Title: Anti-atopic dermatitis effects of *Parasenecio auriculatus* via simultaneous inhibition of multiple inflammatory pathways

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Supplementary Materials and Methods

Western blot

Whole-cell lysates were prepared using passive lysis buffer (Promega, Madison, WI, USA) with a protease inhibitor cocktail (Roche, Basel, Switzerland). Proteins were electrophoresed and transferred to a membrane. The membranes were incubated overnight at 4°C with primary antibodies against phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38, phospho-Lyn, Lyn, phospho-Syk, Syk, phospho-PLCγ1, PLCγ1, phospho-NF-κB, and NF-κB (Cell Signaling Technology, Beverly, MA, USA), GAPDH (Trevigen, Gaithersburg, MD, USA), ZO-1, and occludin (Invitrogen, Camarillo, CA, USA). The membranes were then washed and incubated with the appropriate secondary antibodies for 1 hour at room temperature. Western blot images were analyzed using a LAS 4000 mini camera (Fujifilm, Tokyo, Japan). The relative density of each band was quantified using ImageJ software (NIH).

Immunofluorescence

Cells grown on slides were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde or 100% methanol for 15 min, and permeabilized in 0.2% Triton X-100 in PBS. Slides were incubated overnight at 4°C with the primary antibody. Slides were then incubated for 1 hour with the appropriate fluorescence-labeled secondary antibody (Life Technologies). All images were collected using an LSM700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Cell viability assay

Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. HEK293 cells, RAW264.7 cells, and HaCaT cells were seeded in 96-well plates at a density of 2×10^4 cells/well and treated with PAE in a dose-dependent manner. MTT (5 mg/ml) was added to the plates and incubated at 37°C for 4 hours. After the formazan crystals were dissolved with DMSO, the OD was measured at 570 nm using a microplate reader.

Animal models

All procedures were approved by the Ethics Committee of the Catholic University of Korea (No. 2020-0233-05). Female BALB/c mice, aged 6 weeks, were purchased from Orientbio (Sungnam, Korea). Mice had free access to diet and water under a 12 hours light/12 hours dark cycle in a temperature-controlled environment. Briefly, the dorsal hair of mice was completely removed before the day of administration. On day 1, 150 μL of 1% dinitrochlorobenzene (DNCB, Merck, NJ, USA) dissolved in a vehicle containing acetone and olive oil (4:1 v/v) was dropped on the dorsal skin. On day 8, 150 μL of 0.2% DNCB solution was applied to the dorsal skin three times a week for 4 weeks (days 8-34). For the experimental group, 150 μL of PAE at a dose of 10 mg/ml and 150 μL of dexamethasone (Merck) at a dose of 10 μM were topically applied to the same area once per day for 2 weeks (days 22–34). When the experiments were completed (day 35), the animals were sacrificed, and dorsal dermal tissue was collected for analysis.

Evaluation of atopic index

The severity of dermatitis was assessed by the scoring AD (modified SCORAD) method. The degree of each symptom was scored as 0 (absence), 1 (mild), 2 (moderate), and 3 (severe). This scoring was based on the severity of 1) erythema/hemorrhage, 2) scaling/dryness, 3) edema, and 4) excoriation/erosion. The sum of individual scores (minimum, 0; maximum, 12) was used as the atopic index.

Histological analysis

To assess the epidermal thickness, basement membrane thickness, and inflammatory cell infiltration, the dorsal skin lesions of the mice were fixed in 10% neutral-buffered formalin (Merck), and 4 μm-thick paraffin sections were prepared. Hematoxylin and eosin (HE) staining, periodic acid-Schiff (PAS) staining, and toluidine blue staining (TBS) were performed for 30 sec each at 37°C to identify the epidermal thickness, basement membrane thickness, and inflammatory cells of each group. Epidermal and basement membrane thicknesses were measured using ImageJ software. To stain macrophages, VECTASTAIN Elite ABC Universal Kit peroxidase (Vector laboratories, CA, USA) was used with antibodies against F4/80 (eBioscience, CA, USA) at a dilution of 1:50, according to the manufacturer's standard protocol. The number of eosinophils, mast cells, and

macrophages in each section was obtained from 10 random views under 200× magnification. The tissue sections were observed and photographed using a digital camera (EOS 300D, Canon, Tokyo, Japan). Each experimental group consisted of six mice.

Extraction procedure and phytochemical analysis

P. auriculatus (family *Compositae*) is a perennial herb that grows at high altitudes. This plant is a native Korean species but is also distributed in Japan, northern China, and Russian Far East. The extract of this plant was obtained from the natural product library of the KIST Gangneung Institute of Natural Products, which contains ethanolic extracts of edible plants native to Korea. Briefly, the aerial parts of the plants were collected in June 2015 on the Bongui Mountain of Chuncheon, Gangwon-do, Republic of Korea. A voucher specimen (accession number BE0675A1) was deposited at the KIST Gangneung Institute of Natural Products. The dried plants (100 g) were ground and extracted twice with 100% ethanol (1 L × 2) under reflux for 2 hours to obtain a dark green ethanolic extract (17.1 g). In addition, 29 other PEs extracted by the same method were provided from this library, and detailed information on the PEs is presented in Supplementary Table 1. PAE was analyzed using an Agilent 1200 HPLC system connected with 6120 quadrupole MSD equipped with a YMC-Pack ODS-A column (3 μm, 50 × 4.6 mm). The injection volume was 10 μL and the column temperature was set at 30˚C. The mobile phase consisted of 0.05% formic acid (Sigma-Aldrich) in water and acetonitrile with a flow rate of 0.7 mL/min. The gradient condition was as follows: 0 - 12 min, 10 - 100% acetonitrile.

Quantitative RT-PCR

Reverse transcription was performed using M-MLV Reverse Transcriptase (Life Technologies). qRT-PCR was performed using SYBR Premix Ex Taq II (Takara Biotechnology, Dalian, China) and QuantStudio 6 Pro System (Applied Biosystems, Carlsbad, CA, USA). The amount of target mRNA was normalized to that of GAPDH mRNA. The primer sequences used are listed in Supplementary Table 2.

NO assay

RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10^4 cells per well and incubated with or without LPS (1 μg/ml) in the absence or presence of various

concentrations of PAE for 24 hours. To analyze nitrite production, the supernatant was collected and mixed with Griess reagent (Sigma-Aldrich) in a 1:1 ratio. After 20 min, the OD was measured at 540 nm using a microplate reader. Nitrite concentrations were calculated using a standard curve.

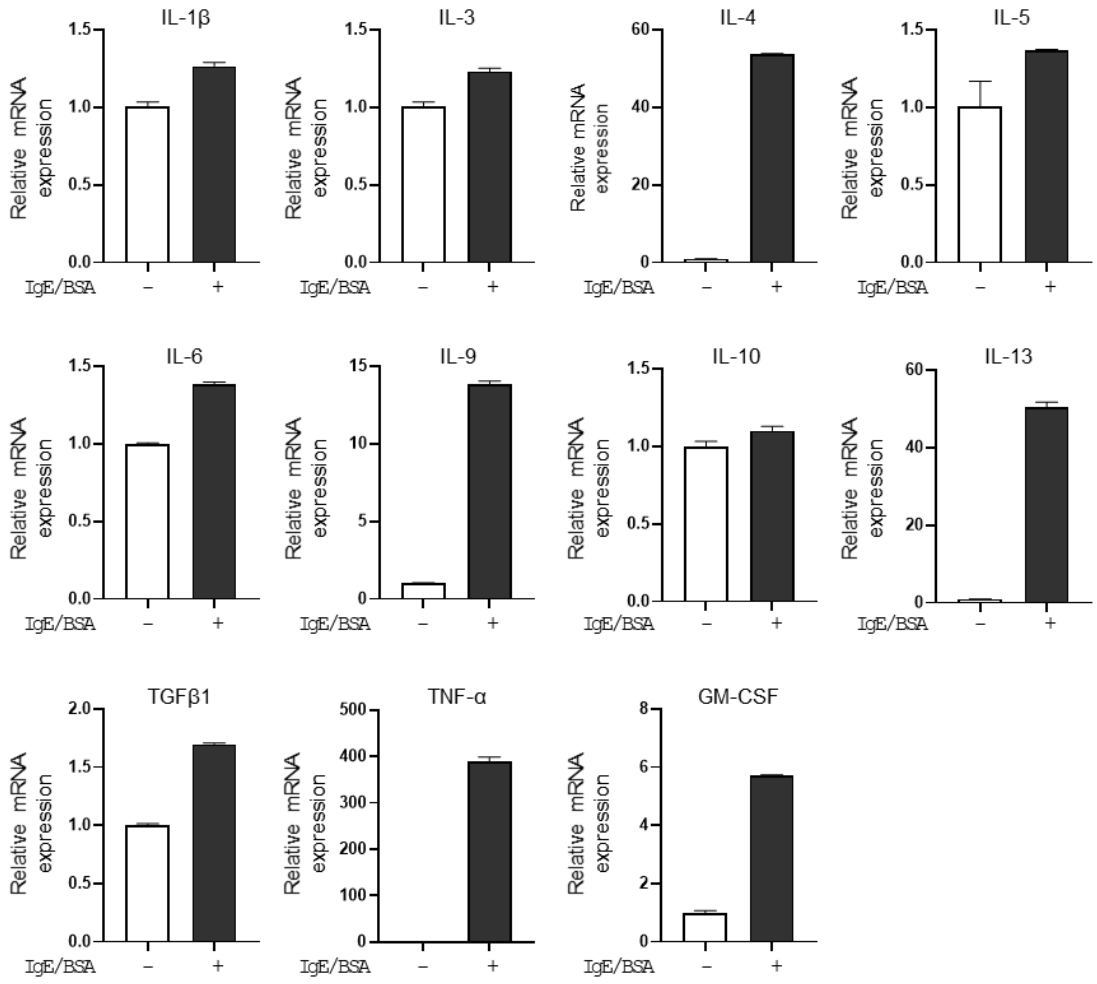
Enzyme-Linked Immunosorbent Assay (ELISA)

After 12 hours of DNP-BSA/IgE stimulation, RBL-2H3 cells were co-treated with PAE (100 μg/ml) and 100 ng/ml DNP-BSA at 37°C for 2 hours. Then, the supernatants from cultured RBL-2H3 cells were collected and the IL-4 concentration was measured with Rat IL-4 ELISA kit (Biomatik, ON, Canada), according to the manufacturer's instructions. RAW 264.7 cells were co-treated with PAE (100 μg/ml) and LPS (1 μg/ml) for 24 hours. Then, the supernatants from cultured RAW 264.7 cells were collected and IL-6 concentration was measured with Mouse IL-6 ELISA MAX Deluxe kit (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions.

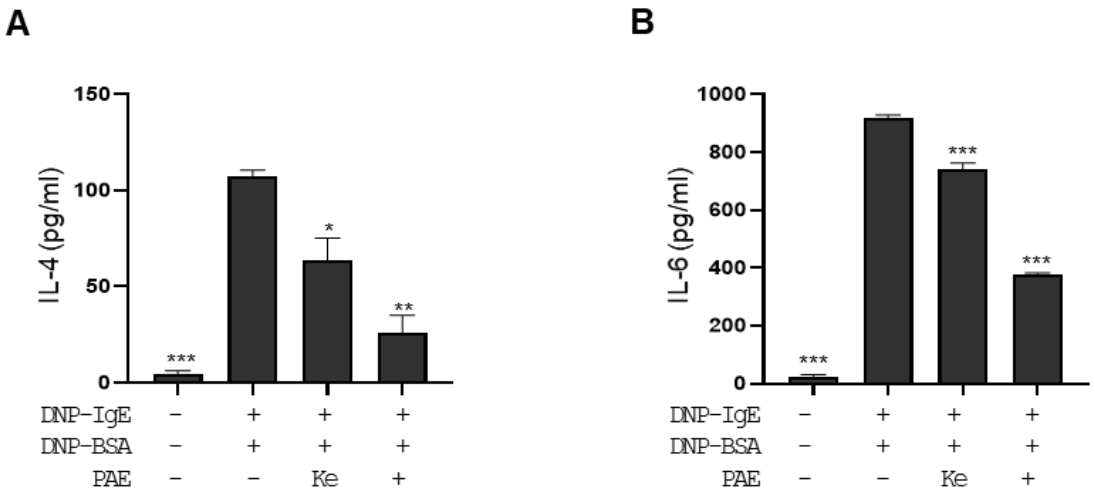
Statistical analysis

Statistical analyses were performed using SPSS software, version 21.0.0.0 for Windows (IBM, Armonk, NY, USA). Data are expressed as mean ± SD, and a one-way analysis of variance with a post hoc test (Bonferroni) was performed to compare multiple means.

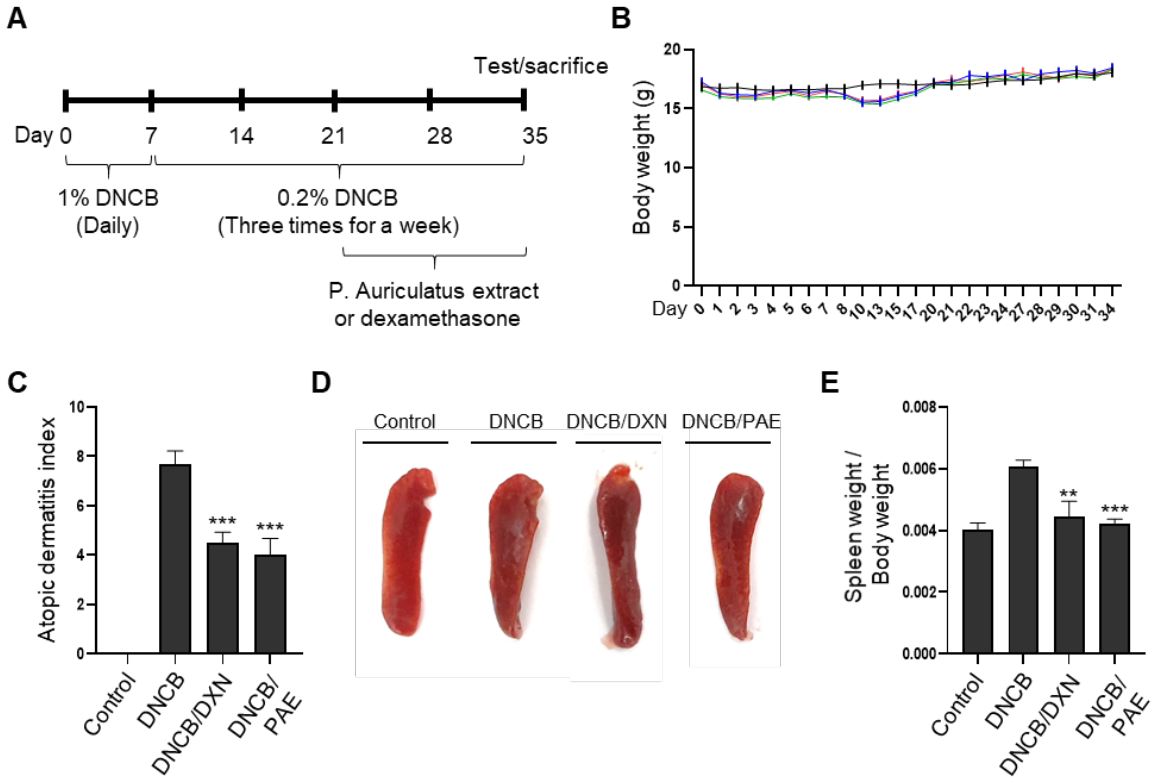
Supplementary Figures



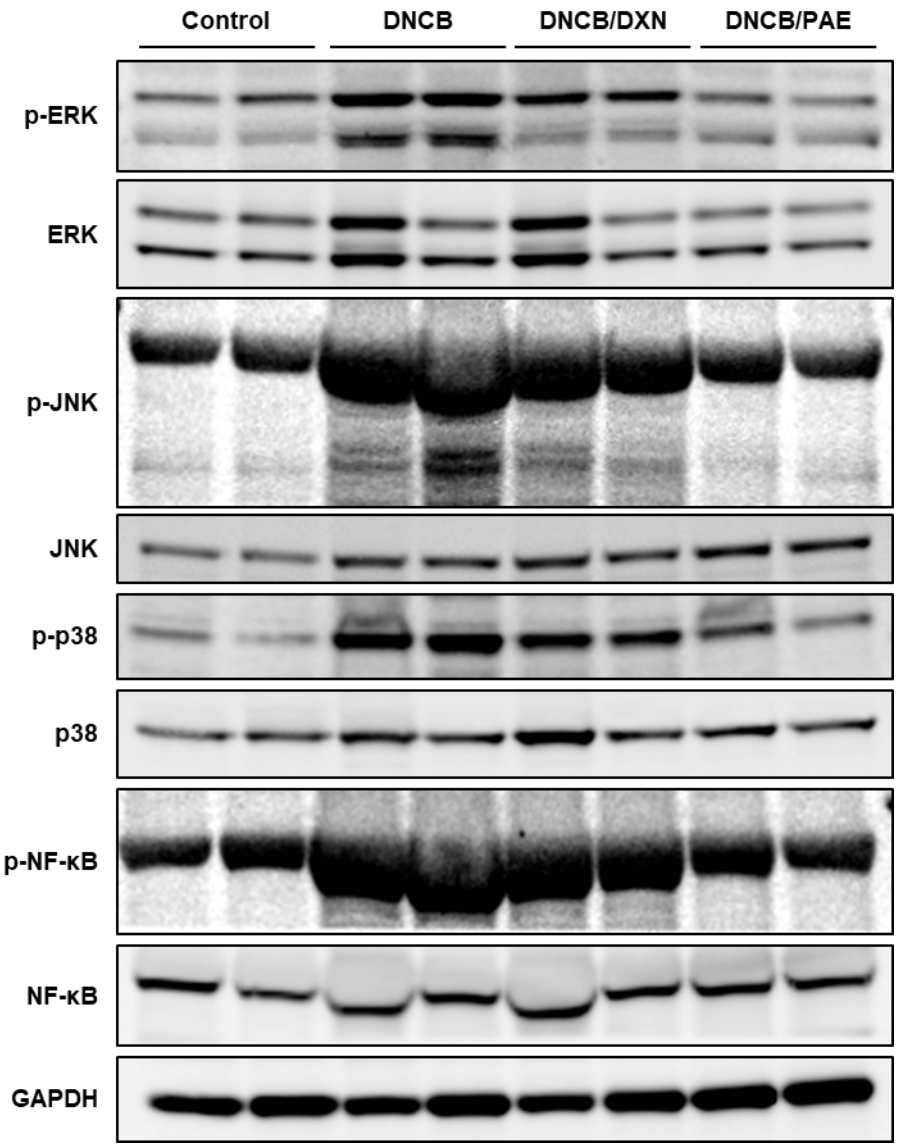
Supplementary Figure 1. qPCR analysis of atopic dermatitis-related cytokines in activated RBL-2H3 cells. To select the cytokines specifically affected by DNP-BSA/IgE stimulation in RBL-2H3 cells, the mRNA expression of IL-1β, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, TGFβ1, TNF-α, and GM-CSF is analyzed by qRT-PCR.



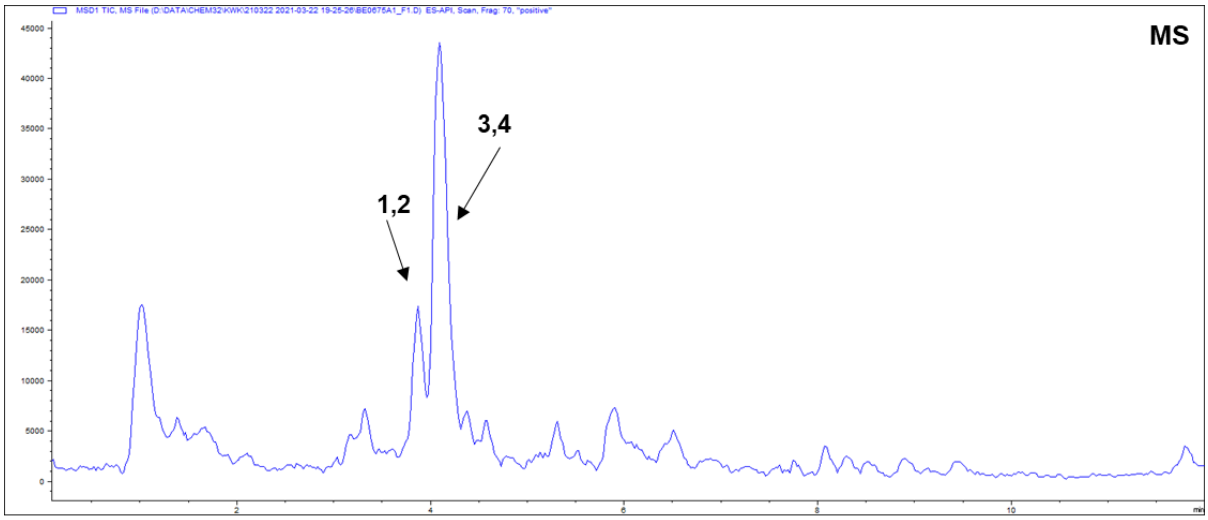
Supplementary Figure 2. ELISA analysis of IL-4 and IL-6 secretion. The secretion of IL-4 and IL-6 was measured in RBL-2H3 and RAW264.7 cells after PAE treatment, respectively.



Supplementary Figure 3. Construct of a 2,4-Dinitrochlorobenzene (DNCB)-induced atopic dermatitis (AD) mouse model. (A) A schematic of AD mouse model construction. Four groups are generated: a control (healthy) group, a DNCB-induced AD group, a DNCB-induced AD group with topical dexamethasone treatment, and a DNCB-induced AD group with topical PAE treatment. Each group consisted of six mice. (B) Body weight is regularly measured in each group, and the mean weight of each group is presented. (C) The atopic index is evaluated by scoring the levels of excoriation/erosion, scarring, erythema, and edema in each group. (D) Spleen weight is measured after mice are sacrificed, and the spleen to body weight ratio is calculated. *p < 0.05; ** p< 0.01; *** p< 0.001.

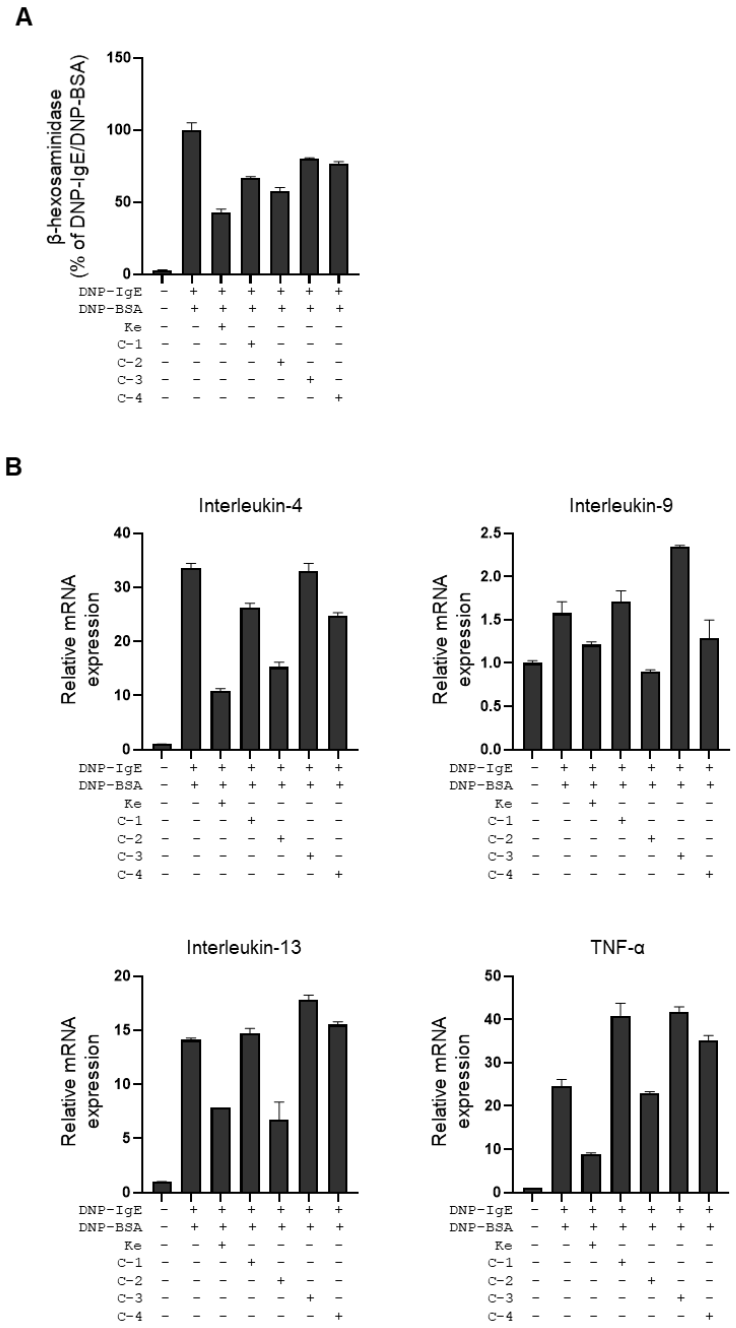


Supplementary Figure 4. Activation status of TLR4 and FcεRI signaling molecules in an AD mouse model. Using mouse skin tissues, western blot was performed to evaluate the effect of PAE on the expression of ERK, phospho-ERK, JNK, phospho-JNK, p38, phospho-p38, NF-kB, and phospho-NF-kB.



1: neoplatyphylline
2: integerrimine
3: neoplatyphylline N-oxide
4: integerrimine N-oxide

Supplementary Figure 5. Identification of major components in PAE. Mass chromatogram of PAE is displayed. Compounds 1 and 2 were detected at 3.8 min and compounds 3 and 4 were detected at 4.1 min. Compounds 1 and 2 were identified as neoplatyphylline and integerrimine, respectively. Compounds 3 and 4 were identified as neoplatyphylline N-oxide and integerrimine N-oxide, respectively.



Supplementary Figure 6. Analysis of anti-inflammatory activity of major compounds derived from PAE. (A) β-hexosaminidase release is measured in stimulated RBL-2H3 cells treated with neoplatyphylline, integerrimine, neoplatyphylline N-oxide, and integerrimine N-oxide (100 μM). (B) The expression of IL-4, IL-9, IL-13, and TNF-α is measured by qRT-PCR in the same samples used in (A). Ketotifen was used as a positive control at a concentration of 100 μM.

Supplementary Table 1. Detailed information of 30 plant sources.

No	Name	Part	Extraction method
1	*Ulmus macrocarpa* Hance	bark	100% EtOH (100 g/L); reflux
2	*Sambucus sieboldiana* (Miq.) Blume ex Graebn.	stem	100% EtOH (100 g/L); reflux
3	*Houttuynia cordata* Thunb.	aerial	100% EtOH (100 g/L); reflux
4	*Sambucus williamsii* var. *coreana* (Nakai) Nakai	twig, leave	100% EtOH (100 g/L); reflux
5	*Hydrangea serrata* f. *acuminata* (Siebold & Zucc.) E.H.Wilson	aerial	100% EtOH (100 g/L); reflux
6	*Cirsium japonicum* var. *maackii* (Maxim.) Matsum.	aerial	100% EtOH (100 g/L); reflux
7	*Dystaenia takesimana* (Nakai) Kitag.	aerial	100% EtOH (100 g/L); reflux
8	*Aruncus dioicus* var. *kamtschaticus* (Maxim.) H. Hara	whole plant	100% EtOH (100 g/L); reflux
9	*Boehmeria nivea* (L.) Gaudich.	whole plant	100% EtOH (100 g/L); reflux
10	*Nelumbo nucifera* Gaertn.	seed	100% EtOH (100 g/L); reflux
11	*Rehmannia glutinosa* (Gaertn.) Libosch. ex Steud.	root	100% EtOH (100 g/L); reflux
12	*Hydnocarpus anthelmintica* Pier.	seed	100% EtOH (100 g/L); reflux
13	*Viscum album* f. *rubroauranticum* (Makino) Ohwi	aerial	100% EtOH (100 g/L); reflux
14	*Patrinia scabiosifolia* ex Trevir.	aerial	100% EtOH (100 g/L); reflux
15	*Xanthium strumarium* L.	rruit	100% EtOH (100 g/L); reflux
16	*Fraxinus rhynchophylla* Hance	twig, leave	100% EtOH (100 g/L); reflux
17	*Chelidonium majus* var. *asiaticum* (H. Hara) Ohwi	aerial	100% EtOH (100 g/L); reflux
18	*Sorbus commixta* Hedl.	bark	100% EtOH (100 g/L); reflux
19	*Akebia quinata* (Houtt.) Decne.	fruit	100% EtOH (100 g/L); reflux
20	*Oenothera biennis* L.	aerial	100% EtOH (100 g/L); reflux
21	*Parasenecio auriculatus* var. *matsumurana* Nakai	aerial	100% EtOH (100 g/L); reflux
22	*Philadelphus schrenkii* Rupr.	twig, leave,	100% EtOH (100 g/L); reflux
23	*Justicia procumbens* L.	whole plant	100% EtOH (100 g/L); reflux
24	*Angelica decursiva* (Miq.) Franch. & Sav.	aerial	100% EtOH (100 g/L); reflux
25	*Paeonia lactiflora* Pall.	root	100% EtOH (100 g/L); reflux
26	*Glehnia littoralis* F.Schmidt ex Miq.	aerial	100% EtOH (100 g/L); reflux
27	*Trichosanthes kirilowii* Maxim.	root	100% EtOH (100 g/L); reflux
28	*Ligularia fischeri* (Ledeb.) Turcz.	aerial	100% EtOH (100 g/L); reflux
29	*Dendranthema zawadskii* var. *latiloba* (Maxim.) H.C.Fu	whole plant	100% EtOH (100 g/L); reflux
30	*Dryopteris crassirhizoma* Nakai	aerial	100% EtOH (100 g/L); reflux

Supplementary Table 2. Primers used in this study

Cell line	Target	Direction	Sequence
RBL-2H3	IL-1β	Forward	5'- GCTGTGGCAGCTACCTATGTCTTG
		Reverse	5'- AGGTCGTCATCATCCCACGAG
	IL-3	Forward	5'- CCAGATTTCAGACAGGGGCTC
		Reverse	5'- CAGGTTTACTCTCCGCAAGGT
	IL-4	Forward	5'- GAAAAAGGGACTCCATGCAC
		Reverse	5'- TCTTCAAGCACGGAGGTACA
	IL-5	Forward	5'- TGTTGACGAGCAATGAGACGA
		Reverse	5'- ATGGTATTTCCACAGTGCCCC
	IL-6	Forward	5'- TCCTACCCCAACTTCCAATGCTC
		Reverse	5'- TTGGATGGTCTTGGTCCTTAGCC
	IL-9	Forward	5'- CCCTAAAGAGCAACAAGTGTCA
		Reverse	5'- GTGTTGCCTGCTGTGGTCT
	IL-10	Forward	5'- TGCCAAGCCTTGTCAGAAATGATCAAG
		Reverse	5'- GTATCCAGAGGGTCTTCAGCTTCTCTC
	IL-13	Forward	5'- CCACAGGACCCAGAGGATATTGA
		Reverse	5'- TAGCGGAAAAGTTGCTTGGAGTAA
	TGFβ1	Forward	5'- ATTCCTGGCGTTACCTTGG
		Reverse	5'- AGCCCTGTATTCCGTCTCCT
	TNFα	Forward	5'- ATGGGCTCCCTCTCATCAGT
		Reverse	5'- GAAATGGCAAATCGGCTGAC
	GM-CSF	Forward	5'- AGACCCGCCTGAAGCTATACAA
		Reverse	5'- CTGGTAGTGGCTGGCTATCATG
	GAPDH	Forward	5'- GAGAAGGCTGGGGCTCAC
		Reverse	5'- GTTGTCATGGATGACCTTGGCC
RAW264.7	IL-1β	Forward	5'- GCAACTGTTCCTGAACTCAACT
		Reverse	5'- ATCTTTTGGGGTCCGTCAACT
	IL-6	Forward	5'- CCAAGAGGTGAGTGCTTCCC
		Reverse	5'- CTGTTGTTCAGACTCTCTCCCT
	TNFα	Forward	5'- AGCCCCCAGTCTGTATCCTT
		Reverse	5'- CTCCCTTTGCAGAACTCAGG
	COX-2	Forward	5'- TGAGTACCGCAAACGCTTCTC
		Reverse	5'- TGGACGAGGTTTTTCCACCAG
	iNOS	Forward	5'- GGCAGCCTGTGAGACCTTTG
		Reverse	5'- GCATTGGAAGTGAAGCGTTTC
	GAPDH	Forward	5'- ATGGTGAAGGTCGGTGTGAAC
		Reverse	5'- GCCGTGAGTGGAGTCATACTG
HaCaT	IL-6	Forward	5'- CCACTCACCTCTTCAGAACG
		Reverse	5'- CATCTTTGGAAGGTTCAGGTTG
	IL-8	Forward	5'- CAAACCTTTCCACCCCAAAT
		Reverse	5'- ACCCTCTGCACCCAGTTTTC

	CCL5	Forward	5'- AGTGTGTGCCAACCCAGAGA
		Reverse	5'- AGCAAGCAGAAACAGGCAAA
	CCL17	Forward	5'- GTCTTGAAGCCTCCTCACCC
		Reverse	5'- GGATCTCCCTCACTGTGGCT
	CCL22	Forward	5'- CCTGCCAAAAGGCAGTTACA
		Reverse	5'- TGGGGGAAGAAAGGTGAATC
	GAPDH	Forward	5'- GGAGCGAGATCCCTCCAAAAT
		Reverse	5'- GGCTGTTGTCATACTTCTCATGG