COMPARATIVE STUDY ON THE ANTIOXIDANT PROPERTIES OF FLAVONOIDS OF *GARCINIA* KOLA SEEDS

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ABSTRACT

Objective: Oxidative damage has been suggested to be a contributory factor in the development and complications of atherosclerosis, and of recent the beneficial effects of antioxidants against some pathologies have gained considerable interest. We evaluated the protective effects of flavonoids from *Garcinia* kola seeds on the oxidation of human low- density lipoprotein (LDL) and their ability to scavenge reactive oxygen species (ROS) *in vitro*.

Setting: Department of internal medicine, Faculty of Medicine & Health Sciences, UAE University, Al Ain, UAE.

Methods: Human low- density lipoprotein (LDL) was prepared from fresh blood donated by volunteers with informed consent. The inhibitory effect of varying concentrations of flavonoids from *Garcinia* kola seeds, namely; Kolaviron (KV), *Garcinia_biflavanone* (GB) 1, GB 2 and kolaflavanone (KF) on Fe/ ascorbate- induced peroxidation in LDL was investigated. Likewise the scavenging effect of these flavonoids on superoxide radicals and hydrogen peroxide *in vitro* was examined.

Results: In the present study, KV, GB1, GB2 and KF exhibited marked scavenging effect (41- 93 %) on H_2O_2, *in vitro* and the scavenging activities of these flavonoids were concentration dependent. Furthermore, KV, GB1, GB2 and KF showed marked activity as superoxide radicals ($O_2^{\cdot}$) scavenger. At 1 mg/ ml, KV, GB1, GB2 and KF scavenged $O_2^{\cdot}$ by 71%, 52 %, 66 % and 48 %, respectively. In addition, KV, GB1, GB2 and KF showed marked reducing property on potassium ferricyanide *in vitro*. KV, GB1 and GB2 were effective at preventing LDL lipid peroxidation (LPO) induced by Fe/ ascorbate system. Specifically at 0.01 mM, 0.1 mM and 1.0 mM, KV inhibited LPO by 29 %, 61 % and 79 %, while GB1 inhibited LPO by 29 %, 56 % and 86 %, respectively. At these concentrations, GB2 reduced LPO by 41 %, 72 % and 91 %, respectively. However, the inhibition of LDL peroxidation by KV, GB1 and GB2 were concentrations dependent. KF showed significant ($p<0.05$) inhibition of LDL peroxidation at 1.0 mM only. The inhibition of LDL peroxidation by these flavonoids follow the trend GB2 > GB1 > KV > KF at 1.0 mM.

Conclusion: These flavonoids are active scavengers of H_2O_2 and $O_2^{\cdot}$ radicals *in vitro* and their anti-lipoperoxidative effect might be linked to free radical scavenging ability, and this could be a pointer to their possible anti- atherogenic property.

KEYWORDS: Flavonoids, *Garcinia* kola, lipid peroxidation, reactive oxygen species, Antioxidant

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INTRODUCTION

Active oxygen and free radicals are related to various physiological and pathological events, such as inflammation, aging, mutagenicity and carcinogenicity.[1]

During the last decade, evidences abound to show that the free radical- mediated lipid peroxidation plays a crucial and causative role in the pathogenesis of atherosclerosis.[2] It is currently believed that lipid peroxidation is involved in the oxidative modification of low-density lipoprotein (LDL).[3]

It is important to note that oxidized LDL contains highly cytotoxic lipid peroxidation products such as certain aldehydes, which can

be considered as diffusible toxins.[4] Oxidized LDL deposited in the arterial wall may release these biologically active compounds continuously, irritating the endothelial cell layer and causing a range of other effects that may contribute toward the development of several pathologies.[5] LDL oxidation can be initiated *in vitro* by incubating LDL with macrophages, endothelial cells, smooth muscle cells and lymphocytes or in cell- free systems utilizing a variety of pro- oxidants.[6,7] The mechanism of LDL oxidation *in vivo* is largely a matter of speculation. LDL oxidation as a lipid peroxidation process leads to increase atherogenicity of LDL. It follows that antioxidant status should have a major impact not only on the rate of LDL oxidation but perhaps also on the development of atherosclerosis.[8,9] Since oxidized LDL seems to play a role in the development of atherosclerosis, antioxidants may be a therapeutic option. Probucol[10], ß- carotene[11] and vitamin E[12] are known to influence the antioxidation of LDL.

The natural antioxidants discovered recently are expected to replace the synthetic antioxidants that are widely used at present time. Antioxidants from natural substances such as plants, spices and herbs that are consumed as foods or ingredients have been widely investigated for several biochemical and pharmacological properties.[13,14] A number of naturally occurring antioxidant compounds have been found to strengthen the resistance of LDL to oxidative modification *in vitro* and *in vivo*.[15,16]

Garcinia kola seeds are eaten as refreshing past time in West and Central Africa, and are known to contain high content of biflavonoid compounds.[17] Seeds of *Garcinia* kola Heckel (family; *Guttiferae*) are known to have a general antidotal effect in folk medicine in Africa. The seeds (known as "bitter kola" or false kola) are believed to possess aphrodisiac properties and are used for the treatment of catarrh and abdominal colicky pain. In addition, their use is believed to improve the singing voice and relieve cough.[18] Likewise, extracts from bark, stem and seed of *Garcinia* kola have been reported to inhibit the growth of *plasmodium falciparum* by well over 60% *in vitro* at a concentration of 6- mg/ml.[19]

Kolaviron (KV)- a biflavonoid complex extracted from the kola seeds, contains *Garcinia biflavanone* GB 1, GB 2 and kolaflavanone in an approximate ratio of 2: 2: 1 as shown in figure 1. KV has been reported to modulate the hepatotoxicity of carbon tetrachloride, galactosamine, amanita toxin, paracetamol, thioacetamide and 2- acetylaminofluorene in various experimental animal models.[20-24] KV has also been reported to prevent accumulation of lipid peroxidation products and protect biomembranes against oxidative damage by acting as *in vivo* antioxidant in animal studies.[25] Similarly, KV has also been demonstrated to be a scavenger of reactive oxygen species *in vitro*.[26]

Following our interest on the pharmacological mechanism of action of kolaviron, the present study was designed to compare and establish which of the fractions (GB1, GB2 or KF) are responsible for the effects of KV observed on the reactive oxygen species and lipid peroxidation.

MATERIALS AND METHODS

Chemicals: Phenazine methosulfate (PMS) and reduced Nicotinamide adenine dinucleotide (NADH) were obtained from E. Merck AG. Darmstadt (Germany). Nitro blue tetrazolium (NBT), thiobarbituric acid (TBA) and ascorbate were procured from Sigma chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and trichloroacetic acid (TCA) are products of Hopkins and Williams, England. Potassium ferricyanide was procured from BDH Chemicals Ltd. (Poole, Dorset, UK).

All other reagents were of analytical grade and the purest quality available.

Extraction of Kolaviron: *Garcinia* kola seeds were obtained commercially in Ibadan, Nigeria. 3 kg of peeled seeds were sliced, pulverized with an electric blender and then air-dried in the laboratory (25- 28^{0}C). Extraction of KV was achieved by the method of Iwu *et al.*,[22]

Briefly, Powdered seeds were extracted with light petroleum ether (bp 40- 60^{0}C) in a soxhlet extractor for 24 hours. The defatted, dried marc was repacked and then extracted with methanol. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate (6 x 250 ml). The concentrated ethyl acetate fraction gave a yellow solid known as kolaviron, a mixture of *Garcinia biflavanones* GB1, GB2 and kolaflavanone (KF) (Figure 1). KV was separated by thin layer chromatography (TLC) using Silica gel GF 254 coated plates and solvent mixture (Methanol and Chloroform in ratio 1:4 v/v). The separation revealed the presence of three main compounds designated FI, FII and FIII. They were identified by their RF values as GB 2, GB 1 and KF, respectively. Using a molecular weight of 558,[27] three different concentrations (0.01 mM, 0.1 mM and 1.0 mM) of KV, GB1, GB 2 and KF were prepared and tested for their free radical scavenging ability and anti-lipoperoxidative effect in LDL.

Preparation of LDL: Fresh blood was obtained from thirty volunteers (with informed consent) into EDTA tubes. About 2-3 ml of blood was obtained from each volunteer after due approval from the ethical committee of the Faculty of Medicine and Health Sciences, UAE University, Al Ain and then centrifuged at 3,000 g for 10 min to obtain plasma. The plasma

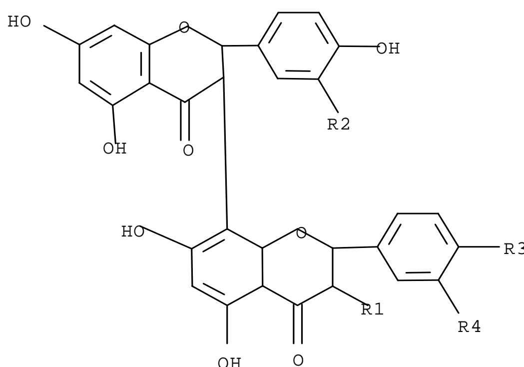

Figure-1: Structure of kolaviron

	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

was pooled and then used to isolate LDL according to the method of Chung *et al.*,[28]

Briefly, about 300 ml of plasma was spun at 20,000rpm for 20min in a BECKMAN T1 - 70 Rotor (ultra - centrifuge). The tubes were removed and the chylomicrons (upper white fraction) discarded. The plasma was spun again at 50, 000 rpm for 24 h in T1-70 Rotor. The lower layer (greenish–pellet) containing glycogen and fibrinogen was discarded. The supernatant (LDL) was adjusted to a density of 1.06 using KBr. The adjusted supernatant was spun at 40,000rpm for 48 h in TI – 70 Rotor. The uppermost fraction (pure LDL) was collected and dialyzed extensively against 0.9% (w/v) NaCl and 0.004% (w/v) EDTA, pH 7.4. It was stored at 4^{0}C. Prior to use, LDL was dialyzed against phosphate- buffered saline, pH 7.4, to remove the EDTA.

Scavenging of superoxide radical: The influence of extracts on the generation of superoxide radical was achieved by spectrophotometric measurement of the product formed on reduction of nitro blue tetrazolium.[29]

Briefly, superoxide radical was generated in a non - enzymic system and the reaction mixture containing, 200- 1000 mg/ ml of extracts in methanol, 1ml of PMS (60 mM) in phosphate buffer (0.1 M, pH 7.4), 1ml of NADH (468 mM) in phosphate buffer and 1ml of NBT (150 mM) in phosphate buffer, was incubated at ambient temperature for 5 min and the colour was read at 560 nm against blank.

Scavenging of hydrogen peroxide: The ability of extracts to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*,[30] Briefly, a solution (4 mM) of H_20_2 was prepared in a phosphate - buffered saline (PBS, pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm using molar absorptivity 81 $M^{-1}cm^{-1}$.[31] 200- 1000- mg/ ml of extracts in methanol was added to H_2O_2 solution (0.6 ml). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing extracts in PBS without H_2O_2.

Reducing property of extracts: The reducing property of extracts was determined according to the method of Oyaizu.[32] Extracts (200 – 1000 mg/ml) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and $K_3Fe(CN)_6$, 2.5 ml, 1%); the mixture was incubated at 50^0C for 20 min. A portion (2.5 ml of TCA, 10%) was added to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture is indicative of increase in reducing power of extracts.

Determination of lipid peroxidation: Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) produced according to the method of Walls *et al*.[33] Briefly, 1 mg/ml final concentration of LDL was incubated for six hours at 37^0C in a shaking water bath with or without 1 mM FeSO4, 0.2 mM H_2O_2, 1 mM ascorbate and 0.05 ml of extracts (0.01 mM, 0.1 mM, 1.0 mM) were added. 0.5 ml of 0.75% TBA in 0.1 M HCl was added to 0.5 ml of the incubation mixture already quenched with 0.5 ml of 10% TCA. The mixture was heated at 90- 95^0C for 25 min in a boiling water bath and then cooled.

The mixture was then centrifuged at 3,000 rpm for 10 min and the supernatant transferred to cuvette and absorbance taken at 532 nm after standing for 20 min.

Statistics: Results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for the data analysis. Significant differences between groups were detected in the ANOVA using Duncan's Multiple Range test at $p<0.05$.

RESULTS

1. *Effect of flavonoids of Garcinia kola seeds on reactive oxygen species*

• The scavenging effect of flavonoids of *Garcinia* kola seeds on hydrogen peroxide is given in Figure 2. The scavenging of H_2O_2 by KV was comparable with butylated hydroxytoluene (BHT) especially at higher concentrations (800 and 1000- mg/ml). At lower concentrations (200 and 400-mg/ml), BHT exhibited greater scavenging effect (66% and 71% respectively) when compared with the scavenging effect of these flavonoids. The scavenging effect of GB1 at the tested doses was comparable with BHT. Among the flavonoids of *Garcinia* kola

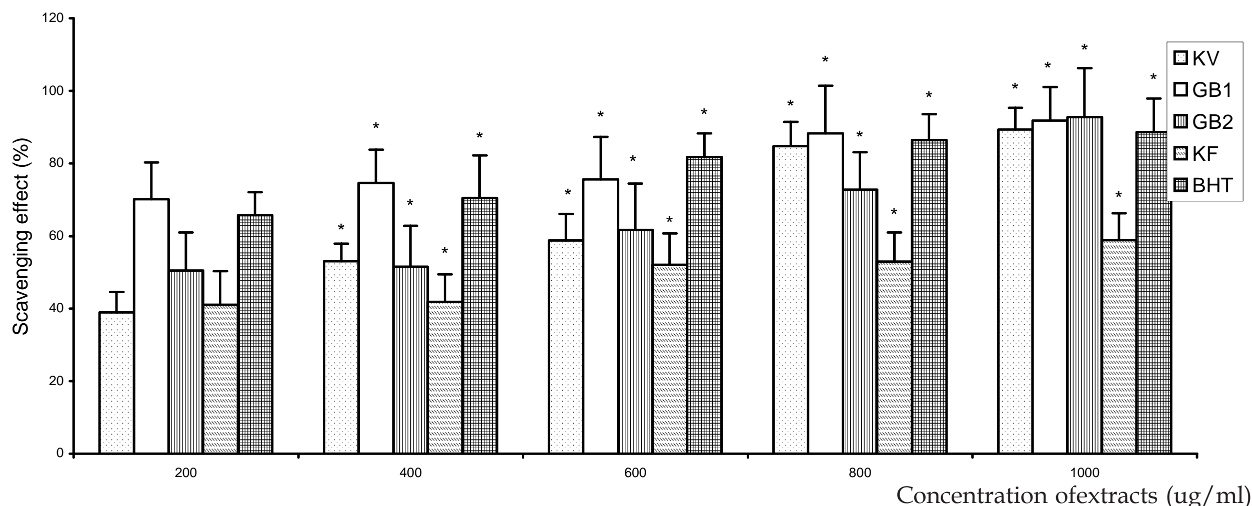

Figure-2: The scavenging activity of flavonoids from Garcinia kola seeds on hydrogen peroxide in vitro.
Values are means ± S.D. of five determinants.
* Significant and dose- dependent increase from 200 ug/ml at p< 0.05
KV= kolaviron, GB1= Garcinia biflavanone1, GB2= Garcinia biflavanone2,
KF= kolaflavanone, BHT= Butylated hydroxytoluene

seeds, KF has the least scavenging effect on H_2O_2 *in vitro*.

• The scavenging activity of flavonoids of *Garcinia* kola seeds on superoxide anion radicals (O_2) generated non- enzymically using phenazine methosulfate- NADH system is shown in figure 3. BHT at concentrations 200- 1000 mg/ ml scavenged superoxide radicals dose- dependently. At lower concentrations (200- 400 mg/ ml), the scavenging effect of KV, GB2, KF and BHT on O_2^- was below 50%. At these concentrations, GB1 exhibited better scavenging effect (greater than 50%) than BHT. However, at higher concentrations (800- 1000 mg/ ml), the scavenging effect of between 50- 71 % was obtained for KV, GB1, GB2 and BHT. At the tested concentrations, the scavenging effect of KF was less than 50 %. However, at 1 mg/ ml, KV exhibited better scavenging ability (71%) than BHT (68%).

2. ***The reducing power of flavonoids of Garcinia kola seeds on potassium ferricyanide in vitro***

Figure 4 depicts the reducing power of flavonoids of *Garcinia* kola on potassium ferricyanide at an absorbance of 700 nm. At the tested doses, the reducing power of KV, GB1and GB2 was observed to be greater than BHT. It was also found to be dose-dependent for GB1 and GB2. KF has the lowest reducing power among the flavonoids studied.

3. ***Effect of flavonoids of Garcinia kola seeds on the oxidation of LDL in vitro***

Figure 5 shows the effect of different concentrations of flavonoids of *Garcinia* kola on iron/ ascorbate- induced oxidation of human low- density lipoprotein (LDL) *in vitro*. KV, GB1 and GB2 at concentrations 0.01 mM, 0.1 mM and 1.0 mM inhibited the accumulation of lipid peroxidation (LPO) products in a dose dependent manner. At these concentrations, KV inhibited lipid peroxidation (LPO) by 29%, 61% and 79%; GB2 also inhibited LPO by 41%, 72% and 91%, while GB1 inhibited LPO by 29 5, 56% and 86%, respectively. However at 0.01 mM and 0.1 mM of KF, the values of LPO was higher than control but not statistically significant ($p>0.05$). While at a dose of 1.0 mM KF, LPO was significantly inhibited when compared to the control ($p<0.05$).

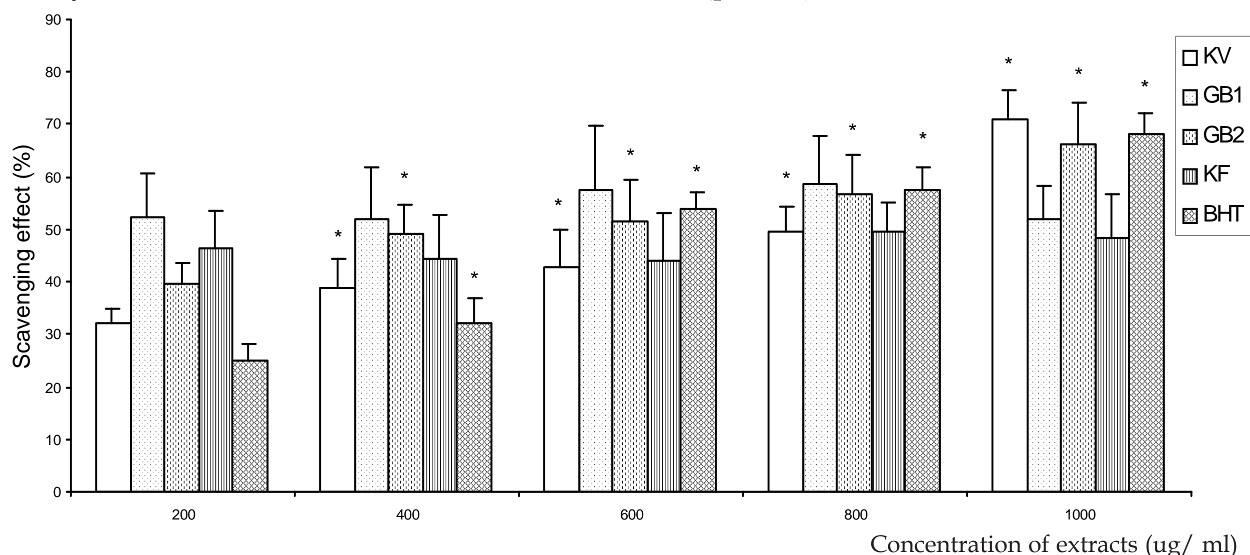

Figure-3: The scavenging activity of flavonoids from Garcinia kola seeds on superoxide anions radical in vitro.
Values are means ± S.D. of five determinants.
* Significant and dose- dependent increase ($p< 0.05$) from 200-ug/ml
KV= kolaviron, GB1= Garcinia biflavanone1, GB2= Garcinia biflavanone2,
KF= kolaflavanone, BHT= Butylated hydroxytoluene

DISCUSSION

Peroxidation of low- density lipoprotein (LDL) within the vessel wall under increased oxidative stress is a major factor in atherogenesis and neuro- degenerative diseases such as Alzheimer's diseases.[34,35] This peroxidation of LDL molecule renders it immunogenic and causes monocyte recruitment, foam cell formation and cytotoxicity to various cells including neurons.[36,37] Modified LDL is recognized by scavenger receptors and is taken up with enhanced efficiency.[38] Steinberg *et al.*,[39] has shown that arterial endothelial cells and smooth muscle cells are capable of oxidizing LDL *in vitro* so that macrophages will internalize it faster. It is now known that a relationship exist between lipid peroxidation and atherosclerotic lesion.[40]

In the present study, KV, GB1 and GB2 at the tested concentrations prevented iron/

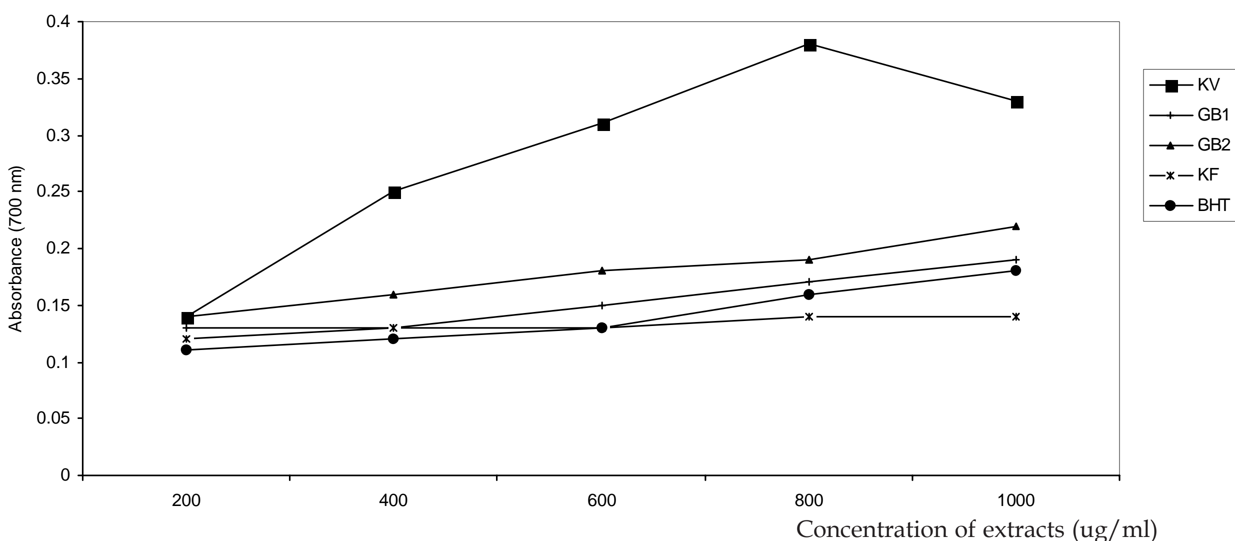

Figure-4: The reducing property of flavonoids from Garcinia kola seeds on potassium ferricyanide in vitro. Values are means ± S.D. of five determinants.
KV= kolaviron, GB1= Garcinia biflavanone1, GB2= Garcinia biflavanone2, KF= kolaflavanone,
BHT= Butylated hydroxytoluene

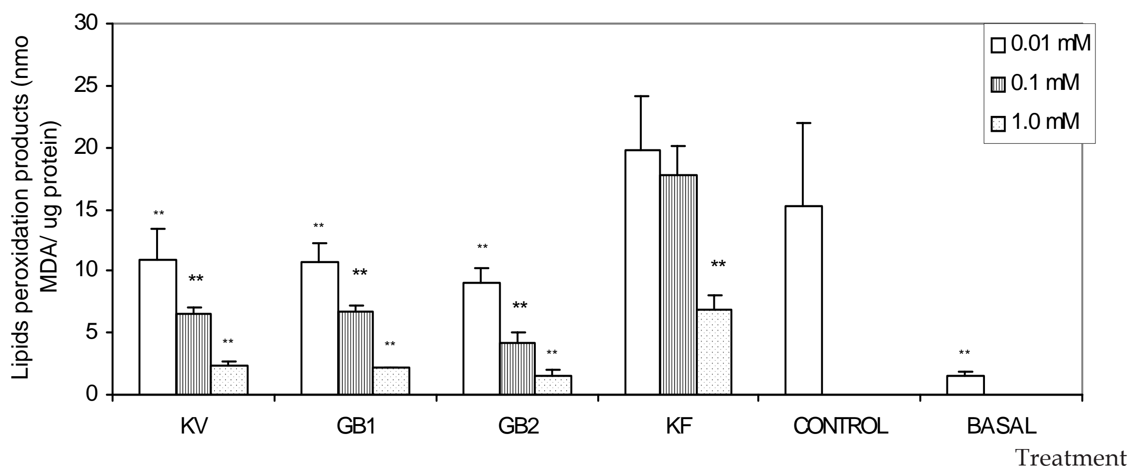

Figure-5: Inhibitory effect of flavonoids from Garcinia kola seeds on Fe/Ascorbate-induced oxidation of lipids in LDL in-vitro.
Values are means ± S.D. of five determinants.
** Dose- dependent, and significantly different from control ($p < 0.05$)
KV= kolaviron, GB1= Garcinia biflavanone1, GB2= Garcinia biflavanone2, KF= kolaflavanone.

ascorbate catalyzed oxidation of LDL *in vitro*. The inhibition of LDL oxidation by these flavonoids was significant and concentration dependent. It has been postulated that regular intake of diets rich in fruits, vegetables and beverages (rich sources of flavonoids) may protect against a variety of diseases, particularly cardiovascular disease and cancer.[41,42] Hertog *et al*.,[43] and Knekt *et al*.,[44] found an inverse relationship between dietary flavonoids intake and mortality from cardiovascular disease. Aviram [45] supported this inverse relationship by stating that flavonoids such as isoflavan from licorice root, quercetin and catechin from red wine, ginger, pomegranate and grapefruit peels are absorbed directly into the system, bind to plasma LDL in circulation and prevent LDL oxidation by acting as an *in vivo* antioxidant.

Although kolaviron, a biflavonoid complex from *Garcinia* kola seeds has been shown to exhibit marked inhibition of microsomal lipid peroxidation *in vivo* and *in vitro*,[24-26] however, the inhibition of purified human LDL oxidation *in vitro* by flavonoids of *Garcinia* kola is reported here for the first time. Among the flavonoids tested, GB2 exhibited the highest inhibitory effect on the oxidation of LDL *in vitro*. Precisely, GB2 inhibited LPO by 72% and 91% at concentrations of 0.1 mM and 1.0 mM, respectively. Hussain *et al*.,[46] has reported that the overall antioxidant effect of flavonoids on lipid peroxidation might be due to scavenging of hydroxyl and superoxide anions radicals at the stage of initiation and termination.

Our data suggest that KV, GB1 and GB2 scavenged both hydrogen peroxide and superoxide anions radicals effectively. The scavenging effect of these flavonoids was more than 50% at concentrations of 800 and 1000 mg/ ml for both reactive species, and dose- dependent for hydrogen peroxide. This observation is in consonance with the findings of Farombi *et al*.,[26] who reported marked scavenging effect of KV on hydrogen peroxide and superoxide anion radicals *in vitro*. In the present work, the scavenging effect of KV, GB1 and GB2 on the reactive oxygen species was comparable with the synthetic antioxidant, BHT. However, KF has the lowest scavenging effect on these species at the tested concentrations. BHT is one of the most effective, commonly used, harmless industrial antioxidants used in foods, drugs and cosmetics.[47] In the present study, the reducing property of these flavonoids followed the order KV> GB2> GB1> BHT> KF at a dose of 1000-mg/ml. The greatest reducing power was observed in KV relative to the other flavonoids and may be due to the synergistic effect of GB1, GB2 and KF. The reducing power shows that flavonoids of *Garcinia kola* seeds are electron donors and could react with free radicals to convert them to stable products thereby terminating radical chain reaction.[48]

The structure- activity relationship of flavonoids and the inhibition of lipid peroxidation indicate that the presence of 3- hydroxyl group in the C- rings of GB1 and GB2 make them potent inhibitors of LPO.[49] Similarly, antilipoperoxidative effect of KV, GB1 and GB2 as seen in this study may be linked to the numbers of hydroxyl groups on the A and B rings of these flavonoids.[50] The least scavenging activity obtained for KF might be due to the methoxy group in its C- ring. Cholbi *et al.*[51] attributed the reduced anti- peroxidative efficiency of flavonoids to steric hindrance caused by the presence of methoxy group in their structures. Thus, it is apparent that KV, GB1 and GB2 inhibit oxidation of LDL *in vitro* by acting as scavengers of reactive oxygen species, and this effect may be linked to their possible anti- atherogenic properties (Adaramoye *et al*., personal communication).

Overall, our data demonstrate that flavonoids of *Garcinia* kola seeds, particularly KV, GB1 and GB2, act as potent antioxidant and scavengers of reactive oxygen species. Furthermore, the present study revealed that GB1 and GB2, and not KF are responsible for the protective effects of KV earlier reported by Farombi *et al*.[26] These effects may be related to the suppression of the oxidation of LDL *in vitro*. These flavonoids may therefore act as a preventative agent in pathologies where reactive oxygen species have been implicated. However, further work is required to extend our observa-

tions to the *in vivo* situation. There is also the need to determine whether binding relationship may exist between these flavonoids and native LDL thereby affecting the lag- phase during oxidation.

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