**Original Research Article**

The bi-flavonoid complex kolaviron reduces cadmium-induced cytotoxicity and production of reactive oxygen species by suppressing inflammatory response

**Abstract**

The effect of kolaviron (a bi-flavonoid complex extracted from *Garcinia kola*) on cadmium (Cd)-induced cell death/production of reactive oxygen species (ROS) in monocyte U937 cells and production of inflammatory markers/antioxidant enzymes in U937-derived macrophages was investigated. In the first experiment, U937 cells were incubated with or without kolaviron for 24 h before exposed to Cd (10 µM). Cell viability and ROS production were later assessed via MTT reduction and DCHF assays respectively. In the other experiment, U937 cells were transformed to the macrophage form using phorbol 12-myristate 13-acetate and incubated with or without kolaviron for 24 h before exposed to Cd. Subsequently, production of TNF-α, IL-6 were analysed via cytokine ELISA and the expression of NF-κB, iNOS, SOD and catalase were assessed using RT-PCR. Results revealed that Cd caused significant cell death and production of ROS in U937 cells which were reduced by kolaviron in a dose-dependent manner ($p < 0.05$). Kolaviron also reduced Cd-mediated secretion of TNF-α and IL-6 in U937-derived macrophages which was concentration-dependent for the reduction of TNF-α ($p < 0.05$). The bi-flavonoid complex also reduced Cd-mediated expression of the transcription factors (NF-κB and iNOS) and the antioxidant enzymes (SOD and catalase) but the observation was only concentration-
dependent for the reduction of catalase \((p < 0.05)\). These shows that kolaviron reduced Cd-mediated alterations in monocyte U937 cells and macrophages. Implications are discussed.

*Keywords: Garcinia kola; kolaviron; cadmium; macrophages; cell viability*

1. **INTRODUCTION**

Cadmium (Cd) is one of the most toxic environmental pollutants whose levels are raised due to anthropogenic activities [1-2]. Human activities that produce cadmium include use of automobiles, batteries, paints, etc. These activities seem to be indispensable to life as such human exposure to cadmium will be continuous [3]. Human beings are exposed to Cd via food consumption, smoking and probably contaminated drugs however toxicity depends on the amount ingested, entry rate, distribution and excretion [4-7]. Following uptake, cadmium is mainly retained in the kidney where it binds to albumin and cystein-rich protein metallothionein for excretion however the toxicant causes significant renal tubular damage [5]. Cadmium also causes liver damage, teratogenic effects, neurotoxic effects and cancer [8-10].

Plants are widely used in traditional medicine for the treatment of various ailments thus have attracted a lot of attention all over the world. *Garcinia kola* Heckel (Clusiaceae) is a medium sized plant valued in many part of Western and Central Africa whose seeds are consumed to treat cough, liver diseases, laryngitis, infections and inflammation [11-13]. Kolaviron is a biflavonoid complex isolated from *Garcinia kola* with immense antioxidant power. It has been reported that the complex has anti-nephrotoxic, anti-diabetic, anti-diabetogenic, anti-inflammatory and anti-microbial effects [11, 14-17]. This current work investigates the effect of kolaviron on cadmium-induced toxicity and production of reactive oxygen species (ROS) in the monocyte cell line U937. The effect of kolaviron on cadmium-mediated alterations of
some transcription factors and antioxidant enzymes in U937-derived macrophages was also
reported.

2. MATERIALS AND METHOD

2.1 Materials

Cadmium chloride, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2’ ,7’ -dichlorohydrofluorescein diacetate (DCHF-DA) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (USA). Penicillin, streptomycin and all antibodies were purchased from Gibco (USA). All other chemicals were of analytical grade and commercially available.

2.2 Maintenance of cell culture

The monocyte cell line U937 obtained from the American Type Culture Collection (Rockville, MD, USA) was maintained in RPMI-1640 medium (Sigma-Aldrich, USA) as described [18]. Cells were seeded in flasks and grown in RPMI-1640 containing 10 % heat inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL streptomycin and penicillin (100 mg/mL). Cells were kept at a temperature of 37°C in an atmosphere of 5 % CO₂/95 % air and maintained at 5 x 10⁴/mL.

2.3 Extraction of kolaviron

Kolaviron was extract from Garcinia kola seeds according to Iwu [19]. Briefly, 4 kg of the powdered seeds were soxhlet-extracted with light petroleum ether (bp 40–60°C) for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and re-extracted with ethylacetate (6×300 ml). The ethylacetate fraction was concentrated to give a golden yellow solid known as kolaviron.
2.4 Cell viability and ROS production

Two hundred microlitres of cells (at 5 x 10⁴ cells/mL) were delivered into the wells of culture plates. In order to evaluate the effect of kolaviron on cell viability, cells were incubated with or without kolaviron (10 µg/mL or 25 µg/mL) for 24 h before exposure to 10 µM Cd (as cadmium chloride). For controls, cells were supplemented with equivalent volumes of RPMI-1640. One hour after addition of Cd, cell viability was assessed via the MTT reduction assay as reported [20]. Briefly, MTT was added to each culture to a final concentration of 0.5 mg/mL and incubated for 1 h at 37°C. MTT was aspirated and culture supplemented with DMSO. Absorbance was finally measured at 570 nm using a microplate reader.

The production of ROS was assessed based on the oxidation of 2',7'-dichlorohydrofluorescein by intracellular peroxides as reported [21] with a slight modification. Twenty-four hours following the addition of kolaviron (10 µg/mL or 25 µg/mL), medium was removed and replaced with RPMI-1640 supplemented with 50 µM DCHF-DA and incubated for 30 min at 37°C. Cells were washed with 0.02 M phosphate buffered saline (pH 7.4) and incubated with Cd (10 µM) for 1 h. Fluorescence of cells was measured at excitation and emission wavelength at 485 nm and 530 nm respectively. Antioxidant activity was expressed as percent inhibition of intracellular ROS following Cd exposure.

2.5 Secretion of cytokines

Cells were transformed to the macrophage form using PMA as described [18]. Medium was removed and replaced with kolaviron (10 µg/mL or 25 µg/mL). Twenty-four hours later, Cd (10 µM) was added and the supernatant of each cell culture analyzed for the production of tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) 1 hr later via cytokine ELISA as reported [18].
2.6 RNA isolation and RT-PCR

Following the transformation and treatment of cells with kolaviron and Cd (as in 2.5 above), total RNA was extracted from cells (after removing supernatants) using TRIzol reagent (Invitrogen) and quantified by measuring absorbance at 260 nm. The cDNA was synthesized using a Revert Aid cDNA synthesis kit according to the manufacturer’s protocol. For RT-PCR, 1 µg of the resulting cDNA was used to amplify regions specific to nuclear factor kappa B (NF-κB), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD) and catalase (CAT) in an ABI Prism 7500 system (Applied Biosciences) with primer pairs listed in table 1. Real-Time PCR data was analyzed and presented as fold change in expression to the GAPDH housekeeping gene of same sample.

Table 1. Primers pairs for RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5’-3’)</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>FP: GTGCCACCTCCAGTCCAG</td>
</tr>
<tr>
<td></td>
<td>RP: GCTGCCCCAGTTTTTGATCC</td>
</tr>
<tr>
<td>NF-κB</td>
<td>FP: GCCCTTCATCTAGCCACAGAG</td>
</tr>
<tr>
<td></td>
<td>RP: GATGTCAGCACCAGCTTCAG</td>
</tr>
<tr>
<td>SOD</td>
<td>FP: GACTGAAGGCCTGCGATGGATTC</td>
</tr>
<tr>
<td></td>
<td>RP: CACATCGGCCACACCATCTTTG</td>
</tr>
<tr>
<td>CAT</td>
<td>TTTCGCCACACACCATG</td>
</tr>
<tr>
<td></td>
<td>GATAATTGGGTCCCAGGCGATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: GTCGGAGTCAACGGATTTTGGTC</td>
</tr>
<tr>
<td></td>
<td>RP: CTTCCCGTCTCACGCCTTGAC</td>
</tr>
</tbody>
</table>

2.7 Statistical analysis
Where appropriate, data were expressed as mean ± SEM of six replicates. Data analysis was done using ANOVA followed by Tukey’s range test. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Cytotoxicity and ROS production

The effect of kolaviron on Cd-induced cell death is shown in figure 1. Treatment of U937 monocytes with Cd resulted in significant cell death (16.73 ± 4.12 % viability) when compared to untreated controls ($p < 0.05$). However kolaviron reduced Cd-induced cell death which was concentration-dependent ($p < 0.05$). Following the DCHF-DA assay, the results further revealed that kolaviron reduced Cd-induced production of ROS in a concentration-dependent manner ($p < 0.05$) (Figure 2).

Figure 1. Effect of kolaviron on Cd-induced cell death in U937 monocytes assessed by MTT reduction assay. Cd, cells treated with cadmium only. 10 µM KVR, cells treated with 10 µM kolaviron; 25 µM KVR, cells treated with 25 µM KVR before incubating with Cd.
Each column represents mean ± SEM of six replicates. Values having different superscript letters differ significantly (p < 0.05).

Figure 2. Effect of kolaviron on Cd-induced production of ROS in U937 monocytes. Cd, cells treated with cadmium only. 10 μM KVR, cells treated with 10 μM kolaviron; 25 μM KVR, cells treated with 25 μM KVR before incubating with Cd. Each column represents mean ± SEM of six replicates. Values having different superscript letters differ significantly (p < 0.05).

3.2 Effect of kolaviron on cytokine secretion

In order to investigate the effect of kolaviron on the production of the cytokines, the U937 monocytes were treated with PMA (transforming them to the macrophage form) before exposed to kolaviron and Cd. The results revealed that kolaviron significantly reduced Cd-
mediated release of the cytokines i.e. TNF-α and IL-6 (Figures 3 and 4). While the reduction of TNF-α secretion was concentration-dependent ($p < 0.05$), the effect on IL-6 secretion was not ($p > 0.05$).

Figure 3. Effect of kolaviron on Cd-induced secretion of TNF-α in U937-derived macrophages. Cd, cells treated with cadmium only. 10 µM KVR, cells treated with 10 µM kolaviron; 25 µM KVR, cells treated with 25 µM KVR before incubating with Cd. Each column represents mean ± SEM of six replicates. Values having different superscript letters differ significantly ($p < 0.05$).
Figure 4. Effect of kolaviron on Cd-induced secretion of IL-6 in U937-derived macrophages. Cd, cells treated with cadmium only. 10 µM KVR, cells treated with 10 µM kolaviron; 25 µM KVR, cells treated with 25 µM KVR before incubating with Cd. Each column represents mean ± SEM of six replicates. Values having different superscript letters differ significantly (p < 0.05).

3.3 Expression of transcription factors and antioxidant enzymes

The effect of kolaviron on Cd-induced expression of the transcription factors (NF-κB, iNOS) and antioxidant enzymes (SOD and CAT) was assessed via RT-PCR (Figure 5). The findings reveal that Cd significantly up-regulated the expression of NF-κB, iNOS, SOD and CAT when compared to untreated cells (p < 0.05). Incubating the macrophages with kolaviron prior to the treatment with Cd reduced the expression levels closer to control levels. However, the effect of kolaviron was only concentration-dependent in the expression of CAT (p < 0.05).
Figure 5. Expression of iNOS transcription factors and antioxidant enzymes in U937-derived macrophages. Values are % fold expressions over housekeeping gene GAPDH for RT-PCR of mRNA isolated from U937-derived macrophages. Cd, cells treated with cadmium only; 10 µM KVR, cells treated with 10 µM kolaviron; 25 µM KVR, cells treated with 25 µM KVR before incubating with Cd. Each column represents mean ± SEM of six replicates. Values having different superscript letters differ significantly (p < 0.05). (Comparisons not made between different markers)

4. DISCUSSION

Cadmium is considered a significant hazard because of its high toxicity at very low levels of exposure coupled with its prolonged half-life (over 20 years) [22]. The heavy metal interferes with several essential biological processes such as uptake of essential metals (e.g. calcium, zinc, Fe), oxidative phosphorylation and basal respiration and the consequences could be cell death [23-25]. This is in accord with the preliminary results of the toxic nature of cadmium. However, the results of the study also revealed that kolaviron reduced cadmium-induced cell death which was concentration-dependent (Figure 1). Flavonoids are cytoprotective because they chelate cadmium thereby reducing its cellular accumulation and enhancing the uptake of
essential elements [26]. Cd-induced cell death has been linked to the generation of ROS [27-28]. Cadmium is not a Fenton-like metal so its induction of ROS might be via an indirect mechanism as suggested [23]. The toxicant interferes with the uptake of essential metal ions which are key co-factors to important scavenger proteins such as superoxide dismutase, peroxidase, catalase therefore inactivates them [29]. The metal also stimulates the production of ROS by binding to complex III of the electron transport chain [30-31].

Several reports have it that flavonoids are cytoprotective due to their direct antioxidant potential [32-33]. However, flavonoids also possess indirect antioxidant activity as they could induce the up-regulation of glutathione, γ-glutamylcystein ligase, glutathione s-transferase and NAD(P)H:quinine oxidoreductase in different cell systems [34]. Another way flavonoids inhibit cell death is by suppressing oxidative stress [17].

Macrophages secrete cytokines (e.g TNF-α and IL-6) when activated and produce NF-κB and iNOS as part of the inflammatory response [35-36]. Thus Cd activates macrophages as part of the inflammatory response since it induced the secretion of pro-inflammatory cytokines TNF-α and IL-6 and expression of NF-κB, iNOS in the transformed U937 cells (otherwise known as U937-derived macrophages) (Figures 3 - 5). The excessive release of these factors has been implicated in many pathophysiological responses [37-38]. Nuclear factor kappa-B is a transcription factor that is up-regulated during inflammation and implicated in some disorders [39-40]. The activation of NF-κB is predisposing to cell death and linked with oxidative stress [41-43]. Thus the induction of NF-κB may accompany cadmium-induced oxidative damage. Though the monocyte form of the cell line could also release the cytokines, treatment with phorbol esters enhances their transformation to the macrophage form and enables them to maintain cell numbers [44].
Nitric oxide is an important ROS produced from arginine in a reaction catalyzed by inducible nitric oxide synthase (iNOS) thus upregulation of iNOS expression is key to oxidation. The expression of iNOS is dependent on the translocation of NF-κB into the nucleus and their co-expression has been linked to various tissue injuries. Excessive release of pro-inflammatory cytokines and other transcription factors by macrophages has been implicated in organ failure, septic shock, rheumatoid arthritis, asthma, cancer, chronic obstructive pulmonary diseases, viral infections, autoimmune diseases, hypotension and other systemic responses which could be traceable to the production of ROS [45-46]. Thus the inhibition of their expression is therapeutic [47-49].

The results from the present investigation revealed that kolaviron reduced Cd-mediated production of the cytokines TNF-α and IL-6 and expression of NF-κB, iNOS (Figures 3–5). Kolaviron has been shown to reduce inflammatory responses by inhibiting the secretion of various cytokines, nitric oxide and downregulation of transcription factors [50-53]. Flavonoids also suppress inflammatory processes by inhibiting NF-κB activation [54].

Since treatment of cells with cadmium resulted in the production of ROS, we thought this may also be due to the reduction in the levels of antioxidant enzymes thus the expression of superoxide dismutase (SOD) and catalase (CAT) in U937-derived macrophages was investigated. The results from this current study reveal that Cd (at 10 µM) increased the expression of both enzymes in the U937-derived macrophages (Figure 5). This seems to be at variance with some reports that Cd suppresses the activity of these enzymes via direct interaction [55-56]. In this experiment, the Cd-mediated elevation of the enzymes could be ascribed to their induction to compensate with the increase in ROS production which has been suggested [57]. The biflavonoid kolaviron reduced the cadmium-mediated production of
the antioxidant enzymes via an indirect mechanism which is attributable to the direct
scavenging of cadmium. However, this could be further investigated.

5. CONCLUSION

The study reveals that kolaviron reduced Cd-induced cell death/ ROS production in the
monocyte cell line U937 and activation of U937-derived macrophages. The production of the
cytokines and transcription factors by macrophages is part of the inflammatory response
which is implicated in some disorders. Thus the ability of kolaviron to reduce cell death may
involve the suppression of the inflammatory response. Since some diseases are traceable to
the oxidation by ROS and inflammation signals, kolaviron (and perhaps *Garcinia kola*) could
be considered in their management. Even though further follow-up studies are still required,
*Garcinia kola* is a valuable resource and should be exploited both nutraceutically and
pharmacologically.

CONFLICT OF INTEREST

The authors declare that they have no conflict in interest.

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