



Anti-Asthmatic Effects of an *Amomum compactum* Extract on an Ovalbumin (OVA)-Induced Murine Asthma Model

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Despite ongoing intensive asthma research, the incidence of asthma is increasing worldwide. We investigated in this study the effects of *Amomum compactum* on ovalbumin (OVA)-induced asthma in a mouse model, and studied the possible mechanism for its anti-asthmatic action. Our data show that an *A. compactum* treatment markedly decreased the number of infiltrating eosinophils and the hypersecretion of mucus when compared with the effects on mice treated with OVA alone. The *A. compactum* treatment dose-dependently decreased the levels of reactive oxygen species (ROS) and T helper (Th)2 cytokines, including interleukin (IL)-4 and IL-5, in the bronchoalveolar lavage fluid (BALF), and a high dose of *A. compactum* effectively reduced the level of total immunoglobulin (Ig)E in the serum. Taken together, these data indicate that the administration of *A. compactum* may have potential therapeutic value when used as an adjuvant for the immunomodulatory treatment of allergic asthma.

Key words: asthma; *Amomum compactum* soland ex Maton; reactive oxygen species (ROS)

Asthma is a chronic inflammatory disease characterized by airway inflammation and remodeling, bronchial hyper-responsiveness, variable airflow obstruction, and mucus hypersecretion.¹⁾ The latter characteristic is associated with increased mucin secretion and increased secretion from inflammatory cells.²⁾

Oxidative stress is induced by a large variety of free oxygen radicals, including reactive oxygen species (ROS). An increasing amount of clinical and experimental evidence suggests that ROS perform an essential function in the pathogenesis of airway inflammation.^{3,4)} The secretion of ROS by eosinophils and other leukocytes during airway infiltration is extremely relevant to the airway tissue injuries and remodeling processes associated with asthma. Furthermore, increased levels of ROS are thought to initiate the lung inflammatory response by activating such transcription factors as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), as well as by activating signal transduction and increasing the expression of genes encoding pro-inflammatory mediators.⁵⁾

The asthmatic response to allergen exposure is associated with immunoglobulin (Ig)E-mediated mast cell activation which leads to the accumulation of leukocytes, including eosinophils and T helper (Th)2 lymphocytes, in the airway.^{6,7)} Th2 lymphocytes play important roles in the initiation and progression of allergic diseases (including asthma) through their ability to release interleukin (IL)-4 and IL-5.⁸⁾ These Th2 cytokines induce various inflammatory responses, including airway infiltration, eosinophil activation, IgE production, and mucus secretion.^{9,10)} IL-4 is important to the induction of isotype-class switching which is required for B cells to express IgE.¹¹⁾ IL-4-deficient (IL-4 -/-) mice immunized with aerosolized OVA have shown markedly reduced eosinophilia and inflammation when compared with wild-type mice.^{12,13)} IL-5, on the other hand, is involved in eosinophil activation and is synthesized not only by Th2 lymphocytes, but also by mast cells and eosinophils.¹⁴⁾

Amomum compactum soland ex Maton is widely distributed in Korea, and has been commonly used in Korean traditional medicine to resolve dampness, disperse stuffiness, move qi, warm the middle, increase appetite, and promote digestion.¹⁵⁾ However, the use of *A. compactum* has not previously been investigated in the context of allergic asthma. We examined here the potential therapeutic effects of *A. compactum* on an OVA-induced murine model of allergic asthma.

Materials and Methods

Preparation of the A. compactum ethanolic extract (ACEE). *A. compactum* plants were purchased in October 2008 from HMAX (Chungbuk, Korea), and a voucher specimen has been deposited at the Korean Institute of Oriental Medicine (KIOM; Daejeon, Korea). Dried seeds (200 g) were suspended in 70% (v/v) ethanol (2 liters) and extracted with sonication for 1 h. This process was conducted three times. The extract was passed through filter paper and allowed to evaporate to dryness; 5.85 g of the product was obtained in a yield of 2.93%.

Animals. Six-week-old female BALB/c mice were purchased from Dae Han Biolink (Eumseong, Korea). The mice were randomly distributed to the various experimental groups (n = 7). The mice were kept for one week prior to the experiment in air-conditioned and

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Abbreviations: ACEE, *Amomum compactum* ethanolic extract; ALT, alanine aminotransferase; AP-1, activator protein-1; AST, aspartate aminotransferase; BALF, bronchoalveolar lavage fluid; DCF-DA, 2',7'-dichlorofluorescein diacetate; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; Ig, immunoglobulin; IL, interleukin; NF-κB, nuclear factor-kappaB; OVA, ovalbumin; PAS, periodic acid Schiff; ROS, reactive oxygen species; Th, T helper

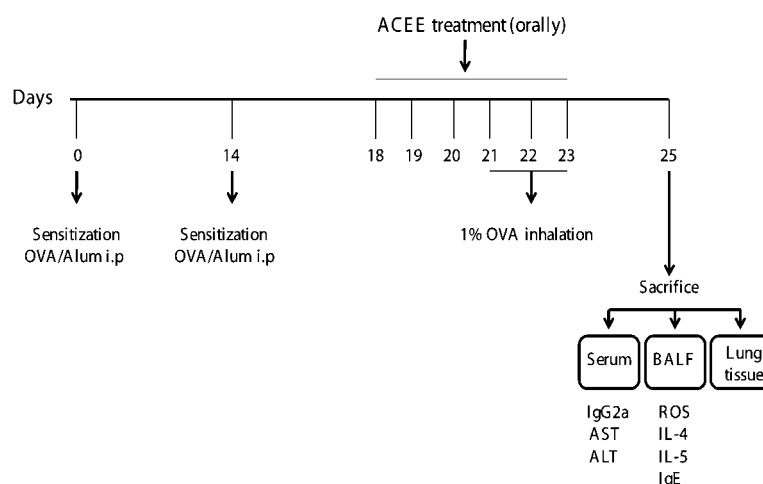


Fig. 1. Mouse Model of Airway Inflammation and Treatment with ACEE.

specific pathogen-free isolators, and maintained under conditions of $23 \pm 2^\circ\text{C}$, $55.5 \pm 10\%$ humidity, and a regulated 12 h light/dark cycle. The mice were cared for and treated in accordance with the guidelines established by the Public Health Service Policy for the Human Care and Use of Laboratory Animals.

OVA-sensitization, challenge and administration of ACEE. On day 0 and day 14, the mice were sensitized with an intraperitoneal (i.p.) injection of $20\mu\text{g}$ of OVA (Sigma-Aldrich, MI, USA) and 2 mg of aluminum hydroxide (Sigma-Aldrich, MI, USA) dissolved in 0.2 ml of phosphate buffered saline (PBS; Invitrogen, CA, USA). On days 21–23, the mice were challenged through the airway with OVA (1% dissolved in PBS) for 1 h by using an ultrasonic nebulizer (Omron, Kyoto, Japan). ACEE (100 mg/kg or 200 mg/kg) was administered orally once daily on days 19–23 (five doses in total). Negative and positive control mice were orally treated with PBS and Montelukast (Mon; 30 mg/kg), respectively, once daily on days 19–23 (five doses in total). Montelukast was developed as a cysteinyl leukotriene (cys-LT)-1 receptor antagonist,¹⁶ by which the clinical responses of patients with aspirin-sensitive asthma, and nocturnal exacerbations of asthma and allergic asthma led to Montelukast being successfully introduced into the market.¹⁷ The mice were sacrificed on day 25. A schematic diagram of the treatment schedule is shown in Fig. 1.

Preparation of the bronchoalveolar lavage fluid (BALF). Forty-eight hours after the last OVA challenge, the mice were sacrificed for preparing BALF. Ice-cold PBS (0.6 ml) was instilled into the lungs, and BALF was obtained by three aspirations (total volume of 1.8 ml) via tracheal cannulation. The liquid was centrifuged at 4°C for 5 min. The supernatant (subsequently referred to as BALF) was subjected to enzyme-linked immunosorbent assays (ELISAs) for the presence of the cytokines of interest (see below), as well as for other tests. To count the cells, the pelleted cells were centrifuged on to a microscope slide by using a CellSpin (Hanil Science, Korea), stained with a Diff-Quick staining kit (IMEB, CA, USA), and observed under a microscope (Olympus, Tokyo, Japan).

2',7'-Dichlorofluorescein (DCF) fluorescence assay. The induction of oxidative stress was monitored by using 2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, OR, USA) which is converted into highly fluorescent DCF by cellular peroxides, including hydrogen peroxide. In brief, BALF was treated with $25\mu\text{M}$ DCF-DA for 10 min at 37°C . The intracellular ROS activity was measured by determining the fluorescence at 488 nm excitation and 525 nm emission with a fluorescence plate reader (Perkin-Elmer, MA, USA).

Measurement of Th2 cytokines (IL-4 and IL-5) in BALF. The concentrations of IL-4 and IL-5 in the acquired BALF samples were determined by using an ELISA kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm by a microplate ELISA reader (Bio-Rad Laboratories, CA, USA).

Histological studies. Forty-eight hours after the last OVA challenge and following the collection of BALF, the lungs were removed from the mice. The lung tissues were fixed in 10% neutral buffered formalin at 4°C for 24 h, embedded in paraffin, and sectioned to a thickness of $4\mu\text{m}$. The sections were stained with a hematoxylin and eosin (H&E; IMEB, CA, USA) solution, and with the periodic acid Schiff (PAS; IMEB, CA, USA) reagent for measuring the inflammation and mucus production. The tissues were subsequently mounted and cover-slipped by using a Histo-mounting solution (Invitrogen, CA, USA).

Respective quantification of total IgE and OVA-specific IgG2a in the serum and BALF. Total IgE in the serum and OVA-specific IgG2a in BALF were measured by capture ELISA which was performed according to the manufacturer's instructions. The IgE level was quantified by using a mouse IgE ELISA kit (Serotec, Oxford, UK). An OVA solution ($10\mu\text{g}/\text{ml}$) was dispensed to 96-well plates and allowed to bind overnight at 4°C . The OVA-specific IgG2a level was quantified by using a mouse IgG2a ELISA kit (Bethyl, TX, USA). The absorbance was measured at 450 nm by a microplate ELISA reader (Bio-Rad Laboratories, CA, USA).

Measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in the serum. The ALT and AST levels, which are used to assess the liver function, were measured by using commercial kits (Beckman Coulter, CA, USA) and a CX4 auto-analyzer (Beckman Coulter, CA, USA).

Statistical analysis. Data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical significance was determined by using one-way ANOVA followed by Tukey's *post hoc* test. The critical level for significance was set at $p < 0.05$.

Results

ACEE suppressed the infiltration of eosinophils into BALF

To evaluate the effect of the ACEE treatment on the total cell number and eosinophils in the OVA-sensitized/challenged mice, the cells recruited to BALF were counted 48 h after the last challenge. The numbers of total cells and eosinophils were significantly higher in BALF from the OVA-sensitized/challenged mice than from the OVA-unsensitized mice (negative control group). However, the numbers of total cells and eosinophils were significantly and dose-dependently lower in BALF from the ACEE-treated, OVA-sensitized/challenged mice than from the OVA-sensitized/challenged mice that had not received ACEE (Fig. 2).

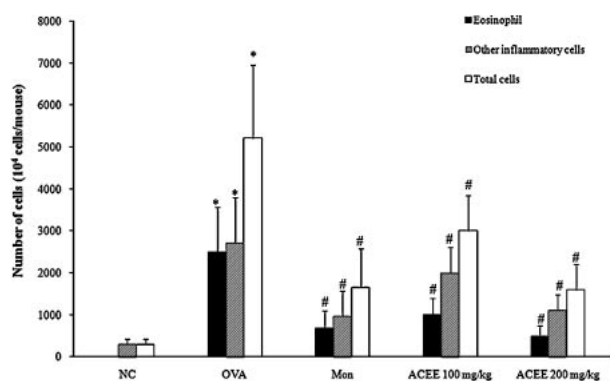


Fig. 2. Effect of ACEE on the Recruitment of Inflammatory Cells to the Bronchoalveolar Lavage Fluid (BALF) in Mice.

BALF samples were collected 48 h after the last OVA challenge, and cell numbers were assessed by direct microscopic counting with a hemocytometer. *Abbreviations:* NC, OVA-uninduced mice (PBS only); OVA, OVA-induced mice; Mon, Montelukast (30 mg/kg)-treated, OVA-induced mice; ACEE, ACEE (100 mg/kg or 200 mg/kg)-treated, OVA-induced mice. * $p < 0.05$ compared with NC, # $p < 0.05$ compared with OVA.

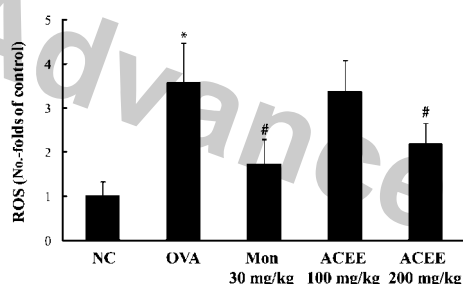


Fig. 3. Effect of ACEE on the ROS Level in BALF Taken from Mice 48 h after the Last OVA Challenge.

Abbreviations: NC, OVA-uninduced mice (PBS only); OVA, OVA-induced mice; Mon, Montelukast (30 mg/kg)-treated, OVA-induced mice; ACEE, ACEE (100 mg/kg or 200 mg/kg)-treated, OVA-induced mice. * $p < 0.05$ compared with NC, # $p < 0.05$ compared with OVA.

The positive control (Mon treatment) showed a similar suppressive effect on eosinophilia in the BALF of OVA-sensitized/challenged mice.

ACEE diminished ROS generation in BALF

We used DCF-DA to investigate whether ACEE affected ROS generation, and found that the ROS level was dose-dependently lower in BALF from the ACEE-treated OVA-sensitized/challenged mice than from the OVA-sensitized/challenged mice that had not received ACEE. This suggests that the administration of ACEE had an inhibitory effect on the generation of ROS (Fig. 3).

ACEE treatment suppressed the production of Th2 cytokines in BALF

ELISA was used to measure the levels of IL-4 and IL-5 in the BALF samples. As shown in Fig. 4A and B, the levels of IL-4 and IL-5 in BALF were significantly increased by the airway challenge with OVA, while the ACEE treatment significantly decreased these levels when compared with those in the OVA-sensitized/challenged mice.

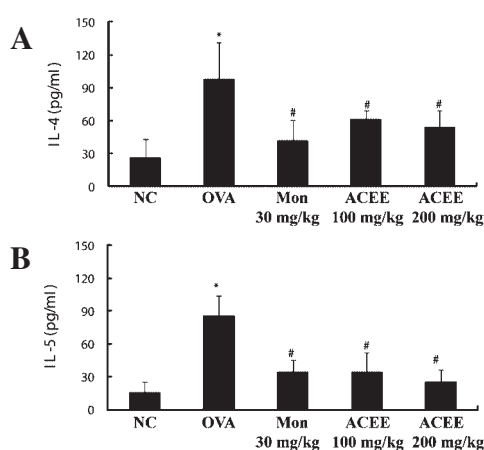


Fig. 4. Effect of ACEE on the IL-4 (A) and IL-5 (B) Levels in BALF.

BALF samples were collected 48 h after the last OVA challenge and analyzed by ELISA. *Abbreviations:* NC, OVA-uninduced mice (PBS only); OVA, OVA-induced mice; Mon, Montelukast (30 mg/kg)-treated, OVA-induced mice; ACEE, ACEE (100 mg/kg or 200 mg/kg)-treated, OVA-induced mice. * $p < 0.05$ compared with NC, # $p < 0.05$ compared with OVA.

ACEE decreased eosinophilia in the lung tissues of the OVA-sensitized/challenged mice

To measure the possible anti-inflammatory or anti-remodeling effect of ACEE, we collected lung tissues 48 h after the last challenge with OVA, and examined sections for the infiltration of leukocytes (mostly eosinophils) into the peribronchiole and the perivascular connective tissues. The infiltration of eosinophil-rich leukocytes was significantly attenuated in the ACEE-treated OVA-challenged mice when compared to that in the OVA-sensitized/challenged mice that had not received ACEE (Fig. 5A and B).

ACEE decreased the release of IgE into the serum and OVA-specific IgG2a into BALF

To determine the effects of ACEE on IgE and the antigen-specific antibodies, we determined the serum and BALF levels of IgE and OVA-specific IgG2a antibodies 48 h after the last OVA challenge. The OVA-unsensitized (negative control) mice produced barely detectable levels of IgE and OVA-specific IgG2a in the serum and BALF, whereas the OVA-sensitized/challenged mice showed significantly higher levels of IgE and OVA-specific IgG2a in the serum and BALF. The treatment with ACEE reduced the levels of IgE and OVA-specific IgG2a antibodies compared to those in the OVA-sensitized/challenged mice, and the treatment with a higher dose of ACEE (200 mg/kg) yielded an IgE level that was lower than that in the Mon-treated (positive control) mice (Fig. 6A and B).

The AST and ALT levels in the serum suggest that ACEE did not show toxicity under our test conditions

We estimated the serum AST and ALT levels to examine the liver function of the ACEE-treated mice to assess the toxicity of ACEE. There was no significant difference in the AST and ALT levels across all groups, indicating that the ACEE treatment did not affect the liver function of the mice (data not shown).

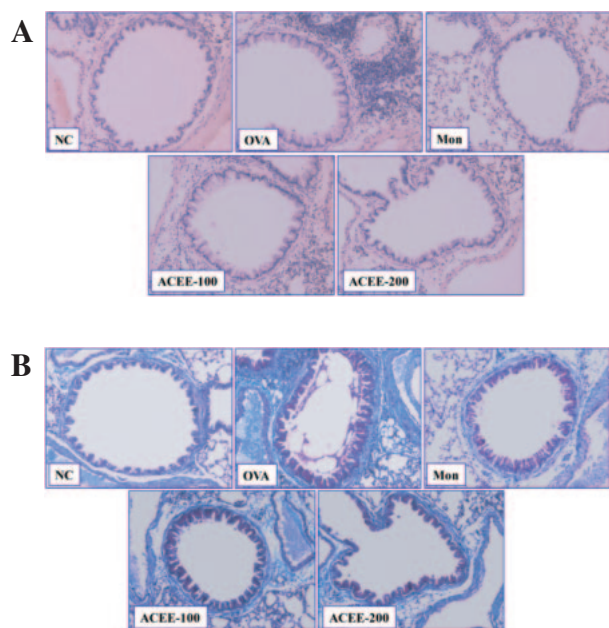


Fig. 5. Effect of ACEE on the Recruitment of Leukocytes to Lung Tissue (A), and on the Production of Mucus (B).

Lung tissues were fixed, sectioned at a thickness of 4 μ m, and stained with the Diff-quick (A) and periodic acid Schiff (PAS) reagent. **Abbreviations:** NC, OVA-uninduced mice (PBS only); OVA, OVA-induced mice; Mon, Montelukast (30 mg/kg)-treated, OVA-induced mice; ACEE-100 or ACEE-200, ACEE (100 mg/kg or 200 mg/kg)-treated, OVA-induced mice. * p < 0.05 compared with NC, # p < 0.05 compared with OVA.

Discussion

Bronchial asthma is a chronic inflammatory disorder of the airways characterized by variable airway obstruction, airway eosinophilic inflammation, and bronchial hyper-responsiveness.¹⁸⁾ One characteristic of asthma is the infiltration of eosinophils and lymphocytes into the airways, and increased numbers of these cells have been found in BALF from asthmatics.¹⁹⁾ Several mediators secreted from eosinophils and lymphocytes can amplify portions of the inflammatory cascade; they can increase airway hyper-reactivity and stimulate mucus secretion, both of which can contribute to airway obstruction and airway remodeling.^{20–22)} The numbers of total cells and eosinophils in BALF from the OVA-sensitized/challenged mice were significantly higher than those in the OVA-unsensitized mice (negative control group) in this study. However, the cell numbers in BALF from the ACEE-treated, OVA-sensitized/challenged mice were significantly and dose-dependently lower than those from the OVA-sensitized/challenged mice, and showed markedly less infiltration of inflammatory cells into the peribronchiolar and perivascular regions. Furthermore, mucus hypersecretion from goblet cells was also reduced in the OVA-challenged/sensitized mice that had been subjected to the ACEE treatment.

Airway inflammation and hyper-reactivity in asthma are likely to involve oxidative stress to the lungs.²³⁾ ROS may affect extracellular matrix remodeling, apoptosis, mitochondrial respiration, alveolar repair responses, and immune modulation in the lung.²⁴⁾ Thus, the secretion of ROS by eosinophils and other leukocytes that infiltrate the airways is extremely relevant to the asthma-associated airway tissue injuries and remodeling proc-

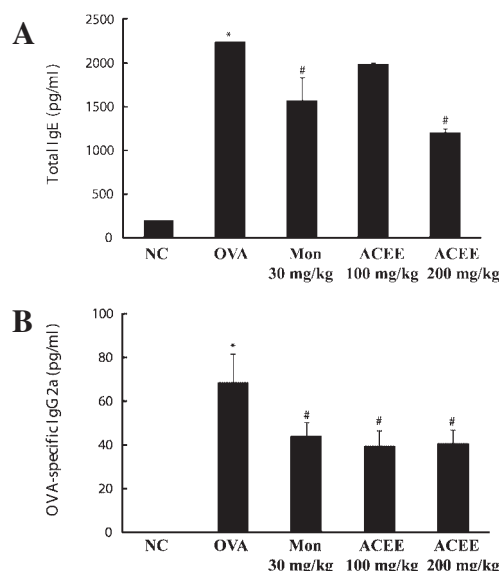


Fig. 6. Effect of ACEE on the Total IgE Level in Serum (A) and on the OVA-Specific IgG2a Level in BALF (B).

BALF and serum were collected 48 h after the last OVA challenge, and samples were analyzed by ELISA. **Abbreviations:** NC, OVA-uninduced mice (PBS only); OVA, OVA-induced mice; Mon, Montelukast (30 mg/kg)-treated, OVA-induced mice; ACEE, ACEE (100 mg/kg or 200 mg/kg)-treated, OVA-induced mice. * p < 0.05 compared with NC, # p < 0.05 compared with OVA.

esses.²⁵⁾ We found here that ROS generation was dose-dependently decreased in BALF from the ACEE-treated OVA-sensitized/challenged mice. Our results therefore collectively suggest that ACEE played a beneficial role in the lung by blocking mucus secretion, eosinophil recruitment, and ROS generation.

Th2 cells play an important role in the initiation and progression of allergic responses (including asthma) by releasing IL-4 and IL-5.²⁶⁾ These Th2 cytokines induce inflammatory responses, including airway infiltration, eosinophil activation, IgE production, and mucus secretion.¹⁰⁾ We have shown here that the ACEE treatment of OVA-sensitized/challenged mice also significantly decreased the levels of IL-4 and IL-5 in BALF. This effect of the ACEE treatment may at least in part be responsible for the decreased recruitment of eosinophils into the lungs of the mice that had been treated with both ACEE and OVA.

An elevated serum IgE level is associated with bronchial asthma which is another disease characterized by eosinophilic inflammation of the airways. The activation of antigen-specific Th2 cells in the lung with the subsequent release of IL-4 and IL-5 is believed to play an important role in the pathogenesis of this disease.⁹⁾ IgE has been closely associated with Th2-type responses, whereas IgG2a has been connected with Th1-type responses.²⁷⁾ Our results show that the oral administration of ACEE to OVA-sensitized/challenged mice significantly decreased the resulting serum IgE level and OVA-specific IgG2a level in BALF. The former could be related to the observed down-regulation of IL-4 and IL-5.

In summary, our present results support the notion that *A. compactum* can suppress the generation of a Th2 immune response in OVA-sensitized/challenged mice, potentially indicating that *A. compactum* may have a therapeutic effect on allergic asthma or inflammatory disease.

Acknowledgments

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