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Evaluation of phenolic contents, free radical scavenging activity and functional group analysis of the leaf extract of a medicinal plant in Niger Delta region

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ABSTRACT

Funtumia africana leaves have been used by the people of Niger Delta region of Nigeria to treat diabetes. This study aims to evaluate the polyphenolic contents, antioxidant, and free radical scavenging activities of the leaf crude extract and fractions. The crude aqueous methanolic leaf extract of *F. africana* was extracted by liquid-liquid partitioning into *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol fractions. The free radical scavenging activity of the fractions was measured *in vitro* by using the ferric-reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picryl dihydrazyl (DPPH), and nitric oxide (NO) assays. Phytochemical screening showed the presence of tannins, saponins, alkaloids, terpenoids, steroids, reducing sugars, cardiac glycosides and flavonoids in the extract and polar fractions. The highest total phenolics, total flavonoids and total antioxidant capacity were found to be 1331.18±41.56 mg GAE/g, 833.96±19.14 mg QUE/g and 83.74±1.88 mgAAE/g respectively in the butanol fraction. The *n*-hexane and butanol fractions demonstrated strong DPPH scavenging activity with IC_{50} values of 0.06±0.57 and 0.06±0.5 µg/ml, respectively. The DCM fraction showed the highest NO scavenging activity with IC_{50} value of 0.741±0.07 mg/ml, while hexane fraction has negligible effect. The butanolic fraction has the highest ferric reducing antioxidant activity with value of 48.46±0.15 mg AAE/g of sample.

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Capsule Summary: The free radical scavenging activities and polyphenolic contents of the methanol leaf extract of *Funtumia africana* was evaluated *in vitro*.

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INTRODUCTION

Several biochemical reactions in the body generate free radicals, which have been implicated as mediators of many diseases, including diabetes, cancer, atherosclerosis and

heart diseases (Al-Dabbas et al., 2006). Diabetes is widely recognised as one of the leading causes of death and disability worldwide (American Diabetes Association, 2010). It is known to affect 3% on average of adult Nigerians (Akinkugbe et al., 1992). In humans, diabetes is normally associated with oxidative stress which gives rise to the generation of free radicals due to high levels of blood sugar (hyperglycemia) (Aruoma et al., 2007). Although these free radicals can be scavenged by the *in vivo* produced antioxidant compounds, the endogenous antioxidants are insufficient to completely remove them and maintain a balance. As a result, dietary antioxidants are required to counteract excess free radicals (Scalbert et al., 2005).

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are effective in their role as antioxidants, are commercially available and currently used in industrial processes. However, since suspected actions as promoters of carcinogenesis and other side effects have been reported, their use in food, cosmetic and pharmaceutical products have been decreasing (Tepe et al., 2005). Thus, there has been an upsurge of interest in naturally-occurring antioxidants from vegetables, fruits, leaves, oilseeds, cereal crops, tree barks, roots, spices and herbs (Terashima et al, 2007). Many medicinal plants have now been recognised as sources of natural antioxidant and free radical scavenging compounds which are mainly phenolic compounds (Aruoma, 2003; Wangensteen et al., 2004).

Funtumia africana (Benth.) Stapf belongs to the family Apocynaceae, which generally contains alkaloids (Gurib-Fakim, 2006). The plant which is known as Lagos Silk-rubber in English, is a medium-sized forest tree up to 8-27 m in height; commonly composing in the second story of moist ever-green rain forests. The latex is used traditionally to treat incontinence and burns in West Africa (Wagner et al., 1987). The leaves of *F. africana* have been used by the people of Niger Delta region of Nigeria to treat diabetes. Given the implication of reactive oxidation species in many diseased conditions such as diabetes mellitus, neurodegenerative and cardiovascular diseases (Halliwell, 1991) and the possibility of polyphenolic compounds providing biological resistance against the destructive effect of free radicals, it was therefore worthwhile to estimate the possible antioxidant properties of *F. africana*. As far as our literature survey could ascertain there was no published information on the antioxidant activity of this plant species. This present study therefore aims to evaluate the *in vitro* free radical scavenging activities and polyphenolic contents of the methanol leaf extract and fractions from the plant using more than five antioxidant assays.

MATERIAL AND METHODS

Chemical and reagents

All reagents used for antioxidant assays were of analytical grade bought from Sigma Aldrich.

Collection of plant material

The fresh leaves of *Funtumia africana* were collected from Amatolo town, Wilberforce Island, in the Niger Delta region of Nigeria. The plant was identified by Prof. K.A Ajibesin of the Department of Pharmacognosy and Herbal Medicine, Niger Delta University, Wilberforce Island, Nigeria. A voucher specimen (NDUP 112) of this collection has been retained at the Niger Delta University herbarium.

Extraction procedure

The powdered leaves of *F. africana* (100 g) were extracted with 20% aqueous methanol (1L) at room temperature for 24 hours and filtered. The filtrate was concentrated *in vacuo* at 40^0C to a small volume to give the crude methanolic extract (CME) which was partitioned into n-hexane (HEF), dichloromethane (DCF), ethylacetate (EAF) and n-butanol (BUF) fractions by solvent-solvent extraction technique.

Qualitative phytochemical tests

The preliminary phytochemical tests on the fractions were carried out using standard techniques described by Sofowora (1993) and Trease and Evans (2000).

FT-IR analysis

FTIR spectra of the extract and fractions were obtained on a FT-IR spectrometer Bruker Alpha-P with diamond ATR (Billerica, MA, USA).

Antioxidant assays

Total phenolic content (TPC): Total phenolic content of crude extract and fractions was determined by using the Folin–Ciocalteu assay method of Singleton and Rossi (1965) as described by Pourmorad et al. (2006). Plant extracts were mixed with diluted Folin–Ciocalteu reagent and 7% sodium carbonate. Absorbance at 765 nm was measured on a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA) after 90min of incubation at room temperature. A standard curve was prepared using different concentrations (50 – 250mg/L) of gallic acid in 50% aqueous methanol. Total phenolic content of the fractions was expressed as milligrams of gallic acid equivalents (GAE) per gram dry weight of plant material (mg GAE/g).

Total flavonoid content (TFC): The determination of total flavonoid content of the plant extract and fractions were based on the aluminium chloride colorimetric assay method described by Neergheen *et al (*2006). An aliquot (1ml) of each fraction was added to 4ml of distilled water in a 10ml volumetric flask. This was followed by the addition of 0.3ml 5% sodium nitrite. After 5mins, 0.3ml of 10% aluminium chloride was added and vortexed. This was immediately followed by the addition of 2ml of 1M sodium hydroxide solution and the volume then made up to 10ml with distilled water. The resulting assay mixture was vortexed and the absorbance was then measured at 510nm against a reagent blank. A calibration curve was prepared by using quercetin solutions at concentration 12.5 to 100µg/ml in methanol.

Total antioxidant capacity (TAC): The large number of antioxidants in tissue makes it difficult to measure each antioxidant separately. Therefore, several methods have been developed to measure the total antioxidant of a biological sample (Ashton et al., 1998). The TAC gave an overall value corresponding to the sum of all antioxidants (Prior and Cao, 1999).

TAC was determined based on the method described by Prieto et al., (1999). To 0.3ml of the fraction or standard solutions of ascorbic acid (100 - 600µg/ml) in a test tube was added 3ml of the reagent solution which consisted of 0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate. The tubes containing the reacting mixture were incubated in a water bath at 95^0C for 90 min.The mixture was then allowed to stand and cool to room temperature and the absorbance measured at 695nm against a blank, which consisted of the reacting mixture and distilled water in place of the fractions. The antioxidant activity was expressed as the number of equivalent ascorbic acid.

Determination of the radical scavenging ability using the 2,2-diphenyl-2-picrylhydrazyl hydrazyl hydrate assay: DPPH· is a stable free radical in aqueous or ethanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). In order to evaluate antioxidant potency through free radical scavenging with the test samples, the change in the optical density of DPPH radicals was monitored. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants (Duh et al., 1999). The radical scavenging activity of crude plant extract and fractions was determined spectrophotometrically from the reduction in the optical absorbance at 517 nm due to scavenging of stable DPPH free radical using the method of Brand- Williams et al., (1995).

Briefly, 1ml of different concentrations (31.25- 125 µg/ml) of the fractions in a test tube was added to 1ml of 0.3 mM methanolic solution of DPPH and ascorbic acid and trolox were used as positive controls. The mixture was vortexed and incubated in a dark chamber for 30 minutes after which the absorbance measured at 517 nm. The absorbance of the DPPH radical without an antioxidant, that is, control, was also measured. The percent inhibition of DPPH scavenging activity was calculated using relation shown in equation 1.

$$\text{DPPH inhibition (\%)} = \left[\frac{A_0 - A_i}{A_0}\right].100 \qquad (1)$$

Where, A_0 is the absorbance of DPPH solution with methanol, A_i is absorbance of a DPPH solution with fraction samples. A plot of the percentage DPPH radical scavenging versus log concentration of each fraction was prepared and the concentration at 50% radical inhibition (IC_{50}) was determined from the linear regression equation. Regression equations had correlation coefficients ≥0.91. The IC_{50} value of the tested compound is the concentration required to scavenge 50% DPPH• free radicals.

Nitric oxide radical scavenging assay: This assay was performed according to the method of Green et al., (1982), as described by Marcocci et al., (1994). Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which are measured by the Griess reagent. The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate buffered saline, and the fractions of the fractions at different concentrations (312.5 -10000 µg/ml) were incubated at 25°C in a water bath for 150 min. About 1.5 ml aliquot of the incubated sample was removed at 30 min intervals and 1.5 ml Griess reagent was added. The absorbance of the chromophore formed was measured at 546 nm. Percentage inhibition of the nitric oxide generated was calculated as expressed above with DPPH radical scavenging.

Determination of ferric reducing antioxidant power (FRAP): This was determined according to the method of Benzie and Strain (1996). A 300 mmol/L acetate buffer of pH 3.6, 10mmol/L 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine and 20 mmol/L $FeCl_3.6H_2O$ were mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. A 50 µl aliquot of the fractions at 0.1 mg/ml and 50 µl of standard solutions of ascorbic acid (20, 40, 60, 80, 100µg/ml) were added to 1ml of FRAP reagent. Absorbance measurements were taken at 593 nm exactly 10 minutes after mixing against reagent blank containing 50 µl of distilled water. Ascorbic acid was used as a positive control and results were expressed in milligrams of ascorbic acid equivalents per milliliter (mg AAE/ml). All measurements were taken at room temperature with samples protected from direct sunlight. The reducing power was expressed as equivalent concentration (EC) which is defined as the concentration of antioxidant that gave a ferric reducing ability equivalent to that of the ascorbic acid standard.

RESULTS AND DISCUSSION

Phytochemical screening of *F. africana* has shown the occurrence of tannins, saponins, terpenoids, steroids, reducing sugars, cardiac glycosides and flavonoids in the CME and polar fractions (Table 1).

The FTIR spectra confirmed the presence of aromatic, phenolic, hydroxyl and carbonyl groups in the fractions as shown in Table 2.

Figure 1 shows the phenolic, flavonoid contents and total antioxidant capacity of the different fractions *F. africana*. The n-butanolic fraction had the highest amount of phenolic and flavonoid compounds, with values of 1331.18±41.56mg GAE/g and 833.96±19.14mg QUE/g respectively while the dichloromethane fraction had the lowest TPC and TFC with values of 692.60±11.83 and 321.82±10.56 respectively.

Table 1: Qualitative phytochemical analysis of the different fractions of Funtumia *africana* leaf extract

Phytochemicals	CME	HEF	DCF	EAF	BUF
Alkaloids	+	-	+	+	+
Saponins	_	+	+	+	+
Tannins	+	+	+	+	+
Flavonoids	+	-	-	+	+
Cardiac glycosides	+	+	+	+	+
Terpenoids & steroids	+	+	-	+	+
Phlobatamin	-	+	+	-	+
Ketonic sugar	+	-	-	-	-
Anthroquinones	+	+	+	-	+
Reducing sugar	+	+	+	+	+

+ detected/present; **-** not detected/present

The ability of the fractions to cause reduction of molybdenum (VI) to molybdenum (V) in total antioxidant capacity(TAC) assay was observed in the following descending order: butanolic > crude extract > DCM > hexane >and ethyl acetate fractions having values of 83.74 ± 0.71, 82.42±1.25, 81.59 ± 0.48, 64.37 ± 2.47 and 39.11 ± 2.44 mg AAE/g of sample respectively.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of *F. africana* is given in Table 2. All the fractions exhibited significant proton-donating ability. The HEF and BUF showed higher activities with IC_{50} values of 0.06±0.57 and 0.06±0.5µg/ml respectively while the DCF and EAF demonstrated low activities. It has been established that the lower the IC_{50} value of a compound, the higher its radical scavenging activity (Maisuthisakul et al., 2007).

Table 3 shows that DCF had the highest NO scavenging activity with IC_{50} value of 0.741 ± 0.07 mg/ml, followed by butanolic, the crude extract and ethyl acetate fractions with IC_{50} of 1.389 ± 0.1, 2.35±0.24 and 2.999 ± 0.19mg/ml respectively while hexane fraction has negligible effect.

The result of the ferric reducing ability of plant fractions, expressed in term of ascorbic acid equivalent is displayed in Table 3. In this study, the n-butanolic fraction had the highest antioxidant activity with the value of 48.46 ± 0.15 mg AAE/g of sample, followed by CME, DCF, HEF and EAF with values of 36.24±0.85, 34.17 ± 0.81, 32.77 ± 1.91 and 7.05 ± 0.51 mg AAE/g of sample respectively.

Natural antioxidants such as flavonoids and other phenolic compounds in fruits, vegetables and other medicinal plants have been found to scavenge free radicals and buffer the effects of pro-oxidants by reducing oxidative stress and preventing oxidative damage (Rimm and Stampfer, 1993).

Polyphenolic contents

The values of the TPC and TFC and of the extract and fractions (Table 3) obtained confirmed that the extract and fractions of *F. africana* are very rich in phenolic and flavonoid contents. A direct relationship has been established between the phenolic content and antioxidant capacity of plants (Al-Mamary et al., 2002). The n-butanolic fraction had the highest amount of phenolic, flavonoid compounds, and antioxidant capacity with values of 1331.18±41.56mg GAE/g and 833.96±19.14mg QUE/g and 83.74±1.88mgAAE/g respectively.

The scavenging property of these phenolic compounds has been ascribed to the hydroxyl moiety in their chemical structures, and their redox properties, (Gulcin et al., 2004) which allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen. In addition, they may also possess metal chelation properties (Liyana-Pathirana and Shahidi, 2006; Rice-Evans, 1995).

Several convincing experimental and clinical evidences have shown that the generation of reactive oxygen species (ROS) increases in both types 1 and 2 of diabetes mellitus and that the onset of diabetes is closely associated with oxidative stress (Rosen et al., 2001; Johansen et al., 2005; Bashan et al., 2009). The possible sources of oxidative stress in diabetes might include auto-oxidation of glucose, shifts in redox balances, decreased tissue concentrations of low molecular weight antioxidants, such as reduced glutathione (GSH) and vitamin E, and impaired activities of antioxidant defence enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Haskins et al., 2003).

Flavonoids are able to inhibit aldose reductase enzyme (that converts sugars to sugar alcohols) and is implicated with diabetic complications, such as neuropathy, heart disease and retinopathy (Alan et al., 2001). Antidiabetic activity of flavonoids and phenolic acids has been reported by several studies (Torres-Piedra et al., 2010; Weisburg et al., 2004; Wongsa et al., 2012).

In this study, four radical scavenging methods were used to assess the determination of potential radical scavenging activities of the fractions from *F. africana* methanolic leaf extract, namely DPPH· radical scavenging, NO radical scavenging, and ferric ion reducing ability (FRAP).

DPPH· radical scavenging activity

Table 2: Qualitative phytochemical analysis of the different fractions of *Funtumia africana* leaf extract

Functional Groups	Functional groups description	HEF	DCF	EAF	BUF
		Absorption frequency (cm^{-1})			
Hydroxyl, amino	OH, NH	3349.86	3392.69	3359.45	3333.77
Carbonyl	C-CO-C stretch	1168.65	1163.37	1167.79	1177.61
Carbonyl	C=O stretch	1691.61	1693.68	1691.11	1726.47
	asy C-O stretch	1241.82	1242.03	1241.44	1270.04
	sy-C-O stretch	1031.61	1031.89	1030.18	1028.82
Nitrile	C≡N stretch; aromatic	-	-	-	2362.04
Alkanes	C-H stretch	2922.28	2922.57	2922.69	2923.20
	sy C-H vibration	2852.23	2852.43	2852.73	2856.30
	aliphatic C-H stretch in CH_3 bend	1376.10	1375.99	1375.78	1363.35
Alkenes	C=C stretch; aromatic	1618.54	1617.42	1607.58	1609.22
	C=C ring stretch; CH_2 bending	1454.67	1455.97	1452.90	1448.75
Aromatics	Aromatic p-disubsitution	-	837.30	835.03	836.13
Alkyl halide	C-Cl	721.44	719.06	-	741.00
	C-OH out- of -plane bend	661.63	668.73	653.50	668.37
Glycogen		557.40	558.04	554.61	-

Source: Sahaya Sathish et al., 2012; Schwanninger et al., 2004; Silverstein and Webster, 2005

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts (Nanjo et al., 1996). Table 3 shows the H-donating activities of the fractions and extract of *F. africana* when compared to those of the standard ascorbic acid and trolox. All the fractions demonstrated excellent H-donor activities and were better than those of the standard ascorbic acid and trolox with values of 12.47±1.85 and 13.56±2.91 µg/ml respectively.

NO radical scavenging activity

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and involved in the regulation of various physiological processes. However, excess concentration of NO is associated with several diseases. Oxygen reacts with the excess nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals (Hemmani and Parihar, 1998). NO inhibitors have been shown to have beneficial effects on some aspects of the inflammation and tissue changes seen in models of inflammation bowel disease and in diabetes mellitus (Aydin et al., 2000). Increasing evidence suggests that oxidative stress and changes in nitric oxide formation or action play major roles in the onset of diabetic complications (Maritim et al., 2003).

In the present study, the fractions of *F. africana* effectively reduced the generation of nitric oxide from sodium nitroprusside by competing with oxygen to react with nitric oxide and thus inhibit generation of the nitrite anions. All the fractions and extract showed stronger nitric oxide scavenging activity (Table 3) than the standard L-ascorbic acid (IC_{50} 61.95±0.89 µg/ml).

Table 3: *In vitro* free radical scavenging activities of the different fractions of *Funtumia africana* leaf extract

Fractions	DPPH	NO	FRAP
	IC_{50} (µg/ml)		mg AAE/g
Crude Extract	0.12±0.73	2.35±0.24	36.24±0.85
n-Hexane	0.06±0.57	-	32.77±1.91
Dichloromethane	0.43±0.29	0.741±0.07	34.17±0.81
Ethylacetate	0.38±1.03	2.999±0.19	7.05±0.51
n-Butanol	0.07±1.12	1.389±0.1	48.46±0.15
L-ascorbic acid	12.47±1.85	61.95±0.89	ND
Trolox	13.56±2.91	ND	ND

Values are expressed as mean± SD of three parallel measurements
ND – not determined

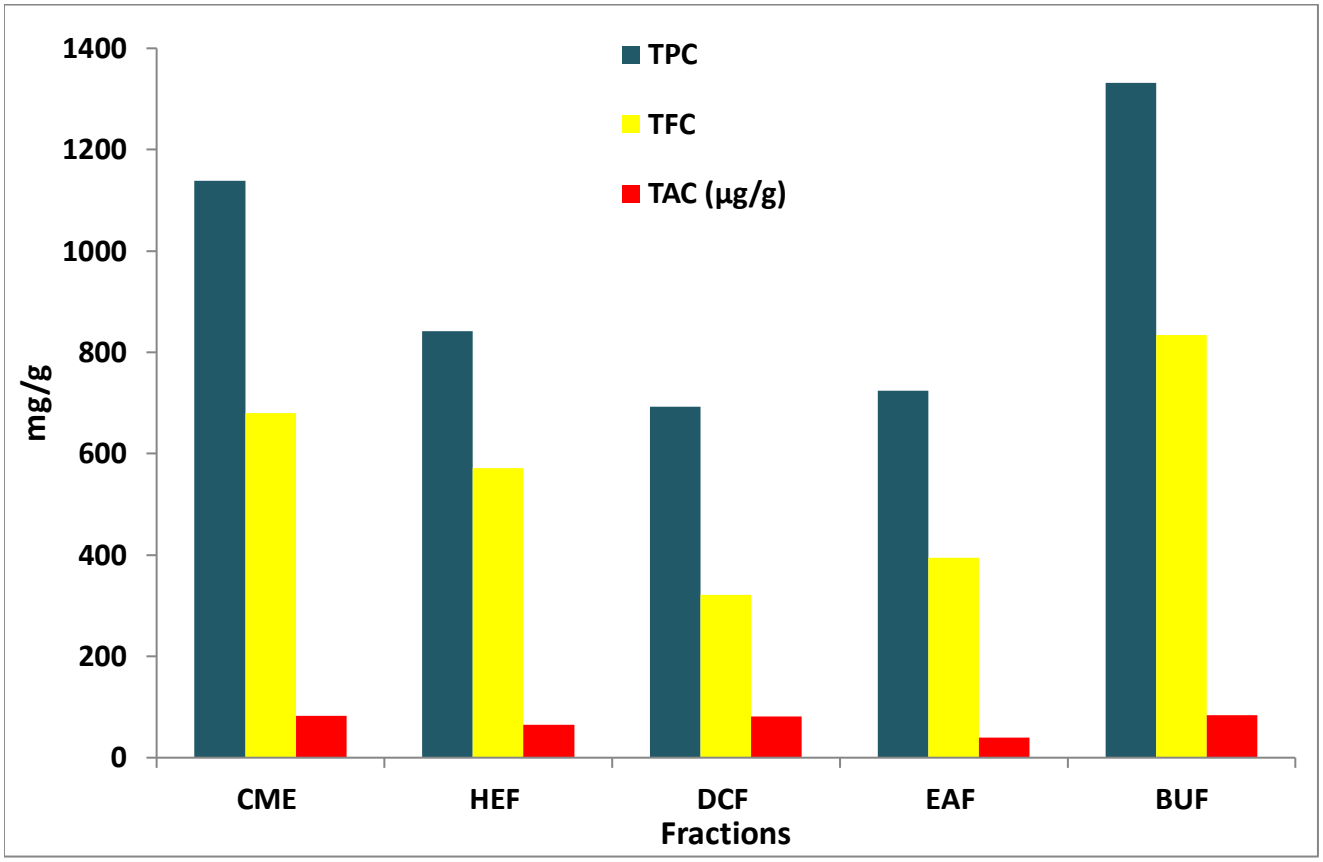

Fig. 1: Total phenolic and flavonoid contents and total antioxidant capacity of the different fractions of *F. africana* leaf extract.

Ferric reducing power ability

FRAP assay measures the ability of various fractions and extract of *F. africana* to reduce a ferric tripyridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}-TPTZ)(Table 3). The n-hexane, DCM, crude and n-butanol fractions showed significant ferric reducing activity with values ranging from 32.77±1.91 to 48.46±0.15mgAAE/g. Earlier authors (Tanaka et al., 1988) have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reductive ability of the fractions was attributed to the presence phenolic compounds in the fractions (Meir et al., 1995).

In general, this study demonstrates that antioxidant and radical scavenging activities were found to be predominant in the highly polar n-butanol fraction of F*untumia africana.*

CONCLUSIONS

Findings revealed that the extract and fractions from the leaves of F*untumia africana* possess many phytochemicals and antioxidant and free scavenging activities. Results also tend to indicate the potential of the leaves of *F. africana* to prevent or slow the progress of various oxidative stress-induced diseases such as diabetes as claimed by herbalists.

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