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Free Radical Scavenging Activity of a 70% Ethanol Extract of the Whole Herb of *Platostoma africanum* P. Beaur. (Lamiaceae)

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Abstract: When oxidation by free radicals exceeds antioxidant systems in the body, oxidative stress usually results. This is common in the underlying processes leading to aging, infertility and of a range of non-communicable diseases including cancers, cardiovascular diseases, neurodegenerative diseases and rheumatoid arthritis. There exist measures to curb oxidative stress, one of which is to take in exogenous antioxidants in form of supplements and in diet. Studies have shown that certain plants have antioxidant and free radical scavenging activity. The aim of this study is to evaluate the free radical scavenging activity (FRSA) of the crude extract of *Platostoma africanum* P. Beauv. (Lamiaceae). The crude extract was partitioned using N-hexane, Di-ethylether, Ethyl acetate and N-butanol in that order. TLC phytochemical screening as well as DPPH bioautographic analysis was done for each fraction as well as the residual aqueous fraction in suitable mobile phase system in order to evaluate their FRSA and secondary metabolites. A DPPH assay was then carried out using ultraviolet spectroscopy to quantify the activity. The fraction with the lowest IC_{50} and highest activity was further separated into fractions by column chromatography. DPPH bioautography and phytochemical screening were done on the resulting fractions. The crude as well as the five fractions had FRSA in varying degrees. Ethyl acetate fraction had the lowest IC_{50} (0.42mg/mL) and the highest activity. The decreasing order of activity was ethyl acetate>diethyl ether>n-butanol>crude>aqueous>n-hexane. Among the fractions obtained from column chromatography, fraction D was the most active. Terpenoids and flavonoids were the main secondary metabolites observed in the plant and were responsible for the FRSA. *Platostoma africanum* P. Beauv. has antioxidant activity which is mostly due to its terpenoid and flavonoid content.

Keywords: *Platostoma africanum* P. Beauv., FRSA, Antioxidant, Phytochemical Screening, Column Chromatography, DPPH Bioautography

1. Introduction

Plastostoma africanum is a plant that is widely found in damp sites and waste places in Tropical African countries including Eritrea, Kenya, Angola, Tanzania, India and Senegal. It naturally grows in forest regions and often in partial shade at forest edges [1]. It has a mildly aromatic mint or sage odour and the leaves are ovate, acute or abruptly acuminate with serrated margins at the upper portion with petioles that are 2 to 4 cm long [2]. The plant has common names such as Akan-Osante (Ghana), Akan-brong, Guere (Ivory Coast), Mani (Liberia) and Mkpri-Ibok-ukpong (Efik-Nigeria). Leaves of *Plastomaafricanum* has been used in the

treatment of rheumatic symptoms and internal heat as well as ahaemostatic agent [2]. It is also sometimes used to treat fever and in Ghana, the leaves and seeds are used for children's coughs also in Liberia; it is used as stomach medicine [3]. It has also been reported to have antioxidant and anti-inflammatory activity [4]. Studies have shown that the hexane and dichloromethane extracts of *P. africanum* contain eight acidic pentacyclictriterpenes namely ursolic acid, oleanolic acid, epimaslinic acid, corosolic acid, hyptadienic, euscaphic acid and tomentic acid with a mixture of β-sitosterol and stigmasterol [3, 4]. Hexane and dichloromethane extracts of the plants have been found to exhibit significant antioxidant and anti-inflammatory activities [4, 5].

Oxidative stress on the other hand refers to a state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them [6]. It often leads to deleterious occurrences in the body including lipid peroxidation, oxidative deoxyribonucleic acid (DNA) change as well as oxidative alteration of protein structure where physiological adaptation phenomena and regulation of intracellular signal transduction occur due to oxidative stress [7]. Oxidative stress is associated with a number of diseases where the presence of free radicals in deleterious amounts could lead to neurodegeneration and excess reactive oxygen species could lead to a number of disorders ranging from Alzheimers disease, ageing to a number of other neuronal disorders [8]. Oxidative stress has also been found to be inextricably linked to non-communicable diseases as it plays a major role in their onset and progression [8, 9]. Antioxidant enzymes in human cells act to counteract the effect of toxic reactive oxygen species and more than one layer of these antioxidant defense systems including the catalases, superoxide dismutase, glutathione, vitamin C and vitamin E offer the first line of defense to scavenge ROS or RNS directly and so prevent or delay initiation of oxidative stress [10]. These enzymes are known to perform damage-removing or repairing functions thereby acting as the last line of defense to regenerate biomolecules damaged for oxidative injury [11].

2. Materials and Methods

2.1. Chemicals and Reagents

Distilled water, Distilled methanol solution, Diethyl ether (Central Drug House Ltd), Ethyl acetate, N-Butanol, N-Hexane, 1% Ethanol Potassium Hydroxide (KOH), AluminiumTrichloride ($AlCl_3$), Iodine, Liebermann Burchard Spray, 2,2-Diphenyl-1-1-Picrylhydrazyl (DPPH), Dichloromethane (Sigma Aldrich®).

2.2. Equipment

The equipments used in this work included separating funnel (500 ml), Test tubes, Alluminium foil, Capillary tubes, Petri dishes, Conical flasks, Sample bottles, Silica gel GF 254 thin layer chromatography (TLC plates (Merck). Rotary evaporator (Stuart ®), Weighing balance (Ohaus ®), Water bath, Thin layer chromatography (TLC) tanks, Hot air oven, Ultraviolet spectrophotometer (Spectrumlab 752s®), Hot air oven, Sephadex LH20 GE Healthcare life services glass column for chromatography.

2.3. Preparation and Extraction of P. africanum Leaf Extract

A 200 g of the pulverized sample was extracted in 70% ethanol for 72 hours and concentrated in a rotary evaporator, lyophilized and preserved for further use according to the method of Akawaet al [12].

2.4. Partitioning of P. africanum 70% Ethanol Extract

Crude extract (10 g) was weighed and dissolved completely in a small quantity of distilled water and then the volume was made up to 100 mL. The solution was then transferred into a separating funnel. Then it was first partitioned with aliquots of n-hexane (3 x 100mL) until the n-hexane phase was colourless. The resulting fractions were pooled together while the mother liquor was re-extracted with aliquots of diethyl ether (7 x 100mL) and the fractions were pooled. The same procedure was repeated for aliquots of ethyl acetate (7 x 100mL) and then n-butanol (7 x 100mL). The pooled fractions except the n-butanol fraction were evaporated to dryness using a rotary evaporator. The concentrated n-butanol fraction was diluted with 50 mL of methanol and then evaporated to dryness on a water bath at reduced temperature (60°C).

2.5. Thin Layer Chromatographic (TLC) Analysis of Extracts and Fractions

Thin layer chromatographic analysis was carried out according to the method of Aschbacher et al [13]. The plates were activated in a hot air oven at 120°C for some minutes before they were used. Solutions of *P. africanum* extract and fractions were prepared at a concentration of 10 mg/mL in suitable solvents. The n-hexane fraction was dissolved in n-hexane, the diethyl ether fraction was dissolved in dichloromethane, ethyl acetate fraction was dissolved in ethyl acetate, the n-butanol fraction was dissolved in methanol, aqueous fraction was dissolved in distilled water, as well as the crude extract. Capillary tubes were used to spot the fractions (10µL) on the TLC plates (silica gel GF240) at the origin. Suitable solvent systems were used as mobile phases. The solvent fronts were marked on the spotted plates and then they were carefully placed in the TLC tank a few minutes after the mobile phase has been put in it. After elution, the plates were visualized in daylight and under ultraviolet (UV) light at wavelengths of 254nm and 365nm.

Stationary phase: Silica gel GF254.

Visualization: Visible light, UV (254nm and 365nm), Liebermann-Burchard spray, ferric chloride spray.

Mobile phase: There were 5 differentelutions of mobile phases for the fractions.

(A): 1.3mL of n-hexane: 0.5mL of ethyl Acetate (72:28)

for n- hexane fraction and diethylether fraction.

(B): 1mL of n-hexane: 1mL of ethyl acetate (50:50) for ethyl acetate fraction (non-polar system).

(C): 0.5mL of dichloromethane: 0.5mL of ethyl acetate: 1mL of methanol (25:25:50) for ethyl acetate fraction (polar system).

(D): 1.75mL of ethyl acetate: 0.5mL of methanol: 0.25mL of water: 1 drop (1µL) of acetic acid (70: 20: 10: 0.04).

(E): 1.25mL of ethyl acetate: 0.5mL of methanol: 0.25mL of water: 1 drop (1µL) of acetic acid (62.5: 25: 12.5: 0.02).

2.6. TLC Phytochemical Screening

TLC phytochemical screening was done according to the method of Behrendoff et al [14]. After the plates were developed, they were sprayed with visualizing reagents to detect different secondary metabolites that could be present. The sprayed plates were viewed at wavelengths of 254nm and 365nm. The reagents used were Liebermann Burchard spray, 10% ethanol KOH spray, Aluminium trichloride (AlCl3) spray and ferric chloride spray. The plates sprayed with Liebermann-Burchard spray were heated in the hot air oven for a few seconds before visualization.

2.6.1. Test for Triterpenoid Derivatives

This was carried out for both polar and non-polar systems. The TLC plate was placed uprightly on a glass wall and then sprayed with Liebermann-Burchard spray. It was then heated in the oven for one to two minutes, air dried and then viewed under visible light and UV at 254nm and 365nm.

2.6.2. Test for Free Anthraquinones

This was carried out for both polar and non-polar solvent systems. The plates were placed uprightly on a glass wall and then sprayed with 10% ethanol KOH. It was then air dried and viewed under visible light and UV at 254nm and 365nm.

2.6.3. Test for Flavonoid Aglycones

This was carried out for both polar and non-polar systems. The plates were placed uprightly on a glass wall and then sprayed with 1% AlCl3. It was then air dried and viewed under visible light and UV at 254nm and 365nm.

2.7. TLC DPPH Bioautographic Analysis for Antioxidant Activity

Solutions of 10mg/mL of different fractions in suitable solvents were prepared, and then TLC plates were carried out for each fraction in their respective mobile phases. Each fraction was spotted in duplicate on the same TLC plate. The plates were air-dried and then sprayed with 0.2% (g/mL) solution of DPPH in methanol. Zones of antioxidant activities appeared as bright yellow or cream zones on a purple background. The plates were then sprayed with spray reagents to determine the secondary metabolites present.

2.8. Antioxidant Activity Assay

The free radical scavenging activity of the fractions against DPPH radical was determined and quantified using UV-spectrophotometry at a wavelength of 517nm. Solutions of each fraction in methanol were prepared at different concentrations. 50µL of each concentration was added to 5mL of 0.004% solution of DPPH in methanol and mixed. A blank solution was also prepared by adding the same volume of methanol to 5mL of the DPPH solution. This was carried out in triplicates for each concentration and the blank. The solutions were then incubated in the dark for 30 minutes before the absorbance was measured using a UV-spectrophotometer at 517nm. The radical scavenging activity was calculated using the formula:

$$\text{Percentage Inhibition} = ((Ab - Aa)/ Ab) \times 100$$

Where Ab=absorbance of blank solution
Aa=absorbance of the fraction

2.9. Column Chromatography of Ethyl Acetate Fraction

The sephadex column was prepared by pouring sephadex (u20g) which had been allowed to swell in dichloromethane (DCM) and methanol mixture (1:1) for several hours into a chromatographic column. The sephadex was allowed to equilibriate for 1 hour by eluting with DCM/Methanol (1:1). Then the sample dissolved in DCM/Methanol (1:1) was carefully loaded onto the top of the sephadex column and eluted with the mobile phase in the following gradient: DCM:Methanol (50:50), DCM/Methanol (25:75) and Methanol (100%). Eluates of 5mL-10mL portions were collected and combined after Thin Layer Chromatographic analysis into 8 combined fractions. The combined fractions were concentrated and dried after antioxidant determination. The yield was recorded.

2.10. TLC-DPPH Bioautographic Analysis for Antioxidant Activity

Using the same procedure in 2.7, DPPH bioautographic analysis was carried for the different pooled fractions. (A) was used for fractions A, B and C. (B) was used for fractions D, E, F and G. (C) was used for fraction H.

(A): 0.5mL of methanol: 4.5mL of ethyl acetate (10:90).

(B): 2.5mL of dichloromethane: 2.5mL of methanol (50:50).

(C): 2.5mL of ethyl acetate: 0.25mL of formic acid: 0.25mL of acetic acid: 0.05mL of water (82: 8.2: 8.2: 1.6).

3. Results

The percentage yield of *P. africanum* fractions are shown in table 1. The fractions are N-hexane, diethylether, ethyl acetate, N-butanol and aqueous. In the result, it was shown that the percentage yield of aqueous had the highest, with 44.60% while the yield of N-hexane had the least with 2.20%. The results of thin layer chromatography phytochemical screening was shown in table 2. Here, it was revealed that several phytochemicals are present in various fractions corresponding to the degree of prevalence. Triterpenoids were shown to be present in N-hexane and

diethyl ether fractions in considerable amount. Similarly, the diethyl ether and ethyl acetate fractions were shown to have flavonoids in trace amounts when sprayed with 10% ethanolic KOH. In the same vein, N-hexane and diethyl ether fractions were suspected to posses double bond containing compounds in them in little amounts when sprayed with iodine while anthraquinones were completely shown to be absent in all the fractions.

The results of the free radical scavenging activity of the N-hexane fractions by bioautography was shown in table 3. The Rf (retardation factor) of the secondary metabolites in N-hexane fraction ranges from 0.07 to 0.95. Secondary metabolites with terpenoid without visualization in UV (365nm) AlCl3 spray had the lowestRf values while the terpenoid with the light pink colour after visualization in UV spray had the highest of values. In the FRSA bioautographic analysis of diethyl ether fraction according to table 4, flavonoid was shown to have the least and highest Rf values which obviously could be due to changes in colour after DPPH spray connoting that DPPH spray could have hindered the values. Table 5 reveals the FRSA bioautographic analysis of ethyl acetate fraction in the non polar mobile phase. Here, it was observed that only flavonoid had a significant Rf values of 0.36 and 0.87 under bioautography. Table 6 as revealed for ethyl acetate fraction in polar mobile phase only. It was observed that terpenoids was the only secondary metabolites withRf values 0.68, connoting the highest. In table 7, terpenoid was observed as the only secondary metabolite detected in N-butanol fraction. The same result was also shown in table 8, where only terpenoid was detected in the aqueous fraction. According to table 9, the free radical scavenging activity of different concentrations of the crude extract of *P. africanum* in various concentrations in mg/ml were determined in variance to the percentage inhibition. Here, concentrations increased with corresponding increase in their percentage inhibition. In table 10, N-hexane fractionshowed a corresponding increase in their percentage inhibition in agreement with their concentrations. Diethyl ether fractions in table 11as revealed, showed that there was no corresponding increase in the percentage inhibition of extract according to concentrations (mg/ml).

Conversely, in ethyl acetate fraction, according to table 12, there was a corresponding increase in the percentage inhibition of ethyl acetate fraction with the concentrations in mg/ml. For N-butanol fraction of extract as shown in table 13, it was observed that a corresponding increase in the concentrations of N-butanol fraction led to a corresponding increase in the percentage inhibition of extract as well. In table 14 as shown, the values of percentage inhibition of extract and concentrations of extract in mg/ml were not correspondingly observed. The inhibitory concentrations of the different fractions against DPPH at 50% maximal concentrations was shown in table 15. The ethyl acetate fraction was shown to have the lowest inhibitory concentrations (mg/ml) against DPPH and N-hexane was revealed to have the highest IC50 against DPPH. The order of increasing IC50 of the different fractions against DPPH is as follows; ethyl acetate<diethyl ether<N-butanol< crude<aqueous<N-hexane while the order of increasing free radical scavenging activity is similarly ethyl acetate>diethyl ether>N-butanol>crude>aqueous>N-hexane. In respect to the result shown in table 15, it was evident that ethyl acetate has the highest activity and was consequently further analysed using column chromatography with sephadex as a stationary phase and mobile phase as methanol and dichloromethane as seen in the various fractions from table 16 to table 23.

Table 1. *Percentage Yield of Plastomaafricanum Fraction.*

Fraction	Colour	Form	Yield (g)	Percentage yield %
(Partitioning solvent)				
N-Hexane	Dark brown	Semi-solid	0.220	2.20
Diethyl ether	Dark brown	Semi-solid	0.796	7.96
Ethyl acetate	Black	Solid	0.670	6.70
N-butanol	Dark brown	Semi-solid	0.810	8.10
Aqueous	Dark brown	Semi-solid	4.460	44.60

Table 2. *Results of TLC Phytochemical Screening.*

Spray Reagent	Group of secondary metabolites	Observation	Inference	Degree
Liebermann Burchard spray	Triterpenoids	Brown colourations were observed in the N-hexane and diethyl ether fractions	Triterpenoids present in N-hexane and diethyl ether fractions	+++
10% ethanolic KOH	Anthraquinones	Nil	Anthraquinones absent	-
Aluminium chloride (AlCl3)	Flavonoids	Faint yellow regions were seen on the plate in the diethyl ether and ethyl acetate fractions	Free flavonoids may be present in the diethyl ether and ethyl acetate fractions	++
Iodine	Double-bond conjugation containing compounds	Tiny regions of yellow were seen in the N-hexane and diethyl ether fractions	Presence of double-bond containing compounds suspected in N-hexane and diethyl ether fractions	++

Key: +++=moderate amount++=little amount+=trace amount-=absent.

Table 3. *FRSA Bioautographic Analysis of n-Hexane Fraction.*

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after Liebermann Burchard spray	Colour after DPPH	Class of SMRf va	Rf values
1	Nil	brown	yellow	terpenoid	0.07
2	yellow glow	Nil	yellow	flavonoid	0.13
3	light purple glow	brown	faint yellow	terpenoid	0.18
4	yellowish green	Nil	faint yellow	flavonoid	0.31
5	light purple	Nil	faint yellow	Nil	0.36
6	pink	Nil	faint yellow	Nil	0.48
7	pink	brown	faint yellow	Terpenoid	0.66
8	light yellow	Nil	faint yellow	flavonoid	0.77
9	light pink	Nil	faint yellow	Nil	0.84
10	light pink	brown	faint yellow	terpenoid	0.95

Key: FRSA=free radical scavenging activity R_f=retardation factor, SM=Secondary metabolites

Table 4. *FRSA Bioautographic Analysis of Diethyl ether fraction.*

Spot	Visualization in UV (365nm) $AlCl_3$	Colourafter Liebermann Burchard spray	Colour after DPPH	Class of SM	Rf values
1	light yellow	Nil	yellow	flavonoid	0.08
2	light pink	brown	yellow	terpenoid	0.15
3	purple	Nil	yellow	Nil	0.21
4	purple	purple	Nil	Nil	0.30
5	red	Nil	Nil	Nil	0.34
6	light yellow	Nil	Nil	flavonoid	0.42

Key: FRSA=free radical scavenging activity R_f=retardation factor, LB=Liebermann Burchard, SM=Secondary metabolites

Table 5. *FRSA Bioautographic Analysis of Ethyl Acetate fraction (Non polar mobile phase).*

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after Liebermann Burchard spray	Colour after DPPH Spray	Class of SM	Rf values
1	Red	Nil	yellow	Nil	0.10
2	blue	Nil	yellow	Nil	0.26
3	blue	Nil	greenish yellow	Nil	0.31
4	faint yellow	Nil	yellow	flavonoid	0.36
5	blue glow	Nil	Nil	Nil	0.83
6	yellow	Nil	Nil	flavonoid	0.87
7	red	Nil	green	Nil	0.93

Key: FRSA=free radical scavenging activity R_f=retardation factor, SM=Secondary metabolites

Table 6. *FRSA Bioautographic Analysis of Ethyl Acetate fraction (polar mobile phase).*

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after Liebermann Burchard spray	Colour after DPPH Spray	Class of SM	Rf values
1	Red	Nil	yellow	Nil	0.10
2	blue	Nil	yellow	Nil	0.17
3	blue	Nil	yellow	Nil	0.22
4	blue	Nil	yellow	Nil	0.34
5	blue	Nil	yellow	Nil	0.46
6	blue	Nil	yellow	Nil	0.51
7	blue	Nil	yellow	Nil	0.56
8	blue	Nil	yellow	Nil	0.61
9	deep blue	brown	yellow	terpenoids	0.68

Key: FRSA=free radical scavenging activity R_f=retardation factor, SM=Secondary metabolites

Table 7. *FRSA Bioautographic Analysis of N-butanol fraction.*

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after Liebermann Burchard spray	Colour after DPPH Spray	Class of SM	Rf values
1	purple	Nil	yellow	Nil	0.10
2	purple	brown	yellow	terpenoid	0.15
3	purple	Nil	yellow	Nil	0.27
4	purple	brown	yellow	terpenoid	0.44
5	purple	Nil	yellow	Nil	0.54

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after Liebermann Burchard spray	Colour after DPPH Spray	Class of SM	Rf values
6	purple	Nil	yellow	Nil	0.80
7	purple	Nil	yellow	Nil	0.92

Key: FRSA=free radical scavenging activity R_f=retardation factor, SM=Secondary metabolites

Table 8. *FRSABioautographic Analysis of Aqueous fraction.*

Spot	Visualization inUV (365nm) $AlCl_3$	Colour after Liebermann Burchard spray	Colour after DPPH Spray	Class of SM	Rf values
1	purple	Nil	yellow	Nil	0.10
2	purple	brown	yellow	Nil	0.16
3	purple	Nil	yellow	Nil	0.21
4	purple	brown	yellow	Nil	0.27
5	purple	Nil	yellow	Nil	0.33
6	purple	brown	yellow	Terpenoid	0.38
7	purple	Nil	yellow	Nil	0.44
8	purple	Nil	Nil	Nil	0.51

Key: FRSA=free radical scavenging activityR_f=retardation factor, SM=Secondary metabolites

Table 9. *Free Radical Scavenging Activity of Different Concentrations of the Crude Extract of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.170	0.171	0.173	0.171	
0.5	0.149	0.164	0.135	0.149	12.87
1.0	0.116	0.108	0.123	0.116	32.16
1.5	0.120	0.094	0.108	0.107	37.43
2.0	0.114	0.088	0.084	0.095	44.44
3.0	0.086	0.061	0.037	0.061	64.33
3.5	0.052	0.044	0.051	0.049	71.35
4.0	0.042	0.030	0.030	0.034	80.12

Table 10. *Free Radical Scavenging Activity of Different Concentrations of the N-hexane fraction of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.268	0.265	0.267	0.268	
0.5	0.254	0.265	0.238	0.252	5.97
1.0	0.278	0.269	0.270	0.272	-1.49
1.5	0.255	0.259	0.240	0.251	6.34
2.0	0.252	0.238	0.218	0.236	11.94
3.0	0.237	0.234	0.232	0.234	12.69
3.5	0.253	0.211	0.234	0.233	13.06
4.0	0.250	0.263	0.257	0.257	4.10

Table 11. *Free Radical Scavenging Activity of Different Concentrations of the Diethyl ether fraction of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.764	0.724	0.730	0.739	
0.5	0.419	0.350	0.302	0.357	51.69
1.0	0.203	0.183	0.226	0.204	72.40
1.5	0.237	0.213	0.108	0.186	74.83
2.0	0.143	0.098	0.088	0.110	85.12
3.0	0.134	0.105	0.101	0.113	84.71
3.5	0.151	0.155	0.153	0.153	79.30
4.0	0.162	0.164	0.164	0.163	79.94

Table 12. *Free Radical Scavenging Activity of Different Concentrations of ethyl acetate fraction of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.553	0.541	0.537	0.544	
0.1	0.499	0.504	0.515	5.33	0.543
0.25	0.359	0.372	0.365	0.365	32.90
0.35	0.417	0.346	0.317	0.360	33.82
0.5	0.246	0.229	0.225	0.233	57.72

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
0.75	0.144	0.161	0.168	0.158	70.96
1.0	0.087	0.095	0.093	0.092	83.09
2.0	0.052	0.032	0.030	93.01	0.038

Table 13. *Free Radical Scavenging Activity of Different Concentrations of N- butanol fraction of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.380	0.386	0.386	0.384	
0.25	0.175	0.280	0.298	0.251	34.64
0.5	0.226	0.224	0.281	0.244	35.46
1.0	0.174	0.180	0.146	0.167	56.51
1.5	0.123	0.118	0.096	0.112	70.83
2.0	0.072	0.038	0.043	0.051	86.72
3.0	0.057	0.034	0.054	0.048	87.50
3.5	0.051	0.106	0.050	0.069	82.03

Table 14. *Free Radical Scavenging Activity of Different Concentrations of Aqueous fraction of Plastosomaafricanum P. Beauv.*

Concentration (mg/ml)	Absorbance				
	1	2	3	Mean	% inhibition
Blank	0.599	0.624	0.619	0.614	
0.5	0.540	0.530	0.466	0.512	16.61
1.0	0.633	0.536	0.516	0.562	8.47
1.5	0.507	0.541	0.579	0.542	11.73
2.0	0.456	0.460	0.471	0.462	24.76
3.0	0.364	0.354	0.404	0.374	39.09
3.5	0.403	0.362	0.399	0.388	36.81
4.0	0.348	0.360	0.349	0.352	42.67

Table 15. *IC_{50} of Different Fractions against DPPH.*

S/N	Fraction	IC_{50} (mg/ml)
1	crude	2.27
2	N-hexane	26.08
3	ethyl acetate	0.42
4	N-butanol	0.77
5	aqueous	4.7

KEY: IC_{50}=Concentration that produced 50% inhibition

Table 16. *TLC DPPH bioautographic analysis of crude ethyl acetate fractions.*

Spot	Visualization inUV (365nm) $AlCl_3$ Spray	Colour after DPPH Spray	Class of Secondary metabolites	Rf values
1	faint yellow	yellow	flavonoid	0.17
2	faint yellow	yellow	flavonoid	0.34
3	pink	Nil	Nil	0.47
4	pink	Nil	Nil	0.49
5	faint yellow	Nil	flavonoid	0.76

Table 17. *TLC bioautographic analysis of fraction A.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after Sulfuric acid spray and heat	colour after DPPH spray	class of secondary metabolites	Rf values
1	pink	Nil	yellow	Nil	0.14
2	faint yellow	Nil	Nil	Nil	0.25
3	faint yellow	Nil	Nil	Nil	0.34
4	faint yellow	brown	Nil	terpenes	0.53

Table 18. *TLC Bioautographic analysis of fraction B.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after sulfuric acid spray and heat	colour after DPPH spray	class of secondary metabolites	Rf values
1	faint yellow	Nil	yellow	flavonoid	0.15
2	pink	Nil	green	Nil	0.42
3	deep pink	Nil	green	Nil	0.64

Table 19. *TLC Bioautographic analysis of fraction C.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after sulfuric acid spray and heat	colour after DPPH spray	class of secondary metabolites	Rf values
1	light yellow	Nil	yellow	flavonoid	0.14
2	light yellow	Nil	yellow	flavonoid	0.41
3	pink	Nil	yellow	Nil	0.51
4	pink	brown	yellow	terpenoid	0.83

Table 20. *TLC Bioautographic analysis of fraction D.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after sulfuric acid spray and heat	colour after DPPH spray	class of secondary metabolites	Rf values
1	yellow	Nil	yellow	flavonoid	0.53
2	bright yellow	Nil	yellow	flavonoid	0.86
3	yellow	Nil	yellow	flavonoid	0.80

Table 21. *TLC Bioautographic analysis of fraction E.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after sulfuric acid spray and heat	colour after DPPH spray	class of secondary metabolites	Rf values
1	yellow	Nil	yellow	flavonoid	0.15
2	Nil	Nil	yellow	Nil	0.56
3	Nil	Nil	yellow	Nil	0.49

Table 22. *TLC Bioautographic analysis of fraction F.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	colour after sulfuric acid spray and heat	Colour after DPPH spray	Class of secondary metabolites	Rf values
1	Nil	Nil	yellow	Nil	0.44
2	Nil	Nil	Nil	Nil	0.14

Table 23. *TLC Bioautographic analysis of fraction G.*

Spot	visualization in UV (365nm) $AlCl_3$ spray	Colour after sulfuric acid spray and heat	Colour after DPPH spray	Class of secondary metabolites	Rf values
1	Nil	Nil	yellow	Nil	0.46

4. Discussion

While partitioning the crude extract, the least polar solvent, n-hexane, was the first solvent used. Then subsequent solvents were used in order of increasing polarity namely n-hexane, diethyl ether, ethyl acetate and n-butanol. The aqueous fraction was collected separately. After all the fractions were concentrated and dried, all the fractions were dark brown semisolids except the ethyl acetate fraction which was a black solid. Ethyl acetate fraction was darker than the others probably because it had a higher proportion of flavonoid, as was seen during thin layer chromatographic analysis. The highest yield was the aqueous fraction which was 44.6% of the entire yield. The order of increasing yield of fractions are as follows. n-hexane<ethyl acetate<diethyl ether<n-butanol<aqueous. N- hexane fraction had the lowest yield which was 2.2%. Based on this result, it is likely that polar compounds occur in a higher proportion than non-polar compounds in the crude extract of *Platostoma africanum*.

In developing suitable mobile phases for thin layer chromatographic analysis of each fraction, it was noted that a single non-polar system (1.3mL of n-hexane: 0.5mL of ethyl acetate) was suitable for both the N-hexane and diethyl ether fractions. However, when the ethyl acetate fraction was developed with the same system, there was still some part of the spot remaining at the origin after the plate was developed. A polar mobile phase system (0.5mL dichloromethane: 0.5mL ethyl acetate: 1mL methanol) was then used for the fraction. The spot moved completely from the origin and separation was good but not as well as the non-polar system. Hence in the bioautographic analysis, the ethyl acetate fraction was developed separately on both the polar and non-polar mobile phase systems. Different mobile phase systems were also used to develop the n-butanol and aqueous fractions as suitable.

Phytochemical screening showed that free flavonoids may be present in the diethyl ether and ethyl acetate fractions. This was indicated from the yellow regions seen on the TLC plates. Iodine spray reagent also revealed that double-bond containing compounds are likely to be present in the n-hexane and diethyl ether fractions. Triterpenoids were observed in n-hexane and diethyl ether fractions. Double-bond containing compounds were also suspected in the polar fractions.

For the DPPH bioautographic analysis of the fractions, 0.2% of DPPH solution was prepared by dissolving 7mg of DPPH in 3.5mL of methanol. The DPPH was well protected from light using aluminium foil to prevent photo degradation of the free radical. Free radical scavenging was observed in all five fractions at different levels. Active zones appeared as bright yellow or cream on a purple background. In the n-hexane

fraction, according to table 3, ten active zones were observed, albeit, with varying intensities of the yellow colour. Two zones (Rf=0.07, 0.13) showed yellow colour but the yellow in the other active zones was faint. In the diethyl ether fraction as shown in table 4, three zones (Rf=0.08, 0.15, 0.21) showed free radical scavenging activity. Ethyl acetate in non-polar mobile phase as seen in table 5, had four active zones (Rf=0.10, 0.26, 0.31, 0.36). Rf=0.31 had a green pigment with the yellow colour, this is likely to be contaminated with chlorophyll. It was observed that in these non-polar fractions, the active zones had the least Rf values which means that the compounds responsible for the free radical scavenging activity had more affinity for the polar phase than the non-polar phase. This is in tandem with the work of Bayaniet al [15].

In the polar mobile phase as shown in table 6, the active spot in ethyl acetate fraction were moved further up the TLC plate, including the greenish yellow zone (Rf=0.83). All seven spots of n-butanol fraction showed free radical scavenging activity according to table 7. The aqueous fraction, on the other hand, had only six active spots (Rf=0.10, 0.16, 0.21, 0.27, 0.33, 0.38), while the other spots were not active.

Ultraviolet spectroscopy was used for the quantitative analysis of the free radical scavenging activity of the crude extract and all the fractions. This was possible because DPPH has chromophores that allowed for absorbance of ultraviolet light [16]. Compounds with free radical scavenging activity neutralized the free radical, DPPH, and after thirty minutes of incubation, the solution in the test tubes had changed from purple to yellow. Concentrations with highest percentage inhibition had the highest intensities of yellow. Concentrations with the least percentage inhibition still had purple colours after incubation. Ethyl acetate fraction had the least IC50 value (0.42mg/mL) and so had the highest activity. N-hexane fraction showed the least activity and the highest IC50 (26.08mg/mL). The increasing order of IC50 of the fractions is as follows: ethyl acetate<diethyl ether<n-butanol<crude<aqueous<n- hexane. The order of increasing free radical scavenging activity is similarly ethyl acetate>diethyl ether>n- butanol>crude>aqueous>n- hexane.

Being the most active fraction, the ethyl acetate fraction was further analysed using column chromatography with sephadex as the stationary phase and the mobile phase varying from methanol: dichloromethane (1:1, and then 3:1), as well as methanol (100%). There were 62 sub fractions which were then pooled to obtain eight fractions depending on the similarities in TLC chromatogram.

The highest yield was fraction D (47.74%) while the lowest yield was fraction F (17.47%). The increasing order of yield is as follows: D>B>C>E>H>A>G>F. The fractions were exposed to air and the solvents were allowed to evaporate. After drying, all fractions appeared as solids of different colours ranging from brown to yellow and green.

DPPH bioautography was carried out for all the fractions. Two solvent systems were used for the mobile phase. Fractions A, B and C were developed in the same mobile phase (10% methanol in ethyl acetate) while fractions D, E, F and G were developed in another mobile phase (dichloromethane: methanol 1:1). There were two zones (Rf=0.34, 0.17) with free radical scavenging activity in the crude ethyl acetate fraction as revealed in table 16. Fraction A as shown in table 17, had one active zone (Rf=0.14). Likewise, fraction B in table 18, also had one active zone (Rf=0.15). All zones in fractions C, D, and E were active according to tables 19, 20, and 21. Fraction D had the highest intensity of yellow colour and is probably the most active of all fractions. The secondary metabolites in the active fractions were observed to be flavonoids. One of the two zones in fraction F as shown in table 22, was active (Rf=0.44). Fraction G as revealed in table 23, had one active zone Rf=0.46. All the fractions had free radical scavenging activity in varying degrees.

Fraction H was developed with a separate mobile phase system (2.5mL of ethyl acetate: 0.25mL of formic acid: 0.25mL of acetic acid: 0.05mL of water). When visualized under UV at 365nm, the spot appeared purple before the plate was developed in the mobile phase. However, after elution, there was no visible zone on the plate when visualized as well as when sprayed with DPPH, vanillin sulphuric acid spray and aluminium chloride spray. It is likely that this fraction did not contain any compound.

5. Conclusion

The free radical scavenging activity of a 70% ethanol extract of the whole herb of *P. africanum* was studied. The crude extract was partitioned using N-hexane, diethylether, ethyl acetate and N-butanol. The TLC phytochemical screening revealed mostly of terpenoids and flavonoids and the DPPH bioautographic analysis were done for each fraction in mobile phase to reveal the secondary metabolites. The fraction with the most activity was revealed to be ethyl acetate fraction with the least IC50 and the secondary metabolites with the highest activity, terpenoids and flavonoids. On the basis of these, flavonoids and terpenoids are known as components of foods that can regulate metabolic processes in humans or animals and also improve health. Beneficial effects include decreasing inflammation, scavenging free radicals and regulating cell signaling pathways. Because of their rich availability, safety and low side effects, use of terpenoids and flavonoids in *P. africanum* is encouraged purposely to reduce the incidence or delay the progression of several disesases such as Type II diabetes mellitus, cardiovascular diseases and cancer cases.

Conflicting Interest

All the authors do not have any possible conflicts of interest.

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