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Polyphenol and Antioxidant Content of *Kigelia africana* Leaves from Ghana

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ABSTRACT

In this study we investigate the polyphenol and antioxidant content of *Kigelia africana* leaves from Ghana, West Africa. *K. africana* is a semi-deciduous tree that grows wild and its leaves and fruit are used as food and medicine by local residents. The aims of this study are to compare the content and tentatively identify the major polyphenols in methanolic (aq.) versus aqueous extracts of *K. africana* leaf and the antioxidant activity of these extracts. The polyphenol content of the methanolic (aq.) (20/80, v/v) extract of *Kigelia* was 1.3-fold greater than that of the aqueous extract and 2-fold greater than that of the methanolic (aq.) extract (80%) of spinach, Chinese cabbage or lettuce. According to the results of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, the methanolic (aq.) extract of *K. africana* leaves contained 6-fold more antioxidant activity than similar extracts of spinach, cabbage or lettuce. Of these four green leafy vegetables, the methanolic (aq.) extract of *K. africana* was the most potent in reducing NO production by LPS-stimulated macrophages (RAW 264.7) in culture. HPLC analysis showed that the dominant phenolic compounds in *K. africana* leaf were ellagic acid and caffeic acid (491 and 70 mg/100 g dry weight, respectively). In conclusion, this study demonstrates that the leaves of *K. africana* contain large amounts of polyphenolic compounds and antioxidants. These findings provide a basis for encouraging efforts to conserve this endangered species and promote further studies on its potential nutritional benefits to populations in sub-Saharan Africa.

Keywords: antioxidants, caffeic acid, ellagic acid, Ghana, *Kigelia africana*, polyphenols

INTRODUCTION

Although the nutritional value for humans of green leafy vegetables such as spinach, cabbage and lettuce is recognized worldwide, the healthful benefits of many other vegetables in the diets of populations in Ghana and elsewhere in sub-Saharan Africa are incompletely understood and underappreciated (Amisah and Aduasah 2002). A series of recently-published studies (Glew *et al*. 2009, 2010a, 2010b) documented the fact that 11 different species of green leafy vegetables which grow in Ghana and many other parts of Africa contain useful quantities of most of amino acids, fatty acids, and minerals and trace elements that are essential to humans. The present report extends those studies to include the content of antioxidants and polyphenols in the dark, green leaves of *Kigelia africana* (Lam.) Benth.

K. africana, called *nufuten* in the Twi language that is spoken in Ghana, grows throughout Africa. The name *nufuten*, translated as "hanging breast" in English, is derived from the long, narrow fruits which hang from the tree. The bark, roots, leaves and flowers of *K. africana* are used by traditional healers in Africa to treat a variety of illnesses including: iron-deficiency anemia, sickle cell anemia, epilepsy, protein-calorie malnutrition, and ailments of the liver and disorders of the digestive tract and cardiovascular and respiratory systems (Irvine 1961; Burkill 1985; Grace and Davis 2002). Birth attendants in rural areas prescribe consumption of *K. africana* leaves to lactating mothers to facilitate lactation. The young leaves of the tree are cooked in a sauce that accompanies the staple foods of the area, such as maize and yam. Herbalists use the bark of the tree to treat sexually-transmitted diseases.

We chose to study the antioxidant properties of *K. africana* since there are few published reports of this aspect of the leaves of this tree and because there is growing concern among conservationists in Ghana and elsewhere in Africa that the plant may be endangered. Furthermore, new knowledge about the biochemical composition of *K. africana* promises to illuminate the nutritional and medicinal significance of such substances contained the various structures of this tree. For example, in Ghana the young leaves of *K. africana* are used to make a palm-nut soup which is given to lactating women "to restore the richness of the blood" after childbirth and to enhance the volume and nutritional quality of their milk.

The information contained in this report builds on the findings of Olaleye and Rocha (2007, 2008) who reported on the free radical scavenging capacity of aqueous extracts of the leaves and fruit of *K. africana* growing in Nigeria. The information we present in this report regarding the antioxidants contained in of *K. africana* leaves should lead to a wider appreciation of the nutritional and medicinal potential of this green leafy vegetable and heighten awareness of the responsibility of national governments to ensure the availability and very perpetuation of this interesting enigmatic plant.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), HPLC standard compounds

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including caffeic acid, ellagic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were analytical grade.

Sources and preparation of plant specimens

The leaves were collected from *K. africana* trees in Kumasi, Ghana, rinsed with tap water to remove extraneous contamination, and sun-dried. The leaves were then ground into powders using a stainless-steel mill. Prior to analysis, the powder was dried for seven days to constant weight in a vacuum desiccator. Chinese cabbage (*Brassica rapa pekinensis*), spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*) were purchased in local markets in Hsinchu, Taiwan, dried for 24 h at 50°C, and finally ground to a fine powder with the aid of a mortar and pestle. *Kigelia* leaf and the three vegetable cultivars were extracted at room temperature with aqueous methanol (20/80, v/v) or distilled water at 1:10 (w/v) ratio with shaking at 100 rpm and 25°C for 24 h. The samples were centrifuged at 1,000 × *g*. The supernatants from the aqueous extracts were freeze-dried, whereas the supernatants from the methanolic (aq.) extracts were taken to dryness using a rotary evaporator at 40°C.

Determination of total phenolic compounds

The total phenolic content of the plant specimens was estimated using the Folin-Ciocalteu colorimetric assay (Singleton *et al*. 1999). After the dried methanolic (aq.) and aqueous extracts had been dissolved in methanol or distilled water, respectively, a 0.1 mL aliquot was transferred to a 10 mL test tube. One-half milliliter of undiluted Folin-Ciocalteu reagent and 1.5 mL of 20% (w/v) Na_2CO_3 in water were then added, followed by sufficient distilled water to bring the volume to 10 mL. Following mixing and a one-hour incubation at room temperature, the absorbance of the solution was measured at 760 nm. Gallic acid was used as the standard and results were expressed as mg/g gallic acid equivalent (GAE). The measurements were performed in triplicate.

The 2,2-diphenyl-1-picrylhydrazyl free-radical scavenging assay

The ability of the two different kinds of extracts of *K. africana* leaves and green leafy vegetables to neutralize the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined using a procedure described in the literature (Shimada *et al*. 1992). The sample extracts were dissolved in distilled water as described above and diluted to the appropriate concentration. Thirty µL of the diluted solutions were added to a 96-well plate and mixed with 0.12 mL of 0.5 mM DPPH reagent. Distilled water served as control and DPPH was replaced with distilled water to provide the blank. The plate was left to stand in the dark at room temperature for 30 min after which time the absorbance was measured by an ELISA reader at 517 nm (Anthos Zenyth 3100, Anthos Labtec Instruments, Austria). The DPPH scavenging activity was calculated as: % DPPH scavenging = [1 – (sample absorbance/ control absorbance)] × 100.

Best-fit lines were drawn through dose-response plots using a computer program and EC_{50} values (e.g., the amount of extract required to scavenge 50% of the DPPH radicals) were calculated: the smaller the EC_{50} value the greater the concentration of antioxidant.

HPLC analysis

The methanolic (aq.) and aqueous extracts of *K. africana* were resuspended in methanol or water, respectively, to a concentration of 5 mg/mL, clarified by centrifugation in an Eppendorf tube, and filtered through a 0.45 µm filter. The mobile phase contained 1.0% (v/v) acetic acid (solvent A) and acetonitrile (solvent B). Samples (10 µl) were chromatographed on a RP-C18 column (250 × 4.6 mm, Thermo Scientific (Waltham, MA, USA)) in a gradient mode: solvent B from 8 to 15% in 40 min, from 15 to 23% in 60 min, and 23 to 8% in 120 min with a flow rate of 0.8 mL/min and detection at 280 nm. Compounds were tentatively identified by comparing retention times and spike with standard compounds.

Cell culture and cell viability assay

The RAW 264.7 macrophage cell line was obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY, USA) with 10% (v/v) fetal bovine serum in an atmosphere containing 5% CO_2. The density of the RAW 264.7 cell suspension was adjusted to 1×10^6 cells/mL and a 0.1 mL aliquot of the cell suspension was transferred into each well in a 96-well culture plate. The methanolic (aq.) extracts were dissolved in ethanol, and the aqueous extracts were dissolved in distilled water and filtered using a Millex HA 0.24-µm filter (Millipore Corp., Bedford, MA, USA). The extracts were then diluted to the appropriate concentration with culture medium prior to the cell proliferation assay. After cell adhesion for 3 h, the cells were treated with different extracts. After 24 h of incubation, the medium in the wells was aspirated and replaced with 0.1 mL of aqueous MTT solution (0.5 mg/mL). The plate was incubated for 2 h after which time the medium in each well was aspirated and replaced with 0.1 mL of 0.04 N HCl/ isopropanol solution. The absorbance of the contents of each well was determined at 540 nm with the aid of an ELISA reader (Anthos Zenyth 3100, Anthos Labtec Instruments, Austria).

Determination of NO production by cultured macrophages

After adjusting the density of the RAW 264.7 cell suspension to 1×10^6 cell/mL, a 0.1 mL aliquot was introduced into each well of a 96-well plate. The cells were incubated for 3 hours and incubated in medium containing samples for another 24 h with or without LPS (0.1 µg/mL). Fifty microliters of cell medium and 50 µL of Griess reagent [1:1 mixture (v/v) of 1% (w/v) sulfanilamide and 0.1% (w/v) naphthylethylenediamine dihydrochloride in 5% H_3PO_4] were added to the wells and the absorbance was determined at 540 nm.

Statistical analyses

The significance of the differences between the sample values was analyzed by ANOVA and Bonferroni's test (SPSS for Windows 10.0; SPSS Inc., Chicago, IL). A *P* value of 0.5 was considered significant.

RESULTS

Comparison of the total phenolic content of extracts of *K. africana* leaves and three common green leafy vegetables

In general, the methanolic (aq.) extract of *K. africana* leaves contained about one-third more phenolic compounds than the corresponding aqueous extract, as determined by the Folin-Ciocalteu reagent (**Table 1**), and the aqueous methanol extract. The methanolic (aq.) and the aqueous extract of *K. africana* leaves contained 1.7 to 2.2-fold and 1.3 to 1.8-fold more total polyphenols, respectively, than those of spinach, cabbage or lettuce (**Table 1**).

Comparison of the DPPH radical scavenging capacity of extracts of *K. africana* leaves, Chinese cabbage, spinach and lettuce

The methanolic (aq.) and aqueous extracts of *K. africana* leaf both contained much larger amounts of free radical scavenging substances than several representative examples of common green leafy vegetables (**Table 2**). Methanolic (aq.) extracts of *K. africana* leaves contained at least 6-fold more free radical scavenging capacity than the corresponding extracts of the three common green leafy vegetables, namely Chinese cabbage, spinach and lettuce.

The aqueous extract of *K. africana* leaves contained precisely half the free radical scavenging activity as the methanolic (aq.) extract of the same plant (**Table 2**). However, the aqueous extracts of Chinese cabbage, spinach and

Table 1 Comparison of the total phenolic contents of *Kigelia africana*, Chinese cabbage, spinach and lettuce.

Sample	Content (GAE, mg/g dry leaves)	
	Methanol: water (80/20) extract	Water extract
Kigelia africana	83.0 ± 2.2 a	60.9 ± 1.5 a
Chinese cabbage	49.5 ± 2.1 b	45.5 ± 1.5 b
Spinach	48.4 ± 1.4 b	41.6 ± 3.5 bd
Lettuce	38.0 ± 0.7 c	34.6 ± 1.0 cd

[a]All data are expressed as mean ± standard deviation of triplicate analyses. Means in the same row with different lower case letters (a, b, c, and d) are significantly different ($P < 0.05$).

Table 2 The DPPH radical-scavenging activity (effective concentration EC_{50}) of extracts of *Kigelia africana*, Chinese cabbage, spinach and lettuce.

Sample	DPPH radical-scavenging activity EC_{50} (mg/mL)	
	Methanol extract	Water extract
Kigelia africana	0.44 ± 0.03 aA	0.88 B
Chinese cabbage	2.80 ± 0.10 b	ND
Spinach	2.79 ± 0.24 b	ND
Lettuce	>4 c	ND

Means in the same row with different upper case letters (A and B) or in the same column with different lower case letters (a and b) were significantly different ($P < 0.05$).

lettuce did not contain detectable antioxidant activity in the DPPH assay. Thus, in general, the methanolic (aq.) extract of *K. africana* was richer in polyphenolic compounds and higher antioxidant activity compared to the aqueous extract.

Characterization of phenolic compounds in extracts of *K. africana* using HPLC

In an effort to identify the major phenolic antioxidants in *K. africana* leaf, the methanolic (aq.) extract of said leaves was analyzed by HPLC (**Fig. 1A**). The most prominent peak co-eluted precisely with the ellagic acid standard (54.6 min). A second, smaller peak eluting at 23.1 min was identified as caffeic acid. The other minor peaks were not identified. Caffeic acid accounted for 7.96% of the total phenolic content and ellagic acid accounted for 36.2% of the total phenolics. The absolute amounts of caffeic acid and ellagic acid in the methanolic (aq.) extracts was estimated by calibrating the HPLC column with standards. The quantities of caffeic acid and ellagic acid in the methanolic (aq.) extract of *K. africana* amounted to 70 mg/g and 491 mg/g dry weight of leaves, respectively. The corresponding aqueous extracts of *K. africana* leaf were also subjected to analysis by HPLC (**Fig. 1B**); however, the peak intensities at 280 nm were barely detectable when compared with the chromatogram obtained when the same mass of methanolic (aq.) extract was put through the column (10 µl of a 10 mg/mL solution). This result indicates that the high content of free radical scavenging substances in aqueous extracts of *K. africana* leaf (**Table 2**) are unlikely to be due to polyphenolic compounds which absorb at 280 nm. Additional studies are required to identify the nature of these compounds.

Inhibition of NO production by cultured macrophages

A preliminary study of the cytotoxicity of *K. africana* leaf extracts showed that the methanolic (aq.) extract reduced the viability of RAW 264.7 macrophages in a dose-dependent manner. Cell viability significantly decreased when the cells were exposed to 200 µg/mL or greater concentrations of the methanolic (aq.) extract of the leaves (data not shown). In contrast, the aqueous extract of *K. africana* leaves was much less toxic to these cultured cells than the corresponding methanolic (aq.) extract; greater than 90% of the cells remained viable when incubated for 24 h with final concentrations of aqueous extract in the 100 to 800 µg/mL range (data not shown).

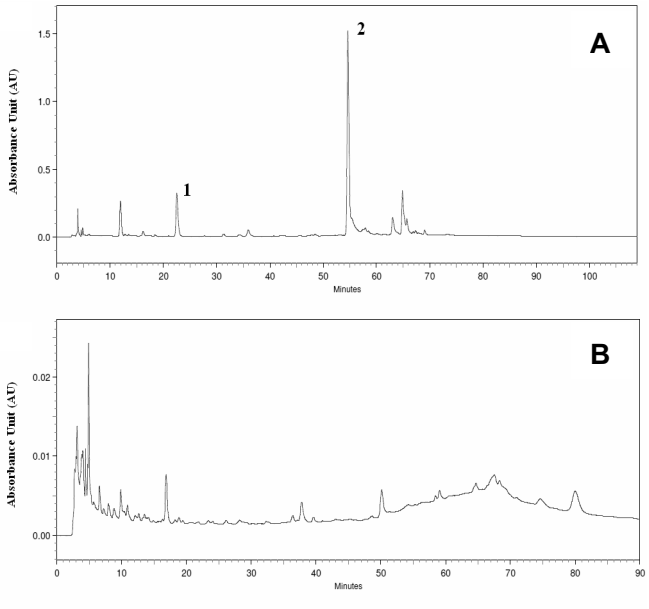

Fig. 1 HPLC chromatogram of *Kigelia africana* phenolics at 280 nm. (**A**) Methanolic (aq) extract of *Kigelia*. Peaks: 1 – caffeic acid; 2 – ellagic acid. (**B**) Aqueous extract of *Kigelia*.

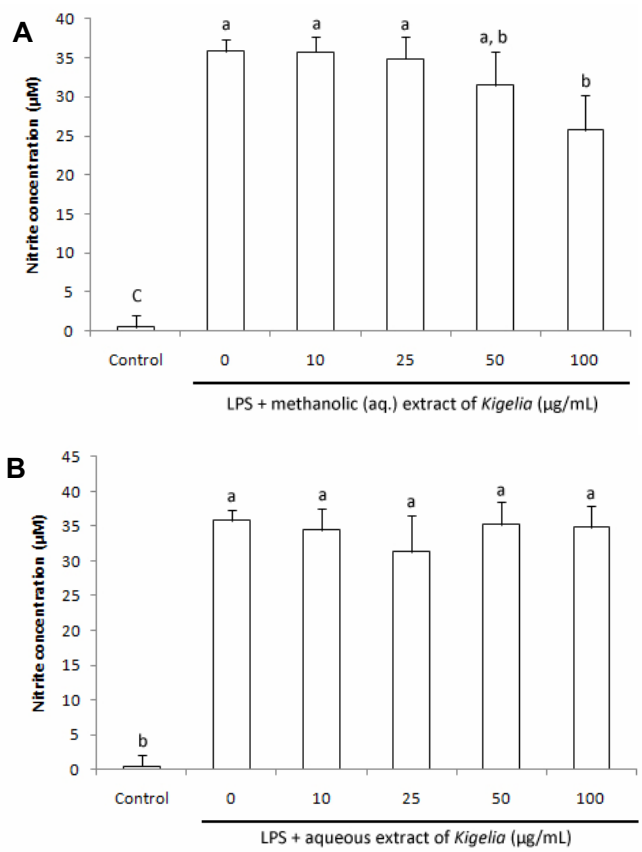

Fig. 2 Effects of *Kigelia africana* extract on NO production in LPS-stimulated RAW 264.7 cells. Cells were incubated with different concentration of (**A**) methanolic (aq.) extract (**B**) aqueous extract. Values are expressed as means ± S.D. ($n = 3$) of triplicate tests. Means with different letters were significantly different ($P < 0.05$).

The addition of LPS to cultured RAW 264.7 cells stimulated NO production (**Fig. 2**). Addition of methanolic (aq.) extract of *Kigelia* to the cells reduced NO production in a dose-dependant manner (**Fig. 2A**); a 35% reduction in NO production was obtained with 100 µg/mL of the methanolic (aq.) extract. However, the addition of aqueous extract of *K. africana* at 100 µg/ml to RAW 264.7 cells did not inhibit NO production (**Fig. 2B**).

DISCUSSION

Since the leaves of *K. africana* are used as a food and medicine and because they appear to contribute to overall human well-being, we considered it timely to begin to explore the basis of these benefits. Furthermore, because the tree is less commonly found in Ghanaian forests, without continued research into its chemical composition local knowledge about its uses may also begin to fade from memory.

The main result of this study was the finding that methanolic (aq.) and aqueous extracts of *K. africana* from Ghana contained large quantities of phenolic compounds and other substances capable of scavenging free radicals. The information in **Table 1** shows that in terms of its content of total phenolic compounds, as determined using the Folin-Ciocalteu reagent, *K. africana* leaf compares favorably to several of the common green leafy vegetables such as Chinese cabbage, spinach and lettuce that are consumed in most places in Africa as well as elsewhere in the world. We also found that the methanolic (aq.) extract of *K. africana* leaves contained more phenolic compounds and antioxidant activity than the aqueous extract (**Table 1**).

The polyphenol content we found for lettuce is in the range of values reported by Liu and colleagues (Liu 2004) who analyzed 12 lettuce varieties. The amount of free radical scavenging substances we found in the methanolic (aq.) extract of spinach also agrees with the results reported by Ismail and colleagues (Ismail *et al*. 2004).

HPLC analysis of the phenolic compounds present in the methanolic (aq.) extract of *K. africana* leaves revealed that ellagic acid was the major phenolic compound and that caffeic acid was the second most abundant phenolic compound, accounting for 36.2% and 7.96%, respectively, of the total phenolic compounds. Ellagic acid is an antioxidant that is commonly found in a variety of berry fruits, such as blackberry, raspberry, strawberry and pomegranate (de Ancos *et al*. 2000). Ellagic acid has several beneficial physiological effects on mammalian cells, tissues and animal models, including inhibition of lipid peroxidation (Osawa *et al*. 1987) and protection of mice against dextran sulfate sodium (DSS)-induced colitis in a microsphere model (Ogawa *et al*. 2002). Caffeic acid, the second most abundant phenolic compound in the methanolic extract of *K. africana* leaf, had also been found in dried fruit of *K. africana* (Picerno *et al*. 2005). Caffeic acid, too, has the capacity to scavenge free radicals (Chen and Ho 1997).

In this study, we also tested the anti-inflammatory activity of the two different kinds of *K. africana* leaf extracts. The NO radical has several important biological functions including vasodilation, neurotransmission, and platelet aggregation (Kim *et al*. 2005), as well as inflammation. However, overproduction of NO has been implicated in atherosclerosis (Naseem 2005) and carcinogenesis (Tamir and Tannenbaum 1996). The ethanolic stem bark extract and the polar fruit extract of *K. africana* already had been shown to have anti-inflammatory activities (Picerno *et al*. 2005; Owolabi and Omogbai 2007). Our cultured cell assay showed that *K. africana* leaves also contained anti-inflammatory compounds: the methanolic extract of *K. africana* leaf was more potent in inhibiting NO production than the aqueous extract of the *K. africana* and the ability of the *K. africana* extracts to inhibit NO production were correlated to their polyphenolic content. As revealed by HPLC chromatography, ellagic acid and caffeic acid were the dominant phenolic compounds in the methanolic (aq.) extract of *K. africana*; their relative contribution to the anti-inflammatory activity will be further analyzed in a future study.

The data generated in this study should provide government officials with a scientific basis for promoting efforts aimed at conserving *K. africana.* In addition, the information in the present report, together with our recently-published study documenting the presence of nutritionally useful quantities of essential amino acids, fatty acids and minerals and trace elements in *K. africana* leaf (Glew *et al*. 2010a), should encourage further research on the plant so nutritionists, dieticians and other kinds of public health professionals may, if warranted, educate the populations they serve about the potential health benefits of including *K. africana* leaves in their diet.

A limitation of the present study is that there were several minor peaks in the HPLC chromatogram (**Fig. 1**) that we did not identify, in part because of the limited number of standards available to us. Phytochemicals such as verminoside and kigelinone have been reported in the stem bark of *K. africana* (Gabriel and Olubunmi 2009). In addition, we did not assess the bioavailability of the phenolic compounds and antioxidants in *K. africana* leaf. However, we plan to address these issues in future studies. We are also interested in investigating the antimicrobial properties of extracts of *K. africana.*

CONCLUSION

We have demonstrated that the leaves of *K. africana* contain considerably greater amounts of polyphenols and antioxidant substances than some common green leafy vegetables. Thus, as a dietary ingredient, *K. africana* may be helpful in decreasing the risk of certain diseases in which free radicals have been implicated, including cardiovascular diseases and cancer (Liu 2004).

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