

RESEARCH ARTICLE

Effects of *Angelica acutiloba* on mast cell-mediated allergic reactions *in vitro* and *in vivo*

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Abstract

The root of *Angelica acutiloba* is a widely used herbal medicine which has been used as a typical therapeutic for allergic diseases in traditional medicine. This study was aimed to investigate the effects of *A. acutiloba* on allergic reactions in *in vitro* and *in vivo* models and its mechanism of action. *A. acutiloba* was extracted by maceration with 80% ethanol (AAE) and standardized by high-performance liquid chromatography. We investigated the effect of AAE on phorbol-12-myristate-13-acetate plus calcium ionophore A23187 (PMACI)-induced cytokine release; phosphorylation of JNK, ERK, and p38 in human mast cell-1 (HMC-1); and compound 48/80-induced release of histamine in rat peritoneal mast cells (RPMCs). We also investigated the effects on Evans blue (EB) extravasation induced by anti-DNP IgE in rats. Treatment with 1, 10 and 100 µg/ml AAE concentration-dependently inhibited the release of cytokines (tumor necrosis factor-α, interleukin (IL) -6, and IL-8) and phosphorylation of ERK and JNK induced by PMACI in HMC-1 cells, but it did not inhibit the phosphorylation of p38. It also inhibited compound 48/80-induced histamine release in RPMCs. Oral administration of 271 mg/kg AAE inhibited EB extravasation in a passive cutaneous anaphylaxis rat model. In conclusion, AAE inhibited mast cell-derived allergic reactions by inhibiting the release of histamine, the production of pro-inflammatory cytokines, and the phosphorylation of ERK and JNK.

Keywords: *Angelica acutiloba*, IgE-mediated allergic diseases, mast cell, passive cutaneous anaphylaxis, cytokine, histamine

Introduction

The root of *Angelica acutiloba*, an herbal medicine distributed in east Asia, has been widely used to nourish blood, and moisten the intestines for the treatment of gynecological diseases, hemodysfunction, and constipation.^(1,2) In the field of traditional medicine, the root of *Angelica acutiloba* can be regarded as an therapeutic choice in the treatment of allergic diseases.⁽³⁾

Allergy is defined as a hypersensitivity disorder caused by environmental substances known as allergens.^(4,5) Mast cells have been well known as the effector cell in IgE-mediated immediate hypersensitivity and allergic

reactions.^(6,7) When mast cells are activated, they release the inflammatory mediators. Among them, cytokines are immunomodulatory substances that control proliferation, differentiation, and activity of immune cells.⁽⁸⁾ Histamine enhances vascular permeability and inflammatory reactions.⁽⁹⁾

To date, *A. acutiloba* has been reported to have anti-tumor,⁽¹⁰⁾ anti-inflammatory,^(11–13) immunostimulatory,⁽¹⁴⁾ anti-complementary,⁽¹⁵⁾ insecticidal,⁽¹⁶⁾ and anti-diabetes activities.⁽¹⁷⁾ Although some studies have demonstrated the anti-inflammatory effects of *A. acutiloba*, the anti-allergic activity has not been studied yet. Considering

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the traditional uses and pharmacological activities, we planned to investigate the effects and mechanism of action of *A. acutiloba* on allergic reactions in the present study.

Lots of studies of allergic reactions have revealed several inducers of mast cell activation. Phorbol-12-myristate-13-acetate (PMA) plus calcium ionophore A23187 and compound 48/80 have been widely used to induce mast cell activation and well documented.⁽¹⁸⁾ Several models have been used in *in vivo* studies, including the well-known passive cutaneous anaphylaxis (PCA) model, which is induced by injection of anti-dinitrophenyl (DNP) IgE and used to investigate the effects of agents on allergic dermatitis.⁽¹⁹⁾

In the present study, we investigated the effect of *A. acutiloba* on PMA plus calcium ionophore A23187-induced cytokine release and the compound 48/80-induced release of histamine in a human mast cell (HMC) and rat peritoneal mast cell (RPMC), respectively. In a further investigation, we focused on the effect of *A. acutiloba* on the production and expression of the components of the MAPK pathways (ERK, JNK, and p38) in HMCs. In the *in vivo* study, we investigated the effects of dorsal skin injection of anti-DNP IgE on Evans blue (EB) extravasation in rats.

Materials and methods

Reagents

Decursin, compound 48/80, anti-DNP IgE, DNP-human serum albumin (HSA), PMA, A23187, and EB were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant interleukin (IL)-6 and IL-8, biotinylated IL-6 and IL-8, and anti-human IL-6 and IL-8 were purchased from BD Biosciences (San Diego, CA, USA). Recombinant tumor necrosis factor- α (TNF- α), biotinylated TNF- α , and anti-human TNF- α were purchased from R&D Systems (Minneapolis, MN, USA). 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the cell culture media Iscove's Modified Dulbecco's Medium (IMDM), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). The histamine kit was purchased from Oxford Biomedical Research (Oxford, MI, USA). All antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Sample preparation and high-performance liquid chromatography (HPLC) analysis

A. acutiloba roots were obtained from Kyung Hee University Medical Center (Seoul, Korea). Samples were identified by Professor Youngmin Bu of Kyung Hee University. Sample specimen (A023) of *A. acutiloba* roots were deposited at the Department of Anatomy, College of Oriental Medicine, Kyung Hee University, Seoul, Korea. One hundred grams of root was extracted by maceration with 80% EtOH (2000 ml), and kept in an airtight container for 2 weeks. The mixture was filtered

using Whatman filter paper, evaporated *in vacuo* at 60°C, and freeze dried to obtain *A. acutiloba* root EtOH extract (AAE, 41.0 g).

For the quantitative analysis of AAE, 10.0 mg of extract was dissolved in 2.0 ml of MeOH and analyzed by HPLC (Waters 2695 Alliance System with a 2996 PDAD detector and a Waters Empower System, Ver. 5.00; Waters, Milford, MA, USA). The separation was performed on a Gemini Reversed Phase C₁₈ column (4.6×150 mm, 5.0 μ m; Phenomenex, CA, USA). The mobile phase consisted of acetonitrile and distilled water (7:3). The flow rate was kept constant at 1.0 ml/min. The sample injection volume was 10 μ l. Absorbance was measured at 330 nm with a run time of 65 min. Decursin was used as a standard for AAE. The standard calibration curve of decursin, $y = 35944x - 621.28$ ($R^2 = 0.9991$), was used. The average content of decursin in *A. acutiloba* was calculated to be 0.980 ± 0.121 mg/g ($N = 2$), from the above formula (Figure 1).

Animals

Eight-week-old male Sprague-Dawley (SD) rats were purchased from Samtako (Ossan, Gyeonggi-do, Korea). Animal care and all experimental procedures were approved by and conducted according to the guidelines of the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-11-024). The rats were housed under controlled temperature ($22 \pm 2^\circ\text{C}$) with constant humidity and 12-hour light/dark cycles (lights on from 0700 to 1900), and food and water were provided ad libitum.

Culture of the HMC-1 cells and sample treatment

The human mast cell line-1 (HMC-1) was grown in IMDM with 10% FBS at 37°C in a 5% CO₂ atmosphere with 95% humidity. The HMC-1 cells (3×10^5 cells in 24-well plates) were pretreated with non-cytotoxic concentrations of AAE (1, 10 and 100 μ g/ml) for 1 h before they were stimulated with 25 nM PMA plus 1 μ M A23187 (PMACI).

MTT assay for cell viability

Cell viability was determined using an MTT colorimetric assay. Briefly, a HMC-1 cell suspension (3×10^5 cells)

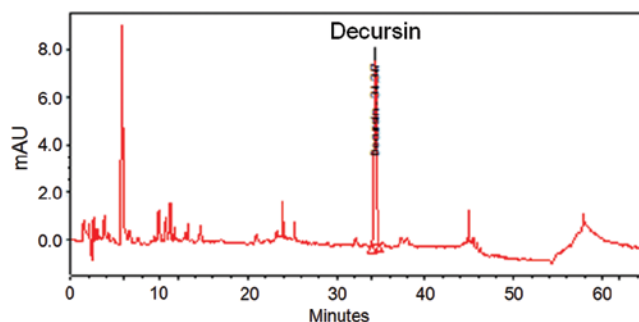


Figure 1. Quantitative analysis of AAE by HPLC. The retention time of decursin was 34.3 min.

was cultured (24-well plates) with AAE (1, 10 and 100 µg/ml). An MTT solution (5 mg/ml) was then added to each well, and the cells were cultured overnight. After washing out the supernatant, the insoluble formazan product was dissolved in DMSO. The optical density (OD) of the culture plates at 540 nm was measured with an ELISA reader. The OD of formazan that formed in untreated control cells was taken to represent 100% viability.

Cytokine assay

After incubation with PMACI for 7 h, each culture supernatant was subjected to ELISA (method previously described.⁽²⁰⁾ Briefly, 100-µl aliquots of the mouse anti-human TNF-α, IL-6, and IL-8 monoclonal antibodies (1.0 µg/ml of PBS at pH 7.4) were coated in 96-well ELISA plates (Nunc, Roskilde, Denmark) and were blocked with PBS containing 1% BSA, 5% sucrose, and 0.05% NaN₃ and incubated with each supernatant or recombinant TNF-α, IL-6, or IL-8 standard. After washing, 0.2 µg/ml of biotinylated anti-human TNF-α, IL-6, or IL-8 was added, and then they were incubated at 37°C for 2 h. After another wash, avidin-peroxidase was added and the antibodies were incubated at 37°C for 30 min. The wells were washed again, and the ABTS substrate (Sigma-Aldrich) was added. Color development was measured at 405 nm with an automated microplate ELISA reader. A standard curve was generated on each assay plate with serially diluted recombinant human TNF-α, IL-6 or IL-8.

Western blotting analysis of MAPK expression

After incubation with PMACI for 30 min, the cells were harvested and washed twice with ice-cold PBS. The washed cell pellets were resuspended in a lysis buffer, and protein fractions were obtained after centrifugation at 13,000 × g and 4°C for 20 min. The protein concentration was determined using the Bio-Rad (Hercules, CA, USA) protein assay reagent according to the manufacturer's instructions. Each protein was separated on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel; the proteins were then transferred to a nitrocellulose membrane. The membrane was blocked with 1% skimmed milk and 1% BSA in PBS containing 0.5% Tween-20 (PBST) for 1 h at room temperature. After the membrane was lightly washed with PBST, it was incubated overnight at 4°C with anti-MAPK antibodies (p-ERK, t-ERK, p-JNK, t-JNK, p-p38, and t-p38; 1:1000 dilutions in the 1% BSA in PBST solution) and then washed 3 times with PBST. Anti-mouse IgG, the secondary antibody (1:2000 dilution in the blocking solution), was incubated for 1 h at room temperature, and the antibody-specific proteins were visualized using an enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia, Piscataway, NJ, USA). Bands were analyzed using Quantity One 1-D analysis software (Bio-Rad). Data were obtained from triplicate experiments.

Histamine assay

RPMCs were obtained using the method previously reported.⁽²⁰⁾ The cells were then suspended in a concentration of 1×10^6 cells/ml in HEPES-Tyrode buffer. The 200 µl of peritoneal cell suspensions was incubated with 25 µl of AAE (final concentration: 1, 10 and 100 µg/ml) for 10 min at 37°C. Histamine release was evoked by adding 25 µl of compound 48/80 (final concentration, 5 µg/ml) and incubating at 37°C for 20 min. The reaction was discontinued 20 min later by cooling the tubes, and the tubes were centrifuged at 3000 rpm for 15 min at 4°C. The histamine content was measured using the *o*-phthalaldehyde spectrofluorometric procedure. The fluorescence intensity was measured at 440 nm (excitation wavelength, 360 nm) with a spectrofluorometer.

PCA and sample treatment

SD rats were injected with 0.5 µg (50 µl) anti-DNP IgE intradermally at three dorsal shaved skin sites. Forty-eight hours later, each rat was injected with 100 µg (100 µl) of DNP-HSA in saline containing 4% EB through the dorsal vein of the penis. AAE was suspended in distilled water and orally administered (160 mg/kg, 4 ml/kg) 1 h before the EB injection; the vehicle-treated group received the same volume of distilled water. The dorsal skin tissues of the rats were removed 30 min after EB injection. An image of each tissue was taken, and the EB-stained area was measured with an image analyzer (Scion Image, version 4.0.3; Scion Corporation, MD, USA). EB extravasation of each tissue was extracted by formamide for 24 h and measured at 620 nm with a spectrophotometer (Beckman, IL, USA), and the amount of dye (µg/ml) was calculated using the calibration curve of EB.

Statistical analysis

Each measured value is presented as the mean ± standard error of mean (S.E.M.). Statistical analysis was performed using the SAS statistical software (SAS Institute, Cary, NC, USA). Treatment effects were analyzed using one-way ANOVA, followed by Duncan's multiple-range test, and $P < 0.05$ was considered to be significant.

Results

Effect of the AAE on cytotoxicity in HMC-1 cells

AAE (1, 10 and 100 µg/ml) had no cytotoxic effect over a 24-h period (Figure 2).

Effect of AAE on IL-6, IL-8 and TNF-α release

IL-6, IL-8 and TNF-α were not detected in the normal group, while their levels were increased in the PMACI-treated control group. Non-cytotoxic concentrations of AAE reduced the release of these cytokines. Treatment with 10 and 100 µg/ml AAE significantly reduced IL-6 release compared with the control group (Figure 3A). Treatment with 1, 10 and 100 µg/ml AAE significantly reduced IL-8 release compared to the control group (Figure 3B). Treatment with 1, 10 and 100 µg/ml AAE

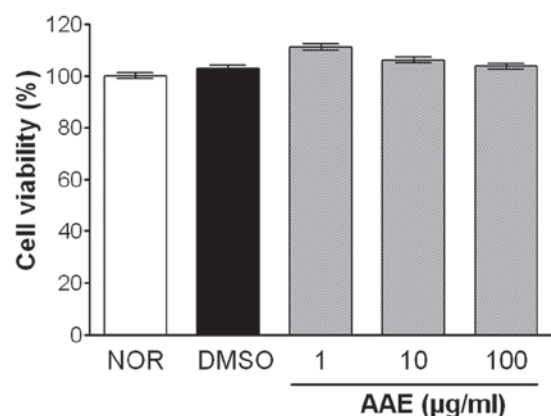


Figure 2. Effect of AAE on cell viability in HMC-1 cells. The optical density of the culture plates at 540nm was measured with an ELISA reader. The value represents the mean $\pm$ S.E.M. from three independent experiments.

significantly reduced TNF- α release compared to the control group (Figure 3C).

Effect of AAE on histamine release from RPMCs

Normal group showed only slight release of histamine, while the histamine release increased over 17 times in the compound 48/80-treated control group. Treatment with 10 and 100 μ g/ml AAE significantly inhibited histamine release compared to the control group (Figure 4).

Effect of AAE on the phosphorylation of MAPKs

ERK, JNK, and p38 were not phosphorylated or were only slightly phosphorylated in the normal group, while PMACI-induced control group showed increased phosphorylation. Treatment with 1, 10 and 100 μ g/ml AAE significantly inhibited p-ERK/t-ERK protein expression (Figure 5A,B) and p-JNK/t-JNK protein expression (Figure 5A,C) compared to the control group. However, 1, 10 and 100 μ g/ml AAE did not inhibit p-p38/t-p38 protein expression (Figure 4A,D).

Effect of AAE on PCA

The normal group showed evident EB-stained blood vessels in the dorsal skin; however, there was no EB extravasation or stained areas (Figure 6). In contrast, the anti-DNP IgE-injected vehicle-treated group showed evident EB extravasation and stained areas compared to the normal group (Figure 6). The group treated with 273mg/kg AAE showed evident reduced extravasation (Figure 6A) and stained areas (Figure 6B) compared with the vehicle-treated group.

Discussion

In the present study, AAE inhibited the release of TNF- α , IL-6, IL-8; PMACI-induced phosphorylation of ERK and JNK in HMC-1 cells; and compound 48/80-induced histamine release in RPMCs. AAE also inhibited EB extravasation in a PCA rat model.

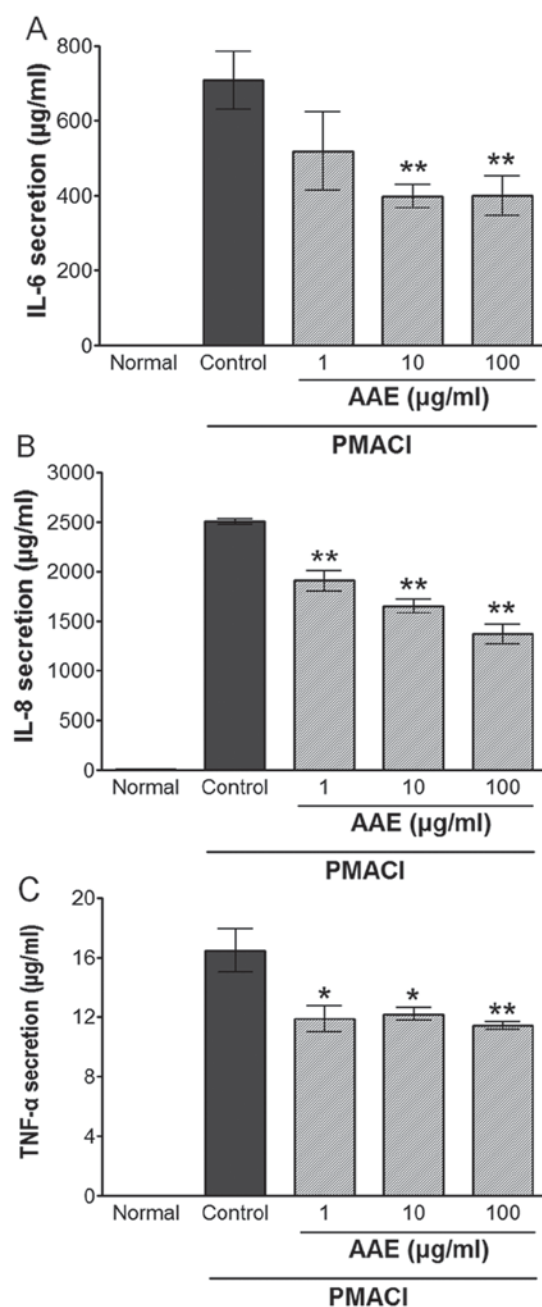


Figure 3. Effect of AAE on PMACI-induced IL-6 (A), IL-8 (B), and TNF- α (C) release in HMC-1. Normal group was not treated with AAE and PMACI. Data represent the mean $\pm$ S.E.M. from three independent experiments. * P < 0.05, ** P < 0.01, compared with the control group.

PMACI-induced TNF- α , IL-6 and IL-8 release was inhibited by AAE in HMC-1 cells. Mast cells release histamine, proteases, prostaglandins, leukotrienes, and several pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-4, IL-6, IL-8 and IL-13.⁽⁶⁾ Of these inflammatory cytokines, TNF- α , IL-6 and IL-8 in particular play key roles in allergic reactions.⁽²¹⁾ TNF- α induces differentiation, proliferation, activation, apoptosis, and release of pro-inflammatory mediators in mast cells.⁽²²⁾ Moreover, the TNF- α inhibitors infliximab and etanercept are regarded as clinically useful therapeutics for allergic inflammation.⁽²²⁾ IL-6

induces cell differentiation, growth, survival and migration during inflammation and immune responses.⁽²³⁾ IL-8 is a potent chemotactic cytokine in localized inflammatory reactions. It is known to be activated by radicals and results in further increase in the number of radicals in inflammatory cells.⁽²⁴⁾ It is suggested that AAE brings about its anti-allergic effects by decreasing the level of TNF- α , IL-6 and IL-8 released from mast cells.

In our study, AAE inhibited the compound 48/80-induced histamine release from RPMCs. Compound

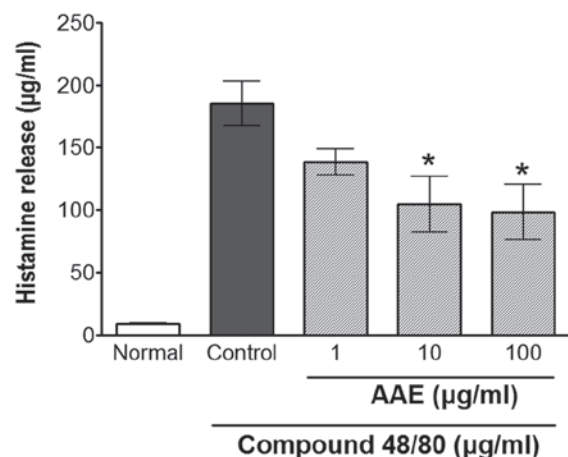


Figure 4. Effects of AAE on compound 48/80-induced histamine release from RPMCs. Normal group was not treated with AAE and compound 48/80. The value represents the mean $\pm$ S.E.M. from three independent experiments. * $P < 0.05$, compared with the control group.

48/80 is a popular inducer of the release of vasoactive mediators, including histamine, from mast cells or basophiles, by increasing the permeability of the lipid bilayer membrane.⁽⁹⁾ Histamine, a typical initiator of allergic inflammatory reactions in response to antigen exposure, causes several clinical symptoms including redness, swelling, and itching.⁽²⁵⁾ Thus, the current results indicate that AAE exhibits its anti-allergic effects partially by inhibition of histamine release from mast cells.

From our *in vitro* studies, we demonstrated that AAE inhibited chemically induced cytokine and histamine release from mast cells. The conditions in an *in vitro* cell are considered to be different from those *in vivo*. Therefore, we confirmed the effect of AAE on an *in vivo* allergy model. Oral administration of AAE inhibited EB extravasation induced by anti-DNP IgE injection. Intradermal injection of anti-DNP IgE induces anaphylaxis in the case of local allergic reactions by increasing mast cell-dependent vascular permeability.⁽¹⁹⁾ Therefore, we proved the anti-allergic effect of AAE in the *in vivo* model. The dose was calculated from the human dose (4 g raw material/60 kg weight), and then this was converted by extraction yield (41.0%), into 27.3 mg/kg. In general, the metabolism of a rat is known to be about 10 times higher than that of a human⁽²⁶⁾; thus, we selected a dose that was 10 times (273 mg/kg) the general human dose (27.3 mg/kg).

We investigated whether the inhibitory effects of AAE affected cytokines and histamine released from mast cells via the MAPK pathway. AAE inhibited PMACI-induced ERK and JNK expression but not p38 expression. MAPK

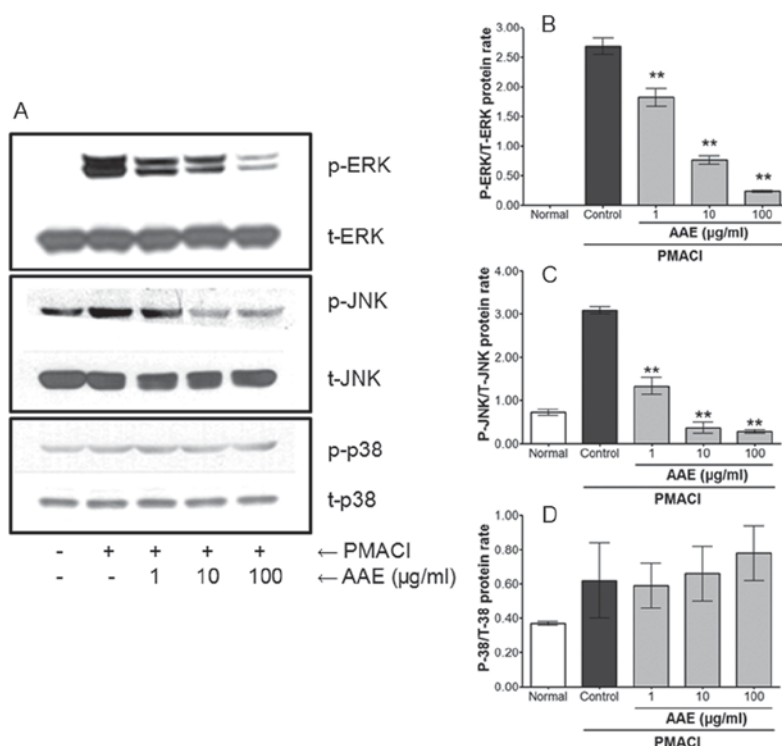


Figure 5. Typical protein expressions of MAPKs and their phosphorylated forms (A) and the protein ratio of p-ERK/t-ERK (B), p-JNK/t-JNK (C), and p-p38/t-p38 (D), respectively. The value represents the mean $\pm$ S.E.M. from 3 independent experiments. ** $P < 0.01$, compared with the control group.

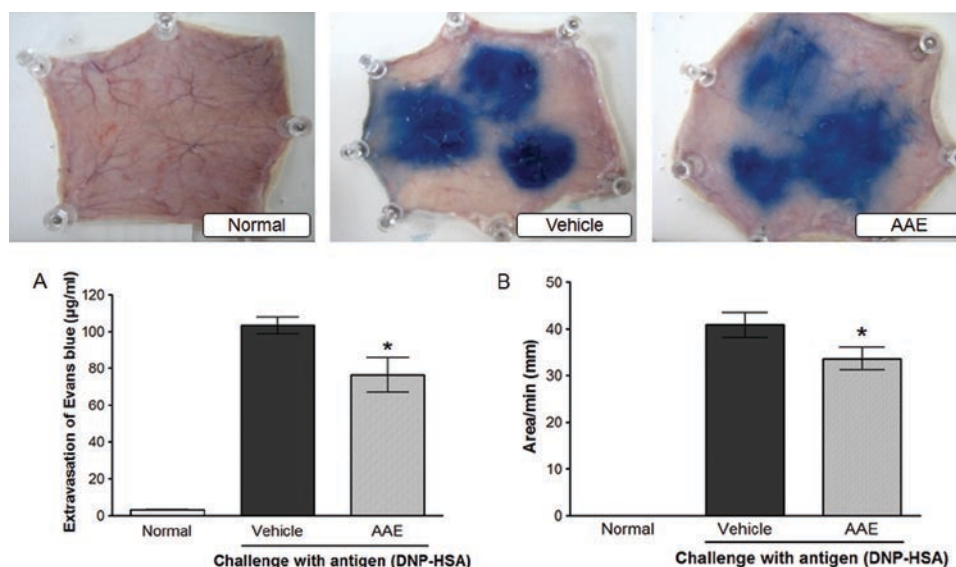


Figure 6. Inhibitory effects of AAE (273 mg/kg) on EB extravasation (A) and EB-stained area (B) in the dorsal skin site of PCA rat model. The stained blue areas indicate EB extravasation. Normal group was not treated with anti-DNP IgE and AAE. The vehicle-treated group was administered distilled water. Value represents the mean $\pm$ S.E.M. of 8 animals each. * $P < 0.05$, compared with the vehicle-treated group. (See colour version of this figure online at www.informahealthcare.com/ipi)

pathways are one of the most important signaling pathways in immune responses; they regulate the production and activity of inflammatory mediators by the activation of transcription factors such as NF- κ B.⁽²⁷⁾ ERK, JNK, and p38 are typical MAPK groups in mammals. The activation or expression of these MAPKs are known to be different due to differences in extracellular stimuli, downstream targets, and disease models.⁽²⁷⁾ Thus, our results suggest that AAE regulated the inflammatory process by inhibiting the phosphorylation of ERK and JNK, but not the phosphorylation of p38. Another study showed that decursin, a major constituent of AAE, inhibited vascular endothelial growth factor-induced phosphorylation of ERK and JNK, but not p38 in vascular endothelial cells.⁽²⁸⁾ Therefore, we think that the anti-allergic effect of AAE may be attributable to decursin.

Conclusion

In conclusion, AAE inhibited the mast cell-derived inflammatory allergic reactions by inhibiting the release of histamine, the production of pro-inflammatory cytokines (TNF- α , IL-6 and IL-8) and the phosphorylations of ERK and JNK.

Declaration of interest

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