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Anti-atopic dermatitis effect of *Scutellaria barbata* D. Don via regulation of MAPK signaling pathways

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Abstract

Background: Atopic dermatitis (AD) is a chronic disease that causes skin itching.

Objective: To investigate the effects of *Scutellaria barbata* D. Don (SBD) on AD-like symptoms induced by trimellitic anhydride (TMA) treatment in the ears of mice and its underlying mechanism.

Methods: To induce AD in mice, the dorsal skin was treated with 5% TMA on day 0, 5% TMA in both ears on day 5, and 2% TMA in both ears from days 6 to 14. From days 9 to 14, SBD was given orally and daily before TMA treatment. Symptom analysis consisted of weighing ear lymph nodes, measuring ear thickness, analyzing scratching behavior, and measuring the levels of immunoglobulin E, tumor necrosis factor-alpha, interleukin-4 (IL-4), and mitogen-activated protein kinase (MAPK) signaling.

Results: Treatment with 100 mg/kg SBD significantly decreased lymph node weight, ear thickness, the skin score, scratching behavior, and the serum immunoglobulin E level. Moreover, 100 mg/kg SBD treatment significantly reduced the levels of IL-4 expression and MAPK phosphorylation in AD mice.

Conclusions: SBD may be useful for treating AD. These results provide information for treating patients with inflammatory diseases such as eczema and asthma and provide a molecular basis for developing new therapeutics.

Key words: *Scutellaria barbata* D. Don, atopic dermatitis, trimellitic anhydride, interleukin-4, immunoglobulin E, mitogen-activated protein kinase

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Introduction

Atopic dermatitis (AD) is a chronic disease that causes skin itching.[1,2] AD is characterized by eczema dermatitis and itching, increased immunoglobulin E (IgE) levels in the blood, and increased infiltration of immune cells, such as skin lymphocytes, eosinophils, neutrophils, and mast cells.[3]

In allergies, T helper 2 (Th2) cells are thought to mediate IgE-dependent mast cell activation and degranulation, while increasing inflammation and stimulating other cells.[4] IgE is a major cause of AD. Th2 cytokines, particularly interleukin (IL)-4, upregulate IgE in the blood, and the immune response of Th2 cytokines is central to the pathogenesis of AD.[5]

AD can be induced in mice in various ways.[6] Trimellitic anhydride (TMA) applied to skin causes allergic reactions and is widely used to evaluate AD in animal models. In mice, TMA also induces T cell-dependent hypersensitivity, T cell infiltration, Th2 cytokine production, and IgE release.[7,8] This method is highly reproducible and the results are very similar to the clinical symptoms, facilitating studies of AD.[9]

Drugs commonly used to treat AD include antihistamines, immunosuppressants, and steroids. However, the use of these drugs can increase infections, suppress the adrenal glands, and cause serious skin damage.[10] Therefore, the use of naturally derived therapeutics may be effective for treating AD with fewer side effects.

Scutellaria barbata D. Don (SBD) is a plant that grows in Asia, where it has been used as a medicinal herb in traditional medicine.[11] According to ancient documents, SBD has been used for various inflammatory diseases, and it is now being investigated for its use in inflammatory diseases and cancer.[12] One study demonstrated the efficacy of SBD extract applied to liver, colon, lung, and breast cancer cells *in vitro*.[13,14] Although there are few studies of SBD in AD, a study discovered that SBD flavonoids inhibit IL-13 and CD40 ligand expression in activated basophils in asthma patients.[15] Since SBD might be effective for atopic disease, this study evaluated the efficacy of SBD in reducing the symptoms of AD in a mouse model of TMA-induced AD and the mechanism of action of SBD.

Materials and Methods

Animals

The male Balb/c mice used in the experiment weighed 28–30 g at 12 weeks of age and were purchased from Samtako Animal (Osan, South Korea). The mice were housed in a sterile animal room under a 12:12 h light–dark cycle and were provided food and water *ad libitum*. The experimental procedures followed the animal protection guidelines of the National Institutes of Health and the Kyung Hee University Institutional Animal Care and Use Committee. All animal experiments started at least 7 days after receiving the mice to allow for acclimation.

Reagents

Acetone (99%) was purchased from Merck, and isopropyl myristate (98%) and TMA (98%) were obtained from Sigma-Aldrich Chemical. Reagents were freshly constituted before use. Dried SBD roots were obtained from an oriental drug store (Jungdo, Seoul, Korea). The crushed materials (120 g SBD) were added to distilled water, and extraction was performed by heating for 4 h at 100°C, concentrating using a rotary evaporator, and freeze-drying. The powdered form of the SBD extract was used in all experiments after dilution in deionized H_2O.

Experimental schedule and drug treatments

AD was induced by treating both ears of Balb/c mice with 5% TMA, applied to the dorsal skin.[17] Five days later, both ears were sensitized with 5% TMA. Then 2% TMA was applied daily from days 6 to 14 (9 days in total). On days 9 to 14, SBD (10, 50, and 100 mg/kg, n = 7) was administered orally 30 min before applying the 2% TMA (**Figure 1**).

Ear skin manifestation

The atopic changes in the ear skin were observed by photographing the ear skin of mice in each experimental group using the Canon 6D camera (Canon, Seoul, Korea). On the last day of the experiment, the mice were anesthetized with 1.2% isoflurane inhalation and sacrificed; ear thickness was measured using a dial gauge (Bosung Scientific, Seoul, Korea), and the parotid lymph nodes were weighed using a digital balance (Coretech, Seoul, Korea).

Scratching behavior

As the most representative symptom of AD, scratching behavior is a very useful indicator of AD. Scratching was assessed by recording all mouse actions for 10 min. During this period, to maintain quiet, no one was allowed in the observation room. A 10-min video was provided to blinded investigators to count the number of times each mouse scratched itself. If there were multiple scratches per second with the hind paw, this was considered one scratch.

ELISA

Blood samples were collected from the retro-orbital plexus from non-anesthetized mice on the day of killing using a capillary tube. Serum was obtained by centrifugation at 6500 rpm for 20 min and stored at −70°C until use. Serum IgE (Bethyl Laboratories, Montgomery, TX, USA), cytokines (tumor necrosis factor-alpha [TNF-α] and IL-4; R&D Systems, Minneapolis, MN, USA), and mitogen-activated protein kinase (MAPK; RayBiotech, Atlanta, GA, USA) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits. In brief, each antibody was diluted in coating buffer, applied to the surface of a 96-well microplate, and left overnight. Each well was washed with wash buffer, and the samples were dispensed. After washing the plate, the samples were treated with avidin–horseradish peroxidase binding antibody and tetramethylbenzidine solution. Finally, the absorbance was measured at 450 nm after treatment with the stop solution (Versa Max, Sunnyvale, CA, USA).

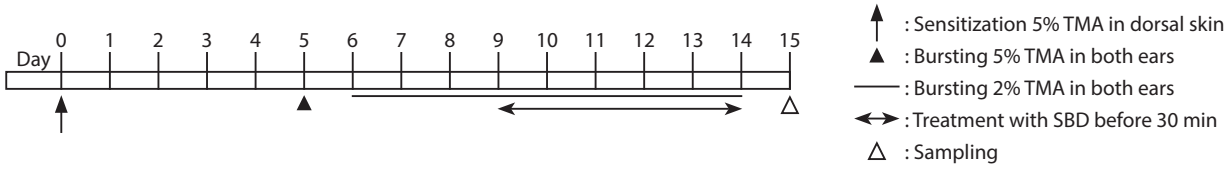

Figure 1. Schematic diagram of the TMA-induced atopic dermatitis experiment.

Statistical analysis

All analyses were performed by blinded investigators. Results are presented as the mean and standard error. Experimental data were analyzed by one-way analysis of variance (ANOVA) using SPSS ver. 13.0 (IBM, Chicago, IL, USA). Statistical differences among groups were analyzed using Tukey's *post hoc* test. All *p*-values less than 0.05 were considered statistically significant.

Results

Ear manifestations and scratching behavior

To confirm the effects of SBD on atopy, the macroscopic clinical response, ear thickness, and lymph node weight were determined in all experimental groups. Skin symptoms such as edema and vasodilation were increased in the AD group compared with the normal (NOR) group. SBD treatment significantly suppressed these TMA-induced atopic changes in a dose-dependent manner, especially in the AD+SBD100 group (**Figure 2A**).

As a macroscopic indicator of AD, ear thickness was significantly reduced by the high-concentration SBD treatment ($p < 0.001$). The reduction in ear thickness was greater in the AD+SBD100 group than that in the AD+SBD50 group (**Figure 2B**). TMA-induced AD can induce an increase in lymph node weight. Lymph node weight increased the most in the AD group, by approximately three times compared with the NOR group (**Figure 2C**). The administration of SBD had a dose-dependent inhibitory effect, with a 30.9% reduction at the highest dose ($p < 0.001$).

The scratching behavior was significantly increased in the AD group compared with the NOR group (**Figure 2D**), while SBD tended to decrease scratching behavior, especially in the AD+SBD100 group.

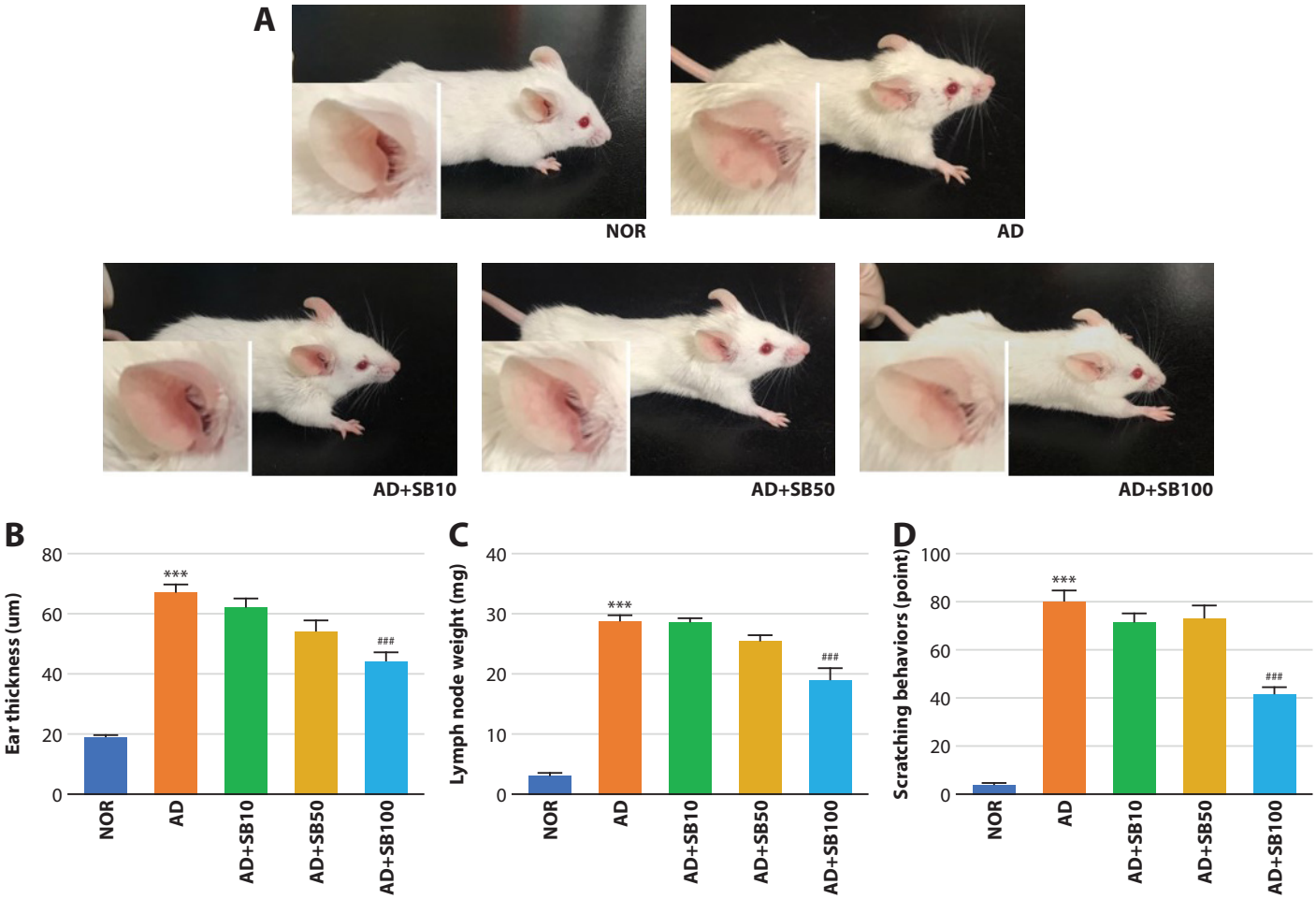

Figure 2. Ear thickness (A), lymph node weight (B), and scratching behavior (C) in TMA-induced AD mice. SBD was given 30 min before TMA treatment. Ear thickness was measured using a dial thickness gauge, and the auricular lymph nodes were weighed using a digital balance. The scratching behavior of all mice was video-recorded for 10 min; the mice generally scratched with their hind paws several times in 1 s, and such a series was counted as one scratching episode. Results are presented as means ± standard error and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{***}p < 0.001$ vs. the NOR group; $^{###}p < 0.001$ vs. the AD group.

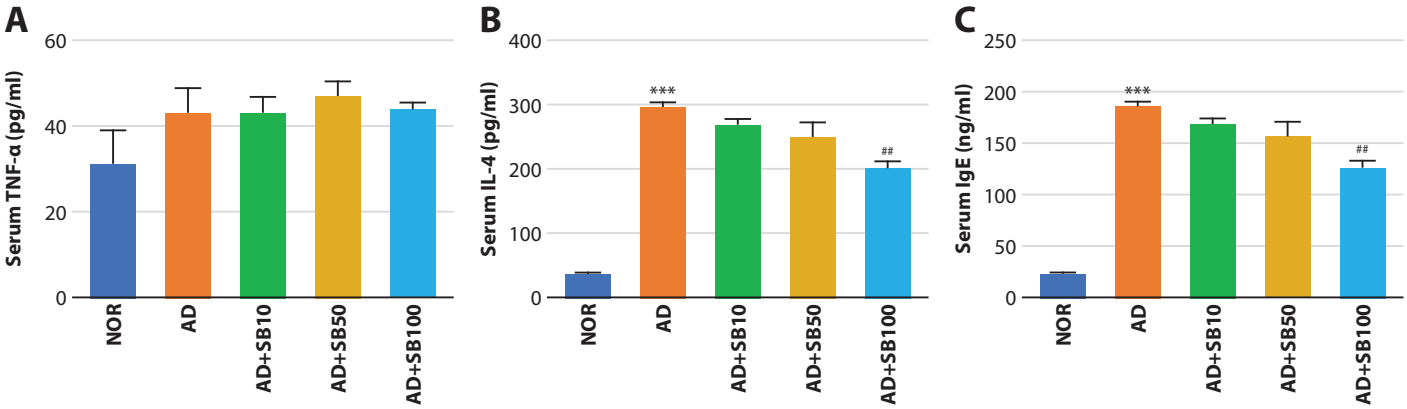

Figure 3. TNF-α (A), IL-4 (B), and IgE (C) levels in TMA-induced AD mice. SBD was given 30 min before TMA treatment. Serum was collected from four mice randomly selected in each group. Serum TNF-α, IL-4, and IgE levels were measured using ELISA and are presented as means ± standard errors. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{***}p < 0.001$ vs. the NOR group; $^{\#\#}p < 0.01$ vs. the AD group.

Serum TNF-α, IL-4, and IgE levels

Because TNF-α, IL-4, and IgE are important markers for diagnosing AD, we also investigated their expression. As shown in **Figure 3**, the IL-4 (approximately six-fold) and IgE levels were increased markedly in the AD group compared with the NOR group. However, AD did not significantly increase the expression of TNF-α (**Figure 3A**). IL-4 expression decreased with SBD administration in a dose-dependent manner and was reduced by 33.3% in the AD+SBD100 group ($p < 0.01$; **Figure 3B**). As an index of AD, the serum IgE level was increased four-fold in the AD group compared with the NOR group. As expected, 100 mg/kg SBD significantly reduced the serum IgE level ($p < 0.01$, **Figure 3C**).

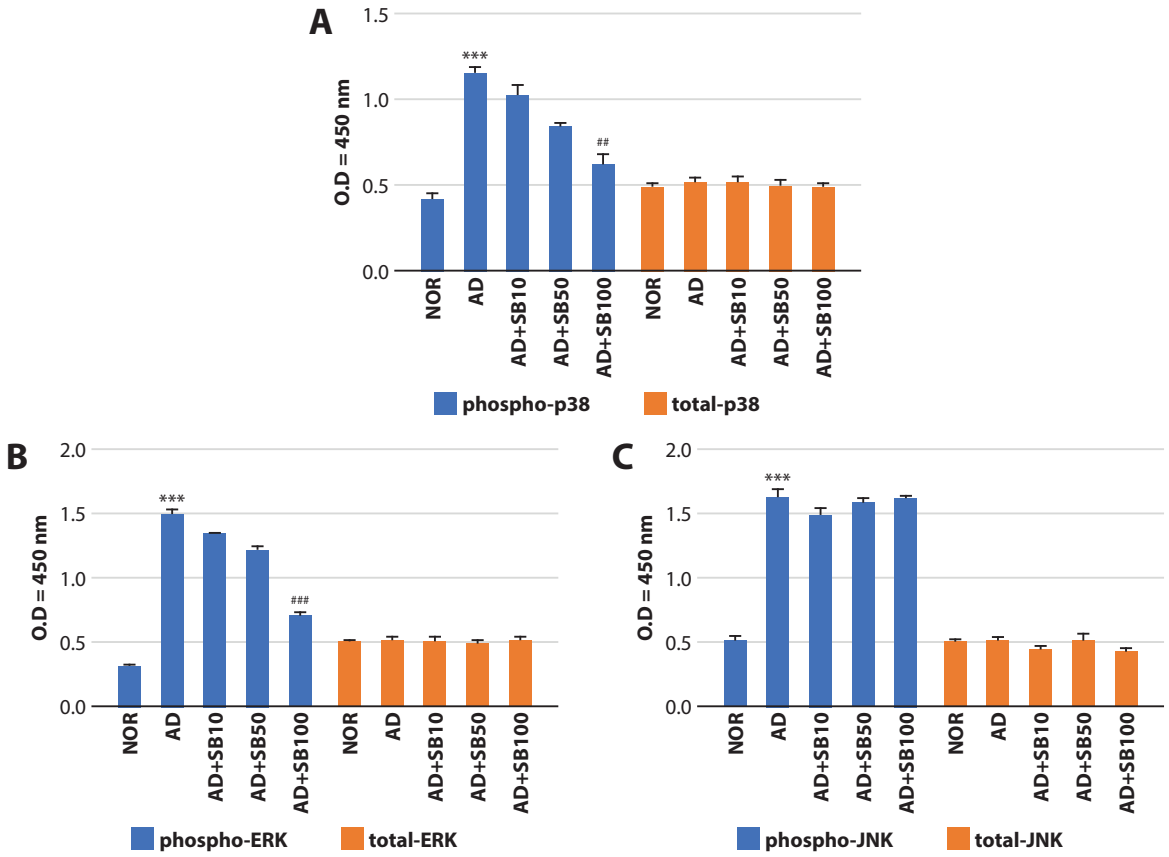

Figure 4. Inhibitory effect of SBD on the phosphorylation of p38 (A), ERK (B), and JNK (C) MAPKs in TMA-induced AD mice. SBD was given 30 min before TMA treatment. Serum MAPK levels were measured using ELISA. Results are presented as means ± standard errors. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{***}p < 0.001$ vs. the NOR group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. the AD group.

MAPK signaling pathways in the mouse

The MAPK pathway regulates cell proliferation and differentiation in AD by mediating extracellular signals. To determine whether SBD treatment affects the MAPK pathway, we analyzed the phosphorylation of p38, extracellular signal regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) (**Figure 4**). No significant MAPK phosphorylation was observed in the NOR group, but TMA-induced AD mice had significantly higher phosphorylation levels of p38, ERK, and JNK. Treatment with 100 mg/kg SBD significantly inhibited p38 and ERK phosphorylation in TMA-treated mice, while there was no change in JNK (**Figure 4**).

Discussion

Studies of SBD have been conducted since 1889. While the anti-inflammatory effects of SBD have been reported, no studies have examined the effect of SBD in a mouse model of AD.[16] Therefore, this study analyzed the anti-AD efficacy of SBD in TMA-induced AD mice and evaluated the underlying mechanism of its effect.

AD induced by this method manifested as increased ear thickness, lymph node weight, and epidermal hyperplasia. Oral SBD markedly improved these characteristics. Inflammation and immune responses are the major cause of AD. Cytokines that respond to AD have been recognized as mediators of AD, and many studies of these cytokines are ongoing.[17] TNF-α has a wide variety of biological effects in humans, while IL-4 can increase FceRI expression on the surface of mast cells, amplify IgE-induced signaling in mast cells, and provide a receptive environment for eosinophil recruitment due to its presence in local tissues.[18] After TMA treatment, the IL-4 level in AD mouse serum was significantly increased in the AD group compared with the NOR group. SBD treatment reduced IL-4 expression in the AD model, especially at 100 mg/kg SBD. More specifically, IL-4 expression was significantly reduced compared with TNF-α expression, suggesting that IL-4 is important for evaluating AD. Several studies have demonstrated that increased pro-inflammatory cytokines, such as TNF-α, induce the scratching behavior of AD and play an important role in AD neuropathology.[19] The TNF-α level also increases rapidly in acute TMA-induced AD but decreases in chronic AD.[19] Therefore, we examined whether the atopy-like symptoms were induced acutely or chronically based on the TNF-α level. We found that TMA infiltration increased serum TNF-α expression, albeit not significantly. Nevertheless, SBD suppressed the scratching behavior of AD by suppressing the increased TNF-α level.

IgE induces mast cell degranulation, resulting in histamine release.[20] Therefore, an increased serum IgE level is an indicator of atopy. IgE levels are increased in most patients with AD.[21] In this study, treatment with a high SBD concentration significantly reduced the IgE level.

To analyze the effects of SBD on intracellular signaling pathways, we evaluated MAPK, a typical inflammatory signaling mechanism that has an important role in the activation of various cells and the subsequent release and transport of inflammatory substances.[22,23] MAPK signaling is triggered by p38, ERK, and JNK. Recently, pharmacological inhibitors of MAPK have been developed and might be very effective for treating allergic diseases.[24] The increased TNF-α expression and histamine induction in rat peritoneal mast cells after substance P stimulation require the p38 and JNK MAPK pathways.[25] The p38, JNK, and ERK signaling pathways play important roles in AD-induced inflammatory diseases.[26] In this study, SBD inhibited mainly the phosphorylation of p38 and ERK and slightly that of JNK. In the AD inflammatory response, the release of signals from lymph node mast cells increased the serum expression of p38, JNK, and ERK. We found that SBD treatment significantly decreased the increased serum p38 and ERK expression due to TMA. However, SBD did not affect the expression of JNK, a signal downstream of MAPK.

Interestingly, the AD+SBD100 group did not recover significantly from the AD-induced increase in the serum TNF-α level, although the same SBD dose significantly reduced the IL-4 and IgE levels. The reason for this difference should be investigated. SBD significantly reduced AD by modulating p38 and ERK cells in this study. However, our results were limited to the induction of AD by TMA treatment in both ears. Future studies must analyze how the induction of AD by TMA affects scratching behavior and pro-inflammatory cytokines throughout the body.

In summary, our AD mouse model was suitable, and SBD suppressed allergic symptoms in this AD model. Moreover, SBD significantly reduced AD by modulating p38 and ERK in cells. Therefore, SBD might be effective for treating allergic diseases, especially AD.

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Author disclosure statement

The authors declare no potential conflicts of interests.

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