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ARTICLE

Protective effects of kolaviron and gallic acid against cobalt-chloride-induced cardiorenal dysfunction via suppression of oxidative stress and activation of the ERK signaling pathway

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Abstract: Cobalt (Co) toxicity is a potential public health problem due to recent renewed use of Co in orthopedic implants, dietary supplements, and blood doping in athletes and horses. We investigated the protective roles of kolaviron (KV), a biflavonoid of *Garcinia kola*, and gallic acid (GA) on cobalt chloride ($CoCl_2$)-induced cardiorenal damage in rats. $CoCl_2$ caused significant increases ($p < 0.05$) in serum creatine kinase–myocardial band (CK-MB), lactate dehydrogenase (LDH), aspartate transaminase (AST), xanthine oxidase (XO), urea, creatinine, malondialdehyde, H_2O_2, nitric oxide, as well as *C*-reactive protein expression, along with significant ($p < 0.05$) reduction in cardiac and renal expression of extracellular signal regulated kinase (ERK) and the activities of superoxide dismutase, catalase, and glutathione *S*-transferase. KV and GA prevented the toxic effects of $CoCl_2$ by stimulating ERK expression and reversing Co-induced biochemical changes. Administration of $CoCl_2$ alone did not significantly alter ECG patterns in the rats, although co-treatment with KV (200 mg/kg) produced QT-segment prolongation and also appeared to potentiate Co hypotension. Histopathology of the heart and kidneys of rats treated with KV and GA confirmed the biochemical data. KV and GA thus protected against cardiac and renal damage in Co intoxication via antioxidant and (or) cell survival mechanisms, possibly involving ERK activation.

Key words: cobalt, heart, kidneys, kolaviron, gallic acid, oxidative stress, ERK, ECG.

Résumé : L'utilisation récemment renouvelée du cobalt (Co) dans les implants orthopédiques, les suppléments alimentaires et les agents utilisés dans le dopage sanguin chez les athlètes et les chevaux font de la toxicité du Co un problème de santé publique éventuel. Nous avons étudié les rôles protecteurs du kolaviron (KV), un biflavonoïde de *Garcinia kola*, et de l'acide gallique (AG) dans un modèle de dommages cardio-rénaux provoqués par le chlorure de cobalt ($CoCl_2$) chez le rat. Le $CoCl_2$ a entraîné une nette augmentation ($p < 0,05$) des taux sériques de l'isoenzyme myocardique de la créatine kinase (CK-MB), de déshydrogénase lactique (LDH), de xanthine oxydase (XO), d'urée, de créatinine, de malondialdéhyde, de H_2O_2, d'oxyde nitrique ainsi que de l'activation de l'aspartate aminotransférase (AST) et de l'expression de la protéine C réactive dans le sérum, avec une diminution marquée ($p < 0,05$) de l'expression de la protéine ERK (pour « extracellular signal regulated kinase ») et de l'activité de la superoxyde dismutase, de la catalase et de la glutathion *S*-transférase dans le cœur et les reins. Le KV et l'AG ont permis de prévenir les effets toxiques du $CoCl_2$ en stimulant l'expression de la protéine ERK et en contrant les variations des paramètres biochimiques provoquées par le Co. L'administration de $CoCl_2$ seul n'a pas entraîné de modification notable des tracés d'ECG chez le rat, même si l'administration concomitante de KV (à 200 mg/kg) a entraîné un allongement de l'intervalle QT et semblé potentialiser l'hypotension provoquée par le Co. L'examen histopathologique du cœur et des reins des rats recevant du KV et de l'AG a confirmé les données biochimiques. Le KV et l'AG ont donc eu un effet protecteur contre les dommages cardiaques et rénaux provoqués par l'intoxication au Co par l'intermédiaire de mécanismes antioxydants ou de survie cellulaire dans lesquels l'activation de la protéine ERK pourrait jouer un rôle. [Traduit par la Rédaction]

Mots-clés : cobalt, cœur, reins, kolaviron, acide gallique, stress oxydatif, ERK, ECG.

Introduction

Cobalt has been used historically in the treatment of pernicious anemia, due to its ability to stimulate the production of red blood cells and hemoglobin (Barceloux 1999). It was also employed in the 1960s as a foam stabilizer in beers (Alexander 1972). The use of this metal in these situations had been accompanied by toxic effects, namely thyroid dysfunction in children and cardiomyopathies in some heavy beer drinkers (Paustenbach et al. 2013). The introduction of more efficacious drugs in the treatment of anemia and the occurrence of adverse effects led to the termination of cobalt use in these situations decades ago.

Recent concerns over cobalt toxicity have emerged from its potential use as a blood doping agent in humans and animals (Jelkmann and Lundby 2011; Lippi et al. 2006; Mobasheri and

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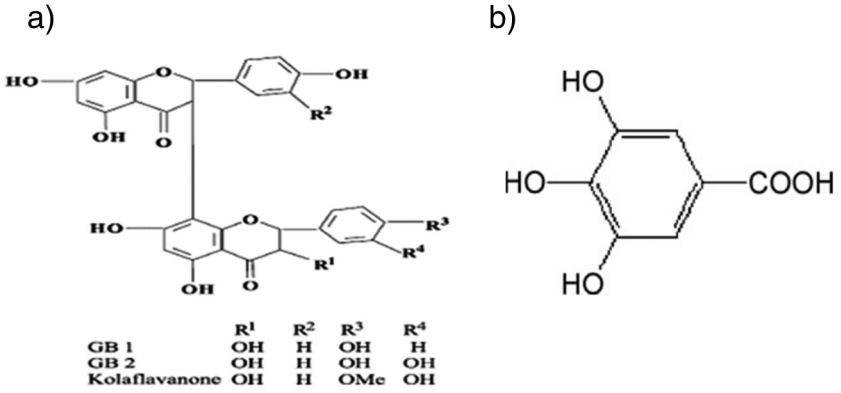
Fig. 1. Structures of (*a*) kolaviron and (*b*) gallic acid.



Proudman 2015), as well as its use in surgical patients with cobalt-containing hip implants (Hasegawa et al. 2012; Macnair et al. 2012). Furthermore, intense mining activities in certain areas have led to environmental contamination with potential widespread exposures to cobalt (Banza et al. 2009; Cheyns et al. 2014). The consumption of cobalt-containing dietary supplements is also believed to be on the rise in different parts of the world (Tvermoes et al. 2013).

Formation of reactive oxygen species, leading to oxidative stress and lipid peroxidation has been shown to be one of the major pathogenetic mechanisms for cobalt-induced tissue injuries (Ahmed and Siddiqui 2007; Catelas et al. 2005; Franco et al. 2009; Kubrak et al. 2011). Oxidative processes result in post-translational modifications of a variety of proteins including kinases (e.g., extracellular signal-regulated kinase, ERK; p38); proteases (e.g., caspases) and transcription factors (e.g., NF-κB, AP-1, p53, Nrf 2) (Franco et al. 2009; Son et al. 2011). These proteins regulate diverse biochemical and cellular functions, ranging from cell survival to cell death (Haagenson and Wu 2010).

Antioxidants may act directly by scavenging reactive oxygen and nitrogen species (ROS/RNS) via direct consumption or chemical modification. Alternatively, antioxidants may act indirectly by controlling gene expression during oxidative stress by up-regulating phase II detoxifying and antioxidant enzymes (Kim and Jang 2014). Stimulation of these enzymes usually occurs by the activation of nuclear transcription factor-erythroid 2-related factor 2 (Nrf-2) via its release from Kelch-like ECH-associated protein 1 (Keap 1)-Nrf-2 complex (Park et al. 2004). In addition, Keap 1-independent activation of Nrf-2 can occur by its phosphorylation by several signal pathways involving ERK, phosphatidyl inositol 3-kinase (PI3K/Akt), and protein kinase C (PKC) (Baird and Dinkova-Kostova 2011; Itoh et al. 2004).

Flavonoids have found valuable therapeutic and prophylactic applications due to their excellent free radical scavenging activities (Havsteen 2002) and their ability to induce the expression of detoxifying and antioxidant enzymes (Hörmann et al. 2012; Mann et al. 2009). Kolaviron (KV) is a de-fatted extract from bitter kola (*Garcinia kola*) seeds, widely reported to contain *Garcinia* biflavonoid 1 (GB1), *Garcinia* biflavonoid 2 (GB2) and kolaflavanone (Fig. 1*a*). Its antioxidant and anti-inflammatory actions have been employed to protect against many pathological conditions (Adedara and Farombi 2012; Olaleye et al. 2000). Its cardioprotective (Adaramoye and Lawal 2015) and nephroprotective (Adedara et al. 2015) actions have been reported. Gallic acid (GA; Fig. 1*b*) is an endogenous plant phenol that is found in many fruits, teas, and wine (Ma et al. 2003; Singh et al. 2004). It has been widely reported for its strong antioxidant (Kroes et al. 1992), anti-inflammatory (Kim et al. 2002), and cardioprotective properties (Priscilla and Prince 2009).

In the present study, we attempted to evaluate the cardio- and nephro-protective effects of KV and GA on cardiac and renal damage induced by cobalt chloride administration in rats. We attempted to demonstrate the possible mechanisms of the therapeutic efficacies of these compounds by studying the biochemical markers of injury to cardiac and renal tissues, antioxidant defense system, electrocardiographic and histopathological changes, as well as immunohistochemical staining patterns of a key signaling protein, ERK.

Materials and methods

Chemicals

Cobalt chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) was obtained from Tianjin Kermel Chemical Reagent Co., China. GA, glutathione, 1, 2-dichloro-4-nitrobenzene (CDNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), sodium hydroxide, xylenol orange, potassium hydroxide, and hydrogen peroxide were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). All other chemicals were of the highest purity commercially available.

Extraction of KV

Seeds of *Garcinia kola* were obtained from a local vendor in Ibadan, Nigeria. They were identified at the Department of Botany herbarium, University of Ibadan, Nigeria. The seeds were peeled, sliced, and dried at room temperature (25–28 °C). KV was extracted according to the method reported by Iwu (1985).

Animal treatment and experimental design

Male Wistar rats aged between 10 and 12 weeks (180–200 g) were obtained from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. They were housed in plastic cages placed in a well-ventilated animal house and were given ad libitum access to rat chow (Ladokun Feeds Ltd., Ibadan, Nigeria) and subjected to natural photoperiod of 12 h light – 12 h dark cycle. All the animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science (2011). The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animal welfare during experiments (PHS 1996).

The rats were divided randomly into 5 groups of 8 rats each. Group A received clean tap water only, while Group B was treated with cobalt chloride ($CoCl_2$, 350 ppm) in drinking water for 14 days. Groups C and D rats were orally treated by gavage with 100 and 200 mg/kg KV (KV1 and KV2), respectively, along with $CoCl_2$ treatment in drinking water. Group E rats were treated by oral gavage with 120 mg/kg GA and also with $CoCl_2$ in the same manner as groups C and D. The doses of cobalt chloride, KV and GA have been carefully chosen based on previous studies (Farombi et al. 2012; Garoui et al. 2011, 2012; Oyagbemi et al. 2015).

Electrocardiography

Standard lead II electrocardiogram was recorded in rats immobilized with xylazine–ketamine combination using a 6/7-lead ECG

machine (EDAN VE-1010, Shanghai, China). The machine was calibrated at 20 mm/mV paper speed and 50 mm/s paper speed. From the electrocardiogram, parameters such as heart rate, PR interval, QRS wave duration, R-wave amplitude, and QT/QTc values were determined.

Blood pressure measurements

After all the treatments, indirect blood pressure parameters, including systolic, diastolic, and mean arterial blood pressures were determined without anesthesia, by tail plethysmography using an electrosphygmomanometer (CODA, Kent Scientific, USA). The average of at least 9 readings, taken in the quiescent state, following acclimatization, was recorded per animal.

Animal necropsy and tissue preparation

Blood was drawn from the retro-orbital plexus into plain vials prior to sacrifice. All rats were sacrificed by cervical dislocation, 24 h after the last treatment. The heart and kidneys were quickly removed, rinsed in 1.15% KCl and homogenized in potassium phosphate buffer (0.1 mol/L, pH 7.4), and homogenates were centrifuged at 10 000*g* for 20 min to obtain the post-mitochondrial supernatant, which was maintained at −4 °C used for the biochemical assays. The collected blood was allowed to clot and the samples were centrifuged at 3000*g* for 10 min. Serum was collected as the supernatant and was also used for some biochemical assays.

Determination of cardiac and renal oxidative stress and antioxidant markers

Protein concentration was determined using the Biuret method as described by Gornal et al. (1949). Hydrogen peroxide (H_2O_2) generation was determined spectrophotometrically at 560 nm as described by Wolff (1994). Lipid peroxidation was evaluated by estimating malodialdehyde (MDA) using the method of Varshney and Kale (1990). MDA content was quantified with a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ and expressed as micromoles per gram of tissue. Superoxide dismutase (SOD) activity was determined by measuring the inhibition of the auto-oxidation of epinephrine at pH 7.2 at 30 °C as described by Misra and Fridovich (1972) with slight modifications from our laboratory (Oyagbemi et al. 2015). Briefly, 100 mg of epinephrine was dissolved in 100 mL distilled water and acidified with 0.5 mL concentrated hydrochloric acid. Then, 0.01 mL of each sample was added to 2.5 mL of 0.05 mol/L carbonate buffer (pH 10.2), followed by the addition of 0.3 mL of 0.3 mmol/L epinephrine. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. One unit of SOD activity represents the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 min. The concentration of GSH was determined at 412 nm using the method described by Jollow et al. (1974). Glutathione-*S*-transferase (GST) activity was estimated by the method of Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Catalase (CAT) activity was determined according to the method of Sinha (1972). Nitric oxide content was measured as described by Olaleye et al. (2007) by indirectly measuring the nitrite concentration. The activity of xanthine oxidase in serum was determined according to the method described by Akaike et al. (1990).

Evaluation of markers of cardiac and renal injury

Activities of creatine kinase–myocardial band (CK-MB), lactate dehydrogenase (LDH), and aspartate transaminase (AST) and the concentrations of urea and creatinine in serum were assayed in in serum using commercial kits purchased from Randox Laboratories Ltd. (Crumlin, UK).

Immunohistochemistry of ERK and C-reactive protein (CRP)

Immunohistochemistry of paraffin-embedded heart and kidney tissues was performed after the tissues were fixed with 10% formalin based on the methods described by Todorich et al. (2011) with slight modifications. Briefly, paraffin sections were melted at 60 °C in the oven. Dewaxing of the samples in xylene was followed by passage through ethanol of decreasing concentration (100%–80%). Peroxidase quenching with 1% H_2O_2/methanol was followed by antigen retrieval performed by microwave heating in 0.01 mol/L citrate buffer (pH 6.0) to boil. All the sections were blocked in normal goat serum (10%, HistoMark, KPL, Gaithersburg, Maryland, USA) and probed with anti-ERK and anti-CRP antibodies, as appropriate (Bioss, San Diego, California, USA), 1:200 overnight at room temperature. Detection of bound antibody was carried out using biotinylated (goat anti-rabbit, 2.0 μg/mL) secondary antibody and subsequently, streptavidin peroxidase (horseradish peroxidase – streptavidin) according to manufacturer's protocol (HistoMark, KPL, Gaithersburg, Maryland, USA).

The reaction product was enhanced with diaminobenzidine (DAB, Amresco, USA) for 2–3 min and counterstained with high definition hematoxylin (Enzo, New York, USA), with subsequent dehydration in ethanol. The slides were covered with coverslips and sealed with resinous solution. The immune-reactive positive expression of ERK- and CRP-intensive regions were viewed starting from low magnification on each slide then with 400 × magnifications using a photo microscope (Olympus) and a digital camera (Toupcam; Touptek Photonics, Zhejiang, China).

Histopathology

Small pieces of cardiac and renal tissues were collected in 10% buffered formalin (pH 7.4) for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5–6 μm in thickness were made and stained with hematoxylin and eosin for histopathological examination (Drury et al. 1976).

Statistical analyses

Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups with Least Significant Difference (LSD) post-hoc analysis, followed by Student's *t* test using GraphPad Prism software (version 6.01). *P* values <0.05 were considered statistically significant.

Results

Effects of $CoCl_2$ exposure and treatments with KV and GA on blood pressure parameters

Cobalt chloride caused significant reduction ($p < 0.05$) in systolic blood pressures in the rats, compared with control. The reductions observed in diastolic and mean arterial pressures were, however, not statistically significant. Rats treated with $CoCl_2$ + KV exhibited further dose-dependent reduction in systolic, diastolic, and mean arterial pressures when compared with those treated with $CoCl_2$ alone. There were also blood pressure reductions in the group exposed to $CoCl_2$ + GA, although these were not as much as the blood pressure reductions caused by the higher dose of KV (Table 1).

ECG waves and intervals

Electrocardiographic measurements for control and experimental rats are presented in Table 2. No statistically significant differences were observed in the heart rate, P wave, PR interval, QRS duration, QT, QT corrected (Bazett), and R amplitude between rats treated with $CoCl_2$ alone and the control rats. Similarly, rats co-treated with KV and GA did not show any significant alterations in most of the ECG parameters including heart rate, P wave, PR interval, QRS duration, and R amplitude. The only noticeable significant ($p < 0.05$) alteration was displayed by rats co-treated with $CoCl_2$ and KV (200 mg/kg), which displayed prolonged QT and QTc intervals, compared with control rats, as well as the rats given $CoCl_2$ alone.

Effect of KV and GA on markers of cardiac and renal injury

In $CoCl_2$-exposed rats, we observed significant increases ($p < 0.05$) in serum levels of all the markers of myocardial injury measured in

Table 1. Effects of kolaviron (KV) and gallic acid on blood pressure parameters in rats exposed to cobalt chloride.

	A	B	C	D	E
Systolic blood pressure (mm Hg)	154.56±5.04	136.10±1.89[a]	129.25±0.55[a,b]	93.95±17.81[a,b]	117.29±4.53[a,b]
Diastolic blood pressure (mm Hg)	125.00±6.36	117.55±11.86	114.08±5.05	82.60±17.94[a,b]	94.50±17.30[a]
Mean arterial blood pressure (mm Hg)	134.44±5.92	123.41±8.05	118.92±3.50	85.98±17.94[a,b]	101.76±12.90[a,b]

Note: Values are presented as mean ± standard deviation ($n = 10$). A, Control; B, $CoCl_2$ alone; C, $CoCl_2$ + KV (100 mg/kg); D, $CoCl_2$ + KV (200 mg/kg); E, $CoCl_2$ + gallic acid (120 mg/kg).

[a]Significant difference at $p < 0.05$, when compared with control.

[b]Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.

Table 2. Effects of kolaviron (KV) and gallic acid on electrocardiographic parameters in rats exposed to cobalt chloride.

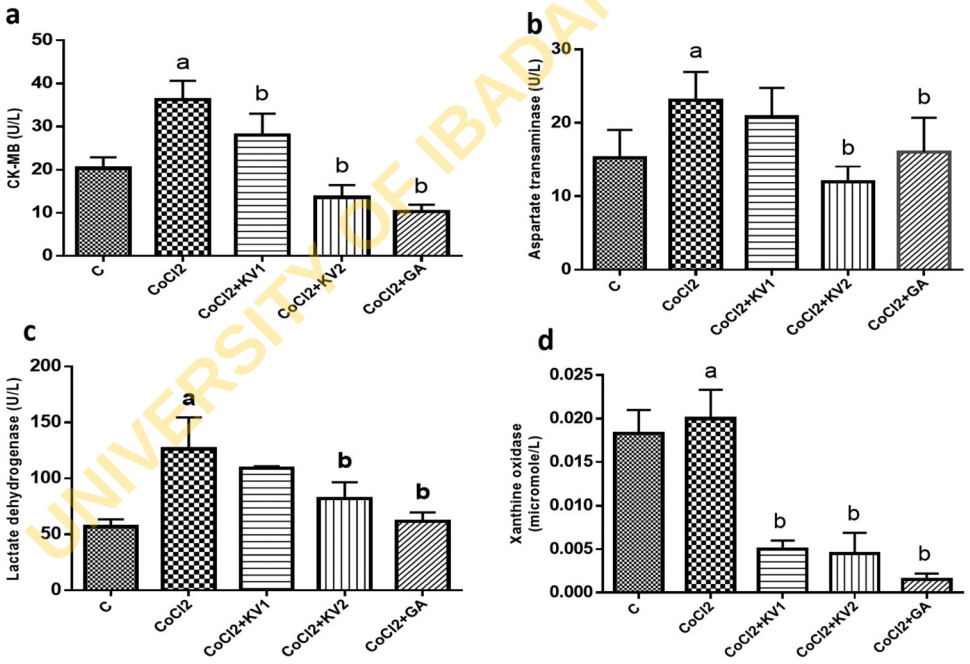
	A	B	C	D	E
Heart rate (per min)	278.00±20.42	291.33±38.42	274.33±19.40	282.00±49.50	303.50±38.89
P wave duration (ms)	21.67±1.53	20.00±7.81	23.67±3.06	17.50±4.95	21.50±12.02
PR interval (ms)	41.00±1.00	40.33±4.62	47.67±7.51	38.00±4.24	36.50±9.19
QRS duration (ms)	16.67±1.53	19.00±5.29	19.33±4.16	21.00±5.66	14.50±9.19
QT segment (ms)	78.67±9.02	76.33±8.51	69.33±1.16	99.50±9.19[a,b]	71.00±5.66
QT corrected Bazett (ms)	168.33±16.44	167.00±10.44	147.67±5.03	214.00±1.41[a,b]	159.50±23.34
R amplitude (ms)	0.24±0.036	0.22±0.056	0.29±0.035	0.31±0.129	0.21±0.066

Note: Values are presented as mean ± SD ($n = 10$). A, Control; B, $CoCl_2$ alone; C, $CoCl_2$ + KV (100 mg/kg); D, $CoCl_2$ + KV (200 mg/kg); E, $CoCl_2$ + gallic acid (120 mg/kg).

[a]Significant difference at $p < 0.05$, when compared with control.

[b]Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.

Fig. 2. Effect of cobalt chloride with or without kolaviron (KV) or gallic acid (GA) on cardiac marker enzymes. Values are presented as mean ± standard deviation ($n = 10$). [a], significant difference at $p < 0.05$, when compared with control (C); [b], Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.



this study: CK-MB, LDH, AST, and xanthine oxidase (XO) (Fig. 2). KV exhibited dose-dependent reductions ($p < 0.05$) in all these parameters, when compared with the $CoCl_2$ group, while GA also showed marked reduction in the activities of these enzymes. There were also significant increases ($p < 0.05$) in the markers of renal injury, urea, and creatinine in the $CoCl_2$ group compared with the control (Fig. 3). The increased levels of creatinine was significantly reversed ($p < 0.05$) by both KV and GA, while significant ($p < 0.05$) amelioration of serum urea concentration was only obtained with KV at 200 mg/kg.

Effect of KV and GA on markers of oxidative stress and antioxidant defense system

Exposure to $CoCl_2$ caused the induction of oxidative stress in cardiac tissues, indicated by significant increases in hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and nitric oxide contents, compared with control (Table 3). Kidney tissues also showed significant elevation in MDA level. KV and GA exhibited significant antioxidant activity towards H_2O_2 in both the heart and kidneys, causing significant reduction ($p < 0.05$) in this reactive oxygen species, compared with the $CoCl_2$ group. KV produced significant reduction ($p < 0.05$) of MDA levels in the kidneys, as well as significant reduction ($p < 0.05$) of nitric oxide in the heart.

From Table 4, no significant alterations were observed in the concentration of reduced glutathione (GSH) in both the heart and kidneys of rats exposed to $CoCl_2$, with or without KV or GA. However, we observed significant reduction ($p < 0.05$) in the activities of SOD, CAT, and GST in the heart of $CoCl_2$-treated rats, while the kidney also showed significant reductions ($p < 0.05$) in CAT and

Fig. 3. Effect of cobalt chloride with or without kolaviron (KV) or gallic acid (GA) on serum urea and creatinine. Values are presented as mean ± standard deviation (n = 10). [a], significant difference at $p < 0.05$, when compared with control (C); [b], Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.

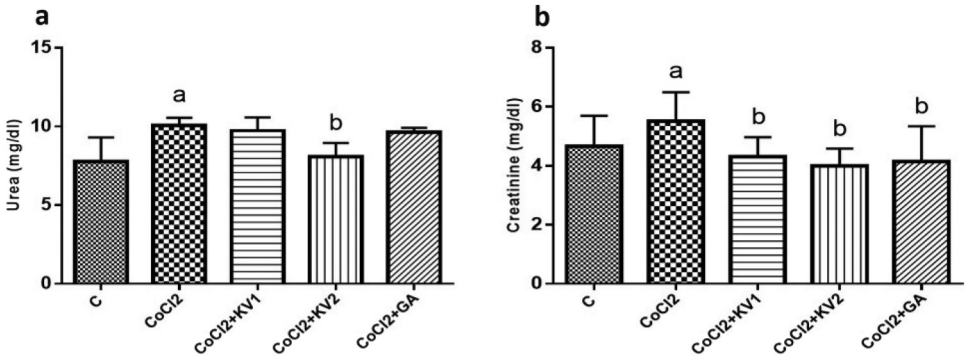


Table 3. Effects of kolaviron (KV) and gallic acid on markers of oxidative stress in cardiac and renal tissues of rats exposed to cobalt chloride.

	A	B	C	D	E
Hydrogen peroxide (μmol/min per mg protein)					
Heart	10.95±0.46	12.08±0.74[a]	11.33±0.35[b]	10.87±0.50[b]	10.77±0.70[b]
Kidney	10.31±0.27	11.17±0.50[a]	9.86±0.40[b]	9.83±0.61[b]	10.15±0.26[b]
Malondialdehyde (μmol/g tissue)					
Heart	0.63±0.04	0.91±0.17[a]	0.90±0.11	0.89±0.04	0.90±0.10
Kidney	1.40±0.47	1.64±0.08	1.10±0.19[b]	1.02±0.04[b]	1.16±0.30
Nitric oxide (μmol/L)					
Heart	0.14±0.03	0.19±0.01[a]	0.19±0.05	0.16±0.02[b]	0.14±0.03[b]
Kidney	0.75±0.10	0.76±0.13	0.69±0.16	0.65±0.09	0.67±0.13

Note: Values are presented as mean ± SD (n = 10). A, Control; B, $CoCl_2$ alone; C, $CoCl_2$ + KV (100 mg/kg); D, $CoCl_2$ + KV (200 mg/kg); E, $CoCl_2$ + gallic acid (120 mg/kg).
[a]Significant difference at $p < 0.05$, when compared with control.
[b]Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.

Table 4. Effects of kolaviron (KV) and gallic acid on antioxidant systems in cardiac and renal tissues of rats exposed to cobalt chloride.

	A	B	C	D	E
GSH (μmol/g tissue)					
Heart	43.80±1.07	43.90±0.37	44.18±0.53	43.95±0.51	44.50±0.61
Kidney	47.57±0.47	47.20±0.71	47.02±0.63	46.54±0.66	47.48±1.53
SOD (units/mg protein)					
Heart	0.19±0.07	0.018±0.007[a]	0.21±0.07[b]	0.24±0.03[b]	0.25±0.06[b]
Kidney	0.085±0.022	0.083±0.026	0.184±0.047[b]	0.265±0.034[b]	0.264±0.066[b]
CAT (μmol H_2O_2 consumed/min per mg protein)					
Heart	31.57±1.36	26.95±1.42[a]	31.78±0.97[b]	31.50±1.31[b]	27.90±1.45
Kidney	89.33±4.10	79.69±2.38[a]	79.18±7.01	78.86±5.11	77.16±4.70
GST (mmol CDNB-GSH complex formed/min per mg protein)					
Heart	0.021±0.004	0.011±0.006[a]	0.021±0.006[b]	0.023±0.009[b]	0.018±0.003
Kidney	0.014±0.005	0.0021±0.0009[a]	0.0065±0.0014[b]	0.0078±0.0045[b]	0.0076±0.004

Note: Values are presented as mean ± SD (n = 10). A, Control; B, $CoCl_2$ alone; C, $CoCl_2$ + KV (100 mg/kg); D, $CoCl_2$ + KV (200 mg/kg); E, $CoCl_2$ + gallic acid (120 mg/kg).
[a]Significant difference at $p < 0.05$, when compared with control.
[b]Significant difference at $p < 0.05$, when compared with $CoCl_2$ group.

GST. KV demonstrated significant ($p < 0.05$) dose-dependent restoration of SOD, CAT, and GST activities in the heart and kidneys, when compared with the $CoCl_2$ group, while GA only exhibited significant elevation of SOD activity.

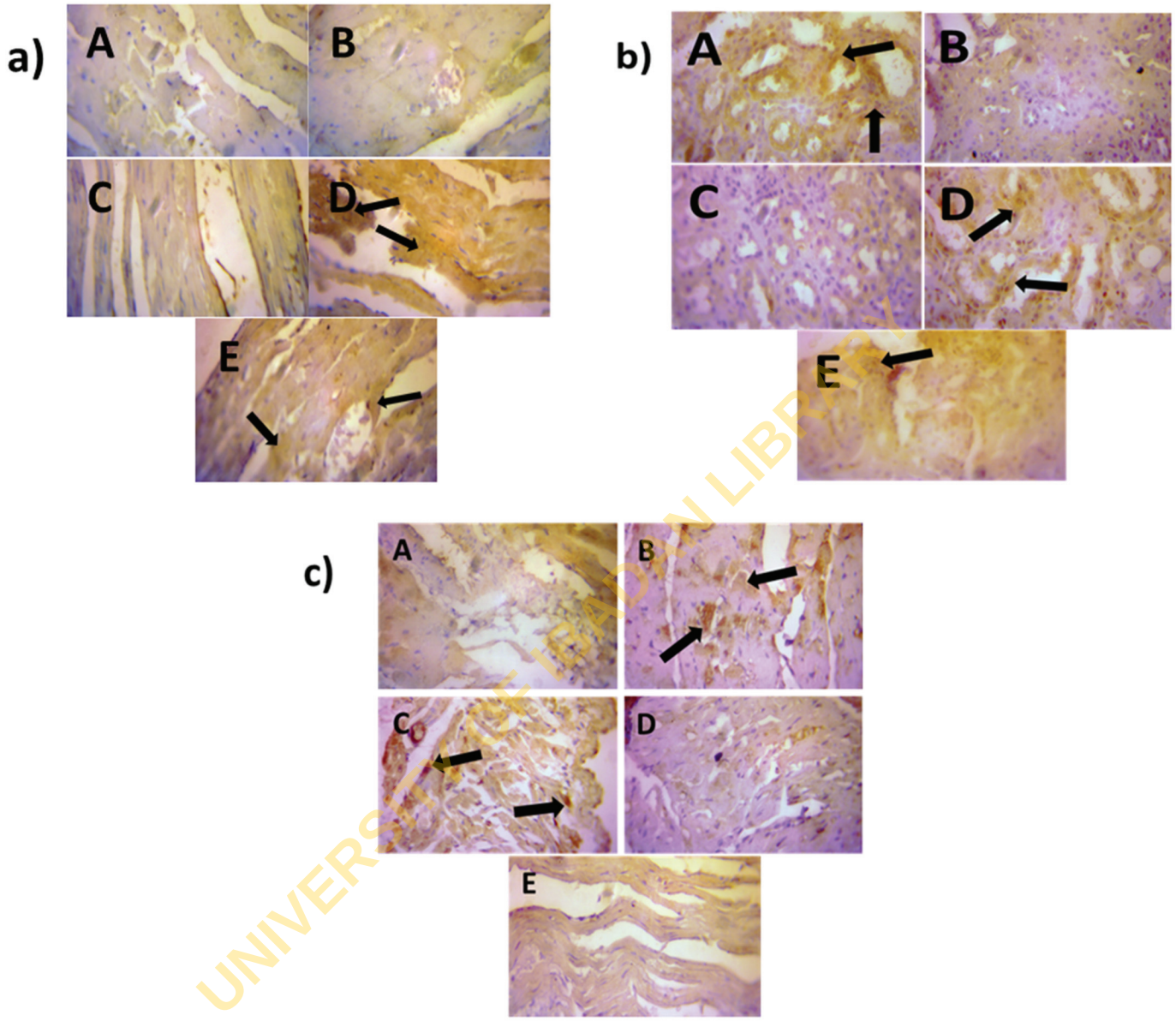
Effect of KV and GA on myocardial and renal histoarchitecture

Histopathological examination of the cardiac and renal tissues confirmed the biochemical findings of cardiac and renal damage due to cobalt intoxication in the rats (Figs. 5 and 6). Hearts from rats that received cobalt chloride alone showed disseminated hemorrhagic lesions with congestion of coronary blood vessels and mild infiltration of the myocardium and atrium by inflammatory cells. Kidney damage was indicated histologically by severe loss of normal morphology, loss of tubular and glomerular outlines with marked peri-tubular inflammatory cell infiltration, and vascular congestion. However, treatment with KV, especially at the higher dose, and GA, prevented much of the damage as the histological appearance of the tissues tended towards that in the control rats.

Expression of ERK and CRP in cardiac and renal tissues

Immunohistochemically, the cardiac tissues in the control group displayed faint ERK staining (Fig. 4*a*), whereas the kidneys exhibited a much greater staining intensity (Fig. 4*b*). Exposure to $CoCl_2$ alone exhibited very weak ERK immunoreactivity in both the heart and kidneys. By contrast, rats treated with either KV or

Fig. 4. Immunohistochemical staining patterns of (*a*) extracellular signal-regulated kinase (ERK) in cardiac tissues, (*b*) ERK in kidney tissues, and (*c*) C-reactive protein (CRP) in cardiac tissues, of rats exposed to cobalt chloride with or without treatment with kolaviron (KV) and gallic acid. (A) Control; (B) $CoCl_2$ alone; (C) $CoCl_2$ + KV (100 mg/kg); (D) $CoCl_2$ + KV (200 mg/kg); (E) $CoCl_2$ + gallic acid (120 mg/kg). Intensity of staining is indicated with black arrows. [Colour online.]



GA, showed a strong staining intensity for ERK in both the heart and kidneys. This effect appeared to be much stronger with KV at the higher dose.

CRP immunoreactivity was monitored only in the heart tissues and this study (Fig. 4*c*). Rats in the $CoCl_2$ group exhibited a strong staining intensity for CRP, compared with all other groups. The higher dose of KV, as well as GA, both demonstrated much lower immunoreactivity for CRP, while control rats only had faint expression of this protein.

Discussion

The chemopreventive property of phenolic antioxidants has become very attractive in their use for protection against toxic and neoplastic effects of many xenobiotics, including heavy metals. Their ability to induce several detoxifying and antioxidant enzymes explains, in large part, the mechanisms underlying their protective actions. In this study, 2 natural phenolics, KV and GA, have been investigated for their protective activities against cobalt toxicity in cardiac and renal tissues of Wistar rats. Cobalt, as a metal, is currently growing in importance due to recent modern applications in medical, industrial, and domestic settings.

Cobalt-induced cardiac damage in the present study was indicated by significant elevation in serum CK-MB, AST, and LDH activities in the group of rats treated with $CoCl_2$ alone. Serum CK-MB, AST, and LDH are well-known diagnostic markers of myocardial damage. Damage to cardiomyocytes by different toxicants causes an increase in membrane permeability or rupture of the cells themselves. As a result, these enzymes, normally found intracellularly, can enter the bloodstream, thereby increasing their activities in serum (Priscilla and Prince 2009). CK-MB is highly specific as a marker of myocardial damage in this regard (Patel et al. 2010).Treatment with KV and GA effectively prevented the elevation in the activities of these enzymes. The protective effect obtained for KV was dose-dependent for all the enzymes.

Fig. 5. Photomicrographs of heart sections of rats exposed to cobalt chloride with or without treatment with kolaviron (KV) and gallic acid. (A) Control; (B) $CoCl_2$ alone; (C) $CoCl_2$ + KV (100 mg/kg); (D) $CoCl_2$ + KV (200 mg/kg); (E) $CoCl_2$ + gallic acid (120 mg/kg). Hemorrhagic lesions in the myocardium are indicated with blue arrows. [Colour online.]

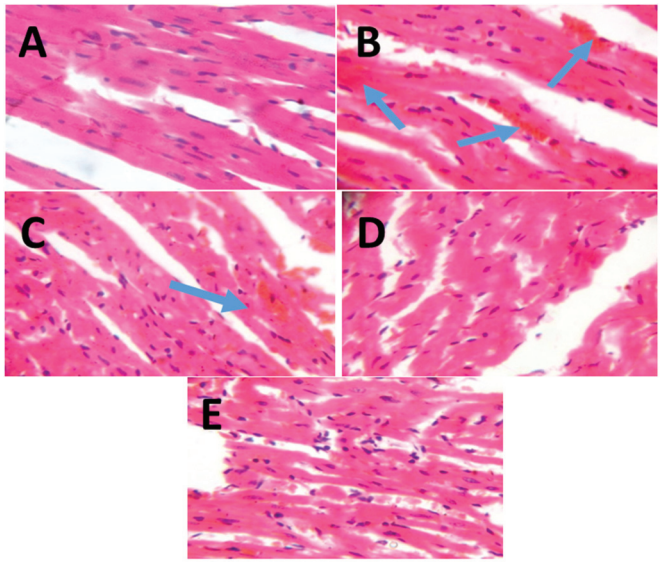
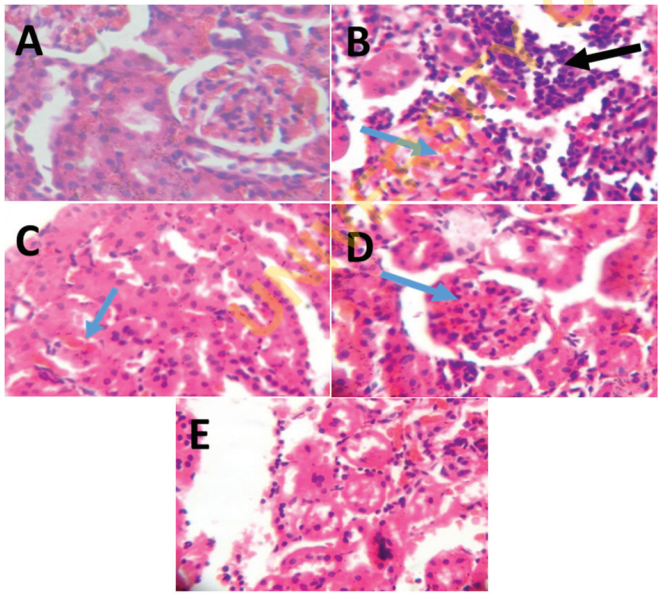


Fig. 6. Photomicrographs of kidney sections of rats exposed to cobalt chloride with or without treatment with kolaviron (KV) and gallic acid. (A) Control; (B) $CoCl_2$ alone; (C) $CoCl_2$ + KV (100 mg/kg); (D) $CoCl_2$ + KV (200 mg/kg); (E) $CoCl_2$ + gallic acid (120 mg/kg). Hemorrhagic lesions and congested vessels are indicated with blue arrows; black arrows show severe inflammatory cell infiltration. [Colour online.]



The kidney is the main site of metabolism and elimination of many metals and other toxicants (Matos et al. 2009). Toxicants that affect the kidneys can interfere with the incorporation of amino acids into proteins can increase the serum concentration of nitrogen-containing products of protein metabolism, including urea and creatinine, due to the failure of their elimination (Atessahin et al. 2005; Cuzzocrea et al. 2002). Our study revealed increased levels of urea and creatinine in the serum of $CoCl_2$-treated rats, an indication of possible renal damage. Similar results were obtained by Garoui et al. (2012), who utilized the same concentration of cobalt chloride as in the present study. With respect to these renal parameters, KV, at the higher dose, produced better amelioration of cobalt-chloride-induced renal damage than GA.

We found evidence for the involvement of ROS as mediators of cardiac and renal damage in $CoCl_2$-treated rats. There were significant increases in hydrogen peroxide levels in heart and kidneys, as well as increased malondialdehyde and nitric oxide levels, especially in cardiac tissues. This pattern of elevation of these parameters clearly indicates the induction of oxidative stress and lipid peroxidation. Increased lipid peroxidation might be responsible for increased cell membrane permeability that led to the leakage of intracellular enzymes such as CK-MB, LDH, and AST into the serum. Further evidence of free radical generation was indicated by the elevated serum activity of XO. XO is an important source of free radicals and an important marker of myocardial damage (Raghuvanshi et al. 2007). Oxidation of sulfhydryl residues in xanthine dehydrogenase can easily give rise to XO, both enzymes being inter-convertible forms of what is collectively known as xanthine oxido-reductase (XOR) (Harrison 2002, 2004).

In ischemic or hypoxic conditions, cellular ATP is degraded to hypoxanthine, which is converted by XO to oxygen free radicals (Hearse et al. 1986), such as superoxide radicals. XO-derived superoxide radicals can also react with nitric oxide forming the highly cytotoxic peroxynitrite (Trujillo et al. 1998), which causes tissue injury. In addition, cardiac damage was indicated by increased myocardial expression of CRP in $CoCl_2$-treated rats. CRP is a prototype inflammatory marker and is used as a predictor of coronary heart disease (Calabro et al. 2012). KV and GA caused reversal of $CoCl_2$-induced increases in oxidative stress parameters and increased CRP expression in the present study, affirming the profound antioxidant and anti-inflammatory activities of these compounds.

To understand the involvement of key signaling pathways in the protection of cobalt-induced injury by KV and (or) GA, we investigated the expression of the extracellular signal-regulated kinase (ERK). ERKs are one of 4 families of proteins called Mitogen-activated protein kinases (MAPKs), which play vital roles in signaling events involved in cell proliferation, differentiation, metabolism, survival, and apoptosis. The other proteins in this class are c-Jun N-terminal kinases (JNK), p38 and ERK5 (or Big MAPK) (Rose et al. 2010). While ERKs are known to be stimulated mainly by growth factors (Ramos 2008), JNK and p38 are activated by stress factors such as UV-light, oxidant stress, infection, and osmotic shock (Kyriakis and Avruch 2001), and are both collectively called Stress-activated MAPKs (SAPKs). With immunohistochemistry, both the heart and kidneys of $CoCl_2$-treated rats consistently exhibited lower expressions of ERK. However, KV and GA produced considerably higher expressions of this protein in both the heart and kidneys.

Stimulation of the ERK pathway by various stimuli has been demonstrated by various studies to produce cardioprotection (Hausenloy and Yellon 2007). Similarly, other studies have suggested that a down-regulation of ERK 1/2 is involved in doxorubicin-induced cardiac damage (Su et al. 2006; Xiang et al. 2009). These studies concluded that administration of substances that increase ERK 1/2 leads to prevention of doxorubicin-induced cardiotoxicity. Based on these studies and the findings from our study, it is reasonable to infer that ERK activation has mechanistic implications for the protection offered by KV and (or) GA.

The explanation for the mechanistic involvement of ERKs, as observed in the present study, may be linked to their ability to directly phosphorylate Nrf-2, causing the stimulation of the transcription factor. Downstream effectors of Nrf-2 include phase II detoxifying enzymes, such as GSTs, as well as antioxidant enzymes like SOD, CAT, and GPx. We found significantly elevated

activities of SOD, CAT, and GST in the cardiac and renal tissues of rats treated with KV and GA, compared with those treated with $CoCl_2$ alone. This points to a likely up-regulation of these enzymes by mechanisms possibly related to the activation of ERK, as observed in this study.

The data on blood pressure indicated a reduction in blood pressure indices and especially the systolic blood pressure, in rats treated with cobalt chloride alone. This is in agreement with previous reports that infusion of Co^{2+} (55 mg/kg) lowered blood pressure in Wistar rats by decreasing total peripheral resistance, suggesting that cobalt acted by vasodilator effects on blood vessels (Dugin et al. 1991). With rats co-treated with KV or GA, we observed further decreases in all blood pressure indices measured, suggesting that these compounds exerted synergistic effects with cobalt chloride to cause hypotension in the rats. Previous reports have identified lowering effects of *Garcinia kola* extracts on systolic and mean arterial blood pressures (Naiho and Ugwu 2009). Like cobalt, the blood pressure-lowering effect of *Garcinia kola* extracts was attributed to its relaxant effects on vascular smooth muscles. Substances that cause vasorelaxation could act by stimulating cyclic AMP, which, by an energy-requiring Ca^{2+}-binding mechanism, may produce a decrease in sarcoplasmic Ca^{2+} and, hence, relaxation. Juxtaposing our findings in the present study with those of Naiho and Ugwu (2009), it may be reasonable to suggest that the hypotension-inducing factor in *Garcinia kola* may indeed be KV. To support this assertion, previous experiments in rat superior mesenteric arteries have provided functional evidence that KV produce vaso-relaxant effects by blockade of influx of extracellular Ca^{2+}, inhibition of intracellular Ca^{2+} release, and opening of K^+ channels (Adaramoye and Medeiros 2009).

Electrocardiography is considered one of the most important clinical tests for diagnosis of cardiac dysfunction (Patel et al. 2010). ECG assessments in this study showed that rats treated with $CoCl_2$ alone did not show any statistically significant differences in the patterns of most ECG parameters, when compared with control rats. However, co-treatment of $CoCl_2$ with KV at 200 mg/kg produced a noticeable alteration in the form of prolonged QT and QTc intervals, compared with control. The QT interval is the time from the start of the QRS complex to the end of the T wave. QT is a measure of the time required for the rapid inflow of Na^+ and Ca^{2+}, resulting in the depolarization of the myocardium and the outflow of K^+, ultimately resulting in repolarization (Moskovitz et al. 2013). Because the rate of depolarization and repolarization depends on a patient's heart rate, QT is usually corrected to the QTc value. Prolonged QT/QTc interval indicates delayed repolarization of the heart, and usually identifies patients with increased risk of acute cardiovascular complications including ventricular dysrhythmias, seizure-like activities, or sudden cardiac death (Witchel and Hancox 2000). Our finding from the present study would suggest that KV at high doses may predispose to cardiovascular risks and must, therefore, be used with caution.

Histopathological findings of rats treated with KV and GA showed a near normal morphology of the heart and kidney tissues, compared with those of rats treated with $CoCl_2$ alone, which showed disseminated hemorrhagic lesions in the myocardium and inflammatory cell infiltration and vascular congestion in the kidneys.

Overall, our study clearly shows the cardioprotective and nephroprotective effects of KV and GA against tissue damage induced by cobalt chloride. Our findings lend further credence to previous reports on the health benefits of these compounds on the heart and kidneys, as have been reported elsewhere (Adaramoye and Lawal 2015; Adedara et al. 2015; Priscilla and Prince 2009). Significant correlation in ERK expression and the activities of antioxidant and detoxifying enzymes in this study provided some mechanistic insight into the protective abilities of these compounds. However, the use of KV in treating conditions involving oxidative stress must be with caution to avoid synergistic drug–drug interactions that may potentiate its effects on lowering of blood pressure, as well as the risk of cardiovascular complications associated with the use of high doses.

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

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