



Nigella sativa L. oil: Study of its toxicity, antiradical activity, and effect on circulating xanthine oxidoreductase

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ARTICLE INFO

Article Type:

Original Article

Article History:

Received: 11 September 2022

Accepted: 5 November 2022

Keywords:

Hepatotoxicity

Total oil

Neutral lipid fraction

Reactive oxygen species

ELISA assay

ABSTRACT

Introduction: *Nigella sativa* L. is a widely used medicinal plant throughout the world. The low toxic effects and low price of this plant make it an excellent treatment choice for many diseases. The present study aims to investigate the hepatoprotective effect of *N. sativa* L. total oil (TO) and its neutral lipid fraction (NLF) via the estimation of circulating xanthine oxidoreductase (XOR) level and anti-XOR antibodies titer.

Methods: Antiradical activities of TO and NLF, *in vitro*, were carried out using three reactive oxygen species (ROS), superoxide anion, hydroxyl radical, and hydrogen peroxide. *In vivo* study was conducted to determine the possible protective effects of TO and NLF against ethanol-induced hepatotoxicity in rats feeding Lieber-DeCarli liquid diet in both sera and liver homogenate. Before conducting the hepatoprotective effects, we assessed the toxicity of our extracts using the same animal model. The administrated doses of 400 mg/kg for TO and 300 mg/kg for NLF showed no toxic effects.

Results: ELISA assay indicated a significant increase ($P < 0.001$) in the level of XOR and the titer of anti-XOR antibodies in rats treated with ethanol compared to the control group. After treatment with TO and NLF, the titer of anti-XOR antibodies and the level of XOR decreased significantly compared to the control group.

Conclusion: XOR plays an important role in alcohol liver pathologies as a major source of free radicals. In addition, TO and NLF have significant potential as liver protective agents and might be utilized as new antioxidant therapeutics.

Implication for health policy/practice/research/medical education:

Nigella sativa total oil (TO) and its neutral lipid fraction (NLF) showed antioxidant potential in both *in vivo* and *in vitro* studies. This plant presented a significant hepatoprotective effect, which highlighted its role as a liver-protecting agent.

Please cite this paper as: Mosbah A, Khither H, Mosbah C, Slimani A, Mahrouk A, Boucherit H, et al. *Nigella sativa* L. oil: Study of its toxicity, antiradical activity, and effect on circulating xanthine oxidoreductase. J Herbmed Pharmacol. 2023;12(1):128-134. doi: 10.34172/jhp.2023.12.

Introduction

In recent years, medicinal plants have required special attention in the treatment of human diseases and are increasingly applied instead of chemical drugs. Many research studies are focusing on medicinal plants' therapeutic potential since only a few plant species have been investigated for their medicinal properties. Amongst these plants is *Nigella sativa* L. (English: black cumin, Sanskrit: Kalonji). Its seeds are commonly called sanoudj

in Algeria. It is an annual flowering plant that belongs to the Ranunculaceae family (1). *N. sativa* L. seeds have traditionally been used in popular medicine as a natural remedy for various diseases related to liver and kidney functions, digestive tract, respiratory, cardiovascular, and immune systems support (2-4).

Alcohol liver pathologies present a major risk factor for chronic disease. Alcohol consumption induced various liver injuries (5-7), such as steatosis, acute alcoholic

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hepatitis, fibrosis, and cirrhosis. Recent estimates indicate that harmful drinking is responsible for one in seven deaths in men and one in thirteen deaths in women aged 15 to 64 years (6).

The mechanisms involved in the development of alcoholic liver diseases remain poorly elucidated. The pathophysiology of alcoholic liver disease is still incompletely understood but relates largely to the direct toxic effects of alcohol and its main intermediate byproduct, acetaldehyde. However, the degree of severity of these diseases is linked to excessive prolonged drinking (7). Numerous arguments show that alcohol is responsible in the liver for oxidative stress (8,9). Most studies evoke the association between biological markers of oxidative stress and liver disorders related to alcoholism (10).

Amongst the sources of oxidative stress, the enzyme xanthine oxidoreductase (XOR) is located in the liver and generates superoxide anion during its metabolism. High rates of XOR have been observed in various liver pathologies (11,12).

The main role of this enzyme is to catalyze the hydroxylation of hypoxanthine and xanthine to xanthine and uric acid, respectively (13). The reduction of oxygen leads to the generation of superoxide anion and hydrogen peroxide. These reactive oxygen species (ROS) indicate widespread interest in this enzyme. In recent years, one of the aims of scientific research has been to find new bioactive molecules from natural resources with significant therapeutic potential. In the present research work, we aim to determine the hepatoprotective effects of *N. sativa* L. seeds oil against ethanol-induced toxicity in the rat model based on its effect on XOR as an important source of ROS in the human body.

Materials and Methods

Collection of plant

Seeds of *N. sativa* L. used in this study were collected from Algerian Sahara, Bechar in 2019. Seeds were cleaned and stored in darkness at 4°C. The plant material was identified by Dr. Houssine Laouar, Setif 1 University, Algeria. A voucher specimen was deposited at the laboratory of Natural Biological Resources Development under the numbers *N. sativa* L. (MNHN-P-P02305329) in Setif 1 University, Algeria.

Preparation of extracts

Nigella sativa L. seeds powder was extracted in methanol solvent using Soxhlet for 2 hours at room temperature. Methanol was evaporated in reduced pressure at 40°C using a rotary evaporator (BÜCHI 461). The resulting extract was mixed with 200 mL of hexane solvent in the Bulb of settling; two phases of methanol and hexane appeared. The hexane extract was fractionated using a silica gel chromatography column and pure chloroform solvent. The resulting extracts were the total oil (TO) and the neutral lipid fraction (NLF) (14).

Experimental animals

Male albino rats weighing 120-180 g, obtained from the animal house of Mentouri University Constantine 1, Algeria, were kept under laboratory conditions (temperature $22 \pm 2^\circ\text{C}$ and 12/12 hours light/dark cycle). The handling of animals and experiments were performed with respect to the principles and procedures of the European Union Animal Care (CEE Council 86/609) guidelines (15).

Antiradical activities

Superoxide anion radical ($\text{O}_2^{\bullet-}$) scavenging assay

Superoxide anion was produced with the xanthine/XOR system according to the Surendra and Ajay method (16). Results were determined by the following relationship:

$$\text{Inhibition \%} = (A_c - A_e / A_c) \times 100$$

A_c : Control absorbance

A_e : Samples absorbance .

Hydroxyl radical (OH^\bullet) scavenging assay

This experiment was carried out following the Wenli et al method (17). The percentage of OH^\bullet inhibition was calculated using the following formula:

$$\text{Inhibition \%} = [1 - (A_1 - A_2) / A_0] \times 100$$

A_0 : Control absorbance (without extract).

A_1 : Extracts absorbance.

A_2 : Absorbance without sodium salicylate.

Hydrogen peroxide (H_2O_2) scavenging assay

The ability of TO and NLF to scavenge hydrogen peroxide was determined according to Sahoo et al method (18) with slight modifications (extension of incubation time). The percentage of H_2O_2 scavenging was calculated using the following formula:

$$\text{Inhibition \%} = (A_c - A_e) / A_c \times 100$$

A_c : Control absorbance (H_2O_2).

A_e : Samples absorbance.

Study of TO and NLF toxicity

Animal treatment

Albino Wistar rats were divided into three experimental groups (6 rats in each group). Extracts of *N. sativa* L. (TO and NLF) were administered orally using a specific probe.

- Control Group: Rats received drinking water and standard food (4 weeks).
- TO Group: Rats received TO (400 mg/kg) and standard food (4 weeks).
- NLF Group: Rats received NLF (300 mg/kg) and standard food (4 weeks).

Study of biochemical parameters

Toxicities of TO and NLF were assessed by plasma biochemical parameters, including liver parameters

(alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase), renal parameters (urea and creatinine) and lipid parameters (cholesterol and triglyceride). Samples were analyzed using Beckman Coulter Synchron (CX 9 PRO) and commercial kit (Spin react, Spain).

Histological analysis

Livers and kidneys were fixed in formaldehyde (10%) and embedded in paraffin. Sections of 5 μm were stained with a hematoxylin and eosin combination (1923). Analysis of the liver and kidney histological sections was performed using an Optika b-500T i-5 microscope (10 and 40x) and digital images were obtained using the Optika camera associated with a microscope.

Hepato-toxicity study

Alcohol hepato-toxicity in rats was induced using Lieber-DeCarli ethanol-containing diet model for a period of six weeks (20). Rats (8 rats per group) were treated daily by gavage with TO and NLF and the positive control group was treated with N-acetyl cysteine (NAC) for four weeks.

- EtOH group: Received ethanol diet
- TO group: Received ethanol diet + TO 400 mg/kg/d.
- NLF group: Received ethanol diet + NLF 300 mg/kg/d.
- NAC group: Received ethanol diet + NAC 1.2 g/kg/d.
- Control Group: Received control diet.

XOR purification and characterization

XOR enzyme used in this work was purified from human milk using ammonium sulphate fractionation, followed by affinity chromatography on heparin according to the method described by members of Professor Harrison's team (21,22). The purity of the purified enzyme was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and by protein/flavine ratio (23).

Anti-XOR antibodies purification

Anti-XOR antibodies were obtained from the white rabbit's immunized sera using purified XOR as an antigen. The work was carried out at the Pasteur Institute of Algiers (Algeria). Purification of anti-XOR antibodies was conducted using sodium sulphate precipitation and immunoaffinity chromatography on sepharose gel (24).

All rabbits were raised under standardized pathogen-free conditions in the Animal Care Facility in Pasteur Institute (Algeria).

Enzyme-linked immunosorbent assay (ELISA) for XOR and anti-XOR antibodies

XOR assay was performed using ELISA sandwich assay, which allowed us to measure the concentrations of XOR and anti-XOR-antibodies in sera and liver homogenate of rats (22). The absorbance was determined using a microplate reader (ELX800, B instruments, INC) at 492 nm.

Statistical analysis

Results were expressed as mean \pm standard deviation. The differences between groups were determined by the one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical analysis for *in vitro* results was undertaken using a student *t* test. All results were analysed using GraphPad Prism version 5.00. Differences were considered significant at $P < 0.05$.

Results

The experimental yields achieved were $25.45 \pm 3.5\%$ percent by weight for TO and $95.30 \pm 0.6\%$ by TO weight for NLF.

Antiradical assays

TO and NLF showed scavenging capacities against superoxide anion radical ($\text{O}_2^{\bullet-}$), hydroxyl radical (OH^\bullet), and hydrogen peroxide (H_2O_2) in a concentration-dependent manner. TO showed the best anti-radical capacity in all assays and this capacity remained lower than that of positive control α -tocopherol (Table 1).

Toxicity of TO and NLF

Toxic potency of TO and NLF was performed using biochemical parameters and histological analysis of liver and kidney sections. Biochemical markers and lipid profiles did not present any significant differences ($P < 0.05$) compared with the control group (Table 2). Histological study of liver and kidney sections of treated rats indicated that TO and NLF did not show any structural modifications and tissue damages compared with the control group (Figure 1). These results suggested that there would be no functional alteration related to

Table 1. Antiradical activities of total oil (TO) and neutral lipid fraction (NLF) of *Nigella sativa*

Extracts/Standard	IC ₅₀ (mg/mL)		
	$\text{O}_2^{\bullet-}$	OH^\bullet	H_2O_2
α -Tocopherol	0.0315 \pm 0.0056	0.130 \pm 0.0034	0.075 \pm 0.0022
TO	0.0525 \pm 0.0074*	0.330 \pm 0.0028*	0.440 \pm 0.0081**
NLF	0.0680 \pm 0.0039**	0.460 \pm 0.004**	0.730 \pm 0.0054***

Data are presented as mean \pm standard error followed by radicals ($\text{O}_2^{\bullet-}$, OH^\bullet , H_2O_2) production inhibition percentage. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$ compared to the standard α -tocopherol.

Table 2. Hepatic, lipid, and renal profiles of groups treated with Total oil (TO: 400 mg/kg) and Neutral lipid fraction (NLF: 300 mg/kg) of *Nigella sativa* seeds for 4 weeks compared to the normal group (control)

Parameters	Control	TO	NLF
Aspartate aminotransferase (U/L)	138 ± 16.36	160.66 ± 22.48 ^{NS}	141 ± 26.62 ^{NS}
Alanine aminotransferase (U/L)	61 ± 5.47	72.34 ± 9.72 ^{NS}	56.16 ± 12.38 ^{NS}
Phosphatase Alkaline (U/L)	194.83 ± 28.43	257.66 ± 27.74 ^{NS}	230.67 ± 22.73 ^{NS}
Cholesterol (g/L)	0.67 ± 0.06	0.9 ± 0.07 ^{NS}	0.77 ± 0.18 ^{NS}
Triglyceride (g/L)	0.65 ± 0.09	0.81 ± 0.09 ^{NS}	0.75 ± 0.11 ^{NS}
Urea (g/L)	0.45 ± 0.25	0.55 ± 0.13 ^{NS}	0.49 ± 0.11 ^{NS}
Creatinine (mg/L)	6.15 ± 0.84	7.81 ± 0.5 ^{NS}	6.53 ± 0.69 ^{NS}

Values are expressed as means ± SEM (standard error of the mean). NS: Non-significant difference.

* $P < 0.05$ compared to the control group; ns – $P > 0.05$ compared to the control group.

AST, aspartate aminotransferase; ALT, alanine aminotransferase, ALP, alkaline phosphatase.

* $P < 0.05$ compared to the control group; ns: $P > 0.05$ compared to the control group. ASAT: Aspartate Aminotransferase; ALAT: Alanine Aminotransferase; PAL: Phosphatase Alkaline.

structural changes in liver and kidney tissues.

Purification of XOR enzyme and anti-XOR antibodies

Purification yielded 5.7 mg/L of XOR per liter of milk. Enzyme purity was estimated using a UV-Visible spectrum (Figure 2a) with a PFR ratio of 5.34 ± 0.24 (mean ± SD, n = 3). Purity was confirmed with SDS-PAGE, which showed a single major protein band with MW of approximately 150 000 KD (Figure 2b).

Alcohol liver toxicity

The present study, conducted according to OECD guideline 407 and CEE Council 86/609, revealed that TO and NLF did not produce any toxicity and behavioral changes in rats during the period of study and under the conditions of the study.

Liver toxicity induced with ethanol diet consumption (Lieber-DeCarli Liquide diet) was assessed in terms of (i) state of oxidative stress markers (GSH, CAT, SOD, LPO), (ii) biochemical markers and lipid profile (iii) histopathology of liver tissue (25-30). Results showed an association between oxidative stress and liver injury caused by alcohol consumption. Thus, these oxidative alterations acted in synergy and determined liver cytotoxicity (26).

Determination of XOR and anti-XOR antibodies

XOR and anti-XOR antibodies levels in sera and homogenate were determined using ELISA assay based on standard absorption curves against known equations ($y = 0.049x + 0.089$ for XOR and $y = 0.063x + 0.247$ for anti-XOR antibodies) (Table 3).

Results indicated that the XOR level in sera was

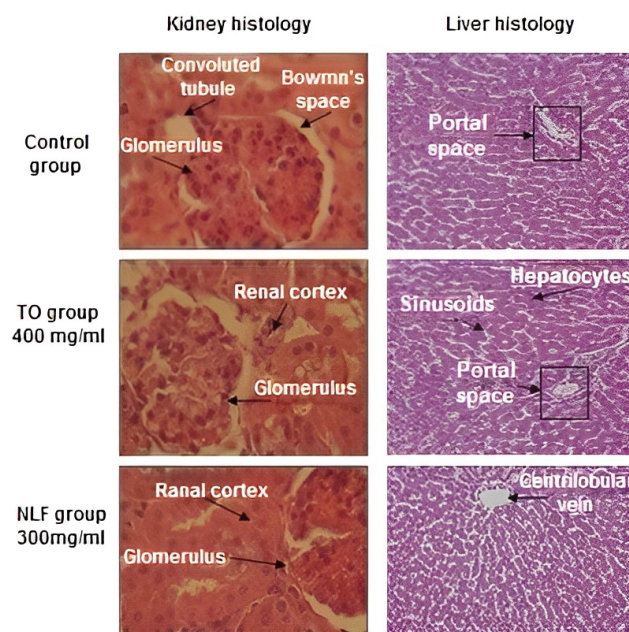


Figure 1. Histopathological analysis of rats' liver and kidney sections treated with Total oil (TO: 400 mg/kg) and Neutral lipid fraction (NLF: 300 mg/kg) for four weeks compared with the control group (x40).

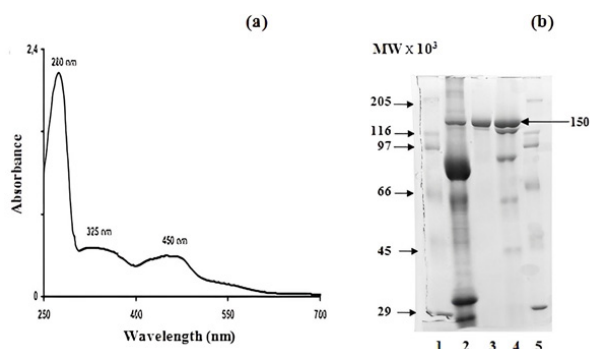


Figure 2. (a) Ultraviolet/Visible spectrum of purified milk xanthine oxidoreductase. (b) SDS-PAGE (10%) of purified milk xanthine oxidoreductase (2: XOR raw fractions, 3: XOR after chromatography, 4: XOR before chromatography, 1 and 5: standard molecular weight proteins).

10.65 ± 2.22 ng/ml and in the liver homogenate was 10.23 ± 5.64 ng/mL. Thus, anti-XOR antibodies titer in liver homogenate was 180.00 ± 16.46 ng/mg and in sera was 120.27 ± 47.90 ng/mg (Table 3).

After ethanol consumption, results showed an increase in XOR level and anti-XOR antibodies titer compared to the control group in both liver homogenate and sera (Table 3).

After treatment with TO (400 mg/kg), NLF (300 mg/kg), and NAC (1.2 g/kg) for four weeks, the level of XOR was decreased and attained values near to those indicated in the control group in both homogenate and sera, respectively (TO=10.03 ± 3.64 ng/mg and 10.43 ± 6.11 ng/mg), NLF (10.88 ± 5.24 ng/mg and 8.4 ± 4.72 ng/mg) and the positive control NAC (11.6 ± 1.60 ng/mg, 10.09 ± 3.11 ng/mg). However, results of anti-XOR antibodies anti-XOR antibodies titer showed a significantly decreased in homogenate and sera, respectively in groups treated with TO (150.22 ± 63.68 ng/mg and 90.88 ± 26.99 ng/mg), NLF (130.44 ± 49.68 ng/mg, 100.22 ± 41.78 ng/mg) and the positive control NAC (200.27 ± 70.33 ng/mg, 80.55 ± 40.01 ng/mg) (Table 3).

Discussion

The present research intended to provide scientific knowledge about the effect of *N. sativa* L. TO and its NLF on alcohol-induced hepatotoxicity and oxidative stress in

rats, focusing on XOR level and anti-XOR antibodies titer (27).

The results of *in vitro* experiments demonstrated that TO exhibited the best antiradical activity against the three radicals produced ($O_2^{\bullet-}$, OH^{\bullet} , H_2O_2). This capacity could be explained by the presence of bioactive lipids such as phytosterols, fat-soluble vitamins, α -tocopherols and β -carotenes, as well as unsaturated fatty acids, in particular ω -3 and ω -6 (14,31). In addition, TO may also contain phenolic compounds with amphiphilic characters (28). These data indicated that the extracts of *Nigella sativa* L. had a considerable ability to react with free radicals and convert them into non-reactive species and thus interrupt the radical reactions chain (29,30), and consequently exert a positive potency on lipid peroxidation (31).

Toxicity study of TO and NLF, *in vivo*, based on biochemical parameters, lipid profile, and histopathological study, which metabolically reflect the physiological status of animals did not show any evidence of adverse effects on liver and kidney in dose of 400 mg/kg/d TO and 300 mg/kg/d NLF for four weeks. These results are comparable to those reported by Sobhi et al using the same fractions for *in vivo* and *in vitro* studies (31). Also, these biochemical parameters and histological results are consistent with the results reported by Dollah et al, which indicated that *N. sativa* L. did not show any toxicity (32).

The study, *in vivo*, was carried out to estimate the antioxidant capacity of *N. sativa* L. extracts using an animal model. Many previous studies have reported the protective effect of the *N. sativa* plant against hepatotoxicity induced by ethanol and other toxic agents (33,34).

To understand the mechanism by which the hepatoprotective effect of the two fractions (TO and NLF) occurs, we used ELISA assay to estimate the XOR level, enzyme generating of ROS, and the anti-XOR antibodies titer in both sera and liver homogenates of ethanol-treated and non-treated rats. Results of the ELISA assay in the control group showed that the XOR level and the anti-XOR antibodies titer in liver homogenate were higher than them in sera. This could be explained by the naturally high distribution of this enzyme in the liver (22) since XOR mRNA was detected in most tissues in the human body and the highest levels of transcription were detected in the liver and intestine organs (35,36).

Table 3. Xanthine oxidoreductase (XOR) concentration and anti-XOR antibodies titer in liver homogenate and sera of experimental groups

Groups	Homogenate (ng/mg)		Sera (ng/mL)	
	XOR	Anti-XOR ab	XOR	Anti-XOR ab
Control	10.65 ± 2.22	180.00 ± 16.46	10.23 ± 5.64	120.27 ± 47.90
EtOH	30.81 ± 3.42	320.77 ± 30.21	18.81 ± 9.42	220.5 ± 23.30
NAC	11.6 ± 1.60*	200.27 ± 70.33*	10.09 ± 3.11	80.55 ± 40.01*
TO	10.03 ± 3.64*	150.22 ± 63.68*	10.43 ± 6.11	90.88 ± 26.99*
NLF	10.88 ± 5.24*	130.44 ± 49.68*	8.4 ± 4.72*	100.22 ± 41.78*

Values are expressed as mean ± SD. * $P < 0.05$ compared to the EtOH group. EtOH: Animals that received an ethanol diet. TO: Animals that received total oil at the dose of 400 mg/mL. NLF: Animals that received neutral lipid fraction at the dose of 300 mg/mL. NAC: Animals that received NAC at the dose of 1.2 mg/mL. Control: Animals that received a control diet.

After ethanol diet consumption for six weeks, the results indicated that XOR level and anti-XOR antibodies titer were significantly increased ($P < 0.001$) in both liver homogenate and sera compared with the control group. Following the application of the treatment with TO, NLF, and NAC, the XOR level and the anti-XOR antibodies titer were decreased significantly and attained values near those of the control group. This finding could be attributed to the natural distribution of XOR in the liver; therefore, the liver was considered the main source of XOR in sera after a large tissue injury and cell damage. Consequently, these results suggested that ethanol would be an important overproduction factor for cytosolic XOR (37). Once released into the plasma, XOR is quickly converted to the xanthine oxidase form responsible for ROS production (38). Circulating XOR can reach distant organs and bind to vascular cells. Our results suggested that the amount of ROS released in blood circulation was produced, in part, by liver XOR. Otherwise, the high titer of anti-XOR antibodies detected, after ethanol diet consumption, could be attributed to the XOR boosting of immune system due to the significant exposure to ethanol. It has been suggested that anti-XOR antibodies can be generated by exposure of XOR to immune system (39). This enzyme can be released during capillary endothelium tissue damage that is rich in XOR (endogenous stimulation).

Another important finding of this study is of the significant increase in XOR levels in hepatic damage, which makes it possible to propose this enzyme as a complementary biological parameter to evaluate liver integrity and for the screening of alcoholic liver lesions (11,40).

Conclusion

The present research work highlighted the antiradical capacity of *N. sativa* L. extracts, TO, and NLF, *in vitro* and *in vivo*. Both extracts showed an antiradical capacity against the three ROS used (O_2^- , OH^\cdot , H_2O_2). *In vivo* study indicated that the two extracts presented a significant potential as liver-protecting agents against alcohol-induced hepatotoxicity, due to their significantly decreasing XOR level and anti-XOR antibodies titer. Evaluation of extracts toxicity, *in vivo*, allowed us to conclude that, on experimental conditions used in the present work (dose and period), the two extracts did not show any toxicity.

Authors' contribution

MA, KH, and MK designed and performed the experiments and collected the data. SA and MA analysed the data. BH and MZ prepared the manuscript. All authors read and approved the manuscript for publication.

Conflict of interest

The authors declare no conflict of interest.

Ethical considerations

The experimental protocol was approved by University Setif 1 Ethics Committee (Article 3 JORA N04, 18 Aout 2013). The handling of animals and experiments were performed with respect to the principles and procedures of the European Union Animal Care (CEE Council 86/609) guidelines adopted by University Setif 1 ethics committee, Algeria. All authors have inspected carefully and cleared the issues related to misconduct, plagiarism, fabrication, and redundancy associated with the manuscript.

Funding/Support

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MERS) (F01220060052).

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