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KOLAVIRON ANTAGONIZES BIOCHEMICAL ALTERATIONS OCCASIONED BY MERCURY CHLORIDE INTOXICATION IN RAT LIVER HOMOGENATES

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ABSTRACT

Kolaviron is a biflavonoid complex isolated from *Garcinia kola* seeds which has been reported to possess multiple bioactivities. Mercury is a heavy metal used in industries and found in home appliances but toxic to man on exposure. The liver is the major organ responsible for toxicant metabolism and its health is crucial for overall well-being. In this study, the protective effect of kolaviron on mercury-induced toxicity in rat liver was evaluated. Rat liver homogenate intoxicated with 0.1% $HgCl_2$ was incubated with/without kolaviron and analyzed for biochemical markers of hepatotoxicity. Results showed that kolaviron at concentrations of 100 and 500 µg/ml reversed mercury chloride incited oxidative stress and hepatotoxicity as adjudged by its effects on levels of reduced glutathione and malondialdehyde, and activities of catalase, superoxide dismutase, alanine aminotransferase, aspartate amino transferase and alkaline phosphatase. These results suggest that kolaviron has potential in the management of toxicities and pathologies arising from exposure to mercury.

Key words: hepatotoxicity, hepatoprotection, kolaviron, mercury, oxidative stress

INTRODUCTION

Exposure to heavy metals is a common phenomenon due to their environmental pervasiveness. Mercury is a heavy metal used in industries and found in home appliances and which induces severe alterations in the tissues of both animals and men (Sener et al., 2003). All forms of mercury can cause toxic effects in a number of tissues and organs depending on the chemical form of mercury as well as the level,

duration and the route of exposure (Clarkson, 2002).. Exposure to mercury compounds can occur by inhalation, ingestion or skin contact. Although the liver is not a primary target for mercury toxicity and most studies have focused on other organs like the brain and the heart, it is reasonable to investigate its effect on the liver because it is the principal site for xenobiotic metabolism and may potentiate the toxicity of the metal. Mercury undergoes extensive biliary-hepatic cycling (Dutczak and Ballatori, 1994). It is secreted into bile and partly reabsorbed into the portal circulation and thereby returned to the liver. Based on the role of liver in mercury biotransformation and cycling, as well as its central roles in the control and synthesis of critical blood constituents that affect whole body physiology; and in view of the prevalence of mercury exposure in humans, understanding of the mechanism of mercury-induced hepatotoxicity is therefore important for elucidating its impact on liver health.

Bioactive components from medicinal plants, especially polyphenolics, have gained prominence as reliable and safer alternatives to synthetic drugs in the management and therapy of diverse pathologies. Kolaviron is a biflavonoid complex isolated from defatted *Garcinia kola* seeds. Pharmacological activities that have been reported for kolaviron include in vivo hepatotoprotection, cardioprotection, neuroprotection and reproprotection via antioxidant, anti-inflammatory and other mechanistic routes (Akinmoladun et al., 2015) as well as potent activity in several in vitro studies (Abarikwu et al., 2011; Abarikwu, 2014).

In this study, we evaluated the impact of mercury chloride on biochemical status of rat liver homogenates and the modulatory effect of kolaviron.

MATERIALS AND METHODS

Chemicals

Mercury chloride ($HgCl_2$) was obtained from BDH Chem Co., England. Quercetin, Ellman's reagent (5,5′-Dithiobis(2-nitrobenzoic acid), reduced glutathione (GSH) and adrenalin were products of Sigma-Aldrich (St-Louis, MO, USA). Other chemicals and reagents were of analytical grade.

Extraction of kolaviron

Extraction of Kolaviron was achieved by the procedure previously described by Iwu (1985) and modified by Braide (1991). Briefly, *Garcinia kola* seeds were peeled, air dried and ground into powdered form. The powdered seeds were extracted with petroleum ether (b.p 40-60°C) in a soxhlet for 24 h. The defatted, dried marc was repacked and then extracted with acetone. The extract was concentrated and diluted to twice its volume with distilled water and extracted with ethyl acetate. The concentrated ethyl acetate yielded a yellow-brown residue that was tested to be kolaviron.

Animals

Adult Wistar rats of both sexes were used for the experiment. Animals were handled in accordance with the guidelines for the care and use of

experimental animal resources (National Institute of Health, NIH publication 85–23, 1985).

Experimental design

Animals were sacrificed by cervical dislocation and the livers excised, washed in ice cold 0.85% saline and weighed. A 5% (w/v) homogenate was prepared in 50 mM sodium phosphate buffer (pH 7.4). The toxicant used was mercury chloride (100 µg/ml). Three concentrations (100-, 500- and 1000 µg/ml of kolaviron) were used. The reference standard was quercetin (500 µg/ml). The experimental groups were: homogenate only; homogenate + toxicant; homogenate + toxicant + extract (100 µg/ml); homogenate + toxicant + extract (500 µg/ml); homogenate + toxicant + extract (1000 µg/ml); homogenate + extract (100 µg/ml); homogenate + extract (500µg/ml); homogenate + extract (1000 µg/ml) and homogenate + toxicant + quercetin. Mixtures were incubated at 37°C for 20 min and samples were withdrawn for biochemical estimations after the period of incubation.

Biochemical estimations

Activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase were determine using assay kits from Randox Laboratories Ltd., Antrim, UK.

Superoxide dismutase activity

Assay for superoxide dismutase activity was performed using the method of Misra and Fridovich (1972). One ml of appropriately diluted supernatant of brain homogenate (sample) was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer and the reaction was started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. Increase in absorbance per minute was calculated using the formula: $(A_3-A_0)/2.5$ where A_3 and A_0 were absorbance after 30 s and 150 s, respectively. Percentage inhibition was calculated using the formula: (increase in absorbance of substrate/increase in absorbance of blank) x100. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 minute.

Catalase activity

Evaluation of catalase activity was carried out using the method described by Sinha (1972). Briefly, appropriately diluted sample was mixed with 0.8 mM of H_2O_2 and phosphate buffer (pH 7.0). A 1 ml portion of the reaction mixture was withdrawn and added to 1 ml dichromate/acetic acid reagent at 60 s intervals. The H_2O_2 content of the withdrawn sample was determined by taking the absorbance at 570 nm. The mononuclear velocity constant K, for the decomposition of H_2O_2 by catalase was determined using the equation:

$$K = {}^{1}/t \log S_0/S$$

where S_0 is the initial concentration of H_2O_2 and S is the concentration of the peroxide at 1 min. The values of K were plotted against time and the velocity constant of catalase K_0 at 0 min

determined by extrapolation. The catalase content of the enzyme preparation was expressed in terms of Katalase feiahigkeit or 'Kat. f.'

Kat. f = K_0 /(mg protein/ml).

Reduced glutathione level

Reduced glutathione (GSH) level was determined following the method of Beutler et al. (1963). Sample (0.2 ml) was added to 1.8 ml of distilled water and 3 ml of the precipitating solution was mixed with sample. The mixture was then allowed to stand for approximately 10 min and then centrifuged at 3000 g for 5 min. Then, 0.5 ml of the supernatant was added to 4 ml of 0.1 M phosphate buffer. Finally 0.5 ml of Ellman's reagent was added. The absorbance of the reaction mixture was read within 30 min of colour development at 412 nm against a reagent blank.

Lipid peroxidation

Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Varshney and Kale (1990). Sample (0.4 ml) was mixed with 1.6 ml of Tris-KCl buffer to which 0.5 ml of 30% trichloroacetic acid (TCA) was added. Then 0.5 ml of 0.75% thiobarbituric acid (TBA) was added and placed in a water bath for 45 min at 80°C. This was then cooled and centrifuged at 3000 g. The absorbance of the clear supernatant measured against a reference blank at 532 nm. Lipid peroxidation in units/mg protein or gram tissue was computed with a molar extinction coefficient of 1.56 x 105 $M^{-1}cm^{-1}$.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical differences between means were determined by one-way analysis of variance (ANOVA) followed by Duncan's test. $P < 0.05$ was considered statistically significant.

RESULTS

Figures 1, 2 and 3 show the effect of kolaviron on the activities of ALT, AST and ALP, respectively, which are biomarkers of hepatocellular injury. Toxicant-induced decrease was observed in all enzyme activities but this was reversed in intoxicated groups treated with extract or standard. In homogenates treated with kolaviron alone, peak activity was observed at 500 µg/ml with declining activity observed in the 1000 µg/ml treated group compared with other concentrations.

SOD activity (Figure 4), catalase activity (Figure 5) and GSH level (Figure 6) were decreased in the positive control group but the decrease was reversed, dose-dependently in mercury chloride challenged groups treated with kolaviron. On the other hand, MDA level was increased in the positive control group but the increase was nullified by treatment with kolaviron. Following the trend observed in the activities of the transaminases and alkaline phosphatase, homogenates treated with 1000 µg/ml kolaviron without mercury intoxication showed decreased values of the antioxidant enzymes and GSH but increased MDA level compared with the preceding concentration (500 µg/ml).

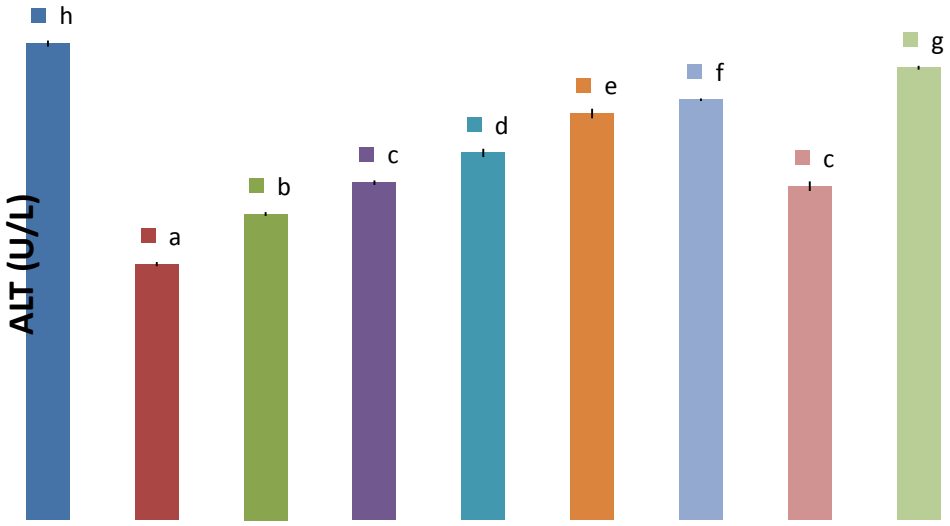

Figure 1. ALT activity in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different ($P<0.05$). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

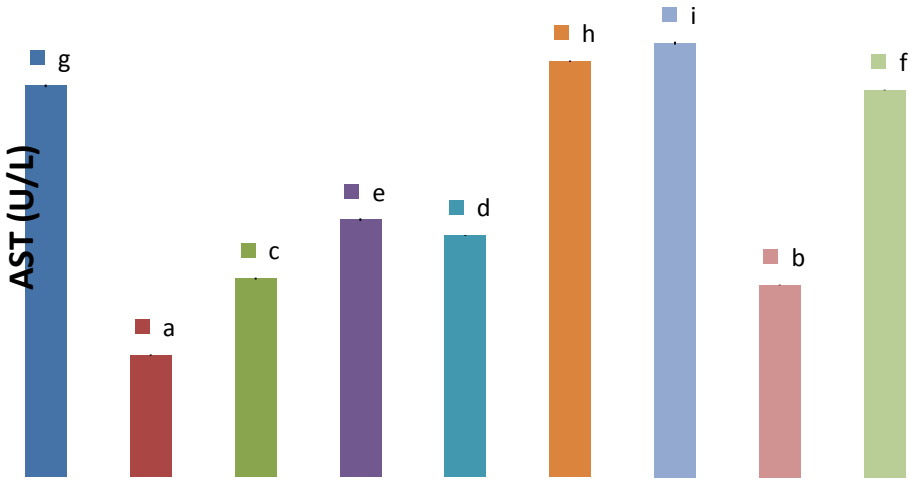

Figure 2. AST activity in homogenates challenged with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

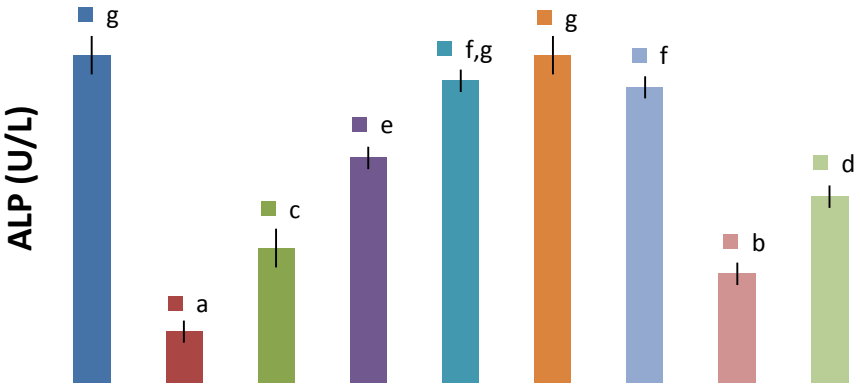

Figure 3. ALP activity in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not

significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

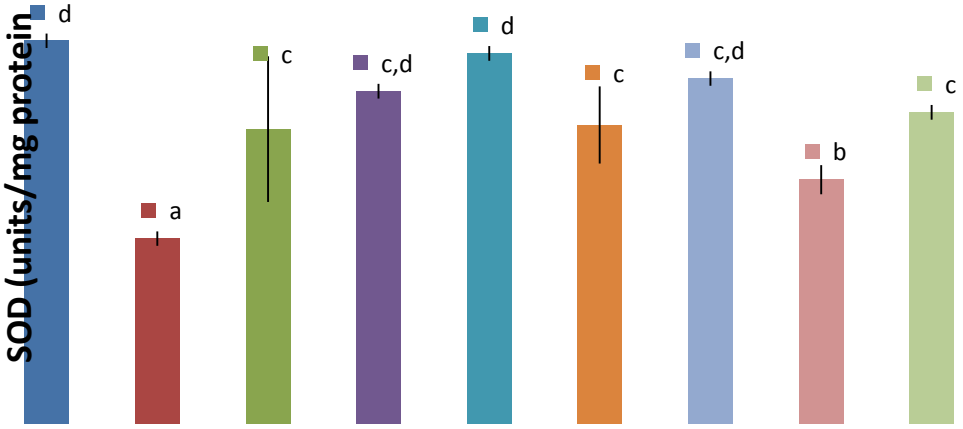

Figure 4. SOD activity in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

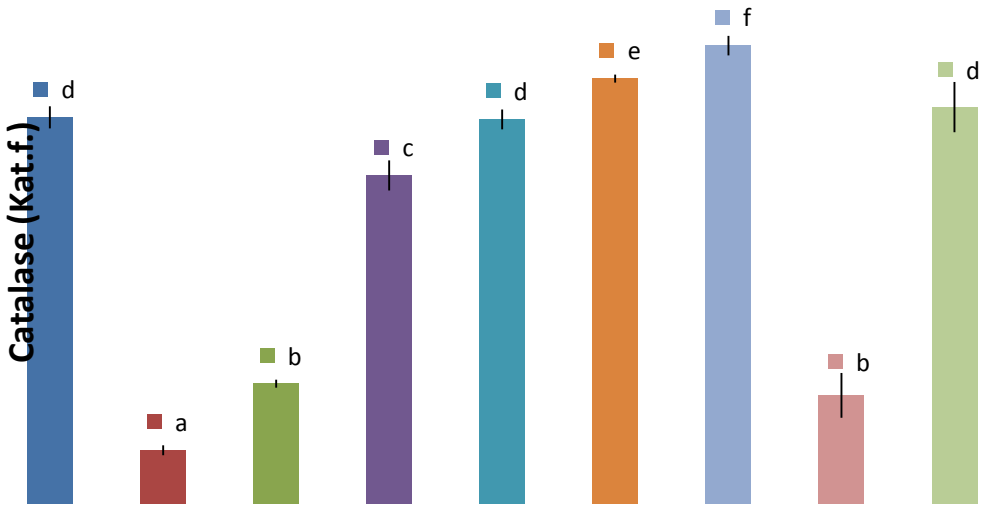

Figure 5. Catalase activity in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

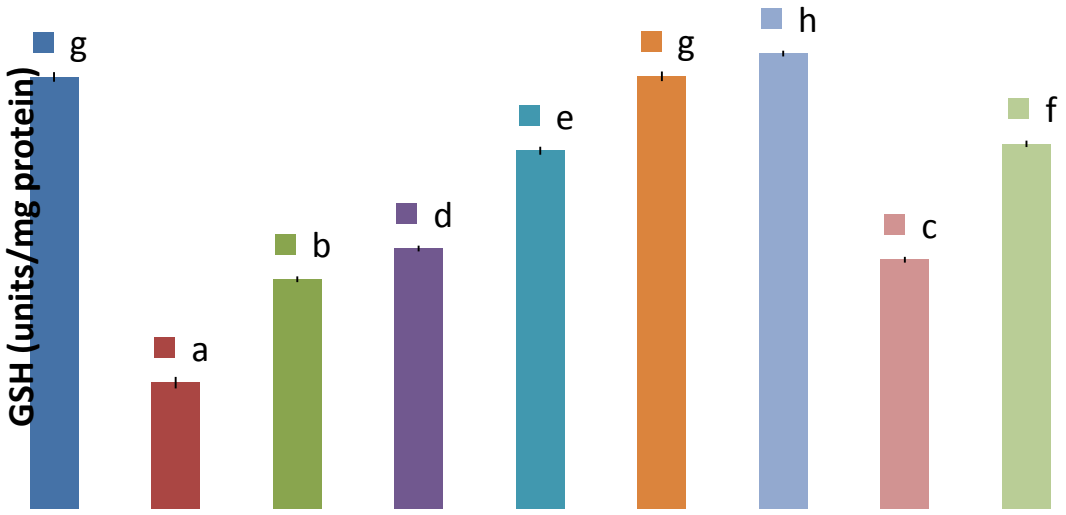

Figure 6. GSH level in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

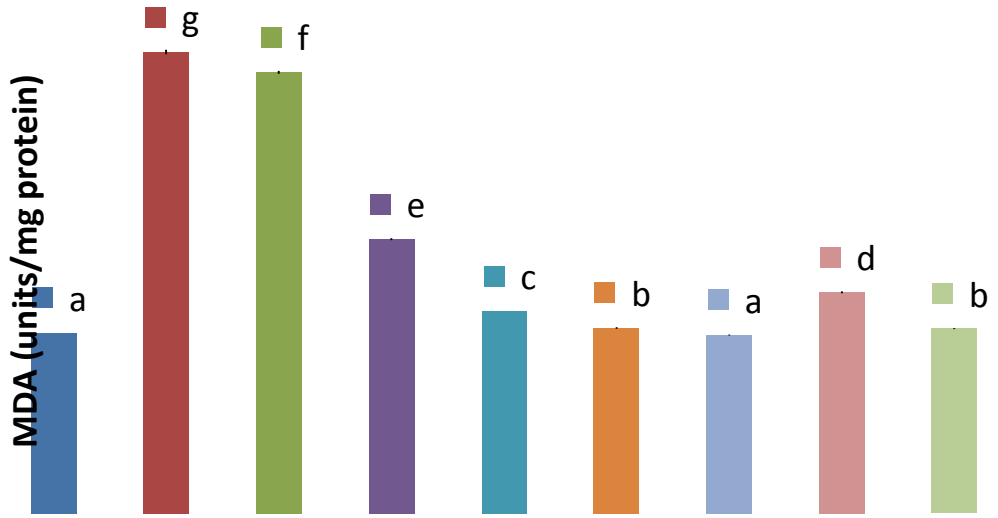

Figure 7. GSH level in homogenates intoxicated with mercury chloride and treated with kolaviron.

Each bar represents mean ± S.D of three replicates. Bars with same alphabets are not significantly different (P<0.05). H: homogenate only; H+T: homogenates challenged with mercury chloride; H+T+E100, H+T+E500 and H+T+E1000: homogenates challenged with mercury chloride and treated with 100-, 500- and 1000 µg/ml kolaviron, respectively; H+E100, H+E500 and H+E1000: homogenates treated with 100-, 500- and 1000 µg/ml kolaviron, respectively without mercury chloride intoxication.

DISCUSSION

The decrease in activities of ALT, AST and ALP observed in mercury chloride intoxicated homogenates (positive control) is suggestive of hepatocellular injury. These enzymes are leaked from hepatocytes following injury to the liver and

their activities are therefore elevated in the serum/plasma but decreased in the hepatic tissue in *in vivo* settings (Olaleye et al, 2010). In both *in vivo* and *in vitro* settings as in the present scenario, probable reason for their decreased activities is their inhibition or inactivation by mercury making them unavailable for reaction with the substrates. Different reports have shown that heavy metals with different mechanisms can inhibit enzymes. Mercuric chloride and sodium selenite can oxidize -SH groups of different enzymes. Reports have indicated that Hg^{2+} inhibits the activity of δaminolevulinate dehydrogenase in mouse liver, kidney and brain by oxidizing -SH groups located at the active center of the enzyme (Farian et al., 2003; Ghaffari and Motlagh, 2011). The propensity of mercury to bind the sulfhydryl (-SH) group of proteins thereby inactivating them could also explain the decreased activity of the enzymic antioxidants, SOD and catalase, since they are proteins as well.

Other mechanisms, including lipid peroxidation have been proposed for the biological toxicity of mercuric chloride ($HgCl_2$), and it has been demonstrated that lipid peroxidation occurs in the kidney, liver and other tissues of rats and mice following parenteral administration of $HgCl_2$ (Mahboob et al. 2001). The induction of lipid peroxidation associated with Hg^{2+} treatment of isolated rat hepatocytes has been reported with suggestion of a causative role of oxidative stress in mercury cytotoxicity (Stacey and Kappas, 1982). Woods et al. (1990) investigated the aetiology of mercury-induced porphyrinuria under *in vitro* conditions. Their findings support the view that Hg^{2+} ions both compromise the antioxidant potential of GSH and promote formation of reactive species via thiol complexation. These observations are in agreement with the results obtained in this study. The reduction in the activities of SOD and catalase, the decrease in the level of GSH and the increased MDA level in the mercury chloride intoxicated group are indicative of oxidative stress. This could be due either to a loss of the cells expressing these enzymes (especially in *in vivo* situations), to a direct effect of reactive oxygen species on the enzymes, or due to a direct inhibition from Hg^{2+} causing impairment of the antioxidant function and hence, increased reactive oxygen species production (Sorg et al., 1998; Sener et al., 2003). From the foregoing, it could be inferred that in addition to depletion of intracellular thiol pools, the oxidant pathway may be a primary mechanism of mercury toxicity. Mercury induced formation of free radicals or lipid peroxides could be coupled to Hg^{2+} induced alteration of protein conformation through covalently binding to sulfhydryl groups, or creating protein adducts through modification of side chains leading to changes in protein shape and activity since such changes are known to be the result of the generation of free radicals by metals (Sanders et al., 1996).

The reversal of mercury chloride induced alterations to evaluated parameters in homogenates treated with kolaviron is consistent with the reported antioxidant and antihepatotoxic properties of the extract (Iwu, 1985; Nwankwo et al., 2000; Farombi et al, 2002; Farombi and Nwaokeafor, 2005). Kolaviron possibly ameliorated mercury toxicity by antagonizing the binding of the metal to the sulfhydryl groups of susceptible proteins and/or prevented free radical triggered mercury toxicity through its antioxidative effect. The observed tendency to prooxidant effect observed at the 1000 µg/ml concentration of kolaviron highlights the need for appropriate dosing in chemoprophylaxis or therapy with phytoextracts.

CONCLUSION

Our results show that kolaviron has potential in ameliorating mercury induced liver injury and related dysfunctions. In view of this finding and previous reports of its hepatoprotective property and other bioactivities, further studies leading to its pharmaceutical formulations are warranted.

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