Longxuetongluo capsule alleviates lipopolysaccharide-induced neuroinflammation by regulating multiple signaling pathways in BV2 microglia cells

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Abstract

Background: Longxuetongluo capsule (LTC), derived from the total phenolic compounds of Chinese dragon's blood, is now used in the treatment of ischemic stroke in convalescence. The aim of this study is to explore the neuroprotective effect of LTC from the perspective of neuroinflammation.

Methods: Cell viability and lactate dehydrogenase (LDH) release were measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and LDH assay kit. Proinflammatory mediators and cytokines production including Nitric Oxide (NO), prostaglandin E2, (PGE2), interleukin (IL-β), IL-6, and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay (ELISA) assay. In addition, western blot was used to detect the expression of inflammatory proteins associated with the mitogen-activated protein kinases (MAPKs), janus kinase/signal transducer and activator of tranions (JAK/STAT), nuclear transcription factor κB (NF-κB), and nuclear factor erythroid-2-related actor 2/heme oxygenase 1 (Nrf2/HO-1) signaling pathways. Moreover, immunofluorescence assay and electrophoretic mobility shift assays (EMSA) were performed to determine the Nrf2 translocation and the binding-DNA activity of NF-κB, respectively.

Results: LTC at 0.5 to 2 μg/mL significantly increased cell viability and decreased LDH, NO, PGE2, IL-1β, IL-6, and TNF-α production in oxygen-glucose deprivation/reoxygenation (OGD/R) and lipopolysaccharide (LPS)-induced BV2 microglia cells. Meanwhile, LTC not only decreased the protein expressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) but also down-regulated phosphorylation of extracellular signal-regulated kinase (ERK)1/2, p38, and up-regulated HO-1 expression via nuclear translocation of Nrf2. LTC can significantly inhibit the phosphorylation of JAK1/STAT3 and reduce the translocation of NF-κB from cytosol to nucleus as well as the binding-DNA activity. PC12 cell pretreated with LTC-condition medium (CM) significantly alleviated LPS-induced neurotoxicity and increased PC12 cell viability in a dose-dependent manner.

Conclusion: The present study showed that LTC exhibited a strong antineuroinflammatory activity and neuroprotective effects on LPS-stimulated BV2 microglial cells and PC12 cells.

Keywords: Dragon's blood; Longxuetongluo; Stroke

1. INTRODUCTION

Chinese dragon's blood, the red resin of *Dracaena cochinchinensis* (Lour.) S. C. Chen (Yunnan, China), has been used to improve blood circulation, alleviate blood stasis, stop bleeding, and relieve pain in traditional Chinese medicine for many years. Evidences based on previous studies have shown that the extracts and pure compounds from Chinese dragon's blood exerted multiple pharmacological activities in radioprotective, antithrombotic, antiplatelet aggregation, antiatherosclerotic, and antioxidant, etc.[1–5] Dragon's blood dropping pills also have been showed to be a benefit in rat focal cerebral ischemia model.[6] Longxuetongluo capsule (LTC), another new drug of Chinese dragon's blood, was approved by China Food and Drug Administration (CFDA) for the treatment of ischemic stroke in convalescence stage after phases II–III of clinical trials.

Ischemic cerebral stroke, a pathological condition characterized by a transient or permanent reduction in cerebral blood flow in a major brain artery, is a leading cause of death and adult disability worldwide. In terms of the mechanism of ischemic stroke, rapid reperfusion as soon as possible is considered to be very important in the treatment of cerebral ischemic disease. However, restoration of blood flow usually causes exacerbation of cerebral damage and an extensive inflammatory response. Although multiple mechanisms are involved in cerebral ischemia/reperfusion, lines of evidence demonstrated that inflammation mainly contributes to stroke progression.[7,8] Microglia, the main type of mononuclear macrophages in the brain, have been proposed to be involved in immune-inflammatory reaction in the central nervous system (CNS), and reportedly, is an important contributor of homeostasis and neuroinflammation after

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cerebral ischemia.[9,10] In addition, numerous studies, including ours, have shown that the proinflammatory mediators, such as Nitric Oxide (NO), prostaglandin E2, (PGE2), tumor necrosis factor-α (TNF-α), IL-6, and interleukin (IL-β), secreted from activated microglial are harmful to the survival of the neurons cells.[11,12] Therefore, inhibition of inflammatory signaling in activated microglial could decrease proinflammatory mediators, thus promoting the survival of neurons.

Until now, whether the effect of LTC on the treatment of ischemic stroke related to inhibition of microglia-mediated neuroinflammation is still unknown. As a classic model of inflammation, lipopolysaccharide (LPS)-induced BV2 microglial cell has been used as a common microglia cell line to evaluate the antineuroinflammatory activity of natural products or mixture from herbs. Therefore, the purpose of this study was designed to investigate the capability of LTC on LPS-induced microglial activation and uncover the molecular mechanism of LTC against inflammatory response.

2. METHODS

2.1 Preparation of LTC

LTC was provided by Jiangsu Kanion Pharmaceutical Co. Ltd. (Jiangsu, China). In brief, 0.5 g total phenolic compounds mixture was placed into volumetric flask and ultrasonically (250 W, 40 kHz) extracted with an appropriate amount of 70% ethanol for 30 min. After the sample was cooled to room temperature, the volume was adjusted to 50 mL, and 2 mL sample was then centrifuged at 2599×*g* for 5 minutes. Subsequently, the supernatant was taken for detection.

2.1 HPLC fingerprint analysis of LTC

The High Performance Liquid Chromatography (HPLC) fingerprinting of LTC was constructed using Agilent 1200 liquid chromatograph (Agilent Technologies, USA). The Kromasil C18 (250 mm × 4.6 mm, 5 μm) column was used with a mobile phase of acetonitrile (A)-0.1% (v/v) formic acid (B) gradient elution. A linear gradient program was set as follows: 0 to 30 minutes, 22% to 40% A; 30–60 minutes, 40% to 48% A. The flow rate was kept constant at 1.0 mL/min, the column temperature was maintained at 35°C, and the detection wavelength was 278 nm (determination of loureirin A and loureirin B) and 325 nm (determination of 7, 4′-dihydroxyflavone and pterostilbene). The sample injection volume was 10 μL.

2.3 Chemical reagents and antibodies

LTC was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL, and further diluted in DMEM media so that the final concentration of DMSO was <0.1% v/v. LPS (Escherichia coli O55: B5) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were purchased from Promega (Madison, WI). Lactate dehydrogenase (LDH) was purchased from Roche (Roche Diagnostics GmbH, HYPERLINK "https://www.baidu.com/link?url=5cKjHrj-OiE5DZy6e60-pX6G5KfzTh-ZS1aXqyE-8JqyV7RIqhkTrW2-afExeRDgKZ48qz8jROFLHJaw1cMFhutH7Dzs4RcjHdwheQcEpo7q&wd=&eqid=8e633a020001f45a000000065e27dcd0"Mannheim Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco (Gibco-BRL, Grand Island, NY). Rabbit anti-Nrf2, HO-1, phospho-/total of extracellular signal-regulated kinase (ERK) 1/2, c-jun NH2-terminal kinase (JNK), p38, JAK1, STAT3, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and horseradish peroxidase–conjugated goat antirabbit secondary antibody were all purchased from Cell Signaling Technology (Beverley, MA). Goat antirabbit immunoglobin (Ig)G-Cruz Fluor 488 secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nuclear transcription factor κB (NF-κB) p65 were purchased from Abcam (Cambridge, UK). TNF-α, IL-1β, and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience (Vienna, Austria). PGE2 ELISA kits were purchased from Enzo Life Science (Farmingdale, NY). NO assay kit was purchased from Beyotime Biotechnology (Jiangsu, China). Light shift chemiluminescent electrophoretic mobility shift assay (EMSA) kit was purchased from Thermo Scientific (Rockford, IL). Hoechst 33258 and cell culture grade DMSO were from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade.

2.4 Cell culture and treatment

PC12 cells and murine BV2 microglial cells that exhibit phenotypic and functional properties of reactive dopaminergic neurons and microglial cells were purchased from Cell Resource Centre of the Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China) and cultured in DMEM medium which contains 10% FBS and 1% penicillin–streptomycin under a humidified 95% air, 5% CO_2 atmosphere at 37°C. Cells between passages 3 and 15 were used in this study.

2.5 OGD/R and drug administration

BV2 cells at a density of 2.5 × 10^5 cells/mL were seeded into 96-well plates and cultured overnight to 80% confluence. The normal cultured cells without any treatment were defined as control, and the other cells were performed in oxygen-glucose deprivation (OGD) experiment as our previous procedure.[11] After 4 hours of OGD, cells were transferred to normal culture conditions and incubated with different concentrations of LTC (0.5, 1, and 2 μg/mL) for an additional 3 hours. Twenty microliters of MTS was added to the cells as soon as the oxygenation ended, and cell viability was evaluated by measuring the absorbance at 490 nm using a microplate spectrophotometer (Molecular Devices Flex Station 3) after 3 to 4 hours incubation. The amount of LDH released was detected using a commercial kit according to the operating instructions.

2.6 Measurement of NO, PGE2, and inflammatory cytokines release

BV2 cells were seeded into 96-well plates at a density of 2.5 × 10^5 cells/mL. After a 24-hour incubation to allow cell attachment, cells were treated with LPS (1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 24 hours, and the cells without any treatment were defined as control. Cell supernatants then were collected and NO, PGE2, IL-1β, IL-6, and TNF-α content were detected using the corresponding assay kit according to the manufacturer's instruction.

2.7 Western blot analysis

BV2 cells were seeded into 100-mm cell culture dish at a density of 1.0 × 10^6 cells/mL and cultured overnight. The cells were then treated with LPS (1 μg/mL) alone or LPS plus three dosage of LTC (0.5, 1, and 2 μg/mL) for different times according to the difference of proteins (18 hours for Cox-2, iNOS, NF-κB p65, nuclear factor erythroid-2-related actor 2/heme oxygenase 1 (Nrf2/HO-1), and MAPKs signaling pathway; 4 hours for Janus kinase/signal transducer and activator of tranions [JAK/STAT] signaling pathway). After that, the cells were harvested and nuclear and cytosolic proteins were separated using Nuclear and Cytoplasmic Extraction kit (BestBio, Shanghai, China), and the protein concentration in each sample was measured by Bradford method. Equal amounts of protein (50 μg) in each sample were separated by

10% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membranes. Standard procedures for western blotting was carried out as described previously.[11]

2.8 Electrophoretic mobility shift assays

For EMSA assay, 10 μg nuclear extracts were incubated with [^{32}P]-labeled oligonucleotide encoding NF-κB consensus binding site (5′-AGTTGAGGGGACTTTCCCAGGC-3′) containing 2 μg poly(dI-dC) in binding buffer at room temperature for 30 minutes. Meanwhile, a 400-fold excess of unlabeled oligonucleotide probe was added to extracts of LTC-treated cells for competition analysis. After incubating for an additional 10 minutes at 4°C, NF-κB–DNA mixture was run on a 5% polyacrylamide gel and visualized on Chemi Doc XRS + detection system (Bio-Rad Laboratories, Hercules, CA).

2.9 Immunofluorescence assays for Nrf2

To investigate the activation of Nrf2/HO-1 signaling in LPS-treated, double-labeled immunocytochemistry was performed to determine the cellular localization of Nrf2. Briefly, BV2 cells (100 μL/well) at a density of 8.0 × 10^4 cells/mL were seeded into black-walled optically clear-bottomed 96-well plates. The cells were untreated (control) or incubated with LPS (1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 2 hours. Subsequently, the cells were washed with 1× PBS three times and fixed with 4% (v/v) formaldehyde (Amresco, LLC, Solon, Ohio 44139, USA) in 1× PBS at room temperature for 30 minutes. Standard procedures for immunofluorescence assays were carried out as the previous description.[13]

2.10 Effect of BV2-conditioned medium on PC12 cell viability

BV2 cells at a density of 2.5 × 10^5 cells/mL were seeded into 96-well plates overnight and treated with LPS (15 μg/mL) and three dosages of LTC (0.5, 1, and 2 μg/ml) for additional 24 hours. The plate was centrifuged at 1000 × g for 5 minutes and cell supernatant was then collected as conditioned media (CM). PC12 cells at a density of 1 × 10^4 cells/well were plated into 96-well plates overnight at 37°C. The CM was then added into PC12 neuronal cells which were further incubated for an additional 24 hours at 37°C and cell viability was then measured by MTS assay.

2.11 Statistical analysis

All experiments were repeated at least three times independently. Data were analyzed using GraphPad Prism 5.0 and expressed as mean ± SD. Statistical differences were analyzed by one-way analysis of variance (ANOVA) and Student's *t* test. Differences were considered to be significant when the values were $p < 0.05$.

3. RESULTS

3.1 HLPC profile of LTC

The major compounds in LTC were analyzed with HPLC fingerprinting technique. The analysis results of LTC are shown in Figure 1. Furthermore, the content of four main phenolic compounds, loureirin A (0.93%), loureirin B (0.79%), 7,4′-dihydroxyflavone (0.70%), and pterostilbene (0.84%), in the total phenol extract of LTC was calculated based on preconstructed standard curves.

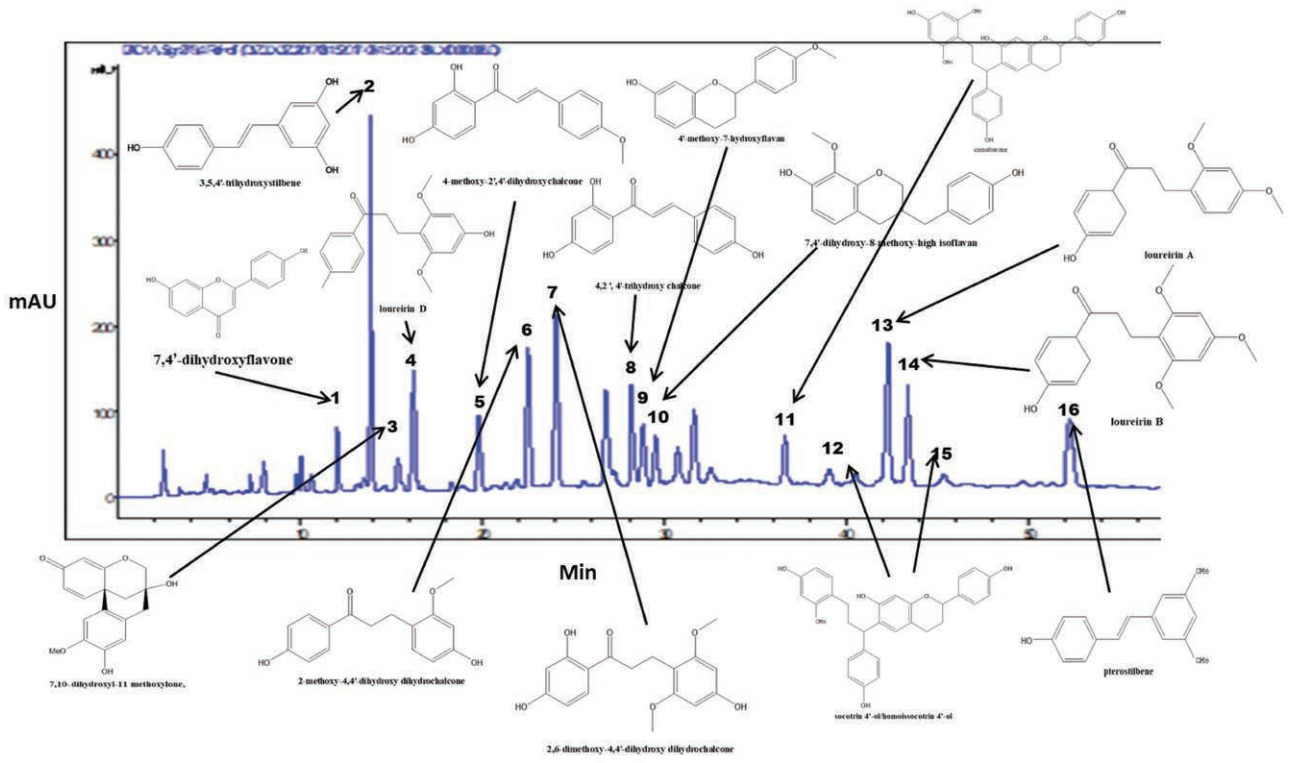

Fig. 1. HPLC chromatograms of Longxuetongluo capsule (LTC) detected at 278 nm and 325 nm. Sixteen peaks corresponding to 1: 7,4′-dihydroxyflavone; 2: 3,5,4′-trihydroxystilbene; 3: 7,10-dihydroxyl-11-methoxylone; 4: loureirin D; 5: 4-methoxy-2′,4′-dihydrochalcone; 6: 2-methoxy-4,4′-dihydroxydihydrochalcone; 7: 2,6-dimethoxy-4,4′-dihydroxydihydrochalcone; 8: 4,2′,4′-trihydroxychalcone; 9: 4′-methoxy-7-hydroxyflavan; 10: 8-methoxy-7,4′-dihydroxy-high-isoflavan; 11: cinnabarone; 12/15: socotrin 4′-ol/homoissocotrin 4′-ol; 13: loureirin A; 14: loureirin B; 16: pterostilbene.

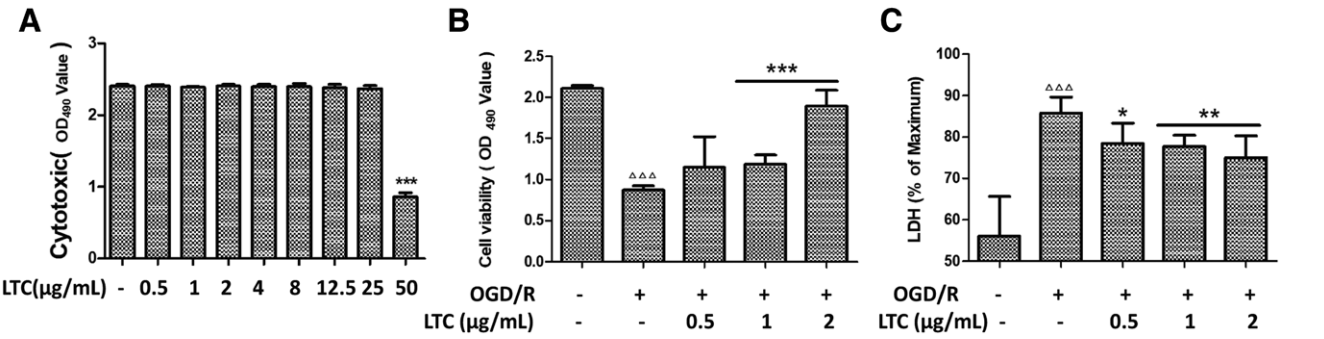

Fig. 2. Effects of Longxuetongluo capsule (LTC) on cell viability and lactate dehydrogenase (LDH) release of BV2 microglial cells exposed to oxygen-glucose deprivation/reoxygenation (OGD/R). A, The cytotoxic effect of LTC on BV2 microglia cells. Cells at a density of 2.5×10^5 cells/mL were treated with different concentration of LTC (0.5–50 μg/mL) for 4 h, cell viabilities were then tested using MTS assay by absorbance at 490 nm. All the results were expressed as OD490 values, $^{***}p < 0.001$ as compared with control group. B and C, Effects of LTC on cell viability and LDH release of BV2 microglial cells exposed to OGD/R. Cells at a density of 2.5×10^5 cells/mL were subjected to 4 h OGD followed by reoxygenation for an additional 3 h with different concentrations of LTC (0.5–2 μg/mL), cell viability and LDH release were then tested using the corresponding assay kit. Each value indicates the mean ± SD and is representative of results obtained from six wells in all experiments. $^{\Delta\Delta\Delta}p < 0.001$ as compared with control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with model group. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

3.2 Effects of LTC on cell viability of BV2 microglial cells exposed to OGD/R

We examined the occurrence of ischemic damage using the optimized oxygen-glucose deprivation/reoxygenation (OGD/R) model by measuring the normal and OGD/R cell viability and evaluated the efficacy of LTC. As the results are shown in Figure 1, treatment with LTC (0.5–2 μg/mL) dose-dependently increased cell viability in BV2 microglia cells exposed to OGD/R (Fig. 2B), while LTC at the dosage range of 0.5 to 2 μg/mL did not produce a cytotoxic effect ($p > 0.05$, Fig. 2A). Correspondingly, the results of cell death detection (LDH assay) were consistent with the results of cell viability in the case of LTC intervention (Fig. 2C). The above results show that LTC may play a protective role in OGD/R-induced cell injury.

3.3 Effects of LTC on NF-κB p65 in LPS-induced BV2 microglial cells

NF-κB is a central mediator which is involved in microglia-related inflammatory processes. Activation of NF-κB led to downstream genes such as IL-1β, IL-6, TNF-α, and iNOS expression. For this reason, it is very necessary to detect whether LTC has inhibitory effect on the activation of NF-κB. As Figure 3A–C shows, high dosage of LTC (2 μg/mL) significantly decreased NF-κB p65 level in nucleus, while increased its level in cytosol. The result was also proved by the experiment of EMSA by detecting NF-κB–DNA binding activity in nuclear extracts of LPS-induced BV2 microglia cells. As shown in Figure 3D, LPS (1.0 μg/mL) treatment caused an obvious increase in the DNA-binding ability of NF-κB to oligonucleotides. LTC dose-dependently suppressed the NF-κB–DNA binding activity after treatment along with LPS for 18 hours, indicating that the activation of NF-κB induced by LPS was likely to be blocked by LTC.

3.4 Effects of LTC on NO, PGE2 production and iNOS, COX-2 expressions

Antineuroinflammatory effects of LTC on NO and PGE2 production induced by LPS in BV2 microglial cells were further examined. As shown in Figure 4A, B, LPS stimulation significantly increases the production of NO (19.91 ± 1.64 μM) and PGE2 (2486.45 ± 728.12 pg/mL) compared with normal cells (NO: 0.29 ± 0.1 μM; PGE2: 1265.98 ± 344.11 pg/mL). As expected, treating the cells with LTC at 0.5 to 2 μg/mL significantly decreased the LPS-induced NO and PGE2 releases in a dose-dependent manner. Further investigation was also performed to validate whether the inhibitory effects of LTC on NO and PGE2 production were associated with the decrease of iNOS and COX-2 expressions. Consistently, the western blot analysis showed that COX-2 and iNOS protein expressions were also significantly decreased in LPS-activated cells along with the LTC treatment (Fig. 4C–E). These findings indicated that LTC can potently suppress the production of NO and PGE2 through inhibiting the expressions of iNOS and COX-2, which was up-regulated by LPS stimulation.

3.5 Anti-inflammatory activity of LTC on BV2 microglial cells

Reactive nitrogen species, PGE2, oxygen radicals, and cytokines have pivotal functions in the development and maintenance of neuroinflammation responses.[14] Anti-inflammatory activity of LTC in LPS-induced BV2 microglia cells was also detected by analyzing the levels of cytokines in cell supernatants. The results showed that LPS (1 μg/mL) could strongly promote IL-1β, IL-6, and TNF-α production above basal levels (Fig. 4, IL-1β: 39.75-fold; IL-6: 1551.43-fold; TNF-α: 312.99-fold vs control group, $p < 0.001$, respectively). However, after being the cells treated by LTC, most cytokines were significantly decreased in a dose-dependent manner, of which the maximum inhibition rate was 42.0% (Fig. 4F), 8.4% (Fig. 4G), 34.3% (Fig. 4H) for IL-1β, IL-6, and TNF-α, respectively. The results indicated that LTC had a good antineuroinflammatory activity.

3.6 Effects of LTC on MAPKs signaling pathways in LPS-induced BV2 microglial cells

It is well recognized that MAPK pathway–associated molecules, including ERK, JNK, and p38, are playing an important role in middle cerebral artery occlusion and reperfusion (MCAO/R) and OGD/R-induced injury in rats and microglia.[15] Therefore, to further assess whether LTC treatment would regulate the MAPK pathways, thus suppressing the release of inflammatory cytokines, we examined the p-ERK1/2, p-JNK1/2, and p-p38 MAPK expression in LPS-induced BV2 microglia cells. As Figure 5 shows, phosphorylation of p38, ERK1/2, and JNK1/2 MAPKs occurred in BV2 microglia cells after LPS stimulation for 18 hours. Three dosages of LTC significantly suppressed the extracellular phosphorylation of p38, ERK1/2 in different degrees, while phosphorylation of JNK1/2 was not affected.

3.7 Effects of LTC on JAK/STAT signaling pathway in LPS-induced BV2 microglial cells

The JAK-STAT signal pathway plays myriad pivotal roles in promoting and modulating immune and inflammatory responses.[16,17]

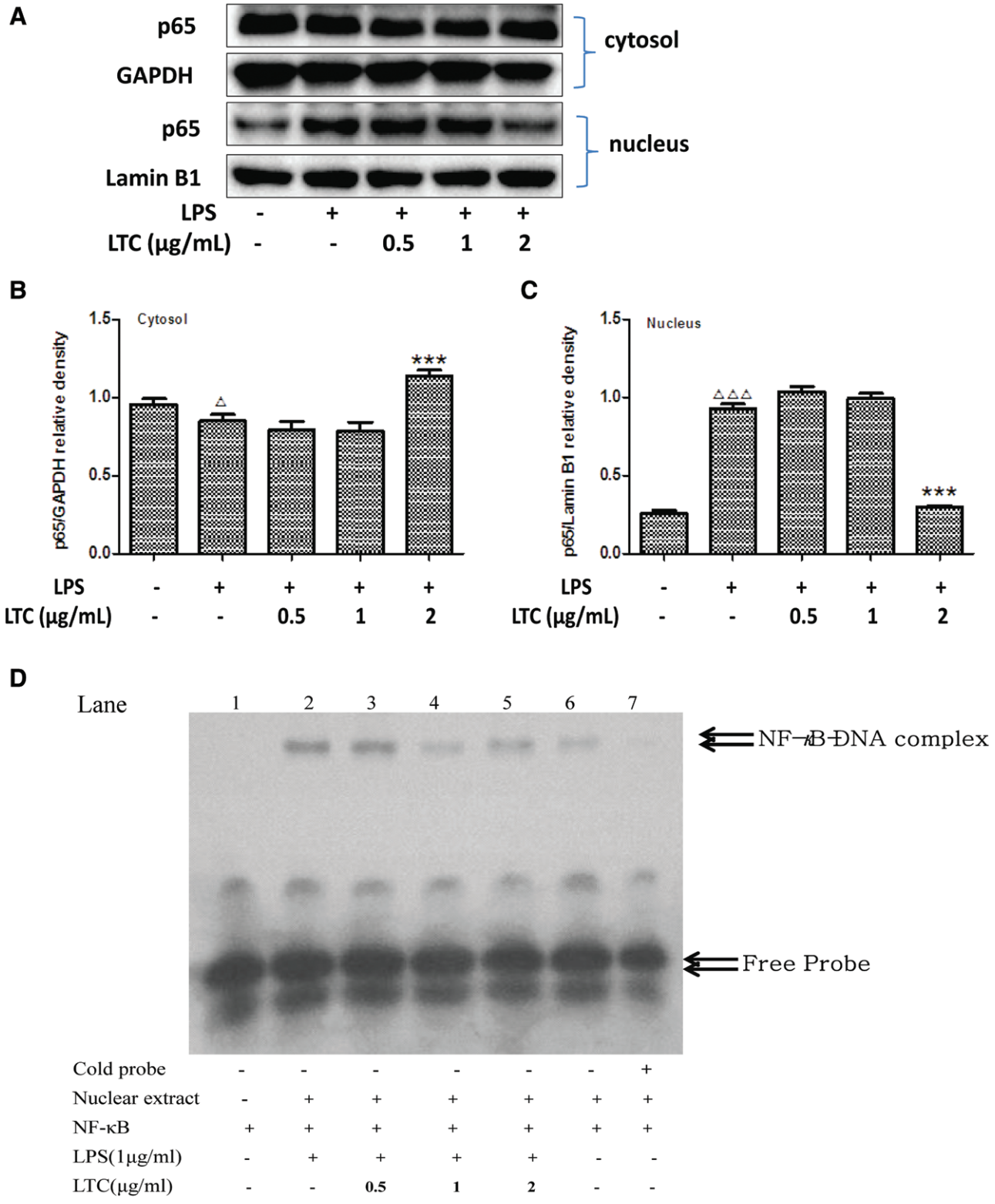

Fig. 3. Effects of Longxuetongluo capsule (LTC) on NF-κB protein expression and DNA-binding activity in lipopolysaccharide (LPS)-induced BV2 microglia cells. The cells at a density of 1.0×10^6 cells/mL were treated with LPS (1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 18 h. The protein levels of NF-κB p65 and DNA-binding activity were detected by Western Blot and electrophoretic mobility shift assay (EMSA). A, Western Blot analysis of NF-κB expression in the nucleus and cytoplasm of BV2 cells. B and C, The gray values of protein expression were quantified by Image Tool 3.0 (UTHSCSA, USA). The figure shows the representative of three independent experiments. $^{\triangle}p < 0.05$, $^{\triangle\triangle\triangle}p < 0.001$ as compared with control group; $^{***}p < 0.001$ as compared with model group. D, Effect of LTC on LPS-stimulated NF-κB–DNA complex formation in BV2 microglia cells. The upper double arrows indicate the specific binding of NF-κB–DNA complex, which competes against in the presence of 400-fold excess of unlabelled oligonucleotide encoding NF-κB consensus binding site (lane 7). Lane 1 indicates only the labeled probes were added to the reaction system. The lower double arrows indicate the free probes. Results are the representative of two separate experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear transcription factor κB.

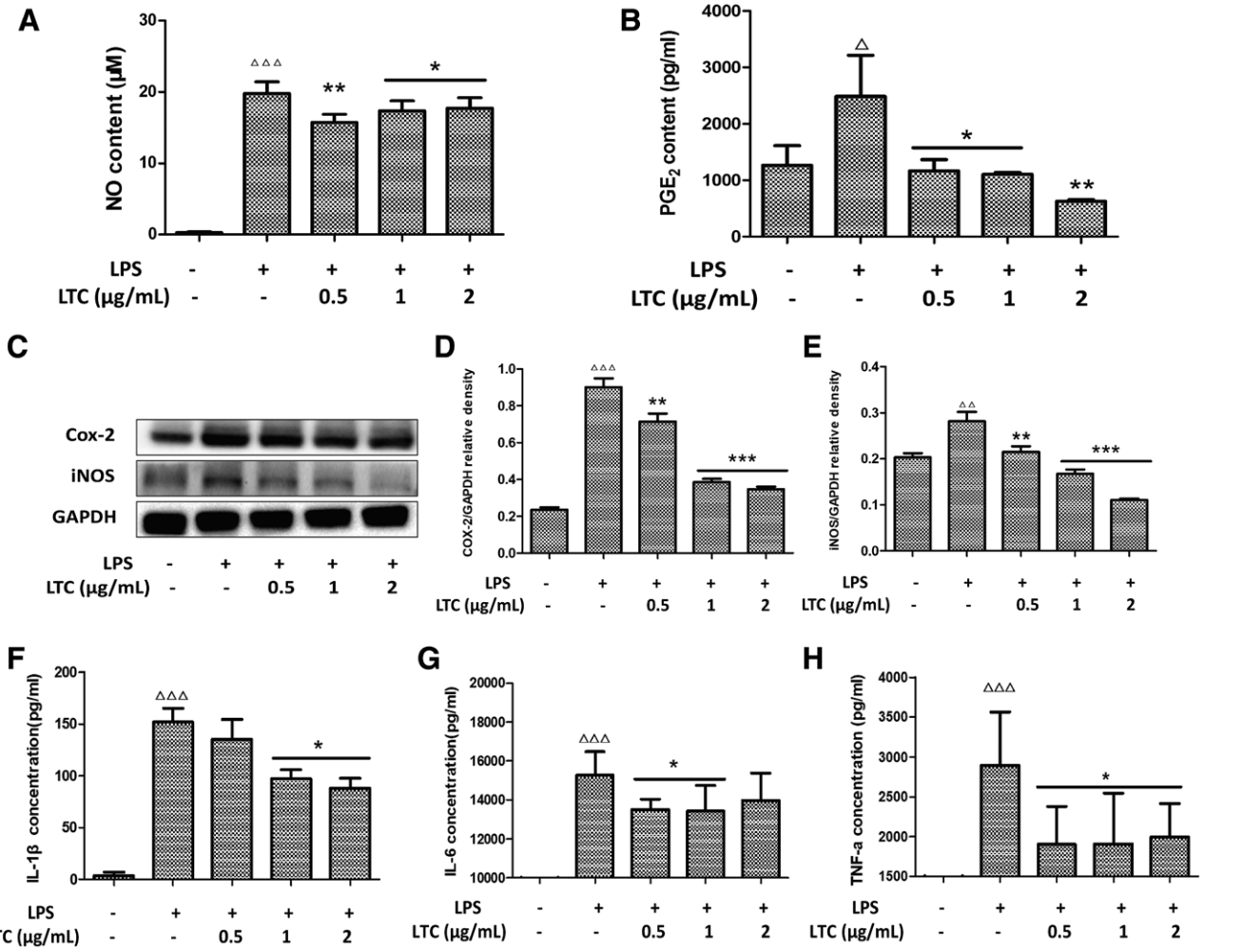

Fig. 4. Effects of Longxuetongluo capsule (LTC) on the production of NO, PGE2, protein expression of COX-2, iNOS, and inflammatory cytokine in lipopolysaccharide (LPS)-induced BV2 microglia cells. The cells at a density of 2.5×10^5 cells/mL (NO, PGE2 assays) or 1.0×10^6 cells/mL (COX-2, iNOS assays) were treated with LPS (1 μg/mL) alone or LPS plus three dosage of LTC (0.5, 1, and 2 μg/mL) for 24 h, NO/PGE2 production, IL-1β, IL-6, and TNF-α levels and COX-2/iNOS protein levels were detected by corresponding assay kits and Western Blot. A and B, NO and PGE2 production. Each value indicates the mean ± SD and is representative of results obtained from six wells in all experiments. C, Western Blot analysis of COX-2 and iNOS protein levels. D and E, The gray values were quantified by Image Tool 3.0. The figure shows the representative of three independent experiments. F–H, IL-1β, IL-6, and TNF-α production. Each value indicates the mean ± SD and is representative of results obtained from six wells in all experiments. $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$, $^{\triangle\triangle\triangle}p < 0.001$ as compared with control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with model group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL, interleukin; PGE2, prostaglandin E2.

Previous study has shown that LPS can stimulate JAK/STAT by phosphorylation of JAK1, JAK2, and STATs including STAT1, STAT3, and STAT5 in BV2 microglia cells.[18] Thus, to further exploring the antineuroinflammatory mechanism of LTC, we examined the effects of LTC on phosphorylation of JAK1 and STAT3 in BV2 microglia cells. As shown in Figure 6, LPS (1.0 μg/mL) significantly up-regulated phosphorylation of JAK1 and STAT3 in BV2 cells. However, middle and high dosage of LTC (1.0–2.0 μg/mL) remarkably inhibited this increase, implying that the anti-inflammatory mechanism of LTC may be, at least in part, related to its inhibition of JAK1/STAT3 signaling pathway.

3.8 Effects of LTC on Nrf2/HO-1 signaling pathway in LPS-induced BV2 microglial cells

HO-1 induction by activated Nrf2 protects cells against oxidative stress, which has been reported to have an anti-inflammatory effect on microglia.[19] As HO-1 is a Nrf2 downstream protein and is mainly responsible for anti-inflammatory property, we first examined whether the HO-1 expression regulated in the presence of LPS and LTC or not. Our results showed that LTC at 0.5 to 2.0 μg/mL significantly increased HO-1 protein expression in a dose-dependent manner (Fig. 7A). Afterward, we investigated the Nrf2 expression in BV2 cells by western blot and immunofluorescence assays. Fortunately, the results showed that treatment with LTC resulted in the nuclear translocation of Nrf2 in the presence of LPS (Fig. 7B). Also, the expression of Nrf2 in LPS-treated BV2 microglia was lower than in normal BV2 microglia, whereas high dosage of LTC (2.0 μg/mL) tends to induce the activation of Nrf2 in inflammatory condition (Fig. 7F). Overall, all the results suggested that LTC activates the HO-1/Nrf2 signaling pathway and enhances the anti-inflammatory potential.

3.9 Effects of LTC on microglia-mediated neurotoxicity in PC12 cells

To ensure sufficient damage to PC12 cells and evaluate the efficacy of LTC, we firstly investigated the effects of conditioned media from BV2 cells which are stimulated by different concentrations of LPS on PC12 cell viability. The result showed that

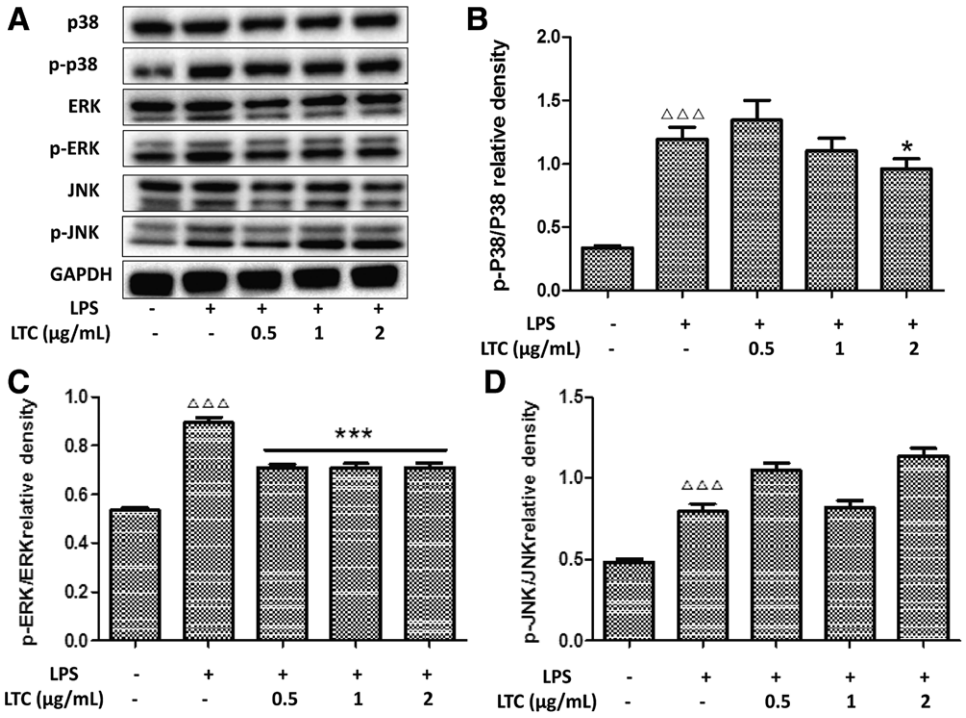

Fig. 5. Effect of Longxuetongluo capsule (LTC) on lipopolysaccharide (LPS)-induced activation of mitogen-activated protein kinases (MAPKs) LPS-induced BV2 microglia cells. The cells at a density of 1.0 × 10^6 cells /mL were treated with LPS (1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 24 h, the protein levels of phosphorylated or total extracellular signal-regulated kinase (ERK)1/2, c-jun NH2-terminal kinase (JNK), p38 were detected by Western Blot with specific antibodies. The gray values were quantified by Image Tool 3.0. The figure shows the representative of three independent experiments. $^{\triangle\triangle\triangle}p < 0.001$ as compared with control group; $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with model group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

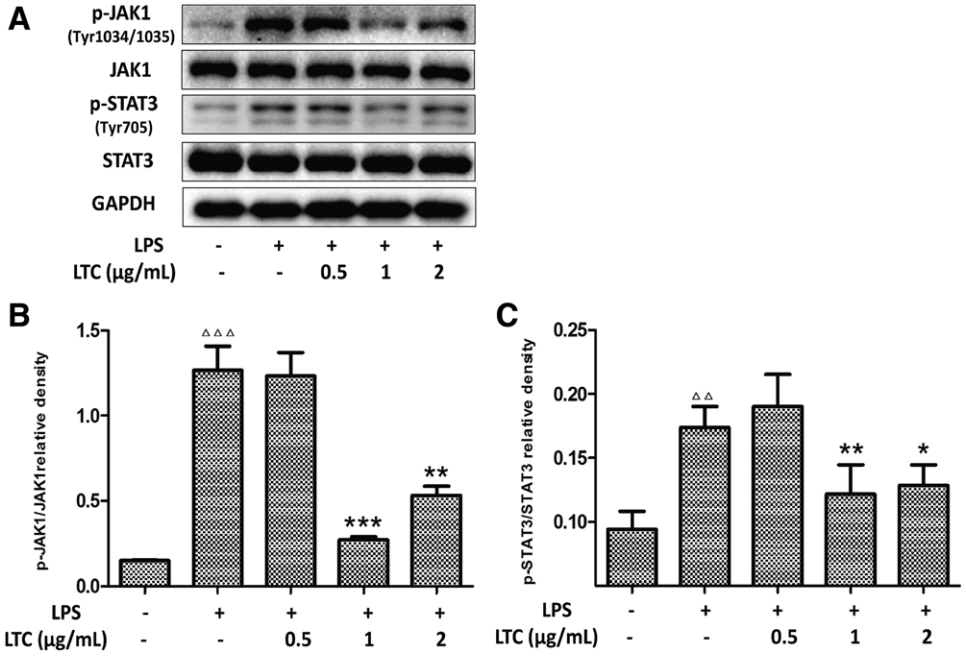

Fig. 6. Effect of Longxuetongluo capsule (LTC) on JAK/STAT signaling pathway in lipopolysaccharide (LPS)-induced BV2 microglia. The cells at a density of 1.0 × 106 cells /mL were treated with lipopolysaccharide (LPS; 1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 4 h, the protein levels of phosphorylated or total JAK1, STAT3 were detected by Western Blot with specific antibodies. The gray values were quantified by Image Tool 3.0. The figure shows the representative of three independent experiments. $^{\triangle\triangle}p < 0.01$, $^{\triangle\triangle\triangle}p < 0.001$ as compared with control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with model group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK/STAT, Janus kinase/signal transducer and activator of tranions.

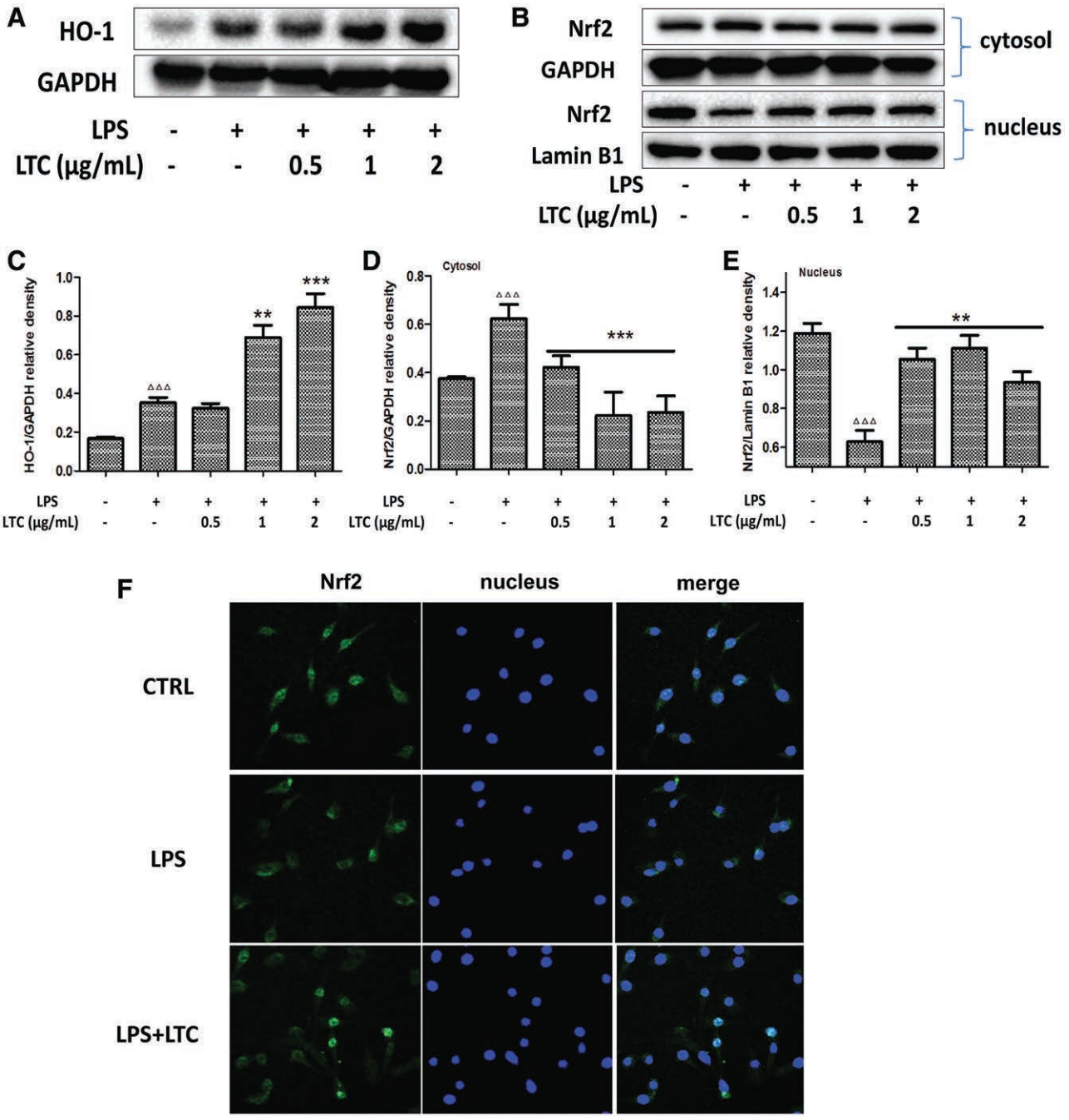

Fig. 7. Effect of Longxuetongluo capsule (LTC) on Nrf2/HO-1 signaling pathway in lipopolysaccharide (LPS)-induced BV2 microglia cells. The cells at a density of 1.0 × 106 cells /mL were treated with LPS (1 μg/mL) alone or LPS plus three dosages of LTC (0.5, 1, and 2 μg/mL) for 18 h (protein assay), the protein levels of HO-1 and Nrf2 were detected by Western Blot with specific antibodies. In the immunofluorescence assay, cells at a density of were 8.0 × 10^4 cells /mL were treated with LPS (1 μg/mL) alone or LPS plus 2 μg/mL LTC for 2 h. A and B, Western Blot analysis of HO-1 and Nrf2 expression. C–E, The gray values were quantified by Image Tool 3.0. The figure shows the representative of three independent experiments. $^{\triangle\triangle\triangle}p < 0.001$ as compared with control group; $^{**}p < 0.01$, $^{***}p < 0.001$ as compared with model group. F, Immunocytochemical analysis of Nrf2 in BV2 microglia cells. Fluorescent images were visualized under a fluorescence reverse microscope (Leica DMI 4000B, LeicaCo., Solms, Germany). From left to right, The Nrf2 protein, the nucleus, and merged images. Nuclei were stained Hoechst 33258 (blue), Nrf2 was detected by DyLight 488-labeled antibody against Nrf2 (green). GAPDH, glyceraldehyde-3-phosphate dehydrogenase, Nrf2/HO-1, nuclear factor erythroid-2-related actor 2/heme oxygenase 1.

conditioned media from 15 μg/mL LPS-treated BV2 cells supernatant (LPS-CM) for 24 hours can produce stable and effective damage to PC12 cells, and the damage rate is nearly 20% when compared to the control conditioned media (C-CM) group, suggesting the neurotoxicity of microglia-mediated inflammation (Fig. 8A). However, conditioned media from LTC treatment group (LTC-CM) dose-dependently increased PC12 cell viability (Fig. 8B), which suggest that LTC suppressed microglial activation induced by inflammation response, thus protecting the nerve cells from subsequent neurotoxicity.

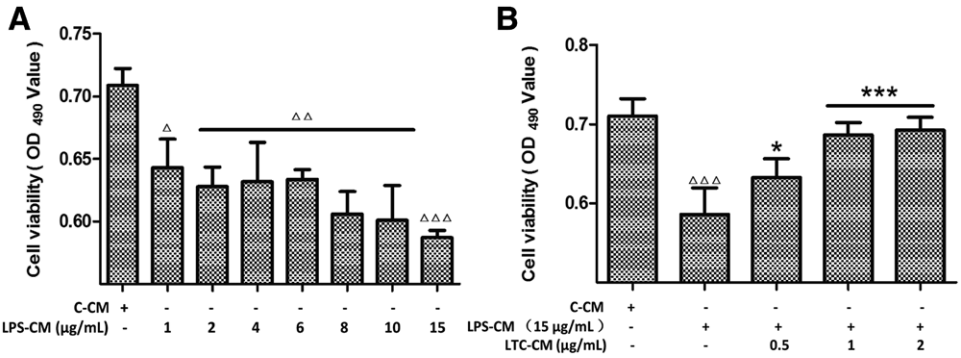

Fig. 8. Effects of conditioned medium on PC12 neurons cell viability. After treatment of the conditioned medium for 24 h, cell viability was measured by the MTS assay. Results are expressed as mean ±SD (n = 3). $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$, $^{\triangle\triangle\triangle}p < 0.001$ as compared with control conditioned media (C-CM) control. $^{*}p < 0.05$, $^{***}p < 0.001$ as compared with lipopolysaccharide (LPS)-CM group. MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

4. DISCUSSION

As a traditional Chinese medicine, Chinese dragon's blood has been used to treat various diseases for a long time. Previous studies have shown that LTC had an excellent anti-inflammatory effect on suppressing aortic endothelial and vascular cell inflammation response in high cholesterol diet (HCD)–induced rat atherosclerosis and ApoE–/– mice model.[1,20,21] However, antineuroinflammatory effect is still not been well understood. In this study, the anti-inflammatory effect of LTC was extensively investigated in LPS-induced neuroinflammation in BV2 microglia cells. The major finding of this study was that treatment with LTC significantly increased cell viability and decreased LDH, NO, PGE2, IL-1β, IL-6, and TNF-α production after the BV2 cells suffered from OGD/R and LPS stimulation. Further study demonstrated that LTC appears to ameliorate some protein expression and regulate multiple signaling pathways associated with inflammatory response. Moreover, LTC can also protect PC12 neuronal cells from the cytotoxicity of conditioned medium from LPS-stimulated BV2 microglia cells. The current study provides the experimental evidence that LTC can suppress inflammation progression, which may contribute to treat ischemic stroke.

It is known that LPS treatment triggers diverse microglial responses, such as reactive oxygen species (ROS) and inflammatory mediator production, and subsequent microglia activation, which play a critical role in the regulation of neuroinflammation. Once microglia is stimulated by LPS, it could release amounts of cytotoxic molecules, oxygen radicals, and inflammatory mediators, such as NO, TNF-α, IL-1β, IL-6, and PGE2, which could induce neuronal injury in brain.[22] NO and PGE2, which generated from L-arginine and arachidonic acid by iNOS and COX-2, have been found to be induced in various types of central nervous injuries and diseases and presumed to be neurotoxic.[18] NF-κB has been considered to be a central transcription factor in controlling many inflammatory genes, including TNF-α, IL-1β, IL-6, iNOS, and COX-2, which respond to the stimulation of LPS in microglia.[11,23] It has been demonstrated that TNF-α, IL-1β, IL-6 produced by activated microglia could initiate the cytokine cascade during the neuroinflammation, thus promoting the cascade of glial cell reactions and facilitating ischemic, traumatic brain injury.[24] Blocking of NF-κB signaling or knocking out NF-κB gene can inhibit the production of inflammatory mediators against LPS in microglia.[25] Other studies also proved that suppression of NF-κB could reduce brain infarct volumes, water content, and attenuate microglial response in mouse ischemic brain injury. Thus, inhibition of NF-κB and NF-κB–related factors (such as inflammatory mediators, iNOS and COX-2) in microglia is very important in the treatment of neuroinflammation diseases and it is also a key indicator to evaluate the anti-inflammatory activity of various drugs. In view of these reasons, we first investigated the changes of NF-κB and found that treatment with 2 μg/mL LTC can significantly reduce NF-κB p65 in cytoplasmic fraction and increase it in nuclear fraction in LPS-induced BV2 cells (Fig. 3A). Furthermore, inflammatory mediators and cytokines (TNF-α, IL-1β, IL-6, NO, and PGE2) production and protein expression (iNOS and COX-2) were significantly suppressed by three dosages of LTC (Fig. 4). To further confirm whether inhibition of the NF-κB target genes by LTC was caused by blocking the formation of NF-κB–DNA complex in nucleus, an EMSA assay was performed to test the interaction between LTC and NF-κB–DNA complex. Our results showed that 1 to 2 μg/mL LTC leads to an obvious inhibition of NF-κB–DNA complex in nuclear extracts after the cells stimulated by 1.0 μg/mL LPS, which strongly supported the above results from another aspect (Fig. 3D).

Focal cerebral ischemia-reperfusion activates mitogen-activated protein kinases (MAPKs) signaling, which plays a critical role in controlling neuronal survival/death.[15] Because the MAPKs is one of the most important signaling molecules involved in the modulation of inflammatory responses, three types of MAPKs including ERK1/2, JNK, and p38 MAPK activated by LPS have especially been implicated in signal transduction pathways which may contribute to the production of neuroinflammatory mediators (such as iNOS, COX-2, TNF-α, IL-1β, and IL-6) in microglia.[26] The present study revealed that LTC significantly inhibited ERK and p38 phosphorylation induced by LPS, indicating that ERK and p38 MAPK, but not JNK MAPK, might be implicated in resisting neuroinflammatory response (Fig. 5). Collectively, the above results suggest that LTC suppresses LPS-induced production of inflammatory mediators through inhibition of the NF-κB, p38, and ERK pathways in BV2 microglia.

Similarly, the JAK/STAT is one of another predominant signaling pathway downstream of cytokine receptors and growth factor receptors. Once cytokines or growth factors bind to their specific receptors, JAK/STAT is activated and regulates the development, differentiation, and function of myeloid and lymphoid cells. A wealth of evidences suggest that activation of JAK/STAT pathway involved in the progress of various CNS diseases, such as spinal cord injury, cerebral ischemia, and brain inflammation.[16,27] Rat cerebral and renal ischemia and reperfusion injury studies also found the JAK/STAT activation in neurons, macrophages/microglia, endothelial cells, and renal tissue after an ischemic insult.[28] Dysregulation of the JAK/STAT pathway, particularly by activating and polarizing myeloid cells and T cells to pathogenic phenotypes, has pathological implications for neuroinflammatory diseases.[29] In this study, we first found that LTC treatment repressed phosphorylation of JAK1, accompanied by downregulation in the phosphorylation of downstream protein

STAT3 (Fig. 6). This might indirectly indicate that the antineuroinflammation of LTC is at least partially dependent on inhibiting the activation of the JAK/STAT signaling pathway.

Nrf2, a main redox-sensitive transcription factor, has been found in the majority of cell types including neurons and microglia, which account for phase II detoxifying and antioxidant enzymes HO-1 and NAD(P)H: quinone oxidoreductase 1 induction after the body stimulated by oxidative stress or xenobiotic. Activation of Nrf2/HO-1 signaling pathway by inhibiting proinflammatory cytokine secretion and suppressing COX-2 and iNOS protein levels has been demonstrated to have antineuroinflammation in microglia or mouse brain.[30,31] Furthermore, Nrf2-activating compounds inhibit LPS-stimulated ERK1/2 and JNK phosphorylation or p38 phosphorylation, thus blocking LPS-induced inflammation in microglia, which suggest that there may be a functional crosstalk between MAPK and Nrf2 signaling.[32] To examine whether the Nrf2/HO-1 signaling is activated and whether LTC regulates Nrf2/HO-1 induction in BV2 microglia under inflammatory conditions, we investigated the Nrf2 and HO-1 protein expression by western blot and immunocytochemistry. Here, we found that LTC evidently up-regulated HO-1 expression and promoted nuclear translocation of Nrf2 in the presence of LPS (Fig. 7). The current study provides an important clue for understanding the extensive mechanism of antineuroinflammatory effects of LTC. Consistent with previous reports,[18] this study also demonstrates that Nrf2/HO-1 and MAPK have opposite regulatory effects on LPS-induced inflammatory responses.

Microglial cells have dual regulatory effects on neurons, which are neuroprotective in resting time and neurotoxic in activated states, respectively. The activated microglial cells act out of normal behavior, which causes neuron death by releasing a large number of inflammatory mediators such as TNF-α, IL-1β, NO, and ROS. In turn, the release and accumulation of these microglia-derived inflammatory mediators, which in turn, further aggravates microglia activation. This vicious cycle will lead to durable neuroinflammation and the progressive neurodegeneration. Moreover, a previous study showed that activation of BV2 microglia by LPS triggers an inflammatory reaction in PC12 cell apoptosis,[33] promoting us to investigate the neuroprotective effects of LTC on neurotoxicity of microglial cells induced by LPS. Our results showed that LTC treatment dose-dependently increased cell viability in PC12 cells and exerted neuroprotective effects on neuronal cells exposed to conditioned medium of LPS-stimulated microglial (Fig. 8). The result demonstrated that LTC may have a neuroprotective function and could probably be further developed for the treatment of neuroinflammatory conditions.

In conclusion, this study shows that LTC displayed excellent antineuroinflammatory effect in vitro by decreasing the production of proinflammatory mediators in LPS-induced BV2 microglial cells via regulating multiple signaling pathways including NF-kB, MAPK, JAK/STAT, and Nrf2/HO-1. Furthermore, LTC exerted significant neuroprotective effects against microglial-mediated PC12 cell injury. In conclusion, this study provides the evidence that LTC might control the functional activation of microglia, which suggests LTC could alleviate neuropathology of neuroinflammatory disease such as stroke. Future studies will focus on in vivo studies to confirm our findings as well as the potential preventive effect on neurodegenerative diseases concern in neuroinflammation such as Parkinson's disease and Alzheimer's disease.

REFERENCES

1. Zhou JM, Wang HM, Lv YZ, Wang ZZ, Xiao W. Anti-atherosclerotic effect of longxuetongluo capsule in high cholesterol diet induced atherosclerosis model rats. *Biomed Pharmacother* 2018;**97**:793–801.
2. Xu H, Zhang Y, Lei Y, Gao X, Zhai H, Lin N, et al. A systems biology-based approach to uncovering the molecular mechanisms underlying the effects of dragon's blood tablet in colitis, involving the integration of chemical analysis, ADME prediction, and network pharmacology. *PLoS One* 2014;**9**:e101432.
3. Choy CS, Hu CM, Chiu WT, Lam CS, Ting Y, Tsai SH, et al. Suppression of lipopolysaccharide-induced of inducible nitric oxide synthase and cyclooxygenase-2 by sanguis draconis, a dragon's blood resin, in RAW 264.7 cells. *J Ethnopharmacol* 2008;**115**:455–62.
4. Yi T, Chen HB, Zhao ZZ, Yu ZL, Jiang ZH. Comparison of the chemical profiles and anti-platelet aggregation effects of two "dragon's blood" drugs used in traditional Chinese medicine. *J Ethnopharmacol* 2011;**133**:796–802.
5. Gurgel LA, Sidrim JJ, Martins DT, Cechinel Filho V, Rao VS. In vitro antifungal activity of dragon's blood from croton urucurana against dermatophytes. *J Ethnopharmacol* 2005;**97**:409–12.
6. Xin N, Yang FJ, Li Y, Li YJ, Dai RJ, Meng WW, et al. Dragon's blood dropping pills have protective effects on focal cerebral ischemia rats model. *Phytomedicine* 2013;**21**:68–74.
7. Long M, Wang Z, Zheng D, Chen J, Tao W, Wang L, et al. Electroacupuncture pretreatment elicits neuroprotection against cerebral ischemia-reperfusion injury in rats associated with transient receptor potential vanilloid 1-mediated anti-oxidant stress and anti-inflammation. *Inflammation* 2019;**42**:1777–87.
8. Lei JR, Tu XK, Wang Y, Tu DW, Shi SS. Resveratrol downregulates the TLR4 signaling pathway to reduce brain damage in a rat model of focal cerebral ischemia. *Exp Ther Med* 2019;**17**:3215–21.
9. Song XY, Hu JF, Sun MN, Li ZP, Zhu ZX, Song LK, et al. IMM-H004, a novel coumarin derivative compound, attenuates the production of inflammatory mediatory mediators in lipopolysaccharide-activated BV2 microglia. *Brain Res Bull* 2014;**106**:30–8.
10. Zhou K, Chen J, Wu J, Wu Q, Jia C, Xu YXZ, et al. Atractylenolide III ameliorates cerebral ischemic injury and neuroinflammation associated with inhibiting JAK2/STAT3/drp1-dependent mitochondrial fission in microglia. *Phytomedicine* 2019;**59**:152922.
11. Zhou JM, Gu SS, Mei WH, Zhou J, Wang ZZ, Xiao W. Ginkgolides and bilobalide protect BV2 microglia cells against OGD/reoxygenation injury by inhibiting TLR2/4 signaling pathways. *Cell Stress Chaperones* 2016;**21**:1037–53.
12. Hou Y, Xie G, Miao F, Ding L, Mou Y, Wang L, et al. Pterostilbene attenuates lipopolysaccharide-induced learning and memory impairment possibly via inhibiting microglia activation and protecting neuronal injury in mice. *Prog Neuropsychopharmacol Biol Psychiatry* 2014;**54**:92–102.
13. Zhou JM, Xu ZL, Li N, Zhao YW, Wang ZZ, Xiao W. Identification of cardioprotective agents from traditional Chinese medicine against oxidative damage. *Mol Med Rep* 2016;**14**:77–88.
14. Ma A, Wang Y, Zhang Q. Tormentic acid reduces inflammation in BV-2 microglia by activating the liver X receptor alpha. *Neuroscience* 2015;**287**:9–14.
15. Jiang M, Li J, Peng Q, Liu Y, Liu W, Luo C, et al. Neuroprotective effects of bilobalide on cerebral ischemia and reperfusion injury are associated with inhibition of pro-inflammatory mediator production and down-regulation of JNK1/2 and p38 MAPK activation. *J Neuroinflammation* 2014;**11**:167.
16. Qin H, Buckley JA, Li X, Liu Y, Fox TH III, Meares GP, et al. Inhibition of the JAK/STAT pathway protects against α-synuclein-induced neuroinflammation and dopaminergic neurodegeneration. *J Neurosci* 2016;**36**:5144–59.
17. Liu Y, Kim S, Kim YJ, Perumalsamy H, Lee S, Hwang E, et al. Green synthesis of gold nanoparticles using euphrasia officinalisleaf extract to inhibit lipopolysaccharide-induced inflammation through NF-κb and JAK/STAT pathways in RAW 264.7 macrophages. *Int J Nanomedicine* 2019;**14**:2945–59.
18. Guo C, Yang L, Wan CX, Xia YZ, Zhang C, Chen MH, et al. Anti-neuroinflammatory effect of sophoraflavanone G from sophora alopecuroides in LPS-activated BV2 microglia by MAPK, JAK/STAT and nrf2/HO-1 signaling pathways. *Phytomedicine* 2016;**23**:1629–37.
19. Kwon YW, Cheon SY, Park SY, Song J, Lee JH. Tryptanthrin suppresses the activation of the LPS-treated BV2 microglial cell line via nrf2/HO-1 antioxidant signaling. *Front Cell Neurosci* 2017;**11**:18.
20. Zheng J, Liu B, Lun Q, Gu X, Pan B, Zhao Y, et al. Longxuetongluo capsule inhibits atherosclerosis progression in high-fat diet-induced apoe-/- mice by improving endothelial dysfunction. *Atherosclerosis* 2016;**255**:156–63.

21. Zheng J, Liu B, Lun Q, Yao W, Zhao Y, Xiao W, et al. Longxuetongluo capsule improves erythrocyte function against lipid peroxidation and abnormal hemorheological parameters in high fat diet-induced apoe-/- mice. *Oxid Med Cell Longev* 2016;**2016**:2603219.
22. Gu JH, Ge JB, Li M, Wu F, Zhang W, Qin ZH. Inhibition of NF-κb activation is associated with anti-inflammatory and anti-apoptotic effects of ginkgolide B in a mouse model of cerebral ischemia/reperfusion injury. *Eur J Pharm Sci* 2012;**47**:652–60.
23. Yan A, Cai G, Xia W, Fu Y. Thromboxane A2 receptor antagonist SQ29548 suppresses the LPS-induced release of inflammatory cytokines in BV2 microglia cells via suppressing MAPK and NF-κb signaling pathways. *Mol Med Rep* 2017;**16**:2491–6.
24. Du RW, Du RH, Bu WG. B-arrestin 2 mediates the anti-inflammatory effects of fluoxetine in lipopolysaccharide-stimulated microglial cells. *J Neuroimmune Pharmacol* 2014;**9**:582–90.
25. Zeng KW, Wang S, Dong X, Jiang Y, Tu PF. Sesquiterpene dimer (DSF-52) from artemisia argyi inhibits microglia-mediated neuroinflammation via suppression of NF-κb, JNK/p38 mapks and jak2/stat3 signaling pathways. *Phytomedicine* 2014;**21**:298–306.
26. Puri R, Kaur Bhatia R, Shankar Pandey R, Kumar Jain U, Katare OP, Madan J. Sigma-2 receptor ligand anchored telmisartan loaded nanostructured lipid particles augmented drug delivery, cytotoxicity, apoptosis and cellular uptake in prostate cancer cells. *Drug Dev Ind Pharm* 2016;**42**:2020–30.
27. Gong P, Zhang Z, Zou Y, Tian Q, Han S, Xu Z, et al. Tetramethylpyrazine attenuates blood-brain barrier disruption in ischemia/reperfusion injury through the JAK/STAT signaling pathway. *Eur J Pharmacol* 2019;**854**:289–97.
28. Si Y, Bao H, Han L, Shi H, Zhang Y, Xu L, et al. Dexmedetomidine protects against renal ischemia and reperfusion injury by inhibiting the JAK/STAT signaling activation. *J Transl Med* 2013;**11**:141.
29. Benveniste EN, Liu Y, McFarland BC, Qin H. Involvement of the janus kinase/signal transducer and activator of transcription signaling pathway in multiple sclerosis and the animal model of experimental autoimmune encephalomyelitis. *J Interferon Cytokine Res* 2014;**34**:577–88.
30. Subedi L, Lee JH, Yumnam S, Ji E, Kim SY, Anti-inflammatory effect of sulforaphane on LPS-activated microglia potentially through JNK/AP-1/NF-kappaB inhibition and Nrf2/HO-1 activation. *Cells* 2019;**8**:17.
31. Li Y, Meng F. Effects of icariside II on brain tissue oxidative stress and nrf2/HO-1 expression in rats with cerebral ischemia-reperfusion injury1. *Acta Cir Bras* 2019;**34**:e201900208.
32. Brandenburg LO, Kipp M, Lucius R, Pufe T, Wruck CJ. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. *Inflamm Res* 2010;**59**:443–50.
33. Dai XJ, Li N, Yu L, Chen ZY, Hua R, Qin X, et al. Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. *Cell Stress Chaperones* 2015;**20**:321–31.