Diospyros lotus leaf extract and its main component myricitrin regulate pruritus through the inhibition of astrocyte activation

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Abstract. *Diospyros lotus* is a deciduous plant native to Asian countries, including Korea, Japan and China, and southeast Europe. In traditional medicine, *Diospyros lotus* is used as an anticancer, antidiabetic and antipyretic agent. The present study aimed to evaluate the effect of *Diospyros lotus* leaf extract (DLE) in ameliorating histamine-independent pruritus. Activation of signal transducer and activator of transcription 3 (STAT3) in astrocytes contributes to pruritus. In this study, the effects of DLE and its main component, myricetin (MC), on the activation of STAT3, expression of glial fibrillary acidic protein (GFAP), and production of lipocalin-2 (LCN2) in IL-6-treated astrocytes and chloroquine-injected mice were investigated through western blot, reverse transcription-quantitative PCR, and immunofluorescence staining. DLE and MC inhibited STAT3 activation, GFAP expression and LCN2 release via inositol 1,4,5-trisphosphate receptor type 1 blockade in astrocytes. DLE and MC ameliorated scratching behavior, expression of GFAP, mast cell infiltration and serum IL-6 levels in chloroquine-injected mice. These results suggested that DLE and MC can be used as oral therapeutic agents for the treatment and management of pruritus.

Introduction

Pruritis is an unpleasant sensation that causes an intense desire to rub or scratch (1). Pruritus is the primary cause of poor quality of life in patients with hypersensitive skin disease (2). The desire to scratch in hypersensitive skin disease is not resolved by scratching and it becomes stronger as the patient scratches (3,4). Repeated scratching weakens the skin barrier and exacerbates skin lesions (5). Therefore, pruritus control may be key to improving the quality of life of patients with hypersensitive skin disease.

Astrocytes are a type of glial cell involved in regulating neuronal excitability in the central nervous system (CNS) (6,7). Astrocytes mediate itching by releasing signaling molecules, such as ATP, that activate C-fibers (8). However, a recent study showed that inositol 1,4,5-trisphosphate receptor type 1 (IP3R1) is important for both the IL-6-induced sustained activation of signal transducer and activator of transcription 3 (STAT3) and expression of genes associated with reactive astrocytes (9). STAT3 serves an important role in the transformation of reactive astrocytes (9). Reactive astrocytes intensify the itching sensation by sensitizing gastrin releasing peptide receptor (GRPR)-positive neurons to GRP itch neuropeptides via lipocalin-2 (LCN2) secretion in hypersensitive skin disease models such as atopic dermatitis (10). Previous studies have showed that pharmacological inhibition of STAT3 decreases reactivity of astrocytes (11,12). Therefore, modulating STAT3 in reactive astrocytes is a useful strategy to control the itch.

Diospyros lotus, also called date plum or Caucasian persimmon, is a deciduous plant native to Asian countries, including Korea, Japan and China, and Southeast Europe. It can be eaten directly or by processing the leaves into herbal tea (13,14). *Diospyros lotus* leaf extract (DLE) has been used in traditional medicine as an antitumor, antidiabetic, sedative, astringent, antipyretic or laxative agent (13). Our previous studies showed that DLE contains flavonoids such as myricitrin (MC), gallic acid, astragalin, myricetin-3-O-galactoside and myricetin with anti-atopic dermatitis, anti-inflammatory, antioxidant and anti-obesity effects (15-17). However, to the best of our knowledge, effects of DLE on histamine-independent pruritus and its mechanism of action in activated astrocytes have not been reported yet.

Therefore, the present study aimed to investigate the effect of DLE and MC on histamine-independent pruritus in IL-6-stimulated astrocytes and chloroquine-injected mouse models.

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Materials and methods

Plant materials. Diospyros lotus leaves were collected in June 2020 from Cheonjam mountain, Jeonju-si, Jeollabuk-do, Republic of Korea. The leaves were washed five times with water and dried in a windy area with shade. Dried leaves (100 g) were mixed with 70% (v/v) ethanol (2 l) at room temperature for 48 h. The extracted sample was filtered using 0.5 μm filter paper (ADVANTEC), Thee extract was concentrated at 45˚C using a vacuum concentrator (EYELA Rotary evaporator N-1100, EYELA) to obtain DLE in powder form.

Materials. MC was purchased from Tokyo Chemical Industry Co., Ltd. Quanti-MAX™ WST-8 Cell Viability Assay kit (cat. no. QM1000) and WestGlow™ FEMTO Chemiluminescent substrate (BWF0100) were obtained from Biomax Co. Ltd. ReadyShield® Protease and Phosphatase Inhibitor Cocktail, cat. no. PPC2020) and Chloroquine diphosphate salt were purchased from Sigma-Aldrich (Merck KGaA). Radioimmunoprecipitation assay (RIPA) buffer and phosphorylated (p)-STAT3 (44-384G), STAT3 (MA1-13042) glial fibrillary acidic protein (GFAP) (14-9892-82), LCN2 (PA5-79590), IP3R1 (PA1-901) and goat anti-mouse IgG Alexa Fluor 488 (A-11001) antibodies were purchased from Thermo Fisher Scientific, Inc. Actin (sc-8432), m-IgGκ BP-HRP (sc-516102) and mouse anti-rabbit IgG-HRP (cat. no. sc-2357) were purchased from Santa Cruz Biotechnology, Inc. Hematoxylin and eosin (H&E) stain kit (ab245880) was purchased from Abcam. ProLong® gold antifade reagent with DAPI mounting solution (8961) was came from Cell signaling (Danvers, MA, USA).

Cell culture. Mouse-origin astrocytes (CRL-2535™) and DMEM (cat. no. 30-2002) were purchased from American Type Culture Collection. Fetal bovine serum (cat. no. 16000044) and Penicillin-Streptomycin-Glutamine (100X; cat. no. 10378016) were purchased from Thermo Fisher Scientific, Inc. Astrocytes were cultured and maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37˚C.

Cell viability. Cell viability was analyzed using Quanti-MAX™ WST-8. Astrocytes ($3x10^5$ cells/ml) were seeded into 96-well plates and cultured at 37˚C for 24 h. Then, DLE (0.0, 12.5, 25.0, 50.0, 100.0 and 200.0 μg/ml) or MC (0, 10, 20, 30, 50, 75 and 100 μM) were treated and further incubated at 37˚C for 20 h. Subsequently, 10 μl Quanti-MAX™ WST-8 reagent was added to each well followed by incubation at 37˚C for 4 h. The absorbance of each well was measured at 450 nm using a spectrophotometer (Tecan Group, Ltd.). The cell viability was calculated based on the absorbance compared with the control group without the sample added.

Protein extraction and western blotting. Astrocytes ($3x10^5$ cells/ml) were cultured in 60-mm cell culture dishes at 37˚C for 24 h, treated with DLE (50 and 100 μg/ml) or MC (10 and 20 μM), and incubated at 37˚C for 1 h. These cells were stimulated with IL-6 (10 ng/ml) at 37˚C for 30 min or 24 h. Total protein was extracted from each sample using protease inhibitor-treated RIPA buffer. Following quantification by Bradford assay, 50 μg protein was loaded in each lane and separated by SDS-PAGE on 10 or 12% gel. Separated proteins were transferred onto a polyvinylidene fluoride membrane, blocked with 5% bovine serum albumin (BSA) (A0100-010; GenDEPOT) at room temperature for 1 h and washed three times with Tris-buffered saline with 1% Tween-20 (TBST) solution (10 min each wash). These membranes were incubated with antibodies against STAT3 (1:1,000), p-STAT3 (1:1,000), IP3R (1:1000), LCN2 (1:2000), GFAP (1:1000) and β-actin (1:500) at 4˚C overnight. After washing five times with TBST, membranes were incubated with anti-mouse (1:5,000; cat. no. sc-516102, Santa Cruz Biotechnology, Inc.) or rabbit (1:5,000) (sc-2357, Santa Cruz Biotechnology, Inc.) horseradish peroxidase-conjugated secondary antibodies containing BSA for 2 h at room temperature. Subsequently, membranes were washed three times with TBST solution (10 min each) and visualized with an imaging system (ALLIANCE LD4; Uvitec, Cambridge, UK) using EZ-Western Lumi Pico Alpha chemiluminescent reagent (DG-WP250; DoGenBio, Seoul, South Korea). Band densities were analyzed using ImageJ 1.53e (National Institutes of Health) with β-actin as the loading control.

Reverse transcription-quantitative (RT-q)PCR. Astrocytes ($3x10^5$ cells/ml) were cultured in 60-mm cell culture dishes at 37˚C for 24 h, treated and incubated at 37˚C for 1 h with DLE (0, 50 and 100 μg/ml) or MC (0 10, and 20 μg/ml) and stimulated with IL-6 (10 ng/ml) at 37˚C for 4 h. Total RNA was extracted and purified using a GeneAll® Ribospin™ II extraction kit (GeneAll Biotechnology Co., Ltd.), according to the manufacturer's instructions. The concentration of total RNA isolated and purified was determined by spectrophotometry (Optizen NanoQ plus; KLAB). The ReverTra Ace™ qPCR RT Master Mix cDNA synthesis kit (Toyobo Life Science) was used to synthesize cDNA using 5 μg each RNA sample. qPCR was performed using the Power SYBR® Green Master Mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 3 min, followed by 40 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 10 sec and extension at 72˚C for 30 sec. The following primer pairs were used for qPCR: LCN2 forward, 5'-CCAGTTCGCCATGGTATTTT-3' and reverse, 5'-GGTGGGGACAGAGAAGATGA-3' and GAPDH forward, 5'-GGCTACACTGAGGACCAGGT-3' GAPDH reverse, 5'-TCCACCACCCTGTTGCTGTA-3'.

Animals and experimental design. Specific-pathogen-free male ICR mice (25 4 weeks-old 21 g) were obtained from Orient Bio, Inc. Mice were housed in a room with standard environmental conditions (temperature of 22±2˚C, 50-60% humidity and a 12/12-h light/dark cycle) and were provided free access to a commercial standard laboratory diet and water. Experimental procedures were performed according to Jeonju University Institutional Animal Care and Use Committee guidelines (18). During the experimental period, the health of animals was monitored daily, and no mice met the humane endpoints specified, such as a weight loss of over 20%, appetite loss for more than 2 days, dyspnea, increased heart rate, self-harm, jaundice, persistent diarrhea/vomiting,

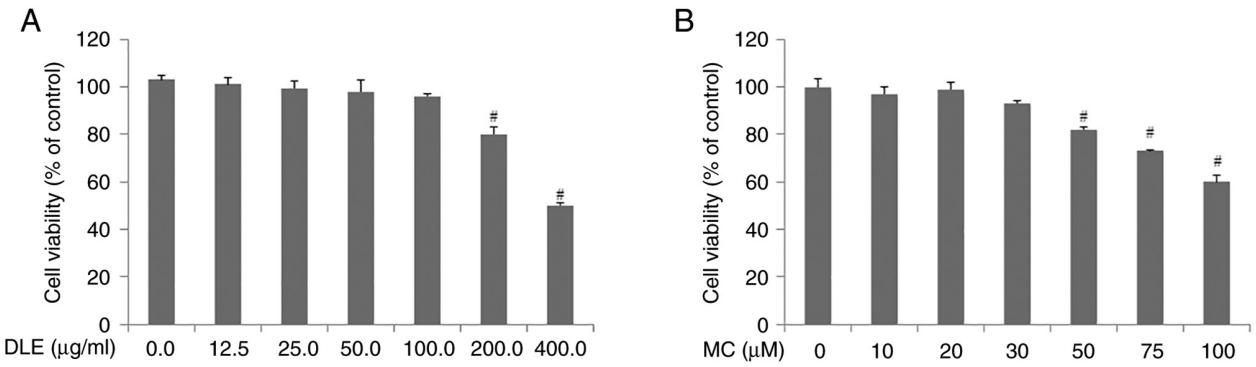

Figure 1. Effects of DLE and MC on astrocyte viability. Astrocytes were pretreated with (A) DLE or (B) MC for 24 h. Cell viability was measured by water-soluble tetrazolium dye assay. Each bar represents the mean ± standard deviation (n=3). #P<0.05 vs. 0. DLE, *Diospyros lotus* leaf extract; MC, myricitrin.

and decreased response to external stimuli. After a 1-week acclimation period, mice were shaved on their back using an electric shaver and then randomly assigned to four groups (n=5/group): 1, Normal control (200 μl of saline); 2, chloroquine (50 μg/site); 3, chloroquine + 200 mg/kg DLE and 4, chloroquine + 20 mg/kg MC. Normal saline, DLE, and MC were administered orally, while chloroquine was administered via subcutaneous injection. After the experiment, mice were anesthetized with a 2-6% isoflurane for induction and maintained at a 1-3% concentration. Skin thickness was measured with vernier calipers and blood samples (600-800 μl) were collected from the orbital venous plexus. Subsequently, mice were euthanized through cervical dislocation and, after confirming the absence of respiratory and heartbeat, the dorsal skin samples were collected.

Scratching behavior analysis. The hair on the back was shaved with a hair clipper 1 day before the start of the experiment. At 1 h before chloroquine injection, mice in groups 1 and 2 were orally administered with saline, whereas those in groups 3 and 4 were orally administered with DLE or MC. After 30 min CCTV recording immediately after chloroquine injection, five researchers evaluated scratching behavior in a double-blind manner. The back skin was collected for histological analysis and the vertebrae were collected for immunofluorescence staining.

Histopathological examination. Tissues were fixed in 4% paraformaldehyde at 4˚C for 24 h and washed five times with phosphate-buffered saline (PBS) at room temperature for a total of 24 h. Samples were dehydrated in ascending ethanol series (60-100%) at room temperature for 30 min at each concentration. Samples were washed twice in xylene (2 h each at room temperature), embedded three times in paraffin (1 h each at 60˚C). Finally, new paraffin was poured into the tissues at 60˚C and solidified at 4˚C for 1 h to form a block. Paraffin-embedded tissue samples were cut into 5-μm-thick sections using a microtome (Leica Microsystems GmbH). Samples were stained using the H&E staining kit (cat. no. ab245880, Abcam) according to the manufacturer's protocol.

Immunofluorescence staining. Astrocytes ($3x10^5$ cells/ml) were cultured in 4-well cell culture slides at 37˚C for 24 h, treated at 37˚C for 1 h with DLE (100 μg/ml) or MC (20 μM) and then stimulated with IL-6 (10 ng/ml) at 37˚C for 24 h. The cells were fixed with ice cold methanol (99.9%) for 20 min, blocked with PBS containing 1% BSA at room temperature for 1 h and then incubated at 4˚C for 16 h after injection with GFAP primary antibody (1:100). After washing three times for 10 min with PBS with 0.1% Tween-20, the cells were incubated in the dark at room temperature for 2 h with goat anti-mouse IgG Alexa Fluor 488 secondary antibody (1:1,000), followed by mounting with a mounting solution containing DAPI. After drying overnight, images were captured at 400x using a fluorescence microscope (Carl Zeiss AG).

Segments of the fifth lumbar vertebra were fixed in 4% paraformaldehyde at 37˚C for 4 h and incubated with PBS containing 30% sucrose at 4˚C for 24 h. Tissues were cut into 30-μm sections using a cryotome (Amos Scientific Pty, Ltd.). Samples were washed three times in PBS (10 min each) and incubated at room temperature for 1 h in PBS containing 0.3% Triton X-100 and 2% BSA. Samples were incubated overnight at 4˚C with GFAP antibody. After washing five times with PBS, sections were washed with PBS and incubated with the goat anti-mouse IgG Alexa Fluor 488 secondary antibody (1:1000) at room temperature for 2 h. After washing five times with PBS, samples were mounted with DAPI-containing mounting medium. After drying overnight, images were captured at 400x using a fluorescence microscope (Carl Zeiss AG).

Statistical analysis. All statistical analysis was performed using SPSS version 26.0 (IBM, Armonk, NY, USA). Data are presented as the mean ± standard deviation (n=3). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of DLE and MC on astrocyte survival. Cell viability assay was performed to investigate the effect of DLE and MC on astrocytes. Results showed that DLE was not cytotoxic at ≤100 μg/ml. However, DLE was cytotoxic at concentrations >200 μg/ml (Fig. 1A). MC was not toxic at concentrations ≤30 μM; however, it was cytotoxic at concentrations >50 μM (Fig. 1B). Therefore, in the subsequent experiments, cells were treated with ≤100 μg/ml DLE and ≤30 μM MC.

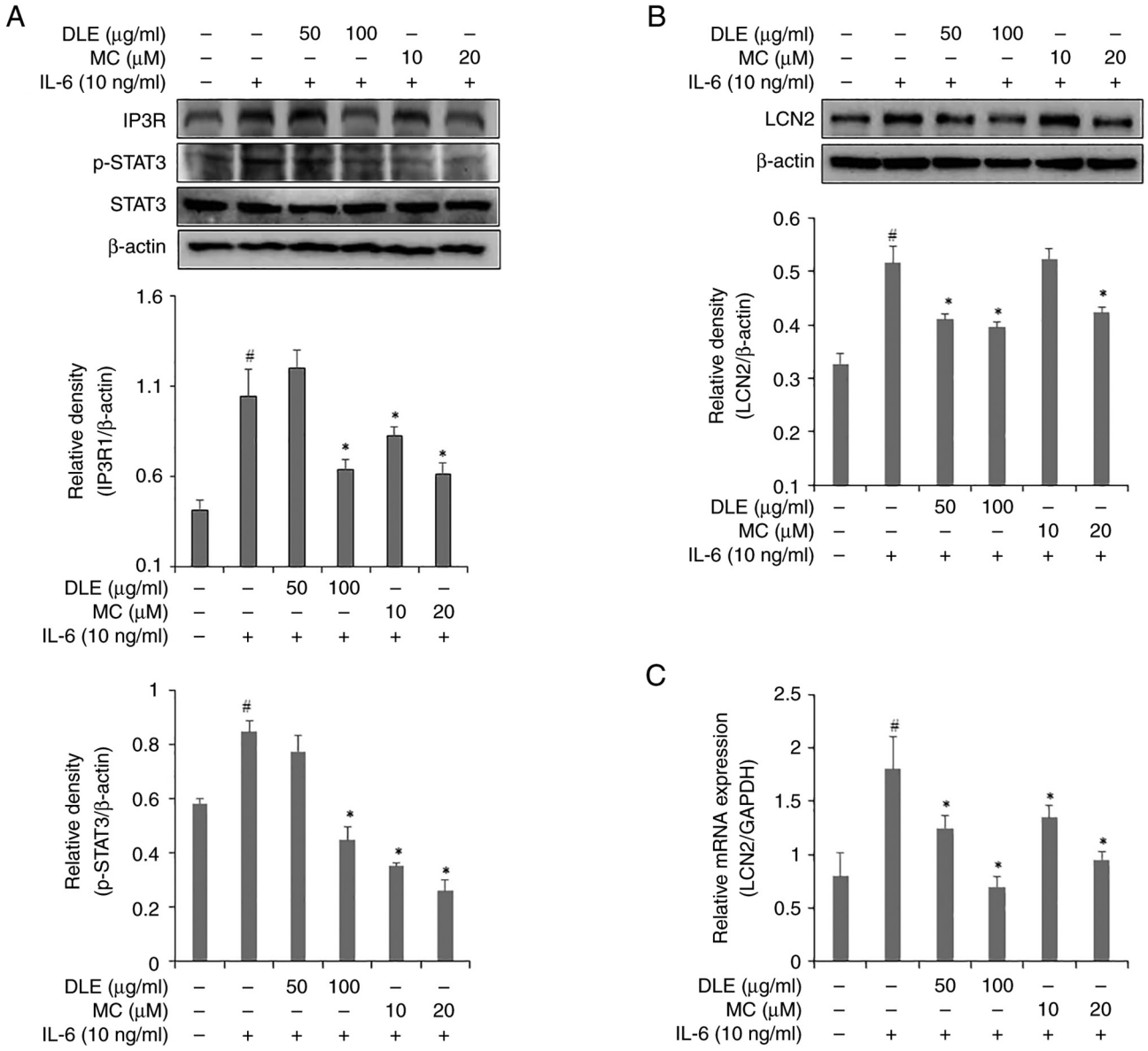

Figure 2. Effects of DLE and MC on activation of STAT3 and LCN2 expression in IL-6-treated astrocytes. (A) Astrocytes were pretreated with DLE or MC for 1 h and subsequently stimulated with 10 ng/ml IL-6 for (B) 24 and (C) 4 h. Each bar represents the mean ± standard deviation (n=3). #P<0.05 vs. untreated cells. *P<0.05 vs. IL-6 alone. IP3R, inositol 1,4,5-trisphosphate receptor type 1; LCN2, lipocalin-2; DLE, *Diospyros lotus* leaf extract; MC, myricitrin; p-, phosphorylated.

Effects of DLE and MC on STAT3 and IP3R1 activation and LCN2 production. To investigate the effects of DLE and MC on STAT3 and IP3R expression, western blotting was performed (Fig. 2A). The expression of IP3R showed a significant decrease compared with the IL-6 alone treated group at all DLE concentrations except 50 μg/ml. Similarly, 50 μg/ml DLE did not inhibit STAT3 expression. However, 100 μg/ml DLE significantly decreased compared to the IL-6 alone treated group expression of STAT3. Expression of STAT3 was decreased in a concentration-dependent manner starting from 10 μM MC. The effects of DLE and MC on LCN2 production were assessed using western blotting (Fig. 2B) and RT-qPCR (Fig. 2C). DLE treatment similarly inhibited LCN2 expression at both 50 and 100 μg/ml concentrations, and the inhibitory effect showed a significant decrease compared to the IL-6 alone treated group. However, MC treatment significantly decreased LCN2 expression only at 20μM compared to the control group. RT-qPCR results showed that both DLE and MC decreased LCN2 mRNA expression in a dose-dependent manner compared to the control group.

Effects of DLE and MC on GFAP expression in IL-6-treated astrocytes. GFAP expression was assessed using western blotting and immunofluorescence staining. Western blotting analysis indicated that 50 μg/ml DLE did not significant decrease in GFAP expression compared with the IL-6 alone treated group but 100 μg/ml DLE significantly suppressed GFAP expression. The treatment with MC resulted in a dose-dependent decrease in GFAP expression in astrocytes and showed a significant reduction compared to the group treated with IL-6 alone (Fig. 3A). Immunofluorescent staining

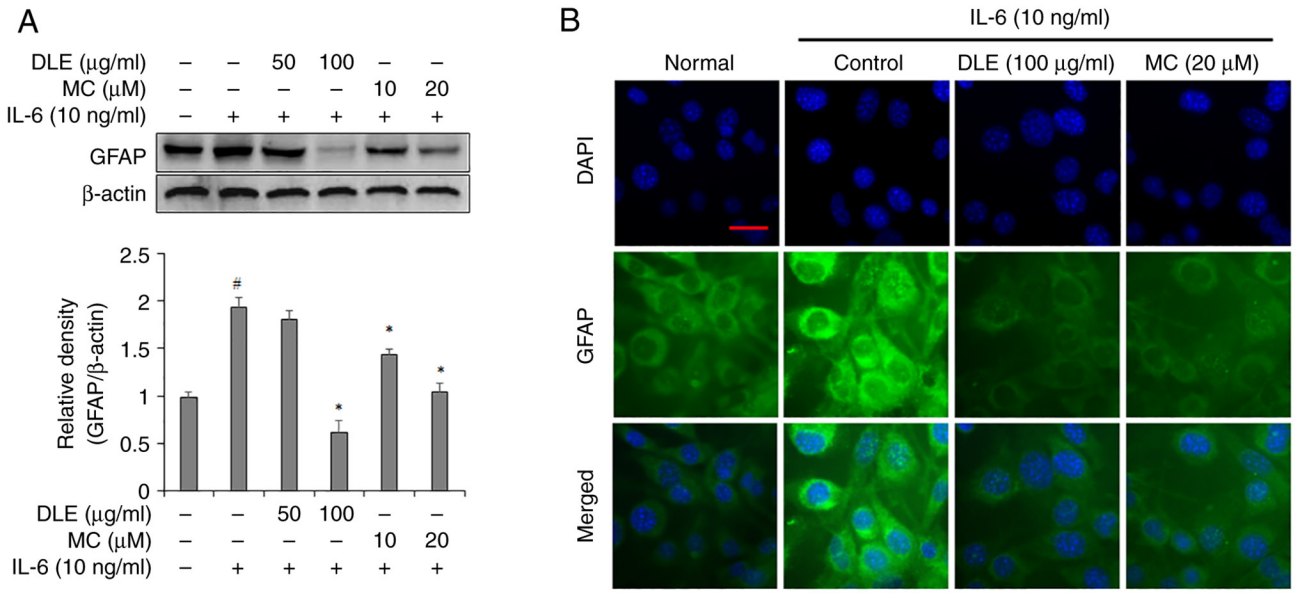

Figure 3. Effects of DLE and MC on GFAP expression in IL-6-treated astrocytes. Astrocytes were pretreated with DLE or MC for 1 h and stimulated with 10 ng/ml IL-6 for 24 h. Expression of GFAP was evaluated using (A) western blotting and (B) immunofluorescence staining (scale bar, 20 μm). GFAP was stained in green, while the nuclei were stained in blue (DAPI). Each bar represents the mean ± standard deviation (n=3). $^{\#}$P<0.05 vs. untreated cells. *P<0.05 vs. IL-6 alone treated cells. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; GFAP, glial fibrillary acidic protein.

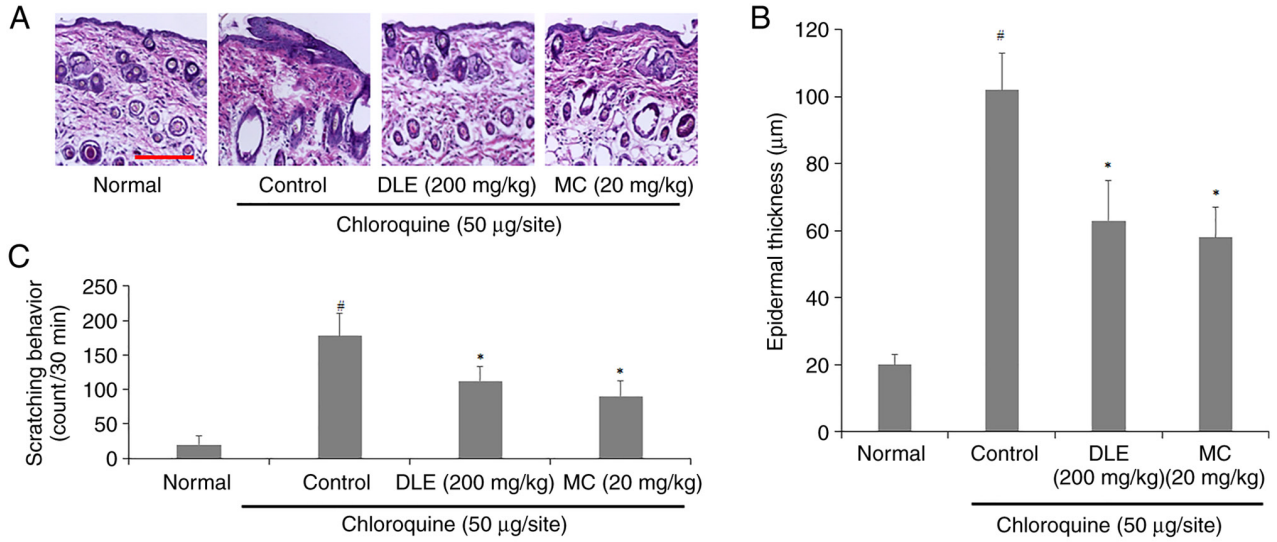

Figure 4. Effect of DLE and MC on chloroquine-induced scratching behavior and histological changes. (A) Skin sections were stained with hematoxylin and eosin. Sections were evaluated using a microscope (scale bar, 200 μm). (B) Epidermis thickness was measured using Vernier calipers. (C) Scratching behavior was measured using a double-blind test. Each bar represents the mean ± standard deviation (n=3). $^{\#}$P<0.05 vs. normal. *P<0.05 vs. control. DLE, *Diospyros lotus* leaf extract; MC, myricitrin.

assay showed that the expression of GFAP was decreased by treatment with DLE and MC, which was consistent with the results of the western blotting assay (Fig. 3B).

Effect of DLE and MC on histological changes in the skin and scratching behavior of chloroquine-injected mice. To investigate the effects of DLE and MC on histological changes of chloroquine-injected mice, the back skin was observed through H&E staining. Severe edema was observed in the back skin of the control group, whereas this was decreased in the DLE and MC-administered groups (Fig. 4A). Additionally, DLE and MC administration resulted in a decrease in epidermal thickness compared to the control group (Fig 4B). The frequency of scratching behavior also significantly decreased in DLE and MC-administered groups (Fig. 4C).

Effect of DLE and MC on mouse spinal dorsal horn (SDH) astrocyte activity. To investigate effects of DLE and MC on SDH astrocyte activity, a GFAP-specific fluorescence staining assay of spinal sections collected from the mice was performed. The expression of GFAP was significantly increased in the control group compared with that in the normal group; however, administration of DLE or MC significantly decreased the expression of GFAP (Fig. 5).

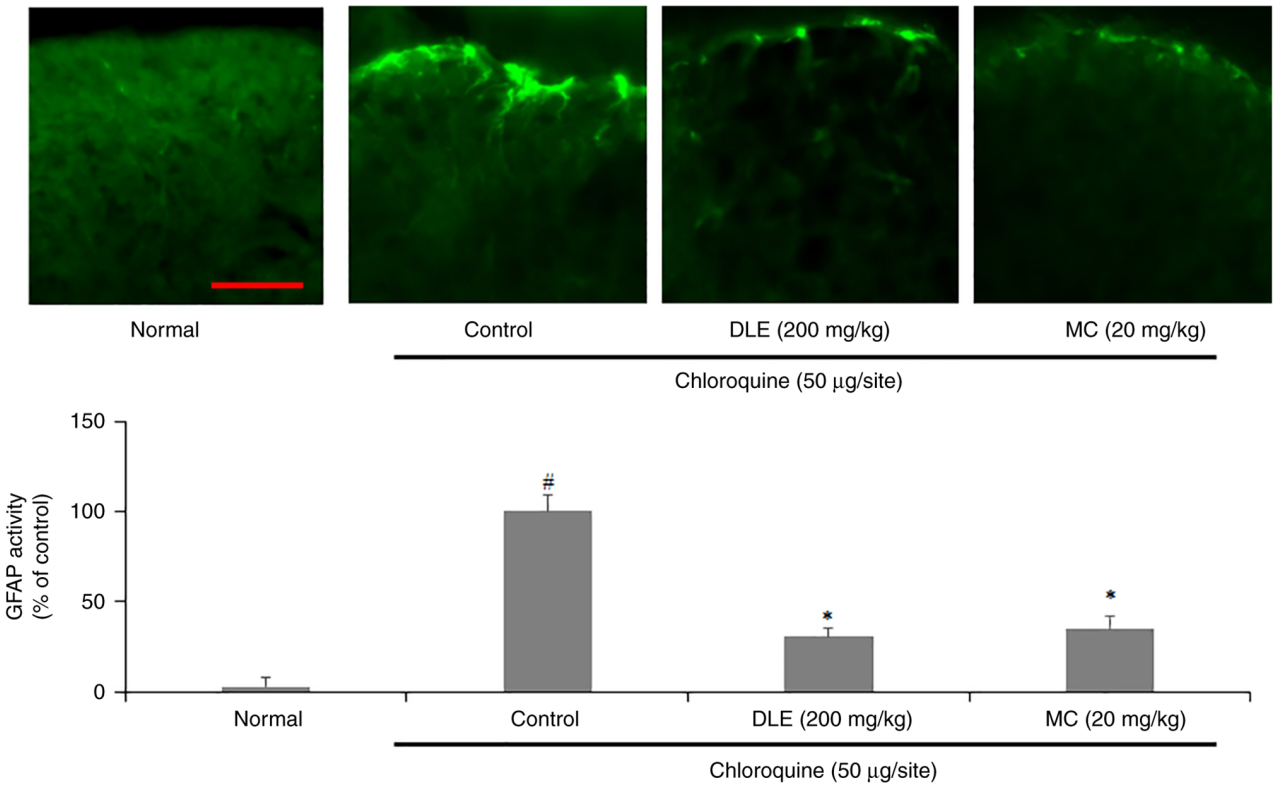

Figure 5. Effect of DLE and MC on SDH astrocyte reactivity in chloroquine-injected mice. Spine sections were stained by immunofluorescence (scale bar, 40 μm). GFAP was stained in green. Each bar represents the mean ± standard deviation (n=3). $^{\#}$P<0.05 vs. normal. *P<0.05 vs. control. DLE, *Diospyros lotus* leaf extract; MC, myricitrin; SDH, spinal dorsal horn; GFAP, glial fibrillary acidic protein.

Discussion

Our previous studies demonstrated that administration of DLE could inhibit biomarkers such as pro-inflammatory cytokines, immune cell infiltration, IgE and clinical score in atopic dermatitis animal models (16,19). However, to the best of our knowledge, it is unclear whether DLE could control the pruritus. Therefore, the present study investigated the antipruritic effects of DLE and its primary component MC in reactive astrocytes and an animal model of histamine-independent itch.

STAT3 is activated by phosphorylation of JAK after activation of the IL-6 family cytokine receptors (20). However, a recent study reported that IL-6 induces sustained STAT3 activation in astrocytes via IP3R1/transient receptor potential cation channel (TRPC)-mediated Ca^{2+} signaling (9). Activation of STAT3 in astrocytes increases expression of LCN2 and enhances pruritus via activation of GRP/GRPR signaling (21). The present study showed that DLE and MC treatment decreased expression of STAT3, IP3R1 and LCN2 in IL-6-stimulated astrocytes. The present results indicated that DLE and MC suppressed pruritus by suppressing the expression of LCN2, which suggested that the antipruritic effect of DLE and MC may be associated with IP3R1/TRPC channel inhibition. Furthermore, based on previous reports of symptom control through the inhibition of receptors by natural substances in allergic disease (22,23), it is hypothesized that DLE and MC could serve as effective itch relievers.

The present study used a mouse model of chloroquine-induced pruritus to investigate the *in vivo* antipruritic effect of DLE and MC. Chloroquine induces itch through the GRP/GRPR signaling pathway and this is not dependent on the histamine release (24,25). Therefore, chloroquine is a suitable tool to induce histamine-independent pruritus. The present results showed a decrease in scratching behavior in mice with chloroquine-induced pruritus following treatment with DLE or MC. Considering that pruritus occurred through GRP/GRPR signaling due to subcutaneous injection of chloroquine, the inhibitory effect of DLE and MC in mice was hypothesized to be related to LCN2 inhibitory effect that was observed *in vitro*.

GFAP is a target gene for STAT3 and is often investigated to determine the reactive state of astrocytes (12,26). Reactive astrocytes have been reported in chronic pain caused by nerve damage; studies reporting an increased expression of GFAP in SDH in pruritus-induced mice demonstrated that this is also an important indicator for pruritus (9,21,27). Thus, decreasing or knocking down expression of GFAP might be beneficial in ameliorating pruritus sensation. In the present study, DLE and MC inhibited the expression of GFAP both *in vivo* and *in vitro*. The inhibitory effect of GFAP expression in cells was hypothesized to be due to the inhibition of STAT3 expression. However, the present study did not find conclusive evidence that the GFAP inhibitory effect of DLE and MC *in vivo* was the result of STAT3 inhibition. Therefore, it is necessary to clarify if DLE and MC inhibit STAT3 expression in a chronic itch model in a future study.

Our previous study confirmed that MC, myricetin, myricetin-3-O-galactoside, gallic acid and astragalin are present in DLE; of these, the dominant component is MC and

its content is 45 mg/g (28). On this basis, it was estimated that 100 μg/ml DLE used in the present study contained ~4.5 μg MC. This concentration was lower than the 10 and 20 μg/ml MC used in the study. Despite this, the results suggested that the antipruritic effect of DLE was not solely attributed to MC as a concentration of 100 μg/ml DLE showed improved performance compared with that 20 μg/ml MC. Further studies are necessary to investigate the components of DLE and their mechanisms of action.

In conclusion, the present results demonstrated that DLE and its primary component, MC, suppressed expression of LCN2 through inhibition of IP3R1 and STAT3 activation in astrocytes. Furthermore, in chloroquine-injected mice, it was confirmed that administration of DLE and MC ameliorated scratching behavior and inhibited SDH astrocyte activity. The results of this study suggest that DLE and MC may be suitable candidates as oral therapeutic agents for the treatment and management of pruritus.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS made major contributions to the study design and manuscript writing. BC was involved in the study conceptualization, manuscript review and editing. JP and EK conducted formal analysis and investigation. YK and SJ contributed to the study conceptualization, data curation, manuscript review and editing, and project management. All authors have read and approved the final manuscript. BC and SJ confirm the authenticity of all the raw data

Ethics approval and consent to participate

The present study was approved by Jeonju University Institutional Animal Care and Use Committee (approval no. JJU-IACUC-2018-3).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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