Journal of Inflammation Research

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

The Protecting Role of Black Seed Oil and Its Nano-Formulation in LPS-Induced Acute Kidney Injury in Mice: Evaluation of Oxidative Stress, Biochemical & Molecular Parameters

Barakat M ALRashdi[1], Diaa Massoud[1], Hager K Rashwan[2], Shahenda Mahgoub[3], Nermeen Z Abuelezz[2], Ali M Nasr[4,5], Rami B Kassab[6], Hatem K Amin[3]

[1]Department of Biology, College of Science, Jouf University, Sakaka, Saudi Arabia; [2]Department of Biochemistry, College of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, Giza, Egypt; [3]Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, Helwan University, Cairo, Egypt; [4]Department of Pharmaceutics, Faculty of Pharmacy, Port Said University, Port Said, 42526, Egypt; [5]Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Galala University, New Galala, 43713, Egypt; [6]Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo, Egypt

Correspondence: Hager K Rashwan, College of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, P.O. Box, 77, Giza, 12572, Egypt, Tel +201066116240, Email hager.khaled@must.edu.eg

Background: Acute kidney injury (AKI) is a medical concern that is accompanied by the rapid deterioration of kidney function. It can be triggered by lipopolysaccharide (LPS) of gram-negative bacteria as it activates a complicated immune response, resulting in widespread inflammation and potential organ dysfunction. Black seed oil (BSO) is rich in beneficial constituents and has been widely used owing to its nutritional advantages.

Purpose: This research is aimed to investigate the potential protective effects of BSO and its nano-formulation on AKI induced by LPS. It also aimed to compare their anti-inflammatory activity with indomethacin, a known synthetic anti-inflammatory drug.

Materials and Methods: Forty-eight mice were placed randomly into 8 groups. A single intraperitoneal (*i.p.*) injection of 2.5 mg/kg B.W. of LPS was used to trigger inflammation, and pretreatment with BSO and its nano-formulation was at 0.2 mL/kg/day for 14 consecutive days. Indomethacin was used as a reference drug and its efficacy was tested alone or in combination with BSO at lower doses. Renal function was assessed using urea, creatinine, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1). Also, oxidative and inflammatory markers were assessed by measuring levels of reduced glutathione (GSH), nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), and toll-like receptor-4 (TLR-4). Histopathological examination of the kidney tissues was also performed.

Results: The study showed that BSO and its nano-formulation had anti-inflammatory effects comparable to or better than those of indomethacin. They greatly decreased the oxidative stress and inflammatory markers induced by LPS. Their protective effect against pathological alterations in kidney tissues was significantly noticed.

Conclusion: BSO and its nano-formulation could be used as nephroprotective and anti-inflammatory supplements.

Keywords: TLR-4, *Nigella sativa*, inflammation, nephrotoxicity, nanoparticles

Introduction

Acute kidney injury (AKI) is characterized by a rapid decline in kidney performance within hours to seven days,[1] which results in the accumulation of waste products, poor electrolyte balance, and altered medication concentration. It is often described by an increase in serum creatinine levels with or without a reduction in the patient's urination as a matter of observable clinical outcomes.[2,3] Approximately, AKI affects 13.3 million individuals worldwide. This has been widely observed in critically ill patients.[2] The reduction of blood flow, nephrotoxic drugs, ischemia, and sepsis are among the common causes of AKI.[3,4] AKI is characterized by a certain extent of renal cell death as a result of inflammatory

Received: 10 February 2024
Accepted: 26 June 2024
Published: 16 July 2024

responses, oxidative stress, and apoptosis.[5,6] Inflammation is usually viewed either as a contributor to the kidney injury or as a result of the injury.[3] Molecular mediators of inflammation activate pattern recognition receptors that drive the hyperinflammatory response. Consequently, dysregulation of immune system activity leads to kidney dysfunction.[7,8]

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria. It has gained significant attention as a causative agent for AKI. When LPS is released into the bloodstream, it causes leukocyte infiltration and consequent systemic inflammation that resembles many of the early clinical characteristics of sepsis, in addition to its direct inflammatory response within the renal cells. The activation of toll-like receptor 4 (TLR-4) recruits NF-κB and MAPK pathways. This ultimately increases the production of inflammatory cytokines, chemokines, and adhesion molecules.[9,10] LPS-induced renal toxicity is also associated with elevated oxidative stress levels, resulting in cellular damage and dysfunction.[11,12]

Black seed (BS) or *Nigella sativa L*. (*Ranunculaceae* family) is a flowering herb native to the Middle East, Far East, West Asia, and parts of Europe. For decades, the Middle Eastern population has utilized BS and its oil as natural treatments for a variety of illnesses. Traditionally, BS has shown beneficial effects in treating headache, common cold, cough, asthma, urticaria, and digestive discomfort. Studies have been expanded to justify and explore the beneficial activities of BS and the possible underlying mechanisms. BS possesses anti-tumor,[13] anti-inflammatory,[14] anti-oxidant,[15] anti-fungal, anti-viral, and anti-bacterial activities,[16] as well as analgesic, antipyretic,[17] and fertility[18] enhancing activities. In addition, it is used to support and protect body's organs against various toxicities.[19–21]

Black seed oil (BSO) has been reported to contain valuable active constituents including thymoquinone, limonene, carvacrol, longifolene, p-cymene, vitamin E, saturated and unsaturated fatty acids (FAs), and sterols.[22,23]

Thymoquinone (TQ) (2-Isopropyl-5-methylbenzo-1,4-quinone) has been identified as the primary bioactive component of black cumin seed oil. It has been extensively studied for its wide array of therapeutic and pharmacological activities, including but not restricted to its anti-oxidant, anti-inflammatory and anti-apoptotic effects.[24–26]

Despite using BSO for many nutritional benefits, its poor physical characteristics such as hydrophobicity and volatility can minimize these benefits.[27]

Nano-formulation technology has revolutionized various industries, and the field of oil-based formulations is no exception. The conversion of BSO into a nano-formulation can improve its stability and bioavailability while maintaining its volatile active ingredients.[27] Hence, we intended to study the possible improved outcomes of using the nano-formulation compared to the conventional formulation.

As best as we know, previous studies explored the therapeutic effectiveness of using the black seeds or their extracts or thymoquinone against different types of nephrotoxic chemicals, drugs, insecticides, and heavy metals. Thus, this is the first report to evaluate the effect of the black seed oil and its nano-formulation on LPS-induced nephrotoxicity in mice.

The purpose of this research was to evaluate the renal protective effects of BSO and its nano-formulation, in addition to the mechanisms underlying this protective effect. The results were compared with those of indomethacin, a well-established synthetic anti-inflammatory medicine used in clinical practice.

Materials and Methods

Chemicals and Kits

Lipopolysaccharide (*Escherichia coli* serotype 0111: B4) was purchased from Sigma Aldrich (USA), black seed oil was purchased from Imtenan company (El Obour City,1st Industrial Area, Egypt), Liometacen® (indomethacin) was purchased from the Nile company for pharmaceuticals and chemical industries (Egypt), soy lecithin and tween 80 were purchased from Alfa Aesar (Lancashire, UK). Colorimetric nitric oxide (NO) kit Cat. No. MBS8243214, and ELISA kits for reduced glutathione (GSH) Cat. No. MBS724815, and cyclooxygenase-2 (COX-2) Cat. No. MBS269104 were obtained from MyBioSource (San Diego, USA). RNA extraction kit (GeneJET RNA purification kit, Cat. No. K0731), and cDNA Synthesis Kit (RevertAid First Strand cDNA Synthesis Kit Cat. No. K1622) and SYBR green kit (Master PowerUp™ SYBR green master mix, Cat. No. A25779) were all obtained from Thermo Scientific/Applied Biosystems (Logan, USA). TE buffer (pH 8.0, Item number: T1120) was purchased from SolarBio (China). Primers were purchased from Willofort (Birmingham, UK).

Black Seed Oil Administration

BSO was emulsified in a 1% Tween 20 solution to provide mice with the recommended dosages of the oil.[28]

Black Seed Oil Characterization

Analysis of the Bioactive Chemical Compounds of BSO using Gas Chromatography Flame Ionization Detector (GC-FID)

At the Central Laboratories Network, National Research Centre (Egypt), the gas chromatography model 7890B (from Agilent Technologies, Inc.) was equipped with a flame ionization detector. The column Zebron ZB-FAME (60 m × 0.25 mm internal diameter × 0.25 μm film thickness) was used in the separation process. The analysis was performed using hydrogen as a carrier gas at a flow rate of 1.8 mL/min in split-1:50 mode, injection volume of 1 μL, and the following thermal program was carried: 3 min at 100°C, rising at 2.5°C/min to 240°C, and holding for 10 min. The injector and flame ionization detector (FID) were maintained at 250°C and 285°C, respectively.

Analysis of the Bioactive Chemical Compounds of BSO Using Gas Chromatography-Mass Spectrometry (GC-MS)

At the Central Laboratories Network, National Research Centre (Egypt), the GC-MS system (Agilent Technologies) was equipped with a mass spectrometer detector (5977A) and gas chromatograph (7890 B). The HP-1MS column was equipped with a GC with specifications of (60 m × 0.25 mm internal diameter and 0.25 μm film thickness). The analysis was performed using helium as a carrier gas at a flow rate of 1.4 mL/min and a splitless injection volume of 1 μL and the following thermal program was carried: 70°C for 0 min, rising at 10°C/min to 220°C, and held for 15 min. The injector and detector were maintained at 220°C, 240°C. Mass spectra were obtained by electron ionization (EI) at 70 eV using a spectral range of m/z 50–550 and a solvent delay of 7 min. The mass temperature was 230°C and Quad 180°C.

Black Seed Oil Nano-Formulation Preparation and Characterization

Preparation of the Nano-Emulsion

The method described by Gumus and colleagues[29] was slightly modified to produce an oil-in-water emulsion with black seed oil (BSO) as the internal phase. Tween 80 and lecithin were used in the one-step procedure. The aqueous phase, having 3% tween 80 and the oily phase, consisting of BSO with 3% lecithin, had a volume ratio of 80:20, respectively. Briefly, the two phases were combined and sonicated for 10 min at a 50% amplitude in an ice bath using a probe sonicator (Vibra Cell, Sonics, USA). Sonication was stopped every minute for 30s, until the nano-emulsion was formed. Subsequently, the produced nano-emulsion was kept out of the light and stored at 4°C until further use.

Characterization of the Nano-Emulsion

Droplet Size Analysis and Surface Charge Measurement

The droplet size and droplet size distribution expressed by the polydispersity index (PDI) of the BSO nano-emulsion were evaluated using dynamic light scattering (DLS) technique. The values were measured using Zetasizer (Nano ZS, Zetasizer, Malvern Instruments Ltd., UK). The electrical charge on the surface of the oil droplets in the nano-emulsions was determined by measuring their Zeta Potential (ZP). The ZP values expressed in millivolts were assessed based on electrophoretic mobility. To avoid multiple scattering effects, a suitable dilution using double-distilled water was performed before measurement. The values were expressed as mean ± SD and the measurements were done in triplicates.

The Morphological Analysis

The morphology of the formed BSO nano-emulsion droplets was evaluated by transmission electron microscopy (TEM) using a JTEM-1010 microscope (JEOL, Tokyo, Japan) by applying the negative staining technique. In brief, one drop of the nano-emulsion was added onto a carbon-coated copper grid, and the filter paper was used to remove excessive droplets. Five minutes later, one drop of uranyl acetate solution (2% w/v) was added to the grid. The sample was then dried in air at room temperature, and the examination was performed at 80 kV.

Animals and Experimental Design

Forty-eight male balb/c mice with an average age of 8 ± 1 weeks and a weight of 30 ± 5 g were used. They were purchased from VACSERA Animal Center (Helwan, Egypt). All the procedures used in this study were in accordance with the ethical standards of the ethical committee of the Faculty of Pharmacy, Helwan University, Egypt (Ethical No.02A2020; date 13/10/2020).

Mice were accommodated to the laboratory conditions around two weeks before the experiment and housed in polypropylene cages with stainless steel grid covers with a 12 h light/dark cycle, at optimum humidity and a temperature of 26 ± 2°C and had access to water and food ad libitum through the experiment.

The experiment was designed as shown in Figure 1 and mice were randomly divided into eight groups (n = 6 mice per group).

- Negative control group: Healthy mice were treated with vehicle only for 2 weeks.
- Black seed oil control group (BSO): BSO (0.2 mL/kg)[30] was administered intraperitoneally to mice for 14 consecutive days.
- Nano-black seed oil control group (nano-BSO): Nano-formulation of BSO (0.2 mL/kg) was administered intraperitoneally to the mice for 14 consecutive days.
- LPS group (LPS): Mice were treated with a single intraperitoneal dose of LPS (2.5 mg/kg)[31,32] six hours before termination.
- Black seed oil + LPS group (BSO + LPS): BSO (0.2 mL/kg) was administered intraperitoneally to mice for 14 consecutive days, followed by a single intraperitoneal injection of LPS (2.5 mg/kg).
- Nano-black seed oil + LPS group (nano-BSO + LPS): nano-formulation of BSO (0.2 mL/kg) was administered intraperitoneally to mice for 14 consecutive days, followed by a single intraperitoneal injection of LPS (2.5 mg/kg).
- Indomethacin + LPS group (Indo. (5 mg/kg) + LPS): mice were treated intraperitoneally for three days with indomethacin (5 mg/kg)[33] before termination, followed by a single intraperitoneal dose of LPS (2.5 mg/kg).
- Black seed oil + indomethacin half dose + LPS group: (BSO + Indo. (2.5 mg/kg) + LPS): BSO (0.2 mL/kg) was administered intraperitoneally to mice for 14 consecutive days, indomethacin (2.5 mg/kg) was injected intraperitoneally for three days before termination followed by LPS intraperitoneal single dose (2.5 mg/kg).

Handling and Processing of Samples

At the end of the experiment, blood samples were collected from the orbital sinus of experimental mice, then sacrificed by cervical dislocation. The kidneys were removed immediately, rinsed, cleaned from any blood, and weighed. One of the kidneys was fixed in 10% formalin for histopathological investigation, and the other was stored at −80°C until homogenization. The homogenates were prepared according to the kidney weights, as each gram was homogenized with 10 mL ice-cold saline. The supernatants of the homogenates were separated at 5000 rpm for 15 min using a cooling centrifuge and divided into aliquots for further analysis and stored at −80°C for further tests.

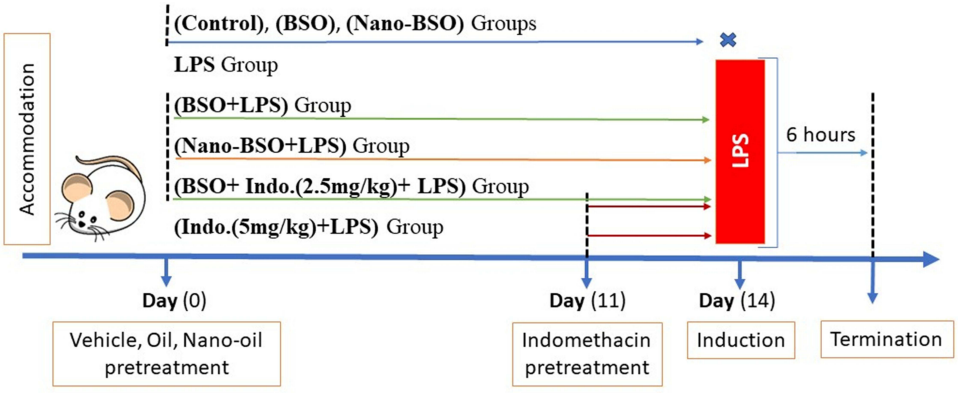

Figure 1 Experimental design and animal groups.

The blood samples were maintained at room temperature for 20 min and then centrifuged for 15 minutes at 3000 rpm. The clear supernatant was isolated and kept at −80°C for further tests.

Histopathological Samples Preparation

The kidney tissues were fixed in 10% buffered formalin. Paraffin slices were prepared from autopsies of mice from each experimental group. The specimens were rinsed with tap water and dehydrated using serial dilutions of ethyl alcohol. The sections were then cleared with xylene and embedded in paraffin. Hematoxylin and eosin (H&E) were used to stain paraffin block sections with an average thickness of 4–5µm.[34] Light microscope (Olympus, Tokyo, Japan) was used to evaluate the histological sections. Micrographs were taken at X400 magnification.

Biological Markers of Assessments

Markers of Renal Function

For assessing renal function, urea and creatinine levels were measured in serum by using Olympus automated chemistry analyzer device (AU400), while Neutrophil Gelatinase-Associated Lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) levels were assessed in kidney tissue using real-time polymerase chain reaction.

Markers Related to Renal Oxidative Stress and Inflammatory Process in Kidney's Tissue

Reduced glutathione (GSH) and Cyclooxygenase-2 (COX-2) levels were assessed using enzyme-linked immunosorbent assay (ELISA), as directed by the manufacturer, through Tecan Spectra Rainbow microplate reader A-5002 (Switzerland). Nitric oxide (NO) levels were assessed using a colorimetric assay.

Quantitative Real-Time PCR (qRT-PCR)

The GeneJET RNA Purification Kit (Thermo Scientific/Applied Biosystems, Logan, UT, USA) was used to extract total RNA from kidney tissues, as directed by the manufacturer. Nanodrop was then used to quantify the concentration and purity of the total RNA samples. They were then reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific/Applied Biosystems, Logan, UT, USA).

To evaluate how BSO and its nano-formulation affect the expression of certain genes, the mRNA levels of *KIM-1* and *NGAL* were assessed as kidney injury-specific markers. *TLR-4* and tumor necrosis factor alpha (*TNF-α*) were assessed as inflammatory markers using the Master PowerUp™ SYBR™ Green Master Mix along with *Actb* as a housekeeping gene. Rotor-Gene Q (QIAGEN Hilden, Germany) was used to perform quantitative PCR, and the results were interpreted using Livak's method 2-(ΔΔCt)[35] and expressed as the mean fold change. Table 1 contains a list of the primer sequences.

Statistical Assessments

Data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used, followed by the Tukey–Kramer multiple comparison test using GraphPad Prism® software (version 5.01). In all analyses, *P* values less than 0.05 were regarded as significant.

Results

Characterization of BSO Using Gas Chromatography

BSO was analyzed using a GC equipped with either two detector systems: flame ionization detection (FID) or mass spectrometry (MS).

Gas Chromatography-Flame-Ionization Detection (GC-FID)

Fatty acids (FAs) were identified by means of comparing their retention times with those of known standards. Approximately, 17 FAs were identified in BSO, as shown in Table 2 in the GC-FID chromatograms (Figure 2). The main unsaturated FAs were linoleic (58.64%) and oleic (21.82%) acids, whereas the main saturated fatty acids were palmitic (12.36%) and stearic (3.16%) acids. The fatty acids obtained were close to those detected in previous studies[36,37] for the cold-pressed form of BSO, which was in accordance with the oil's supplying company (Imtenan) form.

Table 1 List of Primer Sequences of the Genes Analyzed by qRT-PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)	Accession Number*
KIM-1	ACATATCGTGGAATCACAACGAC	ACTGCTCTTCTGATAGGTGACA	NM_001166632.1
NGAL	GCTGTCGCTACTGGATCAGA	TGGCGAACTGGTTGTAGTCC	NM_008491.1
TLR-4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA	NM_021297.3
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG	NM_013693.2
β-Actin	CTCTAGACTTCGAGCAGGAGATGG	ATGCCACAGGATTCCATACCCAAGA	NM_007393.5

Note: *Based on the GenBank Primer-Blast Program, NCBI.
Abbreviations: KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; TNF-α, tumor necrosis factor alpha; TLR-4, toll-like receptor 4; β-Actin, beta Actin.

Table 2 Identified Fixed Oils in the Black Seed Oil

Peak	Rt* (min)	Name	Relative Area %
1	8.276	Capric acid	0.04
2	18.237	Myristic acid	0.21
3	21.138	Pentadecanoic acid	0.03
4	24.075	Palmitic acid	12.36
5	25.131	Palmitoleic acid	0.20
6	26.812	Margaric acid	0.05
7	27.754	cis-10-Heptadecenoic acid	0.04
8	29.630	Stearic acid	3.16
9	30.447	Oleic acid	21.82
10	32.213	Linoleic acid	58.64
11	34.117	Linolenic acid	0.25
12	34.782	Arachidic acid	0.20
13	35.439	*cis*-11-Eicosenoic acid	0.33
14	37.005	*cis*-11,14-Eicosadienoic acid	2.50
15	38.768	Arachidonic acid	0.08
16	39.687	Behenic acid	0.05
17	41.666	*cis*-13,16-Docosadienoic acid	0.04

Note: *Retention time.

Gas Chromatography-Mass Spectrometry (GC-MS)

The different constituents of BSO were identified by comparing their spectrum fragmentation patterns with those stored in Wiley and NIST Mass Spectral Library data. As depicted in Figure 3 and Table 3, approximately 16 compounds were identified. Among these, thymoquinone was the most abundant essential constituent (84.34%).

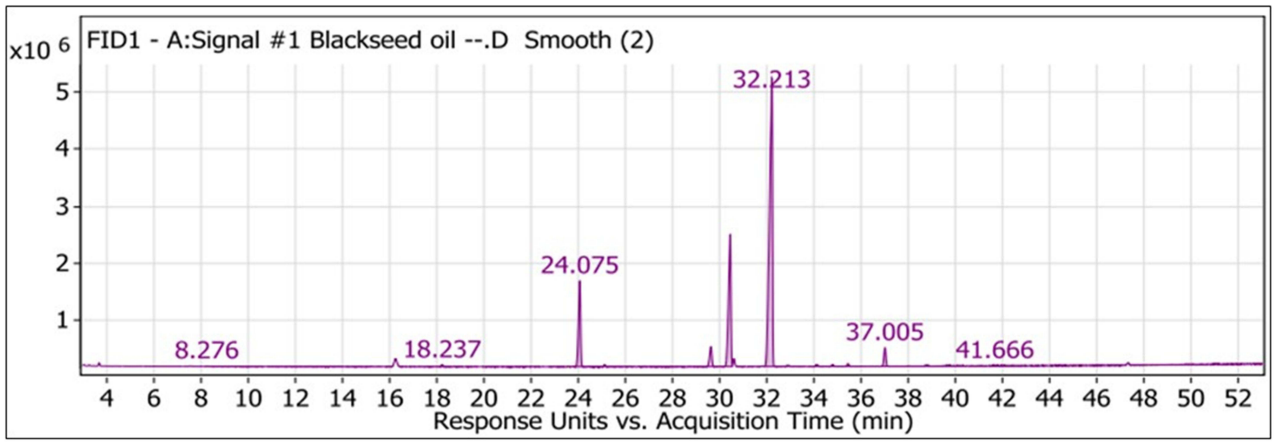

Figure 2 GC-FID chromatogram of fixed oils of black seed oil.

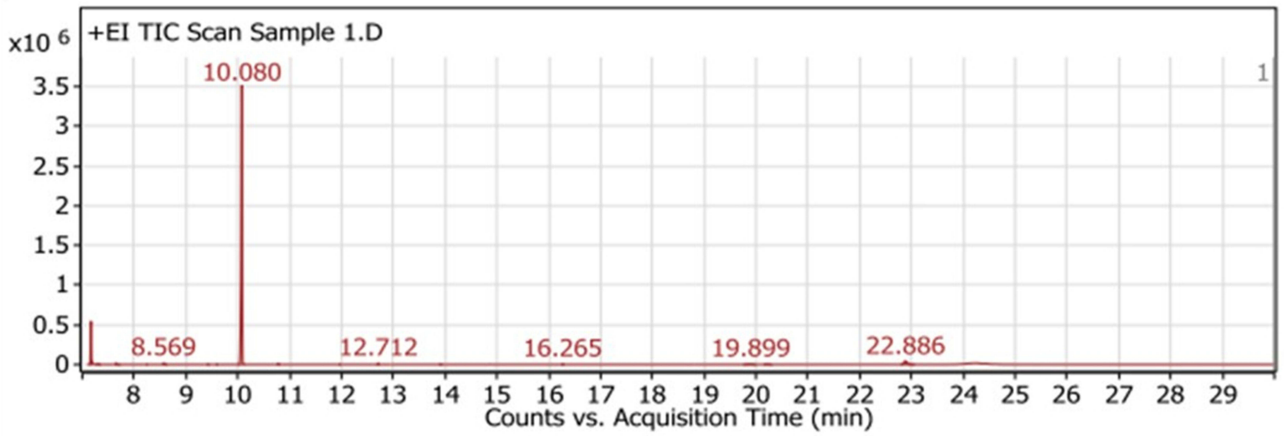

Figure 3 GC-MS chromatogram of volatile oils of black seed oil.

Characterization of BSO Nano-Formulation

Polydispersity Index (PDI), Droplet Size, and Zeta Potential

Regarding the homogeneity of droplets, which is presented by the PDI value (0–1),[38] the obtained PDI was 0.24 ± 0.3, which was less than 0.3, thus indicating a narrow particle size distribution[39,40] and considered acceptable. The mean

Table 3 Identified Volatile Oils in the Black Seed Oil

Peak	Rt* (min)	Name	Formula	Relative Area %
1	7.167	O-Cymene	$C_{10}H_{14}$	9.17
2	7.293	Alpha-Caryophyllene cis-[[1R-(1R*,4E,9S*)]-4,11,11-Trimethyl-8-methylenebicycl	$C_{15}H_{24}$	0.40
3	7.654	Alpha-Thujene	$C_{10}H_{16}$	0.54
4	8.243	3,3-Dimethyl-4-methylenebicyclo [3.1.0] hexan-2-one	$C_9H_{12}O$	0.02
5	8.569	(1R,4R,5S)-1-Isopropyl-4-methoxy-4-methylbicyclo [3.1.0] hexane	$C_{11}H_{20}O$	0.59
6	9.422	Tricyclo [3.2.1.0(2,4)] oct-6-ene (isomer 1)	C_8H_{10}	0.06

(*Continued*)

Table 3 (Continued).

Peak	Rt* (min)	Name	Formula	Relative Area %
7	9.599	Cyclopropylidenepropanol	$C_6H_{10}O$	0.05
8	10.080	Thymoquinone	$C_{10}H_{12}O_2$	84.34
9	10.778	Phenol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{14}O$	0.31
10	11.968	5-Methyl-2-methylidene-3-vinyl-4-hexen-1-ol	$C_{10}H_{16}O$	0.05
11	12.712	(Z, Z)-. alpha-Farnesene	$C_{15}H_{24}$	0.40
12	13.908	2,4-Dideuterio-3-tert-butylanisole	$C_{11}H_{14}D_2O$	0.10
13	16.265	1-(methylsufinyl) oct-2-ene	$C_9H_{18}OS$	0.15
14	19.899	Docosanoic acid	$C_{22}H_{44}O_2$	0.55
15	20.213	7-Decen-1-ol	$C_{10}H_{20}O$	0.30
16	22.886	Dodecanal	$C_{12}H_{24}O$	2.98

Note: *Retention time.

droplet size obtained using the DLS technique was 60.44 ± 4.05 nm. Droplet sizes less than 100 nm offer a large surface area and hence better bioavailability.[41] On the other hand, zeta potential (ZP) value was –32.0 ± 2.17, which indicates a low probability of coalescence or increased size of the prepared nano-emulsion with time. The ZP value is an indicator of the stability of nano-dispersions. The higher the absolute ZP value, the more repulsion occurred between particles or droplets, and the higher the stability of the two-phase nano dispersion.[42] Thus, the more stable the nano-formulation.[43]

The Morphology of the Formed Black Seed Oil Nano-Emulsion Droplets

The obtained negatively stained oil droplets were relatively homogenous and spherical in shape. The droplet size was comparable to that obtained by DLS (Figure 4). No signs of aggregation or coalescence were observed.

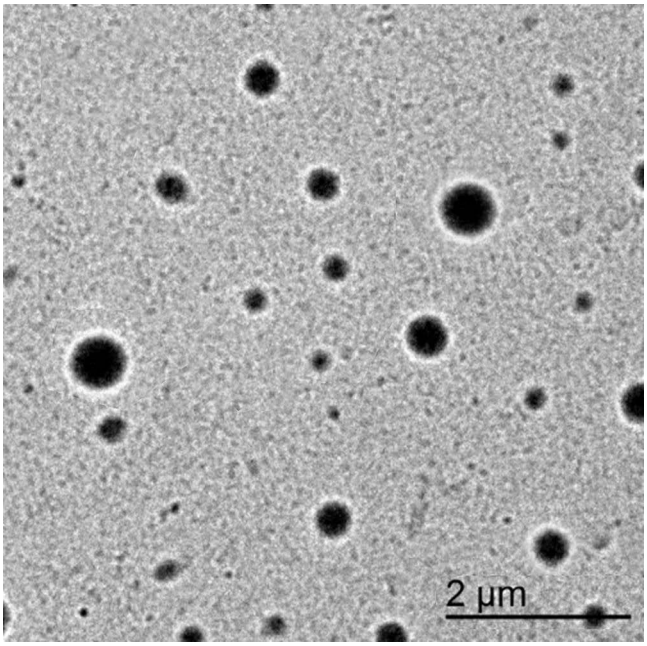

Figure 4 TEM micrograph of BSO nano-emulsion.

Kidney Function/Injury Markers

Serum Urea and Creatinine Levels

The urea levels were significantly higher in the LPS group (95.57%) than in the control group ($p < 0.05$). All pretreated mice showed variably low urea levels. However, a significant reduction (28.60%) ($p < 0.05$) was observed in the BSO-pretreated mice compared to that in the LPS and the (Indo. (2.5 mg/kg)+BSO) pretreated group (47.33%) as compared to the LPS group and the (Indo. (5 mg/kg)) pretreated group (Figure 5). Considering serum creatinine, lower levels were noticed in mice pretreated with BSO and nano-BSO. However, no significant differences were observed between the groups (Figure 5).

Kidney Injury Molecule-1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) Gene Expression

Both *KIM-1* and *NGAL* genes were significantly upregulated in LPS treated mice by (36.89%) and (70.3%), respectively, compared with the control group ($p < 0.05$). As shown in Figure 6, all pre-treatments of mice prior to LPS induction caused a significant downregulation ($p < 0.05$) of both genes compared to the LPS group. Moreover, pretreatment of mice with BSO followed by indomethacin low dose (2.5 mg/kg) significantly ($p < 0.05$) downregulated *KIM-1* expression levels by (68.09%) compared with BSO pretreatment (34.75%) or indomethacin higher dose (5 mg/kg) only pretreatment (36.88%), respectively. The *KIM-1* expression levels in mice pretreated with nano-BSO were significantly ($p < 0.05$) lower at 53.9% compared to mice pretreated with BSO (34.75%). Regarding *NGAL* expression, it was downregulated in the mice pretreated with BSO followed by indomethacin low dose (2.5 mg/kg) by 21.51%, which was close to the BSO only pretreatment

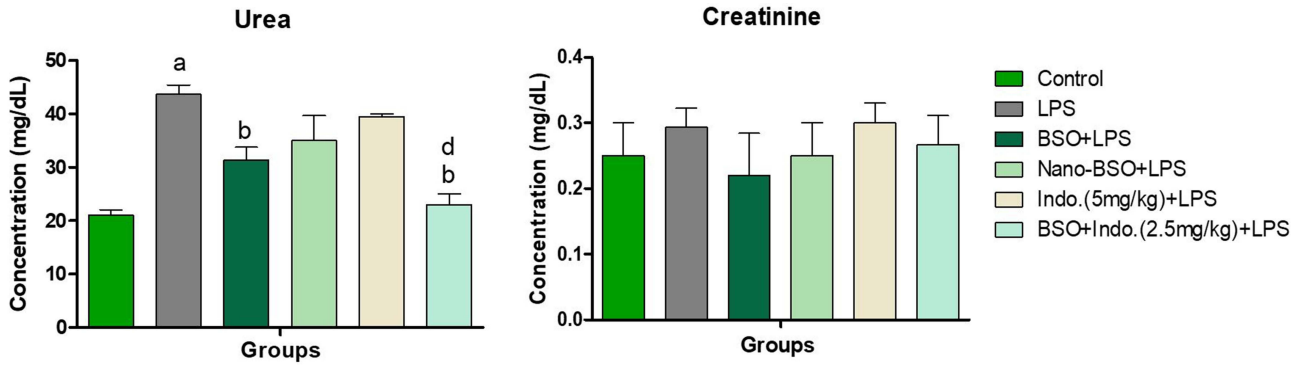

Figure 5 Serum creatinine and urea levels. The values are presented as mean ± SD; a indicates significant differences compared with control group, b indicates significant differences compared with LPS group, c indicates significant differences compared with BSO + LPS group, d indicates significant differences compared with Indo. (5 mg/kg) + LPS group. Results were regarded statistically significant at $p < 0.05$.
Abbreviations: BSO, black seed oil; LPS, lipopolysaccharide; Indo., indomethacin.

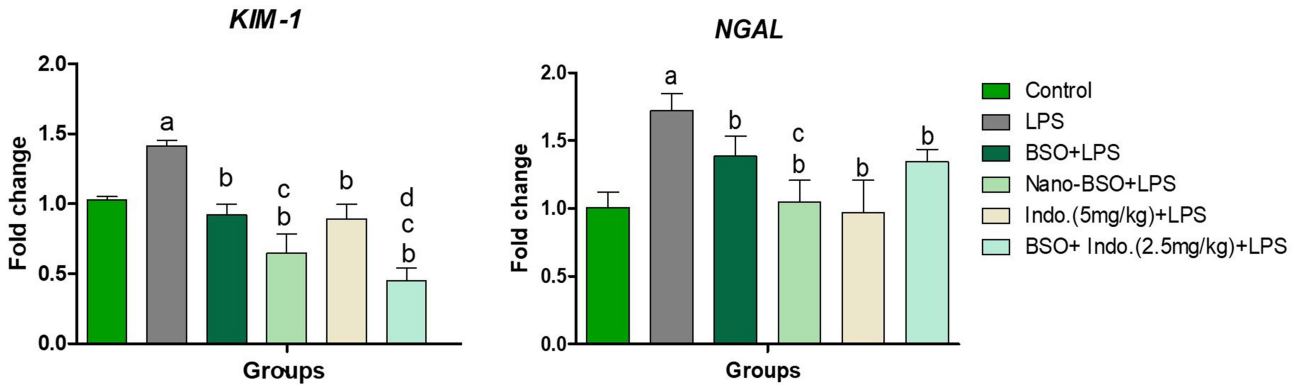

Figure 6 Kidney injury molecule-1 (KIM-1) and Neutrophil gelatinase-associated lipocalin (NGAL) expression levels. The values are presented as mean fold change ± SD; a indicates significant differences compared with control group, b indicates significant differences compared with LPS group, c indicates significant differences compared with BSO + LPS group, d indicates significant differences compared with Indo. (5 mg/kg) + LPS group. Results were regarded statistically significant at $p < 0.05$.
Abbreviations: BSO, black seed oil; LPS, lipopolysaccharide; Indo., indomethacin.

(19.19%), but higher than that in the indomethacin high-dose (5 mg/kg) pretreatment (43.6%). Nano-BSO formulation significantly ($p < 0.05$) lowered the *NGAL* expression levels at 38.95% compared with mice pretreated with BSO (19.19%).

Renal Oxidative Stress Markers

A significant reduction in reduced glutathione (GSH) levels was observed in the LPS treated group (77%) compared to the control group. The groups pre-treated before LPS induction exhibited variable curing effects. Pretreatment with BSO alone or BSO followed by Indo. (2.5 mg/kg) showed higher GSH levels (46.93% and 113.64%, respectively) when compared to LPS group but without significance. In contrast, pretreatment with the nano-BSO formulation or indomethacin (5 mg/kg) resulted in significantly higher levels of GSH than LPS (104.35% and 217.47%, respectively) (Figure 7).

Nitric oxide (NO) levels were significantly ($p < 0.05$) higher in the LPS group (578.98%) compared to the control group. Mice pretreated with the nano-BSO form showed significantly ($p < 0.05$) lower NO levels (57.22%) than BSO-pretreated mice (39.61%). Pre-treatment with BSO followed by indomethacin at a low dose (2.5 mg/kg) lowered NO levels (79.93%), which was greater than the effect observed with BSO alone (39.61%). However, this effect was similar to that observed after pre-treatment with a higher dose of indomethacin (5 mg/kg) (Figure 7).

Inflammatory Markers in Kidney Tissue

Renal Cyclooxygenase-2 (COX-2) Levels

COX-2 levels in kidney tissues were significantly higher in the LPS group (509.07%) than in the control group. The pretreatments of mice in all groups followed by LPS induction markedly reduced the COX-2 levels. They were all significantly ($p < 0.05$) lower than those in the LPS treated group. However, pretreatment with BSO, nano-BSO, or BSO followed by indomethacin low dose (2.5 mg/kg) or indomethacin higher dose (5 mg/kg) lowered COX-2 at variable levels but with no significant differences among them (Figure 8).

Tumor Necrosis Factor Alpha (TNF-α) and Toll Like Receptor-4 (TLR-4) Expression

TNF-α and TLR-4 expression levels were significantly higher in the LPS treated group (404% and 717.17%, respectively) than in the control group. Both genes were significantly downregulated in all pretreated mice groups prior to LPS induction compared to those in the LPS group. Notably, pretreatment of mice with nano-BSO significantly decreased *TNF-α* expression compared with BSO pretreatment, whereas for *TLR-4* expression, there were no significant differences between the two groups. In the remaining groups, *TNF-α* and *TLR-4* were down regulated at similar levels (Figure 8).

Histopathological Examination

Microscopic examination of various sections of kidneys of control mice (Figure 9A) BSO control mice (Figure 9B), and nano-BSO mice (Figure 9C) showed normal histological structure of the renal glomeruli and renal tubules. In contrast, there were marked histological alterations in the kidneys of LPS-treated mice as they showed multiple variable size foci

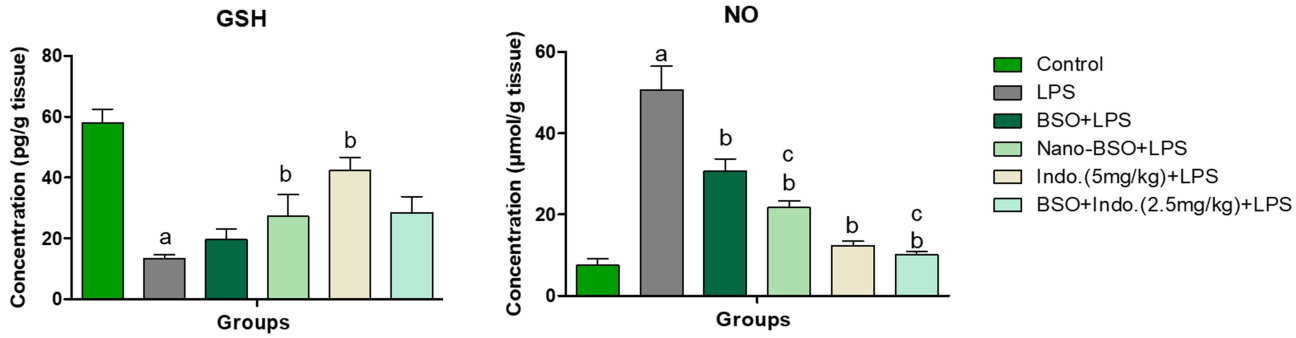

Figure 7 Reduced glutathione (GSH), nitric oxide (NO) levels in kidney tissues. The values are presented as mean ± SD; a indicates significant differences compared with control group, b indicates significant differences compared with LPS group, c indicates significant differences compared with BSO + LPS group, d indicates significant differences compared with Indo. (5 mg/kg) + LPS group. Results were regarded statistically significant at $p < 0.05$.
Abbreviations: BSO, black seed oil; LPS, lipopolysaccharide; Indo., indomethacin.

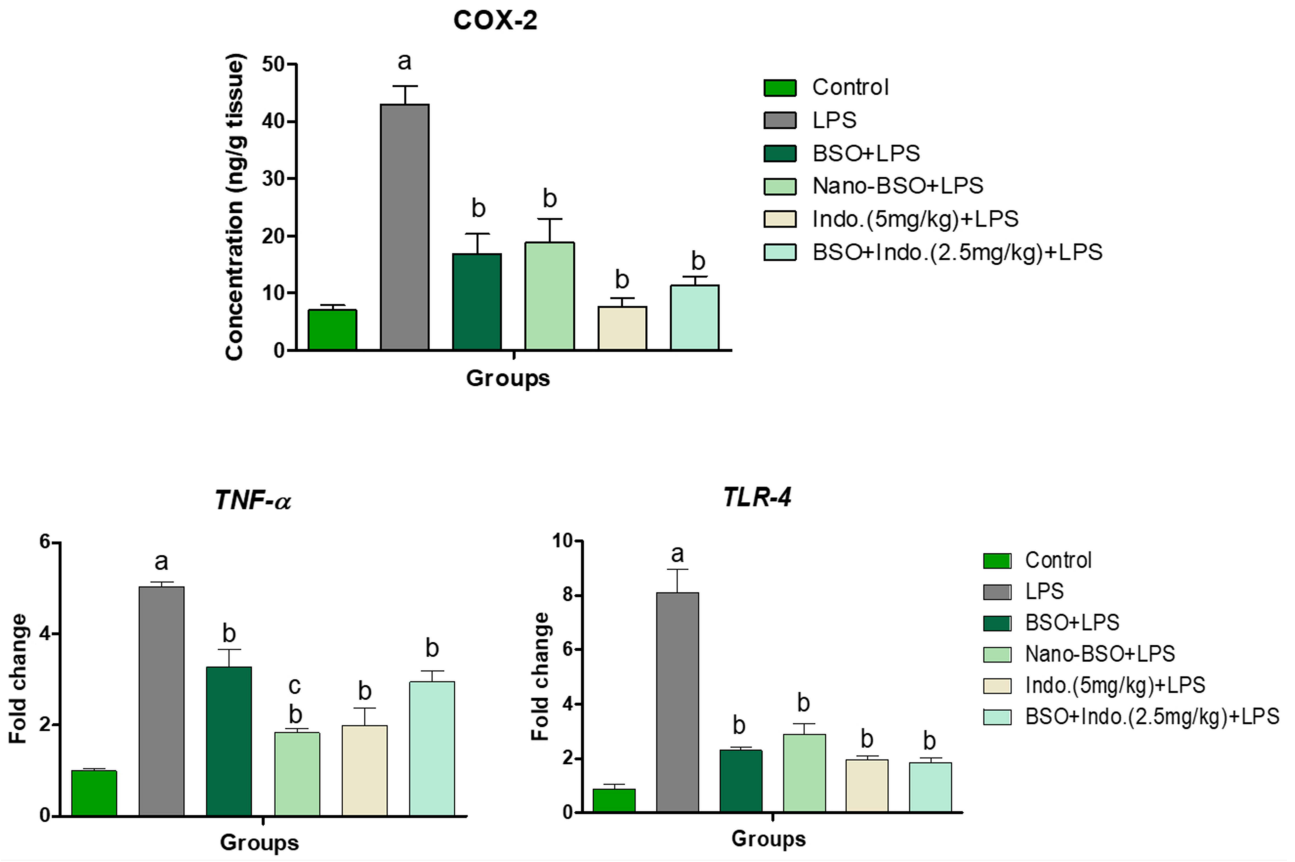

Figure 8 Renal cyclooxygenase-2 (COX-2) levels and tumor necrosis factor alpha (*TNF-α*), toll like receptor-4 (*TLR-4*) gene expression. The values are presented as mean ± SD; a indicates significant differences compared with control group, b indicates significant differences compared with LPS group, c indicates significant differences compared with BSO + LPS group, d indicates significant differences compared with Indo. (5 mg/kg) + LPS group. Results were regarded statistically significant at $p < 0.05$.
Abbreviations: BSO, black seed oil; LPS, lipopolysaccharide; Indo., indomethacin.

of inter-tubular hemorrhages, diffuse eosinophilia of large areas of the renal tubules, with diffuse vacuolar degeneration, necrosis of the tubular linings with the presence of eosinophilic cast formation in the lumen of some tubules (Figure 9D).

Regarding the pretreated groups prior to induction by LPS, the examination of kidneys of mice pretreated with BSO showed mild to moderate degree of vacuolar degeneration, mild swelling of some parietal cells of the Bowman's capsule, single cell necrosis and scarce cast formation (Figure 9E). On the other hand, the kidneys of mice pretreated with nano-BSO showed near to normal appearance of the kidney tissue with higher protection against the action of LPS. Rare casts with a mild degree of degeneration of the tubular linings and scarce necrosis were noticed (Figure 9F). The kidneys of mice pretreated with indomethacin (5 mg/kg) showed good protection of the renal tissue, and only a few tubules showed focal vacuolar degeneration of their lining epithelium with few necrotic cells (Figure 9G). Finally, pretreatment of mice with BSO followed by a low indomethacin dose (2.5 mg/kg) prior to LPS induction showed good protection of the renal tubular epithelium against the harmful effects of LPS (Figure 9H).

Discussion

LPS triggers an overwhelming inflammatory reaction that ends up with potential organ dysfunction. Acute kidney injury is a common complication of sepsis, where the kidneys are susceptible to inflammation and thus consequent endothelial dysfunction.[44] NSAIDs are a class of drugs applied for the treatment of fever, pain, and additional inflammatory processes[45] via inhibition of the cyclooxygenase (COX) enzyme. This inhibition prevents the conversion of arachidonic acid into prostacyclins, thromboxanes, and prostaglandins.[46] However, non-selective NSAIDs have reported adverse effects influencing the gastric mucosa, renal and cardiovascular systems.[47]

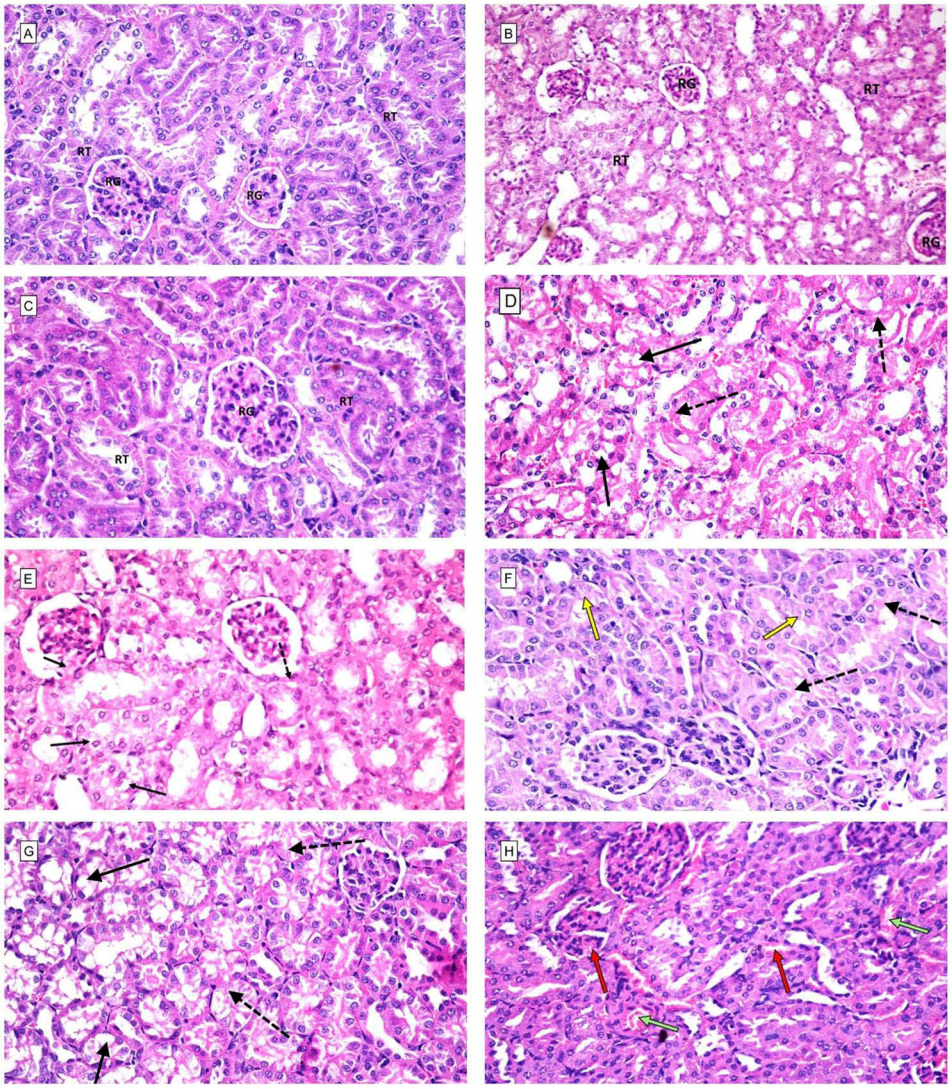

Figure 9 Histopathological examination of nephrotic tissue in all groups (H&Ex400). Control group (**A**), control BSO group (**B**), control nano-BSO group (**C**) showed normal histological structure of real glomeruli (RG) and renal tubules (RT). LPS group (**D**) showed diffuse vacuolar degeneration (arrow) and necrosis (dashed arrow) of the tubular linings. BSO+ LPS group (**E**) showed mild vacuolation (arrow) and single cells necrosis with mild swelling of some parietal cells (dashed arrow) of the Bowman's capsule and scarce cast (short arrow) formation. Nano-BSO+ LPS group (**F**) showed a mild degree of tubular linings' degeneration (yellow arrow) and scarce necrosis (dashed arrow). Indo. (5 mg/kg) + LPS group (**G**) showed vacuolar degeneration (arrow) of some renal tubular epithelial linings (arrow) with few necrotic cells (dashed arrow). (BSO+ Indo. (2.5 mg/kg) + LPS) group (**H**) showed congestion of few inter-tubular vessels (green arrow) and few inter-tubular pockets of hemorrhage (red arrow).

There is an increasing interest in the use of natural products as medicinal or protective supplements against inflammatory conditions, as many of them showed anti-inflammatory properties with limited side effects when compared to synthetic drugs.

Hence, the objective of this study was to investigate the possible protective effects of using black seed oil and its nano-formulation on LPS-induced acute renal toxicity. The BSO nano-formulation was prepared and studied to improve its therapeutic effect for better management of inflammatory disorders. The activity of both forms was compared with that of the anti-inflammatory reference drug indomethacin (5 mg/kg). The effect of combining the oil with indomethacin at a low dose (2.5 mg/kg) was also studied in an attempt to use the oil as adjuvant therapy to reduce the side effects associated with this non-selective COX inhibitor.

In the current work, the major active constituents of black seed oil were analyzed using gas chromatography. Our results showed that black seed fixed oil contained different fatty acids, of which (linoleic and oleic acids) accounted for the largest proportion of unsaturated FAs, whereas (palmitic and stearic acids) for the largest proportion of saturated FAs.

These fatty acids offer a wide range of health benefits, including supporting cellular health, promoting heart health, reducing body fat, and being involved in the lipid composition of cellular membranes.[48,49] Thymoquinone is a promising bioactive compound of BSO and its dominant hallmark owing to its numerous pharmacological features and health benefits.[50] Our results revealed that thymoquinone was the predominant compound in our black seed essential oil, and the protective effect observed in our study can contribute to this.[51]

Considering kidney function assessment, LPS has been recorded to disturb urea and creatinine levels in many studies.[52,53] However, our findings were in agreement with the earlier study[54] who showed that creatinine levels were not significantly changed between groups after 6 hours of LPS induction despite the elevation in urea levels. A possible reason is that serum creatinine is not detectable until renal function has been severely impaired, which makes the early detection of AKI more difficult.[55,56] Blood urea and serum creatinine are not sensitive enough to spot early or minimal damage in animal experiments,[57] which are directly proportional to the LPS dose.[58] Serum urea and creatinine levels are used as a reflection of filtration capacity rather than injury markers.[59]

Kidney injury was evaluated by measuring KIM-1 and NGAL levels. KIM-1 is a type I cell membrane glycoprotein that is primarily expressed in the proximal tubule cells of the kidney in response to injury, ischemia, toxins, or inflammation. In healthy kidneys, there is no evidence of KIM-1 gene or protein expression.[59] NGAL is a 25kD protein that belongs to the lipocalin superfamily. NGAL is expressed at low concentrations in several tissues. However, its levels are markedly raised after ischemic or nephrotoxic injury within 2 hours.[60] Our results showed a marked upregulation of *KIM-1* and *NGAL* by LPS, which agrees with previous studies.[52,61,62] The pretreatment of mice with BSO, nano-BSO, or indomethacin prior to LPS induction significantly reduced the expression of *KIM-1* and *NGAL*, indicating the protective effects of BSO and BSO nano-formulation suggesting that they can be used as prophylactic agents against tubular injury.

Oxidative stress occurs when there is a disturbance between the generated oxidants and the body's natural antioxidant defenses in the oxidants' favor. Reactive oxygen species (ROS) and other oxidants can result in tissue damage by oxidizing lipids, proteins, and DNA. Toxic oxidative products cause cytostatic effects, membrane damage, and thus cell death.[63] Previous studies have showed the effectiveness of using antioxidant supplements (such as herbal derivatives and vitamins) to attenuate the oxidative stress caused by toxins and drugs as cisplatin,[64] methotrexate,[65] acrylamide.[66]

The pathophysiology of LPS-induced nephrotoxicity has been linked to excessive generation of reactive oxygen species.[67] The two enzymes, inducible NO synthase (iNOS) and NADPH oxidase-4 (NOX-4) become active once LPS stresses the renal cells. Their overexpression results in primary redox state by producing intra-cytosolic superoxide anion ($O2^{\cdot}$-) radical and nitric oxide ($NO^{\cdot}$) radical, respectively.[12]

Excessive NO production alters the hemodynamic stability and the glomerular filtration rate.[68] Inducible NOS inhibition might mitigate kidney damage by reducing NO's harmful impact even without affecting intrarenal hemodynamics.[69] In our work, NO was significantly increased by LPS and noticeably inhibited by BSO and its nano-formulation in addition to the BSO and indomethacin low-dose treatment.

Glutathione, a powerful antioxidant thiol that alleviates oxidative damage, has been reported to be suppressed by LPS, which is associated with increased production of inflammatory cytokines and oxidative stress.[70–72] In our study, the nano-formulation of BSO significantly elevated the GSH levels.

Among the types of toll-like receptors, TLR-4 is the most well characterized in AKI.[73] TLR-4 expression in kidney cells is dynamic, with a low level at rest that rises in response to LPS.[44] The activation of TLR-4 by LPS ends up with the increased expression of pro-inflammatory mediators and cytokines such as TNF-α and the inducible COX-2 enzyme.[9] In our study, COX-2 levels, *TLR-4*, and *TNF-α* expression levels were elevated in response to LPS.

COX-2 is a key limiting enzyme in prostaglandin production that increases the levels of inflammatory cytokines and chemotaxis of immune cells. Under pathological conditions, COX-2 inhibition has been shown to ameliorate ischemic AKI induced in mice.[74,75] As a non-selective COX inhibitor, indomethacin pretreatment significantly showed a great inhibition of induced COX-2. Indomethacin is not frequently used as an analgesic or antipyretic because of its side effects, which are linked to either long-term use or high doses.[76] COX-1 primarily preserves the normal physiological activity of the kidney. Therefore, non-selective NSAIDs' suppression of COX-1 may cause ischemic renal damage.[77] BSO and its nano-formulation significantly inhibited COX-2 expression. Moreover, a low dose of indomethacin combined with BSO displayed a close effect to the indomethacin (5 mg/kg) group.

TNF-α is crucial in mediating kidney damage induced by LPS.[78] LPS causes extensive damage to the glomerulus by disrupting the glomerular endothelium surface layer and disrupting the density and diameter of fenestrae. This was suggested to be mediated by TNF-α through TNF receptor-1 on glomerular endothelial cells.[78] TNF-α has a potential role in raising the expression of IL-1 and IL-6 and encouraging the production of chemokines and endothelial adhesion molecules, which drive inflammatory leukocytes to areas of tissue damage.[79] In the present study, BSO and the nano-formulation significantly downregulated the TNF-α expression.

To the best of our knowledge, no studies have examined the enhancing activity of a combination of BSO and indomethacin. Nevertheless, a recent study designed nanoparticles that encapsulated both black seed essential oil and indomethacin to enhance indomethacin's anti-inflammatory and analgesic activity. However, it was only concerned with the characterization and design of the nanoparticles.[80] From the current study, it may be useful to use BSO and lower doses of indomethacin to reduce the possible side effects of indomethacin as ulcers and gastritis.[81] This combination exerted a protective effect similar to or better than that of indomethacin. The most observed effects were observed for serum urea, KIM-1, NO, and TLR-4.

In current research, we synthesized a black seed oil nano-emulsion and assessed its immunomodulatory activity. This may be the first study on this type of formulation in this model. We observed that the BSO nano-formulation had a positive effect as a nephroprotective agent, which was evident by altering the negative impact on KIM-1, NGAL, GSH, NO, and TNF-α levels. Although BSO nano-formulations have not been reported in previous studies, thymoquinone-nanoparticles have been reported to effectively ameliorate cisplatin-induced nephrotoxicity.[82] Also, nanoparticles loaded with doxorubicin and thymoquinone improved the anticancer activity and lowered nephrotoxicity.[83]

Conclusion

Pre-treatment with BSO and its nano-formulation alleviated LPS-induced AKI through their anti-oxidative and anti-inflammatory effects. The preservation of renal histology in treated groups further supports these findings. The nano-formulation offered an enhanced protective effect over the conventional form. Additionally, combining BSO with lower doses of indomethacin may serve as a protective anti-inflammatory supplement against NSAID side effects. These findings suggest that BSO and its nano-formulation could be promising immunomodulators in AKIs.

Abbreviations

AKI, acute kidney injury; BSO, black seed oil; LPS, lipopolysaccharide; TLR-4, toll-like receptor 4; BS, black seed; FAs, fatty acids; GSH, reduced glutathione; COX-2, cyclooxygenase-2; NO, nitric oxide; GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detector; EI, electron ionization; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; TNF-α, tumor necrosis factor alpha.

Institutional Review Board Statement

The animal study protocol was approved by the ethics committee of the Faculty of Pharmacy, Helwan University, Egypt (protocol code 02A2020 and approval date: 13/10/2020).

Funding

The authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia, for funding this research work through project number (223202).

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Kellum JA, Romagnani P, Ashuntantang G, Ronco C, Zarbock A, Anders HJ. Acute kidney injury. *Nat Rev Dis Primer*. 2021;7(1):1–17. doi:10.1038/s41572-021-00284-z

2. Wijayaratne D, Beligaswatta C, Harber M. Acute Kidney Injury Epidemiology and Causes. In: Harber M, editor. *Primer on Nephrology*. Springer International Publishing; 2022: 153–180.
3. McWilliam SJ, Wright RD, Welsh GI, et al. The complex interplay between kidney injury and inflammation. *Clin Kidney J*. 2020;14(3):780–788. doi:10.1093/ckj/sfaa164
4. He FF, Wang YM, Chen YY, Huang W, Li ZQ, Zhang CS-IAKI. From pathogenesis to therapeutic approaches. *Front Pharmacol*. 2022;13:981578. doi:10.3389/fphar.2022.981578
5. Rana A, Sathyanarayana P, Lieberthal W. Role of apoptosis of renal tubular cells in acute renal failure: therapeutic implications. *Apoptosis*. 2001;6 (1):83–102. doi:10.1023/A:1009680229931
6. Tomsa AM, Alexa AL, Junie ML, Rachisan AL, Ciumarnean L. Oxidative stress as a potential target in acute kidney injury. *PeerJ*. 2019;7:e8046. doi:10.7717/peerj.8046
7. Peerapornratana S, Manrique-Caballero CL, Gómez H, Kellum JA. Acute kidney injury from sepsis: current concepts, epidemiology, pathophysiology, prevention and treatment. *Kidney Int*. 2019;96(5):1083–1099. doi:10.1016/j.kint.2019.05.026
8. Chang YM, Chou YT, Kan WC, Shiao CC. Sepsis and acute kidney injury: a review focusing on the bidirectional interplay. *Int J Mol Sci*. 2022;23 (16):9159. doi:10.3390/ijms23169159
9. Pålsson-McDermott EM, O'Neill LAJ. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*. 2004;113 (2):153–162. doi:10.1111/j.1365-2567.2004.01976.x
10. Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine*. 2008;42(2):145–151. doi:10.1016/j.cyto.2008.01.006
11. DeLeo FR, Renee J, McCormick S, et al. Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J Clin Invest*. 1998;101(2):455–463. doi:10.1172/JCI949
12. Quoilin C, Mouithys-Mickalad A, Lécart S, Fontaine-Aupart MP. Evidence of oxidative stress and mitochondrial respiratory chain dysfunction in an in vitro model of sepsis-induced kidney injury. *Biochim Biophys Acta BBA*. 2014;1837(10):1790–1800. doi:10.1016/j.bbabio.2014.07.005
13. Ansary J, Giampieri F, Forbes-Hernandez TY, et al. Nutritional value and preventive role of nigella sativa L. and its main component thymoquinone in cancer: an evidenced-based review of preclinical and clinical studies. *Molecules*. 2021;26(8):2108. doi:10.3390/molecules26082108
14. Amin B, Hosseinzadeh H. Black Cumin (Nigella sativa) and its active constituent, thymoquinone: an overview on the analgesic and anti-inflammatory effects. *Planta Med*. 2015;82:8–16. doi:10.1055/s-0035-1557838
15. Dalli M, Eddine AS, Kandsi F, Gseyra N. Evaluation of the in vitro antioxidant activity of different extracts of Nigella sativa L. seeds, and the quantification of their bioactive compounds. *Mater Today Proc*. 2021;45:7259–7263. doi:10.1016/j.matpr.2020.12.743
16. Perera W, Liyanage J, Dissanayake K, Chandrasiri W, Gunathilaka H. A review on pharmacological activities and anti-microbial properties of Nigella sativa and isolated Thymoquinone. *J Med Plants Stud*. 2021;9(3):118–122. doi:10.22271/plants.2021.v9.i3b.1277
17. Al-Ghamdi MS. The anti-inflammatory, analgesic and antipyretic activity of Nigella sativa. *J Ethnopharmacol*. 2001;76(1):45–48. doi:10.1016/S0378-8741(01)00216-1
18. Mohammad MA, Mohammad MM, Dradka H. Effects of black seeds (Nigella Sativa) on spermatogenesis and fertility of male albino rats. *Res J Med Med Sci*. 2009;4.
19. Khazdair MR. The protective effects of *nigella sativa* and its constituents on induced neurotoxicity. *J Toxicol*. 2015;2015:841823. doi:10.1155/2015/841823
20. Mollazadeh H, Hosseinzadeh H. The protective effect of Nigella sativa against liver injury: a review. *Iran J Basic Med Sci*. 2014;17(12):958–966.
21. Hosseinian S, Khajavi Rad A, Hadjzadeh MAR, Mohamadian Roshan N, Havakhah S, Shafiee S. The protective effect of Nigella sativa against cisplatin-induced nephrotoxicity in rats. *Avicenna J Phytomed*. 2016;6(1):44–54.
22. Farhan N, Salih N, Salimon J. Physiochemical properties of Saudi Nigella sativa L. ('Black cumin') seed oil. *OCL*. 2021;28:11. doi:10.1051/ocl/2020075
23. Dinagaran S, Sridhar S, Eganathan P. Chemical composition and antioxidant activities of black seed oil (Nigella sativa L.). *Int J Pharm Sci Res*. 2016;7:4473–4479. doi:10.13040/IJPSR.0975-8232.7(11).4473-79
24. Behairy A, Elkomy A, Elsayed F, Gaballa MMS, Soliman A, Aboubakr M. Antioxidant and anti-inflammatory potential of spirulina and thymoquinone mitigate the methotrexate-induced neurotoxicity. *Naunyn Schmiedebergs Arch Pharmacol*. 2024;397(3):1875–1888. doi:10.1007/s00210-023-02739-4
25. Behairy A, Elkomy A, Elsayed F, Gaballa MMS, Soliman A, Aboubakr M. Spirulina and thymoquinone protect against methotrexate-induced hepatic injury in rats. *Rev Bras Farmacogn*. 2024;34(1):154–167. doi:10.1007/s43450-023-00470-y
26. Abdo W, Elmadawy MA, Abdelhiee EY, et al. Protective effect of thymoquinone against lung intoxication induced by malathion inhalation. *Sci Rep*. 2021;11:2498. doi:10.1038/s41598-021-82083-w
27. Dawaba AM, Dawaba HM. Application of optimization technique to develop nano-based carrier of nigella sativa essential oil: characterization and assessment. *Recent Pat Drug Deliv Formul*. 2020;13(3):228–240. doi:10.2174/1872211313666190516095309
28. Jerin IA, Begum N. Effect of Nigella Sativa (Kalozira) oil on pain in long Evans rats. *J Bangladesh Soc Physiol*. 2018;13(1):1–7. doi:10.3329/jbsp.v13i1.37840
29. Gumus ZP, Guler E, Demir B, et al. Herbal infusions of black seed and wheat germ oil: their chemical profiles, in vitro bio-investigations and effective formulations as phyto-nanoemulsions. *Colloids Surf B Biointerfaces*. 2015;133:73–80. doi:10.1016/j.colsurfb.2015.05.044
30. Shahroudi MJ, Mehri S, Hosseinzadeh H. Anti-aging effect of nigella sativa fixed oil on D-galactose-induced aging in mice. *J Pharmacopuncture*. 2017;20(1):29–35. doi:10.3831/KPI.2017.20.006
31. Nessem M, Esmat A, Azab S, Zaki E. The experimental effects of lipopolysaccharide-induced renal injury in mice model. *Arch Pharm Sci Ain Shams Univ*. 2021;5:46–51 doi:10.21608/APS.2021.30593.1049.
32. Boaru SG, Borkham-Kamphorst E, Tihaa L, Haas U, Weiskirchen R. Expression analysis of inflammasomes in experimental models of inflammatory and fibrotic liver disease. *J Inflamm*. 2012;9:1–16. doi:10.1186/1476-9255-9-49
33. Zhu SH, Zhou LJ, Jiang H, et al. Protective effect of indomethacin in renal ischemia-reperfusion injury in mice. *J Zhejiang Univ Sci B*. 2014;15 (8):735–742. doi:10.1631/jzus.B1300196
34. Bancroft JD, Gamble M. *Theory and Practice of Histological Techniques*. Elsevier Health Sciences; 2008.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods San Diego Calif*. 2001;25(4):402–408. doi:10.1006/meth.2001.1262

36. Alrashidi M, Derawi D, Salimon J, Yusoff MF. An investigation of physicochemical properties of Nigella sativa L. Seed oil from Al-Qassim by different extraction methods. *J King Saud Univ Sci*. 2020;32(8):3337–3342. doi:10.1016/j.jksus.2020.09.019
37. Piras A, Rosa A, Marongiu B, et al. Chemical composition and in vitro bioactivity of the volatile and fixed oils of Nigella sativa L. extracted by supercritical carbon dioxide. *Ind Crops Prod*. 2013;46:317–323. doi:10.1016/j.indcrop.2013.02.013
38. Sahafi SM, Goli SAH, Kadivar M, Varshosaz J, Shirvani A. Pomegranate seed oil nanoemulsion enriched by alpha-tocopherol; the effect of environmental stresses and long-term storage on its physicochemical properties and oxidation stability. *Food Chem*. 2021;345:128759. doi:10.1016/j.foodchem.2020.128759
39. Furedi P, Kovacs K, Ludanyi K, Antal I, Klebovich I. Development and characterization of voriconazole loaded nanoparticles for parenteral delivery. *Int J Pharm*. 2016;510(1):159–163. doi:10.1016/j.ijpharm.2016.06.027
40. Yang J, Zhou Y, Chen L. Elaboration and characterization of barley protein nanoparticles as an oral delivery system for lipophilic bioactive compounds. *Food Funct*. 2014;5(1):92–101. doi:10.1039/C3FO60351B
41. Sahu P, Das D, Mishra VK, Kashaw V, Kashaw SK. Nanoemulsion: a Novel Eon in Cancer Chemotherapy. *Mini Rev Med Chem*. 2017;17(18):1778–1792. doi:10.2174/1389557516666160219122755
42. Sari TP, Mann B, Kumar R, et al. Preparation and characterization of nanoemulsion encapsulating curcumin. *Food Hydrocoll*. 2015;43:540–546. doi:10.1016/j.foodhyd.2014.07.011
43. Bhattacharjee S. DLS and zeta potential - What they are and what they are not? *J Control Release*. 2016;235:337–351. doi:10.1016/j.jconrel.2016.06.017
44. Anderberg SB, Luther T, Frithiof R. Physiological aspects of Toll-like receptor 4 activation in sepsis-induced acute kidney injury. *Acta Physiol*. 2017;219(3):575–590. doi:10.1111/apha.12798
45. Phillips WJ, Currier BL. Analgesic pharmacology: II. Specific analgesics. *J Am Acad Orthop Surg*. 2004;12(4):221–233. doi:10.5435/00124635-200407000-00003
46. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature New Biol*. 1971;231(25):232–235. doi:10.1038/newbio231232a0
47. Chaiamnuay S, Allison JJ, Curtis JR. Risks versus benefits of cyclooxygenase-2-selective nonsteroidal antiinflammatory drugs. *Am J Health-Syst Pharm AJHP off J Am Soc Health Syst Pharm*. 2006;63(19):1837–1851. doi:10.2146/ajhp050519
48. Farhan N, Salih N, Salimon J. A review on olive oil and Nigella sativa L. black cumin seed oil: composition and biological activity. *Rasayan J Chem*. 2021;14(04):2469–2477. doi:10.31788/RJC.2021.1446525
49. Jandacek RJ. Linoleic acid: a nutritional quandary. *Healthcare*. 2017;5(2):25. doi:10.3390/healthcare5020025
50. Darakhshan S, Bidmeshki Pour A, Hosseinzadeh Colagar A, Sisakhtnezhad S. Thymoquinone and its therapeutic potentials. *Pharmacol Res*. 2015;95-96:138–158. doi:10.1016/j.phrs.2015.03.011
51. Goyal SN, Prajapati CP, Gore PR, et al. Therapeutic potential and pharmaceutical development of thymoquinone: a multitargeted molecule of natural origin. *Front Pharmacol*. 2017;8:656. doi:10.3389/fphar.2017.00656
52. Ban KY, Nam GY, Kim D, Oh YS, Jun HS. Prevention of LPS-induced acute kidney injury in mice by bavachin and its potential mechanisms. *Antioxidants*. 2022;11(11):2096. doi:10.3390/antiox11112096
53. Chen L, Yang S, Zumbrun EE, Guan H, Nagarkatti PS, Nagarkatti M. Resveratrol attenuates lipopolysaccharide-induced acute kidney injury by suppressing inflammation driven by macrophages. *Mol Nutr Food Res*. 2015;59(5):853–864. doi:10.1002/mnfr.201400819
54. Zhang C, Walker LM, Mayeux PR. Role of nitric oxide in lipopolysaccharide-induced oxidant stress in the rat kidney. *Biochem Pharmacol*. 2000;59(2):203–209. doi:10.1016/S0006-2952(99)00324-X
55. Shang W, Wang Z. The update of NGAL in acute kidney injury. *Curr Protein Pept Sci*. 2017;18(12):1211–1217. doi:10.2174/1389203717666160909125004
56. Doi K, Yuen PST, Eisner C, et al. Reduced production of creatinine limits its use as marker of kidney injury in sepsis. *J Am Soc Nephrol*. 2009;20(6):1217. doi:10.1681/ASN.2008060617
57. Wang W, Li Z, Chen Y, Wu H, Zhang S, Chen X. Prediction value of serum NGAL in the diagnosis and prognosis of experimental acute and chronic kidney injuries. *Biomolecules*. 2020;10(7):981. doi:10.3390/biom10070981
58. Nessem M, Esmat A, Azab S, El-Demerdash Zaki E. The experimental effects of lipopolysaccharide-induced renal injury in mice mode. *Arch Pharm Sci Ain Shams Univ*. 2021;5(1):46–51.
59. Bonventre JV. Kidney injury molecule-1 (KIM-1): a urinary biomarker and much more. *Nephrol Dial Transplant*. 2009;24(11):3265–3268. doi:10.1093/ndt/gfp010
60. Corbacıoglu SK, Cevik Y, Akinci E, et al. Value of plasma neutrophil gelatinase-associated lipocalin (NGAL) in distinguishing between acute kidney injury (AKI) and chronic kidney disease (CKD). *Turk J Emerg Med*. 2017;17(3):85–88. doi:10.1016/j.tjem.2017.03.002
61. Tan F, Chen Y, Yuan D, Gong C, Li X, Zhou S. Dexmedetomidine protects against acute kidney injury through downregulating inflammatory reactions in endotoxemia rats. *Biomed Rep*. 2015;3(3):365–370. doi:10.3892/br.2015.427
62. Zhao Y, Feng X, Li B, et al. Dexmedetomidine protects against lipopolysaccharide-induced acute kidney injury by enhancing autophagy through inhibition of the PI3K/AKT/mTOR pathway. *Front Pharmacol*. 2020;11:128. doi:10.3389/fphar.2020.00128
63. Sies H. Oxidative stress: concept and some practical aspects. *Antioxidants*. 2020;9(9):852. doi:10.3390/antiox9090852
64. Soliman A, Elsayed A, Elkomy A, et al. Ameliorating effect of lycopene and N-acetylcysteine against cisplatin-induced cardiac injury in rats. *Pak Vet J*. 2022;2022:2074–7764. doi:10.29261/pakvetj/2021.035
65. Aboubakr M, Elbadawy M, Ibrahim S, et al. Allicin and lycopene possesses a protective effect against methotrexate-induced testicular toxicity in rats. *Pak Vet J*. 2024;2024:1. doi:10.29261/pakvetj/2023.057
66. Aboubakr M, Elmahdy A, Taima S, et al. Protective effects of N acetylcysteine and vitamin e against acrylamide-induced neurotoxicity in rats. *Pak Vet J*. 2023;2023:2074–7764. doi:10.29261/pakvetj/2023.027
67. Plotnikov EY, Pevzner IB, Zorova LD, et al. Mitochondrial damage and mitochondria-targeted antioxidant protection in LPS-induced acute kidney injury. *Antioxidants*. 2019;8(6):176. doi:10.3390/antiox8060176
68. Oliveira FRMB, Assreuy J, Sordi R. The role of nitric oxide in sepsis-associated kidney injury. *Biosci Rep*. 2022;42(7):BSR20220093. doi:10.1042/BSR20220093

69. Millar CGM, Thiemermann C. Intrarenal haemodynamics and renal dysfunction in endotoxaemia: effects of nitric oxide synthase inhibition. *Br J Pharmacol*. 1997;121(8):1824–1830. doi:10.1038/sj.bjp.0701335
70. Mir SM, Ravuri HG, Pradhan RK, et al. Ferulic acid protects lipopolysaccharide-induced acute kidney injury by suppressing inflammatory events and upregulating antioxidant defenses in Balb/c mice. *Biomed Pharmacother*. 2018;100:304–315. doi:10.1016/j.biopha.2018.01.169
71. Islam MS, Miao L, Yu H, Han Z, Sun H. Ethanol extract of illicium henryi attenuates LPS-induced acute kidney injury in mice via regulating inflammation and oxidative stress. *Nutrients*. 2019;11(6):1412. doi:10.3390/nu11061412
72. Raza H, John A, Shafarin J. Potentiation of LPS-induced apoptotic cell death in human hepatoma HepG2 Cells by Aspirin via ROS and mitochondrial dysfunction: protection by N-acetyl cysteine. *PLoS One*. 2016;11(7):e0159750. doi:10.1371/journal.pone.0159750
73. Vázquez-Carballo C, Guerrero-Hue M, García-Caballero C, et al. Toll-like receptors in acute kidney injury. *Int J Mol Sci*. 2021;22(2):816. doi:10.3390/ijms22020816
74. Ranganathan PV, Jayakumar C, Mohamed R, Dong Z, Ramesh G. Netrin-1 regulates the inflammatory response of neutrophils and macrophages, and suppresses ischemic acute kidney injury by inhibiting COX-2-mediated PGE2 production. *Kidney Int*. 2013;83(6):1087–1098. doi:10.1038/ki.2012.423
75. Nørregaard R, Kwon TH, Frøkiær J. Physiology and pathophysiology of cyclooxygenase-2 and prostaglandin E2 in the kidney. *Kidney Res Clin Pract*. 2015;34(4):194–200. doi:10.1016/j.krcp.2015.10.004
76. Taiwo V, Conteh O. The rodenticidal effect of indomethacin: pathogenesis and pathology. *Vet Arh*. 2008;78(2):167.
77. Hörl WH. Nonsteroidal anti-inflammatory drugs and the kidney. *Pharmaceuticals*. 2010;3(7):2291–2321. doi:10.3390/ph3072291
78. Xu C, Chang A, Hack BK, Eadon MT, Alper SL, Cunningham PN. TNF-mediated damage to glomerular endothelium is an important determinant of acute kidney injury in sepsis. *Kidney Int*. 2014;85(1):72–81. doi:10.1038/ki.2013.286
79. Vielhauer V, Mayadas TN. Functions of TNF and its receptors in renal disease: distinct roles in inflammatory tissue injury and immune regulation. *Semin Nephrol*. 2007;27(3):286–308. doi:10.1016/j.semnephrol.2007.02.004
80. Badri W, El Asbahani A, Miladi K, et al. Poly (ε-caprolactone) nanoparticles loaded with indomethacin and Nigella Sativa L. essential oil for the topical treatment of inflammation. *J Drug Deliv Sci Technol*. 2018;46:234–242. doi:10.1016/j.jddst.2018.05.022
81. Lucas S. The Pharmacology of Indomethacin. *Headache J Head Face Pain*. 2016;56(2):436–446. doi:10.1111/head.12769
82. Harakeh S, Qari Y, Tashkandi H, et al. Thymoquinone nanoparticles protect against cisplatin-induced nephrotoxicity in Ehrlich carcinoma model without compromising cisplatin anti-cancer efficacy. *J King Saud Univ Sci*. 2022;34(1):101675. doi:10.1016/j.jksus.2021.101675
83. Zidan AAA, El-Ashmawy NE, Khedr EG, Ebeid EZM, Salem ML, Mosalam EM. Loading of doxorubicin and thymoquinone with F2 gel nanofibers improves the antitumor activity and ameliorates doxorubicin-associated nephrotoxicity. *Life Sci*. 2018;207:461–470. doi:10.1016/j.lfs.2018.06.008