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Modulatory role of Kolaviron (KV), a biflavonoid from *Garcinia kola*, in sodium arsenite-induced hepatotoxicity and haematotoxicity in rats

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Abstract

Arsenic acid ($NaAsO_2$) is an environmental pollutant that is widely distributed in air, water and soil. Exposure to this silent killer in drinking water has posed a major health threat to both developing and developed countries of the world. The use of phytochemicals has gained acceptance in the recent years. Hence, the purpose of this study was to explore the chemopreventive effect of Kolaviron, a biflavonoid from *Garcinia kola* seed on $NaAsO_2$ induced hepatoxicity and haematotoxicity. Rats were pre-treated with KV (100 & 200mg/kg) for 7 days prior to $NaAsO_2$ (10mg/kg; i.p.) intoxication on day 8. Complete blood counts (CBC), liver function tests, hepatic antioxidant, markers of oxidative stress and histological sections of the liver were assessed. $NaAsO_2$ intoxication caused a significant ($p<0.05$) increase in the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Also, the hepatic catalase (CAT), glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD) and reduced glutathione (GSH) content were significantly ($p<0.05$) reduced whereas markers of oxidative stress such as malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and nitric oxide (NO) contents were also severely ($p<0.05$) elevated. $NaAsO_2$ intoxication caused a significant ($p<0.05$) reduction in packed cell volume (PCV), red blood cell (RBC) counts and haemoglobin (Hb) concentration. KV pre-treatment significantly suppressed markers of oxidative stress and prevented the depletion of antioxidant defence system. Normochromic normocytic anaemia precipitated by $NaAsO_2$ intoxication was attenuated by KV administration. Histopathological examination showed that liver tissue injury mediated by arsenic was ameliorated by KV pre-treatment. It was concluded that KV may represent a potential drug candidate to protect against liver damage and other detrimental effects of arsenic toxicity.

Keywords: Hepatotoxicity, arsenite, Kolaviron (KV), oxidative stress, chemoprevention

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1. Introduction

Arsenic (As) is an environmental pollutant that is widely distributed in air, water and soil. Exposure to this silent killer in drinking water has posed a major health problem to both developing and developed countries of the world. The pentavalent form of arsenic is the most toxic as it binds to DNA, RNA and other macromolecules with resultant elevated risk of cancer and vital organ dysfunctions.[1, 2]

It has been reported that liver is one of the major target organs of arsenic toxicity and carcinogenesis.[3, 4] Derangement in liver function has been described as gastrointestinal symptoms (e.g., abdominal pain, indigestion, loss of appetite, etc.) and elevations of markers of liver damage such as alanine amino transferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) following acute and chronic exposure to arsenic in drinking water.[5, 6] Furthermore,

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the development of portal hypertension and liver fibrosis has also been reported in arsenic-exposed populations.[5] However, reactive oxygen species (ROS) production and oxidative stress are the major players in arsenic poisoning.[7] The intracellular antioxidant defence system known as reduced glutathione (GSH) may play a major role in arsenic detoxification in the liver acting as an antioxidant or by facilitating arsenic detoxification via glutathione-arsenic conjugation for cellular efflux and ultimate biliary excretion.[3]

Kolaviron (KV) is a biflavonoid fraction from *Garcinia kola* seed extract. The medicinal properties and other potential benefits of KV have been reported.[8-10] Similarly, anti-oxidant, anti-inflammatory and free radical scavenging activities of KV have been documented.[11-16]

The search for new drugs from medicinal plants has gained acceptance in this millennium. Furthermore, there are avalanche of antioxidants and phytonutrients present in medicinal plant that can ameliorate, prevent and even mitigate toxicity associated with environmental toxicants in via water, air and food. Therefore, this study was conducted to evaluate the antioxidant, hepatoprotective and heamatinic effect of KV in arsenic-induced hepatotoxicity and anemia.

2. Materials and Methods

2.1 Extraction of *Garcinia kola* and Isolation of Kolaviron (KV)

Kolaviron was extracted from the seeds of *Garcinia Kola* according to the method of Iwu *et al.*[17] The seeds were sliced, air-dried and ground to a fine powder using a pestle and mortar. The powdered seeds were defatted by extraction using n-hexane in a Soxhlet extractor apparatus for 24 hours. The defatted dried marc was repacked and extracted with methanol. Kolaviron was fractionated from concentrated methanol extract using chloroform to give Garcinia biflavanones-GB1, GB2 and kolaflavanone.

2.2 Experimental design and animal treatment

Forty male rats were obtained from the Experimental Animal Unit of Faculty of Veterinary Medicine, University of Ibadan, Nigeria and were randomly divided into four groups of ten animals per group. The animals were kept in wire mesh cages under controlled light cycle (12h light/12h dark) and fed with commercial rat chow *ad libitum* and liberally supplied with water.

Group 1 received normal saline. Group 2 received sodium arsenite at 10 mg/kg single dose intraperitoneally on day 8. Rats in Groups 3 and 4 were pre-treated orally with Kolaviron (KV) 100 mg/kg and 200 mg/kg for 7 days.

2.3 Care of animals

All of the animals received humane care according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health. The ethics regulations were followed in accordance with national and institutional guidelines for the protection of the animals' welfare during experiments.[18]

2.4 Chemicals

Sodium potassium tartrate, Copper sulphate, Sodium arsenite, Bovine serum albumin (BSA), Potassium hydroxide, Reduced glutathione (GSH), Trichloroacetic acid, Sodium hydroxide, 1, 2-dichloro-4-nitrobenzene (CDNB), Thiobarbituric acid (TBA), Xylenol orange, and Hydrogen peroxide (H_2O_2), N-(1-naphthyl) ethylenediamine dihydrochloride (NEM), Potassium iodide, 5, 5-Dithiobis-(2-nitrobenzene) DTNB, Thiobarbituric acid (TBA), Sorbitol, Ammonium ferrous sulphate (AFS) were purchased from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade.

2.5 Blood collection and preparation of serum

About five milliliters (5 ml) of blood was collected from the retro-orbital venous plexus of the animals into non-heparinized tubes and allowed to clot. The clotted blood was subsequently centrifuged at 4,000 rpm for 10 minutes and the serum was aspirated using a Pasteur pipette and then stored at -4°C until the time of use.

2.6 Preparation of microsomal fraction from liver tissues

The rats were sacrificed 24 hours after the last administration. The liver was removed, rinsed in 1.15% KCl and homogenized in aqueous potassium phosphate buffer (0.1 M, pH 7.4) and homogenates were centrifuged at 12,000 g for 15 minutes to obtain the post mitochondria

fraction (PMF) of the liver. The PMF obtained were stored at -4^{O}C until the time of use.

2.7 Determination of hepatic markers of oxidative stress

Hydrogen peroxide generation was determined by Wolff.[19]The hydrogen peroxide (H_2O_2) generated was extrapolated from hydrogen peroxide standard curve. Thiobarbituric acid reactive substance TBARS was quantified as Malondialdehyde (MDA) in the hepatic PMF. The MDA was determined according to the method of Varshney and Kale.[20]

2.8 Determination of hepatic antioxidant defence system

Catalase (CAT) activity was determined according to the method of Sinha.[21]The absorbance was read at 570 nm using distilled water as blank. One unit of CAT activity represents the amount of enzyme required to decompose 1 µmol of H_2O_2/minute.The hepatic PMF of superoxide dismutase (SOD) was determined by the method of Misra and Fridovich with modification from our laboratory.[22-24]Briefly, 100 mg of epinephrine was dissolved in 100 ml distilled water and acidified with 0.5 ml concentrated hydrochloric acid. This preparation prevents oxidation of epinephrine and is stable for 4 weeks. 10 µL of hepatic PMF was added to 2.5 ml 0.05M carbonate buffer (pH 10.2) followed by the addition of 300 µL of 0.3mM adrenaline. The increase in absorbance at 480 nm was monitored every 30 seconds for 150 seconds. 1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of adrenaline to adrenochrome during 1 minute. The hepatic glutathione peroxidase (GPx) activity was also measured according to Buetler *et al.*[25]The total hepatic reduced glutathione (GSH) was estimated by the method of Jollow *et al.*[26] Glutathione-S-transferase activity was assessed in the PMF of the hepatic tissue by Habig *e al.*[27]

2.9 Measurement of hepatic nitric acid (NO) contents

Nitric acid (NO) contents were determined as described by Olaleye *et al.*[28] Briefly, 500 µL of hepatic PMF were mixed with 500 µL Griess reagent [0.1% N- (1-naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide and 2.5% H_3PO_4]. After incubation at room temperature for 20 minutes, the absorbance at 540 nm was measured by spectrophotometer. The concentration of nitrite in the sample was determined from a sodium nitrite ($NaNO_2$) standard curve and was expressed as µmol nitrite/mg protein.

2.10 Determination of hepatic protein concentration

Protein concentrations were determined by means of the described by Gornal *et al.*[29] Briefly, 1 ml of diluted serum was added to 3 ml of the biuret reagent. The reaction mixture was incubated at room temperature for 30 minutes. The mixture was thereafter read with spectrophotometer at 540 nm using distilled water as blank. The final value for total protein was extrapolated from total protein standard curve.

2.11 Liver function tests

The activities of Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) were determined using Randox Kits (Random Laboratories Limited, UK). The manufacturer instructions were followed in performing the assay.

2.12 Haematology

The complete blood counts (CBCs) and differentials were measured with 3000 Haematology autoanalyzer (MINDRADY). The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated as described earlier.[30]

2.13 Histopathological examination

Small pieces of liver tissues were collected in 10% buffered formalin for proper fixation. These tissues were processed and embedded in paraffin wax. Sections of 5-6 µm in thickness were made and stained with haematoxylin and eosin for histopathological examination [31].

2.14 Statistical analysis

All values were expressed as mean±S.D. The test of significance between two groups was estimated by Student's t test. "One-way ANOVA with Dunnett's posttest was also performed using GraphPad Prism version 4.00.

Table 1. Effect of Sodium Arsenite and Kolaviron on Haematological parameters of rats

Parameters	Group 1 (n=5)	Group 2 (n=5)	Group 3 (n=5)	Group 4 (n=5)
PCV (%)	46.5±2.38	41.0±2.94[a]	46.6±3.21[b]	44.67±2.08[b]
Hb (g/dl)	14.6±0.74	12.65±0.84[a]	15.28±0.75[b]	13.9±0.59
RBC x10^6/µL)	7.65±0.55	6.43±0.45[a]	7.88±0.53[b]	7.65±0.47[b]
MCV (fl)	60.4±2.88	60.6±2.30	59.2±3.56	58.33±0.58
MCH (pg)	18.8±1.30	18.8±0.45	18.8±1.30	18.03±0.05
MCHC (g/dl)	31.4±0.89	31.4±0.89	31.8±0.45	31.25±0.5
Platelets (x10^3/µL)	739.0±90.55	666.25±76.19	669.5±87.04	633.67±75.27[a]
WBC (x10^3/µL)	12.24±0.64	10.03±3.52	12.02±2.92	9.78±2.16[a]
Lymphocytes (x10^3/µL)	55.0±4.12	58.2±4.09	56.6±1.52	63.5±3.70[b]
Monocytes (x10^3/µL)	6.8±3.96	7.6±0.89	5.67±0.58	6.0±1.63
Neutrophils (x10^3/µL)	35.8±5.89	32.6±4.72	38.4±2.70[b]	30.0±3.37[a]

3. Results

3.1 Effects of KV on $NaAsO_2$ intoxication

From our findings, the result showed that there was a significant ($p<0.05$) reduction in Packed cell volume (PCV), Haemoglobin concentration (Hb) and Red blood cell (RBC) counts in rats administered with $NaAsO_2$ alone compared with the control (Table 1). However, rats pre-treated with 100 & 200 mg/kg of KV showed significant improvement in the aforementioned haematological parameters (Table 1). Also, there was a significant ($p<0.05$) reduction in White blood cell (WBC), platelet and neutrophil counts in rats pre-treated with 100 & 200 mg/kg of KV compared to other treatment groups.

3.2 Liver function tests

The activities of serum Aspartate aminotransferase (ALP), Alanine aminotransferase (AST), and Alkaline phosphatase (ALT) were significantly ($p < 0.05$) elevated in $NaAsO_2$ treated group compared to the control (Table 2). The values of liver function tests were brought to near normal in KV pre-treated animals (Table 2).

3.3 Markers of oxidative stress and antioxidant defence system

The hepatic markers of oxidative stress (MDA, H_2O_2 and nitric oxide) were significantly increased in $NaAsO_2$ treated group (Table 3). The antioxidant activity of KV was however demonstrated by significant ($p<0.05$) reduction in MDA, H_2O_2 and nitric oxide (NO) contents in rats pre-treated with KV (100 & 200 mg/kg), respectively (Table 3). Interestingly, the reduced glutathione (GSH) content increased significantly ($p<0.05$) in sodium arsenite treated rats compared to the control and rats pre-treated with KV (Figure 1). However, the GSH content in rats pre-treated with 100 and 200 mg/kg of KV crashed significantly compared to the arsenic treated rats but better than the control (Figure 1). The activity of the *in vivo* antioxidant enzyme (SOD) declined significantly ($p<0.05$) in $NaAsO_2$ treated rats compared to the control and KV pre-treated rats (Figure 2). Our data show significant ($p<0.05$) reduction in the activity of GST, GPx and CAT due to arsenic acid toxicity whereas KV pre-treatment caused a significant increase in the activity of these enzymes in dose-dependent manner (Figure 3, 4 & 5). The result

Table 2. Effect of Sodium Arsenite and Kolaviron on aminotransferases (ALT & AST) and phosphatase (ALP) rats

Parameters	Group 1	Group 2	Group 3	Group 4
ALP (U/L)	139.84±15.20	275.85±16.64[a]	86.94±9.76[a,b]	82.80±13.80[a,b]
AST (U/L)	208.95±63.81	356.32±32.87[a]	156.32±14.89[b]	187.90±14.89[b]
ALT (U/L)	53.32±9.03	84.93±28.02[a]	48.13±5.34[b]	62.60±19.64[b]

Table 3. Effect of Sodium Arsenite and Kolaviron on level of markers of oxidative stress (Malondialdehyde MDA, Hydrogen Peroxide H2O2 generation and Nitric Oxide NO) in rats

Parameters	Group 1	Group 2	Group 3	Group 4
MDA(µmol of MDA formed/mg protein)	1.96±0.40	5.50±1.84[a]	2.58±0.36[b]	2.61±0.51[b]
H_2O_2(µmol/min/mg protein)	12.29±0.33	14.42±0.95[a]	12.94±0.85[b]	12.05±0.41[b]
NO (µmol/mg protein)	0.90±0.07	1.33±0.11[a]	0.96±0.05[b]	1.02±0.07[a,b]

obtained in this study reveals a significant ($p<0.05$) increase in the liver weight of rats administered with $NaAsO_2$ when compared to the control and animals pre-treated with 100 & 200 mg/kg KV, respectively (Figure 6).

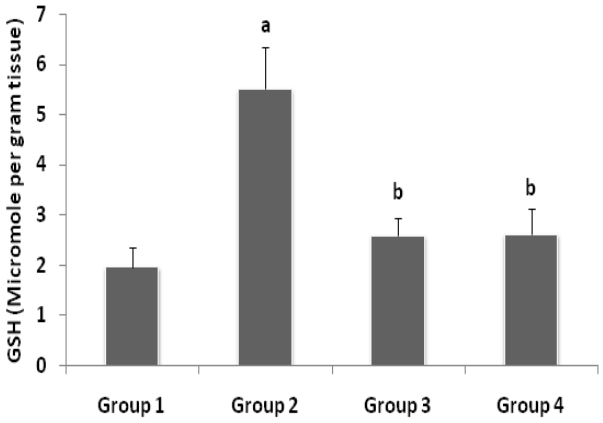


Figure 1. Effect of Kolaviron on reduced glutathione (GSH) of arsenic acid intoxicated rats.

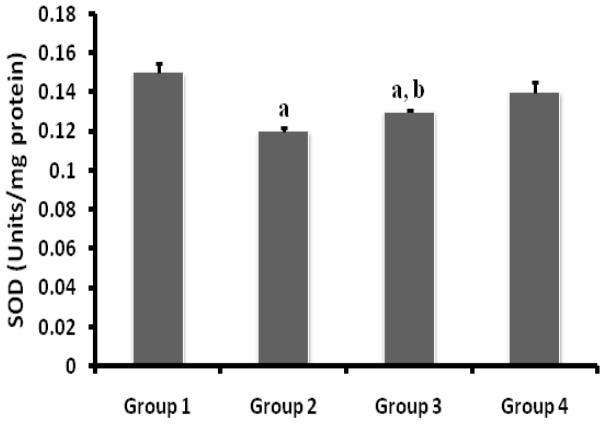


Figure 2. Effect of Kolaviron on superoxide dismutase (SOD) activity of arsenic acid intoxicated rats.

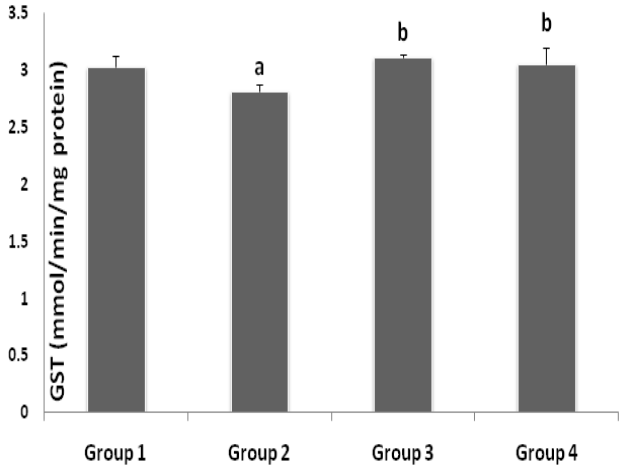


Figure 3. Effect of Kolaviron on glutathione-S-transferase (GST) activity of arsenic acid intoxicated rats.

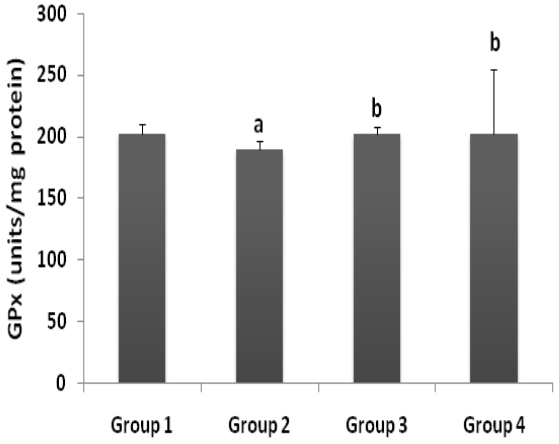


Figure 4. Effect of Kolaviron on glutathione peroxidase (GPx) activity of arsenic acid intoxicated rats.

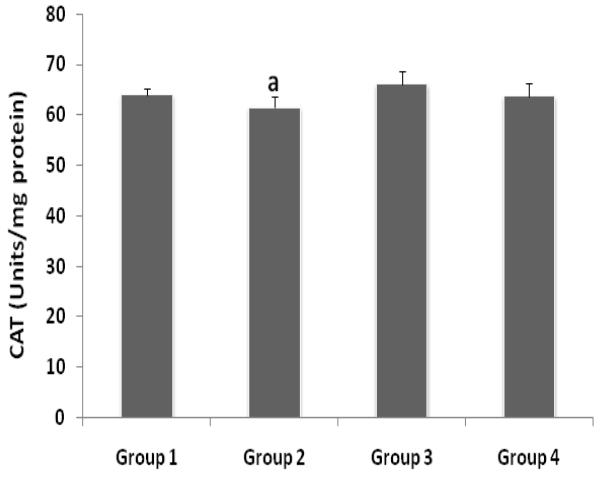


Figure 5. Effect of Kolaviron on catalase (CAT) activity of arsenic acid intoxicated rats.

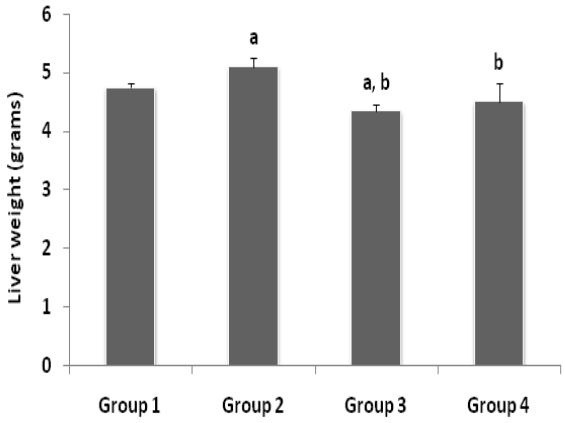


Figure 6. Effect of Kolaviron on liver weight of arsenic acid intoxicated rats.

3.4 Histology

Control rats (Group 1) showed normal architecture of the hepatocytes with mild periportal infiltration by inflammatory cells (arrows). The Liver section of rats intoxicated with $NaAsO_2$ showed fibrous cyst which is infiltrated by inflammatory cells (Group 2). There was congestion of vessels as indicated by arrow (Group 2). Rat pre-treated with 100 mg/kg body Kolaviron (KV) showed no visible lesion but mild infiltration of inflammatory cells was observed as indicated by the arrow (Group 3) and there was mild congestion of vessels (arrow) as well as the sinusoid in group pre-treated with 200 mg/kg of KV (Group 4).

4. Discussion

From our findings, $NaAsO_2$ intoxication led to a significant fall in RBC counts, Hb concentration and PCV. This is suggestive of haemolytic anaemia. Anaemia due to arsenic toxicity has been associated with haemolytic and cytotoxic effects on the blood cells and a suppression of erythropoisis with relatively high dose of $NaAsO_2$ causing bone marrow depression.[32, 33] Haemolysis associated with arsenic acid toxicity could also be linked to the depletion of GSH which is an intracellular antioxidant defence system. This reduction is also linked to the oxidation of sulfhydryl groups in the haemoglobin with resultant reduction in oxygen carrying capacity of red blood cells. We also observed a decrease in white blood cell and platelet counts. This is suggestive of association between oxidative stress and alterations in haeme synthetic pathways. Free radicals generated by $NaAsO_2$ may contribute to peroxidation of the RBC membrane leading to haemolysis and subsequent anaemia. Our findings are in agreement with the study of Mittal and Flora who reported significant depletion in blood delta-aminolevulinic acid dehydratase (ALAD) activity, platelet counts (PLT), glutathione (GSH) level and white blood cell (WBC) counts.[34] The haematological indices from this study showed normocytic hypochromic anaemia as there was no significant difference in the calculated MCV, MCH and MCHC, respectively. Our results therefore supports the findings of Antonio *et al.* [32] and Dwivedi *et al.*[35] that reported normocytic hypochromic anaemia in pregnant and lactating rats exposed to $NaAsO_2$. However, pre-treatment with 100 and 200 mg/kg of KV ameliorated anaemia caused by Na_3SO_3 intoxication with resulted increase in RBC counts, Hb concentration and PCV to near normal. Hence, we propose the anti-haemolytic effect of KV and this might be associated with its antioxidant and free radical scavenging activity as reported by Adedara *et al.* [13]

The activities of serum Aspartate aminotransferase (ALP), Alanine aminotransferase (AST), and Alkaline phosphatase (ALT) were significantly elevated in $NaAsO_2$ treated group compared to the control. In this study, we observed that pre-treatment with KV prior to $NaAsO_2$ intoxication mitigated hepatic damage as indicated in increased activities of ALP, AST and ALT. The liver is known to accumulate $NaAsO_2$ following repeated exposures. Therefore, liver disease is commonly associated with $NaAsO_2$ intoxication. The antioxidant

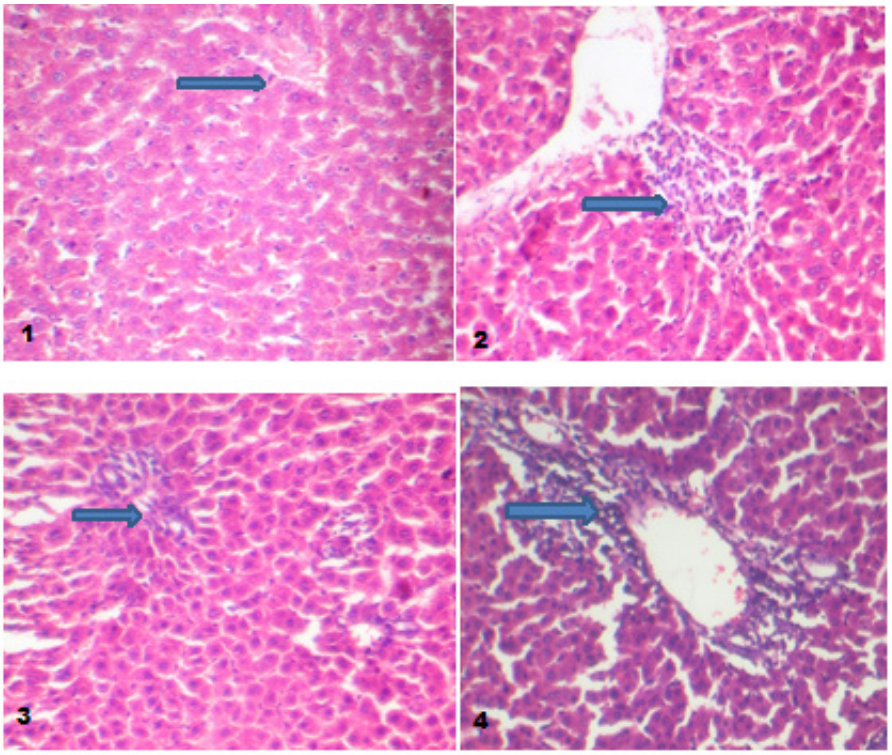


Figure 7. Histological sections of the liver of arsenic acid intoxicated rats.

and hepatoprotective effect of KV was demonstrated in this study as hepatic intoxication caused by $NaAsO_2$ was attenuated by KV administration. The antioxidant and hepatoprotective effect of KV has been reported in relation to various toxicants.[36-39]

The GSH is the most important cellular non-protein thiol and has been shown to play a protective role against oxidant injuries via its antioxidant and reducing equivalent.[3]In the present study, it was surprising to note significant increase in GSH in arsenic intoxication since it is a common knowledge that GSH concentration should crash after being used to mop-up free radicals that escape *in vivo* antioxidant system. It has been reported elevated GSH levels may be influenced by Nrf2 and that Nrf2 promotes the inducible expression of GSH generating enzymes, such as glutamate cysteine ligase (GCL), γ-glutamylcysteine synthetase heavy (γ-GCS) and light subunits (γ--GCS_L), glutathione reductase (GR), and glutathione S-transferases (GSTs).[40, 41]However, $NaAsO_2$ is known to generate reactive oxygen species (ROS) and nitrogen species (RNS). Our study showed increase in markers of oxidative stress (MDA, H_2O_2 and nitric oxide) in animals intoxicated with $NaAsO_2$. Furthermore, the aforementioned markers of oxidative stress were significantly reduced in rats administered with KV (100 and 200 mg/kg) prior to $NaAsO_2$intoxication. The antioxidant activity of KV was painstakingly demonstrated in this study with its ability to quench ROS and RNS generated by $NaAsO_2$.

Induction of oxidative stress and free radical generation has always been a major player in $NaAsO_2$-induced toxicity. SOD quenches superoxide anion radicals whereas CAT and GPx detoxify hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2). This signifies the importance of *in-vivo* antioxidant defence system as protective mechanism against the damaging effects of ROS. Our data showed that $NaAsO_2$ intoxication significantly inhibited SOD, GST, GPx and CAT. Documented evidences have shown that –$NaAsO_2$ inhibited the activities of SOD, GST, GPx and CAT.[42-44]The inhibition of these antioxidant defence system ultimately led to the accumulation of superoxide anion radicals and hydrogen peroxide (H_2O_2) which finally bind to nucleic acid of the hepatocytes. In this study, KV administration was able to quench and ameliorate lipid peroxidation products generated following $NaAsO_2$ intoxication.

The increase in liver weight and fibrous cyst formation

which was infiltrated by inflammatory cells combined with congestion of vessels as indicated by the histology section. The increase in the liver weight might be associated with deposition of $NaAsO_2$ in the parenchyma of the liver and probably followed by the infiltration of inflammatory cells. The resultant effect is hepatitis which is the acute phase of hepatic damge. However, KV was able to ameliorate the architectural anarchy inflicted of the liver cells in response to $NaAsO_2$ intoxication.

5. Conclusion

Combining all, the biflavonoid present in KV might have been responsible for the antioxidant, free radical scavenging and hepatoprotective effects. Furthermore, KV may represent a potential phytochemical to protect the liver tissue from the detrimental effects of arsenic toxicity.

6. Conflict of interest

There is no conflict of interest with this manuscript.

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