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Voluntary Ingestion of Natural Cocoa Extenuated Hepatic Damage in Rats with Experimentally Induced Chronic Alcoholic Toxicity

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ABSTRACT:

Background: Chronic ethanol ingestion causes hepatic damage imputable to an increased oxidative stress engendered by alcoholic toxicity. Polyphenols in cocoa have antioxidant properties, and natural cocoa powder (NCP) contains the highest levels of total antioxidant capacity when compared to all other kinds of edible cocoa products. This study tested the hypothesis that dietary supplementation with NCP mitigates hepatic injury resulting from chronic ethanol consumption. Three groups of eight randomized Sprague-Dawley rats were fed standard rat food and treated daily for 12 weeks as follows: (i) the Ethanol-water group was given unrestricted access to 40% (v/v) ethanol for 12 hours (at night) followed by water for the remaining 12 hours (daytime), (ii) the Ethanol-cocoa group had similarly unrestricted access to 40% ethanol for 12 hours followed by 2% (w/v) NCP for 12 hours, and (iii) the control group was not given alcohol and had unrestricted access to only water which was synchronously replenished every 12 hours as it was for the ethanol treated animals.

Results: Qualitative structural liver damage evidenced by hepatocyte cytoplasmic fatty accumulation, nuclear alterations, and disruption of general liver micro-architecture, was severe in the ethanol-water group when compared with the ethanol-cocoa group of rats. Design-based stereologic assessment yielded a significantly greater volume (Tukey's HSD, p = 0.0005) of undamaged hepatocytes (9.61 ml, SD 2.18 ml) in the ethanol-cocoa group as opposed to the ethanol-water group of rats (2.34 ml, SD 1.21 ml). Control rats had 10.34 ml (SD 1.47 ml) of undamaged hepatocytes, and that was not significantly greater (Tukey's HSD, p=0.659) than the value for the ethanol-cocoa group of rats. Relative to controls, therefore, histomorphometry

showed 93% hepatocyte preservation from alcoholic injury in rats that voluntarily imbibed NCP suspension compared with 23% in animals that drank water.

Conclusions: Ethanol-induced structural liver injury was qualitatively and quantitatively milder in rats which chronically imbibed alcohol then afterward drank NCP beverage in place of water. The antioxidant and anti-inflammatory properties of polyphenols in NCP are postulated in mitigating the damage of rat liver due to chronic ethanol consumption. Thus, it is suggested from these findings that regular drinking of NCP beverage may slow progression of alcoholic liver disease in dipsomaniacs.

Key words: natural cocoa powder, chronic alcoholic toxicity, total antioxidant capacity, polyphenols

BACKGROUND:

Substantial experimental and clinical evidence support a causative role of oxidative stress in the pathophysiological processes of liver injury [1, 2] resulting from excessive alcohol ingestion [3]. The liver is most liable to the damaging effects of chronic alcohol consumption because it is the site of alcohol metabolism via a cascade of biochemical processes [4]. When the metabolic pathway for alcohol is overly taxed, an excessive amount of reactive oxygen species (ROS) is generated [5] which causes oxidative stress consequent to the compromised endogenous antioxidant capacity of hepatocytes [6]. Being the parenchymal cells which make up the bulk of liver structure, hepatocytes are particularly susceptible to the oxidative and inflammatory challenges of both acute and chronic ethanol toxicity [3]. The ultimate damage of hepatocytes due to ethanol metabolism validates concomitant poor growth indices and impairment of liver function in humans and experimental animals [7]. Given the integral involvement of xenobiotic pathways in amplifying the debilitating effect of alcohol on the liver, interventions suggested that to fend off the vulnerability of the liver to oxidative challenges during alcohol consumption one must reinforce the endogenous antioxidant defense systems [6, 8, 9]. Liver damage due to alcoholic toxicity includes elevated serum alanine transaminase (ALT) and aspartate transaminase (AST) levels [10,11]. Other workers [13] have noted that hepatic damage is also characterized by markedly elevated levels of alanine transaminase (ALT), aspartate transaminase (AST) and bilirubin, coupled with a marked hepatic oxidative stress.

Cocoa contains polyphenols, which are beneficial compounds found in many fruits, vegetables, teas, and wines [14]. The polyphenols found in cocoa are called flavanols, which have potent antioxidant properties. These polyphenols also include catechins, epicatechins, and procyanidins [15], which are known to exert anti-inflammatory effects [16]. Epidemiological studies and anecdotal observations have primarily ascribed the health benefits of cocoa consumption to its high antioxidant content. Notable among these many health benefits are cocoa's hypothesized antimalarial prophylactic effect [17]. Cocoa consumption also plays a preventative role in cardiovascular protection [14] and also against tumoural and carcinogenic processes [18, 19]. The modulation of oxidative stress by cocoa polyphenols has been

demonstrated in many experimental models. Furthermore, different mechanisms of activity have been established within this main effect for both cocoa products and for isolated flavanols [20, 21].

The antioxidant properties of flavanols are ascribed partly to their structural characteristics, including the oligomeric chain length and the stereochemical features of the molecule. These structural characteristics of flavanols represent the molecular basis for their radical-scavenging (antioxidative) property [22, 23]. Long term feeding studies of flavanol-rich cocoa showed an increase in total plasma antioxidant capacity and a reduction in susceptibility to oxidative injuries [14]. What's more, cocoa powder and it's extracts have been shown to exhibit a greater antioxidant capacity than many other flavanol-rich foods and food extracts such as green and black tea, red wine, blueberries, garlic, and strawberry *in-vitro* [24].

This study assessed the benefit of natural cocoa powder (NCP) when given as a regular dietary supplement, in preventing liver damage due to chronic ethanol consumption. In the end, we reported that alternating (12-hourly) voluntary ingestion of 40% alcohol and 2% aqueous suspension of NCP for 12 weeks significantly attenuated structural liver damage in experimental rats. This was proven by a 93% preservation of total hepatocytes from an alcohol-induced injury when compared to only 23% in the livers of rats that drank water without NCP.

MATERIALS AND METHODS:

Acquisition and Treatment of Animals: This experimental study was approved by the Ethical and Protocol Review Committee of the University Of Ghana Medical School (UGMS) (reference number MS-Et/M.5-P.2/2008-09). Twenty-four male adult Sprague Dawley rats which were between 12–14 weeks old and weighed 210–270 g, were purchased from the Animal Experimentation Department of the Noguchi Memorial Institute for Medical Research (NMIMR) in Legon, Accra. Female rats, being more susceptible to alcoholic hepatotoxicity [25], were excluded because studies have indicated that they were likely to die before chronic effects became evident. The rats were kept in the animal experimentation laboratory of NMIMR throughout the study under the laboratory conditions of temperature (22 ± 2°C), relative humidity (70 ± 4%), and a 12 hour light/dark cycle. The animals were provided tap water for drinking and given nutritionally-standard rat food pellets obtained from Ghana Agro Food Company Limited (GAFCO), (Tema, Ghana). Procedures involving the care and use of the animals were in compliance with national and international laws and guidelines for the use of animals in biomedical research.

Following one week acclimation, the rats were put into three major weight–matched randomized [26] groups of eight. The average weights per group prior to any treatment were 239.4 (SD, 15.0) g; 240.0 (SD, 13.9) g; and 240.5 (SD, 17.9) g for groups 1, 2, and 3 respectively. There were no significant differences between groups when their mean weights were compared using one-way ANOVA (df = 2, F = 0.01, p = 0.990). On account of limited facilities for individual handling, rats in each group were placed into two groups of four animals per cage (39 cm × 26 cm × 20 cm).

Experimental protocol: One group of rats (designated ethanol-water fed or EWF group) was given unrestricted access to 200 ml of clean tap water for 12 hours during the daytime (6.00 – 18.00 hours GMT). The water was replaced with 200 ml of 40 % (v/v) ethanol [27] for the following 12-hour period (i.e., 18.00 – 6.00 hours GMT) daily. A second experimental group of rats (designated ethanol-cocoa fed or ECF group) had free access to 200 ml of a 2 % (w/v) aqueous suspension of NCP drink for 12 hours during the day (6.00 – 18.00 hours GMT), followed by 200 ml of 40 % (v/v) ethanol drink for the next 12-hour period daily. A third group (designated control or C group) had unrestricted access to 200 ml of tap water in two 12-hourly changes synchronous with fluid changes for the alcohol-treated animals. The control rats were therefore given neither cocoa nor ethanol. All three groups of rats continued to have free access to nutritionally standard GAFCO rat food pellets *ad libitum*. The regimen followed in the treatment of the animals is shown in Fig. 1. The respective daily amounts of water and ethanol drank by the rats were measured. Each individual rat was weighed using an animal weighing balance (Animal scale, Shinano TS-872, TGC, Japan) at the commencement and termination of the experiment.

Preparation of 2% (w/v) aqueous NCP and 40% alcoholic drink: A suspension of GoodFood® unsweetened natural cocoa powder obtained from Kakawa Enterprise Ltd., Accra, Ghana (batch number KK0904A) was freshly prepared daily by weighing out 8.0 g on a chemical weighing balance (Mettler Teledo P1200, Switzerland) and then thoroughly dissolving it in 400 ml of boiling-hot tap water. With alcohol withdrawal every morning, the freshly prepared and cooled 2 % (w/v) aqueous NCP was placed in the cages of the ECF rats. The NCP particles had a tendency to sediment, necessitating intermittent agitation which made night administration in this study impractical. The volume of NCP suspension ingested by the rats was determined every evening before replacement with ethanol. Forty percent ethanol solution was prepared daily by appropriately diluting absolute ethanol (99.5 % [v/v]) (Sigma Chemical Company, Germany). The volume of alcoholic drink consumed daily by each rat was determined.

Blood sampling and serum preparation: At commencement (baseline) and 12 weeks (termination) of experimentation, blood samples were collected from each rat to prepare serum for biochemical determination of their liver function. Approximately 1.5 ml of blood was collected via rat tail snipping and gently milking into plain sample bottles with inert separating gel (Vacuette {4ml}, Z Serum Sep Clot Activator, Austria). The blood collected was allowed to stand at room temperature (26 ^{o}C) for 30 minutes to facilitate clotting and to aid serum preparation. Tubes containing blood samples were centrifuged for 10 minutes at 3000×g to obtain serum. The serum was decanted by pipetting into Eppendorf tubes (101 – 1000 μl) and kept on ice for immediate biochemical analyses.

Biochemical determination of liver function: Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assessed in rats as biomarkers of liver function. The analyses were done with a fully automated biochemical analyzer (Flexor E, Vita scientific,

Netherlands) at NMIMR. The ratio of AST: ALT was calculated and used to evaluate the extent of liver toxicity in all treatment groups.

Harvesting rat liver: Rats were transported from the animal experimentation unit of NMIMR to the Histology Laboratory at the University Of Ghana Medical School, Korle Bu, where they were sacrificed using established laboratory methods in order to harvest their livers. Each rat was weighed and its blood sample was collected prior to being anaesthetized with chloroform (BDH Chemicals Limited, Poole, England). Then, the rats were killed by standard protocol of cardiac perfusion fixation using 10% buffered formalin. Finally, when the liver was dissected out, its absolute volume was determined by fluid displacement [28, 29].

Measurement of hepatosomatic index: The gross liver weight of each rat was determined using a Mettler P1200 (Switzerland) weighing balance immediately after the freshly harvested liver was blotted dry. A hepatosomatic index (HSI) was computed by expressing the liver weight as a percentage of the rat's body weight [30] at the termination of the experiment.

Multistage sampling of rat liver for histomorphometry: Each liver was sampled using a multistage systematic random technique (Fig. 2). Firstly, an improvised fractionation grid with lines 5.0 mm apart was designed for use in this study. The liver of each rat was carefully separated into the right, middle, left and papillary lobes [30]. Starting with the right lobe, followed by the middle, left and papillary (quadrate) lobes, the liver tissues were sliced with a disposable microtome blade into 5.0 mm thick slabs as indicated by a pilot study and the fractionation grid. All the lobes were consistently oriented and fractionated on the grid using a common approach. The liver lobes were fractionated in turns and every third slice was selected, starting from a random starting point (determined by lottery) on the right lobe and working progressively to the papillary lobe. At this first sampling stage, 8–10 slices were obtained from each whole liver.

At the next stage of sampling, the slices were placed across the fractionation grid lines and cut in turns (at right angles to their original orientation) into blocks. These liver blocks were then sampled by picking every 11^{th} block. The first block was randomly selected and every 11^{th} block was picked. By this means, three liver blocks were sampled from each whole rat liver. Because of the unidirectional method of fractionation and sampling, the three final liver blocks sampled were from at least three of the four rat liver lobes. Variation between sampled blocks from each liver frequently came from an exclusion of either the left, or the papillary lobe. In total, 72 blocks were sampled from the 24 rat livers.

The blocks of liver tissues were taken through standard histological processing protocols in order to obtain the appropriate sections necessary. Paraffin-embedded blocks of liver were sectioned at 5.0 μm thickness using a rotary microtome. From a random starting point, a total of 10 sections were systematically sampled by picking every other 20^{th} section from each tissue block, with the distance between successive sections being 100 μm. These liver sections were then placed onto 25.4 mm × 76.2 mm glass slides, 1.0 mm-1.2 mm thick (All Pro, Middlesex,

England) prior to being stained with Haematoxylin and Eosin (H & E) and mounted in DPX (BDH, Poole, England).

The next stage of sampling used the liver sections that were previously prepared from each group of rats. It was accomplished by initially numbering all the slides, and from a random starting point, selecting every 5th slide. Ultimately, a total of six slides were sampled from each rat liver and used for stereological evaluation. Thirty microscopic fields were then systematically and randomly captured [29] from each selected section onto a computer screen with the aid of a digital eye piece (Premiere MA 88). The eye piece was attached to a Leica Galen III light microscope and connected to a computer with Ulead photo explorer 7.0 SE software.

The diagram below shows the step-wise sampling regime, beginning with the whole rat liver through histological blocks and sections for the stereological analysis of micrographs**.**

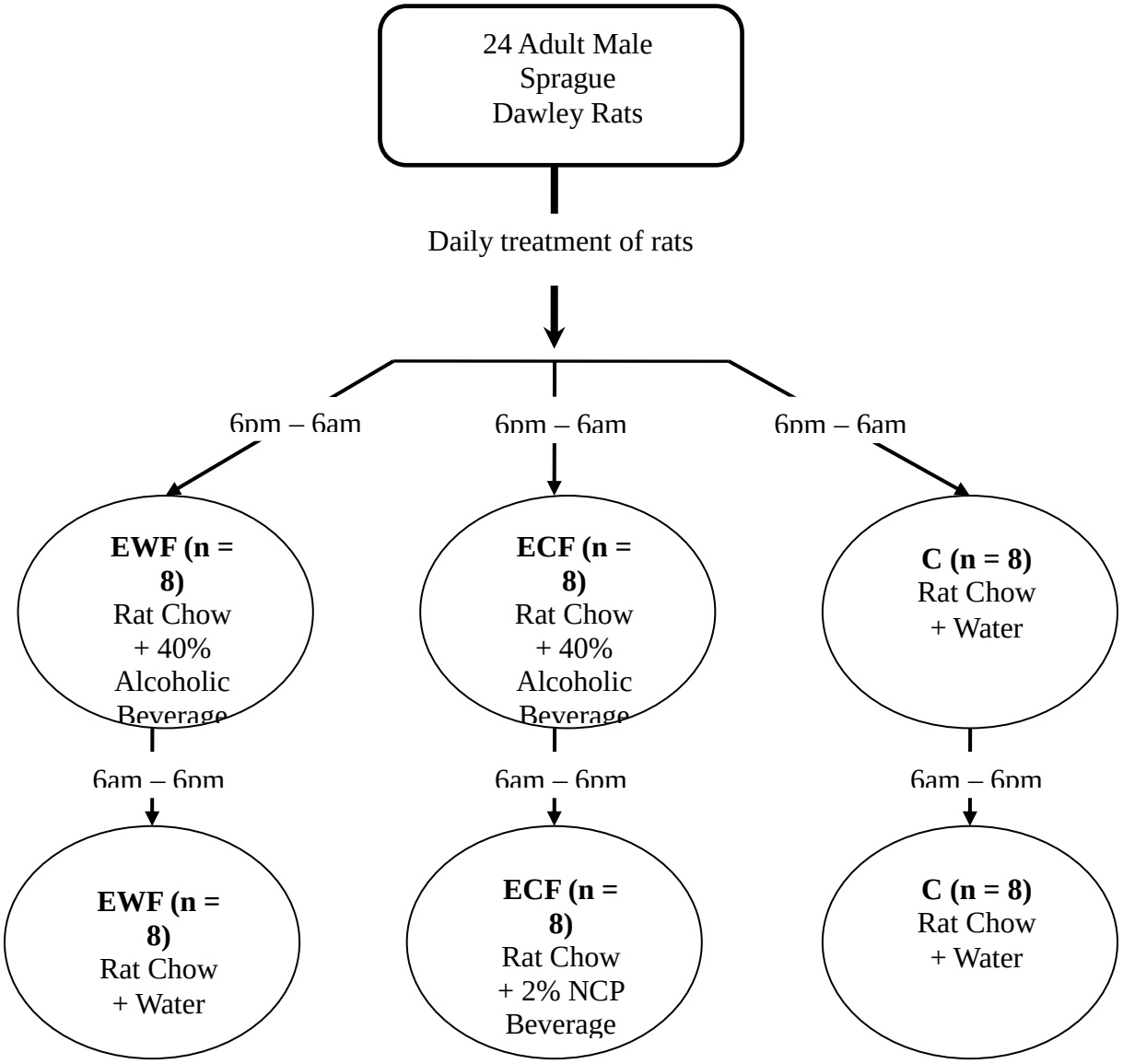

Figure 1: Experimental treatment of rats in the study

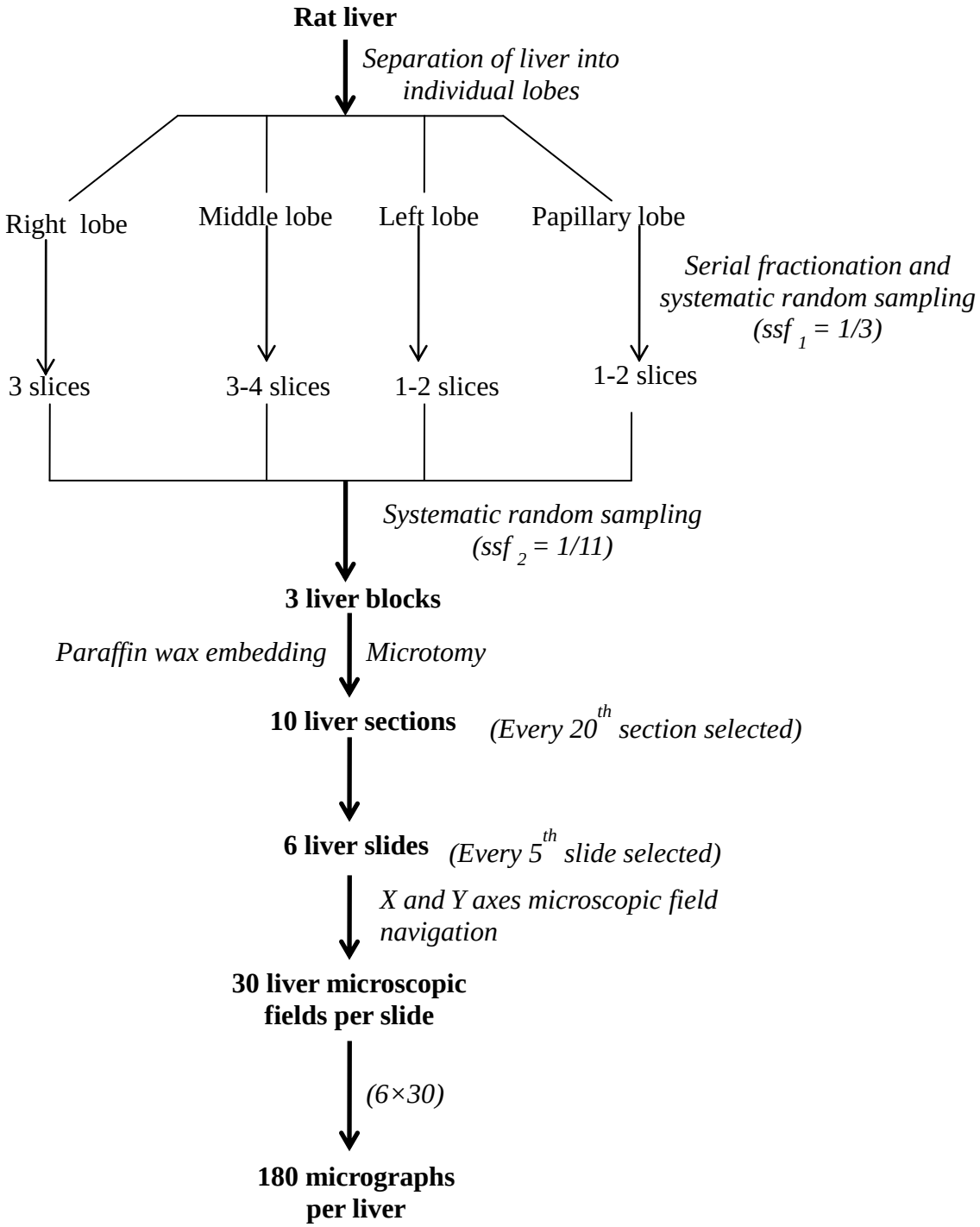

Figure 2: Illustration of multistage sampling adopted in the study

Microscope field sampling was systematized by manually using the X and Y axes of the microscope stage micrometer. By alternately moving two graduations on the X-axis followed by two graduations on the Y-axis and capturing the image in the field of view anytime a tissue was in focus, the whole section on each slide was exhaustively sampled. Images were captured as the micrographs were saved on the computer in digital format. Thirty micrographs were obtained

from each slide. In all, a total of 180 micrographs were obtained from each rat liver. Colored paper print versions of the micrographs were made for stereological assessment.

Estimation of absolute volume of normal (undamaged) hepatocytes: Determination of absolute volume [31] of undamaged hepatocytes was done by point counting and using the following three main criteria to identify and distinguish visually undamaged hepatocytes from damaged ones: (i) cytoplasmic changes including fatty vesiculation (visualized as histologic vacuolation), ballooning cells, and increased eosinophilia, (ii) nuclear changes such as retraction, swelling with chromatin aggregation, pyknosis, and karyorrhexis, and (iii) presence of inflammatory cells surrounding hepatocytes [32]. A 1.0 cm square lattice system was used for point counting. This test grid system was printed on an A4 transparency and superimposed onto the micrograph printed on paper (Fig.3). The superimposed test grid and the printed micrographs were then secured in position with paper clips during counting. Finally, the square lattice system was randomly placed onto each printed photomicrograph (Fig. 3), the number of test points falling on undamaged hepatocytes (P_x) was recorded. The number of points falling on all hepatocytes was also recorded (P_y). The absolute volume of undamaged hepatocytes (AV_{Unhep}) for each micrograph was estimated as follows:

$$\mathbf{[AV_{Unhep} = (P_x/P_y) \times \text{Absolute liver volume } (cm^3)]}$$

The above equation was used to determine the degree of hepatic preservation in all animal treatment groups in this study.

Statistical analyses: Microsoft Statistical Package for the Social Sciences (SPSS) Version 16.0 for Windows was used for statistical analyses. All analyzed data results were expressed as mean and standard deviation (SD). Confidence intervals (95% CI) for means were appropriately reported, and statistical significance of the differences between group means was performed by a one-way analysis of variance [ANOVA] (between and within subjects) followed by a *Post Hoc* Test (*Tukey's HSD*). Differences with $p < 0.05$ were considered to be significant

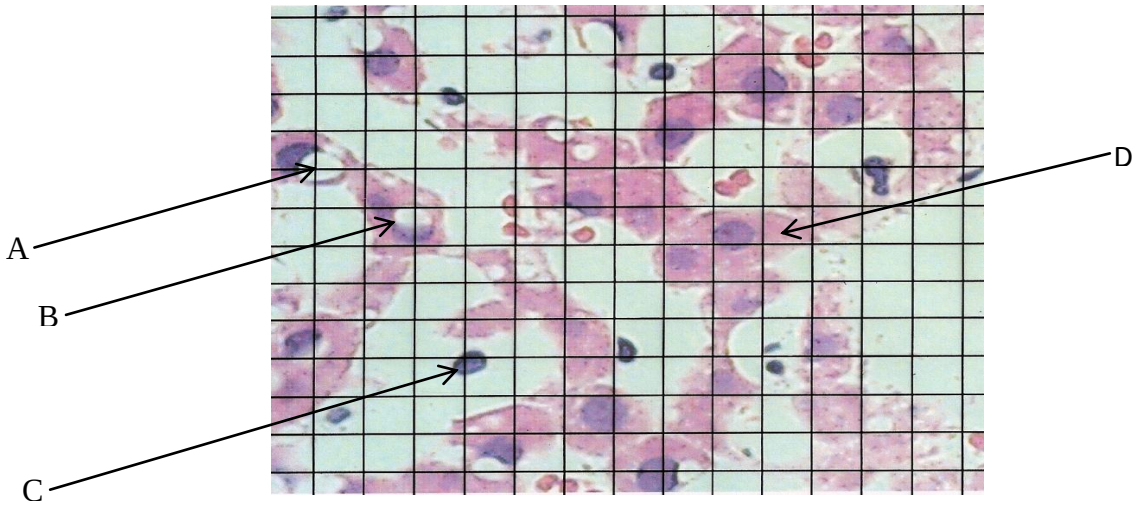

Figure 3: Test lattice grid superimposed on a micrograph of rat liver

A photomicrograph of a test grid with a 1.0 cm^2 lattice superimposed on a micrograph of rat liver for the stereological estimation of undamaged hepatocytes. A- Test point hitting a damaged hepatocyte. B- Hepatocyte with a laterally displaced nucleus, C- Nucleus of leucocyte, and D- Binucleated undamaged hepatocyte.

RESULTS:

Fluid intake by rats: On a daily basis, ECF rats imbibed a median average of 30 ml of alcohol per animal compared with 25 ml for EWF rats which gave a Levene's test p = 0.001. Mann-Whitney U-test comparison yielded U = 1301.0, N1 = 70, N2 = 70, p = 0.0005, two- tailed. EWF rats drank a median average of 30 ml water per rat per day as against 25 ml by Control rats and had Levene's test p = 0.001. Mann-Whitney U = 793.0, N1 = 7-, N2 = 70, p = 0.0005, two-tailed. Each ECF rat imbibed a median volume of 45 ml per day.

Biomarkers of liver function: Animals given access to ethanol had elevated serum AST and ALT ratios. At the termination of the experiment, the AST/ALT ratios were significantly different among the three rat groups studied (one-way ANOVA: df = 2, F = 11.747; $p < 0.0005$). Multiple comparison tests (Tukey's HSD) on AST/ALT ratio data are summarized in Table 1.

Table 1: Results of statistical comparisons of serum AST/ALT ratio of rats in 3 groups studied

Treatment group	Mean	SD	95% CI for the mean	*p*-value (Tukey's HSD)
EWF [a]	1.83	0.38	1.51 – 2.14	0.095 [ab]
ECF [b]	1.51	0.23	1.31 – 1.71	0.039 [bc]
Control [c]	1.13	0.22	0.95 – 1.32	0.0005 [ca]

SD = standard deviation of the mean; CI = confidence interval; Superscript ab = comparison between groups a and b; applies respectively to bc and ca.

Body weight (Bwt) of rats: The average weights of the rats at the end of the experiment were significantly different among the three rat groups (one-way ANOVA: df = 2, F = 3.902, p = 0.036). Mean weight for the EWF group was 302.3 g (SD 22.0 g) (95% CI: 283.9–320.6 g), which for the ECF group was 312.3 g (SD 20.3 g) (95% CI: 295.5–329.0 g), and for the control group was 334.3 g (SD 27.6 g) (95% CI: 311.1–357.4 g). A test of within-subjects effects for duration of alcohol administration at the baseline, midway and end of the experiment, yielded significant differences (one-way repeated measures ANOVA: F = 106.067, p = 0.0005, partial η^2

= 0.822). Tukey's HSD *post hoc* multiple comparison test on the means of rat weight at the termination of the experiment yielded a significantly higher value (p = 0.032) when the control group was compared with the EWF group. A similar comparison of control rats with the ECF group produced a non- significant difference in their mean weights (p = 0.170). When the same statistical test was performed on the terminal weight of the EWF group compared with the ECF group, a p-value of 0.675 was obtained, indicating again a non-significant difference.

Hepatosomatic index (HSI): One way ANOVA yielded an F statistic of 9.020 (p = 0.001) when the hepatosomatic indices studied were finally compared. This indicated significant differences among the three groups. Multiple comparison tests (Tukey's HSD) on HSI data are summarized in Table 2.

Table 2: Synopsis of statistical analyses of hepatosomatic indices among the 3 groups of rats studied.

Treatment group	**Mean (%)**	**SD**	**95% CI for the mean**	***p*-value (Tukey's HSD)**
EWF [a]	4.7	0.8	4.0 – 5.4	0.105 [ab]
ECF [b]	4.1	0.4	3.7 – 4.4	0.113 [bc]
Control [c]	3.4	0.5	3.1 – 3.8	0.001 [ca]

SD = standard deviation of the mean; CI = confidence interval; Superscript ab = comparison between groups a and b; applies respectively to bc and ca.

Gross liver volume at termination of experiment: The average gross liver volume for the control group of rats was 10.9 ml (SD 1.5) ml, of which that of the EWF group was 12.4 ml (SD 1.8 ml), and that of the ECF group was 11.5 ml (SD 1.9 ml). This comparison of gross liver volume in these three groups of rats by ANOVA yielded p = 0.219, indicating a lack of statistical significance among them.

Histologically assessed liver injury: The normal histological structure of the liver parenchyma (Fig. 4A) was observably disrupted in rats that were given ethanol (Figs. 4B & 4C). Qualitatively, the injury to liver micro-structure was less severe in the ECF rats (Fig. 4B) compared with the EWF group (Fig. 4C). Hepatocytes in the liver of EWF rats exhibited more than two of the criteria used to identify and score hepatotoxicity in this study, notably (i) conspicuous cytosolic changes involving fatty vesiculations (appearing as vacuolations), and (ii)

nuclear changes represented by pleomorphism, retraction, and displacement (Fig. 4C). The patterns of morphological changes and structural perturbations seen by observation between the groups were identified in about 60 % of all the treatment groups assessed. Ethanol toxicity also caused noticeable pericentral hepatic disruption of typical radial arrangement of sheets of hepatocytes from the central venule. Periportal assessment of morphological changes revealed general distortion and fatty infiltration in the connective tissue, which was absent in normal controls and minimized in ethanol-cocoa fed rat liver as compared to ethanol-water fed rat liver.

Stereologically assessed liver injury: Stereologically determined absolute volume of undamaged (preserved) hepatocytes yielded the results that are shown in Fig. 5. One way ANOVA indicated that differences in means were significant ($F = 56.127$, $p = 0.0005$). Tukey's HSD *post hoc* multiple comparison tests (Table 3) confirmed that the absolute volume of undamaged hepatocytes was statistically lower in EWF rats compared with control rats. Table 3 also shows that the absolute volume of undamaged hepatocytes was statistically higher in ECF group of rats when compared with EWF group. However, there was no statistical difference between the absolute volume of preserved hepatocytes in ECF and control rats (Table 3).

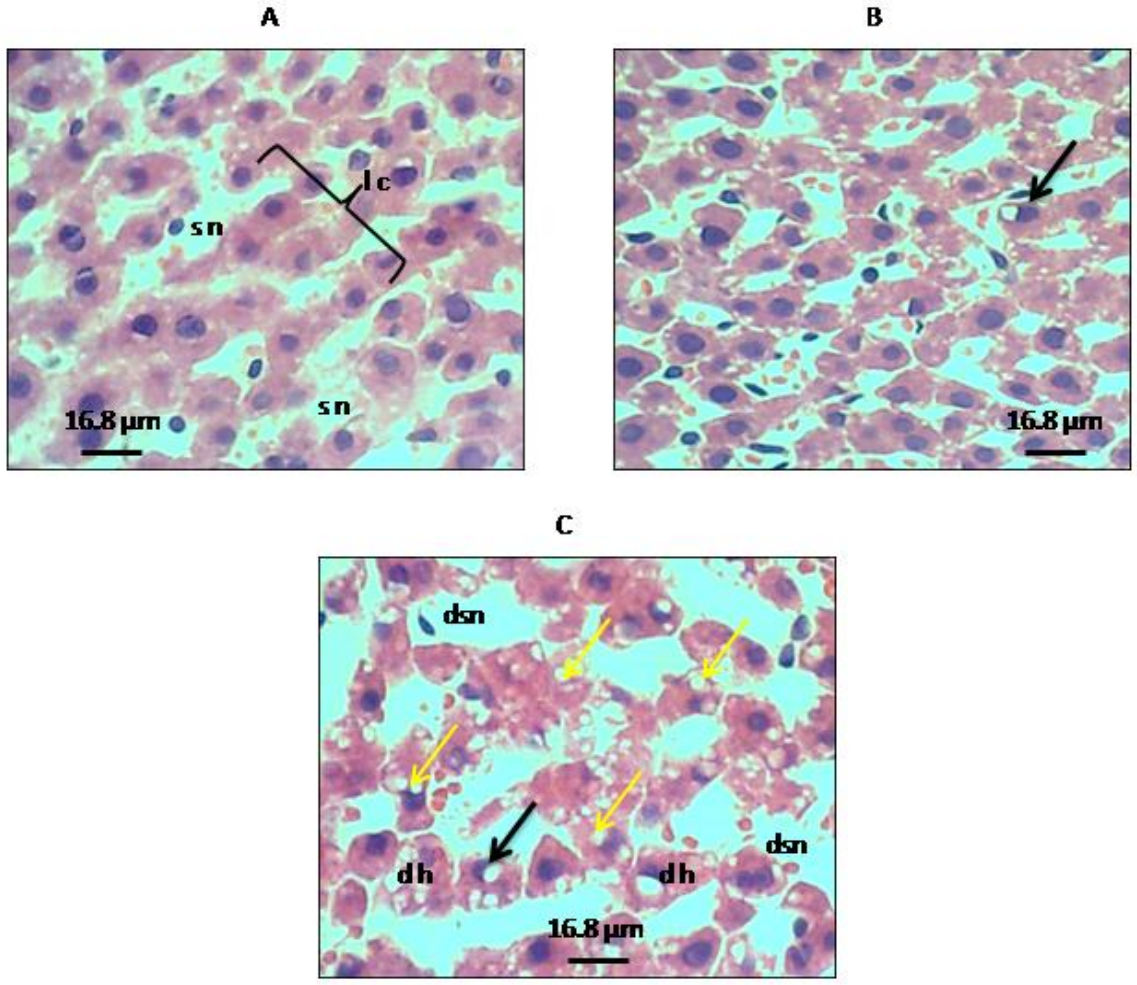

Figure 4 (A-C): Photomicrographs of H & E stained liver sections.

(A) Sinusoids (sn) run between the linear cords (lc) of hepatocytes in the liver of a control rat. (B) Apparently unperturbed sinusoids (sn) and mildly perturbed linear cords (lc) of hepatocytes in the liver of an ECF rat. (C) Apparently dilated sinusoids (dsn), distortions in the linear arrangement of hepatocytes, an increased number/size of cytoplasmic vesiculations (yellow arrows), and nuclear displacement (dark arrows). Note that dispersed small vesiculations are normal in the liver and represent endogenous stores of fat as energy depots [61].

Table 3: Summarized analyses of absolute volume of undamaged hepatocytes among the 3 groups of rats studied.

Treatment group	Mean in ml	SD	95% CI for mean	*p*-value (Tukey's HSD)
EWF [a]	2.34	1.21	1.33 – 3.35	0.0005 [ab]
ECF [b]	9.61	2.18	7.79 – 11.43	0.659 [bc]
Control [c]	10.34	1.47	9.11 – 11.58	0.0005 [ca]

SD = standard deviation; CI = confidence interval; Superscript ab = comparison between groups a and b; applies respectively to bc and ca.

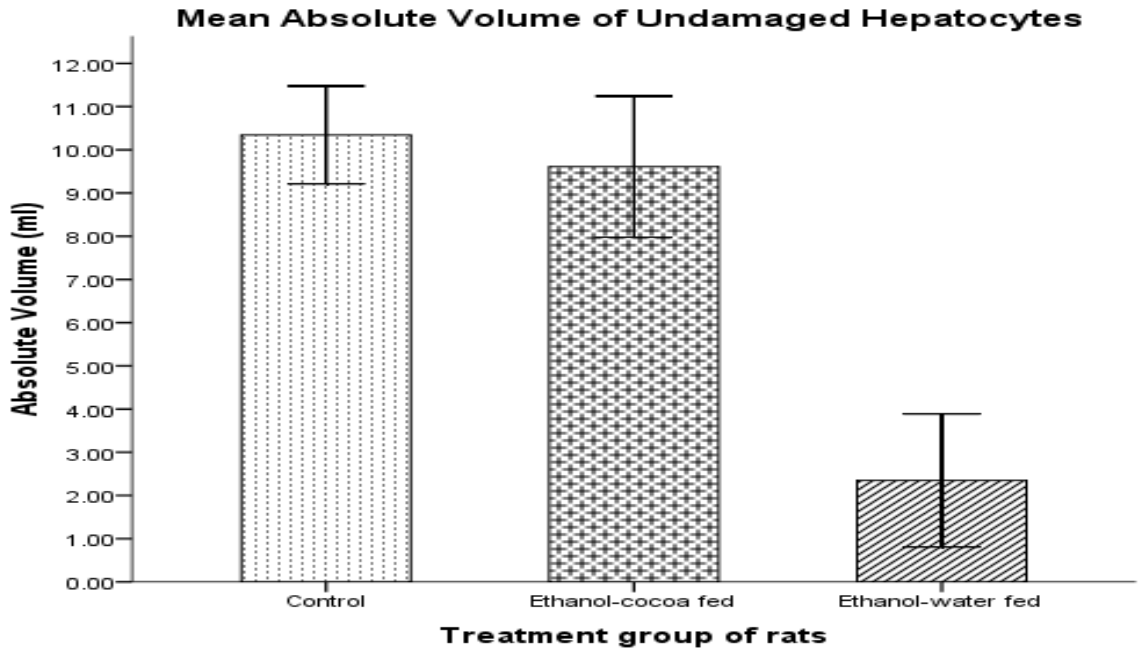

Figure 5: Mean absolute volume of undamaged hepatocytes

Absolute volumes of undamaged hepatocytes in all groups of rats assessed by design-based stereology from micrographs such as those presented in Fig. 4. Error bars represent standard deviations; data was obtained from 180 micrographs per liver and eight animals in each group.

DISCUSSION:

The main focus of this study was to test the hypothesis that natural cocoa ingestion would minimize alcoholic liver damage in experimental rats. Given the established pathogenic contribution of oxidative stress in the development of alcoholic liver disease [3, 7, 21], it was expected that the antioxidant properties of cocoa polyphenols [33] would ameliorate experimentally- induced ethanol hepatotoxicity in rats. The plausibility of this hypothesis was elicited by the previously reported benefits of resveratrol, a red wine polyphenol, in attenuating the oxidative stress effect on alcohol metabolism in the liver of experimental animals [6, 25]. Natural cocoa was used in the present study because it contains the highest levels of Total Antioxidant Capacity (TAC) and procyanidins when compared with other edible cocoa products [34]. The 2% (w/v) of NCP used in this study is the most widely tolerated concentration for human consumption without sugar or any other additive (FKA, unpublished observations).

In agreement with earlier studies, our study showed that the serum concentration of AST and ALT was raised in rats given ethanol, demonstrating that these liver enzymes significantly increased during excessive consumption of alcohol [6, 35, 36]. The diagnostic significance of a high AST/ALT ratio for alcoholic liver disease was emphasized in the Practical Guidelines for Alcoholic Liver Disease, published by the American College of Gastroenterology in 1998 [37]. When the AST/ALT ratio is ≥ 2, it indicates advanced liver disease [38]. Although the EWF rats in the present study had the highest AST/ALT ratio of 1.83 (SD = 0.38), it was still not ≥ 2, implying that only moderate liver damage was caused. This is probably because the voluntary oral mode of ethanol administration in this experiment did not achieve a high and sustained blood alcohol level which is a prerequisite for the establishment of advanced alcohol-induced liver injury [39].

It is important in the context of the main focus of the present study, that regular NCP consumption attenuated an increase of the AST/ALT ratio in ECF rats. This group's relatively lower AST/ALT ratio [1.25 (SD 0.18)] compared with EWF rats suggests that cocoa ingestion limited pathologic release of serum AST and ALT *in vivo*. The increased activities of the enzymes AST and ALT in serum indicate hepatocellular damage since the levels of these enzymes are raised in acute hepatoxicity [40]. Our findings that ALT and AST levels were lower in ECF rats compared to EWF rats affirms the implied hepatoprotective activity of components in cocoa. Furthermore, it has been reported [41] that antioxidants reduce AST and ALT levels in experimental rats and mice with liver disorders. For example, the extract of *Antrodia camphorata* (an antioxidant) has been reported to ameliorate the increase of ALT and AST levels caused by chronic repeated CCL_4 intoxication in mice by mediating antioxidative and free radical scavenging activities [42]. Our finding that serum AST and ALT elevation was apparently milder than quantified hepatocyte injury, invites an inference that these functional markers are less acute indicators of hepatic damage than structural disruption, and/or may lag behind histological damage to the liver. The presumed mechanism of action in this respect may

be anti-inflammatory [15] and antioxidant [21] mediation by cocoa flavanols which thus reduces a subsequent generation of free radicals during alcohol metabolism [5]. Support for this postulate is obtained from studies similar to the present report. One such study involved streptozotocin (STZ) –induced diabetic rats that were given 2% NCP over 13 weeks [43]. In the latter work, the concentration of plasma isoprostane (F_2-IsoPs), an oxidative marker, rose by 43.82% in STZ-induced diabetic rats and 9.14% in STZ-induced diabetic rats that voluntarily ingested cocoa as a drink, signifying a 34.68% decrease in the plasma oxidative marker.

After being fed a cocoa-enriched diet, the total antioxidant capacity (TAC) and activity of antioxidant enzymes (superoxide dismutase and catalase), has been shown to be enhanced in the liver tissue of young rats that were chronically exposed to ethanol [44]. In the STZ-induced diabetic rats study cited above [43], total antioxidant power (TAP) of testicular tissue measured as a uric acid equivalent, decreased by 80.5% in animals not given cocoa and 43.4% in animals given 2% NCP over 13 weeks. This indicated that ingestion of NCP was associated with 37.1% retention of TAP in the testicular tissue of the STZ-induced diabetic rats.

The antioxidant defense of quercetin, a constituent of natural cocoa, against oxidative stress action is the main mechanism of its protective effect on ethanol-induced liver injury [45]. An assessment of the protective effect of quercetin on ethanol-induced acute liver injury in rats concluded that hepatoprotectivity can be achieved by eating foods rich in quercetin [45]. Therefore, it is noteworthy that quercetin had the highest peak in the preliminary tests for antioxidant content in the NCP used for the present study [17].

Rats in all treatment groups gained weight throughout the experiment. However, EWF rats gained the least weight when compared with ECF and control rats. Ingested cocoa can prevent high-fat diet–induced obesity by modulating lipid metabolism, especially by decreasing fatty acid synthesis and transport systems, and enhancing part of the thermogenesis mechanism in liver and white adipose tissue [46]. It is therefore reasonable to infer that the weight gained by ethanol-cocoa treated rats was not due to cocoa ingestion (caloric effect). Rather, it is speculated that NCP intake was in fact able to limit the toxic effect of alcohol, which would in most cases lead to systemic dehydration, reduced food intake, and ceased or reduced growth rate [47]. The latter effect of alcohol ingestion may partly account for the lower weight of EWF rats at the termination of the present study.

A common variable, hepatosomatic index (HSI), is usually used to assess change in gross liver weight with respect to the overall body weight [27]. The liver indices of the experimental rats were markedly affected by ethanol ingestion and regular consumption of natural cocoa drink. The results of this study showed significantly higher HSI in EWF rats when compared to controls. Conversely there was an absence of difference in HSI between ECF rats and controls. It is also notable that even though there was a significantly greater volume of alcohol consumption; ECF rats produced milder hepatic damage than EWF rats. This was confirmation that cocoa ingestion did not eliminate or prevent but *minimized* liver damage caused by alcohol.

In this study, the oral administration and voluntary drinking of alcohol by rats may account for the absence of pathology beyond hepatic fatty changes. This is because simple inclusion of ethanol in drinking fluid rarely causes high and sustained elevation of blood ethanol levels and only a moderate rise in liver triglycerides is observed [48]. Given that it was the aim of this study

to mimic human alcoholism well enough in experimental rats, the mode of ethanol administration used is defensible. On the other hand, considering the close resemblance of a rat liver's microstructure to that of a human's [49], results obtained from hepatological studies of this kind in experimental rats may be extrapolated to humans with potential application in clinical trials.

In humans, chronic ethanol consumption leads to a characteristic set of changes in the metabolism of lipids in the liver. This is referred to as an alcoholic fatty liver [50]. This condition is characterized by an increase in liver weight [51], an accumulation of triglycerides, and changes in expression of genes involved in lipid metabolism [52]. In severe cases, these changes eventually lead to inflammation [53] and steatohepatitis [54]. Most of these changes are also observed in rodents [55], justifying the use of chronic alcohol exposure in rodents as a model to understand the human condition.

In agreement with the documented antioxidant, anti-inflammatory, and anti-obesity properties of cocoa, it was histologically observed that ECF rats had more undamaged (preserved) hepatocytes in their liver that EWF rats in spite of a chronic consumption of alcohol. Due to the fact that EWF rats were not given NCP drink, their liver parenchyma was significantly more damaged structurally in comparison to the control rats and the ECF group (Figure 5). The structural evidence affirmed the functional deficits in AST and ALT as discussed above. It is suggested that the anti-inflammatory and antioxidant effects of NCP, minimized cellular damage from ethanol metabolism by reducing the intensity of fatty vesiculation in the livers of ECF rats compared with EWF rats. This was a result of beneficial modulation of the *de novo* fatty acid biosynthetic pathways.

In recent years, researchers have used design-based stereology for the study of rat livers [56, 57, 58]. This has established appropriate quantification strategies for obtaining unbiased estimates of liver cells and structure. This constitutes a new solid quantitative background in normal and pathologic conditions. This is relevant for establishing morphofunctional correlations and for monitoring the progression of inflammatory and fibrotic liver conditions [59]. In fact, although advances in cell isolation and molecular biology have contributed significantly to the current understanding of the role of both parenchymal and non-parenchymal cells in hepatic injury, in vivo morphological examination continues to be the most effective and powerful means for investigating the cellular interplay between structure and function [60].

The systematic and random approach used in sampling and subsequent quantification of hepatocyte preservation made it possible to study and stereologically evaluate liver damage in all four liver lobes of the rats in the treatment groups. A decreased amount of normal hepatocytes in the liver of the ethanol-water treated rats rationalizes the reduced functionality, delineated by a high AST/ALT ratio, and undesirable alteration in their liver structure. This was a result of the alcoholic insult. It is inferred from the present study that voluntary NCP intake significantly reduced the steatotic effect of the alcoholic drink used to establish ethanol toxicity. Therefore, judging from the results of the stereological assessment (Table 3), it is affirmed that regular intake of NCP drink exerted hepatoprotective benefits. This slowed the rate of structural damage and alcohol induced indications of liver damage. Concomitantly, NCP also improved the lipid metabolism in the liver of the ECF rats.

Conclusion: Ethanol-mediated pathologic alterations in hepatic morphology and functional deficits due to voluntary ingestion of 40% (v/v) of ethanol in rats have been established. At the same time, the possible prophylactic benefits of NCP in slowing down liver injury arising from chronic ethanol consumption have also been demonstrated. Inferentially, the early phase of alcohol-induced liver damage may be significantly attenuated by NCP consumption. This is possibly afforded by anti-inflammatory and antioxidant augmentation against damage to hepatocyte morphology and metabolism.

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List of abbreviations: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase **AVUnhep**: Absolute volume of undamaged hepatocytes; Bwt: Body weight; ECF: Ethanol Cocoa fed**;** EWF: Ethanol water fed**;** GAFCO: Ghana Agro Food Company Limited**;** HI: Hepatosomatic Index; NCP: Natural cocoa powder; NMIMR: Noguchi Memorial Institute for Medical Research; ROS: Reactive oxygen species; TAC: Total Antioxidant Capacity; TNF-α: Tumor necrotic factor alpha; UGMS: University of Ghana Medical School.

Competing Interest: F.K. Addai is nationally (Ghana) and regionally (West Africa) known as a researcher and promoter of natural cocoa consumption for better health. He is the founder/director of Kakawa Enterprise Limited (KEL), a nascent ultra-micro scale company that produces GoodFood® Unsweetened Natural Cocoa Powder. KEL made no financial contribution towards the study or publication of this paper.

Authors' Contribution: F.K. Addai originated the idea of the project, and enlisted R.K Gyasi (Medical Doctor & Pathologist) and K.A. Bugyei (Veterinary Doctor & Pharmacologist) who together with J. Ahenkorah and B.A. Hottor conceptualized the research. G. Sokpor performed all experiments reported in this study as part of his graduate studies supervised by FKA and RKG. All authors reviewed the manuscript and contributed to interpretation of the data.

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REFERENCES:

1. Lieber CS: Alcohol and the Liver: Metabolism of alcohol and its role in hepatic and extrahepatic diseases. Mt Sinai J of Med 2000, 67:84–94.
2. Albano E: Oxidative mechanisms in the pathogenesis of alcoholic liver disease. Mol Aspects. Med 2008, 29:9–16.
3. Castilla R, Gonzalez R, Fouad D, Fraga E, Muntané J: Dual effect of ethanol on cell death in primary culture of human and rat hepatocytes. Alcohol Alcohol 2004, 39: 290–296.
4. Zakhari S: Overview: How is alcohol metabolized by the body? Alcohol Res Health 2006, 29:245–254.
5. Koop DR: Alcohol Metabolism's damaging effects on the cell: a focus on reactive oxygen generation by the enzyme cytochrome P450 2E1. Alcohol Res Health 2006, 29:274–280.
6. Kasdallah–Grissa, A, Mornagui B, Aouani E, Hammami M, El May M, Gharbi N, Kamoun A, El–Fazaâ S: Resveratrol, a red wine polyphenol, attenuates ethanol-induced oxidative stress in rat liver. Life Sci 2007, 80:1033–1039.
7. Nath ABB, Szabo G: Alcohol-induced modulation of signalling pathways in liver parenchymal and nonparenchymal cells: implications for immunity. Semin Liver Dis 2009, 29:166–176.
8. Koch O, Farré S, De Leo ME, Palozza P, Palazzotti B, Borrelo S, Palombini G, Cravero A, Galeotti T: Regulation of manganese superoxide dismuatse (MnSOD) in chronic experimental alcoholism: effects of vitamin E-supplemented and-deficient diets. Alcohol and Alcohol 2000, 35:159–163.
9. Ozaras R, Tahan V, Aydin S, Uzun H, Kaya S, Santurk H: Nacetylcysteine attenuates alcohol-induced oxidative stress in the rat. World J Gastroenterol 2003, 9:125–128.
10. Husain K, Mejia J, Lalla J, Kazim S: Dose response of alcohol induced changes in BP, nitric oxide and antioxidants in rat plasma. Pharmacol Res 2005, 51:337–343.
11. McCullough AJ, O'Connor JFB: Alcoholic liver disease: Proposed recommendations for the American College of Gastroenterology. Am J Gastroenterol 1998, 93:2022–2036.
12. Edington GM, Gilles HM: Pathology in the Tropics. London: Edward Arnold, 1976.
13. Tirkey N, Pilkhwal S, Kuhad A, Chopra K. Hesperidin: A citrus bioflavonoid, decreases the oxidative stress produced by carbon tetrachloride in rat liver and kidney. BMC Pharmacol. 2005,5:2.
14. Keen CL, Holt RR, Oteiza PI, Fraga C, Schimitz HH: Cocoa antioxidants and cardiovascular health. Am J Clinl Nutr 2005, 81:298S–303S.

15. Hopper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, Ryder JJ, Hall WL,Cassidy A: Falvonoids, flavonoid-rich foods, and cardiovascular risk: a meta-anlysis of randomized controlled trials. Am J Clin Nutr 2008, 88:38-50.
16. Min-Hsiung P, Ching-Shu L, Chi-Tang Ho: Anti-inflammatory activity of natural dietary flavonoids. Food Funct 2010, 1, 15-31.
17. Addai FK: Natural cocoa as diet-mediated antimalarial prophylaxis. Med Hypotheses, 2010, 7:825–830.
18. De S, Chakraborty J, Chakraborty RN, Das S: Chemopreventive activity of quercetin during carcinogenesis in cervix uteri in mice. Phytother Res 2000, 14:347–351.
19. Erden Inal M, Kahraman A: The protective effect of flavonol quercetin against ultraviolet A induced oxidative stress in rats. Toxicology 2000, 154:21–29.
20. Scalbert A, Johnson IT, Saltmarsh M: Polyphenols: antioxidants and beyond. Am J Clin Nutr 2005, 81:215S–217S.
21. Jeon SE, Choi–Kwon S, Park KA, Lee HJ, Park MS, Lee JH, Kwon SB, Park KC: Dietary supplementation of (-) Catechin protects against UVB-induced skin damage by modulating antioxidant enzyme activities. Photodermatol Photoimmunol Photomed 2003, 19:235–241.
22. Kofink M, Papagiannopoulos M, Galensa R: (-)-Catechin in cocoa and chocolate: occurrence and analysis of an atypical Flavan-3-ol Enantiomer. Molecules 2007, 12:1274–1288.
23. Radojčić RI, Delonga K, Mazor S, Dragović-Uzelac V, Carić M, Vorkapić-Furač J: Polyphenolic content and composition and antioxidative activity of different cocoa liquors. Czech J Food Sci 2009, 27:330–337.
24. Roy, H., Lundy, S. and Brantley, P: Cocoa Polyphenols. Pennington Nutrition Series No.1: 2005, [http:// www.pbrc.edu/Division_of_Education/pdf/PNS_Cocoa_Polyphenol]
25. Kono H, Wheeler MD, Rusyn I, Lin M, Seabra V, Rivera CA, Bradford BU, Forman DT, Thurman RG: Gender differences in early alcohol-induced liver injury: role of D14, NF-kB, and TNF-α. Am J Physiol Gastrointest Liver Physiol 2000, 278:652–661.
26. Sun GY, Xia J, Xu J, Allenbrand B, Simonyi A, Rudeen PK, Sun AY: Dietary supplementation of grape polyphenols to rats ameliorates chronic ethanol-induced changes in hepatic morphology without altering changes in hepatic lipids. J Nutr 1999,129:1814–1819.
27. Aguiar AS, Da-Silva VA, Boa Ventura GT: Behavioral toxicity of increasing doses of ethanol in malnourished rats. Nutri Neurosci 2006, 9:113–119.
28. Delahunty G de Vlaming VL: Seasonal relationship of ovary weight, liver weight and fat stores with body weight in the goldfish, *Carassius auaratus*. J Fish Biol 1980, 16:5–13.
29. Scherle W: A simple method for volumetry of organs in quantitative stereology. Mikroskopie 1970, 26:57–60.
30. Low D, Thomas NW, Fry JR: Lobar variation of carbon tetrachloride hepatotoxicity in the rat. Toxicol Lett 1995, 81:1–4.
31. Mayhew TM, Huppertz B, Kaufmann P, Kingdom JC: The 'reference trap' revisited: examples of the dangers in using ratios to describe fetoplacental angiogenesis and trophoblast turnover. Placenta. 2003, 24:1–7.

32. Altunkaynak BZ, Ozbek E: Overweight and structural alterations of the liver in female rats fed a high-fat diet: A stereological and histological study. Turk J Gastroenterol, 2009, 20: 93–103.
33. Gu L, House SE, Wu X, Ou B, Prior RL: Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. J Agric Food Chem 2006, 54:4057–4061.
34. Wollgast J, Anklam E: Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Res Int 2000, 33:423–447.
35. Jurczuk M, Brzóska MM, Moniuszko-Jakoniuk J, Galażyn-Sidorczuk M, Kulikowska-Karpiñska E: Antioxidant enzymes activity and lipid peroxidation in liver and kidney of rats exposed to cadmium and ethanol. Food Chem Toxicol 2004, 42:429–438.
36. Husain K, Mejia J, Lalla J, Kazim S: Dose response of alcohol induced changes in BP, nitric oxide and antioxidants in rat plasma. Pharmacol Res 2005, 51:337–343.
37. McCullough AJ, O'Connor JFB: Alcoholic liver disease: Proposed recommendations for the American College of Gastroenterology. Am J Gastroenterol 1998, 93:2022–2036
38. Nyblom H, Berggren U, Balldin J, Olsson R: High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. Alcohol Alcohol 2004, 39:336–339.
39. French SW, Nash J, Shitabata P, Kachi K, Hara C, Chedid A, Mendenhall CL Pathology of alcoholic liver disease. Semin Liver Dis 1993, 13:154–169.
40. Obi E, Orisakwe OE, Asomugha LA, Udemezue, OO. The Hepatotoxic effect of halofantrine in guinea pigs, Indian J. Pharmacy. 2004; 36:303-305.
41. Ko K, Ip S, Poon M, Wu S, Che C, Ng K, Kong Y. Effect of a lignan-enriched fructus schisandrae extract on hepatic glutathione status in rats: protection against carbon tetrachloride toxicity, Planta Med. 1995; 61:134-137.
42. Hsiao G, Shen M, Lin K, Lan M, Wu L, Chou D, Lin C, Su C, Sheu J. Antioxidative and hepatoprotective effects of *Antrodia camphorata* extract, J Agric Food Chem. 2003;51:3302-3308.
43. Etsey AN: Effect of regular ingestion of natural cocoa on testicular histology in streptozotocin-induced diabetic rats. M.Phil thesis. University of Ghana, Anatomy Department; 2009.
44. Ramiro-Puig E, Urpí-Sardà M, Pérez-Cano FJ, Franch À, Castellote C, Andrés-Lacueva C, Izquierdo-Pulido M, Castell M: Cocoa-enriched diet enhances antioxidant enzyme activity and modulates lymphocyte composition in thymus from young rats. J Agric Food Chem: 2007, 55:6431–6438.
45. Chen X: Protective effec*ts of quercetin* on liver injury induced by ethanol. Pharmacogn Mag 2010, 6:135–141
46. Matsuia N, Itoa R, Nishimura E, Yoshikawaa M, Kato M, Kamei M, Shibatab H, Matsumoto I, Abe K, Hashizume S: Ingested cocoa can prevent high-fat diet–induced obesity by regulating the expression of genes for fatty acid metabolism. Nutrition 2005, 1:594–601.

47. Patek AJ, Toth IG, Saunders MG, Castro GA, Engel JJ: Alcohol and dietary factors in cirrhosis. An epidemiological study of 304 alcoholic patients. Arch Intern Med 1975, 135:1053–1057

    Lieber CS, Decarli LM: Liquid diet technique of ethanol administration: update. Alcohol Alcohol 1989, 24:197–211
48. Kogure K, Ishizaki M, Kuwano H, Makuuchi M: A comparative study of the anatomy of rat and human liver. J Hepatobilliary Pancreat Sci 1999, 6:171–175.
49. Kusunoki J, Kanatani A, Moller, DE: Modulation of fatty acid metabolism as a potential approach to the treatment of obesity and the metabolic syndrome. Endocrine 2006, 29:91–100.
50. Baillie M: Alcohol and the liver. Gut 1971, 12:222–229.
51. Mantena SK, King AL, Andringa KK, Eccleston HB, Bailey SM: Mitochondrial dysfunction and oxidative stress in the pathogenesis of alcohol- and obesity induced fatty liver diseases. Free Radic Biol Med 2008, 44:1259–1272.
52. Forgione A, Miele L, Cefalo C, Gasbarrini G, Grieco A: Alcoholic and nonalcoholic forms of fatty liver disease. Minerva Gastroenterol Dietol 2007, 53:83–100.
53. Yeh MM, Brunt EM: Pathology of nonalcoholic fatty liver disease. Am J Clin Pathol, 2007, 128: 837–847.
54. Kohgo Y, Ohtake T, Ikuta K, Suzuki Y, Torimoto Y, Kato J: Dysregulation of systemic iron metabolism in alcoholic liver diseases. J Gastroenterol Hepatol 2008, 23: S78–S81.
55. Marcos R, Rocha E, Henrique RM, Monteiro RA: A new approach to an unbiased estimate of the hepatic stellate cell index in the rat liver: an example in healthy conditions. J Histochem Cytochem 2003, 51:1101–1114.
56. Marcos R, Monteiro RAF, Rocha E: Design-based stereological estimation of hepatocyte number, by combining the smooth optical fractionator and immunocytochemistry with anticarcinoembryonic antigen polyclonal antibodies. Liver Int 2006, 26:116–124.
57. Santos M, Marcos R, Santos N, Rogério MF, Monteiro AF, Rocha E: An unbiased stereological study on subpopulations of rat liver macrophages and on their numerical relation with the hepatocytes and stellate cells. J Anat 2009, 214:744–751.
58. Pinzani M, Rombouts K, Colagrande S: Fibrosis in chronic liver diseases: diagnosis and management. J Hepatol 2005, 42:S22–S36.
59. Tsukamoto H, Lin M: The role of Kupffer cells in liver injury. In: Cells of the Hepatic Sinusoid, Volume 6. Wisse E, Knook DL, Balabaud C. (Eds.) Leiden: Kupffer Cell Foundation; 1997:244–250.
60. Martin WH III, Klein S: Use of endogenous carbohydrate and fat as fuels during exercise. Proc Nutr. Soc. 1998, 57:49–54.