Original Article

Nitrate-induced Biochemical and Histopathological Changes in the Liver of Rats: Ameliorative Effect of Hyparrhenia hirta

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Abstract

Objective The present study investigated the protective role of Hyparrhenia hirta (H. hirta) against sodium nitrate (NaNO₃)-induced hepatotoxicity.

Methods Male Wistar rats were randomly divided into three groups: a control group and two treated groups during 50 d with NaNO₃ administered either alone in drinking water or co-administered with H. hirta.

Results NaNO₃ treatment induced a significant increase in serum levels of glucose, total cholesterol and triglyceride while serum total protein level decreased significantly. Transaminases and lactate deshydrogenase activities in serum were elevated indicating hepatic cells’ damage after treatment with NaNO₃. The hyperbilirubinemia and the increased serum gamma glutamyl transferase activities suggested the presence of cholestasis in NaNO₃ exposed rats. In parallel, a significant increase in malondialdehyde level along with a concomitant decrease in total glutathione content and superoxide dismutase, catalase and glutathione peroxidase activities were observed in the liver after NaNO₃ treatment. Furthermore, nitrate caused a significant induction of DNA fragmentation. These modifications in NaNO₃-treated rats corresponded histologically with hepatocellular necrosis and mononuclear cells infiltration. H. hirta supplementation showed a remarkable amelioration of the abnormalities cited above.

Conclusion The results concluded that the treatment with H. hirta had a significant role in protecting the animals from nitrate-induced liver dysfunction.

Key words: Antioxidant; Hyparrhenia hirta; Liver; Nitrate toxicity; Oxidative stress; Rat

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INTRODUCTION

Nitrate is the most common chemical contaminant in the world’s groundwater aquifers. The majority of an individual’s nitrate intake are from vegetables, especially from spinach, lettuce, and beetroot. Nitrogenous fertilizer used in agricultural area is the major source; however, nitrogen from human waste appears to be the most important source in urban areas without sufficient centralized water and sanitation systems. Nitrate can penetrate through soil and remains in groundwater for decades. In addition inorganic nitrates are used in glass making and as oxidizing agents in chemical industries, food preservatives and explosives. Consumption of cured meats is another potential route of human exposure to nitrate. Nitrate levels in drinking water are below the World Health Organization drinking water guideline value of 50 mg/L.

Health risks associated with nitrate intake are still the subject of an ongoing debate. The main risk of nitrate stems from its reduction to nitrite, and subsequently to the possible occurrence of methemoglobinemia among bottle-fed infants under the age of 6 months. In this situation, normal hemoglobin is oxidated and converted to methemoglobin, which fails to bind and to carry oxygen. Possible relationships between nitrate intake and effects on the thyroid have also been reported. In animals, experimental studies have shown that inorganic nitrate is at short term a goitrogenic agent. In human, consumption of water with nitrate levels was associated with thyroid hypertrophy, increased blood pressure and acute respiratory tract infection. Adverse reproductive outcomes of nitrates in drinking water have been also studied. Other possible outcomes of nitrate exposure are increased infant mortality, central nervous system birth defect, diabetes, spontaneous abortion, and changes to the immune system. Nitrate contamination of drinking water may increase cancer risk, because nitrate is endogenously reduced to nitrite and subsequent nitrosation reactions lead to a rise in N-nitroso compounds.

Nitrates can be endogenously synthesized in the organism from L-arginine to form nitric oxide as an intermediate. The latter has a variety of biological functions. Its presence can cause metabolic, physiological and pathological modifications in hepatic cells. The liver plays a central role in nitrogen metabolism. It is known that the nitric oxide is formed by different liver cell types, including hepatocytes, during endotoxemia and inflammation. In the liver, the nitric oxide known as a prooxidant could lead to secondary formation of highly oxidising molecules. Nitric oxide and superoxide anion react to form a peroxynitrite anion. Peroxynitrite is able to oxidize aminoacids and proteins, lipids and deoxyribose.

Oxidative stress plays a central role in liver pathologies and their progression, the use of antioxidants have been proposed as therapeutic agents as well as drug coadjuvants to counteract liver damage. Therefore, herbal products and traditional medicines with better effectiveness and safe profiles are needed as a substitute for chemical therapeutics.

Recent studies have shown that various herbal extracts could protect the liver against oxidative stress by altering the increased levels of lipid peroxidation and by enhancing the decreased activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). More attention has been paid to the protective effects of natural antioxidants against chemically induced toxicities which are increasingly used to treat a wide variety of clinical diseases.

Hyparrhenia hirta (H. hirta) (Coolati grass) is used in our experiment as a plant candidate to provide a natural source of antioxidants. This plant is a tufted perennial grass growing to 1 m in height with owned, hairy, grey green spikelets carried in many pairs of racemes, in an open panicle. Each raceme has a leaf-like bract at the base. It is distributed mainly in the shore regions of the Mediterranean and across the African continent.

The plant is known for its diuretic properties but its phytochemistry had not been investigated until relatively recently when triterpenes, a β-ketone and aromatic aldehydes, were described.

To our knowledge, No investigations on the protective effect of H. hirta against nitrate-induced toxicity in vivo have been conducted. The present study was performed to evaluate the potential protective effect of H. hirta methanolic extract in NaNO3-induced liver injury in male wistar rats.

MATERIAL AND METHODS

Plant Material

H. hirta grows in highest densities in central and southern parts of Tunisia. Aerial parts of plants...
growing near Sfax were collected in November 2012. A voucher specimen authenticated by Prof. Mohamed Chaieb (Faculty of Sciences, Sfax University), was deposited at the herbarium of the Botanic Laboratory in the Sciences Faculty of Sfax (voucher n° PT-453).

**Preparation of Hyparrhenia Hirta Methanolic Extract**

Aerial parts of *H. hirta* once collected, were stored at room temperature in a dry place prior to use. The plants were washed quickly in running water, dried in the shade at ambient temperature until total dehydration. Dried aerial parts of plants were blended into fine powder and stored in the dark at a dry place. The powdered aerial part of plants was extracted in 1/10 dichloromethane and methanol (1w/10v) for 48 h under a continuous reflux set-up in a Soxhlet extractor (hot). The extract was filtered with N°1 whatmann Millipore filter paper (0.45 µm Ref HAWPO4700, MA, USA) and concentrated to a small volume in order to remove all the methanol using rotary evaporation (Büchi Rotavapor; Büchi Laboratories, Switzerland) at 40 °C under vacuum. The yield of extraction was determined. The small volume was later freeze-dried. All the dried extracts were preserved in the refrigerator until further use.

**Identification of Phenolic Compounds Using HPLC/MS**

High-performance liquid chromatography (HPLC) was performed as described. Briefly, HPLC analysis was performed by using an 1100 series LC system installed with a G1322A degasser, a G1312A pump, a G1313A autosampler, and a G1316A oven (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was carried out on a Zorbax StableBond Analytical SB-C18 column (4.6×250 mm, 5 µm; Agilent Technologies). The binary solvent system was made up of 0.1% aqueous formic acid (A) and methanol/acetonitrile (1:1) (B). The conditions for analyzing the methanol extract of *H. hirta* L were: 0-4 min, 20% B; 4-50 min, 20%-80% B; 50-55 min, 80% B; 55-65 min, 80%-20% B followed by 10 min of linear gradient 20% B. The flow rate was 0.3 mL/min in column temperature of 35 °C and an infusion volume of 10 µL in each experiment.

Chromatographic data were collected and controlled using a ChemStation, Rev.B.0301 (Agilent Technologies). Spectral data were collected (200-400 nm, 2 nm resolution) for the entire progression, and the flavonoids were separated by extracting each chromatogram at 350 nm (Figure 1). Tandem mass spectrometry (MS/MS) experiments were conducted using a 3200QTRAP LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) with a Turbo V-source and a Turbo Ion Spray probe (Applied Biosystems). The mass spectrometer was operated in the positive mode with selected ion monitoring (SIM). The electron spray voltage was set at 5.2 kV and the source temperature at 500 °C. The mass spectra were recorded between m/z 100 and m/z 1000 with a step size of 0.06 amu.

The diode array detection (DAD) was set at 140, 254, 280, and 350 nm to provide real time chromatograms and the UV/Vis spectra from 190 to 650 nm were recorded for plant component identification. Mass spectra were simultaneously acquired using electrospray ionization in the positive (PI) and negative ionization (NI) modes, at low (70 V) and high fragmentation voltages (250 V) for both ionization modes. In brief, the high and low fragmentation voltages of the PI and NI modes will be identified as PI250, PI70, NI250, and NI70 in the text. The mass spectra were recorded for the range of m/z 100-1000. The LC system was directly connected with MSD without stream splitting.

**Animals and Experimental Design**

Adult male rats of Wistar strain weighing approximately 200 g were from the breeding center of Tunisia (SIPHAT). 24 rats were acclimatized under laboratory condition for 1 week prior to the experiments. They were maintained under standard conditions of temperature (22±3 °C) and humidity (50±10%) with an alternating 10 h/14 h light/dark cycle. A standard pellet diet (SICO; Sfax, Tunisia) and distilled water were given *ad libitum* for all animals. After acclimatization to the laboratory conditions, rats were divided into three groups of eight each and treated as follows: Group I (control group) was given distilled water; group II (NaNO$_3$) was given daily NaNO$_3$ through intragastric tubes at the rate of 400 mg/kg·bw and group III (NaNO$_3$·HH) received daily NaNO$_3$ by oral gavage at a dose of 400 mg/kg·bw and *H. hirta* methanolic extract at a dose of 200 mg/kg·bw via drinking water in order to have a protective effect spread evenly throughout the day. The dose of NaNO$_3$ used in this study represented 1/12 of LD$_{50}$ (4800 mg/kg·bw)$^6$. In a preliminary dose-response study, three *H. hirta* methanolic extract concentrations (200, 300, and 400 mg/kg·day) were assessed for
antioxidant effects. The results of the three concentrations were similar (data not shown). Therefore among these three concentrations, 200 mg/kg·day was chosen as the experimental study dose of *H. hirta* methanolic extract. Animal handling and experimental procedures were in accordance with the local Institute Ethical Committee Guidelines and the Principles of Laboratory Animal Care published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996).

**Figure 1.** HPLC-MS chromatogram at 350 nm and chemical structures of the compounds isolated from methanolic extract of *Hyparrhenia hirta*: 3-O-methylquercetin (1), luteolin-7-O-glucoside (2), luteolin (3), apigenin-7-O-glucoside (4), apigenin-8-C-glucoside (5), luteolin-8-C-glucoside (6), and luteolin-6-C-glucoside (7).
Samples’ Preparation

At the end of the experimental period (50 d), the animals in different groups were killed by cervical decapitation to avoid stress. Blood samples were collected without heparin and centrifuged at 2200xg for 10 min. Serum samples were drawn and stored at -20 °C until analysis.

The rats’ livers were dissected out, cleaned and weighed. Some samples were rinsed and homogenized (10%, w/v) in an appropriate buffer (pH=7.4) and centrifuged. The resulting supernatants were stored at -80 °C for biochemical assays. Other liver samples were immediately removed, cleaned, fixed in 10% formalin solution and embedded in paraffin for histological studies.

Biochemical Assays

Biochemical Markers of Hepatotoxicity

Serum levels of total proteins were determined by colorimetric method using a commercial kit (Diays, Germany, ref: FS 12311021). Glycemia was assayed with a commercial kit (Biomaghreb, Tunisia, ref: 20121) and determined by enzymatic colorimetric method using glucose oxidase enzyme.

Serum lipid parameters such as total cholesterol (TC), triglyceride (TG) and high density lipoprotein-cholesterol (HDL-C) levels were determined by enzymatic methods using commercial kits from Biomaghreb (Ariana Tunis, Tunisia, Ref 20111, 20131, and 20113 respectively). The low density lipoprotein-cholesterol (LDL-C) fraction and atherogenic index (AI) were determined according to the Friedewald equation [20].

\[
LDL-C = \text{TC} - (\text{HDL-C} + \text{TG}/2.2)
\]

(1)

\[
AI = (\text{TC}-\text{HDL-C})/\text{HDL-C}
\]

(2)

Serum total ALP was determined by the enzymatic colorimetric method using kit from Elitech diagnostics, France (Ref: PASL-0500). The direct bilirubin level in serum was evaluated by colorimetric method using kit from Biomerieux, France (Ref: 61037). Serum LDH, AST, ALT, and GGT activities were assayed spectrophotometrically according to the standard procedures using commercially available diagnostic kits (Biomaghreb, Ariana, Tunisia, Ref, 20012, 20043, 20047, and 20021 respectively).

Lipid Peroxidation

The concentration of malondialdehyde (MDA) in tissues, an index of lipid peroxidation, was determined spectrophotometrically according to Draper and Hadley [21]. An aliquot of 0.5 mL of liver extract supernatant was mixed with 1 mL of trichloroacetic acid solution and centrifuged at 2500xg for 10 min. One milliliter of a solution containing 0.67% thiobarbituric acid (TBA) and 0.5 mL of supernatant were incubated for 15 min at 90 °C and cooled. Absorbance of TBA–MDA complex was determined at 532 nm using a spectrophotometer. Lipid peroxidation was expressed as nmoles of thiobarbituric acid reactive substances (TBARS), using 1,1,3,3-tetra-ethoxypropane as a standard.

Protein Quantification

Protein content in the liver was determined according to Lowry et al. using Bovine serum albumin as a standard [22].

Liver Glutathione (GSH) Levels

GSH in the liver was determined by the method of Ellman [23] modified by Jollow et al. [24]. The method is based on the development of a yellow color when DTNB (5,5-dithiobis-2 nitro benzoic acid) was added to compounds containing sulfhydryl groups. Five hundred microliters of tissue homogenate in phosphate buffer were added to 3 mL of 4% sulfosalicylic acid. The mixture was centrifuged at 1600xg for 15 min. Five hundred microliters of supernatants were taken and added to Ellman’s reagent. The absorbance was measured at 412 nm after 10 min. Total GSH content was expressed as µg/g tissue.

Determination of Antioxidant Enzyme Activities

SOD activity was estimated according to Beauchamp and Fridovich [25]. The reaction mixture contained tissue homogenates in potassium phosphate buffer (50 mmol/L; pH 7.8), 0.1 mmol/L EDTA, 13 mmol/L L-methionine, 2 µmol/L riboflavin and 75 mmol/L nitro blue tetrazolium (NBT). The developed blue color in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as units/mg of protein.

CAT activity was assayed by the method of Aebi [26]. Enzymatic reaction was initiated by adding an aliquot of 20 µL of the homogenized tissue and the substrate (H₂O₂) to a concentration of 0.5 mol/L in a medium containing 100 mmol/L phosphate
buffer (pH 7.4). Changes in absorbance were recorded at 240 nm. CAT activity was calculated in terms of µmoles H₂O₂ consumed/min/mg of protein.

GPx activity was measured according to Flohe and Gunzler. The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

DNA Fragmentation Analysis

We used three mice replicates in each group to evaluate the DNA fragmentation. The extent of DNA fragmentation in the liver tissue was determined by the method described by Kanno et al. Briefly liver tissue was homogenized in lysis buffer (10 mmol/L Tris-HCl, 50 mmol/L NaCl, 1 mmol/L EDTA, 0.5% SDS, pH 8). The lysate was incubated for 3 h with proteinase K (100 mg/mL) at 56 °C. After incubation, the mixture was centrifuged at 10,000 g for 20 min. DNA was extracted from the supernatant with equal volume of phenol/chloroform/isoamyl alcohol (25: 24:1), incubated in ice and centrifuged at 10,000 g at 4 °C. The clear supernatant containing DNA (aqueous layer) was transferred to another tube and mixed 1/10 volume of 3 mol/L sodium acetate (pH 7.4) and 2.5 fold volume of ethanol were added and centrifuged. The precipitated DNA was washed twice with ethyl alcohol (80%) and finally dissolved in TE buffer (Tris-EDTA buffer 10 mmol/L Tris-HCl and 1 mmol/L EDTA pH 8). RNase A (DNase-free) was added and the mixture was incubated at 37 °C for 1 h and genomic DNA yield was measured spectrophotometrically at 260 nm. DNA samples were carried out at 80 V for 1 h on 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer, containing 0.5 mg/mL ethidium bromide. The gel was observed under an ultraviolet lamp through photography.

Histopathological Studies

Some liver samples, prepared for histological examination by light microscopy, were immediately fixed in formalin solution (10%) and processed in a series of graded ethanol solutions. Then they were embedded in paraffin, serially sectioned at 5 µm and stained with hematoxylin-eosin. Six slides were prepared from each liver. All sections were evaluated for the degree of liver injury.

Statistical Analysis

The data were analysed by using the statistical package program Stat view 5 Software for Windows (SAS Institute, Berkley, CA). Results were expressed as means±SD. Statistical data analysis was assessed using Student’s t test with P≤0.05 as the minimal level of significance.

RESULTS

Determination of Flavonoids

The flavonoid profile of H. hirta extract enriched in polyphenols was shown in Figure 1. Based on analysis by HPLC-MS, the flavonoids found in the present study were 3-O-methylquercetin (1), luteolin-7-O-glucoside (2), luteolin (3), apigenin-7-O-glucoside (4), apigenin-8-C-glucoside (5), luteolin-8-C-glucoside (6), luteolin-6-C-glucoside (7) and their chemical structures are shown in Figure 1.

Evaluation of Relative Liver Weight

Results presented in Figure 2 indicated that the relative liver weight of NaNO₂-treated group increased when compared to controls. Whereas, the relative liver weight of rats which have received H. hirta were similar to those of the controls.

Food and Water Consumption

Death was not observed during the experimental period. Food and water consumption by adult rats in control and tested groups were presented in Table 1. Water consumptions by NaNO₃ and (NaNO₂+HH) treated rats, were similar to those of controls. While, there was a significant decrease in food intake by NaNO₃ treated-rats when compared to that of controls.

Biochemical Markers of Hepatotoxicity

Rats exposed to NaNO₃ reduced serum total protein levels (Table 1). Compared with those of the controls, the NaNO₃-treated rats had a significant increase in serum glucose and bilirubin levels (Table 1). Serum activities of AST, ALT, LDH, ALP, and GGT increased significantly in NaNO₂-treated rats, as compared with those of the control group (Figure 2). Supplementation of H. hirta in drinking water of the NaNO₂-treated group ameliorated all the parameters cited above.

Plasma Lipid Profile

The exposure of rats to NaNO₃ induced a significant increase in total cholesterol and triglyceride levels. Low density lipoprotein-cholesterol (LDL-C)
levels and atherogenic index (AI) were enhanced, while high density lipoprotein-cholesterol (HDL-C) remained unchanged in nitrate-treated rats compared with the controls. Results revealed that the presence of *H. hirta* with NaNO₃ could alleviate the adverse effects of NaNO₃ (Table 1).

**Figure 2.** Relative liver weight (absolute liver weight/body weight × 100) and the activities of serum AST, ALT, LDH, ALP, and GGT in control, NaNO₃-treated and NaNO₃+HH-treated rats. AST: aspartate transaminase; ALT: alanine transaminase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; GGT: gamma glutamyl transferase. Significant differences: values are means±SD for eight rats in each group. NS: no significant difference; * P≤0.05; ** P≤0.01; *** P≤0.001.

**Table 1.** Daily Food, Water Consumption, Serum Total protein, Glucose and Direct Bilirubin Levels, Serum Lipid Profile, Malondialdehyde and Glutathione Levels, Superoxide Dismutase, Catalase and Glutathione Peroxidase Activities in Liver of Control, NaNO₃-treated and NaNO₃+HH-treated Rats

<table>
<thead>
<tr>
<th>Parameters and Groups</th>
<th>Controls</th>
<th>NaNO₃</th>
<th>NaNO₃+HH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water consumption (ml/day/rat)</td>
<td>29.56±2.84</td>
<td>29.97±2.76</td>
<td>30.87±2.06</td>
</tr>
<tr>
<td>Food consumption (g/day/rat)</td>
<td>18.13±2.35</td>
<td>15.52±1.64***</td>
<td>16.95±1.84†††,***</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>60.33±1.96</td>
<td>51.8±1.64***</td>
<td>59.2±2.58**</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>122.8±11.8</td>
<td>158.2±3.9**</td>
<td>143.6±12.9***</td>
</tr>
<tr>
<td>Direct bilirubin (µmol/L)</td>
<td>2.18±0.32</td>
<td>4.57±1.04***</td>
<td>2.91±0.47**</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>0.78±0.05</td>
<td>0.98±0.06***</td>
<td>0.88±0.05**</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.68±0.08</td>
<td>0.88±0.09***</td>
<td>0.73±0.08**</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>0.37±0.09</td>
<td>0.38±0.07</td>
<td>0.39±0.04</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>0.11±0.01</td>
<td>0.21±0.02***</td>
<td>0.15±0.03***</td>
</tr>
<tr>
<td>Atherogenic index</td>
<td>1.11±0.03</td>
<td>1.58±0.05***</td>
<td>1.25±0.04***</td>
</tr>
<tr>
<td>Malondialdehyde (nmoles/g tissue)</td>
<td>62.18±2.08</td>
<td>119.47±11.66***</td>
<td>80.84±4.8***</td>
</tr>
<tr>
<td>Glutathione (µg/g tissue)</td>
<td>80.63±0.46</td>
<td>76.51±0.4***</td>
<td>78.89±0.21***</td>
</tr>
<tr>
<td>Superoxide dismutase (U/mg protein)</td>
<td>199.29±18.75</td>
<td>107.61±7.39***</td>
<td>139.25±8.05***</td>
</tr>
<tr>
<td>Catalase (µmoles H₂O₂ degraded/min/mg protein)</td>
<td>42.61±6.46</td>
<td>20.02±3.74***</td>
<td>29.11±4.46***</td>
</tr>
<tr>
<td>Glutathione peroxidase (nmoles of GSH/min/mg protein)</td>
<td>11.71±1.97</td>
<td>5.55±1.09***</td>
<td>8.11±0.98***</td>
</tr>
</tbody>
</table>

**Note.** Significant differences: values are means±SD for eight rats in each group. NaNO₃ treated group versus control group, ** P≤0.01; *** P≤0.001. NaNO₃+HH-treated group versus NaNO₃ treated group, †† : P≤0.01; ††† : P≤0.001. NaNO₃+HH-treated group versus control group, * P≤0.05; ** P≤0.01.
Estimation of Lipid Peroxidation

In the liver homogenate of NaNO₃-treated rats, MDA levels significantly increased compared to those of the control group (Table 1). Supplementation of *H. hirta* restored the MDA contents in the liver to near-normal values.

Liver GSH Levels

A significant decrease of GSH levels in liver was evident in rats exposed to NaNO₃ (Table 1). Supplementation of *H. hirta* ameliorated GSH levels in (NaNO₃+HH)-group when compared with the NaNO₃ group.

Enzymatic Antioxidant Status in Liver

NaNO₃ treatment led to a significant decrease in all antioxidant enzyme activities when compared with those of the control group. Treatment with *H. hirta* restored the levels of enzymatic antioxidants to near-normal values (Table 1).

DNA Fragmentation

As shown in Figure 3, a smear (hallmark of necrosis) without ladder formation on agarose gel, indicating random DNA degradation, was observed through the lane of DNA in the liver of the NaNO₃-treated rats. *H. hirta* treatment exerted a protective effect against NaNO₃ by reducing the smear formation.

Liver Histopathology

The biochemical alterations can be correlated with histological changes in the liver of rats. In fact, livers of control rats had a regular histological structure (Figure 4A). The livers of the NaNO₃-treated group were characterized by an infiltration of inflammatory leucocyte cells (Figure 4B, 4C) as well as a centrilobular hepatic necrosis (Figure 4D, 4E). The cellular infiltrations were localized in portal canals and migrated in necrose hepatic lobules, resulting from a poor vascularization. The severe liver damages significantly decreased when *H. hirta* was added to the drinking water of (NaNO₃+HH)-treated rats compared with those treated only with NaNO₃ (Figure 4G, 4I).

**DISCUSSION**

It is well known that nitrates are the precursor of NO leading to ONOO⁻ production in particular if oxidative stress is present in biological areas. ONOO⁻ causes damage to many important biological molecules including proteins, lipids, and nucleic acids by a number of mechanisms[29].

Our results clearly showed, in the NaNO₃-treated rats, a significant increase in serum glucose concentration, results confirming those of Boukerche et al. who reported the hyperglycemic effect of ammonium nitrate given to rats by gavage at a concentration of 600 mg/kg of body weight during three weeks[30]. Shelpov et al. reported that in the presence of nitrate ions, the activity of amylase and phosphorylase increases lead to the liberation of glucose from glycogen, so blood glucose increases while liver glycogen decreases[31]. Other findings suggested a stimulation by nitrate of gluconeogenesis and glucose shift from tissue to blood or an impairment of glucose mobilization[32]. We can expect that nitrate produces hyperglycemia due to a deficiency of insulin release. It is known that nitric oxide is formed from nitrate at least by the vascular epithelial cells[33].

Both nitric oxide and nitrate open potassium channels which through closing voltage gated calcium channels decreases intracellular calcium. Calcium is known to trigger insulin secretion and calcium channel blockers are known to produce hyperglycemia[33]. Furthermore, nitroso-compounds can alter the antioxidant system causing disturbance in the metabolic processes leading to hyperglycemia[34].

Among biochemical changes in rats induced by NaNO₃ treatment, we noted a significant decrease in serum total protein levels. Similar results have been
Hyparrhenia hirta and nitrate toxicity

reported in adult rats exposed to sodium nitrate in drinking water at concentrations of 550 mg/L for four months\textsuperscript{35}. A reduction in food intake observed in this study may account for a decreased protein concentration in serum. In addition, the decrease in total protein concentration may result to nitrate toxicity mediated through formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins. In another way, decreased protein content in response to nitrate exposure may contribute to the harmful effect of nitrite, the active metabolite. Previous studies confirmed this hypothesis and further added that the nitrite effect is reflected in the biosynthesis of protein\textsuperscript{36}. It was found that serum protein of rats are decreased due to the toxic effect of nitrite on the thyroid and adrenal glands leading to block protein synthesis, while fast breakdown occurs due to an increase of free amino acids and due to a decrease of protein turnover. It is clear that nitrate decreases total serum protein mainly through its effects on the liver, either through the necrotic changes, especially of the plasma membrane, or through the inhibition of the oxidative phosphorylation process at first and then the availability of energy source for protein synthesis and other metabolic processes\textsuperscript{37}.

The present investigation also revealed a marked increase of serum total cholesterol and triglyceride levels in rats. LDL-C levels and atherogenic index were enhanced, while HDL-C remained unchanged after nitrate treatment. Our results were consistent with previous findings realized on nitrate administered to adult rats\textsuperscript{30,35}. In this regard, a number of investigations have been focused on the influence of nitrate on the thyroid status. Some workers showed thyroid hypothyropthy with decreased thyroid hormone levels in people who consume drinking water with nitrate concentrations below or above the WHO nitrate standard of 50 mg/L\textsuperscript{38-39}. According to Luboshitzky et al., sub-clinical hypothyroidism characterized by a decreased FT4 and an increased TSH concentration was associated with elevated total cholesterol and LDL-C concentrations\textsuperscript{40}. Also, serum triglyceride concentrations were significantly elevated which could be related to a reduced removal rate of triglycerides from plasma in case of hypothyroidism\textsuperscript{40}.

Moreover, nitrate seemed to produce other biochemical defects in the liver as demonstrated in our work by an increase of serum ALT, AST, and LDH activities suggesting hepatic damages and changes in

The arrows indicate:

\begin{itemize}
\item \rightarrow Portal inflammation
\item \bullet \bullet Lobular inflammation
\item \rightarrow Necrotic-inflamatory activity
\item \rightarrow Regeneration cells
\end{itemize}

(basophilic binucleated cells)

\textbf{Figure 4.} Hematoxylin and eosin stained liver sections of adult rats: controls (A), NaNO\textsubscript{3} treated rats (B, C, D, E), NaNO\textsubscript{3}+HH-treated rats (F, G, H, I). Each microphotograph represents a section from an individual liver. Magnification: x200 (B, D, F, H); x400 (A, C, E, G, I).
its function. These enzymes cited above are the most sensitive biomarkers directly implicated in the extent of cellular damage and toxicity because they are cytoplasmic in location and are released into the circulation after cellular damage\cite{41}. Increased activity in these enzymes in the serum of NaNO\textsubscript{3}-treated rats might be due to the leakage from the hepatic tissues as a result of necrosis and an alteration of membrane permeability. Earlier findings reported the increase in the level of AST, ALT and LDH in ammonium nitrate-\cite{30,42} or sodium nitrate-\cite{43} treated rats due to the formation of free radical ONOO\textsuperscript{-} from nitric oxide. Both NO and oxygen radicals could react further to produce other oxidants and nitro compounds such as peroxynitrite to induce liver injury and to play an important role in death of liver cells\cite{44}.

Many enzymes like ALP and GGT tend to be released into plasma in large amounts following hepatocellular damage\cite{45}. GGT is considered to be more specific for liver function tests. Its activity is markedly increased in plasma in both primary and secondary carcinomas of the liver\cite{46}. Another biomarker of hepatotoxicity is bilirubin which increased in the serum of treated rats. Our results were consistent with previous findings of El-Demerdash et al. who suggest that the increase in serum bilirubin is a clear marker of hepatic dysfunction\cite{46}. The increase in bilirubin and in the activities of ALP and GGT enzymes in the serum of NaNO\textsubscript{3}-treated rats could be attributed according to BenSoltane et al.\cite{42} and Ogur et al.\cite{43}, to the toxic effects of nitroso-compounds, formed in the acidic environment of the stomach, causing a severe cholestasis.

In addition to metabolic changes induced by nitrates, further studies have indicated that one of the most established mechanisms of nitrates toxicity is their ability to induce oxidative stress, through reactive oxygen species generation\cite{8}. Moreover, it is now recognized that nitrate is the precursor of nitric oxide leading to tissue damage. The contribution of NO to tissue damage can be a direct effect mediated by NO itself or an indirect effect mediated by ROS, such as peroxynitrite produced by the interaction of NO with superoxide anions or oxygen\cite{47}. NO can interact with ROS to form ONOO\textsuperscript{-}, which is a powerful oxidant and cytotoxic agent and may play an important role in the cellular damage associated with the overproduction of NO.

An imbalance between intracellular production of free radical and the cellular defense mechanisms and MDA level is one of the most important oxidative stress markers\cite{48}. In the present study, nitrate intake significantly increased liver MDA levels. Our results corroborated previous reports which demonstrated that nitrate exposure enhances lipid peroxidation in rat livers\cite{43}. The overproduction of free radicals overwhelms the detoxification and scavenges capacity of cellular antioxidant enzymatic and nonenzymatic, resulting in toxic lipid peroxides formation, which ultimately contributes to cellular hepatotoxicity.

The antioxidant systems in the body contain numerous enzymatic antioxidants, such as SOD, CAT and GSH-Px and non-enzymatic compounds like GSH, protecting the body from oxidative stress\cite{49}. In the current study, the significant decrease in the antioxidant enzyme activities in the liver proved the failure of antioxidant defence system to overcome the influx of reactive oxygen species generated by NaNO\textsubscript{3} exposure. The decrease in the activities of GPx could result directly from decreased levels of GSH following NaNO\textsubscript{3} exposure. The decline in the level of GSH might be related to its increased utilization counteracting the high lipid peroxidation and leading to oxidative stress.

In addition, free radical generation following xenobiotic exposure may lead to extensive DNA damage giving rise to mutations and/or cell death\cite{50}. In the present study, DNA damage was evaluated by electrophoresis of DNA extracted from the liver. In general, DNA intact band appears to be condensed near the application point with no DNA smearing suggesting no DNA fragmentation. Administration of NaNO\textsubscript{3} provoked DNA damage resulting in DNA shearing without a classical DNA ladder pattern seen in apoptosis.

Results of relative liver weight, obtained from this study, showed that nitrate intake could cause hepatomegaly. Boukerche et al. reported an increase in the hepato-somatic ratio in the rats treated by nitrate\cite{30}. In order to evaluate hepatomegaly in more detail, liver samples were examined under light microscopy. In NaNO\textsubscript{3}-treated rats, there was a cell necrosis with infiltration of mononuclear cells occurring in portal canals and particularly in hepatic lobules. These changes could be the results of membrane distribution induced by nitrate. Similar changes in the hepatic tissue of adult rats have been reported by the previous findings of Ogur et al.\cite{43}. In fact, livers of nitrate-treated rats presented hepatocellular degeneration and cell necrosis, varying from the increased intercellular space to the hydropic degeneration.
In our study, the administration of *H. hirta* had a potent protective effect against oxidative stress and liver damage in rats induced by NaNO₃, as revealed by a remarkable decrease in MDA level as well as an elevation of antioxidant enzyme activities and GSH content in the liver. The reversal of the altered antioxidant enzyme status and peroxidative damage in the liver by *H. hirta* extract confirmed its antioxidant, anti-peroxidative properties and its potential role in the defense against free radicals, which could be attributed to flavonoids isolated from *H. hirta* by using HPLC/MS. Presence of luteolin, luteolin-7-O-glucoside, luteolin-8-C-glucoside, luteolin-6-C-glucoside, apigenin-7-O-glucoside, apigenin-8-C-glucoside, and 3-O-methylquercetin, in *H. hirta* was identified by HPLC analysis. The luteolin derivatives are molecules with an antioxidant activity able to scavenge hydroxyl radicals and to eliminate ROS generated by hydrogen peroxid.[51] Further, the antioxidant ability of quercetin and apigenin derivatives has been reported in several studies.[51-52]

In addition, *H. hirta* could ameliorate liver damage to a high degree, as demonstrated by a reduction of serum ALT, AST, LDH, ALP, and GGT activities. This might be due to the flavonoids present in *H. hirta* extract that helped to prevent membrane fragility and subsequently decreased the leakage of marker enzymes into circulation. Furthermore, the administration of *H. hirta* extract in the NaNO₃-treated group improved the histological alterations induced by nitrate. The livers of the NaNO₃+HH-group had a nearly normal appearance. Thus, this confirmed the anti-inflammatory property of *H. hirta*. Indeed, luteolin and quercetin derivatives were present in this extract. They have been shown to have significant anti-inflammatory properties.[52]

*H. hirta* cotreatment was found to be effective and to prevent the NaNO₂-induced smear formation. It could prevent DNA oxidation mainly by quenching free radicals and modulating enzymes metabolism.

**CONCLUSIONS**

We can conclude that the *H. hirta* has a beneficial role in overcoming the occurred adverse effects of sodium nitrate chronic ingestion, probably through its high antioxidant and anti-inflammatory properties.

**CONFLICT OF INTERESTS STATEMENT**

The authors declare that they have no competing interests to disclose.

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