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Antiglycation and Antioxidant Activity of *Funtumia africana* Root Used in the Management of Diabetes

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ABSTRACT

Funtumia africana (Benth.) Stapf (Apocynaceae) is used folklorically for the treatment of various ailments such as dysentery, urinary incontinence, burns and diabetes. The aim of the study was to evaluate *F. africana* root hydroethanolic extract for the ability to inhibit formation of advanced glycation end-products and reactive oxygen species. The anti-glycation activity of the extract was determined using bovine serum albumin (BSA). Total phenolic content, total antioxidant capacity, ferric reducing antioxidant power (FRAP), nitric oxide (NO), lipid peroxide and 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging effects of the extract were also evaluated to determine the antioxidant activity. Result obtained, revealed that the extract significantly inhibited ($P< 0.05$) the formation of advanced glycation end-products (76.77%) at 100 µg/mL compared to aminoguanidine (85.76%). The total phenolic content was found to be 38.25 mg GAE/g and total antioxidant capacity 41.44 mg AAE/g of extract. DPPH, NO radical scavenging ability and FRAP of the extract were found to be concentration dependent (68.85%, 67.46% and 21.52 mg Fe2+/g respectively, at 100 µg/mL). The extract also exhibited good inhibition for lipid peroxidation in a concentration-dependent manner which was not significantly different from ascorbic acid (74.54% at 100 µg/mL). The antioxidant activity exhibited by the extract may be responsible for the observed inhibition of AGEs formation thus justifying the use of *F. africana* root in traditional medicine for the management of diabetes and its complications.

Keywords: *Funtumia africana* root, phenolic content, antiglycation, radical scavenging effect.

Introduction

Herbal treatment for the symptoms of diabetes and its complications have been employed in managing patients with insulin dependent and non-insulin dependent diabetes mellitus (DM). In DM, there is an increase in non-enzymatic glycation of proteins (Maillard reaction) due to persistent hyperglycemia.[1] This leads to several complications such as blindness, heart disease, nerve damage, and kidney failure. The relationship between the glycation of macromolecules and diabetic complications has been established.[2] Incubation of proteins (such as heamoglobin) with glucose leads to their non-enzymatic glycation and formation of Amadori product known as an early glycation product. Oxidative cleavage of Amadori products is considered as a major route to advanced glycation endproducts (AGEs) formation *in vivo*.[3]

AGEs are the final products of the reaction between excess glucose in the blood and the amino groups in proteins, lipoproteins and nucleic acids, through non-enzymatic rearrangement with the amino acids since the cross-links are unstable. However, AGEs are stable, therefore AGEs accumulate in the tissues which results, with time, in a loss of functionality of the affected tissues.[3] Tissues, especially large and small blood vessels, kidneys, eyes and nerves are mainly affected.[4]

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The accumulation of AGEs and diabetic complications are proportional to the increased duration of hyperglycaemia. AGEs accumulation has been considered to play a major role in the pathogenesis of diabetes and its complications.[5] Therefore, products that can prevent or slow down the formation of AGEs are good candidates for the management of diabetes and its complications. Oxidative cleavage of the early glycation product to form AGEs, can be inhibited by antioxidants.[6]

Antioxidants are compounds that help to inhibit the many oxidation reactions caused by reactive oxygen species (ROS) thereby preventing or delaying damage to the cells and tissues. Antioxidants are also capable of chelating metal ions thus preventing free radicals from damaging biomolecules such as proteins, DNA and lipids by breaking the chain reaction of lipid peroxidation at the initiation stage.[1,7-10] Antioxidation have been suggested as one of the mechanisms of action for antidiabetic activity because hyperglycemia induces oxidative stress by increasing the production of oxidants in the tissues which occur as a result of the metabolism of excess glucose, lipids and free fatty acids. Antioxidation represents the ability to inhibit the oxidation of other molecules.[11] Excess ROS induce oxidative stress and can lead to the accumulation of advanced glycation end products (AGEs) and activation of the protein kinase C pathway, all contributing to possible tissue damage.[12] ROS are various forms of activated oxygen which include free radicals (such as superoxide anion, nitric monoxide, hydroxyl radicals, peroxyl radicals) as well as non-radical oxidants such as hydrogen peroxide, peroxynitrite among others.[9-10] Free radicals are very unstable and highly reactive species due to the presence of one or more unpaired electrons. They occur in the body as a result of chemical reactions during normal cellular processes. Whereas non-radical oxidants generate free-radicals in living organisms through special chemical reactions. They have generally more specific reactivity and higher stability due to all-paired electrons.[13,14] ROS have been implicated in the development of many human diseases, including diabetes, rheumatoid arthritis, malaria, cancer, heart disease, stroke,

arteriosclerosis, cataracts, acquired immunodeficiency syndrome, and neurodegenerative diseases.[15,16]

Currently, the incorporation of medicinal plants with antioxidant activities in antidiabetic formulations is being explored.[1] The interest for antioxidants from natural sources and development of plant products as sources of antioxidants is on the increase.[18] Medicinal plants have shown to be good sources of natural antioxidants, as reactive oxygen scavengers and lipid peroxidation inhibitors.[19-22]

Different antioxidant substances occur in different plant tissues with different mechanisms of action and the different methods of measuring antioxidant indices are all prone to limitations. Standard procedures also seem to lack specificity in the stepwise procedure, hence are susceptible to variable results.[23] Therefore, several methods have been developed in recent years to investigate the antioxidant activity of biological samples. These include but not limited to oxygen radical absorption capacity method, ferric reducing antioxidant capacity method, total oxyradical scavenging capacity and lipid peroxidation assay.[24-25] *F. africana*, a perennial, tropical woody plant with rapid growth, is used traditionally for the treatment of dysentery, wounds, urinary incontinence, edema, and diabetes.[26] The leaf extract has been reported to have anti-microbial, anti-inflammatory, radical scavenging and wound healing activities.[25-27] However, no scientific study has been carried out on the anti-diabetic and antioxidant activities of the root. This study was therefore carried out to evaluate the ability of the extract to inhibit the production of advanced glycation end products and antioxidant activities of the extract *in-vitro*.

Materials and Methods

Chemicals

The chemicals and reagents used were of analytical grade obtained from Merck (Germany) unless otherwise stated. Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picryl hydrazyl (DPPH), ferric tripyridyltriazine (TPTZ), Aminoguanidine hydrochloride and Bovine serum albumin (BSA) were obtained from Sigma Aldrich (Germany).

Plant Material and Extraction

Fresh root of *F. africana* was collected in the month of April, 2013 from Onigambari forest reserve, Onipe, Oyo state, Nigeria. The plants were authenticated by a taxonomist and deposited in the herbarium, Department of Botany, University of Lagos, Akoka, Lagos, with voucher number LUH 5647. The root of *F. africana* was washed and dried in hot air oven at 50ºC for 4 days. The pulverized plant material (200 g) was extracted with 600 mL 90 % ethanol twice by cold maceration for 3 days with intermittent stirring. It was filtered with muslin cloth and Whatman filter paper. The filtrates were combined, concentrated *in vacuo* and dried to constant weight over a regulated water bath at 40°C. The crude extract was stored in a freezer at -20°C until it was used for the study. The extract was reconstituted as appropriate for this study.

Care and Use of Animals

Rats used for the experiment were obtained from the Laboratory Animal Center, College of Medicine, University of Lagos, Idi-Araba and kept under standard environmental condition, maintained on standard animal pellets (Pfizer Feeds Plc, Nigeria), with access to water *ad libitum*. They were allowed to acclimatize to the laboratory conditions for 7 days, prior to use. The care and use of the animals were in strict compliance with the principles of laboratory animal care.[28] The experimentation protocol (Protocol ID: CMUL/HREC/05/17/181) was approved by the Health Research Ethics Committee of the College of Medicine, University of Lagos, Nigeria.

Anti-glycation Assay

Ability of the extract to inhibit the formation of advanced glycated endproducts was determined *in vitro* using bovine serum albumin (BSA), adopting the method described by Schmidt *et al*.[29] Briefly, 0.50 mL BSA (10 mg mL^{-1}) was incubated with 0.46 mL of 500 mM fructose in 100 mM phosphate buffered-saline (PBS) (5 mL total volume, pH 7.4) containing 0.02 % sodium azide at 37°C for 21 days. Before incubation, 0.04 mL of various concentrations of the extract ((0.1 – 0.5 mg mL^{-1}) and aminoguanidine hydrochloride (AG), a standard inhibitor (0.01 - 0.1 mg/mL) were added into the reaction mixtures with final concentrations of BSA (2 $mgmL^{-1}$) and fructose (40 mM). Blanks without any inhibitor were also setup. The formation of fluorescent AGES was measured using a Spectrofluorometer. All the reagents and samples were filtered through 0.2 µm membrane filters. The fluorescent intensity was measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The percentage inhibition of fluorescent AGE formation was calculated as follows:

$$\%\ \text{Inhibition of fluorescent AGEs} = \frac{(FS - FCB) - (FS - FSB)}{FC - FCB} \times 100$$

Where, FC = fluorescent intensity of control with fructose; FCB = fluorescent intensity of control blank without fructose; FS and FSB = fluorescent intensity of sample with fructose; FSB = fluorescent intensity of sample blank without fructose.

Estimation of Total Phenolic Content

Total phenol content in the extract was determined by a modified Folin-Ciocalteu method.[30] Briefly, 0.5 mL of extract and 0.1 mL of Folin Ciocalteu reagent (0.5 N) were mixed and incubated at room temperature for 15 min. After this, 2.5 mL sodium carbonate solution (7.5% w/v) was added and further incubated for 30 min at room temperature. The absorbance was measured spectrophotometrically at 760 nm. A standard curve was generated using different concentrations of gallic acid and total phenol content was extrapolated from the generated calibration curve. Results were expressed as milligram gallic acid equivalent per gram of extract (mg GAE/g).

Total Antioxidant Capacity Determination

Total antioxidant capacity of the extracts was determined using the method described by Prieto *et al*.[31] A sample of the extract (0.3 mL of 20-200 µg/mL) was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95ºC for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm. The total antioxidant capacity was expressed as milligram ascorbic acid equivalent per gram of extract (mg AAE/g).

DPPH Radical Scavenging Assay

The free radical scavenging activity of the extract, based on the scavenging of the stable 1,1 -diphenyl-2-picryl hydrazyl (DPPH) free radical was estimated according to the procedure as described by Cuendet *et al*; Burits and Bucar.[32,33] An aliquot of 0.5 ml of extract in methanol (95%) at different concentrations (20, 40, 60, 80, 100 µg/ mL) was mixed with 2.0 ml of reagent solution (0.004 g of DPPH in 100 mL methanol). The control contained only DPPH solution in place of the sample while methanol was used as the blank. The mixture was vigorously shaken and left to stand at room temperature. After 30 minutes the decrease in absorbance of test mixture (due to quenching of DPPH free radicals) was read at 517 nm. The experiment was repeated three times and ascorbic acid (20, 40, 60, 80, 100 µg/ mL) used as standard. The scavenging effect was calculated using the expression:

$$\%\ \text{DPPH scavenging effect} = \frac{(A0 - A1)}{A0} \times 100$$

Where, A0 is the absorbance of the standard and A1 is the absorbance of sample.

Nitric Oxide Radical Scavenging Assay

In this study, NO radical inhibition was estimated by the use of a two-step diazotization Griess illosvay reaction.[34,35] The reaction mixture (3 mL), containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL) and extract (20, 40, 60, 80, 100 µg/mL, respectively) was incubated at 25°C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) and allowed to stand (10 min) for complete diazotization. Then 1 mL of naphthylethylenediamine dihydrochloride was added, mixed and allowed to stand for 30 min. The absorbance of the pink coloured chromophore that formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550 nm against the corresponding blank solution. Ascorbic acid was

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used as standard. The percentage (%) scavenging effect was calculated from the following equation:

$$\% \text{ Inhibition} = [(A0 - A1)/A0] \times 100$$

Where, A0 is the absorbance of the Control and A1 is the absorbance of the extract or standard.

Ferric Reducing Antioxidant Power (FRAP)

The method of Benzie and Strain[36] was adopted for the FRAP assay. A 300 mM sodium acetate buffer of pH 3.6, 10 mM 2, 4, 6-tri-(2-pyridyl)-1, 3, 5-triazine and 20 mM $FeCl_3.6H_2O$ were mixed together in the ratio of 10:1:1, respectively, to give the working FRAP reagent. A 50 µL aliquot of the extract at 0.1 mg/mL and 50 µL of standard solutions of ascorbic acid (20, 40, 60, 80, 100 µg/mL) were added to 1 mL of FRAP reagent. Absorbance measurements were taken at 593 nm exactly 10 min after mixing against reagent blank containing 50 µL of water. Ascorbic acid was used as the positive control. The calibration curve was prepared with $FeSO_4.7H_2O$ and it was linear between 20-100 µg/mL. All measurements were taken at room temperature and samples protected from direct sunlight. Results obtained were expressed in milligram Fe^{2+} per gram of extract (mg Fe^{2+}/g) and compared with that of ascorbic acid.

In vitro Lipid Peroxidation Inhibition (TBARS Assay)

Lipid peroxidation was induced by Ferrous (II) chloride-ascorbate system in liver homogenate and estimated as thiobarbituric acid reacting substances (TBARS), adopting the method described by Meir *et al*; Oyesola *et al*.[37, 38] Freshly excised rat liver was rinsed, sliced and homogenized in ice cold 50 mM KCl−Tris−HCl buffer pH 7.5 to obtain 10% homogenate. The reaction mixture contained liver homogenate, Tris−HCl buffer (20 mM pH 7.0), $FeCl_2$ (2 mM), ascorbic acid (10 mM), and 0.5 ml plant extract (25−100 µg/mL) in a final volume of 1 mL. The reaction mixture was incubated at 37°C for 1 h. Lipid peroxidation was measured as malondialdehyde (MDA) equivalent using trichloroacetic acid (TCA), thiobarbituric acid (TBA) and HCl (TBA-TCA reagent: 0.375% w/v TBA; 15% w/v TCA and 0.25 N HCl). The incubated reaction mixture was mixed with 2 mL of TBA-TCA reagent and heated in a boiling water bath for 15 minutes. After cooling, the solution was centrifuged at 10,000 g for 5 min and the precipitate obtained was removed. Finally, malondialdehyde concentration in the supernatant fraction was determined spectrophotometrically at 535 nm. Ascorbic acid was used as standard.

Statistical Analysis

Results are presented as mean ± standard deviation (SD) and analyzed using Graphpad prism 6.0 (Graphpad prism Software Inc., San Diego, CA, USA).

Results and Discussion

Total Phenolic Content and Total Antioxidant Capacity

The total phenolic content of *F. africana* root, calculated from the regression equation of gallic acid calibration curve (y = 3.5695x + 0.0062; R^2 = 1.0), was found to be 38.25 mg GAE/g. Total antioxidant capacity expressed in terms of milligram ascorbic acid equivalent per gram of extract (mg AAE/g) was found to be 41.44 mg AAE/g. (Figure 1). Result from this study showed that the extract contains an appreciable amount of phenolic compounds. Plant phenolics remain a prominent class of compounds with free radical scavenging properties and have been implicated in the antidiabetic activity of plants which arise from their high reactivity, as proton donors.[20-22]

Anti-glycation activity

Figure 2 showed that the extract significantly inhibited ($p < 0.05$) the formation of advanced glycation end-products (76.77%) at 100 µg/mL compared to aminoguanidine (85.76%). Aminoguanidin is a known inhibitor of advanced glycation reactions (protein modification by the advanced Mailard reaction) *in vitro* and inhibits the development of diabetic complications in animal models.[6] Any substance or compound that has potential to block the formation of AGEs can prevent the production of excess glucose in the plasma and hence serve as a good antidiabetic agent.[17]

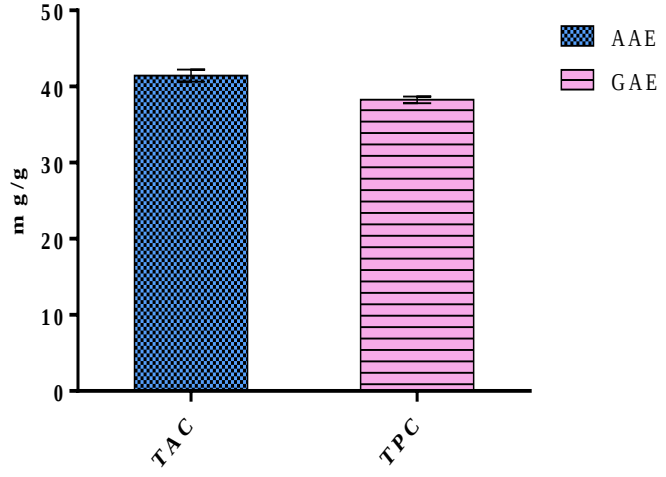

Figure 1: Total antioxidant capacity and Total phenolic content of *Funtumia africana* root ethanolic extract.

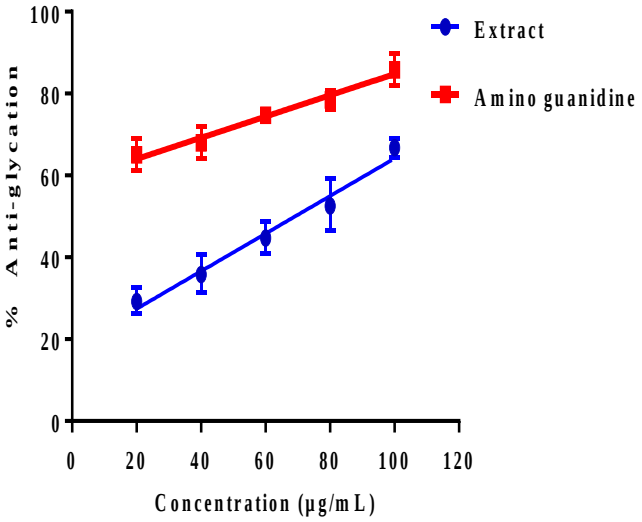

Figure 2: Inhibition of advanced glycated endproducts by *F. africana* root ethanolic extract.

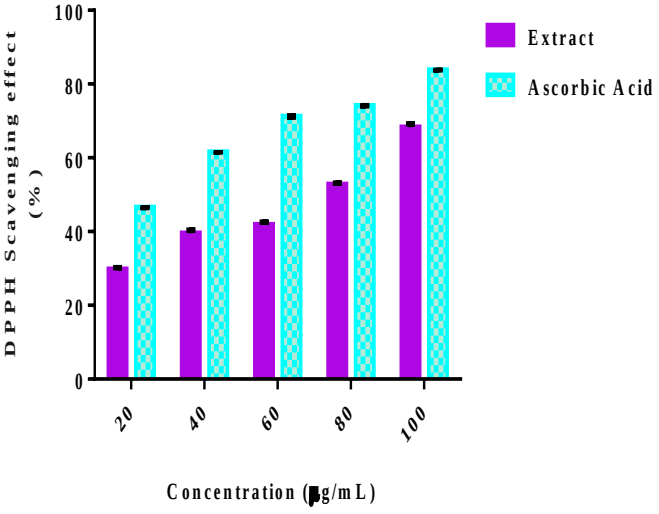

Figure 3: DPPH radical scavenging effect of *F. africana* root ethanolic extract.

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DPPH Radical Scavenging Activity

Figure 3 shows that the radical scavenging ability of *F. africana* root extract and Ascorbic acid on DPPH radical, increased as concentration increased. DPPH radical scavenging assay is suitable for the investigation of the radical-scavenging activity of hydrogen-donating compounds, especially phenolics. Ascorbic acid is a potent antioxidant as an electron donor showing free radical scavenging ability, hence it is commonly used in DPPH radical scavenging assay as a reference compound for measuring antioxidant activity. Free radical scavengers are proton donors that help to mop up excess free radicals.[14] According to the reports of Jimoh *et al*; Frankel and Meyer,[15,20] phenolic constituents of the extract may have contributed to its scavenging ability.

Nitric Oxide Radical Scavenging Activity

From this study, the extract showed high nitrite oxide (NO) radical scavenging activity comparable to ascorbic acid in a concentration dependent manner (Figure 4). NO is produced by the endothelial cells lining the arteries which plays important role in overall blood circulation and various physiological processes such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell-mediated toxicity.[39] NO is very unstable (a reactive radical), therefore under aerobic conditions, NO interacts with oxygen to produce stable nitrite and peroxynitrite anions that are deleterious to human health.[40] Nitric oxide radicals have been implicated in a number of disease states including inflammatory diseases, diabetes mellitus, ischaemia-perfussion injury and neurodegenerative diseases.[41] NO radical inhibition can be estimated by the use of Griess illosvay reaction. Scavenging of NO radical is one approach to the treatment of diseases where there is an overproduction of NO. Sodium nitroprusside is a known nitric oxide donor *in vitro*, which spontaneously generates NO in aqueous solution, at a physiological pH (7.2).[42] The extract showed high NO scavenging activity by effectively reducing the generation of nitric oxide from sodium nitroprusside. by competing with oxygen to react with nitric oxide and thus inhibiting the generation of toxic nitrite anions. The study revealed the radical scavenging ability of the extract as a therapeutic approach to nitric oxide mediated diseases.

Ferric Reducing Antioxidant Power (FRAP)

FRAP assay was used to measure the ability of *F. africana* root extract to reduce a ferric tripyridyltriazine (TPTZ-Fe^{3+}) to its ferrous form (TPTZ-Fe^{2+}). The study shows that the extract exhibited significant FRAP comparable to ascorbic acid. Figure 5 shows the reducing power of the extract compared to the standard, which was concentration dependent. The extract significantly ($P < 0.05$) reduced $Fe3^{+}$ at 40 µg/mL. However, the reducing power of ascorbic acid at the lowest concentration used was significantly ($P < 0.05$) higher than that of the extract. The absorbances were found to be 6.42, 20.72 and 17.90, 22.87 for the extract and ascorbic acid at the concentration of 20 and 40 µg/ml respectively. Meir *et al*[38] reported a direct correlation between antioxidant activity and reducing power; the report also attributed reducing power to the presence of phenolic compounds. This present study has revealed that the extract certainly has proton donating ability and could serve as free radical inhibitor or scavenger acting possibly as primary antioxidants.

Lipid Peroxidation Inhibitory Activity

From the study, it was observed that the TBARS values decreased in a dose-dependent manner as the concentration of the extract increased (Figure 6), indicating low levels of malondialdehyde (MDA) and a reduction in lipid peroxidation. Plant phenolics can react with active oxygen radicals, such as hydroxyl radical superoxide anion radicals and lipid peroxy radicals, and inhibit lipid oxidation at an early stage.[43-44] Ascorbic acid used as positive control, is a water soluble antioxidant that helps to regenerate vitamin E which scavenges lipid peroxyl radicals *in vivo* as well as *in vitro* systems. This helps to protect low density lipoprotein (LDL) from unwanted oxidations. The ability of iron to generate free radicals from peroxides by Fenton reactions has been implicated in cardiovascular disease.[38,45] Reduction of Fe^{2+} concentrations in the Fenton reaction, protects against oxidative damage caused by oxyradical production and lipid peroxidation. Therefore, antioxidants that inhibit lipid peroxidation are protective in the atherogenic process.[46-47] The study revealed that the extract exhibited high inhibition of lipid peroxidation which was not

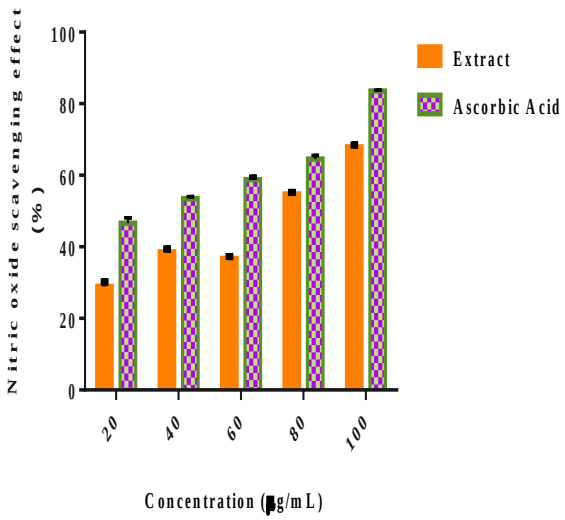

Figure 4: Nitric oxide radical scavenging activity of *F. africana* root ethanolic extract.

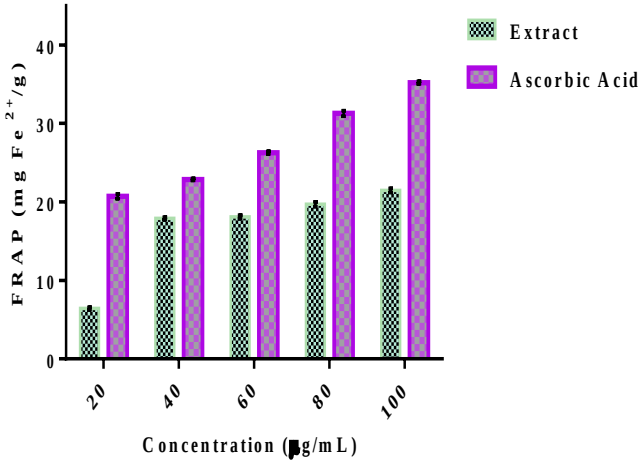

Figure 5: Ferric reducing antioxidant power of *F. africana* root ethanolic extract.

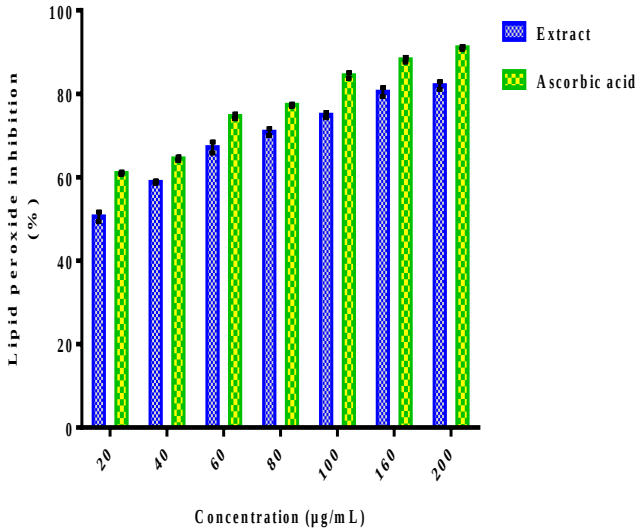

Figure 6: Lipid peroxidation inhibitory activity of *F. africana* root ethanolic extract.

significantly different from that of Ascorbic acid. This further lends credence to the use of *F. africana* root as a pharmacological remedy to ameliorate cardiovascular disease and complications of diabetes, which have placed great economic burden on both the developed and developing countries.

Conclusion

Findings from this study showed that *F. africana* root has the ability to inhibit the formation of advanced glycation end products (AGEs). It also has good antioxidant activity particularly exhibiting very good inhibition for lipid peroxidation which is mainly responsible for cardiovascular disease and tissue damage. The antioxidant activity exhibited by the extract may be responsible for the observed inhibition of AGEs formation thus justifying the use of *F. africana* root in traditional medicine for the management of diabetes and its complications. In addition, the extract could be useful in the prevention and treatment of other diseases, as well as a value-added ingredient for stabilizing herbal preparations against oxidation reactions.

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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