Journal of Intercultural Ethnopharmacology
www.jicep.com
DOI: 10.5455/jice.20170603094811

Original Research

Tigernut (*Cyperus esculentus* L.) "milk" as a potent "nutri-drink" for the prevention of acetaminophen-induced hepatotoxicity in a murine model

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Received: March 11, 2017
Accepted: May 26, 2017
Published: June 09, 2017

ABSTRACT

Aim/Background: Given the prevalence of toxicants in foods, beauty products, etc., and the increasing demand for "green" products, there is a need for the development of "nutri-drinks" with hepatoprotective properties. The usefulness of tigernut milk (TNM) in preventing acetaminophen (APAP)-induced liver injury was, therefore, investigated. **Materials and Methods:** A total of 25 rats were randomized into five equal groups. Four groups were treated with 0, 500, 1000, and 2000 kg/mg body weight (bw) TNM, respectively, *per os* for 2 weeks before they were challenged with 2500 mg/kg bw APAP. Biochemical markers of hepatotoxicity and oxidative stress were determined in the sera of the rats at the end of the study. **Results:** Serum alanine aminotransferase concentrations decreased significantly ($P < 0.001$) and dose-dependently from 334.3 ± 16.1 in the negative control group to 65.4 ± 8.3 in the 2000 mg/kg bw TNM group. Other studied liver enzymes were similarly dose-dependently reduced. These data are corroborated by histological findings. Superoxide dismutase activity (U/mg protein) was increased significantly ($P < 0.001$) from 108.0 ± 7.4 in the negative control group to 291.0 ± 11.3 in the 2000 mg/kg bw TNM group, and indeed all the test groups. The malondialdehyde concentrations in the test rats were slightly lower than that of the negative control group. **Conclusion:** TNM at the tested concentrations significantly prevented liver injury. Phytochemicals in TNM, working directly as antioxidants or indirectly by inducing the synthesis of glutathione, may be responsible for the observed effect.

KEY WORDS: Acetaminophen, hepatotoxicity, prevention, tigernut milk

INTRODUCTION

Tigernuts (*Cyperus esculentus* L.), Cyperaceae, also called yellow nut-grass, earth almond, flats edge, water grass, and chufa [1], is a perennial plant which grows to 24-55 cm in height [2]. It is not a "nut" as it, in fact, produces hard spherical tubers at the base of its scaly rhizomes [3]. Although there are three known edible varieties of tigernuts – yellow, brown and black – the yellow variety (which is larger than the others) is often preferred because of its esthetic, succulence, sensory, and shelf-life superiority. In addition, it is reported to contain less fat and antinutrients, and more protein [4]. Tigernut "milk" (TNM) is popular throughout West Africa and in parts of Southern Europe. It is consumed more during warm periods of the year as a refreshing non-alcoholic beverage [5].

Bioactive phytochemicals and nutrients in tigernut, including salicylic acid, alkaloids, terpenoids, saponins, steroids, vitamins C and E, phosphorous, and potassium [6], have been reported to possess a wide range of health promoting properties, including anti-inflammatory and immunostimulatory properties [7]; heart disease and thrombosis prevention properties [8]; and lowering of colon cancer risk [9]. Its anti-obesity, antidiabetes, anti-diarrheal/dysentery, and gastrointestinal disorders modulatory properties have also been reported [10-13]. Without prejudice to the above, tigernut is considered nutritive and its milk a "nutri-drink" and "health food" due to its high amounts of fiber, antioxidants and microelements [14].

Interestingly, tigernut is consumed as snack in most parts of Nigeria, and there are no reports of any adverse effects associated with its consumption. TNM is also very well tolerated and our preliminary toxicity studies (unpublished laboratory data) agree with Oladipipo *et al.* [6] who after a 4 weeks study on the toxicity of the aqueous extracts of tigernuts, concluded that "the oral lethal dose of *C. esculentus* for rats is well above 5000 mg/kg and may be considered safe within the (tested) doses and period of investigation." These, therefore, make TNM a veritable candidate for study as a possible hepatoprotective "green" or "clean label" "nutri-drink" especially in this age when

large swathes of our population are exposed to hepatotoxicants in processed foods, cosmetics, drugs, and workplaces.

Acetaminophen (N-acetyl-para-aminophenol, APAP or paracetamol) a widely used analgesic and antipyretic drug, is commonly used in studying liver toxicity. Its toxicity to hepatocytes is linked to the conversion of some of it (at high doses) by several P450 cytochromes into N-acetyl-p-benzoquinone imine (NAPQI) – a highly reactive toxic intermediate [15]. Although conjugation with reduced glutathione (GSH) efficiently eliminates substantial amounts of NAPQI, large doses of APAP overwhelms the system by depleting the GSH in the liver. The 3-(cysteine-S-yl) APAP adducts formed by the binding of unconjugated NAPQI to cysteine groups on proteins, induces oxidative stress, rapid cell death and necrosis, and ultimately liver failure [16].

This paper, therefore, examines the hypothesis that given the known phytochemical constituents of tigernut, TNM will be a potent hepatoprotective drink against APAP-induced hepatotoxicity in Wistar rats.

MATERIALS AND METHODS

Preparation of TNM

Fresh tigernuts were bought from the Ogige Main Market, Nsukka. They were sorted to remove bad tubers, and washed thoroughly in tap water. 190 g of the clean tubers were then blended with 500 ml of distilled water into a slurry using a clean personal blender. Thereafter, the slurry was pressed exhaustively using a muslin cloth to extract the milk. The milk was then bottled in clean screw-cap bottles and stored in a refrigerator until use.

Animals and Experimental Design

A total of 25 adult male Wistar rats were obtained from a commercial vendor and acclimatized to the animal house environment for 1 week. Thereafter, they were randomized into five groups of five rats each. Four groups were each given 500 mg/kg body weight (bw) TNM, 1000 mg/kg bw TNM, and 2000 mg/kg bw TNM and distilled water only, *per os* using an intragastric gavage, for 14 days. Thereafter, they were given 2500 mg/kg bw APAP as a hepatotoxicant [Table 1]. One group served as the normal control and received neither the test milk nor the toxicant, but only distilled water [Table 1].

Throughout the study, the rats were housed in standard cages, groupwise, in a properly ventilated animal house, following standard internationally accepted procedures for the care of laboratory animals. They were exposed to 12 h light/dark cycles under humid tropical conditions. All the rats had access to water and feed *ad libitum*. At the end of the study, the rats were fasted overnight, and each was subsequently humanely dazed and bled exhaustively from the retro-orbital plexus. The sera were separated from the cells and used immediately for biochemical analyses. The livers of the rats were carefully harvested for histological analysis.

Biochemical Analyses

The enzymatic colorimetric methods of Reitman and Frankel [17] and Rec [18] were used to determine the serum concentrations of alanine and aspartate aminotransferases (ALT and AST) and alkaline phosphatase (ALP), respectively. The determination of serum total bilirubin and total proteins was done following the methods of Jendrassik and Grof [19] and Tietz [20], respectively. Serum superoxide dismutase (SOD) activity was assayed by spectrophotometrically monitoring the inhibition of the auto-oxidation of epinephrine in the presence of Fenton reagent [21]. The concentration of the product of the reaction between malondialdehyde (MDA) (a proxy for lipid peroxidation) and thiobarbituric acid – thiobarbituric acid reactive substances (TBARS) – was spectrophotometrically measured [22]. Assay kits procured from reputable companies were used for all the determinations and assays, following the manufacturers' instructions.

Histological Studies

The harvested livers of rats from the different groups were cleaned of external fasciae, rinsed in normal saline, blotted with filter paper and fixed immediately in formal saline. The tissues were then dehydrated in grades of ethanol, cleared in xylene, then infiltrated with, and embedded in paraffin. Sectioning was done at 5 μm using a microtome and the sections stained with hematoxylin and eosin. They were subsequently viewed and their photomicrographs taken (×400).

Statistical Analysis

The data generated were analyzed statistically. Means and standard deviations for each parameter per group were calculated, and differences between means were separated by one-way ANOVA test followed by *post hoc* multiple comparisons (using the least significant difference). Differences between means were considered statistically significant at $P < 0.05$. Data analyses were performed using the statistical software IBM-SPSS version 20 (IBM Corp. Atlanta, GA). The results are presented in bar charts.

RESULTS

Pretreating the rats with TNM significantly ($P < 0.001$) and dose-dependently lowered the serum ALT concentration of the

Table 1: Experimental design and treatment of animals

Treatment day	Test one	Test two	Test three	Neg. control	Norm. control
1-14	500 mg/kg bw TNM	1000 mg/kg bw TNM	2000 mg/kg bw TNM	Distilled water	Distilled water
15	2.50 g/kg bw APAP	2.50 g/kg bw APAP	2.50 g/kg bw APAP	2.50 g/kg bw APAP	-

Treatments were given *per os*. Neg: Negative, Norm: Normal, bw: Body weight, APAP: Acetaminophen, TNM: Tigernut milk. APAP was procured from Juhel Nigeria Ltd

test rats relative to the negative control group. Interestingly, at the maximum administered dose (2000 mg/kg.bw) the TNM reduced the ALT concentration so considerably that the mean was statistically similar ($P > 0.05$) to that of the rats that were not challenged with APAP (normal control) [Figure 1]. Akin to the observation made on the serum ALT concentrations, the serum AST concentrations of the test rats were significantly lowered, relative to the negative control, as a result of treatment with TNM before induction of hepatotoxicity. Again, the mean serum AST concentration of rats that received the highest dose of TNM administered was not significantly different ($P > 0.05$) from that of the normal control group [Figure 2]. Serum ALP concentrations were dose-dependently and significantly ($P < 0.001$) lower in test rats, relative to the negative control group. At doses of 1000 and 2000 mg/kg bw, the TNM caused the lowering of the serum ALP concentrations to mean values that were statistically similar ($P > 0.05$) to that of the normal control group [Figure 3].

Figure 4 shows the serum total protein concentrations of rats. The test rats had significantly ($P < 0.01$) higher serum total protein concentrations compared to the negative control group.

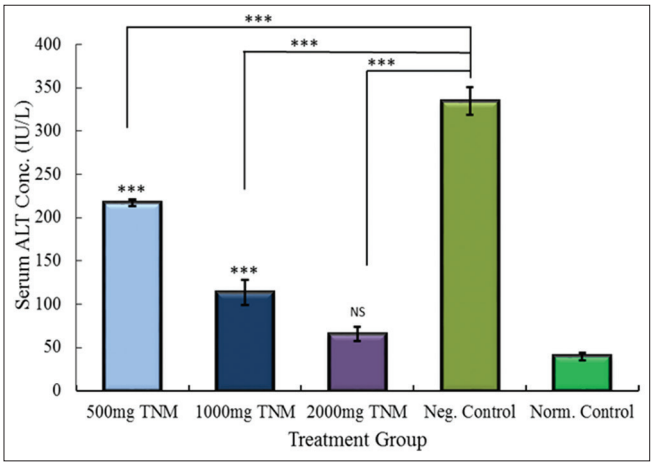

Figure 1: Serum alanine aminotransferase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., NS and ***represent tigernut milk, negative, normal, "not significant" and significant at $P < 0.001$, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

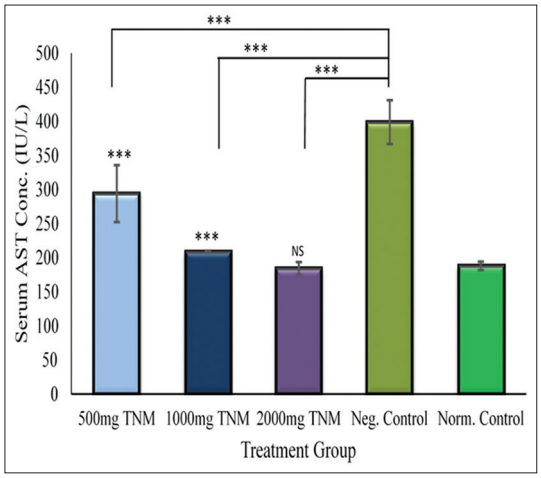

Figure 2: Serum aspartate aminotransferase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., NS and *** represent tigernut milk, negative, normal, "not significant" and significant at $P < 0.001$, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

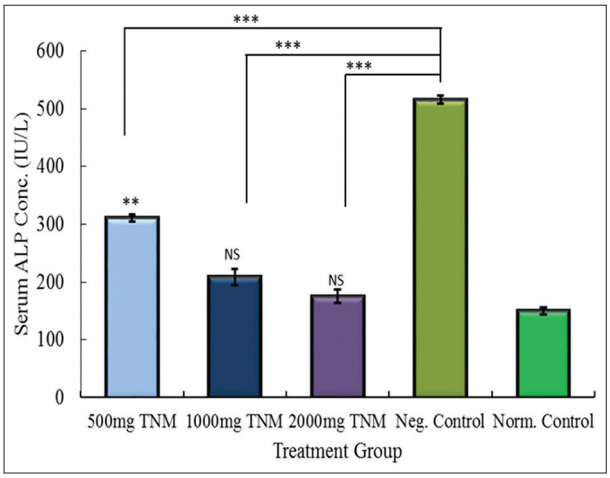

Figure 3: Serum alkaline phosphatase concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. ** and *** indicate significance at $P < 0.01$ and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

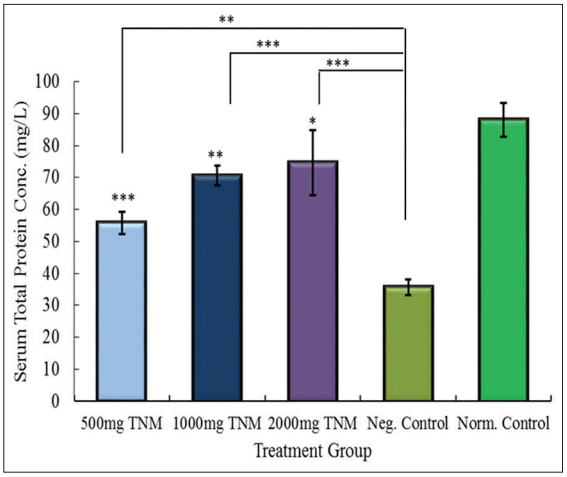

Figure 4: Serum total protein concentrations in rats treated with different doses of tigernut milk before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. *, **, and *** indicate significance at $P < 0.05$, < 0.01 and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

The test values were nonetheless significantly ($P < 0.05$) lower than that of the normal control group. Serum total bilirubin concentrations were dose-dependently and significantly ($P < 0.001$) lower in the test groups treated with TNM before APAP challenge, compared to the negative control group. In fact, at the maximum administered dose, the serum total bilirubin concentrations of the test rats were statistically similar ($P > 0.05$) to that of the normal control group [Figure 5]. From the histological data presented in Figure 6, it is seen that TNM at the tested concentrations prevented or attenuated the severe necrosis and lesions caused by APAP. Clearly, the architecture of the hepatocytes of the test groups differed markedly from those of the negative control and approximated the normal control group considerably. The results corroborate the biochemical observations of a potent hepatoprotective activity of the milk [Figure 6].

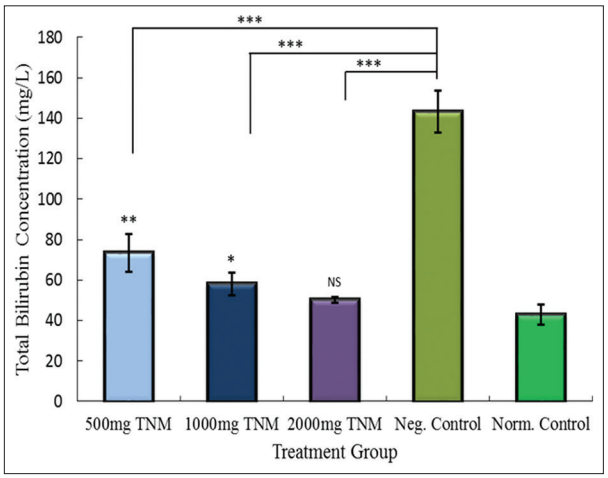

Figure 5: Serum total bilirubin concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent tigernut milk, negative, normal, and "not significant," respectively. *, ** and *** indicate significance at $P < 0.05$, < 0.01 and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

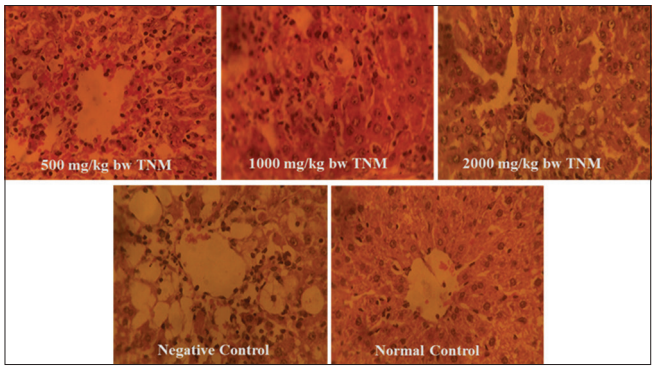

Figure 6: Photomicrographs of liver sections of rats treated with different doses of TNM before acetaminophen challenge (H and E stain, ×400). TNM represents tigernut milk. Necrosis and severe lesions are clearly observed in the negative control group. Improvement in the architecture of the hepatocytes is observed in the test groups relative to the normal control

SOD activities in serum of the test rats were significantly ($P < 0.001$) higher than that of the negative control group. They were nonetheless significantly ($P < 0.05$) lower than that of the normal control rats [Figure 7]. Figure 8 shows that there were no significant differences ($P > 0.05$) between the mean MDA concentrations of the test rats compared to each of the controls.

DISCUSSION

The liver is one of the organs of the body that is impacted first by ingested xenobiotics because it is the organ responsible for the bulk of the detoxification and biotransformation of ingested

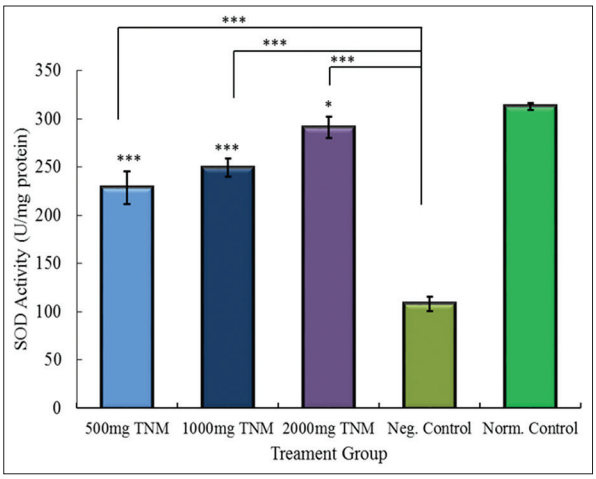

Figure 7: Superoxide dismutase activity in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent Tigernut milk, negative, normal, and "not significant," respectively. * and *** indicate significance at $P < 0.05$ and < 0.001, respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

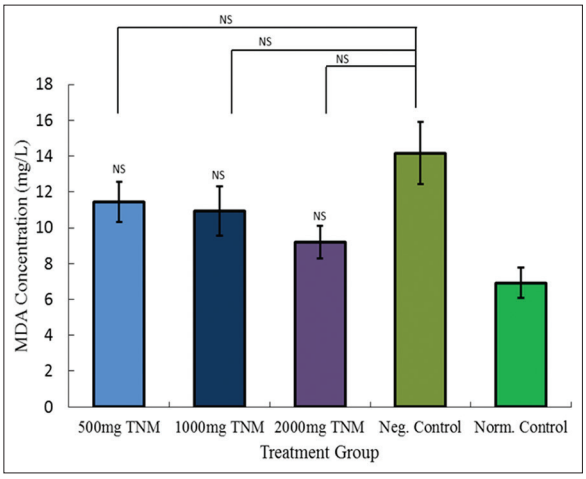

Figure 8: Malondialdehyde concentrations in rats treated with different doses of TNM before acetaminophen challenge. TNM, Neg., Norm., and NS represent Tigernut milk, negative, normal, and "not significant," respectively. Indicators on the bars are for comparisons to the normal control while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control. *N* for each group = 5

xenobiotics [23]. It is therefore logical that toxicity to the liver is an important index for overall toxicity to the system. Given that APAP is known to induce damage to hepatocytes, at high doses, through the exhaustion of GSH reserves by NAPQ1 [15], it is used for the induction of hepatocellular injury in animal models. Data from the negative control group of this study show clearly that liver damage was indeed induced in the rats exposed to APAP. The model used for these studies was therefore effective for use in testing the hypotheses.

The heightened oxidative stress within hepatocytes, due to NAPQ1 accumulation, and the subsequent membrane permeability disruption due to lipid peroxidation and ultimately necrosis [16] cause enzymes that are ordinarily localized within the cell membranes of hepatocytes to leak out into the bloodstream [24]. It is therefore interesting to observe from the results that TNM was effective in lowering the biochemical markers of liver injury studied. At 2000 mg/kg bw pre-treatment with TNM reduced the ALT and AST concentrations so considerably that the mean was statistically similar ($P > 0.05$) to that of the normal control rats. Serum ALP for test rats was not just significantly lower than negative control, they were in fact statistically similar to the normal control group. It is important to note that the concentration of these enzymes in the blood is directly proportional to the degree of liver damage [25], hence their use as "liver function enzymes." Therefore, it is interesting to find that TNM at the tested concentrations, significantly and dose-dependently lowered the concentrations of these enzymes when compared to the negative control group. More interesting is the observation that at 2000 mg/kg bw the TNM resulted in a lowering of the concentrations of the studied enzymes such that they were statistically similar to those of rats that were not exposed to the toxicant (normal control) – a case of complete hepatoprotection.

The findings from the studied liver enzymes are corroborated by the data for serum total bilirubin and serum total proteins. At 2000 mg/kg bw pre-treatment with TNM reduced the bilirubin concentration so considerably that the mean was statistically similar to that of the normal control rats. Iyanagi and Accoucheur [26] reported that elevations in the concentrations of total bilirubin in serum are a clinical marker of liver and/or biliary tract disease. In fact, it is known that increased biliary synthesis occurring concomitantly with increased biliary pressure (which often arises in the face of toxicants) causes elevation in serum ALP concentrations. Not surprisingly, therefore, improvements in the secretory mechanisms of the liver are usually sign-posted by the effective control of serum bilirubin concentrations and ALP activity [27]. Clearly therefore, TNM prevented the APAP-induced damage to the secretory mechanisms of the liver.

GSH is a major nonenzymatic endogenous antioxidant that participates in redox reactions and replenishes the antioxidant enzymes. It also directly mop up free radicals [28]. Dietary antioxidants, mainly those rich in polyphenolic compounds, help to restore the balance between the endogenous antioxidants and free radicals generated due to aerobic respiration, or the xenobiotic transformation of drugs such as APAP [29]. It appears therefore that some bioactive(s) in the TNM are able to neutralize the ROS that accumulates as a result of GSH depletion by APAP, thereby complementing the endogenous antioxidants. Conceivably too, the bioactive compound(s) act by stimulating the production of GSH, thereby increasing its concentration. Increased GSH concentrations would result in higher antioxidant enzymatic activity as seen in the increased activities of SOD in the test rats. This is plausible as Han *et al*. [28] reported that GSH is restored by phytochemicals with antioxidant properties. Furthermore, GSH is known to induce the higher concentrations of antioxidant enzymes such as SOD [30,31]. Interestingly, the serum activities of SOD in this study were higher in test rats compared to negative control, but lower than normal control. One is nonetheless mindful not to ascribe the observed activity to any given phytochemical present in the TNM as bioactive compounds often act in synergy to give the beneficial effects attributed to them.

As noted earlier, APAP disrupts a major mechanism of peroxide detoxification by depleting hepatocellular GSH which is the cofactor for GSH peroxidase. APAP overdose understandably leads to increased intra-hepatocellular peroxide levels, and the attendant increased oxidative stress, via a Fenton mechanism [16]. The monitoring of MDA concentrations via TBARS tests is useful in confirming oxidative stress in the test rats because cell membranes are lipid-rich, and the lipids are often the first macromolecules to be attacked by peroxides, leading to lipid peroxidation. Antioxidants are known to counter the effects of oxidants, and TNM is rich in antioxidant phytochemicals [7,14]. It is therefore plausible that the antioxidant phytochemicals present in TNM were capable of attenuating considerably the oxidative stress induced by the APAP challenge. MDA concentrations in the test groups were lower than that of the negative control group, though not statistically so. This suggests that breakdown products of oxidative stress were less in the test groups compared to the negative control. Clearly, the APAP-induced lipid peroxidation was very severe, yet it was considerably limited by pre-treatment with TNM as seen in Figure 8. One may, therefore, speculate that the test rats, given the considerable protection they received from the TNM would benefit more from treatment if they were to be treated.

This study is limited by a few factors. First, it would have benefitted from an extended panel of antioxidant enzymes/molecules assayed/determined. We were unfortunately limited by resources, but are positive that the deductions from the SOD assay make a clear representation for antioxidant enzymes in the studied model. Second, a positive control group may have been useful in illumining the mechanism(s) of action of TNM. The objectives of this study did not, however, make such a group mandatory, yet subsequent studies may have to include such a group.

In conclusion, the potentials of TNM in preventing liver injury were studied in a rat model in which hepatotoxicity was induced using APAP. The results show that TNM is a potent "nutri-drink" useful in the preventing liver injury arising from drug use or abuse. The findings are interesting especially as a popular saying suggests that prevention is better than therapy.

ACKNOWLEDGMENTS

The authors are grateful to staff of the Department of Home Science, Nutrition and Dietetics, University of Nigeria, for their useful criticisms of the design and protocol of this study. The contributions of the technical staff in the execution of the study, especially the histology, are appreciated.

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Source of Support: Nil, Conflict of Interest: None declared.