

## High-Performance Liquid Chromatography (HPLC) Fingerprinting and Comparative Antioxidant Properties of Fruit and Leaf Extracts of *Kigelia africana*

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### ABSTRACT

The objective of this research was to evaluate and compare the phytoconstituents and antioxidant activities of methanol extract of *Kigelia africana* leaves and fruits. *Kigelia africana* was found to contain flavonoids, terpenoids, alkaloids, isoprenoids, cardiac glycosides, steroids, saponins and tannins. Alkaloids, steroids and anthraquinones were absent in the fruit while only anthraquinones was absent in the leaf. Total flavonoids, total phenolic and total antioxidant capacity of the fruit extract was found to be 9055.5µg/mg quercetin equivalent, 180.7692µg/mg tannic acid equivalent and 299.6215mM/mg ascorbic acid equivalent respectively while those of the leaf extract were 23222.2µg/mg quercetin equivalent, 598.7192 µg/mg tannic acid equivalent and 296.6826 mM/mg ascorbic acid equivalent respectively. The leaf extract showed better antioxidant activity with respect to reducing power assay, DPPH, ABTS and NO radical scavenging activities. Relatively, the fruit extract showed better antioxidant activity in terms of Fe<sup>2+</sup> chelating ability, hydroxyl radical scavenging activity, anti-denaturation activity, anti-hemolytic and anti-lipoperoxidative. Both extracts exhibited significant free radical scavenging and antioxidant characteristics, these may be due to the substantial amount of phenol and flavonoids that were noticed. The results of this work suggest that leaves and fruits of *Kigelia africana* are promising sources of natural phytochemicals and antioxidants that can combat oxidative stress caused by free radicals.

**Keywords:** *Kigelia africana*, Antioxidants, Phytoconstituents, Radical scavenging activities.

### INTRODUCTION

Plants have provided man with most of his needs in terms of shelter, clothing, food, flavors and fragrances and not least the basis of health care throughout the world since the earliest days of humanity and are still internationally used<sup>1</sup>. Plants remain a vital source of medicines for a large proportion of the world's population, particularly in the developing countries<sup>1</sup>. Nature has been a good supply of various medicinal plants for thousands of years and a number of modern drugs have been isolated from plants. A range of medicinal plants have been discovered and used for many years on a daily basis for treatment of different diseases all over the world<sup>2</sup>. Herbal medicine is now globally accepted as a legal, alternative system of therapy for treatment and cure of various diseases and physiological conditions in traditional treatments in the form of pharmaceuticals<sup>3</sup>. Various plant species are used by many ethnic groups around the world for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, and malaria and a range of other indications. Numerous prescriptions, folk drugs

and herbal drugs play an important role in maintaining people's health. These are also the sources for new drug research and development<sup>4</sup>. Most developing countries around the world still rely on the use of indigenous plants for the treatment of various diseases because they are unable to afford pharmaceutical drugs. But the problem is that in most cases very little scientific information is available about the safety, active ingredients and toxicity of such indigenous medicine. There is a great need to harness scientific and clinical research in order to investigate the quality, safety and efficiency of these herbal therapies<sup>5</sup>. The use of medicinal plants and their secondary metabolites and scientific knowledge about them should help researchers determine the efficacy, stability, best drug delivery systems and quality controls of plants commonly used in traditional medicine. Through scientific knowledge, various methods have been developed to test, isolate, purify and characterize these secondary metabolites from medicinal plants. *Kigelia africana* is a tropical tree used in traditional African medicine for its medicinal properties due to the

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presence of numerous secondary metabolites. These secondary metabolites present in *Kigelia africana* provide a basis for its traditional uses. The plant is an endemic species in many parts of Africa. The fruits of the plant have been reported for their traditional use as dressing for ulcers, purgative and as a lactagogue, while the bark has been reported for its antimicrobial cytotoxicity and anti-implantation activities<sup>6</sup>. An unpublished account from local users in Nigeria revealed that the bark of *Kigelia africana* is commonly used for various anticancer and anti-inflammatory preparations while the anti-inflammatory activities of the fruits have been confirmed. The extract of the plant has been shown to possess antioxidative property which apparently makes it useful in the treatment of diseases especially the liver-borne disease<sup>7</sup>. The ethno medicinal plant bark is used for the treatment of rheumatism, dysentery and venereal diseases. It is also used as ringworm and tapeworm expellant, while other uses include treatment of haemorrhages, diabetes, pneumonia and toothache<sup>8,9</sup>. Phytochemical analysis of the plant indicates the presence of naphthoquinones, coumarins, flavonoids and iridoids<sup>8,9</sup>. The present work was designed to determine and compare the phytochemical constituents and antioxidant activities of the methanol extracts of leaves and fruits of *Kigelia africana*.

## MATERIALS AND METHODS

### *Plant material and extraction*

The leaves and fruits of *K. Africana* were collected from a farm settlement in Ikere-Ekiti, Ekiti State, Nigeria and were authenticated in the herbarium of Botany Department, University of Ibadan, Oyo State, Nigeria. Leaf sample was cleaned with distilled water and air dried, and then powdered. The leaves were dried under active ventilation at room temperature. The dried leaves were milled. The pulverized sample was extracted in 80% methanol by continuous agitation for 72 hours. The fruit sample was cleaned with distilled water and cut into smaller pieces to increase the surface area and then extracted in 80% methanol for 72 hours. The methanol extracts were concentrated in a rotary evaporator, freeze dried and thereafter preserved for further use in a refrigerator.

### *Principle of extraction*

The solution of extract and solvents were put into the rotary evaporator flask and the machine was set up, the temperature used was 37°C. When solution was heated to the required temperature, the solvents started to evaporate and were distilled with the use of a distiller attached to the evaporator, and the pure solvent was collected in the provided flask also in the rotary evaporator. The process continued until all the solvents were removed from the solution and only the pure bioactive components of the plants that are been sought are remaining. After the whole process, the extracts that were able to air dry were left in the open air in the laboratory and the others were freeze dried with a freeze drier until a concentrated extract was obtained. The extracts were then transferred into different

bottles of known weights and kept in a refrigerator until further use.

### *Chemicals*

Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), DPPH (2,2-diphenyl-1-picrylhydrazyl) radical, Tannic acid, Quercetin, Ascorbic acid, Mannitol and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich, USA. All other chemicals and reagents used were of analytical grade and obtained from standard suppliers.

### *Chemical, apparatus and general procedures for HPLC*

All chemical were of analytical grade. Acetonitrile, formic acid, gallic acid, chlorogenic acid, ellagic acid, rosmarinic acid and caffeic acid purchased from Merck (Darmstadt, Germany). Quercetin, isoquercitrin, rutin, catechin and kaempferol were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, acetonitrile (ACN) and ethyl acetate (EtAc) purchased from Merck (Darmstadt, Germany). Tocopherol,  $\beta$ -carotene and lycopene were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

### *Quantification of compounds by HPLC-DAD*

Reverse phase chromatographic analyses were carried out under gradient conditions using C<sub>18</sub> column (4.6 mm x 250 mm) packed with 5 $\mu$ m diameter particles. The mobile phase was water containing 1% formic acid (A) and acetonitrile (B), and the composition gradient was: 13% of B until 10 min and changed to obtain 20%, 30%, 50%, 60%, 70%, 20% and 10% B at 20, 30, 40, 50, 60, 70 and 80 min, respectively (Boligon et al., 2012) with slight modifications. *Kigelia africana* (leaves and fruits) methanolic extract was analyzed dissolved in ethanol at a concentration of 20 mg/mL. The presence of ten antioxidants compounds was investigated, namely, gallic acid, chlorogenic acid, caffeic acid, ellagic acid, rosmarinic acid, catechin, quercetin, isoquercitrin, rutin and kaempferol. Identification of these compounds was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards. The flow rate was 0.6 ml/min, injection volume 50  $\mu$ l and the wavelength were 254 nm for gallic acid, 280 nm for catechin, 327 nm for caffeic, ellagic, rosmarinic and chlorogenic acids, and 366 nm for quercetin, isoquercitrin, rutin and kaempferol. All the samples and mobile phase were filtered through 0.45  $\mu$ m membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.030 – 0.250 mg/ml for kaempferol, quercetin, isoquercitrin, catechin and rutin; and 0.030 – 0.250 mg/ml for gallic, caffeic, rosmarinic, ellagic and chlorogenic acids. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to

400 nm). Calibration curve for gallic acid:  $Y = 13548x + 1259.7$  ( $r = 0.9999$ ); catechin:  $Y = 12730x + 1265.3$  ( $r = 0.9997$ ); rosmarinic acid:  $Y = 11947x + 1493.6$  ( $r = 0.9995$ ); caffeic acid:  $Y = 13248x + 1359.3$  ( $r = 0.9990$ ); chlorogenic acid:  $Y = 12661x + 1375.6$  ( $r = 0.9995$ ); ellagic acid:  $Y = 11985x + 1265.9$  ( $r = 0.9998$ ); rutin:  $Y = 12845 + 1365.7$  ( $r = 0.9999$ ); quercetin:  $Y = 13560x + 1192.6$  ( $r = 0.9991$ ), isoquercitrin:  $Y = 14273x + 1275.4$  ( $r = 0.9998$ ) and kaempferol:  $Y = 14253x + 1238.9$  ( $r = 0.9997$ ). All chromatography operations were carried out at ambient temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10  $\sigma/S$ , respectively, where  $\sigma$  is the standard deviation of the response and  $S$  is the slope of the calibration curve<sup>10,11</sup>.

#### Quantification of carotenoids HPLC-DAD

Carotenoids analysis was carried at reverse phase chromatographic analyses were carried out under gradient conditions using  $C_{18}$  column (4.6 mm x 150 mm) packed with 5  $\mu$ m diameter particles. The mobile phase consisted of mixtures of ACN:  $H_2O$  (9:1, v/v) with 0.25% triethylamine (A) and EtAc with 0.25% triethylamine (B). The gradient started with 90% A at 0 min to 50% A at 10 min. The percentage of A decreased from 50% at 10 min to 10% A at 20 min. The flow-rate was 0.8 ml/min and the injection volume was 40  $\mu$ l. Signals were detected at 450 nm, following the method described by Janovik et al. (2012) with slight modifications. Solutions of standards references (tocopherol,  $\beta$ -carotene and lycopene) were prepared in HPLC mobile phase at a concentration range of 0.035 - 0.350 mg/ml. The samples were analyzed at a concentration of 20 mg/mL, carotenoids were identified and quantified in the extracts by comparison of retention times and UV spectra with the standard solution. All chromatography operations were carried out at ambient temperature and in triplicate.

#### Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves. LOD and LOQ were calculated as 3.3 and 10  $r/S$ , respectively, where  $r$  is the standard deviation of the response and  $S$  is the slope of the calibration curve<sup>10,11</sup>.

#### Phytochemical Screening

The extracts were screened for the presence of alkaloids, saponins, tannins, phlobatannins, anthraquinones, steroids, terpenoids, isoprenoids, flavonoids and cardiac glycosides<sup>12-15</sup>.

#### In vitro phytochemical and antioxidant test

##### DPPH (1, 1, Diphenyl 2-Picryl Hydrazyl) assay

The antioxidant activity by DPPH assay was assessed using the stable free radical DPPH. To various concentration (25, 50, 100, 200, and 400  $\mu$ g/ml) of the extract and standard (tannic acid), 1 ml of 0.1mM DPPH was added to the test tubes. After incubation 30 min in the dark at room temperature, absorbance was recorded at

517nm. The percent DPPH radical scavenging was calculated with the equation:

$$\% \text{ DPPH radical scavenging} = [(\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance}] \times 100.$$

The experiment was carried out in triplicate<sup>16</sup>.

##### Nitric Oxide Radical Scavenging

The nitric oxide radical scavenging capacity of the extract was measured by Griess reaction<sup>17</sup>. Various concentrations (25, 50, 100, 200, and 400  $\mu$ g/ml) of the extract and standard were prepared. Sodium nitroprusside (2.5 ml, 10 mM) in phosphate buffered saline (PBS) was added to 0.5ml different concentrations of extract and standard. The reaction mixture was incubated at 25°C for 150 min. After incubation, 0.5 ml aliquot was removed and 0.5 ml of Griess reagent: (1% (w/v) sulfanilamide, 2% (v/v)  $H_3PO_4$  and 0.1% (w/v) naphthylethylenediamine hydrochloride) was added. The absorbance was measured at 546 nm. Ascorbic acid was used as reference standard and was treated the same way as that of extracts. Sodium nitroprusside in PBS (2 ml) was used as control. The nitric oxide radicals scavenging activity of the extracts and ascorbic acid was calculated according to the following equation:

$$\text{Percentage of inhibition} = [(A_0 - A_1) / A_0] \times 100.$$

Where  $A_0$  is the absorbance of sodium nitroprusside in PBS (without extract or ascorbic) and  $A_1$  is the absorbance in the presence of the extract or ascorbic acid.

##### Reductive Potential

##### Ferric cyanide ( $Fe^{3+}$ ) reducing antioxidant power assay

Reducing power of the extracts were measured by the direct reduction of  $Fe^{3+}(CN^-)_6$  to  $Fe^{2+}(CN^-)_6$  and was determined by absorbance measurement of the formation of the Perl's Prussian Blue complex following the addition of excess  $Fe^{3+}$ <sup>18</sup> as described previously by<sup>19</sup>. Different concentrations (25, 50, 100, 200, and 400  $\mu$ g/ml) of extract and standard (quercetin) in 0.5ml of distilled water were mixed with 1.25 ml of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 ml of potassium ferricyanide [ $K_3Fe(CN)_6$ ] (1%) the mixture was incubated at 50°C for 20 min. After 20 min incubation, the reaction mixture was acidified with 1.25 ml of trichloroacetic acid (10%). Finally, 0.5 ml of  $FeCl_3$  (0.1%) was added to this solution and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates greater reduction capability<sup>20</sup>.

##### Hydroxyl Radical Scavenging Activity Assay

The hydroxyl radical ( $\cdot OH$ ) scavenging activity was measured by the method of Jin et al.<sup>21</sup>. The hydroxyl radical was generated in a mixture of 1.0 ml of 0.75 mM 1,10-phenanthroline, 2.0 ml of 0.2 M sodium phosphate buffer (pH 7.4), 1.0 ml of 0.75 mM  $FeSO_4$  and 1.0 ml of  $H_2O_2$  (0.01%, v/v). After addition of 1.0 ml extract at various concentrations (25, 50, 100, 200, and 400  $\mu$ g/ml), the mixture was incubated at 37°C for 30 min. Then, the absorbance of the mixture at 536 nm was measured. Mannitol was used as reference standard and was treated the same way as that of extracts. Distilled water and VC were used as the blank and positive control respectively. The scavenging activity on  $\cdot OH$  was calculated by the following equation:

Scavenging activity (%) = (Abs sample – Abs blank)/ (Abs0 – Abs blank) \*100

Where, Abs0 is the absorbance of the distilled water instead of H<sub>2</sub>O<sub>2</sub> and extract in the assay system.

#### *Metal (Fe<sup>2+</sup>) Chelating Ability Assay*

The *in-vitro* Fe<sup>2+</sup> chelating ability of the extract was assayed according to the method of Minnoti and Aust<sup>22</sup> with slight modification<sup>23</sup>. Briefly, 900µl of aqueous FeSO<sub>4</sub>(500 µM) and 150µl of the extract (25, 50, 100, 200, and 400µg/ml) were incubated for 5min at room temperature. Seventy eight micro liter (78µl) of 1, 10-phenanthroline (0.25 %, w/v, aqueous) was added. The absorbance of the orange colour solution was read at 510nm with a spectrophotometer. EDTA was used as reference standard was treated the same way as the extract. The principle of the assay is based on disruption of o-phenanthroline-Fe<sup>2+</sup> complex in the presence of chelating agent. The *in vitro* of Fe<sup>2+</sup> chelating ability of the sample was calculated by using the following formula:

Chelating ability (%) = (A control - A sample)/ A control x 100.

Where; A control = the absorbance of the control (reaction mixture in the absence of sample) (FeSO<sub>4</sub> alone). A sample = the absorbance of the reaction mixture (sample, FeSO<sub>4</sub> and 1, 10-phenanthroline)

#### *In-vitro Inhibition of Lipid Peroxidation Assay*

*In-vitro* inhibition of lipid peroxidation was estimated according to the method of Ruberto and Baratta<sup>24</sup>. In this assay, egg yolk homogenate served as lipid rich medium and FeSO<sub>4</sub> acts as initiator of lipid peroxidation. Briefly, 50µl of plant extract (25, 50, 100, 200, and 400µg/ml) was mixed with 0.25ml 10% egg yolk. This was followed by the addition of 10µl FeSO<sub>4</sub> (0.07M, aqueous). The mixture was incubated at room temperature for 30min. This was followed by the addition of 0.75ml of glacial acetic acid (5%I, v/v aqueous) and 0.75ml of thiobarbituric acid 0.8% in Sodium dodecyl sulphate (SDS) 1.1%. The mixture was incubated in a boiling water bath (90<sup>0</sup>C) for 60 min, cooled and centrifuged at 3000rpm. One milliliter (1ml) of the pink coloured supernant was read at 532 nm on a spectrophotometer. Ascorbic acid was used as reference standard was treated the same way as the extract.

*In vitro* inhibition of lipid peroxidation = [(Abs control – Abs sample)/Abs control] X 100

#### *Determination of Total Phenolic Content*

The total phenolic content of the extract was determined according to the method described<sup>25</sup>. In this assay, the phenolic group present in plant extract interacts with Folin – Ciocalteu in alkaline medium using Na<sub>2</sub>CO<sub>3</sub> solution giving a blue colour, which has maximum absorption at 685nm and correlates with total phenolic content. 0.1ml of aqueous extract (1mg/ml) of the plant was rapidly mixed with 0.1ml of FolinCiocalteu reagent, followed by the addition of 0.3ml sodium carbonate (15%, w/v) solution. The mixture was incubated in the dark for 30mins. The absorbance of the blue colour was read at 760 nm after 30mins on a spectrophotometer. The total phenolic content was extrapolated from a standard

curve using tannic acid (graded doses, 50 – 250ug/ml) as a standard phenol.

#### *Determination of Total Flavonoid*

Total flavonoid of the extracts was determined using the method described by Kumaran and Karunakaran<sup>26</sup> with slight modification. Briefly, 0.5 ml of extract solution (1 mg/ml) and standard (quercetin) at different concentrations (12.5 - 100µg/ml) were taken in test tubes. 3.0 ml of methanol followed by 0.1 ml of 10% aluminum chloride solution was added into the test tubes. Two hundred ml of 1M potassium acetate solution was added to the mixtures in the test tubes. Furthermore, each reaction test tube was then immediately diluted with 2.8 ml of distilled water and mixed to incubate for 30 min at room temperature to complete reaction. The absorbance of pink colored solution was noted at 415 nm using a spectrophotometer against blank methanol. TFC of the extract was expressed as quercetin equivalents (QE) after calculation using the following equation:

C= (cV)/m, where, C =total flavonoid contents, mg/g plant extract in QE, c = concentration of quercetin obtained from calibration curve (mg/ml), V =the volume of the sample solution (ml), m= weight of the sample (g). All tests were conducted in triplicate.

#### *Total Antioxidant Activity*

The total antioxidant capacity of the extracts was determined with phosphomolybdenum using ascorbic acid as the standard. The assay was based on the reduction of Mo (VI) to Mo (v) by the extracts and the subsequent formation of a green phosphomolybdate (v) complex at acidic pH. 0.1ml of the extract (1000µg/ml) solution was combined with 3ml of reagent (0.6M sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95<sup>0</sup>C for 90min. After the samples had cooled to room temperature, absorbance of the aqueous solution of each was read at 695nm against blank in a spectrophotometer. The blank solution contained 3ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under the same conditions as the rest of the sample. The antioxidant capacity was expressed as the equivalent of ascorbic acid<sup>27</sup>.

#### *Anti-Hemolytic Assay*

Anti-hemolytic activity was assessed by following the spectrophotometric method<sup>28-30</sup>. From a normal healthy individual 5 ml of blood was taken and centrifuged at 1500 rpm for 3 min. The pellet of blood was washed three times in sterile phosphate buffer saline solution (pH 7.2). Obtained RBC pellets were washed twice and then Diluted to 20% cell suspension with PBS.

#### *Protocol*

500µl of RBC + 500 µl of 400µg/ml extract + 250 µl of HgCl<sub>2</sub> (5 µM) in a test tube was incubate at 37<sup>0</sup>C for 3hrs in a water bath. Thereafter, 2ml of PBS was added and centrifuge at 2000g for 10mins. The absorbance of the supernatant was read at 540nm. For control, the extract was replaced with PBS.

Calculation; % inhibition = {(A<sub>Control</sub> – A<sub>Test</sub>)/A<sub>Control</sub>} x 100

Tannic acid was used as reference standard and was treated the same way as that of extracts

#### Anti-Denaturation Activity

The method of Williams et al.<sup>31</sup> was employed for the anti-denaturation assay. A solution of 0.2% w/v of BSA was prepared in Tris buffer saline and pH was adjusted to 6.8 using glacial acetic acid. 400 µg/ml of the extract was prepared. 50 µl of this concentration was transferred to Eppendorf tube. 5 ml of 0.2 % W/V BSA was added to the above Eppendorf tube. The standard consist 10 µg/ml of Diclofenac Sodium in methanol with 5 ml 0.2% W/A BSA solution, Quercetin, Tannic acid. The test tubes were heated at 72°C for 5 minutes and then cooled for 10 minutes. The absorbance of these solutions was determined by using UV/Vis Double beam spectrophotometer (Elico SL -196) at a wave length of 660 nm. The % inhibition of precipitation (denaturation of the protein) was determined on a % basis relative to the control using the following formula.

% Inhibition of Denaturation = (Abs of control – Abs of extract)/Abs of control X 100

#### ABTS radical cation decolorization assay

ABTS also constitutes a relatively stable free radical, which decolorizes in its non-radical form. The spectrophotometric analysis of ABTS<sup>•+</sup> scavenging activity was determined according to the method of Re et al.<sup>32</sup>. In this method, an antioxidant was added to a pre-formed ABTS radical solution and after a fixed time period, the remaining ABTS<sup>•+</sup> was quantified spectrophotometrically at 734 nm<sup>33</sup>. ABTS<sup>•+</sup> was produced by reacting 2 mM ABTS in H<sub>2</sub>O with 2.45 mM potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), and allowing the mixture to stand in the dark at room temperature for 6 h before use. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Prior to the assay, the solution was diluted in phosphate buffer (pH 7.4) to give an absorbance at 734 nm of 0.700±0.02 in a 1 cm cuvette and equilibrated to 30°C, the temperature at which all the assays were performed. Then, 1 mL of ABTS<sup>•+</sup> solution was added to 3 mL of resveratrol solutions in ethanol at different

concentrations (10-30 µg/mL). The absorbance was recorded 30 min after mixing and the percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger. The extent of decolorization was calculated as percentage reduction of absorbance. For the preparation of a standard curve, different concentrations of ABTS<sup>•+</sup> (0.033–0.33 mM) were used. ABTS<sup>•+</sup> concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r 2:0.9899):

Absorbance ( $\lambda_{734}$ ) = 2:5905 × ½ABTS<sup>•+</sup>

The scavenging capability of test compounds was calculated using the following equation:

ABTS<sup>•+</sup> Scavenging effect (%) = [1 - (As = Ac)] × 100

where A<sub>c</sub> is absorbance of a control lacking any radical scavenger and A<sub>s</sub> is absorbance of the remaining ABTS<sup>•+</sup> in the presence of a scavenger<sup>17</sup>.

## RESULTS AND DISCUSSION

### HPLC analysis

HPLC fingerprinting of *Kigelia africana* (leaves and fruits) methanolic extract revealed the presence of the gallic acid (t<sub>R</sub> = 10.15 min; peak 1), catechin (t<sub>R</sub> = 15.09 min; peak 2); chlorogenic acid (t<sub>R</sub> = 21.56 min; peak 3), caffeic acid (t<sub>R</sub> = 25.73 min; peak 4), ellagic acid (t<sub>R</sub> = 29.88 min; peak 5), rosmarinic acid (t<sub>R</sub> = 35.12 min; peak 6), rutin (t<sub>R</sub> = 39.73 min; peak 7), isoquercitrin (t<sub>R</sub> = 44.91 min; peak 8), quercetin (t<sub>R</sub> = 51.26 min; peak 9) and kaempferol (t<sub>R</sub> = 57.04 min; peak 10) (Fig. 1 and Table 1). The HPLC analysis revealed that flavonoids (quercetin, isoquercitrin, rutin and kaempferol), tannins (catechin) and phenolics acids (gallic, chlorogenic, ellagic, rosmarinic and caffeic acids) are present in the extract of *Kigelia Africana*.

### HPLC analysis for Carotenoids

#### Calibration curve

Tocopherol was: Y = 34721x + 1046.9 (r = 0.9999); t<sub>R</sub> 13.81 min. LOD = 0.023 µg/mL and LOQ = 0.075 µg/mL.

β-carotene: Y = 27732x + 1156.8 (r = 0.9988); t<sub>R</sub> 23.59 min. LOD = 0.031 µg/mL and LOQ = 0.099 µg/mL.

Lycopene: Y = 31058x + 1527.1 (r = 0.9999); t<sub>R</sub> 29.14 min. LOD = 0.028 µg/mL and LOQ = 0.091 µg/mL.

Table 1: Composition of *Kigelia africana* methanolic extract.

Compounds	leaves	fruits	LOD	LOQ
	mg/g	mg/g		
Gallic acid	0.97 ± 0.02 a	0.29 ± 0.01 a	0.024	0.079
Catechin	0.34 ± 0.01 b	1.23 ± 0.01 b	0.019	0.063
Chlorogenic acid	1.29 ± 0.01 c	6.59 ± 0.01 c	0.007	0.023
Caffeic acid	1.45 ± 0.03 d	2.08 ± 0.03 d	0.035	0.115
Ellagic acid	2.87 ± 0.01 e	3.35 ± 0.01 e	0.042	0.138
Rosmarinic acid	4.96 ± 0.01 f	1.28 ± 0.02 b	0.021	0.070
Rutin	1.71 ± 0.02 g	1.25 ± 0.01 b	0.028	0.091
Isoquercitrin	1.25 ± 0.03 c	1.67 ± 0.02 f	0.010	0.032
Quercetin	2.76 ± 0.01 e	3.38 ± 0.01 e	0.015	0.049
Kaempferol	4.69 ± 0.01 f	2.93 ± 0.01 g	0.013	0.042

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at p < 0.05.

Table 2: Carotenoids compositions.

Carotenoids	<i>K. Africana</i>	<i>K. africana</i> (leaves)
	(fruits)	
	mg/g	mg/g
Tocopherol	1.93 ± 0.01 a	2.45 ± 0.01 a
β-Carotene	1.75 ± 0.01 b	1.79 ± 0.03 b
Lycopene	0.52 ± 0.01 c	-

Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at  $p < 0.05$ .

Table 3: Phytochemicals in methanol fruit and leaf extracts of *Kigelia Africana*.

Constituents	Result in	Result in
	Fruit	Leaf
Saponins	+	+
Tannins	+	+
Flavonoids	+	+
Alkaloids	-	+
Phlobatannins	-	-
Steroids	-	+
Isoprenoids/Terpenoids	+	+
Anthraquinones	-	-
Cardiac glycosides	+	+

+ = present, - = absent

(Figure 2, table 2). Table 3 reveals the phytochemicals in methanol fruit and leaf extracts of *Kigelia africana*. Alkaloid and Steroids are conspicuously absent in the fruit of the studied plant. Phytochemicals are known to support bioactive activities in medicinal plants and may therefore be responsible for the antioxidant activities of the fruit and leaf of *Kigelia africana*. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity<sup>34</sup>. Alkaloids have been documented to possess analgesic, antispasmodic and bactericidal effects. Tannins hasten the healing of wounds and inflamed mucous membrane<sup>35</sup>. Tannins are generally known to be useful in the treatment of inflamed or ulcerated tissues and have remarkable activity in cancer prevention<sup>36</sup>. Flavonoids are potent water-soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity<sup>37,38,35</sup>. Flavonoids also lower the risk of heart diseases. Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. Cardiac steroids are widely used in the treatment of congestive heart failure. They help in increasing the force of contraction of the heart (positive inotropic activity) in heart failure patients. Thus, the presence of these constituents in *Kigelia africana* supports the common traditional use of the plant in the treatment of various diseases. The total antioxidant capacity (TAC) of the fruit and leaf extracts was found to be 299.62 mM and 296.68 mM AAE per mg respectively. This points out that the fruit and leaf of *Kigelia africana* are good source of natural antioxidants (Table 4). The fruit extract contains 180.77 µg TAE per mg of the total soluble phenolic content, which is far lower than 598.72 µg

TAE/mg determined in the leave extract (Table 4). Total flavonoid content was determined and expressed as microgram quercetin equivalents (QE). Also, the total flavonoid content of the fruit extract is 9055.5 µg/mg QE; this is less than the flavonoid content 23222.2 µg/mg QE of the leaf extract (Table 4). This suggests that the leaves of *Kigelia africana* possess higher antioxidant activities. The presence of flavonoids and tannins in the leaf and fruit of *Kigelia africana* is likely to be responsible for the free radical scavenging effects. Flavonoids serve as health promoting compound as a results of its anion radicals<sup>39</sup>. Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers. Flavonoids have been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2, and this property may possibly explain the mechanisms of antioxidative action of *Kigelia africana* fruit and leaf extracts. The reducing power of a compound is related to its electron transfer ability and may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow color of the test solution changes to green and blue depending on the reducing power of test specimen. Greater absorbance at 700 nm indicated greater reducing power. Figure 3 presents the reductive capabilities of the extracts. In the concentration range investigated, all the extracts demonstrated reducing power that increased linearly with concentration, it also demonstrated that the extracts are more potent than the quercetin which served as the standard. The reducing power of the extracts might be due to their hydrogen-donating ability. Possibly, *Kigelia africana* fruits and leaves contain high amounts of reduction, which could react with radicals to stabilize and terminate radical chain reactions. Further study is advised in this respect. The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage in adjacent biomolecules<sup>3</sup>. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins<sup>40</sup>. In the present study, figure 4 shows that the hydroxyl radical-scavenging effect of the extracts was found to be increasing with increase in concentration. The IC50 values of the fruit extract, leaf extract and mannitol were found to be 245.15µg/ml, 268.60µg/ml and 211.45µg/ml respectively. In terms of hydroxyl radical scavenging ability, the IC50 values show that mannitol (standard) is the most potent, follow by *Kigelia africana* fruits and then the leaves. Hence, the fruit and leaf extracts can be considered as a good scavenger of hydroxyl radicals. Being a stable free radical, DPPH is frequently used to determine radical scavenging activity of natural compounds. In its radical form, DPPH absorbs at 517 nm, but upon reduction with an antioxidant, its absorption decreases due to the formation of its non-radical form, DPPH-H. Thus, the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution. Figure 5 shows the DPPD radical scavenging activities of the extracts. The radical

Table 4: Total phenols, flavonoid contents and total antioxidant capacity of *Kigelia africana*<sup>S</sup>.

<i>Kigelia africana</i>	Total Phenols ( $\mu\text{g TAE/mg}$ ) <sup>A</sup>	Total Flavonoids ( $\mu\text{g QE/mg}$ ) <sup>B</sup>	Total Antioxidant Capacity (mM AAE /mg) <sup>C</sup>
Fruit	180.7692	9055.5	299.6215
Leaves	598.7192	23222.2	296.6826

<sup>S</sup>Values are expressed as mean  $\pm$  SD (n =3); <sup>A</sup>TAE - Tannic acid equivalents; <sup>B</sup>QE-Quercetin equivalents; <sup>C</sup>AAE – Ascorbic acid equivalents

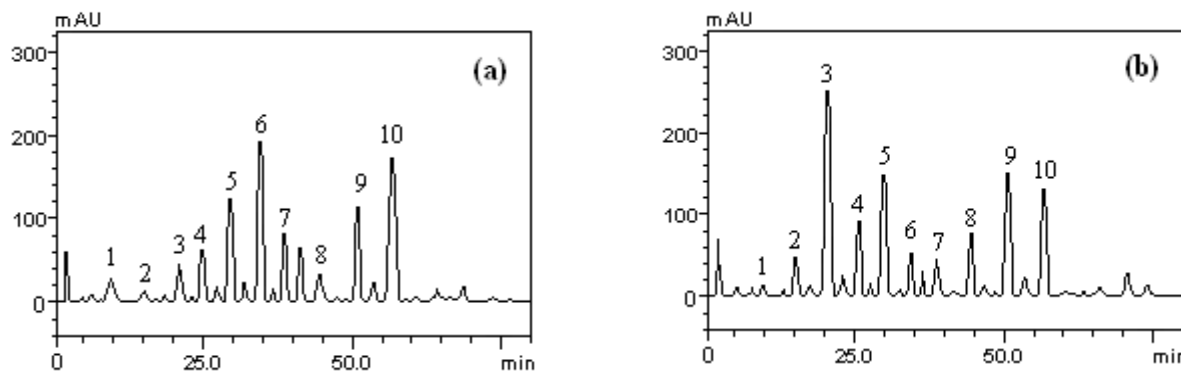
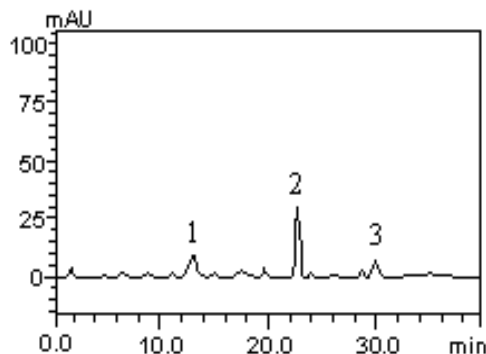


Figure 1: Representative high performance liquid chromatography profile of *Kigelia africana* leaves (a) and fruits (b), detection UV was at 327nm. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), ellagic acid (peak 5), rosmarinic acid (peak 6), rutin (peak 7), isoquercitrin (peak 8), quercetin (peak 9) and kaempferol (peak 10).

*Kigelia africana* (leaves)



*Kigelia africana* (fruits)

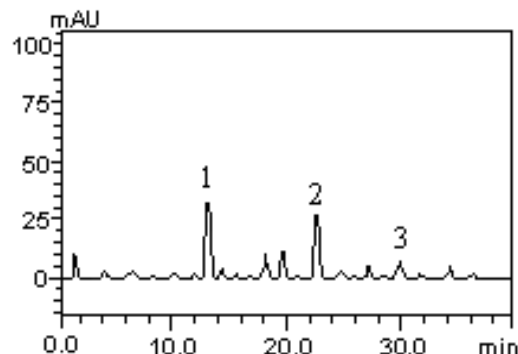


Figure 2: High performance liquid chromatography plants carotenoids profile. Tocopherol (peak 1),  $\beta$ -carotene (peak 2) and lycopene (peak 3), detection UV was at 450 nm.

scavenging activity of the extracts increased with increasing concentrations. The IC<sub>50</sub> values of the fruit and leaves extracts of *Kigelia africana* are 321.27 $\mu\text{g/mg}$  and 160.47 $\mu\text{g/mg}$  respectively as compared to 126.29 $\mu\text{g/mg}$  of tannic acid (standard). The obtained results, in terms of free radical scavenging ability, compared the activities of the fruit and leaf extracts to tannic acid standard. This indicated that the fruit and leaf of *Kigelia africana* exhibited the ability to quench the DPPH radical, which indicated that the extracts were good antioxidant with radical scavenging activity; leaf extract being more potent. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanisms, since it reduces the concentration of the catalyzing transition metal in LPO<sup>41</sup>. The chelating effects of the extracts on ferrous ions increased with

increasing concentrations (Figure 6). At concentration of 400 $\mu\text{g/ml}$ , the fruit and leaf extracts exhibited chelating effects of 59.27% and 52.87% respectively. The obtained results, in terms of metal ion chelating effect, compared the activities of both extracts and EDTA standard and showed them to be good source of metal radical chelating agent. Thus, this study suggests that the fruits of *Kigelia africana* exhibit better chelating activity on ferrous ions than the leaf. Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, and inhibition of platelet aggregation and regulation of cell mediated toxicity<sup>42</sup>. The extracts showed strong nitric oxide-scavenging activity. The percentage inhibition of nitric oxide generated by the fruit and leaf extracts was found to be 76.42  $\pm$  1.22 and 82.62  $\pm$  0.47 respectively at 400 $\mu\text{g/ml}$  (Figure 7). On the other hands, ascorbic acid at the same concentration showed 88.27  $\pm$  0.74 % inhibition

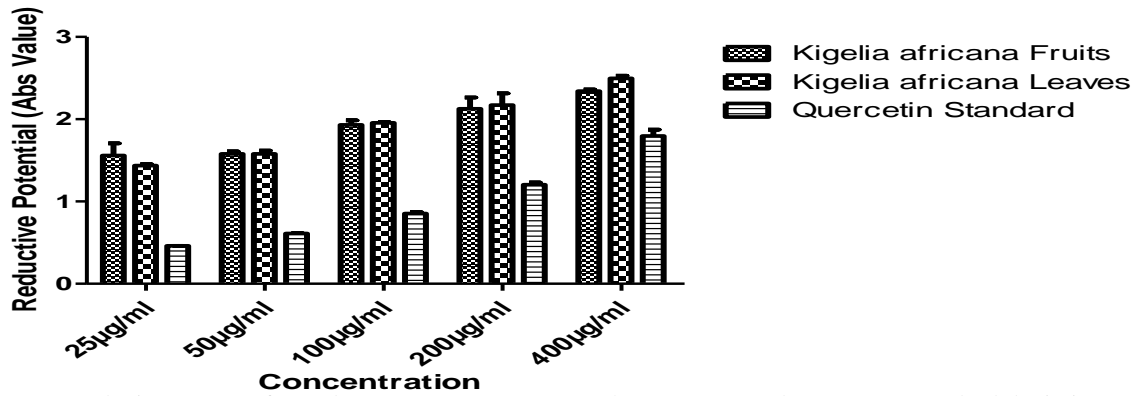


Figure 3: Reducing power of *Kigelia africana* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations\*.

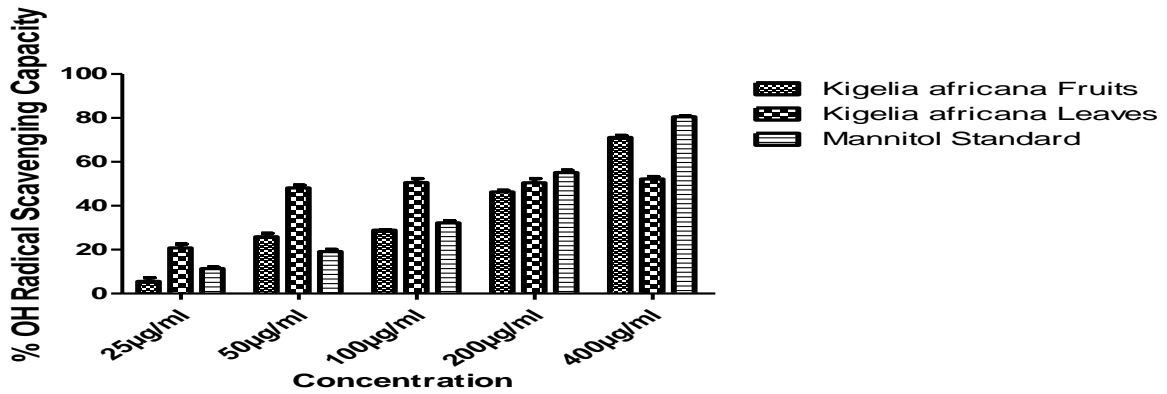


Figure 4: Hydroxyl radical scavenging activity of *Kigelia africana* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations\*.

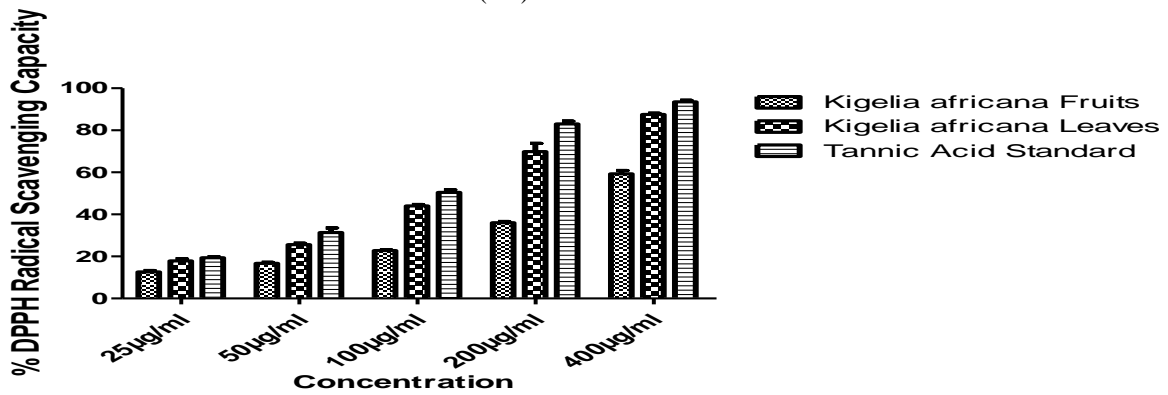


Figure 5: DPPH radical scavenging activity of *Kigelia africana* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations\*.

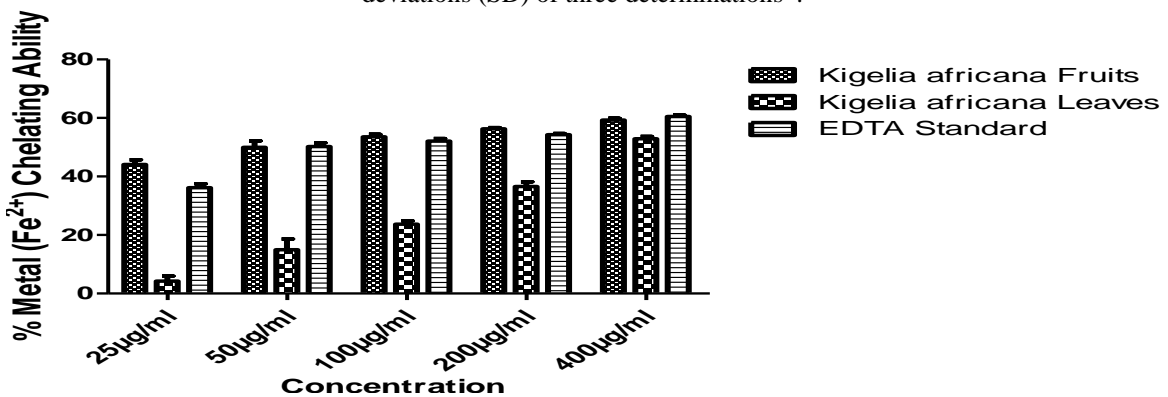


Figure 6: Chelating activity of *Kigelia africana* extracts. Results are expressed as mean ± standard deviations (SD) of three determinations\*.



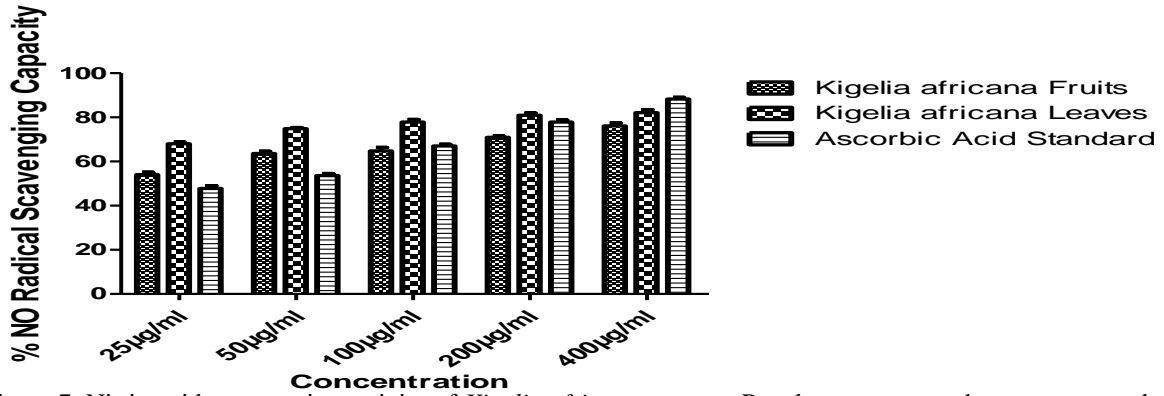


Figure 7: Nitric oxide scavenging activity of *Kigelia africana* extracts. Results are expressed as mean  $\pm$  standard deviations (SD) of three determinations\*.

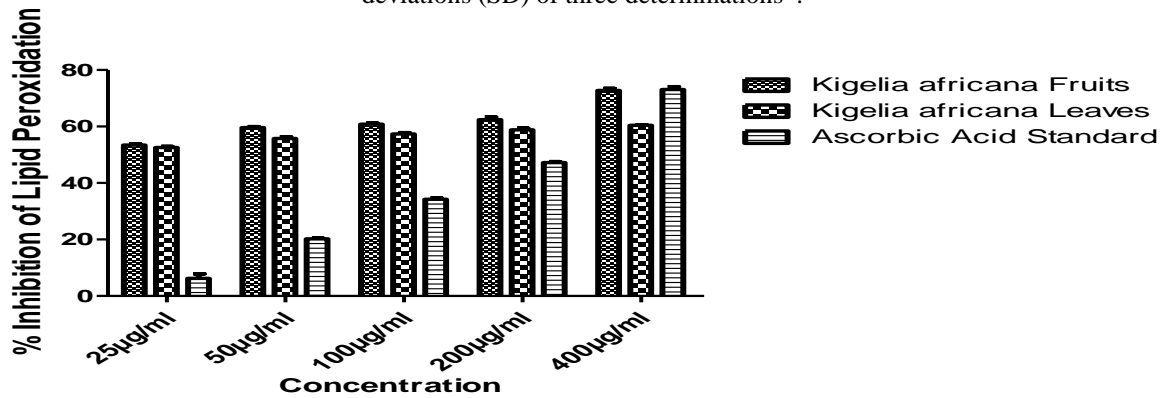


Figure 8: Inhibition of Lipid Peroxidation activity of *Kigelia africana* fruit and leaf. Results are expressed as mean  $\pm$  standard deviations (SD) of three determinations\*.

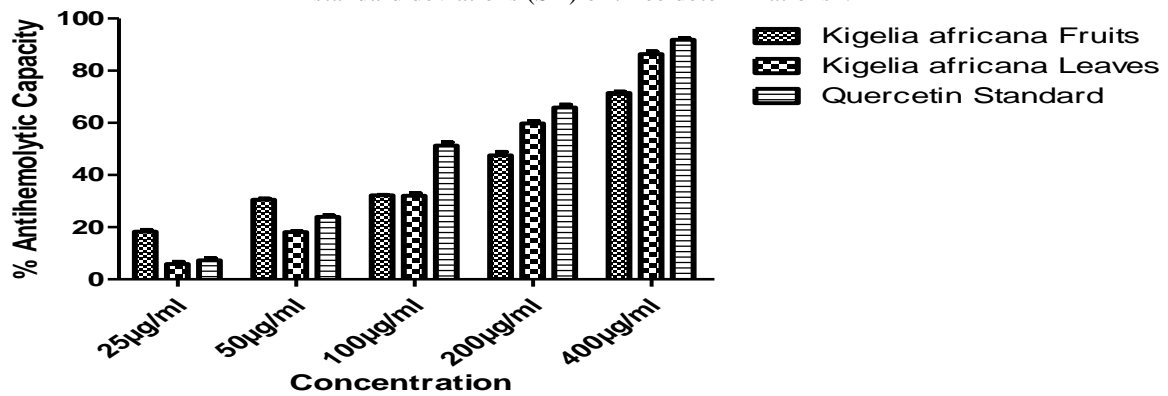


Figure 9: Antihemolytic activity of *Kigelia africana* extracts. Results are expressed as mean  $\pm$  standard deviations (SD) of three determinations\*.

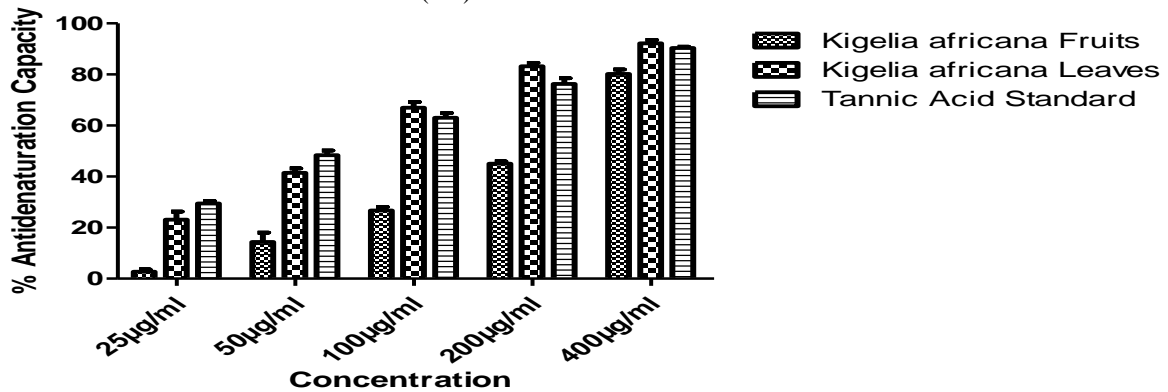


Figure 10: Antidenaturation activity of *Kigelia africana* extracts. Results are expressed as mean  $\pm$  standard deviations (SD) of three determinations\*.

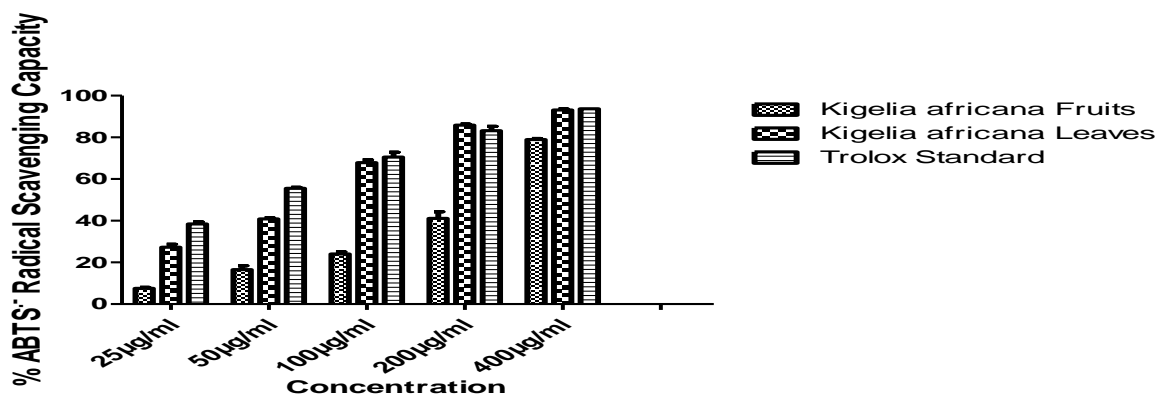


Figure 11: ABTS radical scavenging activity of *Kigelia africana* extracts. Results are expressed as mean  $\pm$  standard deviations (SD) of three determinations\*.

of nitric oxide. Hence, the extracts showed strong nitric oxide-scavenging activity when compared with the standard antioxidant, ascorbic acid. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like brain and liver. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage. Therefore, much attention has been focused on the use of natural antioxidants to inhibit lipid peroxidation and to protect from damage due to free radicals. In the present study, the methanol fruit and leaf extracts of *Kigelia africana* were investigated in comparison with the known antioxidant, ascorbic acid. The fruit extract of *Kigelia africana* was found to have higher lipid peroxidation inhibition than the leaf extract of *Kigelia africana* as shown in figure 8. The anti-hemolytic activities of *Kigelia africana* extracts, obtained in this study compared favorably with standard antioxidant (quercetin). Quite a number of extracts have been demonstrated to protect and stabilize red blood cells that were exposed to a combined hypotonic and hemolytic induced stress. The fruits of *Kigelia africana* exerted very high degree of hemolytic inhibition when compared with that of leaves and standard antioxidant, quercetin (Figure 9). The findings of this study supported the traditional used of the plant for treatment and management of inflammatory related disease as reported in literature. The extracts of *Kigelia africana* effectively inhibited the denaturation of Bovineserum albumin (BSA). The degree of inhibition of BSA denaturation increased with the decrease in the concentration of both the extracts as stated by Williams *et al.*, that the anti-denaturation of the drug will be more at lower concentration. As shown in figure 10, among the two extracts under study, the leaf extract demonstrated a better inhibition of BSA denaturation as compared to fruit extract, the leaf extract even showed more potency in this regard than the standard antioxidant, Tannic Acid. Figure 11 also described the percentage of ABTS<sup>-</sup> radical scavenged by the extracts, the leaf demonstrated a better activity than the fruit extract, and at higher concentrations, the leaf extract even competed favourably with the Trolox standard in scavenging this radical. This ability shows that the extract are undoubtedly excellent

free radical scavengers, which makes them fit in as good source for drug development.

## CONCLUSION

In the present study, the methanol fruit and leaf extracts of *Kigelia africana* were found to possess marked antioxidant and radical scavenging activities, as assayed through various *in-vitro* models; DPPH, Nitric Oxide radical, ABTS radical, OH radical, reducing power, chelating effect on ferrous ions, anti-denaturation activity, anti-hemolytic activity and total antioxidant activity. Comparing the antioxidant and radical scavenging activities of the extracts, it was found that both extracts are concentration dependent. In terms of free radical scavenging ability, the leaf extract has higher ability to quench DPPH radical, scavenge ABTS radical and inhibit NO radical. The leaf also has better reducing power and inhibition of BSA denaturation. On the other hands, the fruit has better ability to scavenge OH radical and chelate ferrous ions. The fruit also show better anti-hemolytic and anti-lipid peroxidation activities. Although, both extracts show equal total antioxidant capacity, the leaf of *Kigelia africana* contain more total phenolic and flavonoids than the fruit. Thus, from the data obtained in this study, it can be concluded that leaf of *Kigelia africana* is a more potent antioxidant than the fruit. It is recommended that this plant should be used as an additive in the food industry providing good protection against oxidative damage. Further studies are warranted for isolation and characterization of the active components of this promising plant species as well as *in vivo* evaluation of toxicity of these active components in animals' studies.

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