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Original Research Article

Carum carvi Linn (Umbelliferae) Attenuates Lipopolysaccharide-Induced Neuroinflammatory Responses via Regulation of NF-κB Signaling in BV-2 Microglia

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Abstract

Purpose: *To investigate the anti-neuroinflammatory properties of Carum carvi Linn. (CCE, Umbelliferae) aqueous extract in stimulated BV-2 microglial cells and explore its underlying mechanisms.*
Methods: *Cell viability assessment was performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay. Lipopolysaccharide (LPS) was used to activate BV-2 microglia. Nitric oxide (NO) levels were measured using Griess assay. Inducible NO synthase (iNOS) and cyclooxygenase (COX) levels were evaluated by Western blot analysis. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) production were evaluated by enzyme-linked immunosorbent assay (ELISA).*
Results: *CCE alone did not exhibit any signs of cytotoxicity to BV-2 cells up to 200 µg/ml concentration. The LPS-activated excessive release of NO in BV-2 cells was significantly inhibited by CCE ($p < 0.001$ at 100 µg/mL). CCE also inhibited the production of inflammatory mediators such as iNOS, COX-2, IL-6 and TNF-α ($p < 0.05$, $p < 0.01$ and $p < 0.001$ at 25, 50 and 100 µg/mL, respectively). Further mechanistic study revealed that CCE acts by regulation of nuclear factor kappa-B (NF-κB) signaling pathway in LPS-stimulated BV-2 microglial cells.*
Conclusion: *The results reveal that CCE exhibited its anti-neuroinflammatory effects via regulation of NF-κB signaling. This can be developed as a potential therapeutic target in ameliorating microglia-mediated neuroinflammation.*

Keywords: *Carum carvi, Anti-oxidant, Neuroinflammation, Microglia, Nitric oxide, Interleukin*

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INTRODUCTION

Carum carvi Linn from the family Umbelliferae is a globally distributed spice with a long history as a traditional medicinal plant [1]. Traditionally, the dried ripe fruits and leaves of the plant are used in folk medicine especially in the treatment of digestive disorders and as a vegetable in the preparation of soups, salads and sauces [2]. Experimental studies have revealed several benefits of *C. carvi* such as anti-dyspeptic, anti-spasmodic, anti-ulcerogenic, anti-tumor, anti-oxidant, anti-hyperglycemic, anti-hyperlipidemic and anti-inflammatory properties [3, 4]. Earlier reports from our group demonstrated that the aqueous extract of *C. carvi* fruit exhibited strong anti-oxidant, adaptogenic and memory enhancing effects in *in vitro* and *in vivo*

experimental models [5, 6]. However, till date the effect of *C. carvi* on microglia-mediated neuroinflammation was not elucidated.

Microglia cells are ubiquitously distributed immune cells in the central nervous system (CNS) acting as first-line defense in neuronal injury [7]. Several factors disrupt the internal environment of the brain and trigger microglia activation. Once activated, microglia undergoes morphological changes simultaneously releasing a number of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, nitric oxide (NO), prostaglandin E2 and toxic free radicals leading to a condition known as neuroinflammation [8]. Neuroinflammation is well known to be closely related with the pathogenesis of several neurodegenerative disorders including Parkinson's and Alzheimer's diseases [8,9]. Thus, the control of microglial activation has been suggested to be promising therapeutic target in combating neuroinflammatory-mediated neurodegenerative diseases.

It is well documented that microglia can be activated *in vitro* by lipopolysaccharide (LPS) which enhances the production of immune-related cytotoxic factors and pro-inflammatory cytokines [10] which resembles the *in vivo* conditions in the CNS. Therefore, LPS-activated BV-microglia is recognized as an important tool for evaluating neuroinflammatory mechanisms [11]. In the present investigation, aqueous extract of *C. carvi* was evaluated for its protective effects on LPS-activated neuroinflammatory processes in BV-2 microglial cells and explored its mechanisms involved.

EXPERIMENTAL

Plant material and preparation of *C. carvi* extract

The dried fruit material of *C. carvi* procured in the month of September 2008 was obtained from Chemiloids, Vijayawada, India. The fruit material was authenticated by Dr Vidyadar, a Botanist at VJ College of Pharmacy and Research Institute, Rajahmundry, India and a voucher specimen (CCE-VJ/08) was deposited in Pharmacognosy Department of VJ College of Pharmacy Herbarium, India. The fruit material (1 kg) was powdered and extracted with boiling water (5 L) for 30 min. The filtrate of *C. carvi* was evaporated under vacuum below 70 °C in a vacuum drier to give a final yield of 66.63 g. The *C. carvi* extract (CCE) was dissolved in sterile distilled water, filtered on 0.22 µm filters and stored at -20 °C until use. All reagents used in this study were of highest grade available commercially.

Cell culture and viability assay

Cells were cultured in DMEM supplemented with 5 % FBS and 100 U/mL P-S in a 37 °C humidified incubator with 5 % CO_2 and 95 % O_2. The cells were seeded at a density of 5 × 105 cells/mL and pretreated for 1 h with various concentrations of CCE followed by incubation with LPS (1 µg/mL). For viability assay, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) assay was used as described previously [12]. The cells were incubated with various concentrations of CCE for 24 h followed by MTT for 4 h, and then 100 µl of isopropanol (in 0.04 M hydrochloric acid) was added to dissolve the formazan crystals. The absorbance was read at 570 nm using the Anthos 2010 spectrophotometer (Salzburg, Austria). Each experiment was conducted in triplicate. Percentage of the cell viability was calculated as (O.D. of extract treated sample/O.D. of non-treated sample) x 100 %.

Nitric oxide assay

Production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent [13]. BV-2 cells (2 x 10^5 cells/mL) were seeded in 6-well plates in 500 µL complete culture medium and treated with the CCE extract at indicated concentrations for 1 h prior to stimulation with LPS (1 µg/mL) for 2 h. Culture supernatant (50 µL) was reacted with an equal volume of Griess reagent (0.1 % naphthylethylenediamine and 1 % sulfanilamide in (5 % H_3PO_4) in 96-well plates at room temperature in the dark. Nitrite concentrations were determined by using standard solutions of sodium nitrite prepared in the culture medium. The absorbance at 540 nm was read using a PowerWavex Microplate Scanning spectrophotometer (Bio-Tek Instrument, Winooski, VT, USA).

IL-6 and TNF-α production assay

BV-2 microglia cells (1 x 10^5 cells/well) were cultured on 96 well plates and treated with the CCE extract at indicated concentrations (25, 50 and 100 µg/mL) for 1 h and stimulated with LPS (1 µg/mL). At 4 h post-LPS treatment, the cells were collected and the supernatants were evaluated for TNF-α and IL-6 levels individually, using respective murine TNF-α and IL-6 ELISA

kits from BD Biosciences (San Jose, CA, USA) according to the manufacturer's instructions.

Nuclear protein extraction and Western blot analysis

Cells were washed in cold PBS three times and lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1 % (v/v) Tergitol- type NP-40 (NP-40), 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 2 mM Sodium orthovanadate (Na3VO4) and protease inhibitor cocktail (Complete MiniTM, Roche, Mannheim, Germany) at 4 °C. The lysate was clarified by centrifugation at 10,000 g for 20 min at 4 °C to remove insoluble components. Cell lysates were normalized for protein content using bicinchoninic acid reagent (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded onto 10 % polyacrylamide gel electrophoresis (PAGE) and separated by standard sodium dodecyl sulphate-PAGE procedure. The proteins were transferred to an NC membrane (S&S, Dassel, Germany) and blocked with 5 % non-fat dry milk in TBS. To detect protein expression, the blots were probed with the specific antibodies followed by the secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Herculus, CA, USA) with β-actin as internal control. The antibodies against inducible nitric oxide synthase (iNOS), cyclooxegenase (COX)-1, COX-2, nuclear factor kappa-B (NF-κB), I kappa B-alpha (IκB-α) and β-actin were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). The immunoreactive proteins on the membrane were detected by chemiluminescence using the West-Save substrate (Lab-Frontier, Seoul, Korea) on X-ray film.

Statistical analysis

All data are presented as mean ± SEM of at least three independent experiments. Statistical analyses were performed with SAS statistical software (SAS Institute, Cray, NC, USA) using one-way analysis of variance, followed by Dunnett's multiple range tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of CCE on BV-2 microglial cell viability

As shown in Fig. 1, CCE treatment for 24 h at various concentrations (10-200 µg/mL) did not exhibit any significant cytotoxicity on BV-2 microglial cells. Further, LPS treatment alone (1 µg/ml) also did not show cytotoxicity to BV-2 cells. Therefore the non-toxic concentrations of CCE (25-200 µg/mL) were further used to evaluate the inhibitory properties of CCE in NO production in LPS-stimulated BV-2 cells.

Effect of CCE on LPS-induced NO production in BV-2 cells

As shown in Fig. 2, cells treated with LPS alone (1 µg/mL) significantly increased the NO levels. Pretreatment with CCE (25, 50, 100 and 200 µg/mL) significantly ($p < 0.001$ at 100 and 200 µg/mL) and concentration dependently suppressed the LPS-stimulated increase in NO release in BV-2 cells.

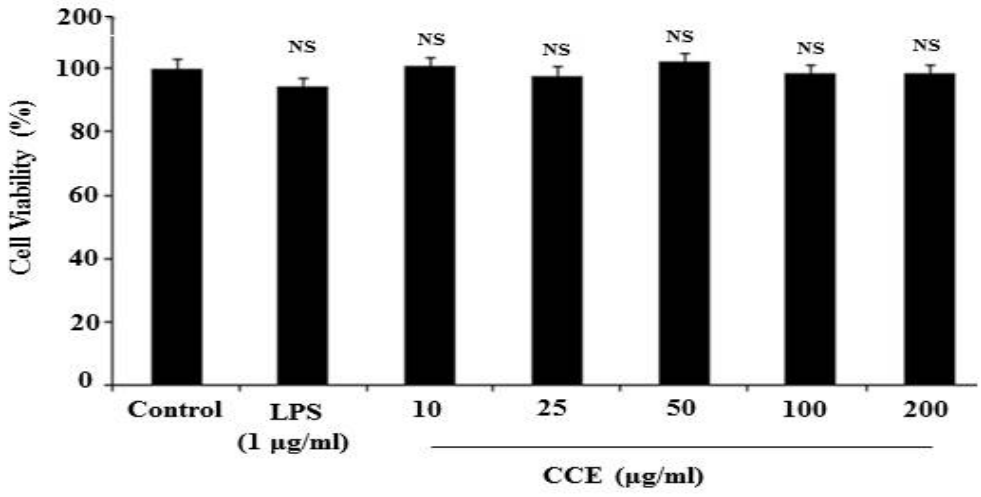

Figure 1: Effects of CCE on the viability of BV- 2 microglial cells. Viability in CCE treated cells was determined using MTT assay. The results are depicted as percentage of control samples. Data are presented as the mean ± S.E.M. (n = 3). NS: Not significant; CCE: *Carum carvi* extract

The maximum effect was observed at a concentration of 100 µg/mL (p < 0.001). Therefore for further experiments we used 25, 50 and 100 µg/mL concentrations of CCE to obtain statistically significant data.

Effect of CCE on LPS-induced expression of iNOS and COX levels

CCE (25, 50 and 100 µg/mL) exhibited a marked inhibitory effect on the expression of iNOS and COX-2 mediators in LPS-stimulated BV-2 cells. The LPS-stimulated increase of protein expression such as iNOS and inducible COX-2 were inhibited in a concentration-dependent manner. However, constitutive COX-1 protein expressional levels were uninterrupted (Fig. 3).

Effect of CCE on IL-6 and TNF-α production in LPS-stimulated BV-2 cells

LPS stimulation increased the IL-6 and TNF-α level in BV- cells, respectively (p < 0.001). However, pretreatment with CCE significantly (p < 0.01 and p < 0.001 at 50 and 100 µg/mL) and concentration dependently decreased the LPS-induced IL-6 (Fig. 4A) and TNF-α (Fig. 4B) levels in BV-2 microglial cells.

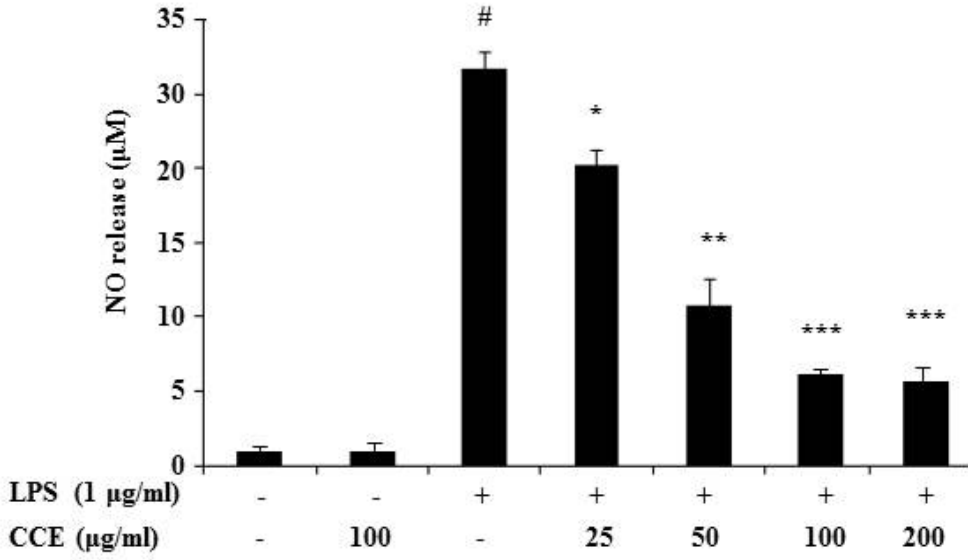

Figure 2: Effect of CCE on NO Production in LPS-stimulated BV-2 microglial cells. BV-2 cells were treated with CCE at various concentrations (25, 50, 100 and 200 µg/mL) with or without LPS (1µg/ml) for 4 h. The nitrite in the culture supernatant was evaluated using Griess reagent. Data are presented as the mean ± S.E.M. (n = 3). $^{\#}p$ < 0.001, when compared with control group. *p < 0.05, **p < 0.01 and ***p < 0.001, when compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. CCE: *Carum carvi* extract

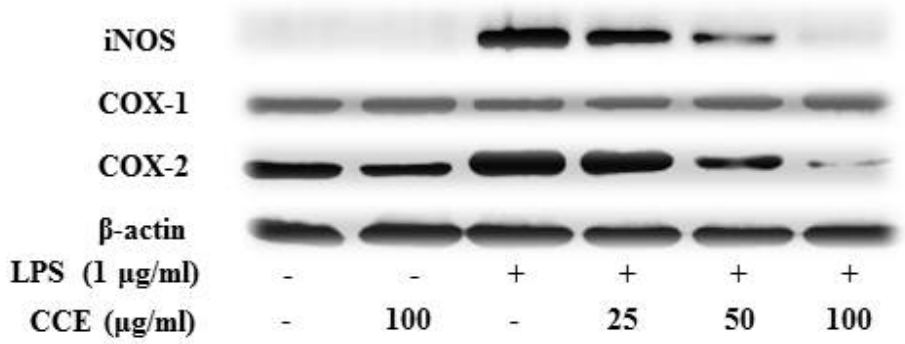

Figure 3: Effect of CCE on iNOS, COX-1 and COX-2 protein expressional levels in LPS-stimulated BV-2 microglial cells. The expression levels of iNOS, COX-1 and COX-2 production in the LPS-stimulated BV-2 cells by various concentrations (25, 50 and 100 µg/ml) of CCE was monitored by immunoblot analyses with the specific antibodies against iNOS, COX-1 and COX-2. The internal control used was β-actin. CCE: *Carum carvi* extract

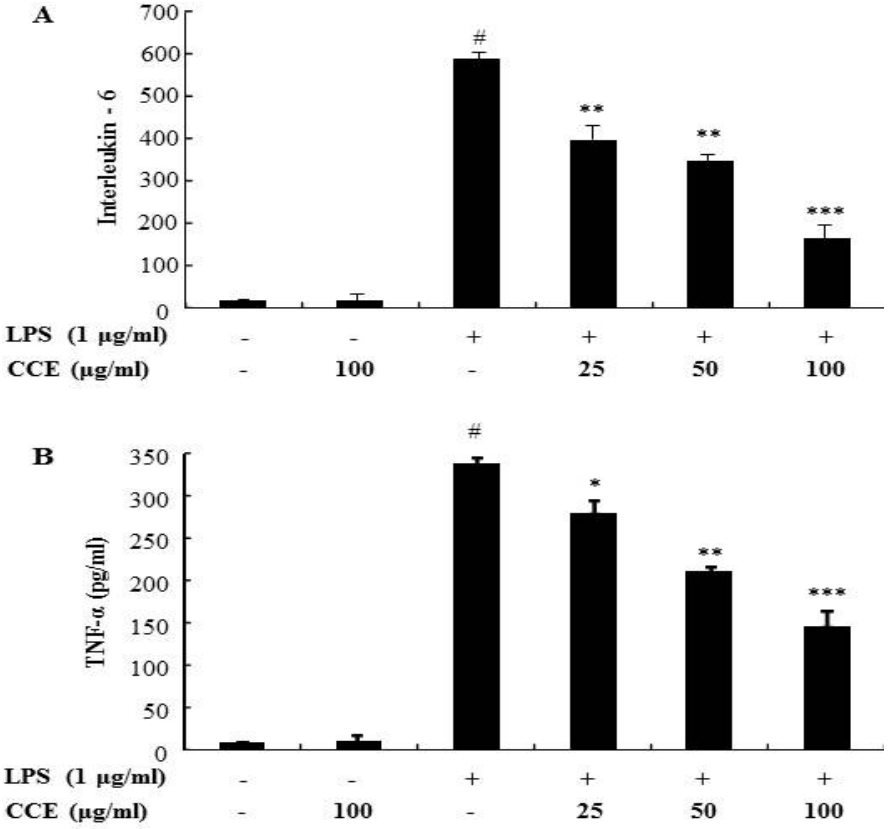

Figure 4: Effect of CCE on pro-inflammatory cytokine IL-6 and TNF-α levels in LPS-stimulated BV-2 cells. BV-2 cells were treated with CCE at indicated concentrations (25, 50 and 100 µg/mL) with or without LPS (1 µg/mL) for 4 h. The IL-6 (A) and TNF-α (B) level in the culture supernatant were evaluated using respective murine ELISA kits according to the manufacturer's instruction. Data are presented as the mean ± S.E.M. (n=3). $^{\#}p < 0.001$, when compared with control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with LPS alone treated group by one-way analysis of variance, followed by Dunnett's multiple range tests. CCE: *Carum carvi* extract

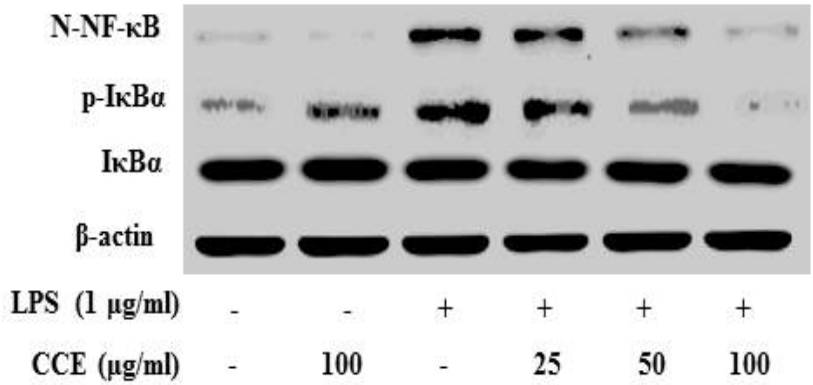

Figure 5: Effect of CCE on NF-κB activity in LPS-stimulated BV2 microglia. The expression levels of IκB-α and nuclear translocation of p65 NF-κB in the LPS-stimulated BV-2 cells by indicated concentrations (25, 50 and 100 µg/ml) of the CCE was analyzed by immunoblot analyses with the specific antibodies. The internal control used was β-actin. CCE: *Carum carvi* extract

Effect of CCE on NF-κB levels in LPS-stimulated BV-2 cells

CCE inhibited the LPS-induced phosphorylation and degradation of IκB-α, and nuclear translocation of p65 NF-κB in a concentration-dependent manner (Fig. 5).

DISCUSSION

Mounting evidence suggest that activation of microglia is the hallmark of neuroinflammation observed in several neurodegenerative diseases and pathological conditions of CNS [8,9]. Studies have shown that anti-inflammatory agents may suppress microglial activation and thus protect neuronal cell death observed in various neurodegenerative diseases [14,15]. Activated microglia can produce several potentially neurotoxic substances, including NO, iNOS and COX-2.

It is well documented that microglia produces NO in response to pro-inflammatory stimuli, and iNOS, an important mediator of inflammation speeds up the production of NO [16]. NO which is a neurotransmitter in the CNS has protective functions in anti-inflammatory pathways but at high concentrations it produces nitrite free radicals therefore being neurotoxic [16]. It was reported that COX-2 was markedly up regulated in rodent brain microglia and in BV-2 microglial cells after LPS treatment [17]. Unlike COX-1, COX-2 is normally not present in most cells, but its expression is induced in response to inflammatory cytokines linked to pathological events [18]. In our study, it was observed that CCE significantly suppressed nitrite production in LPS-stimulated BV-2. Further CCE attenuated the increased iNOS and COX-2 expressions without altering the COX-1 expression in LPS-stimulated BV-2 cells.

Pro-inflammatory cytokines such as TNF-α and IL-6 cause potent activation of iNOS gene expression in rodent glial cells and muscle cells [19]. The likelihood of the involvement of CCE in attenuating such factors is supported by our observations that proinflammatory mediators produced by LPS treatment such as TNF-α and IL-6 were suppressed by CCE in BV-2 microglial cells suggesting that CCE has potent anti-neuroinflammatory activity through the inhibition of NO, iNOS, COX-2 and proinflammatory cytokines such as in BV-2 cells TNF-α and IL-6.

NF-κB is an essential and ubiquitous transcription factor for the expression of many inflammation-related genes, including iNOS, COX-2, TNF-α, IL-1β and IL-6. LPS has also been reported to activate NF-κB in microglia [20]. Here, we confirmed that LPS significantly activated NF-κB in BV-2 microglial cells and this activation was inhibited by CCE. It is well established that the nuclear accumulation of NF-κB relies in large part upon IκB kinase-dependent phosphorylation and subsequent degradation of the cytosolic inhibitor, IκB-α. Our result showed that CCE inhibited the LPS-induced phosphorylation/degradation of IκBα and translocation of NF-κB/p65 sub-unit in a concentration-dependent manner. Therefore we believe that CCE majorly targets in regulating NF-κB signaling.

The main constituents of *C. carvi* are the volatile oils including carvone (40–60 %), limonene, carveol, terpinene, pinene, linalool, cymene, dihydrocarveol and thymol in addition to other glycosides and flavonoids [21,22]. *C. carvi* and its active constituents are reported to possess strong anti-oxidant properties [3,5,6]. Furthermore, carvone which is majorly present in *C. carvi* is known to exhibit anti-inflammatory properties by inhibiting the synthesis of leukotrienes and prostaglandins [3]. In light of such reports, our present data support the notion that CCE might play a promising role in exhibiting anti-neuroinflammatory properties in LPS-stimulated BV-2 cells.

CONCLUSION

This study revealed that CCE inhibits neuroinflammatory responses in LPS-stimulated BV-2 microglial cells via regulation of NF-κB signaling and has the potential to be developed into a therapeutic agent for the treatment of several neuroinflammatory diseases.

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