



## Original Article

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## Anti-inflammatory potential of black cumin seed oil and its nanoemulsion formulation against lipopolysaccharide-induced liver injury in mice

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## ABSTRACT

**Objective:** To evaluate the anti-inflammatory effect of black cumin seed oil (BCSO) and its nanoemulsion on lipopolysaccharide (LPS)-induced liver injury in mice.

**Methods:** LPS-induced acute liver injury mouse model was used to evaluate the effects of BCSO and its nanoemulsion formulation on liver function. Hepatic inflammatory markers including Toll-like receptor 4 (TLR4), interleukin(IL)-1 $\beta$ , heme-oxygenase 1, BAX, and BCL-2 were assessed using real-time PCR. Additionally, protein levels of reduced glutathione, tumor necrosis factor- $\alpha$ , and IL-6 were measured using ELISA, and histological analysis was performed. Indomethacin was used as a standard positive control for comparison.

**Results:** BCSO reduced LPS-induced liver injury and exhibited strong anti-inflammatory effects by downregulating the expression of TLR4, IL-1 $\beta$ , IL-6, tumor necrosis factor- $\alpha$ , and heme-oxygenase 1. Additionally, BCSO demonstrated antioxidant properties by increasing reduced glutathione protein levels and decreasing key apoptotic markers BAX and BCL-2 in hepatocytes. The nanoemulsion formulation further enhanced these anti-inflammatory, antioxidant, and anti-apoptotic effects, and histological examination confirmed this effect. Combining BCSO with indomethacin at a lower dose improved efficacy, thereby reducing its potential side effects.

**Conclusions:** The investigation reveals the anti-inflammatory impact of BCSO and its nanoemulsion formulation on LPS-induced liver oxidative stress, inflammation, and apoptosis.

**KEYWORDS:** Black cumin seed oil; Liver inflammation; Lipopolysaccharide; Oxidative stress; IL-6; TLR4; Apoptosis

## 1. Introduction

Inflammation is the body's reaction to tissue damage caused by several factors, such as physical injury, insufficient blood flow to the organ, infections, and exposure to various toxins. Neutrophils are the first inflammatory cells attracted to the site of inflammation, followed by monocytes, which later undergo a differentiation process to become macrophages secreting cytokines. Several animal toxicity studies showed that inflammatory reactions are initiated by the production of interleukin-1 beta (IL-1 $\beta$ ), interleukin-6 (IL-6),

## Significance

We have previously reported that black cumin seed oil possesses many biological activities, including anti-inflammatory effects; however, its effects on induced liver injury have not been reported. Our study demonstrated that black cumin seed oil and its nanoemulsion formulation inhibited lipopolysaccharide-induced liver oxidative stress, inflammation, and apoptosis.

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cyclooxygenases (1 and 2) (COX-1, COX-2), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), causing systemic inflammation[1].

Liver inflammation is characterized by the invasion of inflammatory cells that produce cytokines, including IL-6 and TNF- $\alpha$ , and is caused by many aggravators, such as many drugs, alcohol consumption, viruses, pregnancy complications, and some metabolic diseases. Alcohol-induced acute liver damage causes liver inflammation more frequently in developed countries, whereas hepatitis A, B, and E are the most frequent causes of acute liver injury in developing countries. Acute liver failure can lead to several serious complications, including cardiovascular and pulmonary failure, neurological issues such as encephalopathy, and renal dysfunction[2].

Bacterial lipopolysaccharide (LPS) is a lipid and polysaccharide-containing molecules known as endotoxin that make up the majority of the outermost layer of Gram-negative bacteria. Several studies used LPS both experimentally and clinically to trigger systemic and hepatic inflammation. LPS induces inflammation through binding to the Toll-like receptors (TLRs) on Kupffer cells and encourages the release of pro-inflammatory cytokines, which are crucial factors in liver damage. Also, LPS activates TNF- $\alpha$  to interact with its cognate receptor, causing activation of the nuclear factor-kappa B pathway with the induction of inflammatory and anti-apoptotic genes. Hence, production of reactive oxygen species, inflammation, and apoptosis are the final complicated and pathophysiological changes that LPS results in the course of septic liver failure[3].

Indomethacin is one of the potent nonsteroidal anti-inflammatory drugs that is widely used in several inflammatory cases and a well-known positive standard control in many inflammation-induced models[4]. However, indomethacin has several serious hepatic and non-hepatic toxicities that limit its uses[5]. Therefore, a continuous need for safe anti-inflammatory new leads is required in drug development.

Black cumin seed oil (BCSO) is a natural herbal oil that has been widely used as a medicinal supplement for various health conditions due to its protective effect against renal and liver complications. Many investigations have shown that crude black seed or its active components have various biological activities, including anti-cancer, anti-oxytotic, anti-tussive, anti-pain, antipyretic, analgesic, and antioxidant activities[6]. BCSO has many active ingredients that have powerful anti-inflammatory properties and could inhibit inflammatory cytokine production, such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . Additionally, BCSO has a broad range of microbiological uses, including antimicrobial, anti-viral, anti-parasitic, and anti-fungal activity. Also, BCSO has been shown to alleviate the symptoms of different disorders, such as hypertension, dyslipidemia, diabetes, asthma, convulsions, and natural and chemical toxicities[6]. Many studies discussed that the anti-inflammatory characteristics of BCSO are attributed to inhibiting leukotriene, histamine production, superoxide scavenging, and immunomodulatory effects[7,8].

In addition, using nanotechnology may open new horizons for enhancing the bioavailability parameters of BCSO and so the efficacy and activity. Droplets containing drugs in nanoemulsion

have a diameter of between 10 and 200 nm, and emulsifier molecules surround each droplet to act as a barrier[9]. Therefore, nanoemulsions will provide a lot of advantages, such as possessing a higher solubilization capacity, greater kinetic stability, enhancing long-term stability by protecting the droplets from harsh environmental conditions, and drug targeting[10,11]. Therefore, the present study aims to evaluate and compare the anti-inflammatory effect of BCSO and its nanoemulsion formulation on LPS-induced inflammation in mice's liver. Additionally, the combinatorial effect of indomethacin with BCSO will be evaluated to investigate the synergetic effect and to minimize side effects.

## 2. Materials and methods

### 2.1. Animals and ethical statement

Forty-two Swiss albino male mice 5 weeks old weighing 25 g were used. Experiments were performed in a pathogen-free area. The care and handling of the animals were carried out according to standard guidelines and under Animal Research: Reporting of *In Vivo* Experiments guidelines. All procedures are approved by the Scientific Research Ethics Committee in Faculty of Pharmacy at Helwan University (Ethical committee approval number: 01A2020).

All animals were maintained for a week for acclimatization and kept at 24°C, 12 hours of light and 12 hours of darkness. Several doses of LPS were evaluated (2, 2.5, and 5 mg/kg) at different time intervals (4–24 h), and 2 mg/kg for 6 h was selected for induction of acute inflammation with minimal animal mortality based on our previous study[12]. We conducted a pilot study on 42 mice receiving different doses of BCSO, tween 20, indomethacin, and LPS. Results showed tween 20 treated animals had minimal toxicity without change in animal behavior, survival rate, or biological markers compared to the untreated group, suggesting its suitability as a solvent for BCSO. Also, a pilot study showed that optimum doses of BCSO (0.2 mL/kg/day)[13] or BCSO nanoemulsion treatment (1 mL/kg/day) for 14 d before LPS-induction has no animal toxicity with stable animal behavior, feeding, and survival rate. Finally, a pilot study showed indomethacin at a dose of 5 mg/kg/day[14] for 3 d prior to LPS induction at 10:00–11:00 a.m. has minimal animal toxicities. The dose of indomethacin in combination with BCSO was reduced to 2.5 mg/kg/day.

### 2.2. Nanoemulsion formulation of BCSO

#### 2.2.1. Preparation of nanoemulsion formulation of BCSO

The BCSO nanoformulation was prepared as an oil-in-water nanoemulsion following literature[15]. Briefly, the oily phase, which constituted 20% of the nanoemulsion, was composed of BCSO with 3 wt% lecithin dissolved. The aqueous phase made up 80% of the formulation and contained 3 wt% Tween 80. The two phases were combined and sonicated for 10 min using a probe sonicator (Vibra Cell, Sonics, USA), with sonication occurring for 30 s followed by

a 30-second pause each minute. The process was carried out at 50% amplitude in an ice bath. The resulting nanoemulsion was stored in a refrigerator at 4 °C for further use.

### 2.2.2. Characterization of BCSO nanoemulsion

The droplet size, the polydispersity index (PDI), and the surface charge expressed by Zeta potential (ZP) were measured using the dynamic light scattering technique by Malvern Zetasizer (nano ZS, Zetasizer, Malvern Instruments Ltd., UK).

### 2.3. Animal groups

The mice were divided randomly into seven groups: 1) The negative control group received tween 20 (solvent for BCSO) for 2 weeks (1 mL/kg/day) and represented the negative control (C group); 2) The positive control group received BCSO (0.2 mL/kg/day) dissolved in tween 20 for 2 weeks; 3) The LPS-induced group (LPS group) received tween 20 (1 mL/kg/day) for 2 weeks followed by LPS (2 mg/kg for 6 h); 4) LPS-induced group pre-treated with BCSO (LO group) received BCSO dissolved in tween 20 (0.2 mL/kg/day) for 2 weeks followed by LPS (2 mg/kg for 6 h); 5) LPS-induced group pre-treated with BCSO nanoemulsion (NL group) received BCSO nanoemulsion (1 mL/kg/day) for 2 weeks followed by LPS (2 mg/kg for 6 h); 6) LPS-induced group pre-treated with indomethacin received tween 20 (1 mL/kg/day) for 11 d followed by indomethacin (5 mg/kg) for 3 d followed by LPS (2 mg/kg for 6 h) and 7) LPS-induced group pre-treated with BCSO (0.2 mL/kg/day) dissolved in tween 20 for 11 d followed by reduced dose of indomethacin (2.5 mg/kg) for 3 d followed by LPS (2 mg/kg for 6 h).

### 2.4. Sample collection

At the end of the experiment, after 6 hours of LPS injection, animals were anesthetized by an intraperitoneal injection of ketamine-xylazine, and peripheral blood was collected through medial canthus of eyeballs with a fine capillary tube. Animals were killed by cervical dislocation. The liver was then harvested. The livers were dissected into 2 portions. One was kept in 10% buffered neutral formalin for histopathological examination and the other portion was rinsed in cold saline, and rapidly kept at -80 °C for further biochemical investigations.

### 2.5. GC-MS analysis of BCSO oil extract

BCSO was purchased from the Wilko™ company for natural products as 100% pure oil. The GC-MS system (Agilent Technologies) is equipped with a gas chromatography system (7890B) and a mass spectrometer detector (5977A) and carried out at a suitable institute. Sample was dissolved in chloroform and then injected into a DB-624 column (30 m × 320 µm internal diameter and 1.8 µm film thickness). Analysis was carried out using helium as the carrier gas at a flow rate of 3.0 mL/min at a split 1:20 of injection volume of 1 µL and the following temperature program: 40 °C for 1

min; rising at 7 °C/min to 250 °C and held for 5 min.

The injector and detector were held at 250 °C. Mass spectra were obtained by electron ionization at 70 eV using a spectral range of  $m/z$  30-440 and a solvent delay of 6 min. Identification of different constituents was performed by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

### 2.6. Analysis of liver function

Serum aspartate transaminase (AST) and alkaline phosphatase (ALP) are sensitive enzyme markers for liver inflammation[16]. Serum AST and ALP concentrations were measured by colorimetry using BIODIAGNOSTIC™ Kits for diagnostic and research reagents, AST (Cat. No. AT1034) and ALP (Cat. No. AP1020).

### 2.7. Determination of TNF- $\alpha$ , IL-6, and glutathione (GSH) using ELISA kits

The weighed liver tissue sections of all groups were collected and washed in 0.1 mol/L phosphate-buffered saline, then tissue protein extraction reagent was added (5 mL/1 g tissue), mixed on ice, homogenized, and centrifuged for 10 min at 5000-10000 rpm. The supernatants were taken, and TNF- $\alpha$  (Cat. No. MBS026635), IL-6 (Cat. No. MBS2023471), and GSH (Cat. No. MBS026635) were determined using ELISA kits from My BioSource Company, USA, Science and Technology Centre, Egypt.

### 2.8. Real-time PCR analysis of key inflammatory and apoptosis-related mediators

Total RNA was extracted from liver tissues using ThermoScientific GeneJET RNA Purification Kit (Cat. No. #K0731) from Thermofisher Scientific Company, USA, Analysis for Life, Egypt. cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Cat. No. #K1622) from Thermofisher Scientific Company, USA, Analysis for Life, Egypt. HERA plus qPCR SYBR® Green master mix kit (Cat No. WF10306001) from Willow Fort Company, UK, Sigma Scientific Service, Egypt was used for real-time PCR reaction that was conducted in a MultiGene gradient thermal cycler from National Labnet Company, Woodbridge, NJ. The cycling condition was as follows: 95 °C for 10 min, then denaturing at 95 °C for 10 s, annealing temperature at 66 °C for 10 s, and extension at 72 °C for 20 s with 40 cycles.  $\beta$ -actin was used as the housekeeping gene.

For validation of the work, relative expression ( $\Delta Rn$ ) was displayed against the number of cycles in a typical amplification plot produced by a real-time PCR experiment, creating a sigmoid-shaped pattern for assessment of sensitivity (limit of quantification: LOQ 95%). The amount of starting cDNA template in each sample can be calculated using a calibration curve that was constructed using a reference gene with different starting concentrations of cDNA from all groups to determine the equivalent starting cDNA, which was determined as 9.4 ng/µL. Analysis of dissociation curves was used to confirm the identity

and specificity of PCR amplicon product without primer-dimer using its expected melting temperature ( $T_m$ ), where after the end of the PCR experiment, the temperature was gradually raised from 65 °C to 95 °C to conduct the dissociation melting curve study. Then the  $2^{-\Delta\Delta C_t}$  method was used to analyze the relative quantification of mRNA levels. The primers used are mentioned in Table 1.

## 2.9. Hematoxylin–eosin staining

The freshly preserved liver tissue samples in 10% buffered neutral formalin were immediately prepared into paraffin blocks, sectioned (4–5  $\mu$ m), and stained with hematoxylin-eosin for histological investigation[20].

## 2.10. Statistical analysis

A one-way ANOVA test was used to compare multiple independent groups using GraphPad Prism 7 software. Dunnett's multiple comparison test was used as *post hoc* test. Statistical data were presented as mean  $\pm$  standard error of mean (SEM) The plotting figures were drawn with GraphPad Prism 7 software. A *P*-value < 0.05 was considered statistically significant.

# 3. Results

## 3.1. BCSO GC–MS analysis

Table 2 shows 11 main active components according to percentage

**Table 1.** Primer sequence of hepatic inflammatory and apoptotic genes for real-time PCR assay.

| Gene name                       | Primer sequence                                                         | Gene ID | Amplicon length (bp) | Reference |
|---------------------------------|-------------------------------------------------------------------------|---------|----------------------|-----------|
| <i>TLR4</i>                     | F: 5'-TGGTGTCCCAGCACTTCATC-3'<br>R: 5'-GATACCAGCAGCACTGCTCA-3'          | 21898   | 92                   | [17]      |
| <i>IL-1<math>\beta</math></i>   | F: 5'-GAGCTGAAAGCTCTCCACCTC-3'<br>R: 5'-CTTTCCTTTGAGGCCCAAGGC-3'        | 16176   | 123                  | [18]      |
| <i>HMOX1</i>                    | F: 5'-GCGAAACAAGCAGAACCCA-3'<br>R: 5'-GCTCAGGATGAGTACCTCCCA-3'          | 15368   | 185                  | NCBI      |
| $\beta$ -actin (Reference gene) | F: 5'-CTCTAGACTTCGAGCAGGAGATGG-3'<br>R: 5'-ATGCCACAGGATTCCATACCCAAGA-3' | 11461   | 163                  | [18]      |
| <i>BAX</i>                      | F: 5'-ATCCAAGACCAGGGTGGCT-3'<br>R: 5'-CTTCCCCCATTCATCCAGG-3'            | 12028   | 197                  | [19]      |
| <i>BCL-2</i>                    | F: 5'-CTGAGTACCTGAACCGGCAT-3'<br>R: 5'-GGTATGCACCCAGAGTGATG-3'          | 12043   | 174                  | NCBI      |

**Table 2.** Chemical composition of black cumin seed oil analyzed by GC-MS.

| Peak | Retention time (Minute) | Name                                                 | Molecular formula                              | Area sum (%) |
|------|-------------------------|------------------------------------------------------|------------------------------------------------|--------------|
| 1    | 10.04                   | Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)- | C <sub>10</sub> H <sub>16</sub>                | 13.77        |
| 2    | 10.24                   | $\alpha$ -Pinene                                     | C <sub>10</sub> H <sub>16</sub>                | 3.17         |
| 3    | 11.34                   | Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- | C <sub>10</sub> H <sub>16</sub>                | 1.08         |
| 4    | 11.42                   | $\beta$ -Pinene                                      | C <sub>10</sub> H <sub>16</sub>                | 2.86         |
| 5    | 12.63                   | <i>D</i> -Limonene                                   | C <sub>10</sub> H <sub>16</sub>                | 1.59         |
| 6    | 12.72                   | <i>p</i> -Cymene                                     | C <sub>10</sub> H <sub>14</sub>                | 36.65        |
| 7    | 15.03                   | <i>cis</i> -4-methoxy thujane                        | C <sub>11</sub> H <sub>20</sub> O              | 4.77         |
| 8    | 18.49                   | Thymoquinone                                         | C <sub>10</sub> H <sub>12</sub> O <sub>2</sub> | 17.53        |
| 9    | 20.12                   | Phenol, 2-methyl-5-(1-methylethyl)-                  | C <sub>10</sub> H <sub>14</sub> O              | 1.52         |
| 10   | 21.13                   | Longifolene                                          | C <sub>15</sub> H <sub>24</sub>                | 1.80         |
| 11   | 25.50                   | <i>p</i> -Cymene-2,5-diol                            | C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> | 15.25        |

weight components. The four major components with the highest peak area were *p*-cymene (36.65%), thymoquinone (TQ) (17.53%), *p*-cymene-2,5-diol (15.25%), and bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl) derivative (13.77%).

## 3.2. Nanoemulsion formulation

### 3.2.1. Droplet size, PDI and ZP

The mean droplet size was (60.44  $\pm$  4.05) nm, while PDI was 0.24 $\pm$ 0.03. ZP value was (–32.0  $\pm$  2.17) mV. The PDI value less than 0.3 is considered acceptable and an indication of a narrow particle size distribution. ZP values higher than  $\pm$  30 mV are considered highly stable. It indicated that the droplet was small and stable, and droplet size distribution was moderately narrow.

### 3.2.2. Morphological analysis

The uranyl acetate-stained droplets were spherical in shape and relatively homogenous. The droplet size was well correlated with the results from particle size analysis using the dynamic light scattering technique, and there were no signs of aggregation or coalescence.

## 3.3. Behavior and mortality rate

The positive control group showed no death, while the mortality of the negative control group and the LPS group was 20% and 50%, respectively. The mice in the positive control group showed the highest activity and healthy behavior compared to other groups.

### 3.4. Effect on liver enzymes

The LPS group had significant increases in AST and ALP levels in comparison to the negative control group that received tween 20 only ( $P<0.05$ ). BCSO pre-treatment and BCSO nanoemulsion pre-treatment showed significant downregulation of both serum AST and ALP levels compared to the LPS group ( $P<0.05$ ). The combination of BCSO and indomethacin in relatively lower doses (Indomethacin+oil group) also restored the downregulation of both serum AST and ALP levels ( $P<0.05$ ) (Figure 1A).

### 3.5. Effect on inflammatory and antioxidant proteins

The LPS group also showed significant increases in IL-6 and TNF- $\alpha$  ( $P<0.001$ ) and significant decreases in GSH protein level ( $P<0.001$ ). BCSO pre-treatment and BCSO nanoemulsion pre-treatment showed significant downregulation of hepatic TNF- $\alpha$  and IL-6 protein levels compared to the LPS group. Hepatic GSH protein level was downregulated significantly after LPS induction, while BCSO was able to restore its level to normal. The combination of BCSO and indomethacin in relatively lower doses (Indomethacin+oil group) also showed similar effects (Figure 1B).

### 3.6. Effect on inflammatory and apoptotic gene expression

RT-PCR analysis showed significant upregulation of expression of *IL-1 $\beta$* , *TLR4*, *HMOX1*, *BAX*, and *BCL-2* in the LPS group compared to the negative control group (Figure 2). Results showed that BCSO and nanoemulsion formulation significantly downregulated *IL-1 $\beta$* , *TLR4*, *BAX*, and *BCL-2* compared to the LPS group (Figure 2). Indomethacin pre-treatment did not downregulate *IL-1 $\beta$* , *BAX*, and *BCL-2*. Neither BCSO nor indomethacin was able to downregulate *HMOX1* gene expression compared to the LPS group. BCSO nanoemulsion formulation and the combination of BCSO and indomethacin in relatively lower doses (Indomethacin+oil group)

significantly downregulated *IL-1 $\beta$* , *HMOX1*, *TLR4*, and *BCL-2* (Figure 2).

### 3.7. Histological results

Histopathology analysis of control healthy liver tissues showed normal histological structures of parenchymal hepatocytes and central veins arranged as cords of cells linking the portal tracts in the periphery to the central veins; tiny lobules with portal tracts at the apices and inside the lobules (Figure 3A). The structure of the hepatic lobule was clear and complete; hepatocyte cords were arranged tightly with a centered nucleus. The hepatic hilar region and the vicinity of the central vein did not have any signs of inflammatory cell infiltration or hepatocyte necrosis. The liver of the LPS group showed marked hepatocellular vacuolar degeneration (ballooning degeneration) with cytoplasmic reticulation, and most showed a signet ring appearance with some atrophic cells and scattered single-cell necrosis (Figure 3B). The hepatic cells and lobules had structural damage and disorganization. The liver of BCSO pre-treatment mice groups showed some degree of liver injury; however, the hepatic lobule and hepatocyte cord structures were intact and well-defined. The liver tissue injury was greatly improved from cell infiltration caused by inflammation. Furthermore, there was mild to moderate swelling of the hepatic cells with a mild degree of vacuolation and a mild increase in bi-nucleated cells (Figure 3C).

The liver of the mice group treated with a nanoemulsion formulation of BCSO showed good restoration of the hepatic parenchyma with minimal scattered degenerated cells with minimal cell necrosis (Figure 3D). Moreover, the livers of indomethacin-treated mice showed mild focal vacuolar degeneration of the hepatocytes with minimal necrotic cells (Figure 3E). The liver of the mice treated with BCSO+indomethacin showed minimally scarce inflammatory cells in the portal tract, with very few degenerated hepatic cells and some bi-nucleated cells (Figure 3F).

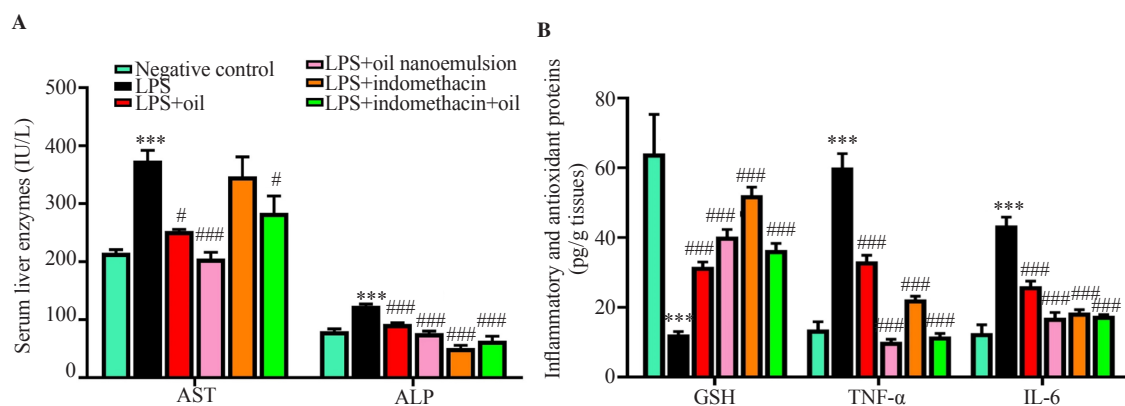


**Figure 1.** Effect of black cumin seed oil on liver enzymes, inflammatory, and antioxidant proteins. A) AST and ALP; B) GSH, TNF- $\alpha$ , and IL-6 protein concentrations. Data are shown as mean  $\pm$  SEM ( $n = 6$ ). \*\*\* $P<0.001$  compared to the negative control group; # $P<0.05$ , ### $P<0.001$  compared to the LPS group. One-way ANOVA test was used to compare multiple independent groups using GraphPad Prism 7 software. Dunnett's multiple comparison test was used as *post hoc* test. AST: aspartate transaminase; ALP: alkaline phosphatase; GSH: glutathione; IL-6: interleukin-6; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .

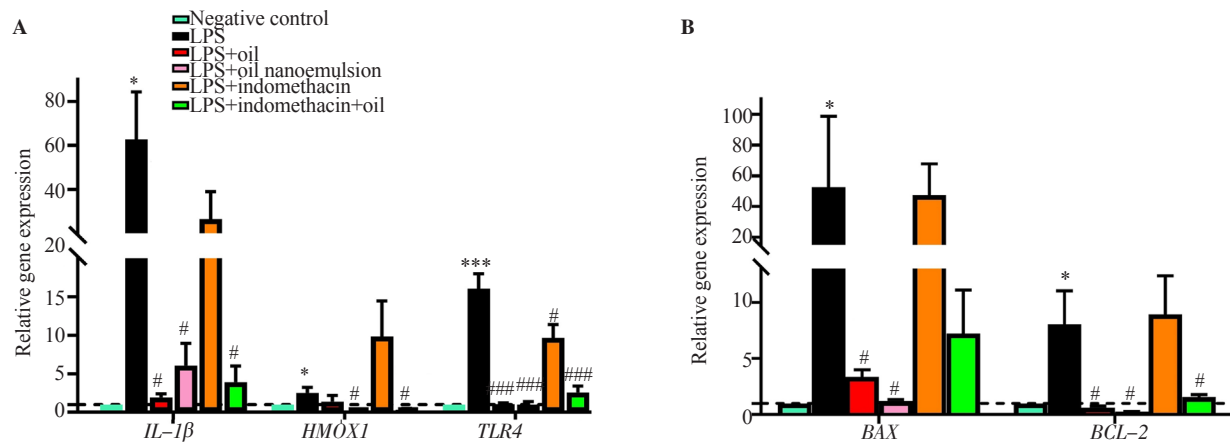


**Figure 2.** Effect of black cumin seed oil on inflammatory and apoptosis-related genes. A) *IL-1β*, *HMOX1*, and *TLR4* genes; B) *BAX* and *BCL-2* genes. Data are shown as mean  $\pm$  SEM ( $n = 6$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$  compared to the negative control group; # $P < 0.05$ , ### $P < 0.001$  compared to the LPS group. One-way ANOVA test was used to compare multiple independent groups using GraphPad Prism 7 software. Dunnett's multiple comparison test was used as *post hoc* test.

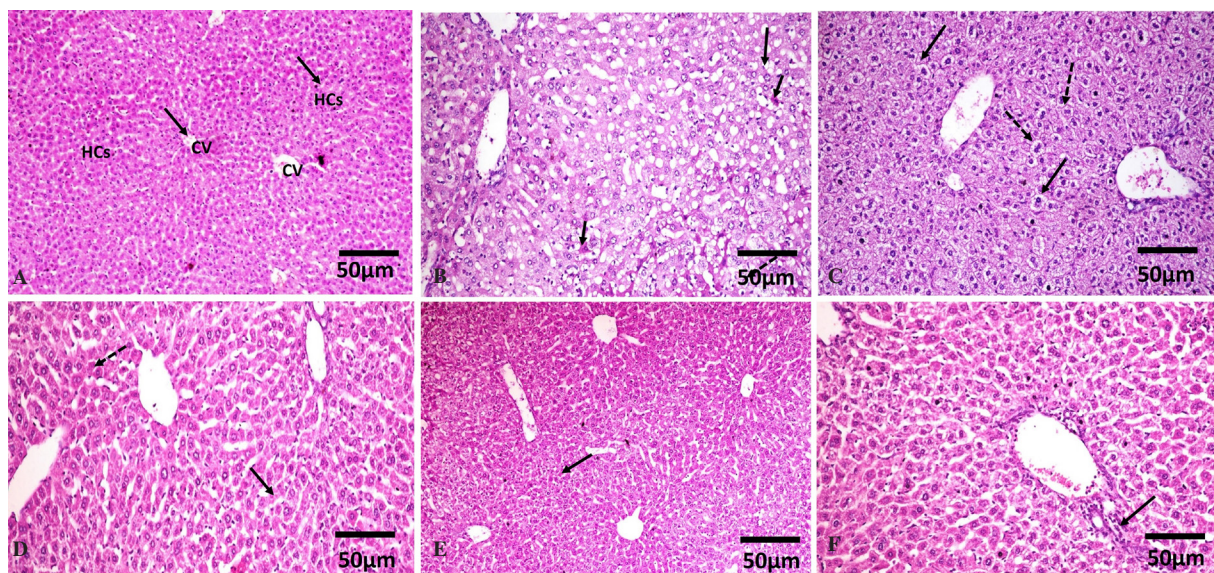


**Figure 3.** Histological examination of liver tissues. A) Liver of control mice treated with tween 20, showing normal histological structure of the central vein (CV) and parenchymal hepatic cells (HCs), B) liver of lipopolysaccharide-induced mouse model, showing marked hepatocellular vacuolar degeneration, some showing ballooning degeneration with cytoplasmic reticulation (dashed arrow) and the most showing signet ring appearance (arrow) with some atrophic cells (short arrow) and scattered single cell necrosis, C) liver of black cumin seed oil (BCSO) pre-treatment mouse model, showing mild to moderate swelling of the hepatic cells with mild vacuolation (arrow) with mild increase in bi-nucleated cells (dashed arrow), D) liver of nanoemulsion formulation of BCSO pre-treatment mouse model, showing few scattered degenerated cells (arrow) and single cell necrosis (dashed arrow), E) liver of indomethacin pre-treatment mouse model, showing mild focal vacuolar degeneration of the hepatocytes (arrow) and minimal necrotic cells, and F) liver of BCSO and indomethacin combination pre-treatment mouse model, showing scarce inflammatory cells (arrow) in the portal tract with very few degenerated hepatic cells and some bi-nucleated cells.

#### 4. Discussion

Liver disease has been classified according to the progression of the disease and damage into 4 main stages, where stage 1 is the early-stage (acute) liver injury characterized by acute liver inflammation[21]. Early-stage liver damage in animals is considered a helpful model to replicate human impact in order to investigate the pathologic origins of this illness and the therapeutic advantages of various medicines[22]. This study was to evaluate the therapeutic impact of BCSO pre-treatment on liver damage and inflammation in the early stages in LPS-treated mice as well as the synergistic

effect of BCSO with indomethacin therapy at a decreased dose. The BCSO-treated negative control group showed no mortality rate, and mice were very healthy and active, which indicates that BCSO is generally considered safe and has no adverse side effects[6].

LPS induction for 6 h induced early-stage liver injury[23] with elevation of several hepatic pro-inflammatory cytokines such as IL-6, TNF- $\alpha$ , and IL-1 $\beta$ [24,25] which agrees with our previous work results that showed that LPS induction caused overexpression of pro-inflammatory cytokine COX-2 on protein and gene level in hepatic tissues[12]. Increased IL-6 secretion exacerbates local or systemic inflammation and impairs liver function by altering insulin

sensitivity[26], while IL-1 $\beta$  and TNF- $\alpha$  have a significant impact on various stages of liver diseases and also increase cytokine production from macrophages, phagocyte oxidative metabolism, and nitric oxide generation[27]. COX-2 is a potent enzyme that can initiate inflammation and promote the synthesis of prostaglandins after being stimulated, and it plays a critical role in the onset and development of liver fibrosis[28].

Also, similar to Ye and Liu's findings[29], the current study showed that LPS induction significantly increased hepatic TLR4 levels which mediates overexpression of HMOX1 in hepatic tissues. It has been shown that TLR4 is a key pattern recognition receptor for LPS, mediating pro-inflammatory signals in parenchymal and non-parenchymal cells that are amplified in the early stages of liver inflammation[30]. LPS administration was shown to reduce hepatic GSH protein levels, which is known as a highly effective intracellular antioxidant first-line defense against free radicals and plays a key role in determining how susceptible tissue is to oxidative damage[31].

This is consistent with research that shows a connection between elevated levels of inflammatory mediators and the formation of reactive oxygen species, which causes early oxidative stress impairment to liver function[32]. Apoptosis is also involved in acute liver injury through DNA fragmentation and caspase-dependent activation mechanisms. DNA damage and cell death are caused by altering the tumor necrosis factor receptor 1 signaling pathway by increasing TNF- $\alpha$ . Current study results showed that LPS induced a significant upregulation in hepatic *BAX* and *BCL-2* genes. Prior studies showed that overexpressed pro-inflammatory components stimulate the overexpressed *Bax* gene, which consequently induces its pro-apoptotic proteins to migrate from the cytosol to the mitochondria and trigger caspase activation and apoptosis[33].

It was interesting in our study to observe that the *BCL-2* gene was overexpressed after LPS induction, and its level was downregulated after BCSO and its nanoemulsion formulation treatment. Previous studies showed that *BCL-2* is overexpressed in both acute and chronic inflammation, especially after LPS induction. Elevated inflammatory mediators such as IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in addition to activation of TLR4 and inflammatory cytokines that mediate IL-13 production, cause *BCL-2* overexpression[34]. Hence, *BCL-2* level will be regulated by an inflammatory effect rather than apoptosis.

LPS induction activates cellular inflammation, and apoptosis leads to cellular leakage and loss of the functional integrity of the liver cell membrane, causing liver cell damage that leads to elevated levels of the liver enzymes AST and ALP. This large-scale enzyme release is linked to the liver's ballooning, cellular degeneration, and infiltration of leukocytic inflammatory cells typical to hepatic injury, which was confirmed by our histopathological results.

To understand and correlate the potential effect of BCSO with the anti-inflammatory effect, GC-MS analysis showed main active ingredients such as *p*-cymene, longifolene, TQ, *D*-limonene,  $\alpha$ -pinene, and  $\beta$ -pinene. *p*-Cymene is a potent anti-inflammatory agent and can decrease the production of intestinal oxidative stress cytokines and serum inflammatory cytokines[35]. Longifolene, as an

active component in many herbs, has immunosuppressive effects on inflammation through inhibitory impact on the expression levels of IL-4 and IL-13 and the secretion of  $\beta$ -hexosaminidase[36]. TQ inhibits inflammation and oxidative stress through a number of molecular mechanisms, including cytokine release, COX-2 activation, nuclear factor erythroid 2-related factor 2 activation, phosphatidylinositol 3-kinase/protein kinase B activation, and nuclear factor kappa-light-chain enhancer of activated B activation[37]. *D*-limonene has been shown in studies to be useful in preventing a number of chronic and degenerative disorders due to its antioxidant, anti-diabetic, anti-cancer, anti-inflammatory, cardioprotective, gastroprotective, hepatoprotective, immunological modulatory, anti-fibrotic, and anti-genotoxic properties[38]. In rats with inflammation treated with isoproterenol,  $\alpha$ -pinene improved the status of antioxidants and reduced the expression of inflammatory markers[39].  $\beta$ -Pinene is a multifunctional product that acts as gastroprotection, anxiolytic, cytoprotectant, anti-convulsant, and neuroprotection. Besides, it also has defense against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, pancreatitis, stress-induced hyperthermia, and pulpal pain[40]. All these activities for BCSO components individually may be attributed to its powerful anti-inflammatory and antioxidant properties.

Pre-treatment with BCSO has been shown to attenuate the effect of LPS-induced inflammation through different suggested mechanisms, including its known anti-inflammatory mechanism[41]. BCSO reduced the level of the *TLR4* gene, which in turn may suppress hepatic cells' TLR4/MyD88 pathway, leading to a reduction in hepatic pro-inflammatory cytokines IL-6, TNF- $\alpha$ , and IL-1 $\beta$  on both gene and protein levels, as what happened with COX-2 in our previous work, as BCSO had an inhibitory action on hepatic COX-2 on protein and gene levels after LPS treatment[12]. In addition, BCSO restored the hepatic GSH levels to their respective normal values, which proved its cytoprotective properties against acute or ongoing inflammation either due to the *de-novo* synthesis of GSH, its regeneration, or both. Moreover, apoptosis and cell death were reversed by downregulation of all apoptotic key markers reflected with decreased serum levels of ALP and AST toward their corresponding normal values, as well as histological signs of liver cell membrane stabilization and repair of hepatic tissue damage.

BCSO nanoemulsion formulation in droplet sizes ranging from 10-200 nm offered exceptional properties for BCSO in the delivery system, such as a large surface area for action and absorption, robust stability, tuneable rheology, high kinetic stability, high solubilization capacity, protection of encapsulated active compounds, and enhancement of the bioactivity[10,11]. Consequently, nanoemulsion formulation showed better effects regarding downregulation of serum AST level, hepatic TNF- $\alpha$  and IL-6 proteins, and hepatic *HMOX1* and *TLR4* genes compared to BCSO alone or indomethacin. The same results were shown with hepatic COX-2 protein and gene in our previous work[12].

Indomethacin belongs to nonsteroidal anti-inflammatory drugs and is widely used in different inflammatory diseases. However, indomethacin has numerous side effects on cardiovascular, renal, gastric, and hepatotoxic effects[42]. Literature showed that prolonged

use of indomethacin decreased hepatic GSH, superoxide dismutase, and catalase activities in the liver with elevation of serum AST due to liver toxicity[43]. Indomethacin had a limited therapeutic effect on the reduction of pro-inflammatory and pro-apoptotic markers compared to BCSO and its nanoemulsion formulation. However, combined therapy with indomethacin and BCSO was also able to enhance activity and regain function and integrity of the liver by enhancing its anti-inflammatory effect, suggesting BCSO's role is promising as adjuvant therapy with its resulting cytoprotective effect.

There are some study limitations. Clinical research is necessary since the response to treatments in mice may not precisely mirror the reaction in people due to species-specific changes in immune response. Prior to being used in human consumption, *in-vivo* research is necessary to determine the safety and bioavailability of the BCSO nanoemulsion formulation. Additional *in-vitro* and *in-vivo* research is necessary to isolate the pure BCSO compounds in order to confirm the main constituents responsible for anti-inflammatory properties and the precise mechanisms of the action, particularly at the level of cellular death. Additional confirmational assays, such as Western blotting, are required to confirm the expression level of proteins such as BCL-2, TLR4, and other inflammatory mediators. In animal models, anti-inflammatory potency is frequently assessed over very short periods, which could not accurately represent the long-term safety and efficacy needed for chronic illnesses like hepatitis in humans.

In conclusion, the current findings indicated that BCSO exhibits anti-inflammatory and antioxidant potential in an experimental animal model in response to LPS-induced injured liver tissues. BCSO exerts these effects *via* downregulating the expression of TLR4 and key inflammatory mediators such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and HMOX1 on a genetic and protein level and upregulation of GSH protein. This may suggest its potential role in mitigating inflammation through the TLR4 activation pathway. The histological examination confirmed the improvement in injury of liver tissue from cell infiltration caused by inflammation. The nanoemulsion formulation showed enhanced activity over both individual BCSO and indomethacin. Additionally, the combination therapy of BCSO and indomethacin at a reduced dose showed enhanced activity compared to indomethacin. Based on these findings, BCSO can be used to develop an anti-inflammatory natural agent in adjuvant therapy with indomethacin for protection of the liver against acute liver injury.

### Conflict of interest statement

The authors declare that there is no conflict of interest.

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### Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

### Authors' contributions

HH and HA developed the study's conception and design. HH and RH performed experimental study, data collection, and analysis. SS prepared the nanoformulation of BCSO. RH and HH wrote the first draft of the manuscript. HH, RH, SS, and HA contributed to reviewing, editing, and investigation on previous versions of the manuscript. All authors read and approved the final manuscript.

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