Black cumin seed oil and its nano-form ameliorate lipopolysaccharide-induced brain inflammatory injury in mice

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Backgrounds and objectives
Microglia play a regulatory role in central nervous system inflammatory diseases, such as Alzheimer's, Parkinson's, and multiple sclerosis. Natural remedies like black cumin seeds (*Nigella sativa*) are rich in bioactive compounds that potentially can modulate inflammatory processes in the brain. In the current work, we studied the protective and anti-inflammatory properties of black cumin seed oil (BCSO) and its nano-form on lipopolysaccharide (LPS)-induced neurotoxicity in mice.
Materials and methods
Forty-eight mice were divided randomly into eight groups (n=6), three control groups (negative control, BCSO control, nano-BCSO control), LPS group, and four treatment groups [BCSO+LPS, nano-BCSO+LPS, indomethacin (5 mg/kg) +LPS, BCSO+indomethacin(2.5 mg/kg)+LPS]. At the end of the experiment, the brain tissues were removed for histopathological and biochemical assessments. Malondialdehyde and interleukin (IL)-10 were assessed using enzyme-linked immunosorbent assay while the gene expression of IL-6, toll-like receptor-4, brain-derived neurotrophic factor, nerve growth factor, cyclooxygenase-2, and B-cell lymphoma-2 were assessed by real-time PCR. IL-1β was quantified immunohistochemically along with the histopathological studies of the cerebral cortex of mice brains.
Results and conclusions
In our study, BCSO and its nano-form demonstrated a reduction in LPS-induced neurotoxicity, exhibiting comparable or better anti-inflammatory effects to indomethacin. These treatments significantly elevated the gene expression levels of neuroprotective factors brain-derived neurotrophic factor and nerve growth factor in LPS-treated mice. Pretreatment with BCSO and its nano-form reduced the malondialdehyde levels, in addition to gene expressions of cyclooxygenase-2, toll-like receptor-4, IL-6, and B-cell lymphoma-2. Immunohistochemical analysis indicated a decrease in IL-1β with BCSO and the lowering effect of the nano-form was superior. The histopathological studies corroborated with biochemical and molecular findings, suggesting that BCSO and its nano-form attenuated the inflammation and enhanced the microglial antioxidative and anti-inflammatory status. BCSO could enhance the anti-inflammatory activity of indomethacin, so lower doses of indomethacin with BCSO may be suggested for protecting against the adverse effects of high doses of NSAIDs as gastritis. Consequently, BCSO can serve a potential stimulatory supplement of the immunity for neurodegenerative conditions.

Keywords:
black seed, inflammation, microglia, nanoparticles, neurodegenerative, toll-like receptor-4

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Introduction

The inflammatory processes and immune system are involved in a widespread diversity of physical and mental health ailments, which was found to be one of the most significant medical findings [1,2]. Nowadays, chronic inflammation-related diseases account for 50% of all deaths globally [3]. Currently, the research is directed toward the central nervous system (CNS) inflammatory diseases, which are also known as focal diseases, such as multiple sclerosis (MS), Alzheimer's, and Parkinson's diseases [4–6].

It was found that when microglia, an indispensable part of the brain's innate immune system and serve a number of regulatory roles during CNS inflammation [7–9], become activated in response to inflammatory stimuli (e.g. microbes and toxins), a cascade of inflammation in the CNS microenvironments occurs

accompanied with the release of inflammatory mediators such as inducible NO synthase, nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in the CNS, eventually leading to the progression of neurodegenerative diseases [9–11]. From the well-recognized inflammatory stimuli, lipopolysaccharide (LPS), a main component of the outer surface of Gram-negative bacteria [12], acts on toll-like receptor-4 (TLR-4), leading to the release of proinflammatory and neurotoxic agents. Moreover, the differentiation of macrophages (MQs) into inflammatory type 1 macrophages (MQ1) consequently occurs [13–15]. Normally these MQs own a vital role against injuries and harmful stimuli. However, overactivation of this type of cells can trigger several inflammatory ailments, like neurodegeneration, MS, and other autoimmune diseases [15]. On the other side, type 2 macrophage (MQ2) cells are known as healing MQs, through the production of anti-inflammatory mediators involving IL-4 and IL-10 [11,14,15].

The immune system deregulation is the main cause of several diseases; therefore, managing the immunological responses could present a beneficial therapeutic approach for handling these diseases. Medicinal plants could influence the immune system through modulation of its function like the production or release of cytokines, the actions of immune cells, in addition to the expression of cellular receptors [16]. The natural products as well as essential oils are well known for their anti-inflammatory and immunomodulatory effects [17].

Nigella sativa, also known as black seed or black cumin, is considered a herbal medicine. It grows in southwest Asia and belongs to the family *Ranunculaceae*. *N. sativa* seeds are used as traditional remedies for the treatment of a variety of neurological disorders [18,19]. The analysis of black cumin seed phytochemical compounds showed an existence of fatty acids [20], ascorbic acid [21], phospholipids [22], and vitamins [23], along with thymoquinone, dithymoquinone, carvacrol, and thymol, which demonstrated antioxidant [24], analgesic [25], and anticancer therapeutic effects in addition to the immunomodulatory activities [26,27]. Furthermore, the anti-inflammatory impacts of black cumin seed extract and its active constituents have been reported on several inflammation models like paw edema induced by carrageenan [28], rheumatoid arthritis in rats [29], eicosanoid generation in white blood cells [30], allergic lung inflammation in mice [31], allergic encephalomyelitis as a model for MS [32], ulcerative colitis [33], and NO production by murine macrophages [34].

Hence, the current research was meant to investigate the impact of black cumin seed oil (BCSO) and a prepared nano-form of BCSO on the LPS-induced neurotoxicity in mice. The study highlighted the signaling pathways involved in inflammation-mediated brain toxicity in mice. Indomethacin was used as a positive control with its regular dose. In addition, another tailored dose of indomethacin in combination with the oil was investigated to decrease the adverse effects of synthetic NSAIDs.

Materials and methods

Kits, reagents, and chemicals

The BCSO was purchased from Imtenan (El Obour City, Egypt). LPS (*Escherichia coli*, 0111: B4) was obtained from Sigma, Aldrich (St. Louis, MO, USA). Indomethacin (Liometacen) was obtained from the Nile Co. for pharmaceuticals and chemical industries (Cairo, Egypt). TE buffer was bought from Solarbio (Beijing, China). Malondialdehyde (MDA) (Cat No. MBS741034) and IL-10 (Cat No.: MBS018124) enzyme-linked immunosorbent assay (ELISA) kits were all purchased from MyBioSource Company (San Diego, USA). RNA extraction kit: GeneJET RNA purification kit (Cat. No.: K0731), cDNA synthesis kit: RevertAid first-strand cDNA synthesis kit (Cat. No.: K1622), and SYBR Green kit: Maxima SYBR qPCR green master mix (K0252) were purchased from Thermo Scientific/Applied Biosystems (Logan, Utah, USA). The primers were obtained from Willofort (Birmingham, UK).

Black cumin seed oil administration

To deliver the appropriate doses of BCSO to mice, the oil was emulsified in a 1% solution of Tween 20 [35]. The obtained suspension had a yellowish-white appearance and was stored in a dry, cold place in a dark-tinted container. Based on the lot number, a certificate of analysis was obtained for the purchased oil. The standardized BCSO fixed constituents include linoleic acid (58.64%), oleic acid (21.82%), palmitic acid (12.36%), stearic acid (3.16%), eicosadienoic acid (2.5%), myristic acid (0.21%), arachidic acid (0.2%), and trace amounts of capric acid, pentadecanoic acid, palmitoleic acid, margaric acid, and behenic acid. The oil also contains essential constituents that include thymoquinone (84.34%), o-Cymene (9.17%) and dodecanal (2.98%) and traces of alpha-thujene and alpha-farnesene.

Antioxidant assay of black cumin seed oil, 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical scavenging activity

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical assay was done following the method of Boly *et al.* [36]. A measure of 100 µl of freshly prepared DPPH reagent (0.1% in methanol) was mixed in 96-well plates (n=6) with 100 µl of serial dilutions of BCSO or reference compound Trolox (Cat. No. 53188-07-1; Sigma Aldrich), and the reaction was incubated in dark for 30 min at room temperature. Finally, the color intensity decline of DPPH was measured at 540 nm using a microplate reader FluoStar Omega (BMG LABTECH, Offenberg, Germany). The percentage of inhibition was calculated according to the following formula:

% inhibition=[(average blank absorbance–average absorbance of the test)/average absorbance of blank]×100.

The IC_{50} was calculated through converting the concentrations to their logarithmic value and applying nonlinear inhibitor regression equation [log (inhibitor) vs. normalized response–variable slope equation] using GraphPad prism software (San Diego, California, USA).

Black cumin seed oil nano-form preparation

Preparation of the nano-emulsion

An oil-in-water emulsion containing BCSO as an internal phase was prepared following the method by Gumus *et al.* [37] with slight modifications. It was prepared using a single-step method involving the use of lecithin and Tween 80. The ratio of the oily phase in form of BCSO with 3% lecithin to the aqueous phase containing 3% Tween 80 was 20 : 80 by volume, respectively. Briefly, both phases were mixed and sonicated with a probe sonicator (Vibra cell, Sonics, USA) at 50% amplitude in an ice bath for 10 min with a 30-s period off following each minute of sonication till the formation of the nano-emulsion. Then, the formed nano-emulsion was stored at 4°C protected from light till the investigation.

The characterization of black cumin seed oil nano-emulsion

Droplet size analysis and surface charge measurement

The mean droplet size and droplet size distribution expressed by the polydispersity index (PDI) were evaluated using the dynamic light scattering (DLS) technique. ZP value was expressed in mV and measured based on the electrophoretic mobility. All values were measured using Zetasizer (Nano ZS; Zetasizer, Malvern Instruments Ltd, UK). To avoid multiple scattering effects, suitable dilution was done for the nano-emulsion before measurement using double distilled water. The measurements were done in triplicates and mean±SD was calculated.

Transmission electron microscopy

The morphology of the droplets of BCSO in water nano-emulsion was evaluated using transmission electron microscopy with JTEM-1010 microscope (JEOL, Tokyo, Japan) by the application of negative staining technique. In brief, one drop of the nano-emulsion was put onto a carbon-coated copper grid coating. Then, the excess liquid droplets were removed gently using filter paper. After 5 min, one drop of uranyl acetate solution (2% w/v) was then dropped onto the grids. The sample was then dried by air at room temperature and the examination was done at 80 kV.

Animals

Forty-eight male BALB /C mice aged 8±1 weeks and weighing (30±5 g) were purchased from VACSERA animal center (Helwan, Cairo, Egypt). Mice were left to adapt to the laboratory conditions for 2 weeks before starting the experiment. They were housed in polypropylene cages with stainless steel grid covers in a 12-h light/dark cycle at optimum humidity and a temperature of 25±2°C and had access to water and food ad libitum through the experiment. All procedures in the current research were compliant with the ethical standards of the Ethics Committee of Faculty of Pharmacy, Helwan University, Egypt. (Ethical No.02A2020; date 13/10/2020). The study was performed following the guidelines of directive 2010/63/EU for animal experiments.

Induction of inflammation in mice and experimental design

The inflammation was induced in mice by a single intraperitoneal injection (i.p.) of 2.5 mg/kg body weight of LPS [38]. The studied groups were as follows: control group: healthy mice were treated with vehicle only for 2 weeks. BCSO control group: mice were (i.p.) treated with BCSO (0.2 ml/kg/day) for 14 consecutive days [39]. Nano oil control group (nano-BCSO): mice were (i.p.) treated with nano-BCSO formula (0.2 ml/kg/day) for 14 consecutive days. LPS group: mice were treated with a single (i.p.) dose of 2.5 mg/kg body weight LPS 6 h before termination. BCSO+LPS group: mice were (i.p.) treated for 14 consecutive days with BCSO followed by a single intraperitoneal injection of LPS. Nano-BCSO+LPS group: mice were (i.p.) treated for 14 days with BCSO nano formula followed by a single intraperitoneal injection of LPS. Indomethacin

(5 mg/kg)+LPS group: mice were (i.p.) treated with indomethacin (5 mg/kg) for 3 days before termination followed by LPS (i.p.) single dose. Indomethacin (2.5 mg/kg)+BCSO+LPS group: mice were intraperitoneally treated for 14 consecutive days with BCSO (0.2 ml/kg/day), then with i.p. of indomethacin half dose (2.5 mg/kg) for 3 days before termination followed by LPS (i.p.) single dose (2.5 mg/kg body weight).

At the end of the experiment course, mice were sacrificed by cervical dislocation after blood sample collection from their retro-orbital sinus veins.

Sample preparation

Brain tissues were collected, rinsed, and weighed. Cerebral cortices of the brain tissue were either fixed in 10% buffered formalin for histopathological investigations or stored at −80°C for investigating biochemical parameters after homogenate preparation.

Homogenate preparation

The cerebral cortices were homogenized with a proportion of 1 g to 5 ml cold saline using high-speed glass Teflon Dounce homogenizer (Glas-Col homogenizer). The resulted homogenate was centrifuged for 15 min at 5000 rpm at 4°C, and the supernatant was separated and divided into aliquots and was kept at −80°C for further ELISA and quantitative real-time PCR tests.

Quantitative detection of lipid peroxidation and anti-inflammatory markers in brain using enzyme-linked immunosorbent assay

MDA and IL-10 levels in the brain tissue were assayed using mice-specific ELISA kits (MyBioSource Company, San Diego, California, USA) following manufacturer's procedures.

Quantitative real-time PCR

The total RNA was isolated from brain tissue homogenates using GeneJET RNA purification kit, Thermo Scientific/Applied Biosystems following the directions of the manufacturer. The concentration of extracted RNA was evaluated using nanodrop, after that, it was reverse transcribed into cDNA by using RevertAid first-strand cDNA synthesis kit, Thermo Scientific/Applied Biosystems following the provider's protocol. To assess the effect of BCSO and its nano-form on the expression of some related genes, mRNA of nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) as brain injury specific markers, TLR-4, IL-6, and cyclooxygenase-2 (COX-2) as inflammatory markers, in addition to B-cell lymphoma-2 (BCL-2) as an apoptotic marker were all determined in brain tissue using Maxima SYBR qPCR green master mix along with beta-actin gene (Actb) as the housekeeping gene. Quantitative PCR was performed using Rotor-Gene Q (QIAGEN, Hilden, Germany). The relative gene expression levels were evaluated using the $2^{-(\Delta\Delta Ct)}$ method [40], and the results were expressed in the form of the mean fold change of three experiments. The primers sequences are listed in Table 1.

Immunohistochemical examination of interleukin-1β in the cerebral cortices of the brain tissues

Immunohistochemistry technique was used to examine the IL-1β expression on the prepared brain paraffin slices of control and treated mice groups using avidin–biotin peroxidase (A-B peroxidase) in accordance with the method mentioned by El-Rahman and Fayed [41]. In brief, at a dilution of 1 : 200, a monoclonal antibody for IL-1β (Abcam, Cambridge, USA) was incubated with tissue sections along with the peroxidase kit, Vectastain ABC, Vector Laboratories) for the revelation of the complex constituted by the union of antigen and antibody. 3,3-Diaminobenzidine tetra hydrochloride (Sigma Chemical Co.) was used as a chromogen to visualize each marker's expression. The immune-stained slides were assessed using image analysis tools in seven microscopic fields of high-power magnification (Image J, 1.46a, NIH, USA).

Table 1 Primer sequences of the genes investigated by RT-PCR

Gene	Forward 5′-3′	Reverse 5′-3′	Accession number*
Actb	CTCTAGACTTCGAGCAGGAGATGG	ATGCCACAGGATTCCATACCCAAGA	NM_007393.5
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC	NM_031168.2
TLR-4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA	NM_021297.3
BDNF	CATACTTCGGTTGCATGAAGG	AGTGTCAGCCAGTGATGTC	NM_007540.4
NGF	CAGATAGCAATGTCCCAGAAGG	AGTGATGTTGCGGGTCTGC	NM_013609.3
COX-2	ACACACTCTATCACTGGCACC	TTCAGGGAGAAGCGTTTGC	NM_011198.5
BCL-2	CTGAGTACCTGAACCGGCAT	GGTATGCACCCAGAGTGATG	NM_009741.5

BCL-2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase-2; NGF, nerve growth factor; TLR-4, toll-like receptor-4. *According to GenBank Primer-Blast Program, NCBI.

Brain tissue staining protocol and histopathological examination

The histopathological examination was performed by an expert at the Department of Pathology Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. Mice cerebral cortices of brain tissues were fixed in 10% buffered formalin. Tissue specimens were rinsed with tap water, then serial dilutions of ethyl alcohol were used to dehydrate them. They were cleared in xylene and lastly embedded in paraffin. The thickness of paraffin block sections were averaged between 4 and 5 μm, and they were stained with hematoxylin and eosin [42]. The histological sections were examined under light microscope (Olympus, Tokyo, Japan), and their photomicrographs were taken at X 400 magnification.

Statistical analysis

Data were depicted as means±SD and analyzed by GraphPad Prism 5 software (GraphPad San Diego, California, USA). Analysis of variance (ANOVA) test followed by Tukey–Kramer multiple comparison post-hoc test were used to draw comparisons between groups. *P* values lower than 0.05 were regarded as statistically significant.

Results

The characterization of nano-black cumin seed oil

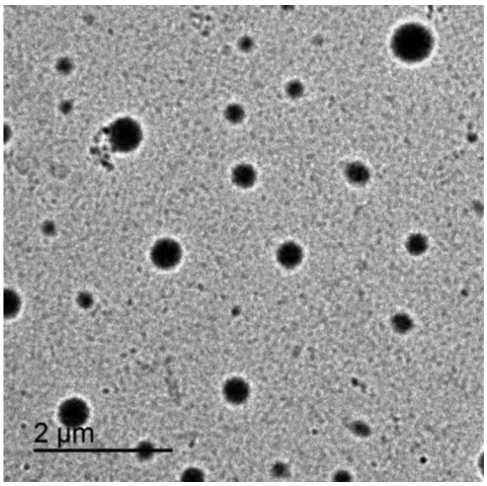
Droplet size, polydispersity index, and zeta potential

The mean droplet size obtained using the DLS technique is 60.44±4.05 nm. Droplet size of very small size (smaller than 100 nm) offers a large surface area and hence better bioavailability [43]. Similar droplet size was obtained by Gumus *et al.* [44], who prepared the nano-emulsion using the same method. The PDI represents the homogeneity of droplets, and it ranges from 0 to 1 [45]. The obtained PDI value was 0.24±0.3. When the PDI value is smaller than 0.3, it is considered acceptable and indicates a narrow particle size distribution [46,47]. High ZP value is an indication of good stability of colloids. ZP values higher than +30 mV or more negative than −30 mV are considered highly stable [48]. The ZP value was −32.0±2.17 indicates low probability of coalescence and size increase of the prepared nano-emulsion with time.

Morphological analysis

As seen from Fig. 1, the negatively stained oil droplets are relatively homogeneous and spherical in shape. There are no signs of coalescence or aggregation. A comparable droplet size was obtained using the DLS technique.

Figure 1



TEM micrograph of BCSO nano-emulsion. BCSO, black cumin seed oil; TEM, transmission electron microscopy.

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate radical scavenging impact of black cumin seed oil

The data represented in Table 2 shows that the BSCO has an efficient scavenging power against DPPH when compared with Trolox. The lower IC_{50} value of the sample indicates a stronger ability to neutralize free radicals [49].

The effects of black cumin seed oil and its nano-form on lipid peroxidation in lipopolysaccharides-induced brain toxicity

Considering MDA levels, a significant elevation of MDA ($P<0.05$) was noticed in the LPS-treated group (19.20±2.35 ng/g tissue) when compared with the control group (6.92±0.90 ng/g tissue). However, pretreatment with BCSO or its nano-form before LPS induction caused a significant reduction in MDA levels ($P<0.05$) in all animal groups. Moreover, MDA levels did not significantly differ between the groups pretreated with indomethacin (5 mg/kg) only (9.04 ±1.01 ng/g tissue) and that pretreated with BCSO followed by half indomethacin dose (2.5 mg/kg) (10.73±1.18 ng/g tissue) before LPS induction (Fig. 2).

The effects of black cumin seed oil and its nano-form on interleukin-10 in lipopolysaccharides-induced brain toxicity

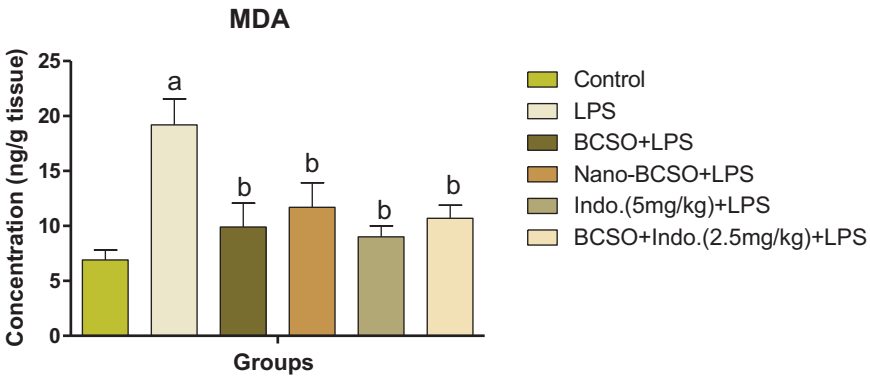
Regarding the levels of IL-10, there were no significant variations between mice in all groups. However, IL-10

Table 2 2,2-diphenyl-1-picryl-hydrazyl-hydrate scavenging effect for black cumin seed oil

Test	IC_{50} (μg/ml)
BCSO	39.24±4.26
Trolox	63.69±0.87

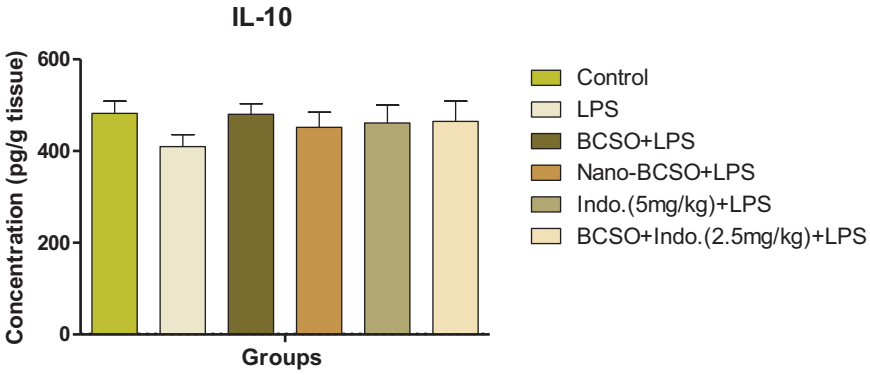
Data are displayed as mean±SD.

Figure 2



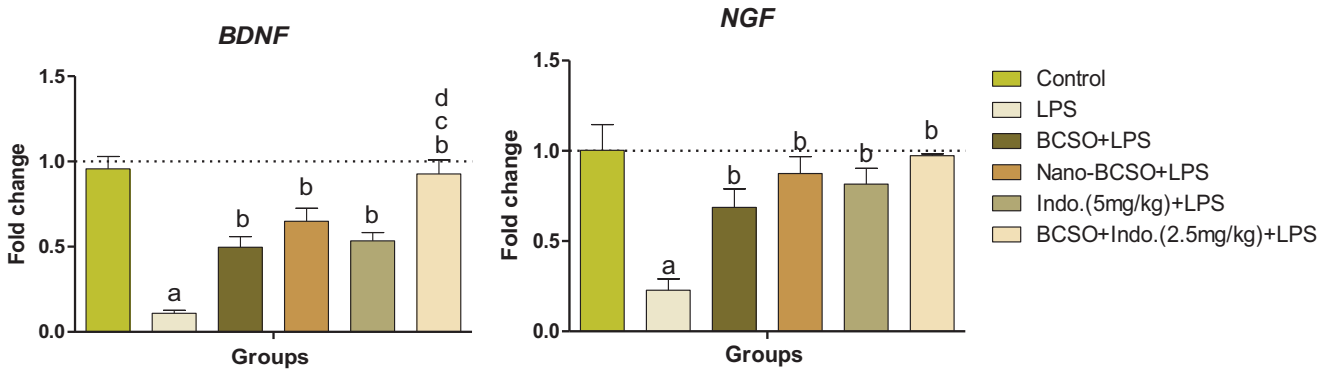
Effect of BCSO and its nano-form and/or indomethacin on lipid peroxidation (MDA) levels in LPS-induced brain toxicity. BCSO, black cumin seed oil; MDA, malondialdehyde; LPS, Lipopolysaccharides; Indo., Indomethacin. The values are expressed as mean ± SD. a, b, c, or d indicate a significant difference from control, LPS, BCSO + LPS, and indomethacin (5 mg/kg)+LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

Figure 3



Effect of BCSO and its nano-form and/or indomethacin on interleukin-10 (IL-10) levels in LPS-induced brain toxicity. BCSO, black cumin seed oil; LPS, lipopolysaccharides; Indo., Indomethacin. The values are expressed as mean ± SD. a, b, c, or d indicate a significant difference from control, LPS, BCSO + LPS, and indomethacin (5 mg/kg)+LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

Figure 4



Effect of BCSO and its nano-form and/or indomethacin on brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) gene expression levels in LPS-induced brain toxicity. BCSO, black cumin seed oil; LPS, lipopolysaccharides; Indo., Indomethacin. The values are expressed as mean fold change ± SD. a, b, c, or d indicates a significant difference from control, LPS, BCSO+LPS, and indomethacin (5 mg/kg) +LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

level in the LPS group (409.60±26.17 pg/g tissue) was lower than that in the control group (482.20±26.64 pg/g tissue) (Fig. 3).

Effects of black cumin seed oil and its nano-form on gene expression of brain-derived neurotrophic factor and nerve growth factor in lipopolysaccharides-induced brain toxicity

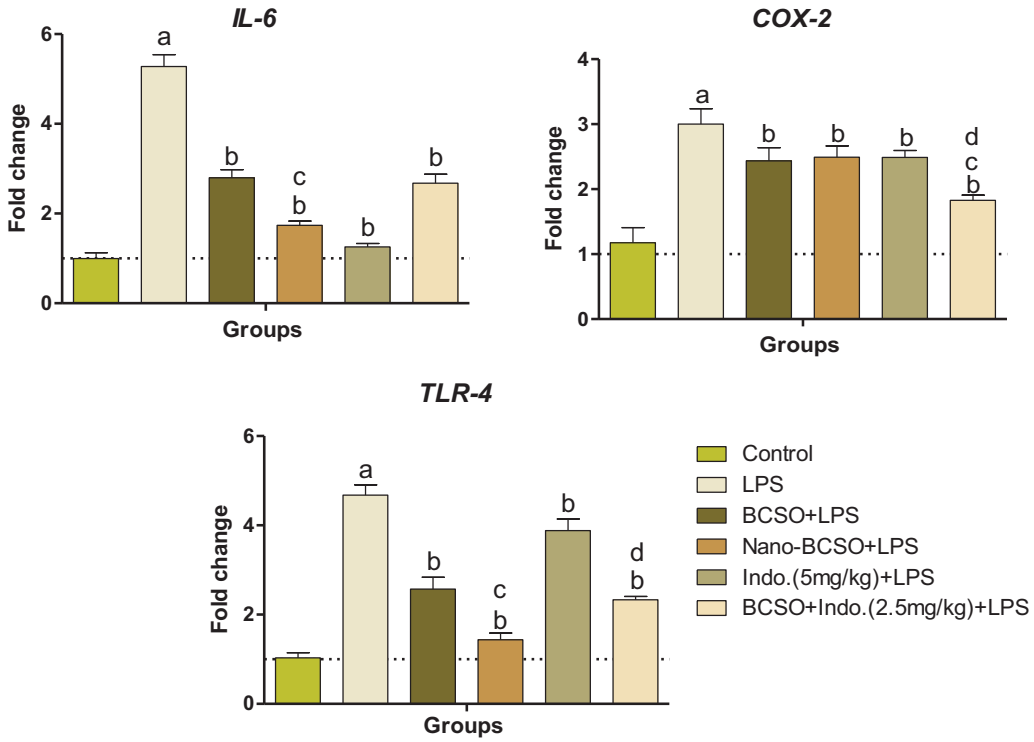
As indicated in Fig. 4, BDNF and NGF gene expression levels were significantly decreased in the LPS-treated mice compared with the normal control group. In all pretreated mice groups before LPS induction, both genes expression levels were significantly ($P<0.05$) improved. It was noticed that pretreated animals with BCSO followed by half indomethacin dose (2.5 mg/kg) showed increased BDNF and NGF expression levels more than those treated with indomethacin (5 mg/kg) only. However, this increase was significant only for BDNF expression levels.

Effects of black cumin seed oil and its nano-form on gene expression of cyclooxygenase-2, toll-like receptor-4, interleukin-6, and B-cell lymphoma-2 in lipopolysaccharides-induced brain toxicity

Regarding the expression levels of inflammation-related genes such as COX-2, TLR-4, and IL-6, LPS treatment induced an inflammatory reaction indicated by statistically significant elevation in expression levels ($P<0.05$) of these genes compared with the normal control group. In contrast, BCSO, BCSO nano-form, and indomethacin pretreatment before LPS induction diminished the inflammation through significant reduction ($P<0.05$) in these genes' expression levels compared with the LPS group. Remarkably, BCSO nano-form pretreatment significantly reduced TLR-4 and IL-6 expression levels compared with BCSO pretreatment ($P<0.05$). But both treatments showed almost the same effect on COX-2 expression levels. Furthermore, pretreatment of animals with BCSO followed by half indomethacin dose (2.5 mg/kg) declined COX-2 and TLR-4 expression levels significantly ($P<0.05$) compared with those treated with indomethacin only (5 mg/kg). However, this effect was not seen for IL-6 (Fig. 5).

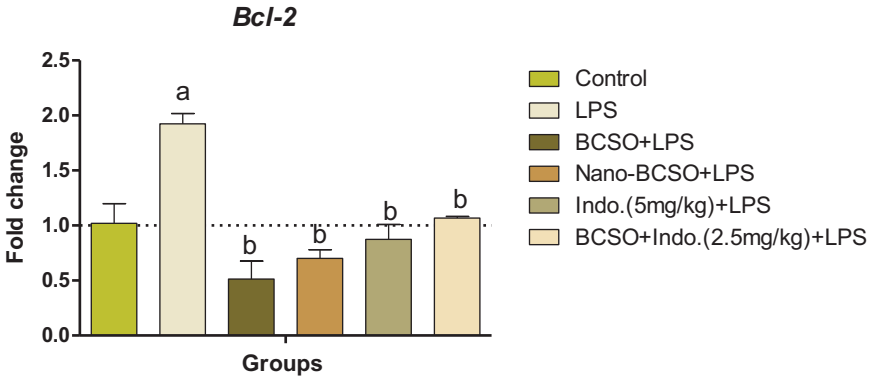
Finally, the expression level of BCL-2 was measured in the brain tissues of all animal groups, and we found that LPS treatment significantly upregulated BCL-2 expression compared with the normal control group. It was noted that BCSO, BCSO nano-form, and indomethacin pretreatment before LPS induction significantly downregulated the expression of BCL-2

Figure 5



Effect of BCSO and its nano-form and/or indomethacin on cyclooxygenase-2 (COX-2), toll-like receptor-4 (TLR-4) and interleukin-6 (IL-6) gene expression levels in LPS-induced brain toxicity. BCSO, black cumin seed oil; LPS, lipopolysaccharides; Indo., Indomethacin. The values are expressed as the mean fold change ± SD. a, b, c, or d indicates a significant difference from control, LPS, BCSO+LPS, and indomethacin (5 mg/kg)+LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

Figure 6



Effect of BCSO and its nano-form and/or indomethacin on B-cell lymphoma 2 (BCL-2) gene expression levels in LPS-induced brain toxicity. BCSO, black cumin seed oil; LPS, lipopolysaccharides; Indo., Indomethacin. The values are expressed as mean fold change ± SD. a, b, c, or d indicates a significant difference from control, LPS, BCSO+LPS, and indomethacin (5 mg/kg)+LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

($P<0.05$) in the brain tissues compared with the LPS group (Fig. 6).

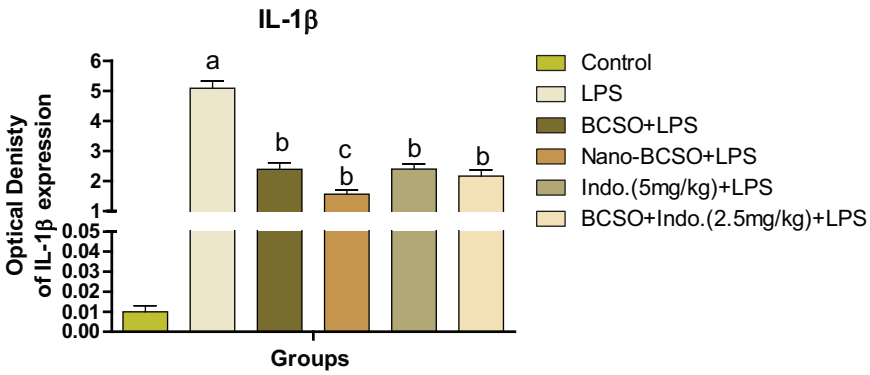
Immunohistochemical quantification of interleukin-1β

As shown in Figs 7 and 8, mice of the normal control group, BCSO, and nano-BCSO control groups showed negative expression of IL-1β in the cortical neurons (Fig. 8a–c), respectively. The LPS induction demonstrated marked expression of IL-1β in the mice cerebral cortical neurons (arrow) (Fig. 8d). Regarding the groups pretreated with BCSO, BCSO nano-form, and indomethacin before LPS induction, they showed variable degrees of marked decreased expression of IL-1β (Fig. 8e–h). The uppermost decrease was noticed in the BCSO nano-form-treated mice as designated by the quantitative analysis of the positive brown color using the image analysis software.

Histopathological examination of hematoxylin and eosin-stained brain tissue sections

As depicted in Fig. 9, microscopic examination of various sections of cerebral cortices of normal control mice (Fig. 9a), BCSO control mice (Fig. 9b), and nano-BCSO control mice (Fig. 9c) demonstrated normal histological structure of the cerebral neurons and normal organization of cerebral layers. Examination of cerebral cortices of LPS-treated mice showed intracellular edema of the cerebral neurons, marked neuronophagia, vacuolar degeneration, and nuclear pyknosis (Fig. 9d). However, pretreatment of mice with BCSO before induction with LPS showed mild degree of protection with scarce apoptotic cells and vacuolation in some neurons with few necrotic ones (Fig. 9e). Likewise, cerebral cortex of mice pretreated with BCSO nano-form or indomethacin (5 mg/kg) before induction

Figure 7



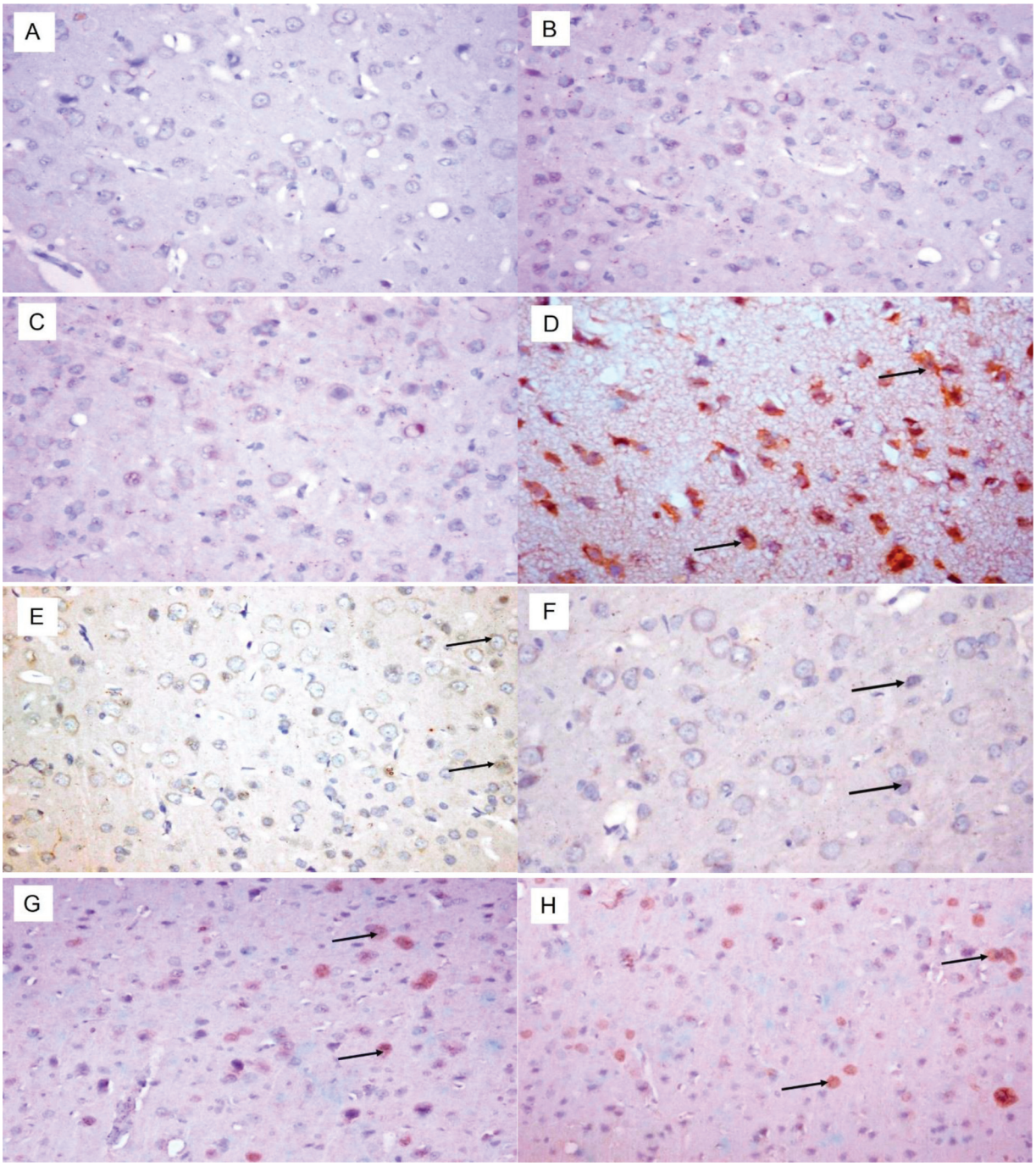
Effect of BCSO and its nano-form and/or indomethacin on interleukin-1β (IL-1β) immune expression in LPS-induced brain toxicity. BCSO, black cumin seed oil; LPS, lipopolysaccharides; Indo., Indomethacin. The values are expressed as mean ± SD. a, b, c, or d indicates a significant difference from control, LPS, BCSO+LPS, and indomethacin (5 mg/kg)+LPS groups, respectively. Results are considered significant when *P* value less than 0.05 using one-way parametric ANOVA followed by Tukey–Kramer multiple comparison post-hoc test.

with LPS showed a great degree of protection of the cerebral neurons against the action of LPS with only very few degenerated and pyknotic neurons (Fig. 9f and g), respectively. Cerebral cortices of mice pretreated with BCSO followed by half indomethacin dose (2.5 mg/kg) and induction with LPS showed few scattered neuronophagia and mild degree of neuronal cell degeneration and pyknosis (Fig. 9h).

Discussion

Previous studies have shown that stimulation of microglial cells results in the production of several

Figure 8

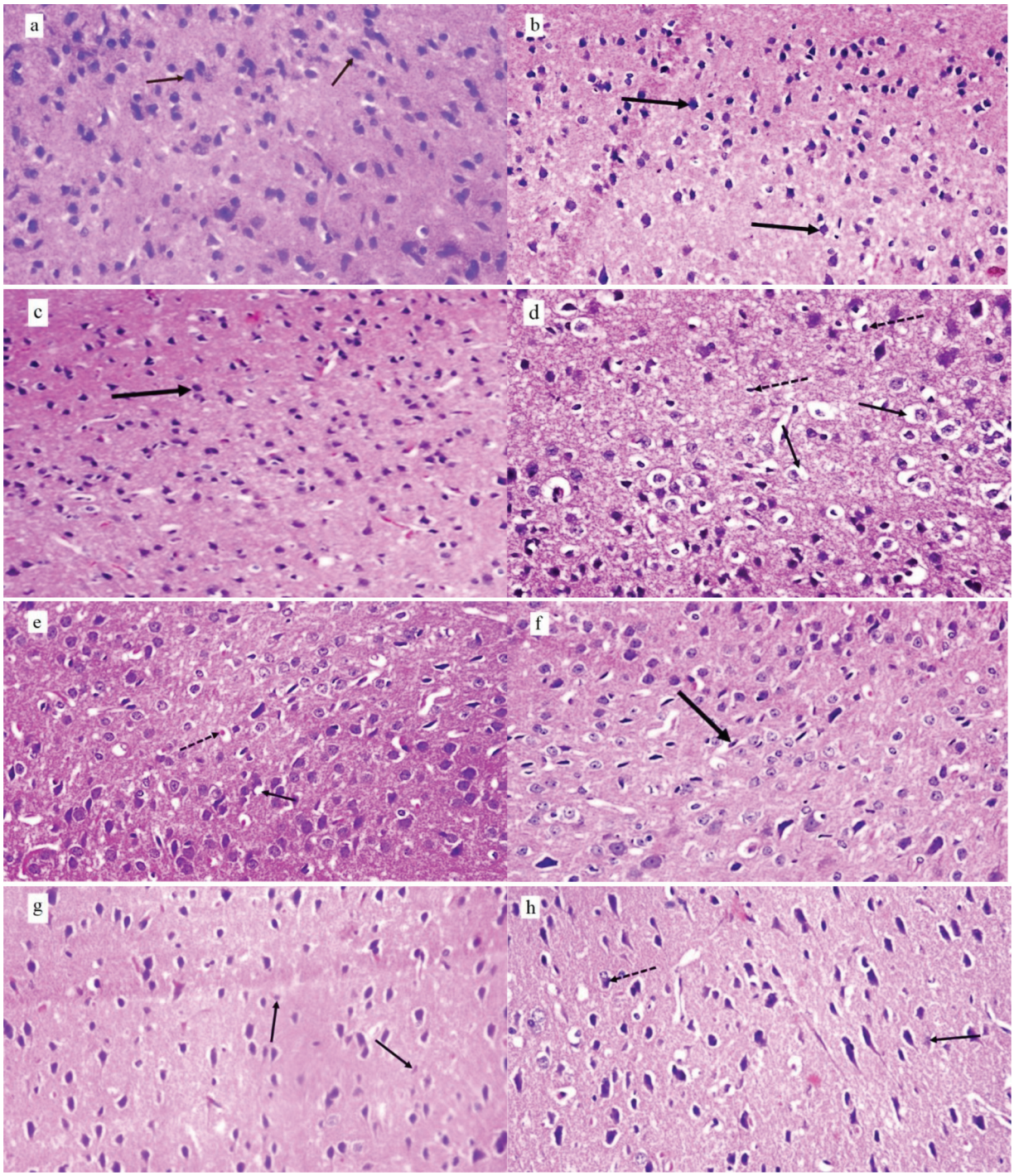


Immunohistochemical expression of IL-1β in cerebral cortices of the brain (×400). a, b, c: mice of control, black cumin seed oil, nano-black cumin seed oil groups, respectively, showed negative expression of IL-1β among the cerebral neurons. d: LPS-administrated mice showed a marked expression of IL-1β in the cytoplasm of most of the cerebral neurons (arrow). e: Black cumin seed oil followed by LPS induction mice group showed mild expression of IL-1β in scattered cerebral neurons (arrow). f: Nano-black cumin seed oil followed by LPS induction mice group showed scarce expression of IL-1β (arrow). g: Indomethacin (5 mg/kg)+LPS mice group showed moderate expression of IL-1β (arrow). h: Black cumin seed oil+indomethacin (2.5 mg/kg)+LPS mice group showed moderate expression of IL-1β (arrow). BCSO, black cumin seed oil; IL, interleukin; LPS, lipopolysaccharides.

proinflammatory cytokines, such as TNF-α, ILs, NO, and reactive oxygen species (ROS) [50,51] causing neuroinflammation and neuronal death, which has a crucial role in the etiology of some neuroinflammatory ailments like Parkinson's, Alzheimer's, and MS diseases [14,52].

In the present work, we studied the protective and anti-inflammatory effects of BCSO and its nano-form on LPS-induced inflammatory neurotoxicity in mice. Indomethacin was used as a reference anti-inflammatory drug at a dose of 5 mg/kg. Moreover, the effect of a combination of BCSO with a

Figure 9



Histopathological examination of cerebral cortices of the brain (hematoxylin and eosin, ×400). a, b, c: Mice of control, black cumin seed oil, nano-black cumin seed oil groups, respectively, showed normal histological structure of the cerebral neurons (arrow) and normal organization of cerebral layers. d: LPS-administrated mice showed intracellular edema (arrow) of the cerebral neurons and many pyknotic cells (dashed arrow). e: Black cumin seed oil followed by LPS induction group showed mild vacuolar degeneration of few neurons (arrow), scarce apoptotic cells (dashed arrow) with general good degree of protection. f: Nano-black cumin seed oil followed by the LPS induction mice group showed few degenerated and pyknotic neurons (arrow) g: Indomethacin (5 mg/kg)+LPS mice group showed good degree with only few degenerated cells (arrow). h: Black cumin seed oil+indomethacin (2.5 mg/kg)+LPS mice group showed mild degree of neuronal cell degeneration (arrow), few scattered neuronophagia (dashed arrow), and few pyknotic neurons. LPS, lipopolysaccharides.

reduced dose of indomethacin (2.5 mg/kg) was also studied.

Generation of ROS was reported in LPS-stimulated microglia [53]. Our results have showed that LPS has induced lipid peroxidation demonstrated by elevated levels of MDA. Changes in MDA levels have been indicated in several inflammatory disorders such as human immunodeficiency virus, cystic fibrosis, and acute respiratory distress syndrome [54]. Furthermore, the NADPH oxidase role was reported in LPS-stimulated microglial activation. NADPH oxidase activation was shown to increase the intracellular ROS levels, which sequentially increased the proinflammatory gene expression [55]. In the current research, significant reduction of lipid peroxidation was seen by the administration of BCSO or its nano-form. Thymoquinone, the major BCSO component, has shown antioxidant properties, which may be attributed to the redox capability of its quinone structure and the unrestricted ability of thymoquinone to go across extensive obstacles to cell niches [56]. This effect is consistent with our results in which BCSO has demonstrated good in-vitro scavenging activity against DPPH along with its impact on reducing lipid peroxidation in mice brains.

However, groups treated with BCSO alone or in combination with indomethacin demonstrated a comparable outcome. Some reporters suggested that ROS are a major contributor in gastric injuries induced by indomethacin as it has a prooxidant action [57]. The use of vitamins C and E as well as sodium selenate was found to reduce indomethacin-induced side effects on gastric mucosa, possibly due to their free radical scavenging action [58]. Other studies suggested that the induction of antioxidant enzymes through the formation of in-vivo metal complexes is another mechanism for the anti-inflammatory effect of indomethacin [59].

Notably, activated microglia also generate IL-10, a strong anti-inflammatory cytokine. In the initial phases of neuroinflammation, IL-10 is present in modest amounts, yet its role in influencing the inflammatory response is crucial. As time progresses, microglia increases the production of IL-10, contributing to the resolution of inflammation [60], which aligning with our findings.

Also, increased expression of neurotoxic and inflammatory factors, such as IL-6, TLR-4, and COX-2, in addition to IL-1β in response to LPS was found in the current work.

Focusing on the TLRs, activation of the microglial TLR-2 and TLR-4 has been related to neuroinflammation and neuronal cell death [61,62]. Therefore, governing the LPS-stimulated microglia activation through downregulation of the TLR-4 receptor and its mediated signaling pathway proteins in addition to the suppression of the production of neurotoxic proinflammatory cytokines would be an efficient therapeutic approach for neuroinflammatory ailments. The current results indicated that BCSO and its nano-form can repress the gene expression of TLR-4 with a better effect than indomethacin. A recent study has shown that indomethacin augmented the LPS-induced expression of *Nos2* gene and inducible NO synthase protein in mice brains [63].

Regarding COX-2, its production is known to be stimulated by LPS in microglial cells *in vitro* [64,65]. COX-2 is a major player in the brain inflammatory responses, and elevated COX-2 expression has been thought to promote neurodegeneration [66]. BCSO, BCSO nano-form, and indomethacin have significantly reduced COX-2 gene expression in the LPS-treated mice. However, their effect on levels of COX-2 was not prominent. There is ongoing debate on the precise role of COX-1 inhibition in systemic inflammation induced by LPS and their impacts on neuroinflammation. Indomethacin is an inhibitor of COX-1 and COX-2 with higher selectivity toward COX-1 [67]. There was evidence indicating that COX-1 and COX-2 have separate roles in the brain in comparison to peripheral tissues. Both COX-1 and COX-2 are constitutively expressed in the brain. COX-1 is mainly expressed in the microglia and can be stimulated in the endothelium in brain injuries [68]. COX-1 has an inflammatory role in the brain, which was indicated by the lower inflammatory reaction after intracerebral injection of LPS in COX-1 lacking mice in comparison to the wild-type mice [69]. COX-2 is specifically expressed in the cortical glutamatergic neurons and the hippocampus [70]. In spite of the direct neurotoxic effects of COX-2, there are some implications that the effect seen in the brain after injection of LPS may be attributed to a role of COX-1 as well [71].

Furthermore, LPS caused significant decrease in BDNF and NGF mRNA expression in animal brains. Many studies linked BDNF and NGF with neuronal maintenance and survival, in addition to the regulation of neurotransmitters. It was found that patients with neurodegenerative disorders have decreased BDNF levels in their brains. The

abnormal BDNF concentrations may be attributed to the inflammatory state of the brain [72]. Moreover, a marked reduction in *NGF* gene expression was described in the cerebral cortex of experimental allergic encephalomyelitis rat model [73].

The anti-inflammatory agents may provide protection for the neuronal cells through the inhibition of microglial stimulation [74]. In the present study, BCSO and its nano-form have reduced LPS-induced inflammation and showed a comparable or better effect than indomethacin. This effect was established through downregulation of COX-2, TLR-4, and IL-6 mRNA. The studies have shown that the black cumin seed extracts have reduced inflammation in rat glial cells [75]. Likewise, black cumin seed and its oil have diminished inflammation in LPS-treated rats [76]. Moreover, thymoquinone, the primary active constituent of BCSO, was shown to reduce LPS toxicity in stimulated microglial cells [77] through reducing cytokines such as NF-κB, TNF-α, IL-1β, IL-6, and IL-10 [78], lipoxygenase [79], and COX-2 [80] activities.

Our findings showed a high expression of the antiapoptotic gene BCL-2 in LPS-inflamed brain tissues. BCSO and its nano-form caused downregulation of BCL-2. This finding is most likely explained by the fact that different cytokines and signaling molecules affects the regulation of BCL-2. For instance, minimal concentrations of LPS activate TLR-2 and TLR-4 receptors, which in turn triggers mast cell production of IL-13, which raises the expression levels of BCL-2 [81]. It was recorded that BCL-2 inhibits NF-κB activation and consequently the upregulation of proinflammatory genes. Correspondingly, the dual role of BCL-2 as antiapoptotic and anti-inflammatory accounts for its cytoprotective function [82].

Notably, the LPS-induced histopathological modifications in mice brains were alleviated by BCSO and its nano-form. Thus, the histological studies have supported the biochemical and molecular effects shown in the current work.

The limitations of therapeutic substances to get to the CNS restricts the effectiveness of noninvasive treatment for neurological disorders. So far, various nano-forms have been developed and used to treat neurological disorders [83]. The thymoquinone nanoparticles were used for brain targeting, and they displayed more brain targeting than conventional thymoquinone [84]. The biochemical evaluation indicated that thymoquinone nanoparticles demonstrated neuroprotective effects in rats with occluded cerebral arteries through the reduction of lipid peroxidation and raising the antioxidant enzymes [85]. In this work, we have prepared a nano-emulsion form of the BCSO and evaluated this nano-form for its protective effect against LPS-induced neurotoxicity. We found that BCSO nano-form showed a promising neuroprotective potential. The uppermost effect was noticed in reducing IL-1β, TLR-4, and IL-6 expression in mice brains. Also, the histopathological examination of mice cerebral cortices showed a great degree of protection of the cerebral neurons against the action of LPS in those treated with the BCSO nano-form.

Conclusion

In a nutshell, BCSO pretreatment has contributed to improve LPS-induced brain injury. The BCSO has shown its ameliorative influence through its positive effects on lipid peroxidation and inflammatory markers. The BCSO has suppressed the inflammatory response induced by LPS with comparable or better effect than indomethacin. Moreover, a combination of BCSO with a reduced dose of indomethacin can be recommended as an anti-inflammatory supplement to protect against NSAIDs side effects.

Furthermore, BCSO nano-form has exerted a neuroprotective effect against LPS-induced inflammatory brain injury, which may offer a useful neuroprotective brain targeting tool. The biochemical, molecular, and histopathological results suggested BCSO and its nano-form may be a potential immunomodulator for various neurodegenerative ailments.

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Conflicts of interest

There are no conflicts of interest.

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