Anti-neuroinflammatory effects of *Cleistocalyx nervosum* var. *paniala* berry-seed extract in BV-2 microglial cells via inhibition of MAPKs/NF-κB signaling pathway

Sakawrat Janpaijita, Pattawika Lertpatipanpong, Chanin Sillapachaiyaporn, Seung Joon Baek, Somsri Charoenkiatkul, Tewin Tencomnao, Monruedee Sukprasansap

**Ph.D Program in Clinical Biochemistry and Molecular Medicine, Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, 10330, Thailand**

**Laboratory of Signal Transduction, Department of Veterinary Medicine, College of Veterinary Medicine and Research Institute for Veterinary Science, Seoul National University, Seoul 08826, South Korea**

**Institute of Nutrition, Mahidol University, Salaya Campus, Phutthamonthon, Nakhonpathom, 73170, Thailand**

**Natural Products for Neuroprotection and Anti-Aging Research Unit, Chulalongkorn University, Bangkok 10330, Thailand**

**Department of Clinical Chemistry, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok 10330, Thailand**

**Food Toxicology Unit, Institute of Nutrition, Mahidol University, Salaya Campus, Phutthamonthon, Nakhonpathom, 73170, Thailand**

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**ABSTRACT**

Neuroinflammation is an essential contributor to multiple neurodegenerative disorders. *Cleistocalyx nervosum* var. *paniala*, an edible berry, has been reported to exhibit a neuroprotective effect. However, only limited research is available on this fruit seed, which is classified as agricultural food waste. We therefore focused on the anti-neuroinflammatory effects and mechanisms of *C. nervosum* var. *paniala* seed extract (CNSE) on lipopolysaccharide (LPS)-induced inflammatory response in BV-2 mouse microglial cells. HPLC analysis showed that CNSE consists of resveratrol (RESV). For cell-based studies, BV-2 cells were pre-treated with CNSE or RESV, followed by LPS. We found that CNSE and RESV inhibited LPS-induced inflammation in a dose-dependent manner. CNSE and RESV inhibited gene expression and activity of iNOS, leading to a decrease in nitric oxide production. Both CNSE and RESV suppressed the gene expression and the activities of TNF-α, IL-1β, and IL-6. Our results revealed that LPS stimulated the protein levels of MAPKs (JNK, ERK1/2, and p38), while pretreatment of cells with CNSE or RESV attenuated these protein expressions. CNSE also suppressed NF-κB activation. These results suggest that CNSE and RESV can inhibit LPS-induced inflammatory response through MAPKs/NF-κB pathways in BV-2 cells. Taken together, CNSE have potential as a functional anti-neuroinflammatory agent.

**1. Introduction**

The excessive inflammatory response in the central nervous system (CNS), known as neuroinflammation, is one of the factors strongly linked to several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and other brain diseases (Ransohoff, 2016). This abnormality is chiefly related to the brain's immune cells, called microglial cells. In general, microglial cells manage brain development by promoting myelination, neurogenesis and synaptic formation (Hagemeyer et al., 2017; Miyamoto et al., 2016; Shigemoto-Mogami et al., 2014). Moreover, microglial cells also critically regulate the immune responses by modulating the inflammatory process to eliminate harmful substances, repair tissue damage and also promote tissue remodeling to maintain the brain homeostasis (Tang and Le, 2016). However, persistent activation of microglial cells leads to excessive production of a wide variety of inflammatory mediators, including reactive oxygen species (ROS), reactive nitrogen species (RNS), nitric oxide (NO), prostaglandins (PGs), and pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), ultimately resulting in neurodegeneration (Hanisch, 2002; Iizumi et al., 2016; Smith et al., 2012).

* Corresponding author.
** Corresponding author.
E-mail addresses: tewin.t@chula.ac.th, tewintencomnao@gmail.com (T. Tencomnao), monruedee.suk@mahidol.ac.th (M. Sukprasansap).

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Lipopolysaccharide (LPS), the major constituent of the outer membrane in gram-negative bacteria, is widely employed as a potent inducer of the inflammatory characterization in several cell and animal models. On the surface of glial cells, there is receptor which is recognized by LPS, namely toll-like receptor 4 (TLR4), which is responsible for activating the inflammatory signal cascades through several transcription factors and proteins. Among these, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is one of the major transcription factors that effectively control the inflammatory processes by regulating the expression and generation of a large number of pro-inflammatory proteins and enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for the generation of NO and PGE2, respectively (Arias-Salvatierra et al., 2011; Lima et al., 2012).

2. Materials and methods

2.1. Antibodies and reagents

Dulbecco’s Modified Eagle’s Medium (DMEM), LPS from *Escherichia coli* O 55:B5, resveratrol (purity ≥99%), and Bradford reagent were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Fetal bovine serum, 0.25% trypsin-EDTA and 10X Penicillin-Streptomycin were obtained from Gibco BRL (Life Technologies, Paisley, Scotland, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Bio Basic (Markham, ON, Canada). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). 10X RIPA buffer and antibodies against p65, p-IκB-α, IκB-α, p-JNK, JNK, p-ERK1/2, ERK1/2, p-p38, p38, iNOS, β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Preparation of CNSE

CN fruits were collected in July–August 2018 from the Plant Genetic Conservation Project under the Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn (Lampang, Thailand). The CN was identified and authenticated as the scientific name by Asst. Prof. Dr. Thaya Jenjittikul (Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand). The voucher specimen (No. 9428) was deposited at Suan Luang Rama IX Herbarium, Bangkok, Thailand. The seeds were separated from the ripe fruit, dried in a hot air oven at 50 °C for 3 days, then ground into a fine powder, using a food blender, before extraction. About 40 g of lyophilized powders were extracted with 400 mL of 95% ethanol using Soxhlet apparatus for 24 h and the extract was subsequently concentrated by using a rotary evaporator at 45–50 °C. The dried raw material of the extract was calculated to be the yield of the sample which was 6.75% (w/w). Then, the dark-green colored CNSE was dissolved in DMSO and filtered through 0.2 μm pore size paper to obtain the stock solution at 100 mg/mL which was protected from light and stored at −20 °C until use.

2.3. Identification of resveratrol (RESV) in CNSE

The amount of RESV in the CNSE was identified by high-performance liquid chromatography with diode array detection (HPLC-DAD) using a SHIMADZU LC-10 HPLC equipped with an analytical C18 reversed-phase column (Zorbax Eclipse XDB-C18, 4.6 × 150 mm) and UV detector at 520 nm. This procedure of HPLC analysis was determined according to the method described (Nantacharoen et al., 2022). The mobile phase was composed of A (2% acetic acid dissolved in DI water) and B (absolute methanol) and the gradient of solvent was set to 90% of A and 10% of B from 0 to 40 min, 50% of A and 50% of B from 40 to 45 min and 90% of A and 10% of B from 45 min to 60 min, respectively. The chromatography was performed at a flow rate of 1 mL/min and all peaks in the samples were identified by comparing the retention time with the commercially standard resveratrol. Quantification of resveratrol in the CNSE was calculated and represented as mg/100 g dry weight (DW).

2.4. Cell culture and treatment

The mouse BV-2 microglia cell line (Cat. #ABC-TC212S) was purchased from AcceGen Biotech (Fairfield, NJ, USA). BV-2 cells were maintained in high glucose DMEM containing 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified incubator with 5% CO₂ at 37 °C. After growing to 80% confluence, the BV-2 cells were used for the experiment. To investigate the anti-inflammatory effects of samples, the cells were pre-treated with indicated doses of CNSE or RESV in serum-free media for 24 h, followed by adding LPS at 1 μg/mL in various times depending on the type of experiments, to mimic the inflammatory characterization.

2.5. Cell viability assay and morphological analysis

To investigate the cytotoxicity of CNSE or RESV, an MTT assay was performed. Briefly, BV-2 cells at a density of 1.5 × 10⁴ were seeded in each well of 96 well culture plates. After 24 h of incubation, cells were treated with different concentrations of the extract ranging from 0 to 100
μg/mL or RESV at 5 and 10 μM, for another 24 h, in the presence or absence of LPS for another 24 h. Then, 5 mg/mL of MTT solution were added to each well and cells were incubated at 37 °C for 4 h to induce the purple formazan production. The formazan crystal products were dissolved in DMSO and then the optical density was measured at a wavelength of 570 nm to determine the cell viability by using an EnSpire® Multimode Plate Reader (Perkin-Elmer, Waltham, MA, USA). Data were shown as a percentage relative to control (untreated) cells. For morphological analysis, cells were imaged at 10X magnification by using a Zeiss Model Axio Observer A1 phase-contrast microscope (Carl Zeiss, Jena, Germany) and the morphological changes were analyzed by ImageJ software and expressed as a percentage of inactivated cells compared to activated cells.

2.6. Nitric oxide (NO) measurement

To determine the generation of NO, nitrite (NO$_2$) level, one of the stable products of NO was measured using a Griess reagent kit (Promega, Madison, WI). Briefly, cells (4 x 10$^5$) were plated on 6 well culture plates, followed by treatment with the extract or RESV for 24 h, in the presence or absence of LPS for another 24 h. According to the manufacturer’s protocol, cell supernatants were collected by centrifugation and then followed by adding Sulfanilamide and NED solution, respectively. After incubation, the absorbance was measured at 540 nm and the levels of nitrite in the culture medium were evaluated from the calibration curve of sodium nitrite standard.

2.7. RNA isolation and quantitative real-time PCR analysis

BV-2 cells were seeded into 6 well culture plate, pre-treated with CNSE or RESV for 24 h, followed by LPS for another 3 h. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA by using 1 μg of total RNA with Maxime RT Premix Oligo dT18 primer (Intron Biotechnology). cDNA samples were used as templates for SYBR Green quantitative real-time PCR using ExiCycler™ 96 (Bioneer, Korea). The process was started with Pre-denaturation; (95 °C for 10 min), Denaturation; (95 °C for 20 s), and Annealing; (58 °C for 40 s), followed by melting temperature analysis. The primer sequences of all genes are shown in Table 1. The 2$^{−\Delta\Delta$Ct} method was used to calculate the fold change of the target genes expression, which was normalized with β-actin.

2.8. Immunofluorescence analysis

BV-2 cells were seeded on round -coverslips on a 12 well culture plate, followed by pre-treatment with the extract for 24 h and LPS for another 1 h. Then, all coverslips were moved to a new plate for immunostaining. Briefly, cells, which were attached to coverslips, were washed with PBS 3 times for 5 min, fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, and then permeabilized with 0.25% Triton X-100 for 10 min. After that, 5% BSA in PBS was used to block any non-specific proteins for 1 h, followed by incubation overnight with primary antibody at 4 °C. Next, cells were rinsed with PBS before incubating with secondary antibody at room temperature for 45 min. After that, cells were again washed with PBS and incubated with DAPI for 10 min. Finally, samples were washed and mounted on a glass slide using a mounting reagent and an anti-fade reagent was used for long-term storage. Fluorescence signals were visualized on LSM900 confocal laser-scanning microscope (CLSM, ZEISS, Jena, Germany).

2.9. Western blot analysis

BV-2 cells were plated on a 6 well culture plate and then pre-incubated with CNSE or RESV for 24 h, in the presence or absence of LPS for 30 min (for measuring the phosphorylated expressions of MAPKs and IκB-α) or 24 h (for measuring iNOS), respectively. After treatment, the whole proteins were lysed using cold 1X RIPA buffer containing proteinase inhibitor and phenylmethylsulfonyl fluoride (PMSF) and subsequently centrifuged at 12,000 rpm for 10 min at 4 °C. Then, the concentration of protein lysates was measured by Bradford protein assay. An equal number of protein samples were added to 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. Next, membranes were blocked with 5% blocking solution and then incubated with primary antibodies against p-IκB-α (1:1000), IκB-α (1:1000), iNOS (1:1000), pERK (1:1000), ERK (1:1000), p-p38 (1:1000), p38 (1:1000), p-JNK (1:1000), JNK (1:1000) and β-actin (1:1000). Then, all membranes were incubated with HRP-conjugated secondary antibody for 45 min at room temperature and targeted proteins were visualized using chemiluminescence (CL) reagent for Western blotting detection (GE Healthcare, Marlborough, MA, USA). The enhanced signal from CL was analyzed and quantified using ImageJ software. All protein bands were normalized with β-actin.

Table 1. All Primer sequences used in Real-time PCR.

| Gene   | Forward primer (5 ’ → 3’)                               | Reverse primer (5 ’ → 3’) |
|--------|----------------------------------------------------------|---------------------------|
| IL-1β  | GAAATTGCGACCTTTTGAGAGTG                                  | CTGGATGCTTCATCAGGAGACA    |
| TNF-α  | GATGCTGCCCCAACAGGGATG                                    | TAGCAAATCGGCTGAGGGTG      |
| IL-6   | TCTGGAGGATGCTGGTG                                        | CACGGTCGGGTTGGAGGTGTA     |
| iNOS   | ACACTCCGCGGCTCAAGAT                                       | CAGAGGGGTCAGCTGTCT        |
| β-actin| GGCCTGATATGCCCCTCCATG                                    | CCAAGTTGTTAACATGCGATGT    |

2.10. Enzyme-linked immunosorbent assay (ELISA)

BV-2 cells were plated on a 6 well culture plate followed by treatment with CNSE or RESV for 24 h and in the presence or absence of LPS for another 24 h. Next, the culture medium was collected by centrifugation and the production of cytokines was measured from cell supernatants using the commercial kit TNF-α, IL-1β and IL-6 ELISA from Thermo Scientific (Rockford, IL, USA), following the manufacturer's method.

2.11. Dual-luciferase assay

BV-2 cells were transfected with the plasmid, pNF-κB-Luc and pRL-null using PolyJetTM in vitro DNA transfection reagent (SignaGen, Frederick County, MD, USA). After transfection, cells were treated with CNSE or RESV for 24 h, followed by LPS for another 1 h. The relative luciferase activity was measured using a SynergyTM HTX multi-mode microplate reader (Biotek Instruments, Winooski, VT, USA).

2.12. Statistical analysis

All results are presented as the means ± SD of at least three independent experiments. The statistical significance of each experiment was analyzed using the one-way ANOVA and Tukey’s multiple comparison analysis of the SPSS statistics version 19 software, with a p-value < 0.05 considered statistically significant.

3. Results

3.1. Determination of RESV content in the CNSE

To identify whether RESV is one of the active compounds within CNSE, the extract and RESV standard compound were analyzed by HPLC analysis. The chemical structure, formula, and molecular weight of RESV are shown in Figure 1A. The HPLC chromatogram of the sample and standard compound are presented in Figure 1B. The amount of RESV in CNSE was approximately 3.93 ± 0.09 mg/100 g DW, with 2.53 % Relative Standard Deviation (RSD).
Figure 1. (A) Chemical structure of resveratrol (RESV) and (B) HPLC chromatograms of CNSE and RESV standard compound.

Figure 2. Effects of CNSE and RESV on cell viability and morphological phenotypes in BV-2 cells. For cytotoxicity assay, (A) cells were treated with CNSE at the concentration (0–100 μg/mL) or RESV at doses of 5 and 10 μM for 24 h. (B) Cells were incubated with indicated doses of CNSE or RESV for 24 h, followed by LPS 1 μg/mL for another 24 h. Cell viability was examined using MTT assay. For morphological analysis, after treatment cells with CNSE or RESV for 24 h, followed by LPS induction, (C) morphological changes were imaged by phase-contrast microscopy at 10× magnification. The red arrow shows the spindle cells. (D) The percentage of the number of cells was represented by a histogram graph. Data represent the mean ± SD from three independent experiments. The significant difference was represented by p-value less than 0.05 (**p < 0.0001 vs. non-treated control, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs. LPS-treated control).
3.2. Effects of CNSE and RESV on BV-2 cell viability and morphological phenotypes

To determine the cytotoxicity of CNSE and RESV in BV-2 cells, the cells were treated with CNSE or RESV alone for 24 h, followed by MTT assay. Results showed that, above 50 μg/mL, CNSE significantly decreased the viability of cells compared to DMSO control, while RESV did not induce toxicity in BV-2 cells up to 10 μM (Figure 2A). Moreover, we also examined the cytotoxicity of both CNSE and RESV in combination with LPS, which was used as an inflammatory inducer in this study. The result showed that the number of viable cells slightly reduced both in CNSE and RESV treatment after LPS activation (Figure 2B). Therefore, doses of CNSE at 5, 10, and 25 μg/mL, and RESV at 5 and 10 μM, were selected for study in the subsequent experiments. We also observed microglial activation by examining morphological phenotypes of BV-2 cells after LPS stimulation. The results demonstrated that BV-2 cells exhibited two morphological shapes without any treatment: a short-round body and an elongated spindle shape. Typically, the short round cells were more numerous than the long cells. However, in the presence of LPS, cell bodies were enlarged with longer bipolar and multipolar branches. Additionally, pretreatment of cells with CNSE or RESV could restore LPS-induced morphological changes in BV-2 cells by decreasing the number of spindle cells in a dose-dependent manner, compared to the LPS-treated group (Figure 2C and D).

3.3. Effects of the CNSE and RESV on NO and iNOS production

NO is synthesized by the catalytic activity of iNOS, a molecule that plays an essential role in the inflammatory processes. iNOS is also one of the main downstream targets of NF-κB, a key transcription factor for regulating inflammation. Thus, to investigate the effects of CNSE and RESV on the production of NO, the Griess reagent method was conducted. In addition, the mRNA and protein expressions of iNOS were examined by real-time PCR and Western blot analysis. The release of NO was determined after treatment with LPS (1 μg/mL) for 24 h. The results indicated that LPS induced the production of NO (by approximately 40 μM) by measuring the level of nitrite, which is a stable product of NO, compared to the control group. CNSE and RESV significantly decreased NO generation, in a dose-dependent manner, compared to LPS treatment (Figure 3A). Interestingly, the highest concentration of CNSE (25 μg/mL) completely suppressed NO secretion. Furthermore, we examined the mRNA and protein expression of iNOS after stimulation with LPS for 6 and 24 h, respectively. These expressions were greatly decreased by both CNSE and RESV, compared to the LPS group (Figure 3B and C). These data demonstrate that CNSE and RESV have inhibitory effects on the generation of NO through suppression of iNOS.

3.4. Effects of CNSE and RESV on pro-inflammatory cytokines production

It is known that pro-inflammatory cytokines, mainly TNF-α, IL-1β, and IL-6, are expressed and secreted in greater quantities during microglial activation caused by LPS (Hanisch, 2002). To determine whether CNSE and RESV reduce the expressions of TNF-α, IL-1β and IL-6 in response to LPS; therefore, stimulation of LPS for 3 and 24 h was used to detect the mRNA and protein levels, respectively. Results showed that LPS could induce mRNA expression levels of TNF-α, IL-1β and IL-6 after activation for 3 h (Figure 4A–C) as well as their protein levels after stimulation for 24 h (Figure 4D–F). Conversely, pretreatment of cells with CNSE or RESV could significantly decrease mRNA levels of all these cytokines in a dose-dependent manner compared to LPS treatment group.
LPS-exposed cells also enhanced the production of these cytokines in the culture medium of BV-2 cells, whereas both CNSE and RESV could reverse these changes, compared to LPS group (Figure 4D–F). However, it was noticed that CNSE at 25 μg/mL did not significantly reduce the mRNA level of IL-6, and slightly decreased the release of IL-6, as shown in Figure 4C and F, respectively. These data suggest that both CNSE and RESV attenuate neuroinflammation by suppressing pro-inflammatory molecules at mRNA and protein levels.

**Figure 4.** CNSE and RESV reduce pro-inflammatory cytokines production in BV-2 cells. BV-2 cells were pre-treated with CNSE or RESV for 24 h with or without LPS 1 μg/mL for 3 h. The levels of mRNA expression of (A) IL-1β, (B) TNF-α, and (C) IL-6 were detected by real-time PCR, and the relative expression of these genes was normalized to β-actin and showed a fold change compared to the non-treated control. ELISA kit was also used to observe the protein releasing of (D) IL-1β, (E) TNF-α, and (F) IL-6. The cell supernatant was collected after CNSE and RESV treatment, followed by LPS 1 μg/mL for 24 h. Data represent the mean ± SD from three independent experiments. The significant difference was represented by p-value less than 0.05 (**p < 0.01, ***p < 0.001 vs. non-treated control, *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 vs. LPS-treated control).

**Figure 5.** CNSE and RESV suppress MAPKs activation. BV-2 cells were incubated with CNSE or RESV for 24 h, followed by LPS 1 μg/mL for 30 min. The relative protein expression of (A) p-JNK, (B) p-ERK1/2 and (C) p-p38 was observed by Western blot analysis and normalized with β-actin. Data represent the mean ± SD from three independent experiments. The significant difference was represented by p-value less than 0.05 (##p < 0.01, ###p < 0.001 vs. non-treated control, *p < 0.05, **p < 0.01 vs. LPS-treated control).
3.5. Effects of CNSE and RESV on MAPKs activation

MAPKs, including the JNK, ERK1/2 and p38, are well-known to be associated with NF-κB in the regulation of numerous inflammatory mediators. To investigate the effects of CNSE and RESV on LPS-induced MAPKs activation, BV-2 cells were treated with CNSE or RESV for 24 h in the presence or absence of LPS for 30 min. The protein levels of the active form of these MAPKs (phosphorylated (p); p-JNK, p-ERK1/2, and p-p38) were determined by Western blot analysis. Results showed an induction of all active forms of MAPKs (p-JNK, p-ERK1/2 and p-p38) in LPS-stimulated cells, compared to the non-treated control. After treatment of CNSE or RESV, levels of these proteins were downregulated, as shown in Figure 5A-C. Our data suggest that CNSE and RESV inhibit neuroinflammation by regulating MAPKs activation.

3.6. Effects of CNSE and RESV on NF-κB activation

NF-κB is one of the central pathways playing an essential role in the inflammatory response. Numerous reports reveal that LPS can stimulate NF-κB activation (Hobbs et al., 2018; Sakai et al., 2017; Zhao et al., 2022). RESV has been previously reported to inhibit this transcription factor (Xu et al., 2018). Therefore, to investigate the underlying mechanism of CNSE and RESV in response to the induction of LPS in BV-2 cells, we performed immunofluorescence analysis of the nuclear translocation of NF-κB (p65). With 1 h of LPS stimulation, cells displayed an increase in nuclear translocation of p65, whereas CNSE at 25 μg/mL distinctly reduced the level of p65 in the nucleus as shown in Figure 6A. Moreover, both CNSE and RESV also inhibited the protein expression of phosphorylation of IκB-α (p65 inhibitor), compared to LPS control (Figure 6B). To further confirm these results, NF-κB binding activity was also investigated. Our results showed that both CNSE and RESV significantly suppressed NF-κB binding activity, compared to LPS treatment as shown in Figure 6C. These data indicate that CNSE and RESV can reduce LPS-induced neuroinflammation through NF-κB modulation.

4. Discussion

Typically, neuroinflammation is relevant to the innate immune response against various pathogenic conditions in the CNS, including infection and tissue injury for resolving brain damage and maintaining brain homeostasis. However, chronic neuroinflammation can cause uncontrolled inflammatory processes which result in detrimental consequences to the brain and ultimately lead to neurodegeneration (Chitnis and Weiner, 2017). Microglia, the resident macrophages found in the brain, play a vital role in the regulation of the inflammatory cascades for eliminating pathogenic events by releasing several inflammatory-related molecules such as ROS, NO, and pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (Smith et al., 2012; Wang et al., 2015). Based on studies of the neuroinflammatory-associated pathologies, LPS is a well-known substance for research. This toxin is an outer membrane component of gram-negative bacteria, and has been widely used to mimic the inflammatory characterization both in vitro and in vivo models of neurodegenerative disorders (Batista et al., 2019). Previous studies have reported on the enhancement of pro-inflammatory cytokines after treatment with LPS in epithelial cells, macrophages, and microglial cells (Liu et al., 2018; Lively and Schlichter, 2018). Thus, LPS was selected to stimulate the neuroinflammatory effects using microglial BV-2 cells.

Figure 6. CNSE and RESV inhibit NF-κB activation in BV-2 cells. (A) BV-2 cells were incubated with CNSE at 25 μg/mL for 24 h, followed by LPS 1 μg/mL for 1 h to observe the nuclear translocation of p65 using immunofluorescence. The red color from Alexa Fluor 555 staining represents p65 in the cells and the blue color from DAPI staining represents the nucleus. (B) The level of p-IκB-α and IκB-α was measured by performing Western blot analysis. The relative expression of p-IκB-α and IκB-α was normalized with β-actin. (C) NF-κB (p65) binding activity was examined by dual-luciferase assay and the relative expression of p65 binding activity was normalized through the activity of p8L-null. Data represent the mean ± SD (n = 6). The significant difference was represented by p-value less than 0.05 (**p < 0.01, ****p < 0.0001 vs. non-treated control, *p < 0.01, ***p < 0.001 and ****p < 0.0001 vs. LPS-treated control).
Targeting neuroinflammation and related pathways is one of the most common therapeutic approaches for the prevention and treatment of microglial-mediated neuroinflammatory diseases. Due to the adverse effects of NSAIDs and clinical trial failures in developing medicines without side effects, natural plants have been gaining attention in neuroinflammatory research for anti-neuroinflammatory agents (Nunes et al., 2020). Berry fruits have been investigated and reported on their various biological properties in the prevention of neurodegenerative disorders (Agarwal et al., 2019; Selvaraju et al., 2014). *C. nervosum* var. *paniala*, locally known as “Ma-kiang”, is one of the fruits that has been noted for its neuroprotective effects, such as antioxidant, anti-apoptosis and anti-aging properties. However, the anti-neuroinflammatory effects of this plant have not been fully investigated, especially those of the seed. Therefore, this study has primarily focused on the anti-neuroinflammatory properties of CNSE in response to LPS stimulation in BV-2 cells. In the current report, we demonstrated the suppressive effects of the CNSE and its active component, RESV, against LPS-induced neuroinflammation in BV-2 cells.

**Figure 7.** The proposed underlying mechanisms of CNSE in response to lipopolysaccharide in BV-2 mouse microglial cells (Created by BioRender.com).
cells by regulating the inflammatory-related signaling cascades, including MAPKs and NF-κB. Pretreatment of cells with CNSE was observed to suppress LPS-induced morphological changes in BV-2 cells (Figure 2C and D). It is well known that the generation of NO results from the conversion of L-arginine by nitric oxide synthase (NOS), particularly iNOS, which is abundantly expressed in glial cells during microglial activation (Saha and Pahan, 2006). Our study showed that CNSE decreased the secretion of NO and completely inhibited it at the highest dose of CNSE (Figure 3A). Moreover, the mRNA and protein expression of iNOS were also suppressed after CNSE treatment in the presence of LPS (Figure 3B and C). Subsequently, we examined whether CNSE could inhibit the expression and secretion of inflammatory proteins. In our present study, we found that pre-treatment of BV-2 cells with CNSE could reduce the mRNA levels of TNF-α, IL-1β and IL-6 and also suppress the release of these cytokines in a dose-dependent manner (Figure 4A–F). It was noticed that the highest concentration of CNSE did not significantly decrease the gene expression of IL-6 and only slightly inhibited the production of IL-6 (Figure 4C and F). Studies with other berries, including blueberries and mulberry fruits, showed a reduction in IL-6 expression in macrophages (Qian et al., 2015; Xie et al., 2011), while consumption of goji berries showed an increase in serum IL-6 in healthy subjects (Dries-sche et al., 2021). Collectively, these data demonstrate the inhibitory effects of CNSE on LPS-induced neuroinflammation in BV-2 cells.

Regarding the inflammatory mechanism of action, it is widely known that the dysregulation of NF-κB has been implicated in the progression of several diseases, including autoimmune and neurodegenerative disorders (Courtois and Gilmore, 2006; Singh and Singh, 2020). The transcription factor NF-κB has been recognized as one of the key mediators in a wide variety of biological functions such as cell proliferation, cell survival, and immune response (Fan et al., 2008; Kopitar-Jerala, 2015; Radhakrishnan and Kamalakaran, 2006). In the normal state, NF-κB is in an inactive form, binding to its inhibitor, IκB-α, within the cytoplasm. During the inflammatory response, IκB-α is phosphorylated and eliminated by ubiquitination and proteasomal degradation leading to the subsequent nuclear translocation of NF-κB. In the nucleus, NF-κB regulates its targeted gene expression of several inflammatory mediators such as pro-inflammatory cytokines, chemokines, and adhesion molecules (Liu et al., 2017). Our investigation showed that LPS induced the nuclear translocation of p65, a member of the NF-κB family, and the phosphorylation of IκB-α which were inhibited by CNSE treatment (Figure 6A and B). This was confirmed by the suppressive effect of CNSE against LPS-mediated NF-κB binding activity (Figure 6C). Furthermore, MAPKs, including JNK, ERK1/2, and p38 were also stimulated by inflammatory inducers and stress, which, in turn, activate the inflammatory cascades (Kyriakis and Avruch, 2001). Previous studies have reported that MAPKs were induced after LPS induction in macrophages and mouse models (Meng et al., 2014; Yang et al., 2019). In the present study, we found that CNSE treatment could significantly suppress protein expression of all active forms of MAPKs, including JNK, ERK1/2 and p38 in LPS-treated BV-2 cells (Figure 5A–C). Therefore, our findings suggest that CNSE inhibits LPS-induced inflammatory features by modulating NF-κB and MAPKs signaling pathways.

In addition, we determined and identified RESV as the active constituent in CNSE by HPLC analysis and investigated the anti-inflammatory properties of RESV. The result showed that RESV could inhibit the expression and the secretion of inflammatory mediators, including TNF-α, NO, and iNOS (Figures 3 and 4). Moreover, NF-κB and MAPKs were suppressed after RESV treatment in response to LPS in BV-2 cells (Figures 5 and 6). These were relevant to previous reports which showed that RESV could ameliorate LPS-induced inflammation through both NF-κB and MAPKs in RAW 264.7 macrophage cells (Tong et al., 2020; Zong et al., 2012). Additionally, some studies confirm the anti-inflammatory effects of RESV in BV-2 cells. RESV also inhibited LPS-induced phagocytosis, reduced the production of NO and iNOS and suppressed the mRNA levels of TNF-α, IL-1β and IL-6 via TNF Receptor Associated Factor 6 (TRAF6)/NF-κB signaling cascade (Ge et al., 2019; Park et al., 2012). Hence, our study shows that CNSE and RESV are involved in inhibiting the inflammatory molecules and responses which link to the downregulation of MAPKs and NF-κB signaling pathways.

5. Conclusion

The present study reported for the first time that CNSE and its active component, RESV, exerts suppressive effects on LPS-induced neuro-inflammation via MAPKs and NF-κB-mediated mechanisms in BV-2 mouse microglial cells. CNSE and RESV could inhibit gene and protein expressions or protein levels of several inflammatory factors such as TNF-α, IL-1β, IL-6, NO, and iNOS. These suppressive effects were regulated through MAPKs and NF-κB signaling cascades as shown in Figure 7. Interestingly, our research suggests that the anti-neuroinflammation effects of CNSE might be the synergistic effect of several compounds within CNSE because it is a crude extract and RESV is only one of its active components. Further studies are needed for investigation of these other compounds. The seed extract of CN fruit should be more thoroughly investigated to determine the cellular mechanisms of anti-neuroinflammation in animal models and human studies. The findings of this study support the potential of CNSE as a functional anti-neuroinflammatory agent for treating neuroinflammatory-related diseases.

Declarations

Author contribution statement

Sakawrat Janpajit: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pattawika Lertpatipanpong: Performed the experiments.

Chanin Sillapachaiyaporn: Performed the experiments; Analyzed and interpreted the data.

Seung Joon Baek; Somsri Charoenkiatkul; Tewin Tencomnao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Monrueedee Sukprasansap: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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