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Some bioactive potentials of two biflavanols isolated from *Garcinia kola* on cadmium-induced alterations of raw U937 cells and U937-derived macrophages

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ABSTRACT

Objective: To investigate the abilities of two flavonoids – *Garcinia* biflavanol-1 (GB-1) and *Garcinia* biflavanol-2 (GB-2) from *Garcinia kola (G. kola)* in reducing cadmium-induced effects on raw U937 cells and U937-derived macrophages. **Methods:** Macrophage U937 cells were incubated with cadmium followed by treatment with the flavonoids and cell viability assessed via trypan blue staining. In the other experiment, the U937 cells were transformed to the macrophage form and treated with cadmium in order to activate them. The cells were later incubated with the flavonoids and finally the supernatant of each cell culture was analysed for the secretion of nitric oxide, catalyse activity, and the release of tumour necrosis factor-alpha, interleukin-1 and interleukin-2 as indices of macrophage activation. Quercetin (a flavonol) was used as the reference flavonoid in all experiments. **Results:** It revealed that the flavonoids significantly increased the viability of the cells and also reduced the cadmium-induced activation of the macrophage cells in a concentration-dependent manner. The flavanols GB-1 and GB-2 possessed higher activities than quercetin in all cases ($P<0.05$). *Garcinia* biflavanol-2 possessed a higher bioactivity than GB-1 significantly ($P<0.05$). **Conclusions:** In addition to corroborating the several reported importance of *G. kola* as a potential neutraceutical and pharmacological condiment, the study also clearly indicates the role hydroxylation especially at the 3′- position of polyphenols could play in enhancing bioactivities of flavonoids.

1. Introduction

Cadmium is one of the most toxic occupational and environmental heavy metals present in soil, air, sediment and water. Even though the concentration of cadmium in natural ecosystems is quite low, its level is increasing due to industrialisation and urbanisation over the years. As a result of its increase in the natural environment coupled with its high rate of soil-to-plant transfer, it can accumulate in the body via food consumption, cigarette smoking, and other activities[1-3]. In fact, much of the cadmium which enters the body is by ingestion due to the consumption of terrestrial foods[4]. To date, cadmium has no known established function in the human body rather it causes a lot of pathological imbalances due to its long half-life (~30 years)[5]. However, the effect of cadmium on a species depends on the dose, period of exposure, and the physiology of the species involved in many cases[6,7]. It has been demonstrated that cadmium targets the liver, kidney, brain, the endocrine system and the immune system[8-10]. Even at very low levels in the natural environment, it is toxic to plants and animals[11]. The body however can detoxify cadmium and other heavy metals by using metallothionein-a cysteine-rich low molecular weight protein. Metallothionein is involved in multiple cellular functions such as transport, storage and detoxification of metals. Metallothionein captures heavy metals within the liver and kidneys and keeps them in non-toxic forms thereby protecting cells against metal toxicity[12]. This implies that the body has the propensity of tolerating low amounts of cadmium. Because of continuous exposure to water, food and air that may have been contaminated

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by cadmium, serious pathological consequences may arise if the level of the heavy metal in tissues overwhelms the capacity of metallothionein and other endogenous detoxification processes[13]. Recently, it has been shown that cadmium accumulates in various ocular tissues such as lens, retina, vitreous humour which is associated with gender, age and pathological status[5].

Flavonoids are a large group of secondary metabolites in plants with low molecular weights. Fruits, vegetables and flowers are claimed to be the most important sources of these polyphenolic compounds[14]. These natural products have been subjected to myriad of investigations due to their potential beneficial properties. Research has it that flavonoids possess anti-inflammatory, anti-carcinogenic, anti-oxidant, anti-viral, and anti-hypertensive potentials[15-17]. Based on these properties, consumption of fresh fruits and vegetables has been seriously encouraged. Among these flavonoids of interest is quercetin (Figure 1). This flavonol is one of the most abundant naturally occurring flavonoids thus has been used as a model for the study of these group of polyphenols. The content of quercetin in some plants is shown in Table 1.

Garcinia kola (G. kola) Heckel (Clusiacaea) is a medium-sized evergreen tree which is revered in major parts of Sub-Saharan Africa. The seeds of the plant have been consumed as cough medicine and also used for several other purposes[18]. Chemical research has indicated that many of the reported uses of the plant are attributed to the possession of flavonoids especially bi-flavonoid compounds. Two flavonoids in *G. kola* which have been characterised are 5,7,5′,7′-Tetrahydroxy-2,2′-bis-(4-hydroxy-phenyl)-2,3,2′,3′-tetrahydro-[3,8′]bichromenyl-4,4′-dione (*Garcinia* biflavanol 1, GB-1), and 2′- (3,4-Dihydroxy-phenyl)-2,3,2′3′-tetrahydro-[3,8′]bichromenyl-4,4′-dione (*Garcinia* biflavanol 2, GB-2) (Figure 1)[19,20]. These compounds have also been shown to possess significant antioxidant and bioactive potentials[21,22].

This current study investigates the effect of purified GB-1, and GB-2 (at different concentrations) on cadmium-induced modulation of raw U937 and U937-derived macrophage cells. Quercetin is used as a reference flavonoid. Cadmium activates macrophages which is a primary event in inflammation. This predisposes the phagocytes to release vasoactive amines, nitric acid, and cytokines many of which are chemotactic[23,24].

Table 1
Content of quercetin in some foods and herbal spices before acid hydrolysis.

Sample	Quercetin content (mg/kg dry weight)
White onion bulps	2 604.0
Spring onion leaves	450.0
Spinach leaves*	403.0
Apple peel	21.0
Herpericum perforatum leaves	1.8
Sambacus nigra flowering tops	0.4

Table modified from Wach *et al*[25]. *From Dehkharghanian *et al*[26].

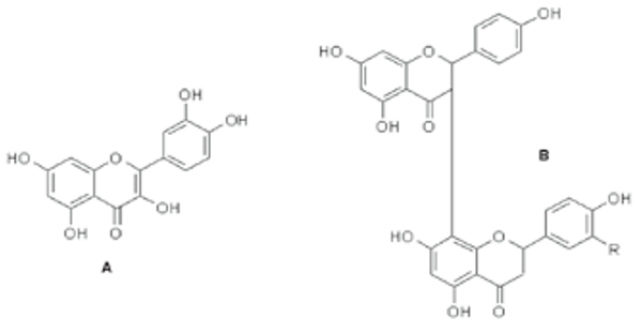

Figure 1. Structures of the flavonoids.

A is 2-(3,4-Dihydro-phenyl)-3,5,7-trihydroxy-chromen-4-one (Quercetin); for B if R = H, is 5,7,5′,7′-Tetrahydroxy-2,2′-bis-(4-hydroxy-phenyl)-2,3,2′,3′-tetrahydro-[3,8′]bichromenyl-4,4′-dione (*Garcinia* flavanol-1, GB-1); if R = OH, it is 2′- (3,4-Dihydroxy-phenyl)-2,3,2′3′-tetrahydro-[3,8′]bichromenyl-4,4′-dione (*Garcinia* biflavanol-2, GB-2).

2. Materials and methods

2.1. Materials

Foetal calf serum (heat inoculated), quercetin, phorbol 12-myristate, 13-acetate (PMA), tween-20 and L-glutamine were purchased from Sigma. The human macrophage U937 cell line was purchased from the European Collection of Cell Cultures, culture media RPMI-1640 was obtained from Invitrogen. All cytokines used in the study were products of Pharmingin. Penicillin was purchased from Euroclone. All other reagents and chemicals were of analytical grade and were commercially available. Buffers were prepared in 18.2 Ωm/cm water. Experiments were performed in a biosafety cabinet unless otherwise stated.

2.2. Preliminary cell culture

The cell line U937 was grown in RPMI-1640 medium supplemental with foetal calf serum (heat inoculated), L-glutamine, penicillin/streptomycin solution (known as the complete medium) as described[17]. In all cases, cells were re-suspended in RPMI-1640 to desired density before experiment.

2.3. Cell viability study

Cells (in complete RPMI-1640) were re-suspended at a density of 5×10^5 cells/mL and 5 mL portions were delivered into sterile tubes. Cadmium (as cadmium chloride, 16 μM, 5 μL) was delivered into each culture with or without quercetin, GB-1, GB-2 at different concentrations (2-16 μM) according to the scheme below.

Set A Cells with cadmium only
Set B Cells with cadmium with quercetin (5 μL)
Set C Cells with cadmium with GB-1 (5 μL)
Set D Cells with cadmium with GB-2 (5 μL)

However, cells were treated with cadmium for 24 h before the addition of the flavonoids. Each culture was kept in the incubator set at 37 ℃, gassing up to 5% CO_2. Seventy-two hours later, each culture was gently agitated and cell viability determined via trypan blue staining and expressed as:

$$\% \text{ Viability} = \frac{\text{Cell count (treated with cadmium)}}{\text{Cell count (untreated with cadmium)}} \times 100\%$$

2.4. Cell activation study

Cells were transformed via treatment with PMA in 96-well coaster plates as described[17]. Thereafter, supernatants were removed and cadmium (16 μM), quercetin, GB-1 and GB-2 were added to the cells according to scheme below.

Plate A PMA, cadmium (100 μL) and RPMI-1640 (100 μL)
Plate B PMA, cadmium (100 μL) and quercetin (100 μL)
Plate C PMA, cadmium (100 μL) and GB-1 (100 μL)
Plate D PMA, cadmium (100 μL) and GB-2 (100 μL)

Different concentrations of the flavonoids were used ranging from 2 μM to 16 μM. Twenty-four hours later, the supernatants of the cultures were assessed for the activation of macrophages.

2.5. Biochemical analysis

The activation of macrophages was measured by assessing the nitric acid production, catalase activity, and the secretion of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-2 (IL-2). Nitric acid production of each supernatant was determined according to the method of Hwang *et al*[27] as modified[28]. The activity of catalase was also measured according to Aebi[29] as modified by Okoko and Oruambo[17]. The levels of TNF-α, IL-1, and IL-2 were also determined via cytokine ELISA as described[17]. However, each result was converted to % inhibitory activities expressed as:

$$\% \text{ Inhibition} = \frac{\text{Amount released in cells treated with cadmium only}}{\text{Amount released in cells treated with cadmium and the flavonoids}} \times 100\%$$

2.6. Statistical analysis

Where applicable, data were presented as mean±S.E.M. Where appropriate, analysis of the data was performed using either student's *t*-test or analysis of variance followed by the Duncan's multiple test. A confidence level exhibited at $P<0.05$ was considered statistically significant.

3. Results

The effect of the flavonoids on cadmium-induced alterations in the viability of the cells is shown on Table 2. There was a concentration-dependent increase in the viability of the cells when they were treated with the flavonoids following the incubation with cadmium. For instance, when the cells were incubated with cadmium (16 μM) alone, cell viability was only (14.14±2.42)%. Further treatment with GB-1 and GB-2 (2 μM each) increased the viability of the cells to (19.78±2.19)% and (23.23±1.08)% respectively (Table 2). Both flavanols (GB-1 and GB-2) significantly increased the viability of the cells more than the flavonol quercetin ($P<0.05$). However, GB-2 had the highest potential.

Figures 2 to 6 show the inhibitory potential of the flavonoids on cadmium-mediated activation of U937-derived macrophages. All the flavonoids inhibited the cadmium-mediated activation (all the indices of macrophage activation that was assessed) in a concentration-dependent manner. The *G. kola*-derived flavonoids possessed significant abilities to reduce the cadmium-mediated effects more than quercetin in all cases ($P<0.05$).

Table 2
Effect of flavonoids on cadmium-induced viability of U937 cells.

Concentration (μM)	Q (%)	GB-1 (%)a	GB-2 (%)ab
2	17.34±0.28	19.78±2.19	23.23±1.08
4	23.66±2.75	32.45±2.31	47.76±4.42
8	37.82±1.54	43.93±2.87	62.83±2.77
16	46.57±3.22	55.54±2.53	70.94±2.07

Values are expressed as mean±SEM ($n=4$). Viability was (14.14±2.42)% when cells were treated with only cadmium (16 μM). Q is quercetin; GB-1 is *garcinia* biflavanol-1, GB-2 is *garcinia* biflavanol-2. aSignificantly different from Q; bSignificantly different from GB-1 ($P<0.05$). The values for the different concentrations also differ significantly ($P<0.05$).

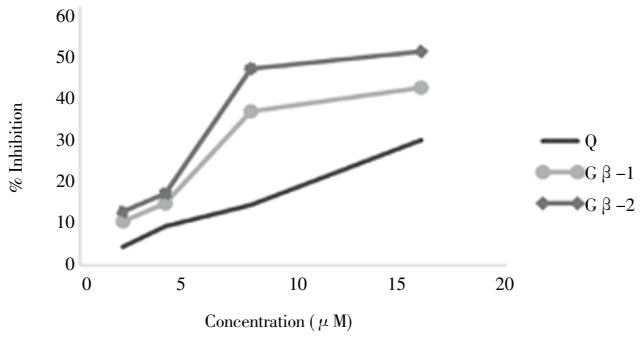

Figure 2. Inhibitory activities of the production of nitric oxide by macrophage cells incubated with the various flavonoids; Q is quercetin, GB-1 is garcinia biflavanol-1, and GB-2 is garcinia biflavanol-2. Each experiment had 12 replicates.

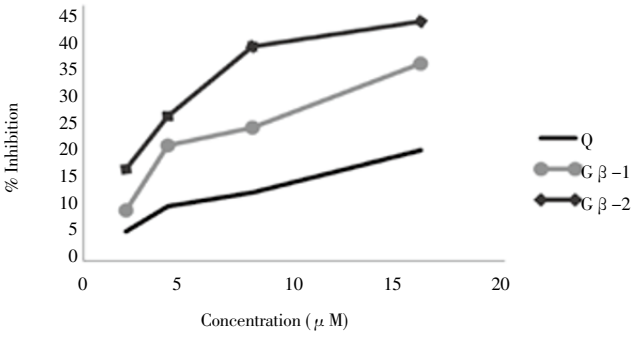

Figure 3. Inhibitory potential on catalase activity of macrophage cells incubated with the various flavonoids.

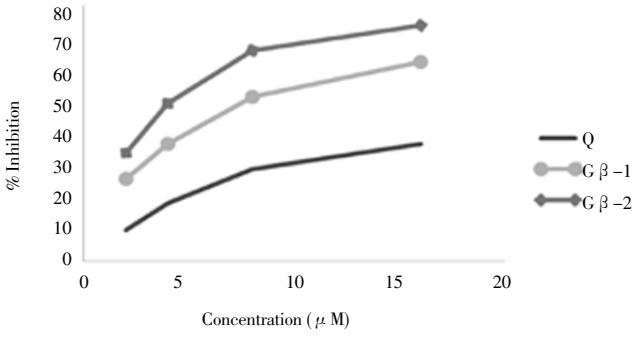

Figure 4. Inhibitory activities of flavonoids on production of interleukin-1 by macrophage cells incubated with the various flavonoids.

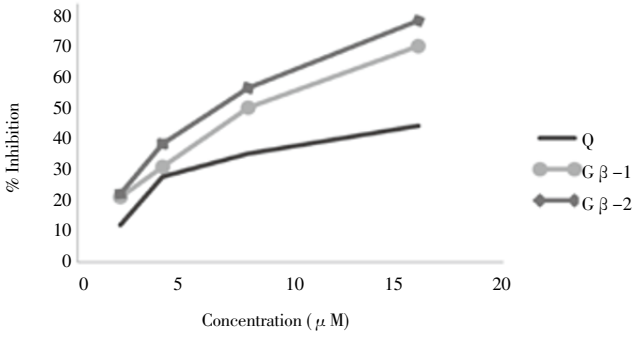

Figure 5. Inhibitory activities of flavonoids on production of interleukin-2 by macrophage cells incubated with the various flavonoids.

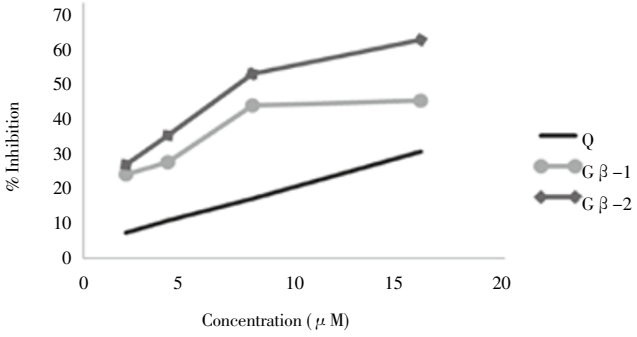

Figure 6. Inhibitory activities of flavonoids on production of tumour necrosis factor-alpha by macrophage cells incubated with the various flavonoids.

4. Discussion

Cadmium has been classified as a human carcinogen by the International Agency for Research on Cancer. One of the major mechanisms of cadmium-mediated effects is its ability to enhance apoptosis and immune suppression which may involve the generation of free radicals especially in the mitochondrion[30]. Being the power house of the cell, the compromised mitochondrial membrane integrity may result in the release of organelle's contents and may precipitate to necrosis and apoptosis. Recently, it has been shown that the loss of the mitochondrial transmembrane potential changes the mitochondrial permeability and this is a critical stage in apoptosis which often may involve caspases that cleave various proteins[31]. Cadmium depresses the level of glutathione thereby stimulating the generation of the superoxide radical[31-32]. Flavonoids act as antioxidants via various mechanisms. It has been argued that flavonoids may trigger reactions that tend to block the formation of a permeability transition pore in mitochondria thus prevent apoptosis[33]. Flavonoids (especially quercetin) participate in reactions to increase the level of glutathione in cells via the up-regulation of the gene that codes for γ -glutamyl-cysteine synthetase which is the pacemaker for the biosynthesis of glutathione[34]. These may be partly responsible for the increase in the viability of the cadmium-treated cells after incubation with quercetin, GB-1 and GB-2.

The cell line U937 changes to the macrophage morphology after treatment with phorbol-12, myristate-13, acetate which peaks after 48 h[17,35]. Activation of macrophages results in the release of tumour necrosis factor-alpha (TNF- γ), vasoactive amines and other pro-inflammatory cytokines. This induces inflammation which can precipitate into kidney and liver damages[36]. The release of cytokines (which are chemotactic) may lead to permanent tissue damage through the continuous production of endogenous oxidants such as nitric oxide and toxic proteases and this is considered to be one of the main mechanisms of cadmium-mediated responses to the lungs[37]. Nitric oxide reacts with superoxide in the vascular epithelium to generate peroxynitrite which induces the oxidation of LDL and also interacts with pro-inflammatory cytokines to cause myocardial dysfunction[38]. The flavonoids (quercetin, GB-1 and GB-2) significantly reduced the cadmium-mediated release of nitric oxide, catalase, interleukin-1 (IL-1), interleukin-2 (IL-2) and TNF- α which are the indices of the activation of macrophages assessed. Thus the flavonoids could also inhibit the oxidation of LDL hence possess unique cardioprotective effects. The biflavanols GB-1 and GB-2 were more powerful in reducing the cadmium-induced

activation than the reference compound quercetin. This may be as a result of the differences in the structures of the compounds. Flavonoids chemical resemble chain breaking antioxidants due to the possession of one or more aromatic-hydroxyl groups[39] and this may account for the greater activity for the flavanols GB-1 and GB-2 over quercetin. It has been shown that there is no obvious consistent correlation between 2-3 unsaturation (as seen in quercetin) and antioxidant activity[40] hence the use of quercetin as a control in this case is justifiable. However in some cases, 2-3 unsaturation seem to confer a higher activity as seen in quercetin[41]. But in the current work, the second flavanol group in GB-1 and GB-2 greater overwhelmed the ability of quercetin to capatalise on that advantage. In all cases, GB-2 was more powerful that GB-1. The difference between these compounds is the presence of a hydroxyl moiety in the 3′position of the second flavanol group. Hydroxyl groups in flavonoids are one the major determinants of antioxidant potentials especially at the 3′position[41-45]. It has been shown that the 3′,4′, catechol structure in the B-ring strongly enhances the inhibition of lipid peroxidation. Ammar *et al*[46] also reported that superoxide ion and xanthine oxidase scavenging activity of flavonoid compounds is enhanced following 3′-hydroxylation in their structures. All these attest to the greater activity of GB-2 over GB-1.

There has been no report of the isolation of GB-1 and GB-2 from any other plant. A lot is yet to be known about the medicinal nature of *G. kola*. The presence of the plant is definitely nature's generosity in providing mankind with cheap and affordable source of neutraceuticals which could even be exploited for pharmacological advantages. Further work on the bioactivities of GB-1 and GB-2 are in progress. Attempts are also being made to characterise other bioactive compounds from *G. kola*.

Conflict of interest statement

We declare that we have no conflict of interest.

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