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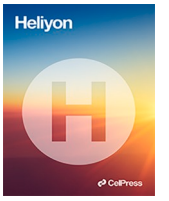

Research article

Effects of Earthworm (*Pheretima communisima*) extract on atopic dermatitis: An in vitro and in vivo study

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ABSTRACT

Earthworm (*Pheretima communisima*) is used as a traditional medicine for the management of allergic airway inflammation. Atopic dermatitis (AD) is a persistent, recurrent disorder marked by allergic inflammation and skin barrier dysfunction. However, the pharmaceutical effects of earthworms on AD have not been defined. Our study examined the anti-allergic and anti-inflammatory actions of earthworm ethanolic extract (EWE) on allergic skin inflammation in a *Dermatophagoides farinae* mite antigen-induced AD mice model, TNF-α/IFN-γ-treated human keratinocytes, and compound 48/80-treated mouse mast cells. Oral administration of EWE in AD mouse reduced inflammatory cell accumulation, epidermal hyperplasia, and dermatitis severity in AD skin lesions and thymic stromal lymphopoietin (TSLP) and immunoglobulin (Ig) E concentrations in serum. EWE administration in AD mice also reduced secretion of Interleukin (IL)-4, IL-13, IL-5, and IFN-γ in cultures of isolated splenic cells. Immunohistofluorescence staining of skin lesions from AD mice revealed that EWE induced expression of claudin-1, filaggrin, and SIRT1. In HaCaT keratinocytes cotreated with IFN-γ and TNF-α, EWE inhibited secretion of the chemokine Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) in a dose-dependent manner. In addition, EWE inhibited histamine release in activated MC/9 mast cells. These results show that EWE might be therapeutics for the management of AD.

1. Introduction

Atopic dermatitis (AD) is a chronic skin condition commonly related to respiratory allergies and is one of the most public dermatological illnesses in children [1]. The prevalence of AD is 15 %–20 % among children and up to 10 % among adults [2]. AD is closely connected to allergic rhinitis, asthma, and increasing levels of circulating immunoglobulin (Ig) E in a phenomenon known as atopic march [3]. Immune dysfunction and skin barrier abnormalities are significant to the onset of AD. The main regulators involved in the progress of epidermal skin barrier dysfunction include reductions of ceramides, filaggrin (filament aggregating protein), antimicrobial peptides, and serine protease inhibitors; disordered tight junctions between adjacent cells; and an increase of serine protease [4]. The pathogenesis of immune dysfunction in AD involves sensitization to allergens; increased IgE levels; predominance of Th2 (IL-13, IL-4, IL-5, and IL-31) cytokines; increased high-affinity cell surface IgE receptor (FcεRI) expression in dermal dendritic cells and Langerhans cells; and increased expression of the chemokines Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES/CCL5) and Thymus and activation-regulated chemokine (TARC/CCL17) and the cytokine Thymic stromal lymphopoietin

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(TSLP) [4–7]. Current treatment of AD is symptomatic relief through avoidance of triggers and long-term disease management, and recovering the skin barrier and improving inflammation is an urgent need [8]. For anti-inflammation, topical calcineurin inhibitors (pimecrolimus and tacrolimus) and topical corticosteroid (dexamethasone) are widely used as first-line treatment drugs [9]. Nevertheless, long-term treatment of these immunosuppressive drugs can lead to serious adverse effects, including immunosuppression, organ dysfunction, and hypothalamic-pituitary-adrenal suppression [10]. Hence, there is an urgent need to advance effective and safe therapeutic interventions for the management of AD.

Earthworm (Jilyong in Korea, *Pheretima communisima*) is commonly used in traditional herbal drugs for the management of asthma, bronchitis, cough, fever, hypertension, myocardial infarction, and stroke because of its anti-asthmatic, anti-inflammatory, thrombolytic, diuretic, hypotensive, and anti-oxidative effects [11,12]. Moreover, earthworm extract (EWE) enhances wound healing and bone tissue regeneration by stimulating osteoblast activity and inhibiting osteoclast differentiation [13]. In the inhaled ovalbumin-induced model of allergic asthma, an earthworm decoction suppressed lung inflammation by suppressing the nuclear factor-κB (NF-κB) pathway [11]. The inhibitory activity of EWE on inflammation has been attributed to the high content of polyphenol compounds in earthworms [14]. Despite these previous studies have shown that earthworm can improve allergic inflammatory disease, the pharmaceutical activity of earthworms on AD-like skin inflammation have not been defined.

Therefore, we measured the biological activities of EWE on the allergic skin inflammatory disease AD. In vitro inhibitory activities of EWE on allergy and inflammation were assessed using chemokine assay in keratinocytes and histamine assay in mast cells. *In vivo* anti-inflammatory and anti-atopic activities were assessed in house dust mite *Dermatophagoides farinae* extract (DfE)-stimulated AD model mice.

2. Materials and methods

2.1. Extraction

Dried earthworm (Omniherb, Daegu, Korea) was extracted twice with 70 % ethanol (with a 2-h reflux). The extracts were filtered, concentrated under reduced pressure, dried, and deposited at 4 °C. The extracts were maintained in the herbarium of the Korean Institute of Oriental Medicine.

2.2. In vivo study

2.2.1. Animal experiment

AD was developed in mice by a previously described method [15]. The backs of the mice were epilated prior to the start of treatments. To make AD, the back skin of the mouse was treated a total of six times over a three-week period as follows. First, sodium dodecyl sulfate (4 %, 150 μL) was sprayed to disrupt the skin barrier. Then, after the dorsal skin was allowed to completely dry for 2–3 h, 100 mg of an ointment containing *Dermatophagoides farinae* (DfE) extract from the house dust mites (Biostir, Hyogo, Japan) was topically treated. Control mouse was treated with vehicle without DfE application. Beginning one week after the start of AD induction, EWE and dexamethasone were prepared in saline solution and administered to the mice every day for two weeks. An equal amount of saline was administrated to the control groups. The dermatitis severity (edema, erythema/hemorrhage, scaring/dryness, and excoriation/erosion) was assessed with macroscopic scoring by a previously described method [16], and the thickness of the ear was measured with a Mitutoyo micrometer (Kanagawa, Japan).

Starting one week before the start of the experiment, 7-week-old male NC/Nga mice (Central Lab Animal Inc., Korea) were kept in the experimental surroundings. The animals were separated into 5 groups (n = 6/group): normal (untreated) control, DfE-applied (DfE), DfE-dexamethasone (5 mg/kg) treated, DfE-EWE (200 mg/kg) treated, and DfE-EWE (400 mg/kg) treated. The experimental dosage was determined from preliminary dose-response experiments. Dexamethasone, a glucocorticoid, was used as a positive control because it is a commonly used chemical drug for inflammatory conditions. The experimental scheme is presented in Fig. 1. This study was permitted by the Animal Care and Use Committee of Daejeon University (Ethical approval code. DJUARB2019-042) and conducted according to the Guide for Care and Use of Laboratory Animals and the guidelines regarding animal care and use (National

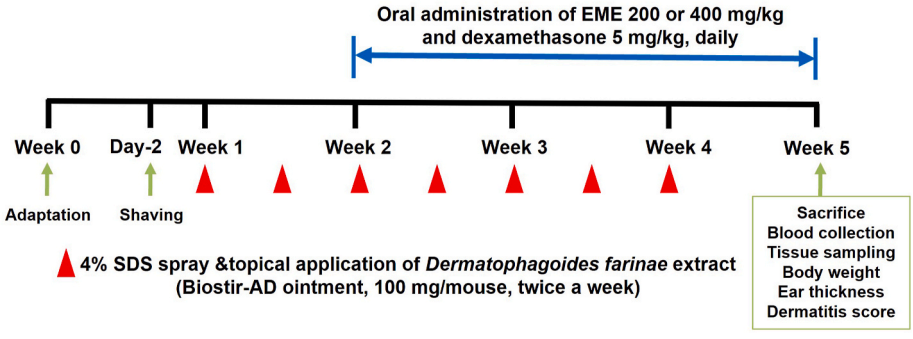

Fig. 1. Experimental scheme for atopic dermatitis induction and oral administration of earthworm (*P. communisima*) extract (EWE) in NC/Nga mice.

Research Council of USA, 1996).

2.2.2. Histological examination

After the animal experiment, the mouse was sacrificed and the lesioned dorsal tissue was cut, fixed, embedded, and cut into 4-μm thick slices. The degree of inflammation in the paraffin-embedded skin sections was assessed using hematoxylin and eosin (H&E) or toluidine blue (TB) staining. Image was observed by light microscopy and photographed (Olympus CX31/BX51 and DP70, Olympus Optical Co., Tokyo, Japan). To measure mast cells accumulation, mast cells were counted in five randomly selected spots from TB-stained slide.

2.2.3. Measurement of white blood cells

Blood was obtained from the mice through a needle inserted into the heart. The number of in the extracted blood was determined by a hematology analyzer CELL-DYN 3200 (Abbott, Santa Clara, CA).

2.2.4. Splenocyte culture and cytokine release from cultured splenocytes

The spleen of the mouse was isolated, and single-cell suspension of splenocytes was created to remove red blood cells. The isolated splenocytes were then cultured for 24 h with or without 0.5 μg/mL CD3 antibody (eBioscience, San Diego, CA). The supernatants were then recovered from the splenocyte cultures to evaluate the secretion of cytokines.

2.2.5. Enzyme-linked immunosorbent assay (ELISA)

Total IgE concentrations from mouse serum and cytokine (IL-13, IL-5, IL-4, and IFN-γ) levels from the supernatant of cultured splenocytes were evaluated using mouse IgE, IL-5, IL-4, IL-13, or IFN-γ ELISA kits (R&D Systems, Minneapolis, MN).

2.2.6. Immunofluorescence staining

Mouse skin section was blocked with 5 % bovine serum albumin for 50 min. After nuclei staining with Hoechst 33342 for 0.5 h, the sections were treated at 4 °C overnight with immunofluorescence-labeled primary antibodies (Abcam, Cambridge, MA) for sirtuin (SIRT)1, filaggrin, or claudin1. The section was then treated with secondary antibodies for 3 h, and immunofluorescence was imaged using a confocal microscope (Nikon, Tokyo, Japan).

2.3. In vitro study

2.3.1. Analysis of chemokine production in HaCaT keratinocytes

HaCaT human immortalized keratinocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) and 100 U/mL penicillin/100 μg/mL streptomycin. Then, 1×10^6 cells/well were laid into 6-well plates, and the cells were incubated with or without EWE at various concentrations for 24 h with tumor necrosis factor (TNF)-α (10 ng/mL) and interferon (IFN)-γ (10 ng/mL). The concentrations of secreted TARC, RANTES, and TSLP from the supernatant were obtained with human ELISA kits (R&D, St. Louis, MO). Dexamethasone (1 μM) was applied as a positive control.

2.3.2. Secretion of histamine from MC/9 mouse cells

MC/9 mouse mast cells (CRL-3616, American Type Culture Collection, Manassas, VA) were maintained in DMEM with 10 % T-STIM (BD Biosciences, CA, USA), 0.05 mM 2-mercaptoethanol, 10 % FBS, and 2 mM L-glutamine. The cells were then treated with 100 μg/ml or 200 μg/ml EWE and 25 μg/mL compound 48/80 (Sigma, St. Louis, MO, USA) for 25 min. The cell density was estimated by measuring absorbance at 650 nm with an absorbance reader (BioRad, Hercules, CA), and histamine secretion into the cell supernatant was evaluated using an ELISA kit (Oxford Biomedical Research, Oxford, MI).

2.3.3. UPLC-PDA analysis

The chemical constituents from EWE (5 mg/mL) were evaluated using an AQUITYTM Ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) equipped with a binary solvent manager and a photodiode array detector. Sample (2.0 μL) was separated on a C18 BEH column (2.1 × 100 mm, 1.7 μm) at a flow rate of 0.4 mL/min and eluted using a linear gradient of two mobile phases (A: water with 0.1 % formic acid; B: acetonitrile with 0.1 % formic acid). A chromatographic gradient was optimized as follows: 0 min, 15 % B; 0–9 min, 15–90 % B; 9–11 min, 90–100 % B; 11–13.3 min, 100 % B; 13.4 min, back to 15 % B. β-sitosterol, a standard compound for quality control, was obtained from ChemFaces (Wuhan, Hubei, China). The solvents were HPLC grade (Merck Millipore, Burlington, MA, USA).

2.3.4. Statistics

Data are shown as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and multiple comparisons by Dunnett's test conducted in GraphPad Prism 7.0 (GraphPad Software, San Diego, CA) were used to compare groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Effect of Ewe on AD mice

Symptoms of AD such as dryness, scarring, erosion, erythema, hemorrhage, and excoriation were visible on the back skin of mice that were treated with topical DfE for three weeks (Fig. 2a). Compared with untreated control mice, DfE-treated mice exhibited a dramatic rise in dermatitis score, which was reduced meaningly by administration of 400 mg/kg EWE or 5 mg/kg dexamethasone (Fig. 2b). The thickness of the ear was also increased in the DfE-treated mice, and treatment with 200 mg/kg or 400 mg/kg EWE decreased the ear thickness in DfE-treated mice (Fig. 2c). The body weight was not affected by any of the treatments (Fig. 2d). Serum levels of IgE, as an AD biomarker were increased in the DfE-treated mice, and administration of 5 mg/kg dexamethasone or 400 mg/kg EWE reduced the IgE levels in DfE-treated mice (Fig. 2e). Serum TSLP levels were also raised in the DfE-treated mice, and these levels were decreased by administration of 200 mg/kg or 400 mg/kg EWE (Fig. 2f). These results demonstrate that EWE im-proved symptoms of AD in DfE-treated mice.

3.2. Effects of Ewe on hematological parameters

Changes in the distributions of immune cells, particularly eosinophils and neutrophils, due to inflammation provide important clues about the initiation of AD. DfE-treated mice had higher total numbers of eosinophils and neutrophils in their blood than untreated control mice, and treatment with EWE reduced the numbers of both types of immune cells in DfE-treated mice (Fig. 3a and b).

3.3. Effects of Ewe on the release of Th1/Th2 cytokines in splenocytes

Next study investigated the suppressive effect of EWE on the secretion of Th2 cytokines (IL-13, IL-4, IL-5) and Th1 cytokine (IFN- γ) in cultured spleen cells isolated from experimental mice. The secreted concentrations of IL-4, IL-5, IL-13, and IFN-γ were elevated in cultures of spleen cells from DfE-treated mice compared with those from control mice, and these levels were reduced in cultures of spleen cells from mice administrated to 200 mg/kg or 400 mg/kg EWE or 5 mg/kg dexamethasone (Fig. 4a, b, 4c, and 4d). These results demonstrate that EWE improves AD by down-regulating Th2-mediated and Th1-mediated inflammatory responses.

3.4. Histopathological examination

Histopathological examination of skin lesions with H&E (Fig. 5a) or TB (Fig. 5b) staining revealed that topical DfE treatment on the back skin caused epidermal thickening and increased mast cell infiltration, and treatment with EWE reduced the epidermal thickening

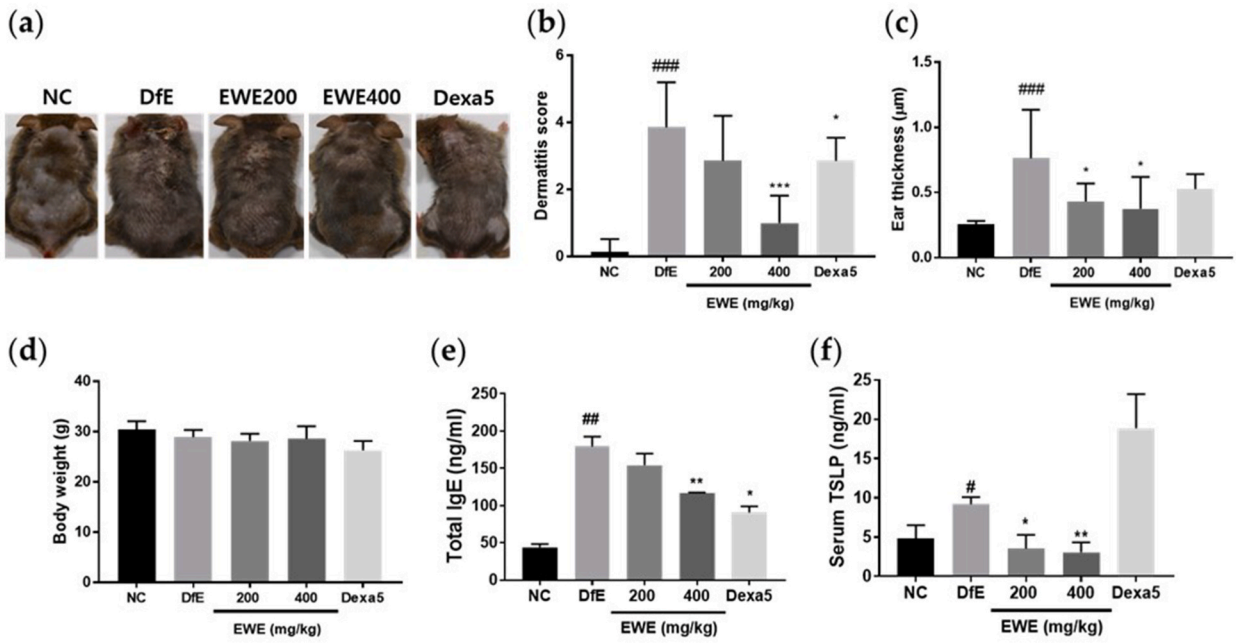

Fig. 2. The effects of earthworm (*P. communisima*) extract (EWE) on atopic dermatitis symptoms in mice. (a) Macroscopic examination, (b) dermatitis severity score, (c) ear thickness, (d) body weight, (e) serum total IgE, and (E) TSLP levels. Results are presented as mean ± SEM (n = 6). #p < 0.05, ##p < 0.01, and ###p < 0.001 in comparison with the normal control (NC) group; *p < 0.05, **p < 0.01, and ***p < 0.001 in comparison with the group treated with Dermatophagoides farinae extract (DfE).

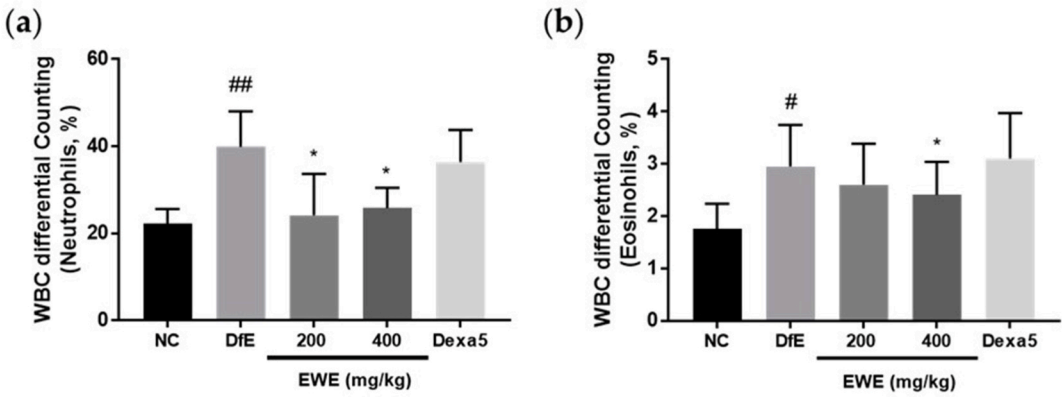

Fig. 3. Differential counts of white blood cells. WBC, white blood cell; Dexa, dexamethasone (5 mg/kg); EWE, earthworm (*P. communisima*) extract. #p < 0.05 and ##p < 0.01 show significant differences between the normal control (NC) and D. farinae extract (DfE)-treated groups. *p < 0.05 shows significant differences between the DfE-treated group and the Dexa-treated or EWE-treated groups.

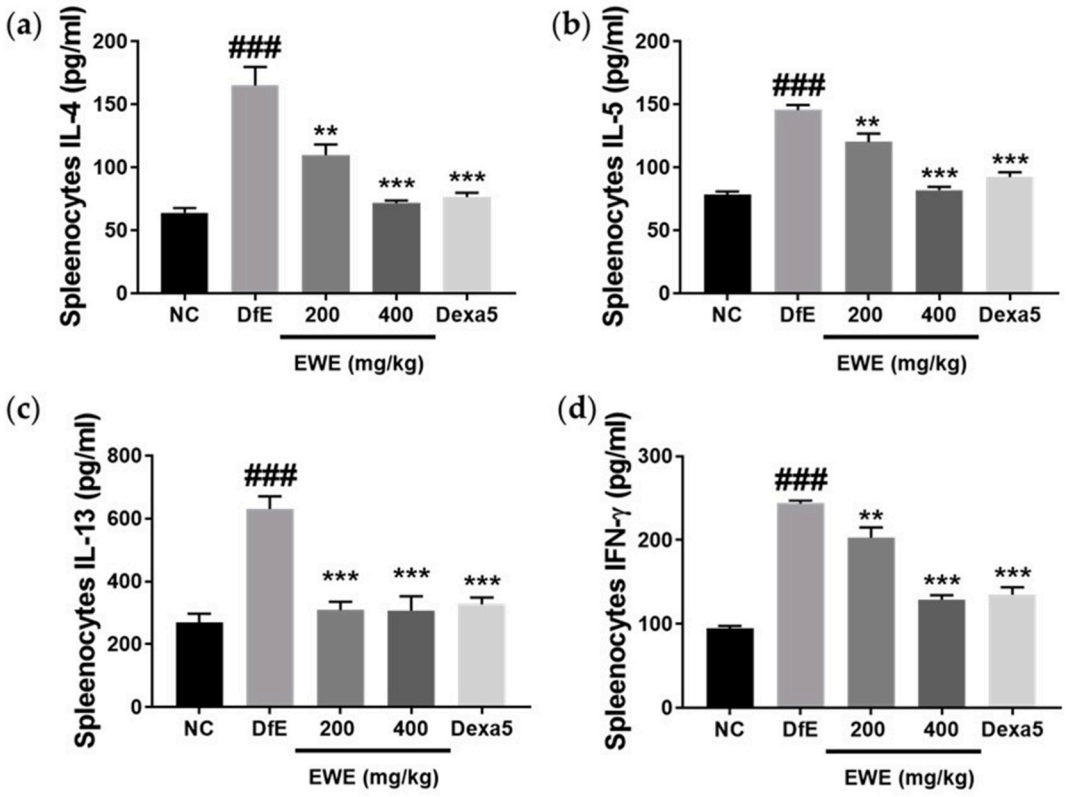

Fig. 4. The effects of earthworm (*P. communisima*) extract (EWE) on the release of Th1 and Th2 cytokines in cultured splenocytes. Secretion of (a) IL-4, (b) IL-5, (c) IL-13, and (d) IFN-γ in the su-pernatant of cultured splenocytes isolated from experimental mice was evaluated. Spleen cells were incubated for 48 h at a density of 1 × 105 cells/well on CD3 antibody-coated 96-well plates. Values are presented as mean ± SEM (n = 6). ###p < 0.001 compared with the normal control (NC) group; **p < 0.01 and ***p < 0.001 compared with the group treated with D. farinae extract (DfE).

(Fig. 5a) and mast cell infiltration (Fig. 5b). These histological consequences show that EWE administration improved skin inflammation in AD mice.

3.5. Effects of Ewe on the protein expression of filaggrin, SIRT1, and claudin1

To identify the effect of EWE on function of skin barrier, we detected the epidermal skin barrier proteins filaggrin, SIRT1, and

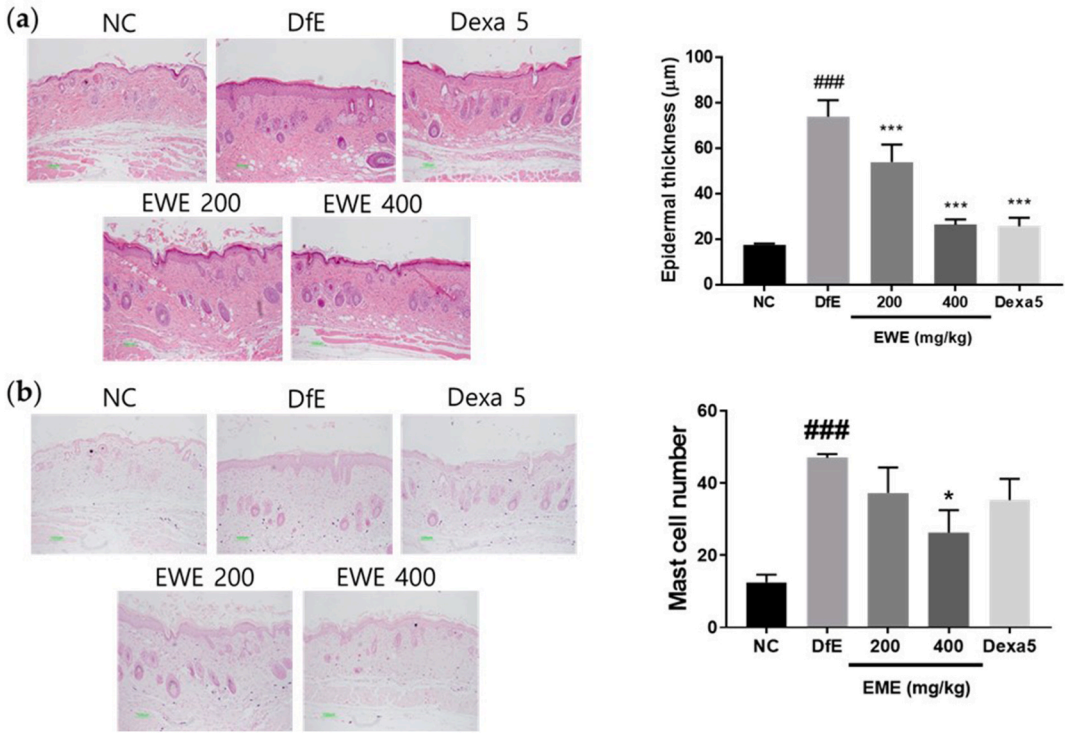

Fig. 5. Dorsal skin sections were stained with (a) hematoxylin and eosin (H&E) or (b) toluidine blue (TB). The thickness of the ear epidermis and the number of mast cells in the stained dorsal skin sections were measured. Results are presented as mean ± SEM (n = 6). ###$p < 0.001$ compared with normal control (NC); *$p < 0.05$ and ***$p < 0.001$ compared with the D. farinae extract (DfE)-treated group.

claudin1 by immunohistofluorescence staining in dermal skin sections (Fig. 6). Filaggrin expression was significantly increased in skin sections from mice administrated to 400 mg/kg EWE or 5 mg/kg dexamethasone compared with that in skin sections from untreated control mice or mice treated with DfE alone (Fig. 6a). SIRT1 expression was reduced in skin sections from mice treated with DfE alone compared with that in skin sections from untreated control mice, and administration to 400 mg/kg EWE or 5 mg/kg dexamethasone increased SIRT1 expression in skin sections from DfE-treated mice (Fig. 6a). Claudin1 expression was increased in skin sections from mouse treated with 400 mg/kg EWE compared with that in skin sections from un-treated control mice or mice treated with DfE alone (Fig. 6b).

3.6. Effects of Ewe on chemokine and histamine release in cultured cells

Secretion of the inflammatory chemokine RANTES was increased in IFN-γ/TNF-α–stimulated HaCaT cells and suppressed in a dose-dependent manner by further action with 25 μg/ml, 50 μg/ml, or 100 μg/ml EWE (Fig. 7a). In addition, histamine release was increased in compound 48/80-treated MC/9 mouse mast cells and reduced by further treatment with 200 μg/ml EWE (Fig. 7b).

3.7. Chemical profiling of Ewe by UPLC-PDA

UPLC-PDA analysis was achieved to find the presence of compounds in EWE. UPLC-PDA provides the quantitation of compounds in complex matrices of plant extracts by using both the retention time and ultraviolet spectrum of each peak, especially those in low concentration, and with a high degree of accuracy. Fig. 8a shows the UPLC-PDA chromatogram of EWE. β-sitosterol was identified as one the main chemical components in EWE from UPLC analysis (Fig. 8b).

4. Discussion

Earthworms have been used as a traditional herbal remedy to manage allergic inflammatory illnesses such as asthma. Nevertheless, the anti-allergic or anti-inflammatory properties of earthworms on AD have not been defined. Thus, we evaluated the activities of EWE on AD allergic skin inflammation. Our results showed that oral administration of EWE improved AD symptoms (such as dermatitis severity, ear edema, elevated serum IgE level, and eosinophilia) induced by DfE antigen. In addition, EWE treatment effectively regulated the enhancement of Th1- and Th2-related pathways by suppressing the Th2 cytokines IL-13, IL-5, and IL-4 and Th1 cytokine IFN-γ and in cultured splenocytes from AD mice, thus down-regulating the immune responses in the AD. The serum total IgE

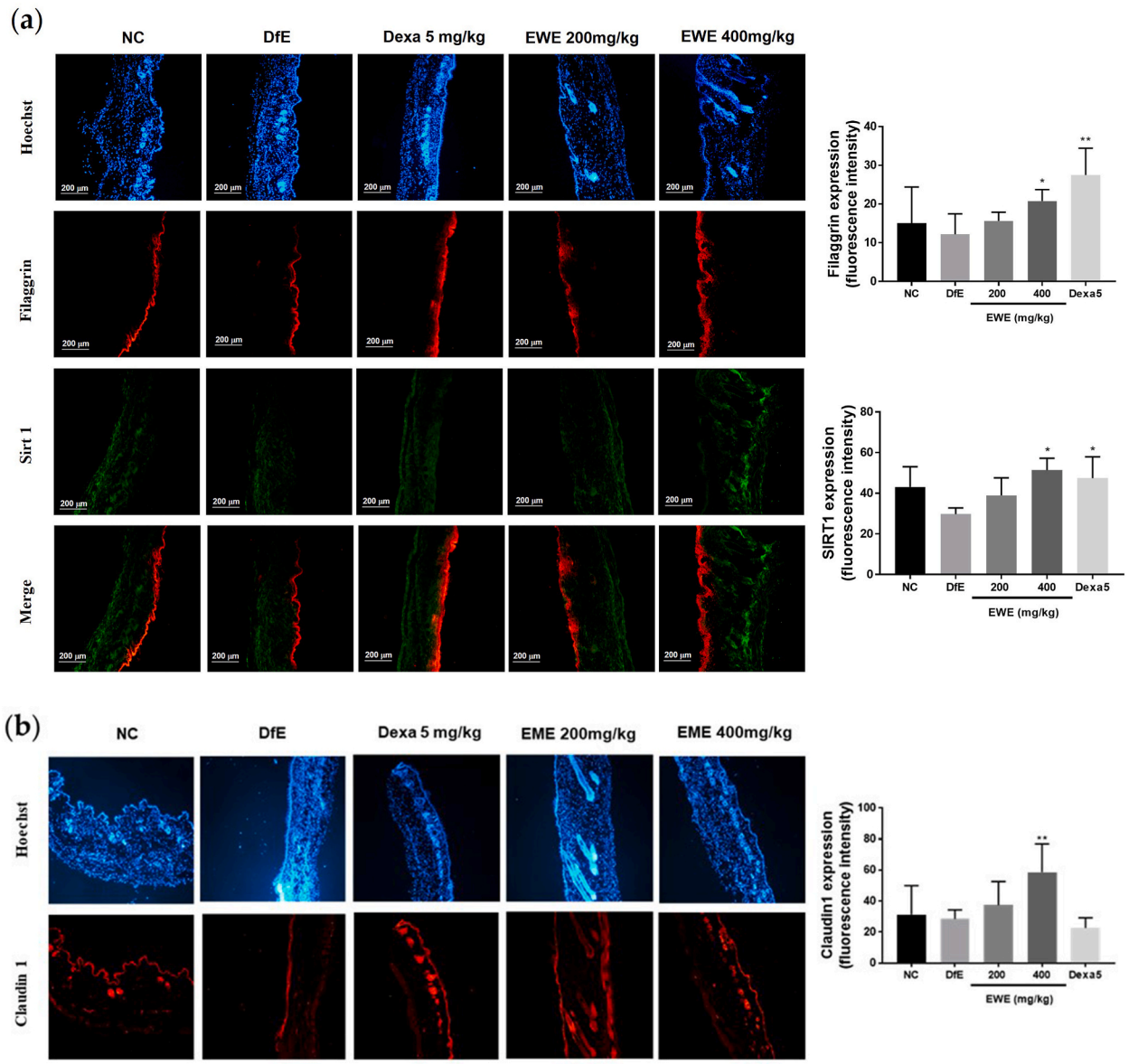

Fig. 6. Immunohistofluorescence analysis of filaggrin, SIRT1, and claudin 1 in the dorsal skin of NC/Nga mice. Densitometry findings on immunohistofluorescence of Hoechst (blue) with (a) filaggrin (red) and SIRT1 (green) and (b) claudin1 (red) were quantified using the Image J program. Results expressed as mean ± SEM (n = 6). #$p < 0.05$ compared with normal control (NC); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with D. farinae extract (DfE)-treated mice. EWE, earthworm (*P. communisima*) extract.

concentrations have been associated directly with AD severity, so AD inflammation contributes to the increased IgE [17]. Consistent with our research, previous studies have shown that earthworm (*Pheretima aspergillum)* treatment in the allergic asthma mice reduced the mRNA and protein levels of IL-13, IL-5, and IL-4 in the lung tissue and downregulated serum IgE [11]. AD is generally regarded to be a Th2-dominant allergic disease. T helper cells in early AD lesions produce IL-13, IL-4, and IL-5, the key cytokines of Th2 cells, and the accumulation of these Th2 cytokines in the skin accelerates skin inflammation with IgE production from B cells [18]. Mast cells and eosinophils cause skin inflammation via Th2 cytokines, and the secretion of histamine from mast cells initiates itchiness that leads to additional damage to the skin barrier [19]. Whereas the acute point of AD is marked by an infiltrate consisting of Th2 and Th22 cells, the chronic point of AD mainly involves skin hypertrophy (induction of epidermal hyperplasia) caused by the secretion of the inflammatory cytokine IFN-γ by Th1 cells [20,21].

EWE decreased TSLP expression and eosinophil numbers in the blood with decreased Th2 cytokines in dermal skin. TSLP is a main regulatory cytokine that activates mast cells, eosinophils and dendritic cells for the onset and development of chronic Th2 inflammatory response in AD and contribute to skin barrier dysfunction [21,22]. In our study, EWE decreased the mast cells number in the

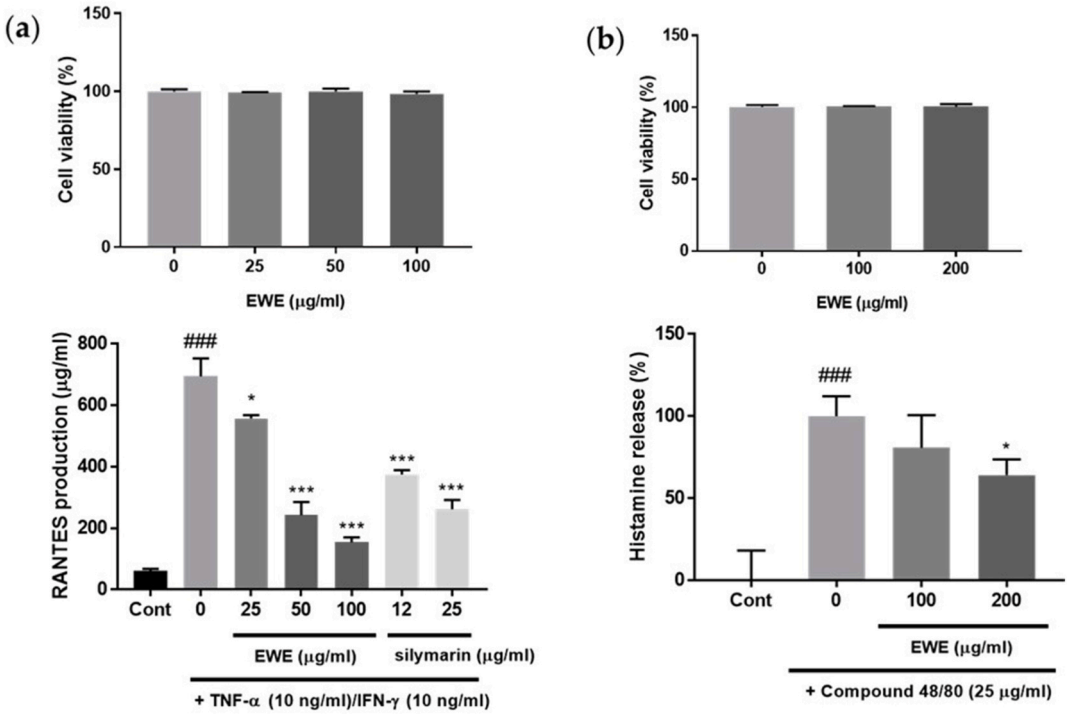

Fig. 7. Effects of earthworm (*P. communisima*) extract (EWE) on chemokine release from HaCaT keratinocytes and histamine release from activated MC/9 mast cells. (a) Cell viability and chemokine (RANTES) production in IFN-γ/TNF-α–cotreated HaCaT cells. (b) Cell viability and histamine release in compound 48/80-stimulated MC/9 cells. Values presented as mean ± SEM (triplicate). ###$p < 0.001$ compared with untreated control; *$p < 0.05$ and ***$p < 0.001$ compared with TNF-α/TNF-γ–stimulated or compound 48/80-stimulated AD control.

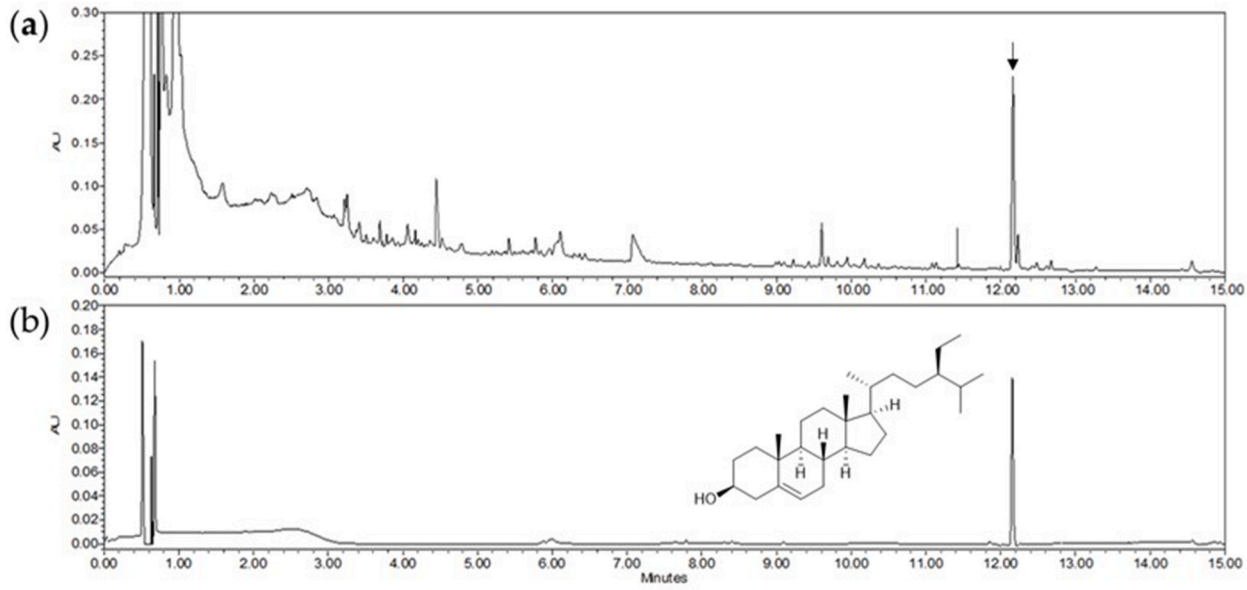

Fig. 8. UPLC-PDA chromatogram of EWE (a) and β-sitosterol (BS) of standard compound (b). (Inset) Chemical structure of BS. The BS concentration was 1 mg/mL.

dermal skin of AD mouse as well as the histamine secretion from activated mast cells. These results suggest that EWE ameliorates the AD clinical signs in mice, that could possibly be derived from downregulation of TSLP.

We found that EWE treatment resulted in increased claudin1 expression in dermal skin and reduced serum IgE and peripheral blood

eosinophilia in AD mice. The skin has two epidermal barrier structures: the stratum corneum and the tight junction via the paracellular pathway. Tight junctions provide a gateway for the transport of water, ions, and solutes and are composed of transmembrane proteins such as junctional adhesion molecules, claudin family proteins, occludin, and zonulae occludens scaffolding proteins [23]. Tight junction is important for skin barrier function. Decreased claudin-1 expression has been reported to contribute to skin barrier dysfunction in nonlesional skin of AD patients [24]. Bebedetto et al. reported that decreased expression of the tight junction transmembrane protein claudin1 in skin lesions of patients with AD was inversely correlated with biomarkers of Th2 polarization such as circulating total IgE and peripheral blood eosinophilia, suggesting that reduction of claudin1 protein expression may affect features of the inflammation and immune response to external allergens in the development of AD [25]. These results suggest that EWE improves skin barrier function and decreases inflammation in AD by increasing expression of the tight junction protein claudin-1.

EWE caused increased filaggrin expression and decreased Th2 cytokine (IL-4, IL-5, and IL-13) expression in dermal skin with inhibition of RANTES production in keratinocytes. Filaggrin, as a major epidermal protein supporting the structure and function of the stratum corneum, is a critical genetic risk factor for the pathogenesis of AD [26]. Loss of filaggrin function is a major factor in allergic rhinitis, asthma, and AD, and AD in patients with filaggrin mutation has more severe symptoms than AD in patients without filaggrin mutation [27,28]. AD skin lesions in patients without filaggrin mutation showed lower filaggrin expression and less overexpression of the Th2 cytokines IL-13 and IL-4 compared with normal skin lesions [29]. It was confirmed that abnormality of the skin barrier induces the Th2-mediated inflammatory response with infiltration of eosinophils and increased thymus activation, which in turn regulates chemokine (TARC) and RANTES expression [30]. It was also reported that expression of the skin barrier proteins filaggrin, involucrin, and loricrin is downregulated by Th2 cytokines in differentiated skin keratinocytes [29,31]. Those results together with our findings suggest that EWE ameliorates the inflammatory response by increasing filaggrin expression and thus improving skin barrier dysfunction.

SIRT1 is a NAD-dependent protein deacetylase that is critical for metabolism, survival, senescence, and stress response in various cells and organisms [32]. SIRT1 prevents proliferation and promotes differentiation in normal keratinocytes [33]. In addition, SIRT1 deletion in mouse skin and normal keratinocyte cells down-regulates filaggrin expression, and mice with epidermal SIRT1 deletion were sensitive to percutaneous allergen-induced response [34]. Those findings suggest that SIRT1 may play a role in skin barrier maintenance and the development of AD. SIRT1 may therefore be a mechanism-based therapeutic target for AD prevention. Our immunohistofluorescence results show that EWE increased SIRT1 expression in AD lesions and effectively relieved AD symptoms, suggesting that EWE ameliorates AD by reversing skin barrier dysfunction and suppressing the inflammatory immune response.

We did not attempt to identify the bioactive chemical constituents of EWE because of its complex components. Other studies showed that purine, proteins, peptides, fatty acids, amino acids, minerals, microelements, enzymes, vitamins, choline, cholesterin, terrestrolum brolysin, lumbrofebrin, lumbrifebrine, lumbricusin, lumbritin, and other compounds are present in earthworms [35,36]. Choline supplement attenuated immune inflammation by reducing inflammatory cytokines (IL-5, IL-4, and TNF-α) and blood levels of eosinophils and total IgE and suppressed oxidative stress in asthma patients [37]. Lumbricusin was informed to show anti-inflammatory properties by reducing cytokines in the activated microglia neural cells [38]. Phytochemical profiling by liquid chromatography-quadrupole time of flight (LC-QTOF) mass spectrometry analysis identified amino acids and twenty oligopeptides in EWE as active components that contributed to anti-asthmatic effects [39]. These findings on inflammatory diseases suggest that these compounds may contribute to the anti-inflammatory properties of earthworms from AD inflammatory disease. Our study showed that β-sitosterol is a main component of EWE. The compound β-sitosterol inhibited the allergic inflammation in a rat asthma model [40]. Also, the anti-inflammatory effect of β-sitosterol on AD mouse has been reported. The β-sitosterol ameliorated effectively the AD symptom through down-regulation of TSLP [41]. This indicates that β-sitosterol may play an important role as a bioactive compound of EWE. However, further research on other compounds is also needed.

Plant derived-natural compounds are can affect different molecular signaling pathways in various inflammatory diseases. Curcumin reduced the expression of transforming growth factor-beta (TGF-β) as a pro-fibrotic cytokine to decline airway inflammation in allergic asthma disease [42]. Apigenin, a member of the flavonoid family, reduced inflammation and oxidative stress by downregulating NF-κB pathway in animal models of lung injury [43]. Among flavonoids, naringenin inhibited inflammatory mediators (TNF-α IL-6, and NF-κB), and oxidative stress markers (CAT, SOD, and MDA) in autoimmune disorder model [44]. Resveratrol ameliorated fibrosis by reducing inflammation and suppressed cancer cell proliferation and migration via promoting downregulation of TGF-β [45]. Previous study showed that EWE improved skin would healing via downregulating IL-6 and TGF-β in mice [46]. Therefore, to find the detailed mechanism of action of EWE and its ingredients for improving AD, it is necessary to study the modulation of various signaling mechanisms such as oxidation, NF-κB, and TGF-β.

However, because of the complication of the biochemical composition of earthworms, the bioactive compounds that are responsible for the anti-allergic and anti-inflammatory properties of EWE are still largely indefinite and need to be explored in further studies. Despite the limitations of this study, our findings show that EWE could be a powerful therapeutic to prevent or treat AD by improving the skin inflammation caused by house dust mite antigen. Based on our current results, human clinical trials on EWE will be conducted in the future for the development of AD treatments or human health foods.

5. Conclusions

EWE suppressed AD responses in NC/Nga mouse and in keratinocytes and mast cells. EWE improved skin barrier function by restoring normal epidermal thickness and decreasing penetration of inflammatory cells in NC/Nga mice with mite antigen-treated AD. Moreover, EWE inhibited TNF-α/IFN-γ–induced chemokine production in keratinocytes and compound 48/80-induced histamine secretion in mast cells. EWE ameliorates AD by improving skin barrier abnormality and immune regulatory dysfunction. These findings

suggest that EWE may be a promising candidate for the prevention or treatment of AD. Further investigations are needed to find the active compounds and to clarify the exact mechanisms of action of the anti-inflammatory effects.

CRediT authorship contribution statement

Yoon-Young Sung: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis. **Heung-Joo Yuk:** Validation. **Seung-Hyung Kim:** Investigation. **Dong-Seon Kim:** Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability statement

The data presented in this study are available in the article.

Ethics approval and consent to participate

This study was approved by the Animal Care and Use Committee of Daejeon University (Ethical approval code. DJUARB2019-042) and conducted according to the Guide for Care and Use of Laboratory Animals and the guidelines regarding animal care and use (National Research Council of USA, 1996).

Consent for publication

Not applicable.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dong-Seon Kim reports financial support was provided by KIOM. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

AD	Atopic dermatitis
BS	β-sitosterol
DfE	*Dermatophagoides farinae* extract
EWE	Earthworm extract
NF-κB	Nuclear factor-κB
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
SIRT 1	Sirtuin 1
TARC	Thymus and activation-regulated chemokine
TSLP	Thymic stromal lymphopoietin
UPLC-PDA	Ultra-high-performance liquid chromatography coupled with ultraviolet/photodiode array

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