

Anti-inflammatory effect of *Amomum xanthioides* in a mouse atopic dermatitis model

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Abstract. Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disorder. The present study investigated the effects of *Amomum xanthioides* extract (AXE) on AD-like skin inflammation using a *Dermatophagoides farinae* extract (DFE) and 2,4-dinitrochlorobenzene (DNCB)-induced mouse AD model. Hematoxylin and eosin staining results demonstrated that repeated DFE/DNCB exposure markedly increased the thickening of the dermis and epidermis, in addition to the infiltration of eosinophils and mast cells. However, oral administration of AXE reduced these histopathological alterations in a dose-dependent manner. Elevated serum histamine, total and DFE-specific immunoglobulin E (IgE), and IgG2a were also decreased by treatment with AXE. In addition, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results demonstrated that the mRNA expression of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , interleukin (IL)-4, IL-13, IL-31 and IL-17A was reduced in ear skin following AXE administration in AD mice. Fluorescence-activated cell sorting demonstrated that the population of CD4⁺/IL-4⁺, CD4⁺/IFN- γ ⁺ and CD4⁺/IL-17A⁺

cells in draining lymph nodes was also significantly decreased in AXE-treated mice compared with AD mice without AXE treatment. Furthermore, keratinocytes that were stimulated with TNF- α and IFN- γ exhibited increased gene expression of pro-inflammatory cytokines and chemokines, including TNF- α , IL-1 β , IL-6, IL-8, C-C motif chemokine ligand (CCL)17 and CCL22, as determined by RT-qPCR. However, upregulation of these genes was reduced by AXE pretreatment. Based on these results, we hypothesize that AXE may be useful in the treatment of allergic skin inflammation, particularly AD.

Introduction

Atopic dermatitis (AD) is a pruritic and chronic inflammatory skin disease caused by dysfunction of the skin barrier and immune response. Initially, skin dysfunction may be caused by increased protease activity, irritant exposure or genetic mutation (1). Disturbed skin tissues exhibit an increased permeability to external antigens or allergens, an increased release of innate immune cytokines from keratinocytes and increased infiltration of type 2 helper (Th2) T cells (2). Infiltrated Th2 cells secrete various inflammatory cytokines, including interleukin (IL)-4, IL-5 and IL-13, which are considered to increase immunoglobulin E (IgE) levels. Th2-promoted pro-inflammatory mediators further impair epidermal differentiation and integrity, and subsequently induce the release of pro-inflammatory and pruritogenic mediators by keratinocytes (2).

AD develops in early childhood up to five years old and is observed in 60% of adults who have a personal family history of similar skin diseases or asthma during childhood (3). Methods of treating this disorder generally include the application of topical steroids or antibiotics and general skin care. As the number of available treatments is limited and several side effects are reported, certain patients opt for alternative treatments such as natural products (4,5). Various pharmacological compounds from natural products have been developed that

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may have the potential to treat various symptoms in chronic diseases including atopic dermatitis (AD) (6).

Amomum xanthioides is the seed of *Amomum villosum* Lour, which is grown throughout Asia. The seed of *A. xanthioides* has traditionally been used to treat indigestion, diarrhea, flatulence, toothache and sepsis in China and Vietnam (7). In addition, the fruit is effective against asthma and also functions as an antiemetic agent (7). Various studies have demonstrated that the seed extract of *A. xanthioides* (AXE) exerts various pharmacological effects, including hepatoprotective and anti-gastritis effects, and the prevention of diabetes mellitus (8,9). Furthermore, our previous studies demonstrated that seed AXE inhibited immediate-type hypersensitivity by reducing mast cell degranulation (10,11). The present study investigated the effects of AXE on allergic skin inflammation, specifically AD, and the underlying mechanism of action.

Materials and methods

Animals. Six-week-old BALB/c mice (female, 20 g) were purchased from Japan SLC, Inc (Hamamatsu, Japan). A total of 30 mice were housed with 5-10 mice per cage in a laminar air flow room and were maintained at a temperature of 22±2°C with a relative humidity of 55±5% throughout the study. The food and water were provided ad libitum, and mice were kept on a 12-h light/12-h dark cycle. 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 (Kyungpook National University, Daegu, Republic of Korea) and was approved by the Institutional Animal Care and Use Committee in Kyungpook National University, Daegu, Republic of Korea.

Preparation of AXE. The AXE was prepared as previously described (11). Briefly, *A. xanthioides* seeds were purchased from Bohwa Dang (Jeonju, Korea) and identified by Dr D.K. Kim at the College of Pharmacy, Woosuk University (Samrye, Korea). A voucher specimen (no. WSP-16-04) was deposited at the Herbarium of Woosuk University. Seeds were ground (1,000 rpm for 30 sec) at room temperature using a Micro Hammer-Cutter Mill (Culatti AG, Zurich, Switzerland). The particle size after grinding was 0.5-2 mm. The plant sample (60 g) was extracted twice with purified water (500 ml) at 70°C for 5 h in a water bath. The extract was filtered through Whatman grade 1 filter paper and the filtrate was lyophilized using 0.45 µm syringe filters. The dried extract yield from crude materials was ~5.2%. The dried extract was dissolved in saline or Tyrode's buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose and 0.1% bovine serum albumin (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA) prior to use.

Drugs and chemicals. All reagents were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) unless otherwise stated. House dust mite (*Dermatophagoides farinae*) extract (DFE; Greer Laboratories, Inc., Lenoir, NC, USA) was used as an antigen and 2,4-dinitrochlorobenzene (DNCB) was used as a sensitizer to induce AD-like skin inflammation. Freeze-dried crude DFE powder was dissolved in PBS

containing 0.5% Tween-20. DNCB (1%) was dissolved in an acetone/olive oil (1:3) solution. Recombinant TNF-α and IFN-γ were purchased from R&D systems, Inc. (Minneapolis, MN, USA).

Cell culture and viability. A human keratinocyte cell line, HaCaT (American Type Culture Collection, Manassas, VA, USA), was maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc) and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) at 37°C in 90-95% humidity and 5% CO₂. Cell viability was determined using a MTT assay. HaCaT cells were treated with various concentration of AXE (0, 0.01, 0.1, 1, 10 and 100 µg/ml) and incubated for 24 h at 37°C in 5% CO₂. Then, MTT (5 mg/ml) was added to each sample well and incubated for 2 h at 37°C. Dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance of each sample was at a wavelength of 570 nm compared with the control and expressed as a percentage.

Induction of AD-like skin inflammation in the mouse ear. The induction of AD-like skin inflammation by DFE and DNCB was performed using methods based on our previous research (12). Total 30 mice were divided into the following six groups (n=5 each group): Vehicle (mice were treated only with PBS); DFE/DNCB + vehicle; DFE/DNCB + AXE (2, 10 and 50 mg/kg) and tacrolimus (Tac; 1 mg/kg) (Sigma-Aldrich; Merck KGaA). The surfaces of both ear lobes were stripped very gently using surgical tape to remove foreign matter or scabbing. After stripping, 20 µl DNCB (1%) was applied to each ear, which was followed by 20 µl DFE (10 mg/ml) 4 days later. Treatment with DFE/DNCB was repeated once a week alternatively for 4 weeks. At 1 week after the first DFE/DNCB treatment, AXE or Tac was orally administered 5 times weekly between days 7 and 27. Ear thickness was measured 24 h after DFE or DNCB application with a dial thickness gauge.

On day 28, blood samples were collected via celiac artery puncture. Whole blood was incubated at 4°C overnight, centrifuged at 400 x g for 10 min at 4°C, and serum was collected. After mice were sacrificed, ears were removed and used for histopathological analysis. IgG2a levels were measured using an ELISA kit (cat. no. 552576, BD Biosciences, Franklin Lakes, NJ, USA). Total IgE and DFE-specific IgE level were assayed with the same kit (cat. no. 555248). Total IgE levels were measured by concentration calculation. Mite-specific IgE levels were detected as optical density values.

Histological observations. The ears were fixed with 10% formaldehyde for one week at room temperature and embedded in paraffin. Sections of 5 µm thickness were stained with hematoxylin solution for 5 min and eosin solution for 1 min at room temperature. Cellular infiltration of eosinophils and thickening of the epidermis and dermis were observed via light microscopy. Eosinophils were also counted from 10 different fields at a magnification x400 in a blinded manner. Dermal thickness in H&E-stained sections was visualized and analyzed at a magnification x200. Thickness was measured from five randomly selected fields from each sample. For measurement of mast cell infiltration, skin sections were stained with 0.1% toluidine blue solution for 2-3 min at room temperature and

mast cells from 10 different fields were counted at a magnification x400 in a blinded manner.

Histamine assay. Histamine content was measured via the *o*-phthaldialdehyde spectrofluorometric procedure based on the method described by a previous report (13). Blood from the mice was centrifuged at 400 x g for 10 min at 4°C and serum was withdrawn to measure histamine content. For serum histamine assay, 50 µl serum was used. Fluorescence was measured on an LS-50B fluorescence spectrometer (PerkinElmer, Inc., Waltham, MA, USA) using 355 nm excitation and 450 nm emission filters.

Fluorescence-activated cell sorting. At the end of the experiment, mice were euthanized with CO₂ and both auricular lymph nodes were collected from each mouse. Auricular lymph nodes were ground using 70 µm nylon cell strainers to isolate single cells. Cells were subsequently stained using a Mouse Th1/Th2/Th17 phenotyping kit (BD Biosciences) according to the manufacturer's protocol, and fluorescence intensity was detected using a FACSCalibur flow cytometer (BD Biosciences). Population of cells was analyzed by BD CellQuest™ Pro software (version 5.1, BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For the quantification of cytokine expression, qPCR was performed using the TP850 Thermal Cycler Dice Real Time System (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. At the end of the *in vivo* experimental period, the ears were excised and total RNA was isolated. Total RNA was isolated using RNAiso Plus (Takara Bio, Inc.). For the *in vitro* analysis, HaCaT cells were pretreated with 0, 0.1, 1 and 10 µg/ml AXE or 10 µg/ml Tac for 1 h at 37°C, and subsequently stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 6 h at 37°C. Total cellular RNA was isolated from cells (2x10⁵ cells/24-well plate) according to a method previously described (14). Complementary (c)DNA was synthesized with 1 µg of total RNA under condition of 1 h at 45°C and 5 min at 95°C using Maxime RT PreMix Kit (Intron Biotechnology, Inc., Seongnam, Korea). A total of 2 µl cDNA (100 ng), 1 µl sense and antisense primer solution (0.4 µM), 12.5 µl SYBR Premix Ex Taq (Takara Bio, Inc.) and 9.5 µl dH₂O were mixed together to obtain a 25 µl reaction mixture in each reaction tube. The relative transcription levels of the mRNAs were calculated according to the 2^{-ΔΔC_q} method (15). β-actin was used as an internal control. The primers used are shown in Table I. The conditions for amplification of DNA were 95°C for 30 sec, 45 cycles of 95°C for 5 sec, and 60°C for 30 sec. A melting curve analysis was done after amplification. Normalization and quantification of mRNA expression were performed using the TP850 software supplied by the manufacturer.

Nuclear protein extraction. HaCaT cells were pretreated with AXE for 1 h, and then stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 30 min. After stimulation, cells (1x10⁶ cells/6-well plate) were washed in 1 ml of ice-cold PBS, centrifuged at 1,200 x g for 5 min at 4°C, resuspended in 400 µl of ice-cold hypotonic buffer (10 mM HEPES, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at

5,000 x g for 5 min at 4°C. Pelleted nuclei were resuspended in 50 µl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, centrifuged at 15,000 x g for 5 min at 4°C, and supernatant was collected.

Western blot analysis. Samples for western blotting were prepared as previously described (14). HaCaT cells (1x10⁶ cells/6-well plate) were stimulated for 20 min with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs) and signal transducer and activator of transcription 1 (STAT1), and 30 min for nuclear factor-κB (NF-κB) after pretreatment with 10 µg/ml AXE or 10 µg/ml Tac for 1 h. Whole-cell extracts were prepared by washing cells twice with ice-cold PBS and incubating them in lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 100 µM DTT] with the addition of phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) for 30 min at 4°C. The lysates were collected by centrifugation at 13,000 x g for 15 min at 4°C, and protein quantification was performed with a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). For western blot analysis, 30 µg of total protein was separated using 8-12% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were stained with reversible Ponceau S stain for 10-20 sec at room temperature to ascertain equal loading of samples in the gel. All blots were blocked in 3% blocking buffer [3% w/v skim milk, 0.1% Tween-20, Tris-buffered saline (pH 7.4)] at room temperature for 1 h and incubated in primary antibody (1:1,000 dilution) at 4°C for overnight. Then, secondary antibody (1:2,000 dilution) were incubated at room temperature for 1 h. Detection was performed using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). β-actin and lamin B were used as internal loading controls for cytosolic and nuclear extraction, respectively. Phospho-ERK (Thr202/Tyr204; cat. no. 9101), phospho-p38 MAPK (Tyr180/Tyr182; cat. no. 9211), phospho-STAT1 (Tyr701; cat. no. 9171), ERK (ERK1/2; cat. no. 9102), p38 MAPK (cat. no. 9212) and STAT1 (cat. no. 9172) were from Cell Signaling Technology Inc. (Danvers, MA, USA). NF-κB p65 (cat. no. sc-109), IκB-α (cat. no. sc-371), lamin B (cat. no. sc-6217), β-actin (cat. no. sc-8432), and horseradish peroxidase-linked anti-rabbit IgG (cat. no. sc-2004) antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Statistical analysis. Statistical analyses were performed using GraphPad Prism 7 (La Jolla, CA, USA). Treatment effects were analyzed using one-way analysis of variance followed by Dunnett's test. Data are presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of AXE on ear thickness and histopathological changes in AD-like skin inflammation. To investigate the

Table I. Sequences of oligonucleotide primers.

Mouse	Primer sequence (5'-3')	Position	GenBank accession number
TNF- α			
Forward	GGCAGGTCTACTTTGGAGTCATTGC	796-1,095	NM_001278601.1
Reverse	ACATTTCGAGGCTCCAGTGAATTCG G		
IFN- γ			
Forward	TCAAGTGGCATAGATGTGGAAGAA	224-315	NM_008337.4
Reverse	TGGCTCTGCAGGATTTTCATG		
IL-4			
Forward	ACAGGAGAAGGGACGCCAT	180-274	NM_021283.2
Reverse	GAAGCCGTACAGACGAGCTCA		
IL-13			
Forward	GCAGCATGGTATGGAGTGTG	226-451	NM_008355.3
Reverse	TGGCGAAACAGTTGCTTTGT		
IL-31			
Forward	TCGGTCATCATAGCACATCTGGA	308-637	NM_029594.1
Reverse	GCACAGTCCCTTTGGAGTTAAGTC		
IL-17A			
Forward	TCCCTCTGTGATCTGGGAAG	321-474	NM_010552.3
Reverse	CTCGACCCTGAAAGTGAAGG		
β -actin			
Forward	TAGACTTCGAGCAGGAGATG	771-1,092	NM_007393.5
Reverse	TTGATCTTCATGGTGCTAGG		
Human	Primer sequence (5'-3')	Position	GenBank accession number
TNF- α			
Forward	CCTACCAGACCAAGGTCAAC	660-937	NM_000594.3
Reverse	AGGGGGTAATAAAGGGATTG		
IL-1 β			
Forward	GCTGATGGCCCTAAACAGATGAA	163-271	NM_000576.2
Reverse	TGAAGCCCTTGCTGTAGTGGTA		
IL-6			
Forward	AAAGAGGCACTGGCAGAAAA	365-776	NM_000600.4
Reverse	ATCTGAGGTGCCCATGCTAC		
CCL17			
Forward	ACTGCTCCAGGGATGCCATCGTTT	296-565	NM_002987.2
Reverse	ACAAGGGGATGGGATCTCCCTCAC		
CCL22			
Forward	AGGACAGAGCATGGCTCGCCTACA	25-386	NM_002990.4
Reverse	TAATGGCAGGGAGGTAGGGCTCCT		
β -actin			
Forward	GGA CTTCGAGCAAGAGATGG	747-980	NM_001101.3
Reverse	AGCACTGTGTTGGCGTACAG		

effects of AXE on AD-like skin inflammation, AXE was orally administered to BALB/c mice 5 times weekly from days 7 to 27. Repeated application of DFE/DNCB on the ear lobes of mice increased ear thickness, swelling, reddening and scaling of the skin. Ear thickening was unaffected until 1 week after initial oral administration of AXE (2,

10 and 50 mg/kg). Thereafter, thickening was suppressed in a dose- and time-dependent manner by AXE (Fig. 1A). Ear photographs at the end of the experiment demonstrated the amelioration of the skin lesion, including swelling and reddening, by AXE treatment (Fig. 1B). Tac, also termed FK-506 or fujimycin, is an immunosuppressive drug used for

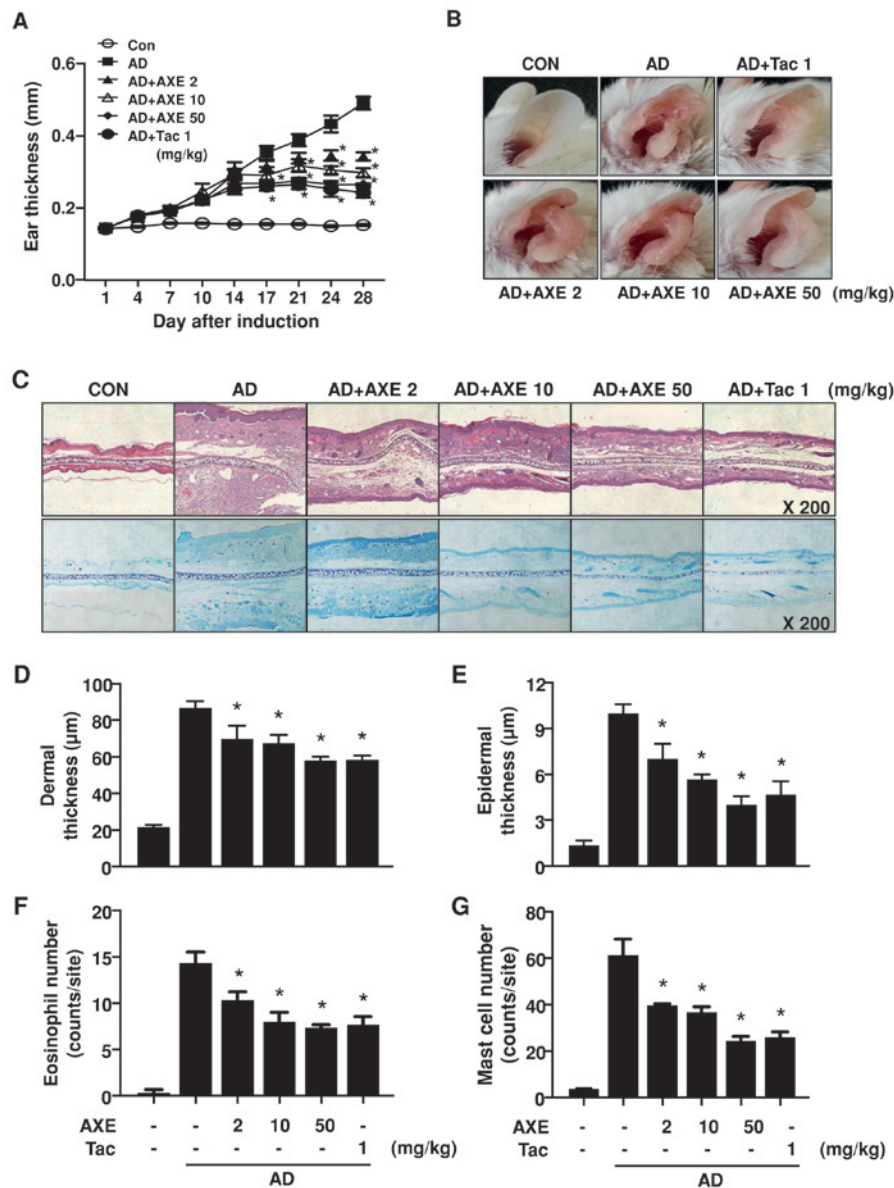


Figure 1. Effects of AXE on DFE/DNCB-induced AD mice. (A) To measure the effects of AXE (2, 10 and 50 mg/kg) or Tac (1 mg/kg) on DFE/DNCB-induced AD, ear thickness was measured 24 h after DFE or DNCB application with a dial thickness gauge. (B) Gross observation of morphological recovery at day 28. (C) Ear tissues were stained with H&E or toluidine blue for histological analysis and the determination of dermal and epidermal thickness, and eosinophil and mast cell infiltration. Original magnification, x200. (D) Dermal and (E) epidermal thickness. Number of (F) eosinophils and (G) mast cells. Data are presented as the mean \pm standard error of the mean of five determinants. * $P < 0.05$ vs. AD group. AXE, *Amomum xanthioides* extract; DFE, *Dermatophagoides farinae* extract; DNCB, dinitrochlorobenzene; AD, atopic dermatitis; Tac, tacrolimus; CON, control.

the treatment of inflammatory skin diseases such as AD and psoriasis. It was used as a positive control.

To analyze the effects of AXE on skin hypertrophy and infiltration of eosinophils and mast cells, which are important effector cells in AD, tissue sections of ears were stained with H&E or toluidine blue (Fig. 1C). Quantitative values based on microscopic observations were analyzed (Fig. 1D-G). Compared with the control, repeated DFE/DNCB exposure caused a marked increase in the thickening of the dermis and epidermis, in addition to the infiltration of eosinophils and mast cells. However, oral administration of AXE reduced these histopathological changes in a dose-dependent manner (Fig. 1D-G).

Effect of AXE on serum histamine and Ig levels. To evaluate the serum levels of histamine and Igs in AD, serum was

isolated following blood collection. Excessive production of histamine, a representative symptom of AD, was detected in DFE/DNCB-sensitized mice (Fig. 2A). In addition, elevation of total IgE, DFE-specific IgE and IgG2a (Fig. 2B-D) was also observed in the DFE/DNCB-sensitized mice. These increased levels of histamine and Igs were significantly decreased following AXE application.

Effects of AXE on the polarization of T lymphocytes and expression of cytokines in the ear tissue of AD mice. The progression of AD is characterized by an alteration in the polarization of the T cell population, particularly that of Th1, Th2 and Th17 cells (16). To assess alterations in T lymphocyte polarization, single cells from auricular lymph nodes were isolated and analyzed using specific antibodies for signature cytokines

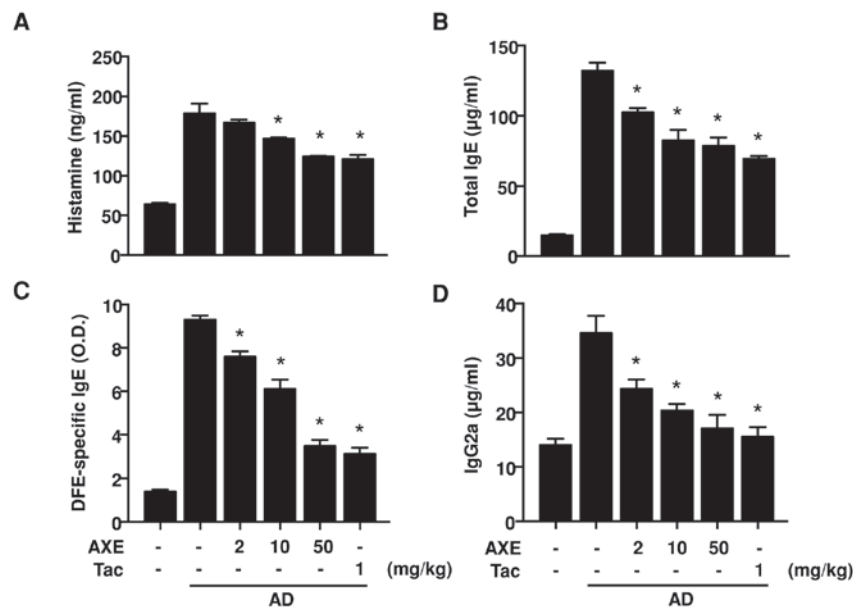


Figure 2. Levels of (A) serum histamine and (B-D) Igs in DFE/DNCB-induced AD mice. Blood samples from vehicle, DFE/DNCB + vehicle, and DFE/DNCB + AXE (2, 10 and 50 mg/kg) or Tac (1 mg/kg) groups were obtained from the celiac artery at day 28, and serum was isolated. Serum histamine and Igs were quantified by ELISA. Data are presented as the mean \pm standard error of the mean of five determinants. * $P < 0.05$ vs. AD group. Ig, immunoglobulin; AD, atopic dermatitis; DFE, *Dermatophagoides farinae* extract; DNCB, dinitrochlorobenzene; AXE, *Amomum xanthioides* extract; Tac, tacrolimus; O.D., optical density.

expressed by polarized lymphocytes, including IFN- γ for Th1, IL-4 for Th2 and IL-17A for Th17 (Fig. 3). The results demonstrated that populations of CD4⁺/IFN- γ ⁺, CD4⁺/IL-4⁺ and CD4⁺/IL-17A⁺ were increased in the DFE/DNCB-sensitized mice, and were dose-dependently decreased following AXE treatment.

In AD, infiltrated immune cells, including lymphocytes and resident keratinocytes, continuously interact via the production of various cytokines, and thus exacerbate the disease (2). To assess the gene expression of cytokines in AD skin, RNA was isolated from ear tissue and qPCR was performed. Repeated application of DFE/DNCB increased the expression levels of IL-4, IL-13, IL-31, TNF- α , IFN- γ and IL-17A. However, treatment with AXE significantly reduced the expression of these cytokines at certain doses (Fig. 4).

Effects of AXE on the activation of keratinocytes. In AD, keratinocytes are an important source of cytokines that accelerate chronic, self-amplifying loops of immune activation. In skin inflammation, the increased production of TNF- α and IFN- γ by keratinocytes promotes the amplification of the inflammatory response. TNF- α and IFN- γ stimulate the synthesis and secretion of various inflammatory mediators by keratinocytes (14,17). To determine the effect of AXE on the production of inflammatory cytokines and its signaling mechanism, HaCaT cells were stimulated with TNF- α /IFN- γ with or without pretreatment with AXE (Fig. 5). Treatment with TNF- α /IFN- γ promoted the gene expression of the pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 and IL-8, CCL17 and CCL22. These increased levels were reduced by pretreatment with AXE (Fig. 5A). Cell viability of HaCaT cells was unaffected by AXE concentrations of up to 100 μ g/ml (Fig. 5B).

To investigate the mechanism responsible for the inhibitory effects of AXE on the expression of cytokines and

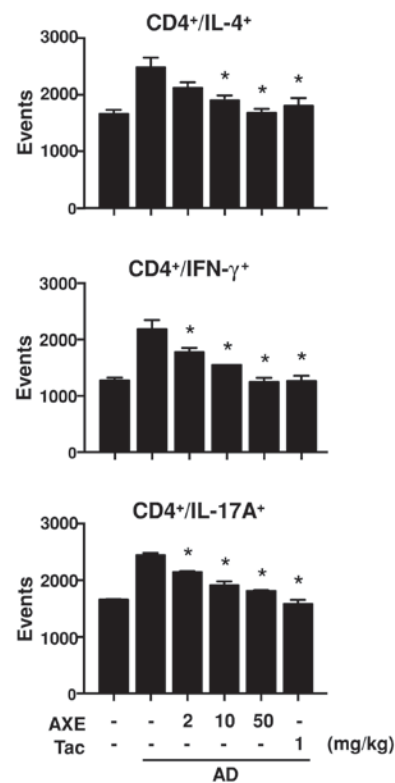


Figure 3. Phenotypes of T lymphocytes in the auricular lymph nodes of DFE/DNCB-induced AD mice. The number of CD4⁺/IL-4⁺, CD4⁺/IFN- γ ⁺ and CD4⁺/IL-17A⁺ cells was detected by fluorescence-activated cell sorting analysis. Data are presented as the mean \pm standard error of the mean of five determinants. * $P < 0.05$ vs. AD group. AD, atopic dermatitis; IL, interleukin; IFN, interferon; AXE, *Amomum xanthioides* extract; Tac, tacrolimus.

chemokines in HaCaT cells, the activation of MAPKs, STAT1, I κ B α and NF- κ B, which regulate the expression of cytokines and chemokines, was analyzed by Western blot (Fig. 5C).

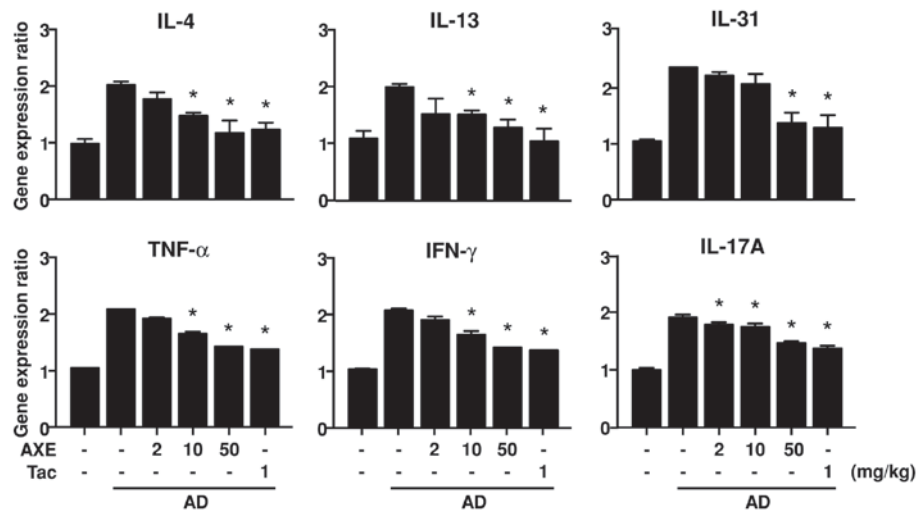


Figure 4. mRNA expression of cytokines in the ear tissue of DFE/DNCB-induced AD mice. Ears were excised at day 28 and total RNA was isolated, then reverse transcription-quantitative polymerase chain reaction was performed. Data are presented as the mean \pm standard error of the mean of five determinants. * $P < 0.05$ vs. AD group. AD, atopic dermatitis; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; AXE, *Amomum xanthioides* extract; Tac, tacrolimus.

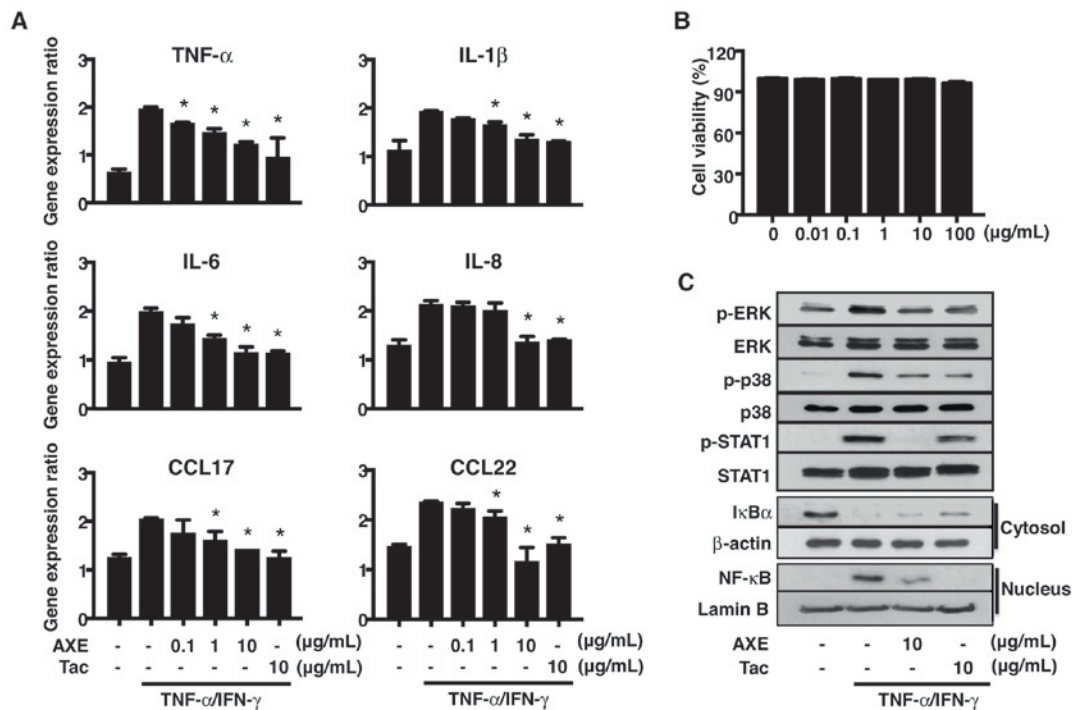


Figure 5. Effects of AXE on TNF- α /IFN- γ -stimulated HaCaT cells. Cells were pretreated with AXE or Tac for 1 h prior to stimulation with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml). (A) Gene expression of TNF- α , IL-1 β , IL-6, IL-8, CCL17 and CCL22 in HaCaT cells were detected by reverse transcription-quantitative polymerase chain reaction. (B) Cell viability in HaCaT cells following AXE treatment was determined by an MTT assay after 24 h. (C) Representative western blot of three independent experiments for the protein expression of p-ERK, p-p38, p-STAT1, NF- κ B and I κ B α . Data are presented as the mean \pm standard error of the mean of five determinants. * $P < 0.05$ vs. TNF- α /IFN- γ stimulation. AXE, *Amomum xanthioides* extract; TNF, tumor necrosis factor; IFN, interferon; Tac, tacrolimus; IL, interleukin; CCL, C-C motif chemokine ligand; p-, phosphorylated-; ERK, extracellular signal-regulated kinase; STAT1, signal transducer and activator of transcription 1; NF- κ B, nuclear factor- κ B; I κ B α , NF- κ B inhibitor α .

TNF- α /IFN- γ -induced phosphorylation of ERK, p38 and STAT1, as well as degradation of cytosolic I κ B α and nuclear translocation of NF- κ B, were inhibited by AXE.

Discussion

A. xanthioides has been used in Asia to treat various disorders, including stomach and digestive disorders, for a long

time (7). The seed of *A. xanthioides* is listed in the Japanese Pharmacopoeia as 'Amomum seed' (18). Although their total composition remains unclear, the seeds commonly contain five primary classes of components, including volatile oils, saponins, flavonoid glycosides, organic acids and inorganic components (19). Specifically, they contain 1-1.5% essential oil that is rich in monoterpenoids (borneol, linalool, camphene and nerolidol), which are reported to exhibit numerous

pharmacological properties, including antimicrobial, anti-inflammatory, anti-oxidant, anti-pruritic, hypotensive and analgesic activities (20).

The present study investigated the pharmacological effects of a water-soluble component of *A. xanthioides* in a mouse AD-like skin inflammation model. AXE alleviated clinical and histopathological alterations in AD-like skin inflammation, including severe ear thickening, ulcers, epidermal thickening and infiltration of immune cells. Gross observation prior to sacrifice demonstrated severe clinical phenotypes, including as hemorrhage, edema, excoriation and scaling in DFE/DNCB-induced skin inflammation. However, the oral administration of AXE ameliorated those symptoms.

The activation of mast cells and eosinophils, and increased IgE levels, are hallmarks of Th2-mediated immune responses (21,22). Mast cells typically accumulate in DFE and/or DNCB-stimulated skin lesions (23,24). They produce histamine, a major contributor to pruritus, which is key symptom of AD (25). Tissue eosinophilia in AD skin is associated with epidermal hyperplasia, spongiosis, skin hypertrophy and disease severity (26). Mast cells and eosinophils express an IgE receptor (FcεRI) on their surface and are activated by IgE (21,27). In general, the IgE level is an appropriate marker for Th2 response, whereas IgG2a level is a marker for Th1 response (28). Excessive Th2 responses are primarily observed in the acute stages of AD, whereas a mixed Th1/Th2 pattern of inflammation is usually observed during the chronic stage. Stimulation with DFE/DNCB for 4 weeks induced a chronic AD-like response in the mouse model of our previous study (17). We hypothesize that the suppressive effect of AXE on DFE/DNCB-induced allergic skin inflammation may be due to a decrease in Th2-mediated immune responses leading to a decrease in Th1 responses.

T cells, as one of the major components of adaptive immunity, have important roles in the pathogenesis of AD. In AD progression, T cell polarization is biphasic, primarily involving Th2 cells in the acute phase and Th1 cells in the chronic phase (16). Each subset is characterized by the expression of specific cytokines. Th2-polarized cells express Th2 cytokines, including IL-4 and IL-13 (29), while Th1 cells express IFN-γ (30). In addition, Th17 cells, as a specific subset of memory CD4⁺ T cells, have been reported to contribute to the pathogenesis of AD. Analysis of the IL-17⁺/CD4⁺ population in peripheral blood mononuclear cells isolated from patients with AD previously revealed that a higher percentage was present in the severely affected group compared with the healthy control group (31). Furthermore, our previous report demonstrated that all three T cell subsets increased in draining lymph nodes following DFE/DNCB exposure (14). In the present study, the results indicated that AXE pretreatment in AD rats reduced Th1, Th2 and Th17 subsets in draining lymph nodes. These findings indicate that AXE may control the differentiation of naive T lymphocytes to effector T cells.

Various immune biomarkers characterize the inflammatory phenotype of lesional skin in AD. IL-4, IL-13 and IL-31 represent the Th2 response, TNF-α and IFN-γ for the Th1 response and IL-17A for Th17 response (32). In the present study, repeated ear skin exposure to DFE/DNCB markedly increased the expression of these biomarkers. IL-4 and IL-13 are the key upstream drivers of the Th2 response in allergic inflammation.

IL-31 originating from inflammatory infiltrates is considered to be critical in the regulation of keratinocyte differentiation and induction of pruritus in AD (33,34). As mentioned above, the chronic stage of AD reveals a mixed Th1/Th2 response pattern. IFN-γ and TNF-α are secreted by activated Th1 cells and keratinocytes, respectively, and affect the fate of keratinocytes and the skin microenvironment. Overexpression of IFN-γ is implicated in skin hypertrophy (35), and TNF-α was reported to induce spongiosis and flatten suprabasal keratinocytes (36). IFN-γ produced by Th1 cells promotes the expression of IgG2a in B cells (37). IL-17 produced by Th17 cells induces the production of certain cytokines, chemokines and antimicrobial peptides by keratinocytes (38). Therefore, the inhibitory effects of AXE on the expression of each biomarker in DFE/DNCB-treated ear skin may occur due to a decrease in the number and activity of infiltrated immune cells.

Keratinocyte activation in inflamed skin has an important role in the pathogenesis of prolonged inflammatory skin disorders through the regulation of innate immunity (39). Stimulation of epidermal keratinocytes with TNF-α and IFN-γ has been reported to induce the expression of various pro-inflammatory cytokines and chemokines, including TNF-α itself, IL-1 and IL-8, and the expression of Th2-attracting chemokines, such as CCL17 and CCL22, was also induced (40,41). These cytokines and chemokines are considered to be important mediators for the development of AD. Cotreatment of keratinocytes with TNF-α and IFN-γ was reported to activate several transcription factors, including NF-κB and STAT1, through regulation of the MAPK signaling cascade (42). Notably, TNF-α/IFN-γ-induced expression of the Th2 chemokines CCL17 and CCL22 was directly altered by specific inhibitors for NF-κB and STAT (43). Therefore, the inhibitory effects of AXE on TNF-α/IFN-γ-stimulated keratinocytes may occur via the suppression of cytokine and chemokine production through the coordination of MAPK, NF-κB and STAT1. We hypothesize that AXE may inhibit chronic and self-amplifying loops of immune activation by blocking the activation of keratinocytes and the subsequent production of inflammatory cytokines and chemokines.

In conclusion, the results of the present study demonstrate that oral administration of AXE suppressed DFE/DNCB-induced AD-like skin inflammation, which may occur due to decreased infiltration of immune cells and subsequent Th1/Th2/Th17 responses in AD skin, and reduced TNF-α/IFN-γ-induced immune activation through the regulation of inflammatory cytokines and chemokines in keratinocytes. The present study employed whole aqueous extract of *A. xanthioides* rather than a purified single compound. Therefore, the biological effects of the individual active components are not clear at present. Efforts to identify the active component from AXE on AD symptoms are ongoing in our laboratory. However, the results presented in this report give an insight into the mechanism that is potentially responsible for the anti-AD activity of AXE. Therefore, these results indicate that AXE may be applied to effectively control AD, as a useful natural pharmacological agent.

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