

Hepato-protective Effects of *Moringa oleifera* Leaves against Sodium Nitrate Toxicity on Rats

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Abstract

Moringa oleifera leaves (MO) contain flavonoids and phenolic acids that can protect against oxidative stress-related chronic illnesses. The current study aimed to evaluate the effectiveness of *Moringa oleifera* leaf powder (MOLP) against the toxic effect induced by sodium nitrate (NaNO₃). Five groups, each of five rats were used. Group I: rats fed on normal diet for 6 weeks; Group II: rats fed on 10% of MOLP mixed with diet; Group III: rats received 500 mg/L of NaNO₃ daily in drinking water for 6 weeks; Group IV: rats received NaNO₃ for 3 weeks in drinking water then fed MOLP 10% mixed with diet; and Group V: rats fed on 10% MOLP ad mixed with diet combined with NaNO₃ in water for 6 weeks. The results showed that administration of NaNO₃ significantly elevates hepatic enzymes ($P \leq 0.05$) as well as oxidative stress biomarkers Malondialdehyde (MDA), Nitric oxide (NO) and Protein carbonyl content (PC), but decreases the antioxidant markers (Superoxide dismutase (SOD), Catalase (CAT) and Glutathione (GSH) levels. The treatment with MOLP significantly reduces liver enzymes activities and hepatic oxidative stress biomarkers but elevated hepatic antioxidants. In conclusion, *Moringa oleifera* leaves powder may have a protecting and therapeutic effects against toxicity induced by NaNO₃ via the improvement of the antioxidant status in NaNO₃ drinking rats.

Keywords: *Moringa oleifera*, Sodium nitrate, liver enzymes, oxidative stress

Introduction

Nitrate (NO₃) is a common pollutant in drinking water and has become a global problem (Fernández-López et al., 2023). Human health is directly impacted by the dangerous practice of drinking water tainted

with NO₃ (Barakat et al., 2020). Nitric oxide (NO), which is readily produced by NO₃, an oxidation product, can interact with superoxide to form the extremely reactive chemical peroxynitrite (Chow and Hong, 2002). Additionally, nitrate can be converted to nitrite, which releases reactive species that affect metabolism, cause hematological changes, and damage the liver (González Delgado et al.,

2018). The functions of antioxidants are the scavenging and suppressing free radicals, protecting people and animals from infections and chronic diseases (**Tan et al., 2018; Yeung et al., 2019).**

Plant natural antioxidants were used in large scale due to their safe therapeutic actions and minimal adverse effects (**Zakaria et al., 2018).** Studies have demonstrated the nutritional and therapeutic effects of strong antioxidant substances like catechin, cyanidin, ellagic acid, luteolin, rosmarinic acid, and rutin in *Moringa oleifera* (**Egbuna et al., 2021; Pareek et al., 2023).** These compounds also assist in decreasing the production of free radicals (**Monraz-Méndez et al., 2022).** Additionally, the antioxidant properties of *Moringa oleifera's* phenolic acids stabilize free radicals by donating a hydroxyl group and creating a stabilized unpaired electron (**Saki et al., 2023).** Also, flavonoids like kaempferol, apigenin and quercetin exhibit a strong antioxidant property (**Olazarán-Santibañez et al., 2021).**

Experimental models have demonstrated that *Moringa oleifera* leaves reduce oxidative damage and boost the body's antioxidant defense system (**Mthiyane et al., 2022).** The purpose of the current study was to evaluate the protective effects of MOLP against sodium nitrate-induced liver injury in rats.

Material and Methods

Animals

Twenty-five adult albino rats male, each weighing between 80 and 100 grams, were purchased from the Egyptian VACSERA Organization in Cairo, Egypt. Rats were housed in from stainless steel cages with a 12-hour light/dark cycle and a temperature of 25±2 °C. Water and a rodent diet were provided to the rats. Animal experiments procedures have been approved by Mansoura University in compliance with EU Directive 2010/EU and the recommendations set forth by the **National Research Council in 2011.**

Experiment design

Rats were acclimatized for a week, then divided randomly into 5 groups each of five rats as follow; GI: rats were fed on normal diet for

six weeks, GII: rats were fed on MOLP (10%) admixed with diet daily for six weeks (**Stephen Adeyemi et al., 2017),** G III: rats were drink 500 mg/L of NaNO₃ daily for six weeks in drinking water (**Anwar and Mohamed, 2015),** GIV: rats were drink NaNO₃ (500 mg/L) in drinking water for three weeks, then, fed on MOLP (10%) mixed with diet daily for three weeks and GV: rats fed on diet mixed with MOLP (10%) combined with NaNO₃ in water for six weeks

Preparing *Moringa oleifera* leaf powder

Fresh leaves from MO were collected from a farm in Mansoura City, Egypt. Leaves are stripped off, washed with tap water, drained, and then spread out to dry for five days in the shade. Leaves were crushed using a blender, purified through a 2-sieve, and kept in refrigerator at a - 4°C, to be used.

Samples preparation

At the end of the experiment, rats were fasting for the whole night, and blood samples were taken and centrifuged in dry, clean tubes to prepare sera. Before being utilized for the ensuing biochemical analyses, sera were kept at -80 °C.

Liver of each rat was immediately removed, washed in saline (0.9%). The known weight of each liver tissue was cut and stored at -80°C for other biochemical analysis.

Liver enzymes (ALT, AST and ALP):

Aspartate transaminase (AST) and alanine transaminase (ALT) kits were purchased from Human Diagnostic Company, Wiesbaden, Germany. Alkaline phosphatase (ALP) kit was bought from AGAPPE Diagnostic Company, Switzerland.

The activity of the enzymes ALT and AST were measured in serum using the **Bergmeyer (1980)** and **Bergmeyer et al. (1976)** methods. Basically, the oxidation of NADH to NAD⁺ causes the absorbance to shift at 340 nm. The concentration of ALT or AST that is present in the sample determines the rate at which absorbance (Abs) decreases.

The activity of ALP enzyme was determined in serum according to **Schlebusch et al. (1974).** The method measures the rate at which P-nitrophenyl phosphate, a byproduct of

ALP, is formed at 405 nm, and that rate is proportional to the ALP concentration present in the sample.

Oxidative stress biomarkers (MDA, NO and PC)

The **Ohkawa et al. (1979)** method was used to measure the concentration of malondialdehyde (MDA) in liver homogenate. MDA and thiobarbituric acid (TBA) reacted for 30 minutes at 95°C, producing a TBA with a wavelength of 534 nm.

The concentration of nitric oxide (NO) in liver homogenate was measured using Montgomery and Dymock's (1961) methodology. A dye with an estimated wavelength of 540 nm was created by combining diazotized sulfanilamide with N-(1-naphthyl) ethylenediamine in an acidic solution.

The protein of hepatic homogenate was tested for carbonyl content (PC) using ELISA kits according to the manufacturer instructions of BioVesion Company (Milpitas, CA, USA) in which the CO in hepatic protein was measured by reacting dinitrophenylhydrazine (DNPH) with protein carbonyls, resulting in the formation of DNP-hydrazone, which was measured at 375 nm.

Antioxidants biomarkers GSH, CAT, and SOD

The concentration of GSH in the liver was measured using the **Beutler et al. (1963)** method, which included reducing 5, 5` dithiobis (2-nitrobenzoic acid) (DTNB) with GSH to generate a yellow color proportionate to GSH content. The absorbance of this solution was assessed at 405 nm.

Superoxide dismutase (SOD) activity was measured spectrophotometrically in hepatic homogenate using the **Nishikimi et al. (1972)** method. This technique assessed the enzyme's capacity to prevent phenazine methosulphate (PMS) from reducing the

nitroblue tetrazolium dye at 560 nm.

The method developed by **Aebi (1984)** was used to measure the catalase (CAT) activity in liver homogenate. The presence of horseradish peroxidase (HRP) 3, 5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS), and 4-aminophenazone (APA) causes the remaining hydrogen peroxide (H₂O₂) to generate a color, the intensity of which is inversely related to the amount of CAT present in the sample. CAT inhibited the reaction with H₂O₂.

Statistical analysis

Version 20 of IBM SPSS Statistics was used to do statistical analysis on the data. A post-comparison Tukey test and an analysis of variance (one-way ANOVA) test were used to identify the groups' differences. $P \leq 0.05$ was taken into account for determining statistical significance, and the results were displayed as mean \pm SE (**Snedecor and Cochran, 1980**).

Results

Liver enzymes ALT, AST, and ALP:

Table 1 showed that the activities of liver enzymes in rats administered sodium nitrate (GIII) were elevated compared with the control group (GI). However, these activities except AST were decreased after treatment with MOLP ($P \leq 0.05$) either after drinking (GIV) or combined (GV).

The ALT activity in rats that were given NaNO₃ and treated with MOLP and rats co-treated with MOLP + NaNO₃ (GV) was significantly reduced compared with NaNO₃ group. However, no improvement was observed in AST activity in rats drinking NaNO₃ and treated with or in rats co-administrated MOLP + NaNO₃.

ALP activity, rats co-treated with MOLP + NaNO₃ (GV) was significantly lower than rats that received MOLP after NaNO₃.

Table1. Liver enzyme activities in different experimental groups

| | G I | G II | G III | G IV | G V |
|-----------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| ALT (U/L) | 38.80 \pm 4.39 ^a | 36.60 \pm 4.23 ^a | 69.80 \pm 5.91 ^b | 44.80 \pm 2.33 ^a | 47.80 \pm 1.43 ^a |
| AST (U/L) | 98.40 \pm 7.70 ^a | 92.60 \pm 6.87 ^a | 231.60 \pm 8.02 ^b | 159.20 \pm 7.79 ^c | 140.40 \pm 6.95 ^c |
| ALP (U/L) | 375.40 \pm 3.56 ^a | 354.20 \pm 4.67 ^a | 719.60 \pm 5.11 ^b | 500.40 \pm 4.95 ^c | 476.60 \pm 6.46 ^d |

Data were expressed as mean \pm SE (n=5), analyzed by one-way ANOVA then by Tukey's test. Values with different letters (a, b, c, d) are significantly different from each other at $P \leq$

0.05. **G I:** Control group; **G II:** MOLP group; **G III:** NaNO₃ group; **G IV:** MOLP/NaNO₃ group; **G V:** MOLP + NaNO₃ group. **ALT:** alanine transaminase; **AST:** aspartate

transaminase; **ALP**: alkaline phosphatase.

Oxidative stress biomarkers (MDA, NO and PC)

The results in **Fig. 1** showed that MDA, NO, and PC concentrations in group III (NaNO₃) were increased considerably ($P \leq 0.05$) when compared to the control group. After treatment with MOLP after/with NaNO₃ in drinking water, MDA, NO, and PC levels were substantially ($P \leq 0.05$) decreased in comparing with NaNO₃ group.

Co-treatment of MOLP with NaNO₃ in rats showed a considerable drop in MDA and PC concentrations compared to MOLP after NaNO₃ treatment to rats, but both treatments were still beyond control rats.

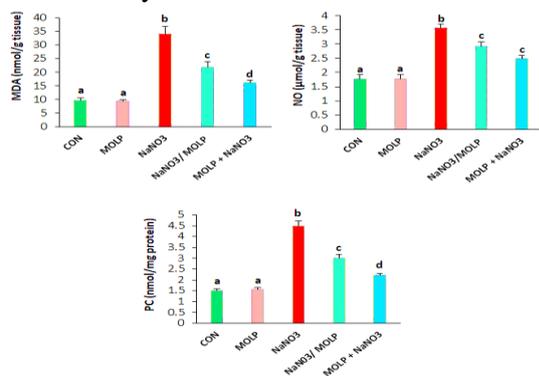


Fig. 1. Effects of MOLP on hepatic oxidative stress markers (MDA, NO, and PC) levels in different experimental groups (**G I**: Control group; **G II**: MOLP group; **G III**: NaNO₃ group; **G IV**: MOLP/NaNO₃ group; **G V**: MOLP + NaNO₃). The values were reported as mean \pm SE (n=5), analyzed by one-way ANOVA and Tukey's test. Different letters (a, b, c, d) are significantly different from each other at $P \leq 0.05$.

Antioxidants biomarkers (GSH, CAT and SOD)

Fig. 2 shows the activity of the hepatic SOD and CAT enzymes, as well as the levels of GSH was significantly ($P \leq 0.05$) decreased in NaNO₃ group compared to control group. However, the activities of SOD, CAT and the levels of GSH were increased after treatment with MOLP after or concomitant of NaNO₃.

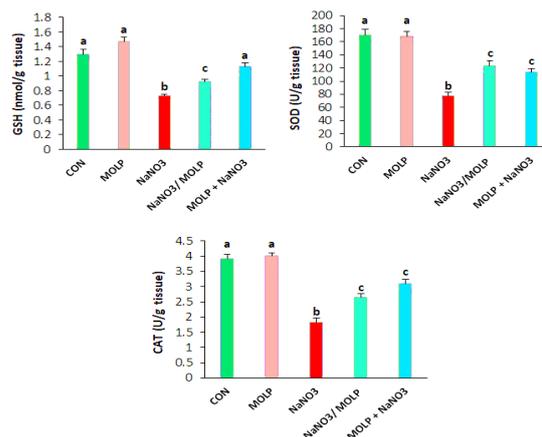


Fig. 2. Effect of MOLP on hepatic antioxidant markers (GSH, SOD, and CAT) activities in different experimental groups (**G I**: Control group; **G II**: MOLP group; **G III**: NaNO₃ group; **G IV**: MOLP/NaNO₃ group; **G V**: MOLP + NaNO₃). The data were reported as mean \pm SE (n=5), then analyzed by one-way ANOVA and Tukey's test. Different letters (a, b, c, d) are significantly different from each other at $P \leq 0.05$.

Discussion

Nitrates are the fundamental components of very reactive compounds, such as peroxynitrite, which upset the equilibrium between oxidants and antioxidants and cause oxidative stress (**Bouaziz-Ketata et al., 2014**).

In the present study the ALT, AST, and ALP activities were increased considerably after NaNO₃ drinking in comparing with the control group. This may be due to hepatotoxicity induced by NaNO₃ as reported by **Orabi et al. (2022)**. Hepatocytes cytoplasmic enzymes are released when liver cells are damaged, resulting in an elevation of their serum levels. The major cause of nitrate toxicity is the production of reactive oxygen species (ROS) and other hazardous transient molecules such as peroxide, peroxynitrite, and superoxide anion hydrogen (**Alrawi, 2016**). These compounds affect hepatocyte integrity and induce hepatocyte membrane peroxidation, which allows injured hepatic cells to release AST, ALT, and ALP into the plasma (**Abd Alla and Badary, 2017**).

The present results showed that simultaneous treatment of rats drinking sodium nitrate with 10% MOLP showed reduction of ALT, AST, and ALP activities in serum. This may be due to the protective effect of MOLP as reported previously by **Mhlomi et al. (2022)**.

The protective effect of MOLP is due to the antioxidant and scavenging of free radical by the phenolic acids (caffeic and gallic acids) and flavonoids contained in MOLP (**El-Hadary and Ramadan, 2019; Ma et al., 2020; Asgari-Kafrani et al., 2020**).

In every cellular and extracellular compartment, the antioxidant system serves as the first line of defense against ROS (**Lafi et al., 2017**). SOD is the first line of protection, converting superoxide ions (O_2^-) into less toxic H_2O_2 . CAT then further breaks down H_2O_2 into water and oxygen molecules (**Elwej et al., 2017**). GSH, a powerful non-protein thiol antioxidant, regulates cellular redox state, protecting cells from lipid peroxides and ROS/RNS and counteracting the deleterious effects of ROS (**Metwaly et al., 2018**).

In the liver tissue homogenate, administration of $NaNO_3$ significantly increases lipid peroxidation (MDA), NO, and PC (oxidative stress biomarkers) while significantly decrease the activities of SOD and CAT (antioxidant enzymes) and the concentration of GSH. The low activities of SOD, CAT, and the level of GSH after drinking $NaNO_3$ indicate the inability of the liver's antioxidant defense mechanism to overcome the increased levels of ROS generated by exposure to $NaNO_3$. **Rouag et al. (2020)** found that $NaNO_3$ increases the generation of free radicals in hepatic cells resulting in increased lipid peroxidation and protein oxidation (MDA and PC) as found in the present study.

$NaNO_3$ can cause a decrease of hepatic GSH level by blocking its thiol function through direct nitrate and/or its metabolite conjugation or by preventing glutamyl-cysteine-synthetase activity and its attempts to combat oxidative stress-causing high lipid peroxidation (**Bouaziz-Ketata et al., 2014; Al Olayan et al., 2020**). According to **Farmand et al. (2005)**, the depletion of SOD promotes the generation of oxidative stress by transforming a very reactive superoxide anion into hydrogen peroxide. Reduced SOD and CAT activities can cause imbalance between antioxidant response and oxidative/nitrosative stress, which compromises cellular function by causing lipid peroxidation, protein carbonylation, and other processes (**Blokhina et al., 2003; Kurutas, 2016**).

The present study showed that treatment of rats drinking $NaNO_3$ with MOLP mixed with diet significantly increase in SOD

and CAT activities and GSH concentration in the liver tissue homogenates but significantly decrease in the concentration of MDA, NO, and PC in comparison with control group; due to its phytochemical contents, which reduce ROS produced by $NaNO_3$ (**Albasher et al., 2020**). These results are consistent with **Stephen Adeyemi et al. (2017)** and **Mousa et al., (2019)**, who highlight the beneficial impact MOLP (10% in diet) and MO leave extract (300 mg/kg BW/) respectively on antioxidant status and oxidative stress indicators in hepatic tissue.

The antioxidant properties of MO leaves are attributed to the presence of polyphenols, flavonoids, and phenolic compounds (**Ma et al., 2020**), which can scavenge free radicals and shield mammalian cells from ROS (**Kurutas, 2016**). In particular, kaempferol, caffeic and gallic acids, and quercetin in MO leave have been reported to significantly prevent oxidative stress in the liver (**Asgari-Kafrani et al., 2020**). Additionally, dietary antioxidants, when present in sufficient amounts, can inhibit the generation of nitrosamines, thereby preventing nitrosamine formation (**Ding et al., 2018**). In conclusion the present study, showed that administration of MOLP in drinking water can protect rats from the hepato-toxic effects of $NaNO_3$ due to its content of antioxidant such as flavonoids and polyphenol compounds that act as ROS scavengers. However, the treatment with MOLP combined with $NaNO_3$ drinking is more effective than treatment after drinking.

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الملخص العربي

عنوان البحث: تأثيرات مسحوق أوراق المورينجا أوليفيرا على شرب الجرذان لنترات الصوديوم

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يحتوي نبات المورينجا أوليفيرا علي الفلافونويد، الجلوكوزينولات، الايزوثيوسيانات، والأحماض الفينولية والتي لها تأثيرات ايجابية علي الصحة. الهدف من الدراسة الحالية هو تقييم التأثير الوقائي والعلاجي لتغذية الجرذان بمسحوق أوراق المورينجا أوليفيرا (MOLP) على وظائف الكبد بعد إعطائها نترات الصوديوم (NaNO₃) في مياه الشرب. تم تقسيم خمسة و عشرين من ذكور الجرذان إلى خمس مجموعات: تم تغذية المجموعة الأولى (الضابطة) بطعام الجرذان القياسي لمدة ستة أسابيع؛ المجموعة الثانية (MOLP) تم تغذيتها بمسحوق أوراق المورينجا بنسبة ١٠ % ممزوجة بطعام الجرذان القياسي لمدة ستة أسابيع ؛ تلقت المجموعة الثالثة 500 (SN) ملجم/لتر من نترات الصوديوم يوميا لمدة ستة أسابيع في مياه الشرب؛ المجموعة الرابعة (NaNO₃/MOLP) تم إعطاؤها نترات الصوديوم في مياه الشرب لمدة ثلاثة أسابيع ثم تغذيتها ب ١٠ % بمسحوق أوراق المورينجا لمدة ثلاثة أسابيع أخرى؛ المجموعة الخامسة (MOLPL + NaNO₃) تم تغذيتها ب ١٠ % من مسحوق أوراق المورينجا ممزوجة بطعام الجرذان القياسي بالإضافة إلى نترات الصوديوم في مياه الشرب لمدة ستة أسابيع. أظهرت النتائج أن الجرذان المعرضة لنترات الصوديوم لها مستويات أعلى بشكل ملحوظ ($P \leq 0.05$) لانزيمات وظائف الكبد (ALT, AST, ALP) في المصل، إنخفاض كبير في مضادات الاكسدة في نسيج الكبد (SOD, CAT, GSH) وزيادة الجهد التاكسدي في نسيج الكبد (MDA, NO, PC). وعندما تمت المعالجة بمسحوق أوراق المورينجا كجرعة وقائية وعلاجية تم تحسين هذه التغيرات بشكل كبير ($P \leq 0.05$). تشير هذه النتائج أن محتوى مسحوق أوراق المورينجا من مضادات الأكسدة قللت من اضطراب وظائف الكبد و الجهد التاكسدي في نسيج الكبد الناتجة عن سمية نترات الصوديوم.