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Unsweetened *Theobroma cacao* powder mitigates oxidative stress and preserves hepatic function in ethanol-fed Sprague Dawley rats

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Abstract

Background: Morphological evidence suggests that unsweetened cocoa powder (UCP) prevents hepatic injury in experimental ethanol-ingesting animal models, possibly due to its antioxidant and anti-inflammatory potentials. However, the functional integrity of hepatocytes and the underlying mechanisms are underexplored.

Objective: This study investigated the protective effect of UCP on liver function in ethanol-ingesting male Sprague-Dawley rats.

Methods: Male rats (200-220 g) were fed daily for 8 weeks with either 30% ethanol, 300 mg/kg UCP p.o, or 30% ethanol and 300 mg/kg UCP p.o. 6 h later. Rats that were neither fed with UCP nor ethanol served as controls. At the experimental endpoint, cardiac blood was collected for liver function test, prothrombin time and C-reactive protein assay. The large lobe of the liver was processed and stained with periodic acid Schiff (PAS) and lipofuschin. PAS-stained livers were used for hepatocyte glycogen estimation. The remaining lobes were homogenised for total antioxidant capacity and catalase activity assays.

Results: Compared to the control, ethanol-only-fed rats showed lower serum total protein, albumin, and globulin, and lower hepatocyte glycogen, and higher prothrombin time. Also, ethanol-only-fed rats showed increased serum AST/ALT ratio, CRP, total bilirubin, and unconjugated bilirubin, but reduced conjugated bilirubin. Additionally, the livers of the ethanol-only-fed rats exhibited increased TAC, low catalase and increased lipofuscin accumulation. However, compared with ethanol-only-fed rats, UCP-treated ethanol-fed rats showed higher serum total protein, albumin, and globulin, and higher hepatocyte glycogen, and a lower prothrombin time. UCP-treated ethanol-fed rats also exhibited higher serum conjugated bilirubin, lower unconjugated bilirubin, less lipofuscin accumulation, and higher TAC and catalase levels compared to the ethanol-only-fed rats.

Conclusion: Ingestion of UCP alongside ethanol not only attenuates hepatocellular damage but also preserves hepatic function, possibly by mitigating inflammation and oxidative stress. Thus, UCP could protect against alcohol-induced liver damage.

Keywords: *Theobroma cacao*, oxidative stress, ethanol, liver

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INTRODUCTION

Alcohol-induced liver damage is a critical global health issue, responsible for approximately half of liver-related mortality worldwide [1,2]. It is characterised by a clinical spectrum ranging from steatosis to hepatocellular carcinoma [3] and also leads to significant social and economic consequences. Alcohol-related liver disease often increases family burden arising from caregiving responsibilities and emotional stress, while stigma related to alcohol abuse can exacerbate mental health issues. This social strain may also lead to reduced productivity at work, absenteeism, and unemployment [4,5]. Economically, the costs of healthcare for the treatment of liver conditions, lost work productivity, and the financial burden on families and social support systems

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are significant [6,7]. Considering the burdensomeness of alcohol-related liver damage, it is expedient that interventions that can delay or prevent this disorder are critically explored. Current clinical management strategies for alcohol-induced liver damage are fraught with significant challenges. The commonly prescribed treatments include corticosteroids such as prednisone, which aim to reduce inflammation in severe alcoholic hepatitis [8,9]. However, corticosteroids have significant side effects, especially when glucocorticoid nonresponsiveness occurs [9]. Pentoxifylline is utilised to improve liver function and mitigate fibrosis, but is also associated with gastrointestinal discomfort [10]. N-acetylcysteine (NAC) has gained attention for its antioxidant properties [11], but N-acetylcysteine may cause mild gastrointestinal discomfort, rash, or headache, and, less commonly, allergic or anaphylactoid reactions—particularly with intravenous administration [12]. Considering the plethora of adverse reactions from the use of orthodox medications, there is a need to explore therapeutic strategies that offer hepatoprotective value through the mitigation of alcohol-induced inflammatory and oxidative damage.

Dietary interventions have emerged as a promising adjunctive approach. Among these, unsweetened cocoa powder derived from *Theobroma cacao* beans has attracted interest as a richer source of antioxidants, even exceeding those of red wine and tea [13], as it contains high levels of flavonoids, particularly epicatechin and proanthocyanidins [14]. These compounds exhibit potent antioxidant and anti-inflammatory properties, potentially counteracting the oxidative stress associated with alcohol metabolism [15]. Moreover, the hepatoprotective potential of unsweetened cocoa drink has also been explored in various oxidative stress-mediated experimental disease models, including alcohol and malaria [16,17,18], reinforcing unsweetened cocoa powder as a beneficial dietary intervention [19]. The prophylactic effect of cocoa on hepatocellular damage has been investigated [16], but the preservation of liver function and the mechanisms underlying cocoa-mediated protection have not been explored. This study, therefore, assessed the protective effects of unsweetened cocoa drink by assaying liver-related biochemical markers, performing histochemical staining, and measuring liver levels of oxidative stress-related variables (total antioxidant capacity and catalase activity).

MATERIALS AND METHODS

Study area

This study was conducted from February to July 2024 at the Animal House of the School of Biological Sciences and the laboratories of the Departments of Biomedical Sciences and Biochemistry at the University of Cape Coast, Ghana.

Preparation of UCP and ethanol

Five percent (5%) UCP drink was prepared by dissolving 5g of UCP (Golden Tree Royal Natural Cocoa Powder, FDA/DK 05-161) in 100 mL of warm distilled water. The warm UCP drink was allowed to cool to room temperature before administering to the rats orally using gavage. Thirty percent (30% v/v) ethanol was prepared from a stock of 96% ethanol by adding 330 mL of distilled water to every 150 mL of ethanol.

Animal grouping

Twenty healthy male Sprague-Dawley (SD) albino rats weighing 200–220 g were obtained from the animal holding facility of the School of Biological Sciences, University of Cape Coast, and acclimatised for 2 weeks. The rats were kept under ambient conditions of temperature 23 – 25°C, relative humidity (60 ± 4%), 12 h light / dark cycle and were provided with a standard pellet diet (Grower Mash, Essaar, Ghana) and water ad libitum. The animals, numbering 5 to 7, were kept in polycarbonate cages measuring 55 cm x 45 cm x 20 cm. The bedding for the animals was made from wood shavings and changed every 2 days. Ethical clearance was obtained from the Ethics and Protocol Review Committee of the University of Cape Coast (UCCIRB/CHAS/2024/90). The experimental procedures followed the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines) and were in full compliance with standard institutional, national, and international guidelines (Guide for the Care and Use of Lab Animals, NIH publication No. 85-23). Furthermore, the experimental design aimed to minimise animal use in accordance with the 3Rs (Replacement, Reduction, and Refinement) principle of animal research ethics (https://nc3rs.org.uk/who-we-are/3rs).

Treatment administration

The SD albino rats were acclimatised for 2 weeks, after which they were weighed and randomly assigned to the following groups: Group C (Control group): rats received neither UCP nor ethanol (N=5); Group X (UCP-only-fed group): rats were fed with 300 mg/kg UCP daily for 8 weeks (N=5); Group A (Ethanol only-fed group): rats were fed with 10 mL/kg 30% (v/v) ethanol daily for 8 Weeks (N=5); and Group XA (UCP-treated ethanol-fed group): rats were fed daily with 300 mg/kg UCP at 8:00 am and 10 mL/kg 30% (v/v) ethanol at 2:00 pm (N=5). The UCP and ethanol were administered orally via gavage to the rats at random. Ethanol was administered to the rats at a dose as described by Altayeb & Salem [20]. Throughout the experiment, the animals had unlimited access to tap water and chow. The rats were monitored daily for signs of distress, pain, or abnormal behaviour (e.g., reduced mobility, ruffled fur, weight loss >20%, or loss of grooming). These were defined as humane endpoint criteria, at which point animals would be humanely euthanised. At the end of the 8-week treatment, the rats were anaesthetised with pentobarbitone (150 mg/kg, i.p.) at random. Blood (5 mL) was obtained by cardiac puncture and collected into gel-separating tubes (4 mL) and citrate tubes (1 mL). The liver was harvested, briefly rinsed in physiological saline, and the large lobe of the liver was preserved in 10% buffered formalin (pH 7.29) for 6 days,

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after which it was used for histochemical staining. The remaining lobes of the liver were frozen at -20°C and later used for total antioxidant capacity and catalase assays.

Biochemical analysis of serum

The blood collected in the gel separator tubes was allowed to clot for 8 minutes, then centrifuged at 3000 rpm for 10 minutes to obtain the serum. All sera were transferred into Eppendorf tubes and 30 µL serum/test was aspirated into the analyser SelectraProXL (SN: 198423) to determine the levels of LFT (liver function test) biomarkers: AST (aspartate aminotransferase), ALT (alanine aminotransaminase), CRP (C-reactive protein), total protein, globulin, albumin, total bilirubin, unconjugated bilirubin, conjugated bilirubin. AST/ALT ratio was determined.

Haematological analysis

1.5 mL of blood was placed in a sodium citrate (3.2%) tube to measure prothrombin time using a Wondfo Fine Care analyser.

Histological assessment of the liver

Slices of the formalin-fixed liver were cut, dehydrated using increasing graded concentrations of ethanol, cleared in two changes of xylene and chloroform. Thereafter, the processed slice was infiltrated with molten wax and embedded to form blocks for sectioning using a Leica RM 2125 microtome (Germany). The tissue blocks were initially trimmed to a thickness of 10 µm to expose the entire tissue profile. The tissue was then sectioned at 5 µm. The first and tenth sections were picked for periodic acid schiff (PAS) staining, and the second and eleventh sections of the block were picked for lipofuschin staining. Photomicrographs of the liver were taken using an Amscope MD500 attached to an Olympus model CXC41 microscope. The photomicrographs underwent blinded assessment by a qualified histopathologist.

Semi-quantitation of glycogen storage by recovered hepatocytes

The glycogen content of hepatocytes was estimated as described by Boye et al. [21]. Photomicrographs of five fields devoid of portal triads or central veins were captured. Each photomicrograph was uploaded to an ImageJ console, and five subfields (upper right, upper left, lower right, lower left, and centre) were randomly selected using the duplicate function. Each subfield was used for the image analysis as follows. In ImageJ, each subfield was colour-deconvoluted and split into colour components (red, blue, and green) using the PAS-hematoxylin defined vectors. Under the edit function, the red component of the processed image was inverted. The inverted image was subjected to automatic thresholding, and the percentage threshold was recorded.

Total antioxidant capacity assay

The total antioxidant capacity of the liver was determined using the phosphomolybdenum method as described by Prieto et al. [22]. A reagent solution consisting of ammonium molybdate (4 mM), sodium phosphate (28 mM) and sulphuric acid (0.6 M) was prepared by mixing the components in a 1:1 ratio. Five hundred microlitres of plasma were mixed with 3 mL of the reagent solution in separate test tubes, and the reaction mixture was subsequently incubated at 95 °C for 70 min. A blank solution was prepared by mixing 0.5 mL of methanol with 3 mL of the reagent solution, then cooled to room temperature. The absorbance of the test mixture was measured at 695 nm against the blank using a spectrophotometer. 500 µL of various concentrations (20-200 µg/mL) of the standard, ascorbic acid, were mixed with 3 mL reagent solution, incubated at 95 °C for 70 min, and cooled. The absorbance was then determined against the blank solution, after which the absorbance (ordinate) was plotted against concentration (abscissa). The absorbance of the test plasma samples was interpolated on the standard line to generate the concentration of antioxidants; total antioxidant capacity (TAC) expressed in ascorbic acid (AscAE) equivalents.

Catalase activity test

One millilitre (1mL) of 4% ammonium molybdate was added to 0.1mL of liver homogenate, followed by the addition of 2mL of 0.01% hydrogen peroxide. The absorbance of the reaction mixture was measured spectrophotometrically at 374 nm [23].

Statistical Analysis

All data followed normal distribution and were presented as mean ± standard deviation (SD). An independent t-test was used to compare two groups, whereas One-way analysis of variance (ANOVA) with Bonferroni post hoc tests was used to compare among three or more experimental groups. GraphPad Prism (version 9) was used for statistical analysis, and $p < 0.05$ was considered statistically significant.

RESULTS

Total protein, Albumin and Globulin

Serum levels of total protein, albumin and globulin were determined to investigate the effect of UCP treatment on the biosynthetic functions of the liver in rats exposed to ethanol. Compared with the control, the mean serum total protein of the UCP-only-fed group was comparable ($p = 0.9239$), whereas that of the ethanol-only-fed group was significantly lower ($p = 0.0087$). Also, the mean serum total protein in the UCP-treated ethanol-fed group was higher than the ethanol only-fed group ($p = 0.0255$) and comparable to the UCP only-fed group ($p = 0.9999$) and the control group ($p = 0.7751$) (Figure 1A). Regarding albumin, the mean serum albumin concentrations of the UCP only-fed group and the ethanol only-fed group were respectively similar ($p = 0.9995$) and lower ($p = 0.0042$) compared to the control. The mean serum albumin concentration in the UCP-treated ethanol-fed group was higher compared to the ethanol only-fed group ($p = 0.0174$) and comparable to both the UCP only-fed group ($p =$

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0.9775) and control group (p = 0.9679) (Figure 1B). Mean serum globulin concentrations of the UCP-only-fed group and the ethanol-only-fed group were respectively comparable (p = 0.9995) and lower (p = 0.0238) than those of the control. Compared to the ethanol-only-fed group, the mean serum globulin concentration of the UCP-treated ethanol-fed group was higher (p = 0.0309), and that of the UCP-only-fed group was higher (p = 0.0466). Also, the UCP-treated ethanol-fed group was lower than that of the control group (p = 0.0403) (Figure 1C). These results suggest that ethanol ingestion impairs the synthetic function of the liver, but UCP, when ingested with ethanol, delays the decline in liver synthetic function. Moreover, UCP does not alter the synthetic function of the liver.

Prothrombin time

The mean prothrombin of the UCP-only-fed group was comparable to that of the control (p = 0.9994), corroborating the non-toxic effect of UCP on liver synthetic function. The mean prothrombin time of the ethanol-only-fed group was higher compared to the control group (p = 0.0187), suggesting that ethanol could impair the synthesis of clotting factors. However, the mean prothrombin time in the UCP-treated ethanol-fed group was lower compared to the ethanol-exposed group (p = 0.0017) and similar to that of both the UCP only-fed group (p = 0.4778) and the control group (p = 0.2627) (Figure 1.D). These results suggest that UCP, when taken with ethanol, could ameliorate the synthesis of clotting factors.

Total, conjugated and unconjugated bilirubin

The UCP-treated ethanol-fed group showed higher mean serum total bilirubin (p = 0.0013) and unconjugated bilirubin (p < 0.001), but comparable conjugated bilirubin (p = 0.0714) compared with the control, suggesting that UCP may increase serum unconjugated bilirubin levels. The ethanol-only-fed group recorded higher serum total bilirubin (p = 0.0032) and unconjugated bilirubin (p < 0.001), but lower conjugated bilirubin (p = 0.0001) than the control, suggesting that ethanol ingestion elevates serum levels of unconjugated bilirubin and impairs the conjugation of bilirubin. This indicates that ethanol ingestion impairs the metabolic function of the liver. However, compared with the ethanol-only-fed group, the UCP-treated ethanol-fed group had comparable serum total bilirubin (p = 0.9504), but higher serum conjugated bilirubin (p = 0.0018) and lower serum unconjugated bilirubin (p = 0.010). This suggests that UCP ingestion promotes the conjugation of bilirubin and thus could ameliorate the ethanol-induced metabolic dysfunction. Also, the UCP-treated ethanol-fed group recorded a comparable serum total bilirubin (p = 0.6532), conjugation bilirubin (p > 0.9999) and lower unconjugation bilirubin (p = 0.013) compared to UCP only-fed group (Figure 1E – G).

AST/ALT ratio and CRP

The serum AST/ALT ratio (also known as the De Ritis ratio) and CRP levels were measured to assess the effect of UCP treatment on liver inflammation in rats exposed to ethanol. A higher AST/ALT ratio together with an elevated CRP level is suggestive of hepatic inflammation [24,25]. The mean AST/ALT ratio of the ethanol-only-fed group was greater than 2, whereas the other groups recorded values less than 2 (Figure 1H). The mean serum AST/ALT ratio of the UCP-only-fed group was comparable to that of the control (p = 0.5165). The mean serum AST/ALT ratio was higher in the ethanol-only-fed group compared to the control (p = 0.0024), suggestive of alcoholic liver disease. The UCP-treated ethanol-fed group recorded a lower AST/ALT ratio (p = 0.0005) compared to the ethanol-only-fed group, suggesting that UCP ameliorates alcoholic liver disease. Also, the AST/ALT ratio of the UCP-treated ethanol-fed group was lower than that of the UCP only-fed group (p = 0.0372) and comparable to the control group (p = 0.2764) (Figure 1H). These results suggest that UCP could prevent the occurrence of alcoholic liver disease.

The mean serum CRP of the UCP-only-fed group was lower than that of the control group (p = 0.0126), suggesting that UCP could attenuate hepatic inflammation. The mean serum level of CRP was elevated, though not statistically significant, in the ethanol-only-fed group compared to the control (p = 0.0623). The UCP-treated ethanol-fed group recorded lower serum CRP levels than the ethanol-only-fed group (p = 0.0416), suggesting that UCP ingestion could attenuate hepatic inflammation. Again, the mean serum CRP of the UCP-treated ethanol-fed group was similar to that of the UCP-only-fed group (p = 0.1438) and the control group (p = 0.5080) (Figure 1I). These results suggest that UCP does not induce hepatic inflammation.

Liver histology

Liver tissues of rats in the control group demonstrated normal plates of polygonal hepatocytes separated by sinusoids. Compared with the control, the hepatocytes from the UCP-only-fed group showed no vesicular fatty acid accumulation and no glycogen deposition around the central vein. Compared to the control group, the hepatocytes from the ethanol-only-fed group demonstrated vesicular fatty, indicative of steatosis. Also, there was glycogen deposition in the central vein. However, relative to the untreated ethanol-exposed rats, the UCP-treated ethanol-fed rats exhibited hepatocytes with minimal steatosis, PAS-positive Kupffer cells in the sinusoids and attenuated deposition of glycogen around the central vein. The UCP-treated ethanol-fed group exhibited more and enlarged PAS-positively stained Kupffer cells in the sinusoids compared to the UCP-only-fed group [Figure 2].

Glycogen storage in the liver

The semi-quantitative analysis of PAS-stained liver sections showed that glycogen content in hepatocytes from the ethanol-only-fed group was lower than in the control group (p = 0.002). The UCP-treated ethanol-fed group exhibited a higher hepatocyte glycogen content compared to the ethanol-only-fed group (p < 0.001) and the UCP-only-fed group (p < 0.001). Thus, UCP ingestion may

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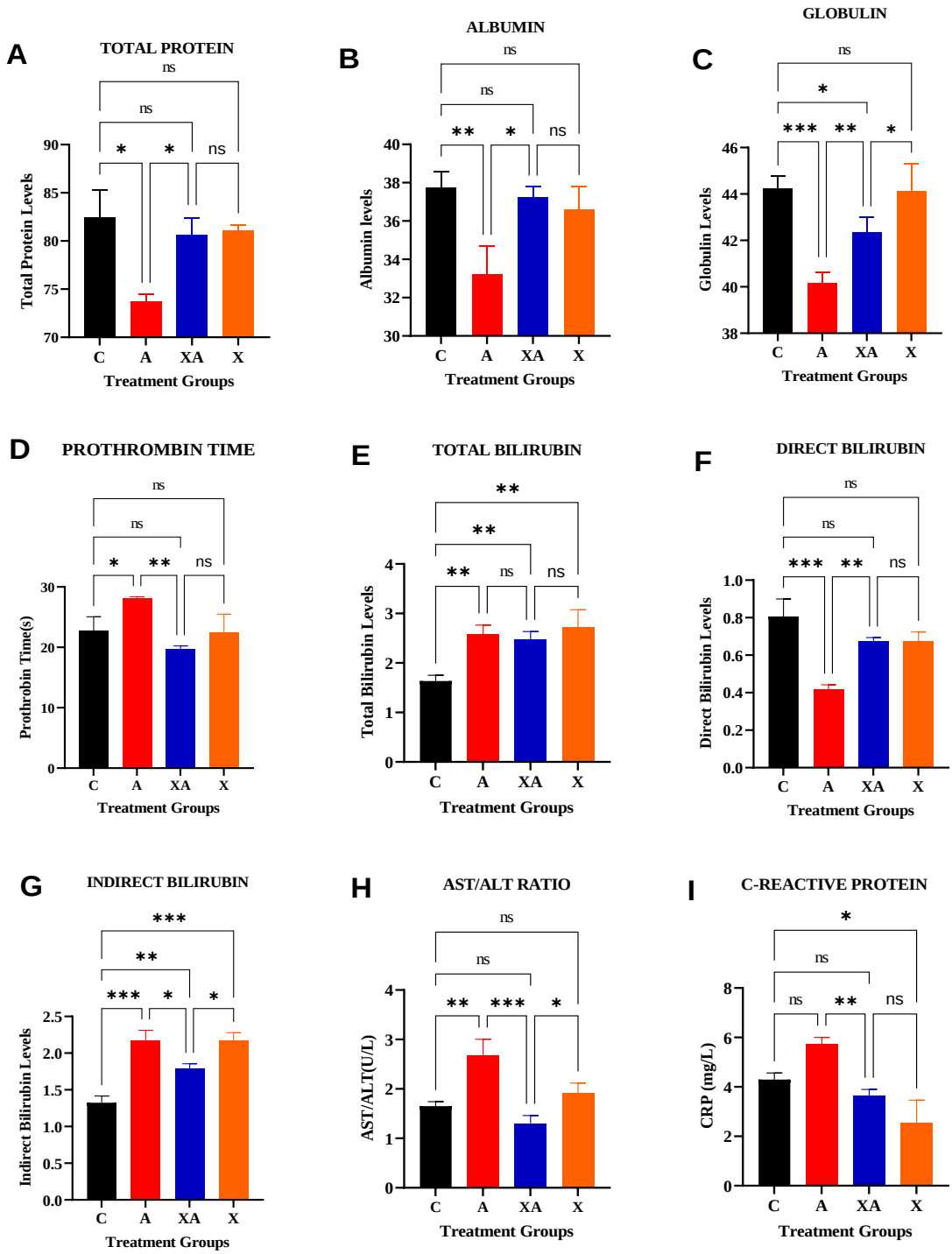

Figure 1. Serum concentration of synthetic and metabolic functions-related liver markers in experimental and control rats. Treatment groups are (C) control group, (A) Ethanol only-fed group, (XA) UCP-treated ethanol-fed group and (X) UCP only-fed group. The symbol * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ and ns represents $p > 0.05$.

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attenuate the ethanol-induced glycogen loss. The glycogen content of hepatocytes from the UCP-treated, ethanol-unexposed group, however, was lower than that of the control.

Liver total antioxidant capacity (TAC) and catalase activity

The liver TAC in the UCP-only-fed group was comparable to that in the control group ($p = 0.9790$), suggesting that UCP does not alter antioxidant levels. However, the liver TAC in the ethanol-only-fed group was higher than in the control ($p = 0.009$), suggesting higher antioxidant levels in the ethanol-only-fed group. The TAC for the UCP-treated ethanol-fed group was higher compared to the ethanol-only-fed group ($p < 0.001$) and the UCP-only-fed group ($p < 0.001$) (Figure 4B). These results suggest that UCP ingestion may enhance antioxidant levels in ethanol-induced increase in antioxidant levels. To partly explain the increase in TAC, the hepatic levels of catalase (an enzymatic antioxidant that metabolises ethanol to acetylaldehyde) were assessed [29]. The catalase absorbance values are inversely proportional to the enzyme level [29]. Compared to the control, the UCP-only-fed group exhibited comparable absorbance in the liver catalase assay ($p = 0.300$), suggesting that unsweetened cocoa drink does not alter liver catalase levels. The absorbance for the liver catalase assay from the ethanol-only-fed group was higher than that of the control ($p < 0.001$), suggestive of low liver catalase levels in the ethanol-only-fed group. The liver from the UCP-treated ethanol-fed group had a lower absorbance for liver catalase activity compared to that of the ethanol only-fed group ($p < 0.001$) and the UCP only-fed group ($p < 0.001$), suggestive of higher catalase levels in the UCP-treated ethanol-fed group and the UCP only-fed group (Figure 4C).

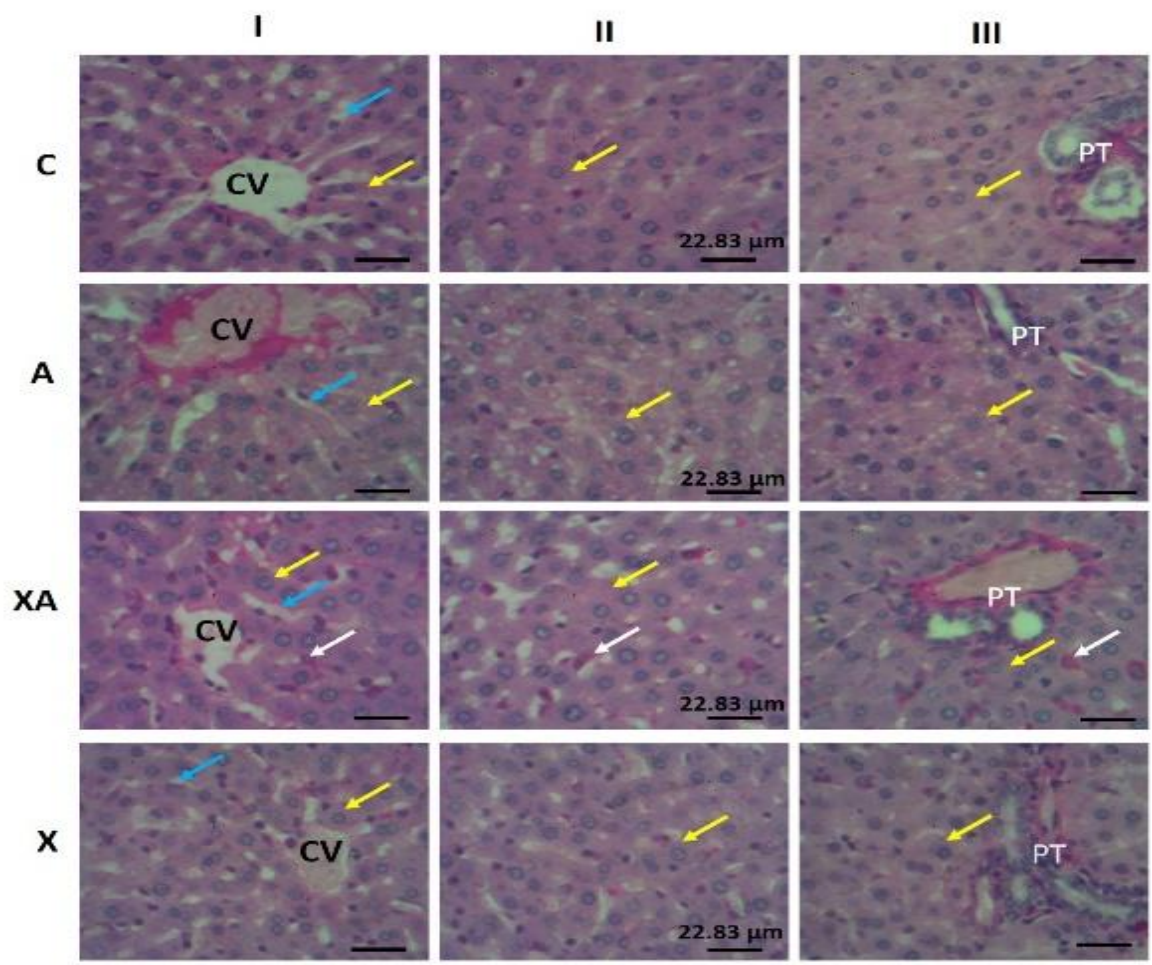

Figure 2. A histological micrograph showing the effects of UCP and alcohol treatment on Periodic Acid Schiff- (PAS-) stained liver parenchyma. Treatment groups are (C) control group, (A) Ethanol only-fed group, (XA) UCP-treated ethanol-fed group and (X) UCP only-fed group. (I) Centrilobular region; (II) Region between centrilobular and periportal regions; (III) Periportalregion. Blue, yellow and white arrows represent sinusoids, hepatocytes and Kuppfer cells, respectively. CV and PT represent central vein and portal triad, respectively. Scale bar is 1 cm = 22.83 µm

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DISCUSSION

This study assessed the protective effects of an unsweetened cocoa drink in an ethanol-ingesting rat model by assaying serum levels of liver-related biochemical markers, using histochemical staining, and assessing the levels of oxidative stress-related variables (total antioxidant capacity and catalase activity) of the liver. The findings of this study suggest that the ingestion of UCP alongside ethanol attenuates hepatocellular damage and preserves hepatic function, possibly by mitigating oxidative stress and inflammation. The synthetic functions of the liver can be assessed by quantifying the serum levels of total protein, albumin and globulin, and the blood's prothrombin time (which is inversely related to the presence of clotting factors) [30]. In this study, ethanol ingestion reduced serum total protein, albumin, and globulin, suggesting a reduction in liver synthetic function [31]. Consistently, ethanol ingestion caused an increased prothrombin time in blood, potentially due to reduced serum levels of clotting factors.

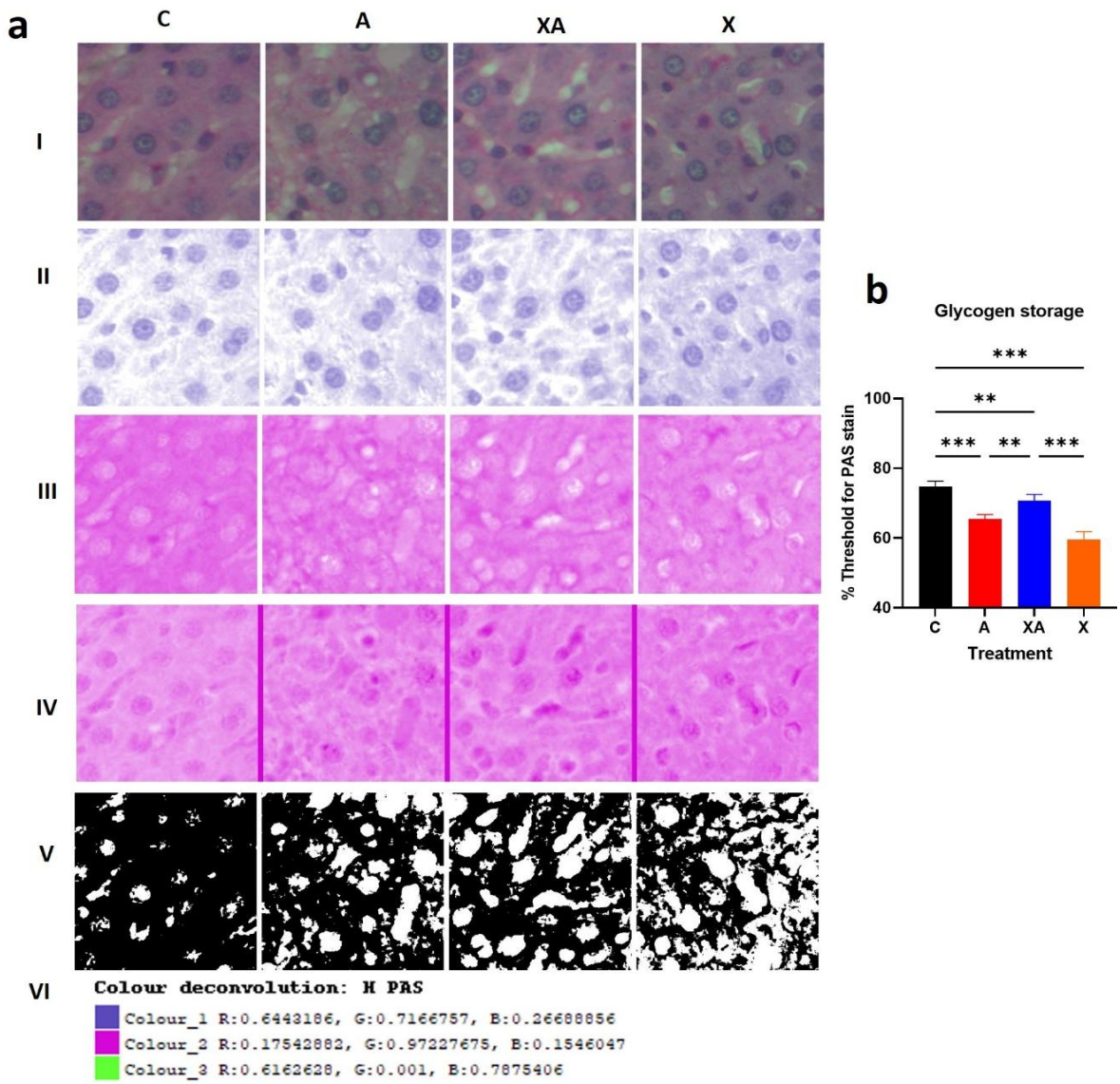

Figure 3. Semiquantitative analysis of glycogen content of hepatocytes in PAS-stained liver of ethanol and/or UCP-fed rats and control. Treatment groups are (C) control group, (A) Ethanol only-fed group, (XA) UCP-treated ethanol-fed group and (X) UCP only-fed group (a) Image processing workflow of periodic acid-Schiff (PAS) stained liver photomicrographs for glycogen storage. (b) is the percentage threshold for glycogen storage content in each group. I is the original image, III and II are the processed image of hematoxylin and PAS respectfully, after color deconvolution using VI as a definitive vector. IV is the inverted image of III. V shows area of glycogen storage within the liver sections. The symbol * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ and ns represents $p > 0.05$.

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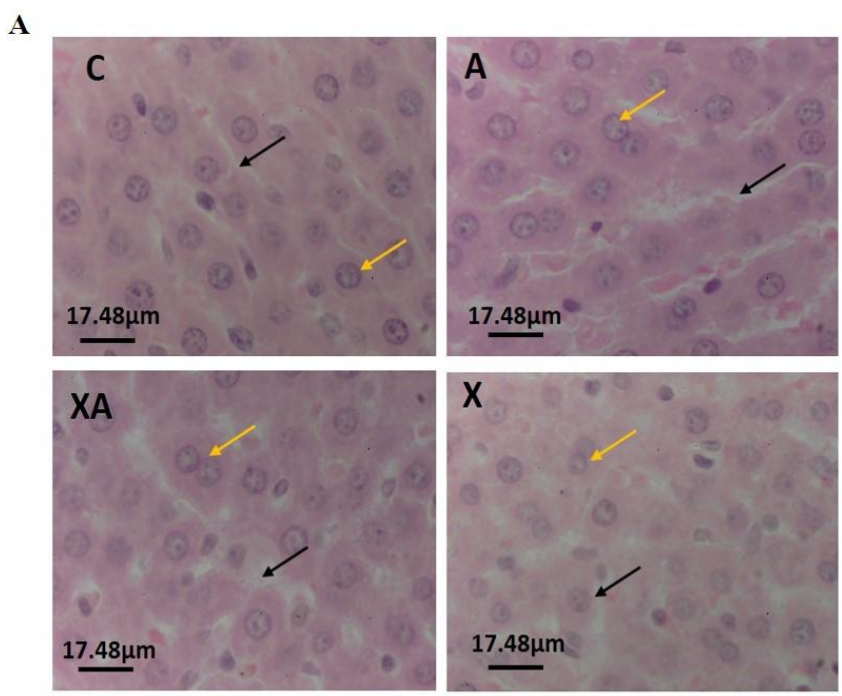

Figure 4. Histology of lipofuscin accumulation (Schinoris method) and Serum concentration of total antioxidant capacity and catalase activity after 8 weeks of administration. Treatment groups are (C) control group, (A) Ethanol only-fed group, (XA) UCP-treated ethanol-fed group and (X) UCP only-fed group. The symbol * represents $p < 0.05$, ** represents $p < 0.01$, *** represents $p < 0.001$ and ns represents $p > 0.05$.Black arrows represent sinusoids, and yellow arrows represents hepatocyte

However, the ingestion of unsweetened cocoa drink alongside ethanol increased serum levels of total protein and albumin and lowered the prothrombin time to a level comparable to that of the control. Serum globulin levels also increased with the ingestion of UCP alongside ethanol but the levels were lower than those of the control. This result suggests that UCP could not have wholly mitigated the ethanol-induced reduction in serum globulin levels. Overall, these results suggest that UCP, when taken along with ethanol, delays the decline in liver function.

Additionally, ingestion of only UCP did not alter serum levels of total protein, globulin or albumin, as well as the prothrombin time, suggesting that an unsweetened cocoa drink may not alter the synthetic functions of the liver. The available literature reports that ethanol ingestion decreases the synthesis of liver proteins [31,32], which may explain the low serum levels of these proteins; however, a previous study indicates that ethanol ingestion enhances the synthesis of liver proteins. However, impairments in the export of proteins to the plasma result in low serum protein levels [33], but neither the expression of the proteins nor their export to the plasma was investigated in this study. These mechanisms could be explored in future studies to explain the protective effect of UCP against ethanol-induced liver damage. Glycogen storage in the liver is known to be affected during alcohol metabolism [26,27]. Ethanol is oxidised to acetate in a two-step process catalysed by the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) using NAD (nicotinamide adenine dinucleotide) as a cofactor. This process results in the accumulation of NADH, lowering the ratio of NAD+/NADH in the mitochondria. The low NAD+/NADH ratio inhibits gluconeogenesis, reducing glucose output from the liver. As gluconeogenesis slows, glycogen stores are not replenished, leading to low hepatic glycogen. Glycogen content of the hepatocytes in a PAS-stained liver can be semi-quantitatively assessed [21].

In this study, ethanol ingestion reduced the glycogen content of the hepatocytes. However, ingestion of UCP alongside ethanol further promoted glycogen accumulation, suggesting that cocoa could preserve the liver's storage function. Another effect of the low NAD+/NADH ratio is a

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reduction of β-oxidation in the mitochondria, resulting in the accumulation of lipids in the hepatocytes, a condition known as steatosis [29,34]. In this study, stained liver sections from untreated ethanol-fed rats showed accumulation of fat in both centrilobular and periportal hepatocytes, indicative of steatosis. However, ingestion of UCP alongside ethanol minimised fat accumulation, suggesting attenuated steatosis.

Additionally, in this study, ingestion of UCP alone decreased glycogen content of the hepatocytes and was not accompanied by lipid accumulation in either centrilobular or periportal hepatocytes, suggesting that cocoa ingestion may promote glycogenolysis and/or inhibit gluconeogenesis and lipogenesis in the liver. In one study, cocoa flavonoids reduced gluconeogenesis by downregulating gluconeogenic enzyme PEPCK (phosphoenolpyruvate carboxykinase) in HepG2 cells [35]. Another study reported that cocoa supplementation attenuated hepatosteatosis in a rat model of non-alcoholic steatohepatitis (NASH) by reducing LFABP (liver fatty acid-binding protein) expression [36]. Further study can assess the expression of these molecules to explain the effect of cocoa on glycogen and lipid accumulation.

In this study, ethanol ingestion elevated serum total and unconjugated bilirubin but reduced conjugated bilirubin compared with the control, as observed in other studies [37]. These observations suggest impaired bilirubin conjugation and metabolism. The ingestion of UCP alongside ethanol, however, did not alter serum total bilirubin as observed in ethanol-ingesting rats, but did raise serum conjugated bilirubin and decrease unconjugated bilirubin, suggesting that UCP ingestion may promote the conjugation of bilirubin and thus enhance the restoration of liver metabolic functions. Compared with the control, ingestion of an unsweetened cocoa drink alone increased serum levels of total and unconjugated bilirubin but did not alter conjugated bilirubin. While these observations suggest increased hemolysis, impaired bilirubin uptake, and/or inefficient bilirubin conjugation, the available literature reports that bilirubin exerts protective roles, such as antioxidant [38] and anti-inflammatory [39], and protects the endothelium [40].

The increase in serum levels of unconjugated bilirubin could be a protective mechanism conferred by UCP. Hepatocellular damage can be assessed by measuring serum levels of liver enzymes, AST and ALT, which are primarily involved in amino acid metabolism. The ratio of AST/ALT is of diagnostic significance in alcoholic liver disease [41], and has been emphasised in the Practical Guidelines forAlcoholic Liver Disease, by the American College of Gastroenterology [42]. An AST/ALT ratio≥ 2 indicates advanced liver disease. In this study, ethanol ingestion increased the serum AST/ALT ratio, suggestive of hepatocellular damage. Ingestion of unsweetened cocoa drink alongside ethanol, however, reduced the serum AST/ALT ratio, suggesting that cocoa may help prevent hepatocellular damage. In this study, ethanol ingestion increased serum CRP levels, suggesting hepatic inflammation. The ingestion of unsweetened cocoa drink, however, reduced serum CRP levels, suggesting that cocoa could mitigate ethanol-induced hepatic inflammation. Compared with the control, ingestion of an unsweetened cocoa drink alone reduced CRP levels and did not alter the AST/ALT ratio, suggesting that unsweetened cocoa drink ingestion may reduce liver inflammation. The microsomal ethanol-oxidising system (MEOS), which involves the enzyme cytochrome P450 2E1 (CYP2E1), becomes more active and contributes to the production of reactive oxygen species (ROS), which can damage liver cells, including the DNA [43]. In this study, hepatic total antioxidant capacity, catalase activity, and lipofuscin were estimated. Formed by the accumulation of oxidised proteins and lipids, lipofuscin is a known oxidative stress biomarker [44,45].

In this study, ethanol ingestion increased hepatic lipofuscin levels. However, ingestion of cocoa alongside ethanol resulted in moderate lipofuscin accumulation, suggesting that cocoa ingestion could attenuate ethanol-induced oxidation. In the study, ethanol ingestion increased the total antioxidant capacity of the hepatocytes, relative to the control. This suggests that antioxidant levels in hepatocytes were elevated upon ethanol ingestion, possibly to mitigate oxidative stress. To partly explain the increase in TAC, the hepatic levels of catalase (an enzymatic antioxidant that metabolises ethanol to acetylaldehyde) were assessed [40]. It was found that hepatic catalase levels were decreased upon ethanol ingestion. However, cocoa ingestion alongside ethanol further increased the liver's total antioxidant capacity, suggesting that cocoa ingestion enhanced the antioxidant levels in the liver. Cocoa ingestion alongside ethanol, however, maintained higher liver catalase levels, and hence, possibly enhanced alcohol metabolism. Other enzymatic and non-enzymatic antioxidants exist and could be assessed in future studies to explain the changes in TAC. Also, compared with the control, ingestion of unsweetened cocoa drink alone did not alter liver TAC or catalase levels, suggesting its non-toxic effects.

Compared to the control, the staining intensity of lipofuschin in the hepatocytes from rats fed with only unsweetened cocoa drink appeared less (though not quantified), suggestive of lower oxidative stress in the hepatocytes. These observations corroborate the protective effects of an unsweetened cocoa drink. Alcohol increases gut permeability, leading to elevated levels of serum endotoxins containing lipopolysaccharides (LPS) [46,47]. LPS binds to LPS-binding proteins and is presented to a membrane glycoprotein called CD14, which then activates Kupffer cells via the toll-like receptor 4. This endotoxin-mediated activation of Kupffer cells leads to the production of cytokines and chemokines and the generation of reactive oxygen species, contributing to oxidative stress [48,49]. In this study, mild Kupffer cell activation was observed in untreated ethanol-fed rats compared with controls.

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However, the liver from UCP-treated ethanol-fed rats exhibited Kupffer cells with increased glycogen accumulation compared to the liver tissue from the ethanol-only-fed rats, suggestive of the cell's involvement in cocoa-conferred protection. Nonetheless, the role of the Kupffer cells in cocoa-mediated liver protection was not assessed in the study. Also, in this study, the treatment of normal rats with unsweetened cocoa drink increased the number of glycogen-laden Kupffer cells, whilst the hepatocytes exhibited low glycogen content. This observation suggests that, in ethanol-fed rats, an unsweetened cocoa drink may activate Kupffer cells to phagocytose glycogen, thereby attenuating glycogen accumulation in hepatocytes. In conclusion, UCP ingestion alongside ethanol attenuated oxidative stress and minimised hepatic inflammation, and may have activated Kupffer cells to protect against ethanol-induced hepatocellular damage and preserve liver function.

This study could not explore the mechanisms underlying the key observations recorded. For example, the discussed role of Kupffer cells as an interferon source is speculative. Also, regarding the hepatic catalase levels, the mechanisms (such as gene expression and cellular degradation of catalase) that could explain the variations in catalase levels were not studied. Furthermore, hepatic levels of "total antioxidant capacity" depend on reactive oxygen species and antioxidant levels. The study did not assess levels of reactive oxygen species or other antioxidants, such as superoxide dismutase and glutathione, to fully explain the variations in total antioxidant capacity. Again, the study only assessed the glycogen levels semi-quantitatively; however, the mechanisms, such as glycogenolysis, glycogenesis and gluconeogenesis that could explain the glycogen levels were not assessed. These limitations will be addressed in future studies.

Conclusion
The ingestion of unsweetened cocoa drink alongside ethanol attenuates hepatocellular damage and preserves hepatic function, possibly by mitigating inflammation and oxidative stress.

DECLARATIONS

Ethical consideration
Ethical clearance was obtained from the Ethics and Protocol Review Committee of the University of Cape Coast (UCCIRB/CHAS/2024/90).

Consent to publish
All authors agreed on the content of the final paper.

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Competing Interest
The authors declare no conflict of interest

Author contribution
EAA and BA conceptualised the research and reviewed and approved the final manuscript. AAA and FT drafted the initial manuscript. FT analysed the data and made inferences. MA performed the liver biochemical assays. JF and BSA assessed liver glycogen storage, lipofuscin staining, and PAS-stained sections. AFW conducted the antioxidant experiments. JBA-Y and DOK administered ethanol and/or UCP to the rats. All authors read and approved the final manuscript.

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Availability of data
Data is available upon request to the corresponding author

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