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Anti-Inflammatory Effect of *Carex scabrifolia* Steud. Extract in RAW264.7 Cells

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This research was designed to evaluate the possible anti-inflammatory effects of *Carex scabrifolia* Steud. extract using RAW264.7 cells. The assessments of these effects were based on cell viability assay, mRNA expression levels of interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), IL-6, tumor necrosis factor alpha (TNFα), and levels of nitric oxide (NO)/prostaglandin E2 (PGE_2) production. Quantitative real-time polymerase chain reaction showed that treatment with *C. scabrifolia* Steud. extract decreased the mRNA levels of iNOS, COX2, IL-1α, IL-1β, IL-6, and TNFα. Furthermore, from the production levels of PGE_2/NO, it can be inferred that *C. scabrifolia* Steud. extract exhibited anti-inflammatory properties. These results suggest that *C. scabrifolia* Steud. extract contains anti-inflammatory compound(s), and consequently, that it may have applications as a potent cosmeceutical material.

Keywords: ***Carex scabrifolia* Steud., inflammation, iNOS, COX2, interleukins, TNFα**

Introduction

Inflammation is an immune response to harmful stimuli both inside and outside the body, including external stimuli, bacteria, and damaged cells [1]. Although it is an immune response to protect cells and tissues of the living body, it is known that excessive inflammation can result in diseases, such as hypertension, diabetic atherosclerosis, neurodegenerative disorders, and cancer [1].

Lipopolysaccharides (LPS) are components of substances that constitute the cell walls of bacteria. They bind to Toll-like receptors, which are cell membrane proteins expressed by macrophages, and activate the nuclear factor kappa beta (NF-κB) and mitogen-activated protein kinase (MAPK) cellular signaling pathways. They also induce the secretion of inflammatory mediators, such as prostaglandin E_2 (PGE_2) and nitric oxide (NO), and increase the secretion of inflammatory cytokines, such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) [2, 3]. Fundamentally, macrophages play important roles in the immune system, and in particular, they carry out three main functions, namely phagocytosis, antigen processing/presentation, and immune regulation. They release various cytokines and inflammatory mediators to facilitate these functions [2, 3]. Importantly, the inflammatory mediators generated in these processes also serve to strengthen immune functions or protect surrounding cells present at the site of inflammation, but excessive PGE_2 and NO can also cause inflammation [4]. NO is a known representative indicator of inflammation and it plays beneficial roles, including the elimination of microorganisms, such as bacteria, or the suppression of tumor growth. However, excessive NO also causes tissue damage and genetic mutation [5–8]. In addition, arachidonic acid is metabolized to PGE_2 by cyclo-oxygenase 2 (COX2), which is known as an important factor in inflammatory responses, along

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with iNOS. When macrophages are activated by LPS, they produce inflammatory cytokines, such as TNF-α, IL-1, and IL-6 [9, 10]. Therefore, the inhibition of PGE_2, NO, and the inflammatory cytokines, is a very important target in preventing or treating inflammatory diseases.

Carex scabrifolia Steud. is a perennial plant that grows in wetlands by the seas. It is a known halophyte that grows wildly throughout Korea, and it is reported to be also distributed in Taiwan, Russia, China, and Japan. Hitherto, the research on *C. scabrifolia* Steud. have been limited to ecological studies, studies on coastal vegetation communities, soil analysis of halophytes, and the antifungal activity of bacteria isolated from the roots of *C. scabrifolia* Steud [11–17]. On the other hand, there is no pathophysiological and pharmacological research on the extracts of *C. scabrifolia* Steud. Presently, there are no reports on the possible inhibition of inflammation in RAW264.7 cells by *C. scabrifolia* Steud. extract (CSE). Therefore, in this study, the anti-inflammatory effect of CSE against LPS-induced inflammatory response in RAW264.7 cells was elucidated. The effects of CSE on cell viability, gene expression levels of macrophage markers, and the generation of PGE_2 and NO in RAW264.7 cells were confirmed by CCK-8 assay, real-time qRT-PCR, and enzyme-linked immunosorbent assay (ELISA) assay, respectively.

Materials and Methods

Experimental materials and culture of RAW264.7 cell line

The *Carex scabrifolia* Steud. used in this study was a sample collected from Yulchon-myeon, Yeosu-si, Jeollanam-do on June 28, 2017, and was distributed from the Marine Extract Biobank of the National Marine Biodiversity Institute of Korea (NP60200003). After freeze-drying of *C. scabrifolia* Steud., its extract, *C. scabrifolia* Steud. extract (CSE), was obtained by sonication with 70% ethanol as a solvent, and was dissolved in DMSO:ethanol (1:1; vol/vol) at a concentration of 100 mg/ml and used for cellular experiments. The RAW264.7 macrophage cell line used in this study was purchased from the Korean Cell Line Bank. Dulbecco's modified Eagle's medium (DMEM; Welgene, Korea) supplemented with fetal bovine serum (Welgene) and penicillin/streptomycin (Life Technologies, USA) were used for culturing RAW264.7 macrophage. RAW264.7 macrophages were cultured in an incubator at 37℃, 5% CO_2, and 100% humidity.

Cell viability assay

Cell viability on treatment with CSE was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Japan). RAW264.7 macrophages were introduced into a tissue culture dish and then stabilized for 24 h. Subsequently, they were treated with CSE at varying concentrations for 24 h. Thereafter, CCK-8 was diluted in a phenol-red-free DMEM (Welgene) medium at a ratio of 10:1 and reacted for 1 h under the conditions of 5% CO_2, 37℃, and 100% humidity. The absorbance of the reagent was measured at a wavelength of 450 nm using a spectrophotometer (BioTek, USA), and cell viability was calculated based on the cell-free medium. The cell viability was expressed as a percentage relative to the control group.

Real-time RT-PCR

Total RNA was extracted from RAW264.7 macrophages using TRIzol® Reagent (Life Technologies). The cDNA was synthesized from RNA using SuperiorScript™ III reverse-transcriptase (Enzynomics, Korea). To confirm the expression pattern of RAW264.7 macrophage markers, gene expression was analyzed using StepOnePlus real-time qPCR machine (Applied Biosystems, USA). The TaqMan® Gene Expression Assay (Applied Biosystems) used in this experiment is summarized in Table 1.

Determination of NO production

RAW264.7 macrophages were introduced into 35 mm tissue culture dishes at a density of 2×10^5 cells/ml and

Table 1. Gene name and assay ID number in real-time RT-PCR analysis.

Symbol	Gene name	Assay ID
NOS2	Nitric oxide synthase 2, inducible	Mm00440502_m1
COX2	Cyclooxygenase 2	Mm03294838_g1
IL1α	Interleukin 1 alpha	Mm00439620_m1
IL1β	Interleukin 1 beta	Mm00434228_m1
IL6	Interleukin 6	Mm00446190_m1
TNFα	Tumor necrosis factor alpha	Mm00443258_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1

then allowed to be stabilized in an incubator at 37℃, 5% CO_2, and 100% humidity for 24 h. After the cells were treated with lipopolysaccharide (LPS; Simga-Aldrich, USA; 100 ng/ml) and CSE at each concentration and cultured for 24 h, the supernatant was harvested and 100 µl of the supernatant was used with a NO Plus Detection Kit (iNtRON, Korea). NO production was measured based on the protocol provided by the manufacturer. To briefly summarize the experimental method, after adding N1 buffer (50 µl) to 100 µl of the supernatant and allowed to react at room temperature for 10 min, then 50 µl of N2 buffer was added to react for 10 min. Thereafter, the absorbance was measured using a spectrophotometer (520–560 nm).

PGE_2-ELISA

In order to assess the effect of CSE on the production levels of PGE_2, RAW264.7 macrophages were stabilized in a cell incubator for 24 h. Subsequently, the cells were treated with LPS (100 ng/ml) and CSE at each concentration and then incubated for 6 h. The PGE_2-ELISA Kit was purchased from Pierce Endogen (USA), and the PGE_2 secreted by RAW264.7 macrophages was measured with it based on the manufacturer's protocol.

Statistical analysis

Statistical analyses were based on one-way analysis of variance. Results are expressed as means ± standard deviation of at least three independent experiments.

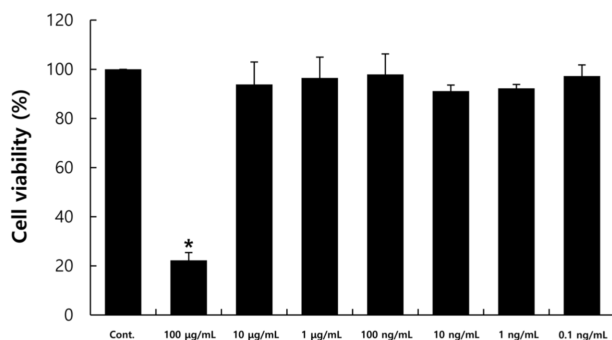

Fig. 1. Cytotoxicity of *Carex scabrifolia* Steud. extract (CSE) in RAW264.7 cells. RAW264.7 cells (5×10^4 cells/well) were seeded in 96-well cell culture plates and treated with CSE for 24 h. The CCK-8 assay was used to perform the cell viability. These results are presented as mean ± S.D. of the percentage of control optical density in triplicate. * Value relative to the control (*$p < 0.05$)

Results and Discussion

Analysis of RAW264.7 cell viability according to the concentration of *C. scabrifolia* Steud. extract

Macrophages are distributed throughout the living body and they are the immune cells that provide defense against inflammatory substances. They are also known to serve as antigen presenting cells that initiate adaptive immunity and they secrete various inflammatory cytokines [18]. RAW264.7 macrophages used in this experimental work produce inflammatory cytokines in response to lipopolysaccharides (LPS). Before confirming the anti-inflammatory effect of *C. scabrifolia* Steud. extract (CSE), the cell viability was determined by CCK-8 assay to confirm the appropriate concentrations of CSE to be applied in the experiments. The control group was not treated with the sample, while the CSE concentrations used to determine cell viability were: 100, 10, 1 µg/ml, 100, 10, 1, and 0.1 ng/ml (Fig. 1). At a 100 µg/ml concentration of CSE, the survival rate of treated RAW264.7 macrophages was significantly reduced. However, at a concentration of 10 µg/ml or lower, the survival rate of RAW264.7 macrophages was similar to that of the untreated group. Therefore, in subsequent experiments, CSE concentrations of 10, 1, and 0.1 µg/ml were used to conduct the experiments.

CSE inhibits LPS-induced dendritic transformation of RAW264.7 cells

It is known that LPS induces morphological transformations in RAW264.7 cells and turns them into dendritic-like cells [19, 20]. In this study, the effects of CSE on the morphological transformations of RAW264.7 cells induced by LPS were investigated. As shown in Fig. 2, LPS induced morphological transformations of RAW264.7 cells into dendritic-like cells, and it was confirmed that the morphological changes were attenuated by treatment with CSE in a concentration-dependent manner. The proportion of cells with dendritic morphology was significantly increased on treatment with LPS to 55.5%, and when these cells were treated with 10 µg/ml of CSE, the number of cells with dendritic morphology decreased to 5.5% (Fig. 2). Based on the results that CSE significantly inhibited the morphological transformations induced by LPS in RAW264.7 cells, the intention to confirm whether CSE can suppress inflammatory

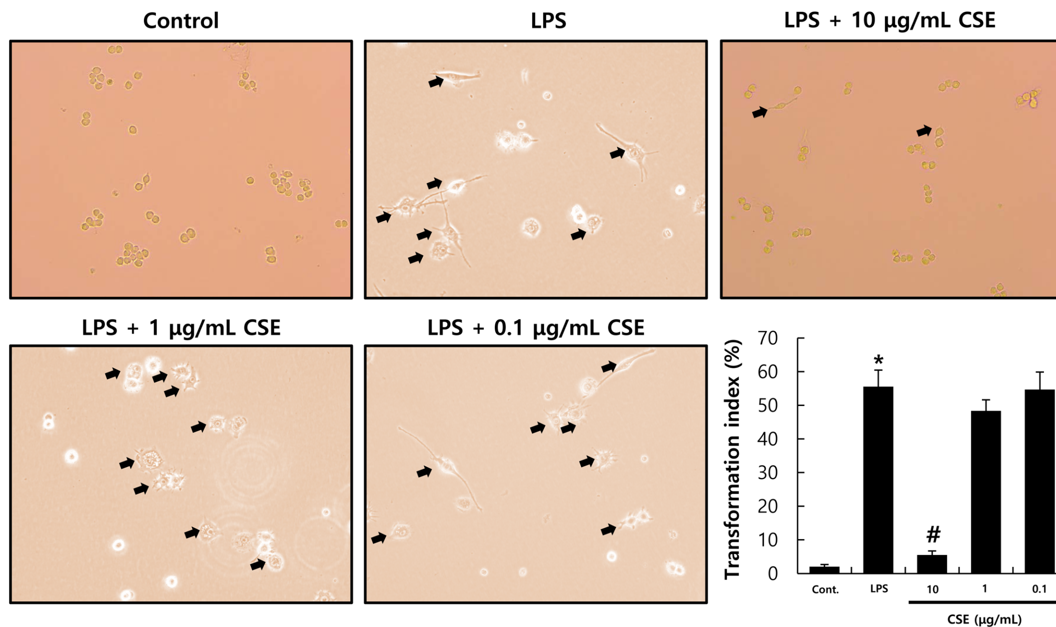

Fig. 2. Effects of CSE on dendritic transformation of LPS-stimulated RAW264.7 cells. RAW264.7 cells were stimulated with 100 ng/ml of LPS and treated with the indicated concentration of CSE in the culture medium for 24 h. Cell morphology was captured under a light microscopy at X200 magnitude. Dendritic transformation of RAW264.7 cells were indicated by arrows. The transformation index percentage was expressed as the number of cells with activated morphology relative to the total number of cells, quantified in 5 random fields. * Value relative to the control; # value relative to the LPS-treated condition; *, # $p < 0.05$

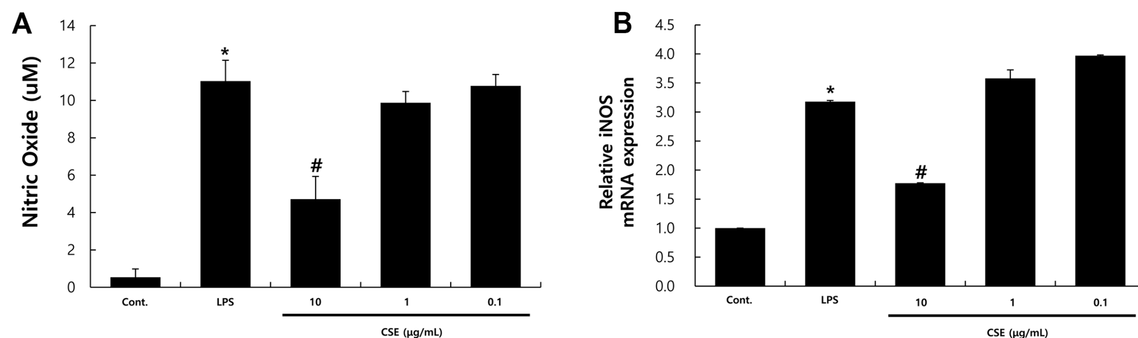

Fig. 3. Effects of CSE on NO synthesis in RAW264.7 cells. RAW264.7 cells were seeded in 35 mm cell culture dishes and treated with CSE for 24 h. The conditioned media were analyzed for NO levels using a NO detection kit (A). Real-time qPCR analysis of iNOS mRNA (B). The data represents the mean ± S.D. of three independent experiments. * Value relative to the control; # value relative to the LPS-treated condition; *, # $p < 0.05$

responses of RAW264.7 cells was necessary through additional experiments.

Effect of CSE extract on NO production

Nitric oxide (NO) is known as an important factor that regulates blood pressure, facilitates the elimination of bacterial and tumor cells, and mediates signal transduction in the living body [7]. However, excessive levels of generated NO causes damage to nerve tissues and induces intracellular gene mutations, or inflammatory responses, such as edema [5, 6, 8]. When the effect of CSE on NO production was assessed in this study, the production of NO, which was increased to a concentration of 11.0 μM by LPS, was significantly reduced to 4.7 μM when treated with 10 μg/ml of CSE (Fig. 3A).

In macrophages, LPS is known to increase the production of NO by inducing an enzyme, called inducible NO synthase (iNOS), that synthesizes it [21]. Quantitative

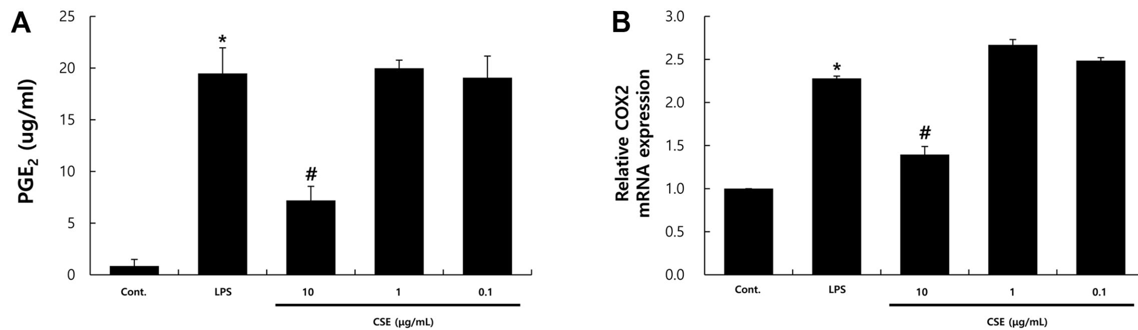

Fig. 4. Effects of CSE on PGE_2 production in RAW264.7 cells. RAW264.7 cells were seeded in 35 mm cell culture dishes and treated with CSE for 24 h. The conditioned media were analyzed for PGE_2 by PGE_2-ELISA (A). Real-time qPCR analysis of COX2 mRNA (B). The data represents the mean ± S.D. of three independent experiments. * Value relative to the control; # value relative to the LPS-treated condition; *, # $p < 0.05$

real-time RT-PCR was performed to ascertain if CSE affects the expression levels of iNOS gene. The expression levels of iNOS were significantly increased in the LPS-treated group as compared to the control group. However, it was confirmed that the expression levels of iNOS were significantly decreased in RAW264.7 cells treated with 10 μg/ml CSE when compared to the LPS-treated group (Fig. 3B).

Effect of CSE extract on PGE_2 production

Prostaglandin (PG) is an unsaturated fatty acid produced from arachidonic acid and it is known as a hormone mediating inflammatory responses [22]. Prostaglandin E2 (PGE_2), one of the PGs, is produced by the enzyme, called cyclooxygenase-2 (COX2), which mediates inflammatory responses. When the COX2 gene is over-activated or expressed, fever, pain, and inflammation are induced [21, 23]. In order to confirm the anti-inflammatory effect of CSE, it was assessed whether the PGE_2 secretion was increased or decreased by treatment with CSE. Specifically, the secretion of PGE_2 in the LPS-treated group was 19.4 μg/ml, which was significantly increased compared to the control group. On the other hand, on treatment with 10 μg/ml of CSE, the PGE_2 secretion reduced to 7.2 μg/ml, which indicates a significantly decreased secretion of PGE_2 as compared to the LPS-treated group (Fig. 4A). In addition, the expression levels of the COX2 gene increased 2.28 times in the LPS-treated group as compared to the control group. However, it decreased by 39% when compared to the LPS-treated group in the RAW264.7 cells treated with 10 μg/ml of CSE. (Fig. 4B).

Inflammation in the skin is known to promote skin aging as well as skin diseases [7, 24, 25]. Specifically, IL-1α and IL-1β are cytokines involved in the inflammatory process, immune response, and hematopoiesis. Furthermore, they are inflammatory mediators produced by macrophages, B cells, and fibroblasts in the dermal layer in which immune responses are induced [26, 27]. In particular, IL-1β has been reported as a cytokine that induces the infiltration of inflammatory cells, such as lymphocytes and neutrophils in the inflammatory sites [28, 29]. It has been reported that IL-6 induces the growth and differentiation of B cells and causes chronic inflammatory diseases, including allergies [5, 7]. On its own, TNFα is a factor that recruits neutrophils to the inflammatory site during the initial inflammatory reactions, causing edema, fever, and an acute immune response [30].

Effect of CSE extract on the expression of inflammatory cytokines

LPS promotes the production of inflammation-related factors, such as various interleukins, iNOS, and TNFα in RAW264.7 macrophages. In this study, after inducing an inflammatory response by treating RAW264.7 macrophages with LPS at 100 ng/ml, it was confirmed whether CSE increased or decreased the levels of inflammatory cytokines. Specifically, the expression level of IL-1α was increased by 48.9 times in the LPS-treated group when compared to the control group, but the LPS-induced increment was decreased by 61% when the cells were treated with 10 μg/ml of CSE (Fig. 5A). Furthermore, the level of IL-1β was increased by 77.3

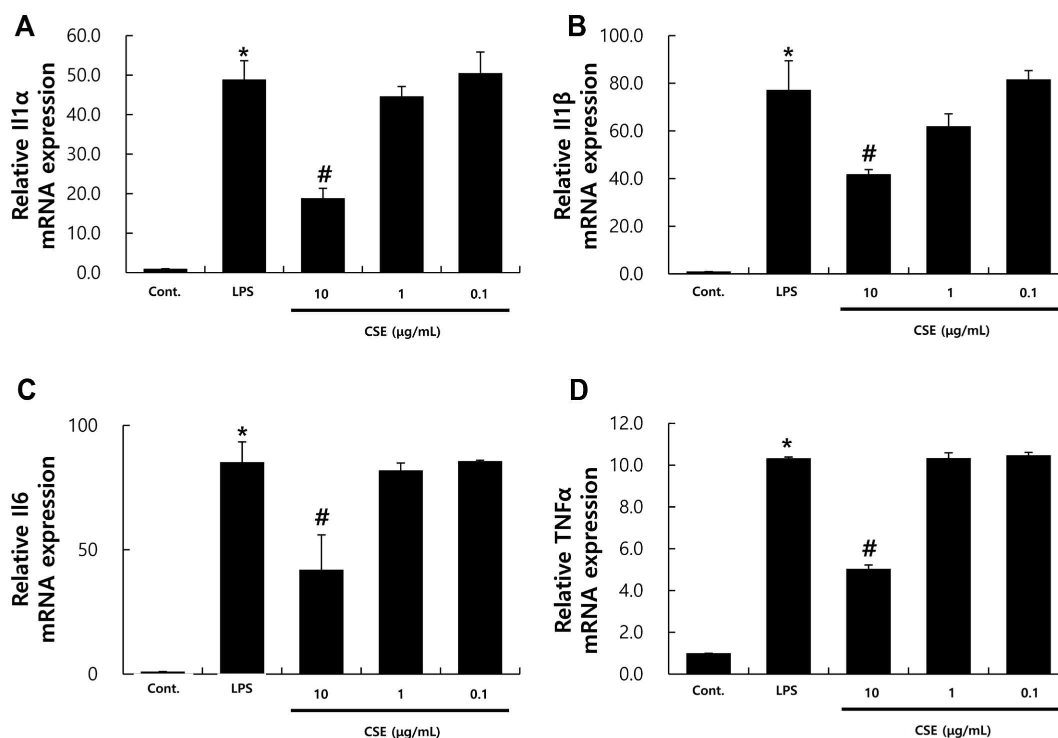

Fig. 5. Effects of CSE on the expression of inflammatory cytokines. Real-time qPCR analysis of inflammatory transcription factors was carried out for IL-1α (A), IL-1β (B), IL-6 (C), and TNFα (D). The graphs are shown as mean ± S.D. of three independent experiments. * Value relative to the control; # value relative to the LPS-treated condition; *, # $p < 0.05$

times in the LPS-treated group, but the increment in expression level was decreased by 46% when the cells were treated with 10 μg/ml of CSE (Fig. 5B). In addition, treatment of the cells with 10 μg/ml of CSE reduced the expression level of IL-6 by 51% when compared to the LPS-treated group (Fig. 5C). When the cells were treated with CSE at a concentration of 10 μg/ml, the expression level of TNFα was reduced by 51% when compared to the LPS-treated group (Fig. 5D). In the current study, CSE suppressed the expression levels of interleukins and TNFα, which are involved in inflammation, and the anti-inflammatory effect was confirmed based on the reduction both in the production levels of PGE_2 and NO in the CSE-treated group at a concentration of 10 μg/ml. The possibility of demonstrating that CSE can be used as a therapeutic agent for improving inflammatory diseases or as a raw material for pharmaceutics for alleviating atopy was confirmed in this study. Especially, in Fig. 3, 4, and Fig. 5, 10 ug/ml of CSE appears to be a concentration that specifically reduces the expression and production levels of NO, PGE_2, interleukins, and TNFα in macrophages induced by LPS inflammatory response and, therefore, it may be used in the composition of functional medicines.

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Conflicts of Interest

The authors have no financial conflicts of interest to declare.

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