Natural Product Sciences
17(3) : 239-244 (2011)

Anti-allergic Effects of *Schizonepeta tenuifolia* on Mast Cell-Mediated Allergy Model

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Abstract – Immediate-type hypersensitivity is involved in many allergic diseases such as asthma, allergic rhinitis and anaphylaxis. The discovery of drugs for the treatment of allergic disease is an important subject in human health. Stimulation of mast cells releases inflammatory mediators, such as histamine and pro-inflammatory cytokines with immune regulatory properties. We investigated the effect of the aqueous extract of *Schizonepeta tenuifolia* (AEST) (Labiatae) on the immediate-type allergic reaction. AEST inhibited compound 48/80-induced systemic allergic reaction. AEST attenuated immunoglobulin E (IgE)-mediated skin allergic reaction and histamine release from human mast cell line (HMC-1) cells. In addition, AEST decreased the gene expression and secretion of pro-inflammatory cytokines in phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore A23187 (A23187)-stimulated HMC-1 cells. Our results indicate that AEST inhibits the mast cell-derived allergic reactions and involvement of histamine and pro-inflammatory cytokines in these effects.
Keywords – *Schizonepeta tenuifolia*, allergic inflammation, mast cell, histamine, pro-inflammatory cytokine

Introduction

Allergic disease is a consequence of exposure to normally innocuous substances that elicit the activation of mast cells. Mast cell-mediated allergic response is involved in many diseases such as anaphylaxis, asthma, sinusitis, atopic dermatitis and allergic rhinitis.

Mast cells have long been recognized for their role in the genesis of allergic inflammation and more recently for their participation in innate and acquired immune responses (Brown *et al*., 2008).

Mast cells have a broad impact on many physiological and pathological processes such as wound healing, tissue remodeling and homeostasis (Rao and Brown, 2008). Typically, mast cells have been considered not only to be associated with immediate-type hypersensitivity but also with late reactions like inflammatory responses (Kemp and Lockey, 2002; Galli *et al*., 2008). Immediate-type hypersensitivity is mediated by histamine released in response to the antigen cross-linking of immunoglobulin E (IgE) bound to FcRI on the mast cells. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several pro-inflammatory and chemotactic cytokines, such as TNF-α, IL-6, IL-8, IL-4, IL-13 and transforming growth factor-β (Galli, *et al*., 1991; Bradding *et al*., 1993). Therefore, modulation of secretion of these cytokines from mast cells can provide a useful therapeutic strategy for allergic inflammatory disease. Anal therapy is a drug delivery system through the anus. It is utilized in patients who have difficulty with oral administration. Absorption of a drug in the rectum avoids the first-pass effect in the liver and allows it to circulate directly in the whole body. Thus, anal therapy is expected to have a better effect than oral therapy due to the increased absorption rate and the strong medical action. AEST was anally administered to mice for high and fast absorption.

Schizonepeta tenuifolia is a major herbal constituent included in treatments for common cold with fever. In addition, it is also used to treat sore throat, allergic dermatitis, eczema and inflammatory diseases (Kimura *et al*., 1986; Fung and Lau, 2002; Kang *et al*., 2008; Kim *et al*., 2008).

In this study, we evaluated the effect of AEST on compound 48/80-induced systemic allergic reaction, anti-DNP IgE antibody-induced local allergic reaction, and histamine release from human mast cell line (HMC-1) cells. Additionally, the effect of AEST on phorbol 12-

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myristate 13-acetate (PMA) plus calcium ionophore A23187 (A23187)-induced gene expression and secretion of pro-inflammatory cytokines were also investigated.

Experimental

Animals – The original stock of male ICR mice were purchased from Dae-Han Biolink Co. Ltd. (Chungbuk, Korea). The animals were housed 5 per cage in a laminar air flow room maintained under a temperature of 22 ± 2 °C and relative humidity of 55 ± 5% throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Reagents and cell culture – Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate and calcium ionophore A23187 were purchased from Sigma Chemical Co.(St. Louis, MO, USA). rTNF-α, rIL-6, rIL-1β and anti-TNF-α, -IL-6, -IL-1β antibodies were purchased from R & D systemic Inc (Minneapolis MN, USA). HMC-1 was grown in Iscove's media (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37 °C in 5% CO_2.

Preparation of AEST – The whole plant of *Schizonepeta tenuifolia* was purchased from the oriental drug store, Bohwa Dang (Jeonbuk, Korea). A voucher specimen was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The sample was extracted with purified water at 70 °C for 5 h (two times) in water bath. The extract was filtered and lyophilized.

Systemic allergic reaction – Compound 48/80-induced systemic reaction was carried out as previously described (Shin *et al*., 2004). Briefly, the mice (n = 10/group) were given an intraperitoneal injection of 8 mg/kg, body weight (BW), of the mast cell degranulator, compound 48/80. AEST (5 - 1000 mg/kg) was anally administered 1 h before the injection of compound 48/80. In the time dependent experiment, AEST (1000 mg/kg) was administered anally at 5, 10, 20 and 30 min after injection of compound 48/80 (n = 10/group). Mortality was monitored for 1 h after induction of anaphylactic shock.

Passive cutaneous anaphylaxis (PCA) reaction – PCA reaction was carried out as previously decribed (Shin *et al.*, 2005). Briefly, mice were injected intradermally with 0.5 μg of anti-DNP IgE. After 48 h, each mouse was given an injection of 1 μg of DNP-HAS in PBS containing 4% Evans blue (1 : 4) via the tail vein. AEST (1 - 1000 mg/kg, BW) was anally administered 1 h before the challenge. Then 30 min after the challenge, the mice were killed and the dorsal skin was removed for measurement of the pigment area. The amount of dye was determined colorimetrically after extraction with 1 ml of 1 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5 : 13) based on the previous report (Shin *et al.*, 2005). The intensity of absorbance was measured at 620 nm in a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, England).

Histamine Assay – Histamine content was measured by the enzyme immunoassay kit (Oxford Biomedical Research, Oxford, MI, USA) according to manufacturer's manual. HMC-1 cells were preincubated with AEST (0.001 - 0.1 mg/ml) for 30 min, and then incubated for 8 h with PMA (20 nM) plus A23187 (1 μM). The cells were separated from the released histamine by centrifugation at 400 × g for 10 min at 4 °C.

Cytotoxicity assay – The 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co.) cytotoxicity assay was used to measure the cytotoxic response to AEST, as previously described (Shin *et al*., 2006; Kim and Shin, 2009). HMC-1 cells were seeded at 1.0×10^5 cells/well in 96-well microplates (Falcon, Becton-Dickinson, Franklin Lakes, NJ). After 24 h of incubation with AEST, 20 μl of MTT (5 mg/ml) was added per well and incubated for 4 h. The formazan crystals dissolved in isopropyl alcohol with 0.04 M HCl and absorbance was read at 570 nm using a Power $Wave_x$ Microplate spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT).

Assay of TNF-α and IL-6 secretion – TNF-α and IL-6 secretion were measured by modification of an enzyme-linked immunosorbent assay (ELISA). HMC-1 cells were cultured with α-MEM plus 10% FBS and resuspended in Tyrode buffer A. The cells were sensitized with PMA (20 nM) plus A23187 (1 μM) for 16 h in the absence or presence of AEST. The ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF-α and IL-6 respectively.

Reverse-transcriptase polymerase chain reaction (RT-PCR) – The total cellular RNA was isolated from the cells (1.0×10^6 /well in a 24-well plate) after stimulation with PMA (20 nM) plus A23187 (1 μM) with or without AEST for 2 h using a TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using the Superscript II reverse transcriptase (Life Technologies). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for TNF-α, IL-6,

Table 1. Effect of AEST on compound 48/80-induced systemic allergic reaction

AEST treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
5	+	100
10	+	80
50	+	70
100	+	40
500	+	0
1000	+	0
1000	–	0

Groups of mice (n = 10/group) were anally pretreated with 200 μl of saline or AEST. Various doses of AEST were given 1 h before the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice × 100/total number of experimental mice.

IL-1β, and β-actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those previously described (Kim and Shin, 2006). The primer sets were chosen with the Primer 3 program (Whithead Institute, Cambridge, MA, U.S.A.). The cycle number was optimized to ensure product accumulation in the exponential range. The amplified products were separated by electrophoresis on 2% agarose gel containing ethidium bromide, documented using a Kodak DC 290 digital camera, and digitized with UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.). The band intensity was normalized to that of β-actin in the same sample.

TLC pattern analysis – TLC pattern analysis was carried out using plate coated with silica gel 60 F_{254} (mobile phase, $CHCl_3$-MeOH-H_2O = 40 : 10 : 1). Detection was performed by spraying with anisaldehyde H_2SO_4 in ethanol followed by heating at 100 - 120 °C for 5 min.

Statistical Analysis – Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Results

Effect of AEST on systemic and local allergic reaction – To determine whether AEST has anti-allergic effects, an *in vivo* model of a systemic reaction was used. Anaphylaxis is a serious allergic reaction that is rapid in onset and causes death (Metcalfe *et al*., 2009). Compound 48/80 (8 mg/kg BW) was used as a model of induction for systemic fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. Injection of compound 48/80 into mice induced fatal shock in 100% of animals. When AEST was anally administered ranging from 5 to 1000 mg/kg BW for 1 h, the mortality with compound 48/80 was dose-dependently reduced (Table 1). In addition, the mortality of mice administered with AEST (1000 mg/kg) 5, 10, 20 and 30 min after compound 48/80 injection increased time-dependently (Table 2).

Another way to test the allergic reaction is to induce PCA. Local extravasation was induced by a local injection of anti-DNP IgE followed by an intravenous

Table 2. Time-dependent effect of AEST on compound 48/80-induced systemic allergic reaction

AEST treatment (mg/kg)	Time (min)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	0	+	100
1000	5	+	10
	10	+	30
	20	+	50
	30	+	100

Groups of mice (n = 10/group) were anally pretreated with 200 μl of saline or AEST. AEST (1000 mg/kg) was given at 5, 10, 20, and 30 min after the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 48/80 injection is represented as the number of dead mice × 100/total number of experimental mice.

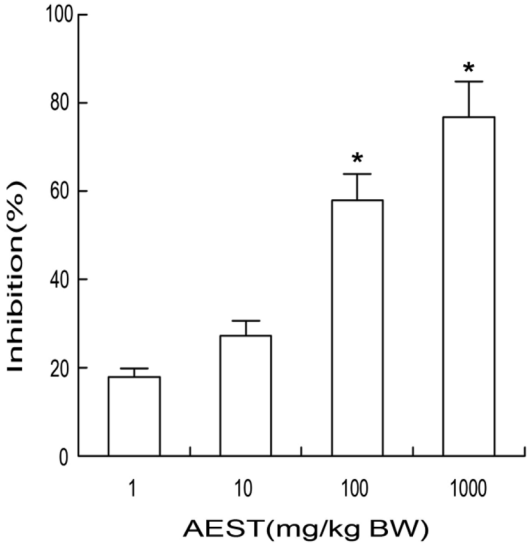

Fig 1. Effect of AEST on the 48 h PCA. AEST was anally administered 1 h prior to the challenge with antigen. Each data represents the mean ± SEM of three independent experiments. *Significantly different from the saline value at $p < 0.05$.

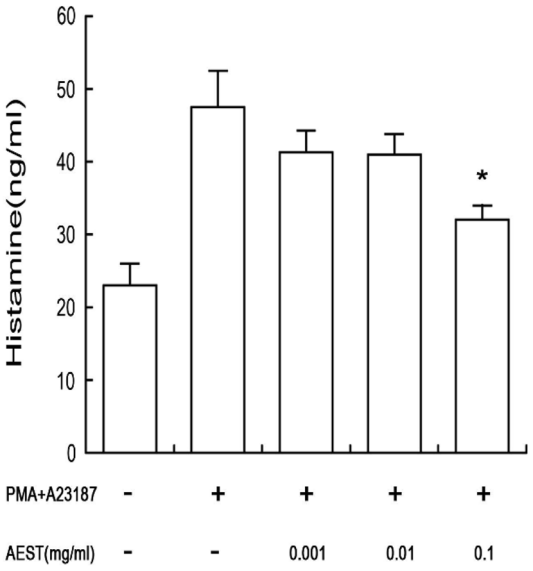

Fig. 2. Effect of AEST on PMA plus A23187-induced histamine release in HMC-1 cells. HMC-1 cells (2.0×10^6 cells/ml) were preincubated with AEST at 37 ℃ for 10 min prior to incubation with PMA plus A23187. Each data represents the mean ± SEM of three independent experiments. *Significantly different from the PMA plus A23187 value at $p < 0.05$.

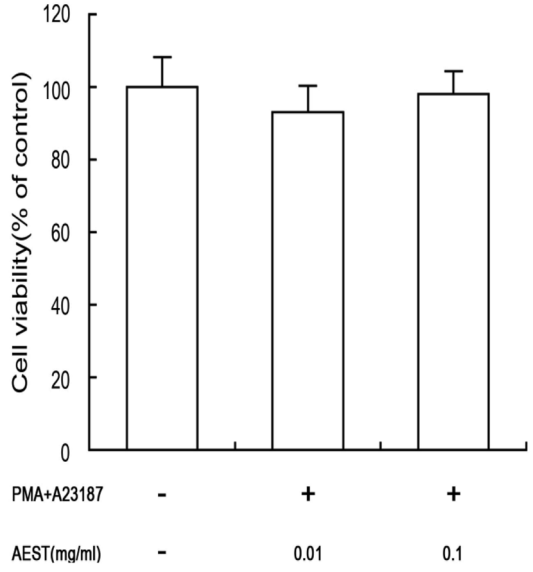

Fig. 3. Effect of AEST on HMC-1 cells viability. Cell viability was evaluated by MTT assay by incubation for 24 h after stimulation with PMA plus A23187 in the absence or presence of AEST. The percentage of viable cells was calculated using 100% for stimulation in the absence of AEST.

antigenic challenge. AEST was administered anally 1 h prior to the challenge with antigen. The administration of AEST (1 - 1000 mg/kg, BW) showed a dose-dependent inhibition in the PCA reaction (Fig. 1).

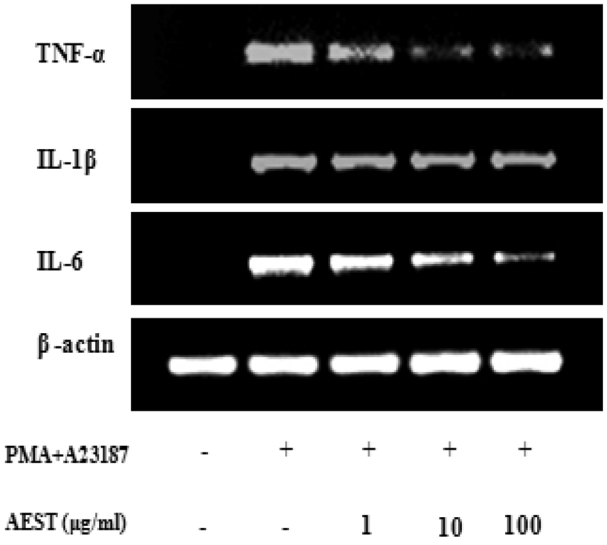

Fig. 4. Effect of AEST on the gene expression of pro-inflammatory cytokines in HMC-1 cells. HMC-1 cells were pretreated with AEST for 30 min prior to PMA plus A23187 stimulation. The levels of TNF-α, IL-6 and IL-1β were determined by RT-PCR.

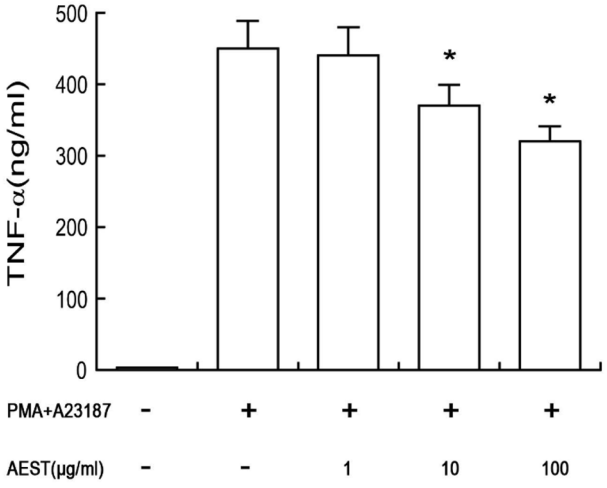

Fig. 5. Effect of AEST on secretion of TNF-α in HMC-1 cells. TNF-α level in supernatant was measured using ELISA. Each data represents the mean ± SEM of three independent experiments. *Significantly different from the PMA plus A23187 value at $p < 0.05$.

Effect of AEST on histamine release from HMC-1 cells – To determine the effect of AEST on allergic reaction, histamine release from HMC-1 cells was evaluated. Treatment with AEST inhibited PMA plus A23187-induced histamine release at concentrations of 0.001 - 0.1 mg/ml (Fig. 2). We examined the cytotoxicity of AEST in HMC-1 cells using MTT assay. AEST did not show cytotoxic effect up to 0.1 mg/ml (Fig. 3).

Effect of AEST on the gene expression and secretion of pro-inflammatory cytokines in HMC-1 cells – TNF-α,

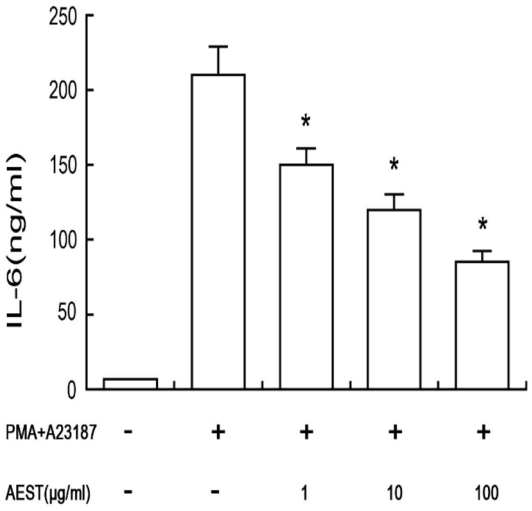

Fig. 6. Effect of AEST on secretion of IL-6 in HMC-1 cells. IL-6 level in supernatant was measured using ELISA. Each data represents the mean ± SEM of three independent experiments. *Significantly different from the PMA plus A23187 value at $p < 0.05$.

Fig 7. TLC pattern of AEST.

IL-6, IL-8 and IL-1β are the most important pro-inflammatory cytokines. Therefore, we examined the effects of AEST on the gene expression of TNF-α, IL-6 and IL-1β induced by PMA plus A23187 in HMC-1 cells. HMC-1 cell line is a useful cell for studying the cytokine activation pathways (Kim and Sharma, 2004; Kim *et al*., 2006). AEST (1 - 100 μg/ml) dose-dependently inhibited PMA plus A23187-induced expression of TNF-α, IL-6 and IL-1β (Fig. 4). To confirm the effect of AEST on the gene expression of pro-inflammatory cytokines, culture supernatants were assayed for TNF-α and IL-6 levels by ELISA. The stimulation of cells with PMA plus A23187 for 16 h induced the secretion of cytokines. AEST (1 - 100 μg/ml) dose-dependently inhibited the secretion of TNF-α and IL-6 in PMA plus A23187-stimulated HMC-1 cells (Fig. 5, Fig. 6).

TLC pattern of the AEST – TLC pattern of the AEST shows that the AEST has plenty of constituents (Fig. 7). So it needs to isolate active compounds in the near future.

Discussion

Immediate-type hypersensitivity (anaphylaxis) is a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine, heparin and various cytokines from mast cells (Kemp and Lockey, 2002). Using an *in vivo* and *in vitro* model, we showed that AEST reduces mast cell-derived allergic inflammatory responses. In the present study, we demonstrated that AESP decreased histamine release and pro-inflammatory cytokine expression. In addition, AESP inhibited systemic and local allergic reaction. Histamine is principal mediator of allergy. Numerous reports established that stimulation of mast cells with PMA plus A23187, compound 48/80 or IgE initiates the activation of a signal transduction pathway which leads to histamine release. Several studies have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins (Mousli *et al*., 1990). Compound 48/80 increases the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This result indicates that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable. AEST might stabilize the lipid bilayer membrane, thus preventing the perturbation being induced by mast cell stimulators.

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions. The mice administered with AEST were protected from IgE-mediated PCA. This finding suggests that AEST might be useful in the treatment of allergic skin reactions.

Mast cell-derived cytokines, especially TNF-α, IL-6 and IL-1β have a critical biological activity in the allergic reaction. It has been reported that mast cells are a principal source of TNF-α in human dermis, and

degranulation of mast cells in the dermal endothelium is abrogated by the anti-TNF-α antibody (Walsh *et al*., 1991). IL-6 and IL-1β are also produced from mast cells and its local accumulation is associated with a PCA reaction (Mican *et al*., 1992; Sillaber *et al*., 1993). These reports indicate that reduction of pro-inflammatory cytokines from mast cell is a one of the key indicator of reduced allergic symptom. The HMC-1 cell is a useful cell for studying cytokine activation pathways (Sillaber *et al*., 1993). In our present study, AEST inhibited the expression and secretion of cytokines in PMA plus A23187-stimulated HMC-1 cells. These results suggest that AEST reduces the allergic responses through decreasing the expression of pro-inflammatory cytokines. The effect of AEST on cytokines production by mast cells *in vivo* and the relative importance of mast cells as a source of cytokines during inflammatory and immune responses are important areas for future studies. In the present report, we provide evidence that AEST inhibits a model of mast cell-mediated allergic inflammation. The results obtained in the present study show that AEST might contributes to the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

Acknowledgements

This work was supported by Woosuk University.

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Received May 12, 2011
Revised August 30, 2011
Accepted September 4, 2011