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<tr>
<th>Journal:</th>
<th>Applied Physiology, Nutrition, and Metabolism</th>
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<tr>
<td>Manuscript ID</td>
<td>apnm-2017-0788.R3</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>17-Apr-2018</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Alabi, Quadri; Obafemi Awolowo University Faculty of Basic Medical Sciences Akomolafe, Rufus; Obafemi Owolowo University, Ile-Ife Adefisayo, Modinat; Ondo State University of Medical Sciences Olukiran, Olaoluwa; Obafemi Awolowo University, Ile-Ife Nafiu, Aliyat; Obafemi Awolowo University, Ile-Ife Fasanya, Micheal; Obafemi Awolowo University Teaching Hospital Complex Oladele, Ayodele; Obafemi Awolowo University Teaching Hospital Complex</td>
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<tr>
<td>Is the invited manuscript for consideration in a Special Issue? :</td>
<td>Not applicable (regular submission)</td>
</tr>
<tr>
<td>Keyword:</td>
<td>antioxidant;, diclofenac;, kidney;, kolaviron;, prostaglandin.</td>
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Kolaviron attenuates diclofenac-induced nephrotoxicity in male Wistar rats

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Abstract

The beneficial effects of kolaviron, a natural biflavonoid from the seeds of *Garcinia kola*, have been attributed to its antioxidant and anti-inflammatory activities. This study was designed to investigate the renoprotective effect of kolaviron in rat model of diclofenac (DFC)-induced acute renal failure. Thirty-five male Wistar rats were divided into seven groups of five rats each as follows: a control group that received propylene glycol orally and treatment groups that received diclofenac, diclofenac followed by kolaviron at three different doses and kolaviron only. Diclofenac treated rats showed sluggishness, illness and anorexia. Their urine contained appreciable protein, glucose and ketone bodies. Histopathological examination of their kidneys revealed profound acute tubular necrosis. Diclofenac treatment significantly increased levels of plasma creatinine, urea, sodium, chloride, potassium ions, and increased renal tissue activities of superoxide dismutase, catalase, levels of malondialdehyde and hydrogen peroxide. Fractional excretion of sodium and potassium and renal tissue levels of reduced glutathione and prostaglandin E$_2$ (PGE$_2$) decreased significantly in DFC treated groups. However, kolaviron administration significantly reduced toxic effect of DFC on PGE$_2$ release, plasma levels of creatinine, urea, glucose, and electrolytes and significantly attenuated renal tubular and oxidative damages. Furthermore, the effects of DFC administration on food consumption, water intake, urine output and urine protein, glucose, ketone bodies and electrolytes, were significantly attenuated in animals treated with kolaviron. The results suggested that kolaviron ameliorated DFC-induced kidney injury in Wistar rats by decreasing renal oxidative damage and restoration of renal PGE$_2$ release back to the basal levels.

**Keywords:** antioxidant; diclofenac; kidney; kolaviron; prostaglandin.
**Introduction**

Non-steroidal Anti-inflammatory Drugs (NSAIDs) are one of the most widely used drugs in medicine and their uses has dramatically increased in recent years. They are a class of drugs that reduce pain, decrease fever and inflammation. They accounted for over 70 million prescription and over 30 billion over-the-counter usage per people annually (Green 2001). They are generally considered to be safe and well tolerated, but studies indicate that these pharmacological agents account for 7% of reported cases of acute renal failure and 35% of drug-induced acute renal failure in the general population (Garella 1993).

Diclofenac (Fig. 1), is a phenylacetic acid derivative, with analgesic, anti-nociceptive, anti-pyretic and anti-inflammatory properties (Ulubay et al. 2017). It is widely used for the treatment of variety of inflammatory and degenerative forms of rheumatism, rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, muscle aches, acute gout attacks, painful post-operative and post-traumatic inflammation and swelling, as well as primary dysmenorrhea (Brogden et al. 1980; Altman et al. 2015). It inhibits the biosynthesis and release of prostaglandin by competing with the arachidonic acid for binding to cyclooxygenase (COX) (Vane and Botting 1996; Quiralte et al. 2007). Normal therapeutic dose of diclofenac is safe, effective and widely used but its extensive use for treatment of pain, inflammation and fever can lead to toxicity and unwanted side effects (Hamza 2007). Recent evidence suggests that diclofenac metabolism plays a role in generation of intra-renal reactive oxygen species (ROS) leading to oxidative stress, inhibition of intra-renal prostaglandin synthesis and genomic DNA fragmentation which are potential mechanisms contributing to development of renal injury (Hickey et al. 2001; Efrati et al. 2007; Inoue 2008; Fattori et al. 2017).
Some medicinal foods or herbs rich in antioxidants such as *Garcinia kola* seeds have been used in treatment of various diseases (Iwu et al. 1990). Kolaviron (KV) (Fig. 1) is a biflavonoid complex of *Garcinia kola* seeds, containing *Garcinia* biflavonoid 1 (GB1), *Garcinia* biflavonoid 2 (GB2) and Kolaflavanone in ratio 2:2:1 as the most dominant secondary metabolites found in *Garcinia kola* species (Iwu 1985). It has been reported to have an antioxidant (Terashima et al. 2002; Farombi et al. 2005), anti-inflammatory (Olaleye et al. 2000), hypoglycemic (Iwu 1993), antigenotoxic (Nwankwo et al. 2000) and anti-hepatotoxicity (Alabi et al. 2017a) effects. Kolaviron beneficial health effects in human and animal models of diseases have been attributed to its antioxidant and anti-inflammatory activities (Adegbebingbe et al. 2008; Alabi et al. 2017a). It showed protective effects against ethylene glycol monoethyl ether (EGEE)-mediated haematotoxicity and renal apoptosis in rats (Adedara and Farombi 2013). The previous observations of the protective effects of kolaviron in different experimental models and its acclaimed ability to suppress oxidative stress indices led us to hypothesize the possible beneficial effect of KV on DFC-induced renal injury in male Wistar rats. Despite the vast therapeutic benefits of KV, studies on its protective effects on drug-induced nephrotoxicity are scarce. This study was therefore designed to investigate the renoprotective effect of KV administration on DFC-induced acute renal injury in rats.
Materials and Methods

Drugs and chemicals

Diclofenac used for this study was manufactured by Wuhan Grand Pharmaceutical Company (Wuhan, China); Propylene glycol and ketamine hydrochloride by Biovision, Milpitas, CA, USA and Rotexmedica, Tittau, Germany respectively. Other reagents used were of analytical grade.

Extraction of kolaviron

Garcinia kola seeds were purchased from Oja Oba, in Ikere Ekiti, Nigeria and certified by a taxonomist at the herbarium of the Department of Botany, Obafemi Awolowo University, with a voucher number (IFE 17540). Kolaviron was isolated according to the method of Iwu et al. (1990) as modified by Farombi et al. (2005). Fresh seeds of Garcinia kola were peeled, air dried and crushed into powder using an electric pulverizer (DIK-2910, Daiki Rika Kogyo Co. Ltd, Tokyo-Japan). Powdered seeds of Garcinia kola (2.1 kg) were defatted with 3.5 liters of petroleum ether (b.p 40 °C - 60 °C) in a Soxhlet extractor for 24 hours. The defatted dry product was further extracted with 3.5 liters of 80 % acetone (1:2 w/v) in a Soxhlet extractor for 24 hours. The extract was concentrated at 40 °C using a Rotary Evaporator, diluted to twice its volume with distilled water and partitioned with 2 litres of ethyl acetate. The concentrated ethyl acetate fraction yielded kolaviron, a golden brown and was freeze-dried in a Lyophilizer (Ilshin Lab. Co. Ltd, Seoul, Republic of Korea) to a solid form. The sample obtained as a product of freeze drying was weighed to calculate for the percentage yield of the plant extract.

Stock solutions of kolaviron

The stock solution for 100 mg/kg was prepared by dissolving 1 g kolaviron in 20 mL of propylene glycol so that each 100 g rat received 0.2 mL of kolaviron to prevent the deleterious effects of extract overload. Accordingly, stock solutions for 200 mg/kg and 400 mg/kg of
kolaviron were prepared by dissolving 2 g and 4 g in each in 20 mL of propylene glycol, respectively.

**Animal care and management**

Thirty-five (35) adult male Wistar rats weighing 110 g - 150 g, purchased from the Animal House of the College of Health Sciences, Obafemi Awolowo University, Ile-Ife, were used for this study. The rats were housed in plastic cages under natural light/dark cycle in the laboratory and allowed to have access to standard rat chow (Ace Feed PLC Ibadan, Nigeria) and water *ad libitum*. Once each week, during the course of the experiment, they were transferred into separate metabolic cages (fabricated by Central Technological Laboratory and Workshops (CTLW), OAU, Ile-Ife, according to Ohaus R Model; Ohaus, Pine Brook, New Jersey, USA) to assess their food consumption, water intake and urine samples. The rats were allowed to acclimatize in the metabolic cage for one week before the commencement of this study, to allow for adaptation to life in metabolic cage. The animal experimental procedures were conducted in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and approved by Obafemi Awolowo University Animal Research Ethics Committee.

**Experimental Design**

The rats were divided into seven (7) groups (*n* = 5 per group) as follows; Group 1, (the control) received 2 ml/kg of propylene glycol daily by oral route for 28 days. Group 2 (DFC) received diclofenac (10 mg/kg/day) i.m for 7 days. Group 3 (DFC recovery) received diclofenac (10 mg/kg/day) i.m for 7 days and were thereafter left untreated for 28 days. Groups 4, 5 and 6 received diclofenac (10 mg/kg/day i.m) for 7 days and they were thereafter treated with 100, 200 and 400 mg/kg/day (p o) of kolaviron respectively for 28 days. Group 7 received 200 mg/kg/day
(p o) of kolaviron for 28 days. At the end of every week of the study period, food consumption, water intake and urine output of each rat in the groups were measured using metabolic cage throughout the study period. Routine urinalysis (using Combi-UriScreen® 10SL urine reagent strips) including determination of pH and specific gravity along with the presence of ketone bodies, protein, and glucose were also carried out on fresh voided urine of each rat in the groups. Twenty-four hours after the end of the experiment, the animals were sacrificed under ketamine hydrochloride anesthesia (10 mg/kg/b.w via intramuscular route). About 5 mL of blood of each animal was collected by cardiac puncture into separate lithium heparinized tubes. It was centrifuged at 4000 revolutions per minute for 15 minutes at – 4°C, using a cold centrifuge (Centurium Scientific, Model 8881). The plasma was collected into separate plain tubes for the assessment of some electrolytes and markers of renal function. The left kidney of each rat was homogenized for the assessment of prostaglandin E₂ (PGE₂), antioxidant and lipid peroxidation status. The right kidney of each rat was carefully excised, weighed and fixed in 10% formal saline for histopathological studies using H&E stain.

**Measurement of percentage body and relative kidney weights**

Percentage body weights of the rats were measured with the aid of a digital weighing balance (Hanson, China) to assess the weight gain or loss in each group.

**Biochemical Assay**

Plasma creatinine, urea and glucose levels were determined by biochemical kits purchased from Randox Laboratories (Crumlin, Co. Antrim, UK). The urine concentrations of the aforementioned parameters were estimated in the last samples of urine collected through metabolic cages from the rats before sacrifice, using Randox kits. Creatinine clearance (C_{Cr}) was
subsequently calculated using the standard conventional formula as a measure of glomerular filtration rate (GFR).

\[(C = U \times V / P)\]

Where;

\(C\) = clearance,

\(U\) = urine creatinine concentration,

\(V\) = urine flow rate (volume of urine/ time),

\(P\) = plasma creatinine concentration.

Creatinine clearance = urine creatinine (µmol/L) × (volume of urine (ml) 24 hrs/1440 min) / Plasma creatinine (µmol/L)

**Determination of urine total protein**

The total protein in the urine of the rats was determined according to the method of Lowry et al. (1951) and as described by Holme and Peck (1998).

**Plasma and urine concentration of electrolytes and estimation of fractional excretion of sodium and potassium.**

The plasma and urine concentrations of sodium (Na\(^+\)), chloride (Cl\(^-\)) and potassium (K\(^+\)) ions were measured. Na\(^+\) and K\(^+\) were measured by flame photometry using PFP7 (Jenway) flame photometer. Cl\(^-\) was assessed by using Teco laboratory kit.

Fractional excretion of sodium (FE\(_{Na}\)) was calculated as follows:

\[FE_{Na} \text{ (%) } = \left[\frac{(NaU \text{ (mmol/L)}/Na^+ \text{ (mmol/L)})}{(CrU \text{ (mg/dL)} / PCr \text{ (mg/dL)})}\right] \times 100\]

\[FE_{Na} \text{ (%) } = \left[\frac{\text{Urine sodium x Plasma Creatinine}}{\text{Plasma Sodium x Urine Creatinine}}\right] \times 100\]

(NaU: urine sodium; CrU: urine creatinine; PCr: plasma creatinine).

Analogous formulas were used to calculate fractional excretion of potassium (FE\(_{K}\)).
Antioxidant and malondialdehyde analysis

The kidney samples of the rats were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15 % potassium chloride, and the homogenate was centrifuged at 10,000 x g for 15 min. at 4°C. The supernatant was collected for the estimation of superoxide dismutase (SOD) and was assayed by the method described by Misra and Fridovich (1972). Catalase (CAT) activity was estimated using hydrogen peroxide as substrate according to the method of Aebi, (1974). Reduced glutathione (GSH) was determined using the method described by Beutler (1963). In addition, the hydrogen peroxide was determined by the method of Wolff (1994) while lipid peroxidation was measured as malondialdehyde (MDA) according to the method described by Ohkawa et al. (1979) and expressed as micromoles of MDA per gram tissue.

Evaluation of kidney PGE$_2$

Renal prostaglandin E$_2$ (PGE$_2$) was determined by using a high-sensitivity peptide enzyme immunoassay, EIA (R&D Systems, USA), based on a competitive binding of the sample PGE$_2$ and the fixed amount of horseradish peroxidase-labelled PGE$_2$ to the sites of a specific monoclonal antibody, according to the manufacturer’s protocol.

Histopathological Studies

The kidney of the rats was fixed in 10% formal-saline, dehydrated in graded alcohol, cleared by xylene and embedded in paraffin wax. The tissues were then cut into 3-4 µm thick sections by a microtome, fixed on the slides and stained with haematoxylin-eosin. The slides were examined under a light microscope (Olympus CH; Olympus, Tokyo, Japan) and photomicrographs were taken with a Leica DM 750 camera at x400 magnifications. All sections were evaluated for the degree of tubular and glomerular injury and necrosis.
**Statistical Analysis**

All values were expressed as means ± S.E.M. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Neumann Keul’s post hoc test for comparison between groups. Differences were considered significant when \( p < 0.05 \). The data were analyzed using the statistical package program Stat Graph Pad 6.0 (Graph Pad Software Inc., CA, USA).
Results

*Effects of kolaviron on food consumption, body weight change and relative kidney weight*

Significant decrease in food consumption and body weight were observed in the DFC treated group when compared with the control and their baseline values ($p < 0.05$). However, DFC recovery group showed a significantly decreased in food consumption and body weight when compared with the control ($p < 0.05$). Treatment with KV significantly increased the food consumption and body weight of rats treated with DFC toward control ($p < 0.05$) (Figures 2 and 3). The relative kidney weight of DFC group was significantly increased when compared with the control ($p < 0.05$). Administration of KV significantly decreased the relative kidney weight of rats treated with DFC toward control (Figure 4).

*Effects of kolaviron on water intake and urine output*

A significant decrease in the water intake and urine output were observed in rats treated with DFC alone group when compared with the control and their baseline values ($p < 0.05$). However, KV alone (at 200 mg/kg) had significant increase in water intake and urine output when compared with the control and its baseline values, but water intake and urine output of DFC+100 mg/kg and DFC+200 mg/kg were increase towards control values ($p < 0.05$) (Figures 5 and 6).

*Effects of kolaviron on Urinalysis*

Leukocyte, nitrite and blood were absent in the urine of rats treated with DFC and all other experimental groups. However, there was a severe indication of urobilinogen and bilirubin in urine of rats treated with DFC when compared with their respective baselines and the control (Table 1). The urine pH was significantly lower in the group of rats treated with DFC when compared with their respective baseline and the control ($p < 0.05$). A significantly higher urine
specific gravity was also observed in group of rats treated with DFC when compared with their respective baselines and the control \((p < 0.05)\) (Table 1). However, DFC and the control groups showed a significantly higher urine protein level when compared with KV groups \((p < 0.05)\). The urine excretion of urobilinogen, bilirubin and protein were reduced in DFC recovery when compared with DFC.

There was a presence of glucose and ketones in the urine of rats treated with DFC when compared with the control and their respective baselines (Table 1). However, administration of KV significantly lower the urine excretion of urobilinogen, bilirubin, protein, glucose and ketone after DFC treatment. Also, KV administration significantly increased urine pH of rats toward normal values \((p < 0.05)\). Kolaviron treatment also normalize the increased in specific gravity after DFC treatment toward normal control (Table 1).

**Effects of kolaviron on kidney functions**

Diclofenac-treated and DFC recovery groups produced a significant increase in plasma creatinine and urea levels as compared to the normal control group \((p < 0.05)\). However, the urine creatinine and urea of DFC-treated and recovery groups were significantly decreased when compared with control. The plasma glucose levels was significantly decreased in DFC-treated group when compared with the control \((p < 0.05)\). However, the plasma glucose of DFC recovery group was significantly increased when compared with the control and other experimental groups \((p < 0.05)\). The urine glucose levels of DFC-treated and recovery groups were significantly increased when compared with the control \((p < 0.05)\). Administration of KV significantly alleviated all these parameters toward normal levels (Table 2).
Treatment of rats with DFC showed a significant increase in plasma Na\(^+\), Cl\(^-\) and K\(^+\) concentration compared to normal control group. The urine concentration of Na\(^+\), Cl\(^-\) and K\(^+\) were significantly decreased in DFC treated group when compared with normal control. On the other hand, treatment with KV significantly alleviated the aforementioned increase in plasma and decrease in urine levels of Na\(^+\), Cl\(^-\) and K\(^+\) respectively toward control (Table 3).

A significant decrease in the renal creatinine clearance was observed in the DFC group, and DFC recovery groups when compared with the control group (\(p < 0.05\)). However, KV treated groups revealed a significant increase in the renal creatinine clearance when compared with DFC and DFC recovery groups (\(p < 0.05\)) (Figure 7).

**Effects of kolaviron on fractional excretion of sodium (FE\(_{Na}\)) and potassium (FE\(_{K}\))**

As shown in (Figures 8 and 9), DFC and DFC recovery treatment caused a significant reduction in the FE\(_{Na}\) and FE\(_{K}\) as compared with the control (\(p < 0.05\)), respectively. However, Administration of KV significantly elevated the diclofenac induced reduction of these parameters to towards control level.

**Effects of kolaviron on renal tissue PGE\(_2\)**

Diclofenac-induced nephrotoxicity was associated with decreased renal tissue PGE\(_2\) level indicating reduction in renal perfusion. Diclofenac recovery had a significantly increased in renal tissue PGE\(_2\) when compared with DFC only, but, PGE\(_2\) in these groups significantly decreased when compared to the control group (\(p < 0.05\)). Post-treatment with KV significantly increased the renal tissue PGE\(_2\) level when compared with DFC group (\(p < 0.05\)). However, there was a significantly increased in PGE\(_2\) at 200 mg/kg KV group when compared with the control and other experimental groups (\(p < 0.05\)) (Figure 10).
Effects of kolaviron on oxidative stress biomarkers

Diclofenac treatment raised MDA and H$_2$O$_2$ levels compared to the normal control group. It also exhibited a significant increase in SOD and CAT activities and decrease in GSH level as compared to the control group. However, KV administration significantly restored the levels of MDA, H$_2$O$_2$ and GSH, SOD and CAT activities towards control values (Table 4).

Effects of kolaviron on renal histopathological examination

Kidney sections from normal control and KV treated only (KV 200 mg/kg/b.w.) rats showed normal glomerulus and tubules with usual morphology (Figure 11 (CN) and (KV 200 mg)). Histological analysis of the kidneys from DFC-treated rats showed distorted renal corpuscles with hyper-infiltration of the glomerulus and increased mesangial matrix as well as severe and wide spread of necrosis of the renal tubules (particularly proximal tubules) which lead to loss of tubular cellular constituents, with dilatation of renal vessels and tubular cell desquamation and intraluminal cast formation and infiltration of inflammatory leucocytes. (Figure 11 (DFC)). While DFC recovery showed signed of improvement in the histoarchitecture of the renal tissues compared to DFC group (Figure 11 (DFC recovery)). Histological analysis of the kidneys from DFC-treated rats post-treated with KV showed less histopathological renal changes (Figure 11 (DFC + KV100 and DFC + KV200 and DFC + KV400)). The histological changes in treated rats were graded and summarized in table 5.
Discussion

Nephrotoxicity is a common global problem that pose great threats to human health and as such requires good attention. In recent years, the science of nutrition has advanced significantly based on food supplements and medicinal herbs employed in controlling and modulating chronic diseases in human beings. In this study, we hypothesized that supplementation with KV, an active antioxidant component of *Garcinia kola* seed might exert beneficial effect against DFC-induced nephrotoxicity.

In this study, DFC treatment significantly decreased food consumption and body weight of rats when compared with the control. These findings are similar to the report of Beun et al. (1987), which confirmed anorexia in patients that received DFC sodium for the treatment of arthritis. Hence, the decrease in food intake and body weight that were observed in these groups could be attributed to loss of appetite due to side effects of DFC on the gastrointestinal tract (GIT) (Beun et al. 1987). However, rats treated with KV significantly ameliorated the adverse effect of DFC on food intake and body weight of rats. These observations are consistent with previous findings of Olaleye et al. (1997) and Alabi et al. (2017b).

The water intake, urinary output volume and urine concentration of $\text{Na}^+$, $\text{Cl}^-$ and $\text{K}^+$ of the experimental groups reduced significantly during the 7 days of treatment with DFC compared with the control. However, the plasma concentration of the electrolytes ($\text{Na}^+$, $\text{Cl}^-$ and $\text{K}^+$) increased significantly in the DFC treated groups when compared with the control. This suggests that DFC might directly or indirectly inhibit the thirst mechanism, thereby reducing water intake, urinary output volume and concentration of urinary electrolytes. In addition, NSAIDs treatment caused disruption of renal physiology by decreasing renal blood flow and glomerular filtration rate and impairing sodium, potassium and water excretion (Brater 1999; Harirforoosh and Jamali...
The significant decrease in the concentration of urinary excretion of Na\(^+\) and K\(^+\) and increase in the plasma levels of these ions in DFC treated groups can also be confirmed by the significant decrease in fractional excretion of sodium and potassium (FE\(_{Na}\) and FE\(_{K}\)) observed in this group. The FE\(_{Na}\) and FE\(_{K}\) are the percentage of filtered sodium and potassium that appears in the urine and they represent Na\(^+\) and K\(^+\) clearance. The mechanism by which DFC decrease fractional excretion of sodium and potassium may be in two processes; firstly, DFC might cause pre-renal effect, evident by the decrease in FE\(_{Na}\) and FE\(_{K}\) parameters. This effect might be as result of myocardial damaged due to DFC induced ROS production in heart tissue (Abdulmajeed et al. 2015), this might result in elevation in cardiac enzymes. As a result of this effect, the heart could be compromised and might result in reduced blood perfusion of the kidney. Reduction in renal perfusion, will decrease GFR and subsequently reduce or decrease clearance rate of water, plasma sodium and potassium ion. Secondly, the effect of DFC on FE\(_{Na}\) and FE\(_{K}\) parameters could be as a result of renal tubular necrosis induced by DFC treatment. The representative photomicrograph of rat treated with DFC showed profound tubular necrosis, particularly proximal and distal tubules of the kidney. This could also be responsible for the reduced secretion and reabsorption of these ions. Thus, the inhibition of renal prostaglandin synthesis, reduction in GFR coupled with tubular necrosis could be the mechanism responsible for the reduction in water and ions (Na\(^+\), K\(^+\) and Cl\(^-\)) excretion following DFC administration. However, administration of KV restored the water intake, normalized the plasma and urine electrolytes and inhibited the oliguria caused by DFC toward control group. These effects indicated that kolaviron induced amelioration of electrolytes balance, evidently, due to its anti-inflammatory property as well as potent modulatory effects on electrolyte channels to bring about restoration of electrolytes homeostasis via the mechanism of FE\(_{Na}\) and FE\(_{K}\) equilibrium. In addition, the
photomicrograph of the groups treated with KV had a significantly improved renal tubular necrosis induced by DFC.

Urinalysis test is a useful indicator to diagnose or detect and manage a wide range of urinary tract infections, kidney disease and diabetes (Wu 2010; McKean et al. 2012). In this study, urinalysis test showed significant presence of urobilinogen, bilirubin, protein, glucose and ketone bodies in the urine of rats treated with DFC for 7 days. Leukocytes, blood and nitrite were not present in the urine of the rats treated with DFC.

Rats injected with DF showed increase in urinary bilirubin and urobilinogen, indicating liver cells function alteration or hemolysis of red blood cells of the rats. Diclofenac metabolites has been reported to cause rapid haemolysis, which resulted in increased plasma bilirubin level (Brune and Lindner 1995; Bort et al. 1999). However, administration of KV reversed the severe presence of bilirubin and urobilinogen in the urine of DFC treated groups toward normal.

The urine specific gravity in DFC-treated group was significantly increased when compared with the control. The higher than normal urine concentration that was observed in DFC group is an indication of reduction in water intake that was observed in this group. However, KV administration restored water intake and subsequently return the urine specific gravity of DFC treated rats back to normal.

Determination of urine pH assesses the effectiveness of the kidney in maintaining acid-base homeostasis. Diclofenac-treated groups had significant decrease in urine pH when compared with the control. The significant decrease in urine pH in DFC-treated group could be as a result of reduction in glomerular perfusion and glomerular filtration rate (GFR). Non-Steroidal Anti-inflammatory Drugs reduced GFR by reducing blood supply to the glomerulus (Brater 1999). A fall in GFR reduced the filtered load of $\text{HCO}_3^-$ in the renal tubules. Since there is reduction of
HCO$_3^-$ in the tubular lumen as a result of a decrease in GFR, more H$^+$ is secreted into the tubular lumen by the tubular epithelial cells than HCO$_3^-$ is filtered by the glomerulus leading to a net loss of hydrogen ion (H$^+$) in the urine (Robert et al. 1972). However, the urine pH of the KV-treated groups was restored to normal. This indicated that kolaviron probably improved the glomerular capillary blood flow by increasing renal PGE$_2$ release.

Presence of glucose, ketone bodies and protein in urine is an indication of uncontrollable or defective handling of these organic compound by the kidney. In this study, urinalysis showed a severe presence of glucose and ketone bodies in the urine of DFC treated rats. This was as a result of distortion in the proximal tubules (Steven and Max 1990). Since, kidney is also the site of DFC excretion, free diclofenac and its metabolites might be taken up by the kidney through organic anion transporter (OAT) on the membrane of the renal proximal tubular cell (Sekine et al. 2006). Hence, accumulation of DFC metabolites in the renal tubules (particularly proximal tubules) could be the cause of impaired of renal glucose re-absorptive capacity. However, severe presence of ketone bodies in the urine of DFC treated rats was as a result of anorexia that occurred in this group. Nam-Seok et al. (2010) reported that caloric reduction could cause ketonuria. In prolonged starvation, fat becomes the predominant body fuel instead of carbohydrates. Presence of ketone bodies (ketonuria) in urine is an indication that fat is the major source of energy (Nam-Seok et al. 2010). Thus, the higher ketones observed in the urine of DFC treated group is an indication that the body of the rat used fat as the major source of energy.

The plasma glucose concentrations in rats that were administered DFC was significantly lower compared with the control. The lower plasma glucose level that was observed in this study was as a result of DFC-induced anorexia and/or enhancement of insulin release from the islets of Langerhans in the rats (Metz et al. 1981). However, KV administration normalized urinary
glucose and ketones levels in DFC treated rats. Kolaviron also normalized plasma glucose levels of DFC treated rats. This indicated that, KV administration reduced DFC toxic effect on renal handling of glucose and ketone bodies, evident by improved structure of renal proximal tubular cell as seen in the photomicrograph of their kidneys.

The urine protein of DFC treated rats and the control group increased significantly when compared with KV treated groups. Perry (1965) reported that excess serum protein was present in the urine of apparently healthy normal rats without renal disease. However, an increase in urinary protein of DFC rats suggest appreciable proteinuria in this group. The mechanism by which proteinuria is expressed in DFC treated rats could be exacerbation of both the integrity of the glomerular filtration barriers and post glomerular reabsorption and/or secretion of plasma proteins. However, KV administration was observed to decrease urine protein level in rats treated with DFC and significantly reversed the proteinuria induced by DFC treatment. These could imply that KV altered the features of the glomerular filtration barrier and significantly modulated the renal handling of the plasma proteins to bring about a decrease in urine excretion of proteins in rats.

This study showed that administration of DFC to rats induced a marked elevation of plasma creatinine and urea and significantly lower urine levels of these parameters, indicating impaired renal function. These results were in agreement with Hussain et al. (2008); and Hickey et al. (2001). However, treatment with KV after diclofenac injection resulted in marked decrease in plasma levels of creatinine and urea and increase in urine levels of these markers, indicating improved kidney function. These results are in accordance with Farombi et al. (2002) who documented that; treatment with KV significantly eliminated ROS in the kidney of rats treated with potassium bromate (KBrO\textsubscript{3}). It has been reported that ROS caused kidney function
impairment, as a result of increase in MDA concentrations in the kidney tissue (Ateşahîn et al. 2005). This effect resulted to kidney tissue necrosis and back leak of creatinine and urea. Interestingly, the protective effect observed for KV in the kidneys of rats treated with DFC imply that this important natural antioxidant may be involved in the elimination of active oxygen species or other reactive bye products generated by DFC toxic metabolites in the kidney tissue. Indeed, the histology score showed evidence of improvement in the histoarchitecture of the kidney of KV treated groups compared with the DFC group.

Diclofenac administration significantly reduced creatinine clearance when compared with the control. This might be due to the overproduction of reactive oxygen species (ROS) generated during DFC treatment causing membrane injury as a result of overproduction of lipid peroxidation, which disrupt the function and integrity of glomerular membranes (Rodriguez-Barbero et al. 2000). Treatment with KV ameliorated DFC-induced alteration in creatinine clearance towards the control level. These suggested that KV restored the structural integrity of the glomerulus by inhibiting the production of LPO.

Injection of rats with DFC in this study significantly decreased renal PGE$_2$ synthesis. However, PGE$_2$ synthesis in renal tissues from animals that received KV following DFC treatment increased back to the levels that were not significantly different from that of the control rats. Moreover, PGE$_2$ in the kidney of the rats that were treated with KV alone was significantly higher than that of the control rats.

Metabolic breakup of arachidonic acid molecule, culminating in PGE$_2$ formation, is a process involving transformation of reduced glutathione (GSH) to its oxidized form GSSG. By this, GSH serves as electron donor in the process of conversion of PGG$_2$ to PGH$_2$ (Asboth et al. 1989; Tsikas et al. 2012). Kolaviron has been reported to increase the concentrations of GSH molecule
in biological system (Omole et al. 2018). Normal renal tissue contains large amount of arachidonic acid and COX. The surplus glutathione provided by KV administration probably resulted in an increased release of PGE$_2$ as manifested in the kidneys of rats subjected to KV administration only. In the kidneys from diclofenac pretreated rats, conversion of arachidonic acid to PGE$_2$ was probably significantly inhibited at the stage of its breakup to PGG$_2$. Therefore less PGG$_2$ was available for conversion to PGH$_2$ and subsequently to PGE$_2$. However, KV administration to DFC treated rats restored PGE$_2$ close to the levels found in normal control kidneys.

Cells maintain a variety of defenses in response to oxidative stress through the induction of antioxidant enzymes. Among these are endogenous enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In this study, SOD and CAT activities were increased in the kidneys of DFC-treated rats when compared to the control. This increase may be due to an increase in the renal tissues H$_2$O$_2$ and OH$^-$ caused by inhibition of peroxidases (Perrin-Nadif et al. 1996; Rohrdanz and Kahl 1997; Hickey et al. 2001). However, the activities of these enzymes were significantly lower by the administration of KV, which can be attributed to the decrease in the renal tissue H$_2$O$_2$ and OH$^-$. A marked decline in renal non-enzymatic antioxidant GSH levels was also observed in DFC-treated rats and this may reflect an impaired antioxidant defense and thus increased the susceptibility of the kidney to oxidative stress. Kolaviron administered to diclofenac treated groups increased kidney GSH level. The normalisation of GSH in KV-treated DFC groups therefore suggested a protective effect of kolaviron against ROS overproduction in the DFC treated rat kidney.

In this study, MDA and H$_2$O$_2$ formation increased in the rats treated with DFC when compared to the control. However, in KV-treated DFC rats, MDA and H$_2$O$_2$ levels were significantly
reduced. The attenuation of MDA and H₂O₂ in KV-treated DFC groups therefore suggested a depleted ROS level by KV, leading to a reduced effect of the hydroxyl radical on antioxidant enzymes and therefore triggered the antioxidant enzymes synthesis which in turn attenuated the oxidative damage in DFC-treated rats.

In conclusion, this study indicated that, DFC induced alteration in body weight and food consumption, markers of renal function, renal handling of electrolytes, physical and chemical properties of urine, antioxidant parameters, prostaglandin release and histology of the kidney. However, KV administration significantly attenuated the adverse effects of DFC on renal parameters. The therapeutic potential of KV could be attributed to its anti-oxidant and anti-inflammatory actions. Most surprisingly, the results further demonstrated that operating mechanisms regulating these kolaviron-induced renoprotective effects involved, in addition to antioxidant effects, augmentation of renal tissue PGE₂ release back to normal physiological state.
Conflict of Interest

The authors of this manuscript declare no conflict of interest
Reference


Olaleye, S.B., Ibironke, G.F., Balogun, W.O., Aremu, A. 1997. Effects of Diets Containing Seeds of *Garcinia kola* (Heckel) on Gastric Acidity and Experimental Ulceration in


Wolff, S.P. 1994. [18] Ferrous ion oxidation in presence of ferric ion indicator xylenol orange
6879(94)33021-2.

<table>
<thead>
<tr>
<th>Table 1 – Effect of KV on physical and chemical properties of the urine of rats treated with DFC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urinalysis</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Leukocyte</td>
</tr>
<tr>
<td>Urobilinogen</td>
</tr>
<tr>
<td>Bilirubin</td>
</tr>
<tr>
<td>Blood</td>
</tr>
<tr>
<td>Nitrite</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>Specific gravity</td>
</tr>
<tr>
<td>Protein (mg/dl)</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Ketone</td>
</tr>
</tbody>
</table>

(-) = None; Nor. = Normal; ± = trace; + = mild; ++ = moderate; +++ = severe.

Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); §= significantly different from baseline (p < 0.05); α= significantly different from Group 2 (p < 0.05); β= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 4-6 (p < 0.05).
### Table 2 – Effect of KV on creatinine, urea, glucose and total protein in DFC – induced nephrotoxicity in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma concentration</th>
<th>Urine concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Creatinine (mg/dl)</td>
<td>Urea (mg/dl)</td>
</tr>
<tr>
<td>1 (Control)</td>
<td>0.76±0.07</td>
<td>19.91±1.02</td>
</tr>
<tr>
<td>2 (DFC)</td>
<td>1.77±0.06*</td>
<td>146.40±15.60*</td>
</tr>
<tr>
<td>3 (DFC Recovery)</td>
<td>1.03±0.07*</td>
<td>103.00±7.48*</td>
</tr>
<tr>
<td>4 (DFC+100 mg/kg KV)</td>
<td>0.74±0.03*</td>
<td>20.68±4.86*</td>
</tr>
<tr>
<td>5 (DFC+200 mg/kg KV)</td>
<td>0.84±0.05*</td>
<td>19.69±4.01*</td>
</tr>
<tr>
<td>6 (DFC+400 mg/kg KV)</td>
<td>0.76±0.07*</td>
<td>45.70±5.30*</td>
</tr>
<tr>
<td>7 (200 mg/kg KV)</td>
<td>0.24±0.08*</td>
<td>15.04±1.11*</td>
</tr>
</tbody>
</table>

Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); = significantly different from Group 2 (p < 0.05); # = significantly different from Group 3 (p < 0.05); = significantly different from Groups 4-6 (p < 0.05).
Table 3 – Effect of KV on plasma and urine electrolytes in DFC – induced nephrotoxicity in rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ Concentration (mmol/L)</td>
<td>Cl⁻ Concentration (mmol/L)</td>
</tr>
<tr>
<td>1 (Control)</td>
<td>136.8±0.80</td>
<td>69.76±2.83</td>
</tr>
<tr>
<td>2 (DFC)</td>
<td>166.0±1.52*</td>
<td>133.1±2.86*</td>
</tr>
<tr>
<td>3 (DFC Recovery)</td>
<td>147.0±1.45*</td>
<td>97.40±3.56*</td>
</tr>
<tr>
<td>4 (DFC+100 mg/kg KV)</td>
<td>118.8±1.72*</td>
<td>74.18±1.90*</td>
</tr>
<tr>
<td>5 (DFC+200 mg/kg KV)</td>
<td>137.8±2.13*</td>
<td>75.14±3.52*</td>
</tr>
<tr>
<td>6 (DFC+400 mg/kg KV)</td>
<td>155.6±1.47*</td>
<td>99.87±3.36*</td>
</tr>
<tr>
<td>7 (200 mg/kg KV)</td>
<td>135.0±0.45*</td>
<td>68.98±2.16*</td>
</tr>
</tbody>
</table>

Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); ω= significantly different from Group 2 (p < 0.05); α= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 4-6 (p < 0.05).
Table 4- Effect of KV on SOD, CAT activities, GSH, MDA and H$_2$O$_2$ levels in DFC – induced nephrotoxicity in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (µ/mg tissue)</th>
<th>CAT (µM/mg tissue)</th>
<th>GSH (µg/mg tissue)</th>
<th>MDA (nM/mg tissue)</th>
<th>H$_2$O$_2$ (nM/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Control)</td>
<td>80.11±1.29</td>
<td>2.24±0.19</td>
<td>5.92±0.47</td>
<td>10.92±0.86</td>
<td>17.77±1.04</td>
</tr>
<tr>
<td>2 (DFC)</td>
<td>111.20±3.36*</td>
<td>4.31±0.28*</td>
<td>1.39±0.15*</td>
<td>25.15±1.05*</td>
<td>34.00±2.03*</td>
</tr>
<tr>
<td>3 (DFC Recovery)</td>
<td>93.82±2.44*</td>
<td>3.49±0.19*</td>
<td>3.71±0.37*</td>
<td>17.66±1.64*</td>
<td>30.25±2.94*</td>
</tr>
<tr>
<td>4 (DFC+100 mg/kg KV)</td>
<td>84.24±2.21*</td>
<td>2.42±0.29*</td>
<td>5.30±0.44*</td>
<td>11.21±1.08*</td>
<td>19.81±0.95*</td>
</tr>
<tr>
<td>5 (DFC+200 mg/kg KV)</td>
<td>83.89±2.07*</td>
<td>2.37±0.18*</td>
<td>5.35±0.47*</td>
<td>10.55±0.60*</td>
<td>19.51±0.64*</td>
</tr>
<tr>
<td>6 (DFC+400 mg/kg KV)</td>
<td>95.03±2.24*</td>
<td>2.79±0.29*</td>
<td>3.71±0.30*</td>
<td>16.74±2.05*</td>
<td>24.28±1.55*</td>
</tr>
<tr>
<td>7 (200 mg/kg KV)</td>
<td>82.93±2.61*</td>
<td>2.01±0.12*</td>
<td>5.87±0.35*</td>
<td>10.22±0.68*</td>
<td>17.74±1.47*</td>
</tr>
</tbody>
</table>

Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); ο*= significantly different from Group 2 (p < 0.05); α*= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 4-6 (p < 0.05).
Table 5 – Grading of the histopathological changes in the renal tissues of DFC or KV rats treated.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Leucocyte infiltration</th>
<th>Necrosis in epithelial cells of the proximal tubules</th>
<th>Hemorrhage in a dilated blood vessel</th>
<th>Tubular dilatation</th>
<th>Markedly lobulated glomeruli</th>
<th>Loss of brush border membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 (DFC)</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3 (DFC Recovery)</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>4 (DFC+100 mg/kg KV)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 (DFC+200 mg/kg KV)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6 (DFC+400 mg/kg KV)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7 (200 mg/kg KV)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Lesion score was noted as follows: none (-), mild (+), moderate (++) and severe (+++).
Figure Legends

Figure 1: Chemical structures of (a) diclofenac and (b) the bioactive compounds present in kolaviron

Figure 2: Effect of KV on food consumption (g) in DFC-induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); δ= significantly different from baseline (p < 0.05); a= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 2-6 (p < 0.05).

Figure 3: Effect of KV on body weight change (%) in DFC-induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); δ= significantly different from baseline (p < 0.05); a= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 2-6 (p < 0.05).

Figure 4: Effect of KV on relative kidney (%) weights in DFC-induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); δ= significantly different from baseline (p < 0.05); α= significantly different from Group 2 (p < 0.05); #= significantly different from groups 3-6 (p < 0.05).

Figure 5: Effect of KV on water volume (ml) in DFC-induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 2 (p < 0.05); δ= significantly different from baseline (p < 0.05); a= significantly different from Group 3 (p < 0.05); β= significantly different from Groups 4 and 5 (p < 0.05); #= significantly different from Groups 2-6 (p < 0.05).

Figure 6: Effect of KV on urine volume (ml) in DFC-induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); δ= significantly different from baseline (p < 0.05); β= significantly different from Groups 4 and 5 (p < 0.05); #= significantly different from Groups 2-6 (p < 0.05).
Figure 7: Effect of KV on creatinine clearance in DFC – induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); ω= significantly different from Group 2 (p < 0.05); α= significantly different from Group 3 (p < 0.05); ß= significantly different from Group 4 (p < 0.05); ß= significantly different from Group 4 and 5 (p < 0.05); #= significantly different from Groups 4-6 (p < 0.05).

Figure 8: Effect of KV on fractional excretion of sodium (FE_{Na}) in DFC – induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); ω= significantly different from Group 2 (p < 0.05); α= significantly different from Group 2 (p < 0.05); α= significantly different from Group 3 (p < 0.05).

Figure 9: Effect of KV on fractional excretion of potassium (FE_{K}) in DFC – induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); α= significantly different from Group 2 (p < 0.05); α= significantly different from Group 2 (p < 0.05); α= significantly different from Group 3 (p < 0.05).

Figure 10: Effect of KV on renal PGE\textsubscript{2} in DFC – induced nephrotoxicity in rats.
Each value is expressed as Mean ± S.E.M (n = 5); *= significantly different from Group 1 (p < 0.05); α= significantly different from Group 2 (p < 0.05); α= significantly different from Group 3 (p < 0.05); #= significantly different from Groups 4-6 (p < 0.05).

Figure 11: Micrograph of kidney of Control (CN), DFC, DFC Recovery, DFC+KV100, DFC+KV200, DFC+KV400 and KV200. CN shows normal renal histoarchitecture. In contrast, DFC shows distorted renal corpuscles with hyper-infiltration of the (green and red arrow) glomerulus, dilated vessels (brown arrow), loss of cellular constituents of the tubules (yellow arrow head) and increased mesangial matrix (red arrow) with infiltration of inflammatory cell. Intact renal corpuscles with normal appearing glomeruli and tubules including the proximal
convoluted tubules and distal convoluted tubules were seen in KV200, DFC+KV100 and DFC+KV200. Inflammatory cells were seen in DFC recovery, and DFC+KV 400 mg/kg rats (yellow arrow head). Sections of kidney tissues of rats were stained with hematoxylin-eosin (Magnification: × 400).
Body Weight Change

1 (Control)
2 (DFC)
3 (DFC Recovery)
4 (DFC+100 mg/kg KV)
5 (DFC+200 mg/kg KV)
6 (DFC+400 mg/kg KV)
7 (200 mg/kg KV)