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Tigernut (*Cyperus esculentus* L.) 'milk' reverses acetaminophen-induced hepatotoxicity in a murine model

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ABSTRACT

The usefulness of tigernut milk (TNM) in reversing acetaminophen-induced liver injury was investigated. Twenty-five rats were randomized into five equal groups. Four groups were challenged on day 0 with 2500 mg/kg bodyweight (bw) acetaminophen. Subsequently, from days 1 to 7, they were treated with 0, 500, 1000 and 2000 kg/mg bw TNM, respectively, *per os*. The fifth group served as the normal control group. On the 8th day, the rats were sacrificed humanely and biochemical markers of toxicity and oxidative stress were determined in their sera. TNM at the tested concentrations significantly reversed liver injury as shown by liver function markers. For example, serum alkaline phosphatase (ALP) concentrations decreased dose-dependently and significantly ($p<0.001$) from 298.9±32.3 in the negative control group to 159.3±22.1 in the 2000 mg/kg bw TNM group. In fact, the serum ALP concentrations of all test rats were statistically similar ($p>0.001$) to those of the normal control rats. These biochemical 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 283.9±20.5 in the 500 mg/kg bw TNM group, and indeed in all test groups. Malondialdehyde concentrations in the test rats suggest less efficient clearance of the break-down products of lipid peroxidation. Phytochemicals in the TNM may have acted directly as antioxidants, or induced the synthesis of glutathione (which exerts downstream positive effects on antioxidant systems), thereby aiding recovery from drug-induced liver damage.

Keywords

Acetaminophen
Hepatotoxicity
Tigernut milk
Treatment

Introduction

Drug-induced liver injury causes substantial morbidity and mortality globally. Acetaminophen (N-acetyl-para-aminophenol, APAP or paracetamol) is a widely used analgesic and antipyretic drug. Because it is sold readily over the counter, its abuse is fairly common. In Nigeria, it is reported to be a widely abused drug, even in children [1], while in the USA it accounts for 56,000 emergency room visits, 2600 hospitalizations and about 500 deaths each year [2]. The toxicity of APAP derives from its metabolism in the liver where it is conjugated with sulphate and glucoronidate, thereby making it inert and water soluble, allowing it to be excreted in the urine. However, a fraction of APAP (depending on the dose) is converted (by several P450 cytochromes) into N-acetyl-p-benzoquinone imine (NAPQI) which is a highly reactive toxic intermediate [3]. Nevertheless, conjugation with reduced glutathione (GSH) efficiently eliminates substantial amounts of NAPQI. Therefore, large doses of APAP deplete the GSH in the liver and beyond the saturation point, resulting in the accumulation of unconjugated NAPQI. Unconjugated NAPQI binds to cysteine groups on proteins (to form 3-(cystein-S-yl) acetaminophen adducts), inducing oxidative stress, rapid cell death and necrosis, and ultimately liver failure [4]. Consequently, APAP can be used for studying oxidative stress-induced liver injury in animal models and for examining agents that are capable of treating such injuries.

Tigernuts (*Cyperus esculentus* L.), commonly known as yellow nut-grass, yellow nutsedge, Earth almond, rush nut, flatsedge, water grass, Zulu nut, edible rush and chufa [5, 6], belongs to the family *Cyperaceae*. It is a perennial plant

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which grows to 24–55 cm in height [7] and produces scaly rhizomes from its base which end in hard spherical tubers (not nuts!) [8]. It is the only tuber known to produce a significant amount of all three major storage reserves – starch, sucrose and lipids [9]. There are three known varieties of tigernut – yellow, brown and black. Although all three are edible, the yellow variety is often preferred because of its larger size, aesthetic appeal, higher milk yield/succulence, better flavour and storage quality. Furthermore, it contains less fat, more protein and fewer anti-nutritional factors [10]. Tigernut 'milk' (TNM) is popular especially in Spain and throughout West Africa. It is consumed more during warm periods of the year as a refreshing non-alcoholic beverage [11].

There are reports that bioactive components in tigernut exhibit anti-inflammatory and immunostimulatory properties [12], prevent heart disease and thrombosis and improve blood circulation [13]. Phytochemicals in the plant are also reported to lower the risk of colon cancer [14], and help in the management of obesity, diabetes and gastrointestinal disorders [15, 16]. TNM is also said to be useful in the treatment of flatulence, indigestion, diarrhoea and dysentery [17, 18]. For these reasons, and because it has high amounts of fibre, antioxidants (such as polyphenols and vitamins C and E) and micro-elements [18, 19], tigernut is considered nutritious and its milk a 'nutri-drink' and 'health food'. Interestingly, our preliminary toxicity studies (unpublished laboratory data) agree with Oladipipo *et al* [20] who after a 4-week 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".

Given the need for natural agents that can ameliorate damage to the liver due to drug use or abuse, and the growing popularity of nutraceuticals and functional foods, the search for such agents has been heightened lately. However, the potential of TNM as a potent 'nutri-drink' with hepato-curative possibilities has not been studied, despite its reported active principles which could have liver-related benefits. This paper therefore describes a study carried out to test the hypothesis that TNM is useful in the treatment of hepatocellular injury induced by APAP in rats.

Materials and methods

Preparation of TNM

One hundred and ninety grams (190 g) of fresh tigernuts were bought from the local market (Ogige main market, Nsukka), sorted to remove bad tubers, and washed thoroughly in tap water. The clean tubers were then blended into a slurry using a clean personal blender, with the addition of 500 ml of distilled water. The slurry was then pressed through a muslin cloth to extract the milk which was bottled in clean screw-cap bottles and stored in a refrigerator until use.

Animals and experimental design

Twenty-five (25) adult male Wistar rats were obtained from a commercial vendor and acclimatized to the animal house environment for 1 week. They were then randomized into five groups of five rats each. Four groups were each given 2500 mg/kg bodyweight (bw) APAP as a hepatotoxicant on day 0. From day 1 to day 7, the groups were given 500 mg/kg bw TNM, 1000 mg/kg bw TNM, 2000 mg/kg bw TNM and distilled water only, respectively, *per os* by intra-gastric gavage daily. The fifth group (the normal control group) received only distilled water (that is, neither APAP nor TNM) (Table 1). Throughout the experiments, the rats were housed in groups in standard cages in a properly ventilated animal house, following standard procedures and international regulations for the care of laboratory animals. They were exposed to 12-hour 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

Serum concentrations of alanine and aspartate aminotransferases and alkaline phosphatase were determined using the enzymatic colorimetric methods of Reitman and Frankel [21] and Rec [22], respectively. The methods of Jendrassik and Grof [23] and Tietz [24] were used for the determina-

Treatment day	Group 1	Group 2	Group 3	Negative control	Normal control
0	2.5 g/kg bw APAP	2.5 g/kg bw APAP	2.5 g/kg bw APAP	2.5 g/kg bw APAP	-
1–7	500 mg/kg bw TNM	1000 mg/kg bw TNM	2000 mg/kg bw TNM	Distilled water	Distilled water

Table 1 - Experimental design and protocol for the treatment of animals

tion of serum total bilirubin and total proteins, respectively. Serum superoxide dismutase (SOD) activity was assayed by spectrophotometrically monitoring inhibition of the auto-oxidation of epinephrine in the presence of Fenton reagent as described by Misra and Fridovich [25]. Serum malondialdehyde (MDA) concentrations were measured as a proxy for lipid peroxidation. This was done by spectrophotometrically measuring the concentration of the product of the reaction between MDA and thiobarbituric acid, thiobarbituric acid reactive substances (TBARS), a pink chromogen [26]. Assay kits procured from reputable companies were used for all 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 processed further by dehydration in grades of ethanol, clearing in xylene, then infiltration with, and embedding in, paraffin. Sectioning was done at 5 µm using a microtome and the sections stained with haematoxylin and eosin. Sections were subsequently viewed and photomicrographs taken (magnification: × 400).

Statistical analysis

The data generated were subjected to statistical analyses. Means and standard deviations for each parameter per group were calculated and differences between means were separated by one-way ANOVA. Multiple comparisons were done using the least significant difference (LSD) test. A significance threshold of $p<0.05$ was adopted for all analyses. Data analyses were done using the statistical software IBM-SPSS version 20 (IBM, Atlanta, GA). The results are presented as bar charts.

Results

Treatment with TNM subsequent to the induction of hepatic injury significantly ($p<0.001$) reduced serum ALT concentrations in the test rats compared with the negative control. However, the mean ALT concentrations of the test groups were significantly higher than that of the normal control rats (Fig. 1). Whereas treatment with TNM significantly ($p<0.001$) lowered the serum AST concentrations of the test rats compared to the negative control, the values were still significantly ($p<0.001$) higher than that of rats in the normal control group (Fig. 2).

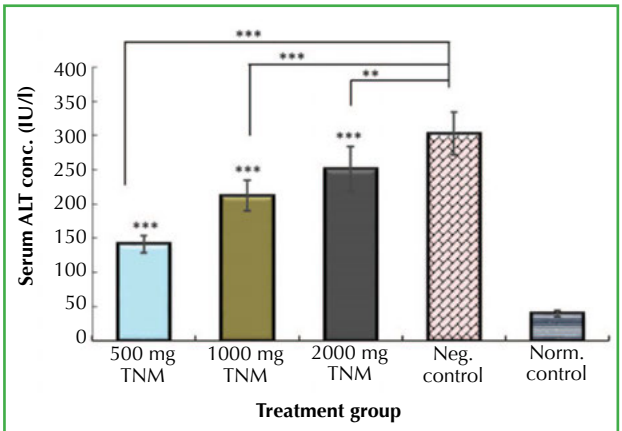

Figure 1 - Serum alanine aminotransferase (ALT) concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg*. negative, *Norm*. normal, *TNM* tigernut milk. $^{**}p<0.01$; $^{***}p<0.001$

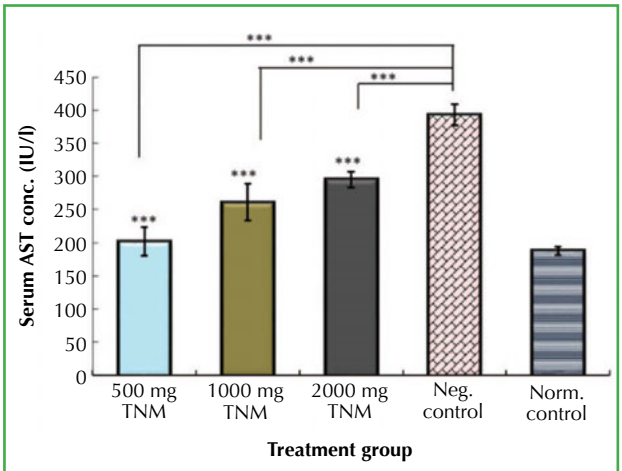

Figure 2 - Serum aspartate aminotransferase (AST) concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg*. negative, *Norm*. normal, *TNM* tigernut milk. $^{***}p<0.001$

Interestingly, for these two key liver enzymes – ALT and AST – there was no apparent advantage in treatment with increased doses of TNM. In fact, a reverse-dose-dependent effect was observed. Conversely, serum ALP concentrations were dose-dependently and significantly ($p<0.001$) lower in test rats treated with TNM relative to the negative control group. The most important findings concerning the serum ALP concentrations of the test rats were the dose-dependent effect seen and the observation that the mean ALP values were each statistically similar ($p>0.05$) to that of the normal control rats (Fig. 3).

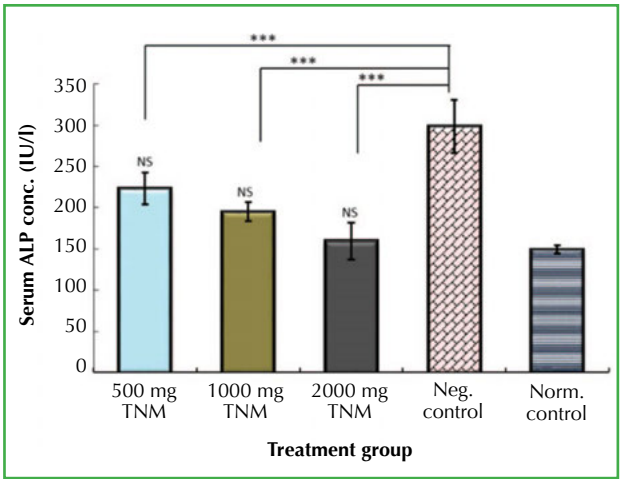

Figure 3 - Serum alkaline phosphatase (ALP) concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg.* negative, *Norm.* normal, *NS* not significant, *TNM* tigernut milk. ***p<0.001

Figure 4 shows the serum total protein concentrations of the rats. The test rats had significantly (p<0.01) higher serum total protein concentrations compared with the negative control group (except the 2000 mg/kg bw group). The test values were nonetheless significantly (p<0.05) lower than that of the normal control group. The serum total bilirubin concentrations of the test rats were significantly (p<0.001) lower than that of the negative control, but significantly (p<0.01) higher than that of the normal control group (Fig. 5).
Data from the histological studies corroborate the biochemical observations of recovery from liver damage induced by APAP upon treatment with TNM. The photomicrographs show a dose-dependent improvement in the architecture of the hepatocytes as the necrosis and lesions observed in the negative control clearly reduced with increase in test doses up to the 2000 mg/kg bw group which closely approximated the normal control group (Fig. 6).
SOD activity in the serum of the test rats was significantly (p<0.01) higher, and lower, than the negative and normal control groups, respectively (Fig. 7). Interestingly, the lowest dose of TNM administered was more effective in increasing the activity of the antioxidant enzyme. Figure 8 shows that there was no significant reduction in the MDA concentrations of the test rats relative to the negative control group. While the 500 mg/kg bw group was significantly (p<0.001) higher, the other two test groups had values that were similar statistically (p>0.05) to the negative control group. The test groups all had MDA values that were significantly (p<0.05) higher than that of the normal control group.

Discussion

The liver is the organ responsible for most of the detoxification of ingested xenobiotics and is therefore highly exposed to the impact of toxicants [27]. Acetaminophen, although tolerable at low doses, induces damage to hepatocytes at high doses, through exhaustion of GSH reserves by NAPQI [3]. This leads to the heightening of oxidative stress within hepatocytes, and subsequent mitochondrial membrane per-

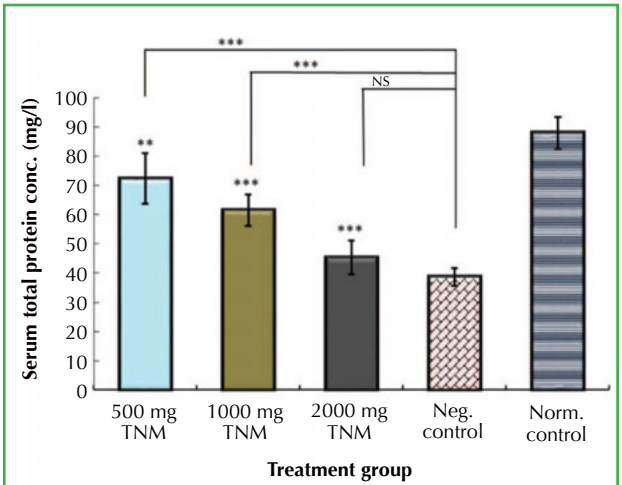

Figure 4 - Serum total protein concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg.* negative, *Norm.* normal, *NS* not significant, *TNM* tigernut milk. **p<0.01; ***p<0.001

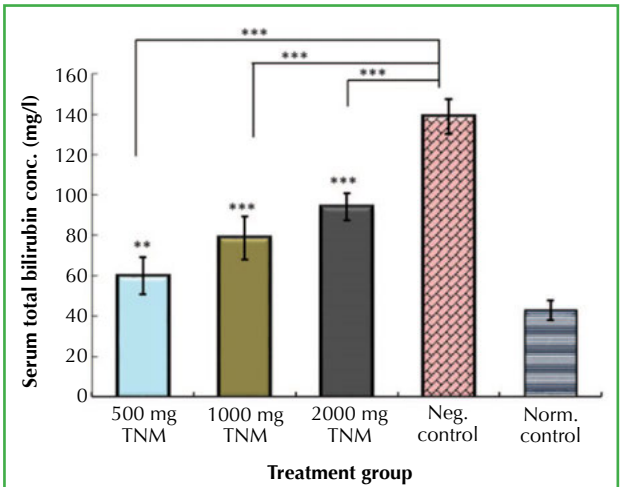

Figure 5 - Serum total bilirubin concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons to the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg.* negative, *Norm.* normal, *TNM* tigernut milk. **p<0.01; ***p<0.001

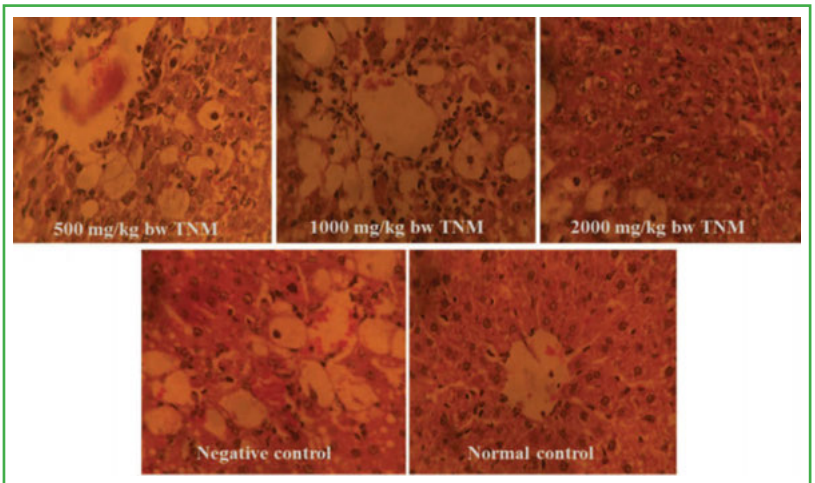

Figure 6 - Photomicrographs of liver sections of rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Necrosis and severe lesions are clearly observed in the negative control group. A dose-dependent improvement in the architecture of the hepatocytes is observed in the test groups relative to the normal control. H&E stain, magnification ×400. *bw* bodyweight, *TNM* tigernut milk

meability disruption due to lipid peroxidation, loss of mitochondrial ATP synthesis, and ultimately necrosis [4]. Data from the negative control group show clearly that liver damage was indeed induced in the rats exposed to APAP. The model used for this study was therefore effective for testing the hypothesis.

Therefore, it is interesting to observe from the results that TNM was effective in reversing the biochemical markers of liver injury studied. At all the tested doses, treatment with TNM reduced ALT, AST and ALP concentrations significantly; for ALP, the reduction was so great that the mean was statistically similar ($p>0.05$) to that of the normal control rats. Usually, these enzymes remain localized within the hepatocytes, and only leak into the circulation as a result of disruption of the hepatocellular architecture [28]. Okonkwo *et al* [29] noted that the production of lipolytic mitochondrial enzymes (in response to the presence of toxicants) and the additional or independent action of peroxides and radicals cause the dissolution of cell membranes, and the membranes of hepatocellular organelles, thereby causing the release of liver enzymes into the blood. The concentration of these enzymes in the blood is therefore directly proportional to the degree of liver damage. This underscores their use as 'liver function enzymes'. Thus, it is interesting to find that TNM at the tested doses significantly lowered the concentrations of these enzymes when compared to the negative control group. It is also interesting to note that the lowest tested dose apparently achieved more than the higher doses, suggesting that there may not be an advantage in increasing the dosage of TNM used to treat liver injury.

The findings from the studied liver enzymes are corroborated by the data for serum total bilirubin and serum total proteins. The test groups had significantly lower bilirubin concentrations compared with the negative control but not the normal control.

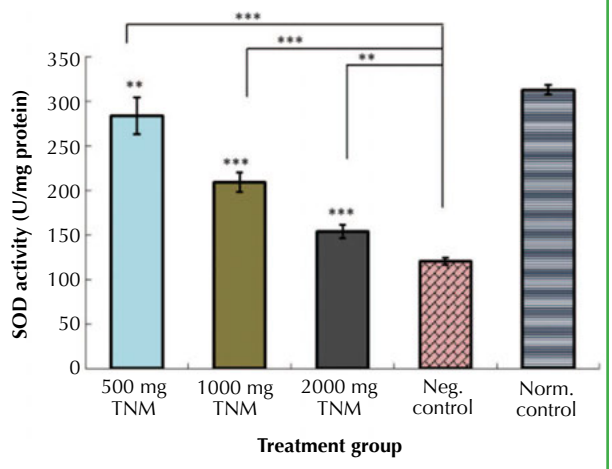

Figure 7 - Superoxide dismutase (SOD) activity in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg.* negative, *Norm.* normal, *TNM* tigernut milk. **$p<0.01$; ***$p<0.001$

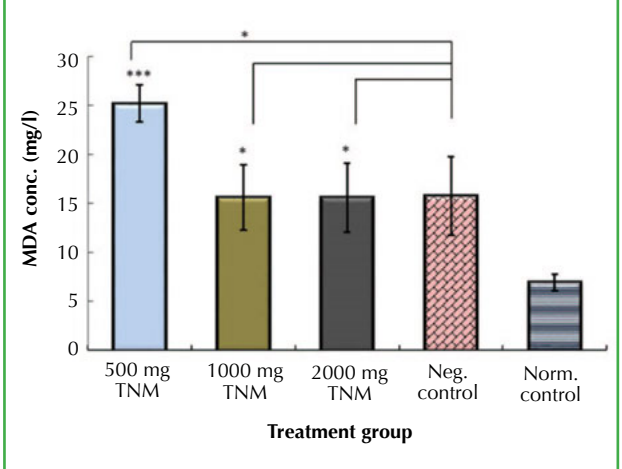

Figure 8 - Malondialdehyde (MDA) concentrations in rats treated with different doses of tigernut milk subsequent to acetaminophen challenge. Indicators on the bars are for comparisons with the normal control, while those on the lines connecting the negative control to the test groups are for respective comparisons to the negative control.
N=5 for each group. *Neg.* negative, *Norm.* normal, *NS* not significant, *TNM* tigernut milk. *$p<0.05$; ***$p<0.001$

An elevated concentration of total bilirubin in serum is a known clinical marker of liver and/or biliary tract disease [30]. In fact, it is reported that elevation in ALP is due to increased synthesis occurring concomitantly with increased biliary pressure. For this reason, effective control of serum bilirubin concentrations and alkaline phosphatase activity is a clinical indication of improvement in the secretory mechanism of the liver [31]. Clearly therefore, TNM 'repaired' the secretory mechanisms of the liver that were damaged by APAP. This position is re-enforced and validated by the data from the histological study where a clear recovery is seen in the hepatocytes of the groups treated with TNM.

Since GSH depletion is central to APAP-induced hepatotoxicity and GSH is the cofactor for glutathione peroxidase, APAP therefore disrupts a major mechanism of peroxide detoxification. Thus, GSH depletion or APAP overdose would lead to increased intracellular peroxide levels, and the attendant increased oxidative stress, via a Fenton mechanism [4]. Given that the membranes of cells are lipid-rich, and the lipids are often the first macromolecules to be attacked by peroxides, leading to lipid peroxidation, monitoring of MDA concentrations via the TBARS test is therefore valid for confirming oxidative stress in the test rats. TNM is rich in antioxidant phytochemicals such as polyphenols and vitamins C and E [12, 18, 19]. This study shows that these antioxidant phytochemicals (apparently) were capable of lowering oxidative stress induced by the APAP challenge. The serum activities of SOD were higher in test rats compared to negative control, suggesting induction to higher concentrations and activities by TNM.

The most important endogenous antioxidant enzymes are SOD, catalase, glutathione peroxidase and glutathione reductase. GSH is a major non-enzymatic endogenous antioxidant that participates in redox reactions which replenish the antioxidant enzymes and directly mop up free radicals [32]. Consequently, dietary antioxidants, mainly those rich in polyphenolic compounds, help to restore the balance between natural antioxidants and the free radicals generated endogenously as a result of electron transfer to molecular oxygen in the electron transport chain, or the xenobiotic transformation of drugs such as APAP [33]. It appears, therefore, that some bioactive(s) in the TNM compete with antioxidant enzymes (here typified by SOD) competitively for the reactive oxygen species (ROS) generated as a result of GSH depletion by APAP. Perhaps, additionally, the bioactive compound(s) act by stimulating the production of GSH, thereby increasing its concentration, and then (stretched a little further) by GSH inducing higher antioxidant enzymatic activity as seen in the increased activities of SOD in the test rats. This is plausible as GSH is known to be restored by phytochemicals with antioxidant properties [32], and is also known to induce the higher concentrations of antioxidant enzymes such as SOD [34]. Molina *et al* [35] have made similar observations in their study of the effect of quercetin on the oxidative state of the liver of mice. However, one should not ascribe the observed activity to any given phytochemical present in the TNM as bioactive compounds often act in synergy to provide the beneficial effects attributed to them. Furthermore, it appears that the SOD subtype discussed here is the Mn-SOD (which is induced by a myriad of agents) not the CuZn-SOD (which is a weakly inducible enzyme) [36].

The concentrations of MDA, one of the biomarkers of lipid peroxidation, in the test rats, in comparison with the negative control, suggest that breakdown products of oxidative stress were similarly present in the rats. It appears that the APAP-induced oxidation of lipids was so severe that treatment with TNM for 7 days was not sufficient to bring down the concentrations of MDA to values that could reach statistical significance. It is also plausible that whereas TNM resulted in recovery from APAP-induced toxicity in the liver, it could not improve the mechanisms required to clean-up the by-products of oxidative stress. Be that as it may, one may speculate that longer treatment with the milk may have resulted in significant reductions in the MDA concentrations. The results agree with the data on SOD activity which was increased relative to the negative control, but did not match the activity found in the normal control rats.

Clearly the studies would have benefitted from an extended panel of antioxidant enzymes/molecules assayed/determined. We were unfortunately limited by resources. Nevertheless, the SOD assay clearly suggests the presence of antioxidant enzymes in the studied systems. Similarly, the studies would have benefitted from a positive control group as this may have thrown more light on the mechanism(s) of action of TNM. We did not include such a group as our objectives did not require it. Subsequent studies will however take a positive control group into account, where necessary.

In conclusion, the potentials of TNM in treating liver injury in a rat model in which hepatotoxicity was induced using APAP was studied. The results show that TNM is a potent 'nutri-drink' useful in the treatment of liver injury arising from drug use or abuse. Further studies are warranted to elucidate the mechanism(s) of action of TNM, and examine the impact of an extended treatment period after induction of hepatocellular injury.

Acknowledgements

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Conflict of Interest

The authors declare that they, singly or collectively, have no real or potential conflicts of interest.

Author Contributions

NOO (first author) conceived, designed and supervised the study; NOO (second author) participated in study design, carried out the experiments and collated the data; NJU participated in study design; CECCE participated in study design, analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the final manuscript.

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