

# ***Diospyros lotus* leaf extract and its main component myricitrin inhibit itch-related IL-6 and IL-31 by suppressing microglial inflammation and microglial-mediated astrocyte activation**

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**Abstract.** *Diospyros lotus* has been traditionally used in Asia for medicinal purposes, exhibiting a broad spectrum of pharmacological effects including antioxidant, neuroprotective and anti-inflammatory properties. While the anti-itch effect of *D. lotus* leaves has been reported, studies on the detailed mechanism of action in microglia and astrocytes, which are members of the central nervous system, have yet to be revealed. The present study aimed to investigate effects of *D. lotus* leaf extract (DLE) and its main component myricitrin (MC) on itch-related cytokines and signaling pathways in lipopolysaccharide (LPS)-stimulated microglia. The effect of DLE and MC on activation of astrocyte stimulated by microglia was also examined. Cytokine production was evaluated through reverse transcription PCR and western blot analysis. Signaling pathway was analyzed by performing western blotting and immunofluorescence staining. The effect of microglia on astrocytes activation was evaluated via western blotting for receptors, signaling molecules and itch mediators and confirmed through gene silencing using short interfering RNA. DLE and MC suppressed the production of itch-related cytokine IL-6 and IL-31 in LPS-stimulated microglia. These inhibitory effects were mediated through the blockade of NF- $\kappa$ B, MAPK and JAK/STAT pathways. In astrocytes, stimulation by microglia promoted the expression

of itch-related molecules such as oncostatin M receptor, interleukin 31 receptor  $\alpha$ , inositol 1,4,5-trisphosphate receptor 1, lipocalin-2 (LCN2), STAT3 and glial fibrillary acidic protein. However, DLE and MC significantly inhibited these receptors. Additionally, astrocytes stimulated by microglia with IL-6, IL-31, or both genes silenced did not show activation of LCN2 or STAT3. The findings of the present study demonstrated that DLE and MC could suppress pruritic activity in astrocytes induced by microglia-derived IL-6 and IL-31. This suggested the potential of DLE and MC as functional materials capable of alleviating pruritus.

## **Introduction**

Itching, commonly known as an itch sensation, is a sensory experience that is universally felt by most animals, including humans. It serves as a protective mechanism against irritants and serves as a defense mechanism against parasites and pests (1). However, when itching becomes uncontrollable and excessive, as often seen in conditions such as atopic dermatitis and allergies, it can lead to skin damage, sleep disturbances and a significant reduction in the quality of life (2). Historically, studies on itching have primarily focused on histamine-related mechanisms. However, in the search for solutions to chronic itching that is not alleviated by antihistamines, research has been conducted on non-histaminergic itching. The field of itch studies has expanded to the central nervous system (CNS) (3). Itch signals can be transmitted from the skin to astrocytes of the spinal dorsal horn (SDH) through primary afferent nerves. Histamine-independent itch can develop (4). Therefore, it is necessary to find substances that can regulate itching not only in the skin, but also in the CNS.

Microglia under normal physiological conditions serve a central role as intrinsic immune cells of the CNS (5), promoting the proliferation and survival of neuronal precursor cells and protecting neurons with limited regenerative abilities (6). However, upon exposure to pathological stimuli, activated microglia can release IL-6 and inflammatory mediators such as nitric oxide, prostaglandin  $E_2$ , IL-1 $\beta$  and TNF- $\alpha$  known

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to contribute to itching (7). Spinal microglia serve a crucial role in both histamine-dependent and histamine-independent itching (8), with a recent study emphasizing microglia-neuron interactions involving the gastrin-releasing peptide receptor (GRPR) in itch sensation (9).

Astrocytes comprising 20-40% of the glial cells in the CNS traditionally provide structural and nutritional support to neurons (10). Historically, astrocytes have been primarily associated with pain in neurodegenerative conditions and inflammation (11,12). However, their relationship with acute or chronic itching remains an uncharted territory. Emerging findings from rodent models of atopic dermatitis and contact dermatitis have now shed light on the activation of astrocytes within the SDH region, indicating their involvement in persistent itching by releasing gastrin-releasing peptide (GRP) (13).

*Diospyros lotus*, a member of the Ebenaceae family and a deciduous tree native to various parts of Asia including South Korea, has a rich history of traditional use in food, folk and traditional medicine (14). Different parts of *D. lotus*, including its fruit, seeds, leaves and bark, have been used for various therapeutic purposes due to their anti-inflammatory (14), sedative (14) and antimicrobial (15) effects. They can also relieve biliousness, diabetes, cancer and fevers (16). In addition, they can provide relief from back pain (17). We have previously identified several key compounds in *D. lotus* extract (DLE), including gallic acid, caffeic acid, chlorogenic acid, myricetin-3-O-galactoside, myricitrin, astragaloside, quercetin and myricetin, with myricitrin standing out as notably abundant among these compounds (18,19). Our previous studies have also demonstrated multifaceted effects of DLE, including anti-inflammatory (18), photoprotective (18), liver-protective (19), anti-obesity (20) and notably, anti-atopic effects (21,22). Particularly, our previous research has demonstrated DLE's efficacy in alleviating itching by inhibiting the activity of SDH astrocytes (23). Despite these promising findings, previous studies have not thoroughly investigated the influence of DLE on cytokine production related to itch in microglia, nor have they explored the complex interplay between microglia and astrocytes in relation to itch mechanisms. Therefore, the primary objective of the present study was to investigate effects of DLE and its major component myricitrin (MC) on microglia when stimulated by lipopolysaccharide (LPS) in relation to itchiness. Additionally, the present study aimed to examine the impact of conditioned media from LPS-treated microglia on astrocytes, particularly in relation to itch mechanisms.

## Materials and methods

**Plant materials.** On June 13, 2022, *D. lotus* leaves were collected from Cheonjam mountain, Jeonju-si, Jeollabuk-do, Republic of Korea. The identification and authentication of the plant were conducted by Professor Hong-Jun Kim (College of Oriental Medicine, Woosuk University, Jeonbuk, Republic of Korea). A voucher specimen (no. 2022-06-04) was deposited in the Department of Health Management, College of Medical Science, Jeonju University (Jeollabuk-do, Republic of Korea). Leaves were washed five times with water and then dried in a well-ventilated shaded area. Dried leaves (100 g) were

extracted with 2 l of 70% (v/v) ethanol at room temperature for 48 h. The resulting extract was filtered using a 5  $\mu$ m filter paper, vacuum-concentrated and then freeze-dried to obtain a powdered form of the DLE.

**Reagents and materials.** The following reagents and materials were purchased from the specified suppliers: LPS, Griess reagent and protease inhibitors from MilliporeSigma; myricitrin from Tokyo Chemical Industry; Quanti-MAX WST-8 Cell Viability Assay Kit and WestGlow FEMTO Chemiluminescent substrate from Biomax Ltd.; radio-immunoprecipitation assay (RIPA) buffer, IL-33, inositol 1,4,5-trisphosphate receptor 1 (IP3R1), lipocalin-2 (LCN2), glial fibrillary acidic protein (GFAP), goat anti-rabbit IgG Alexa Fluor 488 antibodies and goat anti-mouse IgG Alexa Fluor 488 from Thermo Fisher Scientific, Inc.; IL-31 antibodies from Abcam;  $\beta$ -actin, phosphorylated (p-)I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , p-NF- $\kappa$ B, NF- $\kappa$ B, p-JNK, JNK, p-p38, p38, oncostatin M receptor (OSMR), Toll-like receptor 4 (TLR4), IL-6, interleukin 31 receptor a (IL31RA) antibodies and IL-31 short interfering (si)RNA from Santa Cruz Biotechnology, Inc.; and ProLong Gold Antifade Reagent with DAPI, p-I $\kappa$ B, I $\kappa$ B, p-JAK1, JAK1 p-STAT3, STAT3, p-ERK and ERK antibodies from Cell Signaling Technology, Inc.

**Cell culture.** Mouse-origin microglia (CRL-3265) and astrocytes (CRL-2541) were obtained from ATCC. Microglia were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% horse serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Thermo Fisher Scientific, Inc.) in a 5% CO<sub>2</sub> incubator at 37°C. Astrocytes were cultured and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin under the same incubation conditions.

**IL-6 and IL-31 gene silencing.** To silence IL-6 and IL-31 genes in microglia, IL-6 siRNA (cat no. sc-39628), IL-31 siRNA (cat no. sc-146219), control siRNA (cat no. sc-37007), siRNA transfection medium (cat no. sc-36868) and siRNA transfection reagent (cat no. sc-29528) from Santa Cruz Biotechnology, Inc. were used. Gene silencing was performed according to the manufacturer's protocol without modification. Microglia (2 $\times$ 10<sup>5</sup> cells/ml) were cultured in 6-well cell culture plate at 37°C until 60-80% confluence was reached. siRNA duplexes were diluted to a final concentration of 50 pmol in 100  $\mu$ l of siRNA transfection medium. Separately, 5  $\mu$ l of siRNA transfection reagent was diluted in 100  $\mu$ l of the same medium. The two solutions were combined and incubated at room temperature for 30 min to form transfection complexes. Cells were washed once with siRNA transfection medium and the transfection complexes were overlaid onto the cells. After a 6 h incubation period at 37°C, 1 ml of normal growth medium (DMEM) containing double the normal concentration of serum and antibiotics was added without removing the transfection mixture. Cells were further incubated for 24 h and used for experiments within 48 h after transfection. Control siRNAs with scrambled sequences were used to ensure specificity of the siRNA-mediated gene silencing. The manufacturer confirmed the following:

sc-39628: IL-6 siRNA (m) is a pool of 3 different siRNA duplexes:

sc-39628A: Sense: GGCAAUUCUGAUUGUAUGAtt;  
antisense: UCAUACAAUCAGAAUUGCCtt  
sc-39628B: Sense: CCAAGACCAUCCAAUUCAUtt;  
antisense: AUGAAUUGGAUGGUCUUGGtt  
sc-39628C: Sense: CCAGAUGGUUUCUUGGAAUtt;  
antisense: AUUCCAAGAAACCAUCUGGtt

sc-146219: IL-31 siRNA (m) is a pool of 3 different siRNA duplexes:

sc-146219A: Sense: GAACUACAAUUGACCUCUtt;  
antisense: AAGAGGUCAAUUGUAGUUCtt  
sc-146219B: Sense: CAGGCUAAGGACAAUACUAtt;  
antisense: UAGUAUUGUCCUAGCCUGtt  
sc-146219C: Sense: GGUCAUUACUAGUCAUGUtt;  
antisense: AACAUAGACUAGUAAUGACtt.

**Preparation of LPS-stimulated microglia culture medium (LSMCM).** Microglia ( $2 \times 10^5$  cells/ml) were cultured in 60-mm cell culture dishes at 37°C for 24 h. Cells were then stimulated with LPS (1 µg/ml) at 37°C for 3 h. Afterward, the culture medium was replaced with fresh culture medium and cells were cultured for an additional 24 h. Subsequently, the culture medium was collected and centrifuged at 300 x g at 4°C for 2 min, followed by storage at 4°C.

**Enzyme-linked immunosorbent assay (ELISA).** The concentrations of IL-6 and IL-31 contained in LSMCM were analyzed using an ELISA kit. ELISA analysis was performed according to the manufacturer's instructions (IL-6; cat. no. M6000B; R&D Systems, Inc. and IL-31; cat. no. ab243681; Abcam). The optical density was measured at 450 nm using a microplate reader (Tecan Group Ltd.).

**Protein extraction and western blotting.** Microglia ( $2 \times 10^5$  cells/ml) were cultured in 60-mm cell culture dishes at 37°C for 24 h. Cells were then treated with either DLE (50 and 100 µg/ml) or MC (10 and 20 µM) and further incubated at 37°C for 1 h. Subsequently, cells were stimulated with LPS (1 µg/ml) at 37°C for 30 min or 24 h. Astrocytes ( $1 \times 10^5$  cells/ml) were cultured in 60-mm cell culture dishes at 37°C for 24 h. Cells were then treated with either DLE (50 and 100 µg/ml) or MC (10 and 20 µM) and further incubated at 37°C for 1 h. Subsequently, cells were stimulated by LSMCM (mixed with the existing culture medium at a 1:1 ratio) at 37°C for 30 min or 24 h. Total protein was extracted from each sample using RIPA buffer treated with protease/phosphatase inhibitors. The protein concentration was determined using the Bradford assay. SDS-PAGE was performed using 7.5, 10, or 12% gels to separate proteins (50 µg of proteins per lane). Separated proteins were transferred to a polyvinylidene fluoride membrane. Subsequently, membranes were blocked with 5% skimmed milk dissolved in TBST buffer (Tris-buffered saline with 1% Tween-20) for 1 h at room temperature to prevent non-specific binding during subsequent immunoblotting steps. After three washes with TBST for 10 min each, membranes were incubated overnight with primary antibodies against IL-6 (1:500; cat. no. sc-57315), IL-31 (1:2,000; cat. no. ab102750), IL-33 (1:1,000; cat. no. MA5-15773), p-ERK (1:500;

cat. no. sc-81492), ERK (1:500; cat. no. sc-514302), p-p38 (1:500; cat. no. sc-166182), p38 (1:500; cat. no. sc-1972), p-JNK (1:500; cat. no. sc-293136), JNK (1:500; cat. no. sc-7345), p-STAT3 (1:1,000; cat. no. 9145S), STAT3 (1:1,000; cat. no. 9139S), p-JAK1 (1:1,000; cat. no. 44-422G), JAK1 (1:1,000; cat. no. 3344S), p-IKK (1:1,000; cat. no. 2697S), IKK (1:1,000; cat. no. 2682S), p-IκB (1:500; cat. no. sc-52943), IκB (1:500; cat. no. sc-1643), p-NF-κB (1:500; cat. no. sc-271908), NF-κB (1:500; cat. no. sc-8414), OSMR (1:500; cat. no. sc-271695), IL-31RA (1:500; cat. no. sc-515465), IP3R1 (1:1,000; cat. no. PA1-901), LCN2 (1:1,000; cat. no. PA5-79590), GFAP (1:1,000; cat. no. 13-0300) and β-actin (1:1,000; cat. no. sc-8432) at 4°C. Following five washes with TBST, membranes were incubated with HRP-conjugated secondary antibodies anti-mouse (1:5,000; cat. no. sc-525409) and anti-rabbit (1:5,000; cat. no. sc-8432) in 5% skimmed milk for 2 h at room temperature. Subsequently, membranes were washed three times with TBST solution for 10 min each and visualized using an imaging system (ALLIANCE LD4; UVITEC) and WestGlow FEMTO (Biomax). Band densities were analyzed using ImageJ 1.53e (National Institutes of Health) with β-actin serving as the loading control.

**RNA extraction and reverse transcription-quantitative (RT-q) PCR.** Microglia ( $2 \times 10^5$  cells/ml) were cultured in 60 mm cell culture dishes for 24 h. Cells were then treated with either DLE (50 and 100 µg/ml) or MC (10 and 20 µM) and further incubated at 37°C for 1 h. Subsequently, cells were stimulated with LPS (1 µg/ml) at 37°C for 3 h. Cells were used to extract total RNAs using an RNA-spin Total RNA Extraction Kit obtained from iNtRON Biotechnology. Subsequently, cDNAs were synthesized from extracted RNAs using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.). and a T100TM Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc.). Resulting cDNAs were then amplified using a SYBR kit from TOYOBO. To quantify gene expression levels, reverse transcription PCR was conducted using a StepOne Real-Time PCR system from Thermo Fisher Scientific, Inc. Sequences of primers used for reverse transcription PCR in the present study are listed in Table I. The thermal profile consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of amplification at 95°C for 30 sec and 60°C for 30 sec. Expression levels were normalized to GAPDH using the  $2^{-\Delta\Delta C_q}$  method (24). All protocols were conducted in accordance with the manufacturer's instructions without any modifications.

**Immunofluorescence staining.** Microglia ( $2 \times 10^5$  cells/ml) were cultured on cell culture slides at 37°C for 24 h. These cells were then treated with either DLE (50 and 100 µg/ml) or MC (10 and 20 µM) and further incubated at 37°C for 1 h. Subsequently, cells were stimulated with LPS (1 µg/ml) at 37°C for 30 min. Slides were fixed with 4% formaldehyde at room temperature for 15 min. Slides were then rinsed three times with PBS for 5 min each. Subsequently, slides were blocked with a solution containing 5% FBS and 0.3% Triton X-100 in PBS for 1 h. They were then incubated with a primary antibody against p-NF-κB (1:500; cat. no. sc-271908) or p-STAT3 (1:1,000; cat. no. 9145S), which were diluted in PBS containing 1% bovine serum albumin (BSA) and 0.3%

Table I. List of primer sequences used for reverse transcription PCR in the present study.

Primer	Forward	Reverse
Mouse IL-6	5'-TCCATCCAGTTGCCTTCTTG-3'	5'-AAGCCTCCGACTTGTGAAGTG-3'
Mouse IL-31	5'-CCTACCCTGGTGTCTGCTTTG-3'	5'-CTGACATCCCAGATGCCTGC-3'
Mouse IL-33	5'-ACTGTGGTGCCTGCTCTTCT-3'	5'-TTGGCTTACGATGTTGTGGA-3'
Mouse GAPDH	5'-GGCTACACTGAGGACCAGGT-3'	5'-TCCACCACCCTGTTGCTGTA-3'

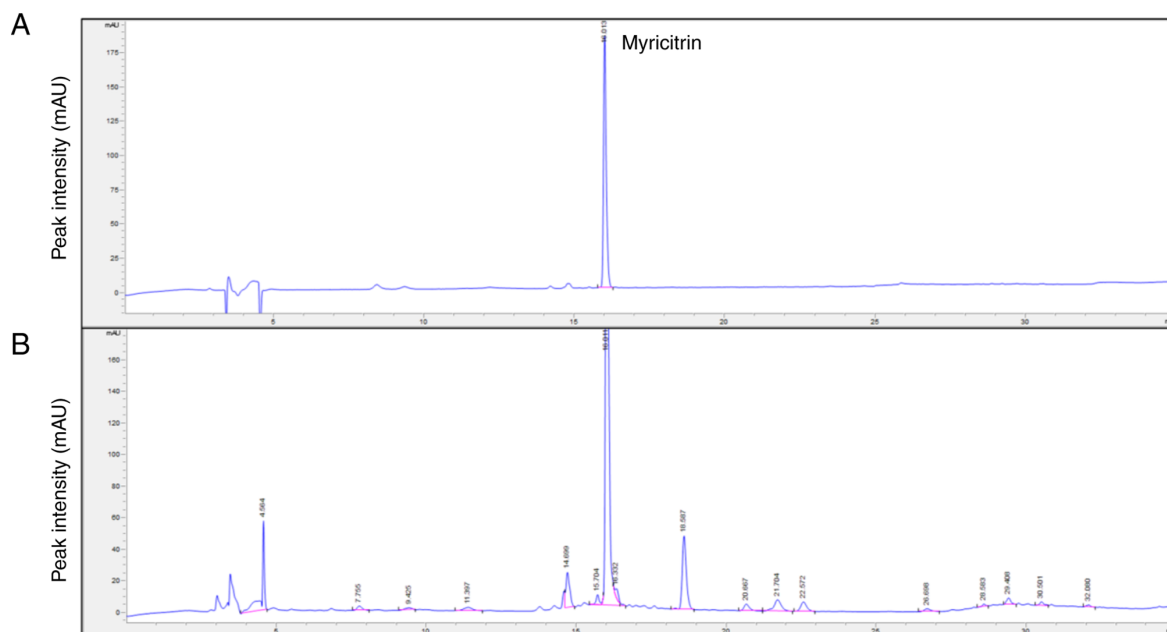


Figure 1. Myricitrin identified in DLE. Comparative chromatograms of DLE by HPLC. (A) Myricitrin in the standard solution, (B) DLE was profiled with an HPLC at 280 nm. DLE was compared with conventional compounds by HPLC-DAD. Peak represents the standard compound myricitrin. HPLC analysis was performed as described in the material and method section. DLE, *Diospyros lotus* leaf extract; HPLC, high-performance liquid chromatography; DAD, diode-array detection.

Triton X-100, at 4°C overnight. Afterward, slides were rinsed three times with PBS for 5 min each and incubated with secondary antibodies anti-mouse (1:2,000; cat no. A-11001) or anti-rabbit (1:1,000; cat no. A-11008) purchased from Thermo Fisher Scientific, Inc., which were diluted in antibody buffer at room temperature for 2 h. Finally, slides were covered with a coverslip using a mounting solution containing DAPI. Afterwards, the slide was incubated for 24 h at room temperature in the dark. Immunofluorescence images were observed at x400 magnification using a Zeiss Axioskop 50 microscope and images were captured with an AxioCam ICm1 camera (Carl Zeiss AG).

**High-performance liquid chromatography (HPLC) analysis.** An Agilent 1100 series (Agilent Technologies, Inc.) was employed for the identification of primary active compounds in DLE using an HPLC method. The setup included a binary pump delivery system, a degasser (cat. no. G1379A), an autosampler (cat. no. G1313A) and a diode array detector (cat. no. G1315B; all from Agilent Technologies, Inc.). The separation was carried out using an Agilent Eclipse XDB-C18 column (Agilent Technologies, Inc.; 4.6x250 mm; 5 mm particles) through gradient elution with 0.5% aqueous formic acid (A) and acetonitrile (B).

The gradient elution profile was: 0 min, 5% B; 10 min, 10% B; 50 min, 40% B; 54 min, 100% B and then hold for 10 min before returning to initial conditions. The gradient elution involved varying the proportion of solvent A (water, containing 0.2% acetic acid) to solvent B (acetonitrile). The mobile phase flow rate was set at 1 ml/min. UV detection was performed at a wavelength of 280 nm. The column temperature was maintained at 30°C and the sample injection volume was 20 µl. Identification of the standard was based on retention time. The concentration of the main isolated compound was determined by comparing its peak area with that of a standard. Standard stock solutions (1,000 ppm) were prepared using methanol and a calibration curve was constructed using six different concentrations (0, 20, 40, 60, 80, 100 ppm). The integration of each component on the chromatogram was processed using Agilent Chemstation software (Agilent Technologies, Inc.).

**Statistical analysis.** All statistical analyses were performed using SPSS version 26.0 (IBM Corp.). Data are presented as mean ± standard deviation (n=3). For statistical analysis, one-way ANOVA followed by Tukey's post hoc test was performed for comparisons involving three or more groups, while independent samples t-tests were used for comparisons

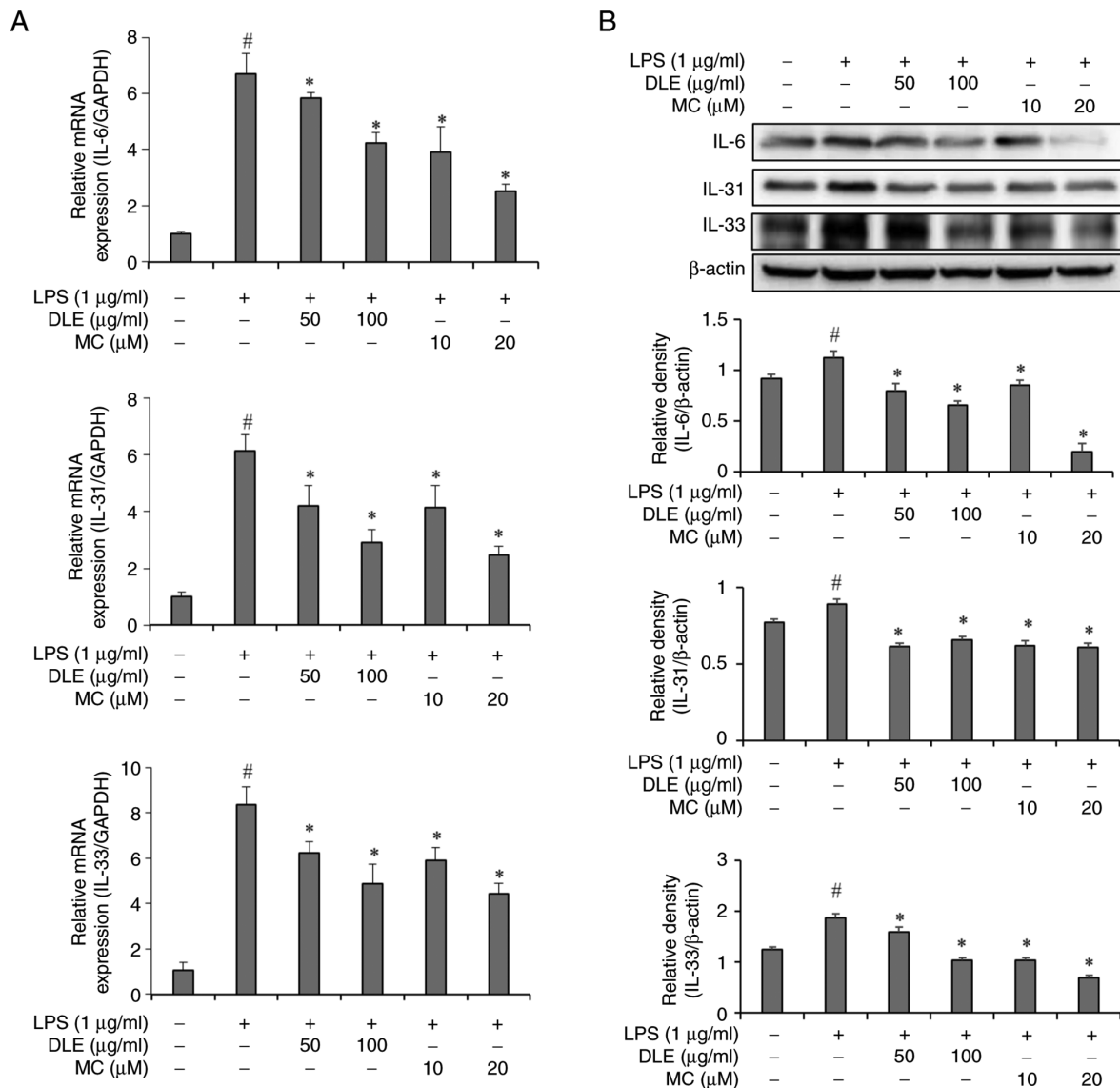


Figure 2. Effects of DLE and MC on IL-6, IL-31 and IL-33 expression in LPS-stimulated microglia. Microglia were pretreated with DLE or MC at the indicated concentrations and stimulated with LPS for 24 or 3 h. (A) IL-6, IL-31 and IL-33 mRNA expression levels were determined by reverse transcription PCR. (B) IL-6, IL-31 and IL-33 protein expression levels were determined by western blot analysis. Each bar represents the mean  $\pm$  SD (n=3). <sup>#</sup>P<0.05 vs. untreated cells; <sup>\*</sup>P<0.05 vs. LPS alone treated cells. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LPS, lipopolysaccharide.

between two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Quantitative analysis of MC content in DLE using HPLC.** HPLC analysis was conducted to measure the content of MC, known to be the main active compound of DLE. As a result, MC (retention time: 16.013 min) was detected. The concentration of MC, the main peak, was determined by establishing a strong linear regression between the peak area and the concentration of myricitrin standard. Based on this analysis, the concentration of myricitrin was found to be  $86.68 \pm 3.36$  µg/mg (Fig. 1).

**Effects of DLE and MC on cytokine expression in LPS-stimulated microglia.** To investigate effects of DLE on cytokine production related to itch in activated microglia,

PCR and western blot analysis was conducted. Results showed that mRNA levels of IL-6, IL-31 and IL-33 were significantly increased in the microglia after 3 h of LPS treatment. However, pre-treatment with DLE or MC before LPS stimulation led to significant reductions of IL-6, IL-31 and IL-33 mRNA expression levels (Fig. 2A). Following 24 h of LPS stimulation, intracellular protein expression levels of IL-6, IL-31 and IL-33 were significantly increased. Pre-treatment with DLE or MC before LPS exposure resulted in significant decreases in the expression of these proteins across all treatment concentrations (Fig. 2B).

**Effects of DLE and MC on MAPKs activation in LPS-stimulated microglia.** DLE and MC effectively suppressed cytokine production in LPS-stimulated microglia. To delve into its mechanism of action, an experiment was conducted into the influence of DLE and MC on phosphorylation of MAPKs using western blot analysis. LPS treatment



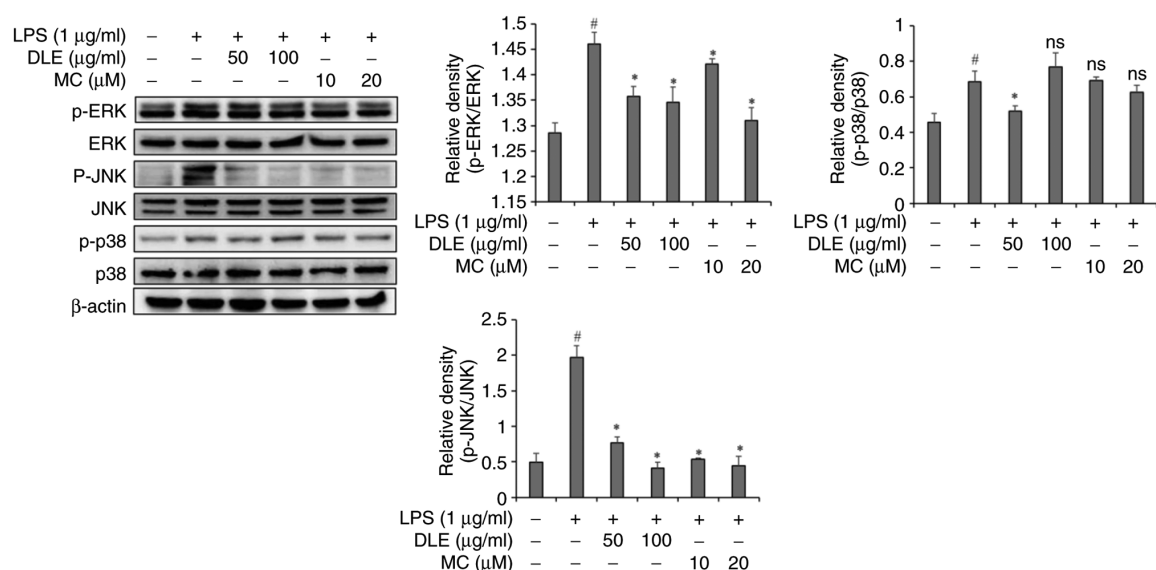


Figure 3. Effects of DLE and MC on MAPKs signaling pathway in LPS-stimulated microglia. Microglia were pretreated with DLE or MC at indicated concentrations and stimulated with LPS for 30 min. Relative expression levels of phosphorylated ERK, JNK and p38 with their non-phosphorylated counterparts were evaluated by western blot analysis. Each bar represents the mean  $\pm$  SD (n=3). <sup>#</sup>P<0.05 vs. untreated cells; <sup>\*</sup>P<0.05 vs. LPS alone treated cells; ns, no significant difference compared to LPS alone treated cells. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LPS, lipopolysaccharide; p-, phosphorylated.

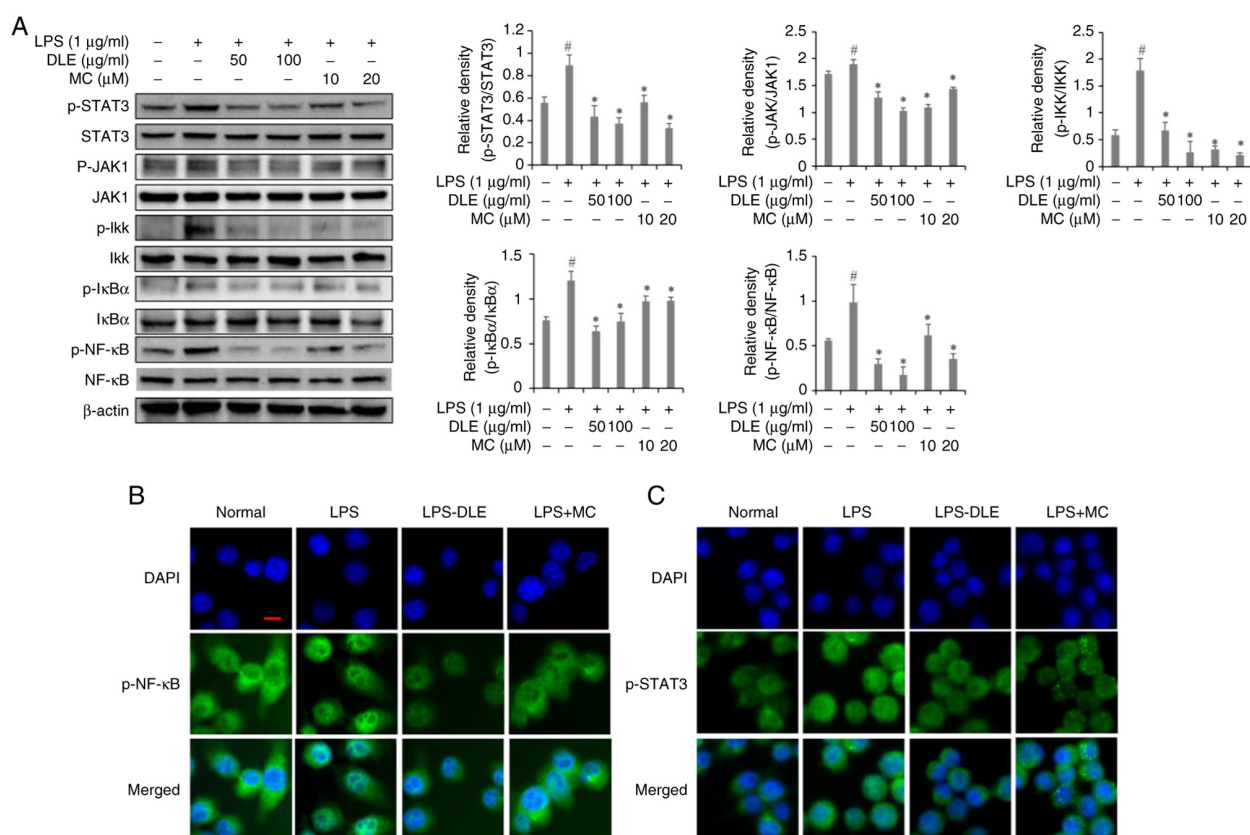


Figure 4. Effects of DLE and MC on NF- $\kappa$ B and STAT3 signaling pathway in LPS-stimulated microglia. Microglia were pretreated with DLE or MC at indicated concentrations and stimulated with LPS for 30 min. (A) Relative expression levels of phosphorylated STAT3, AKT, IKK, IkB and NF- $\kappa$ B with their non-phosphorylated counterparts were evaluated by western blot analysis. (B) Nuclear translocation of p-NF- $\kappa$ B and (C) p-STAT3 were evaluated using immunofluorescence staining (scale bar, 20  $\mu$ m). p-NF- $\kappa$ B and p-STAT3 were stained in green, while nuclei were stained in blue (DAPI). Each bar represents the mean  $\pm$  SD (n=3). <sup>#</sup>P<0.05 vs. untreated cells; <sup>\*</sup>P<0.05 vs. LPS alone treated cells; DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LPS, lipopolysaccharide; p-, phosphorylated.

significantly increased the phosphorylation of ERK, JNK and p38 in microglia (Fig. 3). However, pre-treatment with

DLE or MC before LPS exposure led to significant reductions of the phosphorylation of ERK and JNK. Notably,

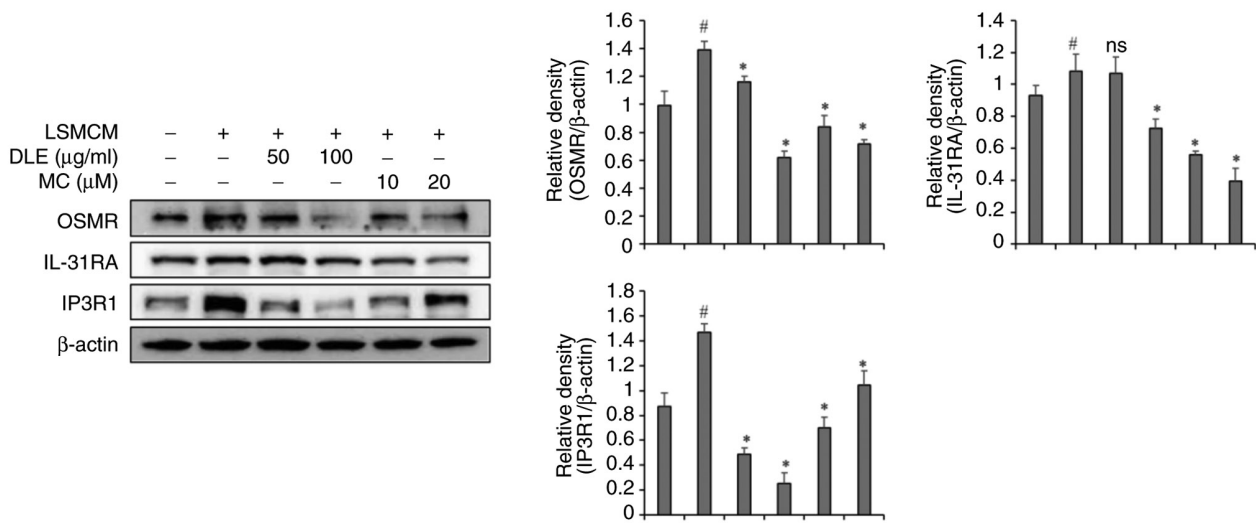


Figure 5. Effects of DLE and MC on expression of OSMR, IL-31RA and IP3R1 in LSMCM-stimulated astrocytes. Astrocytes were pretreated with DLE or MC at indicated concentrations and stimulated with LSMCM for 24 h. Relative expression levels of OSMR, IL-31RA and IP3R1 were evaluated by western blot analysis. Each bar represents the mean  $\pm$  SD (n=3). \*P<0.05 vs. untreated cells; #P<0.05 vs. LSMCM alone treated cells; ns, no significant difference compared to LSMCM alone treated cells. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LSMCM, LPS-stimulated microglia culture medium; LPS, lipopolysaccharide; OSMR, oncostatin M receptor; IL-31RA, interleukin 31 receptor a; IP3R1, inositol 1,4,5-trisphosphate receptor 1.

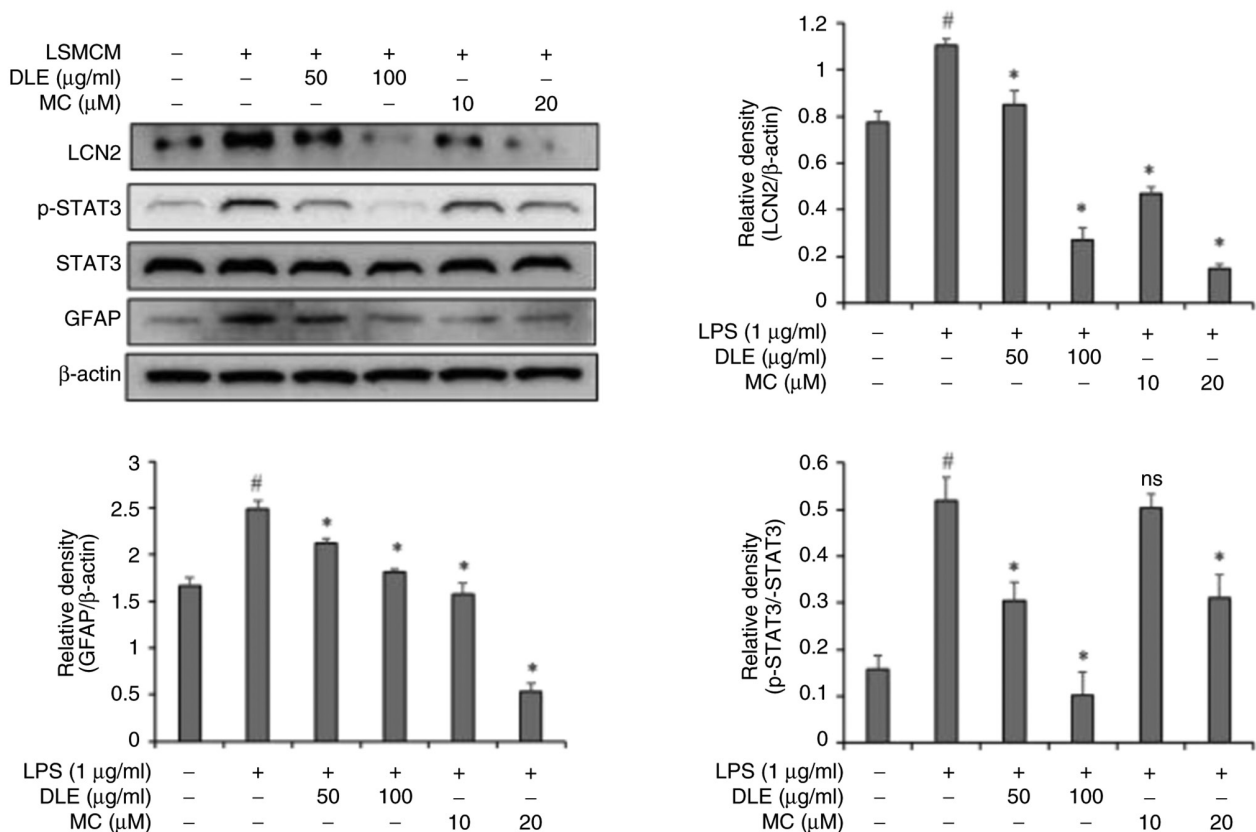


Figure 6. Effects of DLE and MC on expression of LCN2, STAT3 and GFAP in LSMCM-stimulated astrocytes. Astrocytes were cultured in stimulated microglia conditioned media for 24 h. Relative expression levels of LCN2, STAT3 and GFAP were evaluated by western blot analysis. Each bar represents the mean  $\pm$  SD (n=3). \*P<0.05 vs. untreated cells; #P<0.05 vs. LSMCM alone treated cells; ns, no significant difference compared to LSMCM alone treated cells; DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LCN2, lipocalin-2; GFAP, glial fibrillary acidic protein; LSMCM, LPS-stimulated microglia culture medium LPS, lipopolysaccharide.

both DLE and MC demonstrated effective inhibition of JNK phosphorylation (Fig. 3). However, phosphorylation of p38 did not significantly decrease except in the group treated with DLE at 50 μg/ml.

*Effects of DLE and MC on STAT3 and NF-κB Signaling pathways in LPS-stimulated microglia.* Since DLE inhibited the phosphorylation of MAPKs in LPS-stimulated microglia, the effect of DLE on cellular signaling molecules associated with

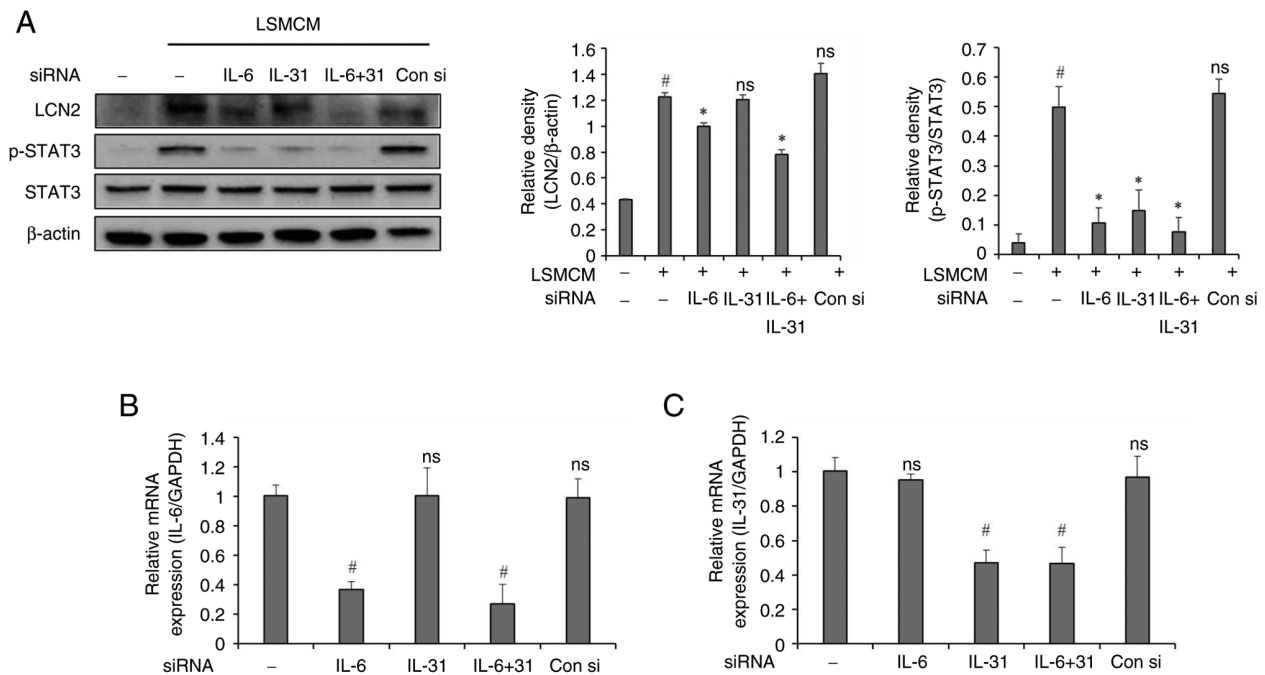


Figure 7. Effect of IL-6 and IL-31 gene silencing on LCN2 and STAT3 activation of astrocytes by LSMCM. Astrocytes were cultured in stimulated microglia conditioned media for 24 h. Prior to receiving LPS-stimulation, microglial cells were silenced for IL-6 or IL-31 or IL-6 plus IL-31 genes. (A) Relative expression levels of LCN2 and STAT3 were evaluated by western blot analysis. Transfection efficiency was confirmed by reverse transcription PCR. (B) IL-6 mRNA and (C) IL-31 mRNA expression levels. Each bar represents the mean  $\pm$  SD (n=3). <sup>#</sup>P<0.05 vs. untreated cells; <sup>\*</sup>P<0.05 vs. LSMCM alone treated cells; ns, no significant difference compared to LSMCM alone treated cells; DLE, *Diospyros lotus* leaf extract; MC, myricitrin; LSMCM, LPS-stimulated microglia culture medium; LPS, lipopolysaccharide; LCN2, lipocalin-2; si, short interfering.

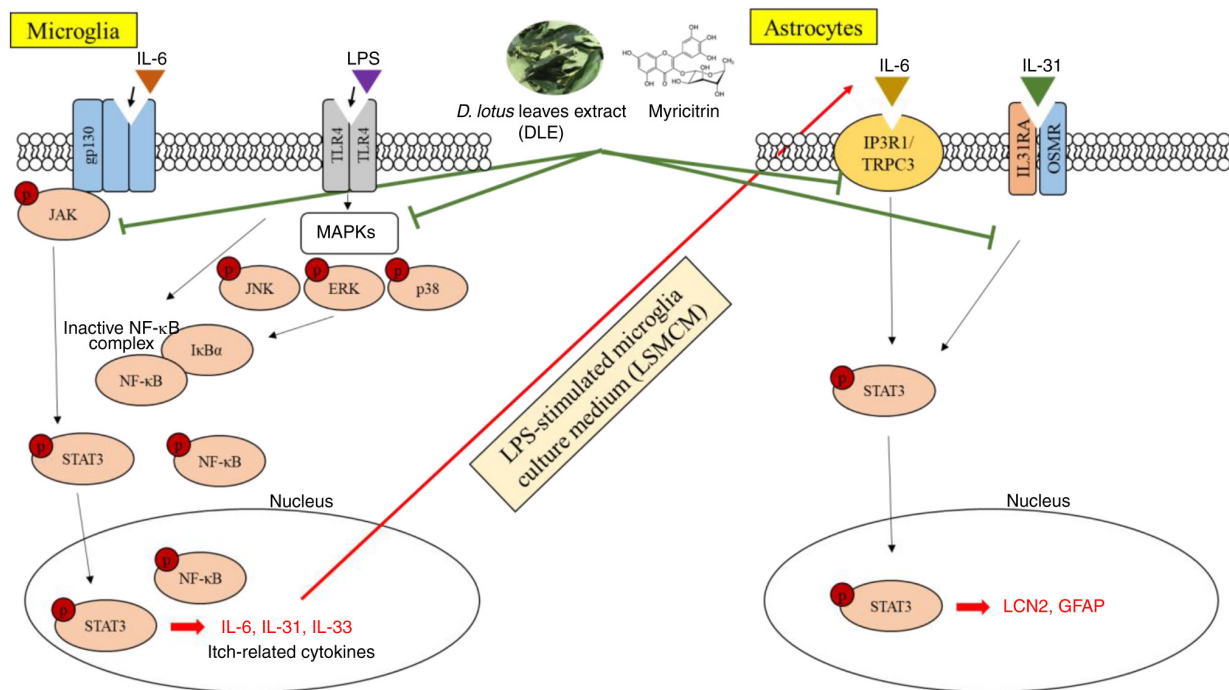


Figure 8. Schematic diagram of the mechanism involved in the anti-itch effect of DLE and MC on astrocytes and microglia. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; gp130, glycoprotein 130; TLR4, toll-like receptor 4; JAK1, Janus kinase; p38, p38 mitogen-activated protein kinases; LSMCM, LPS-stimulated microglia-conditioned media; IP3R1, inositol 1,4,5-trisphosphate receptor 1; TRPC, transient receptor potential canonical; IL-31RA, interleukin 31 receptor  $\alpha$ ; OSMR, oncostatin M receptor; LCN2, lipocalin-2; GFAP, glial fibrillary acidic protein.

MAPKs was investigated. A 30 min LPS treatment significantly increased the phosphorylation of STAT3, AKT, IKK, I $\kappa$ B and NF- $\kappa$ B in microglia (Fig. 4A). However, pre-treatment

with DLE or MC effectively suppressed the phosphorylation of these factors. To investigate the inhibition of NF- $\kappa$ B and STAT3 nuclear translocation, immunofluorescence staining



was performed (Fig. 4B and C). Results showed that stimulation with LPS led to nuclear translocation of NF- $\kappa$ B and STAT3. However, pretreatment with DLE and MC resulted in a reduction of their nuclear translocation.

**Effects of DLE and MC on itch-related receptor expression in LSMCM-stimulated astrocytes.** Levels of IL-6 and IL-31 in LSMCM were measured using an ELISA kit. Results indicated that IL-6 level was  $\sim 345$  pg/ml, while IL-31 level was measured at  $\sim 78$  pg/ml (Fig. S1). Whether cytokines produced by activated microglia activated astrocytes was investigated and the effect of DLE and MC on this process in LSMCM-stimulated astrocytes assessed. Expression levels of itch signaling receptors OSMR, IL-31RA and IP3R1 were significantly increased in LSMCM-stimulated astrocytes. However, pre-treatment with DLE and MC led to decreased expression levels of these receptors (Fig. 5).

**Effects of DLE and MC on LCN2, STAT3 and GFAP expression in LSMCM-stimulated astrocytes.** To further investigate the effect of DLE and MC, downstream signaling and associated proteins were assessed in LSMCM-stimulated astrocytes. Expression levels of LCN2, STAT3 and GFAP were significantly increased in LSMCM-stimulated astrocytes. However, in groups pre-treated with DLE and MC, expression levels of LCN2, STAT3 and GFAP were decreased by DLE and MC in a concentration-dependent manner (Fig. 6).

**Effects of IL-6 and IL-31 gene silencing in microglia on astrocyte activation.** The present study conducted experiments using conditioned media from microglia in which IL-6 and IL-31 genes were silenced to investigate whether the activation of astrocytes by LSMCM was mediated by cytokines IL-6 and IL-31 known to be associated with itching. Astrocytes cultured in LSMCM exhibited activation of LCN2 and STAT3. However, in groups with IL-6 or IL-6 plus IL-31 gene silenced, LCN2 expression was suppressed, while it showed no significant decrease in the IL-31 alone inhibition group. STAT3 activity was inhibited in all gene-silenced groups, although it showed no significant changes in the control siRNA group (Fig. 7).

## Discussion

LPS is a well-established macrophage stimulant. It has been widely used to study molecular mechanisms underlying microglial activation (25,26). The current study found that LPS induced the activation of microglia, leading to the generation of cytokines IL-6, IL-31 and IL-33. This observation agreed with our earlier research, where we initially demonstrated that LPS stimulation led to the production of IL-31 and IL-33 by microglia (27). In chronic itching, an increase in IL-6 has been observed, although its precise function remains to be elucidated (28). However, it has been reported that IL-6 causes itching by acting on dorsal root ganglion (DRG) in uremic itching (29) and that upregulation of IL-6 can improve the pruritic response of SDH astrocytes in a chronic itching mouse model (30). IL-31, a significant contributor to itching in conditions such as atopic dermatitis, acts as a crucial neuroimmune link between T helper 2 cells (Th2) and sensory neurons (31). IL-33, on the other hand, has been associated with the activation of Th2 cells, mast cells and eosinophils, leading to

the production of various Th2 cytokines and mediating allergic immune responses (32). Based on these findings, it can be inferred that DLE and MC may alleviate pruritic conditions by inhibiting the production of IL-6, IL-31 and IL-33.

To elucidate the mechanisms underlying inhibitory effects of DLE and MC on cytokine production in microglia, the present study investigated key signaling pathways associated with cytokine production. The NF- $\kappa$ B pathway is well-known for upregulating the expression of inflammatory cytokines. It is traditionally considered a classical inflammatory signaling pathway (33). Following LPS stimulation, I $\kappa$ B, an NF- $\kappa$ B inhibitor, is phosphorylated and degraded by IKK, leading to nuclear translocation of NF- $\kappa$ B and subsequent transcription of target genes (34). In the present study, DLE and MC effectively inhibited the phosphorylation of IKK and I $\kappa$ B, ultimately suppressing nuclear translocation of NF- $\kappa$ B. Additionally, the MAPKs pathway encompassing JNK, ERK and p38 serves a crucial role in regulating the production of cytokines and chemokines in LPS-stimulated microglia (35,36). Activation of MAPKs can stimulate kinase proteins, leading to nuclear translocation of NF- $\kappa$ B (37). DLE and MC were able to significantly inhibit the phosphorylation of ERK and JNK. However, suppression of p38 phosphorylation was not observed. These results suggested that inhibitory effects of DLE and MC on LPS-induced NF- $\kappa$ B nuclear translocation are attributed to suppression of the MAPK signaling pathway excluding p38. Cytokine production signaling is also mediated through the JAK/STAT pathway (38). LPS can bind to TLR4 on the cell membrane, leading to phosphorylation of JAK1, which subsequently phosphorylates STAT3, ultimately inducing transcriptional responses and increasing the expression of relevant inflammatory mediators (39,40). To further verify the effect of DLE and MC on the inhibition of NF- $\kappa$ B and STAT3 phosphorylation, immunofluorescence staining was performed. Consistent with the western blotting results, translocation of NF- $\kappa$ B and STAT3 to the nucleus was observed upon LPS stimulation, as evidenced by overlap of green fluorescence and nuclear staining. These results are consistent with the results of other previous studies (41,42). By contrast, treatment with DLE or MC significantly reduced the fluorescence intensity in the nuclear location, indicating that translocation of NF- $\kappa$ B and STAT3 was inhibited. This suggests that DLE and MC may reduce the production of inflammatory mediators by inhibiting the activation of NF- $\kappa$ B and STAT3 in microglia.

DLE and MC were found to suppress the expression of OSMR, IL-31RA, IP3R1, phosphorylated STAT3 and LCN2 in LSMCM-stimulated astrocytes (Fig. 5). IL-31 mediates itch-related signaling through the heterodimeric receptor composed of OSMR and IL-31RA (43). Considering the reported involvement of IL31RA/OSMR through SDH in sensitizing CNS-related itching and spinal neurons (44), DLE and MC might be able to alleviate CNS-related itching. Additionally, DLE and MC suppressed the phosphorylation of STAT3 and the expression of LCN2 in LSMCM-stimulated astrocytes. In chronic itching, SDH astrocytes exhibit sustained STAT3 activation due to IL-6, leading to upregulation of LCN2, which can exacerbate itch (13). GFAP, a target gene of STAT3, is commonly used as a marker for the activation status of astrocytes (45). Reports of increased GFAP expression in the SDH of itch-inducing mice suggest that GFAP activation serves as an indicator of itch (13,46,47). DLE and MC were

found to inhibit the expression of GFAP in LSMCM-stimulated astrocytes. These inhibitory effects are considered to result from the suppression of STAT3. Considering these results, it is hypothesized that DLE and MC can suppress the activity of astrocytes by microglia in relation to itching.

Additionally, the present study demonstrated the role of microglial IL-6 and IL-31 in the activation of STAT3 and LCN2 in astrocytes. Treatment with IL-6-silenced LSMCM significantly reduced the activation of LCN2 and STAT3 in astrocytes. This agreed with previous research reporting the crucial role of IL-6 in the activation of STAT3 and LCN2 in astrocytes (13). Although silencing the IL-31 gene did not significantly inhibit the expression of LCN2, it did suppress the activation of STAT3. Considering that IL-31 activates STAT3 and subsequently increases GRPR activity in DRG neurons (48), it is plausible to assume that IL-31 also enhances the activity of molecules associated with itch in astrocytes. Translating these findings into *in vivo* models will be crucial to validating the relevance of the *in vitro* results of the present study in a more physiological setting. Future studies should aim to explore effects of IL-6 and IL-31 gene silencing in animal models, allowing for a more comprehensive understanding and implications of itching-related neuroinflammatory responses.

In our previous research, various flavonoid compounds within DLE, including the most abundant MC, were identified (18,19). MC has been reported to alleviate carbon tetrachloride-induced toxicity in rats (49), inhibit periodontitis (50) and alleviate acute lung injury in rats by blocking the JAK/STAT pathway (51). Based on these preceding studies, it is hypothesized that the demonstrated itchy cytokine inhibition effect and mechanism of action of DLE in the present study are attributable to its high content of MC. However, considering that DLE is a complex mixture of various flavonoids as plant extracts, it is worth investigating whether MC interacts with other compounds within DLE to exert anti-inflammatory effects, taking into account the possibility of synergistic effects among these compounds. Through such investigation, it is expected that effects of DLE can be expanded beyond MC alone.

In conclusion, the present study demonstrated that DLE and MC can exert significant inhibitory effects on the production of key pruritic cytokines, IL-6 and IL-31, in LPS-stimulated microglia. This suggested a potential therapeutic role of DLE and MC in alleviating pruritic conditions. Mechanistically, the findings of the present study revealed that DLE and MC could modulate critical signaling pathways involved in cytokine production, such as the NF- $\kappa$ B, MAPK and JAK/STAT pathways. The present study also extended to the role of DLE and MC in astrocyte activation, a crucial component in CNS-related itching. DLE and MC were found to suppress the expression of receptors and signaling molecules associated with itching, such as OSMR, IL-31RA, phosphorylated STAT3 and LCN2, in LSMCM-stimulated astrocytes. This inhibitory effect suggested that DLE and MC might alleviate CNS-related itching by modulating astrocyte activity (Fig. 8). Moreover, the present study elucidated the involvement of microglial IL-6 and IL-31 in the activation of STAT3 and LCN2 in astrocytes, providing insights into intricate neuroinflammatory responses underlying itching. In relation to this, subsequent research is required to apply it to *in vivo* models and validate the relevance of these *in vitro* results.

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## Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

## Authors' contributions

JYS contributed to the conceptualization, methodology, validation, writing the original draft and visualization. BOC contributed to the conceptualization and validation. JHP, ESK, JHK and HYH contributed to performing the experiments and analyzing the data. YSK contributed to the conceptualization, validation and writing the review and editing. SIJ contributed to the conceptualization, validation, review and editing, supervision, project administration and funding acquisition. JYS and SIJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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