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Improved Anti-Atopic Dermatitis Effect of *H. macrophylla* through the Generation of Hydrangenol by Microwave Processing

Youngseok Kim [1,2†], Ha-Neul Ju [1,2†], Pilju Choi [1], Taejung Kim [1], Young-Tae Park [1], Dae-Hee Lee [2*] and Jungyeob Ham [1*]

[1]*Natural Products Research Institute, Korea Institute of Science and Technology (KIST), Gangneung 25451, Republic of Korea*

[2]*Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung 25456, Republic of Korea*

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Abstract: The compounds hydrangenol and hydrangenol 8-O-glucoside are abundant in *Hydrangea macrophylla*. This study investigated the effects of hydrangenol and hydrangenol 8-O-glucoside derived from *H. macrophylla* on atopic dermatitis (AD). Compared with hydrangenol 8-O-glucoside, hydrangenol resulted in more potent inhibition of interleukin 4 (IL-4) gene expression and β-hexosaminidase release, as well as more potent inhibition of the phosphorylation of signal transducer and activator of transcription 6 (STAT6). Ultra High-Performance Liquid Chromatography (UHPLC), the concentrations of hydrangenol were found to be lower than the concentration of hydrangenol 8-O-glucoside in *H. macrophylla* extracts. To increase the hydrangenol content of *H. macrophylla* extract, component conversion methods were studied. Conclusion, microwave processing was found to be the optimal method for deglycosylation.

Keywords: *Hydrangea macrophylla*; hydrangenol; hydrangenol 8-O-glucoside; atopic dermatitis; microwave irradiation.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by severe itching, dry skin, exudative eczema, and erythematous plaques [1]. The prevalence of AD is approximately 1%–3% in adults and 10%–20% in children. Compared with rural areas, AD is more prevalent in children in industrialized areas [2]. Atopic diseases are classified as either extrinsic or intrinsic. Compared with intrinsic AD, extrinsic AD is more prevalent as it arises from hypersensitivity reactions to substances, such as food, pollen, and house dust mites [3]. These reactions are mediated by immunoglobulin E (IgE). Extrinsic AD is characterized by elevated serum levels and mRNA expression of interleukin-4 (IL-4; an inflammatory cytokine produced by T helper 2 cells) [4,5]. Treatment with

* Corresponding authors:E-Mail neogene@gwnu.ac.kr; Phone +82-33-640-2347 (D. Lee); ham0606@kist.re.kr; Phone +82-33-650-3501 (J. Ham)

† These two authors contributed equally to the work described in this study.

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calcineurin inhibitors, such as cyclosporine A, can relieve atopic skin symptoms through the inhibition of IL-4 expression [6]. IL-4 is critical for the differentiation of naive CD4+ T cells into TH2 cells.

Epidermal barrier defects play an essential role in AD pathogenesis by causing allergen penetration, water loss and infection [7]. In AD, elevation of Th2 cytokines may dysregulate barrier proteins [8]. A major pathway for IL-4 signaling in keratinocytes is the Jaks-STAT6 pathway. Th2 cytokine, IL-4, induces activation of the STAT6 signaling pathway through phosphorylation of Janus kinase (JAK)-1 and JAK3 [9].

Allergic diseases appear as hypersensitivity to allergic environmental substances in various target organs of the body (skin, nose, lungs, gastrointestinal tract, etc.), and include both IgE-mediated and non-IgE-mediated components [10]. When exposed to an allergen, B cells produce a number of antibodies called antigen-specific immunoglobulins (Ig) [11]. The allergic reaction is initiated when immunoglobulin E binds to specific receptors (FcRI) on mast cells and basophils [12]. This in turn leads to cell degranulation and release of mediators such as β-hexosaminidase, histamine, leukotriene, serotonin and platelet activating factor [13].

Hydrangea macrophylla is a deciduous shrub that belongs to the family saxifragaceae; it is abundant throughout Korea, China, Japan, and Indonesia, and it is commonly used in traditional herbal medicine [14]. In Japan, *H. macrophylla* flowers are dried and used as an antipyretic [15]. Furthermore, studies have reported the diverse pharmacological actions of hydrangeas, including antimalarial, anti-inflammatory, and antidiabetic effects [16–18]. Many studies have identified the most abundant compounds from *H. macrophylla* as hydrangenol and its derivative, hydrangenol 8-O-glucoside [19–22]. However, there has been no previous study of the effects of *H. macrophylla* on AD. Therefore, this study aimed to investigate the ability of the ethanolic extract of the aerial parts of *H. macrophylla* to inhibit the release of *β*-hexosaminidase from rat basophilic leukemia (RBL)-2H3 cells.

Microwave technology has been widely used to process dielectric materials such as food products currently, its usage is being expanded to include high-temperature material processing applications [23]. Microwave heating has competitive performance when compared with conventional methods as energy is introduced into the target material in a volumetric manner, which reduces the processing time and leads to a highly efficient process [24]. In this study, efforts were undertaken to apply this technology to the isolation of hydrangenol, which is the most abundant phytochemical in *H. macrophylla*, and to investigate its anti-AD effects.

2. Materials and Methods

2.1. Plant material, Extraction, and Isolation

Whole *H. macrophylla* was purchased from a local Herbmaul in Cheongju and it was identified by a taxonomist (Nakdonggang National Institute of Biological Resources, Dr. Jeong-Ki Hong). A voucher specimen of the species was deposited at Natural Product Research Center of Korea Institute of Science and Technology Gangneung (NP-F-195). The dried aerial parts of *H. macrophylla* (50 g) were extracted twice in 2 L of ethanol and then evaporated under reduced pressure to yield the extract (14 g).

The extract was resuspended in 1 L of distilled water and successively partitioned with 1 L of hexane and 1 L of ethyl acetate, which yielded 2.0 g and 0.9 g of residue, respectively. To identify the active ingredients that were responsible for the anti-AD activity, the activity of each fraction was evaluated in RBL2H3 and HaCaT cells. The active ethyl acetate-soluble fraction (0.9 g) was purified by semi-preparative HPLC (Gilson Inc, Middleton, WI, USA) using an acetonitrile/water mobile phase (20/80 to 60/40, v/v) and a Phenomenex Luna C18 column (250 × 10 mm, 5 μm, Phenomenex, Torrance, CA, USA) at a flow rate of 4 $mL \cdot min^{-1}$ to obtain hydrangenol 8-O-glucoside (123.8 mg, purity 97.1%) and hydrangenol (47.0 mg, purity 98.6%). The chemical structures of the two compounds were determined through comparison with previously reported references [25,26].

2.2. Analysis

The *H. macrophylla* extract was dissolved in methanol and then filtered through a 0.45-µm polytetrafluoroethylene (PTFE) membrane filter. An analytical reversed-phase Shimadzu Nexera X2 Ultra High-Performance Liquid Chromatography (UHPLC, Shimadzu, Kyoto, Janpan) system comprising of a solvent degassing unit (DGU−20A, Shimadzu), binary pump (LC−30AD, Shimadzu), autosampler (SIL−30AC, Shimadzu), system controller unit (CBM−20A, Shimadzu), photodiode array detector (SPD−M20A, Shimadzu), and column oven unit (CTO−20AC, Shimadzu) was used for qualitative and/or quantitative analyses. Electrospray ionization (ESI)-mass spectrometry (MS) was performed using a Shimadzu LCMS-2020 system for qualitative analysis. A Phenomenex Luna Omega polar C18 column (150 × 2.1 mm, 1.6 µm, Phenomenex, Torrance, CA, USA) was used. The mobile phase consisted of binary gradients of solvents A (water) and B (acetonitrile). The flow rate was set to 0.3 mL·min^{-1} and the solvent gradient was as follows: 0 min, 10% B; 20 min, 60% B; 26 min, 10% B. The detection wavelength was 200 nm and the injection volume was 3 µL. The analysis was performed three times.

2.3. Microwave Irradiation

The ethyl acetate fraction of *H. macrophylla* was processed using a microwave. For each 1 mg of dry extract, 1 mL of 50% EtOH was added into a 10 mL microwave reactor container (model no.: 908005) manufactured by CEM (Matthews, NC, USA). The dry extract was exposed to microwave irradiation in sealed containers at temperatures of 80°C, 90°C, 100°C, 110°C, 120°C, 130°C, and 140°C at a power of 150 W (a frequency of 2450 MHz) for 10, 20, 30,40, 50, 60, 70, 80, 90, 100, 110, and 120 min. The microwave-irradiated dry extracts were freeze dried to obtain the microwave-irradiated products. The microwave irradiation was conducted at a pressure of 20 atm.

2.4. Cells

RBL-2H3, which is a rat basophilic leukemia mast cell line (Korea Cell Line Bank, Seoul, Korea), was grown in Dulbecco Modified Eagle Medium (DMEM, GenDEPOT, TX, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, MA,USA) 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified 5% CO_2/air atmosphere at 37°C [27]. Human keratinocyte (HaCat) cells (German Cancer Research Center, Heidelberg, Germany) were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified 5% CO_2/air atmosphere at 37°C.

2.5. Expression of IL-4 from RBL-2H3 Cells

RBL-2H3 cells were seeded in DMEM (with 10% FBS) containing either dimethyl sulfoxide (DMSO), *H. macrophylla* extract (10 µM), hydrangenol (10 µM), or hydrangenol 8-O-glucoside (10 µM) for 1 h. Then, treatment with phorbol 12-myristate 13-acetate (PMA, sigma aldrich, MA, USA)/ionomycin (PI, sigma aldrich, MA, USA) was applied for 8 h to induce an AD-like condition. The cells were then harvested to synthesize complementary deoxyribonucleic acid (cDNA), after which real-time quantitative polymerase chain reaction (qPCR) was used to measure IL-4 mRNA expression. Total RNA was isolated from cells using TRIzol Reagent (Thermo Fisher Scientific, MA,USA) in accordance with the manufacturer's instructions. The accumulation of PCR products was observed directly by checking for increases in the reporter dye. At each time point, cytokine expression levels in the treated cells were compared with those in controls using the comparative cycle threshold (Ct) method. The TaqMan probes and primers used were Rn01456866_m1 for IL-4 and Hs03003631_g1 for the reference gene, 18s RNA; all probes and primers were obtained from Life Technologies, Carlsbad, CA, USA.

2.6. β-Hexosaminidase Release Assay

RBL-2H3 cells were treated overnight with anti-dinitrophenyl (anti-DNP) IgE (sigma aldrich, MA, USA), rinsed with Siraganian buffer (119 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 25 mM PIPES, 5.6 mM glucose, 0.1% bovine serum albumin, pH 7.2, biosolution, Seoul, Korea) and then incubated in buffer for 10 min. The cells were then treated with DMSO or separately with each of the isolates of *H. macrophylla* extract, hydrangenol, or hydrangenol 8-O-glucoside (10, 30 μM) for 20 min, and then sensitized with DNP-bovine serum albumin (BSA, sigma aldrich, MA, USA) antigen (10 μg/mL) for 30 min to induce degranulation. The supernatant was then transferred to a 96-well plate and incubated with 1 mM 4-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate in 0.1 M citrate buffer for 1 h at 37°C. The absorbance at 405 nm was measured using a microplate reader [28].

2.7. Expression of STAT6 in HaCaT Cells

HaCaT cells were seeded in DMEM (with 10% FBS) containing either dimethyl sulfoxide (DMSO), *H. macrophylla* extract (10 μM), hydrangenol (10 μM), or hydrangenol 8-O-glucoside (10 μM) for 1 h and then treated with human recombinant IL-4 protein (hIL-4; R&D systems, MN, USA) for 30 min. To analyze the protein expression, HaCaT cells were harvested and homogenized at 4°C in lysis buffer. After centrifugation, the cell debris was discarded, and the protein concentration was determined using the bicinchoninic acid (BCA) assay. Then, 20 mg of protein was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA).

The membranes were blocked with 5% skimmed milk at room temperature for 1 h and then incubated with primary antibodies. For western blotting, anti-STAT6 and anti-phospo-STAT6 (Cell Signaling Technology, Beverly, MA, USA) were used as the primary antibodies. Anti-rabbit IgG-horseradish peroxidase (HRP) and anti-mouse IgG-HRP were purchased from Cell Signaling Technology and used as secondary antibodies. The protein expression signal was detected with Pierce® ECL Western Blotting substrate (Thermo Fisher Scientific, MA, USA). The immunoreactive bands were visualized using an enhanced Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA). An anti-β-actin antibody was used to monitor protein loading in each lane. The densitometric analysis was performed using ImageJ software.

2.8. Statistical Analysis

All quantitative data for this study were obtained through at least two independent experiments. *In vitro* data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-hoc analysis.

3. Results and Discussion

3.1. Conversion of Hydrangenol 8-O-glucoside into Hydrangenol using Microwave Irradiation

The chromatograms for hydrangenol and hydrangenol 8-O-glucoside derived from the prepared *H. macrophylla* extracts are shown in Figure 1. Then, we investigated the effect of processing temperature and reaction time on the content of hydrangenol in the *H. macrophylla* extract. When microwave irradiation was performed at temperature between 80°C and 110°C and a reaction time between 10 and 90 min, the content of the major constituent (hydrangenol 8-O-glucoside) gradually decreased, whereas that of the minor constituent (hydrangenol) increased by deglycosylatio n. The generated amounts of hydrangenol reached a maximum after microwave irradiation at 90°C for 90 min (see Supporting Information for details). In addition, thermal decarboxylation of the dihydroisocoumarin skeleton of hydrangenol occurred after microwave irradiation at temperatures above 120°C.

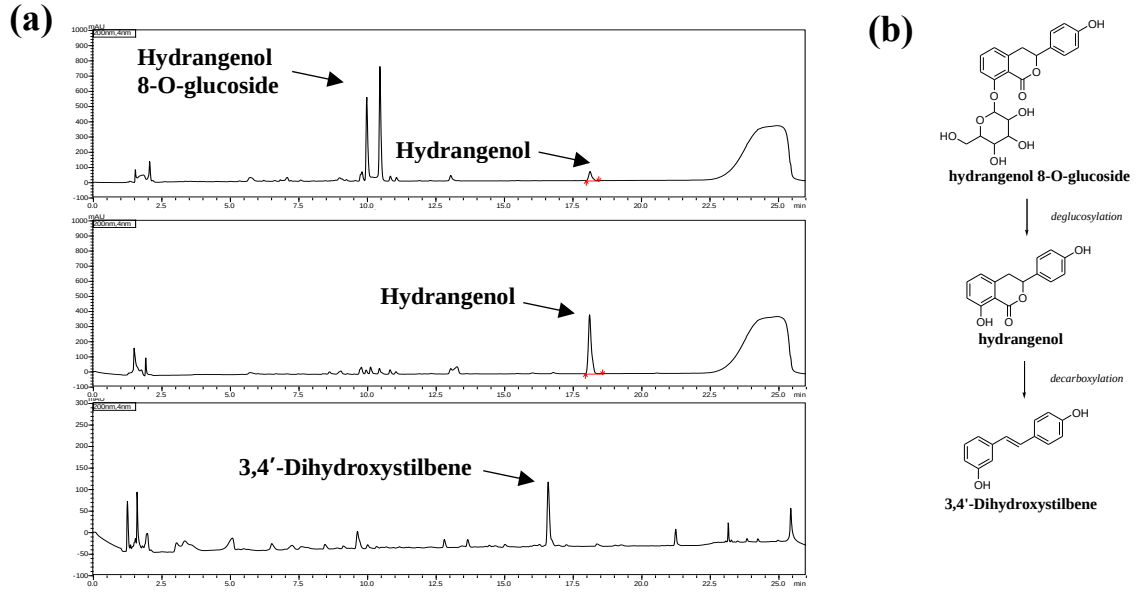
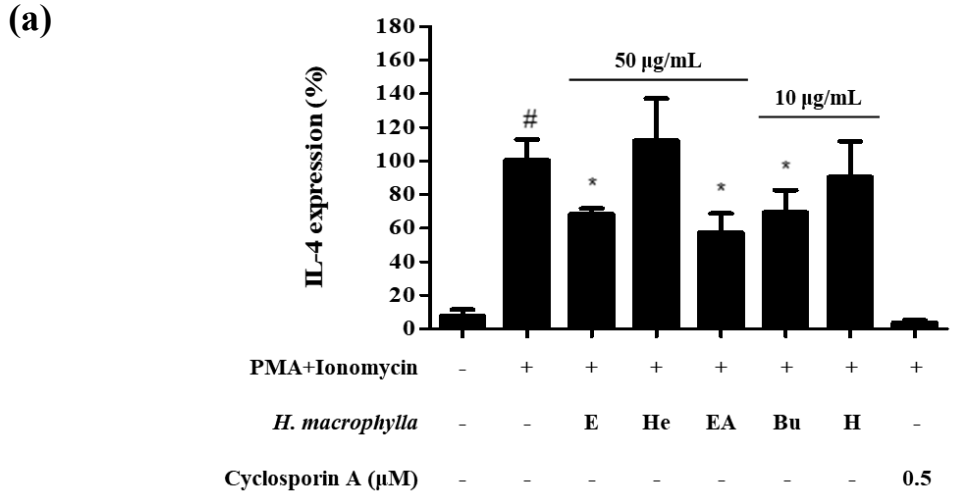


Figure 1. Effect of microwave irradiation on the conversion of hydrangenol 8-O-glucoside to hydrangenol. (a) Ultra high-performance liquid chromatography (UHPLC) chromatograms of *H. macrophylla* and the microwave-irradiated extracts. (b) Chemical structures of the main compounds in *H. macrophylla* and the microwave-irradiated extracts.

3.2. Effects of H. macrophylla Extracts on IL-4 Expression in RBL-2H3 Cells

IL-4 mRNA expression was significantly increased by PI treatment for 8 h (6.7-fold versus vehicle controls). As shown in Figure 2, IL-4 mRNA expression was lower in cells that were pre-treated with *H. macrophylla* EA extract and hydrangenol and hydrangenol 8-O-glucoside and then stimulated with PI, compared with the PI-treated controls. Both compounds exhibited concentration-dependent inhibitory effects on IL-4 expression, with hydrangenol showing a better overall effect.



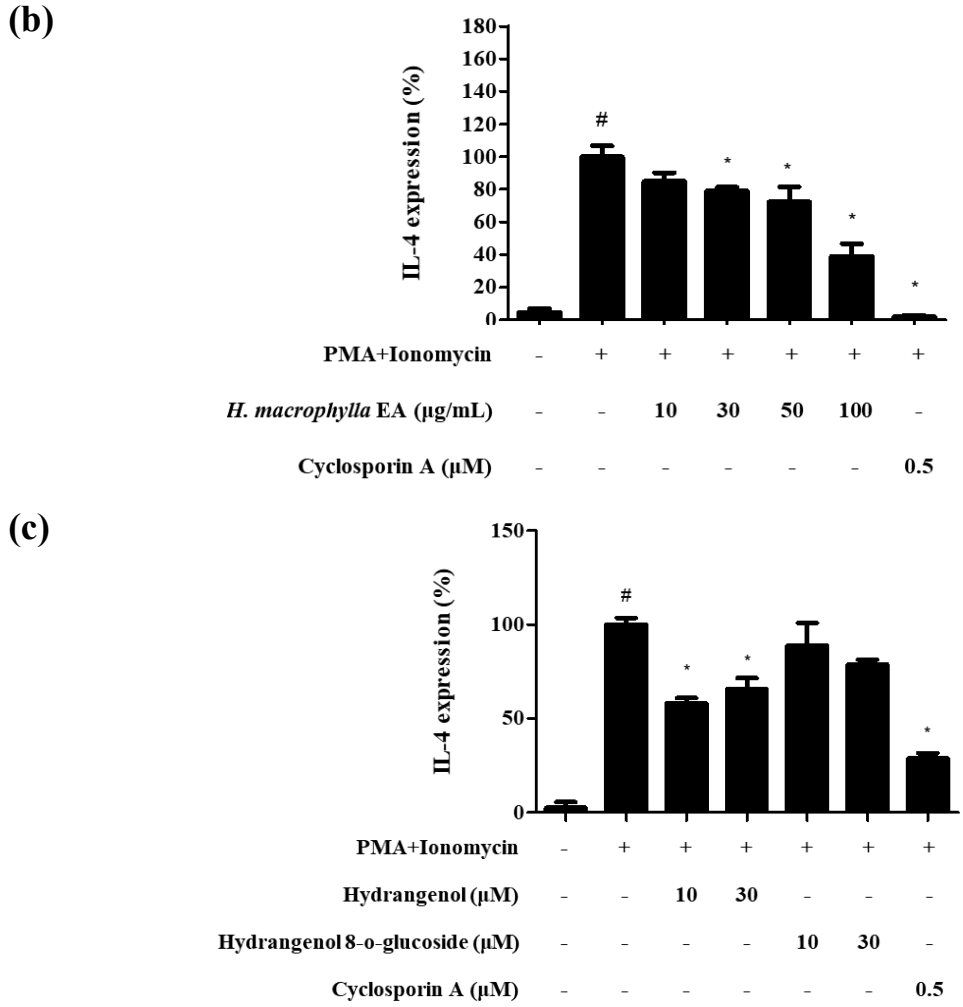


Figure 2. Anti-inflammatory effects of *H. macrophylla* extract and fraction in rat basophilic leukemia cell l ine (RBL-2H3) cells. (a) Effects of *H. macrophylla* extract and fraction on interleukin 4 (IL-4) m RNA expression in PMA/ionomycin (PI)-mediated RBL-2H3 cells. (b) Effects of *H. macrophylla* EA fraction on interleukin 4 (IL-4) mRNA expression in PMA/ionomycin (PI)-sensitized RBL-2 H3 cells. (c) Effects of hydrangenol and hydrangenol 8-O-glucoside on interleukin 4 (IL-4) mRN A expression in PMA/ionomycin (PI)-sensitized RBL-2H3 cells. Results are expressed as the mea ns ± SD of two independent experiments. # $p < 0.05$ vs. vehicle control; * $p < 0.05$ vs. PI. [#]E : EtOH extract, He : Hexane fraction, EA : Ethyl acetate fraction, Bu : Butanol fraction, H : Water fraction

3.3. Effects of H. macrophylla Extracts on β-Hexosaminidase Release in RBL-2H3 Cells

DNP-BSA was previously shown to increase β-hexosaminidase release in IgE-sensitized RBL-2H3 cells [29]. As shown in Figure 3, the release of β-hexosaminidase was lower in cells pretreated with *H. macrophylla* EA extract and hydrangenol and hydrangenol 8-O-glucoside than in the control. In particular, hydrangenol markedly inhibited β-hexosaminidase release in RBL-2H3 cells. DNP-BSA–IgE alone caused significant β-hexosaminidase release (3.5-fold versus untreated controls), but treatment with 30 μM hydrangenol reduced this increase by 42%.

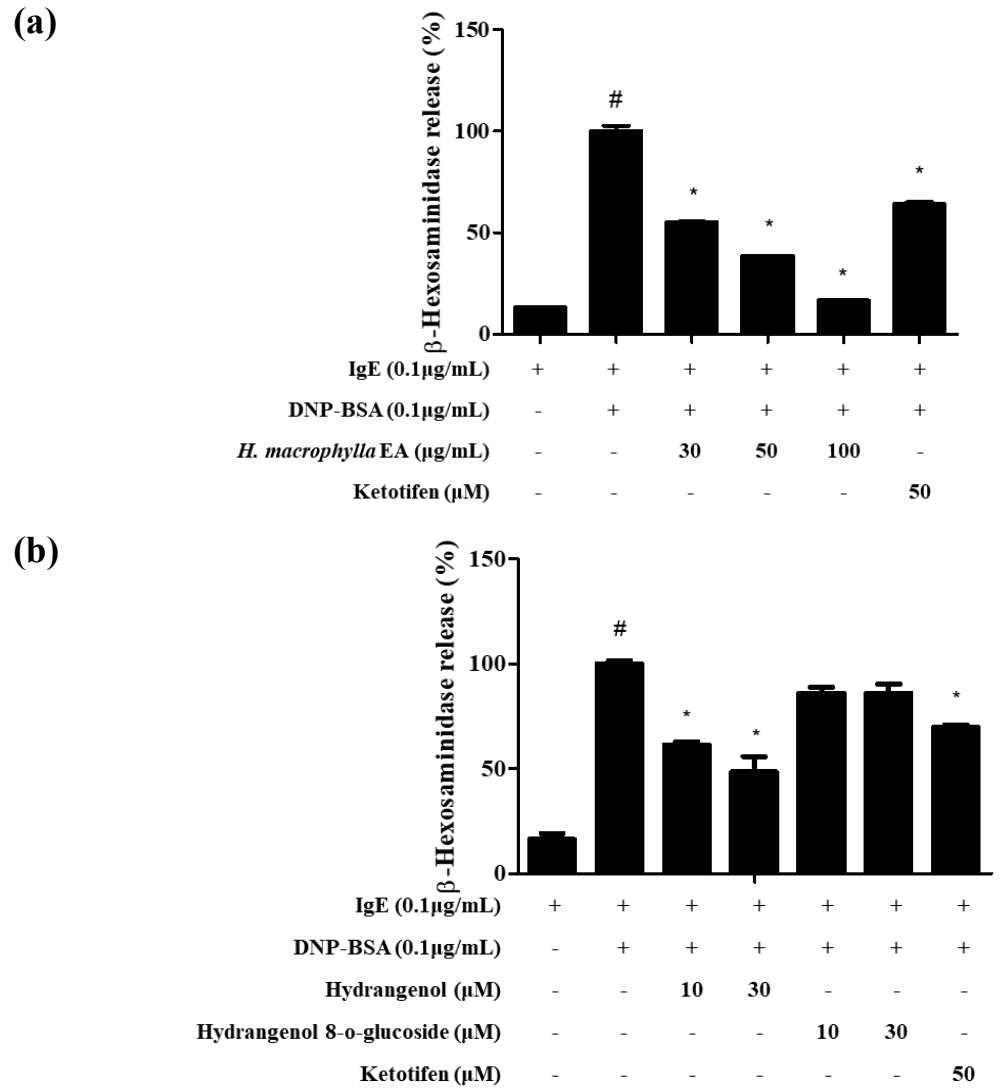


Figure 3. Anti-inflammatory effects of *H. macrophylla* extract and fraction in rat basophilic leukemia cell line (RBL-2H3) cells. (a) Effects of *H. macrophylla* extract on β-hexosaminidase release from immunoglobulin E (IgE)-sensitized RBL-2H3 cells. (b) Effects of hydrangenol and hydrangenol 8-O-glucoside on β-hexosaminidase release from immunoglobulin E (IgE)-sensitized RBL-2H3 cells. Results are expressed as the means ± SD of two independent experiments. # $p < 0.05$ vs. vehicle control; * $p < 0.05$ vs. DNP-BSA
[#]DNP-BSA : Dinitrophenyl bovine serum albumin

3.4. Effects of H. macrophylla Extracts on STAT6 Phosphorylation in HaCaT Cells

STAT6 phosphorylation was significantly increased by recombinant hIL-4 treatment for 15 min (6.7-fold versus the vehicle control). As shown in Figure 4, STAT6 phosphorylation was lower in cells that were pre-treated with *H. macrophylla* EA extract and hydrangenol and hydrangenol 8-O-glucoside and then stimulated with recombinant hIL4 compared with the controls treated with recombinant hIL4. Hydrangenol had a stronger inhibitory effect on STAT6 phosphorylation than hydrangenol 8-O-glucoside.

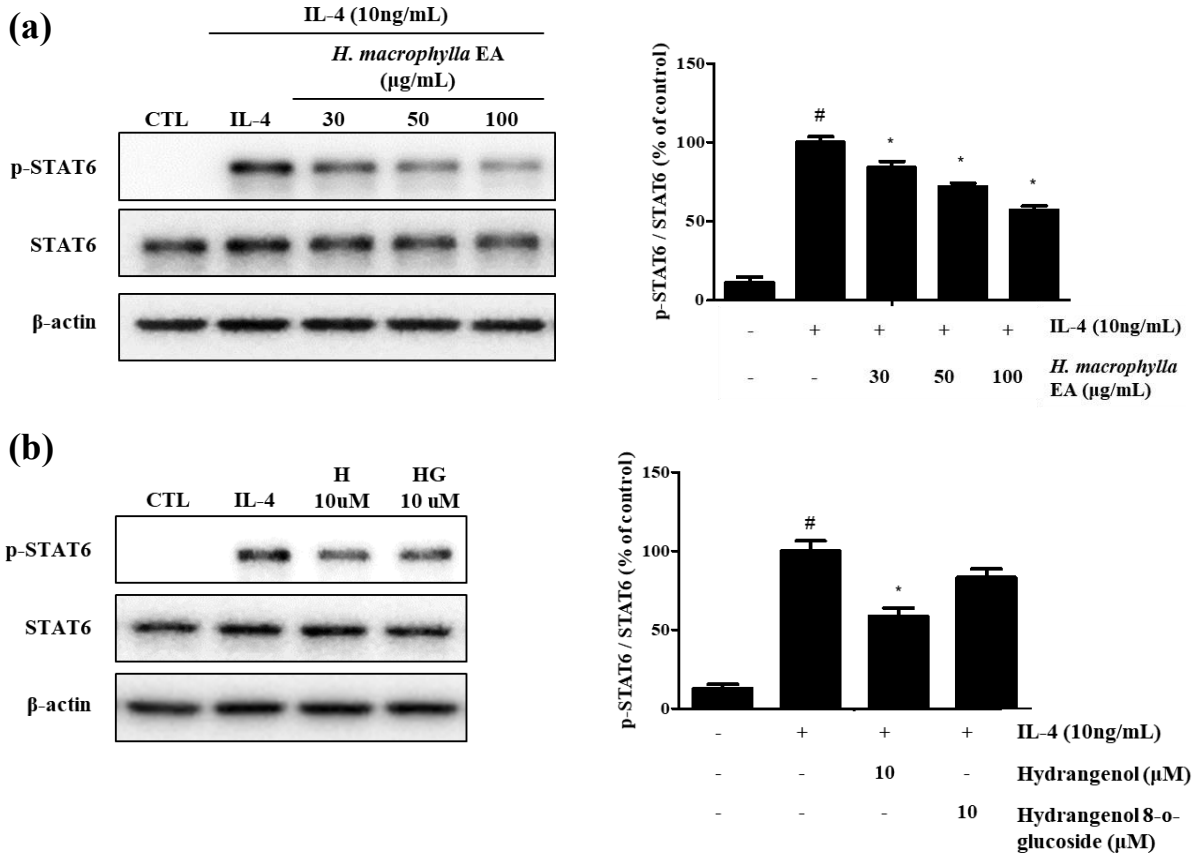


Figure 4. STAT6 phosphorylation of *H. macrophylla* extract and fraction in Human Keratinocyte (HaCaT) cells. (a) Effects of *H. macrophylla* extract on STAT6 phosphorylation from human recombinant IL-4-mediated HaCaT cells. (b) Effects of hydrangenol and hydrangenol 8-O-glucoside on STAT6 phosphorylation from human recombinant IL-4-mediated HaCaT cells. Results are expressed as the means ± SD of two independent experiments. # $p < 0.05$ vs. vehicle control; * $p < 0.05$ vs. IL-4.

[#]STAT6 : Signal transducer and activator of transcription 6, H : Hydrangenol, HG : Hydrangenol 8-O-glucoside

In conclusion, this study aimed to investigate the two constituents of *H. macrophylla* extract with effective anti-atopic dermatitis. The results showed that the minor constituent of *H. macrophylla* extract, hydrangenol, exhibited significant activity, indicating that hydrangenol was the active constituent of *H. macrophylla* that exerted immunosuppressive effects on inflammation. It suppressed the expression of IL-4, a Th2 cytokine, suppressed cell degranulation, and suppressed the expression of STAT6, a major pathway of IL-4 signaling in keratinocytes. This can alleviate the imbalance of Th2 cytokine, a major cause of atopy, and increase the function of the skin barrier protein. Furthermore, several methods to increase the hydrangenol content of *H. macrophylla* extracts were investigated, and microwave irradiation was found to be the optimal method for deglycosylation. This may be useful for the prevention and amelioration of AD, and also established a basis for the future utilization of hydrangenol.

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Supporting Information

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ORCID
Youngseok Kim: 0000-0002-1599-3258
Ha-Neul Ju: 0000-0002-3405-8679
Pilju Choi: 0000-0003-2838-941X
Taejung Kim: 0000-0002-9449-4763
Young-Tae Park: 0000-0003-1787-1577
Dae-Hee Lee: 0000-0002-8960-1632
Jungyeob Ham: 0000-0003-3046-9267

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