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Cudratricusxanthone L Suppresses Lipopolysaccharide-induced Activation of BV2 and Primary Rat Microglial Cells by Inhibiting JNK, p38 MAPK, and NF-κB Signaling

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Abstract

Neuroinflammatory responses are implicated in the pathogenesis of neurodegenerative diseases. In neurodegenerative diseases, neuroinflammatory reactions to neuronal injury are modulated by microglial cells, which are vital innate immune cells in the central nervous system. Activated microglial cells release proinflammatory cytokines, mediators, and neurotoxic factors that induce fatal neuronal injury. The present study investigated the anti-neuroinflammatory effects of cudratricusxanthone L (**1**), which was isolated from *Cudrania tricuspidata*. This compound reduced the levels of lipopolysaccharide-stimulated inflammatory mediators and cytokines, including nitric oxide, prostaglandin E2, interleukin (IL)-1β, tumor necrosis factor-α, IL-6, and IL-12. These effects suggested that cudratricusxanthone L (**1**) suppressed the nuclear factor-kappa B (NF-κB) signaling pathway. Specifically, cudratricusxanthone L (**1**) also attenuated the phosphorylation of Jun kinase and inhibited p38 mitogen-activated protein kinase (MAPK) signaling in BV2 and rat primary microglial cells. These results indicated that cudratricusxanthone L (**1**) effectively repressed neuroinflammatory processes in BV2 and rat primary microglial cells by inhibiting NF-κB and the MAPK signaling pathway.

Keywords: Cudratricusxanthone L; *Cudrania tricuspidata*; Neuroinflammation; Nuclear factor-kappa B (NF-κB); Mitogen-activated protein kinase (MAPK).

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1. Introduction

Neuroinflammation plays a vital role in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, HIV-associated dementia, multiple sclerosis, and stroke [1]. Chemokines, cytokines, nitric oxide (NO), reactive oxygen species, and prostaglandin E2 (PGE_2) play a pivotal role in modulating immune responses [2]. Microglia, the resident of macrophages within the central nervous system, are the primary effectors of neuroinflammation [3]. Microglial cells are activated by lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, and generate neuroinflammation by releasing inflammatory mediators and cytokines such as NO, PGE_2, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-12 (IL-12), and interleukin-6 (IL-6) [4, 5]. Accordingly, regulation of microglial cell activation and production of these proinflammatory cytokines and mediators could provide a useful therapeutic approach to neurodegenerative diseases.

Nuclear factor-kappa B (NF-κB) is a crucial transcription factor that mediates inflammatory responses through regulation of the proinflammatory cytokines and chemokines in microglial cells [6, 7]. NF-κB is normally located in the cytoplasm as an inactive form, which is inhibited by inhibitor of kappa B-α (IκB-α). Following LPS stimulation, IκB-α is phosphorylated and ubiquitinated; the unbound NF-κB then translocates to the nucleus, where it binds to kappaB (κB) sites. NF-κB thus induces the transcription of target genes [8, 9]. In addition, NF-κB activity is modulated by mitogen-activated protein kinases (MAPKs) [10, 11]. MAPKs are one of the major kinase families involved in inflammatory processes. MAPKs such as extracellular signal regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK have all been shown to regulate NF-κB activation [12]. Previous research showed that JNK and p38 MAPK were associated with inflammation in the immune system [13].

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Cudrania tricuspidata (Moraceae), a deciduous broadleaf thorny tree, grows in Korea, China, and Japan. The root and cortex of *C. tricuspidata* have been used in traditional medicine for the treatment of inflammation and neuritis [14]. *C. tricuspidata* contains high levels of flavonoids, xanthones, and glycoproteins [15] reported to have a range of biological effects; these include antioxidant [16], hepatoprotective [17], anti-atherosclerotic, anti-inflammatory [18], neuroprotective [19], and monoamine oxidase A inhibitory activities [20]. The present study investigated the effects of a compound isolated from *C. tricuspidata*, cudratricusxanthone L (**1**), on the MAPK and NF-kB signaling pathways.

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2. Results

*2.1. Chemical structure of cudratricusxanthone L (**1**) and its effect on BV2 microglial cell viability*

The chemical structure of cudratricusxanthone L (**1**), isolated from *C. tricuspidata* (Figure 1), was determined in a previous study [17]. To identify the cytotoxic effects of cudratricusxanthone L (**1**), we conducted an MTT assay to investigate the viability of BV2 microglial cells. No cytotoxic effects were observed in BV2 cells exposed to 1.3-10.0 μM **1** (Figure 2).

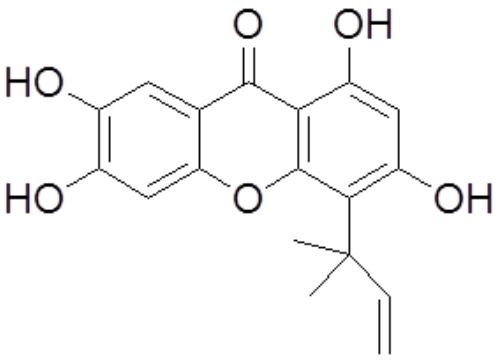

Figure 1. Chemical structure of cudratricusxanthone L (**1**).

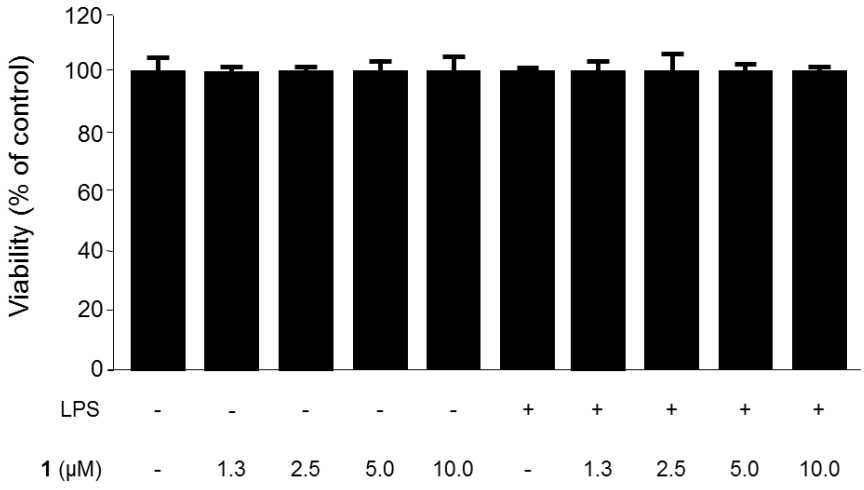

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Figure 2. Effects of cudratricusxanthone L (**1**) on cell viability in BV2 microglial cells. BV2 microglial cells were incubated for 24 h with various concentrations of cudratricusxanthone L (1.3-10.0 μM). Data represent the mean ± SD of three experiments.

*2.2. Effects of cudratricusxanthone L (**1**) on the production of TNF-α, IL-1β, IL-12, and IL-6 by LPS-stimulated BV2 microglial cells*

We evaluated the effects of cudratricusxanthone L (**1**), obtained from a methanol extract of *C. tricuspidata*, on the levels of TNF-α, IL-1β, IL-12, and IL-6 in the media of LPS-treated BV2 microglial cells (Figure 3). The levels of these pro-inflammatory cytokines in media conditioned by LPS-treated BV2 microglial cells were reduced in cells exposed to cudratricusxanthone L (**1**) at a concentration range of 1.3-10.0 μM for 12 h. As shown in Figure 3A-3D, cudratricusxanthone L (**1**) reduced the levels of TNF-α, IL-1β, IL-12, and IL-6 in a concentration-dependent manner.

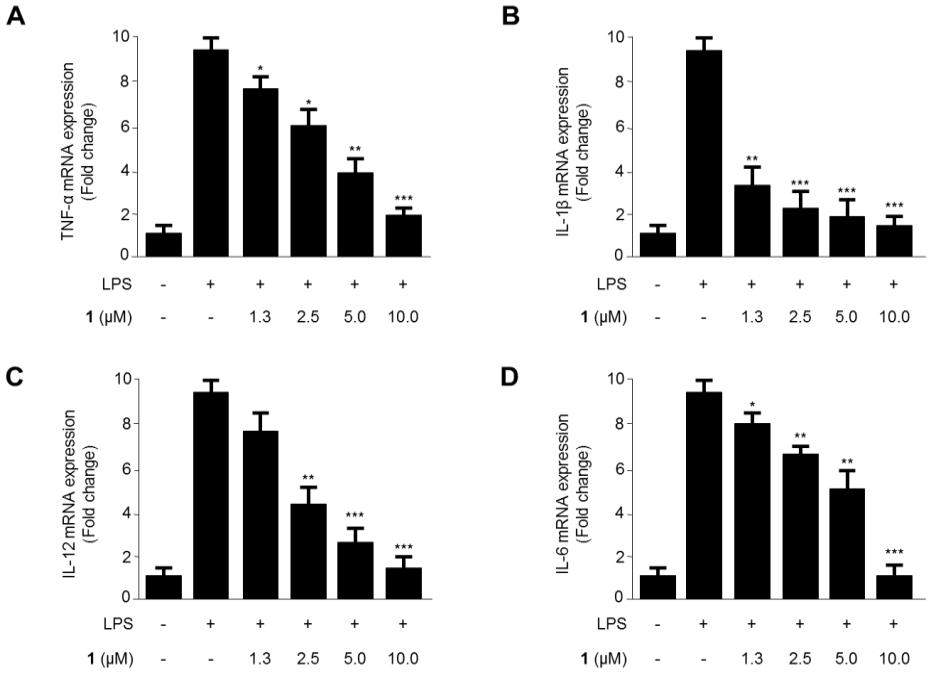

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Figure 3. Effects of cudratricusxanthone L (**1**) on TNF-α (A), IL-1β (B), IL-12 (C), and IL-6 (D) in LPS-stimulated BV2 microglial cells. (A-D) Cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 12 h with LPS (1 μg/mL). The concentrations of TNF-α, IL-1β, IL-12, and IL-6 were determined as described in the Materials and Methods section. Data represent the mean ± SD of three experiments; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, as compared to the LPS-treated cells.

*2.3. Effects of cudratricusxanthone L (**1**) on nitrite and PGE_2 production, and on iNOS and COX-2 protein expression in LPS-stimulated BV2 microglial cells*

Cells were treated with or without LPS (1 μg/mL) in the presence or absence of cudratricusxanthone L (**1**) for 24 h (Figure 4). LPS-mediated upregulation of nitrite (Figure 4A) and PGE_2 (Figure 4B) levels, and of iNOS and COX-2 protein expression (Figure 4C), were significantly repressed by cudratricusxanthone L (**1**) in a concentration-dependent manner. Additionally, these inhibitory effects of cudratricusxanthone L (**1**) did not involve cytotoxic effects in BV2 microglial cells.

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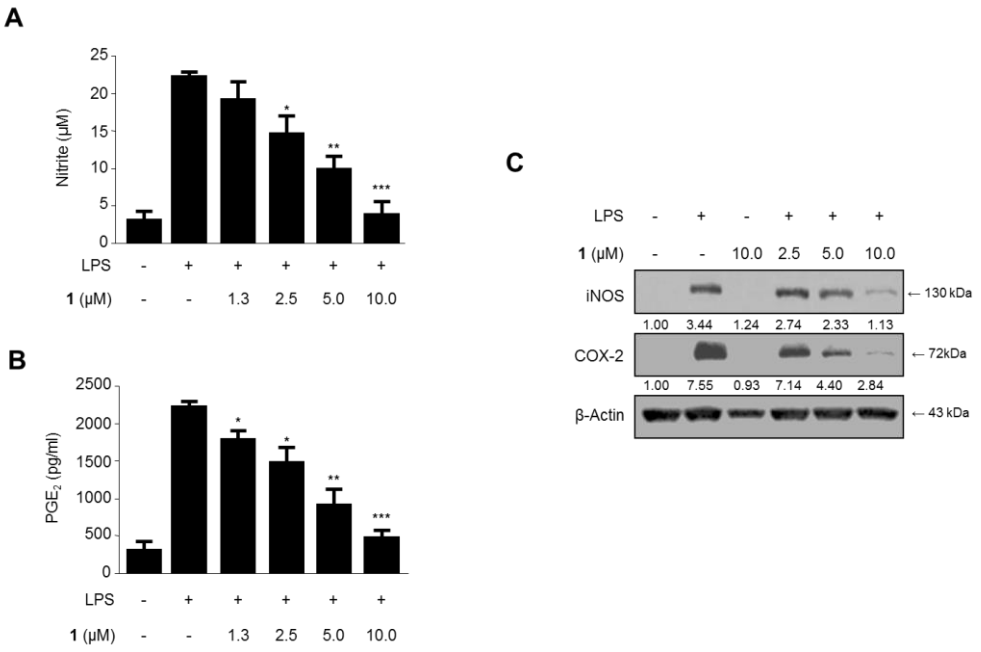

Figure 4. Effects of cudratricusxanthone L (**1**) on nitrite (A) and PGE_2 (B) levels, and iNOS and COX-2 expression (C) in LPS-stimulated BV2 microglial cells. (A-C) Cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 24 h with LPS (1 μg/mL). Nitrite and PGE_2 assays and western blots were conducted as described in the Materials and Methods section. Data represent the mean ± SD of three experiments. Band intensity was quantified by densitometry and normalized to β-actin; the normalized values are presented below each band; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, as compared to the LPS-treated cells.

*2.4. Effects of cudratricusxanthone L (**1**) on IκB-α levels, NF-κB nuclear translocation, and NF-κB DNA binding activity in LPS-stimulated BV2 microglial cells*

Firstly, we investigated the effects of cudratricusxanthone L on IκB-α degradation and phosphorylation. Moreover, we checked how this influenced NF-κB (p50 and p65) nuclear

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translocation. As shown in Figure 5A, IκB-α was degraded in BV2 microglia exposed to LPS for 1 h. However, cudratricusxanthone L (**1**) pretreatment (2.5-10.0 μM) significantly repressed the phosphorylation of IκB-α (Figure 5A) in LPS-stimulated BV2 microglial cells. We next determined the effects of cudratricusxanthone L (**1**) on the nuclear translocation of NF-κB in LPS-induced BV2 microglial cells. NF-κB translocation was blocked in BV2 microglial cells exposed to cudratricusxanthone L (**1**) (Figure 5B and 5C). Moreover, we evaluated the NF-κB DNA binding activity in nuclear extracts from BV2 microglial cells challenged with LPS. This induced an approximately 10-fold increase in NF-κB DNA binding activity, which was repressed by cudratricusxanthone L (**1**) in a concentration-dependent manner (Figure 5D). Confocal microscopy indicated that NF-κB/p50 protein was almost exclusively in the cytoplasm in unstimulated BV2 microglial cells. After treatment with LPS, NF-κB/p50 was observed in the nucleus, indicating that it had translocated (Figure 5E).

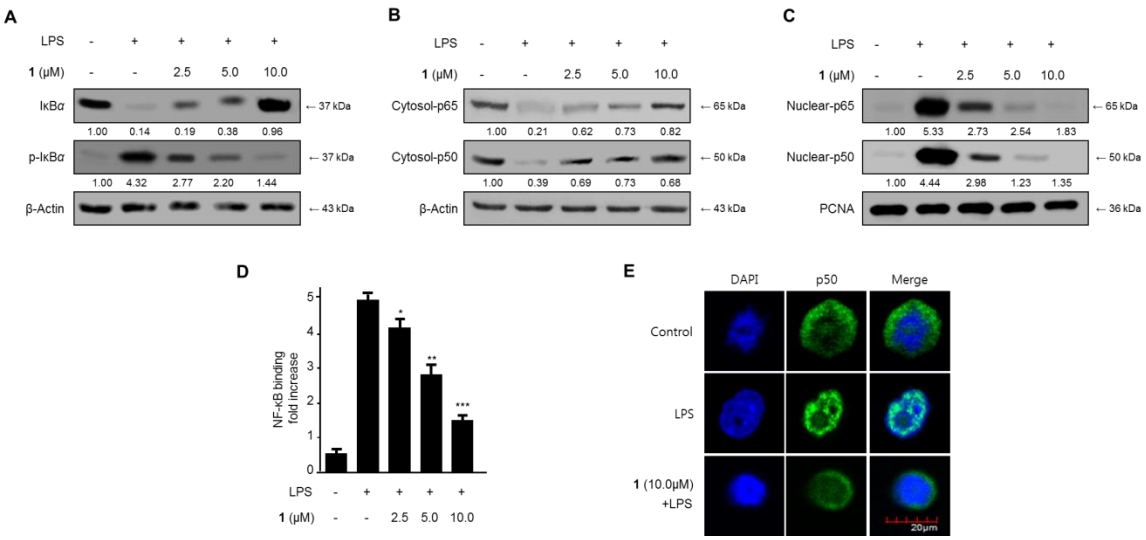

Figure 5. The effects of cudratricusxanthone L (**1**) on IκB-α phosphorylation and degradation (A), NF-κB activation (B, C), NF-κB DNA binding activity (D), and NF-κB localization (E) in LPS-stimulated BV2 microglial cells. (A-E) The cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated

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for 1 h with LPS (1 μg/mL). Western blot analysis, NF-κB DNA binding activity, and immunofluorescence were investigated as described in the Materials and Methods. Data represent the mean ± SD of three experiments. Band intensity was quantified by densitometry and normalized to β-actin or PCNA; the normalized values are presented below each band; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, as compared to the LPS-treated cells.

*2.5. Effects of cudratricusxanthone L (**1**) on MAPK phosphorylation in BV2 microglial cells stimulated with LPS*

As shown in Figure 6, phosphorylation of ERK, JNK, and p38 were increased in BV2 microglial cells treated with LPS for 1 h. Moreover, 2.5-10 μM cudratricusxanthone L (**1**) treatment appeared to inhibit JNK and p38 MAPK phosphorylation in a concentration-dependent manner (Figure 6B and 6C).

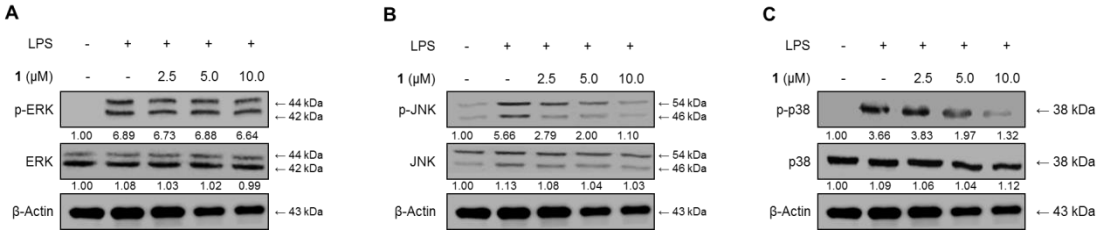

Figure 6. Effects of cudratricusxanthone L (**1**) on ERK (A), JNK (B), and p38 (C) MAPK phosphorylation and protein expression. (A-C) Cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 1 h with LPS (1 μg/mL). The levels of phosphorylated-ERK (p-ERK), phosphorylated-JNK (p-JNK), and phosphorylated-p38 MAPK (p-p38 MAPK) were determined by western blot analysis. Representative blots from three independent experiments

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with similar results and densitometric evaluations are shown. Band intensity was quantified by densitometry and normalized to β-actin, and the values are presented below each band.

*2.6. Effects of cudratricusxanthone L (**1**) on nitrite production, and iNOS and COX-2 protein expression, in LPS-stimulated rat primary microglial cells*

Cells were treated with or without LPS (1 μg/mL) in the presence or absence of cudratricusxanthone L (**1**) for 24 h. LPS-mediated upregulation of nitrite levels (Figure 7A) and iNOS protein expression (Figure 7C) were significantly repressed by cudratricusxanthone L (**1**) in a concentration-dependent manner. Additionally, the inhibitory effects of cudratricusxanthone L (**1**) on nitrite and PGE_2 levels, and on iNOS and COX-2 protein expression, did not involve cytotoxic effects in rat primary microglial cells.

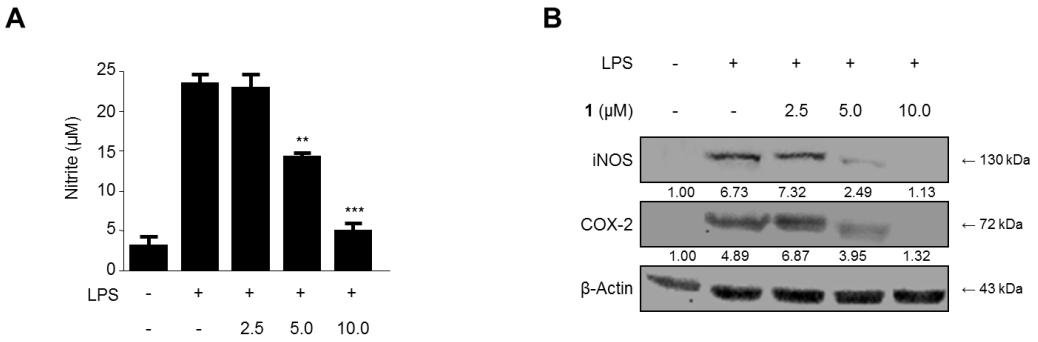

Figure 7. Effects of cudratricusxanthone L (**1**) on nitrite (A) production, and iNOS and COX-2 expression (B) in LPS-stimulated primary rat microglial cells. (A, B) Cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 24 h with LPS (1 μg/mL). Nitrite assays and western blots were conducted as described in the Materials and Methods section. Data represent the

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mean ± SD of three experiments. Band intensity was quantified by densitometry and normalized to β-actin, and the normalized values are presented below each band; $^{**}p < 0.01$; $^{***}p < 0.001$, as compared to the LPS-treated cells.

*2.7. Effects of cudratricusxanthone L (**1**) on the production of TNF-α, IL-1β, IL-12, and IL-6 by LPS-stimulated rat primary microglial cells*

We checked the the levels of TNF-α, IL-1β, IL-12, and IL-6 mRNA expressions in LPS-treated rat primary microglial cells (Figure 8). The levels of these pro-inflammatory cytokines in media conditioned by LPS-treated rat primary microglial cells were reduced in cells exposed to cudratricusxanthone L (**1**) at a concentration range of 1.3-10.0 μM for 6 h. As shown in Figure 8A-8D, cudratricusxanthone L (**1**) decreased the expressions of TNF-α, IL-1β, IL-12, and IL-6 in a dose-dependent manner.

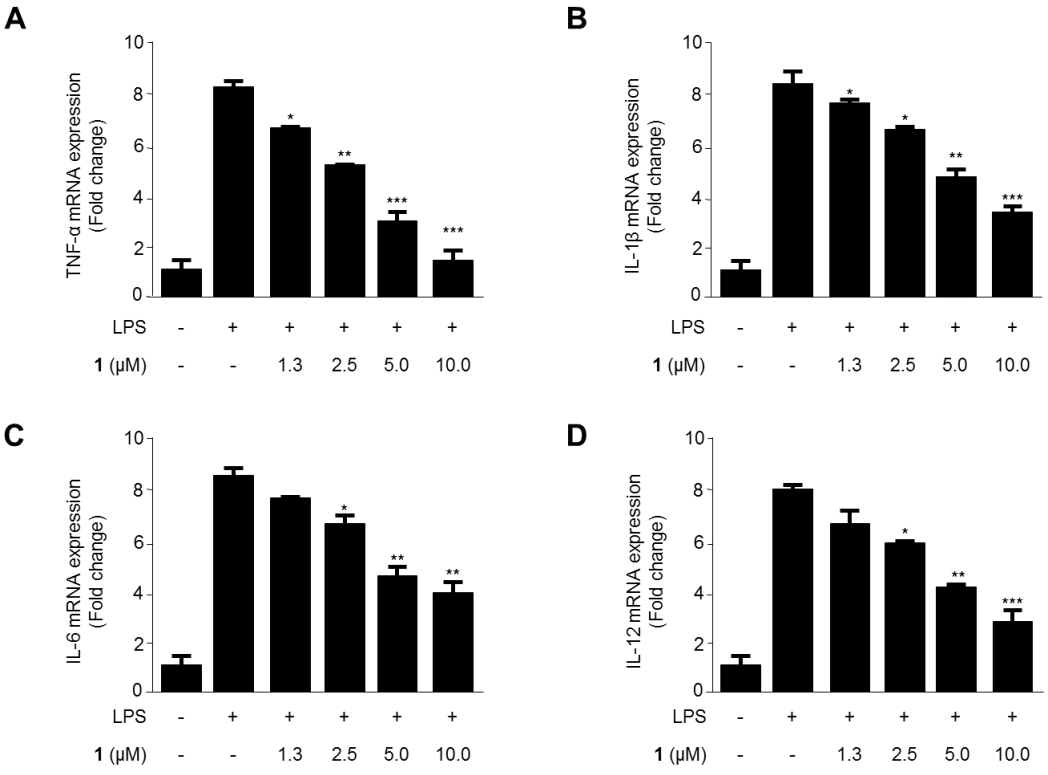

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Figure 8. Effects of cudratricusxanthone L (**1**) on TNF-α (A), IL-1β (B), IL-6 (C), and IL-12 (D) in LPS-stimulated primary rat microglial cells. (A-D) Cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 12 h with LPS (1 μg/mL). The concentrations of TNF-α, IL-1β, IL-6, and IL-12 were determined as described in the Materials and Methods section. Data represent the mean ± SD of three experiments; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, as compared to the LPS-treated cells.

*2.8. Effects of cudratricusxanthone L (**1**) on IκB-α levels, NF-κB nuclear translocation, and NF-κB DNA binding activity in LPS-stimulated rat primary microglial cells*

As shown in Figure 8A, IκB-α was degraded after exposure of rat primary microglial cells to LPS for 1 h. However, cudratricusxanthone L (**1**) pretreatment (2.5-10.0 μM) significantly repressed the phosphorylation of IκB-α (Figure 9A) in LPS-stimulated rat primary microglial cells. NF-κB translocation was also blocked in rat primary microglial cells treated with cudratricusxanthone L (**1**) (Figure 9B and 9C). Confocal microscopy showed that NF-κB/p50 protein existed almost exclusively in the cytoplasm in unstimulated rat primary microglial cells. After treatment with LPS, NF-κB/p50 was observed in the nucleus, indicating that it had translocated (Figure 9D).

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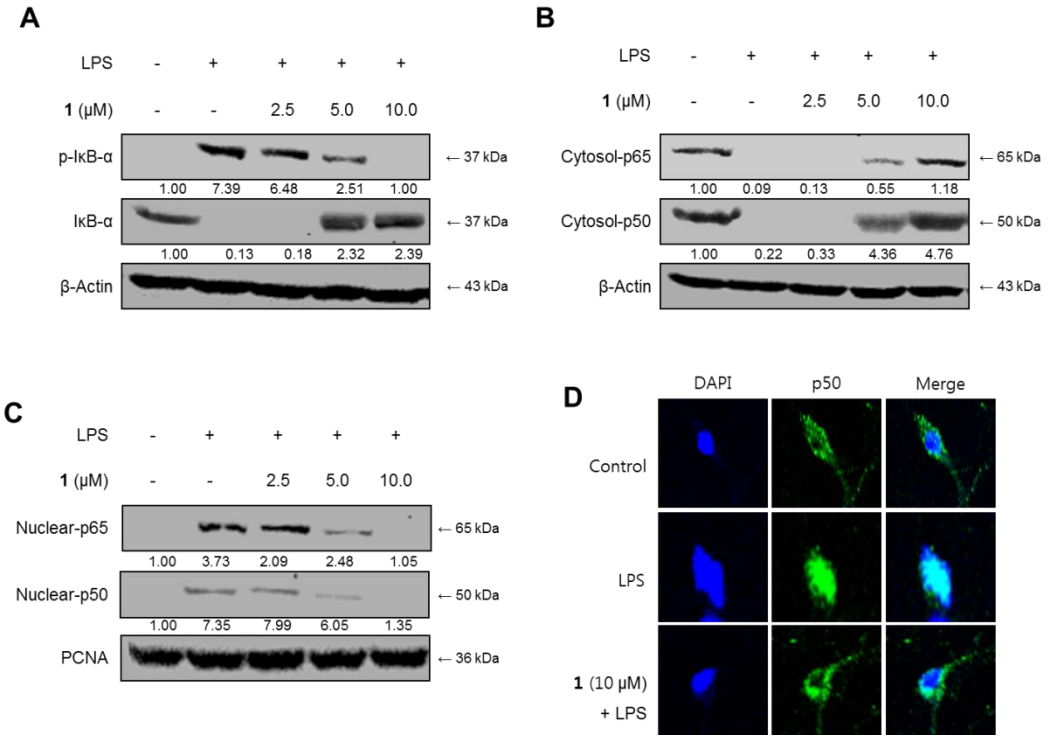

Figure 9. The effects of cudratricusxanthone L (**1**) on IκB-α phosphorylation and degradation (A), NF-κB activation (B, C), and NF-κB localization (D) in LPS-stimulated primary rat microglial cells. (A-D) The cells were pre-treated for 3 h with the indicated concentrations of **1** and then stimulated for 1 h with LPS (1 μg/mL). Western blot analysis and immunofluorescence were investigated as described in the Materials and Methods. Data represent the mean ± SD of three experiments. Band intensity was quantified by densitometry and normalized to β-actin or PCNA; the normalized values are presented below each band.

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3. Discussion

The present investigation demonstrated that cudratricusxanthone L, isolated from *C. tricuspidata*, appeared to exert anti-neuroinflammatory effects in both BV2 and primary microglial cells, by inactivating NF-κB and MAPKs pathways. Plant-derived natural products and their bioactive constituents have been shown to have anti-inflammatory and antioxidant activities, and they could protect the brain against inflammatory damage [24, 25]. *C. tricuspidata* is used as one of the traditional medical herbs for the treatment of inflammation, oxidative stress, and hepatosis [17, 27, 28]. Therefore, the present study investigated the anti-neuroinflammatory effects of a small-molecule constituent of *C. tricuspidata* (cudratricusxanthone L) in microglial cells.

Microglial cells act as the macrophages of the central nervous system [29]. BV2 and primary microglial cells are used as *in vitro* models, in order to elucidate inflammatory reactions [30, 31]. The BV2 cell line is an immortalized murine microglial cell line, and primary microglial cells were isolated from rat cerebral cortices. Although these cells have some similar properties, the BV2 cell line does not have all the characteristics of microglial cells [32]. Thus, we tested the anti-inflammatory effects of cudratricusxanthone L in both BV2 and primary microglial cells, in order to determine the suitability of cudratricusxanthone L as an anti-neuroinflammatory agent for the treatment of various neurodegenerative diseases.

The expression of iNOS and COX-2 proteins is essential for immune-activated inflammatory cells, including microglial cells, because iNOS generates NO and COX-2 produces PGE_2 [33]. Thus, inhibition of iNOS and COX-2 can produce significant anti-neuroinflammatory effects. Pre-treatment with cudratricusxanthone L attenuated LPS-mediated stimulation of iNOS and COX-2 protein expression, as well as reducing the

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production of NO and PGE_2, in BV2 cells (Figure 4). Cudratricusxanthone L also appeared to inhibit the production of NO and iNOS/COX-2 expression in primary microglial cells (Figure 7).

LPS-activated microglial cells show increased production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12; these cytokines are associated with inflammatory responses [34]. Therefore, we investigated whether cudratricusxanthone L altered the production of pro-inflammatory cytokines in BV2 and primary microglial cells. This analysis found that cudratricusxanthone L attenuated the LPS-induced production of these pro-inflammatory cytokines in both BV2 (Figure 3) and primary microglial cells (Figure 8).

Activated NF-κB promotes cellular signal transduction pathways that are related to the regulation of iNOS, COX-2, and various cytokines [35-37]. Therefore, blocking NF-κB transcriptional activity could provide an important tool for the treatment of neuroinflammatory diseases [38]. Pre-treatment with cudratricusxanthone L inactivated NF-κB pathways by inhibiting the phosphorylation and degradation of IκB-α, reducing nuclear translocation of p65 and p50 dimers, and suppressing the DNA binding activity of p65 in both BV2 (Figure 5) and primary microglial cells (Figure 9).

Furthermore, MAPK pathways are also involved in LPS-induced iNOS and COX-2 expression through regulation of NF-κB activation in microglial cells [39]. Thus, the effect of cudratricusxanthone L on LPS-induced MAPK activation was examined. This analysis indicated that cudratricusxanthone L inhibited JNK and p38 MAPK, leading to a reduction in LPS-induced iNOS and COX-2 expression (Figure 6).

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4. Materials and Methods

4.1. Chemicals and reagents

Cudratricusxanthone L was obtained as described in our previous study [17, 21]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies, including mouse/goat/rabbit anti-cyclooxygenase 2 (COX-2), anti-inducible NO synthase (iNOS), anti-β-actin, anti-IκB-α, anti-phosphorylated IκB-α, anti-p50, anti-p65, and anti-proliferating cell nuclear antigen (PCNA), and secondary antibodies, were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-phosphorylated ERK, anti-ERK, anti-phosphorylated JNK, anti-JNK, anti-phosphorylated p38, and anti-p38 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

4.2. Cell culture and viability assay

BV2 microglial cells were obtained from Prof. Hyun Park at Wonkwang University (Iksan, Korea). These cells were maintained at 5×10^6 cells/100-mm diameter dish (5×10^5 cells/mL) in DMEM supplemented with 10% (*v/v*) heat-inactivated FBS, penicillin G (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. To determine cell viability, cells were plated in 96-well plates (2×10^4 cells/well) prior to incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL for 4 h; the formazan produced was then dissolved in acidic 2-propanol. Optical density was measured at 590 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The optical density of the

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formazan formed in control (untreated) cells was considered to represent 100% viability. The assay was conducted three times independently.

4.3. Primary microglial culture

Cells dissociated from the cerebral hemispheres of 1 day-old postnatal rat brains (Sprague–Dawley strain) were seeded at a density of 1.2×10^6 cells/mL in DMEM (Gibco) containing 10% FBS and 1% penicillin-streptomycin in a T-75 flask (SPL Life Sciences, Pocheon, Korea). Two weeks later, microglia were detached by mild shaking and filtered through a cell strainer (BD Falcon, Bedford, MA) to remove astrocytes. After centrifugation ($1000 \times g$) for 5 min, cells were resuspended in fresh DMEM containing 10% FBS and 1% penicillin-streptomycin before plating at a final density of 1.5×10^5 cells/well on a 24-well culture plate. After 2 h, the medium was changed for DMEM containing 5% FBS and 500 μM B27 supplement (Gibco).

4.4. Nitrite determination

As an indicator of NO production, the nitrite concentration in the medium was measured using the Griess reaction. Three independent assays were performed. Each aliquot of conditioned medium (100 μL) was mixed with an equal volume of Griess reagent (Solution A: 222488, Solution B: S438081; Sigma-Aldrich), and the absorbance of the mixture at 525 nm was determined using a microplate reader.

4.5. PGE_2 assay

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The level of PGE_2 present in cell culture medium was determined using a commercially available kit from R&D Systems (Minneapolis, MN). Three independent assays were performed according to the manufacturer's instructions. Briefly, BV2 microglial cells were cultured in 24-well plates, pre-incubated for 3 h with different concentrations of cudratricusxanthone L, and then stimulated for 24 h with LPS. Cell culture media were collected immediately after treatment and spun at 13,000 × *g* for 2 min to remove particulate matter. The medium was added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for PGE_2. An enzyme-linked polyclonal antibody specific for PGE_2 was added to the wells and incubated for 24 h, followed by a final wash to remove any unbound antibody. A substrate solution was then added and the intensity of color produced, measured at 450 nm, was proportional to the amount of PGE_2 present.

4.6. Preparation of cytosolic and nuclear fractions

BV2 or primary rat microglial cells were homogenized in M-PER™ Mammalian Protein Extraction Buffer (1:20, *w/v*) (Pierce Biotechnology, Rockford, IL, USA) containing freshly added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA) and 1 mM phenylmethylsulfonylfluoride. The cytosolic fraction of the cells was prepared by centrifugation at 16,000 × *g* for 5 min at 4 °C. The nuclear and cytoplasmic cell extracts were prepared using NE-PER® nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA), respectively.

4.7. DNA binding activity of NF-κB

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BV2 microglial cells were pre-treated for 3 h with the indicated concentrations of cudratricusxanthone L prior to stimulating for 1 h with LPS (1 μg/mL). The DNA-binding activity of NF-κB in nuclear extracts was measured using the TransAM® kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The assay was conducted three times independently.

4.8. Western blot analysis

BV2 and primary rat microglial cells were harvested and pelleted by centrifugation at 16,000 rpm for 15 min. The cells were then washed with phosphate-buffered saline and lysed in 20 mM Tris-HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethylsulfonylfluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). The protein concentration was determined using a Lowry protein assay kit (P5626; Sigma-Aldrich). An equal amount of protein from each sample was resolved using 7.5% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred onto a Hybond™ enhanced chemiluminescence nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% (*w/v*) skim milk before sequential incubation with the primary antibody (Santa Cruz Biotechnology, CA, USA) and the horseradish peroxidase-conjugated secondary antibody, followed by detection using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The signal intensities were quantified using densitometric ImageJ software (National Institutes of Health, Bethesda, MD, USA). Molecular weight markers were used, as were the internal standards, β-actin and PCNA. The analysis was conducted three times independently.

4.9. NF-κB localization and immunofluorescence

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BV2 or primary rat microglial cells were grown on Lab-Tek II chamber slides and treated as described in the Figure legends. The cells were treated with 10.0 μM cudratricusxanthone L for 1 h. Cells were then fixed in formalin and permeabilized using cold acetone. The cells were probed with an anti-NF-κB antibody and a fluorescein isothiocyanate-labeled secondary antibody (Alexa Fluor 488; Invitrogen, Carlsbad, CA, USA). To visualize the nuclei, cells were then treated with 4',6-diamidino-2-phenylindole (1 μg/mL) for 30 min, washed with phosphate-buffered saline for 5 min, and treated with 50 μL VectaShield (Vector Laboratories, Burlingame, CA, USA). Stained cells were visualized and photographed using a Zeiss fluorescence microscope (Provis AX70; Olympus Optical Co., Tokyo, Japan).

4.10. Statistical analysis

The data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. To compare three or more groups, one-way analysis of variance followed by Tukey's multiple comparison tests was performed. Statistical analysis was conducted using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

The present study showed that cudratricusxanthone L had anti-neuroinflammatory effects in BV2 and primary microglial cells. This compound inhibited the overexpression of pro-inflammatory mediators such as iNOS, COX-2, NO, PGE_2, and pro-inflammatory cytokines; these effects were clearly related to an inactivation of the NF-κB pathway. In addition,

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cudratricusxanthone L repressed the phosphorylation of JNK and p38 MAPK in BV2 cells. Therefore, cudratricusxanthone L isolated from *C. tricuspidata* has the potential to be used as a therapeutic agent for the treatment of neuroinflammation and related neurodegenerative diseases.

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