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Original Research Article

Crassocephalum rubens mitigates hepatic damage in 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary gland toxicity in rats

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ABSTRACT

Crassocephalum rubens has several medicinal properties, making it effective against several ailments, including its local use for treating liver problems and breast cancer. The effect of crude extract of *Crassocephalum rubens* (CECR) against liver damage in 7, 12 - dimethylbenz[a]anthracene (DMBA)-induced mammary gland toxicity was investigated in female Wistar rats. Rats were divided into group of 5 (n = 8). Group 1 served as control. Animals in groups 2 to 4 were administered a single intraperitoneal dose of DMBA at 20 mg/kg. Groups 3 and 4 rats were thereafter treated with 250 mg/kg and 500 mg/kg b.w CECR, respectively, for 12 weeks. Rats in group 5 were given 500 mg/kg b.w. CECR only for 12 weeks. Effect of CECR on DMBA-induced anomalies was investigated by using various biochemical parameters, including liver marker enzymes, oxidative stress, and tissue histology and cell count. CECR ameliorated the hepatic damage accompanied with DMBA-induced mammary gland toxicity by mitigating the abnormal changes noted in the levels of the parameters investigated. This could be proved by a significant ($p<0.05$) reduction in the levels of serum transaminases, dehydrogenase (LDH), gamma-glutamyl transferase (GGT), lactate malondialdehyde (MDA) and estrogen, and a significant ($p<0.05$) increase in the levels of superoxide dismutase (SOD) and liver cell count when compared with DMBA-intoxicated rats. The CECR, mostly at 500 mg/kg. b.w. dose, could serve as an alternative therapy against liver inflammation and oxidative stress associated with DMBA-induced mammary gland toxicity.

Keywords: 7, 12-dimethylbenz[a]anthracene, *Crassocephalum rubens*, hepatic damage, mammary gland, toxicity

Introduction

Medicinal plants have been identified and screened over centuries to uncover their various actions against several human diseases, and numerous compounds have been isolated from these plants for the development of pharmaceutical agents in managing or treating ailments.[1, 2] Various studies have shown the chemopreventive activities of plants and plant products against several diseases by using in vivo and in vitro models among others. *Crassocephalum rubens* is believed to have several medicinal properties that could be linked to the presence of secondary active compounds including alkaloids, anthraquinones, anthrocyanidins, flavonoids, coumarins, mucilage, polyphenols, proanthocyanidin, saponins and steroids.

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Its traditional use for stomach inflammation, liver dysfunction, breast cancer, burns, ear and ocular aches, and leprosy treatment has been reported. [3] Although, *C. rubens* has reportedly been active against breast and colorectal cancers,[3, 4] its action against possible hepatic damage associated with breast carcinogenesis should be considered. This is necessary because liver is a major metastatic target in breast cancer, which can result in acute liver injury or acute liver failure.[5, 6] Polycyclic aromatic hydrocarbons, most especially 7, 12-dimethylbenz(a)anthracene (DMBA) have been studied for their ability to induce breast cancer in experimental animals. Cytochrome P450 system of the liver is responsible for DMBA biotransformation, and this results in generation of diol epoxides and reactive oxygen species (ROS). During metabolic activation of DMBA in the liver, ROS and DMBA-DNA adducts formed react with biomolecules such as lipids and proteins, thereby inducing hepatic damage.[7] In addition, hepatotoxic effect of DMBA has also been reported.[8] Acute liver failure, resulting from metastatic breast carcinoma, has been reported to cause rapid and increased mortality.[5] This is because liver is the central organ that performs important functions such as biotransformation, metabolism, storage, and excretion. This study was designed to investigate the ameliorative effect of crude extract of *Crassocephalum rubens* (CECR) leaf against liver damage associated with DMBA-induced breast carcinogenesis in Wistar rats.

Materials and methods

Chemicals

Trichloroacetic acid, thiobarbituric acid (TBA) and DMBA were sourced from Sigma Aldrich, St. Louis, MO, USA. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH) kits were obtained from Randox Laboratories Ltd (Ardmore, Crumlin, Co- Antrim, UK). All the reagents used were of analytical grade.

Plant extraction

Leaves of *Crassocephalum rubens* were obtained from a farmland in Ora-Igbomina, Osun State, Nigeria in October 2022, authenticated (with voucher number: FHI 112047 at Forestry Research Institute of Nigeria, Ibadan), and extract was prepared as described in our previous study.[9]

Animals

Forty (40) female Wistar rats, weighing 40–60g (5-week-old), were sourced from Department of Veterinary Medicine (Central Animal house), University of Ibadan, Nigeria. The rats were housed in plastic cages bedded with food shavings and maintained at 25 ± 3°C. Rats were fed on standard animal feed and water *ad libitum*. Animal handling procedures were carried out following the guidelines of international ethics. The approved procedures by the College of Sciences Animal Research Ethics Committee, Afe Babalola University, Ado-Ekiti, Nigeria (approval number – ABUAD/SCI19/020) with the guidelines were strictly followed.

Induction of tissue toxicity and treatment

Rats were randomly grouped into five (n = 8), and animals were acclimatized for 2 weeks. Group 1 served as the control. Group 2 animals received DMBA (20 mg/kg) only, and groups 3 and 4 rats received DMBA (20 mg/kg) followed by CECR (250 and 500 mg/kg body weight, respectively), while group 5 rats received 500 mg/kg CECR only. Rats were administered a single intraperitoneal dose of DMBA at the onset of the experiment. Various treatments began after 24 h of DMBA exposure. CECR was administered orally 3 times per week for 12 weeks. Twenty-four (24) hours after the treatment, the overnight-fasted rats were sacrificed by mild exposure to diethyl ether.

Measurement of body weights

Rats were weighed weekly and the weight gain (%) was calculated using equation 1 below:

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad \text{(Equation 1)}$$

Preparation of serum

Blood was collected from animals, after loss of sensory, via cardiac puncture, into plain tubes for 1 h to clot. Blood was centrifuged (3000 x *g* for 10 min in a Beckman bench centrifuge) to obtain serum. This was stored at 4 ºC and used for various biochemical assays.

Preparation of tissues

Rats' liver was harvested, washed in ice-cold physiological saline solution to remove blood, and excess solution was mopped with tissue paper and weighed. A part of each liver was fixed in formalin (10%) for histology, while 1 g of the other part was homogenized in 0.05 M phosphate buffer (pH 7.4, 4 mL) and centrifuged (10,000 x *g*, 15 min). The resulting supernatant was used for biochemical assays.

Biochemical analyses

Liver's functional integrity was determined by assessing the levels of serum ALT AST, GGT and LDH. Serum ALT and AST was determined as described by Reitman and Frankel [10] Serum γ-GT and LDH activities were determined following the method described by Szasz [11] and Weisshaar *et al.* [12], respectively. SOD activity was assessed based on the method of McCord and Fridovich [13] and the level of malondialdehyde (MDA), a marker of lipid peroxidation, was determined by the method of Buege and Aust [14], while serum estrogen level was estimated following the protocol of estrogen Elisa kit (BioVision, Waltham, MA USA).

Histopathological assessment

Fixed liver sections were dehydrated in graded alcohol, cleared in xylene, and were embedded in paraffin. Obtained sections were mounted on microscopic glass slides and stained with hematoxylin and eosin (H&E) and were viewed under light microscope by a histopathologist.

Quantitative liver cell count

For stereological assessment of the hepatocytes, 10 sagittal sections of the liver were analyzed serially. Images of liver cells were captured by an OPTO-Edu industrial camera light microscope with a computer. Liver sections (5 μm width) were captured and processed using an image-processor and analyzed using software Image-J (Version 1.52). The mean of total hepatocytes per section was determined at every 10-section counting.

Statistical analysis

Data was analyzed by GraphPad Prism software (version 5.0) for Windows using one-way analysis of variance (ANOVA). Values were presented as Mean ± (standard deviation) SD, and intergroup comparison were done using Turkey's test. In each instance, values of $p < 0.05$ were reported to be statistically significant.

Results and Discussion

In developing countries, about 80% of the population depends on the use of plant-based medicine in managing or treating various kinds of ailments.[15] This is because many medicinal plants possess bioactive compounds which contribute to their several biological activities, thereby functioning as potential therapeutic agents for several diseases, which include malaria, atherosclerosis, cancer, diabetes, helminthic and inflammation.[16-19]

Polycyclic aromatic hydrocarbons, such as DMBA, can induce breast cancer in experimental rats. This potent carcinogen (DMBA) plays a significant role in cellular damage as DMBA-3,4-dihydrodiol-1,2-epoxide (DMBA-DE) (its reactive intermediate) adds adenine and guanine residues to DNA.[20, 21] DMBA-induced mammary gland cancer in rats is a standard animal model in preclinical studies during drug development for breast cancer.[22] In addition, DMBA damages many other organs in the body, which include the liver (primary site of metabolism), by initiating ROS production, formation of DNA-adduct, thereby having significant effect on the activities of several liver enzymes, including the antioxidant and serum enzymes.[23]

Effect of CECR on body weight gain and relative liver weight of DMBA-treated rats is shown in figures 1 a and b, respectively. Treatment of rats with DMBA caused a significant ($p < 0.05$) decrease in the body weight gain and significant ($p < 0.05$) increase in relative liver weight when compared with control. Treatment of DMBA-intoxicated rats with 500 mg/kg b.w. CECR caused a significant ($p < 0.05$) increase in the body weight gain and a significant decrease in relative liver weight of rats compared to rats treated with DMBA only. However, there was no significant difference in body weight gain and relative liver weight in DMBA rats treated with 250 mg/kg CECR compared to rats treated with DMBA only. Also, rats treated with only 500 mg/kg CECR revealed no significant difference in body weight and relative liver weight gain when compared with control. The noticeable reduction in body weight gain and relative liver weight of DMBA-treated rats compared to the control is an indication of the toxic effect of DMBA on the liver. In contrast, treatment of DMBA-intoxicated rats with 500 mg/kg CECR attenuated reduction in body and relative liver weights, indicating the ameliorative effect of the plant. This is similar to a study by Avtandilyan *et al.* [20], where the weight of DMBA-

intoxicated rats treated with NG-hydroxy-nor-L-arginine (nor-NOHA) was almost similar to control and saline-administered rats.

Overproduction of ROS from DMBA could result in lipid peroxidation, thereby altering the functional and structural integrity of cell membrane,[24] eventually leading to release of cytoplasmic enzymes to the bloodstream.[8] These enzymes serve as biomarkers of hepatic damage, and any abnormal increase in the serum levels indicates damage to the cell and compromised functional integrity of membrane architecture of the liver.[25] Effect of CECR on serum ALT and AST in DMBA-intoxicated rats are shown in figures 2 a and b. A significant ($p < 0.05$) elevation in the levels of liver marker enzymes (ALT and AST) was noted in rats injected with DMBA only in comparison with the control. A significant ($p < 0.05$) reduction was noted in the ALT levels of DMBA rats treated with 500 mg/kg CECR (figure 2a) while a significant ($p < 0.05$) decrease in the levels of AST was noted when the rats were treated with CECR (250 or 500 mg/kg) (Figure 2b) in comparison with rats administered with DMBA only. In this study, elevated levels of serum transaminases in DMBA treated rats indicate hepatic damage, and treatment of these intoxicated rats with CECR, mostly at 500 mg/kg doses, suggests the ameliorative potential of the plant extract against the effect of DMBA on the liver.

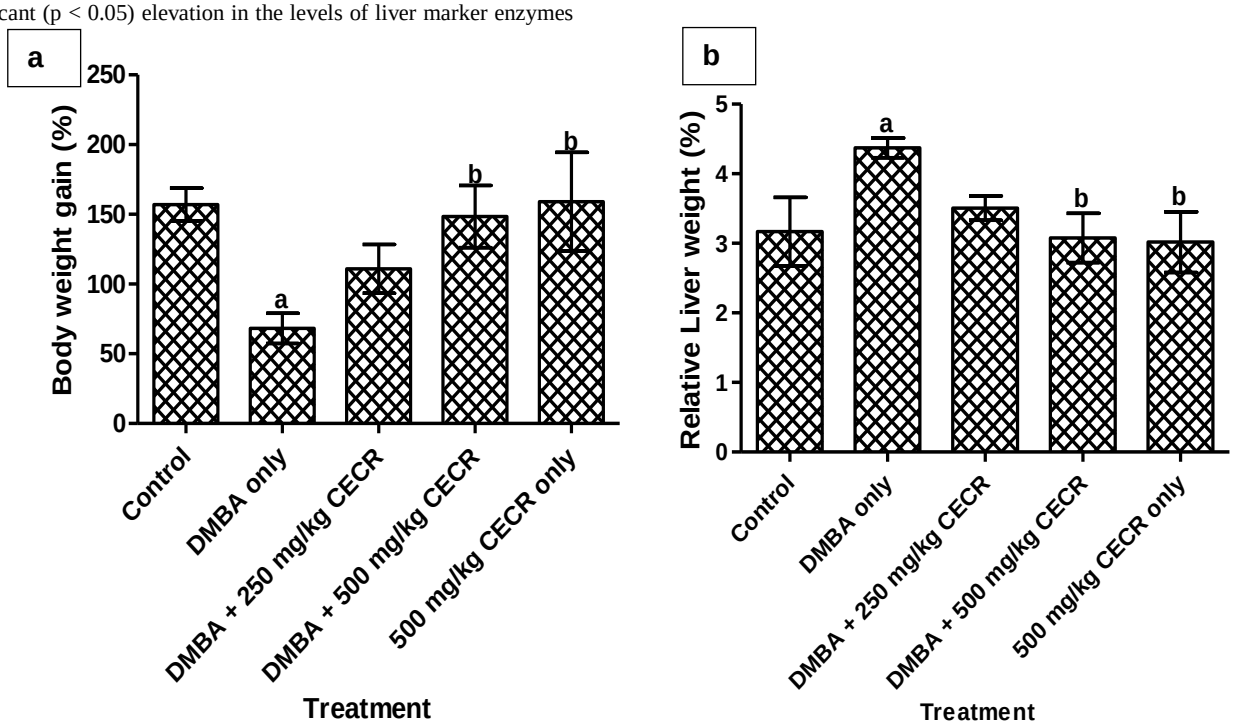

Figure 1: Effect of CECR on body weight (a) and relative liver weight (b) of rats treated with DMBA
Values are expressed as Mean ± SD (n=6). [a]$p < 0.05$ when compared to the control group; [b]$p < 0.05$ when compared to DMBA group.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

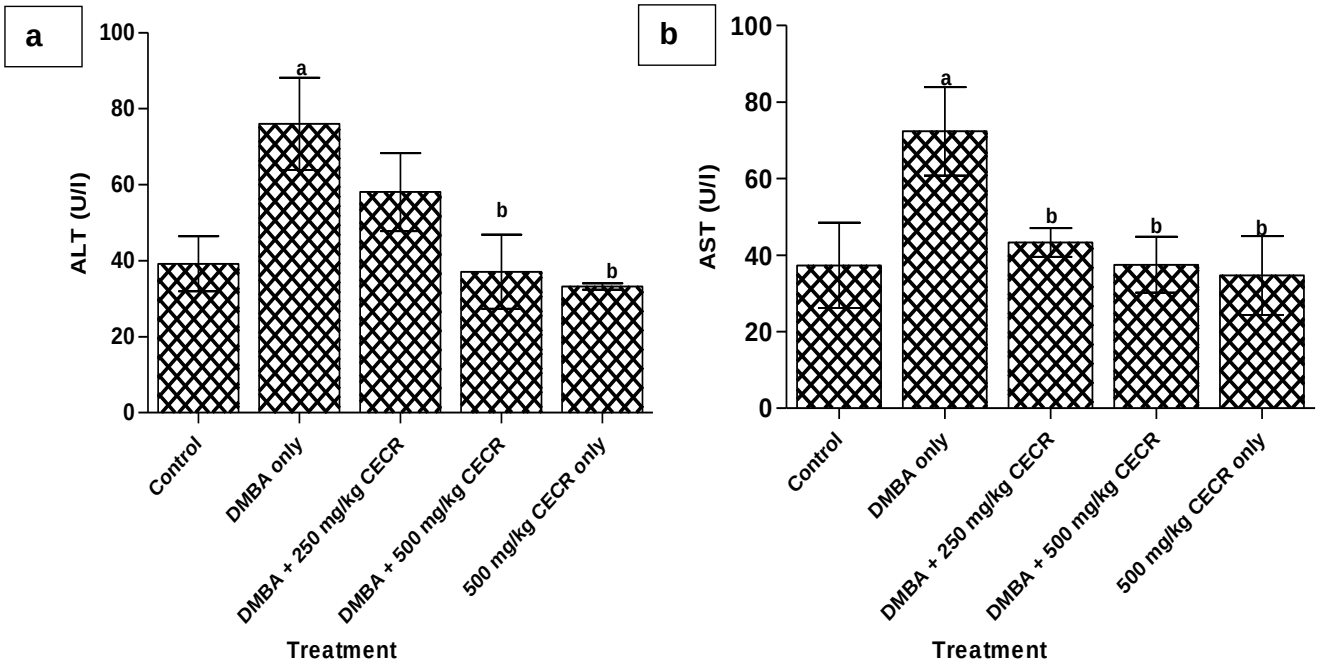

Figure 2: Effect of CECR on serum levels of alanine transaminase (a) and aspartate ransaminase (b) in rats treated with DMBA
Values are expressed as Mean ± SD (n=6). [a]$p < 0.05$ when compared to the control group; [b]$p < 0.05$ when compared to DMBA group.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

The effect of CECR on GGT and LDH in DMBA-intoxicated rats is presented in figures 3 a and b. Activities of serum GGT and LDH was significantly ($p < 0.05$) elevated in rats administered with DMBA only compared to the control. Treatment of DMBA intoxicated rats with 250 and 500 mg/kg b.w. CECR resulted in a significant ($p < 0.05$) reduction in the level of these enzymes when compared with the DMBA only. Increased level of GGT in DMBA treated rats indicates a possible damage to the liver and as well as possible presence of cancerous cells in the rats. Doses of CECR tested caused reduction in the levels of both GGT and LDH, therefore suggesting the potentiality of CECR against hepatic damage occasioned by DMBA. The efficacy of the CECR in the amelioration of abnormal changes in the activities of these enzymes is in agreement with a study by Wang and Zhang [26], where levels of these liver marker enzymes were reduced when compared with breast cancer rats induced with DMBA were treated with Honokiol. This observation is also similar to a study by Arora *et al.* [23], where Erucin, which was isolated from *Eruca sativa* extract, normalized the abnormal changes in the level of these enzymes in DMBA-induced hepatic dysfunction.

Several antioxidant enzymes present in human cells help prevent and combat the harmful effects of ROS, which emerges mostly during oxidative stress. Example of these antioxidants include catalase, glutathione peroxidase and SOD. Antioxidant enzymes, such as SOD, is reported as first line of defense against the hazardous effect of radicals of molecular oxygen. SOD is an intracellular enzyme involved in catalyzing the breakdown of superoxide radicals, and it is an important antioxidant defense enzyme against cellular oxidation. [27] The effect of CECR on activity of SOD in rats intoxicated with DMBA was demonstrated in Figure 4. The SOD level of rats injected with only DMBA was significantly ($p < 0.05$) reduced in comparison with the control. Treatment of the DMBA intoxicated rats with 500 mg/kg CECR resulted in a significant ($p < 0.05$) increase in SOD levels in comparison with DMBA-intoxicated rats, and there was a significant ($p < 0.05$) difference in the level of SOD in DMBA-intoxicated rats treated with CECR at 250 mg/kg in comparison with the control.

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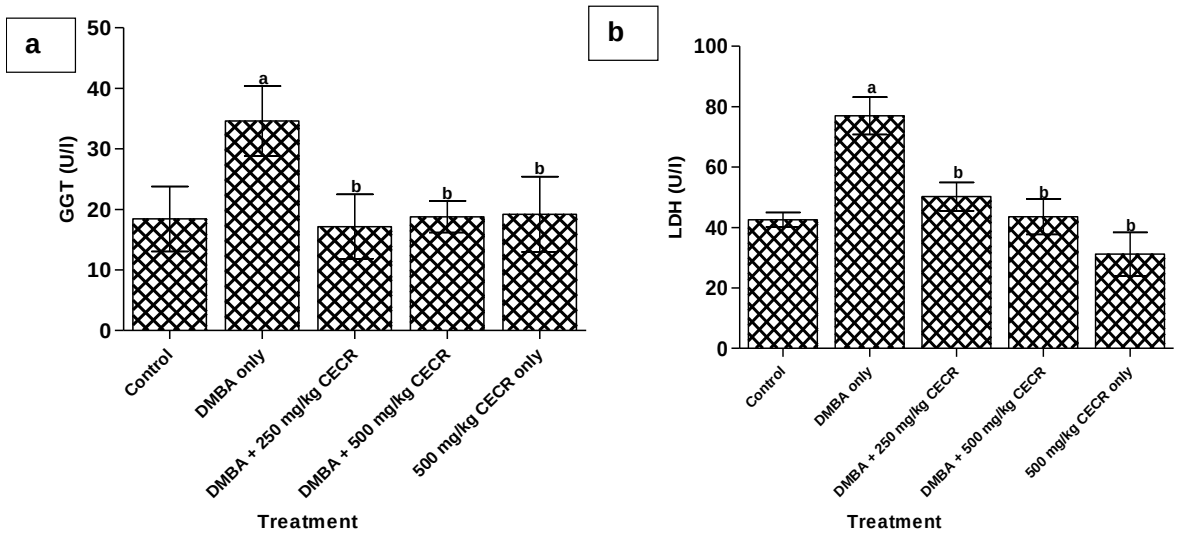

Figure 3: Effects of CECR on serum levels of (a) GGT and (b) LDH in rats treated with DMBA
Values are expressed as Mean ± SD (n=6). $^{a}p < 0.05$ when compared to the control; $^{b}p < 0.05$ when compared to DMBA only.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

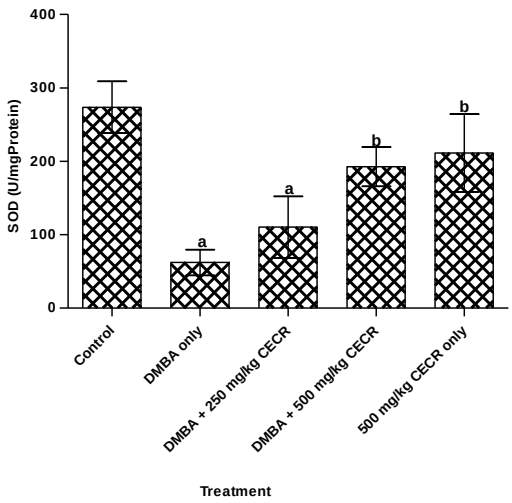

Figure 4: Effect of CECR on tissue SOD of rats treated with DMBA
Values are expressed as Mean ± SD (n=6). $^{a}p < 0.05$ when compared to the control; $^{b}p < 0.05$ when compared to DMBA only.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

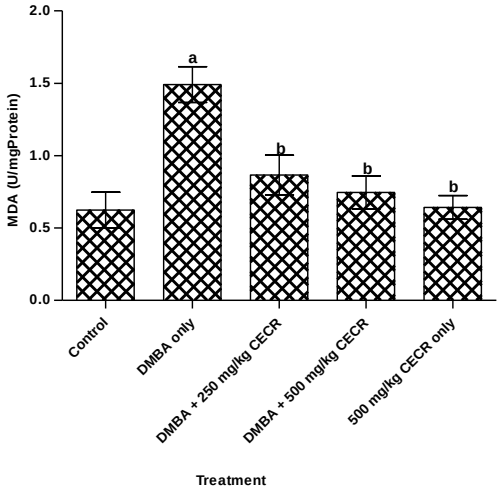

Figure 5: Effect of CECR on liver malondialdehyde level of rats treated with DMBA
Values are expressed as Mean ± SD (n=6). $^{a}p < 0.05$ when compared to the control; $^{b}p < 0.05$ when compared to DMBA only
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

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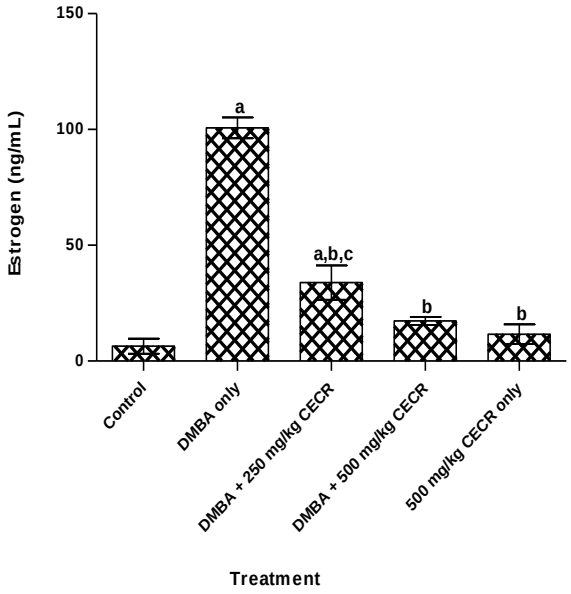

Figure 6: Effect of CECR on serum levels of estrogen in rats treated with DMBA
Values are expressed as Mean ± SD (n=6). [a]$p < 0.05$ when compared with the control; [b]$p < 0.05$ when compared with DMBA only, [c]$p < 0.05$ when compared with DMBA + 500 mg/kg CECR.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

Furthermore, intoxication of rats with DMBA resulted in a significant ($p < 0.05$) elevation in rat's liver MDA level compared to control (Figure 5). This increment was significantly ($p < 0.05$) reduced when treated with CECR (at both doses) in comparison with the DMBA untreated group. Treatment with 500mg/kg CECR showed no significant difference in MDA level in comparison with the control. In this case, it was noted that the 500 mg/kg CECR substantially reversed the significant reduction in the functional activities of SOD and noted lipid peroxidation. It can be suggested that CECR has the potential to restore the altered antioxidant level, and as well reduced the peroxidation of lipids in hepatic cells.

Figure 6 shows the effect of CECR on estrogen in DMBA-intoxicated rats. Exposure to DMBA has been reported to induce estrogen-dependent breast cancer in rats.[28] However, regular consumption of plant-based foods has been linked to reduction in the risk of breast cancer.[29] A significant ($p < 0.05$) rise in the level of estrogen was noted in rats intoxicated with DMBA compared to control. Treatment of DMBA intoxicated rats with CECR, at both tested doses, caused significant ($p < 0.05$) fall in the level of estrogen in comparison with rats exposed to DMBA only. A significant ($p < 0.05$) increase was also noted in estrogen level in DMBA rats treated with 250 mg/kg CECR when compared with the control, and significant ($p < 0.05$) difference was noted in this hormone in DMBA rats treated with 250 mg/kg

CECR compared to rats treated with 500 mg/kg CECR. No significant difference was, however, noted in estrogen level of rats administered with 500 mg/kg CECR only when compared with the control. Therefore, DMBA-induced breast cancer, as indicated by increased levels of this hormone, was ameliorated by CECR, mostly at 500 mg/kg.

These results were also supported by light microscopic analysis of liver histology as shown in Figure 7 (a-e). Histopathological investigation revealed normal arrangement of the hepatocytes and a normal portal vein in control group (Figure 7a) and rats treated with 500 mg/kg CECR only (Figure 7e). DMBA administration to rats resulted in vacuolar degeneration and shrunken portal area (Figure 7b). DMBA intoxicated rats treated with 250 mg/kg CECR resulted in dilated with congested portal vein (Figure 7c) while normal arrangement of the hepatocytes and normal portal vein were seen in DMBA rats treated with 500 mg/kg CECR (Figure 7d).

In the same vein, the hepatocytes count (Figure 8) showed significant ($p < 0.05$) difference in untreated DMBA exposed rats and DMBA-intoxicated rats treated with 250 mg/kg CECR in comparison with control, while a significant ($p < 0.05$) difference was noted in liver cell count in DMBA intoxicated rats treated with CECR (at both doses) compared to rats administered with DMBA only.

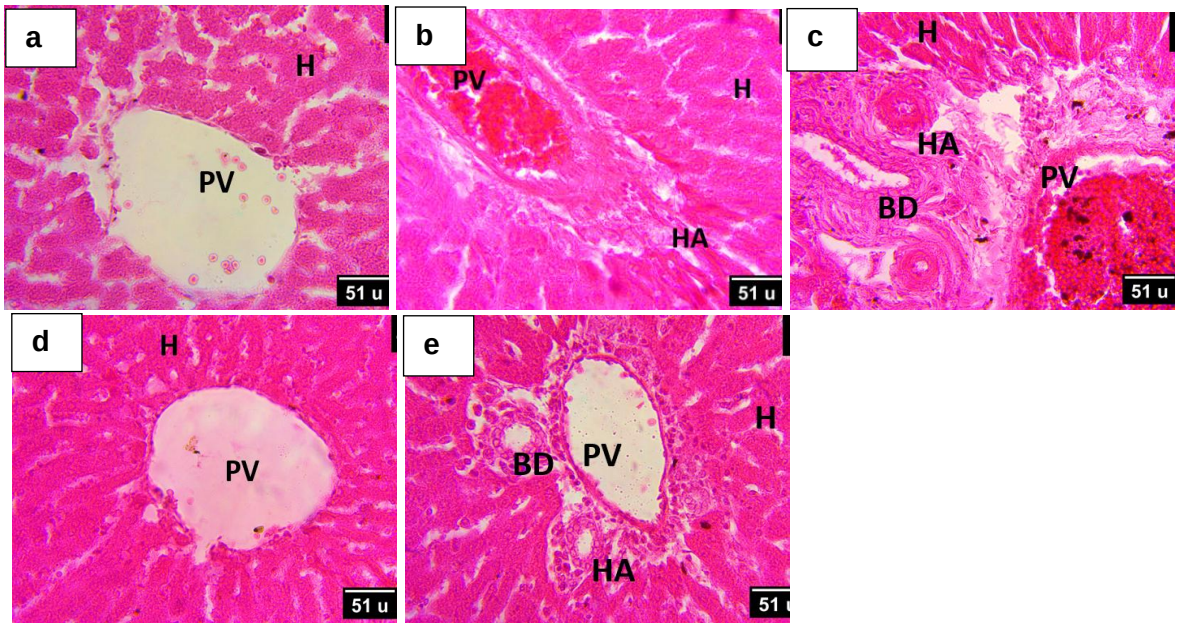

Figure 7: Histoarchitecture of the liver showing the portal triad and the hepatocytes. (a) Control (b) DMBA only (c) DMBA + 250 mg/kg CECR (c) DMBA + 500 mg/kg CECR (e) 500 mg/kg CECR only
Stained with H&E, (Scale bar; 51 µm). (H: Hepatocyte, PV: Portal vein: BD: Bile duct, HA: Hepatic artery).
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

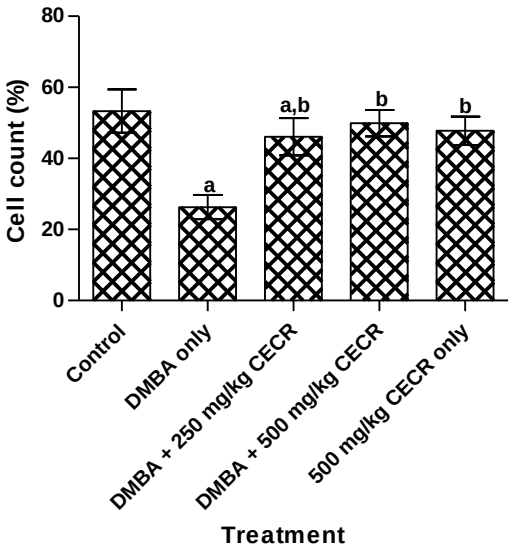

Figure 8: Liver cell count
Values are expressed as Mean ± SD (n=6). [a]$p < 0.05$ when compared to the control; [b]$p < 0.05$ when compared to DMBA only.
CECR – Crude extract of *Crassocephalum rubens*, DMBA – 7,12-dimethylbenz[a]anthracene

Conclusion

In this study, results from body weight increment, relative liver weight, ALT, SOD, as well as liver histology and hepatocyte count suggested that the 500 mg/kg CECR showed more efficacy in the treatment of liver damage associated with DMBA-induced mammary gland toxicity relative to 250 mg/kg b.w. CECR. It can therefore be concluded that CECR possesses compounds capable of ameliorating hepatic damage accompanied with DMBA-induced mammary gland carcinogenesis in female rats. Further study investigating the potential use of CECR against breast cancer and possible mechanisms of action is suggested.

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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