Original Article

Asian Pacific Journal of Tropical Biomedicine

apjtb.org

doi: 10.4103/2221-1691.393578

Foeniculum vulgare Mill. inhibits lipopolysaccharide-induced microglia activation and ameliorates neuroinflammation-mediated behavioral deficits in mice

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ABSTRACT

Objective: To investigate the effect of *Foeniculum vulgare* extract against lipopolysaccharide (LPS)-induced microglial activation *in vitro* as well as cognitive behavioral deficits in mice.

Methods: LPS-activated BV-2 cell viability was measured using MTT assay and reactive oxygen species (ROS) was studied using DCF-DA assay. The antioxidative enzymes and pro-inflammatory mediators were analyzed using respective ELISA kits and Western blotting. For *in vivo* testing, LPS (1 mg/kg, *i.p.*) was given daily for five days in male Swiss albino mice to produce chronic neuroinflammation. Cognitive and behavioral tests were performed using open-field, passive avoidance, and rotarod experiments in LPS-induced mice.

Results: *Foeniculum vulgare* extract (25, 50 and 100 μg/mL) significantly attenuated the LPS-activated increase in nitric oxide (NO), ROS, cyclooxygenase-2, inducible NO synthase, IL-6, and TNF-alpha ($P < 0.05$). Moreover, LPS-induced oxidative stress and reduced antioxidative enzyme levels were significantly improved by *Foeniculum vulgare* extract ($P < 0.05$). The extract also regulated the NF-κB/MAPK signaling in BV-2 cells. In an *in vivo* study, *Foeniculum vulgare* extract (50, 100, and 200 mg/kg) markedly mitigated the LPS-induced cognitive and locomotor impairments in mice. The fingerprinting analysis showed distinctive peaks with rutin, kaempferol-3-*O*-glucoside, and anethole as identifiable compounds.

Conclusions: *Foeniculum vulgare* extract can ameliorate LPS-stimulated neuroinflammatory responses in BV-2 microglial cells and improve cognitive and locomotor performance in LPS-administered mice.

KEYWORDS: *Foeniculum vulgare*; Microglia; Lipopolysaccharide; Antioxidant; Neuroinflammation; MAPK signaling; Cognition

1. Introduction

Neuroinflammation, although largely protective, is implicated in numerous conditions that affect the brain and nervous system. Mounting evidence indicates that long-term inflammation of the central nervous system (CNS) is a major contributor to the development of neurodegenerative disorders[1,2]. Neuroinflammatory responses involve the activation of microglia, which are immunological cells residing in the CNS, releasing large amounts of cytokines, abnormally high production of reactive oxygen species (ROS), and a depleted antioxidative protective mechanism[2]. Activated microglia release mediators that have a

Significance

Foeniculum vulgare Mill., is a tropical herb used in Asian and Mediterranean countries with immense pharmacological benefits. However, the effects of this herb on neuroinflammation were not elaborately studied. In this study, *Foeniculum vulgare* aqueous extract significantly attenuated LPS-stimulated neuroinflammation in BV-2 cells by regulating the NF-κB/MAPK pathways. It also ameliorated the LPS-induced cognitive and locomotor impairments in mice, indicating its potential in the management of neuroinflammation.

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How to cite this article: Koppula S, Alluri R, Kopalli SR. *Foeniculum vulgare* Mill. inhibits lipopolysaccharide-induced microglia activation and ameliorates neuroinflammation-mediated behavioral deficits in mice. Asian Pac J Trop Biomed 2024; 14(1): 28-39.

Article history: Received 12 September 2023; Revision 19 October 2023; Accepted 26 December 2023; Available online 23 January 2024

role in the death of neurons in a number of neurological disorders[1]. Furthermore, microglia activation causes neuroinflammatory reactions, neuronal injury, and cognitive impairment[3]. Therefore, proper regulation of microglia activation and inhibiting molecules that respond to inflammation is considered a major therapeutic criterion in the management or prevention of neurodegenerative and neuroinflammatory disorders[4]. Although clinically important anti-inflammatory drugs, including steroidal and non-steroidal agents, possess potent beneficial effects, long-term exposure might cause serious adverse pharmacological effects. Therefore, substituting with natural products with fewer or no side effects might be an ideal therapeutic approach in the treatment of neuroinflammation-mediated neurodegenerative disorders[5].

Foeniculum vulgare (*F. vulgare*) Mill., from the family Apiaceae, is a tropical aromatic herb cultivated in several countries, including Asia and Mediterranean regions[6]. Traditionally, the fruit extract of *F. vulgare* is known to exhibit carminative, digestive, and diuretic properties[7]. Furthermore, the infusions made by *F. vulgare* fruits are used in the treatment of menstrual problems, and endocrine, reproductive, and respiratory ailments, and as a galactagogue for lactating mothers[6,7]. Due to its aromatic nature, it is used in culinary practices as an additive and a seasoning ingredient in various food products and cosmeceutical preparations[8]. Ethnopharmacologically, *F. vulgare* has been reported to possess various properties, including antibacterial, antipyretic, antispasmodic, hepatoprotective, hypoglycemic, anticancer, anti-inflammatory, antimutagenic, and antinociceptive properties[6,8]. In relation to its beneficial effects on CNS disorders, *F. vulgare* was reported to reduce stress, enhance memory, and potently attenuate stress-induced disorders in experimental rats[9]. Phytochemical investigations of the whole *F. vulgare* herb indicated the presence of flavonoids, polyphenols, carotenoids, minerals, vitamins, and essential oils as major chemical components. In addition, the fruits or seeds of *F. vulgare* possess phytochemically active constituents like estragole, anethole, alpha-phellandrene, fenchone, and aglycons[7].

Despite the numerous biological effects present in *F. vulgare*, the anti-neuroinflammatory and cognitive-enhancing properties of regulating neuroinflammatory microglial activation and the underlying mechanisms were not elaborately studied. Lipopolysaccharide (LPS), the component found in the external walls of Gram-negative bacteria has been shown to activate microglial cells and is considered a valid approach to investigate the effectiveness of natural substances in attenuating neuroinflammation-mediated neurodegenerative disorders[10,11]. Moreover, administration of LPS is known to induce neuroinflammatory responses in microglial cells *in vitro* and impair behavioral and cognitive functions in experimental animal models when given as a systemic injection, intracerebral microinjection, or continuous infusion[11]. Furthermore, it has been suggested that oxidative damage to brain tissue plays a significant role in memory and behavioral problems caused by LPS[12].

In view of the above literature, in the present work, the anti-neuroinflammatory properties of *F. vulgare* and its underlying mechanism were investigated *in vitro* using LPS-stimulated BV-2 microglial cells and *in vivo* using LPS-mediated cognitive behavioral impairments in mice.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), LPS, and thiobarbituric acid (TBA) were acquired from Sigma-Aldrich, St. Louis, MO, USA. The supplier of Dulbecco's Modified Eagle Medium (DMEM) was from Invitrogen, Carlsbad, CA, USA. Fetal bovine serum (FBS) was obtained from Hyclone, Logan, UT, USA. Every other reagent employed in the study was of the highest grade available commercially.

2.2. *F. vulgare* extract preparation and high–performance thin layer chromatography (HPTLC)

F. vulgare dried fruit was purchased from M/s Laila Impex in Vijayawada, India, and its validity was confirmed by taxonomists at the Chemiloids R & D Center in India. The material was deposited in the herbarium of our department (specimen no: FVE-2022). Extraction procedures were carried out in accordance with the method described in our previous article[9]. *F. vulgare* fruit extract had a final yield of 12.14% and was readily soluble in distilled water for use in further studies. In order to perform HPTLC fingerprint analysis, a quantity of 200 mg of *F. vulgare* fruit extract was accurately weighed and thereafter transferred into a 10 mL volumetric flask. The flask was then filled to the mark with methanol, and the resulting solution was subjected to sonication to facilitate complete dissolution of the *F. vulgare* fruit extract in the methanol solvent. The solution was passed through a membrane filter with a pore size of 0.45 μm, and then collected in an autosampler vial. HPTLC analysis was conducted using a Camag automated TLC sampler 4 (ATS 4) equipped with a Camag TLC scanner 4 device and WinCATS software. To facilitate identification, the mobile phase was transferred into a stationary phase (Silica Gel 60F254; 10 cm × 10 cm) TLC chamber. The system was then allowed to reach equilibrium at room temperature for 30 min. A 15 μL sample was applied to the TLC plate, and the plate was developed in the mobile phase [ethyl acetate:methanol:water (8.2:1:0.8)] inside the TLC chamber about 8 cm from the application point. Following the development process, the TLC plate was dried to eliminate any residual solvents. Subsequently, the plate was subjected to examination under UV light at a wavelength of 254 nm.

2.3. Cell culture and viability assessment

BV-2 microglia cells were grown in DMEM with 5% FBS and 1% penicillin/streptomycin (Invitrogen) in a 5% CO_2 incubator at 37℃. After seeding cells at a density of 5×10^4 cells/mL in serum-free DMEM, they were pretreated with *F. vulgare* fruit extract at concentrations ranging from 10 to 200 μg/mL for 1 h before LPS (1 μg/mL) was added. All control treatments received an equivalent amount of sterile water. The MTT assay was used to evaluate cell viability. *F. vulgare* fruit extract at the indicated concentrations (10-200 μg/mL) was subjected to incubation for 24 h, followed by further incubation in dark at 37℃ with MTT for an additional 2 h. The solution was gently removed from the wells and the resulting blue formazan product was dissolved in DMSO. The experiment was repeated thrice and the absorbance was measured at a wavelength of 570 nm using a microplate reader (Tecan Trading AG, Switzerland).

2.4. Nitric oxide (NO) determination

The quantification of NO generation was conducted by evaluating the concentrations of nitrite in the supernatant by a colorimetric reaction employing Griess reagent[13]. Briefly, BV-2 cells seeded at a density of 2×10^5 cells/mL in 500 μL of complete culture media in 6-well plates were treated with varying concentrations of *F. vulgare* fruit extract for 1 h. Following the pre-treatment, the cells were stimulated with LPS (1 μg/mL) for 2 h. The supernatant was removed (50 μL) and was reacted with an equivalent amount of Griess reagent. The concentrations of nitrite were measured using standard solutions of sodium nitrite that were made in the growth medium. Employing a PowerWavex Microplate Scanning spectrophotometer (Bio-Tek Instrument, Winooski, VT, USA), the absorbance at 540 nm was measured.

2.5. Intracellular ROS detection

ROS production was quantified using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay (Sigma, St. Louis, MO, USA). For the assay, BV-2 cells were treated with *F. vulgare* fruit extract (25, 50, and 100 μg/mL) and LPS. Following the treatment, DCF-DA (20 μM) was added to cells and incubated for 30 min at 37℃. Using trypsin, cells were separated thoroughly and washed with PBS thrice (pH 7.4). ROS production was measured by a fluorescence microplate reader (SpectraMax Gemini EM; Molecular Device) at excitation and emission wavelengths of 485 nm and 538 nm.

2.6. Measurement of antioxidant enzyme levels

In the *in vitro* experiment, BV-2 cells (1×10^5 cells/well) were treated with *F. vulgare* fruit extract (25, 50, and 100 μg/mL) for 1 h and exposed to LPS (1 μg/mL) for 4 h. The liquid portion was carefully removed and the expressions of catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) were tested using kits available commercially according to the manufacturer's instructions (Sigma Aldrich). For antioxidant enzyme activity assay in mouse brain homogenates, the level of SOD and malondialdehyde (MDA) were measured using the respective commercial assay kits (Sigma Aldrich). The SOD activity was measured as U/mg protein and the MDA activity as μmol/g protein.

2.7. Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 production

Briefly, BV-2 cells were treated with *F. vulgare* fruit extract (25, 50, and 100 μg/mL) for 1 h, and then they were exposed to LPS (1 μg/mL) for 4 h. The supernatants were collected and assessed for TNF-α and IL-6 levels using the appropriate ELISA kits and following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA).

2.8. Western blotting

The BV-2 cells were subjected to a series of washes using cold PBS and then lysed in a buffer solution consisting of 50 mM Tris-HCl at pH 7.4, with a 1% (*v/v*) concentration of NP-40, 0.25% of sodium deoxycholate, 150 mM concentration of NaCl, 1 mM concentration of EDTA, 25 mM concentration of NaF, 2 mM concentration of Na_3VO_4 and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) at 4℃. To eliminate insoluble components, the lysate underwent clarification using centrifugation at 10 000 ×*g*. The protein content of cell lysates was standardized by using a BCA reagent (Pierce, Rockford, IL, USA). Proteins were separated using the standard 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (S&S, Dassel, Germany). A 5% skim milk was used to block the membrane to avoid non-specific binding and the blots were incubated with primary antibodies against the target proteins that detect iNOS (#SAB5700636, Sigma Aldrich, 1∶2 000), COX-2 (#SAB5700833, Sigma Aldrich, 1∶2 000), NF-κB (F-6 #sc-8008, Santa Cruz Biotechnology, 1∶1 000), IκB-α (#4812S, cell signaling, 1∶1 000), phospho (p)-IκB-α (#2859S, cell signaling; 1∶1 000), β-actin (#C4; Santa Cruz biotechnology, 1∶10 000), JNK (#9253S, cell signaling, 1∶1 000), p-JNK (#9251S, cell signaling, 1∶1 000) ERK (#91012S cell signaling, 1∶1 000), p-ERK (#9101S, cell signaling, 1∶1 000), p38 (#9212S, Cell signaling, 1∶1 000), and p-p38, (#9201S, Cell signaling, 1∶1 000). After incubation, horseradish peroxidase-conjugated secondary antibodies (1∶2 000) were incubated for 1 h at room temperature. The blots were normalized using β-actin. The blots were identified using chemiluminescence on the West-Save substrate (Lab-Frontier, Seoul, Korea) on X-ray film. The immunoreactive bands were imaged using AlphaImager gel documentation system (FluorChem E; Cell Biosciences, USA).

2.9. Animals

Male Swiss albino mice, aged 4-6 weeks, weighing between 20-25 g were acquired from Vishnu Institute of Pharmaceutical Education and Research, Hyderabad, India. They were housed in cages within an air-conditioned animal room maintained at a temperature of (23 ± 2)℃ with a 12 h light/dark photoperiod. The humidity in the room was maintained at 40%. The mice had unrestricted access to both food and water and acclimatized to the lab environment for 7 d.

2.10. Experimental design

Mice were randomly divided into six groups (n = 10), *i.e.* vehicle, LPS, *F. vulgare* fruit extract (200 mg/kg), LPS plus *F. vulgare* fruit extract 50 mg/kg, LPS plus *F. vulgare* fruit extract 100 mg/kg and LPS plus *F. vulgare* fruit extract 200 mg/kg. In order to produce chronic neuroinflammation, mice were injected with LPS (1 mg/kg, *i.p.*) for 5 d in a row (day 1-5) as described previously[14,15]. On day 1 (1 h before LPS administration), selected doses of *F. vulgare* fruit extract (50, 100, and 200 mg/kg) were given orally by gavage (*p.o.*) for 21 d. The dosages were made freshly by dissolving in distilled water. The doses of *F. vulgare* fruit extract were chosen based on earlier reports[9]. The locomotor behavioral paradigm was assessed after the injection of lipopolysaccharide (LPS) on day 4, as well as during the final phase of the investigation on day 20. On days 5 and 21, we conducted an open-field test to evaluate the cognitive paradigm. On days 20 (acquisition) and 21 (retention), the passive avoidance test (PAT) was conducted. Following the behavioral tests, mice were sacrificed by cardiac perfusion, and the brain tissues were extracted as previously reported[16]. In brief, brain tissues were thoroughly washed and homogenized using ice-cold PBS (pH 7.4). Subsequently, the resulting mixture was subjected to centrifugation (1 200 ×g) at −4℃ for 15 min. Aliquots of homogenates were utilized for the estimation of antioxidant enzyme levels.

2.11. Rotarod test

Coordination of mice locomotory behavior was evaluated through the utilization of the rotarod test (Acceler Rota-Rod 7650, Ugo, Basile, Varese, Italy), with slight modifications as previously described[17]. The experiment measures the length of time (s) mice can stay balanced on an accelerating rotating rod that is 3 cm in diameter at a speed of 30 rpm for a maximum of 5 min. The experiment was carried out on days 4 and 20, during which the mean time for each mouse was determined and then used for comparative analysis.

2.12. PAT

The effects of *F. vulgare* fruit extract on cognitive abilities were evaluated *via* the use of a PAT apparatus (GEMINI, Model PACS-30, San Diego, USA). Minor adjustments were made to the apparatus in accordance with previously reported protocols[18]. Briefly, the animals underwent acclimatization in the PAT apparatus for 2-3 min. The acquisition trial was executed by positioning the mouse inside the illuminated chamber for a specific duration (30 s), after which the guillotine door was opened with the assistance of a computer. Upon the entry of mice into the dark chamber, the door was immediately closed, followed by the administration of an electrical foot shock with an intensity of 1 mA *via* the grid flooring, lasting for a duration of 3 s. Twenty-four hours following the acquisition trial, the mice were carefully positioned within the light chamber, and the latency time (s) for the mice to enter the dark chamber was recorded for a duration of 600 s (retention trial). The animals that remained in the light chamber for the entire duration were given a latency time value of 600 s.

2.13. Open-field test

The open-field test apparatus (Coulbourn Instruments L.L.C., Holliston, MA, USA) constituted a wooden box containing a 60 cm × 60 cm × 60 cm square arena divided into 16 squares—four in the center and twelve along the sides. The mouse was placed in the middle of the arena and examined for 5 min for line crossings, rearing, grooming, and immobility as described previously[19]. Following every test, cotton pads were used to wipe out the equipment with 70% ethanol.

2.14. Statistical analysis

Results are shown as mean ± standard error of mean (SEM). The data were computed using one-way ANOVA, followed by Dunnett's tests (GraphPad Software Inc., San Diego, CA, USA). A statistically significant value was defined as $P < 0.05$.

2.15. Ethical statement

Institutional Animal Ethics Committee (Regd.No: 1358/ERe/S/10/CPCSEA), Hyderabad, India, approved all animal research to ensure compliance with globally recognized ethical standards.

3. Results

3.1. HPTLC fingerprinting profile of F. vulgare fruit extract

The results obtained from the HPTLC analysis of the *F. vulgare* fruit extract revealed the presence of five distinct peaks, each exhibiting different retention factor (Rf) values (range: 0.01 to 0.60). However, peaks 1, 3, and 5 exhibited clear distinctions and

were successfully characterized as rutin (Rf: 0.04), kaempferol-3-*O*-glucoside (Rf: 0.37), and anethole (Rf: 0.52), respectively (Figure 1A).

3.2. Effect of *F. vulgare* fruit extract on LPS-stimulated BV-2 cell viability, NO, and ROS production

In order to determine the appropriate concentration of *F. vulgare* fruit extract and its cytotoxicity to BV-2 microglial cells, we conducted preliminary studies in which we examined the impact of *F. vulgare* fruit extract at different concentrations (10, 25, 50, 100, and 200 μg/mL) with or without LPS (1 μg/mL). Although 200 μg/mL of *F. vulgare* fruit extract showed mild cytotoxicity, *F. vulgare* fruit extract up to 100 μg/mL with or without LPS did not influence the overall cell viability or exhibit any signs of cytotoxicity to BV-2 microglia cells (Figure 1B). As a result, concentrations of 25, 50, and 100 μg/mL were utilized in subsequent tests. Furthermore, the NO level released in the medium measured by Griess assay showed a significant ($P < 0.001$) increase in LPS-treated BV-2 cells when compared with control cells [(2.69 ± 0.67) μM *vs.* (34.43 ± 0.91) μM]. Pretreatment with *F. vulgare* fruit extract (25, 50, and 100 μg/mL) reduced NO generation significantly ($P < 0.05$) in a dose-dependent manner (Figure 1C). Additionally, a fluorescence-based ROS assay was conducted to determine the ROS production in BV-2 cells. Treatment with *F. vulgare* fruit extract significantly ($P < 0.05$) suppressed the increased percentage of ROS production caused by LPS stimulation in BV-2 cells (Figure 1D).

3.3. Effect of *F. vulgare* fruit extract on iNOS and COX-2

LPS stimulation increased the iNOS and COX-2 expression in BV-2 cells. *F. vulgare* fruit extract markedly suppressed the iNOS and COX-2 protein levels (Figure 2A). Relative intensities (percentage of LPS) of iNOS were reduced to (84.16 ± 0.89)%, (35.24 ± 4.23)% and (29.14 ± 3.69)%, and COX-2 levels to (99.06 ± 3.10)%, (86.78 ± 3.64)%, and (54.13 ± 2.95)% at 25, 50 and 100 μg/mL, respectively compared with the group treated with LPS (Figure 2A).

3.4. Effect of *F. vulgare* fruit extract on NF-κB and IκB-α expression

To further understand the underlying mechanisms of *F. vulgare* fruit extract, NF-κB and IκB-α levels were estimated in LPS-induced BV-2 cells (Figure 2B). LPS treatment significantly ($P < 0.001$) increased the NF-κB activation and phosphorylation of IκB-α. However, *F. vulgare* fruit extract treatment markedly inhibited NF-κB activation and phosphorylation of IκB-α in a dose-dependent

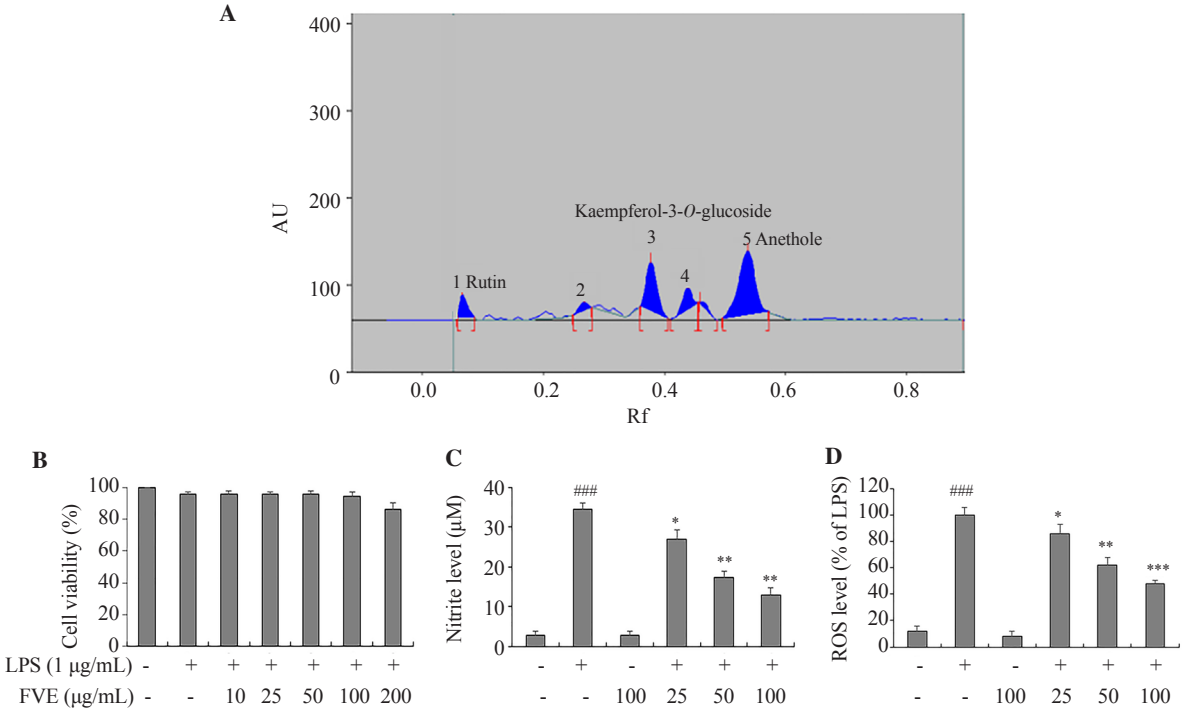

Figure 1. HPTLC fingerprinting analysis of *Foeniculum vulgare* extract, and cell viability, nitric oxide, and ROS assay in LPS-stimulated BV-2 cells. (A) HPTLC chromatogram of *Foeniculum vulgare* extract shows five major peaks of phytoconstituents with peaks 1, 3, and 5 identified as rutin, kaempferol-3-*O*-glucoside and anethole, respectively. (B) Cell viability was evaluated using the MTT assay and shown as a percentage of control, (C) nitrite levels were measured using the Griess reaction, and (D) ROS production was measured using DCF fluorescence assay. Data are expressed as mean ± SEM (n=6) and analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group. HPTLC: high-performance thin layer chromatography, AU: area under curve, Rf: retention factor, ROS: reactive oxygen species, LPS: lipopolysaccharide, FVE: *Foeniculum vulgare* extract.

manner ($P < 0.01$) (Figure 2B).

3.5. Effect of *F. vulgare* fruit extract on pro–inflammatory cytokines

In order to evaluate the effect of *F. vulgare* fruit extract on the generation of pro-inflammatory cytokines in LPS-stimulated BV-2 microglial cells, IL-6 and TNF-α levels were determined. A significant ($P < 0.001$) increase in the expression of IL-6 and TNF-α was observed in LPS-stimulated BV-2 cells. *F. vulgare* fruit extract (50 and 100 μg/mL) significantly ($P < 0.05$) suppressed the increased expression of IL-6 and TNF-α in LPS-stimulated BV-2 cells (Figure 2C and D).

3.6. Effect of *F. vulgare* fruit extract on antioxidative enzyme status

To determine the effects of *F. vulgare* fruit extract on antioxidative enzyme status in LPS-stimulated BV-2 cells, ELISA was applied to assess the levels of GSH, CAT, and SOD (Figure 3). The levels of GSH, CAT and SOD were significantly ($P < 0.001$) decreased compared with the control group, indicating that LPS stimulation caused oxidative damage to BV-2 cells. However, *F. vulgare* fruit extract at the indicated concentrations attenuated the LPS-induced altered expression (fold change) of GSH (0.82 ± 0.06, 0.89 ± 0.03 and 0.94 ± 0.02), CAT (0.75 ± 0.03, 0.79 ± 0.05 and 0.91 ± 0.07) and SOD (0.76 ± 0.04, 0.87 ± 0.06 and 0.92 ± 0.03) significantly ($P < 0.05$) in BV-2 cells (Figure 3). These results indicated that *F. vulgare* fruit extract could reverse LPS-stimulated oxidative damage in BV-2 cells.

3.7. Effect of *F. vulgare* fruit extract on the MAPK signaling

To examine whether the *F. vulgare* fruit extract regulates pro-inflammatory cytokine production in LPS-stimulated BV2 cells *via* the abrogation of the MAPKs signaling pathway, the phosphorylation of JNK, p38, and ERK MAPKs was measured by Western blot analysis. The increased expression of JNK, p38, and ERK phosphorylation was significantly reduced by *F. vulgare* fruit extract treatment (Figure 4A). Relative intensities (percentage of

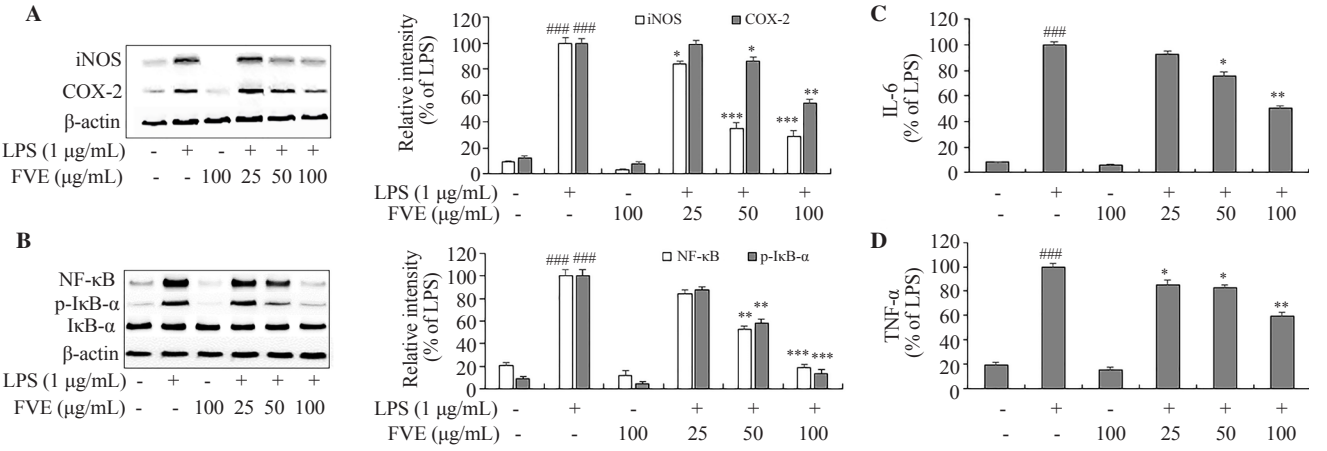

Figure 2. Effect of *Foeniculum vulgare* extract on pro-inflammatory cytokine expression in LPS-stimulated BV-2 cells. (A-B) The expression levels of iNOS, COX-2, NF-κB, and IκB-α in LPS-stimulated BV-2 cells were evaluated by Western blot analysis and normalized against β-actin. (C-D) IL-6 and TNF-α levels were measured using ELISA kits. Data are expressed as mean ± SEM (n=6) and analyzed by ANOVA followed by the Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group. iNOS: inducible nitric oxide, COX-2: cyclooxygenase-2, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, NF-κB: nuclear factor kappa-B, IκB-α: inhibitor of nuclear factor kappa B.

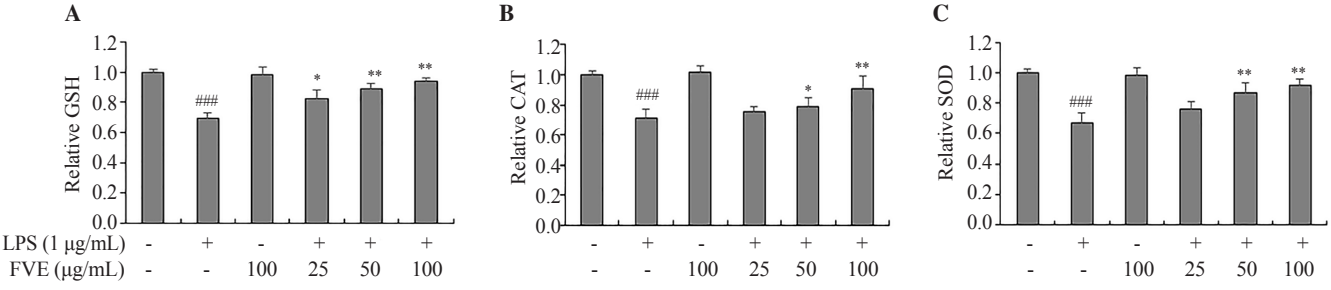

Figure 3. Effect of *Foeniculum vulgare* extract on (A) GSH, (B) CAT, and (C) SOD activities in LPS-stimulated BV-2 microglial cells. Data are expressed as mean ± SEM (n=6) and analyzed by ANOVA followed by the Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs.* the LPS-treated group. GSH: glutathione, CAT: catalase, SOD: superoxide dismutase.

LPS) showed that *F. vulgare* fruit extract significantly ($P < 0.05$) attenuated the increased levels of JNK [(89.15 ± 3.15)%, (62.23 ± 3.68)% and (15.21 ± 2.95)%], p38 [(92.11 ± 3.61)%, (74.02 ± 2.94)% and (21.18 ± 2.54)%], and ERK [(81.24 ± 6.12)%, (76.68 ± 4.82)% and (17.21 ± 5.45)%] at 25, 50 and 100 μg/mL, respectively (Figure 4B-4D). *F. vulgare* fruit extract exhibited a potent anti-neuroinflammatory response *via* inactivation of JNK, p38, and ERK MAPK signaling pathways.

3.8. Effect of F. vulgare fruit extract on locomotory behavior in mice

Compared with the control group mice, the LPS-induced group significantly ($P < 0.001$) reduced locomotor activity by reducing retention time on day 4 [(49 ± 6) s] and day 20 [(59 ± 8) s]. *F. vulgare* fruit extract at different dosages did not affect the LPS-induced locomotor impairments on day 4. In contrast, the LPS-induced reduced retention time was increased in a dose-dependent manner on day 20 [(93 ± 5) s, (112 ± 7) s, and (132 ± 10) s] at 50, 100 and 200 mg/kg, respectively by *F. vulgare* fruit extract (Figure 5A).

3.9. Effect of F. vulgare fruit extract on LPS–induced PAT in mice

In the acquisition trial, when the foot-shock stimulus was absent, the delay associated with entering the dark chamber was insignificant across all groups. When compared to the acquisition trial [(49 ± 11) s] in the control group, the delay time was significantly ($P < 0.001$) higher in the retention trial [(254 ± 25) s]. A statistically significant ($P < 0.001$) decrease in latency time to [(117±14) s] was seen after LPS treatment compared to the control group. When compared to the LPS-induced group, the latency time increased significantly ($P < 0.05$) after administration of *F. vulgare* fruit extract at the specified

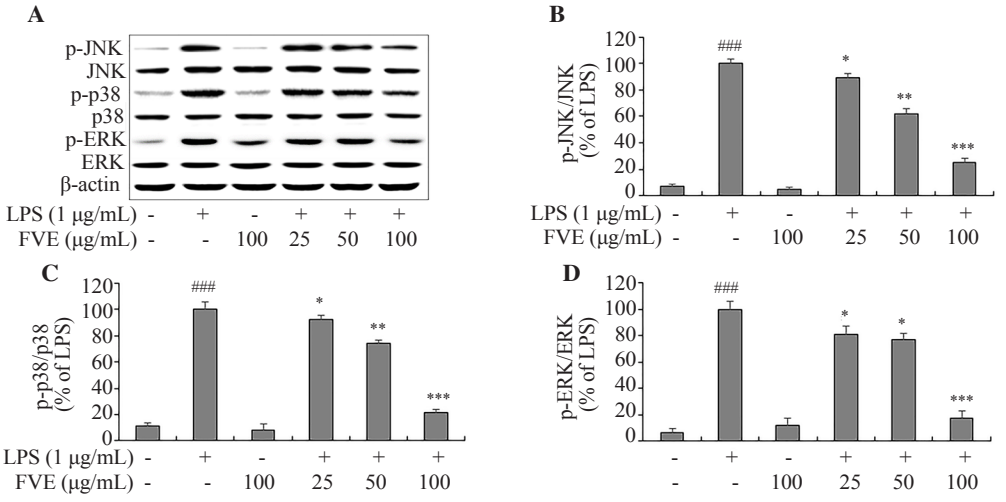

Figure 4. Effect of *Foeniculum vulgare* extract on JNK, p38, and ERK MAPK expression in LPS-stimulated BV-2 cells. (A) The expression levels of p-JNK, JNK, p-p38, p38, p-ERK, and ERK MAPKs in the LPS-stimulated BV-2 cells were analyzed by Western blot analysis and normalized against β-actin. (B-D) Relative intensity levels of JNK, p38, and ERK MAPKs (percentage of LPS) are shown. Data are expressed as mean ± SEM (n=6) and analyzed by ANOVA followed by the Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group.

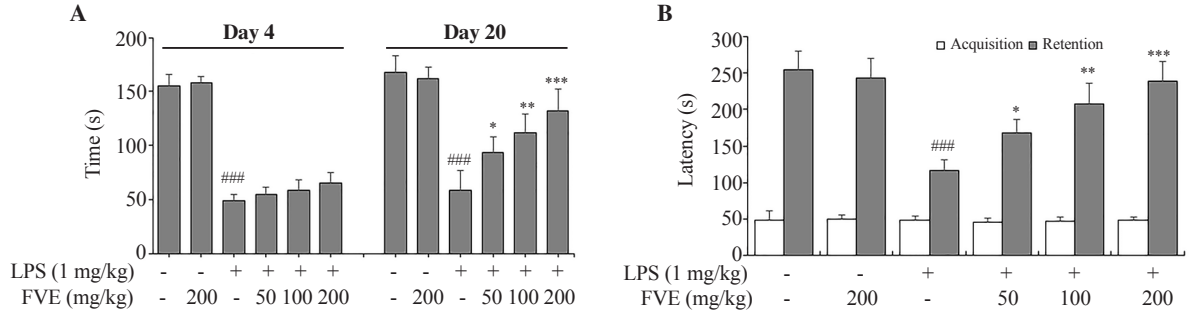

Figure 5. Effect of *Foeniculum vulgare* extract on locomotory and cognitive behavior of LPS-induced mice by rotarod and passive avoidance tests. (A) Rotarod's performance in different experimental groups on day 4 and day 20 is shown. (B) For passive avoidance task, the latency time (s) in acquisition (trial 1) was carried out on day 20, and retention (trial 2) was carried out 24 h after trial 1. Data are expressed as mean ± SEM (n = 10) and analyzed by ANOVA followed by the Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group.

dosages, indicating an improvement in retention [(168 ± 19) s, $P < 0.05$; (208 ± 29) s, $P < 0.01$; (239 ± 28) s, $P < 0.001$ at 50, 100 and 200 mg/kg, respectively] (Figure 5B).

3.10. Effect of *F. vulgare* fruit extract in the open field test

In the open field test, various parameters were observed, such as line crossing, grooming, immobility, and rearing (Figure 6A-6D). Compared with the control group on days 5 and 21, the activities of the four parameters evaluated were significantly altered in LPS-administered mice ($P < 0.001$). On day 21, however, administration of *F. vulgare* fruit extract significantly ($P < 0.05$) raised the decreased numbers of LPS-administered mice engaging in line crossing, rearing, and grooming behaviors and mitigated the increased immobility time dose-dependently. Compared with the LPS-administered group on day 5, no changes were observed in these parameters in the group treated with LPS plus *F. vulgare* fruit extract. In addition, there was no significant difference observed in any of the parameters assessed on day 5 and day 21 between the group treated with *F. vulgare* fruit extract alone and the control group.

3.11. Effect of *F. vulgare* fruit extract on antioxidative enzymes in mouse brain tissue

The concentrations of MDA, a byproduct of lipid peroxide, and SOD, a prominent antioxidant enzyme responsible for protecting nervous tissue against oxidative stress, were determined in brain tissue of mice. Compared with the control group, the LPS-treated group exhibited a substantial reduction in SOD activity ($P < 0.001$) (Figure 7A). Moreover, in the LPS-induced group, MDA concentrations increased significantly ($P < 0.001$) (Figure 7B). However, a significant ($P < 0.05$) reduction in MDA concentration along with a marked increase in SOD activity was observed

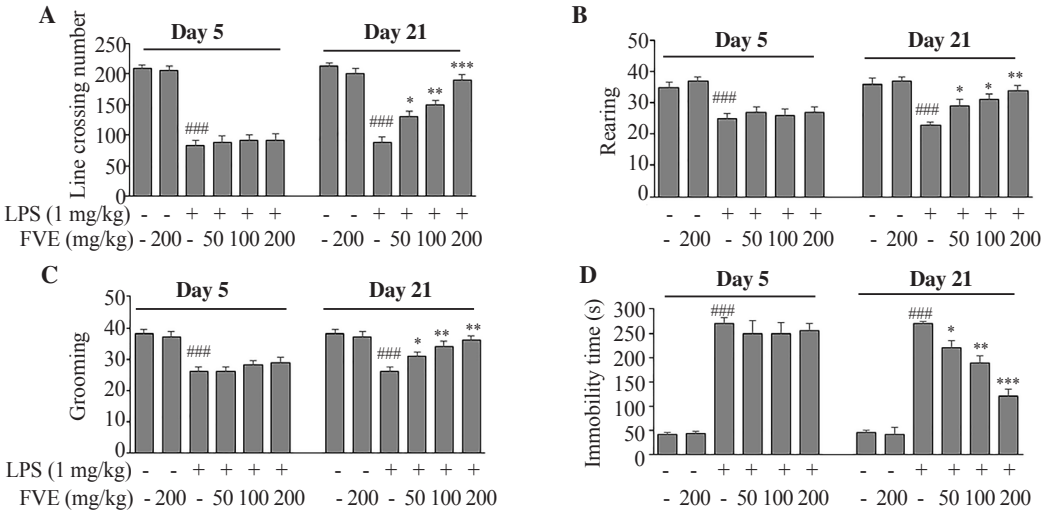

Figure 6. Effect of *Foeniculum vulgare* extract on the open-field performance in LPS-induced mice. Cognitive behavior parameters in open field tests including the (A) line crossing, (B) rearing, (C) grooming, and (D) immobility time were measured in different experimental groups. Data are expressed as mean ± SEM ($n = 10$) and analyzed by ANOVA followed by Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group.

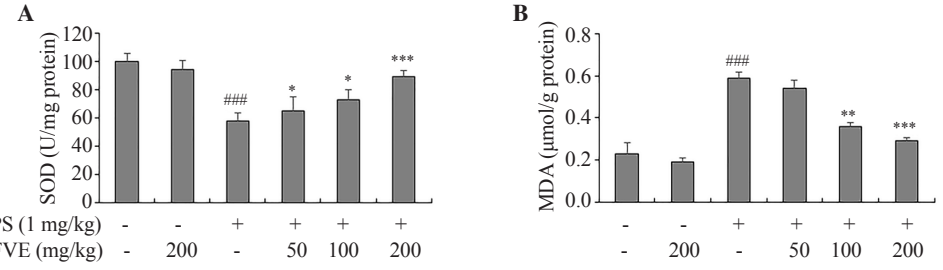

Figure 7. Effect of *Foeniculum vulgare* extract on (A) SOD and (B) MDA levels in LPS-induced mouse brain samples. Data are expressed as mean ± SEM ($n = 10$) and analyzed by the Dunnett's tests. $^{###}P < 0.001$ *vs.* the control group and $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the LPS-treated group. SOD: superoxide dismutase; MDA: malonaldehyde.

following treatment with 100 and 200 mg/kg of *F. vulgare* fruit extract.

4. Discussion

Microglia, the primary immune effectors in the CNS are resident macrophages performing multiple functions[20]. Activated microglia migrate to the site of injury in the CNS thereby secreting pro-inflammatory cytokines and other deleterious toxic molecules such as NO and ROS, ultimately leading to neuronal cell loss[21]. Characterization of the cellular processes that result in the activation of microglia, as well as the regulation of the pro-inflammatory mediators and toxic free radicals holds promise as a target for the development of agents aimed at combating neuroinflammation[4]. Increasing evidence indicated that spices with immense pharmacological benefits including anti-inflammation and neuroprotection are a valuable source of therapeutic agents in ameliorating microglia-mediated neuroinflammatory disorders and other brain pathologies[22,23]. In particular, the tropical herb, *F. vulgare* has been shown to have beneficial therapeutic properties in various neuropathological disorders, including epilepsy, anxiety, sleeplessness, and stress, and enhance memory and cognitive functions[6,8]. In this work, *F. vulgare* fruit extract demonstrated potent anti-neuroinflammatory actions against LPS-stimulated BV-2 microglial cells in varied aspects.

Evidence suggests that LPS at a concentration of 1 μg/mL, stimulated microglial cells and caused the release of excessive inflammatory neurotoxic mediators, and is classified as a valid *in vitro* model to evaluate the mechanisms involving activated microglia-mediated neuroinflammation[24,25]. Our current study confirmed earlier findings by showing that stimulating BV-2 microglial cells with 1 μg/mL of LPS led to a considerable increase in both NO and ROS levels. Furthermore, inflammation-related enzymes such as iNOS and COX-2 expression were also increased by LPS stimulation in BV-2 microglial cells. It is well-documented that iNOS and COX-2 play a crucial role in the inflammatory cascade and are the most appropriate targets in the management of neuroinflammation[26]. Excessive production of neuroinflammatory mediators such as cytokines, ROS, and nitrogen species, increases COX-2 expression in both neurons and microglia[26]. In the present study, *F. vulgare* fruit extract treatment significantly ameliorated the LPS-stimulated increase in NO and ROS and also suppressed the enhanced expression of iNOS and COX-2 levels.

Microglial activation-mediated oxidative damage and neuroinflammation play a crucial role and act as an important pathological event that aids in the onset and development of several neuroinflammation-related neurodegenerative disorders[2,27]. In the present study, *F. vulgare* fruit extract significantly attenuated the ROS generation in LPS-stimulated BV-2 cells. A previous report showed that *F. vulgare* fruit extract exhibited significant anti-stress and memory-enhancing effects based on its antioxidant effects in *in vitro* and scopolamine-induced experimental rats[9]. *F. vulgare* fruit extract exhibited potential free radical scavenging effects including the superoxide, hydrogen peroxide, and lipid peroxide radicals in mouse and rat brain homogenates[28]. Therefore, to understand the beneficial effects of *F. vulgare* fruit extract on LPS-stimulated alterations in the antioxidative enzymes in BV-2 cells, we evaluated the key antioxidative enzyme levels such as SOD, GSH, and CAT majorly involved in the progression of neuroinflammation. In agreement with the strong antioxidant properties exhibited by *F. vulgare* fruit extract, the decreased levels of GSH, CAT, and SOD in LPS-activated BV-2 microglial cells were significantly improved by *F. vulgare* fruit extract. Our study indicated that *F. vulgare* fruit extract not only inhibited the excessive release of ROS but also enhanced antioxidant enzymes in LPS-stimulated BV-2 microglial cells. In particular, *F. vulgare* fruit extract treated at 100 μg/mL concentration showed a greater effect in attenuating these altered enzyme levels.

Inflammatory cytokines including IL-6, IL-1β, and TNF-α are known to be released by activated microglia, and their role in inflammation is well established[29]. Consistent with the above report, LPS stimulation significantly upregulated the expression levels of IL-6, and TNF-α, and treatment with *F. vulgare* fruit extract dose-dependently suppressed the increased production of IL-6 and TNF-α in BV-2 microglial cells. This suggests that *F. vulgare* fruit extract might strongly regulate the excessive production of pro-inflammatory mediators, thereby controlling neuroinflammation. It is well reported that LPS stimulation to microglial cells activates the downstream signaling pathways such as NF-κB and MAPKs[30,31]. NF-κB, a transcription factor implicated in a broad range of biological processes is predominantly involved in regulating various inflammatory cytokines. NF-κB controls inflammatory responses in microglial cells by promoting the expression of pro-inflammatory genes such as TNF-α, IL-6, IL-1β, COX-2, and iNOS[32]. Moreover, microglial cells undergo NF-κB signaling activation in response to LPS stimulation, which in turn causes the phosphorylation and degradation of IκB-α[33]. In view of the above reports, regulation of NF-κB activation and release of pro-inflammatory factors are important strategies in ameliorating microglia-mediated neuroinflammation. In the current study, *F. vulgare* fruit extract significantly reduced the overproduction of pro-inflammatory factors in LPS-stimulated BV-2 microglial cells by inhibiting the activation of NF-κB and the phosphorylation of IκB-α.

Numerous studies have shown that MAPKs are essential for a wide range of cellular activities, including proliferation, differentiation, development, and survival[34,35]. In addition, it is well known that MAPKs play an important role in immunological and inflammatory

responses and regulate the generation of pro-inflammatory cytokines[34]. Phosphorylation of the MAPK signaling molecules including JNK, p38, and ERK was observed in the LPS-stimulated macrophages and microglial cells, thereby activating the production of pro-inflammatory cytokines[35]. As expected, LPS stimulation resulted in the activation of the p38, JNK, and ERK MAPKs in BV-2 microglial cells, and treatment with *F. vulgare* fruit extract at the given dosages markedly suppressed this activation. These results indicate that suppression of MAPK signaling is critical for the beneficial effects of *F. vulgare* fruit extract in LPS-stimulated BV-2 cells.

Mounting evidence suggests that neuroinflammation has a prominent role in the development of neurodegenerative disorders as well as cognitive impairment[36,37]. LPS induces systemic inflammatory processes similar to bacterial infections and exhibits negative cognitive and behavioral consequences including deficits in memory and locomotor performances[38,39]. In the present work, LPS injections (1 mg/kg) daily for 5 d to mice induced cognitive and behavioral impairments when compared with control mice, supporting that inflammation might play an important role in impairing cognition. The cognitive and behavioral deficits seen in LPS-induced mice on the rotarod, PAT, and open field tests were mitigated by *F. vulgare* fruit extract treatment. To evaluate the ability to regulate motor balance, a locomotor behavioral test utilizing a rotarod apparatus was employed. A notable decrease in retention time on an accelerating rotarod was observed in mice injected with LPS, suggesting compromised balance and muscle coordination. Furthermore, poor performance on PAT and open-field tasks indicates that LPS administration significantly altered cognitive performance. Nevertheless, *F. vulgare* fruit extract significantly enhanced motor, behavioral, and cognitive performance in LPS-induced mice. While in the rotarod test and the open field test, no significant change in behavior was observed on days 4 and 5, respectively, significant behavioral improvement was observed on days 20 and 21, indicating that *F. vulgare* fruit extract has a marked therapeutic benefit on LPS-induced cognitive and behavioral deficits in mice.

Oxidative stress plays a crucial role in neuroinflammation and ROS is thought to be involved in the mechanism of LPS toxicity[2,40]. LPS promotes the generation of pro-inflammatory mediators, which leads to oxidative stress[40]. As a result, assessing the level of antioxidant enzymes in brain tissues may be critical in identifying the extent of LPS toxicity in mice. In the current investigation, the activity of SOD and MDA levels in brain tissues was used to quantify the oxidative stress generated in LPS-induced mice. Treatment with *F. vulgare* fruit extract in LPS-induced mice restored the altered levels of SOD and MDA, thereby decreasing LPS-caused oxidative damage. The reduced oxidative stress in the brain after *F. vulgare* fruit extract treatment provided strong evidence for the improved behavioral and cognitive abilities of LPS-induced mice.

The quality of the traditional medicinal herbs used for various pharmacological benefits is mainly based on the presence of numerous active chemical constituents[41]. Analytical techniques including HPTLC help in understanding the basic knowledge of phytochemical variability and availability by generating a fingerprint of the individual herbal extracts[42]. In the present study, to assess the active constituents present in *F. vulgare* fruit extract, we performed the HPTLC fingerprint analysis. HPTLC data of *F. vulgare* fruit extract revealed five distinctive peaks with rutin (Rf: 0.04), kaempferol-3-*O*-glucoside (Rf: 0.37) and anethole (Rf: 0.52) as identifiable compounds. Some previous reports revealed that the compounds rutin, kaempferol, and anethole delivered strong protective effects against neuroinflammation and provided neuroprotective effects by regulating various signaling pathways in both cellular and animal models[43–45]. Based on the available literature and current scientific data, it can be inferred that the physiologically active components found in *F. vulgare* fruit extract may exert their effects either independently or harmoniously, hence potentially mitigating LPS-induced microglia-mediated neuroinflammation and cognitive impairments.

In conclusion, the present study demonstrated that *F. vulgare* fruit extract exhibited strong inhibitory effects on microglial activation-mediated neuroinflammatory processes in LPS-stimulated BV-2 cells *via* the regulation of NF-κB/MAPK signaling pathways. Additionally, *F. vulgare* fruit extract significantly attenuated the behavioral and cognitive impairments in LPS-induced mice. However, future studies are warranted to determine the precise roles of active ingredients from *F. vulgare* fruit extract, as well as the effectiveness and underlying intrinsic mechanisms of *F. vulgare* fruit extract in other cellular and neuroinflammatory animal models.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by Konkuk University in the year 2022.

Funding

The authors received no extramural funding for the study.

Data availability statement

The data supporting the findings of this study are available from the

corresponding authors upon request.

Authors' contributions

SK designed the work and was involved in data collection. Both SRK and RA were involved in data analysis and interpretation. SK and SRK were involved in drafting the article and critical revision of the article. SK, RA and SRK finally approved the version to be published.

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Edited by Liang Q, Tan BJ