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Protopine attenuates inflammation stimulated by carrageenan and LPS via the MAPK/NF-κB pathway

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ABSTRACT

We investigated the anti-inflammatory activity of protopine (PTP) and sought to determine its mechanism of action in LPS-stimulated BV2 cells and a carrageenan (CA)-induced mouse model. Treatment with PTP (5, 10, and 20 μM) significantly suppresses the secretion of NO and PGE_2 in a concentration-dependent manner without affecting cell viability by downregulating iNOS and COX-2 expression in LPS-induced BV2 cells. PTP also attenuates the production of pro-inflammatory chemokines, such as MCP-1, and cytokines, including TNF-α, IL-1β and IL-6, and augments the expression of the anti-inflammatory cytokine IL-10. In addition, PTP suppresses the nuclear translocation of NF-κB by hindering the degradation of IκB and downregulating the expression of mitogen-activated protein kinases (MAPKs), including p38, ERK1/2 and JNK protein. Furthermore, PTP treatment significantly suppresses CA-induced paw oedema in mice compared to that seen in untreated mice. Expression of iNOS and COX-2 proteins is also abrogated by PTP (50 mg/kg) treatment in CA-induced mice. PTP treatment also abolishes IκB phosphorylation, which hinders the activation of NF-κB. Collectively, these results suggest PTP has potential for attenuating CA- and LPS-induced inflammatory symptoms through modulation of MAPKs/NF-κB signaling cascades.

1. Introduction

Neuroinflammation is an important defense mechanism against infection, injury, and trauma in the central nervous system (CNS). Microglia are immune-surveillance macrophages resident in the CNS that play an important role in brain development as well as tissue maintenance and repair (Michell-Robinson et al., 2015). Mounting evidence suggests that microglial activation is involved in a complex array of inflammatory responses (Park et al., 2007, 2011; Suzumura, 2013). Activated microglia are thought to exhibit two distinct phenotypes: phagocytic and antigen-presenting. Imbalances between these states may cause microglial activation, which may be either favourable or injurious to neurons (Dheen et al., 2007). During neuroinflammation, activated microglia secrete immunomodulatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), proinflammatory cytokines and reactive oxygen species (ROS) that enhance and maintain inflammatory responses (Yu et al., 2018). Excessive production of such inflammatory molecules may lead to the development of various neurodegenerative disorders such as Alzheimer's disease, septic shock, Parkinson's disease, and multiple sclerosis (Santiago et al., 2017; Wilms et al., 2007). Being able to control microglial activation could alleviate the progression of neurodegeneration, and developing anti-inflammatory agents that suppress the inflammatory pathways activated by microglia could be beneficial for the treatment of neurodegenerative disorders.

Carrageenan (CA)-induced inflammation is considered a biphasic, nonimmune, acute, age and weight dependent, highly reproducible and well established mouse model (Posadas et al., 2004; Winter et al., 1962). The immediate development of oedema, hyperalgesia and erythema are primary characteristics of CA-induced inflammation, with various inflammatory mediators such as histamine, serotonin and bradykinin becoming detectable in the early phase, while prostaglandins emerge in the late phase of CA-insulted inflammation. Increasing levels of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 also contribute to this inflammation (Cuzzocrea et al., 1999; Posadas et al., 2004). Furthermore, CA-induction also causes neutrophil infiltration and activation, resulting in the production of reactive oxygen species (ROS) (Salvemini et al., 1996). The inflammatory response is usually measured by paw oedema, which is maximal at approximately 4–5 h post-CA injection and is lessened by inhibitors of specific molecules

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Abbreviations

COX-2	cyclooxygenase
ERK	extracellular signal-regulated kinase
IκB	inhibitory κB
iNOS	inducible nitric oxide synthase
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-10	interleukin 10
JNK	c-Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
NF-κB	nuclear factor-κB
NO	nitric oxide
PCR	polymerase chain reaction
PGE_2	Prostaglandin E_2
PTP	protopine
ROS	reactive oxygen species
SD	standard deviation
TNF-α	tumor necrosis factor-α

within the inflammatory cascade. Therefore, this model has had, and will continue to have, a vital role in drug development.

Corydalis yanhusuo WT Wang (Fig. 1A) is a well-known medicinal herb, officially listed in the Chinese Pharmacopoeia. Both powder and decoction forms of this herb have been widely used as a remedy for pain and inflammation (Wang et al., 2016). It remains popular today because of its therapeutic effects on circulation, energy and relief from headache pain, chest pain, epigastric pain, abdominal pain, backache, arthralgia and trauma (Leung et al., 2003; Sagare et al., 2000). As a traditional Chinese medicine, it has been used in the treatment of cardiac arrhythmia, gastric and duodenal ulcer and menorrhagia (Wang et al., 2010). Phytochemical analysis revealed that *C. yanhusuo* plants are abundant in tertiary and quaternary alkaloids, which may account for its biological activities. Nearly 20 such alkaloids have been isolated from *C. yanhusuo* so far and among them, l-tetrahydropalmatine (l-THP) has been found to be a neuroactive alkaloid with analgesic and hypnotic activity through its blockade of D2 dopamine receptors in striatum (Wang et al., 2016; Zhang et al., 2014). Corydaline, dehydrocorybulbine (DHCB) and protopine have also been found to be effective on alleviating pain. Neuroinflammation and its associated pain is difficult to manage; anticonvulsants and antidepressants are sometimes used, but with limited results (Crofford, 2010). Therefore, the search for new natural compounds that present therapeutic alternatives is important.

Protopine (PTP) is an isoquinoline alkaloid (Fig. 1B) and one of the main constituents of *C. yanhusuo* (Fig. 1C). It exhibits several pharmacological effects such as antispasmodic and relaxant activities (Hiller et al., 1998), hepatoprotective activity (Rathi et al., 2008), anticholinesterase activity (Kim et al., 1999), anticancer activity (Chen et al., 2012) in addition to its well-known analgesic and anti-inflammatory activities (Saeed et al., 1997). Previous studies have shown that PTP attenuates inflammatory symptoms via suppression of the mitogen-activated protein kinase (MAPK)/nuclear factor kappa B (NF-κB) signaling pathways in RAW264.7 cells (Bae et al., 2012). However, the inhibitory effects of PTP on neuroinflammation have not been investigated. Moreover, the details of the underlying molecular mechanisms of PTP's neuro-inflammatory inhibition are not completely understood. In this study, we demonstrate that PTP inhibits neuroinflammation in lipopolysaccharide (LPS)-stimulated BV2 cells through the suppression of the MAPK/inhibitory kappa B (IκB)/NF-κB signaling pathway.

2. Materials and methods

2.1. Determination of cell viability and NO level

The effects of PTP on the viability of cells were evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Alam et al., 2017). Briefly, BV2 cells (1×10^5 cells/mL) were

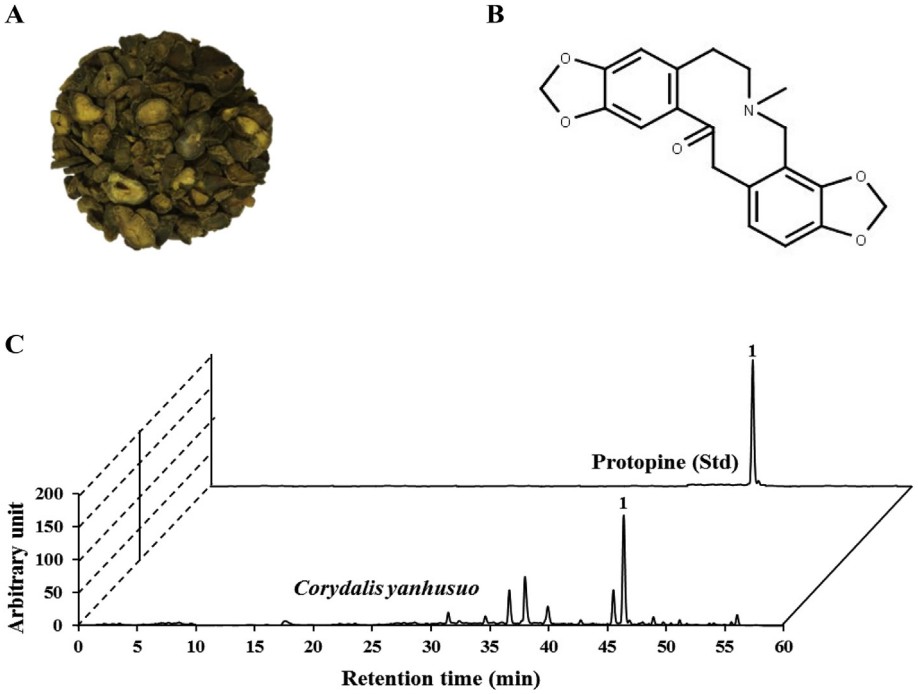

Fig. 1. A classical feature of *Corydalis yanhusuo* WT Wang tuber (A). Chemical structure of protopine (B). HPLC profile of *Corydalis yanhusuo* tuber (C).

seeded onto a 96-well plate and incubated at 37 °C for 24 h. Cells were pretreated with the indicated concentrations of PTP (5–160 μM) or vehicle alone in the presence of LPS (1 μg/mL) for 20 h. After incubation, aliquots (100 μL) of the cell-free culture medium were dispensed for NO level measurement. The cells were evaluated for viability by incubation with 100 μL of 10% MTT at 37 °C for 1 h. The colored formazan crystals were dissolved in 100% dimethyl sulfoxide (DMSO), and the optical density (OD) was measured at 590 nm using a microplate reader (Victor3, PerkinElmer, Waltham, MA, USA). NO production was indirectly measured by determining the level of nitrite using Griess reagent assay (Bajpai et al., 2018).

2.2. Interleukin (IL)-1β, IL-6, IL-10, monocyte chemoattractant protein (MCP)-1, tumour necrosis factor (TNF)-α and PGE_2 assays

Cells were treated with indicated concentrations of PTP (5, 10 and 20 μM) or vehicle alone in the presence of LPS (1 μg/mL) for 24 h. After LPS treatment, cell culture supernatants were collected by centrifugation at 3000 × *g* for 5 min at 4 °C. An enzyme-linked immunosorbent assay (ELISA) was used to quantify the levels of IL-1β, IL-6, IL-10, MCP-1, TNF-α and PGE_2 according to the manufacturer's guidelines.

2.3. Reporter assay for NF-kB

BV2 cells (1×10^5 cells/mL, seeded in 12-well plates) were transfected with a mixture containing a pNF-κB-luc reporter gene (Beyotime Biotechnology, Nantong, China), the reporter construct pRL SV40 (*Renilla* luciferase driven by SV40) and ViaFect™ Transfection Reagent (Promega, Madison, WI, USA) for 24 h. Following incubation, cells were treated with different concentrations of PTP for 30 min and stimulated with LPS. Luciferase assay was performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) (Bajpai et al., 2018).

2.4. Carrageenan (CA)-induced paw oedema

ICR mice (8 weeks old; 25–30 g) were purchased from Central Lab Animals, Inc. (Seoul, Korea). They were housed in stainless steel cages in an air-conditioned room kept at 23 ± 1 °C, humidity of 55 ± 5%, and 12 h/12 h light/dark cycle. The animals were acclimatised for 1 week with *ad libitum* access to water and standard rodent chow. Experiments were conducted in accordance with the guidelines for animal experiments issued by Kyungpook National University and approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU-2018-0015). Mice (N = 25) were randomly divided into five groups (5 animals/group): the treatment naïve control group (group-1), the CA control group (group-2), the indomethacin group (group-3), the 25 and 50 mg/kg (p.o.) PTP treatment groups (group-4 and group-5, respectively). PTP was dissolved in DW and administered orally to mice at 25 or 50 mg/kg once per day for four consecutive days. Indomethacin, an anti-inflammatory drug, was used as a positive control. To induce acute phase inflammation in paw,

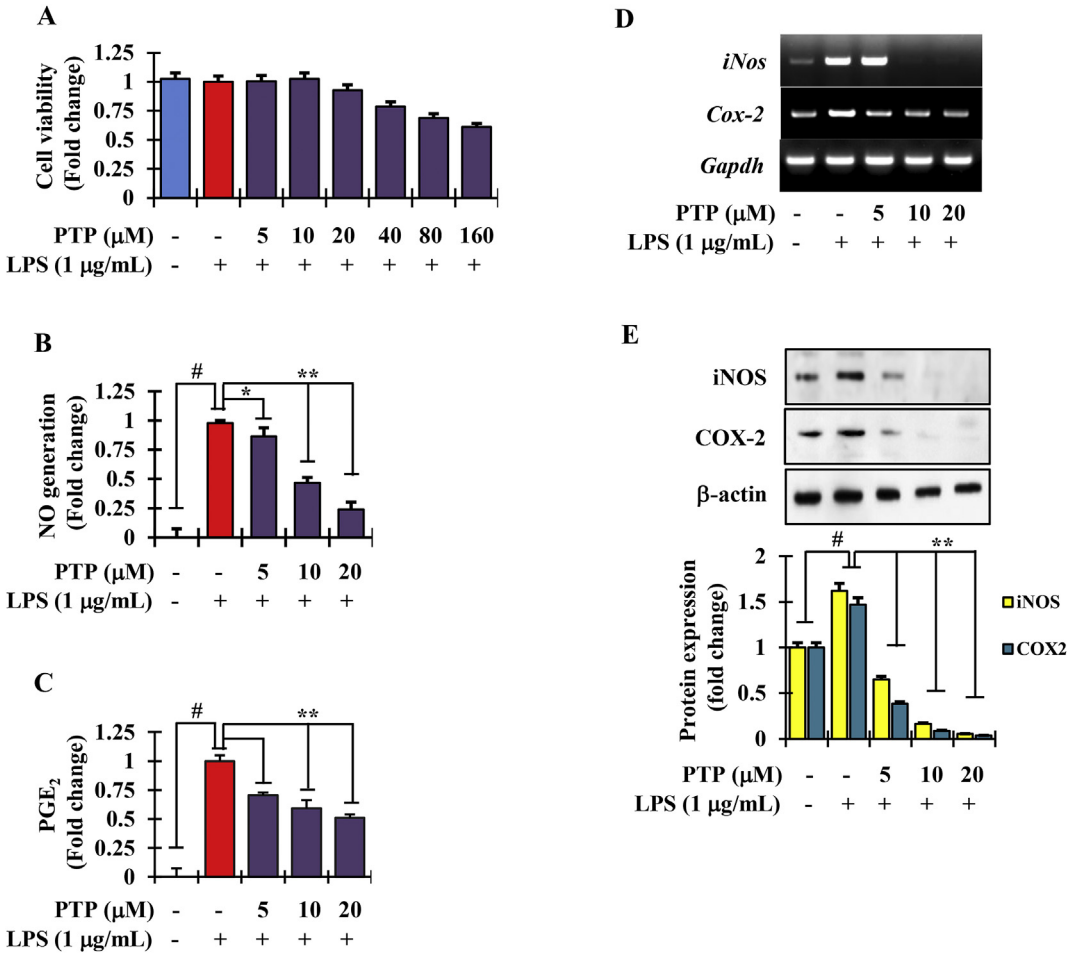

Fig. 2. Anti-inflammatory effect of protopine (PTP). Effects of PTP on cell viability (A); the productions of NO (B), PGE_2 (C) and mRNA (D); and protein expression of iNOS and COX-2 (E) in LPS-induced BV2 cells were examined. Cells (5×10^5 cells/mL) were treated with various concentrations (5, 10, and 20 μM) of PTP for 1 h and incubated with LPS (1 μg/mL) for 20 h. Results are expressed as the mean ± SD of three separate experiments. #$p < 0.01$ as compared with the vehicle-treated control; *$p < 0.05$ and **$p < 0.01$ as compared with LPS alone.

a subcutaneous injection of 1% carrageenan in saline (60 μL per animal) was administered into the right hind paw 1 h after vehicle or PTP treatment. Paw volumes were measured hourly for 4 h after injection using a plethysmometer (UGO BASILE; Comerio, VA, ITALY). Afterward, mice were euthanised, and the right hind paw skin was expunged and immediately frozen in a liquid nitrogen for Western blot analysis.

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared using TRIzol Reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (2 μg) was used to prepare cDNA using RT & GO Mastermix (MP Biomedicals, Seoul, Republic of Korea) that served as the PCR template. A PCR Thermal Cycler Dice TP600 (Takara Bio Inc., Otsu, Japan) was used to carry out RT-PCR using primer sequences (Supplementary Data, Table S1). A 2% agarose gel in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (TAE buffer) was used to isolate the PCR products, which were visualised by ethidium bromide staining and evaluated by Image Lab™ software (version 5.2.1; Bio-Rad Laboratories, CA, USA).

2.6. Protein extraction and Western blot analysis

Cultured cells were harvested and lysed using cold radioimmunoprecipitation assay (RIPA) buffer with a phosphatase and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were centrifuged at 3000 × *g* for 10 min at 4 °C to remove insoluble materials. Protein content was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Nuclear and cytosolic protein extracts were prepared by nuclear and cytoplasmic extraction kit (Sigma-Aldrich Co. St. Louis, MO, USA) according to the manufacturer's protocol. Total protein (50 μg) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols and the separated bands were transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany), followed by blocking with 5% skim milk in TBST buffer. The membranes were then blotted with each primary antibody (1:1000) and the corresponding secondary antibody (1:5000). The antigen-antibody reaction was detected using a SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific, Rockford, IL, USA) and bands were analyzed by Image Lab™ software (version 5.2.1) (Bio Rad Laboratories, CA, USA).

2.7. Statistical analysis

GraphPad Prism Software (GraphPad Software, Inc, San Diego, CA, USA) was used for the analysis of data. All data are expressed as the mean ± standard deviation (SD; n = 3) and analyzed using one-way analysis of variance (ANOVA), followed by Dennett's test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effect of PTP on cell viability and the inflammatory mediator production in LPS-induced BV2 cells

To evaluate the toxic effects of PTP on BV2 cells, cells were pretreated with various concentrations of PTP (5–160 μM) for 2 h, followed by stimulation with LPS for 20 h and evaluation of cell viability by MTT assay. As shown in Fig. 1A, PTP at 5, 10 and 20 μM had no toxic effect but induced significant toxicity at 40 μM and higher. Thus, we used the

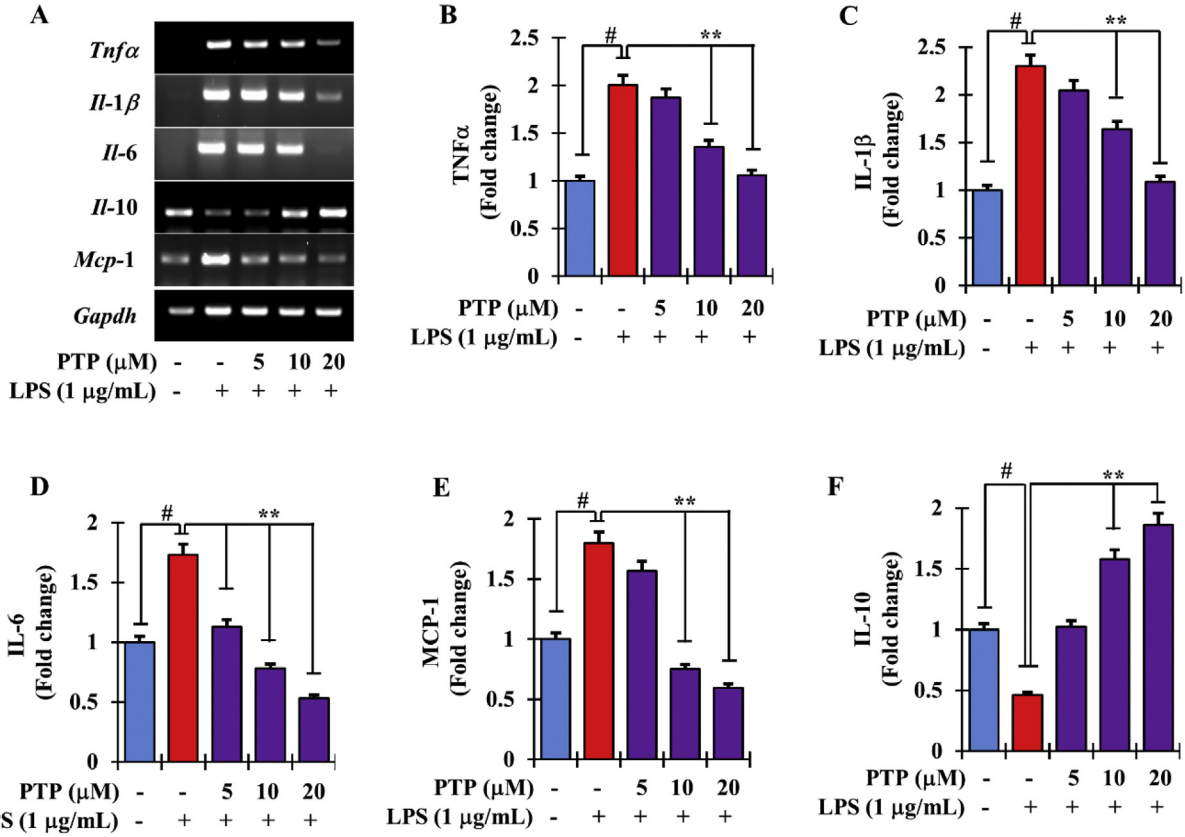

Fig. 3. Inhibitory effects of PTP pretreatment on LPS-induced expression of various cytokines and a chemokine. mRNA expression of TNF-α, IL-1β, IL-6, MCP-1 and IL-10 (A) and protein levels of TNF-α (B), IL-1β (C), IL-6 (D), MCP-1 (E) and IL-10 (F) in BV2 cells were measured as described in materials and methods. Results are expressed as the mean ± SD of three separate experiments. #$p < 0.01$ as compared with vehicle-treated control; *$p < 0.05$ and **$p < 0.01$ as compared with LPS alone.

nontoxic concentrations in our experiments.

To assess the attenuation effect of PTP on LPS-induced NO and PGE_2 production, cells were treated with the PTP (5, 10 and 20 μM). In comparison with the untreated control, cells treated with LPS show a significant increase in the levels of NO and PGE_2 (column 2 of Fig. 2B and C, respectively). PTP treatment results in a significant suppression in the production of both NO and PGE_2 in a dose-dependent manner (columns 3–5 in Fig. 2B and C, respectively). These observations suggest that PTP suppresses the LPS-stimulated inflammatory response without affecting cell viability.

To examine whether the attenuation effect of PTP on the production of NO and PGE_2 was associated with the inhibition of their corresponding genes, RT-PCR and Western blot analysis were carried out to measure the mRNA and protein levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2), respectively. As shown in Fig. 2D and E, LPS treatment increases the mRNA and protein levels of both iNOS and COX-2, whereas PTP treatment significantly attenuates the LPS-stimulated expressions of iNOS and COX-2 at both mRNA and protein levels in a concentration-dependent manner. These data suggest that the suppressive effects of PTP on the production of NO and PGE_2 are associated with regulation at the level of gene expression in BV2 cells.

3.2. Attenuation of pro-inflammatory cytokine and chemokine production by PTP

We evaluated the effects of PTP on the production of pro-inflammatory cytokines and chemokines in LPS-activated BV2 cells by RT-PCR and immunoassay. Compared to untreated cells, cells treated with LPS show significantly upregulated mRNA expression of TNF-α, IL-1β, IL-6 and MCP-1 and downregulated expression of the anti-inflammatory cytokine IL-10 (Fig. 3A). However, pretreatment of cells with PTP results in a significant downregulation of TNF-α, IL-1β, IL-6 and MCP-1 mRNA and upregulation of IL-10 mRNA in a concentration-dependent manner (Fig. 3A). Furthermore, cells pretreated with show a significant decrease in the production of TNF-α, IL-1β, IL-6 and MCP-1 and markedly increased production of IL-10 (Fig. 3B–F) compared to cells exposed to LPS without PTP pretreatment. These results suggest

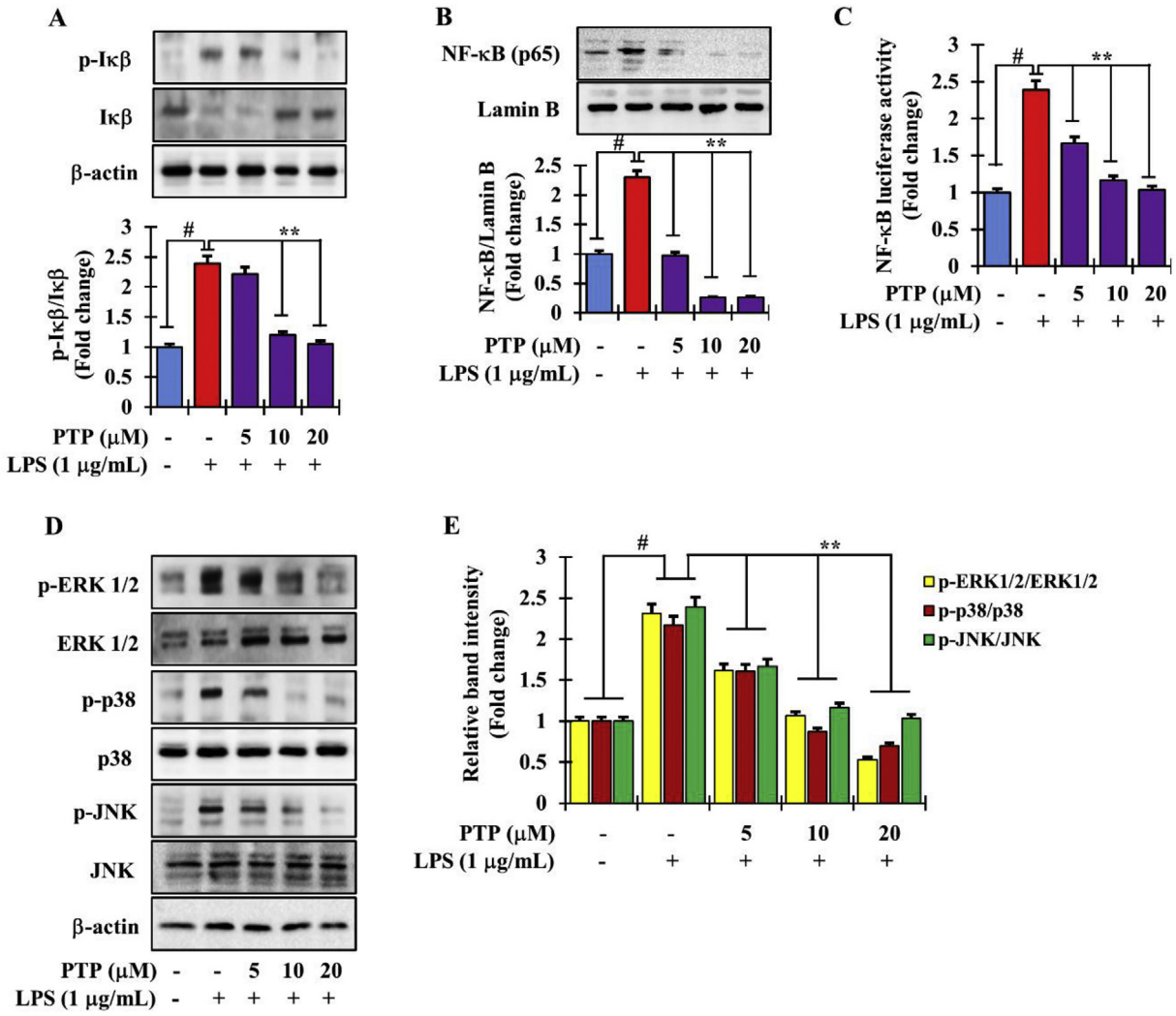

Fig. 4. Attenuation of MAPK phosphorylation and NF-κB signaling by PTP. BV2 cells were treated with PTP in presence or absence of LPS (1 μg/mL) for 1 h. Effects of PTP on IκB phosphorylation (A) and nuclear translocation of NF-κB p65 subunit (B) in LPS-stimulated BV2 cells were analyzed by western blotting. PTP inhibits the LPS-induced promoter activity of NF-κB in BV2 cells (C). A pNF-κB-luc reporter construct containing four NF-κB binding motifs (GGGAATTTCC) and a pRL-SV40 reporter construct were transiently transfected into BV2 cells. Following the indicated treatments, the promoter activity was detected using the Dual Luciferase® Reporter Assay System. Effects of PTP on MAPK phosphorylation in LPS-stimulated BV2 cells (D). Cellular proteins assayed to detect either the phosphorylated form or total amount of the MAPK proteins ERK1/2, p38 and JNK1/2. Quantification of the relative band intensities from three independent experimentals was determined by densitometry (E). Results are expressed as the mean ± SD of three separate experiments. #$p < 0.01$ as compared with vehicle-treated control; *$p < 0.05$ and **$p < 0.01$ as compared with LPS alone.

that PTP may attenuate the production of pro-inflammatory cytokines via regulation of their mRNA expressions in activated microglial cells.

3.3. Blocking of NF-κB signaling by PTP in LPS-induced BV2 cells

We examined whether PTP suppresses the activation of the NF-κB pathway, which is involved in the transcriptional regulation of inflammatory mediators in LPS-induced BV2 cells. Phosphorylation of inhibitory kappa B (IκB) and its subsequent degradation is an important event in the activation of NF-κB in response to different stimuli (Lu et al., 2012). We performed Western blot analysis to investigate the effect of PTP on LPS-stimulated phosphorylation of IκB and its subsequent degradation. LPS treatment significantly induced the phosphorylation of IκB from 30 min to 6 h, with the maximum activity observed at 1 h (Supplementary Data S1), while pretreatment with PTP significantly attenuated the LPS-stimulated IκB phosphorylation in a concentration-dependent manner (Fig. 4A). Detachment of IκB from NF-κB allows the translocation of activated free dimer subunits of NF-κB (p50/p65) into the nucleus from the cytosol. We evaluated the effect of PTP on the translocation of NF-κB into the nucleus by western blotting for NF-κB using nuclear extracts of LPS-induced BV2 cells. Upon exposure to LPS, the level of NF-κB in the nucleus significantly increased from 30 min to 6 h, with the peak level observed at 3 h (Supplementary Data Fig. S2). PTP treatment, on the other hand, significantly suppressed the LPS-induced nuclear translocation of NF-κB in a concentration-dependent manner (columns 3–5, Fig. 4B). Furthermore, we used a reporter gene assay to evaluate the transcription factor activity of the NF-κB protein in LPS-stimulated BV2 cells and found that LPS treatment increases the expression of genes activated by NF-κB binding to promoter sequences (Supplementary Data Fig. S3), a finding which is consistent with the results of a previous study where LPS treatment significantly increased NF-κB-mediated luciferase reporter activity (Khan et al., 2012). Pretreatment with PTP for 3 h significantly suppresses the ability of NF-κB to stimulate reporter gene transcription in a concentration-dependent manner (Fig. 4C), indicating that PTP may attenuate the binding of NF-κB to its target promoters. Together, these results suggest a prospective role for NF-κB in the attenuation of pro-inflammatory mediators by PTP in activated microglial cells.

3.4. PTP attenuated MAPK phosphorylation in LPS-induced BV2 cells

We investigated whether the MAPK pathway may affect the NF-κB signaling during microglial activation. The effects of PTP on the LPS-stimulated phosphorylation of MAPKs such as extracellular signal-regulated kinase (ERK)1/2, p38, and c-Jun N-terminal kinase (JNK) were studied in BV2 cells. LPS treatment augmented the phosphorylation levels of ERK1/2, JNK, and p38 (Fig. 4D and column 2, Fig. 4E), while pre-treatment with PTP markedly repressed the LPS-induced phosphorylation of ERK1/2, p38, and JNK. This result indicates that MAPK signal transduction could be effectively blocked by PTP in activated microglial cells.

3.5. Inhibitory effects of PTP on carrageenan-induced mouse hind paw oedema

CA insult results in significantly increased paw swelling compared with untreated mice. Meanwhile, daily administration with either 25 or 50 mg/kg PTP significantly suppresses the volume of paw oedema (Fig. 5A) similar to that seen with indomethacin administration (10 mg/kg per day) at 4 h post-insult. As expected, PTP treatment significantly mitigates the protein expression of iNOS and COX-2 compared to levels in the CA insult groups (Fig. 5B). Successively, protein expression of IκB and NF-κB was also suppressed in the PTP-treated group compared to CA-induced groups (Fig. 5C). These data suggest that PTP lessened carrageenan-induced inflammation in mice, likely via suppression of NF-κB signaling.

4. Discussion

PTP, a benzylisoquinoline alkaloid, is widely distributed in various plants such as those belonging to the *Papaveraceae*, *Elaeocarpaceae*, and *Fumariaceae* families (Chen et al., 2012; Munoz et al., 2011; Rathi et al., 2008). While the inhibitory effects of PTP on inflammation have been studied, the activity of PTP against CA-induced inflammation and neuroinflammation and its underlying molecular mechanism of action remains unclear. In this study, we evaluated the anti-inflammatory effects of PTP against CA-induced inflammation and neuroinflammation using LPS-stimulated BV2 microglial cells and explored the underlying mechanism.

Previous studies have suggested the association between various neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis; post-traumatic brain injuries; cerebral ischemia and neuroinflammation (Rock and Peterson, 2006; Santiago et al., 2017; Wilms et al., 2007). Uncontrolled activation of microglia results in the release of various inflammatory mediators such as NO, PGE_2 and ROS, all of which are closely associated with neuronal toxicity and death (Rock and Peterson, 2006). In particular, the excessive production of NO by iNOS in microglia is thought to be cytotoxic to neurons (Gresa-Arribas et al., 2012; Li et al., 2016). Moreover, PGE_2 acts as a pleiotropic mediator produced by COX-2 at inflammation sites,

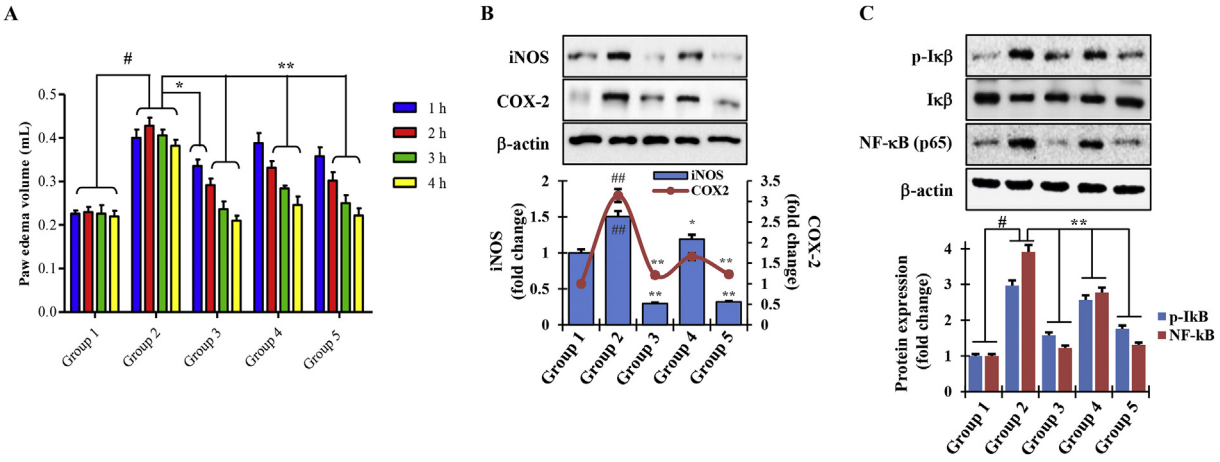

Fig. 5. Inhibition of carrageenan (CA)-induced paw oedema by PTP. Paw volumes were measured 0–4 h after carrageenan injection (A) as described in materials and methods. Protein expressions of iNOS, and COX-2 (B) as well as IκB and NF-κB (C) in CA-induced mice were examined by immunoblotting. Results represent the mean ± S.D. of five animals. #$p < 0.01$ compared with vehicle-treated control; *$p < 0.05$ and **$p < 0.01$ compared with CA insult group.

resulting in pain, swelling and stiffness (Moon et al., 2007). Isoquinoline alkaloids from various plant sources exert anti-inflammatory effects by suppressing the production of NO/iNOS, PGE_2/COX-2, and various cytokines or chemokines and downregulating their mRNA expression in microglial cells (Cordeiro et al., 2016; Dinesh and Rasool, 2017; do Santos et al., 2018). Ample evidence indicates that CA insult triggers the production and release of NO at the injured site. Perfusion of various selective and non-selective NOS inhibitors including NG-monomethyl-L-arginine acetate (L-NMMA) and aminoguanidine hemisulfate (AG) suppress the release of NO at approximately 3–4 h after a CA insult. The production and release of NO by these NOSs are thought to contribute to tissue injury and inflammation-induced oedema and hyperalgesia (Handy and Moore, 1998; Omote et al., 2001). In this study, PTP significantly attenuates the production of NO and PGE_2 in LPS-stimulated BV2 cells (Fig. 2B and C). Furthermore, PTP inhibits the expression of iNOS and COX-2 in LPS-induced BV2 cells and CA-induced inflammation in mice (Figs. 2E and 5B). Our results demonstrate that the suppression of NO and PGE_2 production by PTP may be attributed to the attenuation of iNOS and the upregulation of COX-2 in LPS-stimulated activated microglia as well as in a CA-induced inflammatory model.

Microglial activation and the subsequent overexpression of pro-inflammatory cytokines such as TNF-α and IL-1β are considered histopathological hallmarks of CNS disorders such as Alzheimer's disease, Parkinson's disease and cerebral ischemia (Kim and Joh, 2006). IL-6 is associated with various immunological responses and acts on diverse cell types (Corsi et al., 2011). Our results show that the pretreatment with PTP significantly represses the mRNA and protein levels of pro-inflammatory cytokines in LPS-induced cells (Fig. 3A and 3B-D). Furthermore, MCP-1 is a strong chemoattractant pro-inflammatory chemokine that promotes monocyte recruitment at inflammatory sites and boosts the production of additional pro-inflammatory mediators, thereby accelerating the process of inflammation (Park et al., 2011). Several studies report that LPS treatment significantly augments the production of MCP-1 (Park et al., 2011; Wang et al., 2000). Here, we also find LPS-induced production of MCP-1 and show that PTP significantly suppresses protein and mRNA expression of MCP-1 in LPS-induced BV2 cells (Fig. 3A and E). However, IL-10 is a classic immunoregulatory and anti-inflammatory cytokine that plays a pivotal role in the control of immune response. IL-10 may suppress the pro-inflammatory cytokine production by activated macrophages (de Waal Malefyt et al., 1991). Several studies report that IL-10 successively attenuates the production of pro-inflammatory mediators in LPS-stimulated microglial cells (Ledeboer et al., 2000; Molina-Holgado et al., 2001). Our results reveal upregulated mRNA and protein levels of IL-10 following PTP treatment (Fig. 3A and F). Thus, PTP may offer novel strategies for the treatment of neurodegenerative disorders. The neuroprotective effects of PTP observed in animal models of brain disorder (Durairajan et al., 2015, 2017) and our results are in accordance with other studies reporting the immunomodulatory activity of PTP (Kim et al., 1999).

NF-κB is a pleiotropic regulator of broad-spectrum pro-inflammatory genes such as iNOS and COX-2 as well as various cytokines and chemokines (Moon et al., 2007). Multiple studies report increases in the expression of pro-inflammatory mediators in response to the binding of NF-κB to their gene promoter regions (Khan et al., 2012; Li and Verma, 2002; Togashi et al., 1997). In a latent cell, IκB retains NF-κB in the cytoplasm by preventing its nuclear localisation. Various inflammatory stimuli such as IL-1β, TNF-α and LPS induce NF-κB activation via the phosphorylation or ubiquitination and the subsequent degradation of the IκB protein. The NF-κB dimer is then free to translocate to the nucleus, where it binds to DNA-binding sites in the promoter regions of many genes, leading to transcription activation (Li and Verma, 2002). On the other hand, CA exposure stimulates an inflammatory response through the generation of the ROS and/or BCL10-mediated signaling cascades, leading to either canonical or non-canonical activation of NF-κB (Bhattacharyya et al., 2011). Our study indicates that PTP inhibits both the LPS- and CA-induced IκB degradation and suppresses the nuclear translocation of the p65 subunit of NF-κB (Figs. 4 and 5). Our results are further supported by previous studies that validate the PTP-targeted NF-κB activation through the inhibition of NF-κB nuclear translocation and the phosphorylation and rapid degradation of IκB (Bae et al., 2012). Therefore, the suppression of the NF-κB signaling pathway by PTP may result in the down-regulation of pro-inflammatory mediators, eventually leading to anti-inflammatory effects.

MAPKs such as p38, JNK and ERK have significant roles in the regulation of pro-inflammatory enzyme, cytokine and chemokine production in LPS-stimulated microglia (Kaminska, 2005; Park et al., 2011). JNK and p38 play pivotal roles in the regulation of pro-inflammatory pathways in microglia, while ERK signaling pathways are involved in the regulation of iNOS in interferon-gamma-stimulated

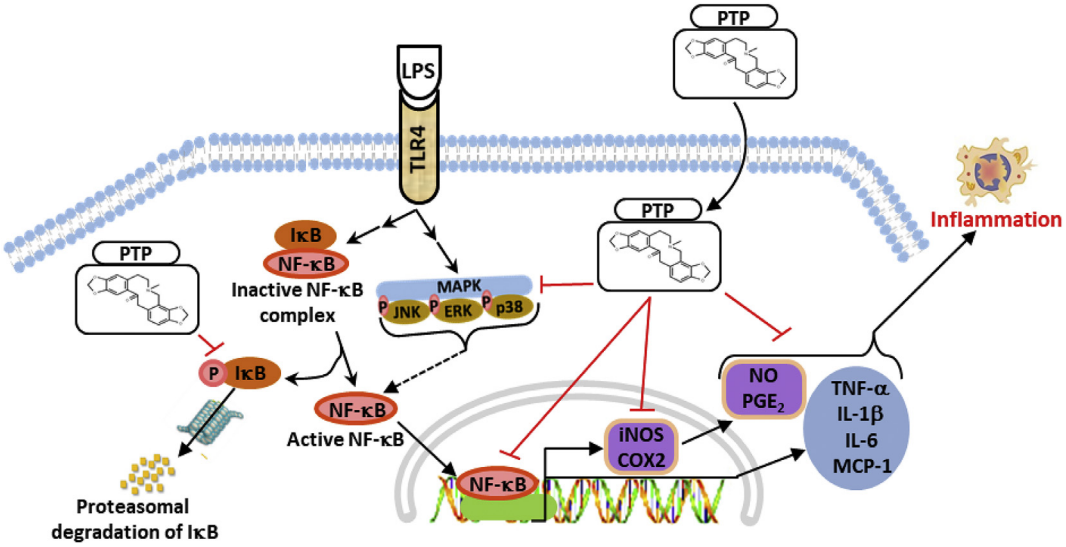

Fig. 6. Proposed mechanism of the anti-inflammatory effect of protopine (PTP). PTP pretreatment actively blocks the phosphorylation of IκB, thereby suppressing translocation of NF-κB to the nucleas, resulting in successful inhibition of the production of NO and PGE_2, various pro-inflammatory cytokines such as TNF-α (2 fold), IL-1β (2.5 fold) and IL-6 (2.5 fold) and chemokine MCP-1 (2.5 fold) compared to LPS-stimulation in the absence of PTP pretreatment.

microglia (Park et al., 2005; Schieven, 2005; Waetzig et al., 2005). Thus, this pathway may serve as a strategic focus for treating LPS-induced neuroinflammatory diseases. Moreover, cumulative studies suggest that the activation of MAPKs can further stimulate other kinase proteins, followed by nuclear translocation of NF-κb, which in turn activates the transcription of pro-inflammatory genes whose promoters have NF-κb binding sites (Coskun et al., 2011; Vallabhapurapu and Karin, 2009). Overexpression of the MEK-ERK pathway negatively regulates NF-κB transcriptional activity by modulating the phosphorylation and activation of TATA-binding protein (Carter and Hunninghake, 2000; Carter et al., 1999). Furthermore, CA-induced inflammation also triggers phosphorylation of spinal p38 MAPK which participates in the maintenance of inflammatory heat hyperalgesia (Schafers et al., 2003). Several isoquinoline alkaloids have been shown to exert anti-inflammatory properties and may competently block the LPS-induced phosphorylation of MAPKs (Chen et al., 2017; Li et al., 2017). We determined the role of PTP on the activation of tightly regulated expression of MAPKs in LPS-stimulated microglia by Western blot analysis. Our results demonstrate that the LPS-induced increase in MAPK phosphorylation is suppressed by PTP in BV2 microglial cells (Fig. 4), indicating that PTP probably exerts its anti-inflammatory effects through the suppression of MAPK signaling pathways. It is known that PTP suppresses NF-κB-induced iNOS and Cox-2 gene expression and subsequently inhibits the production of NO and PGE_2 in RAW 264.7 cells (Bae et al., 2012). To elucidate the mechanism underlying the anti-inflammatory effect of PTP in the present study, we examined the status of MAPK and NF-κB signaling pathways in activated microglia. As expected, PTP significantly suppresses LPS-stimulated phosphorylation of all MAPKs, leading to the inhibition of inflammatory mediator production in BV2 cells.

Abnormal microglial activation has been associated with the production of pro-inflammatory enzymes, cytokines and chemokines, all of which are closely associated with many neurodegenerative disorders. Thus, understanding the underlying mechanism that controls the activation of microglia and downregulates the expression of pro-inflammatory mediators may be important in the development of new strategies for the treatment of neurodegenerative disorders. Our results confirm the anti-inflammatory role of PTP through its suppression of pro-inflammatory enzymes (iNOS and COX-2), cytokines (TNF-α, IL-1β and IL-6), and a chemokine (MCP-1). This effect is exerted by attenuating IκB phosphorylation, which activates the nuclear translocation of NF-κB (Fig. 6). Several anti-inflammatory agents have been shown to attenuate neuronal degeneration via the inhibition of microglial activation and the subsequent production of pro-inflammatory mediators. However, the complete blockage of microglial activation has not been accomplished, as the inflammatory response of microglia is dependent on various factors, including cytokines, chemokines, ROS, matrix metalloproteinase and arachidonic metabolites in inflammatory brain diseases. LPS-induced BV-2 microglial cells may reflect the pathological state and guide future studies on the neuroprotective role of PTP in animal models of neurodegenerative disorders.

5. Conclusions

In summary, we demonstrate that PTP attenuates the release of inflammatory mediators such as NO, and PGE_2 and downregulates their corresponding genes iNOS and COX-2, both in vivo, in a CA-induced inflammation model in mice, and in vitro, in LPS-induced BV2 cells. PTP treatment not only inhibits the degree of TNF-α, IL-1β, IL-6, and MCP-1 production but also suppresses their mRNA expression and increases the mRNA levels of the anti-inflammatory cytokine IL-10 in LPS-stimulated BV2 microglial cells. The anti-inflammatory effects of PTP are mediated through the repression of IκB degradation and NF-κB activation, both in vitro and in vivo. Furthermore, MAPK phosphorylation is significantly decreased when BV2 cells are pretreated with PTP prior to LPS-stimulation. Taken together, our data indicate that PTP targets MAPKs and NF-κB in activated microglia as well as peripheral inflammatory tissue, thereby inhibiting the expression of iNOS, COX-2, pro-inflammatory enzymes, cytokines and chemokines, which is suggestive of its potential role as a therapeutic agent for the treatment of numerous neurodegenerative disorders.

Author contributions

M.B.A. and M.K.J. contributed to the study concept and design, data acquistion, analysis and interpretation and drafting the manuscript; M.B.A., M.K.J. and Y.G.K contributed to data acquisition, analysis and interpretation; S.H.L. was responsible for supervising the study, from its concept and design through critical review and revision of the manuscript. All authors reviewed and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110583.

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