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PHARMACOGNOSTIC AND PHYSICOCHEMICAL EVALUATION, HPLC ANALYSIS AND ANTIPROLIFERATIVE PROPERTIES OF *KIGELIA AFRICANA* (Lam.) Benth

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ABSTRACT

Introduction/Objective of study: *Kigelia africana* (Bignoniaceae) is enriched with bioactive constituents and has thus found various uses in African folklore. This study aims to evaluate the pharmacognostic, physicochemical, chromatographic and antiproliferative properties of *K. africana*.
Methodology: Standard methods were used to determine the qualitative microscopy, moisture content, ash and extractive value. Furthermore, HPLC analysis was conducted on the samples in order to detect and quantify some phenolic compounds (gallic acid, chlorogenic acid, rutin, ferulic acid, caffeic acid and quercetin). The *Sorghum bicolor* model was used for the antiproliferative assay. All experiment was carried out in triplicates.
Results: Microscopy revealed amphistomata with the presence of non-glandular unicellular, uniseriate trichomes on *K. africana* leaf. Cellulose, tannins, calcium oxalate crystals on the leaf and stem bark, while the roots lacked calcium oxalate crystals. Ash contents were leaf (21.8 ± 0.1) %w/w, stem bark (4.8 ± 0.03)%w/w and root (3.9 ± 0.2)%w/w. Moisture content was (10.5 ± 0.5) %w/w and (9.5 ± 0.2) %w/w for the root and leaf parts, respectively. All values were within WHO limits for crude drugs. The stem bark and root parts contained more water-soluble constituents than alcohol soluble constituents. From the results of HPLC analysis the leaf, stem bark and root extracts gave 24 peaks, 16 peaks and 30 peaks, respectively, a few peaks matched with reference compounds- quercetin, caffeic acid, ferulic acid and rutin. Results of antiproliferative assay showed that methotrexate was significantly ($p < 0.05$) more effective than the stem bark (from 2-64 mg/mL) with inhibitions ranging from 72.0 ± 1.4% - 90.0 ± 2.4% and root extracts (from 4 – 64 mg/mL) that had inhibitions ranging from 50.3 ± 1.5% - 97.7 ± 0.4% but comparable with leaf extract (from 16 mg/mL - 64 mg/mL) with inhibitions ranging from 68.4 ± 0.8% - 99.0 ± 0.1%.
Conclusion: Further information which can be included in an official monograph of the plant for its proper identification and quality control has been provided by this study. *Kigelia africana* exhibited effective antiproliferative activities and the presence of phenolic compounds.

Keywords: Antiproliferative, *Kigelia africana*, Phenolics, standardization, Chromatography
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INTRODUCTION

In developing countries such as Nigeria, the cost of carrying out research have led to the development of simple bench top assays that can be used in the preliminary screening of medicinal plants for various assays e.g. screening for antiproliferative properties as a preliminary test for anticancer agents in the absence of expensive and difficult to procure cell lines.

Cancer cells are characterized by uncontrollable rapid proliferation, apoptotic evasion, angiogenesis as well as metastasis [16]. Current options for therapy bedevilled with setbacks include radiotherapy, chemotherapy and surgery. The costs of all these drastically deplete the income and savings of many patients and their relations. More so, the treatment often times is associated with unbearable side effects. Hence there is the need to go back to nature, the laboratory of bioactive constituents, to search for newer drugs with lesser or no side effects to manage cancer. The World Health Organization (WHO) has since identified cancer as one of the most significant causes of death presently. Present postulations reveal that there might be an escalation of cancer mortality to about 15 million by 2030, if nothing is done to arrest the disease [31, 14].

Kigelia africana Lam. Benth (Bignoniaceae) is a tropical plant widely, grown as an ornamental plant in tropical regions for its decorative flowers [25, 4], and distributed in Southern, Central and West Africa. It is commonly called the Sausage tree because of its huge fruits suspended from its lengthy stalks (11). In Nigeria, it is called 'Pandoro' (Yoruba), 'uturubien' (Ibo) and 'Hantsargiiwaa' (Hausa) [8] and used in ethno-medicine for gynaecological disorders, tumors, male infertility, topical application for wound healing [27] bacterial infections [15, 17], fungal infection [3, 7], malaria, diabetes, pneumonia, ulcers, rheumatism [30, 26] and cancer [19]. Pharmacologically, the plant has been reported to have anticancer, antinociceptive, anti-inflammatory, diuretic and antimicrobial activities [30, 2, 21, 23, 9, 28, 10]. Constituents such as iridoids, coumarins, naphthoquinones, flavonoids and sterols have been isolated from *K. africana* [12, 20, 26, 18, 23].

Evaluation of pharmacognostic and physicochemical properties of medicinal plants are essential to enable standardization and preparation of monographs for these plants. Moisture content is a quantitative measurement which provides information needed for processing, preservation and storage of medicinal plants, while ash value, which is the total of the residue remaining after all moisture has been removed as well as the organic material (such as fat, protein, carbohydrates, vitamins and organic acid) on incineration, give information on the purity of the crude drug [13].

The aim of this study is to evaluate the pharmacognostic, physicochemical and antiproliferative properties of the leaf, stem bark and root parts of *K. africana* as a crude drug; qualitatively and quantitatively analyze extracts from the morphological parts using High-Performance Liquid Chromatography (HPLC) and carry out antiproliferative studies on the morphological parts.

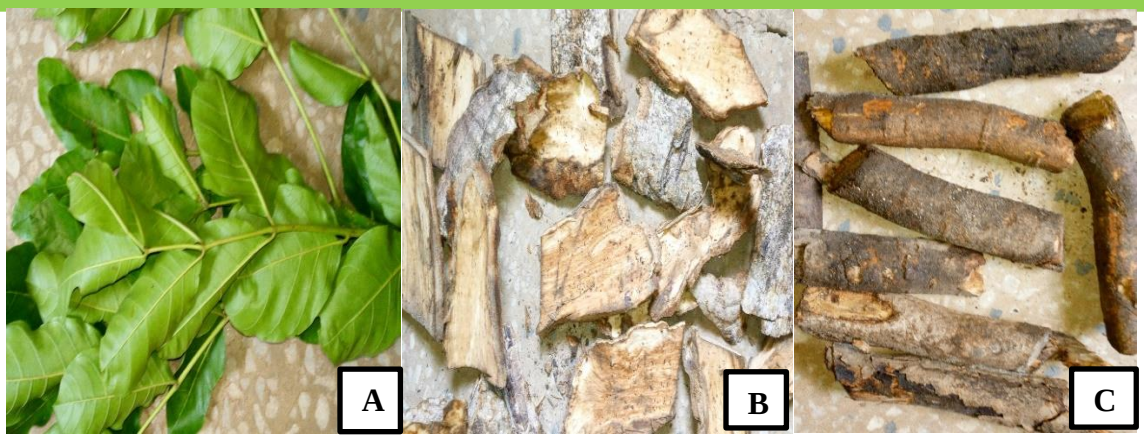

Figure 1: Morphological parts of *Kigelia africana* showing A - Leaf; B -Stem bark and C – Root

MATERIALS AND METHODS

Sample collection

The plant (leaf, stem bark and root) parts were collected at Chaza (Suleja) North Central area of Niger State. It was identified, authenticated and deposited at the National Institute of Pharmaceutical Research and Development (NIPRD) herbarium with voucher number NIPRD/H/6764.

Extraction

The different morphological parts were separately, air dried and pulverized to powder and kept in a desiccator for further use. Hot water infusions of *K. africana* leaf, stem bark and root parts were prepared separately as described by Ayinde *et al.,* [6] and Grace *et al.,* [15]. Each powdered sample (50 g) was stirred in 1L of boiling water for about 10 minutes, allowed to stand, filtered after 24 hours to give hot infused extracts. Extracts were lyophilized and stored at 4˚C prior to use.

Morphological evaluation

Leaf, stem and root samples of *K. africana* were subjected to macroscopic analysis, viz., organoleptic characteristics such as appearance, taste, colour, odour, shape and texture as specified by WHO guidelines [1, 32].

Microscopy

Microscopic analysis was carried out on the pulverized root, stembark and leaf samples separately. Whole leaf samples were cut into 1cm^2 portions and soaked in nitric acid overnight. Epidermal surfaces were separated and preserved in 50% alcohol. Each surface was mounted in glycerin-water after staining with safranin O and viewed under the microscope. A quantity of each pulverized sample was cleared in chloral hydrate, mounted in glycerin-water (1:1) and viewed under the microscope at different magnifications [13].

Physicochemical evaluation

Various physicochemical parameters such as moisture content; total ash; water and alcohol extractive values were determined following WHO guidelines [1, 32].

Chemomicroscopic analysis

Chemo-microscopic studies of the pulverized leaf, stem and root were carried out using reagents and stains like iodine, concentrated Sulphuric acid, ferric acid, Sudan III, ruthenium red and phloroglucinol in concentrated HCl (1:1) to test for the presence of various parameters [13].

Chromatographic analysis

High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto Sampler (SIL-20A), equipped with Shimadzu LC-20AB reciprocating pumps connected to the degasser DGU 20A3 with integrator CBM 20A, UV-VIS detector DAD (diode array detector) SPD-M20A and Software LC solution 1.22 SP1. The following gradient conditions were used (Table 1)

Table 1.0: Gradient composition used for the HPLC analysis

Time (mins)	Composition of A (%)	Composition of B (%)
0 – 10	82	18
10 – 30	80	20
30 – 40	75	25
40 – 50	70	30
50 – 60	90	10

HPLC grade solvents were used. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and sonicated before use. Chromatographic conditions under gradient elution include: Column- C18 (4.6 x 150 mm x 5 µm) mobile phase was 1% formic acid in water (A) and 1% formic acid in Acetonitrile (B); flow rate was 0.6 mL/min; injection volume, 3µL and the wavelength was 254 nm. HPLC fingerprinting of the leaf, stembark and root parts using the standard references as listed above was carried out. Identification of compounds was performed by comparing their retention time and chromatographic peaks (UV absorption spectrum) with those of the standard reference compounds.

Stock solutions of standards references (gallic acid, chlorogenic acid, rutin, ferulic acid, caffeic acid and quercetin) (Sigma) were prepared in methanol at different concentrations. A calibration curve was developed for each compound as shown in Table 5.0 *Kigelia africana* (leaves, stem bark and root) extracts were dissolved in 80% methanol at a concentration of 20 mg/mL. The quantification of the compounds

in the samples was done by using the calibration curve equation to get the values of x with y being the peak area, then calculating the concentration with respect to the plant extract using the formals below;

Amount constituent in plant extract (sample) = $\frac{X \times V}{Wt}$ where X= concentration obtained from curve (mg/mL); V= volume of solvent used in mL and Wt= weight of extract (g)

Anti-proliferative assessment

Guinea corn (*Sorghum bicolor*), procured from Karmo market, FCT Abuja was subjected to viability screening before use by placing it in a beaker containing distilled water. The floating seeds were discarded, while the submerged ones were air dried. The bench top assay method described previously by Ayinde *et al.*, [6] was employed for this study with some modification. Petri dishes were layered with cotton wool and filter paper (Whatman No. 1). In each petri dish twenty (20) viable seeds of *S. bicolor* were placed, in even spaces. The test seeds were treated with fifteen (15 mL) each of different concentrations of *K. africana* stem bark, leaf and root extracts (2, 4, 8, 16, 32 and 64 mg/mL). The negative control seeds were treated with 15 mL distilled water, while the positive control seeds were treated separately with 15 mL of 50 µg/mL methotrexate injection, 15 mL of 166.6 µg/mL of methotrexate injection and tablets according to manufacturer's instructions. All seeds in the different petri dishes were incubated in a dark room. The mean radicle lengths (mm) from the seeds were measured after 24, 48, 72 and 96 hours. A minimum of 10 seeds were counted. The percentage inhibition was calculated as $(K_0 - K)/K_0 \times 100\%$.

Where K = radicle length of test samples/positive controls; K_0 = radicle length of negative control.

Statistical analysis

The data were expressed as mean ± standard deviation and subjected to statistical analysis using Graph pad prism® (6th version). ANOVA-multiple comparisons using two-way analysis of variance was used to test for significance wherein $p < 0.05$ was considered significant.

RESULTS

The results of the macroscopic analysis, viz., organoleptic characteristics of the leaf, stem and root samples of *K. africana* are presented in Table 2.0 below. The appearance, taste, colour, odour, shape and texture of the samples are reported as specified by WHO guidelines.

Table 2.0: Organoleptic Properties of *Kigelia africana* leaf

Properties	**Characteristics**
Leaf odour	Fruity
Colour	Green
Type	Simple
Margin	Entire
Apex	Obtuse-rounded
Shape	Linear-lanceolate
Base	Rounded
Venation	Reticulate
Length (n=20)	113.33 mm
Width (n=20)	75.67 mm

The microscopic analysis of the pulverized root, stem bark and leaf samples showing the distinctive features of the different parts of plants are shown in Figure 1, 2 and 3. This is the first study to extensively describe the unique features of the root, stem and leaf of *K. africana*.

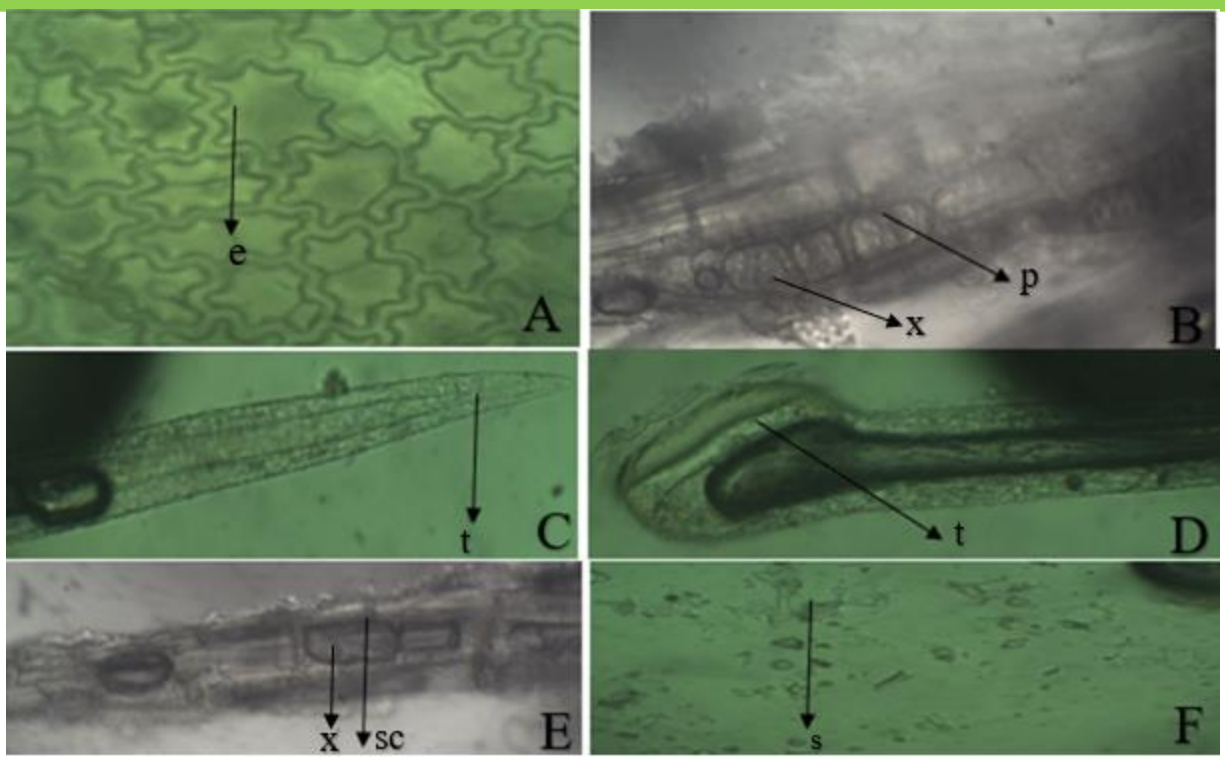

Figure 1: Powder microscopy of powdered Leaf (A,C,D) stem bark (B) and root (E,F) of *Kigelia africana* showing A – irregular/wavy epidermal cells; B – Parenchyma (p) with microcrystals of calcium oxalate (x); C- unicellular and non-glandular trichome tip (t); D- trichome base (t) E- Schlerenchyma (sc) with simple calcium oxalate crystals (x); F- starch grainules Magnification: x 400.

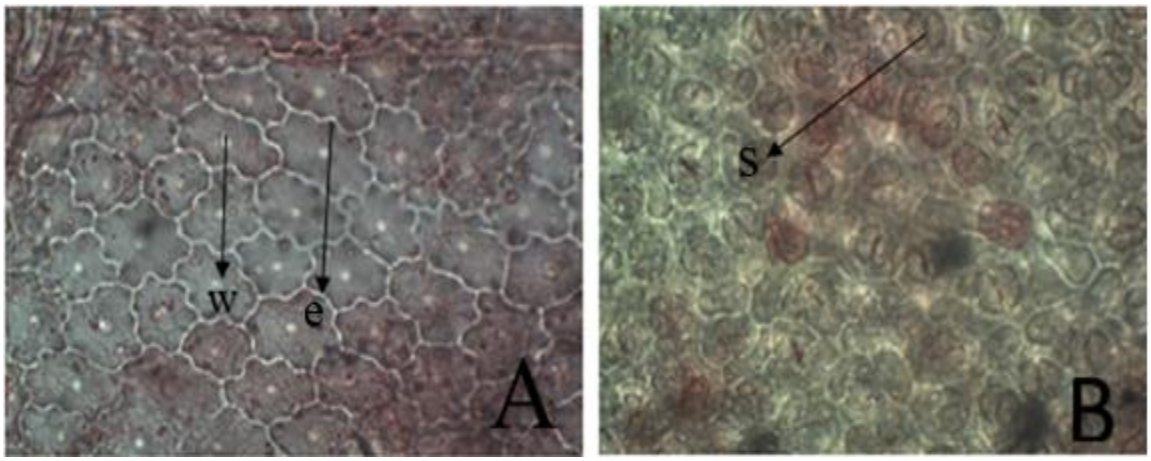

Figure 2: Adaxial leaf epidermal surface of *K. africana* showing: A – irregular wavy epidermal cells (e) with idioblast oil globules (w); B - diacytic stomata (s). Magnification: x 400

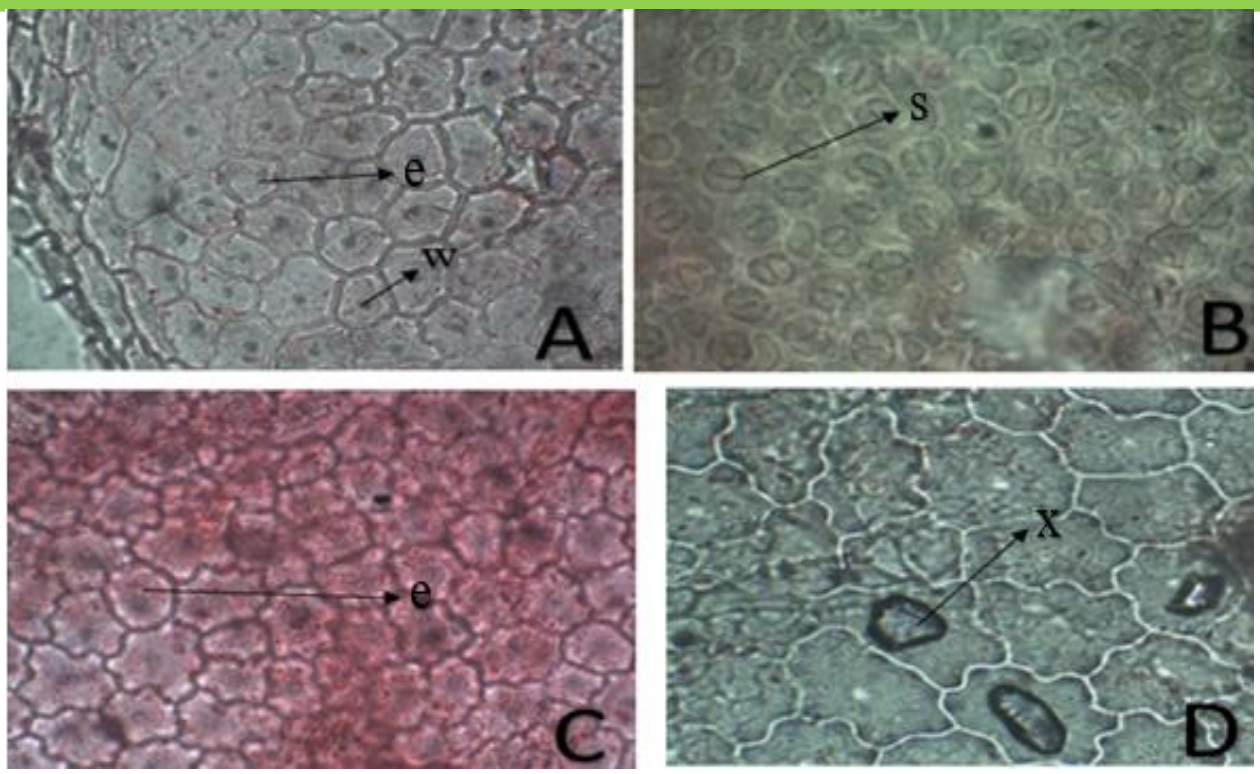

Figure 3: Abaxial leaf epidermal surface of *K. africana* of showing: A/C– irregular angular epidermal cells (e) with idioblasts-oil globules (w), B - diacytic stomata (s); D - simple crystals (x). Magnification: x 400

The results of the physicochemical properties of the root, stem and leaf of *K. africana* is presented in Table 3.0. The moisture content; total ash; water and alcohol extractive values of the samples conformed to the WHO guidelines. The chemo-microscopic studies of the pulverized leaf, stem and root of the plant shows the presence of lignin, cellulose, tannins, starch and protein in all samples. Similarly, the leaf sample contains all the parameters analysed (Table 4.0).

Table 3.0: Physicochemical properties of *Kigelia africana* parts

Parameters	**Leaf (%w/w)**	**Stem bark (%w/w)**	**Root (%w/w)**
Total Ash (n=3)	21.80 ± 0.07	4.80 ± 0.03	3.90 ± 0.20
Moisture content (n=3)	9.54 ± 0.20	9.69 ±0.06	10.50 ± 0.50
Alcohol extractive value (n=3)	15.49 ± 1.04	27.75 ± 1.58	21.14 ± 0.31
Water extractive value (n=3)	11.98 ± 2.33	33.96 ± 2.76	23.19 ± 0.66

Table 4.0: Chemomicroscopic evaluation of plant parts

Parameters	Leaf	Stem bark	Root bark
Lignin	+	+	+
Mucilage	+	+	-
Cellulose	+	+	+
Tannins	+	+	+
Starch	+	+	+
Calcium oxalate crystals	+	+	-
Oils	+	-	+
Proteins (Picric test)	+	+	+
Proteins (Millions test)	+	+	+

Key: + depicts the presence of metabolite and – depicts the absence of metabolite

Results of HPLC analysis

The Calibration curve of gallic acid, chlorogenic acid, caffeic acid, rutin, ferulic acid and quercetin is shown in Table 5.0 below. The regression coefficients of the reference standards used in the chromatographic analysis HPLC analysis was between 0.972 to 0.999.

Table 5.0: Calibration curve of the reference standards used in the chromatographic analysis

Compound	Calibration curve equation	R^2	Linear range (µg/mL)
Gallic acid	Y = 9248.1x + 39471	0.999	10 – 250
Chlorogenic acid	Y = 5586.5x + 196030	0.998	7.812 – 250
Caffeic acid	Y = 11103x + 15442	0.977	25 – 500
Rutin	Y = 7892x + 67898	0.997	3 – 500
Ferulic acid	Y = 6301x + 29944	0.972	10 – 550
Quercetin	Y = 12819x +37596	0.996	60 – 250

y= peak area; x= the concentration of each reference compound (**µg** /mL); R, correlation coefficient of regression equations;

Table 6.0: Quantitative chromatographic analysis of the plant parts

Compound	Amount in extract		
	Leaf	**Stem bark**	**Root**
Gallic acid	1.346mg/g	1.733mg/g	4.204 mg/g
Chlorogenic acid	3.818mg/g	0.959mg/g	1.357 mg/g
Caffeic acid	-	1.292mg/g	17.23 mg/g
Rutin	Trace*	-	-
Ferulic acid	0.081mg/g	-	3.625 mg/g
Quercetin	0.012mg/g	2.816mg/g	Trace*

Trace* = below the lowest limits of calibration curve

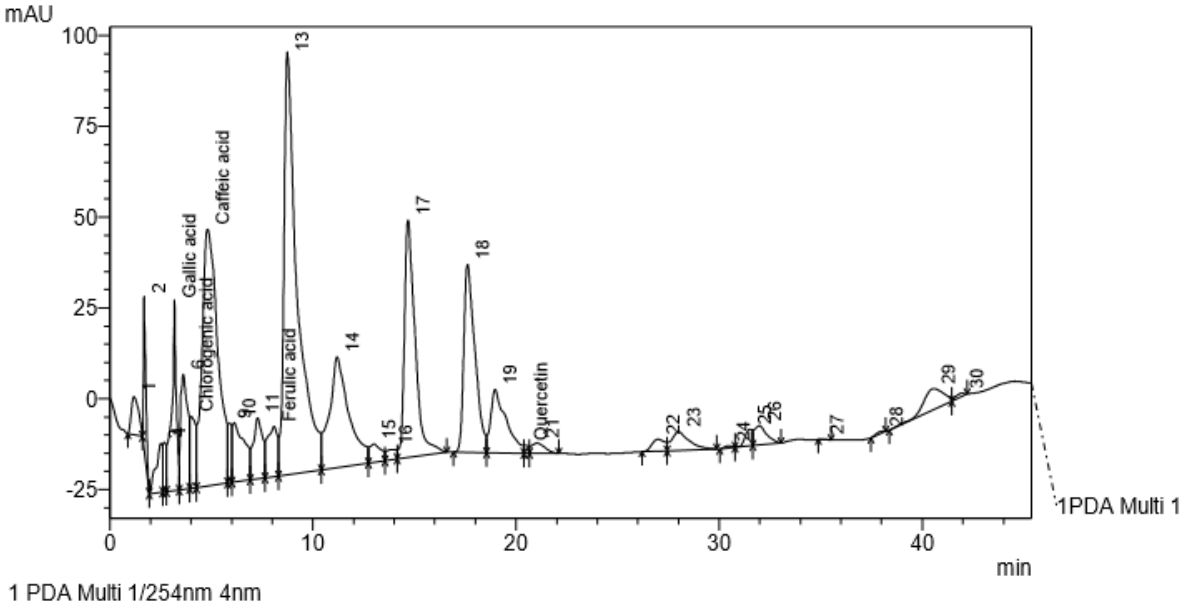

Figure 4: HPLC Chromatogram (fingerprinting) of *K. africana* root extract

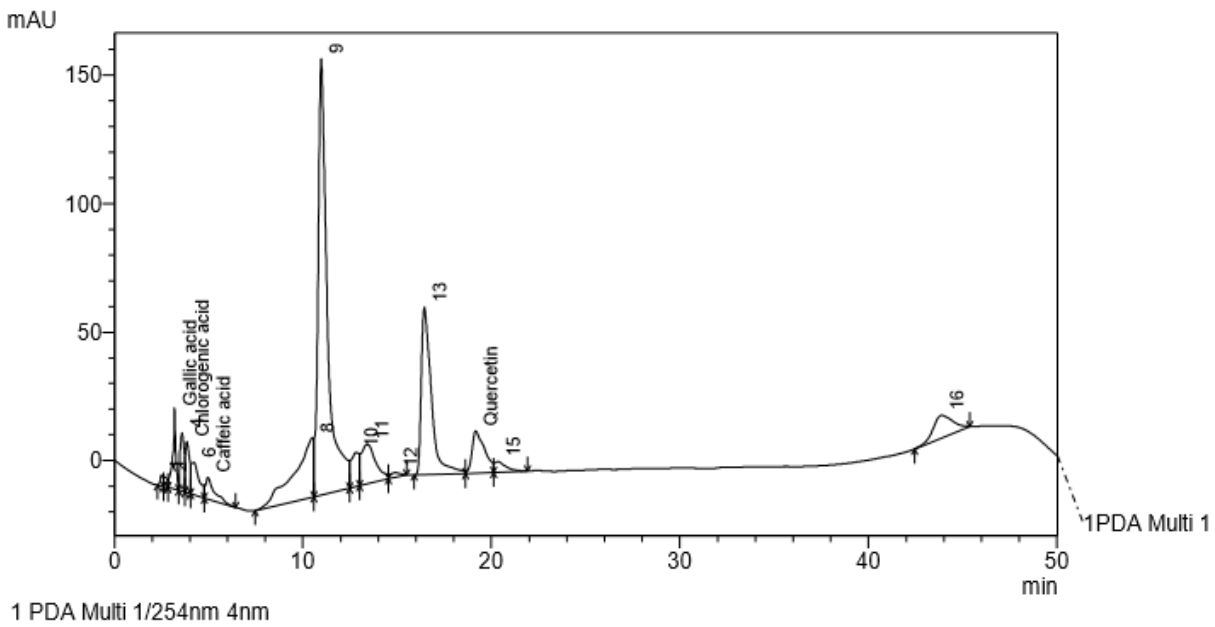

Figure 5: HPLC Chromatogram (finger-printing) of *Kigelia africana* stem extract

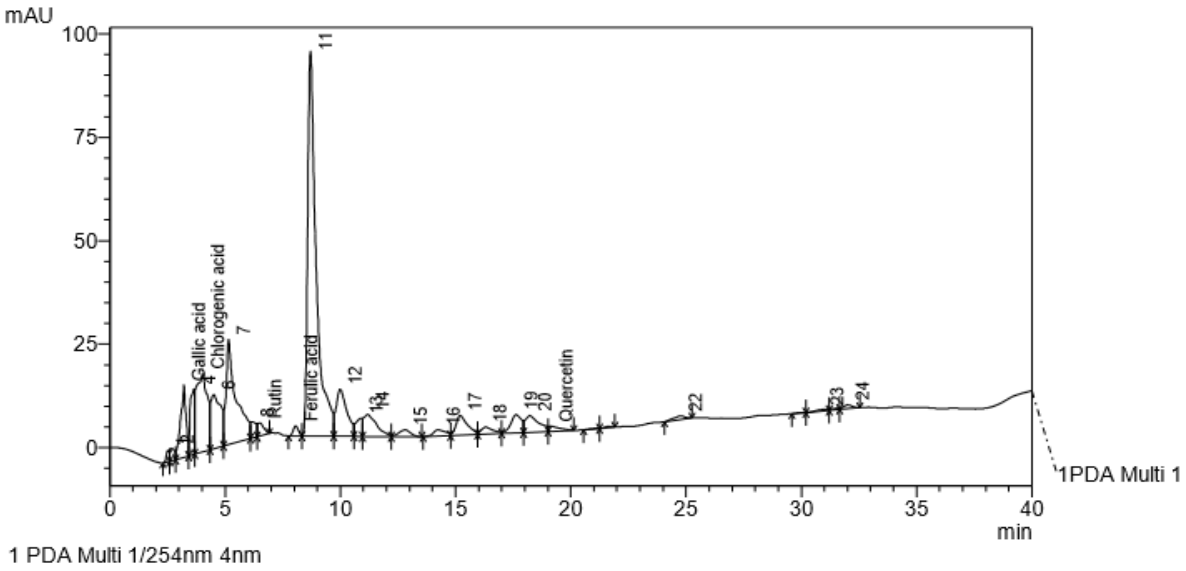

Figure 6: HPLC Chromatogram (finger-printing) of *K. africana* leaf extract

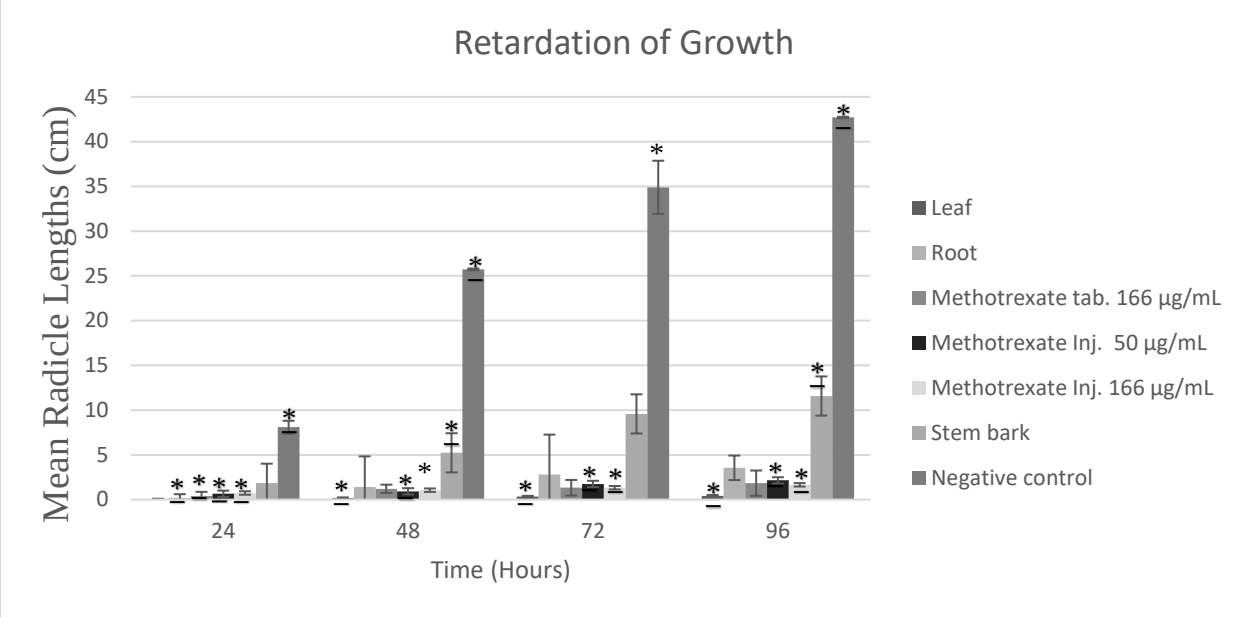

Figure 7.0: Mean radicle lengths of extracts

KEY: Leaf, stem bark and root extracts at 64 mg/mL; Negative control: distilled water and positive controls: methotrexate (tablet -166 µg/mL and Injectable - 166 µg/mL and 50µg/mL). * depict significant difference in activity at $p < 0.05$

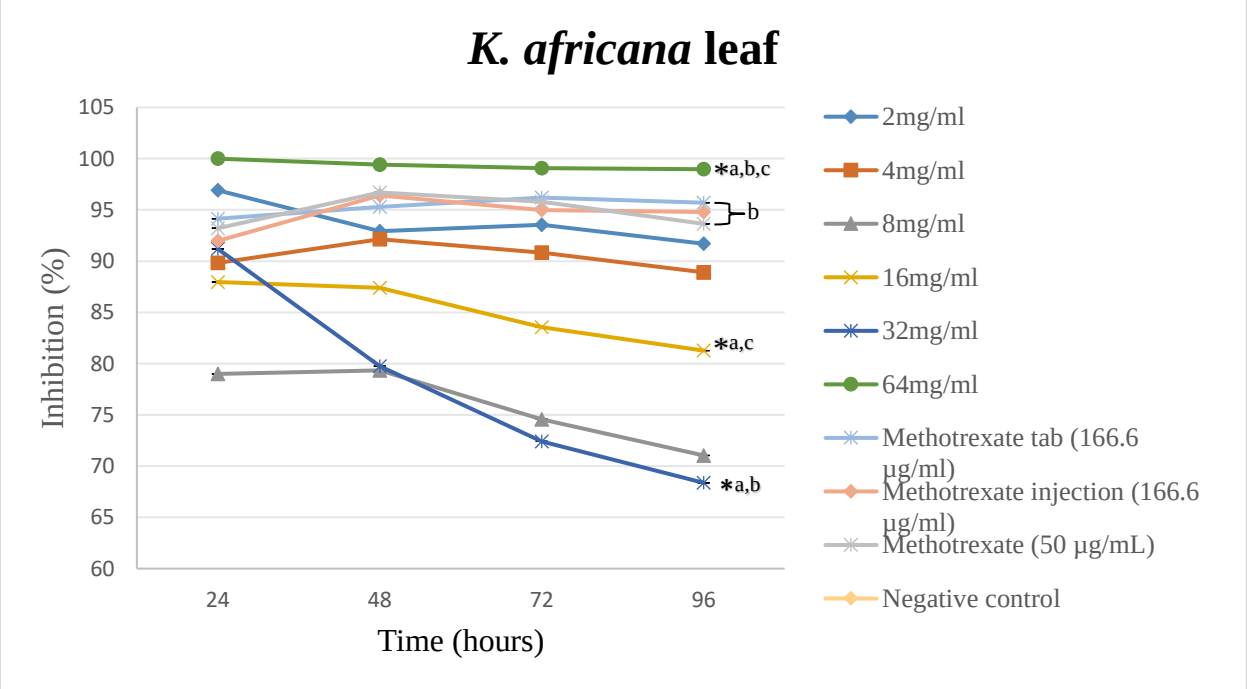

Figure 8: Antiproliferative potency (Inhibition) of *K. africana* leaf. Positive control- methotrexate; Negative control- distilled water. * depict significant difference in activities at p < 0.05

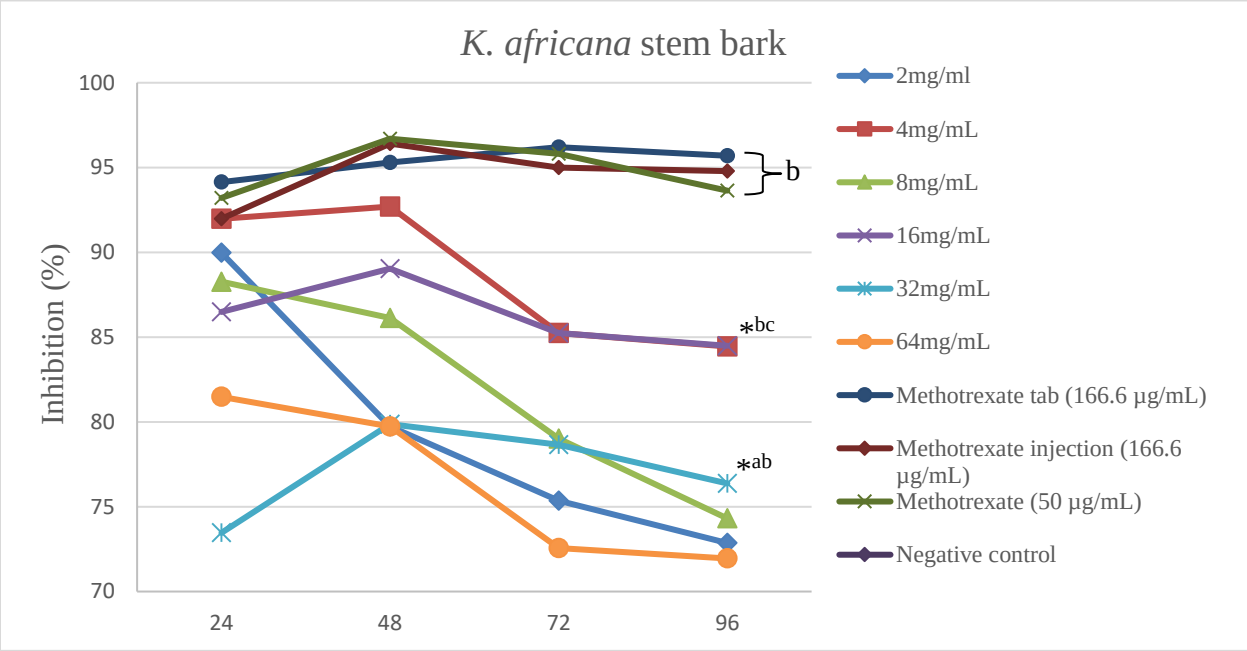

Figure 9: Antiproliferative potency (Inhibition) of *K. africana* stem bark. Positive control- methotrexate; Negative control- distilled water. * depict significant difference in activities at p < 0.05.

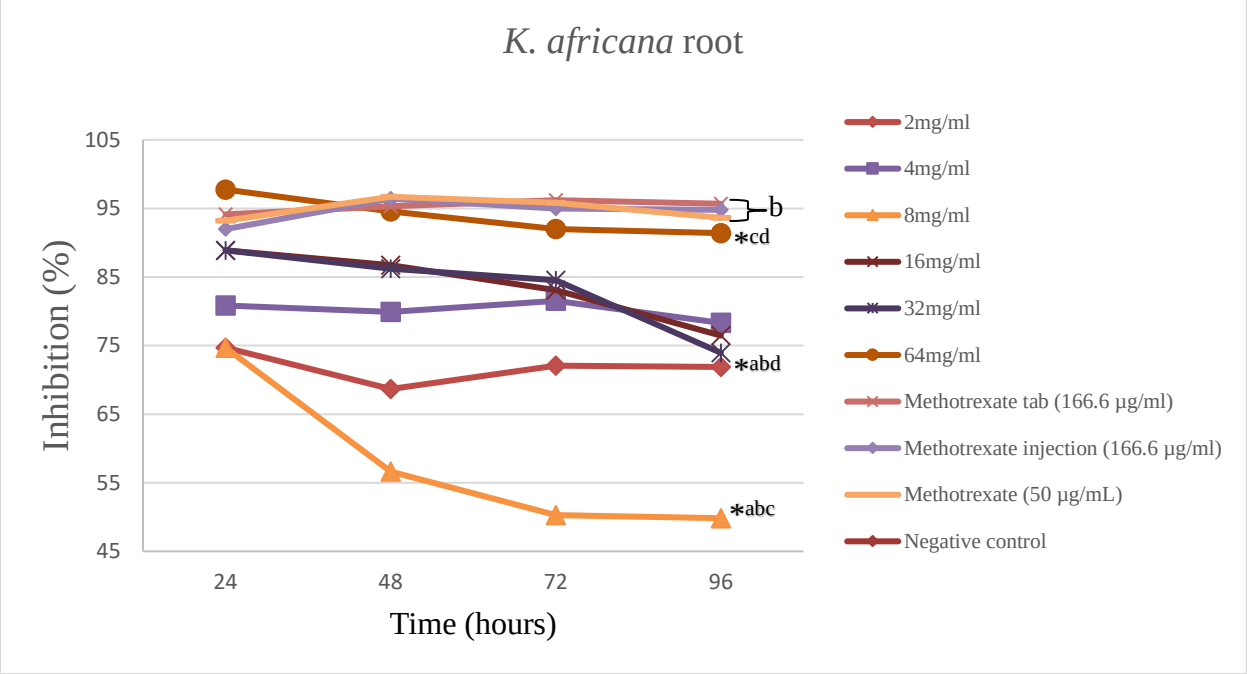

Figure 10: Antiproliferative potency (Inhibition) of *K. africana* root. Positive control- methotrexate; Negative control- distilled water. * depict significant difference in activities at $p < 0.05$

DISCUSSION

On physical examination, the leaf was found to have a green colour, fruity smell, simple leaf structure with entire margin, glaborus surface, obtuse to rounded apex, rounded base, linear to lanceolate shaped, reticulate venation (Table 1). Qualitative microscopy revealed the presence of non-glandular unicellular trichomes which may reduce transpiration, scatter incoming light and deter herbivory. Trichome architecture can be said to be species-specific. *Kigelia africana* leaf was observed to be amphistomatic with the presence of diacytic stomata surrounded by irregular epidermal cells on both surfaces. These features are similar to findings by Ugbabe and Ayodele, 2008, who reported the macroscopy and microscopy of members of the Bignoniaceae family. Chemomicroscopic analysis of the pulverized plant parts showed that the leaf and stem bark contained lignin, mucilage, cellulose, tannins, starch, calcium oxalate crystals and proteins, while the root contained lignin, cellulose, tannins, starch, oils and proteins lacking calcium oxalate crystals (Table 3). Physicochemical tests showed that the leaf (21.8 ± 0.1) %w/w contained more inorganic component than the stem bark (4.8 ± 0.03) %w/w and the root, which gave the lowest ash value (3.9 ± 0.2) %w/w of the three plant parts. The root (10.5 ± 0.5) %w/w had the highest moisture content, while the leaf had the least moisture content of (9.5 ± 0.2) %w/w and thus the root might be more prone to microbial degradation. The values obtained are however within WHO

limits for crude drugs. The stem bark and root parts contained more water-soluble constituents than organic alcohol soluble constituents as observed from their extractive values however, the leaf contained more alcohol soluble constituents (Table 2).

From the HPLC analysis it was observed that the leaf extract gave 24 peaks, the stem extract gave 16 peaks and the root extract gave 30 peaks, some of these peaks corresponds to some of the phenolic reference compounds. A total of 6 phenolic compounds were identified across the different extracts analysed: Gallic acid, chlorogenic acid and quercetin were identified in the leaf, stem and root extract, caffeic acid was seen in the stem and root extract, ferulic acid was seen in the leaf and root extract, and rutin is only present in the leaf extract, as can be seen in Figures 2-4 respectively. In an earlier report by Falode *et al.*, [12], the presences of quercetin, rutin, kaempferol, catechin, gallic acid, chlorogenic acid, ellagic acid, rosmarinic acid and caffeic acid in *Kigelia africana* leaf was established. However, this is the first work to identify these phenolics in the stem and root of *Kigelia africana.*

Furthermore, the quantitative analysis, of these phenols shows that the leaf extract had the least amount of gallic acid (1.346mg/g) while the root had the highest amount of gallic acid (4.204mg/g), chlorogenic acid was least in stem bark (0.959mg/g) and highest in the leaf extract (3.818mg/g), caffeic acid content was higher in the root extract (17.23mg/g) than in stem bark extract (1.292mg/g). Rutin was only present in the leaf and in trace amount. Ferulic was higher in the root extract (3.625mg/g) than in the leaf extract (0.081mg/g). Quercetin showed highest concentration in the stem bark (2.816mg/g), while it was detected in trace amount in the root extract.

Cancer cells exhibit high degree of proliferation, the effect of suspected anticancer agents on proliferation is now a commonly used parameter in the preliminary screening for potential anticancer agents especially in low income countries. The use of various bench top procedures involving rapidly proliferating meristematic cells of seed radicles as parameter in the screening of suspected potential antiproliferating agents have been previously employed [5, 29]. The results from this study showed that the seed radicle controls had a high growth rate throughout the study and has thus further shown that at favourable conditions *S. bicolor* seed radicles are capable of rapid multiplication (as seen in Table 3). The extracts and positive controls at different concentrations inhibited proliferation by reducing the rate of germination of the seeds as observed from the reducing seed radicle lengths within the incubation period of 24-96 h when compared to those of the negative control seeds which increased progressively. A correlation between

rate of germination and inhibition of proliferation has been reported earlier [22].

Kigelia africana extracts exhibited various degrees of antiproliferative activity. The antiproliferative effect of the *K. africana* leaf extract was not significantly time dependent from 2-8 mg/mL. However, a significant change in inhibitory activity with time was observed at 16 mg/mL, 32 mg/mL and 64 mg/mL at 95% confidence interval with 64 mg/mL having the highest inhibitory activity (98.9 ± 0.7) % at 96 h. The antiproliferative activity of *K. africana* leaf extract at 16 mg/mL, 32 mg/mL and 64 mg/mL was comparable to the activity of Methotrexate (positive controls) at 95% confidence interval (Fig. 8). The stem bark and root exhibited non-linear time and non-linear concentration dependent antiproliferative activities as no significant differences in activities with changes in concentration/time was observed at $P < 0.05$. All concentrations showed effective activities ranging from 72.0 ± 1.4% - 90.0 ± 2.4% for stem bark and 50.3 ± 1.5% - 97.7 ± 0.4% for the root samples. Methotrexate was however found to be significantly more ($p < 0.05$) effective than the stem bark (from 2-64 mg/mL) and root extracts (from 4 – 64 mg/mL) (Fig. 9 and 10).

The results of the study showed that the leaf, stem bark and root parts of *K. africana* are rich in phenolic acids. All morphological parts have been reported to contain phenolic compounds implicated in free radical scavenging and antiproliferation [24, 17]. In this study, the leaf of *Kigelia africana* exhibited the highest antiproliferative properties, this could be ascribed to the polyphenols contents of the leaf. Falode *et al.*, [12] reported the presences of quercetin, rutin, kaempferol, catechin, gallic acid, chlorogenic acid, ellagic acid, rosmarinic acid and caffeic acid in it. All morphological parts of *K. africana* have been reported to exhibit antiproliferative properties due to the presence of phytoconstituents such as kigelinone, norviburtinal, β-sitosterol, and isopinnatal richly contained and isolated from the stem bark of *K. africana* [20]. These constituents have been reported to have cytotoxic effects which in turn will reduce the rate of germination and also inhibit proliferation [20]. The root bark contains sterols such as stigmasterol, β-sitosterol, phenolic acids such as ferulic acid, the naphthoquinones such as kigelinone, isopinnatal, dehydro-alpha-lapachol and the phenylpropanoids-isocoumarins: 6-methoxymellein, kigelin and 6-demethylkigelin, p-coumaric acid, all of which are essential secondary metabolites implicated in antitumor properties of *K. africana* [26, 18].

CONCLUSION

The pharmacognostic data provided on *Kigelia africana* may be included in an official monograph of the plant for its proper identification and quality control. The HPLC analysis revealed the presence of phenolic compounds. The leaf, stem bark and root of the plant showed antiproliferative activity by

inhibiting the growth of *Sorghum bicolor* seed radicle.

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