

Full Length Research Paper

Chromatographic characterisation, *in vitro* antioxidant and free radical scavenging activities of *Garcinia kola* seeds

Tebekeme Okoko

¹Biochemistry Division, Department of Chemical Sciences, Niger Delta University, PMB 71, Wilberforce Island, Bayelsa State, Nigeria. E-mail: tebebuddy@yahoo.com.

Accepted 1 October, 2009

***Garcinia kola* Heckel, a tropical plant which grows in moist forest, has found wide applications in traditional medicine especially in the West and Central African sub-region. The seeds have been demonstrated to possess numerous bioactivities but research is highly limited on the link between its fractions and the bioactivities. In this work, the methanolic extract of *G. kola* seeds was subjected to silica gel column chromatography into five fractions ME1 – ME5 and the free radical scavenging activities and antioxidant potentials were determined for each fraction using various *in vitro* models. The ME4 fraction possessed the greatest activities. It was also demonstrated that the ME4 fraction strongly inhibited nitric oxide production in lipopolysaccharide-activated macrophage U937 cells. Chromatographic fractionation and spectroscopic analysis of the ME4 fraction revealed the presence of four compounds namely garcinia biflavonoids GB1 and GB2, garcinal and garcinoic acid. These findings show that these four compounds are partly responsible for the great antioxidant potential of *G. kola* seeds. This gives further evidence to the nutraceutical and pharmaceutical potential of *G. kola*.**

Key words: *Garcinia kola*, bioactivities, antioxidant, nitric oxide, nutraceutical.

INTRODUCTION

The use of plants as alternative/complementary medicine has been in existence for since antiquity. It is believed that the use of plants for medicinal purposes has been associated with less frequent side effects. Nigeria and the West African sub-region are blessed with a lot of floral diversity thus natural medicinal potentials abound.

Garcinia kola Heckel (Gutifera) commonly called bitter kola is a medium-sized evergreen tree which grows to about 15 – 17 m (Plowden, 1972). A more vivid description of the plant is made by Iwu (1993). The seeds are highly valued and consumed as stimulant because of the bitter taste (Atawodi et al., 1995). The seeds have been consumed as ailment for bronchitis, diarrhoea, and throat infections (Adesina et al., 1995; Orié and Ekon, 1993). Other traditional uses include the consumption as aphrodisiac, antipurgative, and as remedy against cough (Madubunyi, 1995; Okunji and Iwu, 1991). The seeds of the plant have been reported to have various bioactivities such as antihepatotoxic (Akintowa and Essien, 1990; Adaramoye and Adeyemi, 2006), antinephrotoxic (Okoko

and Awhin, 2007), and antimicrobial (Adefule-Ositelu et al., 2004). All these have been ascribed principally to flavonoids especially the biflavanones and kolaflavanones whose antioxidant properties have been profusely reported (Adaramoye and Adeyemi, 2006; Terashima et al., 2002). Most of the studies on *G. kola* activities have been done using crude extracts. In this current study, the crude extract was further fractionated and the antioxidant potentials of the various fractions determined using various antioxidant models. This stems from the fact that there is little research on the potencies of the various fractions.

The ability of some of the purified extracts to inhibit lipopolysaccharide – induced release of nitric oxide from the macrophage cell line U937 was also determined. Lipopolysaccharide is a glycolipid and its ability to induce the release of nitric oxide from macrophages has been reported. The release of nitric oxide is a primary event in the activation of macrophages as part of the inflammatory process (Meng and Lowell, 1997).

MATERIALS AND METHODS

Materials

Culture media RPMI-1640 were obtained from the European collection of cell cultures (Salisbury, UK). All other chemicals were of the highest grade thus were used without further purification and are available commercially. All dilutions were made in double glass-distilled water unless otherwise stated.

G. kola seeds were obtained fresh from the Obunagha forest, Bayelsa State, Nigeria. The seed coats were removed and the seeds were cut to smaller bits and sun-dried. The dried seeds were later ground using a warring blender and extraction done in a soxhlet extractor using absolute methanol and concentrated in a rotary evaporator at 40°C. A residue of 153 g was finally obtained.

Fractionation of extract

A 100 g portion of the extract was dissolved in methanol : water (50:50) and subjected to silica gel column chromatography and eluted with methanol/water by 0/100 – 100/0 gradient elution into 5 sub-fractions (ME1 – ME5). Thereafter, the fraction ME4 (400 mg) was later subjected to high performance liquid chromatographic (HPLC) analysis and separated. Elution was effected with acetonitrile/water gradient (50/50, 70/30, 90/10, 100/0) at 15 min intervals at a flow rate of 3.5 mL/min under room temperature and detected by a UV detector.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of the fractions ME1 – ME5 was determined by titrimetric method of Zhao et al. (2006). Briefly, 1 mL of 0.1 mM hydrogen peroxide was mixed with 1 mL of each concentrated fraction (1%), 2 drops of ammonium molybdate (3%), 10 mL of sulphuric acid (2 M) and 7 mL of potassium iodide (1.8 M). This solution was titrated with 5.09 mM sodium thiosulphate until the yellow colour disappeared. The hydrogen peroxide scavenging activity was expressed as a percentage thus:

% scavenging activity = $[(V_{\text{control}} - V_{\text{sample}})/V_{\text{control}}] \times 100$ Ascorbic acid was used as a reference antioxidant.

Metal chelating activity

The metal chelating potential of all the fractions was determined according to Dinis et al. (1994) with some modification. Assay mixture was made up of 0.90 mL of sample (1%), and 0.04 mL of ferrous chloride (2 mM). After 30 s, 0.06 mL of ferrozine (5 mM) was added to the mixture to start the reaction at room temperature. The absorbance was read at 562 nm spectrophotometrically. The metal chelating activity was expressed as per cent ferrous ion chelating activity. Ethylenediaminetetraacetic acid (0.02 M) was used as a reference metal chelator.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of each fraction was determined based on the Fenton reaction as described by Yu et al. (2004) with a slight modification. The assay mixture was made up of 0.02 mL of ferrous chloride (0.02M), 0.5 mL of 1, 10 phenanthroline (0.04 M), 1 mL of phosphate buffer (0.2 M, pH 7.2), and 1 mL of the sample. The reaction was initiated by the addition of 0.05 mL of hydrogen peroxide (7 mM). After 5 min incubation under room temperature, absorbance was measured at 560 nm. Hydroxyl radi-

cal scavenging activity was expressed as described for the hydrogen peroxide scavenging activity. Vitamin C was used as a reference also in this case.

Cell culture and nitric oxide inhibitory assay

The macrophage cell line U937 was cultured in RPMI-1640 supplemented with 50 mL heat-inactivated fetal calf serum, 5 mL of 100 U/mL penicillin, 5 mL of 0.02 M L-glutamine and 5 mL of 100 µg/mL streptomycin (otherwise called the complete medium) and kept in the incubator gassing up to 5% carbon dioxide at 37°C. Cells were later seeded at 5×10^5 cells/mL and 200 µL was delivered into the wells of 96-well coaster plate. Thereafter, 20 µL of phorbol 12-myristate, 13-acetate (100 ng/mL) was added to each well and incubated for 48 h. The supernatants were removed and the cells were incubated with or without 100 µL of lipopolysaccharide (5 µg/mL) in the presence or absence of the various fractions. Twenty four hours later, the supernatants of each of the cell culture was analyzed for nitric oxide inhibitory activities by the Greiss reaction according to Hwang et al. (2002). Values were expressed as % nitric oxide inhibition.

Statistical analysis

The antioxidant and free radical scavenging studies for each experiment was done four times. Where appropriate, the data were subjected to either analysis of variance (ANOVA) or two-tailed students' t- test. Significance was set at $P < 0.05$.

RESULTS

The hydrogen peroxide scavenging activities of the fractions and vitamin C are shown in Figure 1. All the fractions exhibited significant hydrogen peroxide scavenging activities which increased with concentration. The fraction ME4 had a higher hydrogen peroxide scavenging activity than the other fractions though vitamin C which is a known and well-studied antioxidant had the highest activity. The hydrogen peroxide scavenging activities is in the order Vitamin C > ME4 > ME5 > ME2 > ME1 > ME3 and this ability was significantly different from one another ($P < 0.05$).

The metal chelating activities of vitamin C and the various purified extracts are also shown in Figure 2. Ethylenediaminetetraacetate (EDTA) is a well-known metal chelating agent and possessed 100% activity on chelating Fe^{2+} ions (result not shown in Figure 2). The metal chelating activity is in the order EDTA > ME4 > ME5 > ME1 > ME2 > ME1 > ME3 with significant differences among the groups ($P < 0.05$).

There were significant differences in the hydroxyl radical scavenging activities of the purified fractions ($P < 0.05$). The order of the hydroxyl radical scavenging activity was ME4 > ME5 > vitamin C > ME3 > ME2 > ME1 (Figure 3).

Since the fractions ME4 and ME5 possessed higher activities than the other purified fractions, the capacity of these purified fractions to reduce lipopolysaccharide-induced release of nitric oxide in macrophage U937 cell

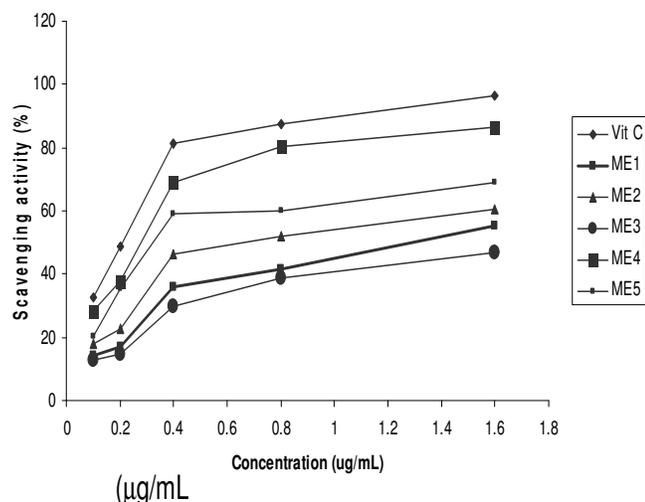


Figure 1. Hydrogen peroxide scavenging activities of the fractions and vitamin C. Each value is the mean \pm SEM of three replicates.

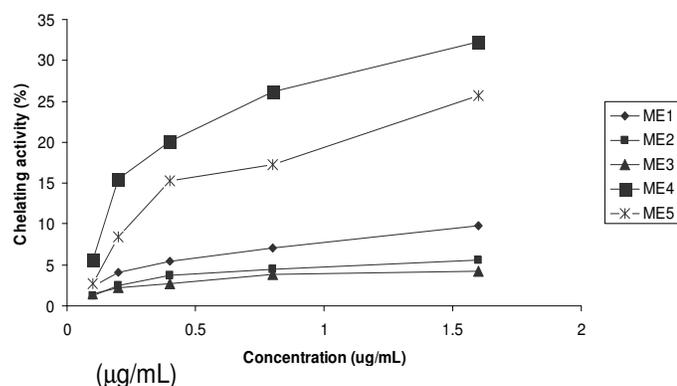


Figure 2. Metal chelating activities of the fractions. Each value is the mean \pm SEM of three replicates.

was also investigated. As expected, there was a concentration-dependent inhibition of nitric oxide production with ME4 possessing a significantly higher activity ($P < 0.05$) (Table 1).

DISCUSSION

Antioxidants such as dietary flavonoids work by scavenging free radicals, chelation of metal ferrous ions, and decomposition of peroxides such as lipid peroxides (Dufresne and Farnworth, 2001). It has been suggested that the antioxidant effect of *G. kola* is ascribed partly to the possession of biflavonoids (Farombi et al., 2002). Thus they are thought to play important role in the prevention of various human disorders as free radical scavengers. Some of these compounds have been shown to have antioxidant traits 1.5 times that of di- α -

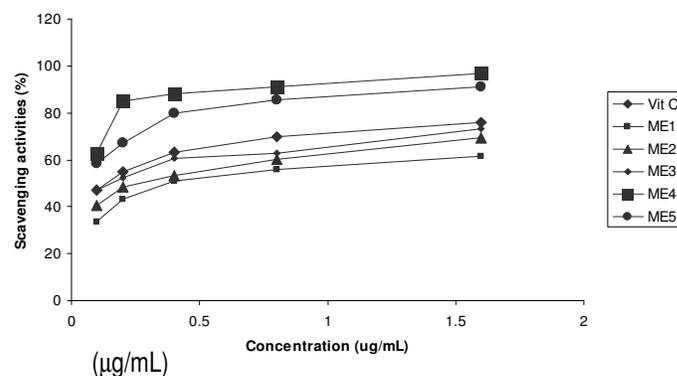


Figure 3. Hydroxyl radicals activities of the fractions. Each value is the mean \pm SEM of three replicates.

Table 1. Nitric oxide inhibitory activity of ME4 and ME5.

Concentration ($\mu\text{g/mL}$)	% Inhibition	
	ME4	ME5*
1.0	12.5 \pm 2.76	9.5 \pm 3.64
2.5	26.9 \pm 3.34	15.6 \pm 3.11
5.0	43.6 \pm 3.07	28.2 \pm 2.48
10.0	56.9 \pm 5.51	40.4 \pm 1.96
20.0	77.7 \pm 4.54	58.8 \pm 2.55

Values are presented as mean \pm SEM. * Significantly different from ME4 ($n = 05$).12, $P < 0$.

ocopherol by the bleomycin-Fe assay (Terashima et al., 2002). Free radicals are generated by some metal catalyzed oxidation reactions and the products have the ability of causing peroxidation of membrane lipids. A classical example is the hydroxyl radical which attacks all proteins, membrane lipids and in fact any biomolecule it touches (Aruoma, 1999). Thus the observed hydroxyl radical scavenging activities of the various fractions of *G. kola* is a clear signal that they may also prevent the biomembranes and biomolecules from being attacked by the free radicals (Farombi, 2000). Nitric oxide is a compound produced by macrophages in the course of the inflammatory response (Surh et al., 2001). It is mutagenic and can interfere with DNA repair processes (Keefer and Wink, 1996). Lipopolysaccharide is a glycolipid which induces the release of proinflammatory cytokines such as TNF- α , IL-6 and the signal compound nitric oxide from macrophages (Park et al., 2000; Meng and Lowell, 1997).

The ability of the fraction ME4 and ME5 to reduce the lipopolysaccharide-induced release of nitric oxide was investigated. Both purified fractions possessed inhibitory activities with ME4 possessing a higher potential significantly ($P < 0.05$, Table 1). Nitric oxide is derived from the oxidation of L-arginine in a reaction catalyzed by nitric oxide synthase which has three isoenzymatic forms;

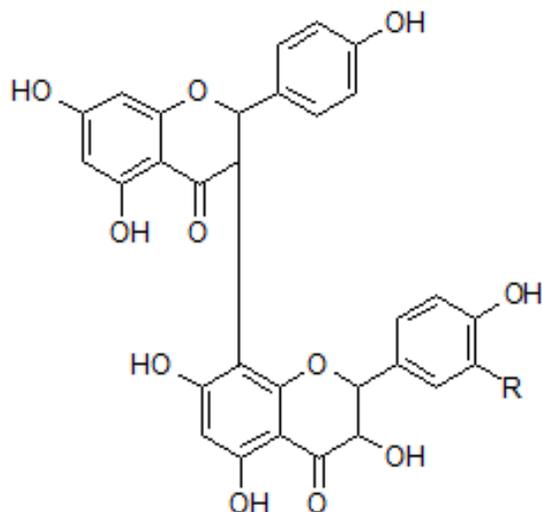


Figure 4. Structure of the garcinia biflavonoids (GB1, R is H; GB2, R is OH).

inducible nitric oxide synthase (iNOS), endothelial NOS, and neuronal NOS; however it is iNOS that exists in macrophages. Abnormal expression of iNOS has been associated with some inflammatory disorders and cancer thus the suppression of its activity may be crucial in the pathogenesis of these disorders. It has been reported that polyphenolic compounds suppress the activity of iNOS (Hsieh et al., 2007) and this may be the mechanism of action of the ME4 and ME5. In fact, the antioxidant activities of flavonoids improve endothelial function by lowering oxidative stress and stimulation of the anti-inflammatory response (Lin et al., 1999). All the above show that the fraction ME4 is more potent in protecting tissues. Thus ME4 was subjected to high performance liquid chromatography. Four compounds were isolated and characterized. After analyzing the compounds via MS and NMR, the compounds were identified as garcinia biflavonoids GB1 and GB2 (Iwu, 1985) (Figure 4), and the chromanols garcinal and garcinoic acid (Terashima et al., 2002). This shows that the famous kolaviron (a mixture of the compounds GB1, GB2, and kolaviron) solicited as progress in this area will extend the frontiers of exploiting the plant for nutraceutical and pharmaceutical purposes. is present in the ME4 fraction. All the compounds are polyphenols hence are suggested to be the major factor responsible for the immense bioactivity of the ME4 fraction. It is clear that all the fractions of *G. kola* Heckel studied have considerable antioxidant activities in the *in vitro* models, however further studies on *G. kola* seeds are

REFERENCES

Adaramoye OA, Adeyemi EO (2006). Hepatoprotection of D-galactosamine-induced toxicity in mice by purified fractions from *Garcinia kola* seeds. *Basic Clin. Pharmacol. Toxicol.* 98: 135-141.

- Adefule-Ositelu AO, Adefule AK, Oosa BO, Onyene PC (2004). Antifungal activity of *Garcinia kola* nut extract as an ocular bacterial isolates in Lagos, Niger. *Qrtly. J. Hosp. Med.* 14: 112-114.
- Adesina SK, Gbile ZO, Odukoya OA, Akinwusi DD, Illoh HC, Jayeola AA (1995). Survey of indigenous useful plants of West Africa with special emphasis on medicinal plants and issues associated with their management. The United Nations University Programme on natural resources in Africa; 2nd edn. pp. 84-85.
- Akintowa A, Essien A (1990). Protective effects of *Garcinia kola* seed extracts against paracetamol-induced hepatotoxicity in rats. *J. Ethnopharmacol* 29: 207-11.
- Aruoma OI (1999). Free radicals and international nutrition; Asia Pacific. *J. Clin. Nutr.* 8: 53-63.
- Atawodi S, Mende P, Pfundstein B, Preussmann R, Spiegelhalter B (1995). Nitrosatable amines and nitrosamide formation in natural stimulants, *Cola acuminata*, *Cola nitida* and *Garcinia kola*. *Food Chem. Toxicol.* 33: 625-630.
- Dinis TCP, Maderia VM, Almeida LM (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch. Biochem. Biophys.* 315: 161-169.
- Dufresne CJ, Farnsworth ER (2001). A review of the latest findings on health promoting properties in tea. *J. Nutr. Biochem.* 12: 404-421.
- Farombi EO (2000). Mechanisms of the hepatoprotective action of kolaviron: studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbon tetrachloride-treated rats. *Pharmacol. Res.* 42: 75-80.
- Farombi EO, Akuru TO, Alabi MC (2002). Kolaviron modulates cellular redox status and impairment of membrane protein activities by potassium bromate in rats. *Pharmacol. Res.* 45: 63-68.
- Hsieh YH, Kuo PM, Chien SC, Shyur LF, Wang SY (2007). Effects of *Chamaecyparis formosensis* Matsumura extractivities on lipopolysaccharide-induced release of nitric oxide. *Phytomed.* 14: 675-680.
- Hwang BY, Lee JH, Koo TH, Hong YS, Ro IS, Lee KS, Lee JJ (2002). Furanoligularenone, an eremophilane from *Ligularia fischeri* inhibits the lipopolysaccharide-induced production of nitric oxide and prostaglandin E2 in crophage RAW264.7 cells. *Planta Med.* 68: 101-105.
- Iwu MM (1985). Antihepatotoxic constituents of *Garcinia kola* seeds. *Experientia* 41: 699-700.
- Iwu MM (1993). Handbook of African medicinal plants. CRC Press, London, pp. 183-184.
- Keefer LK, Wink D (1996). DNA damage and nitric oxide. *Adv. Exp. Med. Biol.* 387: 177-185.
- Lin YL, Tsai SH, Lin-Shiau SY, Ho CT, Lin JK (1999). Theaflavin-3, 3'-digallate from black tea blocks the nitric oxide down regulating the activation of NF- κ B in macrophages. *Eur. J. Pharmacol.* 367: 379-388.
- Madubunyi II (1995). Antimicrobial activities of the constituents of *Garcinia kola* Seeds. *Intern. J. Pharmacog.* 33: 232-237.
- Meng F, Lowell CA (1997). Lipopolysaccharide-induced macrophage activation and signal transduction in the absence of Src family kinases Hck, Fgr, and Lyn. *J. Exp. Med.* 189: 1661-1670.
- Okoko T, Awhin EP (2007). *Garcinia kola* extract reduced cisplatin-induced kidney dysfunction in rats. *Afr. J. Biochem. Res.* 1: 124-126.
- Okunji CO, Iwu MM (1991). Molluscicidal activity of *Garcinia kola* biflavanones. *Fitoterapia* 62: 74-76.
- Orie NN, Ekan EU (1993). The Bronchodilatory Effect of *Garcinia kola*. *East Afr. Med. J.* 70: 143-145.
- Park YC, Rimbach G, Saliou C, Valacchi G, Packer L (2000). Activity of monomeric, dimeric, and trimeric flavonoids on NO production, TNF- α secretion, and NF κ B-dependent gene expression in RAW 264.7 macrophages. *FEBS Letters* 465: 93-97.
- Plowden CC (1972). A manual of plants names. 3rd ed. London. George Ltd. p. 239.
- Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS (2001). Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF- κ B activation. *Mut. Res.* 480: 243-268.
- Terashima K, Takaya Y, Niwa M (2002). Powerful antioxidative agents based on garcinoic acid from *Garcinia kola*. *Bioorg. Med. Chem.* 10:

1619-1625.

Yu W, Zhao Y, Shu B (2004). The radical scavenging activities of radix puerariae isoflavonoids: a chemiluminescence study. Food Chem. 86: 525-52

Zhao GR, Xiang ZJ, Ye TX, Yuan YJ, Guo ZX (2006). Antioxidant activities of *Salvia miltiorrhiza* and *Panax notoginseng*. Food Chem. 99: 676-774.