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ORIGINAL ARTICLE

Methanolic Extracts of *Capparis ecuadorica* Iltis Inhibit the Inflammatory Response in Lipopolysaccharide-Stimulated RAW 264.7 Macrophage Cells

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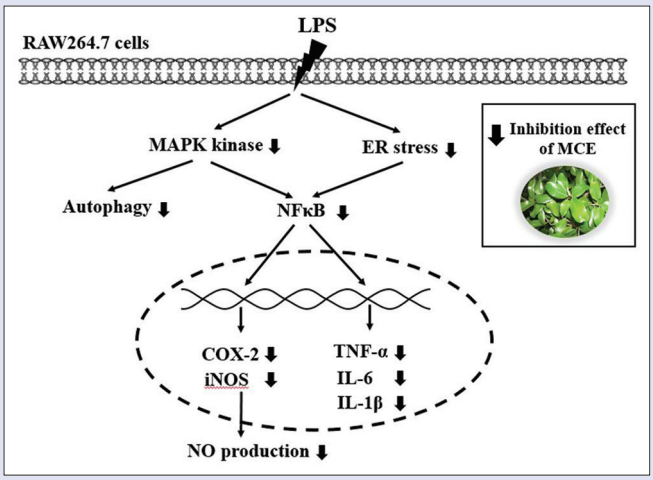
ABSTRACT

Background: Some species of the caper family are known to possess antibacterial, antioxidant, anti-inflammatory, immunomodulatory, and antiviral properties. However, to date, the therapeutic effects of *Capparis ecuadorica* Iltis (*Capparis* L.) have not been studied. **Objectives:** In this study, we investigated the anti-inflammatory activity of a methanolic extract of *C. ecuadorica* leaves (MCE) in macrophages. **Materials and Methods:** Anti-inflammatory responses and mechanisms were assessed in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells after pretreatment with MCE. **Results:** In the MCE + LPS-treated group, the relative mRNA levels of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β, IL-6) were downregulated and protein levels of IL-6 were also decreased as compared to the vehicle + LPS-treated group. Furthermore, the MCE + LPS-treated group showed reduced levels of reactive oxygen species (ROS) production as compared to the vehicle + LPS-treated group; detection of nitrite concentration revealed nitric oxide (NO) to be the reduced ROS. The expression levels of inducible nitric oxide synthase (iNOS)/cyclooxygenase-2 (COX-2) mRNA and the level of phosphorylation of IκBα were decreased in the MCE + LPS-treated group. In addition, MCE + LPS-treated group showed reduced levels of phosphorylation of MAP kinase in comparison to the vehicle + LPS-treated group. Interestingly, MCE also inhibited other inflammatory mechanisms, namely, endoplasmic reticulum (ER) stress and autophagy. **Conclusion:** These results indicate that MCE inhibits inflammatory responses through the inhibition of inflammatory cytokines and NO production, iNOS/COX-2 expression and nuclear factor-kappa B (NF-κB) activation, MAPK inhibition, as well as regulation of ER stress and autophagy in LPS-stimulated RAW 264.7 cells.

Key words: Anti-inflammatory response, *Capparis ecuadorica*, Cytokines, MAK kinase, ER stress, Autophagy

SUMMARY

- Methanolic extract of *C. ecuadorica* leaves (MCE) decreased mRNA levels of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β, IL-6) and also decreased the protein levels of IL-6 in LPS-stimulated RAW 264.7 cells
- MCE downregulated the production of reactive oxygen species (ROS) and mRNA expressions of inducible nitric oxide synthase (iNOS)/cyclooxygenase-2 (COX-2) in LPS-stimulated RAW 264.7 cells through the regulation of phosphorylation of IκBα and MAPK signaling pathway
- MCE inhibited endoplasmic reticulum stress and autophagy triggered by LPS stimulation in RAW 264.7 macrophages.



Abbreviations used: MEC: Methanolic extracts of *Capparis ecuadorica* Iltis leaves; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase-2; LPS: Lipopolysaccharide.

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INTRODUCTION

Inflammation is the reaction of an organism to a variety of substances (including foreign invading micro-organisms) and harmful elements (including transformed cells such as cancer cells).[1] Immune cells recognize these substances and secrete various inflammatory mediators to initiate an inflammatory response. Inflammation is classified as either acute or chronic, depending on the progress of the

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cell's reaction and the inflammatory response.[2,3] Acute inflammation occurs in a relatively short time and leads to increased blood flow and leukocyte migration and activity.[4] Chronic inflammation is caused due to the prolonged inflammation and is associated with cancer, autoimmune diseases, cardiovascular disease, diabetes, obesity, asthma, inflammatory bowel disease, and rheumatoid arthritis.[5]

There is a very high demand for anti-inflammatory drugs because of the increasing number of cases related to chronic diseases such as tissue damage, systemic inflammatory response syndrome (SIRS), and septic shock. Steroids, non-steroids, and biological agents have been used as representative drugs, but their adverse effects and high prices have necessitated the development of new drugs.[6] Recent studies have focused on natural materials, such as medicinal plants, as a source of new anti-inflammatory agents, because they are easy to procure in large amounts at a low cost and have minimal side effects.[7-9]

Caper L. (*Capparis ecuadorica*), a perennial shrub, contains various traditional chemical substances such as ruperin, routine, kerchatin, campenol, stigmasterol, camping, and tocopherol. Formerly, it was used as a cosmetic product, especially in Egypt and Greece, and was also used to treat rheumatism, stomach problems, headaches, and toothache.[10] Recently, the extracts of *Capparis zeylanica*, *Capparis sapinosa*, and *Capparis silkkimensis* have exhibited immune stimulation and anticancer activities in peripheral blood and tumor cells.[11-13] In addition, treatment with *Capparis decidua* and *Capparis spinosa* extracts exerted an antidiabetic activity in the diabetic model animals.[14,15] Extracts from *C. decidua*, *C. zeylanica*, and *C. spinosa* also showed antibacterial activity and antioxidant activity for various micro-organisms.[16] Panico *et al.* reported that the lyophilized extract of *C. spinosa* displays a protective effect on pro-inflammatory cytokine-stimulated human chondrocyte cultures.[17] Moreover, Trombetta *et al.* revealed that the caper extract had a remarkable anti-allergic effect.[18] Studies on the possibility of anti-inflammatory effects of the caper family have long been reported, but so far, no study has clarified the anti-inflammatory effect and the mechanism of action of methanolic extract of *C. ecuadorica* leaves (MCE) in macrophages.

In this study, the anti-inflammatory activities of MCE and their fundamental mechanisms were investigated in RAW 264.7 macrophage cells after stimulation of the cells with lipopolysaccharide (LPS).

MATERIALS AND METHODS

Preparation of methanolic extract of *C. ecuadorica* leaves

The lyophilized sample of MCE (FBM206-086) was provided by the International Biological Material Research Center at Korea Research Institutes of Bioscience and Biotechnology (Dajeon, Republic of Korea). Briefly, the powders grounded from the dried leaves of *C. ecuadorica* were mixed with methanol in a fixed liquor ratio (1:10, powder:water ratio). The extraction was conducted by sonication for 15 min and then incubation for 2 h up to ten times per day for 3 days and then filtered through a filter paper with 0.4-μm pore size. Subsequently, the obtained methanolic extract was concentrated using a rotary evaporator (n = 1000 SWD, EYELA, Bohemia, NY, USA) and lyophilized using a Speed Vacuum Concentrator (Modulspin 40, Biotron Co., Marysville, WA, USA). The final sample of MCE was dissolved in dimethyl sulfoxide (DMSO, Duchefa Biochemie, Haarlem, Netherlands) to the appropriate concentrations for use in experiments.

Mammalian cell culture

RAW 264.7 cells were derived macrophages from the ascites fluid of the Abelson murine leukemia virus-induced tumor model. These cells were provided from the Korea Cell Line Bank (Seoul, Korea) and cultured in Dulbecco Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, S001-01, Welgene, Gyeongsan-si, Korea), L-glutamine (2 mM, Thermo Fisher Scientific), penicillin (100 U/mL, Thermo Fisher Scientific), and streptomycin (100 μg/mL, Thermo Fisher Scientific) using a humidified incubator at 37°C under atmosphere containing 5% CO_2.

Cell viability assay

The viability of RAW 264.7 cells after treatment with MCE and LPS was measured using the tetrazolium compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich Co., St. Louis, MO, USA). Briefly, RAW 264.7 cells were evenly seeded at a density of 2×10^4 cells/0.2 mL of DMEM in each well and grown for 24 h in a CO_2 incubator at 37°C. For cell viability analysis, they were either classified into No treatment group or LPS-treated group (1 μg/mL). Subsequently, LPS-treated group were again treated with either vehicle (DMSO) or were pretreated with aspirin (Asp, 2 mM, Sigma-Aldrich Co.) (Asp + LPS-treated group) or were pretreated with 50 μg/mL MCE (MCELo + LPS-treated group), 100 μg/mL MCE (MCEMid + LPS-treated group) or 200 μg/mL MCE (MCEHi + LPS-treated group). On attaining 70%–80% confluency, RAW 264.7 cells of each group were treated with three different doses of MCE or Asp and subsequently treated with 1 μg/mL of LPS after 2 h. After incubation for 24 h, 200 μL of fresh DMEM and 50 μL of MTT solution (2 mg/mL in 1× phosphate-buffered saline [PBS]) were added to each well with the supernatants discarded. Following incubation at 37°C for 4 h, the formazan precipitate in each well was completely dissolved in DMSO and the absorbance in each well was read at 570 nm using a Vmax plate reader (Molecular Devices, Sunnyvale, CA, USA).

Real-time polymerase chain reaction analysis for cytokine gene expression

Polymerase chain reaction (PCR) for cytokine genes was performed as described in a previous study.[19] Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). After precipitation using ethanol, total RNAs were harvested via centrifugation at 10,000 × g for 15 min, after which their concentration was determined by a Nano-300 Micro-Spectrophotometer (Allsheng Instruments Co. Ltd., Hangzhou, China). Total complementary DNA (cDNA) against mRNA was synthesized using 200 units of Invitrogen Superscript II reverse transcriptase (Thermo Fisher Scientific). PCR was conducted with the cDNA template (2 μL), reaction mixture, and specific primers [Table 1]. All specific genes were amplified in a Perkin–Elmer Thermal Cycler using the following cycle: 30 s at 94°C (denaturation), 30 s at 62°C (annealing), and 45 s at 72°C (extension) for 28–32 cycles. Finally, the PCR products for target genes were separated on 1%–2% agarose gel and detected by staining with ethidium bromide. The density of each band was quantified and represented as related levels using a Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY, USA).

Enzyme-linked immunosorbent assay for interleukin-6 cytokine

RAW 264.7 cells were pretreated with vehicle, Asp, or different concentrations of MCE (50, 100, and 200 μg/mL) for 2 h, and then treated with LPS (1 μg/mL) for 24 h. After the collection of culture supernatant, the concentration of interleukin (IL)-6 was assayed using an IL-6 enzyme-linked immunosorbent assay (ELISA) kit (Biolegend, San Diego, CA, USA) based on the manufacturer's instructions.

Table 1: Primer sequence for real-time polymerase chain reaction

Primer name	Sequence (from 5′ to 3′)	Product size (bp)
iNOS		
Forward	5'-CACTT GGAGT TCACC CAGT-3'	484
Reverse	5'-ACCAC TCGTA CTTGG GATGC-3'	
COX-2		
Forward	5'-CAGGT CATTG GTGGA GAGGT GTATC-3'	654
Reverse	5'-CCAGG AGGAT GGAGT TGTTG TAGAG-3'	
TNF-α		
Forward	5'-G-CCTGT AGCCC ACGTC GTAGC-3'	374
Reverse	5'-TTGAC CTCAG CGCTG ACTTG-3'	
IL-1β		
Forward	5'-GCACA TCAAC AAGAG CTTCA GGCAG-3'	574
Reverse	5'-GCTGC TTGTG AGGTG CTGAT GTAC-3'	
IL-6		
Forward	5'-TTGGG ACTGA TGTTG TTGACA-3'	200
Reverse	5'-TCATC GCTGT TGATA CAATC AGA-3'	
β-actin		
Forward	5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'	540
Reverse	5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'	

iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase-2; TNF: Tumor necrosis factor, IL: Interleukin

Estimation of nitric oxide

The levels of nitric oxide (NO) were determined using Griess reagent (1% sulfanilamide, 5% phosphoric acid, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; Sigma-Aldrich Co.) as described in a previous study.[20] Briefly, RAW 264.7 cells in each well were exposed to vehicle, Asp, or MCE (50, 100, or 200 μg/mL) for 2 h, followed by treatment of LPS (1 μg/mL). After incubating the cells for 24 h, Griess reagent (100 μL) was mixed with the culture supernatant of each well, and the mixture was incubated at room temperature for 10 min. Finally, the optical density was determined using a VersaMax microplate reader (Molecular Devices) at 540 nm. The concentration of NO in culture supernatants was determined by comparing with the standard curve of sodium nitrite ($NaNO_2$).

Detection of intracellular levels of reactive oxygen species

Reactive oxygen species (ROS) levels were measured by staining the cells with the cell-permeant reagent 2',7'-dichlorofluorescein diacetate (DCF-DA) (Sigma-Aldrich Co.). After RAW 264.7 cells reached 70%–80% confluency, they were treated with MCE (50, 100, or 200 μg/mL), vehicle, or Asp for 2 h in an incubator at 37°C, followed by stimulation with LPS (1 μg/mL) for 24 h. Then, the cells were incubated with 100 μM DCF-DA for 15 min at 37°C. After washing with 1x PBS, the green fluorescence was observed at 200x magnification using a fluorescent microscope (Eclipse TX100, Nikon, Tokyo, Japan). The cell morphology was also observed under a microscope (Leica Microsystems, Heerbrugg, Switzerland) at 200x magnification.

Western blot analysis

RAW 264.7 cells were treated with vehicle, Asp, or MCE (50, 100, or 200 μg/mL) for 2 h, followed by LPS (1 μg/mL) stimulation for 15 min. The treated cells were lysed using the Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Seongnam, Korea). After centrifuging at 13,000 rpm for 5 min, the total cell lysate was used to determine protein content using the SMARTTM BCA Protein Assay Kit (Thermo Scientific). Proteins were electrophoresed. Proteins were electrophoresed on 4%–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2 h, and subsequently transferred to nitrocellulose blotting membranes with a 0.45-μm pore size (GE Healthcare, Little Chalfont, UK) for 2 h at 40 V. The membrane was then incubated separately at 4°C overnight with the specific primary antibodies; IκBα antibody (Cell Signaling Technology, Danvers, MA, USA), p-IκBα antibody (Cell Signaling Technology), SAPK/JNK antibody (Cell Signaling Technology), p-SAPK/JNK (Thr183/Tyr185) antibody (Cell Signaling Technology), ERK1 (K-23) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-ERK (E-4) antibody (Santa Cruz Biotechnology), p38 MAPK antibody (Cell Signaling Technology), p-p38 MAP Kinase (Thr180/Tyr182) antibody (Cell Signaling Technology), inositol receptor (IRE) 1α antibody (Novusbio, Littleton, CO, USA), IRE1α (p Ser724) antibody (Novusbio), eIF2α antibody (Cell Signaling Technology), p-eIF2α (Ser51) antibody (Cell Signaling Technology), and anti-actin antibody (Sigma-Aldrich Co.). After this, the chemiluminescence signals derived from specific protein bands were activated with the Amersham™ ECL Select™ (GE Healthcare) Western Blotting detection reagent and detected using FluorChemi®FC2 (Alpha Innotech Co., San Leandro, CA, USA) as analysis for LLC1 cell homogenates.

Autophagy-level measurement

The autophagic vacuoles in RAW 264.7 cells were detected using the Autophagy LC3-antibody-based kit (Millipore, Hayward, CA, USA) based on the manufacturer's protocols. After RAW 264.7 cells reached 70%–80% confluency, they were treated with MCE (50, 100, or 200 μg/mL), vehicle, or Asp and were precultured for 2 h in an incubator at 37°C, following by stimulation by LPS (1 μg/mL) for 24 h. These cells were treated with Autophagy Reagent A in Earle's balanced salt solution and incubated for 5 h at 37°C. After washing with ice-cold Hank's balanced salt solution, the treated cells were stained with anti-LC3 Alexa Fluor®555 (Millipore, Hayward, CA, USA) in 1× Autophagy Reagent B on ice for 30 min in the dark, and unstained solution was washed out with ice cold 1× Assay Buffer. Finally, the fluorescence intensity of stained cells was measured with flow cytometry in a Muse Cell Analyzer (Millipore).

Statistical analysis

Statistical analyses were performed by using SPSS software version 10.10 (SPSS, Inc. Chicago, IL, USA). One-way analysis of variance followed by Tukey's *post hoc* test was performed to identify significant differences between the vehicle- and MCE-treated groups. Experiments yielding $P < 0.05$ were considered statistically significant.

RESULTS

Suppression of pro-inflammatory cytokine expression by methanolic extract of *C. ecuadorica* leaves in lipopolysaccharide-stimulated RAW 264.7 cells

RAW 264.7 cells were incubated with MCE at 50, 100 or 200 μg/mL in the presence of LPS (1 μg/mL) for 24 h, and cell viability was measured via MTT assay. Asp (2 mM) was used as the positive control because its anti-inflammatory activity is well known. Figure 1 shows that at three concentrations, MCE prevented the decrease in viability of LPS-stimulated RAW 264.7 cells. The mRNA expression of tumor necrosis factor (TNF)-α, IL-6, and IL-1β in LPS-stimulated RAW 264.7 cells after exposure to vehicle, Asp, or MCE was measured using the real-time PCR (RT-PCR) assay [Figure 2a]. LPS-stimulated RAW 264.7 cells treated with vehicle or Asp resulted in significant increase

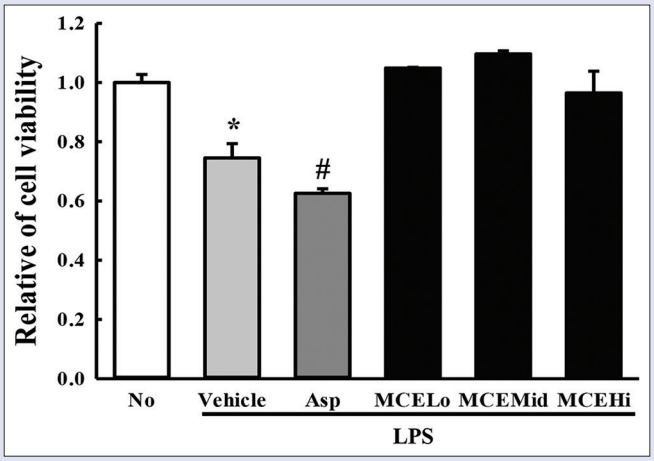

Figure 1: Effect of MCE on the viability of LPS-stimulated RAW 264.7 cells. MCE (50, 100, 200 μg/mL)-pretreated RAW 264.7 cells for 2 h were stimulated with LPS for 24 h. The viability of these cells was estimated using the MTT assay. Data are presented as mean ± SD of two independent. **P* < 0.05 versus No treatment group, #*P* < 0.05 versus vehicle + LPS-treated group. MCE: Methanolic extract of *Capparis ecuadorica* leaves; LPS: Lipopolysaccharide; SD: Standard deviation; MTT: 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

in cytokine expression level as compared to the control (No treatment) group. However, as compared to the vehicle-treated group, the levels of TNF-α, IL-6, and IL-1β remarkably declined in a dose-dependent manner in all the MCE-treated groups (#*P* < 0.05). In addition, the above dose-dependent reduction effect for IL-6 transcript by MCE was observed on the level for IL-6 protein in the culture supernatant using ELISA [Figure 2b].

Suppression of the inducible nitric oxide synthase-mediated cyclooxygenase-2 induction pathway by methanolic extract of *C. ecuadorica* leaves in lipopolysaccharide-stimulated RAW 264.7 cells

NO is produced by inducible nitric oxide synthase (iNOS), and it plays an important role as one of the mediators during inflammatory response. To investigate the role of MCE on ROS levels in LPS-stimulated cells, the DCF-DA staining assay was performed. As presented in Figure 3a and b, treatment with MCE eliminated the presence of active oxygen in the inflammatory response of LPS-induced inflammation. As shown in Figure 3c, the concentration of NO remarkably increased in the vehicle + LPS-treated group as compared to the No treatment group. However, the concentration of NO in cells pretreated with a MCEHi was significantly reduced as compared to the vehicle + LPS-treated group. In the MCEMid and MCELo pretreatment group, the NO concentrations decreased to about 80% and 30%, respectively, compared with the vehicle + LPS-treated group. These results indicate that MCE decreased the production of NO.

We also examined the expression of iNOS to determine whether MCE decreases NO production by exerting its effects on antioxidant scavenging ROS or the expression of iNOS. As expected, a significant expression of iNOS and cyclooxygenase-2 (COX-2) mRNA was observed in the vehicle + LPS-treated group compared to the No treatment group. However, the concentration pretreatment of LPS-stimulated RAW 264.7 cells with MCE showed a decrease in the

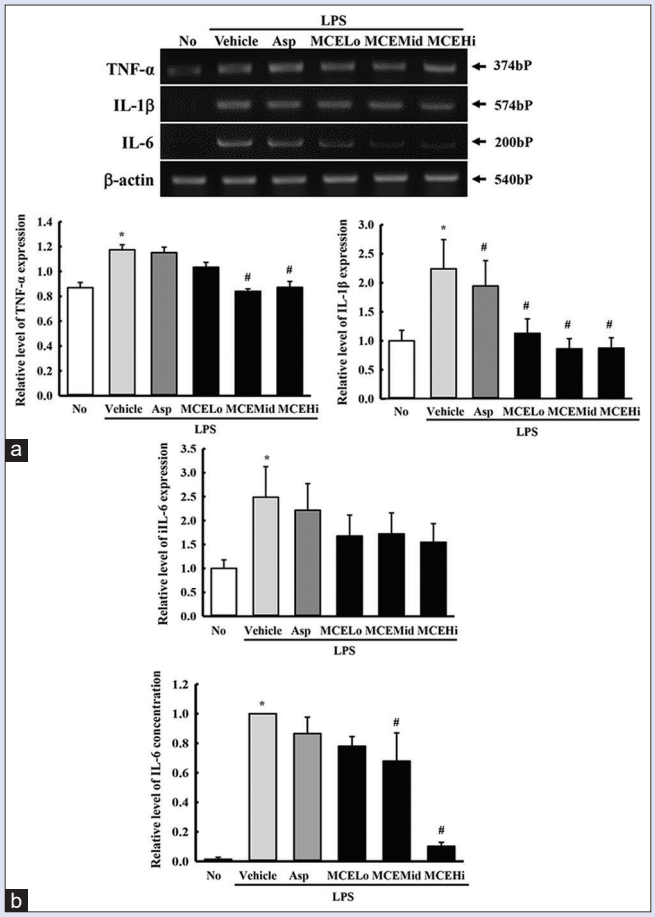

Figure 2: Effects of MCE on LPS-induced cytokine production. (a) LPS-stimulated RAW264.7 cells were pretreated with vehicle, Asp, or varying concentration of MCE for 2 h, and the expression levels of TNF-α, IL-1β, and IL-6 mRNA were determined by RT-PCR. After the intensity of each band was determined using an imaging densitometer, the relative levels of three genes' mRNA were calculated based on the band intensity of β-actin as endogenous control. **P* < 0.05 versus No treatment group, #*P* < 0.05 versus vehicle + LPS-treated group. (b) The level of IL-6 protein in culture supernatants of MCE + LPS-treated RAW264.7 cells was measured by ELISA. Data represent the mean ± SD from duplicates. **P* < 0.05 versus No treatment group, #*P* < 0.05 versus vehicle + LPS-treated group. MCE: Methanolic extract of *C. ecuadorica* leaves; LPS: Lipopolysaccharide; TNF-α: Tumor necrosis factor-α; IL: Interleukin; RT-PCR: Real-time polymerase chain reaction; SD: Standard deviation; Asp: Aspirin; ELISA: Enzyme-linked immunosorbent assay

levels of NO as compared to vehicle + LPS-treated group [Figure 4a]. Thus, it can be said that pretreatment of LPS-stimulated RAW 264.7 cells with MCE suppressed the production of NO, iNOS, and the COX-2 mRNA expression.

Inhibition of nuclear factor kappa activation by methanolic extract of *C. ecuadorica* leaves in lipopolysaccharide-stimulated RAW 264.7 cells

Nuclear factor-kappa B (NF-κB) is an important transcription factor for the induction of iNOS by LPS.[21,22] Therefore, the effect of MCE on the activation of NF-κB was measured by Western blot analysis to determine the phosphorylation of IκBα, which inhibits NF-κB. The low phosphorylation levels of IκBα in unstimulated cells significantly

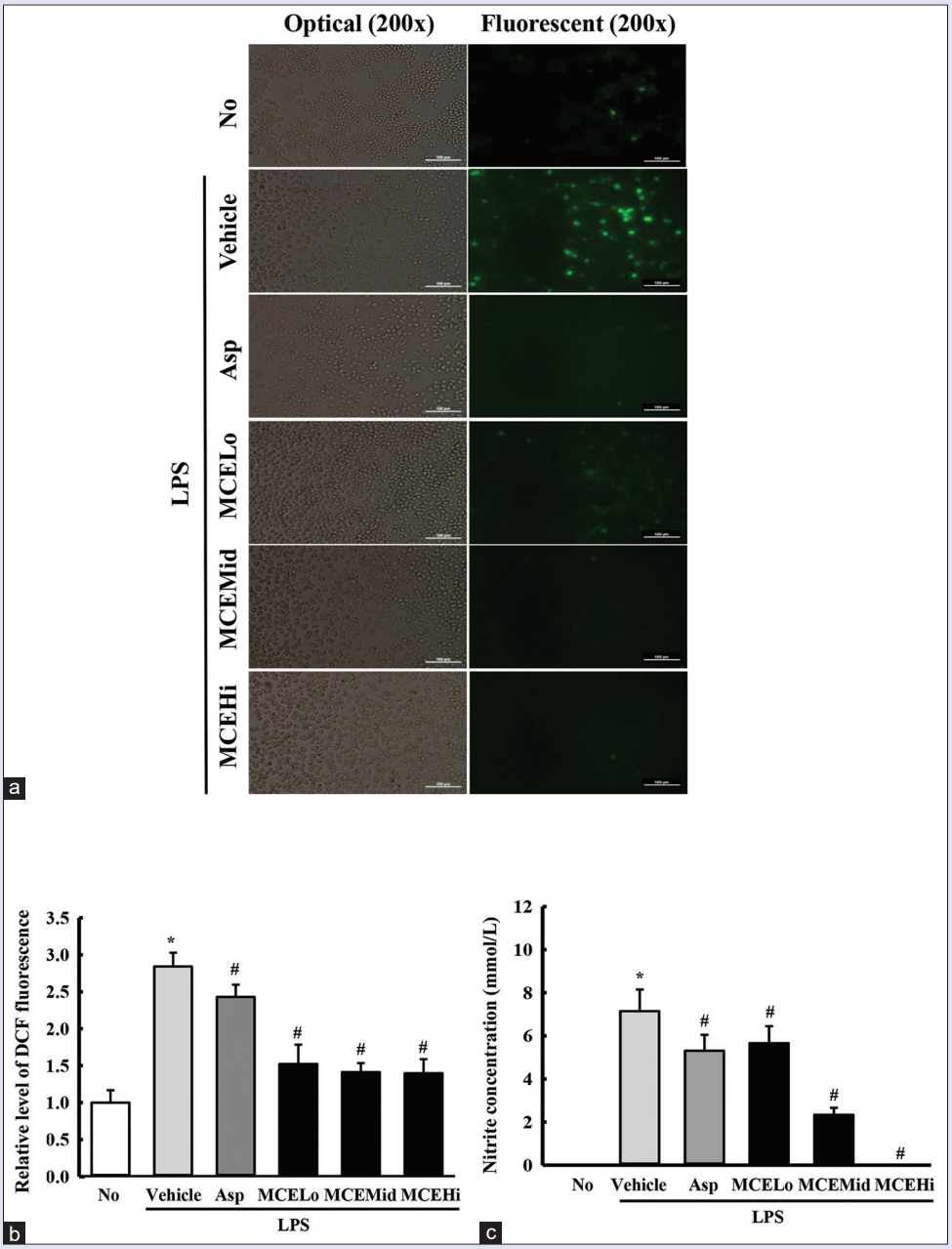

Figure 3: Effect of MCE on oxidative stress in LPS-induced inflammatory response. (a) After DCF-DA treatment, the intensity for green fluorescence in RAW264.7 cells of subset groups was detected at 200× using a fluorescence microscope (Eclipse TX100, Nikon, Tokyo, Japan). (b) After counting total number of DCF-DA stained cells per specific area, the relative level of stained cells in MCE-treated groups was represented based on the stained cell number in No treatment group. Data represent the mean ± SD of duplicates. **P* < 0.05 versus No treatment group, #*P* < 0.05 versus vehicle + LPS-treated group. (c) RAW 264.7 cells (5×10^5 cells/ml) were treated with the vehicle, Asp, or the indicated concentrations of MCE in the absence or presence of LPS (1 μg/ml) for 24 h. After collecting the culture supernatants, NO concentration was measured using Griess reagent. Data represent the mean ± SD of duplicates. **P* < 0.05 versus No treatment group, #*P* < 0.05 versus vehicle + LPS-treated group. MCE: Methanolic extract of *Capparis ecuadorica* leaves; LPS: Lipopolysaccharide; DCF-DA: 2′,7′-Dichlorofluorescein diacetate; NO: Nitric oxide; SD: Standard deviation; Asp: Aspirin

increased after LPS-stimulation. More than 80% of the phosphorylation of IκBα, by LPS was inhibited after exposure of the cells to MCE. This indicates that MCE inhibits the NF-κB-mediated inflammation by groups was detected at 200× using a fluorescence microscope activator of NF-κB [Figure 4b]. These results indicate that the primary mechanism of suppression of inflammatory cytokines, NO levels, COX-2, and iNOS mRNA expression in the LPS-activated RAW 264.7 cells by MCE pretreatment is by modulating the activity of NF-κB.

Inhibition of MAPK phosphorylation by methanolic extract of *C. ecuadorica* leaves in lipopolysaccharide-stimulated RAW 264.7 cells

MAPK pathway is essentially involved in the growth and differentiation of cells. In particular, MAPK signal transduction has a critical role in the regulation of cellular responses to several cytokines, inflammatory responses, and stresses.[23-27] To investigate whether MCE pretreatment affects the activation of MAPK pathway, the

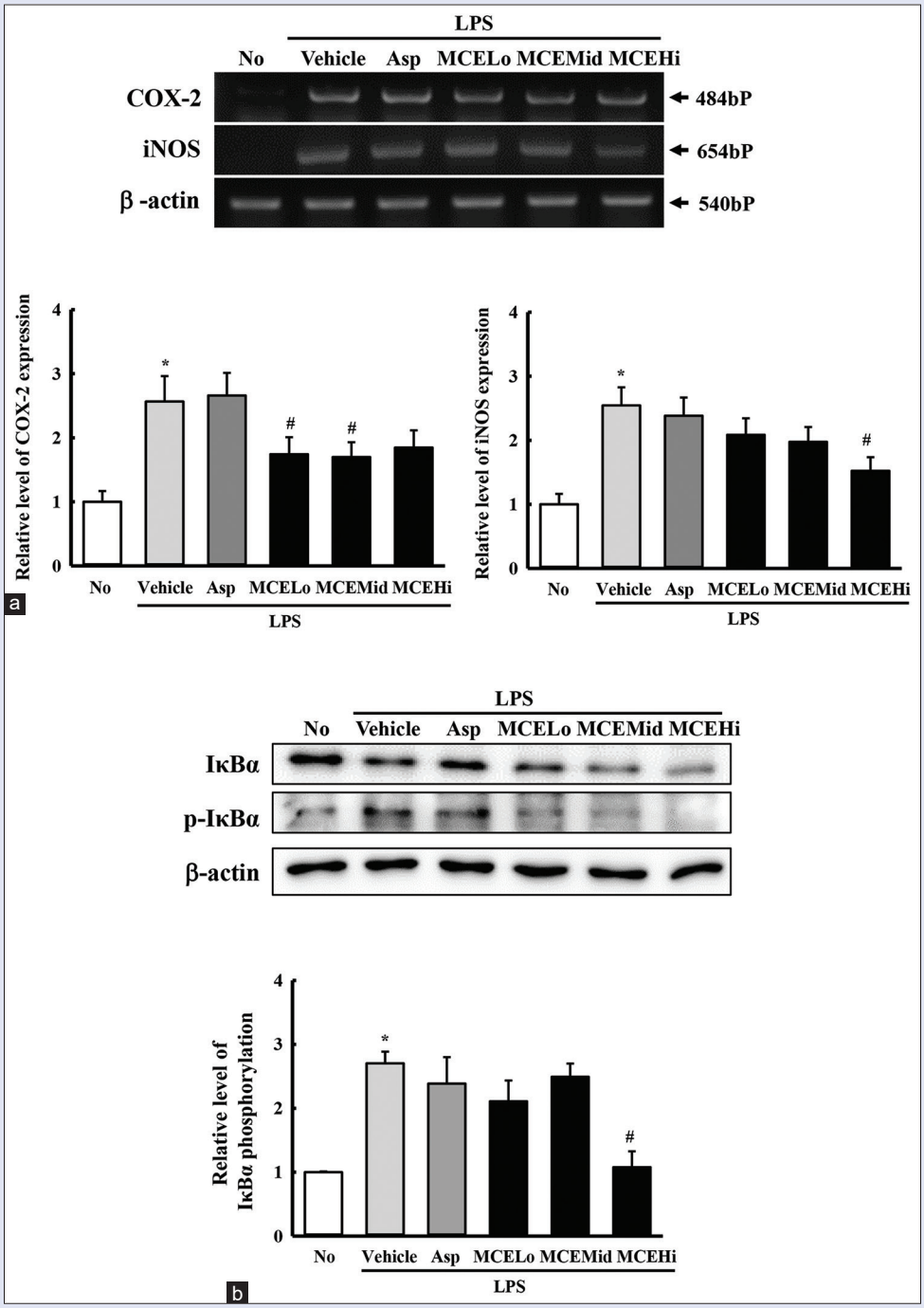

Figure 4: Effect of MCE on inflammatory mediators in LPS-induced inflammatory response. (a) The expression levels of COX-2 and iNOS mRNA were determined by RT-PCR analyses. After determining the intensity of each band using an imaging densitometer, the relative levels of COX-2 and iNOS mRNA were calculated based on the band intensity of β-actin mRNA as endogenous control. Data are presented as the mean ± SD of duplicates. *$P < 0.05$ versus control group, #$P < 0.05$ versus vehicle group. (b) Western blot was used to detect IκBα phosphorylation in the cell lysates. After determining the intensity of each band using an imaging densitometer, the relative levels of IκBα and p-IκBα protein were calculated based on the band intensity of β-actin protein as endogenous control. The relative phosphorylation levels of IκBα in MCE-treated cells were calculated based on the ratio of phosphorylated and nonphosphorylated IκBα protein. Data represent the mean ± SD of duplicates. *$P < 0.05$ versus No treatment group, #$P < 0.05$ versus vehicle + LPS-treated group. MCE: Methanolic extract of *Capparis ecuadorica* leaves; LPS: Lipopolysaccharide; RT-PCR: Real-time-polymerase chain reaction; COX-2: Cyclooxygenase-2; iNOS: Inducible nitric oxide synthase; SD: Standard deviation

phosphorylation levels of ERK, JNK, and p38 were assessed by Western blot analysis in LPS-stimulated RAW 264.7 cells [Figure 5]. The levels of phosphorylation of ERK, JNK, and p38 significantly increased compared to the No treatment group after LPS stimulation (vehicle + LPS-treated group). Exposure of LPS-stimulated RAW 264.7 cells to MCE significantly inhibited the ERK and JNK phosphorylation in a dose-dependent manner as compared to vehicle + LPS-treated group. Although p38 phosphorylation was inhibited by MCE treatment as compared to vehicle + LPS-treated group, it was not dose dependent [Figure 5].

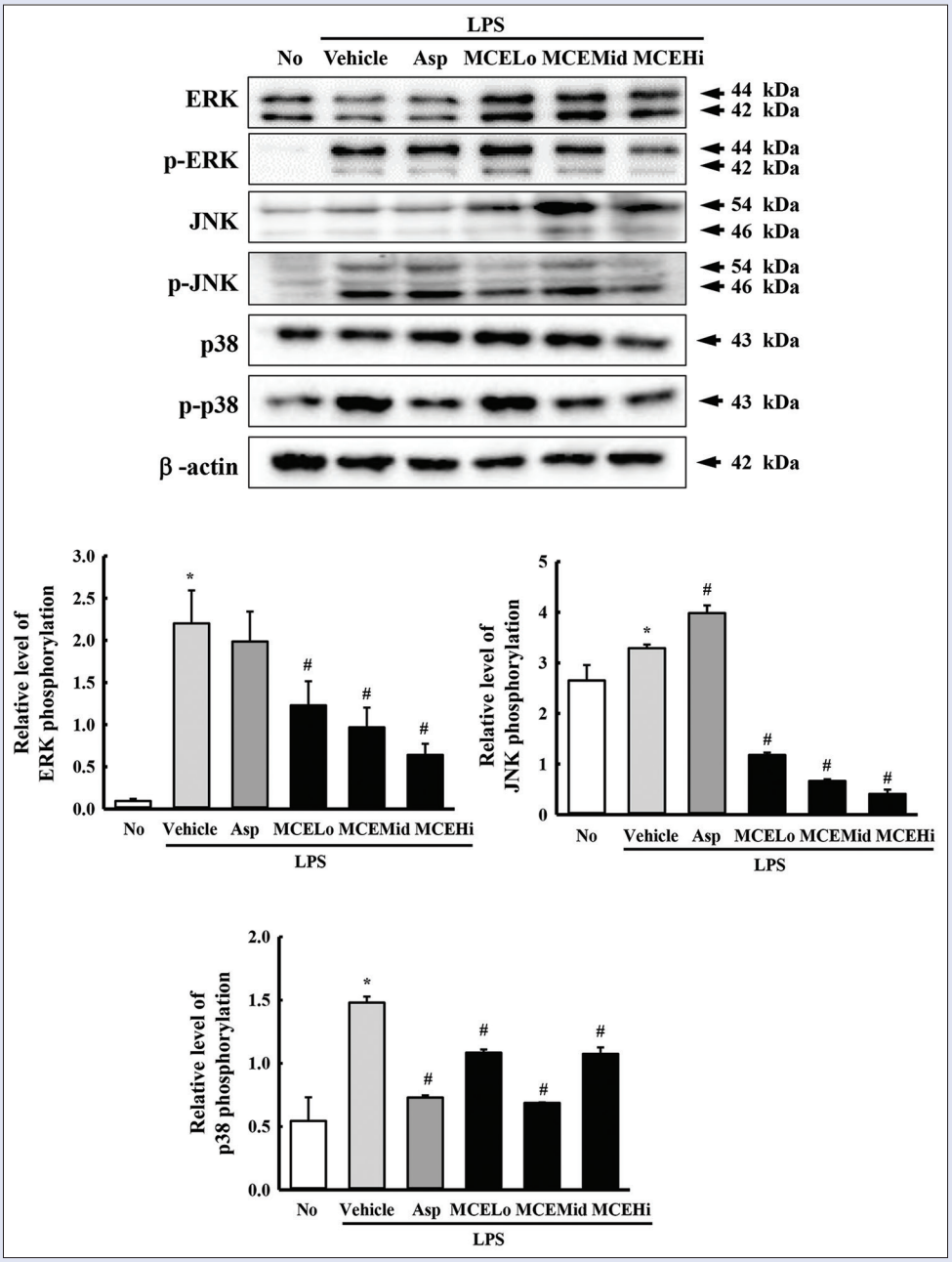

Figure 5: Effect of MCE on LPS-induced MAP kinases. Western blot was used to detect ERK, JNK, and p38 phosphorylation in the cell lysates. After determining the intensity of each band using an imaging densitometer, the relative levels of ERK, p-ERK, JNK, p-JNK, p38, and p-p38 protein were calculated based on the band intensity of β-actin protein as endogenous control. The relative phosphorylation levels of ERK, JNK, and p38 in MCE-treated cells were calculated based on the ratio of phosphorylated and nonphosphorylated protein. Data represent the mean ± SD of duplicates. $^{*}P < 0.05$ versus No treatment group, $^{\#}P < 0.05$ versus vehicle + LPS-treated group. MCE: Methanolic extract of *Capparis ecuadorica* leaves; LPS: Lipopolysaccharide; SD: Standard deviation

Suppression effects of methanolic extract of *C. ecuadorica* leaves on lipopolysaccharide-induced endoplasmic reticulum stress and autophagy

Endoplasmic reticulum (ER) stress is well known as an important mechanism for maintaining cell homeostasis. Several researchers, including Kitamura, have demonstrated its involvement in the inflammatory response such as inflammatory cytokine expression and NF-κB activation.[28-32] To investigate whether MCE has inhibitory effects on LPS-induced ER stress, the phosphorylation level of IRE1α in LPS-stimulated RAW 264.7 cells after MCE pretreatment was evaluated. IRE1α phosphorylation significantly increased in the vehicle + LPS-treated group compared to the No treatment group. However, IRE1α phosphorylation in the vehicle + LPS-treated group was strongly inhibited by the pretreatment of MCE [Figure 6a].

Because several studies suggest the involvement of autophagy in LPS-induced inflammation, we aimed to check the modulation of this process by MCE.[33,34] The inhibitory effects of MCE on LPS-induced autophagy in RAW 264.7 macrophages were measured using an anti-LC3 antibody that detects LC3, a marker that measures autophagy.

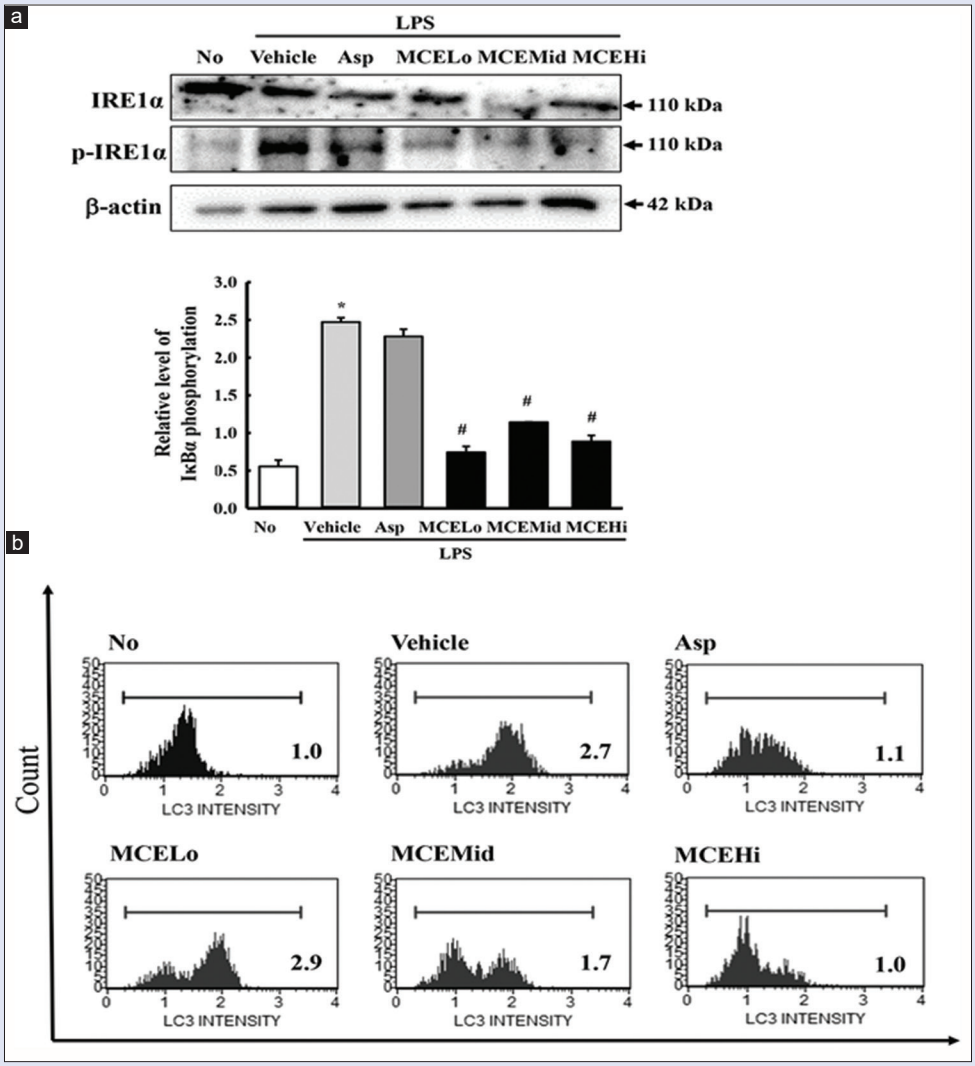

Figure 6: Effects of MCE on LPS-induced ER stress and autophagy. (a) Western blot was used to detect IRE1α phosphorylation in the cell lysates. After determining the intensity of each band using an imaging densitometer, the relative levels of IRE1α and p-IRE1α protein were calculated based on the band intensity of β-actin protein as endogenous control. The relative phosphorylation levels of IRE1α and p-IRE1α in MCE-treated cells were calculated based on the ratio of phosphorylated and nonphosphorylated protein. Data represent the mean ± SD of duplicates.*$P < 0.05$ versus No treatment group, #$P < 0.05$ versus vehicle + LPS-treated group. (b) LC3 fluorescence was detected in MCE + LPS-treated RAW264.7 cells using the Autophagy LC3-antibody-based kit for FACS analysis. The value of fluorescence intensity is taken from the representative histograms of FACS and indicated in the right corner of each panel. *$P < 0.05$ versus No treatment group, #$P < 0.05$ versus vehicle + LPS-treated group. MCE: Methanolic extract of *Capparis ecuadorica* leaves, LPS: Lipopolysaccharide, ER: Endoplasmic reticulum, SD: Standard deviation

We observed a sharp increase in autophagy in the vehicle + LPS-treated group as compared to the No treatment group. However, to the vehicle + LPS-treated group, treatment of MCE inhibited autophagy in a dose-dependent manner [Figure 6b]. These results indicate that MCE treatment inhibits not only normal inflammatory responses such as NF-κB and the MAPK pathway, but also ER stress and autophagy triggered by LPS stimulation in RAW 264.7 macrophages.

DISCUSSION

The pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, play an important role in promoting the inflammatory response; the secretion of these cytokines is measured as a marker of the inflammatory response.[3,35] Excessive production of pro-inflammatory cytokines results in a SIRS, such as septic shock.[6] Hence, it is important to reduce the secretion of pro-inflammatory cytokines during anti-inflammatory therapy. In addition, it is well known that ROS, especially NO, is produced in inflammatory reactions. NO plays an important role in signal transduction and killing bacteria in the normal state, but excessive production of NO causes adverse reactions including tissue damage and gene mutation. Hence, inhibiting the production of NO is important in controlling inflammatory responses, thereby making the production of anti-inflammatory drugs a major target activity for the pharmaceutical industry.[36] In this study, we found that MCE dose dependently inhibited TNF-α, IL-6, and IL-1β expression, as well as NO production, in LPS-stimulated RAW 264.7 cells [Figures 2 and 3]. At these concentrations, MCE suppressed the decrease in viability of LPS-stimulated RAW264.7 cells [Figure 1].

The signaling pathway of NF-κB and MAPKs is involved in the expression of LPS-stimulated pro-inflammatory mediators and cytokines and

plays a crucial role in regulating cell growth and differentiation and in modulating cellular responses to cytokines and stress.[23,24] MAPK is a serine/threonine kinase located in the cytoplasm of macrophages. This pathway consists of three major proteins: ERK1/2, p38, and JNK.[25,26] MAPK is activated by phosphorylation, which ultimately stimulates transcription factors such as NF-κB and AP-1.[27,37] Inhibition of either of the three members of MAPK pathway blocks the LPS-stimulated release of pro-inflammatory mediators and cytokines such as TNF-α, IL-6, and IL-1β.[38,39] It is well known that NF-κB is involved in regulating the expression of cytokines and inflammatory mediators involved in inflammatory responses.[40] In an unstimulated condition, NF-κB is located in the cytoplasm as inactive NF-κB/IκBα complex, and its activity is tightly regulated by the bound inhibitory protein IκBα. Stimulation of the inflammatory response causes phosphorylation of IκBα and cleavage of NF-κB binding, resulting in the migration of the cleaved NF-κB to the nucleus and activation of inflammatory gene expression.[41] Thus, measuring the phosphorylation of IκBα protein is a widely used method in the evaluation of the activation of NF-κB in RAW 264.7 cells stimulated by LPS. LPS induces the inflammation of macrophage, whereas MCE exposure significantly inhibits the phosphorylation of IκBα [Figure 4b].

Several studies have reported that ER stress is involved in diverse inflammatory diseases including cardiovascular disease, Type 2 diabetes, and cancer.[42-44] Although ER stress response is important for normal cell homeostasis, it reportedly plays a key role in the pathogenesis of various diseases.[45] During ER stress, an unfolded protein response (UPR) is initiated as a protective mechanism. Three transmembrane signaling proteins are involved, namely, IRE1, pancreatic ER kinase (PERK), and activating transcription factor (ATF) 6.[46,47] Three pathways of these UPRs have been known to be involved in the activation of NF-κB, which regulates the expression of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β.[32,48] IRE1α binds to IκB kinase and activates it to induce IκB degradation, resulting in the activation of NF-κB.[29] ATF6 induces NF-κB activation through Akt phosphorylation.[30] Endo *et al.* reported that LPS induces ER stress associated with transcription factor CHOP in macrophages.[28] During ER stress, the PERK-eIF2α pathway promotes NF-κB activation by suppressing the IκB translation.[30] However, it has been investigated that LPS-induced ER stress-CHOP pathway is not related to PERK-eIF2α.[31] In this study, we observed that LPS-stimulated ER stress mediated the IRE1α-related pathways [Figure 6].

Autophagy is a process that maintains cell homeostasis. It is thought to be a process of programmed cell death that sacrifices and reuses a portion of the cytoplasm to maintain critical functions during periods of stress such as malnutrition.[49,50] Several studies have reported LPS-induced autophagy through the regulation of TRIF, TLR4, RIP1, and p38 MAPK-related mechanism in macrophages.[33,34] In addition, autophagy helps in eliminating intracellular micro-organisms and is predominantly involved in the presentation of the major histocompatibility complex class II. Furthermore, it assists pattern recognition receptors by transferring the cytosolic pathogen-associated molecular pattern to endosomal toll-like receptors.[51] In this study, we detected that MCE exposure inhibits LPS-induced autophagy [Figure 6c]. However, the signaling mechanism by which MCE regulates autophagy is a subject for further study.

CONCLUSION

The results of this study show that MCE exerts anti-inflammatory activity by inhibiting the inflammatory response to LPS-induced macrophages and the production of inflammatory mediators, such as pro-inflammatory cytokines and NO. In addition, these anti-inflammatory mechanisms regulate the expressions of iNOS and COX-2 mRNA by inhibiting the activation of MAPK and NF-κB. Furthermore, MCE exposure inhibited the ER stress and LPS-induced autophagy. Therefore, it can be said that MCE has excellent anti-inflammatory properties and is a competitive and potential candidate for the development of anti-inflammatory drugs.

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Conflicts of interest

There are no conflicts of interest.

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