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Research Note

Anti-inflammatory effect of *Geranium thunbergii* on lipopolysaccharide-stimulated RAW 264.7 cells

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Abstract *Geranium thunbergii* is a perennial plant commonly used as an oriental medicine for prevention of diarrhea, constipation, and gastrointestinal disorders. However, its anti-inflammatory effect has not been evaluated thus far. Therefore, the present study aimed to investigate the anti-inflammatory effect of *G. thunbergii*. In this study, *G. thunbergii* extracted with methanol; this methanol extract was further partitioned using various solvents, and *G. thunbergii* ethyl acetate fraction (GTEF) was obtained. To determine the anti-inflammatory activity of *G. thunbergii*, the effects of GTEF on nitric oxide (NO) production in lipopolysaccharide-stimulated RAW 264.7 cells were evaluated. GTEF suppressed NO production in a dose-dependent manner without any toxic effects. In addition, western blotting was performed to examine the effect of GTEF on expression of inducible nitric oxide synthase and cyclooxygenase-2. These results suggest that GTEF as a phytoextract may be useful for the prevention or treatment of inflammation.

Keywords: inducible nitric oxide synthase, anti-inflammatory, *Geranium thunbergii*, cyclooxygenase-2, plant

Introduction

Inflammation is a defense mechanism of the body to external harmful stimuli including infections and tissue injury (1). It is a complex process, involving various types of immune cells, clotting proteins, and signaling molecules. It is a multifaceted response mediated by activation of immune cells such as macrophages and neutrophils and is regulated by cytokines such as prostaglandin and nitric oxide (NO) (2). Macrophages are immune cells that are distributed in tissues throughout the body. They play an important role in the inflammatory response via numerous functions such as stimulating NO production and release of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6 (3). NO and prostaglandin E_2 are generated by activation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively. Excess production of NO and inflammatory mediators can lead to diseases such as pulmonary fibrosis, arthritis, asthma, and cancer (4). There are two isozymes of COX, COX-1and COX-2. COX-1 is expressed in almost all tissues whereas COX-2 is overtly induced upon lipopolysaccharide (LPS) stimulation and infection or stress induced damage (5). Therefore, the suppression of inflammatory mediators for prevention of inflammation is essential, particularly in inflammatory diseases. Recently, several studies have demonstrated that inhibition of iNOS and COX-2 gene expression produces anti-inflammatory effects via down-regulation of nuclear factor (NF-κB) and miotgen-activated protein kinases (MAPKs) signaling pathways (6).

Plants are well-known sources of natural compounds with biological activity and diverse functions. They contain various secondary metabolites such as polyphenols and carotenoids. *Geranium thunbergii* also called as ijilpul, and hyuncho, is a perennial plant belonging to the Geraniaceae family and is used as a medicinal plant in East Asia. It is suggested to possess anti-inflammatory activity (7), anti-obesity activity via improvement of lipid metabolism in high-fat diet-induced obese mice (8), and BACE1 (Beta-site APP Cleaving Enzyme 1) inhibitory activity (9). However, *G. thunbergii* as a source of anti-inflammatory compounds and its suppressive effect on inflammation have not been explored extensively. Therefore, in this study, we investigated the anti-inflammatory effect of *G. thunbergii* ethyl acetate fraction (GTEF). The anti-inflammatory effects were evaluated by measuring NO production at a non-toxic concentration and iNOS and COX-2 expression by western blot.

Materials and Methods

Materials and chemicals

Geranium thunbergii was purchased from Omniherb of Gyeongsan (Daegu, Korea) on January 2013. Samples were pulverized to 80 mesh size using Sung Chang Machine (ACM10INCH, Namyangju, Korea) and stored in a freeze at –20°C. Dulbecco's modified Eagle's medium (DMEM), phosphate

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buffered saline (PBS), fetal bovine serum (FBS), and newborn calf serum (NBCS) were purchased from Gibco Inc. (New York, USA). Penicillin and streptomycin were purchased from PAA Inc. Griess reagent, sodium bicarbonate, LPS (*Escherichia coli* O55:B5), and dimethyl sulphoxide (DMSO) was purchased from Wacko Chemical (Tokyo, Japan).

Preparation of sample

The dried sample (100 g) was extracted with 1.0 L of 95% methanol overnight at room temperature for 2 days. The methanol extract was then filtered through filter paper, Advantec 5C (Advantec, Toyo Roshi Kaisha Ltd., Tokyo, Japan) and evaporation by vacuum rotary evaporator (Büchi Rotavapor R-210, Flawil, Switzerland). The methanol extract was partitioned progressively with different solvents (H, *n*-hexane; C, chloroform; E, ethyl acetate; B, butanol; W, water). The solvent-partitioned fractions were stored in −20°C prior to use.

Cell culture and cell viability assay

RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). They were cultured in Doulbeco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 μg/mL of streptomycin 100 U/mL. Then, the cells were incubated at 37°C with 5% CO_2 and subcultured every 2 days after reaching 80% confluence. For each experiment, the cells were treated with varying concentrations of GTEF for 1 h, followed by stimulation with 1 μg/mL LPS. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were cultured in a 96-well plate for 24 h and treated with various concentrations of GTEF (12.5, 25, and 50 μg/mL) for 24 h at 37°C with 5% CO_2. MTT was added in the wells and the cells were incubated for another 4 h. Then, the supernatant was removed and the formazan crystals were dissolved in 100 μL of DMSO. Absorbance was measured at 540 nm with a microreader (Microreader Molecular Devices, Santa Clara, CA, USA).

Measurement of nitric oxide (NO) production

RAW 264.7 cells were seeded (1×10^5) in a 6-well plate and incubated for 12 h at 37°C with 5% CO_2. The cells were washed with PBS and treated with various concentrations (12.5, 25, and 50 μg/mL) of GTEF for 1 h. Then, 1 μg/mL LPS was added to the 6-well plate and incubated for 24 h. NO production was measured at 540 nm using a microplate reader (Microreader Molecular Devices) after the incorporation of 100 μL of Griess reagent.

Western blot

Raw 264.7 cells were plated onto 6-well plates and pre-incubated with various concentrations of GTEF for 1 h before stimulation with LPS (1 μg/mL) for 18 h. The cells were washed twice with ice-cold PBS then resuspended in lysis buffer (50 mM Tri-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM phenylmethylsulfony fluoride, 1% NP-40, 0.5% protease inhibitor cocktail, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4) and clarified by centrifugation at 12,000× *g* for 10 min at 4°C to remove debris. The protein concentration was determined using Bradford assay. Equal amount of total proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBST) for 1 h and incubated with COX-2, iNOS, and β-actin for 3 h at room temperature or overnight at 4°C. After washing with TBST for three times, the blot was incubated with secondary antibody for 1 h. Finally the bands were visualized with the enhanced chemiluminescence (ECL) reagents and exposed to X-ray film.

Statistical analysis

The experimental results were expressed as mean±SD values from triplicate analysis. Statistical comparison was carried out using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA) and analyzed by one-way analysis of variance (ANOVA) with Duncan multiple range test. $p<0.05$ was considered significant.

Results and Discussion

Cell viability and the effect of GTEF on NO production in LPS-stimulated RAW 264.7 cells

In the present study, the anti-inflammatory effects of GTEF were investigated. Prior to evaluating the anti-inflammatory effect of GTEF, viability of LPS stimulated RAW 264.7 cells at various GTEF concentrations was measured with MTT assay. MTT assay is one of the most sensitive and widely used methods for toxicity assessment. MTT solution is reduced to dark blue formazan when cleaved by active mitochondria, and is therefore, used to measure cell survival (10). As shown in Fig. 1A, GTEF at 12.5, 25, and 50 μg/mL has no toxic effects. Therefore, in this study, concentrations of 12.5, 25, and 50 μg/mL were selected to establish the anti-inflammatory effect of GTEF.

NO have three NOS isoforms such as neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). NO is produced by nitric oxide synthase from L-arginine which is involved in inflammation and immunoregulation process. Furthermore, iNOS increases the level of NO and can lead to tissue damage and numerous diseases. Hence, down-regulation and reduction of NO production in inflammatory response is an important pathway for the inhibition of oxidative damage and nitrative stress (11). Therefore, in this study, the anti-inflammatory effect of GTEF on LPS-stimulated RAW 264.7 cells was measured via NO production. The cells were treated with varying concentration of GTEF (without signs of toxicity) and then treated with LPS. NO production was measured after incorporating 100 μL of each cultured medium with 100 μL of Griess reagent. The results are shown in Fig. 1B. GTEF effectively inhibited NO production in a dose dependent manner with more than 60% inhibition occurring at a concentration of 50 μg/mL. Pokharel *et al.* (12) reported the iNOS inhibitory effect of 4-hydroxykobusin isolated from *G. thunbergii* in RAW 264.7 cells.

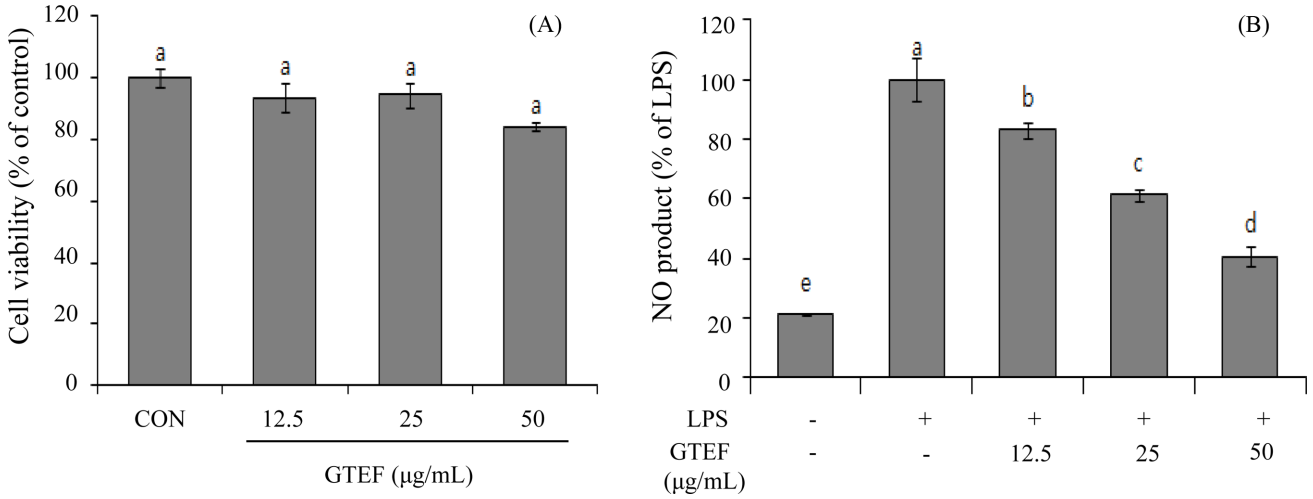

Fig. 1. Cytotoxicity of GTEF against RAW 264.7 cells (A) and inhibitory effect of NO production of GTEF on LPS-stimulated RAW 264.7 cells (B). Cells were pre-treated with GTEF for 1 h then 1 μg/mL of LPS treated for 24 h and cell viability was measured using MTT assay. Data represent the means±SD of three determinations. [a-e]Means the different letters on the bars are significantly different at $p<0.05$.

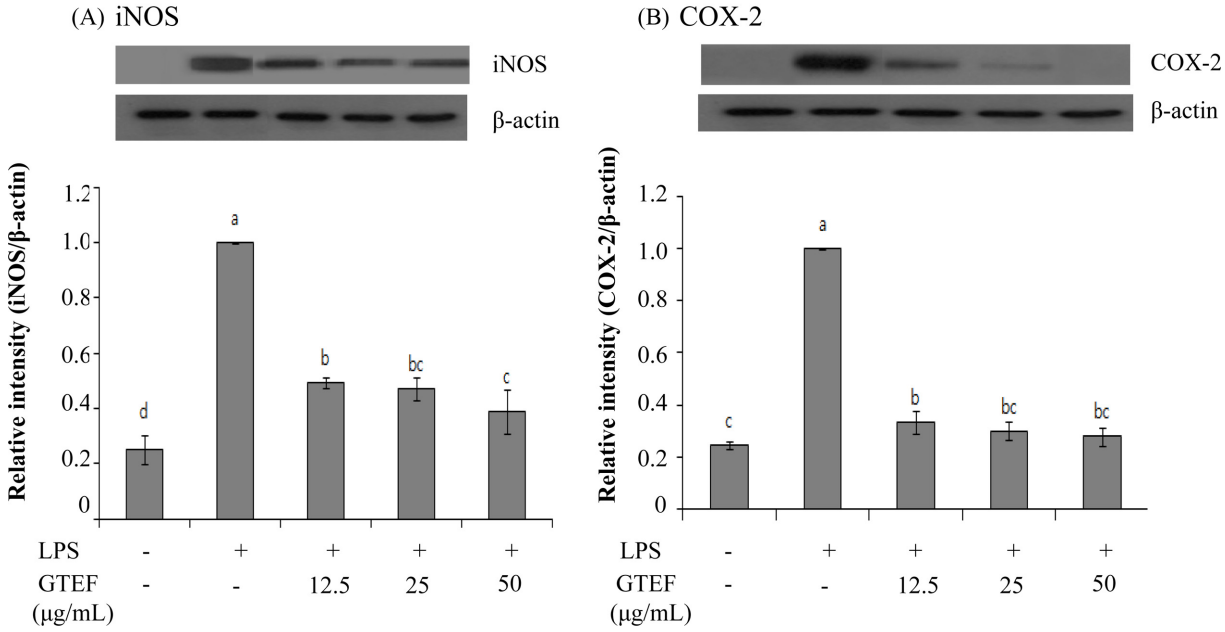

Fig. 2. Effect of GTEF on iNOS (A) and COX-2 (B) on LPS-stimulated RAW 264.7 cells. Cells were pre-treated with GTEF for 1 h then 1 μg/mL of LPS treated for 18 h. And then, cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane, and probed with iNOS and COX-2 antibody for western blot. Data represent the means±SD of three determinations. [a-d]Means the different letters on the bars are significantly different at $p<0.05$.

It was reported that 4-hydroxykobusin significantly suppressed NO production in a dose-dependent manner with approximately 90% inhibition at 100 μM. Based on these results we suggest that *G thunbergii* could be beneficial for prevention of inflammation.

The effect of GTEF on iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells

Inflammation plays a crucial role in the host defense system. The overproduction of inflammatory mediators can lead to complications such as septic shock, cancer, diabetes, atherosclerosis, and obesity (13). NO and prostaglandin E_2 are produced by activation of iNOS and COX-2, respectively. In other words, COX-2 and iNOS-mediated PGE_2 and NO production, respectively, are involved in the progression of inflammatory response. COX-2 is overtly induced upon stimulation with LPS and infection or stress-induced damage (5). The iNOS expression was detected in LPS-stimulated RAW 264.7 cells. Therefore, down- regulation of iNOS expression (an inflammatory-mediator) is important in inhibiting NO production and suppression of inflammatory mediators is an important target in inflammatory diseases.

In this study, to confirm the anti-inflammatory effect of GTEF on LPS-stimulated RAW 264.7 cells, iNOS and COX-2 levels were measured. GTEF effectively inhibited iNOS and COX-2 protein expression in a dose-dependent manner compared

to that in the unstimulated cells (Fig. 2). Pokharel *et al.* (12) reported that pretreatment of RAW 264.7 cells with 4-hydroxykobusin significantly inhibited iNOS protein expression at 30-100 μM. Recently, plant-derived natural compounds have been evaluated. Kang *et al.* (14) reported the anti-inflammatory effect of flavonoids isolated from Korean *Citrus aurantium* L., in LPS-induced RAW 264.7 cells and their results showed that the isolated flavonoids suppressed LPS-induced COX-2 and iNOS protein expression in a dose-dependent manner. Jeong and Jeong (15) reported the inhibitory effect of rheosmin isolated from pine needles via suppression of iNOS and COX-2 expression in LPS-induced RAW 264.7 cells. In addition, Chao *et al.* (16) reported the potential antioxidative and anti-inflammatory properties of polyphenols from *Gynura bicolor* DC. Chao *et al.* (16) imply that polyphenols and flavonoids present in food and plants have diverse properties such as antioxidant, anti-microbial, anti-obesity, and anti-inflammatory. Furthermore, we found that GTEF suppressed NO production by down regulating iNOS and COX-2 expression in LPS-stimulated cells. In conclusion, recently, many research focusing on natural biological activities from plant. In this study, we found that ethyl acetate fraction from *G thunbergii* methanol extract showed the anti-inflammatory effect on lipopolysaccharide-stimulated RAW 264.7 cells through inhibits iNOS and COX-2 expression. Therefore, these results suggest that GTEF may play an important role in contribute to the development of drug and agent of therapeutic for inflammation-related disorders.

Acknowledgments

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