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MICROANATOMICAL EFFECTS OF DOXORUBICIN ON THE LIVER OF WISTAR RATS FOLLOWING THE ADMINISTRATION OF SAPONINS RICH FRACTIONS FROM DIOSCOREA BULBIFERA BULBIS

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Abstract

Background: Doxorubicin extravasation during intravenous administration could result to necrosis and renal failure which are part of side effects of doxorubicin administration. Therefore, this study is to determining the effects of saponins on doxorubicin-induced hepatotoxicity in male Wistar rats. **Methods:** Forty male wistar rats with ages between 12-16 weeks and weights between 120g- 150g were used for this research. The rats were randomly assigned to 5 groups (groups A, B, C, D and E). Group A was the control group and received distilled water, Group B was administered 10mg/kg of doxorubicin only, Group C was administered 50mg/kg of saponin only, Group D was pre- treated with 50mg/kg of saponin for 7 days after which 10mg/kg of doxorubicin was administered, Group E was pre-treated with 100mg/kg of saponin for 7days after which 10mg/kg of doxorubicin was administered. **Results:** Results revealed that there were significant difference in weight change ($F = 42.68$, $p < 0.0001$), ALT ($F = 51.82$, $p < 0.0001$), AST ($F = 47.72$, $p < 0.0001$) and ALP ($F = 18.38$, $p = 0.0001$) across all experimental groups induced by doxorubicin ($p <0.05$). Doxorubicin administration was observed to have a negative effect on the Liver histology and immunohistochemical results. The administration of saponins in group D and E as a pre-treatment counteract the effect of the drug. **Conclusion**: it was noticed that degenerated cells were observed in group B, which were reversed in group D and E by administration of saponin.

Keywords: Hepatic Degeneration; Saponin; *Dioscorea Bulbifera;* Bulbis.

INTRODUCTION

Liver disease refers to any condition that can affect and damage the liver (Moolenaar *et al*., 2022). Overtime liver disease can cause cirrhosis and as more scar tissue replace healthy liver tissue, the liver can no longer function properly (Moolenaar *et al*., 2022). Liver disease accounts for approximately 2 million deaths per year worldwide, 1 million due to complications of cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma (Kareem *et al*., 2022). Hepatotoxicity is a chemically driven liver damage. Drug-inducedced liver injury is a cause of acute and chronic liver disease caused by specific medications (Milano *et al* 2020). Doxorubicin is associated with adverse effects on organs like liver (Milano *et al*., 2020). The molecular mechanisms involved in doxorubicin causing hepatotoxicity are mainly due to the production of the reactive oxygen species (ROS) by the drug during its metabolism in the liver producing an oxidative stress, apoptosis etc. (Milano et al 2020). Doxorubicin in rare instances of with jaundice that can be severe and even fatal which may lead to cirrhosis and hepatitis (Milano *et al.,* 2020).

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Doxorubicin is an anthracycline antibiotic structurally similar to daunorubicin as a natural anti-cancer antibiotic used in cancer treatment (Mohan *et al.*, 2021). It is widely used to treat a broad spectrum of malignancies such as breast, lung, bladder cancers, etc (Yang and Henikoff, 2015). It has been used as a chemotherapeutic agent since the 1960s (Mohan *et al.,* 2021). It was first extracted in 1970 and routinely used in the treatment of several cancers and also soft tissues and bone sarcomas (Mohan *et al.*, 2021). It acts in the cancer cell by intercalation into DNA and disruption of topoisomerase-11-mediated DNA repair (Rossi *et al.*, 2009). Also, generation of free radicals and their damage to cellular membranes, DNA and proteins (Rossi *et al.*, 2009). Doxorubicin is administered via an intravenous (IV) injection through a central line. Iron cheators, such as dexrazoxane, may prevent free radical formation by limiting the binding of doxorubicin with iron (Milano *et al* 2020). It exhibits rapid distribution into tissues and has an elimination half-life of up to 48 hours. It side effects include: Hepatotoxicity, Nephropathy, Neurotoxicity, Cardiomyopathy (Milano *et al* 2020).

Saponin is a large family of amphiphilic glycosides of steroids and triterpenes found in plants and some marine organisms (Savarino *et al.*, 2021). They are classified as Triterpene glycosides, spirostanol glycosides, and steroidal alkaloid glycosides (Furuyaa, 1988). A high saponin decrease blood lipids, lower cancer risks and lower blood glucose response. A high saponin diet can be used in the inhibition of dental caries and platelet aggregation, in the treatment of hypercalciuria in humans and as an antidote against acute lead poisoning (Kareem et al., 2022). This research is aimed to study the effects of saponins rich fraction from *Discorea Bulbifera* Bulbis on Doxorubicin-induced hepatotoxicity in male Wistar rats.

MATERIALS AND METHODS

Animal Care and Management

Forty healthy male wistar rats with ages between 12-16 weeks and weights between 120g- 150g were obtained from the Animal Research Center, Afe Babalola University Ado- Ekiti were used for this research. The rats were allowed to drink as much clean water and eat regular laboratory pellets as they desired. After acclimating for a week, the animals were separated into five groups according to their weight. Research, Ethics, and Grant Committees of the Faculty of Basic Medical Sciences at Afe Babalola University, Ado Ekiti (ABUAD) approved all animal procedures for this study with approval number REC/FBMS/ABUAD/21/35 per the guiding principles for the care and use of animals and the principles of animal research.

Experimental Design

The Wistar rats were randomly assigned to 5 groups (groups A, B, C, D and E). Group A was the control group and it received distilled water, Group B was administered 10mg/kg of doxorubicin only, Group C was administered 50mg/kg of saponin only, Group D was pre- treated with 50mg/kg of saponin for 7 days after which 10mg/kg of doxorubicin was administered, Group E was pre- treated with 100mg/kg of saponin for 7days after which 10mg/kg of doxorubicin was administered. The extract solution of saponin was administered orally using an oral cannula and the doxorubicin was administered intraperitoneally. The duration of the experiment was 14 days. They were all fed a standard laboratory diet and water ad- libitum.

Duration of Experiment

S/N	GROUPS	AGENTS	DOSE	ROUTE	DURATION
1	A	Distilled water	0.15ml	Oral	14 days
2	B	Doxorubicin	10mg/kg/week	Intrperitoneal	14 days
3	C	Saponin	50mg/kg/day	Oral	14 days
4	D	Saponin for 7 days + Doxorubicin	50mg/kg/day + 10mg/kg/week	Oral + Intraperitoneal	14 days
5	E	Saponin for 7 days + Doxorubicin	100mg/kg/day + 10mg/kg/week	Oral + Intraperitoneal	14 days

Plant Material and Preparation of Extract

Freshly harvested bulbils of Dioscorea bulbifera was procured from progresss street, Ado Ekiti. The bulbils was washed, peeled, cut into thin slices, chopped into pieces and air-dried. The chopped dried bulbils were finally pounded into fine powder using electric blender. The extraction of bioactive compound of samples was done using ethanol, one and half bottles of 100ml of acetone was added to a measured amount of grinded *Discorea Bulbifera*. The solution was stirred consecutively until it was evenly distributed after which it was allowed to soak for 48hrs. The mixture was then filtered using Whitman filter paper No 1 to separate the filtrate from the residue. The residue is then aired to remove excess acetone in order to get rid of as much impurities as possible after which it was soaked in methanol for three hours. It was then boiled in a water bath at 100 degree Celsius after which it was left to cool. The filtrate was then filtered into various beakers with the use of filter paper. It was then left to dry at 40 degree Celsius in the oven. It was then transferred into sample bottles where 1ml and 2ml of saponin was being prepared from time to time and administered to the test subjects (Tashi et al., 2016).

Measurement of Body Weight, Blood Collection and Sacrifice of Animals

The body weights of the animals was taken using top loader balance at interval of 7 days. Twenty-four hours after the final administration, the blood samples of the rats were collected through the cardiac puncture to assay for biochemical analysis. The sacrifice of the animals was done via cervical dislocation. The liver was harvested and fixed in 10% formal saline and processed for paraffin wax embedding and sectioned at 5 microns, the blood collected were centrifuged separated the serum from the plasma, and serum were used for biochemical process. Tissue was stained with Hematoxylin and Eosin method which was used to demonstrate the general histoarchitecture of the liver and Gordon and sweet's silver staining method for reticular fibers.

Percentage weight change was calculated using:

$$\frac{Final\ weight - Initial\ weight}{Initial\ weight} \times 100$$

Biochemical Analysis

Biochemical analyses were done to assay the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity. Blood was collected from the heart of the rats and allowed to clot at room temperature. It was then centrifuge for 30 minutes at (3000 r/min). The collected supernatant (serum) was used to analyze hormone measurement. The luteinizing and follicle stimulating hormone were analyzed by ELISA method using rat ELISA kits.

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Photomicrography

Olympus binocular microscope was used. A 5.1-megapixel MV550 research camera for microscopes was mounted in one of the oculars. This was connected to a computer running on image capture and analysis software. The system was adjusted to obtain clarity and resolution and the images were captured and saved on the computer.

Quantification of Staining Intensity

Image analysis and processing for Java (ImageJ), was used to analyze and quantify Gordon and sweet silver staining and CD4 Immunohistochemistry staining intensities. Imported RGB images were converted to grayscale images on ImageJ. The software quantifies staining intensity by measuring the pixel value of each pixel in grayscale images following the threshold of areas of staining activity and converting the pixel value to brightness value or gray value. The values obtained were then analyzed statistically.

Statistical Analysis

One-way ANOVA was used to analyze data, followed by Student Newman-keuls (SNK) test for multiple comparisons. Graph Pad Prism 5 statistical package was used for data analysis. Significant difference was set at $p<0.05$

RESULTS

Effect of Saponin on Body Weight of Rats Administered with Doxorubicin

One way ANOVA revealed that, there was significant difference in weight change across all experimental groups ($F = 42.68$; $p < 0.0001$). Post hoc analysis showed that weight of doxorubicin group was significantly lower than the vehicle treated group. The weight reduction induced by doxorubicin and reversed by concomitant administration of saponin in group D and E ($p<0.05$) (Fig.1).

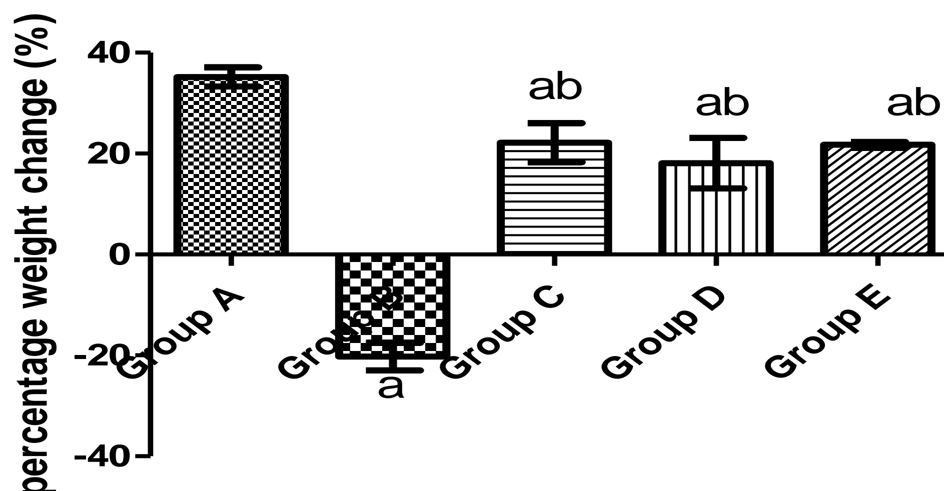

Figure 1: Effect of Saponin on Body Weight of Rat fed with Doxorubicin. n = 7, values are expressed as % weight change ± SEM. a = relative to control at p<0.05; b= relative to group B (doxorubicin only) at p<0.05. Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin

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Biochemical Analysis

One way ANOVA revealed that, there were significant difference in Aspartate Aminotransferase (AST) in Wiatar ($F = 47.72$; $p < 0.0001$), Alanine aminotransferase (ALT)($F = 51.82$; $p < 0.0001$), Alkaline Phosphatase (ALP) ($F = 18.38$; $p = 0.0001$), activities across all experimental groups. Post hoc analysis showed that data in group B (ciplatin only) were significantly lower than the positive control (group A). This reduction induced by doxorubicin and reversed by the treatment of saponins administration in group D and E ($p<0.05$) (Figures 2, 3 and 4).

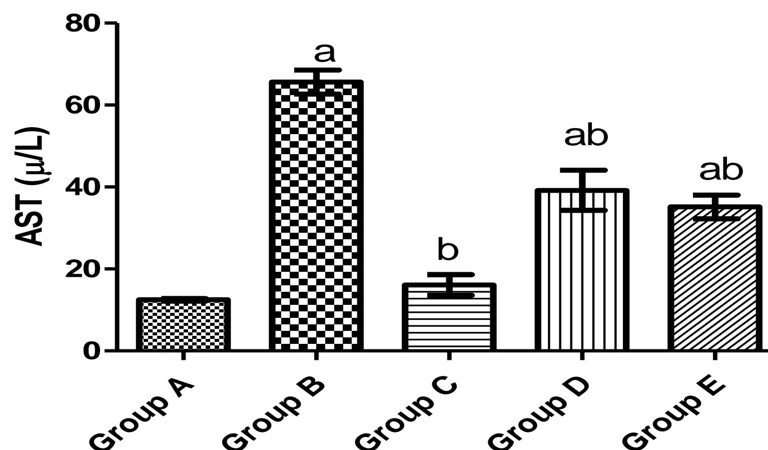

Figure 2: The Effect of saponin on Activity of Serum Aspartate Aminotransferase in Wiatar Rats Administered with Doxorubicin. n = 7, Values are expressed as AST ± SEM. a = relative to control at p<0.05; b= relative to group B (doxorubicin only) at p<0.05. Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin

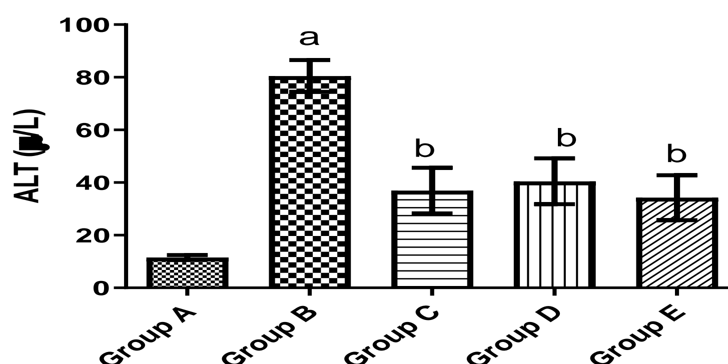

Figure 3: The Effect of saponin on Activity of Serum Alanine aminotransferase (ALT) in Wiatar Rats Administered with Doxorubicin. n = 7, Values are expressed as ALT ± SEM. a = relative to control at p<0.05; b= relative to group B (doxorubicin only) at p<0.05. Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin

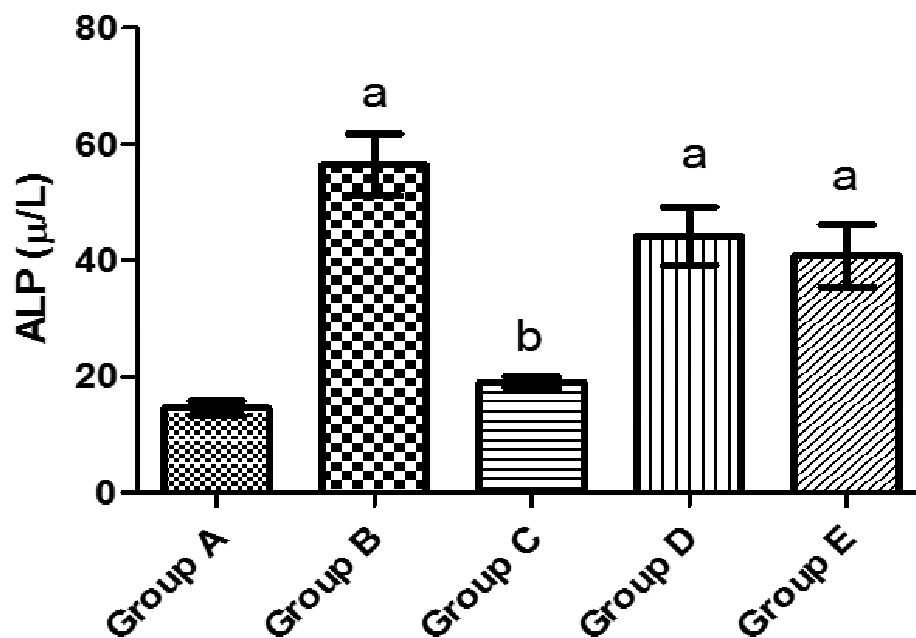

Figure 4: The Effect of Saponin on Activity of Serum Alkaline Phosphatase (ALP) in Wistar Rats Administered with Doxorubicin. n = 7, Values are expressed as ALP ± SEM. a = relative to control at $p<0.05$; b= relative to group B (doxorubicin only) at $p<0.05$. Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin

LIVER HISTOPATHOLOGY

H&E revealed that the liver of the control group is healthy and normal as the section is free from collections and inflammatory cell (figure 5 A and C). Doxorubicin group only showed necrosis of hepatocytes focal area of hepatic necrosis occupied by leucocytic cells infiltration and the congestion of central vein were seen (figure 5 B). Saponin was able to ameliorate the toxic effect of doxorubicin as they were administered together (figure 5 D and E).

There was regular distribution of the reticular fibers around the central vein and the lining of the sinusoids of the liver tissue (figures 6 A and C). The reticular fibers were observed to be sparsely distributed as shown in figure 5 B. Sparse distribution of the reticular fibres around the central vein and lining the sinusoids was evident in figures 6 D and E (figures 6 and 7)

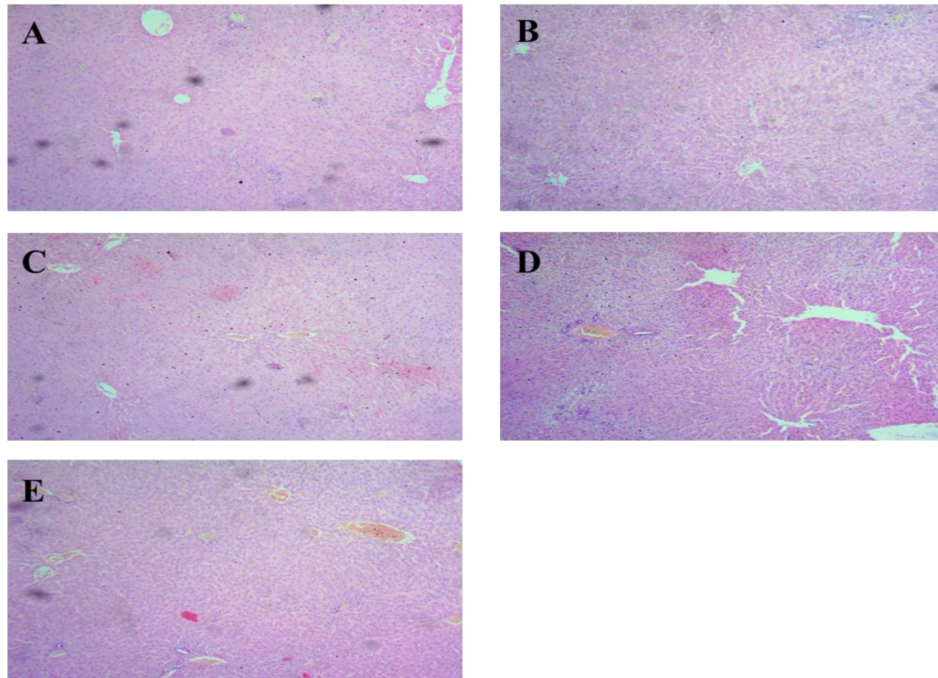

Figure 5: Showing the hepatic tissues composed of hepatocytes (H) disposed in sheet, the hepatocytes were seperated by the sinusoids (S). the central vein (CV) was well outlined and portal vein (PV). Section was free from collections and inflamatory cells in Groups A and C. In group B, blue arrow indicated necrosis hepatocytes (NP). Section was not free from collections and inflamatory cells. Goups D and E: showing the hepatic tissues composed of hepatocytes (H) disposed in sheet, the hepatocytes were seperated by the sinusoids (S). the central vein (CV) is well outlined. Section was free from collections and inflamatory cells. H&E (X 100)

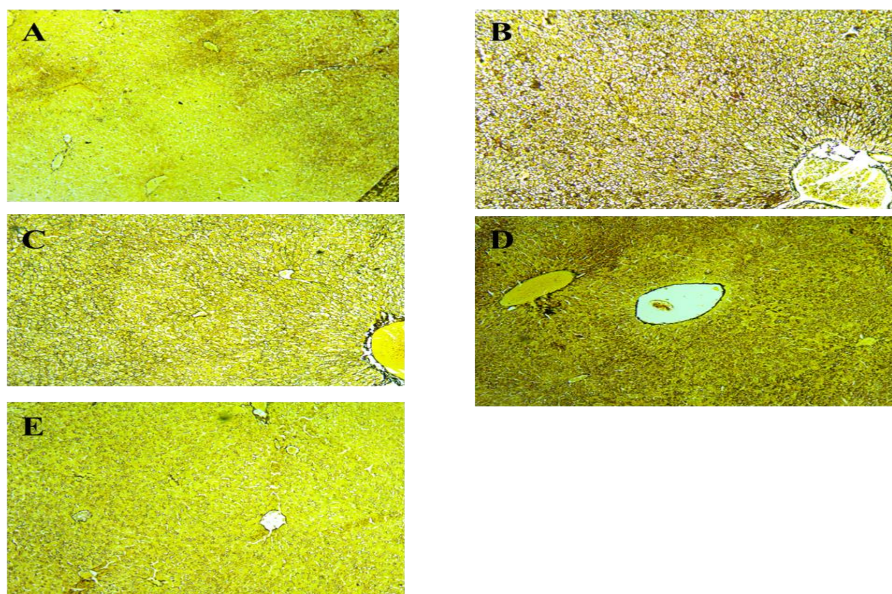

Figure 6: Representative light photomicrographs of the liver of Wista rats stained with Gordon and sweet silver staining in groups A-E. (Gordon and sweet x 100)

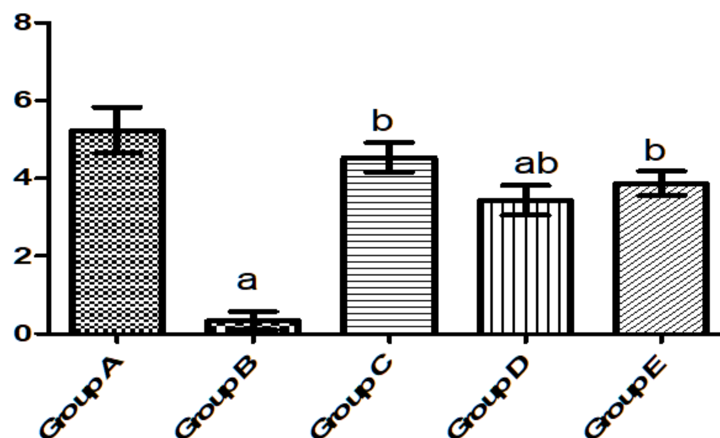

Figure 7: Bar charts depicts reticular fibres content across groups measured by digital densitometry of reticular fibres using Image J software a = relative to control at $p<0.05$; b= relative to group B (doxorubicin only) ($p = 0.0073$; $F = 6.584$). Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin

Immunohistochemical Findings

Sections of the liver from control rats stained for CD4 revealed a normal distribution of T-lymphocyte cell in liver (figure 8 A). Normal distribution of CD4 also appeared in figure 8 C (only saponin were administered). Augmentation of positive staining with the anti-CD4 antibody was seen in the liver from rats that received doxorubicin only (figure 8 B). Sections of the liver from rats treated with doxorubicin and saponin revealed near to normal distribution in CD4 but more reduced in the treatment of saponin groups (figure 8 D and E).

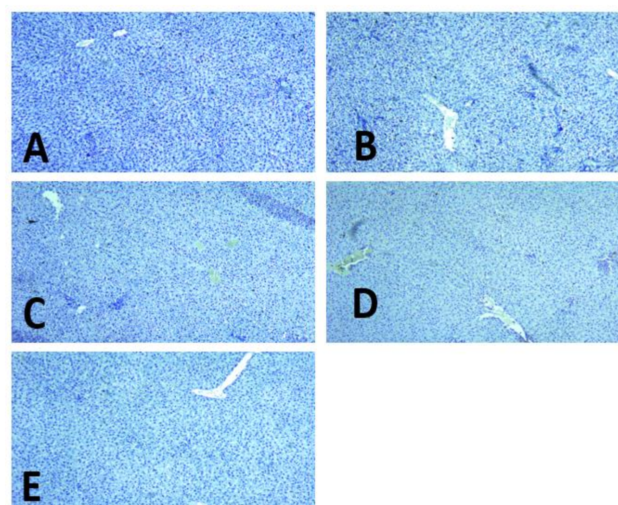

Figure 9: The representative light photomicrographs of the liver. Showing more inflammation in Group B. Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin. (CD 4 immunohistochemical stain × 100)

Table 2: Effects of saponins on doxorubicin in liver in changes on T-lymphocyte. n = 8, values are expressed as % area of CD 4 ± SEM. a = relative to control at p<0.05; b= relative to group B (doxorubicin only) (p < 0.0001; F = 22.17). . Group A = control, Group B = doxorubicin only, C = saponin (100 mg/kg) only, Group D = 10mg/kg of doxorubicin + 50 mg/kg of saponin, Group E = 10mg/kg of doxorubicin + 100 mg/kg of saponin.

Groups	Percentage Area of CD 4 Immunohistochemistry (%)
A (Control)	5.233 ± 0. 593
B (Doxorubicin only)	0.337± 0.232[a]
C (100 mg kg^{-1} of saponins)	4.533 ± 0.384[b]
D (Doxorubicin + 50 mg kg^{-1} of saponins)	3.433 ± 0.384[ab]
E Doxorubicin + 100 mg kg^{-1} of saponins)	3.867 ± 0.318[b]

DISCUSSION

The study investigated the effect of doxorubicin on the liver of male Wistar rats following the administration of saponin isolate, *Dioscorcia Bulbifera*. There was a significant reduction in all experimental groups. Post hoc analysis showed that weight of doxorubicin group (B) was significantly lower than vehicle group. This could be explained by the dosage of doxorubicin administered and this is supported by the research observed by Chaudhary *et al*. (2016). Saponin administration significantly and dose dependently reversed the weight reduction induced by doxorubicin with an agreement of Xing et al., (2019).

Histological findings revealed that the liver of the control group is healthy and normal as the section is free from collections and inflammatory cell (groups 5 A and C). Doxorubicin group only showed necrosis of hepatocytes focal area of hepatic necrosis occupied by leucocytic cells infiltration and the congestion of central vein were seen in figure 5 B. Reticular fibres form thin and extensive network which support structures around the parenchymal cells of the liver within perisinusoidal space (Enye *et al*., 2021). This damage may be responsible for the distorted architecture observed in group B. Junqueira et al. (2005) stated that glycosaminoglycan, a ground substance of extracellular matrix, stabilizes reticular fibres. The groups that were administered with 50 mg/kg and 100 mg/kg of saponin with doxorubicin showed little perivascular and interstitial infilteration of inflammatory cells. Saponin was able to ameliorate the toxic effect of doxorubicin as they were administered together (Wu et al., 2019).

CD4 is a glycoprotein that serves as a co-receptor for the T-cell receptor (Gillespie *et al*., 2021). CD4 is found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells (Steiner *et al*., 2022). It assists the latter in communicating with antigen-presenting cells. The TCR complex and CD4 bind to distinct regions of the antigen-presenting MHC class II molecule (Steiner *et al*., 2022; Al-Moussawy *et al*., 2022). Immunohistochemical analysis for CD4 revealed a normal distribution of T-lymphocytes in group A and C. There were augumentation of positive staining of T-lymphocytes in group B which is the important driver of inflammation (Lacher *et al*., 2018). However, when saponins of was co-administered with doxorubicin, there were decreased in distribution of T-lymphocytes but it was more reduced in the group administered with 100mg/kg of saponins.

CONCLUSION

The result obtained from this experiment showed that doses of saponins rich fraction of *Dioscorea bulbifera* reveals adverse effect on the hepatotoxicity of adult male wistar rats. The study also showed that there was a gain in body weight of groups pre-treated with 100 mg/kg of saponins suggesting the ability of high dosage of saponin administration to significantly increase and reverse the weight reduction induced by doxorubicin. Therefore saponins administration is dose dependent for effects on doxorubicin induced hepatotoxicity.

Conflicts of Interest

Authors detected no conflicts of interest

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