create stock solutions (1.0 mg/mL or 1000 mg/L) before being used in *in vitro* antioxidant tests. From the stock solution, successive dilutions (25, 50, 100, 200, 250, and 300 mg/L) of each extract were created. The antioxidant assays employed ascorbic acid (ASA) as the standard.

Total antioxidant capacity (TAC) assay

The total antioxidant capacity was conducted using the phosphomolybdate method, as previously reported [18]. The extract, fractions, and ascorbic acid were aliquoted at various concentrations (25, 50, 100, 200, 250, and 300 mg/mL), and reagent solution (1.0 mL) (600 mM sulfuric acid, 28 mM sodium phosphate, and 4 mM) was added. The test tubes were incubated for 90 min in a water bath set at 95 ℃ with aluminium foil covering them. The mixture's absorbance was measured at 765 nm using ascorbic acid as a reference and a blank that contained the reagent solution (1.0 mL) after the extract had cooled to room temperature. The assay was performed three times. Ascorbic acid equivalents (AAE) are used to express the antioxidant capacity (TAC) as shown in Equation 1

$$\text{Total antioxidant capacity (\%)} = \frac{\text{Absorbance of control - Absorbance of extract}}{\text{Absorbance of control}} \times \frac{100}{1} \quad \text{---(1)}$$

DPPH free radical scavenging assay

The DPPH assay, with minor modifications, was used to determine the radical-scavenging capacity.[18,19] Briefly, a fresh stock solution of DPPH was created by combining 4.5 mg of DPPH with 100 mL of methanol. Before reading the absorbance at 517 nm, a sample solution (1.0 mL) and DPPH stock solution (3.0 mL) were combined and incubated at room temperature for 30 minutes in the dark. All tests were carried out in triplicate, and ascorbic acid's DPPH radical scavenging activity was

also measured for comparison. The percentage inhibition of radical activity was obtained from Equation 2.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \times \frac{100}{1} \quad \text{--- (2)}$$

Ferric reducing antioxidant power assay

The extract and fractions' ability to reduce ferric iron was assessed using the previous method with minor adjustments [20]. Potassium ferricyanide (2 ml, 10 mg/L), phosphate buffer (2 mL, 0.2 M, pH 6.6), and the extract and fractions solutions (2 mL each) were individually mixed before being incubated at 50 °C in a water bath for 20 min. The mixture then received 2 mL of 100 mg/L trichloracetic acid. In a test tube, distilled water (2 mL), 0.1% ferric chloride (0.4 mL), and 2 mL of each of the solutions were combined. The reaction's absorbance was measured at 700 nm after 10 minutes. The antioxidant capacity of the extract and fractions for reducing ferric was calculated as mg gallic acid equivalent/gram.[21]

Analytical HPLC method

An analytical HPLC column from Jasco (Gross-Umstadt, Germany) was used while analytical HPLC separations were performed. HPLC system (LC Net II ADC Chromatography Data Solutions; pump: PU-2087 plus; diode array detector MD 2018 plus; column thermostat CO 2060 plus; autosampler AS 2055 plus); sample injection loop: 500 µL). An analytical reverse phase column Reprosil 100 C-18 (5 µm, 250 mm × 10 mm) was used for the separations, and binary gradients of the mobile phase (methanol and millipore water) were used. All HPLC analytical separations were conducted using a column-thermostat temperature of 25°C and a flow rate of 0.5 mL/min. A detection between 200 and 400 nm was noted. A 10 cm analytical column containing 20 - 45 µm, 2.5 g of Europrep C18-reverse phase was manufactured. Low pressure was used for the separation process, and a glass chamber with a vacuum control was connected to the column. The following was the gradient system: 0–5 minutes: MeOH content ranges from 20–30%, 5–10 min: 30–70%, 11–15 min: 71–100%, 15–16 min: 100%, and 17–18 min: 100% 20 minutes at 20% MeOH, 100 - 20% MeOH. The sample (1 mg) was dissolved in methanol and water (1:1) before being injected, sonicated for ten minutes in an ultrasonicator, then passed through 0.45 µm Millipore filter paper and put into a 1 mL HPLC vial. For every run, 10 µL of sample was injected.

The produced chromatograms were automatically compared to the database of natural products after each run, searching for the compound or compounds that most closely matched the detected chromatogram. The closest identifier based on retention time (tR), UV wavelengths of maximal absorption, and chromophore match was then found by querying the natural product database.

Analytical Statistics

A two-way analysis of variance (ANOVA) with repeated measures was used to determine the significance of disparity in the data as mean standard deviation (SD) using SPSS v.23. A p-< 0.05 was deemed significant.

Results and Discussion

Phytochemical constituents of A. platyneuron

The methanol extract, *n*-butanol and ethyl acetate fractions showed a high presence of phenolic and flavonoid content (Table 1). The *n*-butanol contained moderate phenolic content and traces of flavonoid content. The TPC in *A. platyneuron* extract and solvent fractions using the Folin-Ciocalteau's reagent was expressed in terms of gallic acid equivalent as shown in Figure 1A. The high TPC in the ethylacetate fraction agreed with previous reports.[30,31] The TPC in plants generally depends on the type of extract and the polarity of the solvent used in the extraction. The high solubility of phenols in polar solvents provides a high concentration of these compounds in the extracts obtained using polar solvents for the extraction[32,33] *A. platyneuron* contained lower TPC than the TFC due to its natural habitat on trees, and hence a lower stress factor.[34] The TFC of the extract and fractions of *A. platyneuron* were determined from the regression equation for the calibration curve (Figure 1B). The values obtained for the concentration of total flavonoids are expressed as mg of quercetin equivalent (QE)/g dry weight of the extract. The *n*-hexane and *n*-butanol fractions contain considerably lesser concentrations of flavonoid when compared to ethyl acetate fraction and methanol extract, which is in agreement with the previous report.[31]

The solubility of polyphenols in the extraction solvent, the kind of solvent, the degree of polymerization of phenols, the interaction of phenols with other plant elements, and the development of insoluble complexes all affect the recovery of polyphenols from plant materials [35]. Differences in extraction yield and antioxidant activity may be explained by variations in the polarity of antioxidants. Additionally, phenolic solubility is significantly increased by solvent polarity.[36,37] As seen in this study methanol extracted a high yield of polyphenolic compounds as previously reported.[38, 3]

Table 1: Phytochemical composition of *A. platyneuron*

Phytochemical constituents	Methanol extract	*n*-Hexane soluble	Ethyl acetate soluble	*n*-Butanol soluble
Phenolics	+	-	+	+
Flavonoids	+	-	+	+

(+) = present, (-) = absent

Table 2: Flavonoids-phenolics ratio for extract and fractions

Concentration (ppm)	Methanol extract	*n*-Hexane soluble	Ethyl acetate soluble	*n*-Butanol soluble
25	8.82	0.00	12.69	0.00
50	6.77	0.00	9.72	25.55
100	10.65	0.00	12.53	34.80
200	18.37	104.93	11.03	28.38
250	20.78	111.86	16.03	25.72
300	27.36	75.98	15.00	10.35

Antioxidant activity of A. platyneuron

The antioxidant activity of *A. platyneuron* was evaluated using the DPPH, TAC and FRAP models. The DPPH radical scavenging activity showed that the ethyl acetate soluble exhibited strong activity with IC_{50} of 1.31 ± 0.68 mg/mL, higher than both the extract and VLC fractions (Table 3). This was also seen in the DPPH inhibition of the radical activity (Figure 3C and D) The significant decline in the *in vitro* activity as a result of fractionation suggested possible synergistic effects of the phytoconstituents. The observation in this study indicates the effective capacity of *A. platyneuron* for scavenging superoxide radicals and associated with total flavonoid content, showing its potential as an antioxidant. The inherent properties of phenolics may be connected to the capacity of extract and fractions for radical scavenging and electron transfer/hydrogen donation which have also been speculated in previous reports.[9, 34]

In the TAC model, similar trends were observed as the ethyl acetate showed significantly higher TAC compared with other samples which is also consistent with previous postulations of synergistic properties of the phytoconstituents (Figure 3A and B). There was also concentration-dependent antioxidant capacity of all the tested samples. The TAC model is known to derive its effect via the reduction of Mo (VI) to Mo (V) by various radicals which are released in the system. The strong antioxidant activity of *A. platyneuron* which compared well with ascorbic acid indicates strong antioxidants in extract and fractions used in this study and these could be attributed to the presence of phenolic compounds and flavonoids.[25,37]

In the FRAP model, the ethyl acetate soluble maintained higher concentration-dependent *in vitro* activity than the other samples tested. In this study, the FRAP assay was expressed as mg GAE/g dry weight of the extract which was obtained from the regression equation (Figure 1C). The model is based on the ability of tested phytochemicals to reduce ferric ions to ferrous ions which later formed a blue ferrous-tripyridyltriazine complex at 590 nm. The decline in the FRAP activity on VLC fractionation of the active ethyl acetate fraction supported our initial postulation of possible synergism in the antioxidant activity of the phytoconstituents of *A. platyneuron*

Table 3: IC_{50} values for DPPH scavenging free radical capacity

Extract/fractions	IC_{50} (mg/mL)
Methanol extract	1.33 ± 0.23
n-Hexane soluble	1.37 ± 0.57
Ethylacetate soluble	1.31 ± 0.68
n-Butanol soluble	1.38 ± 0.48
AP_1 fraction	1.51 ± 1.02
AP_2 fraction	1.43 ± 1.34
AP_3 fraction	1.45 ± 0.09
AP_4 fraction	1.51 ± 0.07
AP_5 fraction	1.43 ± 0.76
AP_6 fraction	1.45 ± 0.02
AP_7 fraction	1.41 ± 1.11
Ascorbic acid	1.02 ± 0.35

AP_1 – AP_7 = VLC fractions of Ethyl acetate soluble of *A. platyneuron*

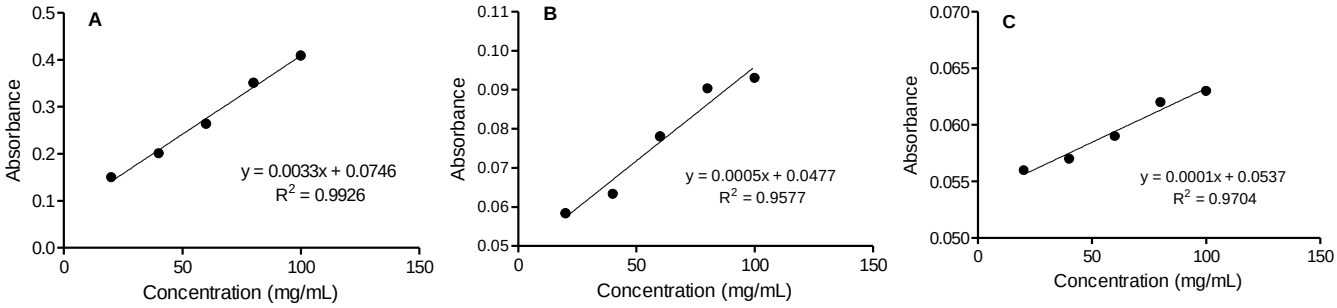

Figure 1: Calibration curves for (A) gallic acid (TPC), (B) quercetin and (C) gallic acid (FRAP)

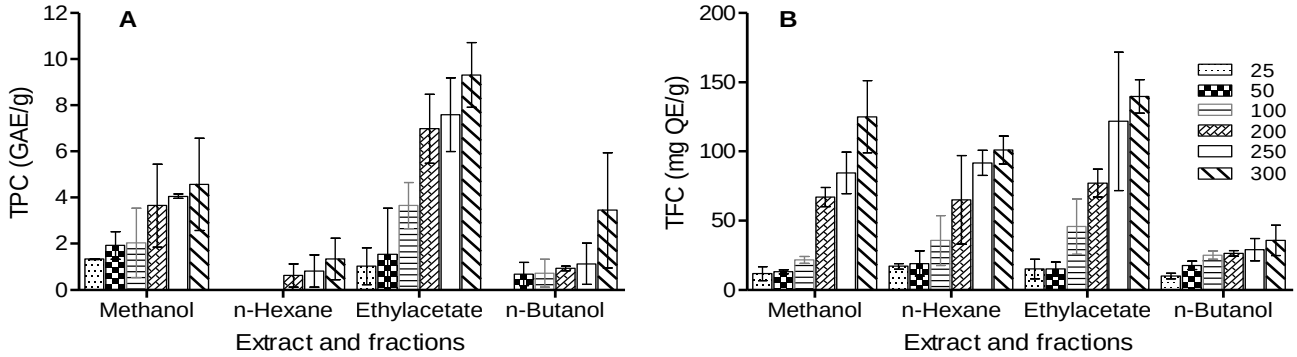

Figure 2: TPC (A) and TFC (B) of *A. platyneuron*.

Table 4: Regression values for various antioxidants models forTPC and TFC

Sample	Total phenolic content			Total flavonoids content		
	FRAP	DPPH	TAC	FRAP	DPPH	TAC
Methanol	0.846	0.938	0.426	0.898	0.872	0.264
n-Hexane	0.932	0.916	0.657	0.950	0.922	0.848
Ethyl acetate	0.873	0.962	0.980	0.866	0.922	0.927
n-Butanol	0.951	0.551	0.533	0.850	0.921	0.909

Polyphenolic compounds such as flavonoids are reputed for their antioxidant activity. The study attempted to correlate the antioxidant activity with the TFC and TPC of *A. platyneuron.* There was a significant correlation between the total phenolic content (TPC) and FRAP for methanol extract, *n*-hexane, ethyl acetate, and *n*-butanol, with regression (R^2) values of 0.846, 0.932, 0.873, 0.951 respectively (Table 4). Some weak correlations R^2 = 0.426, 0.6566, and 0.533 between the TPC and TAC values for methanol extract and *n*-butanol fraction showed variations in the phenolic contents from those which are responsible for TAC scavenging in *A. platyneuron*. There was also a weak correlation R^2 = 0.551 between the TPC and DPPH values for *n*-butanol fraction which suggests that the phenolic compounds are different from those which are responsible for DPPH scavenging in *A. platyneuron*. In this study, it was observed that the *A. platyneuron* extract and fractions that performed the highest antioxidant activity had the highest concentration of phenols. The great antioxidant activity was found to be attributed to the high contents of phenolic compounds, as demonstrated by the considerable linear association between the phenolic compound concentration values and the antioxidant activity. The findings demonstrated the significance of phenolic compounds in the antioxidant behaviour of plant extracts and showed that flavonoids and phenolic acids are the main contributors to the antioxidant activities of fractions of *A. platyneuron* leaves. The significant connection between the various tests demonstrated the viability and complementarity of the antioxidant assays.[31] Taking into account the significant antioxidant activity of this extract and its components, our data also point to a strong link between the total flavonoid concentration and antioxidant capacity. The inclusion of flavonoids and phenolics, which are known to suppress free radicals, is what gives them their antioxidant capacity.[30,40] This finding is consistent with the results of our current investigation, which found high levels of flavonoids and phenolic compounds.

HPLC dereplication of active VLC fraction

Since the ethyl acetate fraction had the highest level of antioxidant activity, its phenolic components were dereplicated using HPLC by comparing the retention time (RT) and UV_{max} spectrum of these compounds with previously isolated compounds (Tables 5, Figures 4 and 5) [23-29]. Thirteen phenolic compounds Kaempferol 3, 7, 4'-*O*-hexaglycoside (1), Mangiferin X^n-*O*-glucoside (2), Vicenin-2 (3), Kaempferol 3,7-*O*-pentaglycoside (4), Kaempferol 3,7-*O*-triglycoside (5), Mangiferin (6), Schaftoside (7), Kaempferol 3-*O*-rhamnoside-7-O-glucoside (8), Kaempferol 3,7-O-tetraglycoside (9), Kaempferol 3-*O*-sophoroside (10), Kaempferol 3-*O*-(caffeoylsophoroside) (11), Isomangiferin (12), Apigenin 6-C-hexoside-8-C-pentoside (13), were identified. Some of the identified compounds are known for anti-inflammatory, anti-diuretic, anti-arthritic, antioxidant and antimicrobial activity which provides leads for a new drug candidate. The phenolic compounds, in particular Apigenin 6-C-hexoside-8-C-pentoside (13), Kaempferol 3-*O*-(caffeoylsophoroside) (11), and Schaftoside (7), could be responsible for the strong activity of the ethyl acetate fraction. However, other compounds may also be involved in the antioxidant activities of *A. platyneuron* leaves.[41]

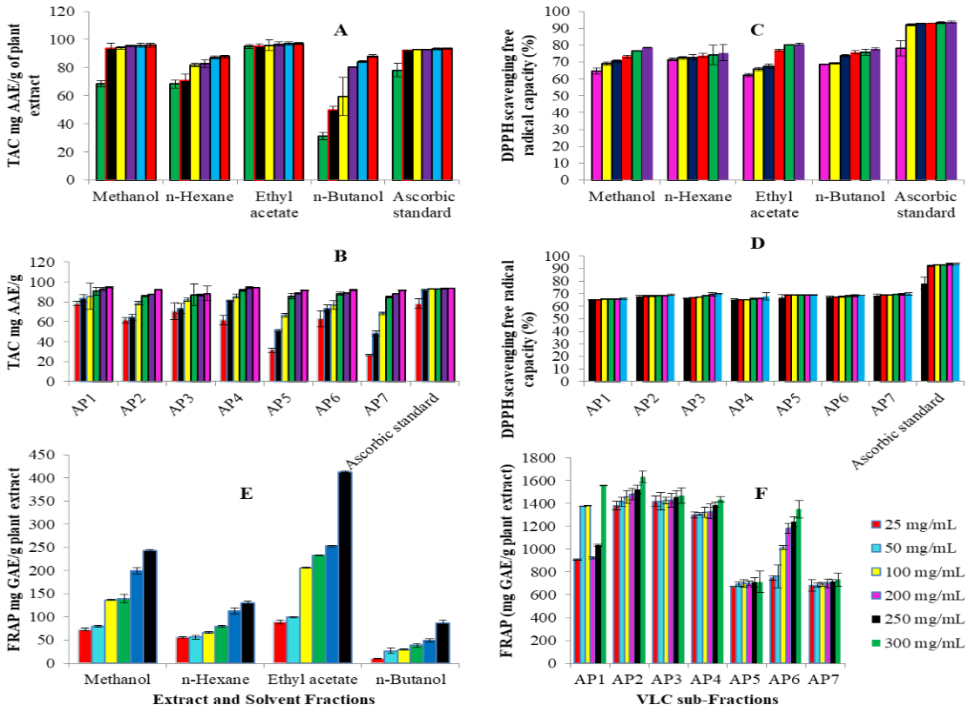

Figure 3: Antioxidant activity showing TAC of (A) extract and fractions (B) VLC fractions; DPPH of (C) extracts and fractions, (D) VLC fractions and FRAP of (E) extract and fractions, (F) VLC fractions

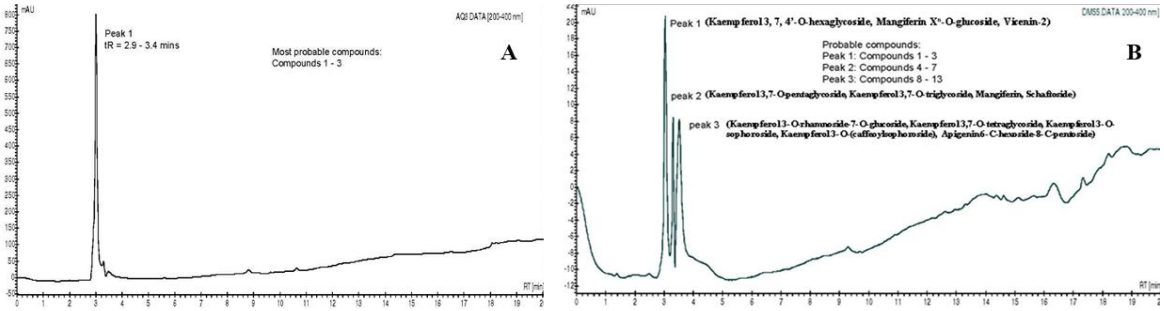

Figure 4: HPLC Chromatogram of (A) AP_2 and (B) AP_3

Table 5: HPLC dereplication analysis of *A. platyneuron*

Fraction AP_3					
RT (min)	**λ_{max} (nm)**	**ID**	**RT (min)**	**UV_{max} (nm)**	**Probable compounds**
2.9 – 3.4	225	1	3.65	269, 327269, 327	Kaempferol 3, 7, 4 -*O*-hexaglycoside (1) [22]
	225	2	3.66	243, 259, 316, 365.	Mangiferin X^n-*O*-glucoside (2) [23-24]
	225	3	3.67	273, 333.	Vicenin-2 (3) [25]
		Fraction AP_4			
2.9 – 3.2	241	1	3.65	269, 327	Kaempferol 3, 7, 4 -*O*-hexaglycoside (1) [22]
2.9 – 3.2	241	2	3.66	243, 259, 316, 365.	Mangiferin X-*O*-glucoside (2) [23-24]
2.9 – 3.2	241	3	3.67	273, 333.	Vicenin-2 (3) [25]
3.2 -3.5	215, 273, 335	4	3.98	243, 267, 331	Kaempferol 3, 7-*O*-pentaglycoside (4) [22]
3.2 – 3.5	215, 273, 335	5	3.70	266, 347	Kaempferol 3,7-*O*-triglycoside. (5) [26]
3.2 – 3.5	215, 273, 335	6	3.70	241, 257, 315, 364	Mangiferin (6) [27]
3.2 – 3.5	215, 273, 335	7	3.84	273, 332	Schaftoside (7) [25,26]
3.5 – 3.8	215, 273, 335	8	4.97	266, 350	Kaempferol-3-*O*-rhamnoside-7-*O*-glucoside (8) [27,28]
3.5 - 3.8	215, 273, 335	9	4.06	245, 268, 330.	Kaempferol 3, 7-*O*-tetraglycoside (9) [26]
3.5 – 3.8	215, 273, 335	10	4.28	267, 332	Kaempferol 3-*O*-sophoroside (10) [28]
3.5 -3.8	215, 273, 335	11	4.95	270, 313	Kaempferol 3-*O* (caffeoylsophoroside) (11) [28]
3.5 – 3.8	215, 273, 335	12	4.34	241, 257, 314, 365	Isomangiferin (12) [28]
3.5 – 3.8	215, 273, 335	13	4.18	273, 332	Apigenin 6-C-hexoside-8 C pentoside (13) [23, 24, 29]

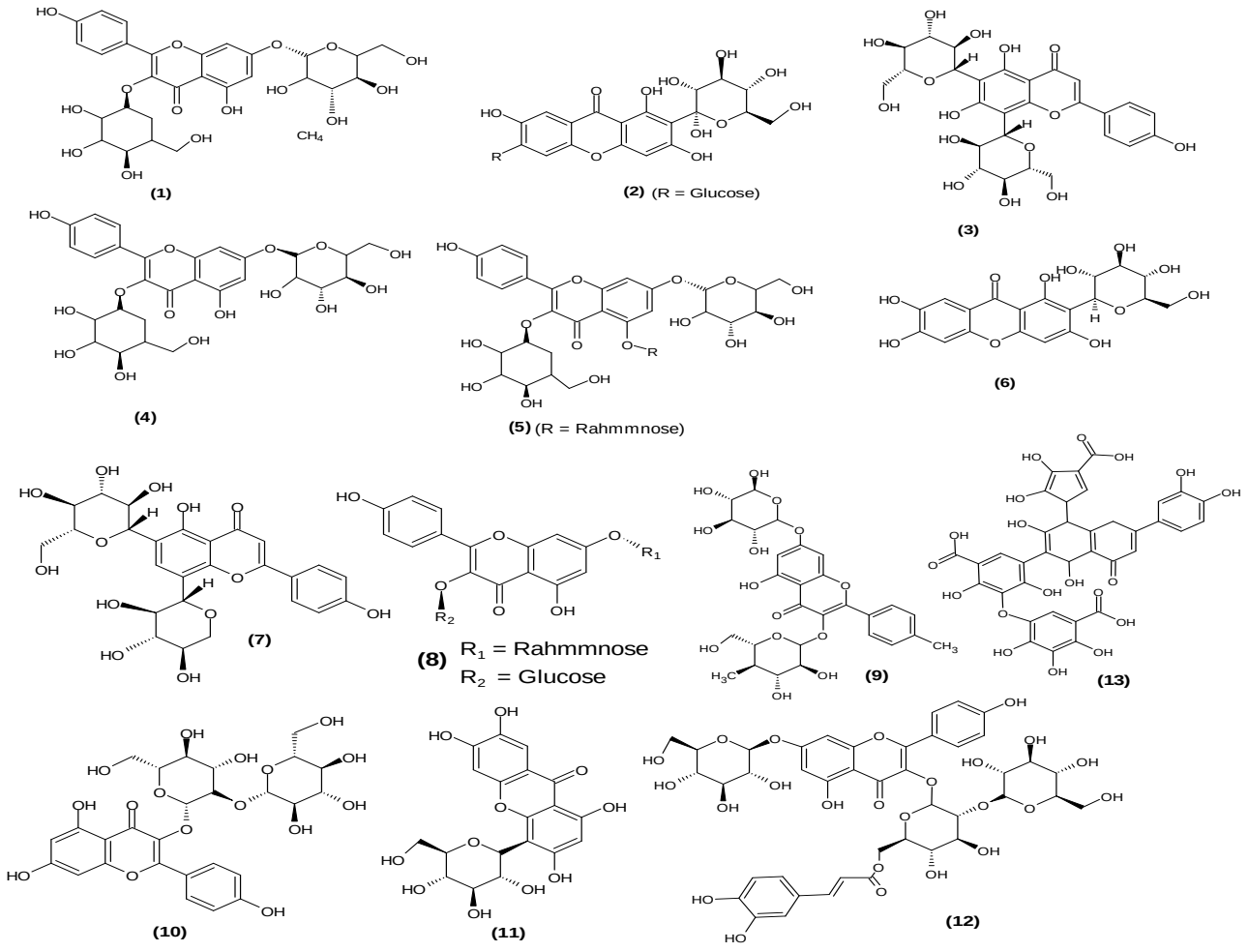

Figure 5: Structures of most probable compounds in *A. platyneuron*

Conclusion

Asplenium platyneuron leaf extract and its ethyl acetate fraction possess significant antioxidant activity in the in vitro DPPH, TAC and FRAP assay models. The quantity of flavonoids and phenolic compounds in these fractions may account for their activity. Phenolic compounds such as apigenin (6-C-hexoside-8-C-pentoside) (13), schaftoside (7) and Kaempferol 3-*O*-(caffeoylsophoroside) (11) present in *A. platyneuron* could be responsible for the antioxidant activity. This work also reports, for the first time, the HPLC investigation of the distribution of bioactive phytochemicals, in the *A. platyneuron*, ethyl acetate fraction. *Asplenium platyneuron* leaves are a promising source of lead antioxidants and this could be obtained when the leaves are consumed as vegetables and as herbal formulation

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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References

1. Nwodo NJ, Nnadi CO, Ibezim A, Mbah CJ. Chapter 10: Plants with Hypolipidaemic Effects from Nigerian Flora In: Antioxidant-Antidiabetic Agents and Human Health. Intech Open Science. 2014; 241-255.
2. Rajan VK, Muraleedharan K. A computational investigation on the structure, global parameters and antioxidant capacity of a polyphenol, gallic acid. *Food Chem.* 2017; 220:93–99.
3. Ayoka TO, Ezema BO, Eze CN, Nnadi CO. Antioxidants for the Prevention and Treatment of Non-Communicable Diseases. J Expl Res Pharmacol. 2022; 7(3): 178-188.
4. Aniya Y. Antioxidants in traditional foods and medicinal plants from Okinawa. In D Itokazu, H Sho, & Y Nakahara. Proceeding of Okinawa International Conference on Longevity. 2002; *p.50.Naha: OICL*.
5. Zhu KX, Lian CX, Guo XN, Peng W, Zhou HM. Antioxidant activities and total phenolic contents of various extracts from defatted wheat germ. Food Chem. 2018; 126: 1122-1126.
6. Pourmorad F, Hosseinimehr SJ, Shahabimajd N. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr J Biotechnol. 2016; 5:1142–1145.
7. Huang D, Ou B, Proir RL. The chemistry behind the antioxidant capacity assays. J Agric Food Chem. 2005; 53: 1841-1856.
8. Sridhar K, Charles AL. In vitro antioxidant activity of Kyoho grape extracts in DPPH and ABTS assays: Estimation methods for EC_{50} using advanced statistical programs. *Food Chem.* 2019; 275:41–49.
9. Ugorji CO, Ihedioha JN, Agbo MO, Ekere NR, Nwafor FI, Odoemelam EI. Antimicrobial and Antioxidant Studies of the Leaf Extract and Fractions of *Sabicea brevipes* Wernham (Rubiaceae). Int J Biochem Res Rev. 2019; 27(2): 9-10
10. Ayoka TO, Nwachukwu N, Ene AC, Igwe CU, Ogara AL, Nnadi CO. In-Vitro Antioxidant Activity and Acute Toxicity of the Alkaloidal Constituents of *Zanthoxylum zanthoxyloides* Leaves. Trop J Nat Prod Res. 2022; 6(2): 276-280.
11. Sirivibulkovit K, Nouanthavong S, Sameenoi Y. Paper-based DPPH Assay for Antioxidant Activity Analysis. *Anal. Sci.* 2018; 34:795–800.
12. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn. Rev*. 2020; 4:118–126.
13. Nnadi CO, Onoja SO, Ezeh GC, Ikpa CB, Ezeja MI, Udem SC, Anaga AO, Asuzu IU. Antioxidant and antidiabetic potentials of a dimethoxylated flavone obtained from *Helianthus annuus* L. leaf extract. Pak J Pharmacol. 2020; 37(2): 59-69.
14. Harbone JB. Phytochemical methods: *A guide to modern techniques of plant analysis, Chapman and Hall New York; 2nd edition*: 1984; pp. 288.
15. Mythili K, Reddy CU, Chamundeeswari D, Manna PK. Determination of Total Phenol, Alkaloid, Flavonoid and Tannin in different Extracts of *Calanthe triplicate.* J Pharmacogn Phytochem. 2014; 2(2): 40-44.
16. Biju J, Sulaiman CT, Satheesh G, and Reddy VRK. Total phenolics and flavonoids in selected medicinal plants from Kerala. Int J Pharma Pharm Sci. 2014; 6: 406-408.
17. Fatiha B, Madani K, Dahmoune F, Rahmani T, Bousbaa K, Oukmanou S, Chibane M. Optimisation of solvent extraction of antioxidants (phenolic compounds) from Algerian Mint (*Mentha spicata L.*). Pharmacogn Commun. 2017; 2(4): 78
18. Onoja SO, Nnadi CO, Udem SC, Anaga AO. Potential antidiabetic and antioxidant activities of a heliangolide sesquiterpene lactone isolated from *Helianthus annuus* L. leaves. Acta Pharm. 2020; 70(2): 215-226.
19. Hoang VD, Hung NK, Duong PQ, Tan CV, Minh TT, & My NT. Chemical Constituents of Dichloromethane Extract from the Leaves of Gardenia angkorensis, their Cytotoxic and α-Glucosidase Inhibition Activities. Trop J Nat Prod Res. 2024; *8*(2), 6267–6271.
20. Agbo MO, Nnadi CO, Ukwueze NN, Okoye FBC. Phenolic Constituents from *Platycerium bifurcatum* and their antioxidant properties. J Nat Prod. 2014; 7: 48-57.
21. Baliyan S, Mukherjee R, Priyadarshini A, Vibhuti A, Gupta A, Pandey RP, Chang CM. Determination of antioxidants by DPPH radical scavenging activity and quantitative phytochemical analysis of *Ficus religiosa*. Molecules. 2022; 27(4): 1326.
22. Ayoka TO, Nwachukwu N, Nnadi CO. Identification of potential antioxidant and hepatoprotective constituents of *Vitex doniana* by UHPLC/+ESI-QQTOF-Ms/Ms analysis. Asian J Pharm Clin Res. 2020; 13(8): 142-148
23. Imperato F. Two new phenolic glycosides in *Asplenium septentrionale.* Am Fern J. 1984; 74: 14-18.
24. Richardson PM, Lorenz-Liburnaw E. C-Glycosylan tones in the *Asplenium adiantum-nigrum* complex. Am Fern J. 1982; 72: 103-106.
25. Obonga WO, Nnadi CO, Chima CC, Okafor SN, Omeje EO. *In-vitro* antioxidant and in-vivo anti-inflammatory potential of *Marantochloa leucantha* (Marantaceae) extracts and fractions. Dhaka Univ J Pharm Sci. 2019; 18(2): 233-240.
26. Iwashina T, López-sáez JA, Kitajima J. Flavonoids from *Osyris alba*. Biochem Syst Ecol. 2008; 36: 146-147.
27. Umikalson Y, Grayer-Barkmeijer RJ, Harbone JB. A comparison of the flavonoids in *Athyriacae* and *Aspleniaceae*. Biochem Syst Ecol. 1994; 22: 587-594.
28. Lu Y, Guo S, Zhang F, Yan H, Qian DW, Wang HQ, Jin L, Duan JA. Comparison of Functional Components and Antioxidant Activity of *Lycium barbarum* L. Fruits from Different Regions in China. *Molecules.* 2019; 24:2228.
29. Iwashina T, Yamaguchi M, Nakayama M, Onozaki T, Yoshida H, Kawanobu S, Ono H, Okamura M. Kaempferol glycosides in the flowers of carnation and their contribution to the creamy white flowers color. Nat Prod Commun. 2010; 5: 1903-1906.
30. Ed-Dahmani I, Kara M, Lfitat A, Touijer H, Bousraf FZ, Slighoua M, Abdellaoui A. Evaluation of Polyphenolic

Content, Antioxidant and Antimicrobial Activities, and Toxicity Study of Ferula communis L. Fruits. Trop J Nat Prod Res. 2024; 8(2): 6423–6432.
31. Agbo MO, Uzor PF, Nneji UNA, Odurukwe CUE, Ogbatue UB, Mbaoji EC. Antioxidant, total phenolic and flavonoid content of selected Nigerian medicinal plants. Dhaka Univ J Pharm Sci. 2015; 14(1): 35-41
32. Ao C, Li A, Elzaawely AA, Xuan DT, Twata S. Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fill extract. Food Control. 2008; 19: 940-948.
33. Mohsen MS, Ammar SMA. Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chem. 2008; 112: 595-598.
34. Chaves JO, De Souza MC, Da Silva LC, Lachos-Perez D, Torres-Mayanga PC, Machado APDF, Forster-Carneiro T, Vazquez-Espinosa M, Gozalez-de-Peredo AV, Barbero GF, Rostagno MA. Extraction of flavonoids from natural sources using modern techniques. Front Chem. 2020; 8: 507887.
35. Smith DM, Harbone JB. Xanthones in the *Apparanchian Asplenium* complex. Phytochem. 1971; 10: 2117-2119.
36. Ayoka TO, Ngwu N, Ene AC, Igwe CU, Nnadi CO. Liquid Chromatography-Mass Spectrometric Analyses of Potential Antioxidant Constituents from Zanthoxylum zanthoxyloides Leaves: Probing into the Role of Alkaloids. Trop J Nat Prod Res. 2020; 4(10): 817-823.
37. Ouryemchi I, Oubihi A, Taibi M, Elbouzidi A, Jaber H, Haida S, Tarfaoui K, Atfaoui K, Chaabane K, El Guerrouj B, Bellaouchi R, Asehraou A, Addi M, Benzakour A, Ouhssine M. GC-MS Characterization, Antioxidant, Antimicrobial and Insecticidal Potential of Moroccan *Cuminum cyminum* L. Essential Oil. Trop J Nat Prod Res. 2024; 8(2): 6108-6114
38. Laohaprapanon S, Jitjum S, Yincharoen K, Wuna P, Siriyong T, Manee S. Evaluation of *In vitro* Antioxidant Activities and Toxicity Effects of a Novel Plant-Based Body Lotion from Thai Medicinal Plants. Trop J Nat Prod. Res. 2024; 8(2): 61-97
39. Hassan V, Ali S, Fatemeh MK, Hossein D, Mir BB. Cytotoxicity, antioxidant activity of phenolic content of eight fern species from North of Iran. Pharm Sci. 2015; 21: 18-24.
40. Sahreen S, Muhammad RK, Rahmat AK. Phenolic compounds and antioxidant activities of *Rumex hastatus* D. Don. Leaves. J Med Plants Res. 2011; 5(13): 2755-2765.