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Physiological and Histological Studies of Moringa Oleifera Leaves against Sodium Nitrate Toxicity in Rats

Taghreed Fouad*[1], El-Sayed Abd El-hady[2], Ayman Hyder[1] and Kadry El-Bakry[1]

[1] Department of Zoology, Faculty of science, Damietta University, Egypt
[2] Department of Zoology, Faculty of science, Mansoura University, Egypt

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*Corresponding author's E-mail: fouadtaghreed83@gmail.com

Abstract

Moringa oleifera, rich in minerals and bioactive substances like phytochemicals such as flavonoids, glucosinolates, isothiocyanates, and phenolic acids, can promote normal body functions and protect from diseases. This study aims to assess the ameliorated effects of *Moringa oleifera* leaf powder (MOLP) on rats given sodium nitrate (SN) in drinking water. Twenty-five male albino rats were divided into five groups: The control group, fed standard diet for six weeks; MOLP group: fed on MOLP (10%) mixed with diet for six weeks; the SN group received 500 mg/L of SN daily for six weeks in drinking water; SN/MOLP group: drink SN for three weeks then fed on MOLP (10%) for another three weeks; and MOLP + SN group: fed on MOLP (10%) mixed with diet and SN in drinking water at the same time for six weeks. We evaluated the biochemical analysis, including total protein, albumin, total bilirubin, cholesterol, and glucose, as well as visualized histopathological changes in the liver tissue. Rats drinking SN showed a significant ($P \leq 0.05$) decrease in total protein and albumin levels, along with an increase in total bilirubin, cholesterol, and glucose levels in serum. As well as a reduction in final body weight and weight gain. Histologically, SN caused hepatic tissue alterations. Otherwise, rats received MOLP as a protective or a treatment showed an improvement in those parameters accompanied by significant improvements in liver tissue of those groups. In conclusion, the study suggests that MOLP feeding may protect and treat the liver damage caused by SN.

Keywords: Sodium Nitrate, Moringa oleifera leaves, total protein, total bilirubin.

Introduction

Hepatic diseases are the major global public health due to the vital function that the liver plays in human health **(Abood et al., 2020).**

Nitrate is a common pollutant in drinking water because it can penetrates through soil and remains in ground water for decades **(Aly et al., 2010; Kalteh et al., 2022).** Drinking water with a high nitrate concentration can induce a variety of harmful effects on the health **(Mohammadpour et al., 2022).** Nitrates and nitrites are converted into N-nitroso compounds which cause cancer in humans and animals such as liver, stomach, rectum, colon, lung, thyroid, and kidney **(Espejo-Herrera et al., 2015; Qasemi et al., 2018; Messier et al., 2019)**.

SN is used as a model to causing liver toxicity and inducing disorders of liver functions in experimental animals because it can dissolves easily in water and generates the nitrate anion. SN causes hyperglycemia, and affects hepatobiliary function through increasing serum bilirubin level **(Rouag et al., 2020)**. Also, SN induce an alteration in protein synthesis function of liver **(Orabi et al., 2022)**, and inducing hyperlipidemic effect **(El-Wakf et al., 2015).**

According to **Wahyuningsih et al. (2021)**, phytochemicals are a highly recommended as alternative synthetic drugs and less expensive medications for the treatment of liver disease due to their pharmacological advantages. These advantages include reduction of necrotic cell death, natural availability, and low or no side effects, restoration of the antioxidant defense mechanism, and limited oxidative stress **(Parthasarathy and Prince, 2021)**. Among the many naturally occurring plants is *Moringa oleifera* (MO). MO, an Indian perennial tree of the Moringaceae family, is cultivated due to its biological properties **(Khalil et al., 2020).**

MO is rich in minerals and other bioactive components such as high concentrations of vitamins, antioxidant, and proteins **(Gopalakrishnan et al., 2016)**; it can promote normal body functions and protect the body because it can serve as antioxidant, anticancer, anti-inflammatory, anti-hepatic disease, neuro-protective and hypoglycemic **(Islam et al., 2021)**. In experimental models MO leaves have protective effect on protein synthesis function of liver and hepatobiliary effect **(El-Hadary and Ramadan, 2019; Abd-Elhakim et al., 2021),** hypoglycemic effect **(Efiong et al., 2013),** and hypo-cholesterolemic effect **(Stephen Adeyemi et al., 2017).** This protective effect is attributed to phytochemicals like flavonoids, glucosinolates, isothiocyanates, and phenolic acids **(El-Hadary and Ramadan, 2019)**. The aim of the present study is the estimation of the protective and therapeutic effects of MOLP on the toxicity of liver induced by SN in male rats.

Materials and methods

Preparation of Moringa oleifera leaf Powder

Fresh leaves from MO trees were collected and rinsed under running water, drained, and then allowed to dry for five days in the shade. After being dried, leaves were powdered with a blinder, put through a 2-sieve, and kept for subsequent use in a refrigerator at -4ºC.

Animal treatment

Twenty-five albino rats' males weighing between 80 and 100 grams were purchased from VACSERA Organization, Cairo, Egypt. Rats were kept in stainless steel cages at Mansoura University's Faculty of Science's animal house. Rats were kept in a 12-hour light/dark cycle at a specific temperature of 25±2°C. Water and normal diet were applied to the rats on unlimited access. Mansoura University Research Ethics Committee approved the use of animals, and their care was conducted in compliance with **National Research Council (NRC, 2011).**

Following a week of acclimatization, rats were randomly divided into five equal groups, each of five rats. The groups were as follows:

- **Control group:** rats were fed on normal meal for six weeks.
- **MOLP group:** rats were fed on a meal containing 10% MOLP daily for six weeks **(Stephen Adeyemi et al., 2017).**
- **SN group:** rats were drink 500 mg/L of SN in water daily for six weeks **(Anwar and Mohamed 2015).**
- **SN/MOLP group:** rats were drink 500mg/L of SN in water daily, and then fed on a meal containing 10% MOLP daily for another three weeks.
- **MOLPL + SN group:** rats were fed on a meal containing 10% MOLP and drink 500 mg/L SN in water at the same time for six weeks.

Rats were weighed at the beginning of the experiment and then once a week after that, every week, to determine changes in body weight.

Blood collection and tissue preparation

At the end of experiment rats were fasted overnight, and blood samples were collected. Sera were prepared by centrifugation for 10 minutes at 3000 rpm. Then sera were separated and storage at -80°C. Liver tissues were removed and washed in 0.9% saline then quickly fixed in a 10% neutral formalin solution for histological preparation.

Physiological studies:

The Methods of the physiological studies were done according to manufacturer instruction of Diamond Diagnostic Company, Cairo, Egypt.

Histopathology of the liver

The liver was removed rapidly, sliced, and then preserved in 10% neutral formalin. After that, the tissues were dehydrated for 30 minutes each of an increasing concentration of ethyl alcohol concentrations (70, 80, 90, and 95%) then in twice for 30 minutes in 100% ethyl alcohol. The tissues were placed in xylene for 20-minute then embedded in paraffin wax. Following preparation, sections were cut at a thickness of 4-5 µm using microtome, and subsequently stained with hematoxylin and eosin **(Bancroft and Gamble, 2008).**

Statistical analysis

SPSS statistical software (IBM SPSS Statistics, version 20) was used to perform statistical analyses of the collected data. The differences between the groups were examined using a one-way ANOVA (analysis of variance) test, and the post-test Tukey test was then utilized. The provided data were presented as the mean ± SE, and statistical significance is defined as $p \leq 0.05$.

Results

I-Body weight

As shown in **Table 1,** rats drinking sodium nitrate (SN) in drinking water daily for six weeks significantly decreased the rats' final body weight and body weight gain compared with the control group ($P < 0.05$). In groups that drink SN and then treated with *Moringa oleifera* leaves powder (MOLP) and that fed on MOLP and drink SN at the same time, the weight loss is significantly decreased ($P < 0.05$).

Table 1. Effect of *Moringa oleifera* powder (10%) on final body weight and weight gain in male rats treated with sodium nitrate (SN)

	Control	MOLP	SN	SN/MOLP	MOLP + SN
IBW (g)	158.60±2.48	155.40±0.75	154.00±1.48	156.20 ± 2.06	158.20 ± 3.37
FBW (g)	250.20±4.50[a]	259.80±4.25[a]	219.40±5.68[b]	242.00 ±3.66[a]	241.00 ±2.35[a]
BWG (g)	91.60± 3.97[a]	104.40±4.90[a]	65.40 ±6.84[b]	82.80 ± 4.48[a]	85.80 ± 2.27[a]

Values are expressed as mean ± SE (n=5).
Different letters (a, b) show significant differences between the groups at $P \leq 0.05$.
MOLP: *Moringa oleifera* Leaf Powder; SN: Sodium Nitrate
IBW: Initial Body weight; FBW: Final Body weight
BWG: Body Weight Gain. Body Weight Gain (g) = Final body weight–Initial body weight

II- Physiological study

Liver functions

Rats that drinking water daily for six weeks resulted in a significant ($P \leq 0.05$) decrease in serum total protein and albumin concentrations compared to the control group (**Fig. 1**). On the other hand, treatment of SN drinking rats with MOLP (SN/MOLP) or at the same time (MOLP + SN) showed significant increase in total protein and albumin ($p < 0.05$).
As shown in **Fig. 2**, the drinking SN daily for six weeks raised serum concentrations of total bilirubin, cholesterol, and glucose in rats significantly ($P \leq 0.05$) when compared to the control group. In groups SN/MOLP and MOLP + SN, serum concentrations of total bilirubin, cholesterol, and glucose were significantly decreased ($P \leq 0.05$).

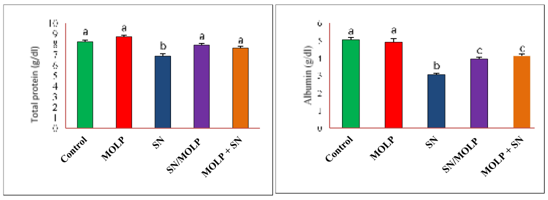

Fig.1. Serum total protein and albumin in the liver of rats treated with MOLP and drink SN. Data were presented as mean ± SE (n=5), a one-way ANOVA was used to analyze them, and Tukey's test was then performed. Values with different letters (a, b, c) are significantly different from each other at $P \leq 0.05$.

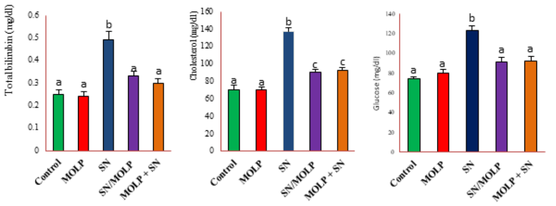

Fig.2. Serum total bilirubin, cholesterol, and glucose in rats drink SN and treated with MOLP. The mean ± SE (n=5) was used to express data. After a one-way ANOVA, Tukey's test was used to assess the data. Different letters (a, b, c) denote significant differences at P ≤ 0.05.

III-Histological study

Rats that drink SN daily in drink water daily for six weeks showed dilated blood sinusoids and cellular infiltrations surrounding the central vein, abnormal hepatocytes with pyknotic nuclei, and enlarged and prominent Kupffer cells. In SN/MOLP group, rats exhibited some normal hepatocytes and others that were still pale, a normal central vein with a normal endothelial lining, normal blood sinusoids and normal Kupffer cells, and cellular infiltrations around the central vein. Co-administration of MOLP with SN displaying normal hepatocytes, normal central vein with normal endothelial lining, and normal blood sinusoids lined with endothelial cells, as shown in **Fig. 3.**

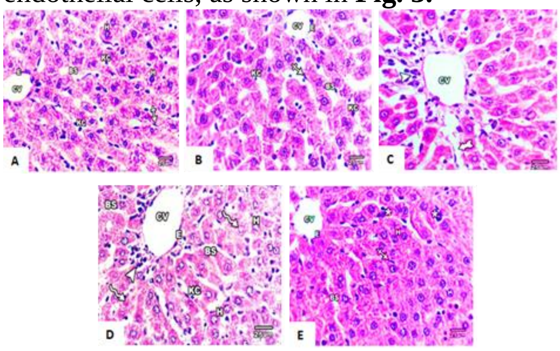

Fig.3. A photomicrograph of a paraffin sections showing histopathological changes in the liver tissue of the experimental groups stained with H&E of the studied groups where A: Control group, B: MOLP group, C: SN (500mg/L) group, D: SN/MOLP group, E: MOLP + SN group. CV: Central Vain, E: Endothelial Lining, BS: Blood Sinusoids, H: Hepatocytes, KC: Kupffer Cells, NA: Necrotic Area.

Discussion

Nitrate produces reactive oxygen species (ROS) and other toxic transient compounds such as hydrogen peroxide and superoxide anion. This leads to unbalance between oxidants and antioxidants, causing oxidative stress **(Alrawi, 2016).** This unbalance alters the metabolism and other functions of hepatic cells **(Bouaziz-Ketata et al., 2014).**

The present study results showed that in comparison to the control group, sodium nitrate (SN) drinking induce a significant reduction in the rats' final body weight and the weight gain. This may be related to harmful effects of SN. This agrees with the findings of **Ogur et al. (2005)** and **El-Wakf et al. (2015).** According to **Zaki et al. (2004)**, nitrate causes loss of body weight by slowing down growth via reduction of plasma T_3 and T_4 levels or by increasing protein catabolism, which is indicated by the low levels of total proteins as in the present study (**Fig 1**).

The present study showed that Co-administration of *Moringa oleifera* leaves powder (MOLP), results in an increase in both final body weight and weight gain in all groups drinking SN daily in drinking water for six weeks. This increase in body weight demonstrates the superior nutritional value of MOLP. These findings are in line with those of **Stephen Adeyemi et al. (2017) and Makkar and Becker, (1997)** who reported that all essential amino acids are present in higher amounts in MO leaves which support growth and health.

A drop in serum total protein and albumin concentrations in rats drinking SN in water suggests a decline in the liver's ability to synthesize protein. Thus, the reduction of total protein concentration could be a sign of increased protein catabolism associated with weight reduction. These results run parallel with those of **Orabi et al. (2022)**. Decreased total protein may be induced by nitrate toxicity through its effects on the liver, due to changes in protein and free amino acid metabolism, such as decreased protein synthesis or increased proteolytic activity or degradation **(Yousef et al., 2008)**, as well as the formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins **(Guzik et al., 2000).** Adrenocortical stimulation may be the cause of albumin breakdown and sodium retention, as shown by the hypoalbuminemia observed in rats receiving SN treatment **(Bhagavan and Ha, 2015).**

Rats co-treated with MOLP showed an increase in serum total protein and albumin concentrations compared to SN-drinking rats, suggesting that MOLP may have an impact on the liver's ability to synthesize protein. The same results were obtained by **Stephen Adeyemi et al. (2017)**. MOLP is used as a protein supplement and has an ameliorative impact as a protein source because it has a suitable amount of protein, fibers, fat, carbohydrates and minerals **(Mhlomi et al., 2022)**. The carotenoids and phenolic content of MO leaves may be associated to alterations in albumin concentration **(Oguntibejua et al., 2020)**.

The present study showed that rats given SN have higher levels of serum total bilirubin, glucose, and cholesterol, suggesting a potential problem with the liver's metabolic processes. As found also by **González Delgado et al. (2018)**.

An increase in bilirubin levels is indicative of hepatic dysfunction **(El-Demerdash *et al,* 2006).** Rats given SN experienced an increase in bilirubin in their serum, emanating from the toxic effect of nitrates by destructing red blood cells **(Rouag *et al.,* 2020)**. Moreover, its increase might represent an attempt to reduce ROS generation in order to protect cells from damage **(Dekker et al., 2011).** Co-administration of MOLP after SN or with SN resulted in a drop in serum total bilirubin concentration, indicating that MOLP's hepatobiliary effects have improved. These results are supported by those of **El-Hadary and Ramadan, (2019)** and **Abd-Elhakim et al. (2021)** and**.** This improvement could be due to presence of proteins, β-carotene, vitamins (A, B, C, and E), riboflavin, nicotinic acid, folic acid and pyridoxine, amino acids, unsaturated fatty acids, minerals, as well as various phenolic compounds in MO leaves.

The liver is essential for the metabolism of cholesterol, and nitrate toxicity can raise cholesterol levels **(Chatterjea and Shinde, 2005; Bishop et al., 2005).** Serum cholesterol serves as an indicator of the extent of damage that free radicals have caused to the body. Rats given MOLP concurrently had lower serum cholesterol concentrations than rats given SN, indicating the hypolipidemic effect of MOLP. These outcomes match those of **Stephen Adeyemi et al. (2017).** The presence of polyphenolic compounds may account for MOLP's hypolipidemic activity. High levels of saponins have been linked to hypo-cholesterolemic effects **(Berhow et al., 2006)** and have been reported to be present in MOLP **(Indriasari et al., 2016).** Two mechanisms may be involved in the cholesterol-lowering actions of saponins: either a decrease in intestinal cholesterol absorption or an increase in bile acid or neutral sterols excretion in feces **(Lee et al., 2005).**

Nitroso-compounds can cause hyperglycemia by interfering with metabolic processes, according to **Bansal et al. (2005)**. Glucose is released from glycogen when nitrate ions are present because amylase and phosphorylase activity increase **(Shelpov et al., 1991)**. Consequently, liver glycogen decreases and blood glucose increases. By transferring glucose from tissue into the circulation, nitrate causes impairment of glucose mobilization or acceleration of gluconeogenesis **(Wiechetek et al., 1993).** Because methemoglobinemia lowers oxygen availability, the rise in glucose levels indicates a metabolic change brought on by SN intoxication. This metabolic adjustment may involve suppressing aerobic metabolism, which is used by cells to break down glucose for the body's use **(Eales et al., 2016).** This study demonstrates the hypoglycemic impact of MOLP by showing a reduction in serum glucose concentrations following SN or in conjunction with SN. These outcomes align with the findings of **Efiong et al. (2013).** In dried MO leaves, chlorogenic acid was shown to be one of the main phenolic acids **(Amaglo et al., 2010)**, which contributes to the metabolism of glucose by blocking the hepatic glycogenolysis and gluconeogenesis caused by the inhibition of glucose-6-phosphate translocase **(Karthikesan et al., 2010).**

Rats given SN show signs of hepatocellular damage, including vacuolations, necrosis, congested central vein, sinusoidal dilation, and inflammatory cellular infiltrations. These changes are consistent with those reported by **Kattaia et al. (2017)**. The histological alterations in liver tissue may be brought on by the generation of ROS, which damage liver cells by causing inflammation, necrosis, fibrosis, apoptosis, or even malignant transformation **(Diesen and Kuo, 2011).** According to **Azeez et al. (2011)**, either the direct relaxing impact of nitrate on the artery wall or the back pressure in the portal space are two potential causes of vascular congestion and sinusoidal dilatation. The current study shows that co-administration MOLP improves hepatocyte damage occurred in liver tissue.

These findings concur with those reported by **Albasher et al. (2020)**. The potential cause of MOLP's reversed impact could be attributed to the veno-activity of flavonoids. According to **Jargin (2018)**, flavonoids are vascular protective agents that are mostly used to treat venous and capillary disorders**.** They strengthen blood vessels by increasing vascular fragility and decreasing capillary permeability **(Rossman et al., 2018).**

Conclusions

The present study concluded that the antioxidant contents of MOLP that mixed with diet can protect from and treat the toxicity induced by sodium nitrate.

Author contributions

Taghreed Fouad: Performance of experiments, Collection of data, writing the manuscript. **El-Sayed Abd El-hady:** Examining histological preparations, interpreting their results. **Ayman Hyder:** Revision of the draft. **Kadry El-Bakry:** Designing the study, helping in manuscript writing. All co-authors permitted the submission of the work for publication.

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الملخص العربي

عنوان البحث: دراسة فسيولوجية ونسيجية لأوراق المورينجا أوليفيرا ضد السمية المستحثه بنترات الصوديوم

تغريد فؤاد[١]*، السيد عبدالهادي[٢]، أيمن حيدر[١]، قدري البكري[١]

[١] قسم علم الحيوان – كلية العلوم - جامعة دمياط.
[٢] قسم علم الحيوان – كلية العلوم - جامعة المنصوره.

يحتوي نبات المورينجا أوليفيرا علي الفلافونويد، الجلوكوزينولات، الايزوثيوسيانات، والأحماض الفينولية والتي لها تأثيرات ايجابية علي الصحه. الهدف من الدراسة الحالية هو تقييم التأثيرالوقائي والعلاجي لتغذية الجرذان بمسحوق أوراق المورينجا أوليفيرا ((MOLPعلى وظائف الكبد بعد إعطائها نترات الصوديوم ((SNفي مياه الشرب. تم تقسيم خمسة و عشرين من ذكور الجرذان إلى خمس مجموعات: تم تغذية المجموعة الأولى (الضابطه) 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 (SN) 500 ملجم/لتر من نترات الصوديوم يوميا لمدة ستة أسابيع في مياة الشرب؛ المجموعة الرابعة ((SN/MOLPتم إعطاؤها

نترات الصوديوم في مياه الشرب لمدة ثلاثة أسابيع ثم تغذيتها ب ١٠% بمسحوق أوراق المورينجا لمدة ثلاثة أسابيع أخرى؛ المجموعة الخامسة (MOLPL + SN) تم تغذيتها 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 مصل أعلى بشكل ملحوظ ($P \leq 0.05$) من إجمالي البيليروبين (total bilirubin) و الكوليسترول (cholesterol) و الجلوكوز (glucose)، إلى جانب إنخفاض كبير في إجمالي البروتين (total protein) و الالبيومين (albumin) في المصل، إنخفاض كبير في وزن الجسم النهائي (final bodyweight) وزيادة الوزن المكتسب (weight gain)، كما أثرت نترات الصوديوم على أنسجة الكبد بشكل ملحوظ. 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، أنسجة الكبد، ووزن الجسم النهائي الناجمة عن سمية نترات الصوديوم.