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ORIGINAL ARTICLE

Antioxidants from stem bark of *Kigelia africana* inhibits free radicals and membrane lipid damage in rat testes *in vitro*

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ABSTRACT

Objective: Bioactive ingredients of plants have been used as a central source of medicine and chemo-therapies for centuries. Nevertheless, the quantification and characterization of phytochemicals in methanol extract stem bark of *Kigelia africana* (MESBKA) will be highly advantageous if found affirmative.

Methods: MESBKA was taken for gas chromatography-mass spectrometry (GC-MS) analysis. Thereafter, its effect on pro-oxidant induced hydroxyl radical and lipid peroxidation in rat's testes was evaluated.

Results: The GC-MS analysis provided four peaks of eighteen different active compounds. Also, MESBKA contains antioxidants (polyunsaturated fatty acids, esters and poly alcohols) which are prophylactics of hydroxyl radicals and inhibitors of cyclooxygenase-2 (COX-2) in the testes. Furthermore, MESBKA inhibited pro-oxidants induced hydroxyl radicals and lipid peroxidation in rat's testes in a dose-dependent manner.

Conclusion: The antioxidant content of *Kigelia africana* could have prevented inflammatory responses to enhance male fertility in rat. Isolation and translation of individual components of the plant would help to find new drugs to cure and/or prevent male infertility.

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Key Words: Bioactive compounds, GC-MS, *Kigelia Africana,* male infertility, pro-oxidant

INTRODUCTION

Notably, medicinal plants had been served as herbal remedies for centuries to prevent and cure several pathologies. World Health Organisation (WHO) estimated that about 90% of the world's population consumes traditional herbal medicines [1]. Also, more than 50% of all modern clinical drugs come from natural product [2]. Interestingly, the isolation and identification of secondary metabolites produced by plants and their use as active principles had considerably gained the attention of many researchers [3]. Reports had indicated that most plants have unlimited capability to synthesize aromatic secondary metabolites, which are phenols or oxygen-substituted derivatives [4, 5]. These metabolites possess active biological performances and several applications such as antioxidants, anti-microbial pharmaceuticals, insecticides, flavours, dyes and fragrances [6, 7].

Lipid peroxidation primarily occurs through a free radical chain reaction. Oxygen is the most important factor on the development of lipid peroxidation of tissues resulting in many pathological processes. This leads to abnormal reproductive functions particularly male infertility and cancer [8]. Hypothetically, ground state oxygen does not have strong enough reactivity, but can be converted to reactive oxygen species (ROS). Free radicals and testicular lipid peroxidation had been recently proposed as the key causes of male infertility in mammals [9].

Kigelia africana (Lam) Benth (sausage plant), belonging to the family *Bignoniaceae*, is abundant in the tropics and widely used in West Africa as a herbal remedy for various ailments such as diarrhea, malaria, rheumatism, retained placenta and dizziness. Reports had also shown that the fruits, roots and leaves of *K.africana* could locally treat sexual complaints such as poor libido, sexual asthenia and impotence [10, 11]. In addition, ointment prepared from this plant had been utilized as disinfectant and curative agent for dermatitis [12, 13]. Although, the plant is employed in the development of growth and for dressing sores and wounds in humans; few investigations were done on the constituents of the stem bark of *K.africana*. Moreover, the possible mechanism on its inhibitory effect on pro-oxidant induced hydroxyl radical and lipid peroxidation has not been properly elucidated. Thus, the quantification and characterization of phytochemicals in methanol extract stem bark of *K.africana* (MESBKA) will be highly advantageous for men that are faced with problems of fertility if found affirmative.

MATERIAL AND METHODS

Sample selection

Fresh sample of stem bark of *Kigelia africana* was purchased from the local market, in Malete metropolis, Kwara State, Nigeria. Authentication of the plant was carried out in the Department of Biology, Obafemi Awolowo University Ile-Ife, Nigeria. Adult male Wistar rats were purchased from the Biochemistry Department animal colony, University of Ilorin, Nigeria and maintained *ad libitum* on commercial diet and water.

Chemicals and reagents

Chemicals and reagents used such as thiobarbituric acid (TBA), DPPH ((1,1-diphenyl-2-picrylhydrazyl)), deoxyribose were procured from Sigma-Aldrich (Steinheim, Germany), acetic acid was sourced from BDH Chemicals (Poole, England), Tris-HCl buffer, sodium dodecyl sulphate, H_2O_2•$FeSO_4$, $NaHCO_3$, Na_2HPO_4, NaH_2PO_4, $K_2S_2O_8$ and all other reagents were of analytical grade while the water was glass distilled.

Stem bark extraction and preparation

The bark of the stem was thoroughly washed in distilled water to remove any contaminant, chopped into small pieces before being milled. The methanol extract of the stem was subsequently prepared by soaking the grinded sample (10 g) in methanol (200 ml) for about 24 h at 37°C; the mixture was filtered and the filtrate was concentrated by rotator evaporator and stored in the refrigerator for subsequent analysis.

Determination of key antioxidants in MESBKA

The total phenol content of MESBKA was determined according to the Folin-Ciocalteu method [14]. Total flavonoid was assessed as reported by Kale *et al* [15]. Determination of tannin content was done using the method of Padmaja [16]. Total saponin content of MESBKA was determined by the method of Hiai *et al* [17], modified by Makkar *et al* [18]. Vitamin C level of MESBKA was estimated using the method of Benderitter *et al* [19].

Gas chromatography-mass spectrum (GC-MS) analysis

The crude extract was subjected to column chromatography over silica-gel (100-200 mesh) and eluted with methanol. The methanol fraction of stem bark of *K.africana* was taken for GC-MS analysis. GC-MS analysis was carried out on a Clarus 500 GC system (Perkin Elmer, Shelton, CT, USA) comprising an auto-sampler and gas chromatography (GC) interfaced to a mass spectrophotometer (MS) instrument employing the following conditions: Elite-1 GC column fused silica capillary column (30 x 0.25 mm x ID 1 EM df, composed of 100% dimethyl polysiloxane), operating in electron impact mode at 70 eV; helium (99.9%) was used as a carrier gas at a constant flow of 1 ml/min and an injection of volume of 0.5 EI was employed (split ratio of 10:1 injector temperature 250°C); ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min) with an increase of 10°C/min, to 200°C then 5°C/min to 280°C, ending with a 9°C/min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da.

Identification of components

Interpretation on mass spectrum GC-MS was conducted using database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and the structure of the components of the test materials were ascertained.

Degradation of deoxyribose (Fenton's reaction)

The ability of the MESBKA to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the method of Halliwell *et al* [20]. Briefly, freshly prepared methanol extract (0-200µl) was added to a reaction mixture containing 120 µl of 20 mM deoxyribose, 400 µl of 0.1 M phosphate buffer (pH 6.9), 40 µl of 500 µm of $FeSO_4$, and the volume were made up to 800 µl with distilled water. The reaction mixture was incubated at 37°C for 30 min and the reaction was the stopped by the addition of 0.5 ml of 2.8% trichloroacetic acid (TCA). This was followed by the addition of 0.4 ml of 0.6% thiobarbituric acid (TBA) solution. The tubes were subsequently incubated in boiling water for 1 h. The absorbance was measured at 532 nm in a spectrophotometer. The decrease in absorbance of hydroxyl radical (OH•) on addition of test sample in relation to the control was used to calculate the percentage OH• following the equation:

$$\%\ OH\bullet = (A_{532\ control} - A_{532\ sample}) / A_{532\ control} \times 100$$

The IC_{50} was calculated from the % OH• *vs* extract concentration non-linear regression curve.

Determination of free radical scavenging ability

The free radical scavenging ability of the MESBKA against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated as described by Ursini *et al* [21]. Briefly, appropriate dilution of the extracts (1 ml) was mixed with 1 ml of 0.4 mM methanolic solution containing DPPH radicals. The mixture was kept in the dark for 30 min and the absorbance was taken at 516 nm. The decrease in absorbance of DPPH on addition of test samples in relation to the control was used to calculate the percentage scavenging ability, following the equation:

$$\%\ scavenging\ ability = (A_{516\ control} - A_{516\ sample}) / A_{516\ control} \times 100$$

The IC_{50} was calculated from the % scavenging ability *vs* extract concentration non-linear regression curve.

Animals

Healthy adult male Wistar rats (n = 2), weighing approx. 150-200 g, were obtained from the Department of Biochemistry, Federal University of Ilorin, Nigeria.

All the animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health (USA). Also, the animals were used according to the institutional guidelines of Nigeria Academy which are in accordance with international guidelines.

Lipid peroxidation and thiobarbituric acid reactions

Animals were decapitated under mild diethyl ether anesthesia and the testes were rapidly isolated and placed on ice and weighed. Each of the tissues was subsequently homogenized in cold saline (1/5 w/v) with about 10-up-and-down strokes at approx. 1200 rpm in a glass teflon homogenizer. The homogenate was centrifuged for 10 min at 3000g to yield a pellet that was discarded and a low-speed supernatant (SI) was kept for the assay.

The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al* [22]. In brief, 100 μl SI fraction was mixed with a reaction mixture containing 30 μl of 0.1 M pH 7.4 Tris-HCl buffers, extract (0-100 μl) and 30 μl of 70 μM freshly prepared sodium nitroprusside. The volume was made up to 300 μl with water before incubation at 37°C for 1 h.The color reaction was developed by adding 300 μl 8.1% sodium dodecyl sulphate (SDS) to the reaction mixture containing SI. This was subsequently followed by the addition of 600 μl of acetic acid/HCl (pH 3.4) mixture and 600 μl 0.8% TBA.This mixture was incubated at 100°C for 1 h. Thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm and the absorbance was compared with that of standard curve using malondialdehyde (MDA). The decrease in absorbance of lipid peroxidation on addition of test sample in relation to the control was used to calculate the percentage MDA following the equation:

$$\% \text{ MDA} = (A_{532\ \text{control}} - A_{532\ \text{sample}}) / A_{532\ \text{control}} \times 100$$

The IC_{50} was calculated from the % MDA *vs* extract concentration non-linear regression curve.

Statistical analysis

The results of the replicates were pooled and expressed as mean ± standard deviation. A one-way analysis of variance (ANOVA) was used to analyze the results and Duncan multiple tests was used for post hoc evaluation [23]. The least significance difference (LSD) was accepted at $P<0.05$.

RESULTS

In order to ascertain the active compounds responsible for the biological activities of the *Kigelia africana* methanol extract *in vitro*, the methanol extract was quantified for various phytochemicals. The result, as presented in Table 1, revealed some important antioxidants such as phenols, saponin, tannin, flavonoids and ascorbic acid. Also, as shown by Figure 1, GC-MS chromatogram provided four peaks of eighteen different active compounds.

Table 1. Quantitative characterization of phytochemicals in methanol extract of *Kigelia africana*

Phytochemical	*Kigelia africana* extract (mg/100 g)
Total phenols	2818 ± 21.21
Tannins	4004 ± 8.49
Saponins	1854.5 ± 24.75
Ascorbic acids	297 ± 8.49
Flavonoids	191

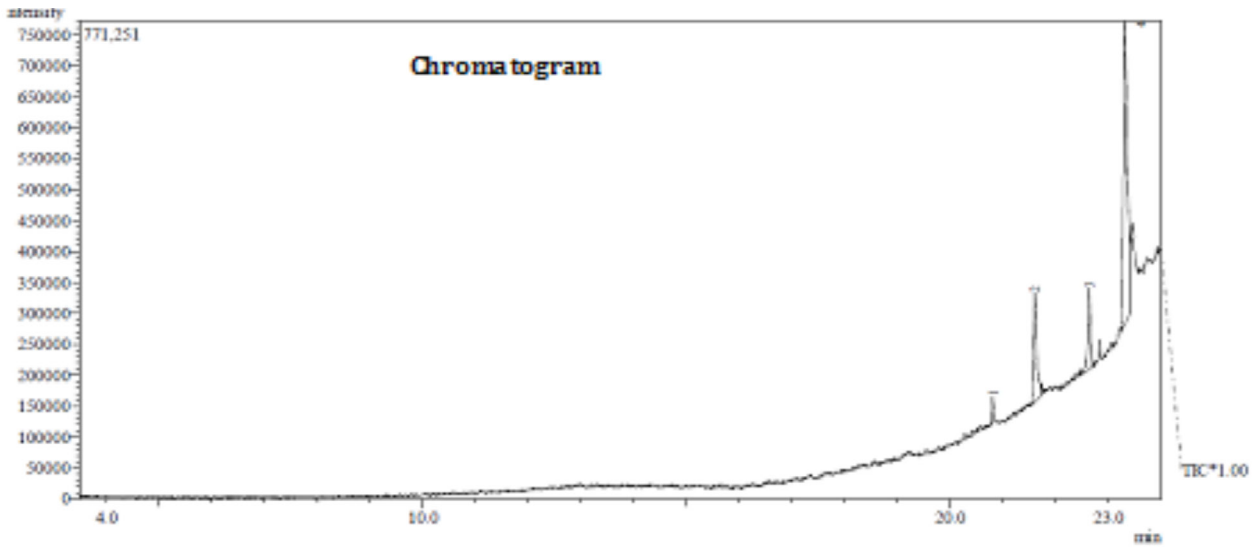

Figure1. GC-MS chromatogram of methanolic extract of *Kigelia africana*

The retention indices, percentage composition, chemical structures and the biological activities of the compounds that were identified in MESBKA are given in Table 2. The compound prediction is based on the phytochemical and ethnobotanical databases of the National Research Institute for Chemical Technology (NARICT). Briefly, the results showed the presence of octanoic acid methyl ester (2.62%), hexadecanoic acid-15-ethyl-methyl ester (2.62%), tridecanoic acid methyl ester (2.62%), pentadecanoic acid, 14-methyl-, methyl ester (2.62%), n-hexanedecanoic acid (19.46%), nonanoic acid (19.46%), octadecanoic acid (19.46%), tetradecanoic acid (19.46%), 13-docosenoic acid methyl ester (11.18%), 11-octadecenoic acid methyl ester (11.18%), cyclopropane pentanoic acid 2-undecyl-methyl ester (11.18%), cyclopropane dodecanoic acid 2-octyl-methyl ester (11.18%), 15-tetracosenoic acid methyl ester (11.18%), 9-octadecenoic acid (66.74%), E-2-octadecadecen-1-ol (66.74%), 9-octadecenal (66.74%), (Z)-10-pentadecen-1-ol (66.74%) and E-9-tetradecenoic acid (66.74%). As observed in the present study, the spectrum profile of GC-MS confirmed the presence of 4 peaks which contain 18 components with retention times of 20.8, 21.6, 22.63 and 23.3 min; peak 1 and 2 contain 4 components while peak 3 and 4 contain 5 components.

The treatment with MESBKA significantly ($P < 0.05$) repressed the hydroxyl radical (OH•) production in a dose-dependent manner against the corresponding control (Figure 3). Also, in the presence of deoxyribose (substrate) and hydrogen peroxide (reacting oxygen species), MESBKA significantly ($P < 0.05$) depleted the OH• production by 71.85, 78.46 and 85.08%, respectively, when compared with the control (Figure 3). Similarly, in the absence of deoxyribose but with the presence of hydrogen peroxide, MESBKA significantly ($P < 0.05$) reduced the OH• production by 39.22, 45.83% and 52.755%, respectively, when compared with the control (Figure 3). The % inhibition or prophylactic effect of MESBKA on OH• was considerably higher in the presence of deoxyribose (IC_{50} = 165 μg/ml) than without deoxyribose (IC_{50} = 30 μg/ml) as shown in Figure 3.

Moreover, the ability of MESBKA to scavenge DPPH stable radical is presented in Figure 4. It was discovered that MESBKA significantly ($P < 0.05$) scavenged DPPH stable radical (IC_{50} = 175 μg/ml) by 16.75, 27.57, 38.28 and 53.2%, respectively, when compared with control. Also, MESBKA treated with rat testes significantly ($P < 0.05$) decreased lipid peroxidation (IC_{50} = 60 μg/ml) in a dose-dependent manner (Figure 5) by 10.78, 46.35, 73.18 and 79.72%, respectively, when compared with the control value.

Table 2. Identification of phyto-active components in methanolic extract of *Kigelia africana*

Compound name	Retention time	Molecular formula	Molecular weight	Peak	Activity
Octanoic acid methyl ester	20.8	$C_9H_{18}O_2$	158	2.62	Nematicide [24]
Hexadecanoic acid-15- ethyl-methyl ester	20.8	$C_{18}H_{36}O_2$	284	2.62	Antioxidant, hypocholesterolemic, hemolytic, 5-alpha reductase inhibitor [25]
Tridecanoic acid methyl ester	20.8	$C_{14}H_{28}O_2$	228	2.62	Nematicide, pesticide [24]
Pentadecanoic acid 14-methyl-methyl ester	20.8	$C_{17}H_{34}O_2$	270	2.62	Antioxidant, cancer preventive, nematicide, hypocholesterolemic, lubricant [26]
n-hexadecanoic acid	21.6	$C_{16}H_{32}O_2$	256	19.46	Antioxidant, nematicide, hypocholesterolemic, hemolytic, 5-alpha reductase inhibitor [26]
Nonanoic acid	21.6	$C_9H_{18}O_2$	158	19.46	Nematicide, pesticide [24]
Octadecanoic acid	21.6	$C_{18}H_{36}O_2$	284	19.46	Antioxidant, cancer preventive, nematicide, hypocholesterolemic, lubricant [27]
Tetradecanoic acid	21.6	$C_{14}H_{28}O_2$	228	19.46	Nematicide, pesticide [24]
13-Docosenoic acid methyl ester	22.63	$C_{23}H_{44}O_2$	352	11.18	Antioxidant [28]
11- Octadecenoic acid methyl ester	22.63	$C_{19}H_{36}O_2$	296	11.18	Antioxidant [28]
Cyclopropane-pentanoic acid-2-undecyl-methyl ester trans	22.63	$C_{20}H_{38}O_2$	310	11.18	Antiviral, antifungal, Anti-HIV, COX-2 inhibitor [29]
Cyclopropane-dodecanoic acid-2-octyl-methyl ester	22.63	$C_{24}H_{46}O_2$	366	11.18	Anticancer, antitumor, antiestrogenic, antimicrobaterial [29]
15-tetracosenoic-acid methyl ester	22.63	$C_{25}H_{48}O_2$	380	11.18	Antioxidant [28]
9-octadecenoic acid	23.3	$C_{18}H_{34}0_2$	282	66.74	Antioxidant [28]
E-2-Octadecadecen-1-ol	23.3	$C_{18}H_{36}O$	268	66.74	Antioxidant, flavor, perfumery [24]
9-Octadecenal	23.3	$C_{18}H_{34}O$	266	66.74	Fatty aldehyde, antioxidant, antimicrobial, anti-inflammatory [24]
Z-10-Pentadecen-1-ol	23.3	$C_{15}H_{30}O$	226	66.74	Antioxidant, antimicrobial [28]
E-9-Tetradecenoic acid	23.3	$C_{14}H_{26}O_2$	226	66.74	Antioxidant [28]

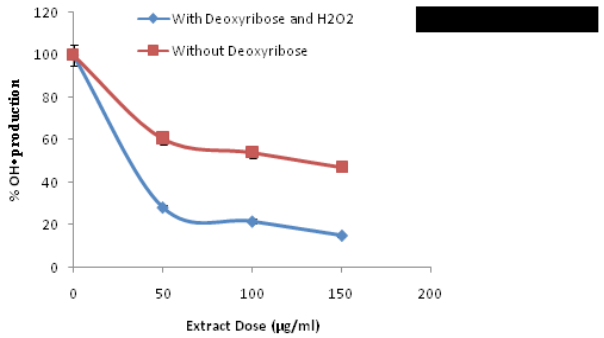

Figure 2. *Kigelia africana* extract (stem bark) scavenged hydroxyl radical OH• production in a dose-dependent manner: IC_{50} for deoxyribose and H_2O_2 =165 µg/ml; IC_{50} for H_2O_2 only =30 µg/ml. The percentage scavenging ability of OH• production was expressed with respect to basal that was taken as 100%.

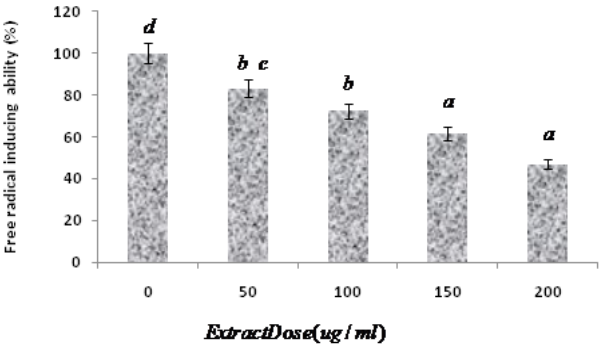

Figure 3. Methanol extract stem bark of *Kigelia africana* (MESBKA) scavenged stable free radical (DPPH) in a dose-dependent manner (IC_{50} = 175 µg/ml). Values with different letters are significantly different ($P < 0.05$).

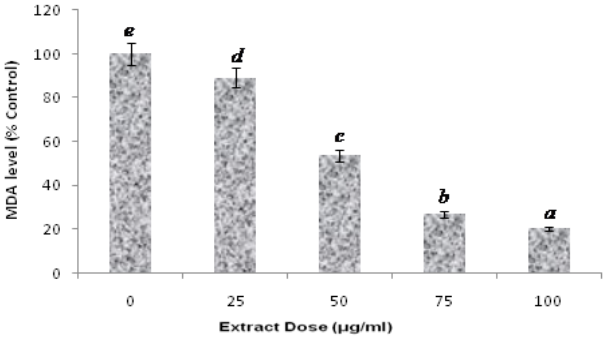

Figure 4. Methanol extracts of *Kigelia africana* inhibits mercury (pro-oxidant) induced lipid peroxidation dose-dependently in rat testes (IC_{50} = 60 µg/ml). The percentage inhibition of malondialdehyde (MDA) production was expressed with respect to basal testicular tissue (without extract and pro-oxidant) which was taken as 100%. Values with different letters are significantly different ($P < 0.05$).

DISCUSSION

As observed in the present study, GC-MS examination of MESBKA showed the presence of 18 compounds. In terms of percentage abundance; 9-octadecenoic acid, E-2-octadecadecen-1-ol, 9-octadecenal, Z-10-pentadecen-1-ol and E-9-tetradecenoic acid were predominant in the extract. These 5 major compounds have all shown to have anti-microbial activity, food flavor, antioxidant and anti-inflammatory potentials [30]. Also,

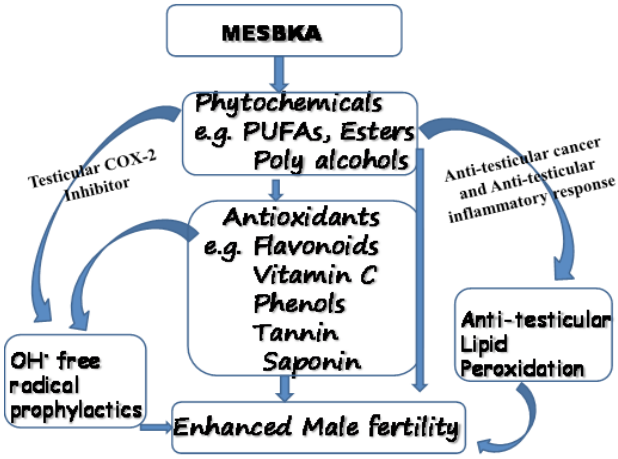

Figure 5. MESBKA contains antioxidants (PUFAs, esters and poly alcohols) which are prophylactics of hydroxyl radicals and inhibitors of cyclooxygenase-2 (COX-2) and lipid peroxidation in the testes. These antioxidants might prevent oncogenesis and inflammatory responses to enhanced male fertility in rat.

anticancer, hypo-cholesterolemic, lubricant activity and inhibitor of hemolytic-5-alpha reductase are shown by n-hexadecanoic acid and octadecanoic acid, while nonanoic acid and tetradecanoic acid showed pesticide and nematicide activity [31]. The esters of 2-octyl methyl cyclopropane dodecanoic acids and 2-undecyl methyl cyclopropane pentanoic acids, which are structurally similar to naturally occurring chrysanthemates, have been implicated as essential classes of agricultural insecticides [32, 33]. These two cyclopropane fatty acids (CPA) have been found to be antifungal, antiviral, anti-HIV, anticancer, antitumor, anti-mycobacteria, anti-estrogenic and cyclooxygenase-2 (COX-2) inhibitor [34, 35] while all other recognized compounds had been involved in prophylactics of free radicals (antioxidant).

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly, and inside the cell, H_2O_2 probably reacts with redox metals, to form the hydroxyl radical (OH•) [36]. As revealed from the present findings, MESBKA inhibited the OH• (Fe^{2+}/H_2O_2)-induced decomposition of deoxyribose in a dose-dependent manner. The inhibitory potential may be linked to the presence of natural antioxidants in the extract. It was discovered that the prophylactic capacity of MESBKA on OH• production was more considerably higher in the presence of deoxyribose (substrate) than without deoxyribose. The reason for this cannot be categorically stated. However, it is likely that the inhibitory complex formed between MESBKA phytochemicals and Fe-induced OH• in Fenton reaction is more active when deoxyribose is used as substrate.

The scavenging capacity of the extract on DPPH-induced stable radical may serve as a significant indicator of its potential antioxidant activity. As observed from the study, the extract scavenged the DPPH radical dose-dependently. The scavenging capacity may be linked to the phenolic compounds contained in the extract. However, earlier report had established that the antioxidant activity of the phenolics was principally due to

their redox properties. This allows them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and free radical abstractors [37]. The high scavenging power of the phenolics suggests that the purified compounds could provide a source of therapeutic antioxidants to health and wellness of humans. This result agrees with earlier reports on the antioxidant/radical scavenging ability of polyphenols from root plants where phenolic compounds had been reported to have higher free-radical scavenging ability [38].

Previous study suggested that active compounds with antioxidant activity have been implicated as therapies and treatment of reproductive dysfunctions and infertility in men [39], while lipid peroxidation had been implicated in testicular cellular membrane damage mediated by ROS. This generates several relatively stable end products, which includes aldehydes, particularly MDA. It proceeds via radical-mediated abstraction of hydrogen atoms from methylene carbons in poly unsaturated fatty acids [40, 41]. Also, spermatozoa have been considered to be highly susceptible to lipid peroxidation in the presence of pro-oxidants [42], due to the abundance of polyunsaturated fatty acids in their membrane [40, 43]. As observed from the study, the MESBKA reversed mercury-induced lipid peroxidation in testicular tissue homogenate in dose-dependent manner. The reversal effects of the extract could be attributed to its high antioxidant activity as reflected by its high free radical scavenging power. This suggests that MESBKA would increase sperm fertility/count (Figure 5), sperm motility, live spermatozoa and decrease in total sperm deformities. This hypothesis supported the previous investigation which stated that increase in lipid peroxidation products had been associated with abnormalities in sperm morphology [44]. Therefore, this *in vitro* experiment supports further investigation of MESBKA inhibition in cultured testicular cells.

In conclusion, methanol extract stem bark of *Kigelia africana* possesses a total of 18 phyto-compounds and could prevent pro-oxidants induced free radicals and lipid peroxidation. This comes to play through the antioxidant activity of its phytochemicals. However, intake of MESBKA could provide a rich source of therapeutic antioxidants to support health and wellness; particularly isolation and translation of individual components would help to find new drugs to cure and/or prevent male infertility. Further studies should also focus on specific underlying molecular mechanisms associated with improvement of spermatogenesis.

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