

Accepted Manuscript

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PII: S0378-8741(18)34484-2

DOI: <https://doi.org/10.1016/j.jep.2019.111850>

Article Number: 111850

Reference: JEP 111850

To appear in: *Journal of Ethnopharmacology*

Received Date: 27 November 2018

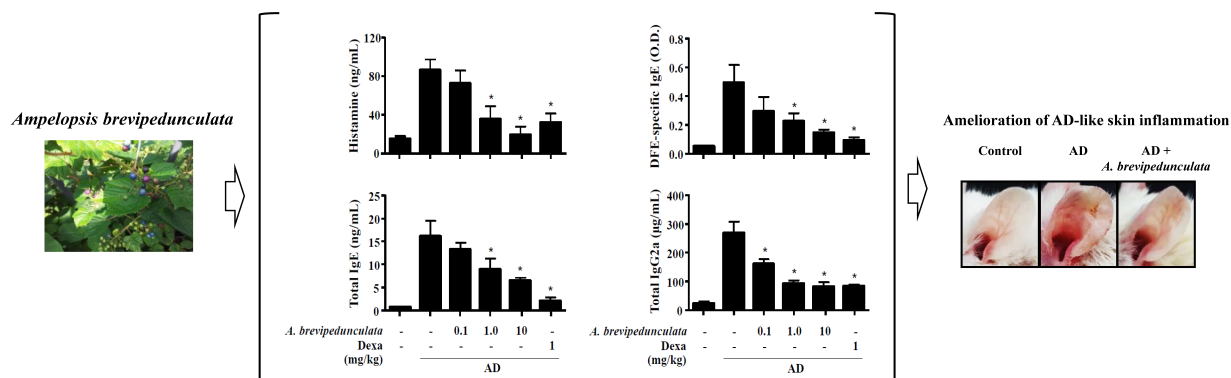
Revised Date: 24 March 2019

Accepted Date: 31 March 2019

Please cite this article as: Choi, Y.-A., Yu, J.-H., Jung, H.D., Lee, S., Park, P.-H., Lee, H.-S., Kwon, T.K., Shin, T.-Y., Lee, S.W., Rho, M.-C., Jang, Y.H., Kim, S.-H., Inhibitory effect of ethanol extract of *Ampelopsis brevipedunculata* rhizomes on atopic dermatitis-like skin inflammation, *Journal of Ethnopharmacology* (2019), doi: <https://doi.org/10.1016/j.jep.2019.111850>.

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**Inhibitory effect of ethanol extract of *Ampelopsis brevipedunculata*
rhizomes on atopic dermatitis-like skin inflammation**

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List of abbreviations:

Ethanol extract of *Ampelopsis brevipedunculata* rhizomes: ABE, Atopic dermatitis: AD,

Dermatophagoides farinae extract: DFE, 2,4-dinitrochlorobenzene: DNCB,

Carboxymethylcellulose: CMC, Fluorescence-activated cell sorting: FACS

Abstract

Ethnopharmacological relevance: Extracts from various parts of *Ampelopsis*

brevipedunculata has been used as anti-inflammatory agents in Asian folk medicine.

Aim of the study: To demonstrate the medicinal effect of the *A. brevipedunculata* in skin inflammation, specifically atopic dermatitis (AD).

Materials and methods: The effect of ethanol extract of *A. brevipedunculata* rhizomes (ABE) on AD was examined using an AD-like skin inflammation model induced by repeated exposure to house dust mite (*Dermatophagoides farinae* extract, DFE) and 2,4-dinitrochlorobenzene (DNCB). The mechanism study was performed using tumor necrosis factor (TNF)- α and interferon (IFN)- γ -activated human keratinocytes (HaCaT). Serum histamine and immunoglobulin levels were quantified using enzymatic kits, while the gene expression of cytokines and chemokines was analyzed using quantitative real time polymerase chain reaction. The expression of signaling molecules was detected using Western blot.

Results: Oral administration of ABE alleviated DFE/DNCB-induced ear thickening and clinical symptoms, as well as immune cell infiltration (mast cells and eosinophils) into the dermal layer. Serum Immunoglobulin (Ig) E, DFE-specific IgE, IgG2a, and histamine levels were decreased after the administration of ABE. ABE also inhibited CD4⁺IFN- γ ⁺ and CD4⁺IL-4⁺ lymphocyte polarization in lymph nodes and expression of TNF- α , IFN- γ , IL-4,

IL-13, and IL-31 in the ear tissue. In TNF- α /INF- γ -stimulated keratinocytes, ABE inhibited the gene expression of TNF- α , IL-6, IL-1 β , and CCL17. In addition, ABE decreased the nuclear localization of signal transducer and activator of transcription 1 and nuclear factor- κ B, and the phosphorylation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase.

Conclusion: Collectively, our data demonstrate the pharmacological role and signaling mechanism of ABE in the regulation of skin allergic inflammation, which supports our suggestion that ABE could be developed as a potential therapeutic agent for the treatment of AD.

Keywords: *Ampelopsis brevipedunculata*, Atopic dermatitis, House dust mite, Keratinocytes, Skin inflammation

1. Introduction

Inflammation is a defense mechanism maintaining health vital. However, uncontrolled and chronic inflammation is a critical risk factor for various human diseases, including inflammatory/autoimmune, metabolic, cardiovascular, and neurodegenerative diseases, and cancers (Chen et al., 2018). Atopic dermatitis (AD) is also a chronic, pruritic, and relapsing inflammatory skin disorder accompanied by allergic features. The disorder occurs and deteriorates due to the complex interaction of multiple factors including genetic mutations, environmental factors and immunological factors (Leung and Bieber, 2003).

Immunologically, the development of AD is well characterized by the predominant CD4⁺ Th2 cell response. Th2 cells secrete Th2-related cytokines such as interleukin (IL)-4, IL-13, and IL-31 in the acute phase of AD (Brandt and Sivaprasad, 2011). Infiltrated Th cells in a local lesion release a variety of cytokines that induce the activation of skin keratinocytes (Auriemma et al., 2013). Keratinocytes in AD lesions exhibit an exaggerated production of cytokines and chemokines modulating the response of immune cells, such as tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and IL-8 (Kim et al., 2014). Consequently, the inflammation of local skin lesions is accelerated and aggravated by self-amplifying loops of immune activation between keratinocytes and immune cells. Therefore, the suppression of keratinocyte activation could be a potential target for the treatment of AD.

Ampelopsis brevipedunculata (Maxim.) Trautv. is one of several deciduous vine plants widely distributed in Asia as well as eastern North America, Mexico, and Guatemala (Byng, 2014). The traditional medicinal usage of *A. brevipedunculata* was well recorded in Korean traditional ancient book (Donguibogam, 1613 by Jun Heo) and Chinese ancient book (Chinese Herbal Manual, 1578 by Shih-Chen Li, registered as a UNESCO World Heritage Site). Based on the contents of these ancient books, Zhou et al., (2003) summarized the application of *A. brevipedunculata*. In these references, the stem and leaf of *A. brevipedunculata* have been used to minimize inflammation and thirst. In addition, new functions of *A. brevipedunculata* were proved by the several researchers; anti-oxidant activity (Wu et al., 2004), preventing bone loss (Kim et al., 2014), and anti-mutagenic activity (Lee and Lin, 1988). Further, it has been reported that vitisinol A, a resveratrol derivative isolated from *A. brevipedunculata*, has an anti-inflammatory effect (Chang et al., 2017).

Because AD progression correlates with oxidative stress and the inflammatory response, we investigated the effect of the ethanol-extract isolated from *A. brevipedunculata* rhizomes on AD-like skin inflammation.

2. Materials and methods

2.1. Animals

Six-week-old female BALB/c mice (20 g) were purchased from Dae-Han Experimental Animal Center (Daejeon, Korea). The mice had *ad libitum* access to standard rodent chow and filtered water during the study. Five mice were housed per cage, maintained in a laminar air flow room at 22 ± 2 °C temperature and $55 \pm 5\%$ relative humidity under a 12 h light/dark cycle throughout the study. 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 and were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

2.2. Reagents

House dust mite (*Dermatophagoides farinae* extract, DFE) was purchased from Greer Inc. (Lenoir, NC, USA) and dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween 20. 2,4-Dinitrochlorobenzene (DNCB) was dissolved in an acetone/olive oil (1:3) solution. Recombinant human TNF- α and IFN- γ were purchased from R&D systems (Minneapolis, MN, USA). All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.3. Preparation of ABE and isolation of compounds

A. brevipedunculata rhizomes were purchased from an herbal store in Seoul, Korea in May 2014. Botanical identification was performed by Dr. Rho (Korea Research Institute of Bioscience & Biotechnology) and a voucher specimen (KRIBB-KR2013-1) was deposited at the laboratory of the Natural Products Research Center. Nine kilograms of dried and pulverized *A. brevipedunculata* rhizomes were extracted with 95% EtOH at room temperature and evaporated *in vacuo* to obtain the 337.8 g of dried EtOH extract. The dried extract was dissolved in 0.5% carboxymethylcellulose (CMC) in saline prior to use.

The isolation and structural identification of the compounds was described in our previous report (Jang et al., 2018). Briefly, the EtOH extract was suspended in H₂O and progressively partitioned with EtOAc. The EtOAc-soluble extract was separated into 38 sub-fractions using silica gel column chromatography with step-gradient solvent system, and the sub-fractions were further purified using MPLC (C18 and silica gel) and semi-preparative HPLC to yield seventeen compounds (**1-17**).

The structures of the isolated compounds were identified by spectroscopic analyses, such as ¹H NMR, ¹³C NMR, and ESIMS. ¹H, ¹³C NMR spectra were recorded on a JNM-ECA600 (Jeol, Tokyo, Japan) instruments using TMS as references. Election spray ionization mass spectrometry (ESIMS) was carried out using a Waters SYNAPT G2-Si HDMS spectrometer (Waters, Milford, MA, USA). The HPLC analysis was performed using an Agilent 1200

series HPLC system (Agilent, Santa Clara, CA, USA) equipped with a quaternary pump.
Semi-preparative HPLC was performed on a Shimadzu LC-6AD instrument (Shimadzu Corp., Tokyo, Japan) equipped with an SPD-20A detector and YMC C₁₈ hydrosphere.

The known compounds were identified as betulin (**1**), betulinic acid (**2**), β -sitosterol (**3**), β -sitosterol glucoside (**4**), dihydrokaempferol (**5**), dihydrokaempferol 3-O-glycoside (**6**), catechin (**7**), gallic acid (**8**), vanillic acid (**9**), ethyl gallate (**10**), ethyl gallate 4-O- β -D-glucopyranoside (**11**), syringic acid (**12**), benzyl 6'-O-galloyl- β -D-glucopyranoside (**13**), ellagic acid (**14**), 3'-O-methylellagic acid 4-O- α -L-rhamnopyranoside (**15**), 3,3'-O-trimethylellagic acid 4-O- β -D-glucopyranoside (**16**), and resveratrol (**17**) by comparison of the NMR data of the isolated compounds with data reported in the literature (Cho et al, 2000; Cichewicz and Kouzi, 2004; Davis et al., 1996; De Winter et al., 2014; Do-Khac et al., 1990; Hui et al., 1976; Inoshiri et al., 1987; Isaza et al., 2001; Kang et al., 2011; Khatun et al., 2012; Mattivi et al., 1995; Neacsu et al., 2007; Nguyen et al., 2013; Yazaki and Hillis, 1976; Yu et al., 2008)

2.4. Cell culture

A human keratinocyte cell line, HaCaT (American Type Culture Collection, Manassas, VA, USA), was maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/m penicillin G,

100 µg/mL streptomycin) at 37 °C in 90-95% humidity and 5% CO₂.

2.5. Cell viability

Cell viability was determined using the 3-(4,5-dimethyl thiazolyl-2)2,5-diphenyl tetrazolium bromide (MTT) assay. HaCaT cells were plated in a 96-well plate at a density of 1×10^4 cells/well ($n = 5$). After exposure to each concentration of ABE treatment for 24 h, 20 µL of MTT (5 mg/mL in PBS) was added into each well and the cells were incubated at 37 °C under 5% CO₂ for 2 h. The formazan crystals were dissolved in 100 µL of DMSO and the absorbance was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. The relative cell viability (%) was expressed as a percentage compared to untreated control cells (100%).

2.6. Induction of AD-like inflammation in the mouse ear

The experimental procedure of AD-like skin inflammation induction is shown in Fig. S1. A total of 30 mice were divided into 6 groups (5 individuals per group): vehicle, DFE/DNCB plus vehicle, DFE/DNCB plus ABE 0.1, 1, or 10 mg/kg, or dexamethasone (Dexa) 1 mg/kg. The surfaces of both earlobes were cleaned by stripping very gently using surgical tape (Nichiban, Tokyo, Japan) to remove foreign matter or scabs. Twenty microliters of DNCB (1%) was painted onto each ear, followed by 20 µL of DFE (10 mg/mL) 3 days later.

DFE/DNCB treatment was repeated once a week orderly for 4 weeks. At one week after the first DFE/DNCB treatment, each dose of ABE or Dexa was orally administered a total of 5 times weekly, until the end of the 4 week induction period. Ear thickness was measured 24 h after DNCB or DFE application using a dial thickness gauge (Mitutoyo, Co., Tokyo, Japan).

On day 28, the animals were euthanized with carbon dioxide. Blood samples were collected from the celiac artery. After blood clotting at 4 °C overnight, serum was collected through centrifugation at 400 *g* for 10 min at 4 °C. Both earlobes were collected for histopathological analysis and RNA extraction.

2.7. Enzyme-linked immunosorbent assay

IgG2a levels were measured using an ELISA kit (cat. no. 552576, BD Biosciences, Franklin Lakes, NJ, USA). Total IgE and DFE-specific IgE levels were assayed with the same kit (cat. no. 555248). Total IgE levels were measured by concentration calculation using the standard curve. Mite-specific IgE levels were measured as optical density values.

2.8. Histological observation

The ears tissues were fixed with 10% formaldehyde for one week at room temperature. The tissues were embedded in paraffin and sectioned into 5 µm slices. For observation of lymphocyte infiltration and the thickness of the epidermis and dermis, the sections were

1 stained with hematoxylin and eosin (H&E). To measure mast cell infiltration, the sections
2 were stained with toluidine blue (TB). The number of infiltrated cells was counted in ten
3 randomly chosen sites at a magnification of $\times 400$. Epidermal and dermal thickness were
4 measured in five randomly selected fields under a magnification of $\times 200$.

5 6 2.9. Histamine assay

7 To determine serum histamine concentrations, 0.1 M HCl and 60% perchloric acid were
8 added to 50 μ L of collected serum before centrifuging. The supernatant was mixed with 5 M
9 NaCl, 5 M NaOH, and *n*-butanol and fractioned. The supernatant was shaken with 0.1 M HCl
10 and *n*-heptane and the final fraction collected. Histamine in the aqueous layer was measured
11 using the *o*-phthalaldehyde spectrofluorometric procedure as previously described (Kim et
12 al., 2017). Fluorescence intensity was measured using 360 nm excitation and 440 nm
13 emission fields using a LS-50B fluorescence spectrometer (Perkin-Elmer, Norwalk, CT,
14 USA).

15 16 2.10. Fluorescence-activated cell sorting (FACS)

17 Both auricular lymph nodes were collected from each mouse at the end of the experiment.
18 The single cells from auricular lymph nodes were isolated using 70 μ m nylon cell strainers
19 (Falcon, Bedford, MA, USA). The cells were stained using a mouse Th1/Th2/Th17

Phenotyping Cocktails (CD4 PerCP-CyTM5.5, IL-17A PE, IFN- γ FITC, IL-4 APC) phenotyping kit (BD Biosciences) according to the manufacturer's instructions. The fluorescence intensity was detected using a FACSCalibur flow cytometer (BD Biosciences). The cellular population was analyzed using BD CellQuestTM Pro software (version 5.1, BD Biosciences).

2.11. Nuclear/cytosol fractionation

HaCaT cells (5×10^5 cells/6-well plate) were pretreated with ABE or Dexa for 1 h, followed by stimulation with TNF- α (10 ng/mL) and IFN- γ (10 ng/mL) for 30 min. The cells were lysed in 400 μ L of ice-cold buffer A (10 mM HEPES, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9) and centrifuged at 5,000 g for 5 min at 4 °C. The supernatant was collected as the cytosolic fraction. Pelleted nuclei were resuspended in 50 μ L of ice-cold buffer B (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), sonicated, and centrifuged at 15,000 g for 5 min at 4 °C. The supernatant was collected as the nuclear fraction.

2.12. Western blot

HaCaT cells were incubated in the same conditions as described, with nuclear/cytosol fractionation. For total cell lysate, cells were lysed in 100 μ L of lysis buffer (20 mM Tris pH

7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 100 μ M DTT) with the addition of protease and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) for 30 min at 4 °C. The total cell lysates were collected through centrifugation at 13,000 g for 15 min at 4 °C. The protein quantification was performed with a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Total protein (10-30 μ g) was separated using 10% SDS-PAGE and then transferred to nitrocellulose membranes (Pall Life Sciences, Port Washington, NY, USA). The membranes were stained with reversible Ponceau S to ensure equal loading of samples onto the gels. Nuclear signal transducer and activator of transcription 1 (STAT1), p65 nuclear factor- κ B (NF- κ B), cytosolic I κ B α were analyzed using anti-STAT1 (Cell Signaling, Beverly, MA, USA), anti-p65 NF- κ B and anti-I κ B α antibody (Santa Cruz Biotech, Santa Cruz, CA, USA), respectively. Phospho- and total-form of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) were detected using the specific antibodies (Cell Signaling, Beverly, MA, USA). Immunodetection was conducted using SuperSignal West Pico Chemiluminescent substrate (Thermo scientific, Waltham, MA, USA).

2.13. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from ear tissue or HaCaT cells treated with TNF- α /IFN- γ (10 ng/mL of each)

and/or ABE or Dexa was isolated using RNAiso Plus (Takara Bio Inc. Japan). The first strand of complementary DNA (cDNA) was synthesized with 1 µg of total RNA at 45 °C for 60 min followed by 95 °C for 5 min using the Maxime RT PreMix Kit (iNtRON Biotech, Sungnam, Korea). qPCR was performed with 200 ng cDNA, 0.4 µM of each of the sense and antisense primer, and 1X SYBR *Premix Ex Taq* (Takara Bio Inc.) using a Thermal Cycler Dice TP850 (Takara Bio Inc.) according to the manufacturer's protocol. The relative transcription levels of the mRNAs were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). β -actin was used as an internal control. The conditions for the PCR were similar to our previous report (Choi et al., 2013). The conditions for amplification of DNA were 95 °C for 30 sec, followed by 45 cycles of 95 °C for 5 sec and 60 °C for 30 sec. Melting curve analysis was performed after amplification. Normalization and quantification of mRNA expression were performed using the TP850 software supplied by the manufacturer. The primer sequences were listed in Supplementary Table S1.

2.14. 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. A value of $p < 0.05$ was taken to indicate a statistically significant difference.

3. Results

3.1. Effects of ABE on the clinical and histopathological aspects of AD-like skin inflammation

The AD skin has various clinical symptoms such as redness, scabs, and keratosis accompanied by inflammation and histopathological changes including epidermal/dermal thickening and infiltration of immune cells within a lesion (Leung and Bieber, 2003). To determine the effect of ABE on the development of AD-like skin inflammation, the indicated dose of ABE was orally administered to DFE/DNCB-challenged BALB/c mice. Repeated stimulation of DFE/DNCB greatly increased the ear thickness, swelling, redness, and scaling of the skin. The administration of ABE significantly reduced ear thickening in a dose- and time-dependent manner (Fig. 1A). DFE/DNCB induced-redness, scabbing, and keratosis accompanied by inflammation was effectively ameliorated by ABE or Dexa (Fig. 1B). In spite of these suppressive effects of ABE and Dexa, they did not affect the mean body weight of any group during the experimental period (Fig. S2). Dexa was used as a control for comparison of the therapeutic effect. To analyze the effect of ABE on skin hypertrophy and immune-cell infiltration, paraffin-embedded tissues were stained with H&E and toluidine blue (Fig. 2A) and quantified through microscopic observation (Fig. 2B-E). DFE/DNCB-stimulated ear tissue showed an increase in the epidermal and dermal thickness and eosinophil and mast cell numbers. However, administration of ABE markedly reduced these histological abnormalities in a dose-dependent manner.

3.2. *Effects of ABE on serum histamine and immunoglobulin levels*

The analysis of serum Ig is a reliable method to assess the efficacy of long-term immunization (Mak and Simard, 2007). To analyze the level of serum Igs and histamine, serum was collected at the end of the experiment (Fig. 3). The excessive production of serum histamine is one of the representative symptoms of an allergic disease such as AD (De Benedetto et al., 2015). Repeated application of DFE/DNCB increased the level of serum histamine, IgE, DFE-specific IgE, and IgG2a. However, administration of ABE or Dexa effectively decreased the elevated levels.

3.3. *Effects of ABE on the T-cell polarization in an auricular lymph node and cytokine expression in the skin lesion*

The skin lesions of AD show a mixed acute and chronic inflammatory cell infiltrate, in which CD4⁺ and Th cells predominate (Su et al., 2017). To assess the effect of ABE on polarization into Th1 and Th2 subsets, T cells were isolated from auricular lymph nodes and signature cytokines were analyzed (Fig. 4A); IFN- γ for Th1 and IL-4 for Th2. The populations of CD4⁺ IFN- γ ⁺ and CD4⁺ IL-4⁺ cells were increased in the DFE/DNCB-sensitized group, while the increased dose-dependently decreased following ABE treatment. Next, the effect of ABE on the expression of immune modulating cytokines by infiltrated

immune cells was assessed using RNA isolated from ear tissues (Fig. 4B). Repeated DFE/DNCB challenge increased the gene expressions of TNF- α , IFN- γ , IL-4, IL-13, and IL-31. However, ABE treatment significantly reduced the expression levels.

3.4. Effects of ABE on the activation of keratinocytes

Keratinocytes constitute the epidermis, interact with infiltrated immune cells, and can be activated to regulate AD progression through the secretion of various inflammatory cytokines and chemokines (Brandt and Sivaprasad, 2011). To investigate the effect of ABE on the activation of keratinocytes, keratinocytes were stimulated with TNF- α and IFN- γ , which promote the amplification of the inflammatory response (Yarilina and Ivashkiv, 2010). Before this experiment, the cytotoxicity of ABE was determined in various concentrations (0, 0.1–1000 μ g/mL) for 24 h using HaCaT, a human keratinocyte cell line. Application of up to 100 μ g/mL of ABE did not reduce the cellular growth (Fig. 5C). TNF- α /IFN- γ stimulation increased the gene expression levels of TNF- α , IL-1 β , CCL17, and IL-6 (Fig. 5A), whereas pretreatment with ABE strongly inhibited their expression levels. The expression of these cytokines is regulated by transcription factors, such as NF- κ B and STAT1 (Yoshimura, 2006) as well as MAPKs (Choi et al., 2013). Therefore, the effect of ABE on their activation was analyzed using Western blotting (Fig. 5B). TNF- α /IFN- γ stimulation in HaCaT strongly induced nuclear translocation of STAT1, p65 NF- κ B, degradation of I κ B α , and

phosphorylation of MAPKs. However, pretreatment with ABE inhibited the nuclear translocation of STAT1 and p65 NF- κ B as well as degradation of cytosolic I κ B α . In addition, ABE reduced phosphorylation of ERK and p38 MAPK but not SAPK/JNK.

3.5. Identification of the compounds using HPLC

To examination the chemical contributors to the anti-allergic inflammatory effects of the ABE, bioactivity-guided fractionation and column chromatography purification of the ethanolic extract were carried out (Fig. 6). Through the subsequent purification as described in section 2.3., a total of 17 compounds, including gallic acid, vanillic acid, and resveratrol, were isolated and identified through comparison with published spectroscopic data.

4. Discussion

In this report, we demonstrated for the first time the pharmacological effects of the ethanol-extract of *A. brevipedunculata* rhizomes on AD-like skin inflammation using *in vitro* and *in vivo* models. Due to the increase of various harmful factors, the prevalence of AD has increased more than twofold during the past several decades. Therefore, the development of a new effective drug with minimal side effects is being actively pursued. In this light, interest in evaluating the effects of natural compounds based on Chinese medicine has been expanding (Tan et al., 2013).

The Th1/Th2 paradigm has been widely accepted as the classical explanation for AD development; in the acute phase, the Th2 cell response predominates, while in the chronic phase, the Th1 response predominates due to switching toward the action of Th1 cells (Auriemma et al., 2013). The DFE/DNCB-induced AD mouse model is widely used and exhibits mixed phenotypes of the Th1 and Th2 responses (Jin et al., 2016; Jung et al., 2017). Co-stimulation with DNCB as an immunogen for the sensitization of skin and DFE as an allergen for mimicking the human AD phenotype leads to reproducible AD-like characteristics such as pruritus, elevation of total and specific IgE, and increased immune cell infiltration including lymphocytes, mast cells and eosinophils (Jin et al., 2016; Jung et al., 2017). In atopic lesion, Th2 polarized cells produce Th2 cytokines such as IL-4, IL-13, and IL-31 in the acute phase, while Th1 cells express IFN- γ (Jutel and Akdis, 2011) in the chronic

phase. Various cytokines released from Th subsets activates mast cells and eosinophils, key effector cells in controlling allergic inflammation through the production of multiple inflammatory mediators, cytokines, and chemokines (Kawakami et al., 2009). Activated mast cells release histamine and cytokines including IL-4 and IL-13, which subsequently induce pruritus and edema, inhibition of epidermal keratinocyte differentiation, and skin barrier dysfunction (De Benedetto et al., 2015; Gschwandtner et al., 2013; Kawakami et al., 2009). Eosinophils acting as multipotential immune cells form a Th2 milieu through the secretion of IL-4, IL-5, and IL-10. IL-4, IL-13, and IL-31 expression are increased in AD lesions and are strongly associated with IgE levels (Wang and Xu Landen, 2015). Eosinophils activated by IL-4 with auto-/paracrine manner contribute to Th2-to-Th1 switching through the production of IL-12 (Di Cesare et al., 2008). Increased numbers of mast cells and eosinophils and high levels of IgE are hallmarks of the Th2 immune response (Stone et al., 2010), whereas elevation of IgG2a is a marker for the Th1 response (Adel-Patient et al., 2000). The production of IgG2a from B cells is promoted by IFN- γ released from Th1 cells (Bossie and Vitetta, 1991). Our results showed that ABE suppressed AD-like characteristics through inhibition of both Th1 and Th2 responses.

In AD skin inflammation, keratinocytes are activated by various immune cell-derived cytokines. Activated keratinocytes produce a variety of pro-inflammatory cytokines including IL-1, IL-6, and TNF- α , which, in turn, aggravate the inflammatory lesion through autocrine

and paracrine mechanisms (Auriemma et al., 2013). In particular, TNF- α , a pro-inflammatory cytokine, is mainly secreted by keratinocytes and controls the fate of keratinocytes (Asahina and Maeda, 2017). To mimic the AD-like skin inflammatory environment, we treated cells with recombinant TNF- α and IFN- γ (Leung and Bieber, 2003; Leung et al., 2004). In our study, we confirm the inhibitory effect of ABE on increased expression of TNF- α , IL-1, IL-6, and CCL17 due to stimulation with TNF- α and IFN- γ . CCL17 is a potent chemoattractant for Th2 cells and correlates well with the degree of severity of AD (Shoda et al., 2014). TNF- α and IFN- γ regulate the expression of cytokines through the activation of the surface receptors, and transcription factors including NF- κ B, STAT1, and MAPKs in keratinocytes (Asahina and Maeda, 2017; Yang et al., 2016). Based on our results, the inhibitory effects of ABE on TNF- α /IFN- γ -stimulated keratinocytes may act through the suppression of cytokine and chemokine production by the inhibition of NF- κ B, STAT1, ERK, and p38 MAPK.

In the general concept of inflammation, the major cells inducing inflammatory responses in the inflammatory lesions are myelogenic lineage immune cells, such as macrophages and neutrophils. To check this point, we measured the effect of ABE on the expression of pro-inflammatory cytokines and chemokine using LPS-stimulated macrophages, and found no effectiveness of ABE. These data imply the specific role of ABE on keratinocytes-involved skin inflammation at least in AD-like condition.

Many plant tissues of *A. brevipedunculata* including stem, root, and berries have been used in folk medicine. Phytochemical researchers investigating specific tissues of *A. brevipedunculata* have identified various bioactive compounds. The extract from the roots of *A. brevipedunculata* contained eight different eight chemicals; β -amyrin, betulin, vanillic acid, ethyl gallate, kaempferol, 3,5-dimethoxy-4-hydroxybenzoic acid, aromadendrol, and resveratrol (Xu et al., 1995). We previously reported that the ethanol-extract from the rhizomes of *A. brevipedunculata* contains 17 compounds, including gallic acid, β -sitosterol, vanillic, and resveratrol (Jang et al., 2018). The beneficial pharmaceutical effects of several compounds have been elucidated. Betulin, betulinic acid, aromadendrin, catechin and ethyl gallate possessed an anti-inflammatory and/or anti-oxidant activity (Laavola et al., 2016; Mehla et al., 2011; Nakanishi et al., 2010; Zhang et al., 2006). Gallic acid and resveratrol ameliorated AD-like skin inflammation (Caglayan Sozmen et al., 2016; Tsang et al., 2016). β -Sitosterol attenuated DNFB-induced AD-like skin lesions in NC/Nga mice through the inhibition of mast cell activation (Han et al., 2014). In addition, vanillic acid and ellagic acid have been reported to have an anti-allergic inflammatory effect through the regulation of mast cell activation (Choi and Yan, 2009; Jeong et al., 2017). Based on these knowledge, we suggest that the each compound of ABE synergistically act to suppress AD-like skin inflammation.

Even now, topical corticosteroids or oral steroids are still the first-line therapy for most AD patients. However, it is well known that these steroid therapies cause various adverse effects, including cracking, bleeding, and thinning of the skin (Barnes, 2001; Wollenberg and Ehmann, 2012). Therefore, researchers are endeavoring to find an effective drug with fewer side effects originating from a natural product, which exhibits anti-allergic and anti-inflammatory properties for treating skin allergic inflammatory disorders such as AD. Based on our research, we expect that ABE as a plant-derived complex compound could be developed as an effective agent for AD treatment possessing a low level of side-effects. However, more detailed study will be required to address the interaction between each compound.

5. Conclusions

The present study demonstrates that oral administration of ABE ameliorated DFE/DNCB-induced AD-like skin inflammation, which may be due to decreased infiltration of immune cells and subsequent Th1 and Th2 responses in AD skin, and reduced TNF- α /IFN- γ -promoted pro-inflammatory cytokines and chemokines in keratinocytes. The immunosuppressive effect of ABE may be due to the synergism of individual bioactive compounds which possess potential anti-inflammatory and anti-allergic activities. Taken together, our data suggest that ABE could be used as a useful pharmacological agent.

Acknowledgements

This research was supported by a grant from the Korea Health Technology R&D Project of the Korea Health Industry Development Institute (KHIDI) and by the Ministry of Health & Welfare, Republic of Korea (HI18C0308).

Conflicts of interest

The authors declare no conflict of interests.

Contributions

Choi Y.A., Yu J.H., and Jung H.D. performed the experiments. Lee S.Y. performed the statistical analyses. Park P.H., Lee H.S., Kwon T.K., and Shin T.Y. designed the study. Lee S.W., Rho M.C., Jang Y.H., and Kim S.H. participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Figure legends

Figure 1. Clinical observation and photographs. (A) Ear thickness was measured using a dial thickness gauge at 24 h after applying DFE or DNCB. (B) Observation of clinical symptom at day 28. Data are presented as the mean $\pm$ SEM of the five determinants. * $p < 0.05$ vs. the AD group. * Significantly lower than those of AD mice at $p < 0.05$. AD, atopic dermatitis; DFE, *Dermatophagoides farinae* extract; DNCB, dinitrochlorobenzene; ABE, ethanol-extract of *Ampelopsis brevipedunculata* rhizomes; Dexam, dexamethasone.

Figure 2. Histopathological analysis of AD mouse ear tissue. (A) Tissue sections were stained with hematoxylin and eosin (H&E, upper panel, original magnification $\times 400$) or toluidine blue (TB, lower panel original magnification $\times 400$). (B) Dermal thickness, (C) epidermal thickness, and numbers of (D) mast cells and (E) eosinophils were analyzed. Data are presented as the mean $\pm$ SEM of the five determinants. * $p < 0.05$ vs. the AD group. *Significantly lower than those of AD mice at $p < 0.05$. AD, atopic dermatitis; DFE, *Dermatophagoides farinae* extract; DNCB, dinitrochlorobenzene; ABE, ethanol-extract of *Ampelopsis brevipedunculata* rhizomes; Dexam, dexamethasone.

Figure 3. Levels of serum histamine and Immunoglobulins in AD mice. Blood samples were obtained from the celiac artery at day 28 and serum was isolated. (A) Serum histamine and

(B-D) Igs were quantified by ELISA. Data are presented as the mean $\pm$ SEM of the five determinants. * $p < 0.05$ vs. the AD group. Ig, immunoglobulin; AD, atopic dermatitis; ABE, ethanol-extract of *Ampelopsis brevipedunculata* rhizomes; Dexa, dexamethasone; O.D., optical density.

Figure 4. Th1 and Th2-type lymphocyte subset distribution in draining lymph node and cytokine production in the AD mouse ear. (A) Both auricular lymph nodes were collected from each mouse at day 28. From the isolated single cells, event numbers of CD4⁺IFN- γ ⁺ and CD4⁺IL-4⁺ cells were quantified by FACSCalibur. (B) Ears were excised at day 28 and total RNA was isolated, then reverse transcription-quantitative polymerase chain reaction was performed as described in the Materials and Methods section. Data are presented as the mean $\pm$ SEM of the five determinants. * $p < 0.05$ vs. the AD group. AD, atopic dermatitis; ABE, ethanol-extract of *Ampelopsis brevipedunculata* rhizomes; Dexa, dexamethasone

Figure 5. Effects of ABE on TNF- α /IFN- γ -stimulated human keratinocyte, HaCaT cells.

Cells were pretreated with ABE (10, 30, or 60 μ g/mL) or Dexa (10 μ g/mL) for 1 h and stimulated with TNF- α (10 ng/mL) and IFN- γ (10 ng/mL). (A) The expression levels of cytokines and chemokine were determined by qPCR. Data are presented as mean $\pm$ SD.

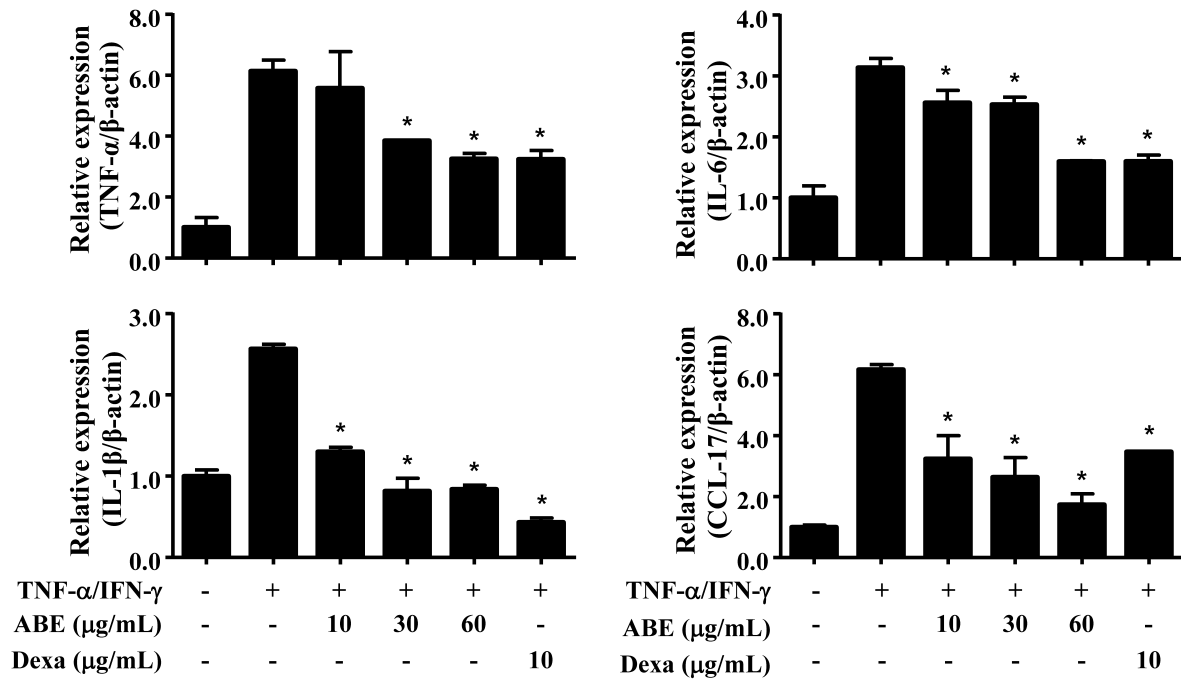
*Significantly lower than the TNF- α /IFN- γ -stimulated positive control at $p < 0.05$. Cytokine

expression was calculated by normalizing to the β -actin value. (B) The protein expression of STAT1, NF- κ B, I κ B α , and phospho-and total-form of ERK, p38 MAPK and SAPK/JNK were detected using Western blot. The data shown represent three independent experiments. β -actin and lamin B were used as loading controls. (C) Effects of ABE on the viability of HaCaT cells were determined using the MTT assay at 24 h after treatment with ABE. ABE, ethanol-extract of *Ampelopsis brevipedunculata* rhizomes; p-, phosphorylated-; Dexa, dexamethasone

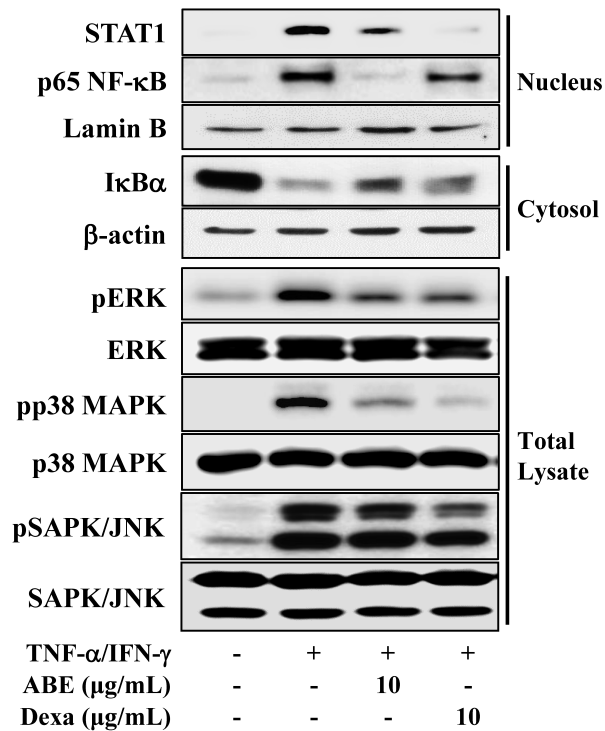
Figure 6. HPLC profiling of ABE. HPLC-DAD analysis was performed at 240 nm. Peaks were confirmed by the addition of standard gallic acid, catechin, ethyl gallate, aromadendrin, and resveratrol in ABE.

Figure 5

A



B



C

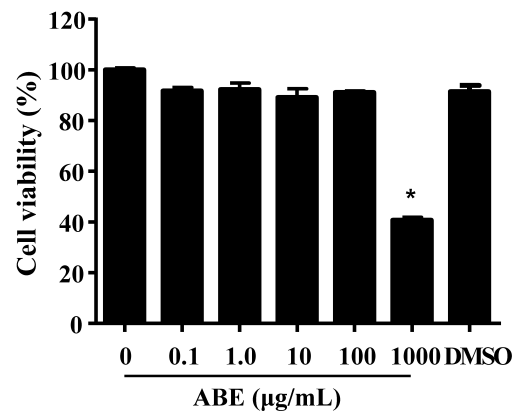


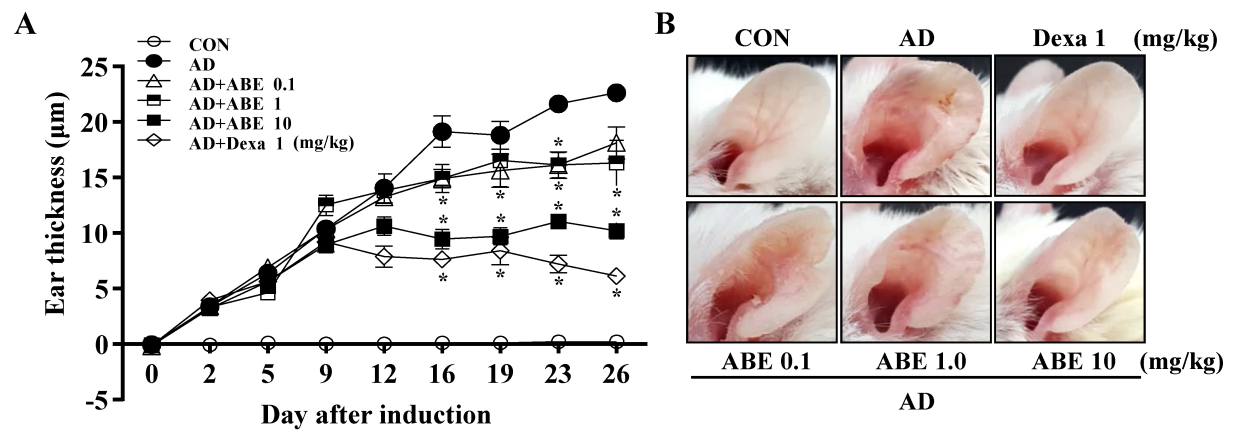
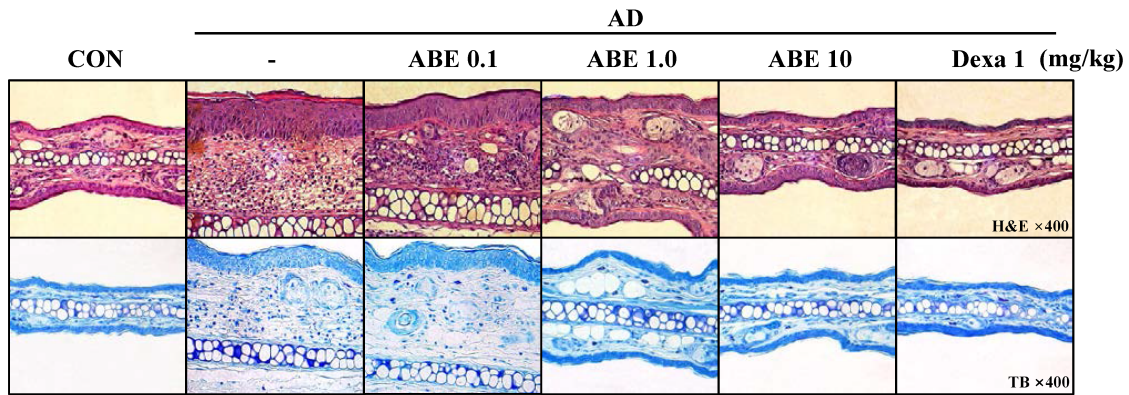
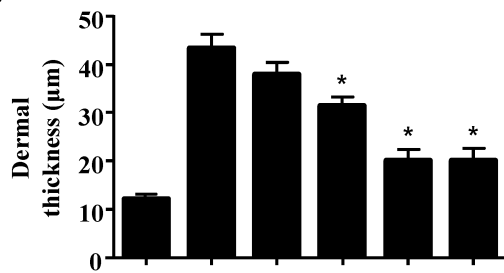
Figure 1

Figure 2

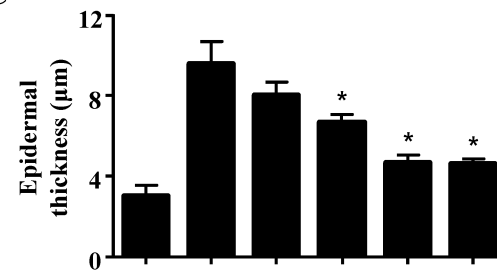
A



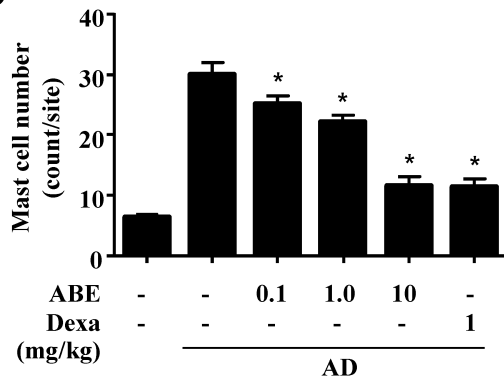
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E

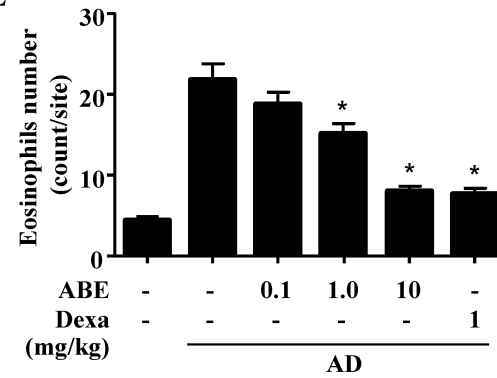


Figure 3

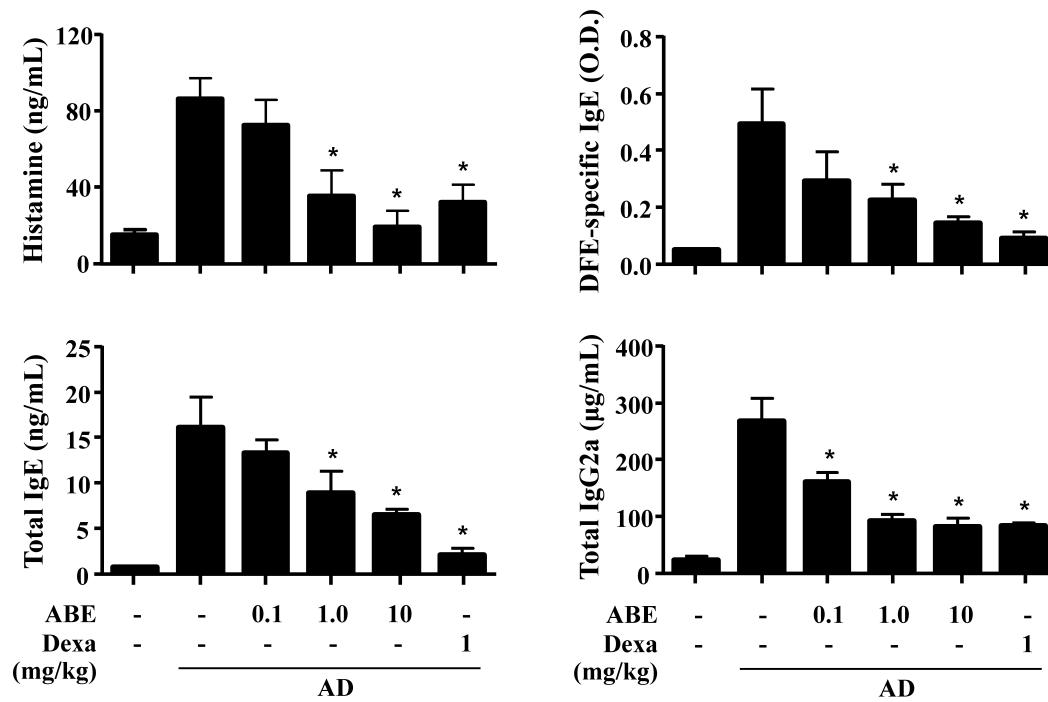


Figure 4

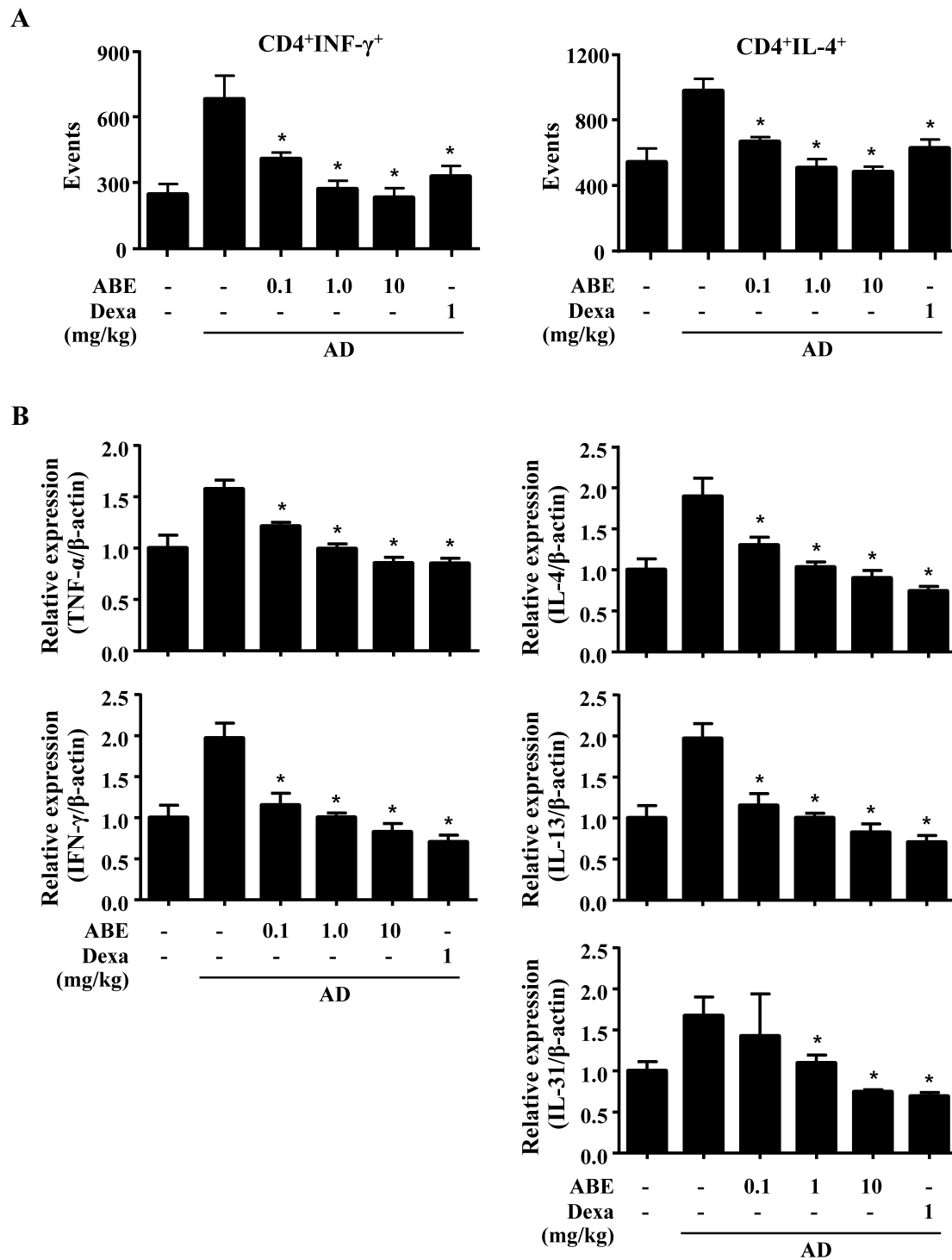
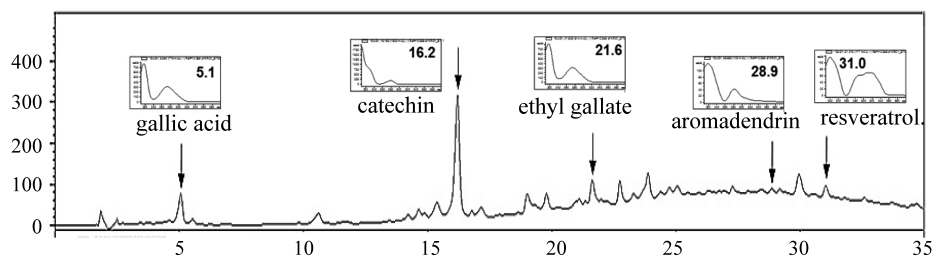


Figure 6



1	Betulin	Patra et al., 1988
2	Betulinic acid	Cichewicz and Kouzi, 2004
3	β -sitosterol	Hui et al., 1976
4	β -sitosterol glucoside	Khatun et al., 2012
5	Aromadendrin	Neacsu et al., 2007
6	3-O-glycoside-aromadendrin	Kang et al., 2011
7	Catechin	Davis et al., 1996
8	Gallic acid	Nguyen et al., 2013
9	Vanillic acid	Cho et al., 2000
10	Ethyl gallate	Yu et al., 2008
11	Ethyl gallate 4-O- α -D-glucopyranoside	De-Winter et al., 2014
12	Syringic acid	Inoshiri et al., 1987
13	Benzyl 6'-O-galloyl- β -D-glucopyranoside	Isasza et al., 2001
14	Ellagic acid	Do-Khac et al., 1990
15	3'-O-methylellagic acid 4-O- α -L-rhamnopyranoside	Yazaki and Hillis, 1976
16	4'-O-(β -D-glucopyranosyl)-3,3',4-tri-O-methylellagic acid	Do-Khac et al., 1990
17	Resveratrol	Mattivi et al., 1995

Inhibitory effect of ethanol extract of *Ampelopsis brevipedunculata* rhizomes on atopic dermatitis-like skin inflammation

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Supplementary figure

Figure S1. Experimental design of the induction of AD. The first treatment with DFE (10 mg/mL, 20 μ L/ear) and 1% DNCB (20 μ L/ear) were rotationally applied to both ears once a week for 4 weeks. After 1 week of induction, ABE (0.1, 1, or 10 mg/kg) or Dexa (1 mg/kg) was orally administered five times per week for 3 weeks ($n = 5$).

Figure S2. Body weight changes in DFE/DNCB-induced AD mice. The body weight of mice was measured on the first day of each week for 4 weeks using a weighing scale. Data are presented as mean $\pm$ SEM ($n = 5$). Dexa, dexamethasone.

Figure S1

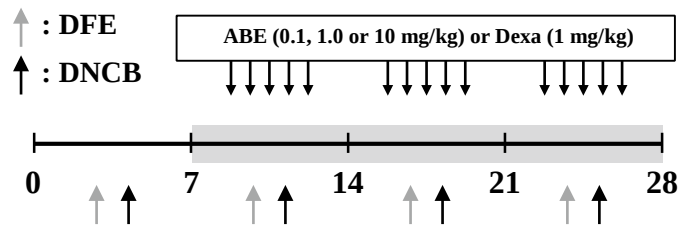
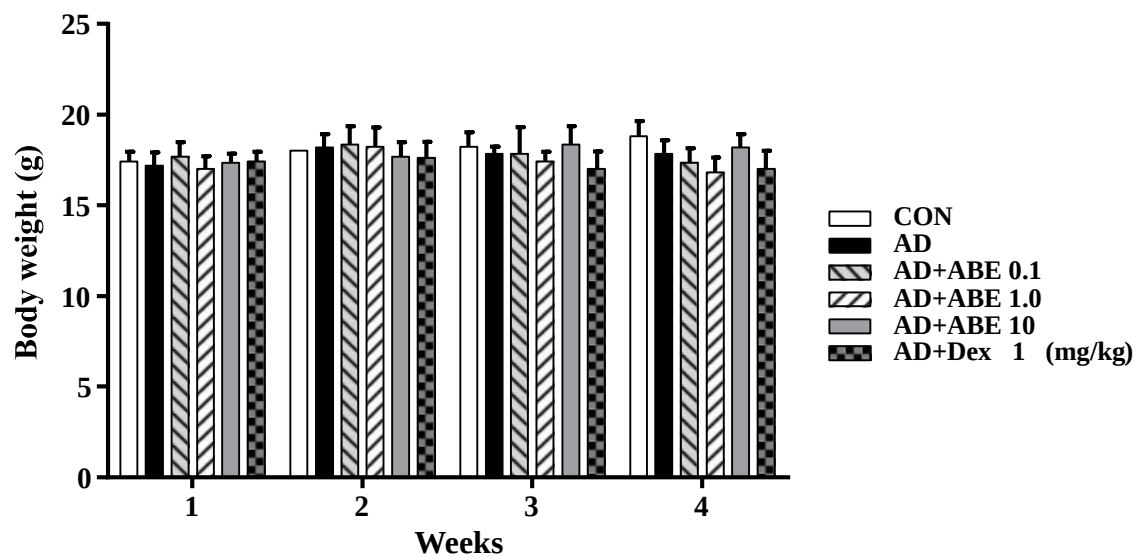


Figure S2



Supplementary Table S1. Primer sequences

Product size		Forward	Reverse
mTNF- α	300	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG
mIFN- γ	201	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
mIL-4	216	TCGGCATTTTGAACGAGGTC	GAAAAGCCCGAAAGAGTCTC
mIL-13	165	GGGACATGGTTTGCTGCCTA	AGACAGGAGTGTTGCTCTGG
mIL-31	330	TCGGTCATCATAGCACATCTGGAG	GCACAGTCCCTTTGGAGTTAAGTC
hIL-1 β	109	GCTGATGGCCCTAAACAGATGAA	TGAAGCCCTTGCTGTAGTGGTG
hTNF- α	237	GAGCTGAGAGATAACCAGCTGGTG	CAGATAGATGGGCTCATACCAGGG
hIL-6	140	CCCCTGACCCAACCACAAAT	CATTTGCCGAAGAGCCCTCA
hCCL17	270	ACAAGGGGATGGGATCTCCCTCAC	ACTGCTCCAGGGATGCCATCGTTT