

Inhibitory Effects of *Schefflera leucantha* Extract on Production of Allergic Mediators by Langerhans Cells and Mast Cells

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■ Abstract

Background: *Schefflera leucantha* Viguier is used as a traditional medicine in Thailand and China to relieve chronic cough and asthma. However, little is known about its anti-allergic effects.

Objective: This study was designed to investigate the effects of *S leucantha* ethanol extract (SLEE) on chemokine production by epidermal Langerhans cells (LCs) stimulated with peptidoglycan (PEG) from *Staphylococcus aureus* and histamine release from mast cells.

Methods: LCs were purified from murine epidermal cells using the panning method with anti-IA^d monoclonal antibody. Chemokine production by LCs was investigated by reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA). Mast cells for histamine release assay were induced by long-term culture of mouse spleen cells. Histamine release from these mast cells was measured by a competitive ELISA.

Results: Production of the eosinophil chemoattractant CCL5 and the type 2 T helper (T_H2)-associated chemokine CCL17 from PEG-stimulated LCs was significantly inhibited by SLEE. Furthermore, SLEE significantly decreased the release of histamine from mast cells by IgE-mediated degranulation.

Conclusion: These results suggest that *S leucantha* may offer a new therapeutic approach for the control of atopic dermatitis associated with *S aureus* colonization through inhibition of the production of allergic mediators.

Key words: Atopic dermatitis. Peptidoglycan. *Schefflera leucantha* Viguier. *Staphylococcus aureus*. Histamine. CCL5. CCL17.

■ Resumen

Antecedentes: *Schefflera leucantha* Viguier se utiliza como medicina tradicional en Tailandia y China para el alivio de la tos y el asma crónicas. No obstante, se sabe poco acerca de sus efectos antialérgicos.

Objetivo: Este estudio fue diseñado para investigar los efectos del extracto etanólico de *S. leucantha* (EESL) sobre la producción de quimiocinas por parte de células de Langerhans (CL) epidérmicas estimuladas con peptidoglucano (PEG) de *Staphylococcus aureus* y liberación de histamina de mastocitos.

Métodos: Se purificaron CL a partir de células epidérmicas murinas mediante el método panning con anticuerpos monoclonales anti-IA^d. Se investigó la producción de quimiocinas por parte de las CL mediante reacción en cadena de la polimerasa en transcripción inversa y enzoinmunoanálisis de adsorción (ELISA). Los mastocitos para el ensayo de liberación de histamina se obtuvieron mediante cultivo prolongado de células esplénicas de ratón. La liberación de histamina de estos mastocitos se determinó mediante ELISA competitivo.

Resultados: El EESL inhibió significativamente la producción del factor quimiotáctico de eosinófilos CCL5 y la quimiocina CCL17 asociada a linfocitos T cooperadores de tipo 2 (T_H2) por parte de CL estimuladas con PEG. Asimismo, el EESL redujo significativamente la liberación de histamina de los mastocitos por desgranulación mediada por IgE.

Conclusión: Estos resultados indican que *S. leucantha* puede ofrecer una nueva estrategia terapéutica para el control de la dermatitis atópica asociada a la colonización por *S. aureus* mediante la inhibición de la producción de mediadores alérgicos.

Palabras clave: Dermatitis atópica. Peptidoglucano. *Schefflera leucantha* Viguier. *Staphylococcus aureus*. Histamina. CCL5. CCL17.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with immunopathologic features that vary depending on the duration of the lesions. Chronic skin lesions in AD patients show an increased number of inflammatory cells such as mast cells, eosinophils, and mononuclear cells in the dermis, as well as superficial *Staphylococcus aureus* colonization [1]. *S aureus* is isolated in 75% to 96% of skin lesions in AD patients, compared to in just 5% to 10% of healthy individuals [2,3]. In a previous study by our group, we found higher detection rates and cell counts for *S aureus* on lesional than on nonlesional skin in AD patients [3]. In the same study, however, we found no significant differences in the detection rate of superantigenic exotoxin production by *S aureus*; this exotoxin has been considered the most important factor in the pathogenesis of skin lesions in AD [1]. Gram-positive bacterial cell walls are composed of highly cross-linked peptidoglycan (PEG) decorated to a variable extent with teichoic acid polymers. These polymers are also linked to plasma membrane phospholipids in the form of lipoteichoic acid (LTA), which is another major cell wall component [4,5]. Gram-positive bacteria do not contain lipopolysaccharide (LPS), and LTA and/or PEG are thought to be the major inflammatory products in the cell walls of these bacteria. We therefore hypothesized that LTA and/or PEG from *S aureus* may have a pathogenic role in AD patients [6-8].

In a previous study, we demonstrated that percutaneous invasion of PEG induced eosinophil migration in the dermis, as also occurs as a result of CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted) production by epidermal Langerhans cells (LCs), in AD patients [9]. Therefore, inhibition of CCL5 production by LCs might provide a therapeutic approach for eosinophilic inflammation in patients with AD with *S aureus* colonization. In order to find an inhibitor of CCL5 production by LCs, 17 medicinal plants from Thailand were screened, and *Schefflera leucantha* Viguier (Araliaceae family) was chosen as the most suitable candidate. Fresh *S leucantha* leaves have been traditionally used in Thailand and China to relieve chronic cough and asthma [10]. Although it has been shown that saponins in the leaves possess bronchodilator activity [11], it has not been clarified whether *S leucantha* actually exerts an anti-allergic effect. In this study, therefore, we investigated the effects of *S leucantha* ethanol extract (SLEE) on the production of CCL5 and other allergy-related chemokines by murine LCs stimulated with PEG. We also explored the effect of SLEE on histamine release from murine mast cells resulting from immunoglobulin (Ig) E-mediated degranulation.

Materials and Methods

Preparation of Plant Extract

Fresh leaves of *S leucantha* were collected in Bangkok, Thailand. The material was identified and a voucher specimen (No. 0307001) was deposited at the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn

University, Bangkok, Thailand. One hundred grams of the leaves were then chopped and extraction was performed by macerating the leaves in 500 mL of 98% ethanol at 25°C to 30°C for 3 days. The ethanolic extract obtained was passed through filter paper and dried under reduced pressure at 50°C. The dark-green residue obtained was then dissolved in dimethyl sulfoxide (DMSO) (50 mg/mL) and stored as SLEE at -20°C until use. The yield of this extract was approximately 2.6% (w/w). Furthermore, the extract was free of detectable endotoxin (negative by the *Limulus* lysate assay at <50 pg endotoxin/mg extract). Anti-mouse TLR4-MD2 monoclonal antibody (MBL, Nagoya, Japan), which neutralizes endotoxin activity in murine systems, did not influence the biological activity of this extract.

Peptidoglycan and Animals

PEG derived from *S aureus* was obtained from Fluka (Buchs, Switzerland). Female specific-pathogen-free BALB/c mice were obtained from Japan SLC (Hamamatsu, Japan) and used when they were 6 to 8 weeks of age. They were housed in plastic cages in a clean, air-conditioned room at 24°C and allowed free access to a standard laboratory diet and water. All procedures performed on the mice were in accordance with the Guidelines of the Animal Care and Use Committee of Meiji Pharmaceutical University, Tokyo, Japan.

Langerhans Cells and Analysis of Chemokine Production

The LCs in the epidermis were separated as described by Tada et al [12]. The LCs were adjusted to 1×10^6 cells/mL in RPMI 1640 medium containing L-glutamine (Sigma, St Louis, Missouri, USA), 10% fetal bovine serum (Sigma), 25 mM Hepes (Sigma), 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco RBL, Grand Island, New York, USA) (RPMI 10), and then incubated in the presence of PEG and SLEE at 37°C in a humidified atmosphere with 5% CO₂. The culture supernatants were collected after incubation for 48 hours, and chemokine concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits for quantification of murine CCL5, CCL17/TARC, and CXCL10/IP-10 (R&D Systems, Minneapolis, Minnesota, USA). The data were expressed as means (SD) and differences between means were analyzed using the 2-tailed *t* test. Statistical significance was set at $P < .05$. In order to determine the levels of type 1 T helper (T_H1)- and T_H2-associated chemokine messenger RNA (mRNA) expression in LCs, mRNA was extracted from LCs treated with PEG and SLEE for 24 hours using a Quick Prep Micro mRNA purification kit (GE Healthcare, Buckinghamshire, UK). Subsequently, cDNA was synthesized from 160 ng of the mRNA using a first-strand cDNA synthesis kit (GE Healthcare). Polymerase chain reaction (PCR) was performed using a GeneAmp PCR System 9700 (Perkin-Elmer, Norwalk, Connecticut, USA) in a 25- μ L reaction volume containing 1.5 μ L of cDNA (corresponding to 16 ng mRNA starting material) as described previously [7], and the PCR products were separated on a 2% agarose gel containing ethidium bromide. The primers used for amplification of CXCL9/Mig [13], CXCL10 [14], CCL17 [15], CCL22/MDC [16], and β -actin have been described elsewhere [7].

Mast Cells and Histamine Release Assay

Mast cells for histamine release assay were induced by long-term culture (16-20 days) of mouse spleen cells in RPMI 10 supplemented with 10 ng/mL tumor necrosis factor α (R&D Systems), as described by Hu et al [17]. To obtain purified mast cells, the mast cells in the culture were separated from dead cells and small lymphocytes by Histopaque-1077 density centrifugation (Sigma) and further expanded in RPMI 10 supplemented with 20 ng/mL interleukin 3 (IL-3) (Peprotech, Rocky Hill, New Jersey, USA) and 20 ng/mL IL-4 (Peprotech) for 2 weeks. These mast cells were then incubated at 1×10^6 cells in 0.2 mL RPMI 10 containing 10 $\mu\text{g/mL}$ anti-dinitrophenol (DNP) monoclonal mouse IgE antibody (Sigma). After incubation for an hour at 37°C in a humidified atmosphere with 5% CO₂, the cells were washed 3 times with RPMI 10. The cells were then suspended in 0.2 mL RPMI 10, preincubated at 37°C for 10 minutes with 25 $\mu\text{g/mL}$ SLEE and subsequently stimulated at 37°C for 20 minutes with 5 $\mu\text{g/mL}$ DNP-human serum albumin (Sigma). Cell stimulation was stopped by cooling in iced water and the supernatant was removed for histamine assay. Histamine release was measured using a competitive ELISA kit for histamine (Immunotech Beckman Coulter, Marseille, France). Data were expressed as means (SD) and differences between means were analyzed using the *t* test as described above.

Results

The first experiments were carried out to examine the influence of SLEE on PEG-induced CCL5 production by murine epidermal LCs. Murine LCs were treated with 10 $\mu\text{g/mL}$ PEG, which signals through toll-like receptor 2 [18,19], in the presence of 3.1 to 50 $\mu\text{g/mL}$ SLEE; the production of CCL5 by LCs was confirmed by ELISA after 48 hours of incubation. Figure 1 shows that SLEE significantly inhibited PEG-induced CCL5 production in a dose-dependent manner at 6.3 $\mu\text{g/mL}$ and above, and the level of inhibition reached a plateau at 25 $\mu\text{g/mL}$ SLEE. Subsequent experiments were thus carried out using 25 $\mu\text{g/mL}$ SLEE.

CXCL9 and CXCL10 are T_H1-associated chemokines that are structurally related and share the common receptor CXCR3 [20]. CCL17 and CCL22, in contrast, are T_H2-associated chemokines that share the receptor CCR4. To investigate whether SLEE influences mRNA expression for these chemokines, semiquantitative reverse transcription (RT)-PCR analysis was performed. After 24 hours of culture in the presence of PEG, the LCs began to express mRNA for CXCL10 and CCL17, but not for CXCL9 or CCL22 (Figure 2A). Furthermore, the expression of CCL17 mRNA but not that of CXCL10 mRNA was inhibited by SLEE. To determine whether CXCL10 and CCL17 were produced at the protein level by cultured LCs and secreted into the culture medium, ELISA was

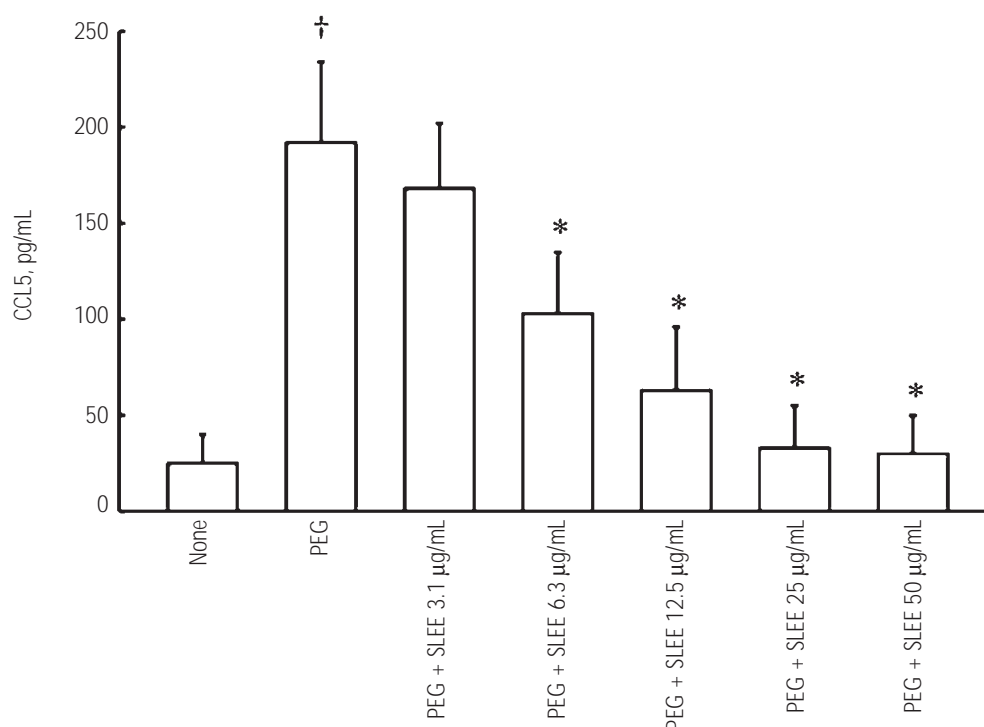


Figure 1. Effect of *Schefflera leucantha* ethanol extract (SLEE) on production of CCL5 by murine Langerhans cells (LCs). LCs ($1 \times 10^6/\text{mL}$) from mouse epidermis were incubated with 10 $\mu\text{g/mL}$ peptidoglycan (PEG) in the presence or absence of 3.1 to 50 $\mu\text{g/mL}$ SLEE. After 48 hours of incubation, culture supernatants were assayed for CCL5 production using enzyme-linked immunosorbent assay. The results are expressed as means (SD) ($n=7$).

* $P < .05$ versus PEG treatment.

† $P < .01$ versus nontreatment.

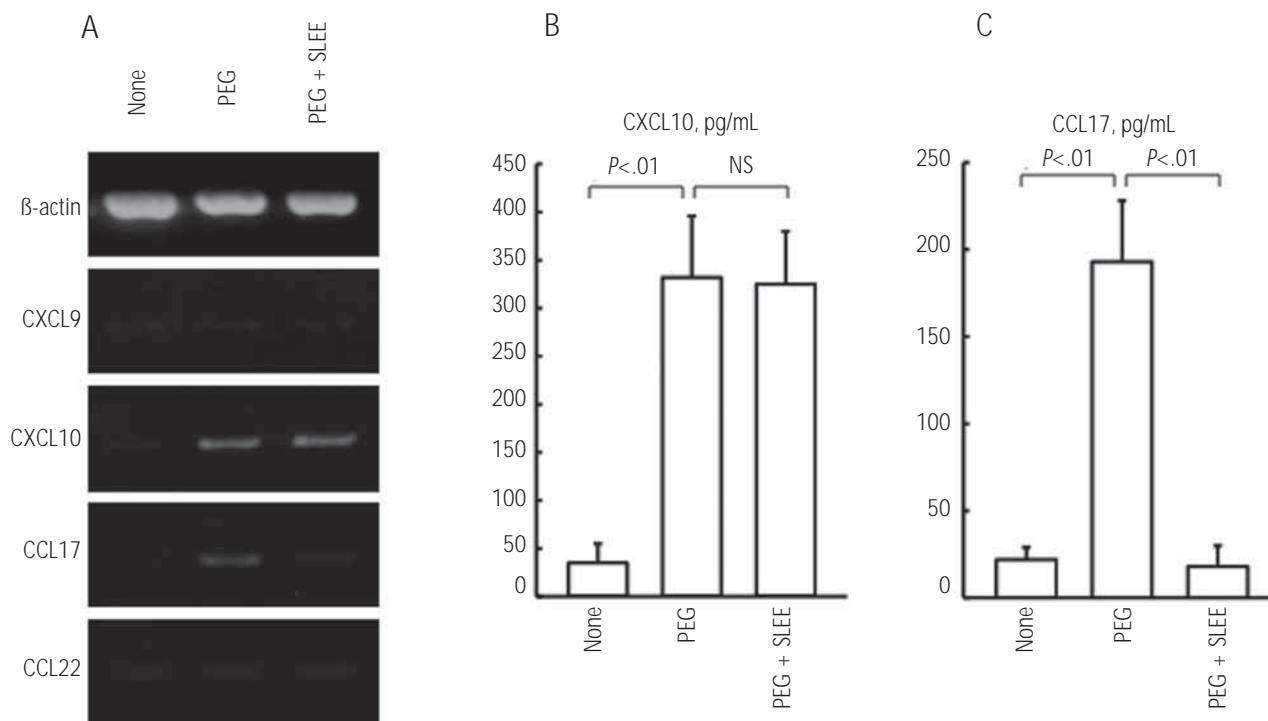


Figure 2. Effect of *Schefflera leucantha* ethanol extract (SLEE) on type 1 T helper (T_H1)- and T_H2 -associated chemokine production by murine Langerhans cells (LCs). LCs (1×10^6 /mL) from mouse epidermis were incubated with 10 μ g/mL PEG in the presence or absence of 25 μ g/mL SLEE. A, After 24 hours of incubation, cytoplasmic messenger RNA was extracted from LCs (5×10^5 cells), reverse-transcribed, and amplified by polymerase chain reaction using primer sets for β -actin, CXCL9, CXCL10, CCL17, and CCL22. The data shown are representative results of 4 independent experiments. B, After 48 hours of incubation, culture supernatants were assayed for CXCL10 production using enzyme-linked immunosorbent assay (ELISA). The results are expressed as means (SD) ($n=7$). C, After 48 hours of incubation, culture supernatants were assayed for CCL17 production using ELISA. The results are expressed as means (SD) ($n=7$).

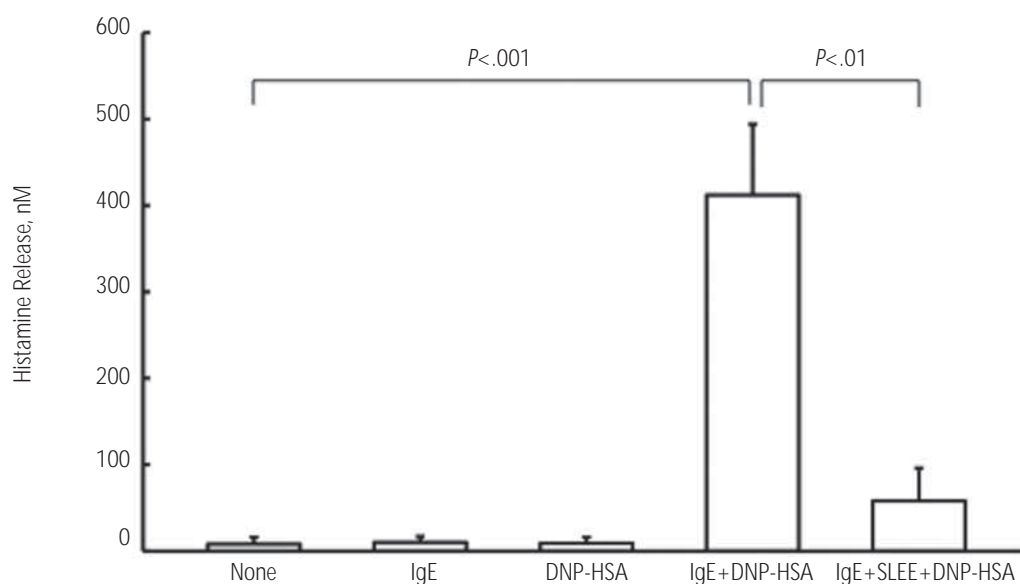


Figure 3. Effect of *Schefflera leucantha* ethanol extract (SLEE) on immunoglobulin (Ig) E-mediated histamine release from murine mast cells. Mast cells sensitized with anti-DNP IgE antibody were pretreated with 25 μ g/mL SLEE, or untreated, for 10 minutes and then stimulated with DNP-human serum albumin for 20 minutes. The supernatants were analyzed for histamine release from mast cells by competitive enzyme-linked immunosorbent assay. The results are expressed as means (SD) ($n=7$).

carried out using medium cultured for 48 hours. As shown in Figures 2B and 2C, PEG-stimulated LCs produced significant levels of CXCL10 and CCL17, while SLEE inhibited CCL17 but not CXCL10 production. These results indicate that the inhibitory effects of SLEE are more specific to T_H2 -associated chemokine production.

When mast cells sensitized with anti-DNP monoclonal IgE antibody were subsequently activated by antigen (DNP-HSA), significant histamine release was elicited (Figure 3). However, brief exposure of the cells to 25 $\mu\text{g}/\text{mL}$ SLEE caused a marked reduction in antigen-induced histamine release. These results suggest that SLEE has the ability to suppress IgE-mediated hypersensitivity reactions through the degranulation of mast cells.

In this study, 0.1% DMSO (Figure 1) or 0.05% DMSO (Figures 2 and 3), used as a solvent control of SLEE, did not inhibit the production of any of the chemokines, the expression of their mRNAs, or IgE-mediated histamine release. Furthermore, SLEE was not toxic to LCs or mast cells at any of the concentrations tested (% cell survival >94%) (data not shown).

Discussion

Chemokines are involved in the recruitment of inflammatory cells to the site of an immune reaction. The chemokine CCL5 has been reported to elicit significant migration of eosinophils [21]. Furthermore, the production of CCL5 is increased in the lesional skin of AD patients, suggesting that localized CCL5 production may be associated with eosinophil infiltration in AD [22]. Since the skin of most AD patients shows superficial *S aureus* colonization and barrier disruption due to reduced ceramide levels [23], PEG from *S aureus* would be expected to penetrate the skin and may play a critical role in perpetuating skin tissue inflammation through eosinophil infiltration [9]. Therefore, the combination of antimicrobial treatment and anti-CCL5 therapy in at least a subgroup of AD patients, irrespective of whether or not they show clinical signs of superinfection, may be considered a possible new therapeutic strategy for AD. Our results demonstrate that SLEE suppressed CCL5 production by LCs stimulated with PEG, suggesting that *S leucantha* may be useful for relieving allergic symptoms caused by eosinophil infiltration associated with *S aureus* colonization.

We found that SLEE suppressed the production of the T_H2 -associated chemokine CCL17, but not that of the T_H1 -associated chemokine CXCL10. A T_H2 response is observed in the acute-phase reaction of AD and is associated with induction of the early allergic reaction [1]. Histamine is also regulated as a primary mediator in early allergic reactions, and histamine released by degranulation of sensitized mast cells/basophils causes allergic symptoms. Since the release of histamine from IgE-sensitized mast cells was also suppressed by pretreatment of mast cells with SLEE, the use of SLEE as a topical agent may be effective for the acute phase of inflammation in AD patients. The current chemotherapy for AD includes the wide use of anti-allergic drugs and corticosteroids. Although topical corticosteroids are effective for the inhibition

of allergic inflammation on the lesional skin of AD patients, major systemic side effects have been frequently observed [24]. Thus, there is a need to develop anti-allergic agents from natural sources such as medicinal plants, and the importance of ethnomedicine may increase in the dermatological field due to the relatively low cost, easy accessibility, and low toxicity of these agents. In our preliminary experiments, SLEE was dissolved in methanol to which water was then added. This aqueous methanol fraction was partitioned with hexane, and then the aqueous methanol extract was partitioned with ethyl acetate. Using bioassay-guided fractionation of SLEE, it was deduced that active components in SLEE would be present in the ethyl acetate extract. These results suggested that the active components would be secondary metabolites of *S leucantha*. The present report is the first to document an inhibitory effect of *S leucantha* on the release of allergic mediators. Our results indicate that *S leucantha* might offer a new therapeutic approach for the control of AD, and suggest that further studies of the anti-allergic activities of compounds purified from *S leucantha* should be undertaken.

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