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Original Research Article

Cytokeratin-7 Expressions and the Protective Roles of *Theobroma cacao* Seed Extract Following Reserpine Induced Hepatotoxicity in Wistar Rats

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Abstract

Background*:** Hepatic damage is a major cause of poor quality of living and mortality worldwide. This study investigates the hepatoprotective effects of cocoa seed extract against reserpine-induced hepatotoxicity in male Wistar rats. ***Methods: Thirty adult male Wistar rats were divided into six groups for a 28-day study: Group A (control), Group B (0.5 mg/kg/B.W reserpine), Group C (20 mg/kg/B.W fluoxetine), Group D (1000 mg/kg/B.W cocoa seed ethanolic extract), Group E (0.5 mg/kg/B.W reserpine + 1000 mg/kg/B.W cocoa seed ethanolic extract), and Group F (0.5 mg/kg/B.W reserpine + 20 mg/kg/B.W fluoxetine). The liver was harvested, placed in 10% neutral buffered formalin, and processed for biochemical, histological analysis. Also, liver tissues for cytokeratin-7 expression was processed according to standard immunohistochemical staining procedures. Data were analyzed using GraphPad Prism 9.0 software and one-way ANOVA. ***Results***: There was a significant decrease in ALT, AST, and ALP levels in animals treated with cocoa extract compared to untreated animals. Meanwhile, cytokeratin expression was higher in the liver of untreated animals relative to the treated groups. ***Conclusion***: *Theobroma cacao* seed extract shows potential in mitigating reserpine-induced hepatotoxicity, contributing to novel therapeutic strategies for liver disorders and providing insights into the mechanisms of cocoa seed bioactive compounds.

Keywords: Reserpine, Hepatotoxicity, Cytokeratin-7, *Theobroma cacao*.

INTRODUCTION

The liver plays a crucial role in various metabolic processes, including the biotransformation and elimination of xenobiotics drugs and other toxic compounds from the body. However, prolonged exposure to certain substances can lead to hepatotoxicity, a condition characterized by liver injury or dysfunction. Despite its therapeutic benefits, reserpine has been associated with various adverse effects, including hepatotoxicity (Benić *et al.*, 2022). More so, hepatotoxicity induced by conventional drugs is a complex phenomenon involving multiple mechanisms, such as oxidative stress, inflammation, and apoptosis (Benić *et al.*, 2022). Excessive ROS can damage cellular components, including lipids, proteins, and nucleic acids, leading to cell death and tissue damage (Benić *et al.*, 2022). Moreover, reserpine has been reported to induce inflammation in the liver, characterized by the infiltration of inflammatory cells and the release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Aryal *et al.*, 2022). Chronic inflammation can further exacerbate

oxidative stress and contribute to the progression of liver injury (Aryal *et al.*, 2022).

Conversely, in recent years, there has been a growing interest in exploring natural products as potential therapeutic agents for various diseases, including liver disorders (Muhammad *et al.*, 2022; Salama *et al.*, 2022). *Theobroma Cacao* is commonly known as cocoa which has attracted attention in recent years for its potential therapeutic properties particularly in the context of mental health disorders such as depression (Tan *et al.*, 2021). Cocoa is rich in bioactive compounds, including flavonoids, polyphenols and alkaloids which have been shown to exert beneficial effects on mood and cognition. In our previous study, cocoa seed extract has been explored for its potential effects on reserpine-induced hematotoxicity and stomach tissue distortions in previous studies (Adetunji *et al.*, 2024).

In the present study, we aimed at investigating the expression patterns of cytokeratin-7 in reserpine-induced liver damage. Furthermore, the potential roles of cocoa in reserpine induced liver dysfunctions were elucidated using biochemical and histopathological methods.

MATERIALS AND METHODS

Chemicals and Drugs

Normal Saline, Phosphate buffer, Formalin, Distilled water, Reserpine injection (0.5 mg/kg/B. W), fluoxetine (20 mg/kg/B. W), cocoa seed ethanolic extract (1000 mg/kg/B. W).

Animal care, conditioning and management

A total number of thirty (30) adult male Wistar rats were kept and cared for at the Babcock University Animal House, Ilisan Remo, Ogun state. They were kept in plastic cages with net covers to keep them cool. They were allowed to acclimatize in the animal house for 7 days (one week). The rats were allocated to six (6) groups with each cage containing five (5) rats. The animals were provided with distilled water and pelletized food daily. The animals' bedding was covered with wood shavings to avoid toxic ammonia accumulation and was replaced every three (3) days. The feed of the rats was measured daily. This was done by measuring the weight of the leftover food per animal group in a cage.

Study Design

	No. of Rats	Treatment Schedule	Rationale
	5	A placebo of water	Control group
Group B	5	Animals treatment with (0.5 mg/kg/B. W) of reserpine intraperitoneal for 14 days	To induce hepatic damage
Group C	5	Animals treatment with (0.5 mg/kg/B. W) of fluoxetine intraperitoneal for 14 days	Fluoxetine control

Administration of solution

Reserpine was administered intraperitoneally for 14 days. The control group (Group A) was given a placebo of water, group B was administered Reserpine (0.5 mg/kg/B.W), group C was administered fluoxetine (20mg/kg/B.W), group D was administered cocoa seed ethanolic extract (1000mg/kg/B.W). Group A was left untreated, groups B, C and D were also left untreated to induce the effects of reserpine, fluoxetine and cocoa ethanolic extracts respectively. Group E was treated with 1000 mg/kg/B.W of *Theobroma Cacao* seed extract orally for seven (7) days using an oral cannula for administration. Meanwhile, group F was treated orally with 20mg/kg/B.W of fluoxetine seven (7) days.

Measurement of body weight and feeding

The body weights of the animals were measured once in three days throughout the duration of administration with the use of a weighing balance. This was done in order to access the weight gain or weight loss in each group. The feed of the rats was measured daily. This was done by measuring the weight of left-over food per animal group in a cage.

Biochemical assay

The cold centrifuge was used to centrifuge 5 ml of whole blood that had been collected into a heparinized tube for 30 minutes at 5000 rpm. The indicators of liver function, such as Aspartate aminotransferase (AST), Alanine transaminase (ALT), and Alkaline phosphatase (ALP) were examined in the plasma after it had been separated (Adewole *et al*., 2023).

Histology

The liver was carefully excised, weighed, and fixed in 10% neutral buffered formalin. Thereafter, the tissues were embedded in paraffin wax, sectioned, and stained using hematoxylin and eosin to demonstrate the general histology and morphology of the liver (Adeoye *et al*., 2022).

Special Stain Procedure

The liver was also stained with Periodic Acid-Schiff (PAS) stained using previously described protocols (Adetunji *et al*., 2024).

Immunohistochemical Procedure for Cytokeratin

The hepatic expressions of cytokeratin 7 was carried out using previously described immunochemical protocol (Adelodun *et al*., 2022).

Animal procurement and ethical consideration

Animals were obtained from Babcock University Animal House for this research following all

rules and regulations in animal research and education as approved by the National Research Council DHHS' Institute of Laboratory Animal Resources. Ethical clearance was obtained from Babcock University Health Research Ethical Committee (BUHREC- 078/22).

Statistical analysis

The results obtained were expressed as mean ± SEM for each group. All data were evaluated statistically through the GraphPad Prism 6.0 software using the one-way analysis of variance (ANOVA). The Student-Newman-Kleus post Hoe test was used to identify differences. The value of ($P < 0.05$) was considered significant.

RESULTS

Morphological analysis

Body weight

As shown below in the Figure below, there was a statistical significance when Group C (fluoxetine), Group D (cocoa), Group E (reserpine + cocoa) and Group F (reserpine + fluoxetine) were compared with the control group. Group C (fluoxetine), Group D (cocoa), Group E (reserpine + cocoa) and Group F (reserpine + fluoxetine) were statistically significant to Group B (reserpine). Group F (reserpine + fluoxetine) was statistically significant to Group C (fluoxetine), Group D (cocoa) and Group E (reserpine + cocoa). *, +, #, $ and @show a statistical significance when the experimental groups are compared at $p < 0.05$.

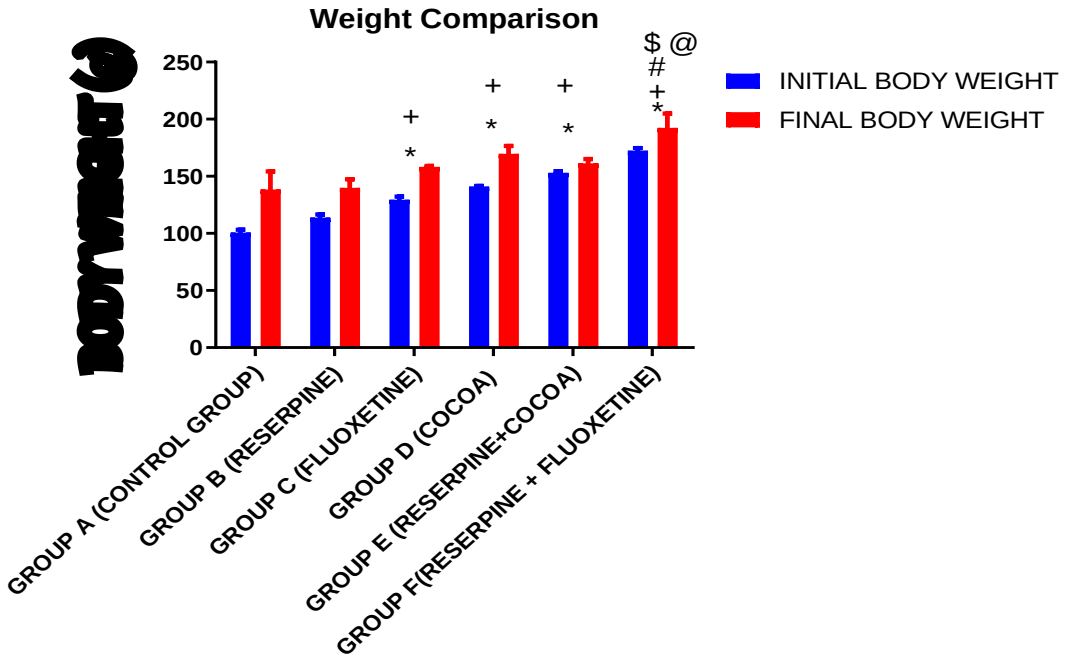

Figure 1: Graph showing the body weight change before and during treatment

Alanine Aminotransferase (ALT)

As represented in Figure 2a Aspartate Aminotransferase levels of Group D (1.2±0.17) showed a significant decrease when compared to Group A (3.6±0.55). When the Alanine Aminotransferase levels were compared to themselves using multiple comparisons, the Aspartate Aminotransferase levels of Group F (2.5±0.11) showed a significant increase when compared to Group D (1.2±0.17). These were the significant differences between groups when compared at a P value of $P<0.05$ (95% confidence interval).

Alanine Aminotransferase (AST)

As represented in Figure 2b, there was a significant decrease in aminotransferase levels of Group D (1.2±0.17 when compared with the Group A (3.6±0.55). However, there was a significant increase in aspartate Aminotransferase levels of Group F (2.5±0.11) when compared to Group D (1.2±0.17).

Alkaline Phosphatase (ALP)

As represented in Figure 2c, there was a significant decrease in alkaline phosphate level in Group C (1.1 ± 0.16), Group D (1.2 ± 0.099), Group E (1.4 ± 0.11), and Group F (1.1 ± 0.13) when compared with the Control group (2.7 ± 0.25).

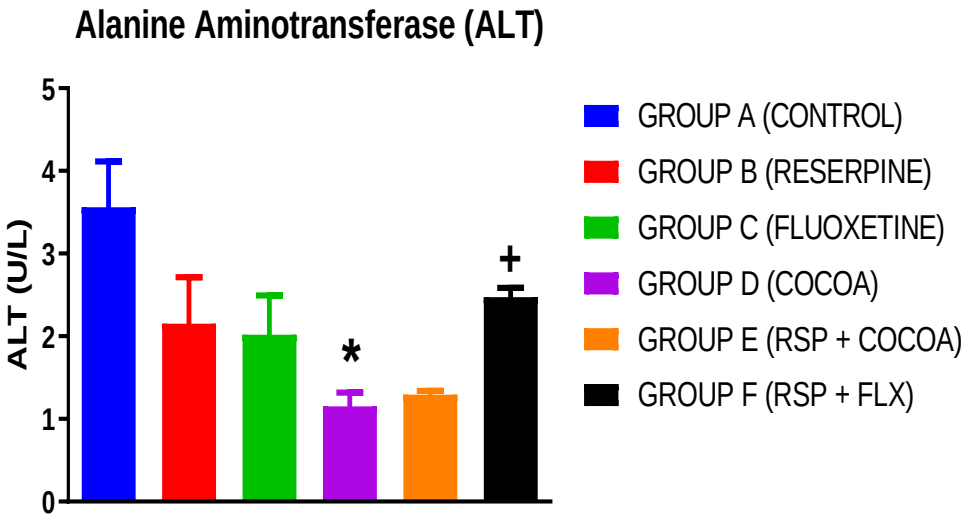

Figure 2a: Graph showing the concentration ALT

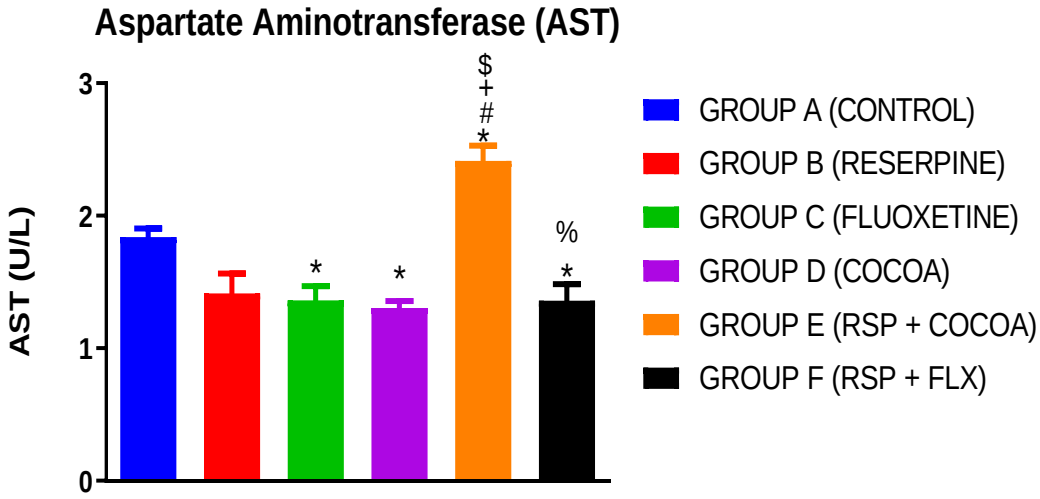

Figure 2b: Graph showing the concentration ASP

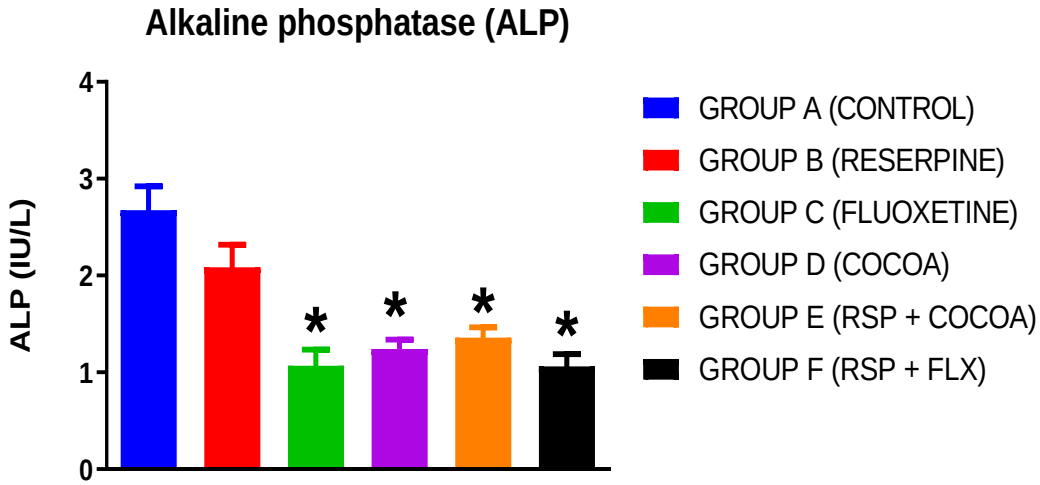

Figure 2b: Graph showing the activity ALP

HISTOLOGICAL RESULTS
Hematoxylin & Eosin

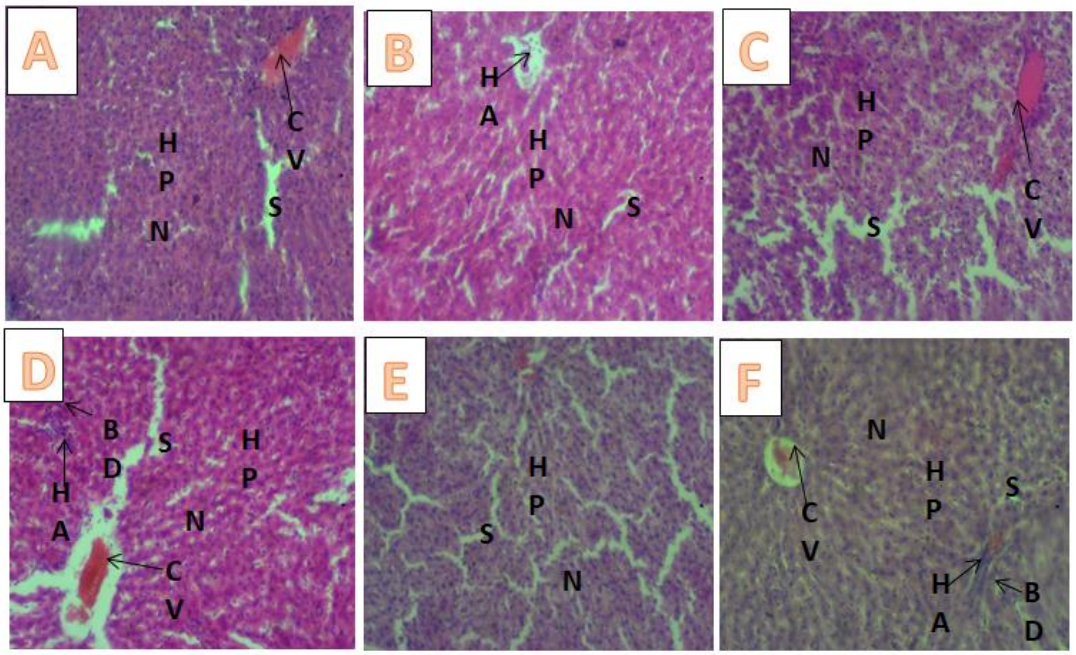

Plate 1: Showing photomicrographs of the general overview of the liver of the rat across the groups. Slides A, B, C, D, E and F at x100. It is stained with H&E to view the cytoarchitecture. This confirms the organ of study which is the liver

Legends:
BD: Bile Duct; HA: Hepatic Artery; PV: Portal Vein; CV: Central Vein; HP: Hepatocytes; S: Sinusoids

PERIODIC ACID SCHIFF'S
PAS X100

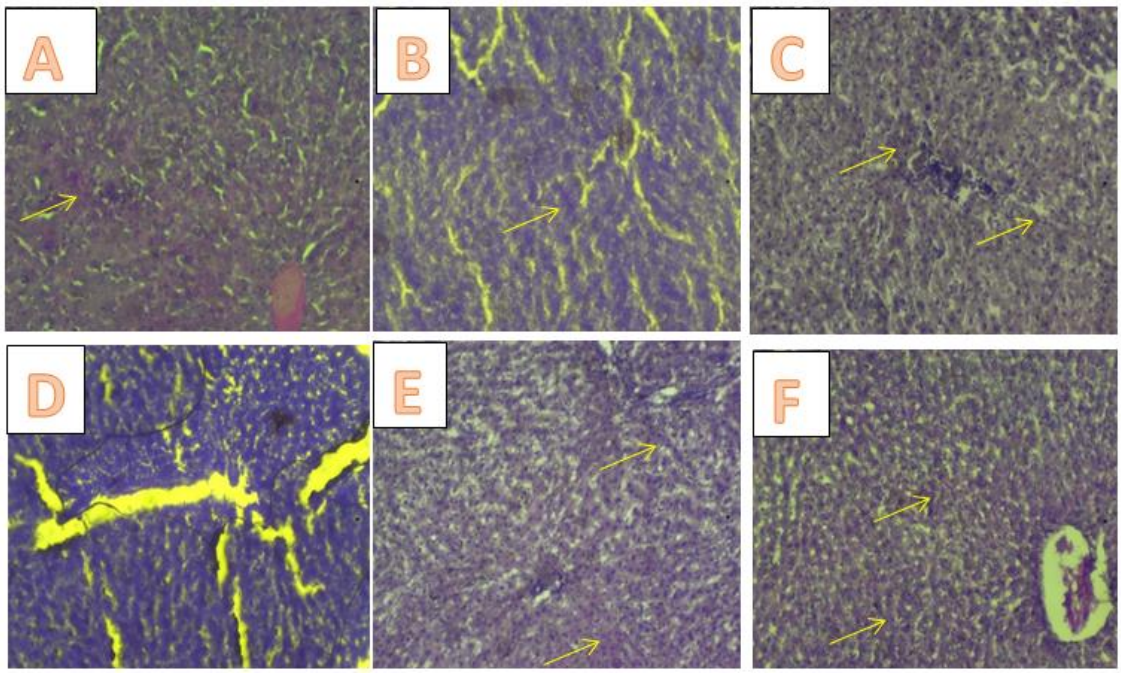

Plate 2: Showing photomicrographs of the general overview of the liver of the rats across all groups, stain with periodic acid sciff's to view intrahepatic glycogen. Periodic Acid-Schiff (PAS) stained liver sections (x100 magnification). Hepatocytes from Group A, C, E & F animals were PAS positive, with glycogen (magenta granules indicated by the arrows) located in the cytoplasm, while these granules were not present in the hepatocytes Group B & D. PAS X400

CYTOKERATIN 7

Plates labeled group A- group F were stained with Cytokeratin7 at x400.
CK7X400

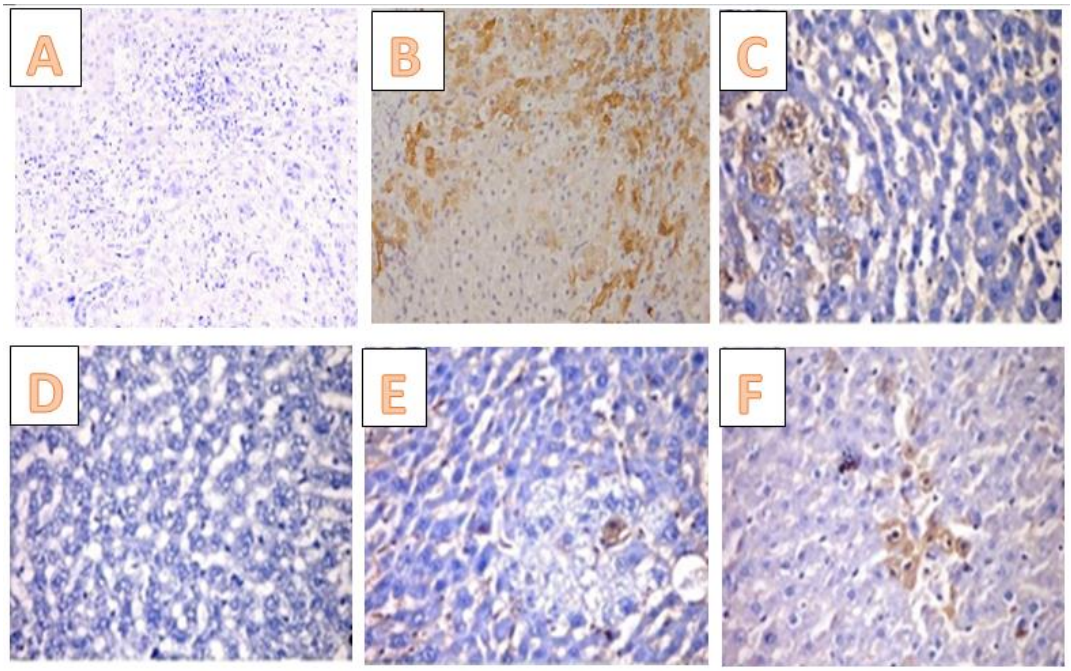

Plate 3: Showing the liver of rats using cytokeratin 7(CK7) at x400 magnification. Immunohistochemistry stained sections: Group A & D showing negative staining reaction of hepatocytes for CK7 (X400). Group C treated group showing moderate positive CK7 expression of most hepatocytes in the altered hepatocellualr foci, Group F showing expression of CK7 in individual hepatocytes. Group E is showing moderate positive CK7 expression of few numbers of hepatocytes, Group B showing positive staining reaction of hepatocytes.

DISCUSSION

Natural products are repositories of bioactive components that can potentially alter the cause of various pathological conditions affecting different organs (Adeoye *et al*., 2022; Tijani *et al*., 2022; Oyerinde *et al*., 2023; Adetunji *et al*., 2022; Adetunji, *et al*., 2024). In the present study, we demonstrated the potential hepatoprotective effects of *Theobroma Cacao* (cocoa) seed extract against reserpine-induced hepatotoxicity in male Wistar rats. Alteration in body weight is a crucial factor that has always been essential in determining the influence of drugs on the body using animal model. However, this study put into consideration the differences in body weight across all groups and this revealed an observable reduction following reserpine administration but there was an observable change after amelioration occurred. To an extent cocoa was able to ameliorate the effects of reserpine on the differences in body weight which wasn't better than fluoxetine. But nonetheless, there was a significant decrease in (RSP + COCOA) and (RSP + FLX) in comparison to the control group which showed weight gain. This observation is in correlation with various studies that have reported depletion in body weight in respect to reserpine exposure (Park *et al*., 2018).

The significant decrease in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels observed in the group treated with cocoa seed extract suggests that cocoa seed extract may have a beneficial effect on liver function. This finding aligns with previous studies that have highlighted the antioxidant and anti-inflammatory properties of cocoa seed bioactive compounds such as flavonoids and polyphenols (Tan *et al.,* 2021; Garcia-Blanco *et al*., 2017). Interestingly, the group treated with reserpine and cocoa seed extract showed a significant decrease in ALT and ALP levels compared to the reserpine-only group indicating that cocoa seed extract may mitigate the hepatotoxic effects of reserpine. This protective effect could be attributed to the ability of cocoa seed bioactive compounds to counteract oxidative stress, inflammation, and apoptosis induced by reserpine, as suggested by previous studies (Benić *et al.*, 2022; Aryal *et al.*, 2022). However, the aspartate aminotransferase (AST) levels in the group treated with reserpine and cocoa seed extract showed a significant increase compared to the cocoa seed extract-only group. This finding suggests that while cocoa seed extract may have a protective effect on liver function, it may not completely eliminate the hepatotoxic effects of reserpine, potentially due to the complex mechanisms involved in reserpine-induced hepatotoxicity (Benić *et al.*, 2022; Aryal *et al.*, 2022).

The histological analysis using Periodic Acid-Schiff's (PAS) staining revealed the presence of

glycogen granules in the hepatocytes of the control group (Group A), fluoxetine-treated group (Group C), reserpine and cocoa seed extract-treated group (Group E), and reserpine and fluoxetine-treated group (Group F). However, these granules were not observed in the hepatocytes of the reserpine-only group (Group B) and the cocoa seed extract-only group (Group D). This finding suggests that reserpine may interfere with glycogen metabolism in the liver while cocoa seed extract alone does not appear to have a significant impact on glycogen storage. Moreover, the immunohistochemical analysis using cytokeratin 7 (CK7) staining revealed varying degrees of positive expression in different treatment groups. Notably, the reserpine-only group (Group B) showed positive staining of hepatocytes, indicating potential cellular alterations or injury. The group treated with reserpine and cocoa seed extract (Group E) exhibited moderate positive CK7 expression in a few hepatocytes, suggesting that cocoa seed extract may partially mitigate the cellular alterations induced by reserpine.

CONCLUSION

Conclusively, the findings of this study suggest that reserpine administration may potentially induce cytokeratin-7 expression in the hepatocytes. Moreover, *Theobroma Cacao* seed extract may have potential hepatoprotective effects against reserpine-induced hepatotoxicity, possibly through its antioxidant and anti-inflammatory properties. However, it has further established that *Theobroma Cacao* seed extract potentially demonstrated hepato-protective effects on implications associated with liver damage.

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Conflict of Interest: Nil

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