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Hepatoprotective Effect of the Methanolic Leaf Extract of *Annona senegalensis* Pers. against 7, 12-dimethylbenz[a]anthracene (DMBA)-Induced Toxicity in Wistar Rats

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ABSTRACT

The liver is critical in the metabolism of 7, 12-dimethylbenz[a]anthracene (DMBA), as a result, DMBA is a strong liver toxin and its hepatotoxicity stems from its conversion to an active carcinogenic metabolite. The study examined the hepatoprotective effect of *Annona senegalensis* in rats induced with DMBA. Ten rats each in eight groups were used. Animals in group A were used as controls; group B was given 35 mg/kg bwt of DMBA intraperitoneally twice monthly for two months; and animals in groups C, D, E and F were induced and treated with 50, 100 and 200 mg/kg bwt of *A. senegalensis* and 15 mg/kg bwt rutin, respectively. Furthermore, group G animals were pre-treated with 100 mg/kg bwt of extract daily for two weeks before induction, while group H was administered 200 mg/kg bwt of extract only. Treatments were administered orally for 14 days. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Malonaldehyde (MDA), creatinine and urea increased ($p< 0.05$), while reduced glutathione (GSH) level and superoxide dismutase (SOD) activity decreased ($p < 0.05$) in the induced-only group compared to the extract and rutin administered groups. Treatment with 100 and 200 mg/kg bwt *A. senegalensis* extract and rutin significantly ($p < 0.05$) reversed these effects. Histological results showed reduced vascular constrictions in the extract and rutin-treated groups. These results show that the extract of *A. senegalensis* was able to bring the antioxidant, liver and oxidative stress biomarkers close to normal while ameliorating hepatocellular lesions observed after DMBA induction.

Keywords: *Annona senegalensis*, Antioxidant, Liver damage, 7,12-dimethylbenz[a]anthracene

Introduction

Contaminants present in the environment include polycyclic aromatic hydrocarbons (PAHs), these PAHs are formed when organic goods and materials are incompletely burned[1]. They are mainly found in automobile exhaust, grilled foods, cigarette smoke, and woodsmoke.[2] 7, 12-dimethylbenz[a]anthracene (DMBA) is a type of PAH with immunosuppressive and carcinogenic effects on various species.[3] The liver is critical in the metabolism of DMBA. As a result, DMBA is a strong liver toxin and its hepatotoxicity stems from its conversion by a family of hepatocyte enzymes called the cytochrome P_{450} (CYP1) to an active carcinogenic metabolite[1]. The bioactivation of DMBA in the liver is the responsibility of the CYP1A enzyme isoforms, while the CYP1B enzymes mainly exert their activity on extra-hepatic tissues, such as the mammary gland[3] this is why DMBA is a known mammary carcinogen.[4,5] The bioactivation process of DMBA leads to free radical generation in tissues which further results in oxidative stress seen in experimental rats.[4]

Free radicals are generated in the course of regular metabolic processes in the body; they can combine to produce oxidative stress as a result of them not being properly eliminated.[6] These ROS can cause damage to tissues, lead to abnormal activation of enzyme and can also be toxic to living cells.

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Some examples of free radicals produced by oxygen include superoxide anion and hydroxyl radicals these of which can lead to oxidative stress which in turn gives rise to several health conditions such as liver damage, ageing, cancer and serious heart conditions.[7,8]

The fundamental roles played by the liver in several metabolic processes, makes it the most important organ in the body; it secretes bile, makes blood-thickening elements, and helps indetoxifying the system.[9] Although, hepatic illnesses (like hepatitis, jaundice, and cirrhosis) have as of late added to significant reasons for high morbidness and death, owing generally to the utilization of hepatotoxic drugs.[10] Other external free radical sources like air contamination, tobacco, pesticides and so forth, contribute to hepatic injury.[11-13]

Plants possess active ingredients that allow them to sustain human health and treat ailments, these compounds are referred to as phytonutrients.[14] Plant products that include many natural bioactive molecules known as phytochemicals that exhibit unique biological functions are known as phytonutrients. These phytonutrients may serve a significant role in maintaining good health by promoting optimal cellular function and combating illness. Phytonutrients are made up of two main compounds, namely the secondary (polysterols, polyphenols, alkaloids, organosulfur compounds and terpenoids) and primary (proteins, carbs and fats) metabolites.[15]*Annonasenegalensis* is a plant with known therapeutic properties, the ethanolic leaf extract of *A. senegalensis* is known to contain phytochemicals such as tannins, flavonoid, alkaloids, saponins and steroids.[16] *The ethanolic leaf* and stem bark contains a high percentage of flavonoids[10,16]andthese flavonoids have shown to be potential preventive and treatment methods in liver damage.[17] According to Omeke *et al*.[10] solvent extracts and isolates of *A. senegalensis* significantly improved the liver parameters in CCL_2-induced liver damage Wistar rats.[10] Thus, this study investigated the hepatoprotective effects of *A. senegalensis* extract in DMBA-induced Wistar rats.

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Materials and Methods

Chemicals
All compounds utilized, including 7, 12-dimethylbenz[a]anthracene (DMBA), were of analytical qualityand purchased from Sigma Chemical Company in St. Louis, Missouri, USA.

Plant collection
Annona senegalensis fresh leaves were acquired from a local farm in Ile-Ife, Osun State, Nigeria. Dr. Jacob O. Popoola, a botanist from Covenant University Ota's Department of Biological Sciences, performed the identification. The plant sample was submitted with the Forestry Research Institute of Nigeria in Ibadan, where it was assigned the voucher number FHI 112597.

Preparation of plant extract
Mashed air dried leaves of *A. senegalensis* (1.4kg) were extracted by maceration for 72 h with 11.3 L of 95% methanol and concentrated with the aid of a rotary evaporator at 50°c which produced a methanol extract and the yield was recorded.[18]

Experimental animals
Female Wistar rats (80) weighing 0.105-0.11 kg were obtained from the University of Lagos Teaching Hospital in Lagos, Nigeria. The animals were housed in large, properly ventilated, polypropylene cages and during the experimental period they had a 12 h light/dark period. Their cages were cleaned, and animal were fed with rodent pelleted diet and water *ad libitum*. Guidelines for laboratory animals' usage and care were adopted and approved by the Covenant Health Research Ethics Committee (CHREC/38/2020).

Experimental design
The animals were divided into eight groups at random; Grp A (normal control) received olive oil, grp B (induced-only) was induced with 35 mg/kg bwt DMBA twice a month for two months intraperitoneally, according to Arora *et al*.[19] with slight modifications, while group C, D, E and F were induced and administered 50, 100 and 200 mg/kg bwt *A. senegalensis* extract and 15 mg/kg of rutin respectively; group F was pre-treated with *A. senegalensis* extract (100 mg/kg) followed by DMBA induction, and group G received *A. senegalensis* extract only (200 mg/kg). Pre- *A. senegalensis*, post- *A. senegalensis*, and rutin treatment were administered orally every day for two weeks.

Collection of blood sample and preparation
Animals were anesthetized using diethyl ether after the experimental trial, and blood was drawn from the heart into sample bottles of plain and ethylenediaminetetra acetic acid (EDTA). The serum used in the biochemical assays, was obtained by centrifuging the blood samples in the plain bottles at 2500 rpm for 15 mins, while haematological evaluation was carried out using whole blood obtained from the EDTA bottles. Liver and kidney tissues were cut, weighed then homogenized using potassium chloride phosphate buffer [10 mM] with EDTA at pH 7.4 and, after that, centrifuged for 10 min at 12,000 rpm. Liver and kidney homogenate was used to assess the antioxidant parameters, while liver tissues excised were used for histopathological analysis.

Biochemical and haematological assay
For all the biochemical parameters assessed, Randox Ltd UKtest kits were utilized. Standard methods in estimating alanine aminotransferase (ALT),[20] aspartate aminotransferase (AST),[21] alkaline phosphatase (ALP),[22] urea,[23] creatinine,[24] and total protein (TP)[25] were carried out as per manufacturer's instructions. An automated system analyzer was used to evaluate haematological parameters.[26]

Measurement of GSH, SOD and MDA in kidney and liver homogenate
Superoxide dismutase (SOD) activity was calculated by measuring 50 % per minute of the amount of SOD needed to inhibit pyrogallol autoxidation,[27] while reduced glutathione (GSH) was determined following the method of Prins and Loose.[28]Malonaldehyde (MDA) level was estimated as described by Ohkawa *et al*.[29]

Histopathological analysis
The excised liver tissues were kept in 10% normal saline for 72 hours before being sliced to 2.1 mm thickness. These were dehydrated in various concentrations of alcohol and tissue sections were cut to 5 um using a microtome. These sections were coated with paraffin wax and formed into blocks, then attached to slides and air dried. These slides were then stained with hematoxylin and eosin and viewed under a photographic microscope to check for any liver damage.[30]

Statistical analysis
The IBM statistical program for social sciences (SPSS) version 21.0 was used for statistical analysis (SPSS Inc., Chicago, IL, USA). ANOVA was used to evaluate the data and p values less than 0.05 were considered significant. The mean ± standard error of the mean was used to illustrate the results.

Results and Discussion

7, 12-Dimethylbenz[a]anthracene has an immunosuppressive and carcinogenic effects on various species.[3] In most liver injury cases AST, ALP and ALT present in the liver leaks into the blood circulation causing a rise in their levels, which may indicate damage to the liver.[31] DMBA induction increased serum AST, ALP and ALT suggesting liver damage[32] (Figure 1a-1c), this is corroborated by the histology results (Plate 1), where hepatocellular lesion marked by liver infiltration of inflammatory cells (lymphocytes) were found in the induced-only; this is in line with the previous analysis[10] on the hepatocellular damaging effect of DMBA induced rats. Treatment with *A. senegalensis* (100 and 200 mg/kg bwt) and rutin decreased the level of serum AST, ALP and ALT when compared to the induced-only group. However, the pre-treatment with *A. senegalensis* for 14 days before DMBA induction prevented elevated liver function biomarkers indicating the maintenance of the tissue cellular membranes functional integrity. Such improvement in plasma enzyme activity may result from the *A. senegalensis* antioxidant properties[15] and the ability of these antioxidants to mop up free radicals thus protecting the integrity of the cell membrane from oxidative damage by DMBA epoxide.
An increase in serum urea and creatinine concentration by DMBA suggests an increase in the catabolism of protein;[33] furthermore, the production of ROS by DMBA epoxide could also be toxic to the kidney, which could deplete the kidney's functioning. Rutin, pre and post administration of *A. senegalensis* for 14 days decreased the levels of urea and creatinine (Figure 2a & 2b), suggesting the treatments may have the therapeutic ability. Oxidative damage to proteins with receptive amino acids is the primary cause of metabolic conditions in disease development.[34] Previous results have also shown the total protein assessment to be a useful index in detecting severe hepatocellular dysfunction.[35] DMBA induction decreased the serum total protein content, as seen by a slight reduction in the induced-only group (Figure 2c) this result is in coordination with the result obtained by El Kholy *et al*.[35]and maybe due to hepatic inflammation and oxidative damage or change in protein synthesis and metabolism as well as increased protein oxidation which may indicate a disturbance in the liver function. Furthermore, administration of *A. senegalensis* (200 mg/kg bwt) increased the level of TP this may be attributed to *A. senegalensis* free radical scavenging ability.
Blood can serve as a pathological marker to assess animals status exposed to a toxicant and other conditions.[36] In the induced-only group, the level of packed cell volume (PCV) was reduced compared to the normal control (Table 1), this could lead to a decrease in oxygen delivered to the tissues and the amount of carbon dioxide reaching the lungs, subsequently leading to anemia.[37] However, there was an increase of the PCV in the groups treated with *A. senegalensis*, which suggests improved oxygen and nutrient transportation in these groups. Platelets (thrombocytes) are components of the blood responsible for blood clotting.[38] The platelet level was reduced in the induced-only group, suggesting that blood clotting time may be extended, leading to increased blood loss in injury cases. Pre- *A. senegalensis* administration increased the platelet level in this group. Furthermore, the platelet level was also increased in the post- *A. senegalensis* treated groups (100 and 200 mg/kg) compared to the induced-only group, although not significant.

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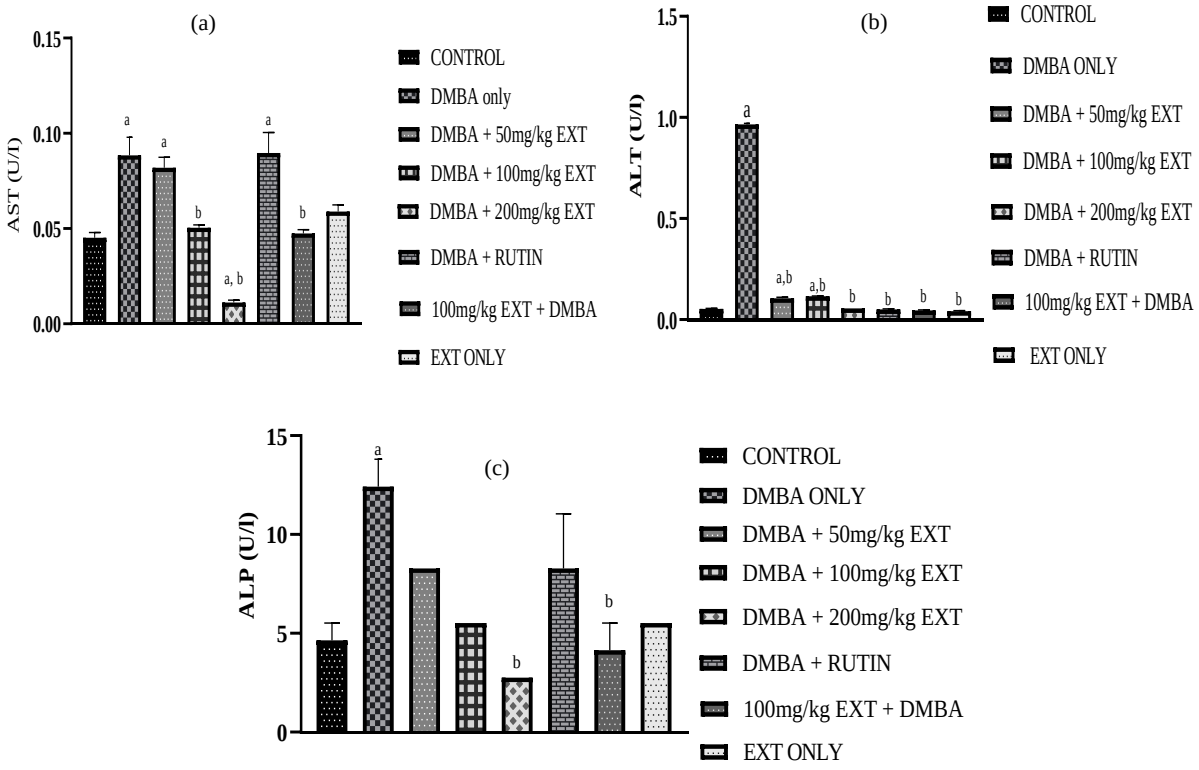

Figure 1: Effect of *A. senegalensis* on (a) AST, (b) ALT and (c) ALP levels. The values are presented as mean ± SEM (n = 6). ($p < 0.05$); [a]Significantly different from the normal control; [b]Significantly different as compared with negative control

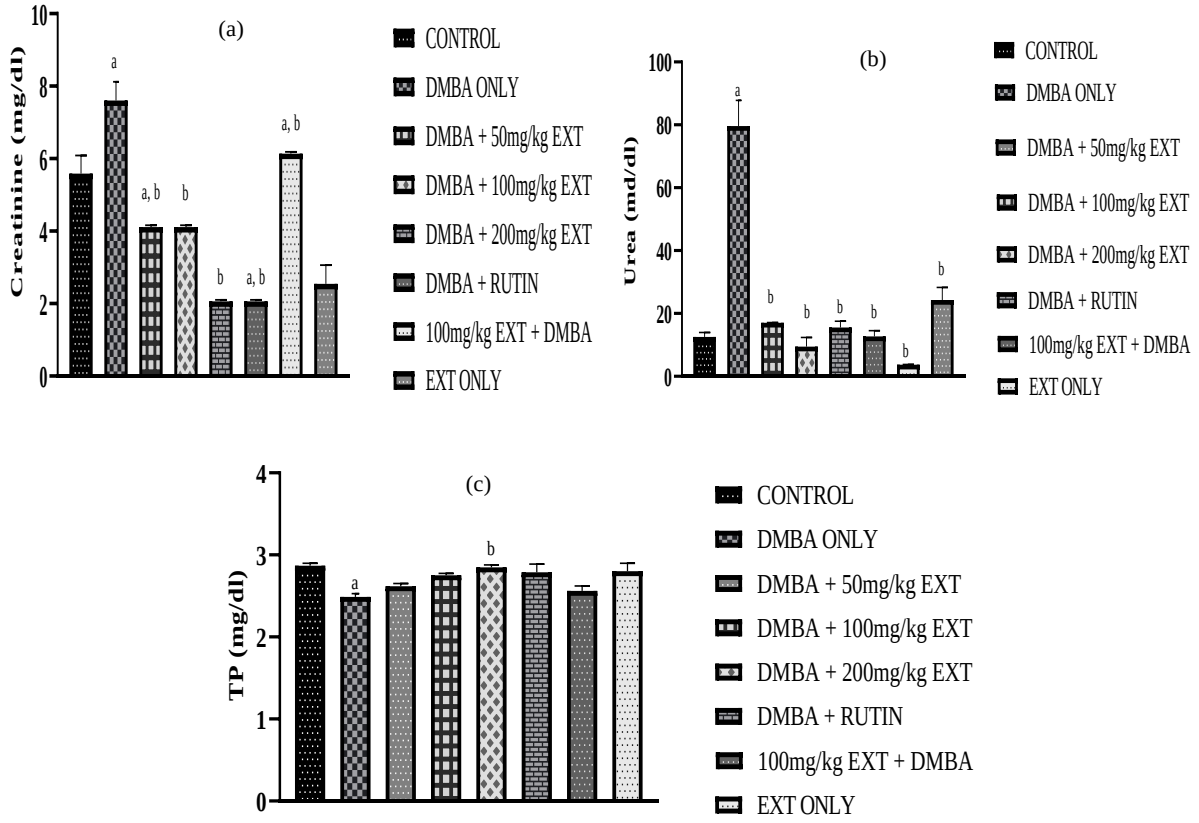

Figure 2: Effect of *A. senegalensis* on (a) creatinine, (b) urea and (c) TP levels. The values are presented as mean ± SEM (n = 6). ($p < 0.05$); [a]Significantly different from the normal control; [b]Significantly different as compared with negative control

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Table 1: Effects of *A. senegalensis* on the haematological parameters

Parameters	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H
PCV (%)	54.33 ± 0.67	37.33 ± 1.45[a]	47.33 ± 1.45	48.67 ± 1.86[b]	51.00 ± 0.58[b]	44.00 ± 2.08	51.67 ± 4.55[b]	42.33 ± 3.18[a]
Platelets ($cmm \times 10^4$)	45.00 ± 1.73	25.33 ± 0.88[a]	26.00 ± 0.577[a]	33.33 ± 2.404	33.67 ± 9.684	51.67 ± 0.88[b]	28.67 ± 3.28	42.67 ± 1.20
WBC (Cmm)	4.70 ± 0.72	4.78 ± 0.55	4.47 ± 0.62	5.33 ± 0.94	5.08 ± 0.67	4.48 ± 0.41	5.47 ± 0.18	5.80 ± 0.35
Neutrophils (%)	49.33 ± 1.33	50.00 ± 7.21	45.00 ± 2.00	40.00 ± 6.11	50.67 ± 2.19	37.00 ± 4.61	53.67 ± 2.91	50.33 ± 2.33
Lymphocytes (%)	25.00 ± 4.04	31.65 ± 3.93	27.67 ± 2.40	33.00 ± 0.58	22.00 ± 5.13	56.33 ± 4.49[ab]	40.33 ± 1.76	23.67 ± 2.60
Basophils (%)	0.33 ± 0.33	0.67 ± 0.33	1.00 ± 0.00	1.67 ± 0.67	2.00 ± 0.58	1.67 ± 0.33	1.00 ± 0.00	1.00 ± 0.58
Eosinophils (%)	24.00 ± 4.00	17.00 ± 3.90	25.00 ± 2.10	23.00 ± 5.60	24.00 ± 4.10	4.30 ± 0.67[ab]	3.70 ± 0.33[a]	21.00 ± 3.40
Monocytes (%)	0.33 ± 0.33	1.00 ± 0.58	1.67 ± 0.44	2.67 ± 0.88	2.33 ± 0.33	1.67 ± 0.33	1.00 ± 0.00	2.67 ± 0.33

The values are presented as mean ± SEM (n = 6). ($p < 0.05$); [a]Significantly different from the normal control; [b]Significantly different as compared with negative control

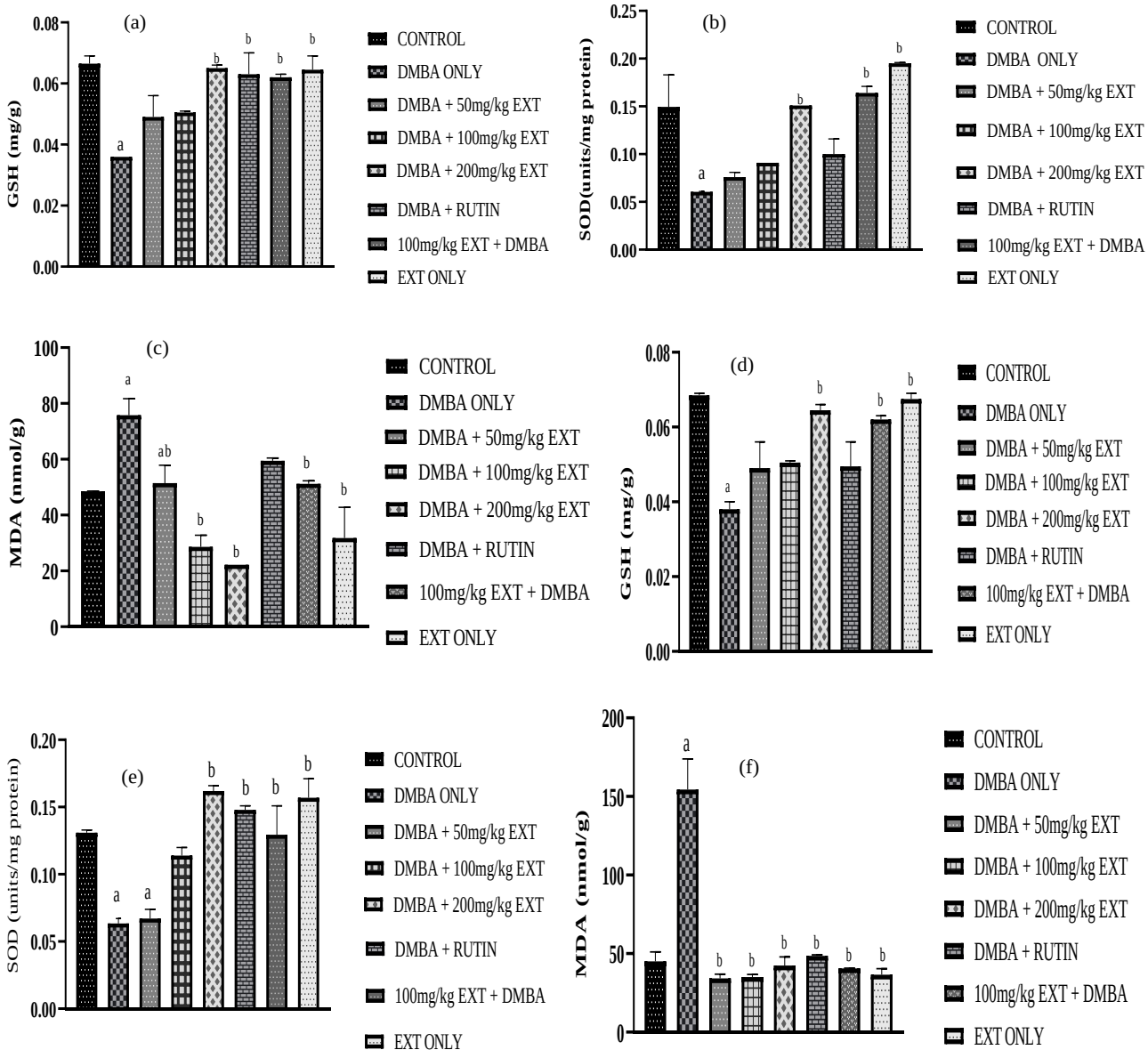

Figure 3: Effect of *A. senegalensis* on the level of kidney (a) GSH, (b) SOD, (c) MDA and liver (d) GSH, (e) SOD, (f) MDA. The values are presented as mean ± SEM (n = 6). ($p < 0.05$); [a]Significantly different from the normal control; [b]Significantly different as compared with negative control

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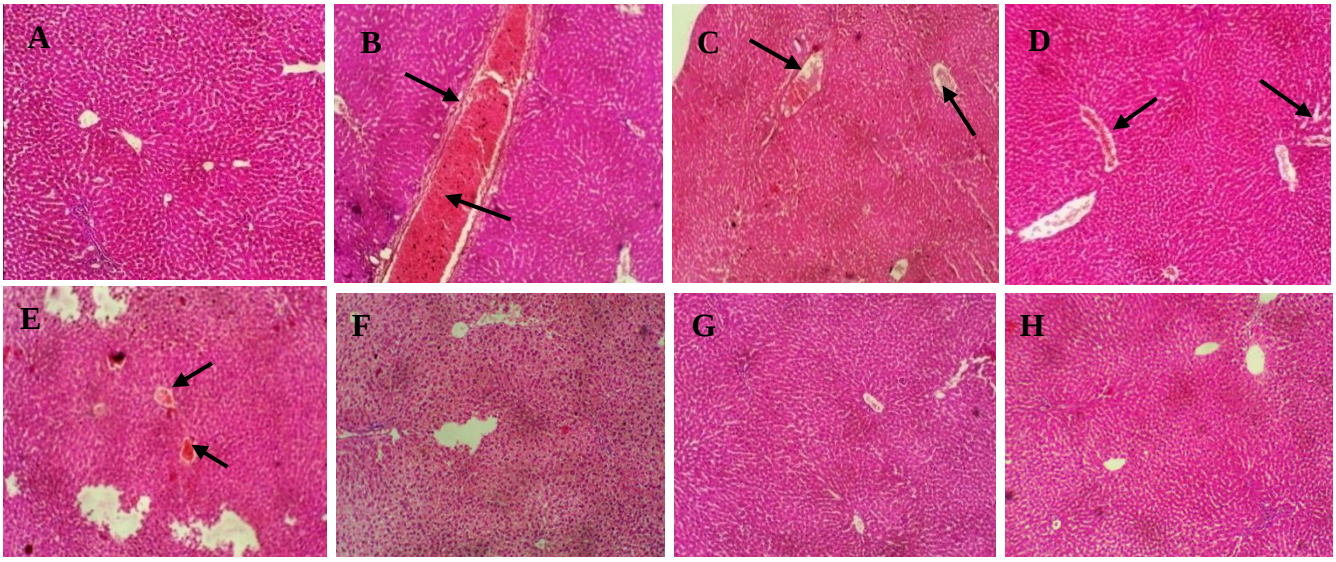

Plate 1: Photomicrograph of liver tissues (H&E × 100) of rats. (a) Control; (b) Induced only; (c) Treated with 50 mg/kg bwt Ext; (d) 100 mg/kg bwt Ext; (e) 200 mg/kg bwt Ext; (f) 100 mg/kg bwt Ext and induced; (g) Rutin; and (h) Ext only

Induction of DMBA caused an increase in the liver and kidney MDA in the induced-only group compared with the normal control, suggesting peroxidation of lipids.[39] Post and pre-administration of *A. senegalensis* and rutin for 14 days reduced the MDA levels, as shown in Figure. 3c & 3d; this denotes that *A. senegalensis* is a potent free radical scavenger, which may be attributed to the plant's leaves' antioxidants.[40] SOD, an antioxidant enzyme that protects the cell against superoxide (O_2^-), was decreased in kidney and liver tissues of the induced-only group. Administration of post- *A. senegalensis* (at dose 100 and 200 mg/kg bwt), pre- *A. senegalensis* (100 mg/kg bwt), and rutin for 14 days increase SOD activity in the liver and kidney tissues' (Figure 3e & 3f). Furthermore, SOD activity in the *A. senegalensis* only group and rutin increased, this could be attributed to the phytonutrient in the plant and their ability to stimulate SOD biosynthesis.[41]

A. senegalensis may overpower the DMBA oxidative effect by inducing antioxidant production, suppressing reactive oxygen species (ROS). Reduced glutathione (GSH) is the next line of defense against reactive oxygen species, it is also an essential non-protein cellular thiol responsible for cell proliferation[42] its concentration in the kidney and liver in the induced-only group decreased compared to the normal control group suggesting an influx of free radicals, thereby affecting the functionality and integrity of the liver and kidney tissue. Anbalagan *et al.*[43] also reported a reduction in GSH level in the buccal mucosa tissue and the hamster's blood plasma in DMBA-induced group compared to the control. The results from our study showed that 14 days post- *A. senegalensis* (100 and 200 mg/kg bwt), pre- *A. senegalensis*, *A. senegalensis* only, and rutin treatment was able to increase the level of GSH when compared to the induced-only group (Figure 3a & 3b). The biological changes in antioxidant levels in the liver and kidney tissues of the induced-only group animals in this study may be due to the induction of lipid peroxidation and free radical production following DMBA induction. The increase in the level of GSH by the *A. senegalensis* could be due to its effect on reducing the regeneration of ROS or increase in glutathione *de novo* synthesis or both.[44] Also, *A. senegalensis* may directly act by scavenging ROS derived from the redox reaction. It may combine with other antioxidants in the body and prevent it from being depleted by DMBA epoxide amelioration oxidative injury.

Conclusion

The rise in antioxidant profiles, namely SOD and GSH, and decrease in MDA, liver, and kidney profiles by *A. senegalensis* and rutin may be related to their biological relevance in removing free radicals created by DMBA, which may interfere with normal hepatocyte function. According to the findings, the effectiveness of *A. senegalensis* against DMBA-induced liver damage increased when delivered at a larger dosage, it is possible to infer that *A. senegalensis* and rutin are beneficial natural products that can ameliorate the hepatotoxicity caused by DMBA induction, and that they could be topics of future research and therapeutic development.

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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