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Ethanol Extract of *Terminalia avicennioides* Root Bark Protects Against Cadmium Toxicities in Rats

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ABSTRACT

Environmental exposure to cadmium is widespread and its toxicity has been linked with generation of reactive oxygen species. Dietary antioxidants therefore hold great promise against cadmium intoxication. We investigated the effects of ethanol extract of *Terminalia avicennioides*root bark (ERTA), a rich source of secondary metabolites, on cadmium-induced hepatotoxicity and clastogenicity in male Wistar rats. Groups of rats, five each group, were treated with either ERTA (200 or 400 mg/kg b.w), cadmium chloride (CdCl2) at 5 mg/kg b.w. or both ERTA and CdCl2. Administration of CdCl2 alone caused significantly ($p<0.05$) higher mean serum aspartate (AST) and alanine aminotransferases (ALT) activities, and mPCEs number in the treated rats compared to the control given distilled water. There was significant ($p < 0.05$) reduction in AST and ALT activities, and mPCEs in the groups given ERTA and CdCl2 compared with group given CdCl2 alone. ERTA alone at 200 mg/kg or 400 mg/kg body weight induced significant ($p < 0.05$) increase in mean serum alkaline phosphatase (ALP) but not AST and ALT activities, compared with the negative control. Results for histopathological analyses of liver samples of rats followed the same trends as what was obtained in the enzyme activities. In addition, CdCl2 alone produced a significantly ($p<0.05$) higher level of thiobarbituric acid index of lipid peroxidation compared with the negative control group which was significantly ($p<0.05$) reduced by ERTA treatment. ERTA therefore displayed protective properties against cadmium-induced toxicities in the male rats though there is possibility of its toxicity at the tested doses.

Keywords; Hepatotoxicity; micronucleated polychromatic erythrocytes (mPCEs); aminotransferases; cadmium chloride; clastogenicity

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INTRODUCTION

Human exposure to cadmium is almost inevitable. The major routes of exposure to cadmium in vertebrates include dermal, gastrointestinal and pulmonary (Jin *et al*., 2002). A high fiber diet has been shown to increase dietary cadmium intake (Jarup *et al*., 1998). Food items like liver, mushrooms, shellfish, mussels, cocoa powder and dried seaweed are rich in cadmium and can therefore greatly increase the cadmium load in human body while low intake of calcium, vitamin D, and trace elements like zinc and copper facilitate the systemic cadmium resorption (Jin *et al*., 2002). Likewise, the human lungs reabsorb 40–60 % of the cadmium in tobacco smoke, thus making cigarette smoke the major source of inhalative cadmium intoxication (Elinder *et al*., 1976).

Carcinogenicity of cadmium has been linked with generation of reactive oxygen species (ROS) during its metabolism (Ajilore and Ayannuga, 2012; Ajilore at al., 2012). On the other hand, antioxidants are promising therapeutic agents against free radical-related diseases. Dietary antioxidants especially hold great promise in contrast to synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which have been shown to have negative side effects on human health (Branen, 1975). The use of plants as sources of natural antioxidants is currently receiving great attention and there is an increasing recognition of traditional medicine as part of primary health care programmes in many countries. There is however a need for scientific evaluation of the medicinal plants for their beneficial as well as toxicological activities (Kyerematen *et al*., 1987; WHO, 1978).

One such important medicinal plant is Terminalia avicennioides, widely used for the treatment of ailments such as diarrhoea, dysentery, dropsy, oedema, gout, leprosy and pains (Mann *et al*., 2011). *Terminalia avicennioides*is a rich source of secondary metabolites like cyclic triterpenes and their derivatives, flavonoids, tannins and other aromatics (Akinyemi *et al*., 2005). We report here, the protective effects of ethanol extract of the root bark of *Terminalia avicennioides*(ERTA) on cadmium-induced hepatotoxicity and clastogenicity in male Wistar rats.

MATERIALS AND METHODS

Chemicals: Cadmium chloride ($CdCl_2$) was product of Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Plant material: The root bark of *T. avicennioides* used was obtained from Oje Market in Ibadan. It was authenticated at the Department of Botany of our university, where a sample was deposited with a herbarium number UIH-22433.

Experimental animals and housing : Thirty-six male rats of Wistar strain weighing between 100-150 g were obtained from the Central Animal Facility, Pre-clinical Section, College of Medicine, University of Ibadan. They were housed in steel metal cages under conditions of 25±2 ^oC temperature and 12 hour light-dark cycle at the Experimental Animal House of our department. They were fed with standard rat pellets and given water *ad libitum.*

Enzyme assay kits: Alkaline phosphatase (ALP) assay kit used was a product of Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany. Assay kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were products of Randox Laboratories Limited, County Antrim, United Kingdom.

Preparation of ethanol extract of the root bark of *Terminalia avicennioides*(ERTA): The extract was prepared according to the modified method of Othman *et al.,* (2007). Briefly, the root bark of *T. avicennioides* was sparingly washed, and air-dried at room temperature. The dried root bark was ground into powder, sieved, weighed and soaked in 50 % ethanol for 72 hours. The extract was filtered through muslin sheet into a conical flask and the filtrate was evaporated to dryness using a rotary evaporator under reduced pressure at 40 ^oC. The extract was stored at 4 ^oC until use.

Experimental rats and treatments: The rats were allowed to acclimatize for seven days before the commencement of the study which lasted for twenty-one days. Experimental animals were treated and sacrificed in accordance with the guidelines of the Ethical Committee on the Care and Use of Laboratory Animals, University of Ibadan, Ibadan. Thirty male rats were randomized into six groups of five animals each as indicated below:

Group 1: Rats served as control and were given distilled water only.

Groups 2: Rats in this group were administered 200 mg/kg body weight of ERTA.

Groups 3: Administered 400 mg/kg body weight of ERTA

Group 4: Rats in this group were given 5 mg/kg body weight of $CdCl_2$ in distilled water.

Group 5: Received 200 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$.

Group 6: Administered 400 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$.

All treatments were carried out daily for three weeks by oral intubation.

Collection and preparation of samples: Twenty-four hours after the administration of the last dose of the test substances, the rats were sacrificed by cervical dislocation. Colchicine (0.04 %) was administered two hours before the commencement of the sacrifice (Seetharama and Narayana, 2005). Blood was collected by cardiac puncture and was allowed to clot for about two hours after which it was centrifuged at 3000 *g* for 30 minutes to separate the serum. The supernatant (serum) was aspirated with the aid of Pasteur pipettes into a clean container and placed on ice for immediate use or stored at -20 ^oC until needed. The liver tissues were excised and thin slices from each animal were stored in 10 % buffered formalin for histopathological analyses.

Serum enzyme assays: Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined using the assay kits and following the instructions of the manufacturer. Serum AST and ALT activities were assayed according to Reitman and Frankel (1957) by monitoring an intensely coloured hydrazone at 546 nm using a Spectronic-20 spectrophotometer (Thermo Scientific, Surrey, UK).

Alkaline phosphatase (ALP) was assayed using kit from Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany by following the manufacturer's enclosed instructions. In the presence of magnesium and zinc ions, *p*-nitrophenyl phosphate is cleaved by phosphatase into phosphate and *p*-nitrophenol. The *p*-nitrophenol released is proportional to the ALP activity and was measured spectrophotometrically.

Assessment of lipid peroxidation: Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) produced during lipid peroxidation using the method of Varshney and Kale (1990). Formed TBARS was measured spectrophotometrically at 532 nm and expressed as nmol malondialdehyde per gramme tissue.

Micronucleus assay: The micronucleus assay was performed according to the method of Heddle and Salmone (1981). The femur of each rat was freed and stripped clean of muscles. The iliac end of the femur was carefully cut until a small opening to the marrow became visible, a pin of an approximate size of the canal was introduced into the marrow canal of the epiphysis end and gently pushed to extrude the marrow out of the opening at the iliac end onto a glass slide to which a drop of fetal calf serum was added from a Pasteur pipette. The edge of a clean slide was used to thoroughly mix the marrow and

serum to homogeneity and then spread as a smear. The slides were air dried and fixed in absolute methanol for five minutes and further air dried for a few minutes to remove the methanol. They were then stained in 5% Giemsa stain that was initially dissolved in 0.01M phosphate buffer pH 6.8, after initial staining with 0.4% May-Grunwald solution. They were thereafter rinsed in distilled water, air dried, mounted in DPX and covered with cover glasses smeared with xylene. After an appropriate coding and screening, the slides were scored using an Olympus XSZ 107 BN microscope to detect the presence of micronucleated polychromatic erythrocytes.

Histopathological analysis: This was carried out by following the method of Germanò *et al.*, (2001). The liver sections were fixed in 4% *p*-formaldehyde and washed in phosphate buffer (pH 7.4) at 4 ^{o}C for 12 hours. After dehydration, the tissue was embedded in paraffin, cut into 5 µm sections, stained with haematoxylin-eosin dye and finally observed under a microscope at x400

Statistical Analysis

Results are expressed as mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was used for data analysis and post-hoc analysis employed the Duncan Multiple Range Test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effects of ethanol extract of the root bark of *Terminalia avicennioides*(ERTA) and CdCl2 on body weight change of rats: All the experimental rat groups showed an increase mean body weight after three weeks of treatment (Figure 1). Group 4 rats, administered CdCl2 alone had the least increase in weight which is significantly ($p<0.05$) different from the control given distilled water. Co-administration of CdCl2 and ERTA at 200 mg/kg body weight (Group 5) or at 400 mg/kg body weight (Group 6) resulted in mean body weight changes that were significantly ($p<0.05$) different from observation made with Group 4 administered CdCl2 only.

Effects of ERTA on CdCl2 –induced hepatotoxicity: The serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) after administration of ERTA and/or $CdCl_2$ are shown in Figure 2. CdCl2 alone significantly ($p<0.05$) caused an increase in mean serum AST and ALT activities in the treated rats compared to the negative control group (Group 1). Co-treatment of rats with ERTA at 400 mg/kg body weight and CdCl2 (Group 6) led to significantly ($p < 0.05$) reduced AST and ALT activities when compared with Group 4, given CdCl2 only. On the other hand, administration of ERTA alone at 200 mg/kg or 400 mg/kg body weight induced a significant ($p < 0.05$) increase in mean serum alkaline phosphatase (ALP) activity as compared with the negative control group (Figure 3). Moreover, administration of CdCl2 alone (Group 4) produced more than five times higher level of ALP activity compared with the control (Group 1).

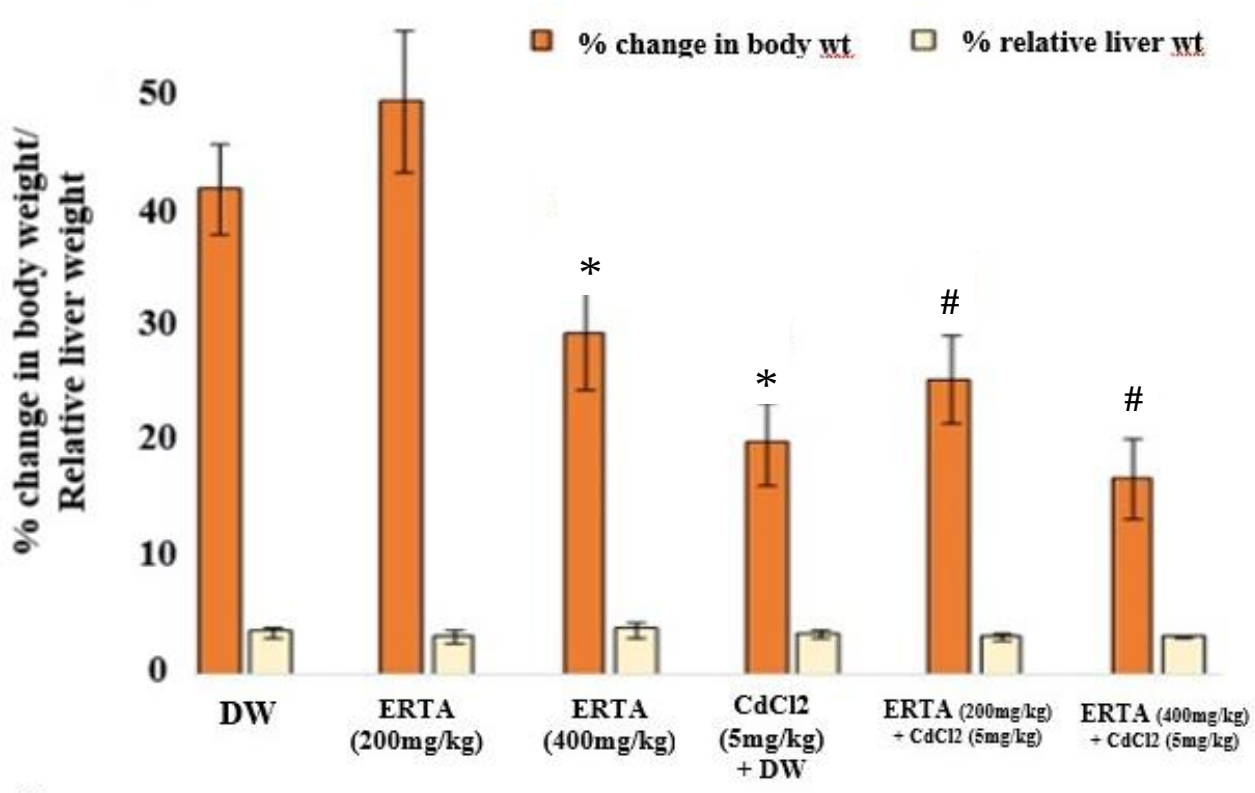

Figure 1:
Effects of ethanol extract of the root bark of *Terminalia avicennioides*(ERTA) and $CdCl_2$ on body weight change of rats
Values are mean + SD (n =5);
** = significantly different (p < 0.05) from control group 1. # = significantly different (p < 0.05) from group 4.*

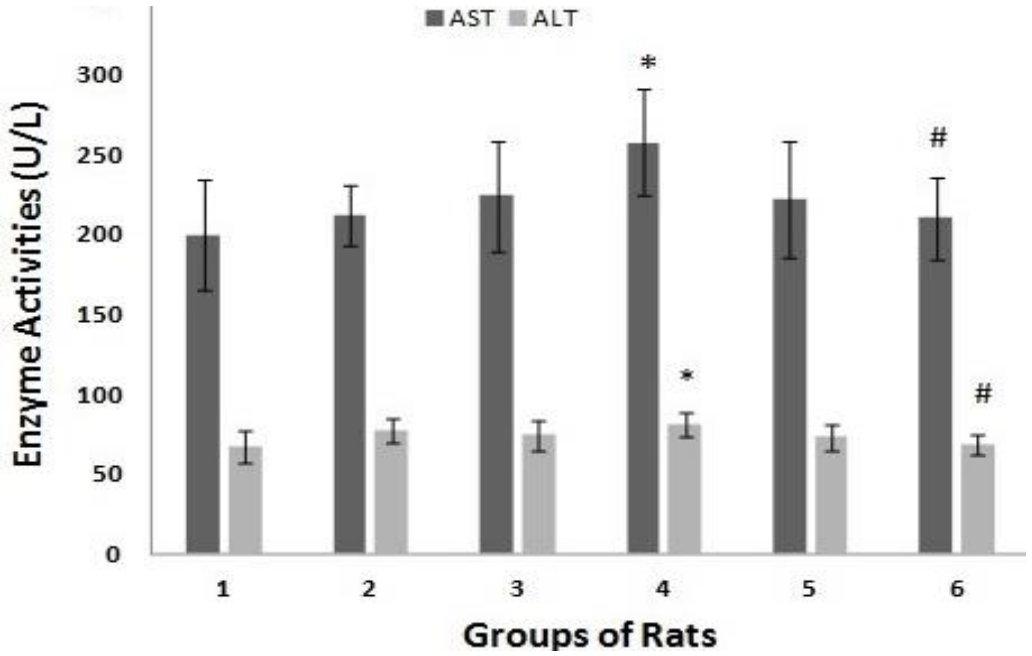

Figure 2: Effects of ERTA on $CdCl_2$–induced activities of serum aminotransferases

*Values are means ± SD (n =5); group 1 represents negative control given distilled water; group 2 rats were given 200 mg/kg body weight of ERTA; group 3 rats were treated with 400 mg/kg body weight of ERTA; group 4 rats were given 5 mg/kg body weight of $CdCl_2$ in distilled water; group 5 rats received 200 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$; group 6 were administered 400 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$. * = significantly different ($p < 0.05$) from control group 1. # = significantly different ($p < 0.05$) from group 4.*

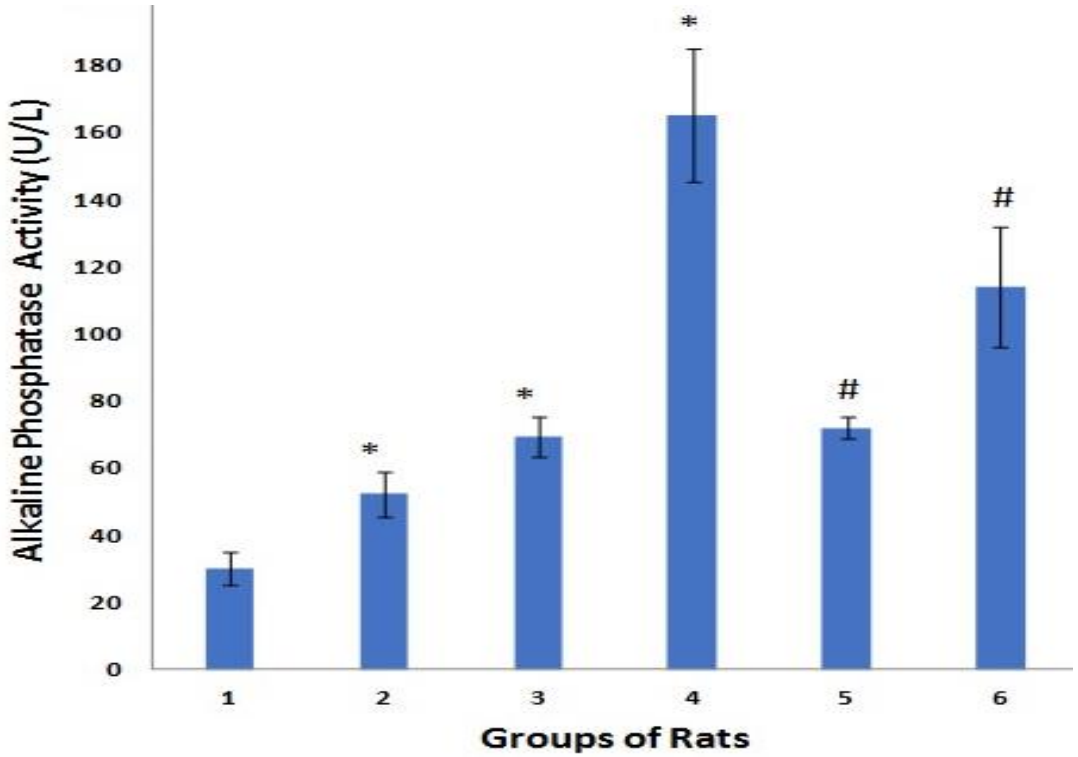

Figure 3:

Effects of ERTA and CdCl2 on activities of serum alkaline phosphatase. Values are means + SD (n =5); group 1 represents negative control given distilled water; group 2 rats were given 200 mg/kg body weight of ERTA; group 3 rats were treated with 400 mg/kg body weight of ERTA; group 4 rats were given 5 mg/kg body weight of CdCl2 in distilled water; group 5 rats received 200 mg/kg body weight of ERTA plus 5 mg/kg body weight of CdCl2; group 6 were administered 400 mg/kg body weight of ERTA plus 5 mg/kg body weight of CdCl2. * = significantly different ($p < 0.05$) from control group 1. # = significantly different ($p < 0.05$) from group 4.

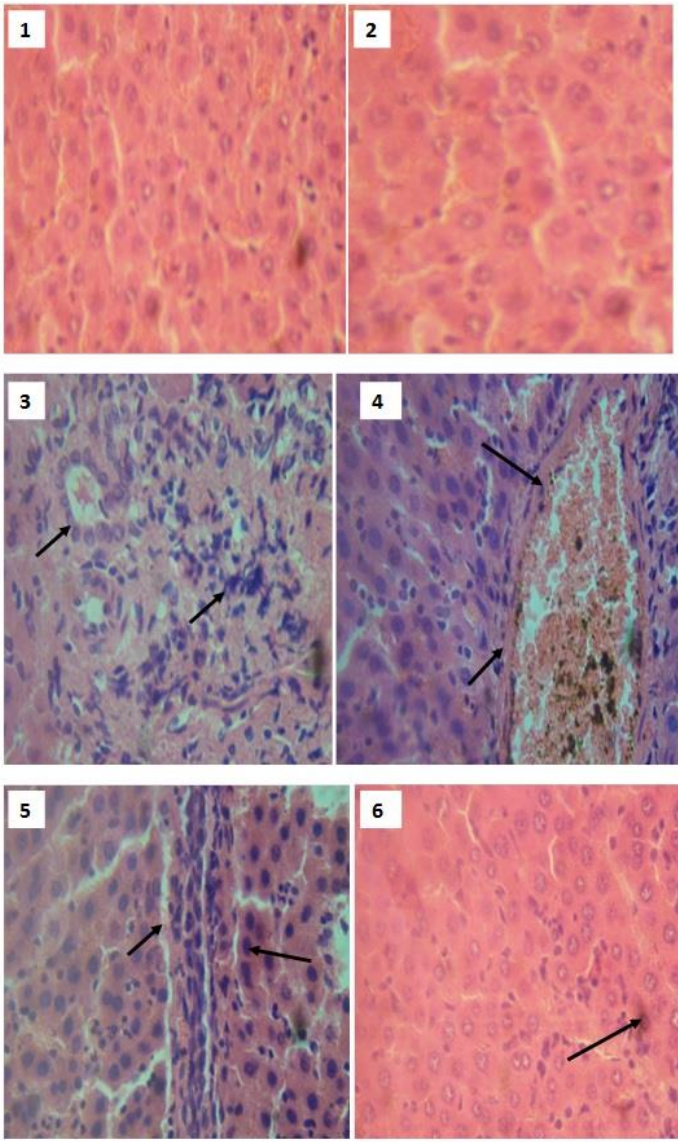

Plate 1: Selected photomicrograph of the liver sections (x40) rats treated with $CdCl_2$ and/or ethanol extract of the root bark of *Terminalia avicennioides*(ERTA).
Negative control group (1) distilled water: There are no visible lesions. Group (2) treated with 200 mg/kg body weight of ERTA: There are no visible lesions. Group (3) treated with 400 mg/kg body weight of ERTA: There were severe portal and central venous congestion. Group (4) treated with 5 mg/kg body weight of $CdCl_2$ in distilled water: There were severe periportal cellular infiltrations by mononuclear cells. Group (5) received 200 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$: There were moderate periportal cellular infiltrations by mononuclear cells. Group (6) administered 400 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$: Very mild diffuse cellular infiltrations by mononuclear cells were observed.

ERTA at 200 mg/kg or 400 mg/kg body weight resulted in significant reduced level of $CdCl_2$-induced ALP activity in Groups 5 and 6 respectively compared with Group 4 given $CdCl_2$ only. Histological analyses of the liver samples of the control and treated rats showed liver lesions with patterns that corroborate observations made with the biochemical enzyme indices (Plate 1). There are no visible lesions in the liver sections of rats in the control group (Group 1) and group treated with only ERTA at 200 mg/kg body weight (Group 2). There were severe portal and central venous congestion in the liver section of rats treated with ERTA alone at 400 mg/kg body weight (Group 3). Severe periportal cellular infiltrations by mononuclear cells were observed in the liver sections of rats treated with $CdCl_2$ only (Group 4). The liver sections of rats treated with ERTA at 200mg/kg body weight plus CdCl2 (Group 5) showed moderate periportal cellular infiltration by mononuclear cells while in the liver sections of rats co-treated with ERTA at 400mg/kg body weight plus CdCl2 (Group 6) were very mild diffuse cellular infiltration by mononuclear cells.

Effects of CdCl2 and/or ERTA on hepatic lipid peroxidation in the treated rats

Administration of ERTA alone at both 200 mg/kg and 400 mg/kg body weight did not elicit any significant ($p > 0.05$) difference in lipid peroxidation index as compared with the negative control (Figure 4). The group that was given CdCl2 alone (5 mg/kg body weight) displayed a significantly ($p<0.05$) higher level of TBAR index of lipid peroxidation compared with the negative control group. Administration of ERTA (only at 400 mg/kg body weight) along with CdCl2 significantly ($p<0.05$) reduced the level of lipid peroxidation compared with the group treated with $CdCl_2$ only.

Effects of ERTA on CdCl2–induced clastogenicity measured as relative number of micronucleated polychromatic erythrocytes (mPCEs) in the rat bone marrow cells

The number of micronucleated polychromatic erythrocytes (mPCEs) scored in the rat bone marrow cells after administration of ERTA and/or $CdCl_2$ is shown in Figure 5. ERTA alone at 200 mg/kg or 400 mg/kg body weight did not induced significant ($p > 0.05$) formation of micronuclei compared with the negative control group. Cadmium chloride induced significant ($p<0.05$) formation of mPCEs compared with distilled water. Co-treatment of rats with ERTA at 200 mg/kg or 400 mg/kg body weight led to significantly ($p<0.05$) decrease number of mPCEs scored in Groups 5 and 6 respectively compared with the group treated with CdCl2 alone (Group 4)

DISCUSSION

Appreciable progress has been made in understanding the roles of environmental factors in the development and progression of many diseases, including cancer. Typical examples of such factors are chemical contaminants like cadmium, a notable clastogen and hepatotoxin (Shaikh *et al.*, 1999; Stohs *et al.*, 2001). In addition, prolonged exposure to cadmium has been shown to result in injury to multi-organ sites such as liver, lung, kidney, and testes (Manca *et al.*, 1991). On the other hand, medicinal plants are proving useful in ameliorating the toxic effects of chemicals.

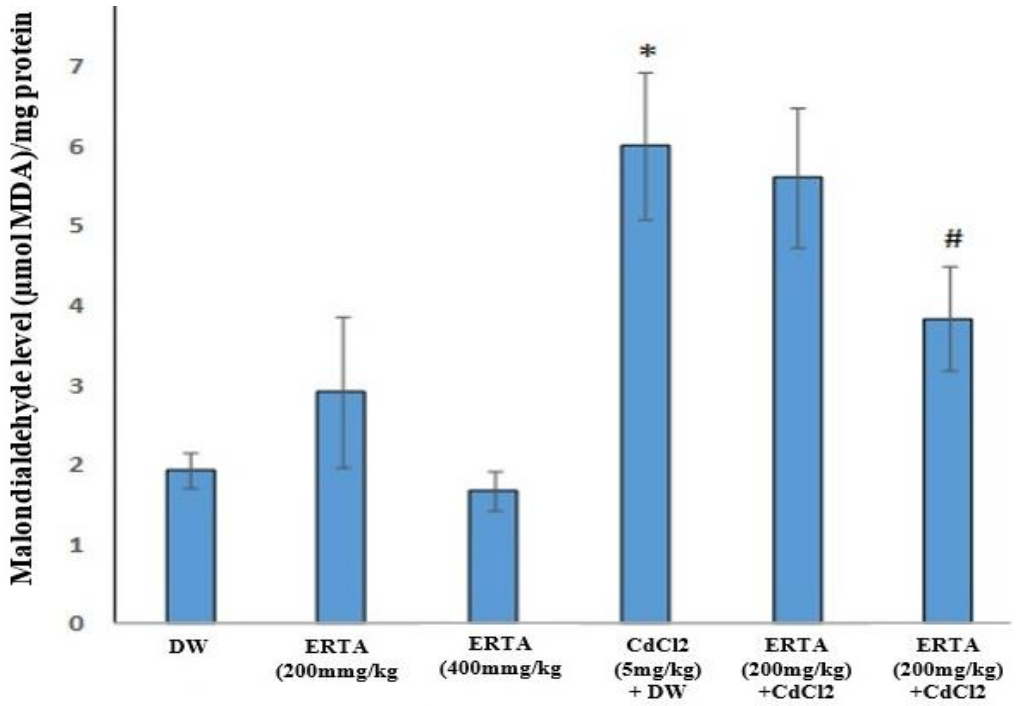

Figure 4: Effects of $CdCl_2$ and/or ERTA on hepatic lipid peroxidation in the treated rats
Values are means ± SD (n =5); group 1 represents negative control given distilled water; group 2 rats were given 200 mg/kg body weight of ERTA; group 3 rats were treated with 400 mg/kg body weight of ERTA; group 4 rats were given 5 mg/kg body weight of $CdCl_2$ in distilled water; group 5 rats received 200 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$; group 6 were administered 400 mg/kg body weight of ERTA plus 5 mg/kg body weight of $CdCl_2$. * = significantly different ($p < 0.05$) from control group 1. # = significantly different ($p < 0.05$) from group 4

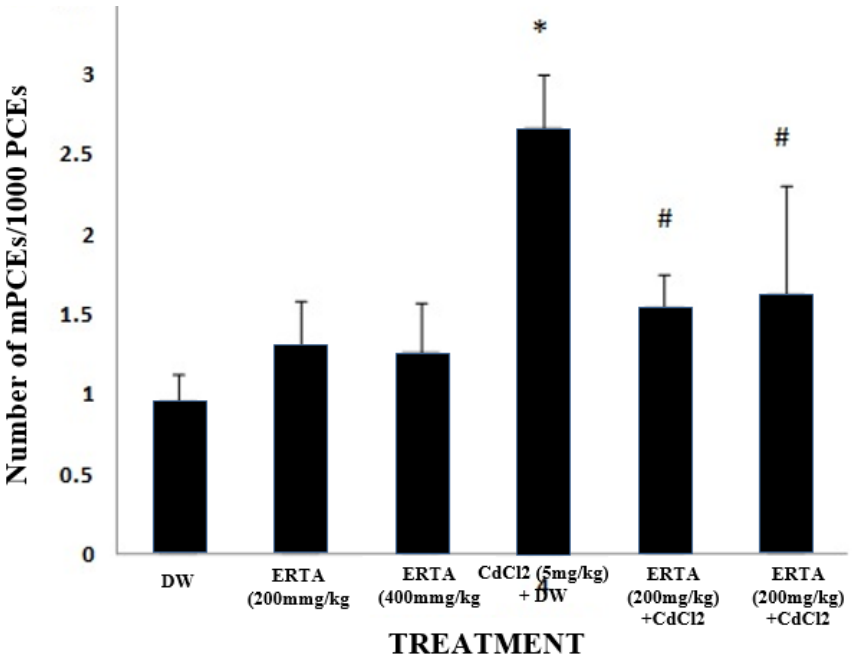

Figure 5:
Effects of ERTA on $CdCl_2$–induced clastogenicity measured as relative number of micronucleated polychromatic erythrocytes (mPCEs) in the rat bone marrow cells. Values are means ± SD (n =5)

The increasing usefulness of medicinal plants as sources of many potent drugs are well documented (Sofowora, 1986; Iwu *et al.*, 1999). Evidence abounds in the African and Asian countries where traditional medicine practitioners use a variety of herbal preparations to treat different kinds of diseases (Sofowora, 1993). *Terminalia avicennioides*is one of the medicinal plants commonly used to treat diverse ailments especially in Nigeria (Atawodi *et al.*, 2003). Extract of the plant roots or stem bark has been applied as medicines for general healing, while the roots have been used in the treatment of diarrhoea, dysentery, dropsy, swellings, oedema, gout, leprosy, and as pain-killers. In the present study, we investigated the effect of ethanol root extract of *Terminalia avicennioides*on $CdCl_2$-induced hepatotoxicity and clastogenicity in male Wistar rats.

Our findings show that $CdCl_2$ significantly ($p<0.05$) reduced the body weight of rats as compared to the control group thereby confirming the earlier reports that cadmium intoxication has negative effects on body weight (Sakr and Nooh, 2013; Ajilore *et al.*, 2012). The observed negative effect on the weight increase of the rats may be due to inflammation and fibrosis of major organs by cadmium (Sadik, 2008).

One of the major targets of chemical toxicity is the liver because of its role in the detoxification of chemical substances (Watkins and Seef, 2006). Therefore, the serum level of liver function marker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) is largely employed to assess liver function and integrity. Loss of functional integrity of hepatocellular membrane and the attendant increase in membrane permeability leads to cellular leakage of these enzymes and consequent elevated levels in systemic circulation. The estimation of the enzymes in the serum is therefore a useful quantitative indicator of the extent and type of hepatocellular damage (Lum and Gambino, 1972; Ideo *et al*., 1972).

In the present study, $CdCl_2$ significantly ($p<0.05$) increased the serum AST, ALT and ALP activities as compared with negative control, whereas the treatment of rats with ERTA and $CdCl_2$ led to significantly reduced ($p<0.05$) activities of the three enzymes above in comparison to the group treated with $CdCl_2$ alone. This is an indication of the ameliorative effect of ERTA in $CdCl_2$-induced hepatotoxicity. This observation may be due to membrane-stabilizing potential of ERTA as also validated by the potent wound-healing activity of ethanol extract of *Terminalia avicennioides*root bark reported by Mann *et al.* (2011). Treatment with ERTA alone induced a dose-dependent increase in the mean ALP values at levels that are significantly ($p<0.05$) different from the control. This is an indication of a possible toxicity of the extract at the tested doses. However, ALP is not as sensitive and specific as AST and ALT as indicator of hepatocellular damage (Hess, 1958).

Histopathological examination of liver sections from the control and treated animals also revealed different level of lesions from no visible in the negative control group and group exposed to only ERTA at 200 mg/kg body weight, to mild lesions in the group given 400 mg/kg body weight ERTA alone. There are more severe liver lesions in group treated with cadmium chloride alone while ERTA drastically reduced the liver lesion severity produced by cadmium. This is consistent with an earlier report in which histological analyses proved that exposure to cadmium could cause liver damage (Ruangyuttikarn *et al.,* 2013; Rikans and Yamano, 2000; Habeebu *et al*., 1998). The reduction in severity of cadmium-induced lesions by ERTA confirms a protective effect of ERTA against $CdCl_2$ hepatotoxicity.

It is well documented that the induction of lipid peroxidation is one of the means by which cadmium induces toxicity in vital organs such as the brain, liver, kidney and testes (Shaikh *et al.,* 1999). Findings in the present study show that ERTA at higher concentrations significantly ($p<0.05$) ameliorated the degree of lipid peroxidation induced by $CdCl_2$. In addition, using the micronucleus assay, one of the preferred and reliably methods for assessing chromosomal damage, measured as chromosome loss and breakage (Fenech, 2000), we found an approximately 3-fold increase in the frequency of induction of micronuclei in the bone marrow cells by $CdCl_2$ as compared with the negative control. This observation is in agreement with earlier reports by Kowalczyk *et al.,* (2002). ERTA at the tested doses did not induced the significant ($p<0.05$) micronuclei formation compared with the negative control group. Co-treatment of rats with ERTA and $CdCl_2$ led to significant ($p<0.05$) decrease in the number of mPCEs scored compared with the group given $CdCl_2$ only. This suggests that ERTA has anticlastogenic activity against $CdCl_2$ induction of clastogenicity in rats.

In conclusion, findings in this study show that ERTA, at the tested doses, displayed both anticlastogenic and hepatoprotective properties against cadmium-induced toxicities in the male Wistar rats. Further study will help to clarify the possibility of the extract toxicity at high doses.

Disclosure of Conflicts of Interest

No conflicts of interest were disclosed by the authors.

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