



Anti-allergic effects and mechanisms of action of the ethanolic extract of *Angelica gigas* in dinitrofluorobenzene-induced inflammation models

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ABSTRACT

To confirm the anti-allergic effects of the ethanolic extract of *Angelica gigas* (EAG), the levels of ear erythema, ear weight, vascular leakage, hematology, tumor-necrosis factor- α , interleukin-6 and immunoglobulin E from mice sensitized with 2,4-dinitrofluorobenzene were examined. The results showed that EAG reduced ear erythema and ear weight; we also found that Evan's blue leakage decreased. Furthermore, the levels of interleukin-6 and immunoglobulin E in the serum were significantly inhibited. In RAW264.7 cells, EAG drastically inhibited the mRNA levels of inducible nitric oxide synthase, tumor-necrosis factor- α and macrophage inflammatory protein-1 β , suggesting that EAG may inhibit the release of pro-inflammatory cytokines and acute neutrophilic inflammation. Western blot analysis showed that EAG inhibited nuclear factor- κ B- and extracellular signal-regulated protein kinase-dependent inflammatory pathways. Interestingly, EAG effectively inhibited the release of β -hexosaminidase, a granule marker from mast cells. Taken together, our results demonstrate that EAG inhibits focal and systemic inflammatory and allergic reactions, and holds great promise for the treatment of several inflammatory diseases.

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1. Introduction

Traditionally, the dried roots of *Angelica gigas* Nakai (Korean angelica), known by the Korean name as 'Cham-dang-gui', have been used in oriental medicine not only for the treatment of anemia and some circulatory disorders, but also as a sedative, an analgesic, and/or a tonic agent (Jung et al., 1991). Recently, several coumarin derivatives, such as decursins, decursinol, decursinol angelate, nodakenin, nodakenetin, and umbelliferone, were isolated from *Angelica gigas* (Chi and Kim, 1970; Kang et al., 2003; Lee et al., 2003a). Additional pharmacological effects of these derivatives include anti-tumor (Ahn et al., 1997; Kim et al., 2005; Jiang et al., 2006), anti-platelet aggregation (Lee et al., 2003b), anti-*Helicobacter pylori* (Bae et al., 1998) and neuroprotective (Lee et al., 2003a; Kang et al., 2005) effects, and

memory-enhancing activities (Kang et al., 2001, 2003; Yan et al., 2004).

Earlier studies demonstrated that another species of angelica, *Angelica acutiloba* Kitakawa (Japanese angelica), had analgesic and anti-inflammatory effects (Tanaka et al., 1971; Cho et al., 1982). Recently, decursinol from *Angelica gigas* was shown to possess anti-nociceptive activity (Choi et al., 2003). New reports have shown that decursin inhibited the induction of inflammatory mediators from lipopolysaccharide-stimulated macrophages (Kim et al., 2006). Moreover, we demonstrated that the ethanolic extract of *Angelica gigas* (EAG) had anti-inflammatory activities in carrageenan-induced air-pouch inflammation and croton oil-induced ear inflammation models (Shin et al., 2009, 2010).

The strong skin sensitizer 2,4-dinitrofluorobenzene (DNFB) is known to provoke hypersensitive responses after repeated topical application (Ahmed and Blose, 1983; Gorbachev and Fairchild, 2001). Mechanically, DNFB is known to mediate allergic contact dermatitis by CD8⁺ cytotoxic T cells, and thus is used to produce skin contact hypersensitivity in mice models (Gorbachev

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Table 1
Primer sequences for real-time RT-PCR.

Gene	Primer	Amino acid sequences	Product size (bp)	Accession
TNF- α	5' Primer	5'-GATTATGGCTCAGGGTCCAA	192	NM_013693
	3' Primer	5'-GAGACAGAGGCAACCTGACC		
iNOS	5' Primer	5'-TGCCCCTGGAAGTTTCTCTT	252	NM_010927
	3' Primer	5'-ACTGCCCCAGTTTTTGATCC		
MIP-1 β	5' Primer	5'-CCCAGCTCTGTGCAACCTA	221	M35590
	3' Primer	5'-TGGAGCAAAGACTGCTGGTC		
β -Actin	5' Primer	5'-CTAGGCACCAGGCTGTGATG	291	NM_007393
	3' Primer	5'-CTACGTACATGGCTGGGGTG		

and Fairchild, 2004). Repeated topical application of DNFB triggers a hypersensitive reaction and induces a shift in the cutaneous cytokine milieu from a Th1 to a Th2 profile (Kitagaki et al., 1997; Tomimori et al., 2005), which might be induced by an alteration in local cytokine levels, augmented eosinophil recruitment, or allergic inflammation. In a cell level, macrophages constitute a major sub-population of cells in the mouse dermis, and it has been reported that RAW264.7 cells (a mouse macrophage cell line) well responded to DNFB (Kim et al., 2008). Therefore, in this study, we examined the effects of EAG on allergic contact dermatitis mice, and signal transductions and mRNA levels in RAW264.7 cells were also investigated to verify whether EAG has a therapeutic advantage on chemical-induced allergic contact dermatitis, which might be related to its anti-inflammatory activity.

2. Materials and methods

2.1. Preparation of extract

The root of *Angelica gigas* was purchased from a local oriental medicine market in Seoul, Korea in 2006. The plant materials were authenticated by an expert at Chungbuk National University. The dried root (5 kg) of *Angelica gigas* was extracted with ethanol in an ultrasonic apparatus. Upon removal of the solvent under vacuum, the ethanolic extract yielded 500 g. The extract was dissolved in 50% ethanol, and orally administered in a volume of 4 ml/kg.

2.2. Animals

Five-week-old male ICR mice were acquired from the Orient Bio Co. (Seongnam, Korea). The animals were housed at the Laboratory Animal Research Center of Chungbuk National University, Korea. The animals ($n=10$ per group) were maintained in a room with a constant temperature of $22 \pm 1^\circ\text{C}$, a relative humidity of $55 \pm 10\%$, and a 12-h light/dark cycle. They were fed standard rodent chow (Samyang, Gapyeong, Korea) and purified tap water *ad libitum*.

The animals were acclimated to the laboratory environment for 1 week before the experiments began. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised in 1996), and the protocol was approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Research Center.

2.3. DNFB-induced ear inflammation

Mice were orally administered EAG (50, 160, or 500 mg/kg) for 12 consecutive days. On the 5th day, 1 h following the administration of EAG, 100 μl of DNFB (0.5% in acetone + olive oil (4:1)) or its vehicle was applied to the mice's clipped and shaven abdominal skin with adhesive tape. On the 10th and 12th days, 20 μl of DNFB (0.2%) was reapplied to both surfaces of the ears.

Table 2
Effect of EAG on the hematology in DNFB-induced ear inflammation.

Treatment (mg/kg)	Control	DNFB alone	+EAG (50)	+EAG (160)	+EAG (500)
WBC ($\times 10^3$ cells/ μl)	2.81 $\pm$ 0.94	4.85 $\pm$ 1.08*	2.68 $\pm$ 0.71#	2.59 $\pm$ 0.59#	3.01 $\pm$ 0.58#
Neutrophils	0.60 $\pm$ 0.39	2.57 $\pm$ 1.05*	1.06 $\pm$ 0.48#	0.88 $\pm$ 0.39#	1.20 $\pm$ 0.97#
Eosinophils	0.06 $\pm$ 0.04	0.03 $\pm$ 0.02	0.06 $\pm$ 0.03	0.08 $\pm$ 0.05	0.05 $\pm$ 0.03
Basophils	0.01 $\pm$ 0.01	0.03 $\pm$ 0.01	0.06 $\pm$ 0.01	0.03 $\pm$ 0.02	0.02 $\pm$ 0.02
Monocytes	0.05 $\pm$ 0.04	0.07 $\pm$ 0.04	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.02
Lymphocytes	1.93 $\pm$ 0.81	2.00 $\pm$ 1.34	1.45 $\pm$ 0.63	1.51 $\pm$ 0.67	1.70 $\pm$ 0.62

DNFB, dinitrofluorobenzene; WBC, white blood cells.

* Significantly different from control ($P < 0.05$).

Significantly different from DNFB alone ($P < 0.05$).

2.4. Measurement of erythema and edema

Twenty-four hour after the final DNFB application, the mice ears were scored for erythema according to a grading system (Oliveria De Melo et al., 2006), and then punctured with a cork borer (6 mm in diameter) and weighed. Separately, to quantify vascular leakage, some of the animals ($n=5$ /group) were intravenously injected with 5 ml/kg of Evan's blue (1% in saline) immediately before DNFB application, and the ear tissues were punctured after transcardial perfusion with cold saline (100 ml/kg). Under anesthesia, the left ventricle of mice was exposed, injected with a 23G needle connected to a bottle containing saline, and perfused in a flow rate of 1 ml/min (Choi et al., 2004). Evan's blue was extracted by immersing ear tissues in 0.5 ml of formamide at 37°C for 3 days, and quantified at 620 nm.

2.5. Blood analysis

Whole blood was collected from the orbital plexus. The number of white blood cells (WBC) and differential counts of neutrophils, eosinophils, basophils, monocytes and lymphocytes were analyzed using an automatic blood cell counter (Bayer, Leverkusen, Germany). Blood tumor-necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were measured with the Mouse Quantikine immunoassay kits (R&D, Minneapolis, USA). Immunoglobulin E (IgE) was analyzed by the ELISA procedures of the manufacturer (Molecular Device, Emax, USA). Fifty μl of each of the standard IgE solutions or 5 μl of the sample and 45 μl of the buffer solution was aliquoted into the wells. After incubation at room temperature for 1 h, the wells were rinsed 3 times with the washing buffer. Next, 50 μl of a biotin-conjugated antibody was added to each well and incubated for 2 h at room temperature. After the reaction occurred and the samples were washed 3 times, a horse radish peroxidase-avidin solution (50 μl) was added to each well and incubated at room temperature for 1 h. After washing 3 times, 50 μl of the chromogenic substrate (3,3',5,5'-tetranethylbenzidine) reagent was added and incubated for 20 min. A reaction stopper (50 μl) was added and the absorbance of each well was measured at 450 nm within 30 min.

2.6. Cytotoxicity assay

We investigated the cytotoxicity of RAW264.7 cells in response to various concentrations of EAG following a 24-h culture period using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The system uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo phenyl)-2H-tetrazolium, monosodium salt (WST-8), which produces a water-soluble colored formazan upon bioreduction in the presence of the electron carrier, 1-methoxy PMS. The reaction plates were measured at 450 nm and the data from triplicate cultures were expressed as percent viability of the non-treated control.

2.7. Quantitative real-time polymerase-chain reaction (PCR) assay

RAW264.7 cells were placed into a 6-well culture plate at a density of 1.25×10^6 cells/ml, and pre-treated with EAG (1, 5, 10 $\mu\text{g}/\text{ml}$) 1 h before DNFB (5 $\mu\text{g}/\text{ml}$) treatment. All cultures were incubated at 37°C and 5% CO_2 . Total RNA extracts from RAW264.7 cells were prepared by the Trizol method. In brief, the cells were lysed using 1 ml Trizol reagent (Invitrogen, Carlsbad, USA), 200 μl chloroform

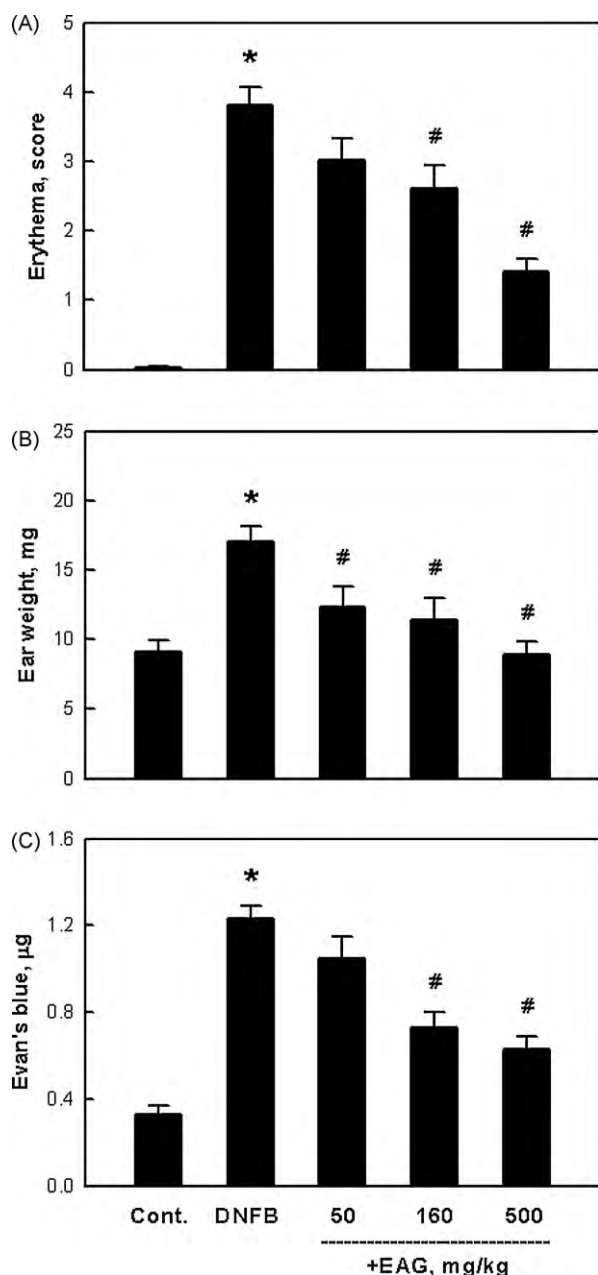


Fig. 1. Erythema score (A), ear weight (B) and Evan's blue leakage (C) in DNFB-induced mouse ear inflammation. EAG was orally administered to mice, and inflammation was induced by applying 100 µl of DNFB [0.5% in acetone + olive oil (4:1) to the abdomen followed by 0.2% to the ears]. Twenty-four hour later, the ears were scored for erythema: 0, no visible; 1, barely perceptible; 2, moderate; 3, pink-red; 4, severe or bright red; 5, very severe. The ears were punctured with a cork borer (6 mm in diameter) and weighed, and Evan's blue was extracted with formamide. *Significantly different from control ($P < 0.05$). #Significantly different from DNFB alone ($P < 0.05$).

was added after 1-min incubation at room temperature, and the mixture was centrifuged at 13,000 rpm for 10 min. The aqueous layer was transferred to another tube and the RNA was precipitated by centrifugation with 500 µl isopropanol at 13,000 rpm for 5 min. RNA pellets were washed in 700 µl cold ethanol (70% in DEPC water) and dried in air. cDNA was synthesized from RNA by the reverse transcription of 1 µg of total RNA using the Improm-II reverse transcription system (Promega, Madison, USA) and oligo dT primers in a total volume of 20 µl. PCR amplification was performed using the primers described in Table 1 (Bioneer, Deajeon, Korea). Quantitative real-time PCR reactions were run on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) using the SYBR Green PCR Master Mix (Qiagen, Valencia, USA) in 20 µl reaction mixtures. Each real-time-PCR master mix contained 10 µl of the 2X enzyme mastermix, 7.0 µl of RNase free water, 1 µl of each primer (10 pM each) and 1 µl of the diluted template. PCR was performed with

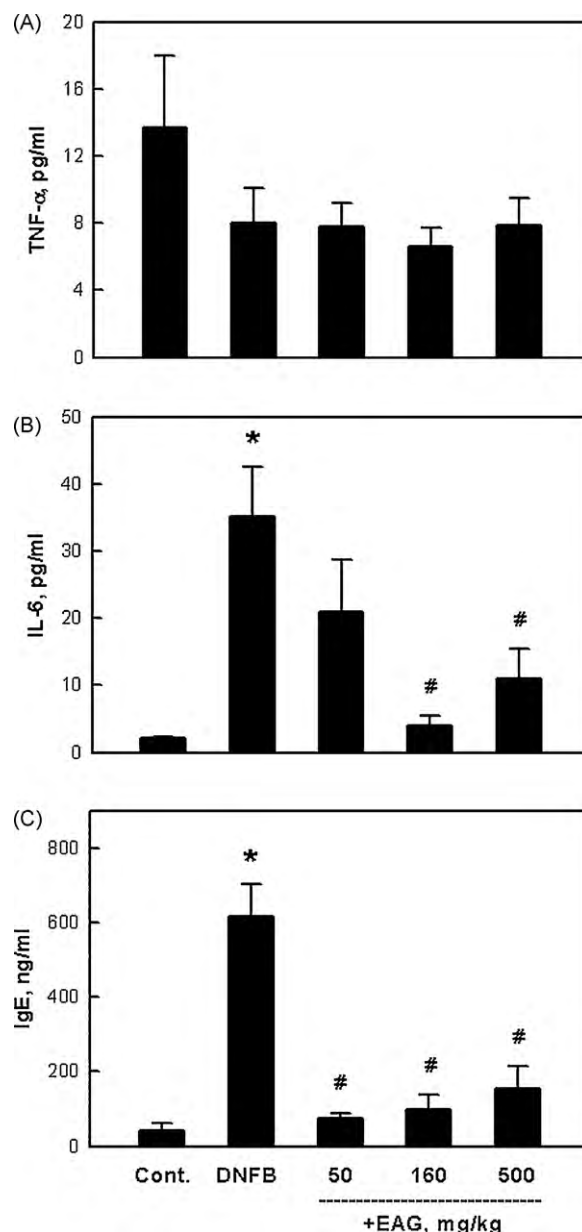


Fig. 2. The inhibition of blood TNF-α (A), IL-6 (B) and IgE (C) in DNFB-induced ear inflammation. EAG was orally administered to mice, and inflammation was induced by applying DNFB as described in Fig. 1 legend. Twenty-four hour later, the serum was analyzed using the Mouse TNF-α and IL-6 Immunoassay kits and Mouse IgE ELISA kit as described in Section 2. *Significantly different from control ($P < 0.05$). #Significantly different from DNFB alone ($P < 0.05$).

an initial pre-incubation step for 10 min at 95 °C, followed by 45 cycles of 95 °C for 15 s, annealing at 52 °C for 15 s and an extension at 72 °C for 10 s. The melting curve analysis was used to confirm the formation of the expected PCR product, and products from all assays were subjected to a 1.2% agarose gel electrophoresis to confirm that they had the correct lengths. An inter-run calibrator was used, and a standard curve was created for each gene to obtain PCR efficiencies. Relative sample expression levels were calculated using Rotor-Gene 6000 Series Software 1.7, and were expressed relative to β-actin and corrected for run variability. Data for the experimental samples were expressed as the percentage of the internal control gene.

2.8. Western blot analysis

RAW264.7 cells were lysed in 1% radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Roche, Mannheim, Germany) and whole cell lysates were separated by 10% SDS-PAGE. After electrophoresis, the proteins were transferred onto PVDF membranes and the membranes were blocked with 5% skim milk in a Tris-buffered saline solution with 0.1% Tween-20.

Immunoblotting with primary antibodies, anti-inhibitor of nuclear factor- κ B (I κ B), anti-phospho-I κ B, anti-extracellular signal-regulated protein kinase 1/2 (ERK1/2), and anti-phospho-ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, USA) was followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Stressgen, Ann Arbor, USA). Blots were developed using an ECL solution. Densitometric measurements were conducted with Gelquant software (MiniBIS Pro, Jerusalem, Israel).

2.9. β -Hexosaminidase secretion assay

Degranulation was determined by measuring the release of a granule marker, β -hexosaminidase (Joo et al., 2009). RBL-2H3 cells were grown on 48-well plates (2.5×10^5 cells/well) and experiments were carried out 3 h after plating. Cells were then treated overnight with 1 μ g/ml of IgE (Sigma, St. Louis, USA). To remove excess IgE before stimulation, cells were washed 4 times with an extracellular buffer (125 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 1.5 mM MgCl_2 , 20 mmol HEPES, pH 7.3). The cells were then stimulated with 400 ng/ml of dinitrophenyl-human serum albumin (DNP-HSA, Sigma), suspended in 500 μ l of extracellular buffer with 0.1% BSA, and incubated at 37 °C for 1 h. Following incubation, 50 μ l of the supernatant was incubated with 200 μ l of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosamine (Sigma) in a 0.05 M citrate buffer (pH 4.5) for 3 h at 37 °C. The enzyme reaction was terminated by the addition of 500 μ l of 0.05 M sodium carbonate buffer (pH 10.0) and the OD of each reaction was read at 405 nm. The effects of the various treatments on β -hexosaminidase release were described as % of the IgE control.

2.10. Statistical analysis

The results were expressed as mean $\pm$ S.E.M. The significance of the differences was analyzed by a one-way analysis of variance followed by a Dunnett's *t*-test correction, with $P < 0.05$ as a criterion of difference.

3. Results

3.1. Effects of topical application of EAG on DNFB-induced allergic contact dermatitis

To evaluate the potential effect of EAG on allergic contact dermatitis, the DNFB-induced ear erythema, ear weight, Evan's blue

leakage, plasma cytokine proteins, and IgE in mice were examined. Fig. 1(A) and (B) showed that EAG significantly reduced the ear erythema and ear weight at around 160 mg/kg ($P < 0.05$), comparing to DNFB control group, in which a profound erythema was induced after a repeated topical application of 0.5% DNFB followed by 0.2% DNFB. We also observed that EAG significantly decreased Evan's blue leakage in DNFB-induced mouse ears when the EAG dose was increased to up 160 mg/kg (Fig. 1(C)). Interestingly, EAG effectively lowered blood WBC and neutrophils when compared to the DNFB-treated group ($P < 0.05$) (Table 2), suggesting the inhibition of DNFB-induced leukocytes response. Although the blood level of TNF- α was not attenuated (Fig. 2(A)), IL-6 was significantly reduced when larger doses (160 and 500 mg/kg) of EAG were administered ($P < 0.05$) (Fig. 2(B)), and the level of IgE was also significantly reduced at all EAG doses (50–500 mg/kg) of EAG ($P < 0.05$) (Fig. 2(C)).

3.2. Effects of EAG on pro-inflammatory signal pathways in DNFB-treated RAW264.7 cells

To investigate whether EAG regulates the inflammatory signal transduction induced by DNFB, the phosphorylation patterns of I κ B and ERK were analyzed, and pro-inflammatory genes, inducible nitric oxide synthase (iNOS) and TNF- α , were also examined in RAW264.7 macrophage cells. Fig. 3(A) shows that EAG concentrations from 0.01 to 10 μ g/ml were non-cytotoxic when exposed for up to 24 h. The greatest cytotoxic effect (>90%) was observed with 100 μ g/ml ($P < 0.05$). In regards to gene expression, 10 μ g/ml of EAG significantly inhibited the levels of iNOS, TNF- α , and macrophage inflammatory protein-1 β (MIP-1 β) mRNA compared to DNFB alone ($P < 0.05$) (Fig. 3(B–D)). Coincidentally, I κ B phosphorylation was well inhibited when treated with 10 μ g/ml of EAG ($P < 0.05$) (Fig. 4(A) and (B)), suggesting that EAG can play a critical role in inhibiting

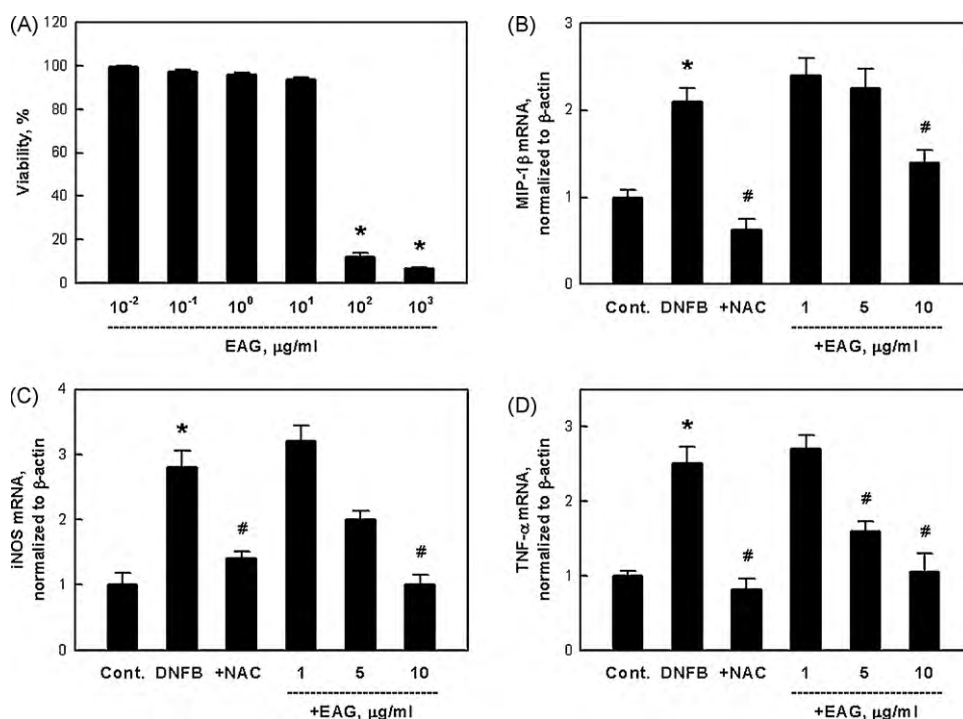


Fig. 3. Pro-inflammatory cytokines and MIP-1 β mRNA levels and cytotoxicity in RAW264.7 macrophage cells. For cytotoxicity, cells were cultured for 24 h after treatment with EAG; for the RT-PCR, cells were pre-treated with EAG 1 h before DNFB treatment. Then, cells were cultured for an additional 2 h. *N*-Acetyl-L-cysteine (NAC) was used as a positive control for mRNA analysis. (A) RAW264.7 cells were exposed to EAG (10^{-2} to 10^3 μ g/ml) for 24 h. Concentration-dependent cytotoxicity was measured as percent viability of the non-treated control. (B–D) Isolated mRNA from RAW264.7 cells treated with DNFB (5 μ g/ml) and/or NAC (10 mM) or EAG (1–10 μ g/ml) were analyzed by real-time RT-PCR for iNOS, TNF- α , MIP-1 β and β -actin. Results were internally confirmed by comparative cycle count (Ct, cycle number threshold) against β -actin as the standard gene. *Significantly different from control ($P < 0.05$). #Significantly different from DNFB alone ($P < 0.05$).

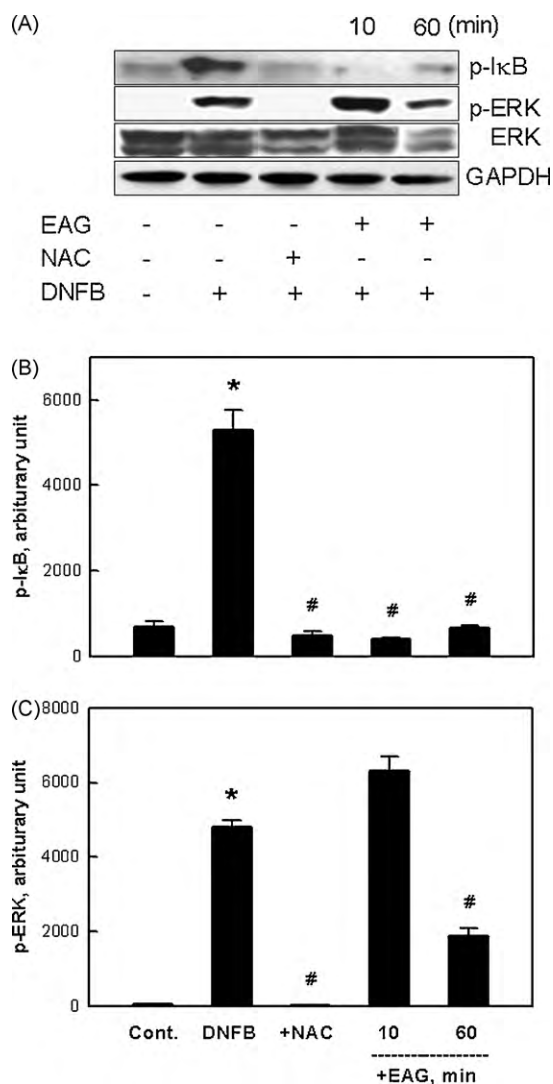


Fig. 4. Inhibition of DNFB-induced IκB and ERK activation in RAW264.7 cells. Cells were treated with DNFB (5 μg/ml) and/or NAC (10 mM) or EAG (1–10 μg/ml) for the indicated times (10–60 min). (A) Cell lysates were analyzed by Western blotting using phosphospecific antibodies to IκB or ERK. (B and C) The amount of p-IκB and p-ERK was analyzed by densitometric quantification as described in Section 2. GAPDH was used as an internal standard and data are mean values from experiments performed in duplicate. *Significantly different from control ($P < 0.05$). #Significantly different from DNFB alone ($P < 0.05$).

the inflammatory process via regulating NF-κB-dependent signal pathways. Furthermore, EAG inhibited the phosphorylation of ERK ($P < 0.05$) (Fig. 4(A) and (C)), which can diminish inflammatory gene expression (Heageman, 2003).

3.3. Effects of EAG on the release of β-hexosaminidase from RBL-2H3 cells

The release of β-hexosaminidase, an indicator of mast cell degranulation, was measured in culture supernatants after treatment with DNP-HSA (Fig. 5). Compared to the cyclosporine A (CsA) positive control, EAG effectively inhibited the release of β-hexosaminidase (49%) from RBL-2H3 cells at 10 μg/ml. EAG alone, without antigen stimulation, did not affect β-hexosaminidase secretion (data not shown).

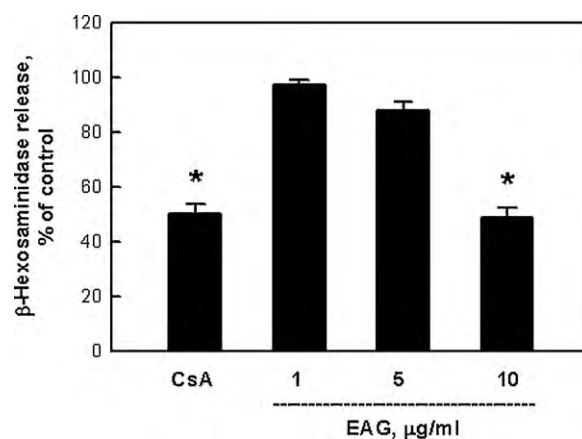


Fig. 5. The inhibition of β-hexosaminidase release in RBL-2H3 mast cells. Cells were cultured for 24 h as described in Section 2, and CsA was used as a positive control for the inhibition of β-hexosaminidase release. RBL-2H3 cells (2.5×10^5 /well) were sensitized with 1 μg/ml of DNP-specific IgE overnight and pre-treated with varying concentrations of EAG (1–10 μg/ml) for 30 min. Cells were then stimulated with 400 ng/ml of DNP-HSA for 1 h. The absorbance was measured using a microplate reader at 405 nm. *Significantly different from DNP-HSA alone (100%) ($P < 0.05$).

4. Discussion

Several coumarin derivatives such as decursins, decursinol, decursinol angelate, nodakenin, nodakenetin, and umbelliferone were isolated from *Angelica gigas* (Chi and Kim, 1970; Kang et al., 2003; Lee et al., 2003a). Decursinol from *Angelica gigas* was recently shown to possess anti-nociceptive activity (Choi et al., 2003) and decursin was shown to inhibit the induction of inflammatory mediators from lipopolysaccharide-stimulated macrophages (Kim et al., 2006). Moreover, in a previous study, we demonstrated that EAG had anti-inflammatory activities in a carrageenan-induced air-pouch inflammation model (Shin et al., 2009). In this experiment, we found that WBC that migrated into the carrageenan-injected inflammation site was markedly inhibited by 1.8-fold relative to the control when administered with EAG at doses higher than 160 mg/kg. In addition, neutrophils were also greatly inhibited by 3-fold relative to the control. In a follow-up study, it was found that decursin and decursinol angelate were the major coumarin derivatives of EAG exerting anti-inflammatory activities in croton oil-induced inflammation model (Shin et al., 2010). Therefore, it is believed that these coumarin derivatives contained in the extract can inhibit both the migration of neutrophils and the activation of macrophages. Moreover, decursin may inhibit cholesteryl ester synthesis in macrophages, and the site of inhibition was one of the steps between the exiting of cholesterol from lysosomes and cholesterol esterification in the endoplasmic reticulum (Ohshiro et al., 2006). These effects of the *Angelica gigas* extract on macrophages might be due to deactivation of the inflammatory signal pathways in the cells. The observation that decursin blocks lipopolysaccharide-stimulated nuclear factor-κB (NF-κB) activation and inhibits cholesteryl ester synthesis supports this hypothesis (Kim et al., 2006; Ohshiro et al., 2006).

The most remarkable efficacy was achieved in the DNFB-induced inflammation model, in which both erythema and edema recovered almost fully following administration of a high dose (500 mg/kg) of the extract of *Angelica gigas*, as measured 24 h after the experiment. DNFB has been used to produce skin contact hypersensitivity in mice (Gorbachev and Fairchild, 2004), and the repeated topical application of DNFB produces a contact hypersensitivity reaction and induces a shift in the cutaneous cytokine milieu from a Th1 to a Th2 profile (Kitagaki et al., 1997; Tomimori et al., 2005). Th2 activation results in type I immediate hypersen-

sitivity, including atopic dermatitis and respiratory asthma, while Th1 leads to type IV delayed hypersensitivity, such as allergic contact dermatitis. Systemically, TNF- α , IL-1 β , and IL-6 are synthesized and released in response to injury (Kushner, 1989). TNF- α is a potent stimulator of iNOS gene expression in certain cell types. It also induces the chemotaxis of neutrophils and T lymphocytes and the expressions of adhesion molecules (Xie et al., 1993). Notably, RAW264.7 cells stimulated for 1 h with DNFB highly expressed TNF- α and iNOS mRNA, although it was demonstrated that DNFB exhibited cytotoxicity in 6 h (Kim et al., 2009). Accordingly, it is believed that tissue or blood macrophages played roles via cytokine release and chemotaxis at early stage, although blood TNF- α did not increase 24 h after exposure to DNFB. IL-6 is a pro-inflammatory cytokine that plays an important role in the acute phase response (Catell et al., 1990; Heinrich et al., 1990). Our results showed that IL-6 increased in parallel with IgE when repeatedly treated with DNFB. However, EAG significantly alleviated the increase in IL-6 and IgE levels, suggesting that EAG effectively inhibited the immune response to DNFB stimulation via T cells or macrophages. Macrophages in skin are crucial host defense element together with Langerhans cells, and thus RAW264.7 macrophage cells were adopted in this study to speculate the signal pathways induced by DNFB (Ohshiro et al., 2006). In regards to gene expression, EAG effectively inhibited the expression of pro-inflammatory genes, such as iNOS, and TNF- α . Moreover, MIP-1 β , which belongs to the family of chemotactic cytokines, was also inhibited when treated with EAG. These results suggest that EAG may inactivate granulocytes (neutrophils, eosinophils and basophils), which can lead to acute neutrophilic inflammation. Signaling experiments conducted in this study confirmed that EAG might be associated with the inhibition of NF- κ B and ERK-dependent inflammation, which are the main signaling pathways that lead to the transcription of most of the pro-inflammatory genes. Furthermore, the inhibition of β -hexosaminidase release from mast cells strongly suggests that EAG is a promising pharmacological candidate for skin disorders, such as allergic contact dermatitis or atopic dermatitis.

In summary, our results demonstrate that EAG may have ideal properties to effectively treat allergic contact dermatitis or atopic dermatitis, as it contains pharmacologically active coumarin derivatives for inflammation. Moreover, due to its lipophilic properties, it may have a high affinity for the skin compartment and a low potential for absorption into systemic circulation (Meingassner et al., 2005). Thus, EAG has the potential to be a good source for developing a topical therapy for allergic dermatitis. Therefore, we conclude that EAG is a promising topical candidate that may effectively suppress transcription of pro-inflammatory repertoires, IgE production, and mast cell activation (a calcium-activated response similar to T cell activation in atopic dermatitis).

Conflict of interest

The authors declare that there are no conflicts of interest.

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