HEPATO- AND NEPHRO – PROTECTIVE EFFECT OF TINOSPORA CORDIFOLIA AGAINST SODIUM NITRITE – INDUCED OXIDATIVE STRESS

Manju Bala¹, Girdhari Lal Gupta²*
¹Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India.
²School of Pharmacy and Technology Management, SVKM’s NMIMS University, Mumbai, Maharashtra, India.

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ABSTRACT

Sodium nitrite (NaNO₂) is widely used as a bacteriostatic and preservative for meats and fish. Meanwhile, NaNO₂ is also associated with detrimental health effects on liver and kidney. Moreover, no scientific validation is available for traditionally used Tinospora cordifolia (Willd.) Hook. f. and Thoms. (Guduchi) in NaNO₂ induced oxidative stress. The objective of the present investigation was to evaluate the effects of ethanolic extract of stems of Tinospora cordifolia (Willd.) (ETC) in NaNO₂ induced oxidative stress, hepatic and renal dysfunction. Twenty-four adult male Sprague–Dawley rats were daily treated with sodium nitrite (80 mg/kg) in the presence or absence of ETC (200 mg/kg) for three months. Biochemical and morphological changes were assessed in liver and kidney sections. ETC significantly restored SOD, catalase and glutathione level compared with sodium nitrite. ETC also reduced serum SGOT, SGPT, urea, creatinine, malondialdehyde and nitric oxide level as compared to sodium nitrite treated group. Therefore, It is suggested that ETC has hepato– and nephro–protective effect against sodium nitrite–induced oxidative stress.

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INTRODUCTION

Food additives are highly specialized ingredients afford us enjoyment of a wide variety of nutritious, fresh and palatable foods in our modern lifestyle by maintaining product quality, freshness and aiding processing and preparation of foods. They are classified into coloring agents, preservatives, stabilizers and flavoring agents [1]. In early ages, salts, or brine solution was added to food for preservation purposes. Sodium nitrite (NaNO₂) is widely used as bacteriostatic and preservative improves flavor and imparts pink color to cured meat. Owing to its properties such as improving food safety, fixing color to meat as well as extending shelf life of products, manufacturers of cured meats prefer the use of NaNO₂ [2]. In addition, NaNO₂ is known to possess antibacterial properties and prevents growth of *Clostridium botulinum*, a Gram–positive anaerobic bacterium responsible for botulism food poisoning [3]. Emerging evidence indicates that nitrite is a potent nitric oxide (NO) donor and may itself possess detrimental biological effects [4]. Excessive oral intake of nitrates and nitrates may be associated with an increased risk of negative health outcomes, causing methemoglobinemia (MtHb) in infants [5,6]. Further evidences suggest that nitrite in food might react with the amines of food in the stomach producing carcinogenic nitrosamines [7]. In addition, nitrosamines also cause excessive generation of free radicals [8]. There has been an increasing thrust worldwide to opt for safer and effective plant–derived agents mentioned in the traditional medical systems.

*Tinospora cordifolia* (Willd.) Hook. f. and Thoms. (Guduchi) is known to possess powerful antioxidant properties. It is a large climbing shrub with greenish–yellow typical flowers belonging to family *Menispermaceae* [9]. Historically, the plant has been exhibited various pharmacological activities such as anti–inflammatory, immunosuppressive, diuretic, antidiabetic, antioxidant and hepatoprotective activity [10, 11]. To our knowledge, no study has yet investigated the potential beneficial effects of ethanolic extract of stems of *Tinospora cordifolia* (Willd.) Hook. f. and Thoms. (ETC) in sodium nitrite – induced toxicities. Therefore, it was considered worthwhile to explore the potential role of ETC in preventing NaNO₂–induced oxidative stress, hepatotoxicity, and nephrotoxicity; by measuring changes in biochemical, hematological and histopathological parameters in rats.

MATERIALS AND METHOD

Animals

Twenty–four adult male Sprague–Dawley rats weighing 110–130 gm were housed separately in groups of five per cage under controlled light (12:12 hrs light: dark cycle, light on at 0700 h) and temperature (25 ± 2°C) environment. Food (Rat chow, Lipton, India) and water was provided *ad libitum*. All procedures were carried out under strict compliance with ethical principles and guidelines of the Institutional Animal Ethical Committee constituted as per CPCSEA (1716/PO/a/13/CPCSEA).

Chemicals

Sodium nitrite was purchased from (Sigma–Aldrich) and bovine serum albumin (BSA), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 5,5–dithionitro bis 2–nitrobenzoic acid (DTNB) were purchased from Himedia lab Ltd, Mumbai. All other chemicals were of analytical grade. The biochemical and hematological parameters were done by using commercial kits purchased from Ranbaxy–RFCL Ltd, India.

Preparation of extract

The experimental plant material was collected from local region Solan, Himachal Pradesh in the month of March. A voucher specimen of the plant was identified by Dr. Yashwant Singh Parmar University of Horticulture and forestry, Nauni, Solan (H.P.). The stems of *Tinospora cordifolia* were washed thoroughly with distilled water and shade dried. The crude drug was pulverized, using a mechanical grinder and passed through sieve no, 40. The powder was defatted with petroleum ether for 6–8 h and further extracted using 70% v/v ethanol by soxhlation for 72 h under regulatory conditions. The extract was subsequently freed from solvent and dried *in vacuum* using a rotary evaporator (Heidolph, Germany) at 50°C. Solid mass was collected after lyophilisation. The final yield was calculated as 12% w/w of crude extract and stored at 4°C for further biological activities.

Acute toxicity study

In our separate experiment acute oral toxicity studies were carried out according to Organisation for Economic Co–operation and Development (OECD) test guidelines 423. ETC was found to be safe up to 2000 mg/kg. There was no mortality and toxic signs presented by animals in any of the groups used in different experimental design.

Experimental design

Three groups were used consisting of eight rats per group (n = 8) and assigned for three months to one of the following treatment regimens.

Group 1: Vehicle (2% w/v gum acacia in distilled water, oral, once daily)

Group 2: NaNO₂ (80 mg/kg, oral, once daily)

Group 3: ETC (200 mg/kg, oral, once daily) and NaNO₂ (80 mg/kg, oral, once daily)

ETC was administered one hour prior to NaNO₂ each day for three months. The body weight, food and water intake of all the animals were recorded. Based on the study by Hassan et al., the dose of NaNO₂ was selected [12]. The drugs were administered between 9.00 am and 11.00 am once a day for three consecutive months. A single dose, route of administration schedule of ETC used in the present experiment was chosen as based on previous results [13].
Serum preparation
Blood samples were collected from the rats under anaesthesia by *retro–orbital plexus* method at the end of the experiment (day 91) for evaluation of hematological and biochemical analysis. All samples were collected in vials containing sodium citrate (0.3% w/v) and centrifuged at 860 g for 20 min. The supernatant was collected and stored at -20°C for further analysis.

Examination of morphological and cellular changes
After collection of blood samples, all rats were sacrificed by cervical dislocation method. Tissue samples of liver and kidney were collected in 10% neutral buffer formalin solution and kept for 24 h for fixation. Thin sections of the tissues were stained with Mayer’s hematoxylin and eosin. Tissue sections were visualized under 100x magnifications. The morphological changes were imaged by digital BX–61 Olympus microscope.

Estimation of biochemical parameters
Lipid peroxidation estimation
The serum (200 µl) was mixed with equal volume of 8.1% of sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% of TBA. MDA, a product of lipid peroxidation reacts with TBA to give a pink colored product. The test tubes were centrifuged at 3500 g for 10 min, lipid layer was collected, and absorbance was noted 532 nm. The concentration of MDA in the samples was calculated using the molar extinction coefficient of 1.56 ×10⁻⁵ M⁻¹ cm⁻¹ [14].

Nitrite estimation
The nitrite content was estimated by using Griess reagent, which involves the formation of dark purple colored azo dye with NO compounds. The samples were mixed with griess reagent (1% sulfanilamide, 2.5% hydrochloric acid, and 0.1% naphthylethylenediamine dihydrochloride) and allowed to stand for 30 min. The absorbance was recorded at 550 nm. 100 µl of sodium nitrite was taken as the standard [15].

Protein estimation
For determination of total protein content, 100 µl of plasma sample was taken, and the same amount of TCA was added to it. A white precipitate thus formed due to denaturation of protein was then centrifuged at 3500 g for 10 min. 0.1 ml of the supernatant was collected, and absorbance was measured at 660 nm. BSA was used as standard [16].

Reduced glutathione estimation
The plasma sample (100 µl) was added with 0.5 ml of TCA to precipitate the protein content. Following centrifugation (10000 g for 5 min), the supernatant was collected and 2.0 ml of Na₂HPO₄ (4.25%) and 0.04 ml of DTNB were added to it. A blank sample was prepared similarly using distilled water in place of the sample. Absorbance was read at 412 nm. The enzymatic activity was calculated using the molar extinction coefficient of 13600 M⁻¹ cm⁻¹ [17].

Superoxide dismutase (SOD) assay
The sample (0.05 ml) was mixed with 1 ml carbonate buffer (0.2 M, pH 10.2) and 0.8 ml KCl (0.015 M). A 0.2 ml of epinephrine (2 mM) was added, and the change in optical density was recorded at 480 nm at intervals of 15 sec for 1 min. A control lacking enzyme preparation was run simultaneously. One unit of enzyme activity is defined as the amount of enzyme required to cause 50% inhibition of auto–oxidation of epinephrine at experimental conditions [18].

Assay of catalase activity
A 0.3 ml of phosphate buffer (0.2 M pH 6.8) and 0.1 ml of H₂O₂ (1 M) were taken in the test tube, and volume was made up to 3.0 ml with distilled water. Plasma sample (0.05 ml) was added, and change in absorbance was noted at 240 nm at intervals of 15 sec for 1 min. Control lacking enzyme preparation was run simultaneously. Catalase activity was expressed as µmoles of H₂O₂ consumed/min/mg protein [19].

Hematological and biochemical analysis
The hematological parameters (Hemoglobin, RBC and WBC count) were measured in the plasma by using commercially available standard kits, while SGOT, SGPT, urea and creatinine were measured in serum using standard kits using the automatic clinical chemistry analyzer (EM 200, Erba Mannheim).

Statistical analysis
All data were expressed as mean ± SEM of each group. The results were analyzed by using Graph pad prism 6.0, Graph Pad Software, San Diego California USA. The one–way analysis of variance (ANOVA) and post hoc Tukey’s test was performed for multiple comparisons among the different groups. Differences were considered to be significant at P<0.05.
RESULTS

Effect of ETC on NaNO₂ induced oxidative stress changes in antioxidant enzymes

Administration of NaNO₂ (80 mg/kg, oral) for a period of 3 months in rats, significantly declined SOD, catalase and GSH activity (P<0.0001) in comparison to the vehicle control group. Prior treatment of ETC with NaNO₂ significantly reversed increase in SOD, catalase and GSH activity (P<0.0001) as compared to NaNO₂ treated rats (Fig. 1. A, B & C).

Effect of ETC on NaNO₂ induced oxidative stress parameters (MDA and NO)

Administration of NaNO₂ (80 mg/kg, oral) for a period of 3 months in rats, showed significant increases in MDA and NO in serum as compared with the control group (P<0.0001). Prior treatment of ETC (200 mg/kg, oral) significantly ameliorated (P<0.0001) NaNO₂ induced effects as compared to NaNO₂ treated rats (Fig. 1. D & E).

Effect of ETC on NaNO₂ induced changes in protein level

NaNO₂ treated group significantly reduced the total protein level compared to the control group (P<0.0001). Furthermore, treatment of ETC (200 mg/kg, oral) prior to NaNO₂ significantly (P<0.001) increased the total protein level as compared to NaNO₂ treated rats (Fig. 1. F).

Effect of ETC on NaNO₂ induced changes in body weight, feed intake and water consumption

Administration of NaNO₂ (80 mg/kg, oral, once daily) for a period of three months in rats, led to a significant decrease in body weight gain, feed intake and water consumption as compared to vehicle control group (P<0.0001). The oral administration of ETC (200 mg/kg, oral) prior to NaNO₂ significantly ameliorated changes in body weight (P<0.01), feed intake (P<0.0001) and water consumption (P<0.0001) as compared to NaNO₂ treated group (Fig. 2. A).

Effect of ETC on NaNO₂ induced changes in hematological parameters

NaNO₂ (80 mg/kg, oral, once daily) for a period of 3 months, lowered values of hemoglobin (P<0.0001), RBC count (P<0.0001) and WBC count (P<0.0001) as compared to the vehicle control group. The values significantly (P values of <0.001^[Hb], <0.05^[RBC], <0.0001^[WBC]) increased in the group receiving oral administration of ETC (200 mg/kg, oral) and NaNO₂ (80 mg/kg) together as compared to NaNO₂ dosed group (Fig. 2. B).

Effect of ETC on NaNO₂ induced changes in biochemical parameters

The levels of liver markers, i.e. SGOT, SGPT and renal parameters urea and creatinine were significantly (P<0.0001) increased in rats dosed with NaNO₂ (80 mg/kg, oral, once daily) for a period of 3 months. Moreover, combined NaNO₂ and ETC treated group significantly ameliorated (P<0.0001) all these effects as compared to NaNO₂ treated group (Table. 1).

Morphological changes in tissues

Morphological changes in the tissues of different treated groups were observed in the liver and kidney sections after 3 months of treatment regimen. The liver sections of the control group showed the normal architecture of hepatocytes and sinusoids around the central vein (Fig. 3A). In contrast, the liver of NaNO₂ treated group displayed moderate congestion and hemorrhages with severe degeneration of hepatic cords (Fig. 3B). A simultaneous effect of NaNO₂ (80 mg/kg, oral) and ETC (200 mg/kg, oral) treated group exhibited normal histology of liver with moderate increase in cellularity (Fig. 3C).

The NaNO₂ administration to rats from first day to the end of the experimental day provoked severe degeneration of tubules and edema with shrinkage of glomeruli (Fig. 3E) as compared to control group (Fig. 3D). Moreover, treatment of ETC (200 mg/kg, oral, once daily) prior to NaNO₂ (80 mg/kg, oral, once daily) for a period of 3 months showed less obvious disturbances in epithelial lining, minimal congestion and minimal increase in cellularity (Fig. 3F) as compared to NaNO₂ treated group.

DISCUSSION

The results of the present investigations revealed that chronic administration of ETC ameliorates NaNO₂ induced hepato– and nephro–toxicities in rats. To our knowledge, no study has yet investigated the potential beneficial effects of ETC in preventing sodium nitrite–induced toxicities. NaNO₂ was associated with increase serum MDA, NO, ALT, AST, urea, creatinine and depletion in concentration of SOD, catalase, GSH; indicative of oxidative stress. NaNO₂ leads to free radical damage via generation of reactive nitrogen species (RNS), lipid peroxidation and direct depletion of antioxidant enzymes [20]. However, it reacts with secondary nitrosoamines, which are carcinogenic in nature. NaNO₂ also increased the level of LPO in comparison to vehicle treated group. The NaNO₂ intoxicated rats produce RNS due to the exposure to nitrite, which in turn overwhelms the oxidant/antioxidant system and triggers LPO of cell membrane. The high level of LPO contributes to pronounced cellular damage under oxidative stress [21]. Our findings are inconsistent with those reported by Krishnamoorthy & Sangeetha [22].

Moreover, NaNO₂ intoxicated rats exhibited significant reduction in SOD and catalase activity. These findings are aligned with those reported by Hassan et al [23]. NaNO₂ treated rats, also showed depleted levels of GSH, which could possibly be due to its enhanced utilization for the scavenging free radicals and the formation of oxidized GSH (GSSG); leading to an imbalance between the ratio of the GSH/GSSG, which is an index of cell damage in NaNO₂ treated groups [23]. Our current findings suggest that maintenance of SOD, catalase and GSH in ETC treated group is probably due to the antioxidant effect of ETC on mitochondrial electron transport chain complex.
Sodium nitrite treated rats showed reduction in weight gain. The mean weight loss in sodium nitrite intoxicated rats might be due to the increased levels of nitrite leading to increase catabolic processes in the body. The weight loss may be attributed to a reduction in food utilization sodium nitrite toxicity. Decreased water intake was also observed in the NaNO$_2$ intoxicated rats. In the present findings, it was confirmed that ETC mitigates the catabolic effects of NaNO$_2$ and improves physiological behavior. A significant low concentration of Hb, RBCs and WBCs count were attributed to the structural alteration and erythrocyte destruction in the blood cells. First time to the best of our knowledge, in the present study, all these effects induced by NaNO$_2$ were reversed by ETC.

The functional and morphological alterations in kidney and liver tissues also support the findings obtained through biochemical parameters and further confirmed the toxic effects of NaNO$_2$. The liver of NaNO$_2$ exposed group showed a marked loss in its characteristic architecture. Severe toxic changes were observed such as congestion, hepatocellular damages, hepatic cord degeneration, and disruption of the endothelial lining of central vein, compared to the vehicle control group. These results, obtained were further confirmed by the significant increase in SGPT and SGOT in serum of sodium nitrite treated rats, indicating a hepatic disorder. It has been assumed that the NaNO$_2$ causes disturbed enzyme syntheses and alters the permeability of liver membrane. Co–administration of ETC (200 mg/kg, oral) prevented increases in the level of these enzymes and exhibited hepatoprotective activity by repairing the structural integrity of cells.

Significant elevation of urea and creatinine was also observed in NaNO$_2$ intoxicated group. Urea and creatinine levels in urine are reliable biochemical indices for the evaluation of renal impairment functions. Impaired renal function could be due to either tubular necrosis or changes in glomerular filtration rate [24]. Histological examination of section of the kidney revealed that treatment of ETC prior to NaNO$_2$ reversed severe degeneration of tubules and edema with shrinkage of the glomeruli of the kidney as observed in NaNO$_2$ treated group. Treatment with ETC significantly decreased the elevated urea and creatinine levels and improved kidney function due to its antioxidant properties. It was also observed in the present study that the total protein level in NaNO$_2$ treated group was significantly lowered compared to control group. Proteins are the fundamental components of all living cells and include many substances such as enzymes. The inhibitory effect on protein synthesis was noticed perhaps due to alteration in protein and amino acid metabolism and the stimulatory effect of the nitrite on the thyroid and adrenal glands. This leads to inhibition of protein synthesis, rapid breakdown, increases in the rate of free amino acids and decrease in protein turnover. These effects were restored after co–administration of ETC indicating its preventive role in protein oxidation. The results thus obtained from the present study provide important evidence that supplementation of ETC (200 mg/kg, oral) during the exposure to NaNO$_2$ may reverse some extent the toxicity induced by NaNO$_2$ in rats.

### Table 1: The effect of ethanolic extract of *Tinospora cordifolia* on renal and hepatic biomarkers in sodium nitrite–induced biochemical changes in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameters</th>
<th>Renal Urea (mg/dl)</th>
<th>Renal Creatinine (mg/dl)</th>
<th>Hepatic SGOT (IU/l)</th>
<th>Hepatic SGPT (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td></td>
<td>26.25 ± 1.02</td>
<td>1.05 ± 0.03</td>
<td>125.78 ± 1.56</td>
<td>38.90 ± 1.34</td>
</tr>
<tr>
<td>NaNO$_2$ (80 mg/kg)</td>
<td></td>
<td>47.56 ± 1.23*</td>
<td>3.18 ± 0.24*</td>
<td>174.50 ± 2.80*</td>
<td>57.98 ± 0.98*</td>
</tr>
<tr>
<td>NaNO$_2$ + ETC (200 mg/kg)</td>
<td></td>
<td>27.34 ± 0.99$^\beta$</td>
<td>1.09 ± 0.09$^\beta$</td>
<td>138.50 ± 1.67$^\beta$</td>
<td>41.67 ± 1.19$^\beta$</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± SEM for eight rats. $^\ast$P<0.0001 vs. respective vehicle control (Tukey’s multiple comparisons test) and $^\beta$P<0.0001 vs. respective NaNO$_2$ treated group (Tukey’s multiple comparisons test).
Figure 1

Fig. 1: A. Effects of ETC on NaNO₂ induced changes in SOD level in rat. B. Effect of ETC and NaNO₂ on catalase level. C. Effects on GSH level. D. Effects on MDA Level. E. Effects on NO level. F. Effects on the level of protein.
**Figure 2.**

A. Effect of NaNO₂ and NaNO₂ + ETC on body weight, feed and water intake.
B. Effects on Hb, RBC and WBC count.

**Figure 3**

Fig. 3. Histopathological sections of liver (A, B and C) and kidney (D, E and F) from rats by H&E staining (magnification × 100). A. Control. B. NaNO₂. C. NaNO₂ + ETC. D. Control. E. NaNO₂. F. NaNO₂ + ETC groups.
CONCLUSIONS
The present study indicates that ETC possesses antioxidant, hepatoprotective and nephroprotective activity against sodium nitrite–induced toxicities, which may be attributed to its free radical scavenging potential. In the same way, identification and isolation of compound(s) responsible for the activity could be used as prototype(s) to design new substances with antioxidant, hepatoprotective and nephroprotective activity.

Abbreviations
ETC– Ethanolic extract of stems of Tinospora cordifolia (Willd.)
NaNO₂– Sodium nitrite
MDA– Malondialdehyde
NO– Nitric Oxide
ALT– Alanine Transaminase
AST– Aspartate Aminotransferase
SOD– Superoxide Dismutase
GSH– Glutathione

Conflict of interest
We declare that we have no conflict of interest.

REFERENCES