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Research Article

INVESTIGATION OF THE EFFECTS OF *KIGELIA AFRICANA* (LAM.) BENTH. EXTRACTS ON TM3 LEYDIG CELLS

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ABSTRACT

Objective: The study reports the effects of acetone, methanol, and aqueous *Kigelia africana* extracts on the TM3 Leydig cells.

Methods: The total phenolic contents were determined using Folin–Ciocalteu's method. Antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl assay and flavonoid content assessed using quercetin as standard. Cell viability and cytotoxicity were assessed.

Results: Total phenolic and flavonoid contents of the extracts were analyzed. Acetone extracts showed highest total phenolic content (105±3.65 mg gallic acid equivalent [GAE]/g) followed by methanol extracts (10.64±0.32 mg GAE/g) and, last, the water extract with the total phenolic content of 4.27±0.65 mg GAE/g. The same trend was observed with total flavonoid content where results recorded for acetone, methanol, and water extracts were 3.63 ±0.20 mg/g quercetin equivalent (QE), 0.16±0.01 mg QE/g, and 0.01±0.00 mg QE/g, respectively. The three extracts showed lower IC_{50} values compared to ascorbic acid (0.143±0.05 mg/ml, 0.023±0.004 mg/l, and 0.043±0.03 mg/ml for acetone, methanol, and water extracts, respectively). At 0.5 mg/ml of acetone extract, there was an increase in the testosterone production compared to the control.

Conclusion: The findings of this study showed that *K. africana* could be recommended for use in the treatment of male infertility.

Keywords: *Kigelia africana* (Lam.) Benth., Antioxidants, Cytotoxicity, TM3 Leydig cells testosterone.

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INTRODUCTION

Medicinal plants are used by millions of people worldwide for alleviation and treatment of various diseases and they have significantly contributed to the improvement of human health [1]. Approximately 80% of world population use traditional medicines for primary health care [2]. Preference of medicinal plants over synthetic medicines is due to the ease of access, low costs, and relatively less side effects. In many developing countries, the use of medicinal plants is profoundly practiced and lot of work is done to verify the efficiency and efficacy of medicinal plant extracts as claimed by traditional practitioners. Medicinal plants are believed to contain a wide range of antioxidants such as polyphenols that maintain the physiological levels of free radicals and subsequently protect tissues from detrimental effects of free radical [3]. The antioxidants present in plant products help in the stimulation of cellular defense system and biological system against oxidative damage [4]. In South Africa, medicinal plants are used to treat and manage various ailments such diabetes, hypertension, diarrhea, and male reproductive disorders [5,6]. Approximately 3000 plant species from more than 30,000 higher plant species are used in traditional medicines for the treatment of various conditions [7]. Traditional medicines are important sources of potentially useful new compounds for the development of chemotherapeutic agents [8].

Kigelia africana (Lam.) Benth. belongs to the *Bignoniaceae* family. The plant is commonly known as African sausage tree, liver sausage tree, or cucumber sausage tree (English), "umfongothi" (Zulu), "modukghulu" (North Sotho), and "muvevha" (Venda). The plant is widespread in Southern Africa, especially in Namibia, Botswana, South Africa, and Swaziland [9]. The different parts of *K. africana* are used traditionally for the treatment of various ailments. The seeds, fruits, stem bark, leaves, and roots are used for the treatment of pneumonia, malaria, diabetes, antifungal, eczema, and waist pain [10]. The leaves are also used to treat dysentery [11]. *K. africana* leaves are mixed with *Hypoxis hemerocallidea* corn, boiled and the decoction is used to treat syphilis and sores [12]. Furthermore, *K. africana* is used to treat wounds, dysentery, ulcer, rheumatism, syphilis, gonorrhea, and purgative [13]. The aim of the study was to determine the effect of *K. africana* (Lam.) Benth. extracts on the TM3 Leydig cells.

METHODS

Collection and preparation of plant materials

The leaves of *K. africana* (Lam.) Benth. were collected, in summer season from labeled trees of Lowveld National Botanical Garden of Mpumalanga Province, South Africa. The plant materials were dried in a cool darkroom temperature, pulverized into fine powder using laboratory electric grinder, and stored in the properly labeled polypropylene bags in a dark cool dry place until further use.

Plant material extraction

Acetone, methanol, and water were used as solvents for extractions. Three grams of the powdered leaves were weighed into 100 ml bottles followed by addition of 30 ml of acetone and methanol. The bottles were shaken for 30 min in the laboratory shaker and supernatants were decanted into centrifuge tubes. The tubes were centrifuged at 4.4 × g for 20 min and filtered through Whatman No. 1 filter paper into pre-weighed labeled glass vials. The bottles were left open under the stream of cold air in the fume cupboard for solvent evaporation. After drying, the vials were weighed again to determine the quantity of crude extracts processed.

The method of extraction with water was different from the acetone and methanol methods. Following addition of water, the concoctions were boiled on the hot plate, cooled, and filtered through Whatman No. 1 filter paper into 50 ml centrifuge tubes. The filtrates were kept overnight in the −80°C freezer and freeze-dried. After drying, the crude extracts were added into pre-weighed labeled vials to determine the quantity of crude extracts processed.

Determination of total phenolic content

The total phenolic contents were determined according to the Folin–Ciocalteu method described by Makkar [13] and modified by Adebayo *et al*. [14]. The crude extracts were prepared to the concentration of 1 mg/ ml, from which 25 µl were added in the test tubes. Extracts were treated with 250 µl Folin–Ciocalteu reagent for 5 min. After 5 min, the reaction was stopped by adding 750 µl 20% anhydrous carbonate. The resultant solution was made up to 5 ml by adding 3975 µl distilled water and incubated at the darkroom temperature for 2 h. Following incubation, 300 µl of solutions were transferred into 96-well plates and absorbance was read at 760 nm with a microplate reader. All the tests were conducted in triplicates. Gallic acid was used for the standard curve. Gallic acid in methanol was prepared to the concentrations (1, 0.5, 0.25, 0.125, 0.063, 0.032, 0.016, and 0.008 mg/ml) through serial dilution. The absorbance was read and absorbance values were plotted versus concentrations. The phenolic contents of each plant extracts were determined from the standard curve and expressed as gallic acid equivalent (GAE) per gram of dried plant extracts (GAE mg/g). The total phenolic contents of the plant extracts were calculated using the formula:

$$\text{Total phenolic content} = \text{GAE} \times V/m$$

Where, GAE is the concentration of gallic acid established from the calibration curve (mg/ml), V is the final volume of extract (ml), and m is the weight of pure plant extract (g) [15].

Determination of total flavonoid content

The total flavonoid content of the extracts was determined according to Yadav and Agarwal [16] and slightly modified by Miliauskas *et al.* [14]. Briefly, the crude extracts were prepared to the concentration of 1 mg/ ml, from which 100 µl were dissolved in 300 µl methanol, to which 20 µl 10% aluminum chloride and 20 µl of 1 M sodium acetate were added. The volume was made up to 1 ml with 560 µl distilled water. The resultant solutions were incubated at room temperature for 30 min and transferred into 96-well microplates. After 30 min incubation, the absorbance was read at 450 nm in the microplate reader. All the tests were conducted in triplicates. Quercetin was used for the standard curve. Different concentrations (2, 1, 0.500, 0.250, 0.125, 0.063, 0.032, 0.016, 0.008, and 0.004 mg/ml) of quercetin were prepared through serial dilution, read and absorbance values were plotted against the concentrations. The flavonoid content of each extract was determined from quercetin standard curve. The values were expressed as mg/g quercetin equivalent (QE). The total flavonoid contents of the plant extracts were calculated using the formula:

$$X = C.\ V/m$$

Where, X is the flavonoid content, mg/g is the plant extract in QE, C is the concentration of QE established from the calibration curve (mg/ml), V is the final volume of extract (ml), and m is the weight of the pure plant extract (g) [17].

Determination of antioxidant activity (AA) using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay

The scavenging activity of the selected plant species was determined using the DPPH assay as previously described by Mensor *et al*. [18] and modified by Hamzah *et al.* [19]. DPPH assay was done through serial dilution of plant extracts and addition of DPPH. The plant extracts were prepared at the concentration of 1 mg/ml. The assay was carried out in the 96-well plates. Initially, 100 µl of methanol were added into each well followed by 100 µl of each plant extracts into the first well. Plant extracts were serially diluted to the concentration of 0.500, 0.250, 0.125, 0.063, 0.032, 0.016, 0.008, and 0.004 mg/ml. DPPH solution (0.02 mg/ml in methanol) was prepared and 100 µl was added and plates were kept at darkroom temperature for 30 min. After incubation, the absorbance was read at 517 nm using microplate reader. Ascorbic acid was used as a positive control. DPPH in methanol served as negative control and methanol as blank. All the samples were carried out in triplicates. Percentage of AA% values was calculated using the formula:

$$AA\% = 1-(Abs_{sample}/Abs_{control})\times 100$$

Where, Abs_{sample} is the absorbance of the sample and $Abs_{control}$ is the absorbance of the negative control. The IC_{50} values defined as the concentration of sample required for 50% inhibition of DPPH radical were determined from the linear regression plots, AA% against the concentrations of the tested plant extracts (mg/ml).

Cell culture conditions

TM3 Leydig cell lines derived from mouse testes were obtained from the University of Western Cape cell culture laboratory. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (complete medium) at 37°C with 5% CO_2 in the incubator [20]. The cells were allowed to reach 80% confluence before passaged.

Passaging cells was carried out according to Hamzah *et al*. [19] with slight modifications. Briefly, the complete medium (DMEM with 10% FBS) was discarded, washed with PBS. Following this, 3 ml trypsin was added and incubated at 37°C with 5% CO_2 until cells detached, about 7 min. To stop trypsin, 7 ml of complete medium was added and then transferred to a 50 ml centrifuge tube to be centrifuged at 1 × g for 5 min at 37°C. Thereafter, the supernatant was removed, the cell pellet was resuspended in 3 ml complete medium and each 1.5 ml of the suspension was transferred into a new 75 cm^2 cell culture flasks added 20 ml of complete medium, written the name and date. Before kept in the incubator, the caps of the flasks were closed but not tight to allow respiration and incubated at 37°C with 5% CO_2.

Cell viability and cytotoxicity assessments

After the cells were treated with trypsin, centrifuged, resuspended in 3 ml fresh complete medium, they were then counted using hemocytometer [21]. The 3-(4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay was carried out according to the method described by Mosmann [22] with slightly modifications. The acetone and methanol crude extracts were reconstituted in acetone and water in DMSO at the concentrations of 100 mg/ml. Then, extracts were prepared to the final concentrations: 0.1, 0.25, 0.5, 0.75, and 1 mg/ml. TM3 Leydig cells (100 µl) were seeded at 5×10^3 cells per well of 96-well plates and treated with 100 µl various concentration of plant extracts (0.1, 0.25, 0.5, 0.75, and 1 mg/ml) and incubated at 37°C with 5% CO_2 incubator. Following 24 h exposure, the medium was carefully removed by aspiration and 25 µl MTT (5 mg/ml in PBS) was added to each well. The plates were then incubated for 2 h. After 2 h, the supernatants were carefully removed and 100 µl DMSO was added to each well to dissolve formazan blue crystals produced. Afterward, the plates were incubated at darkroom temperature for 30 min and absorbance read at 570 nm and 620 nm for reference. Non-treated cells and MTT were used as control and blank, respectively. The samples were tested in triplicates. The percentage cell viability was calculated using the following formula:

$$\%\ \text{cell viability} = \frac{\text{Mean Absorbance of treated cells}}{\text{Mean Absorbance of control}} \times 100$$

The percentage inhibitions were then calculated using the formula:

$$\%\ \text{cell inhbition} = \frac{1 - \text{Mean Absorvance of treated cells}}{\text{Mean Absorbance of control}} \times 100$$

The IC_{50} values were determined from the linear regression graph plotted percentage cell inhibition against test concentrations [23].

Evaluation of testosterone production

The cells (5×10^3 cells/well) were seeded in a 96-well plate, treated with various concentrations of plant extracts range of 0.1, 0.25, and 0.5 mg/ ml in the presence of 1 µl anti-human chorionic gonadotropin (Anti-HCG) hormone and incubated at 37°C with 5% CO_2 for 24 h. After 24 h, the production of testosterone was assessed using Testosterone

ELISA kit according to manufacturer's instructions (ab108666-Testosterone ELISA Kit).

Statistical analysis

The Microsoft Excel 2010 and Origin 6.0 Professional were used for analyzing data. Data were expressed as ±standard deviations.

Ethical consideration

Ethical clearance was approved by the University of South Africa, College of Agriculture and Environmental Sciences Ethics Committee.

RESULTS

Quantity of crude extracts

Three grams of dried powered leaves of *K. africana* were extracted using acetone, methanol, and water as solvents. The percentage yield crude extracts are shown in Table 1. Water extract of *K. africana* yielded highest percentage crude extract (9.7%) followed by methanol crude extract (2.7%). *K. africana* showed lowest percentage crude extract when acetone used as solvent (0.7%).

Total phenolic contents

Table 2 and Fig. 1 show the total phenolic contents of *K. africana* extracts. The results were expressed as GAE per gram of dried plant extract (mg/g GAE). Acetone extract of *K. africana* had the highest total phenolic content (105±3.63 mg/g GAE) followed by methanol extract (10.65±0.32 mg/g GAE). Water extracts showed the least amount of total phenolic content (4.27±0.65 mg/g GAE).

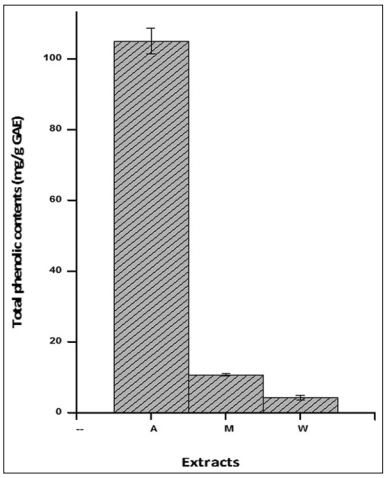

Fig. 1: Total phenolic contents of *Kigelia africana* extracted in acetone (A), methanol (M), and water (W). Error bars represent standard deviations of the data. Values are expressed as mean±standard deviation

Table 1: Percentage crude extracts yield of *K. africana* extracted using acetone, methanol, and water as solvents

Sample	Yield (%)		
	Acetone	Methanol	Water
K. africana	0.7	2.7	9.7

K. africana: *Kigelia africana*

Table 2: The total phenolic contents of *K. africana* extracted using acetone, methanol, and water as solvents. Values represent mean±SD of three determinations

Sample	Total phenolic content (mg/g GAE)		
	Acetone	Methanol	Water
K. africana	105±3.63	10.65±0.32	4.27±0.65

K. africana: *Kigelia africana,* GAE: Gallic acid equivalent, SD: Standard deviation

Total flavonoid contents

Table 3 and Fig. 2 illustrate the total flavonoid contents of *K. africana* extracts. The results showed that the highest total flavonoid content was obtained from acetone extract of *K. africana* (3.63±0.20 mg/ ml QE) followed by methanol extract (0.16±0.01 mg/g QE). Water extract of *K. africana* showed least amount of total flavonoid content (0.01±0.00 mg/g QE).

Antioxidant activities

The AA of *K. africana* extracts was determined using DPPH radical assay and results are summarized in Table 4. The antioxidant activities are expressed as IC_{50} values. The results of the extracts of *K. africana* were compared with positive control, ascorbic acid. From the results, *K. africana* extracts showed lower IC_{50} values than that of ascorbic acid; hence, the extracts were more efficient in scavenging the DPPH radical than ascorbic acid. In terms of solvent comparisons, the methanol extract of *K. africana* had the best AA in relation to other solvents tested.

Cell viability

The TM3 Leydig cells were exposed to increasing concentrations of *K. africana* extracts. The percentage cell viabilities are presented in Fig. 3. Acetone and methanol extracts of *K. africana* showed higher percentage of viable cells at 1 mg/ml, which is the highest concentration. In contrast, water extract had the higher percentage of viable cells at 0.25 mg/ml.

Cytotoxicity

The results of cytotoxic effects of extracts are presented in Table 5 and Fig. 4. The cytotoxic effects are expressed as IC_{50} values. Water extract of *K. africana* showed lowest cytotoxicity against TM3 Leydig cells

Table 3: The total flavonoid contents of *K. africana* extracted using acetone, methanol, and water as solvents. Values represent mean±SD of three determinations

Sample	Total flavonoid content (mg/g QE)		
	Acetone	Methanol	Water
K. africana	3.63±0.20	0.16±0.01	0.01±0.00

SD: Standard deviation, QE: Quercetin equivalent, *K. africana*: *Kigelia africana*

Table 4: DPPH IC_{50} values of *K. africana* extracts in acetone, methanol, and water. Values represent mean±SD of three determinations. Values are compared with positive control, ascorbic acid. Values are expressed as mean±SD

Sample	DPPH IC_{50} (mg/ml)		
	Acetone	Methanol	Water
K. africana	0.143±0.052	0.023±0.004	0.043±0.032

Ascorbic acid: 1.474±0.319. SD: Standard deviation, *K. africana*: *Kigelia africana,* DPPH: 1, 1-diphenyl-2-picrylhydrazyl

Table 5: IC_{50} values of *K. africana* extracts on the TM3 Leydig cells obtained using MTT assay. Values represent mean±SD

Sample	MTT IC_{50} (mg/ml)		
	Acetone	Methanol	Water
K. africana	0.332±0.112	0.209±0.001	0.530±0.096

SD: Standard deviation, *K. africana*: *Kigelia africana,*
MTT: 3-(4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

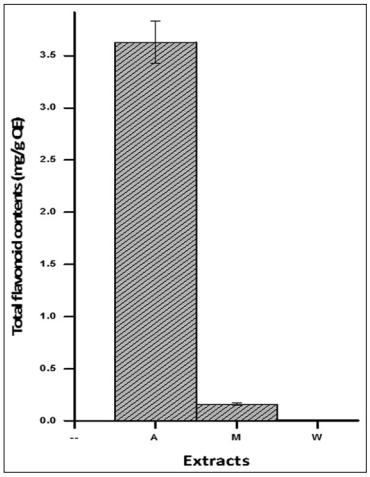

Fig. 2: Total flavonoid contents of *Kigelia africana* extracts extracted in acetone (A), methanol (M), and water (W). Error bars represent standard deviations of the data. Values are expressed as mean±standard deviation

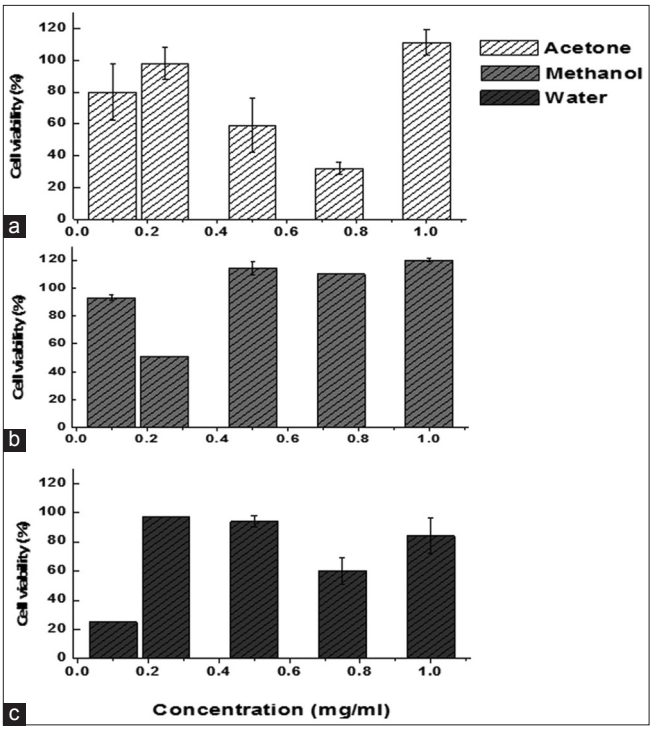

Fig. 3: Percentage cell viability of TM3 Leydig cells treated with different concentrations of *Kigelia africana* extract for 24 h. (a) *K. africana* acetone extract; (B) *K. africana* methanol extract; (C) *K. africana* water extracts. Stimulated treated cells are compared with non-stimulated cells assumed 100% cell viability

at the concentration of 0.530±0.096 mg/ml. In contrast, the highest cytotoxicity was observed with methanolic extract, had lower IC_{50} value (0.209±0.001 mg/ml).

Testosterone production

Table 6 showed the results of testosterone production in the TM3 Leydig cells exposed to selected different concentrations of *K. africana* extracts in the presence of anti-hCG hormone. From the results, the acetone and methanol extracts of *K. africana* showed increase in the testosterone production on the TM3 Leydig cells. At 0.5 mg/ml, acetone extract of *K. africana* showed a significant increase in the testosterone production than in the control A.

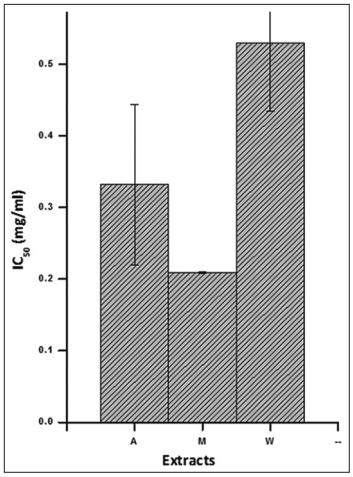

Fig. 4: IC_{50} values of *Kigelia africana* extracts on the TM3 Leydig cells obtained using MTT assay. IC_{50} value represents the concentration of the extract required for 50% inhibition of cells. Error bars represent the standard deviations of the data. Values are expressed as mean±standard deviation. A – Acetone extract, M – methanolic extract, and W – water extract

Table 6: Testosterone production in TM3 Leydig cells exposed to selected varying concentrations of *K. africana* extracts for 24 h in the presence of anti-human chorionic gonadotropin hormone (hCG). Values are compared with control A and B. (-) represent not determined

Sample	Concentration of plant extracts (mg/ml)	Testosterone levels (ng/ml)		
		Acetone	Methanol	Water
K. africana	0.5	1.407	0.084	-
	0.25	0.231	0.032	0.030
	0.1	0.269	0.019	0.004

Testosterone control A: 0.670 ng/ml, testosterone control B: 10.033 ng/ml, (-) not determined. SD: Standard deviation, *K. africana*: *Kigelia africana*

DISCUSSION

Phytochemical screening of this plant was executed with the consideration of the type of solvent used for extraction [24]. The structures of compounds determine their solubility in the solvent polarity [25]. Therefore, it is very important to isolate non-polar compounds with non-polar solvents and polar compounds with polar solvents [26]. In the current study, acetone, methanol, and water were used as solvents for extractions. Water extracted the highest percentage yield of *K. africana* extract. In most studies, methanol yielded highest percentage of crude extract [27]. Our phytochemical screening revealed the presence of phenolic compounds, flavonoids, and antioxidants.

Phenolic compounds constitute one of the main classes of secondary metabolites in plants [28]. They possess the antioxidant properties that protect against damaging effect of free radical scavenging activity [29]. They are believed to account for the AA of most plant species [30]. From the results, acetone extracts of *K. africana* contained a significantly high total phenolic content and total flavonoid content among the extracts tested. The results concur with the findings from Eloff and McGaw [31], where they reported that acetone extracts are considered to be good extracts for many medicinal plant metabolites.

Numerous methods have been developed to measure the radical scavenging activity of antioxidants in food extracts, beverages, and plant extracts. DPPH and 2, 2'-azinobis-3-ethylbenzothiazoline-sulfonic acid scavenging assays are one of the most popular spectrophotometric methods used to evaluate the AA of samples. These methods are simple to use and rapid to assess the AA but

very sensitive to light [32]. In this study, DPPH radical assay was used to determine the AA of *K. africana* extracts. DPPH radical is a purple color compound. On reaction with antioxidant, it decolorizes to light yellow diphenyl-2-picrylhydrazine by accepting hydrogen atom from antioxidants [33]. The degree of color change at the end of the reaction is proportional to the free radical scavenging activity of antioxidants [34]. In our study, methanol extract of *K. africana* effectively reduced the DPPH radical of all the extracts tested and ascorbic acid. The presence of lower IC_{50} value indicated the stronger AA against damaging free radicals [35]. AA could be due to the presence of higher phenolic compounds; however, methanol extract of *K. africana* contained the lowest total phenolic and flavonoid contents than acetone extract. Therefore, the results suggest that there is no relationship between the amount of phenolic compounds and AA. Our findings are in line with the study by Adebayo *et al*. [14] which reported poor relationship between total phenolic contents and AA.

Apart from phenolic compounds, it was reported that the AA could be due to other bioactive compounds in the extracts [36]. Glycosides, phenolic compound, saponin, tannins, alkaloids, flavonoids, and sugar have been found in the methanol and water extracts of *K. Africana* [37]. Moreover, the AA also depended on the chemical structure of the antioxidants and interaction occurring between them and other plant constituents [38,39]. For these reasons, it is important to consider other compounds contributing to curative effect of medicinal plants and their mechanism.

MTT assay is a commonly used assay for the detection of cell viability and cytotoxicity after exposure to toxic substances [40]. MTT is a water-soluble yellow tetrazolium salt which is converted to water-insoluble purple formazan product when incubated with viable cells. The MTT formazan product is impermeable to the cell membrane of dead cells but accumulates in healthy cells. The formazan product is formed when tetrazolium ring is cleaved by mitochondria succinate dehydrogenase enzyme of viable cells. For this reason, the intensity of MTT formazan produced is directly proportional to the number of viable cells tested [22]. Acetone and methanol extracts of *K. africana* showed higher percentage of viable cells at 1 mg/ml, the highest concentration, whereas water extract had the higher percentage of viable cells at 0.25 mg/ml. This was also observed by Opuwari and Monsees [41], who reported the concentration-dependent increased percentage cell viability from 250 to 1000 µg/ml when TM3 Leydig cells were exposed to *Camellia sinensis* (unfermented and unfermented rooibos) and *Aspalathus linearis* (green and black tea), but reduced at 5000 µg/ml.

In terms of cytotoxicity, water extract of *K. africana* showed lowest cytotoxicity against TM3 Leydig cells. Based on this finding, it appears that water extract of *K. africana* is less toxic but may be depended on the type of the cell. Basically, traditional healers use water as solvent to prepare medicinal concoctions [27]. It can, therefore, be conceivable to conclude that traditional healers could be encouraged to continue to use water as solvent for their extractions.

Leydig cells lie between the seminiferous tubules in the testes and their main physiological function is to produce and secrete testosterone [42]. Testosterone maintains and regulates spermatogenesis [43]. The TM3 Leydig cells were used in this present study to examine the effects of varying concentrations of *K. africana* extracts in testosterone production. After 24 h exposure to different concentrations of *K. africana* extracts and anti-hCG, acetone and methanol extracts of *K. africana* caused increase in testosterone production by TM3 Leydig cells. At the higher concentration, acetone extract caused a significant increase in testosterone level than in the control A. Methanol and water extracts of *K. africana* caused increase in testosterone levels in a concentration-dependent fashion. The results concur with findings reported by Petersen *et al*. [44], where *K africana* fruit extracts were found to cause the increase in testosterone levels and follicle-stimulating hormone.

CONCLUSION

K. africana can be regarded as reliable and cheaper source of natural antioxidants. The results showed significant levels of phenolic and flavonoid contents in this plant. Due to low cytotoxicity, the plant can also be recommended to traditional healers to use as additional plant for the treatment of various ailments, especially those that are aggravated by reactive oxygen species, such as diabetes and male infertility.

AUTHORS' CONTRIBUTIONS

Nelisiwe Masuku, as the first author, was involved designing the work, performing experiments, data analysis, data interpretation, and drafting the manuscript. Sogolo Lebelo, as the corresponding author, was involved in conceptualization of the research project, critical revision of the manuscript and approval, and submission of the final manuscript.

CONFLICTS OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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