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Anti-Neuroinflammatory Effects of 50% Ethanolic Curcuma longa Extract in LPS-Stimulated BV2 Microglia: Involvement of Nrf2/HO-1 Signaling and Inhibition of NF-κB/MAPK

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Abstract

Curcuma longa has traditionally been used as a spice, natural colorant, food preservative, and medicinal plant. It has also been applied in treating various ailments, including dyslipidemia, gastrointestinal disorders, arthritis, and liver diseases. This study aimed to investigate the anti-neuroinflammatory properties of a 50% ethanolic extract of *C. longa* (CLE) in lipopolysaccharide (LPS)-activated BV2 microglial cells. The Griess assay was used to quantify nitric oxide (NO) production, while prostaglandin E2 (PGE2) and pro-inflammatory cytokines—interleukin 1-beta (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α)—were measured using commercial ELISA kits. Western blot analysis assessed the expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), heme oxygenase-1 (HO-1), and nuclear factor erythroid 2-related factor 2 (Nrf2). Pre-treatment with CLE reduced both the production and expression of pro-inflammatory mediators, including NO, PGE2, iNOS, COX-2, and cytokines IL-1β, IL-6, and TNF-α, in LPS-stimulated BV2 cells. CLE also suppressed the activation of NF-κB and the three major MAPK pathways. Furthermore, CLE induced HO-1 expression through Nrf2 activation, and blocking HO-1 reversed CLE's anti-inflammatory effects. CLE exhibited anti-neuroinflammatory activity in LPS-stimulated microglial cells by inhibiting the production and expression of pro-inflammatory mediators via negative regulation of NF-κB and MAPK pathways. These effects were mediated through the HO-1/Nrf2 signaling axis. Overall, the findings suggest CLE could be a promising candidate for preventing neuroinflammatory conditions, warranting further in vivo studies for efficacy evaluation.

Keywords: *Curcuma longa*, Anti-neuroinflammation, BV2 microglial cells, Nuclear factor kappa B, Mitogen-activated protein kinases, Heme oxygenase-1

Introduction

Microglia are macrophage-like cells in the central nervous system (CNS), comprising roughly 5–20% of glial cells, and serve as the primary immune defense in the brain [1]. They maintain brain homeostasis and rapidly respond to minor pathological changes, such as pathogen invasion or neuronal injury, by releasing inflammatory mediators, including nitric oxide (NO), prostaglandin E2 (PGE2), and cytokines like ILs and TNFs [2]. Chronic activation leads to excessive production of these mediators, disrupting homeostasis and causing oxidative damage to membranes, proteins, and DNA, ultimately resulting in neuronal death or dysfunction [3]. Such neuronal impairment can contribute to cognitive decline and memory loss, driving the onset of neurodegenerative disorders such as Alzheimer's, Parkinson's, or multiple sclerosis [4]. Therefore, controlling overactive inflammatory responses is critical for preventing neurodegeneration.

Curcuma longa, a perennial herb in the Zingiberaceae family, originates from tropical southwestern India and is cultivated in countries including China, Myanmar, and

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South Korea (mainly Jindo-gun, Jeollanam-do Province). Its rhizome is widely used in food, medicine, beverages, teas, and as a colorant and preservative [5]. It has therapeutic applications for conditions such as dyslipidemia, gastrointestinal issues, arthritis, and liver diseases [6]. Curcuminoids, including curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are the primary bioactive compounds of *C. longa*, with reported antibacterial, anti-HIV, antioxidant, anti-inflammatory, and anticancer properties [7, 8]. Recent studies show that hexane extracts of *C. longa* inhibit neuroinflammation in LPS-induced microglial models via suppression of the ERK MAPK pathway [9].

According to the Ministry of Food and Drug Safety in Korea, *C. longa* is classified as a food material that can be used only partially (up to 50%) in formulations. To overcome this limitation, optimized extraction methods and functional evaluations are needed. Therefore, this study explored the anti-neuroinflammatory potential of a 50% ethanolic extract of Korean *C. longa* in LPS-stimulated BV2 microglial cells.

Materials and Methods

Plant material and preparation of CLE

Curcuma longa rhizomes were collected from Jindo-gun, Jeollanam-do, Republic of Korea. Authentication of the plant material was conducted by Yunji Lee, a senior researcher at the National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong, Republic of Korea. A voucher specimen designated as MPS00 has been deposited in the Herbarium of the Department of Herbal Crop Research, National Institute of Horticultural and Herbal Science, Rural Development Administration, Eumseong, Republic of Korea. The 50% ethanolic extract of C. longa (CLE) was prepared following the procedure outlined in a prior publication that utilized the identical plant material [10].

Chemicals and reagents

Details regarding the reagents, kits, and their respective suppliers are listed in **Table 1.**

Table 1. Reagents and suppliers used in the present study

Product / Reagent	Manufacturer / Supplier
RPMI1640 medium	Gibco BRL Co.
Fetal bovine serum (FBS)	Gibco BRL Co.
Penicillin-streptomycin	Gibco BRL Co.
Phosphate-buffered saline (PBS)	Gibco BRL Co.
Trypsin-EDTA (TE)	Gibco BRL Co.
Lipopolysaccharide (LPS, O55:B5)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich
Ammonium persulfate (APS)	Sigma-Aldrich
Tween-20	Sigma-Aldrich
PGE_2 ELISA kit	ENZO Life Science, Inc.
IL-6 ELISA kit	R&D Systems
TNF-α ELISA kit	R&D Systems
RIPA lysis buffer	Thermo Fisher Scientific
Protease and phosphatase inhibitor cocktail	Thermo Fisher Scientific
NE-PER™ Nuclear and Cytoplasmic Extraction Reagents	Thermo Fisher Scientific
Nitrocellulose (NC) membrane	Bio-Rad Laboratories, Inc.
Bis-acrylamide solution	Bio-Rad Laboratories, Inc.
Tris-HCl	Bio-Rad Laboratories, Inc.
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, Inc.
Tris-glycine SDS buffer	GenDepot
Tris-glycine native buffer	GenDepot
Sodium dodecyl sulfate (SDS)	GenDepot
Tris-buffered saline (TBS)	GenDepot
ECL solution	GenDepot
Skimmed milk powder	BD Biosciences

Anti-iNOS	Cell Signaling Technology Inc.
Anti-COX-2	Cell Signaling Technology Inc.
Anti-IκB-α	Cell Signaling Technology Inc.
Anti-p-IκB-α	Cell Signaling Technology Inc.
Anti-p65	Cell Signaling Technology Inc.
Anti-p-ERK	Cell Signaling Technology Inc.
Anti-ERK	Cell Signaling Technology Inc.
Anti-p-JNK	Cell Signaling Technology Inc.
Anti-JNK	Cell Signaling Technology Inc.
Anti-p-p38	Cell Signaling Technology Inc.
Anti-p38	Cell Signaling Technology Inc.
Anti-HO-1	Cell Signaling Technology Inc.
Anti-Nrf2	Cell Signaling Technology Inc.
Anti-β-actin	Santa Cruz Biotechnology Inc.
Anti-PCNA	Santa Cruz Biotechnology Inc.
Secondary antibodies	Merck Millipore Co.

BV2 microglial cells were cultured at a density of 5×10^5 cells/mL in 100-mm diameter dishes using RPMI1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Cells were maintained at 37 ℃ under a humidified atmosphere containing 5% CO_2.

MTT assay for cell viability

BV2 cells were seeded into 96-well plates and exposed to CLE at concentrations ranging from 12.5 to 200 μg/mL for 24 h. Cell viability was assessed using the MTT assay according to the protocol described previously [11]. Absorbance was recorded at 540 nm with a Multiskan Microplate Reader (ThermoFisher). Viability of the untreated control group was defined as 100%.

Measurement of nitrite (NO production)

BV2 cells were seeded in 24-well plates, pretreated with CLE for 3 h, and subsequently stimulated with LPS (1 μg/mL) for 24 h. Nitric oxide production was quantified by measuring nitrite levels in the culture supernatant, following the method reported earlier [11]. Absorbance was determined at 540 nm using a Multiskan Microplate Reader (ThermoFisher).

Quantification of PGE_2, IL-1β, IL-6, and TNF-α

BV2 cells were plated in 24-well plates, pretreated with CLE for 3 h, and then stimulated with LPS (1 μg/mL) for 24 h. Culture supernatants were collected, and concentrations of PGE_2 were measured using an ELISA kit from ENZO Life Science Inc. (Farmingdale, NY), while IL-1β, IL-6, and TNF-α levels were determined using ELISA kits from R&D Systems Inc. (Minneapolis, MN). All assays were conducted in accordance with the manufacturers' protocols, with three independent experiments performed.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

BV2 cells were seeded in 6-well plates, pretreated with CLE for 3 h, and stimulated with LPS (1 μg/mL) for 6 h. qRT-PCR procedures were carried out as detailed in a previous publication [12]. Primer sequences utilized in this study are provided in **Table 2.**

Table 2. Primer sequences used for qRT-PCR analysis

Gene	Forward primer (5′ → 3′)	Reverse primer (3′→ 5′)
IL-1β	AATTGGTCATAGCC CGCACT	AAGCAATGTGCTG GTGCTTC
IL-6	ACTTCACAAGTCG GAGGCTT	TGCAAGTGCATCAT CGTTGT
TNF-α	CCAGACCCTCACA CTCACAA	ACAAGGTACAACC CATCGGC
GAPDH	TTCACCACCATGGA GAAGGC	GGCATGGACTGTG GTCATGA

Western blot analysis

Expression levels of iNOS, COX-2, NF-κB-related proteins, MAPK-related proteins, HO-1, and Nrf2 were examined by Western blotting. The detailed procedure followed the method described previously [11]. BV2 cells were pretreated with CLE or SnPP and then stimulated with LPS (1 μg/mL). Cells were collected by

centrifugation, washed with PBS, and lysed in RIPA buffer. Protein concentrations were quantified using the Bradford assay (Bio-Rad, CA, USA) to ensure equal loading. Equal amounts of protein (30 µg) were separated by 7.5% or 12% SDS-PAGE, transferred onto nitrocellulose membranes, and blocked with 5% skim milk in TBS-T for 1 h at 4 ℃. Membranes were incubated with primary antibodies overnight or for 90 min at 4 ℃, washed with TBS-T, and then incubated with appropriate secondary antibodies. Protein bands were visualized using chemiluminescence reagent (GenDepot).

Preparation of cytosolic and nuclear extracts

Cytoplasmic and nuclear fractions were isolated using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents. BV2 cells were pretreated with CLE and stimulated with LPS (1 µg/mL) for 1 h. Cells were harvested by centrifugation, washed with PBS, and lysed using cytoplasmic extraction reagent to disrupt the plasma membrane while preserving nuclear integrity. Following centrifugation, the cytoplasmic supernatant was collected and transferred to a chilled tube. The remaining pellet was lysed with nuclear extraction reagent, centrifuged, and the resulting supernatant was collected as the nuclear fraction.

Statistical analysis

All data are expressed as mean ± standard deviation (S.D.) from at least three independent experiments. Comparisons among three or more groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Statistical analyses were performed with GraphPad Prism software (version 3.03, GraphPad Software Inc., USA).

Results and Discussion

CLE effects on BV2 microglial cell viability

To investigate potential cytotoxicity, BV2 cells were exposed to CLE at concentrations between 12.5 and 200 µg/mL for 24 h, and cell survival was evaluated using the MTT assay. Measurements of optical density revealed that CLE concentrations from 12.5 to 150 µg/mL did not adversely affect the viability of BV2 cells **(Figure 1)**.

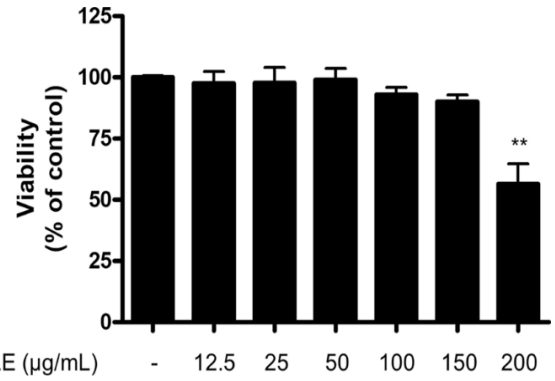
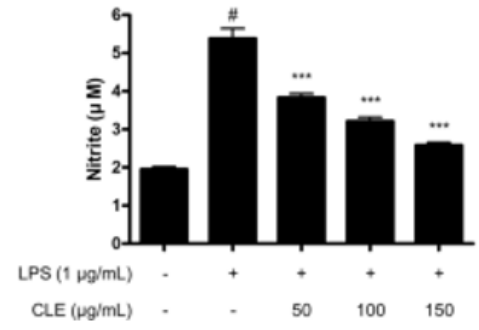


Figure 1. Assessment of BV2 cell viability after CLE treatment. Cells were incubated with varying CLE concentrations for 24 h. Viability was determined via MTT assay.

CLE reduces NO and PGE2 production and downregulates iNOS and COX-2 in LPS-stimulated BV2 cells

The anti-inflammatory activity of CLE was first assessed by measuring NO and PGE2 levels in BV2 cells stimulated with LPS. Cells were pre-treated with CLE for 3 h at non-toxic concentrations and then exposed to LPS (1 µg/mL) for 24 h. LPS treatment caused a marked elevation in NO and PGE2 compared with untreated controls. Pre-treatment with CLE led to a significant, dose-dependent reduction in both NO **(Figure 2a)** and PGE2 **(Figure 2b)** levels.

Subsequently, the expression of iNOS and COX-2, the key enzymes responsible for NO and PGE2 production, was examined. CLE significantly diminished the protein levels of iNOS and COX-2 in a concentration-dependent manner **(Figure 2c).**



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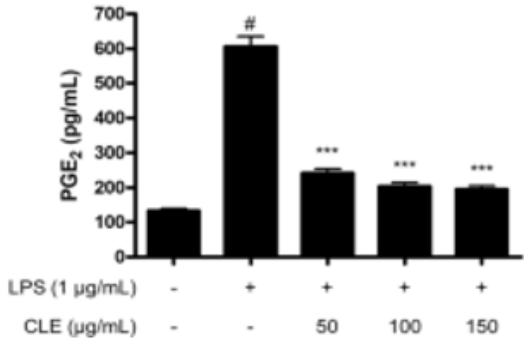

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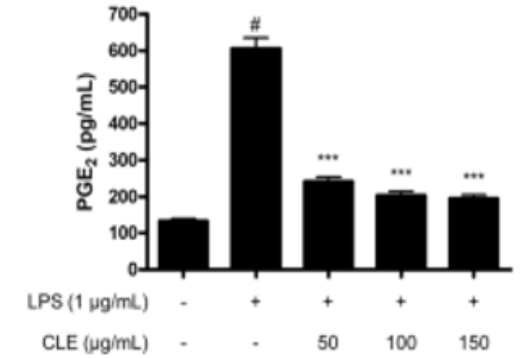


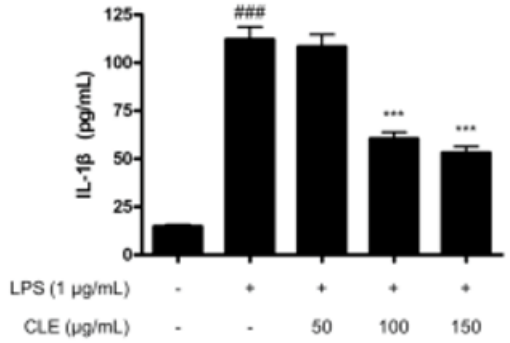
c)

Figure 2. CLE effects on inflammatory mediators in LPS-stimulated BV2 cells. Cells were pre-treated with CLE for 3 h before LPS (1 μg/mL) stimulation for 24 h. (A, B) NO and PGE2 levels were measured by Griess assay and ELISA, respectively. Data represent mean ± SD of three experiments. #p < 0.001 vs. control; **p < 0.01, ***p < 0.001 vs. LPS group. (C) iNOS and COX-2 expression determined by Western blot; β-Actin used as loading control.*

CLE suppresses pro-inflammatory cytokine production and mRNA expression

Excessive activation of microglia results in the overproduction of pro-inflammatory cytokines. To examine CLE's effects, IL-1β, IL-6, and TNF-α levels were quantified in LPS-stimulated BV2 cells using ELISA. Cells pre-treated with CLE for 3 h prior to LPS (1 μg/mL) exposure for 24 h showed a dose-dependent decrease in cytokine release **(Figures 3a–3c)**.

Moreover, CLE also reduced the mRNA expression levels of IL-1β, IL-6, and TNF-α after LPS stimulation for 6 h, indicating transcriptional suppression **(Figures 3d–3f).**



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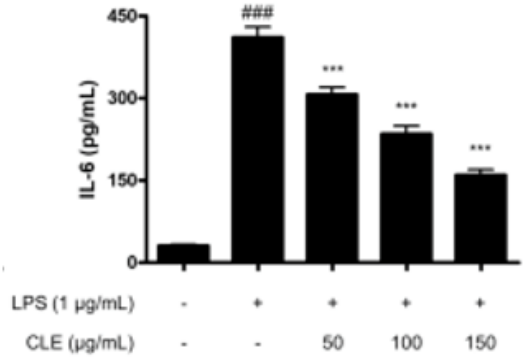

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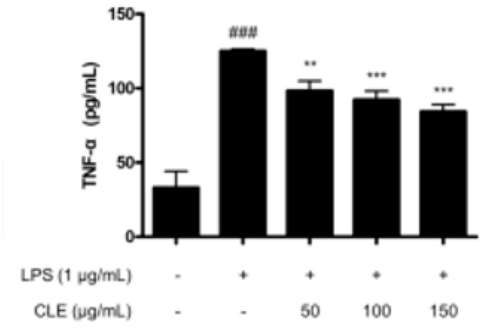

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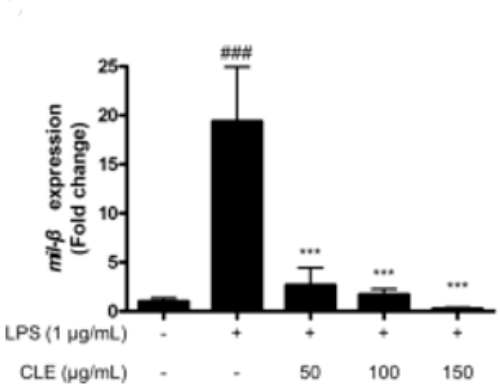

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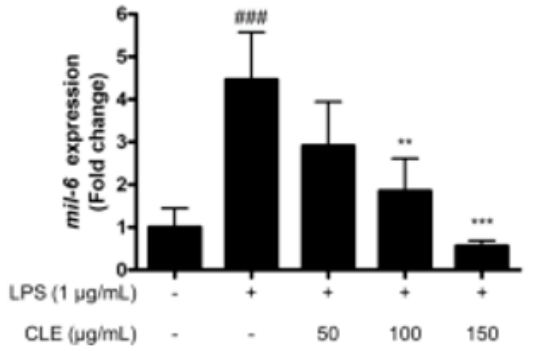

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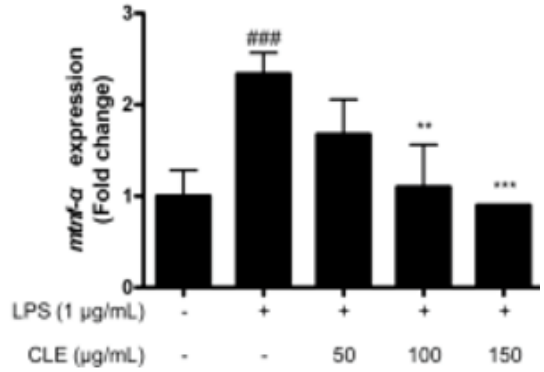
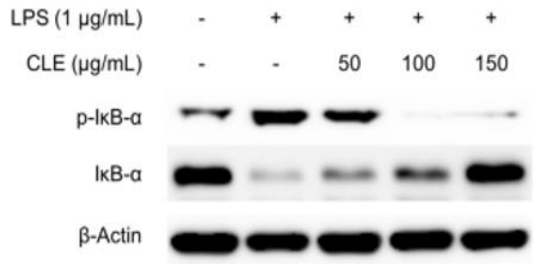


f)

Figure 3. CLE effects on pro-inflammatory cytokines and their gene expression in BV2 cells. Cells were pre-treated with CLE for 3 h, then exposed to LPS (1 μg/mL) for 24 h (protein) or 6 h (mRNA). Cytokines were measured by ELISA, and mRNA by qPCR. #p < 0.001 vs. control; **p < 0.01, ***p < 0.001 vs. LPS group.*

CLE suppresses LPS-induced NF-κB pathway activation in BV2 microglial cells

To explore the mechanisms behind CLE's inhibitory effects on inflammatory mediator production and enzyme expression, we assessed its impact on the NF-κB signaling pathway. BV2 cells were exposed to specified CLE concentrations for 3 h, followed by 1 h stimulation with LPS (1 μg/mL). LPS treatment caused phosphorylation and degradation of IκB-α, whereas CLE pre-treatment attenuated these effects in a concentration-dependent manner **(Figure 4a).** Since p65 is the principal subunit of the NF-κB heterodimer, we further examined whether CLE influenced its nuclear translocation after release from IκB-α. LPS exposure increased nuclear p65 levels, which were reduced by CLE pre-treatment **(Figure 4b).**



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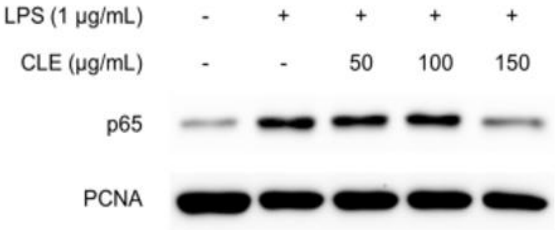
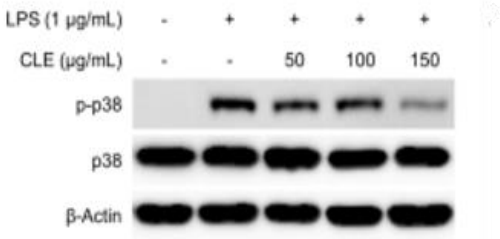


b)

Figure 4. Impact of CLE on NF-κB signaling activation in LPS-stimulated BV2 microglial cells. Cells were pre-treated with the indicated CLE concentrations for 3 h and then exposed to LPS (1 μg/mL) for 1 h. Nuclear and cytoplasmic fractions were separated, and Western blot analysis was conducted to measure p-IκB-α and IκB-α in the cytosol and p65 in the nucleus. β-Actin and PCNA served as loading controls for cytoplasmic and nuclear fractions, respectively. Representative blots from three independent experiments are presented.

CLE attenuates LPS-triggered MAPK pathway activation in BV2 cells

To determine whether CLE modulates MAPK signaling, BV2 cells were pre-exposed to specified CLE concentrations for 3 h before 1 h LPS (1 μg/mL) stimulation. LPS markedly increased phosphorylation of p38, ERK, and JNK, whereas CLE pre-treatment substantially reduced phosphorylation of these MAPKs **(Figure 5).**



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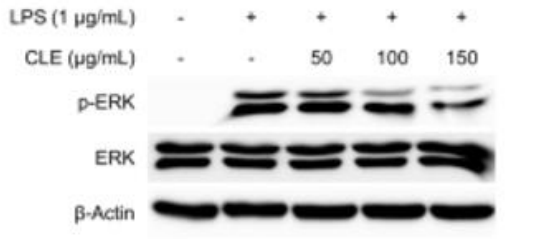

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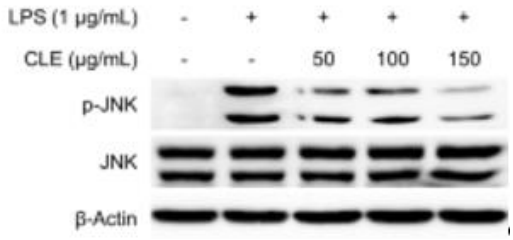


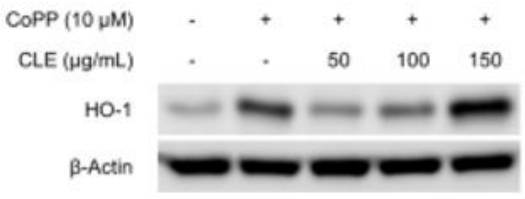
c)

Figure 5. CLE-mediated suppression of MAPK signaling in LPS-treated BV2 cells. Cells were pre-treated with CLE for 3 h, followed by 1 h of LPS (1 μg/mL) stimulation. Western blotting was performed to detect phosphorylated and total p38, ERK, and JNK. β-Actin was used as a loading control. Representative blots from three separate experiments are shown.

CLE upregulates HO-1 via Nrf2 activation in BV2 cells

Western blot analysis was used to investigate CLE-induced HO-1 expression. BV2 cells treated with CLE for 12 h showed increased HO-1 protein levels **(Figure 6a)**, which correlated with enhanced nuclear translocation of Nrf2 **(Figures 6b and 6c).**

To explore the role of HO-1 in CLE's anti-inflammatory effect, cells were pre-treated with 150 μg/mL CLE for 3 h, with or without 1 h pre-treatment with SnPP, then exposed to LPS (1 μg/mL) for 24 h. CLE significantly reduced NO and PGE2 levels and lowered iNOS and COX-2 expression. Blocking HO-1 using SnPP reversed these inhibitory effects of CLE, while SnPP or CLE alone had no independent effect on these inflammatory mediators **(Figure 7).**



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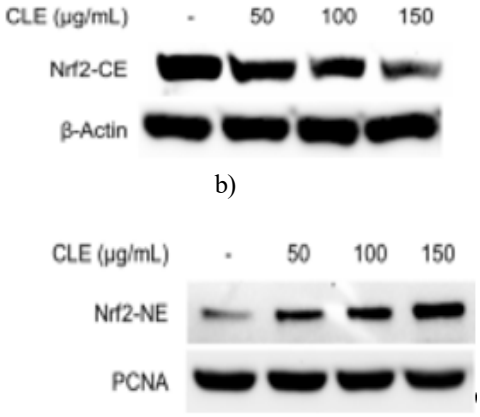
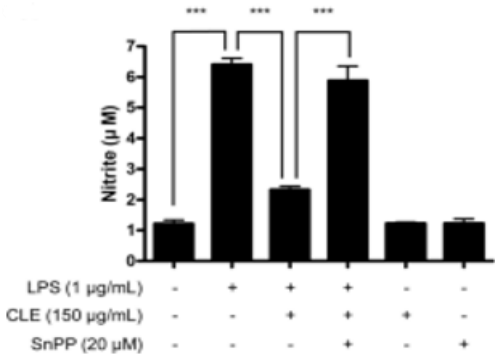


b)

c)

Figure 6. CLE effect on HO-1 expression (A) and Nrf2 nuclear localization (B, C) in BV2 cells. Cells were treated with CLE for 12 h at indicated concentrations. Western blotting was performed to detect HO-1, cytoplasmic Nrf2, and nuclear Nrf2. β-Actin and PCNA were used as loading controls for cytoplasmic and nuclear fractions, respectively. Representative blots from three independent experiments are shown.



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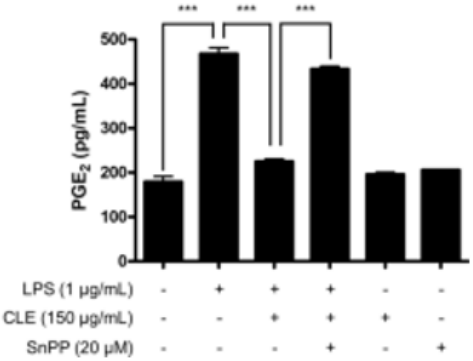

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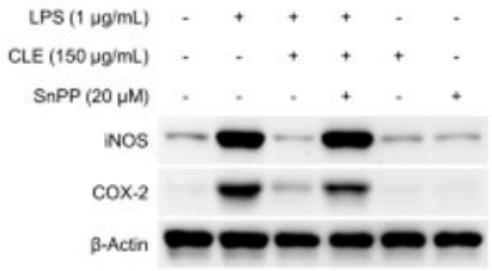

c)

Figure 7. SnPP reverses CLE-induced inhibition of NO, PGE2, iNOS, and COX-2 in BV2 cells. Cells were pre-treated with 150 μg/mL CLE for 3 h, with or without SnPP pre-treatment for 1 h, followed by 24 h LPS (1 μg/mL) stimulation. (A, B) NO and PGE2 levels were quantified using the Griess assay and ELISA, respectively. Data represent mean ± SD of three independent experiments. ***$p < 0.001$. (C) Western blot analysis of iNOS and COX-2 protein expression; β-Actin was used as a loading control. Representative blots from three independent experiments are shown.

This study revealed that the 50% ethanolic extract of *Curcuma longa* (CLE) exhibited notable anti-neuroinflammatory activity in LPS-stimulated BV2 microglial cells. The observed effects were linked to suppression of NF-κB and MAPK (p38, ERK, and JNK) signaling pathways, which consequently decreased the production of pro-inflammatory mediators such as NO, PGE2, iNOS, COX-2, and cytokines including IL-1β, IL-6, and TNF-α. Additionally, CLE enhanced HO-1 protein expression via Nrf2 activation, and inhibition of HO-1 by SnPP reversed CLE's suppressive effects, indicating that its anti-neuroinflammatory properties are mediated through the HO-1/Nrf2 signaling axis.

Neuroinflammation is typically characterized by the excessive generation of pro-inflammatory molecules, including NO, PGE2, iNOS, COX-2, and cytokines [13]. NO production is catalyzed by iNOS, which converts L-arginine to NO and L-citrulline via the intermediate N-hydroxy-L-arginine [14]. PGE2 is produced from arachidonic acid through the activity of COX enzymes and PGE synthases (PGES) [15]. Pro-inflammatory cytokines are small secreted proteins from immune cells that modulate various CNS functions, including sleep, neuronal development, and immune responses against bacterial and viral infections [16]. Therefore, targeting these mediators is a viable strategy for preventing or mitigating neuroinflammatory disorders. In this context, CLE pre-treatment suppressed LPS-induced overproduction of NO and PGE2, as well as protein expression of iNOS and COX-2 **(Figure 2)**. Furthermore, CLE reduced both the secretion and mRNA levels of IL-1β, IL-6, and TNF-α in BV2 cells **(Figure 3)**.

NF-κB is a family of inducible transcription factors that control numerous genes involved in immune and inflammatory responses [17]. It consists of five members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100), which regulate target genes by binding κB enhancer sequences as homo- or heterodimers [18]. NF-κB activation occurs via two principal pathways: the classical/canonical pathway and the alternative/non-canonical pathway, both essential for modulating inflammation [19, 20]. The canonical pathway is triggered by various cell surface receptors, including IL-1 receptor, Toll-like receptors (TLRs), TNF receptor, T-cell receptor, and B-cell receptor [18]. In contrast, the non-canonical pathway is activated by specific ligands of TNF receptor superfamily members such as BAFF, CD40, lymphotoxin β receptor, and RANK [17]. NF-κB responds to diverse stimuli, including viral infection, bacterial toxins, UV radiation, oxidative stress, inflammatory cues, cytokines, carcinogens, tumor promoters, and mitogens [21, 22]. This transcription factor regulates genes encoding iNOS, COX-2, LOX, cytokines, adhesion molecules, cell cycle regulators, and angiogenic factors [23]. Consequently, suppressing NF-κB is considered a crucial strategy for controlling neuroinflammation. In the present study, CLE inhibited LPS-induced NF-κB activation by preventing IκB-α phosphorylation and degradation, as well as p65 nuclear translocation **(Figure 4).**

MAPKs are a family of serine/threonine kinases involved in regulating gene expression, apoptosis, cell proliferation, differentiation, and responses to cellular stress and inflammation [24]. MAPK pathways are typically activated by TLRs, toll-interleukin receptors (TIR), or TNF receptor families in response to primary inflammatory signals and cytokines [25]. In mammals, three major MAPK subgroups exist: p38, ERK, and JNK [26]. ERK1 and ERK2 are highly homologous and ubiquitously expressed, activated by MAPK kinases (MKKs), and play critical roles in cell proliferation, apoptosis, cytoskeletal organization, and motility [25, 26]. JNKs, also called stress-activated kinases (SAPKs), include at least ten isoforms from three genes (JNK1, JNK2, JNK3), and are activated in response to cellular

stress; MKK4 and MKK7 regulate their activation, which is essential for cell survival and viral replication [25, 27]. Mammals possess four p38 isoforms (p38α, p38β, p38γ, p38δ); p38α and p38β are widely expressed, while p38γ and p38δ are restricted to the kidney, skin, and muscle [28]. These isoforms are activated by MKK3, MKK4, and MKK6, leading to phosphorylation of transcription factors that control pro-inflammatory mediator production [29]. MAPK inhibition is considered a promising anti-inflammatory approach because it reduces mediator synthesis at multiple levels and disrupts inflammatory cytokine signaling [30]. In this study, CLE pre-treatment suppressed LPS-induced phosphorylation of p38, ERK, and JNK in BV2 cells **(Figure 5).**

HO-1 and Nrf2 Signaling in neuroinflammation

Heme oxygenase (HO) exists in three isoforms: the inducible HO-1, also called heat-shock protein 32 (Hsp-32), and the constitutively expressed HO-2 and HO-3 [31, 32]. HO-1 functions as a phase II detoxifying antioxidant enzyme and is upregulated under various pathological conditions, including oxidative stress, ischemia, hypoxia, and pro-inflammatory cytokines [33, 34]. Under oxidative and inflammatory stress, HO-1 serves as the rate-limiting enzyme for heme catabolism, producing carbon monoxide (CO), ferrous ions (Fe^{2+}), and biliverdin, which act as antioxidant and anti-inflammatory mediators, mitigating oxidative damage and related disorders [33, 35]. Furthermore, HO-1 expression can be induced by anti-inflammatory cytokines [36], supporting its role as a therapeutic target in neurodegenerative and infectious brain diseases [37].

HO-1 induction is regulated by the Nrf2 signaling pathway. Nrf2, a member of the cap-n-collar (CNC) basic leucine zipper transcription factor family, is critical for maintaining redox homeostasis, controlling oxidative stress, and modulating immune responses [38–40]. At rest, Nrf2 is sequestered in the cytoplasm by Keap1, which promotes its ubiquitination and degradation [40, 41]. Upon oxidative or inflammatory stimuli, Nrf2 dissociates from Keap1, translocates to the nucleus, dimerizes with small Maf proteins, and binds antioxidant response elements (ARE) in the promoter regions of cytoprotective and phase II detoxifying genes such as HO-1, NQO1, PRX, Trx, GST, and GPx [40, 42]. Nrf2-ARE interaction also regulates pro- and anti-inflammatory enzymes, including iNOS and COX-2 [43]. Several natural compounds, including berberine from *Coptidis chinensis*, 7,8-dihydroxyflavone, and tryptanthrin, have been reported to induce HO-1 through Nrf2-ARE activation in astrocytes, myoblasts, and microglial cells [35, 44, 45]. In this study, CLE enhanced HO-1 expression and promoted nuclear accumulation of Nrf2. Pre-treatment with SnPP, an HO-1 inhibitor, abolished CLE-mediated reductions in NO and PGE2 production and iNOS and COX-2 expression, demonstrating that CLE's anti-neuroinflammatory effects are largely mediated through the HO-1/Nrf2 pathway.

C. longa contains various bioactive compounds, including diarylheptanoids (curcuminoids), diarylpentanoids, monoterpenes, sesquiterpenes, diterpenes, triterpenoids, alkaloids, and sterols, with curcuminoids being the most abundant [46]. To quantify curcuminoids, which are the primary bioactive constituents of C. longa, a method using the ethanolic extract of Korean-grown C. longa was developed and validated in a previous study [10]. The ethanolic extract is optimal for analyzing curcuminoid content, and its physiological effects are likely attributed to curcuminoids. However, other compounds, such as monoterpenes and sesquiterpenes, may also contribute to the anti-neuroinflammatory activity, warranting further investigation.

In this study, the maximum CLE concentration of 150 μg/mL was selected based on cytotoxicity assays (**Figure 1)**, showing toxicity at 200 μg/mL but not at 150 μg/mL. Previous research using hexane extracts of C. longa evaluated concentrations up to 500 μg/mL for antioxidant effects in BV2 cells [9]. Additionally, studies assessing anti-neuroinflammatory effects of other natural extracts in BV2 cells have employed concentrations higher than 150 μg/mL [47, 48]. Therefore, 150 μg/mL is considered sufficient to investigate CLE's anti-neuroinflammatory properties.

Conclusion

This study demonstrated that CLE suppresses LPS-induced overproduction of inflammatory mediators in BV2 microglial cells by inhibiting NF-κB, p38 MAPK, ERK MAPK, and JNK MAPK, while activating the HO-1/Nrf2 pathway **(Figure 8)**. These findings suggest that CLE could serve as a promising candidate for the development of anti-inflammatory therapies targeting neurodegenerative diseases.

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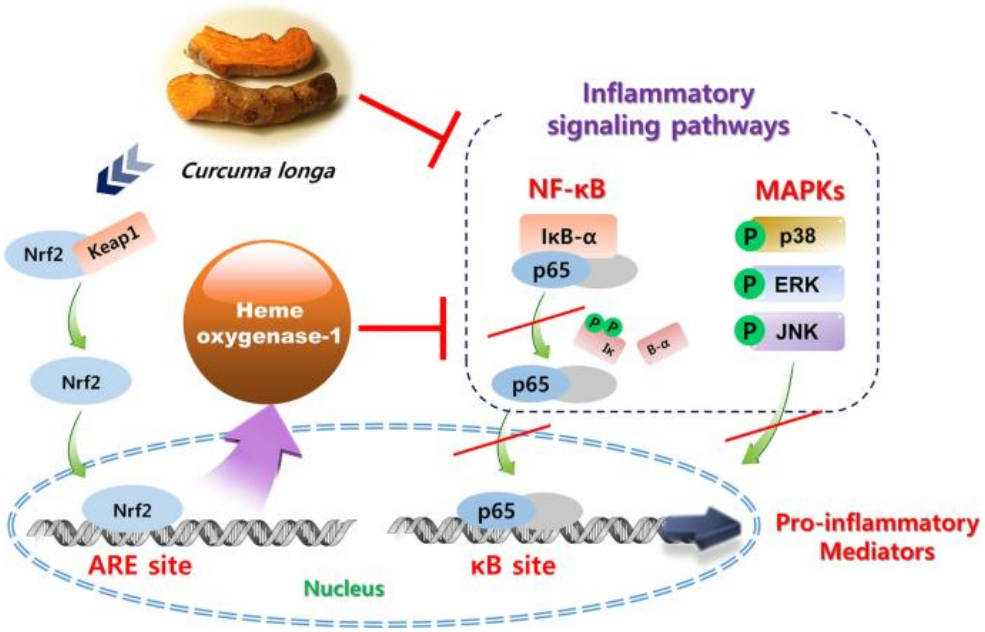

Figure 8. Schematic representation of the molecular pathways through which CLE exerts anti-neuroinflammatory effects.

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