

Alpinia officinarum water extract inhibits the atopic dermatitis-like responses in NC/Nga mice by regulation of inflammatory chemokine production

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ABSTRACT

Alpinia officinarum (AO) has been traditionally used in Asia as an herbal medicine to treat inflammatory and internal diseases. However, the therapeutic effect of AO on atopic dermatitis (AD) is unclear. Therefore, we examined whether *Alpinia officinarum* water extract (AOWex) affects AD in vivo and in vitro. Oral administration of AOWex to NC/Nga mice with *Dermatophagoies farina* extract (DfE)-induced AD-like symptoms significantly reduced the severity of clinical dermatitis, epidermal thickness, and mast cell infiltration into the skin and ear tissue. Decreased total serum IgE, macrophage-derived chemokine (MDC), and regulated on activation, normal T-cell expressed and secreted (RANTES) levels were observed in DfE-induced NC/Nga mice in the AOWex-treated group. These effects were confirmed in vitro using HaCaT cells. Treatment with AOWex inhibited the expression of proinflammatory chemokines such as MDC, RANTES, IP-10 and I-TAC in interferon- γ and tumor necrosis factor- α -stimulated HaCaT cells. The anti-inflammatory effects of AOWex were due to its inhibitory action on MAPK phosphorylation (ERK and JNK), NF- κ B, and STAT1. Furthermore, galangin, protocatechuic acid, and epicatechin from AOWex were identified as candidate anti-AD compounds. These results suggest that AOWex exerts therapeutic effects against AD by alleviating AD-like skin lesions, suppressing inflammatory mediators, and inhibiting major signaling molecules.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is characterized by various clinical phenotypes resulting from complex interactions between several genetic and environmental factors [1,2]. Recently, the rapid increase in the incidence of AD has become a public health problem worldwide [3]. AD has a variety of causes, and several studies have shown that it is related to allergic inflammation of keratinocytes and disturbance of skin barriers [4]. AD is associated with several other allergic diseases. In particular, AD occurs in the early stages of atopic march, which occurs in infancy, followed by allergic rhinitis and asthma [5,6].

NC/Nga mice are well-known animal models for AD, as the clinical symptoms of AD in mice are similar to those in humans [7]. In AD patients, the house dust mite allergen is a major cause of irritation; the *Dermatophagoies farina* mite allergen is the most prevalent in house dust

[8]. It is known to be generally associated with human allergic rhinitis, asthma, and AD [9]. Repeated exposure to *Dermatophagoies farina* extract (DfE) causes AD-like skin lesions in NC/Nga mice under specific pathogen-free conditions [10].

In the epidermal skin region, keratinocytes are a major cell type that plays an important role in the progression of various skin diseases. Inflammatory reactions of keratinocytes occur following the release of proinflammatory mediators such as cytokines and chemokines, which are caused by immunogenic substances including chemical agents, ultraviolet light, and various allergens [11]. Interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α)-stimulated keratinocytes have been reported to produce proinflammatory chemokines, including macrophage-derived chemokine (MDC), regulated on activated normal T-cell expressed and secreted (RANTES), interferon-induced protein of 10 kDa (IP-10), and interferon-induced T cell α -chemoattractant (I-TAC) [12–15]. It was reported that IP-10 and I-TAC are lymphocyte-attracting

Abbreviations: AD, Atopic dermatitis; AO, *Alpinia officinarum*; AOWex, *Alpinia officinarum* water extract; DfE, *Dermatophagoies farina* extract.

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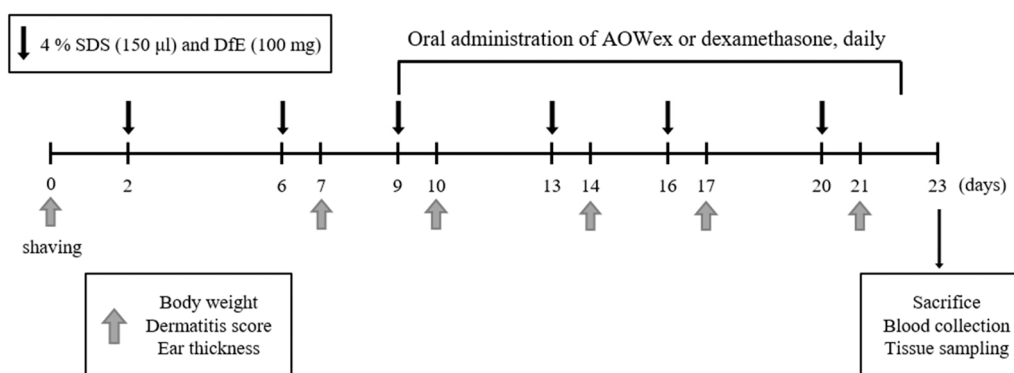


Fig. 1. Experimental design for induction of atopic dermatitis (AD) and *Alpinia officinarum* water extract (AOWex) or dexamethasone (Dexa) administration in mice.

chemokines in vivo and in vitro [16–18]. Furthermore, T helper type 2 (Th2)-related chemokines, such as MDC and RANTES, lead to the migration of Th2 cells into the inflamed skin region [19]. Therefore, the regulation of proinflammatory chemokine production in keratinocytes may be vital to AD treatment.

Alpinia officinarum (AO) is a traditional herbal plant used in Asia to treat several ailments, including pain such as stomachache, inflammation, and cold [20]. According to several pharmacological studies, AO rhizome exerts anti-oxidant, anti-viral, anti-bacterial, anti-fungal, anti-inflammatory, anti-allergic, anti-cancer, and neuroprotective effects [21]. In addition, galangin, a major flavonol in the AO rhizome, is also known to inhibit osteoclastogenesis and proinflammatory cytokine production by reducing NF- κ B levels and the MAPK signaling pathway in collagen-induced arthritis and bone marrow-derived macrophages [22]. However, the inhibitory effect of AO on AD has not yet been investigated. Therefore, in the present study, we investigated the anti-inflammatory activity and the anti-AD effects of AO water extract (AOWex) on an NC/Nga mouse model with DfE-induced AD-like symptoms and IFN- γ /TNF- α -stimulated HaCaT cells.

2. Materials and methods

2.1. Preparation of *Alpinia officinarum* water extract (AOWex)

AOWex was purchased from KOCBiotech (Daejeon, Korea) and stored in the herbarium of the Herbal Medicine Research Division. Dried *Alpinia officinarum* (1 kg) was extracted in 10 L of distilled water under circumfluence for 3 h at 100 ± 2 °C and lyophilized after filtration (53 μ m mesh). Dried AOWex powder was stored at -20 °C until further use.

2.2. Animals and induction of AD

The male NC/Nga mice (8 weeks old) were obtained from Central Lab Animal Inc. (Seoul, Korea). The mice were housed under specific pathogen-free and environmentally controlled conditions (6 mice/cage). The mice were given access to a standard laboratory diet (Purina 38057, Cargill Agri Purina Inc., Sunnam, Korea) and tap water ad libitum and provided with corncob natural bedding material (BioCOB 10384197, BioSystems Corporation Pte Ltd, Peninsula Plaza, Singapore). After acclimatization for 7 d, the mice were randomly divided into different experimental groups. All animal experiments were conducted under approval (No. 20-069) of the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine (KIOM). The shaved dorsal skin and ear barrier of NC/Nga mice were disrupted by 150 μ l of 4% sodium dodecyl sulfate (SDS), and after 3 h, 100 mg of *Dermatophagoies farina* extract (DfE; Biostir Inc., Kobe, Japan) was applied. Mice were treated with DfE twice per week for a total of 3 weeks (Fig. 1). Seven days after the final DfE application, AOWex was dissolved in water, and each dose of AOWex or dexamethasone (Dexa; positive

control) was orally administered daily for 14 d. The NC/Nga mice were randomly divided into six groups: normal (untreated), AD (DfE only), AD-AOWex (30, 100, and 300 mg/kg), and AD-Dexa.

2.3. Measurement of ear thickness and AD severity

Ear thickness was evaluated twice a week using a digital caliper (CAS $\circ$, Seoul, Korea). The dermatitis score was indicated based on four symptoms, namely edema, scarring/dryness, erythema/hemorrhage and excoriation/erosion in dorsal skin and ear lesions. The individual scores were given as 0 (no symptoms), 1 (mild), 2 (moderate) or 3 (severe) for each symptom category. All dermatitis of clinical severity scores was defined as the sum of the individual scores [23].

2.4. Measurement of serum IgE, MDC and RANTES

The mouse blood samples were collected on the 23rd day, and the separated serum was stored at -80 °C until use. The total level of immunoglobulin E (IgE) was determined using a LBIS Mouse IgE ELISA Kit (Fujifilm, Shibukawa, Japan). The serum levels of total MDC and RANTES were measured using a Mouse CCL22/MDC Quantikine ELISA Kit and Mouse/Rat CCL5/RANTES Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). These sera were analyzed in accordance with the manufacturer's instructions.

2.5. Histopathological analysis

Ear and skin tissue samples of NC/Nga mice were removed, fixed with 10% formalin, embedded in paraffin and serially sectioned into 4–6 μ m sections using a Leica RM2135 microtome (Leica Biosystems, Newcastle, UK). Tissue sections were stained with hematoxylin/eosin solution (Sigma-Aldrich, St. Louis, MO, USA) or Toluidine Blue Stain Kit (VitroVivo Biotech, Rockville, MA, USA). After mounting, the tissue samples were observed using a slide scanner (VM1, Motic, Beijing, China). The stained tissue samples were measured from the tissue section analyzed with the Solution for Automatic Bio-Image Analysis (SABIA) software (EBIOGEN, Seoul, Korea).

2.6. Cells and reagents

HaCaT cells, an immortalized human keratinocyte cell line, were obtained from Elabscience (Houston, TX, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1% penicillin/streptomycin and heat-inactivated 10% fetal bovine serum (FBS). Cells were incubated in a 5% CO $_2$ incubator at 37 °C. Interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). High-glucose-containing DMEM was purchased from Hyclone (Logan, UT, USA). Phosphate-buffered saline (PBS), FBS and penicillin/streptomycin were obtained from Gibco-BRL

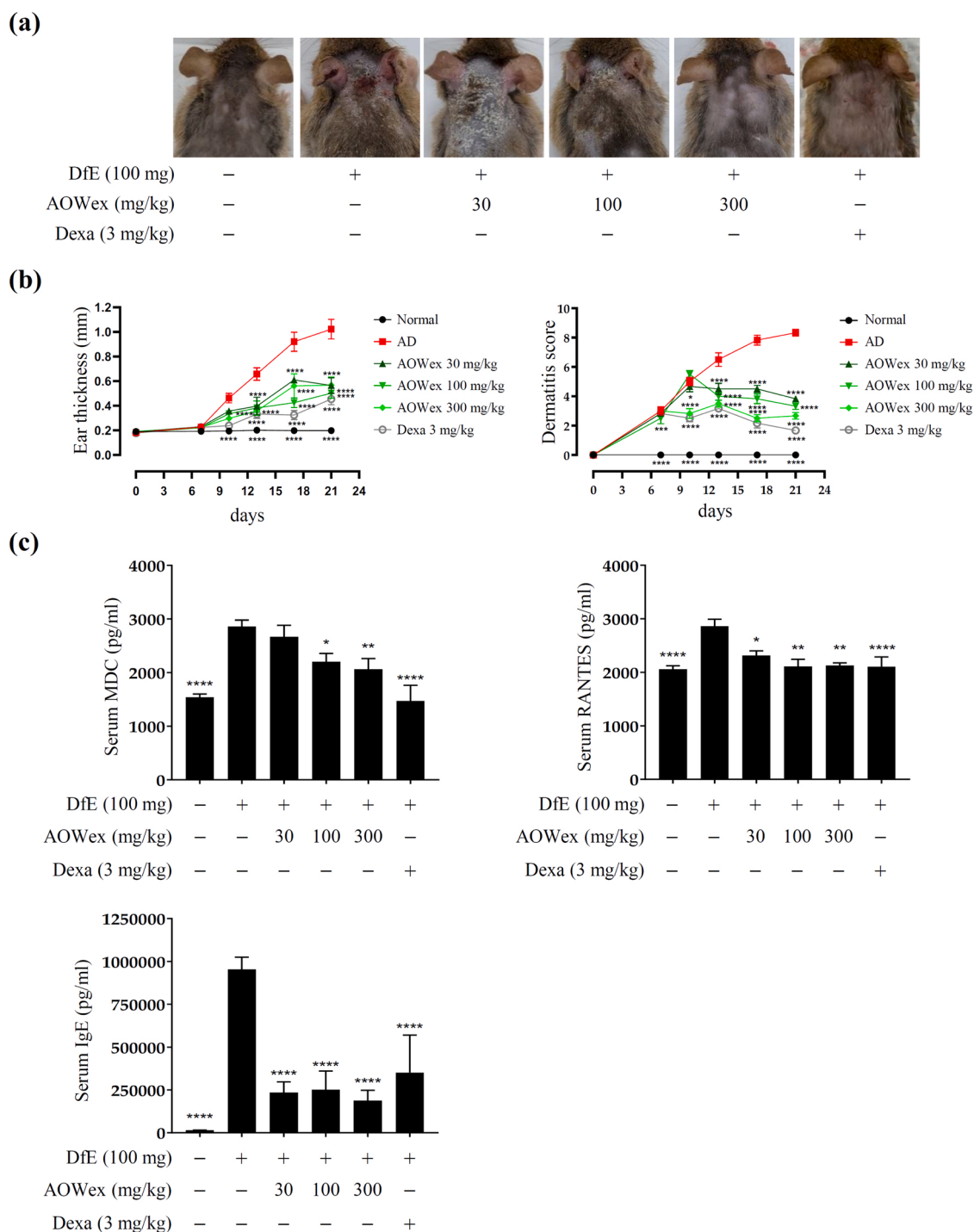


Fig. 2. Effects of AOWex on DfE-induced AD-like skin lesions in NC/Nga mice. (a) Photographs of the back of mice from each group on the 23rd day. (b) The ear thickness and dermatitis score were measured twice a week for 3 weeks. (c) Serum MDC, RANTES and IgE levels were measured by ELISA. Values are represented as the means $\pm$ SEM ($n = 6$). * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ vs. DfE-treated group.

(Gaithersburg, MD, USA). p-STAT1, STAT1, p-p38, p38, p-ERK, ERK, p-JNK, JNK, p65 and β -actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). PCNA, p50 and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dimethyl sulfoxide (DMSO), SDS and Dexa were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.7. Cell viability assay

To confirm the influence of AOWex on HaCaT, cell viability was

determined through the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Cells were seeded in each well of a 96-well plate. AOWex was placed in each well with various concentrations for 24 h at 37 °C in a 5% CO₂ cell incubator. The pre-warmed 20 μ l of assay reagent was added to each well, and the light-blocked plate was incubated in a humidified 5% CO₂ atmosphere chamber at 37 °C for 1 h. Optical density (OD) of response mixtures was detected on a Synergy HTX Multi-Mode Reader (BioTek, Winooski, VT, USA) at 490 nm.

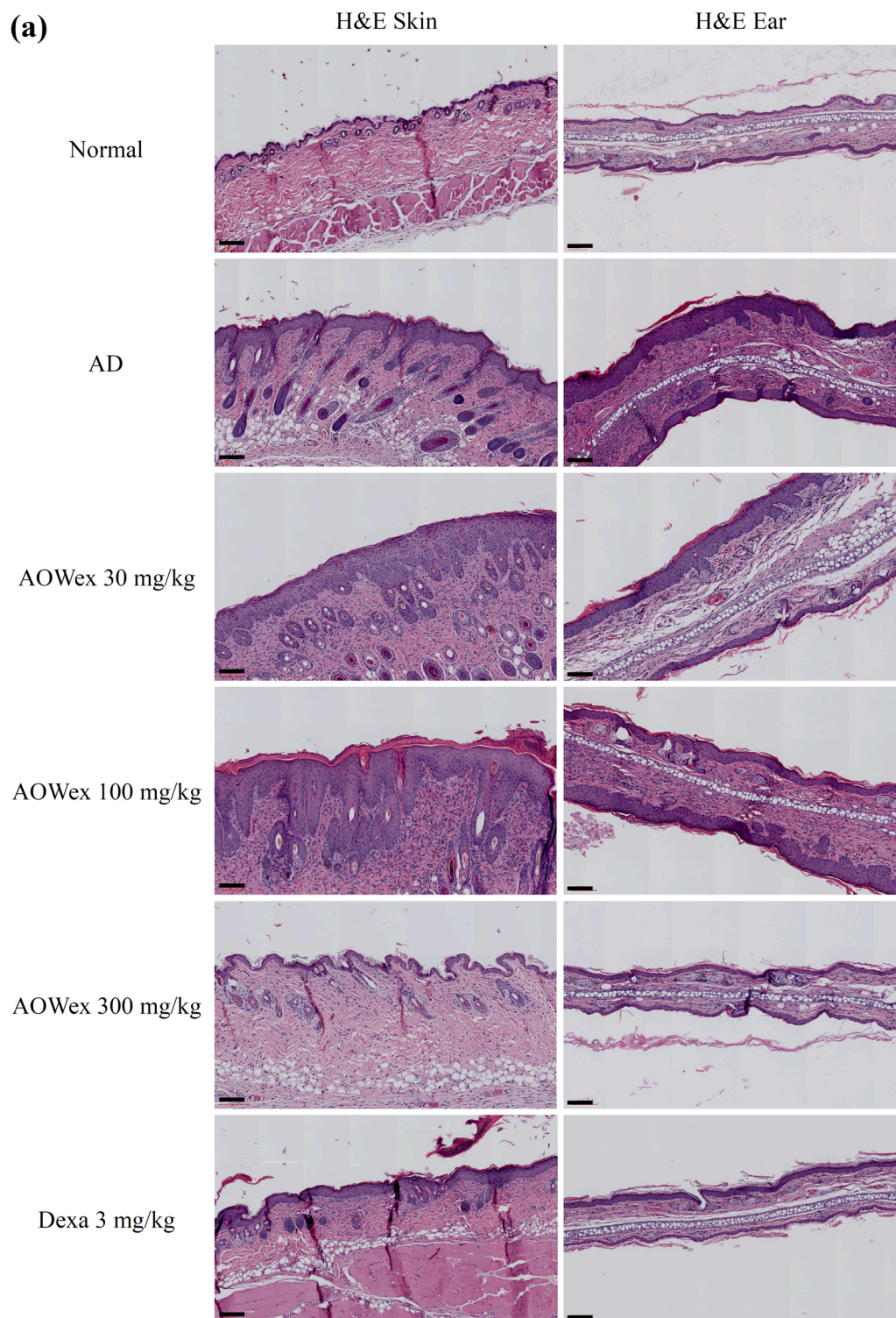


Fig. 3. Effects of AOWex on the histopathological features of the ear and skin tissue of NC/Nga mice with DfE-induced AD-like symptoms. (a) Skin and ear tissue sections were stained with hematoxylin and eosin (H&E) or (b) toluidine blue (TB). The stained sections were observed under a microscope at 100 × (a) and 400 × (b) magnification. Scale bar = 100 μm. (c) The skin epidermal thickness and (d) number of mast cells were analyzed. Values are represented as the means ± SEM (n = 5). *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 vs. DfE-treated AD group.

2.8. Detection of proinflammatory chemokines

Cells were pre-incubated with AOWex for 1 h and then treated with 10 ng/mL of IFN-γ/TNF-α for 24 h at 37 °C. To remove the particulates, cell culture medium was centrifuged at 4000 rpm for 5 min. The levels

of secreted MDC protein in HaCaT cells were detected using Human CCL22/MDC Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA). The released MDC levels in cell culture supernatants were tested according to the manufacturer's protocol. The levels of released RANTES, IP-10 and I-TAC were evaluated using the LEGENDplex™

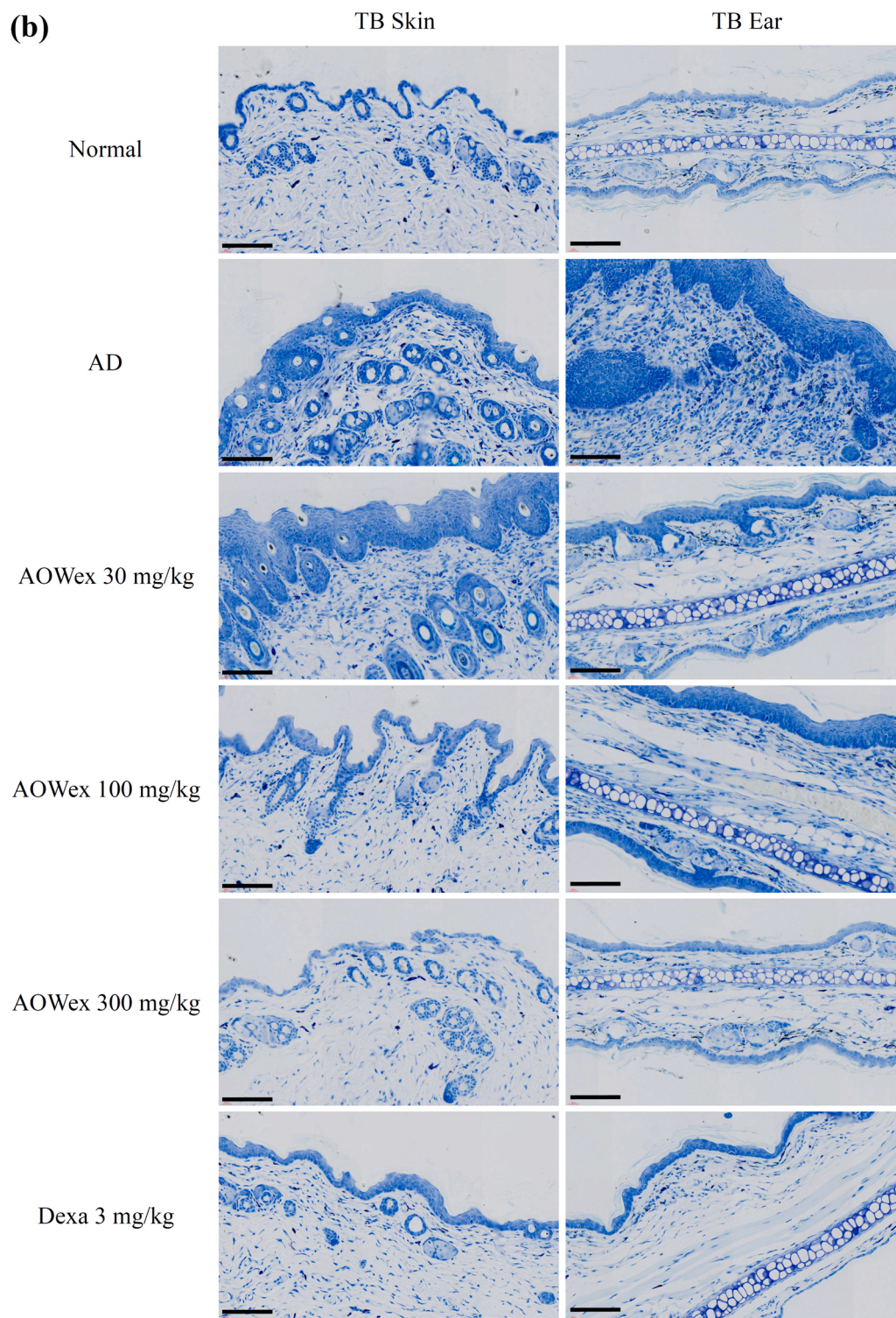


Fig. 3. (continued).

Proinflammatory Chemokine panel (BioLegend, San Diego, CA, USA) bead-based immunoassay according to the manufacturer's instructions. Prepared bead samples were analyzed with a BD LSRFortessa™ Flow Cytometer (BD Biosciences, San Jose, CA, USA) using the BD CellQuest™ software. Data were analyzed using a LEGENDplex™ Software v8.0 (VigeneTech Inc., Carlisle, MA, USA).

2.9. Western blot analysis

HaCaT cells were pre-incubated with AOWex for 1 h and treated with 10 ng/mL IFN- γ and 10 ng/mL TNF- α at 37 °C. The cells were lysed with cold radioimmunoprecipitation assay buffer (Biosesang, Seongnam, Korea) or NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) containing Halt™ Protease

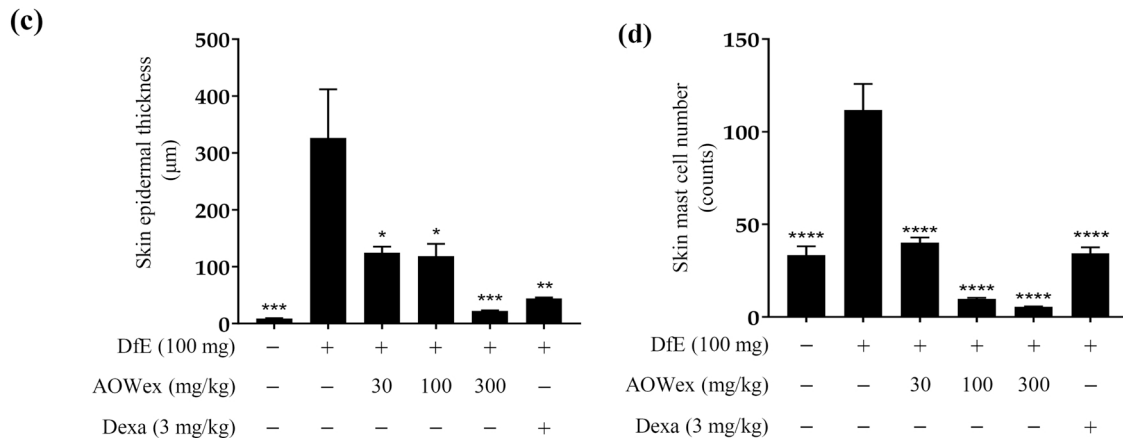


Fig. 3. (continued).

and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Equal amounts of extracted proteins (20 μg) were separated by electrophoresis on 4–15% Mini-PROTEAN TGX Precast Protein Gels (Bio-Rad, Hercules, CA, USA) and then electrophoretically transferred onto Fluoro Trans® PVDF Membrane (Pall Corporation, Dreieich, Germany). Membranes were blocked with 3% bovine serum albumin (MP Biomedicals, Irvine, CA, USA) or 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 2 h. Then, primary antibodies (1:1000 dilution) in blocking solutions were placed overnight with the membrane at 4 °C, followed by incubation with secondary antibodies (horseradish peroxidase-conjugated anti-IgG) for 1 h. Specific proteins were detected using Super Signal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). A ChemiDoc Imaging System (Bio-Rad) was used for detection of protein expression.

2.10. High-performance liquid chromatography analysis

The high-performance liquid chromatography (HPLC) analysis of the three compounds in AOWex was performed with a Waters e2695 liquid chromatography system (Waters Co., Milford, MA, USA), equipped with a Waters 2998 photodiode array detector. The chromatographic separation was carried out using the Phenomenex Luna C18 column (250 mm × 4.6 mm; particle size 5 μm; Phenomenex, Torrance, CA, USA) and detected at 270 nm. The mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B). The gradient conditions were as follows: 0–12 min, 10–40% B; 12–14 min, 40–50% B; 14–16 min, 50–60% B; 16–30 min, 60% B. A wash with acetonitrile was applied for 8 min, followed by equilibration at 90% A and 10% B for 8 min. The flow rate was 1.0 mL/min and the injection volume was 20 μL. For the quantitative analysis of the three compounds, the AOWex was dissolved in methanol (10 mg/mL) by sonication for 10 min and filtered through a syringe filter (0.2 μm). The three reference compounds used in the analysis were purchased from Biopurify Phytochemicals (Chengdu, China), and were ≥ 98% pure.

2.11. Statistical analysis

Data were analyzed using the GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). The data are represented as means ± SEM and calculated using the analysis of variance (ordinary one-way ANOVA, to compare control group, DfE-treated group and IFN-γ/TNF-α). A p-value of < 0.05 was considered to indicate statistical significance. All experiments were performed at least in triplicate.

3. Results and discussion

3.1. Effects of AOWex on DfE-induced AD-like symptoms in NC/Nga mouse skin and serum proinflammatory mediator levels

To investigate the anti-atopic effects of AOWex, we used NC/Nga mice with DfE-induced AD-like symptoms [10]. DfE is one of the main dust mite allergens that lead to allergic diseases [24]. For these reasons, we administered DfE twice a week for 3 weeks to the dorsal skin of NC/Nga mice to determine the effects of AOWex on atopic skin inflammation. Repeated DfE stimulation first led to skin drying in the mice with induced AD, followed by severe scarring, erythema, hemorrhage, excoriation and erosion [25]. Additionally, ear thickness continued to increase in NC/Nga mice after DfE application. However, oral administration of AOWex or Dexa (positive control) significantly reduced these skin symptoms (Fig. 2a, b).

Production of proinflammatory chemokines, such as MDC and RANTES, is induced following mechanical tissue damage [26]. In the early stages of AD, the imbalance between Th1 and Th2 plays an important role in the progression of AD [27]. MDC is one of the ligands of the C-C chemokine receptor type (CCR4), and it is a predominant chemokine receptor in Th2 cells [10]. The increased levels of RANTES in AD patients is related to the migration of mast cells [28]. In addition, reports showed that serum IgE levels were increased in NC/Nga mice with DfE-induced AD-like symptoms [2,7]. IgE secretion levels are closely associated with Th2 allergic inflammation, and through binding to mast cells IgE secretion lead to the production of various inflammatory mediators [29,30]. Thus, inhibition of MDC, RANTES and IgE secretion can lead to reduced inflammatory responses. To evaluate if these AD-related makers were elevated in DfE-treated NC/Nga mice and to confirm the effects of AOWex, blood samples were collected on the 23rd day after treatment. The levels of serum MDC, RANTES and IgE were markedly increased following DfE application compared with those in the non-induced group. Oral administration of AOWex or Dexa significantly reduced the serum levels of MDC, RANTES and IgE. Our data show that AOWex exerts anti-AD effects in NC/Nga mice with DfE-induced AD-like symptoms.

3.2. Effects of AOWex on DfE-induced skin lesions and mast cell infiltration in NC/Nga mice with AD-like symptoms

DfE application leads to several AD symptoms, including epidermal and dermal thickening and inflammatory cell infiltration, in inflamed skin lesions in mice with AD-like symptoms [31]. A key characteristic of AD is the infiltration of mast cells into inflamed skin by inducing proinflammatory mediators such as chemokines and IgE [32,33]. In our

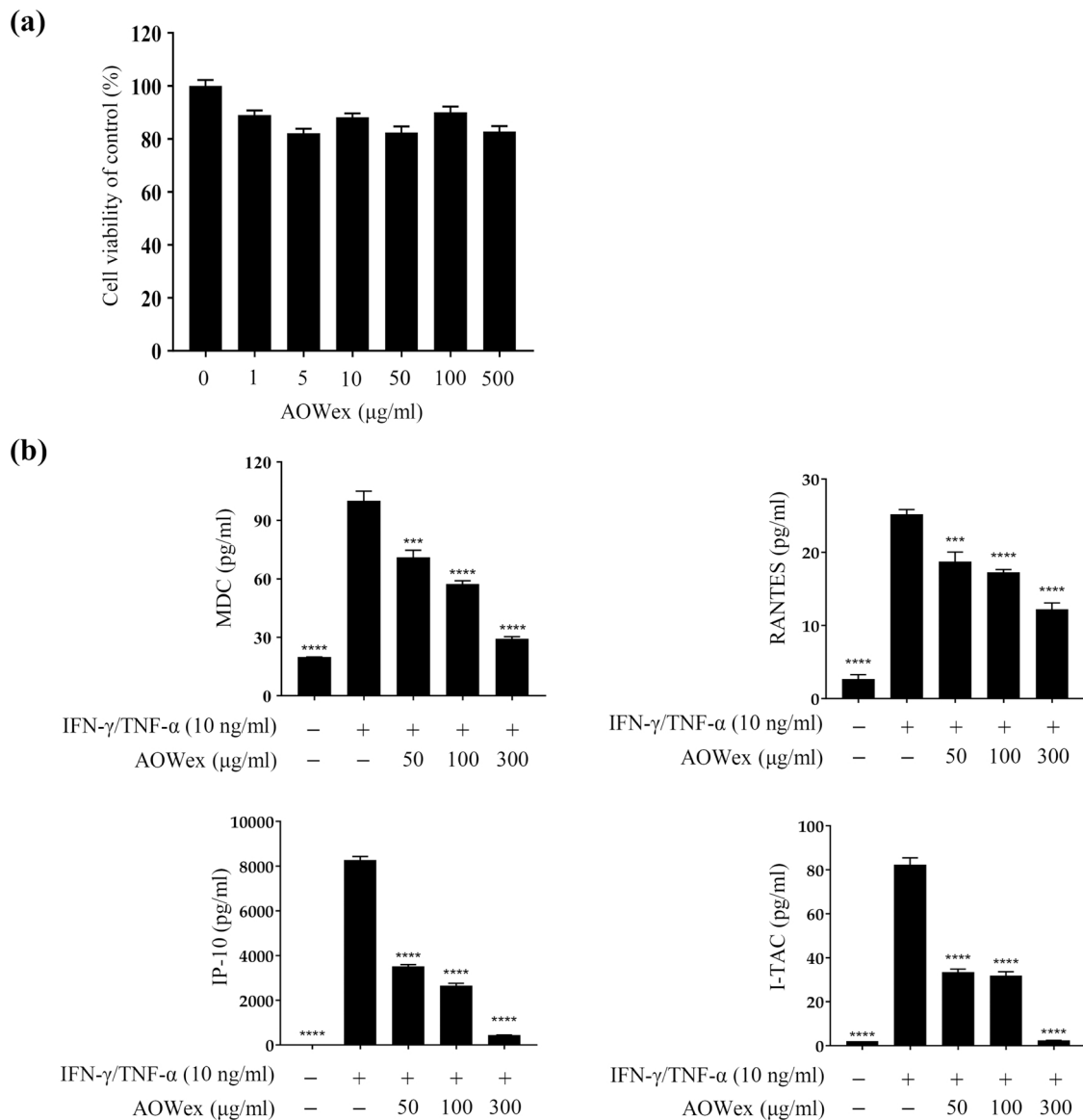


Fig. 4. Effects of AOWex on the secretion of proinflammatory chemokines in IFN- γ /TNF- α -induced HaCaT cells. (a) Effects of AOWex on the viability of HaCaT cells were determined after treatment with AOWex for 24 h. (b) Secreted MDC was detected using ELISA. RANTES, IP-10 and I-TAC levels were measured via a bead-based immunoassay. Cells were pretreated with AOWex for 1 h and stimulated with IFN- γ /TNF- α for 24 h. Data shown as the means $\pm$ SEM of three separate experiments. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ vs. IFN- γ /TNF- α .

histological observation of the skin and ear lesions in NC/Nga mice with AD-like symptoms, the sample tissues were stained with hematoxylin and eosin. The NC/Nga mice with DfE-induced AD exhibited an increase in epidermal and dermal thickness. However, the application of AOWex inhibited these histological changes in both the dorsal skin and ear lesions (Fig. 3a, c). In addition, the skin and ear sections were stained with toluidine blue (TB) to confirm DfE-induced mast cell infiltration. While the number of mast cells in the DfE-induced AD group significantly increased compared with that in normal mice, the administration of AOWex attenuated these increases (Fig. 3b, d). These results indicated that AOWex administration may improve the histological features of AD-like skin lesions and mast cell infiltration in DfE-induced NC/Nga mouse skin lesions.

3.3. Effects of AOWex on IFN- γ /TNF- α -induced proinflammatory chemokine production in HaCaT cells

Activated keratinocytes aggravate the pathogenesis of AD by

secreting proinflammatory mediators such as chemokines [34]. Synergistic effects of IFN- γ /TNF- α , which induced chemokine production in keratinocytes, have been used in the study of inflammatory skin diseases [2,12]. Proinflammatory mediators such as MDC, RANTES, IP-10 and I-TAC play a central role in the progression of inflammation resulting from monocyte activation. These common mediators activate and recruit immune cells, including Th2 lymphocytes in chronic inflammatory sites [5,35–39]. Therefore, we examined the inhibitory effects of AOWex on these induced proinflammatory chemokines in HaCaT cells stimulated by IFN- γ /TNF- α .

First, to confirm the cytotoxicity of AOWex on HaCaT cells, the cells were incubated with AOWex for 24 h at 37 °C. AOWex treatment concentrations ranging from 0 μ g/mL to 500 μ g/mL did not produce any significant cytotoxic effects (Fig. 4a). All subsequent experiments were conducted at nontoxic concentrations. To investigate the effects of AOWex on the production of proinflammatory chemokines in AD keratinocytes, the cells were stimulated with IFN- γ and TNF- α (each 10 ng/mL). As shown in Fig. 4b, IFN- γ /TNF- α stimulation significantly

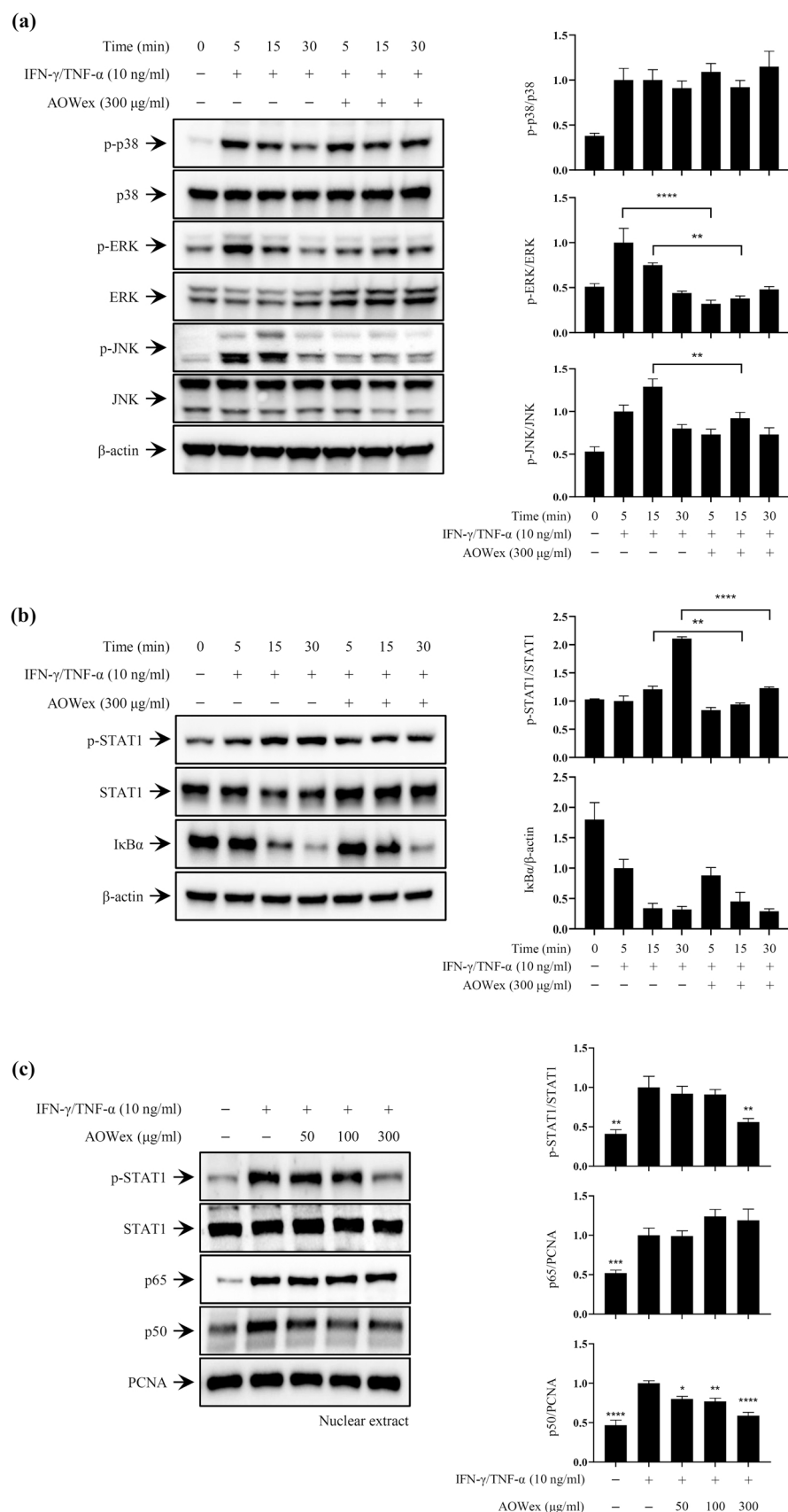


Fig. 5. Inhibitory effects of AOWex on IFN- γ /TNF- α -induced MAPK/NF- κ B/STAT1 signal pathway. Cells pretreated with AOWex for 1 h and then incubated with IFN- γ /TNF- α for 5, 15 and 30 min. Using western blot analysis, phosphorylation or degradation of (a) MAPK, (b) NF- κ B (I κ B α), and p-STAT1 proteins was identified in the total cells. (c) HaCaT cells were pre-stimulated with AOWex for 1 h before IFN- γ /TNF- α treatment. After stimulation with IFN- γ /TNF- α for 1 h, to confirm the nuclear levels of p-STAT1, STAT1, and NF- κ B (p50 and p65), the nuclear extracts were analyzed by western blotting. PCNA was used as a loading control for nuclear extracts. Quantified western blot data shown as the means $\pm$ SEM of three separate experiments. * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001 vs. IFN- γ /TNF- α .

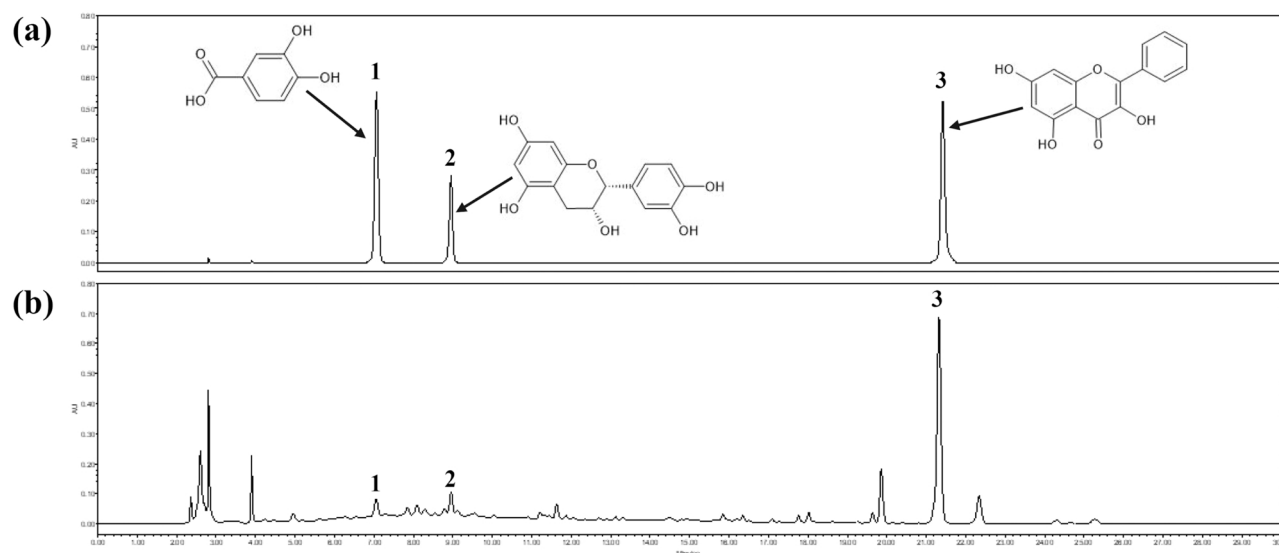


Fig. 6. HPLC chromatograms of the standard mixture solution (a) and the AOWex (b). The compounds are (1) protocatechuic acid, (2) epicatechin, and (3) galangin.

increased the production of MDC, RANTES, IP-10 and I-TAC compared with that in the untreated control group. The secretion of MDC, RANTES, IP-10 and I-TAC was markedly inhibited upon treatment with 50, 100 and 300 $\mu\text{g/mL}$ AOWex. The data showed that nontoxic concentrations of AOWex suppressed the release of IFN- γ /TNF- α -induced proinflammatory chemokines in HaCaT cells.

3.4. Effects of AOWex on IFN- γ /TNF- α -induced MAPK/NF- κ B/STAT1 activation in HaCaT cells

Nuclear factor kappa B (NF- κ B) and signal transducer and activator of transcription 1 (STAT1) together represent part of an important signaling pathway in inflammation. In several previous studies, it has been reported that NF- κ B and STAT1, which are transcriptional factors, are involved in regulating gene expression associated with allergic inflammation such as in asthma, rhinitis, and AD [40–42]. The MAPK cascade plays an important role in immune responses, gene expression, cell proliferation and regulation of various cellular processes and is involved in the production of proinflammatory chemokines [43,44]. Moreover, it is generally well known that production of inflammatory chemokines occurs in the MAPK/NF- κ B/STAT1 pathway in HaCaT cells [39,45–47]. To determine the effect of AOWex on signaling pathways involved in IFN- γ /TNF- α -induced upregulation of proinflammatory chemokines in HaCaT cells, we first investigated MAPK signaling. The results showed that AOWex inhibited the phosphorylation of ERK and JNK in IFN- γ /TNF- α -stimulated HaCaT cells, while the same reduction was not observed for p38 phosphorylation (Fig. 5a).

Several previous studies have also suggested that ERK and JNK/MAPK regulate IFN- γ /TNF- α -induced inflammatory chemokines in HaCaT cells [46–49]. Next, to determine the effect of AOWex on NF- κ B and STAT1 signaling pathways involved in IFN- γ /TNF- α -induced upregulation of inflammatory mediators, the phosphorylation level of STAT1 and degradation level of I κ B α were determined in HaCaT cells stimulated with IFN- γ /TNF- α . Our data showed that IFN- γ /TNF- α -induced

phosphorylation of STAT1 was significantly inhibited by AOWex in HaCaT cells, but there was little inhibition of I κ B α degradation (Fig. 5b). NF- κ B regulates the expression of immune-associated genes in AD through the degradation of I κ B α or NF- κ B activation in the nucleus [41]. Additionally, intracellular STAT1 is phosphorylated and then translocated to the nucleus, resulting in transcription and expression of proinflammatory mediators [50,51]. We further confirmed whether AOWex inhibits the nuclear activation of NF- κ B subunits (p65 and p50) and p-STAT1 in IFN- γ /TNF- α -induced HaCaT cells. Our results showed that IFN- γ /TNF- α induced the activation of STAT1 and NF- κ B subunits in the nucleus, while AOWex prevented it, except for p65 (Fig. 5c). These results indicated that AOWex may inhibit IFN- γ /TNF- α -induced expression of proinflammatory chemokines by downregulating STAT1 and p50 NF- κ B activation via ERK and JNK/MAPK signaling in HaCaT cells.

3.5. HPLC analysis of AOWex

In the HPLC analysis of AOWex, three compounds (protocatechuic acid, epicatechin, and galangin) were detected simultaneously at a UV wavelength of 270 nm. All compounds were separated within 30 min, and the retention times of protocatechuic acid, epicatechin, and galangin were 7.15, 9.05, and 21.42 min, respectively (Fig. 6). The results for calibration curves for the quantitative analysis of the three compounds in AOWex are shown in Table 1. For all three compounds, the coefficient of determination (r^2) of the regression equation was 1.0000, and they all showed excellent linearity. The measurements of the amounts of the three compounds showed that galangin (5.65 mg/g) was found at higher levels than the other components and that protocatechuic acid and epicatechin levels were 0.49 mg/g and 0.86 mg/g, respectively (Table 1).

Table 1
Calibration curves and contents of the three compounds in *Alpinia officinarum*.

Compound	Regression equation				Content (mg/g)	
	Linear range ($\mu\text{g/mL}$)	Slope	Intercept	r^2	Mean	SD
Protocatechuic acid	3.91–250.00	57480	-3220.3	1.0000	0.49	0.00
Epicatechin	3.13–200.00	31273	-15841	1.0000	0.86	0.01
Galangin	3.52–225.00	71466	-38495	1.0000	5.65	0.01

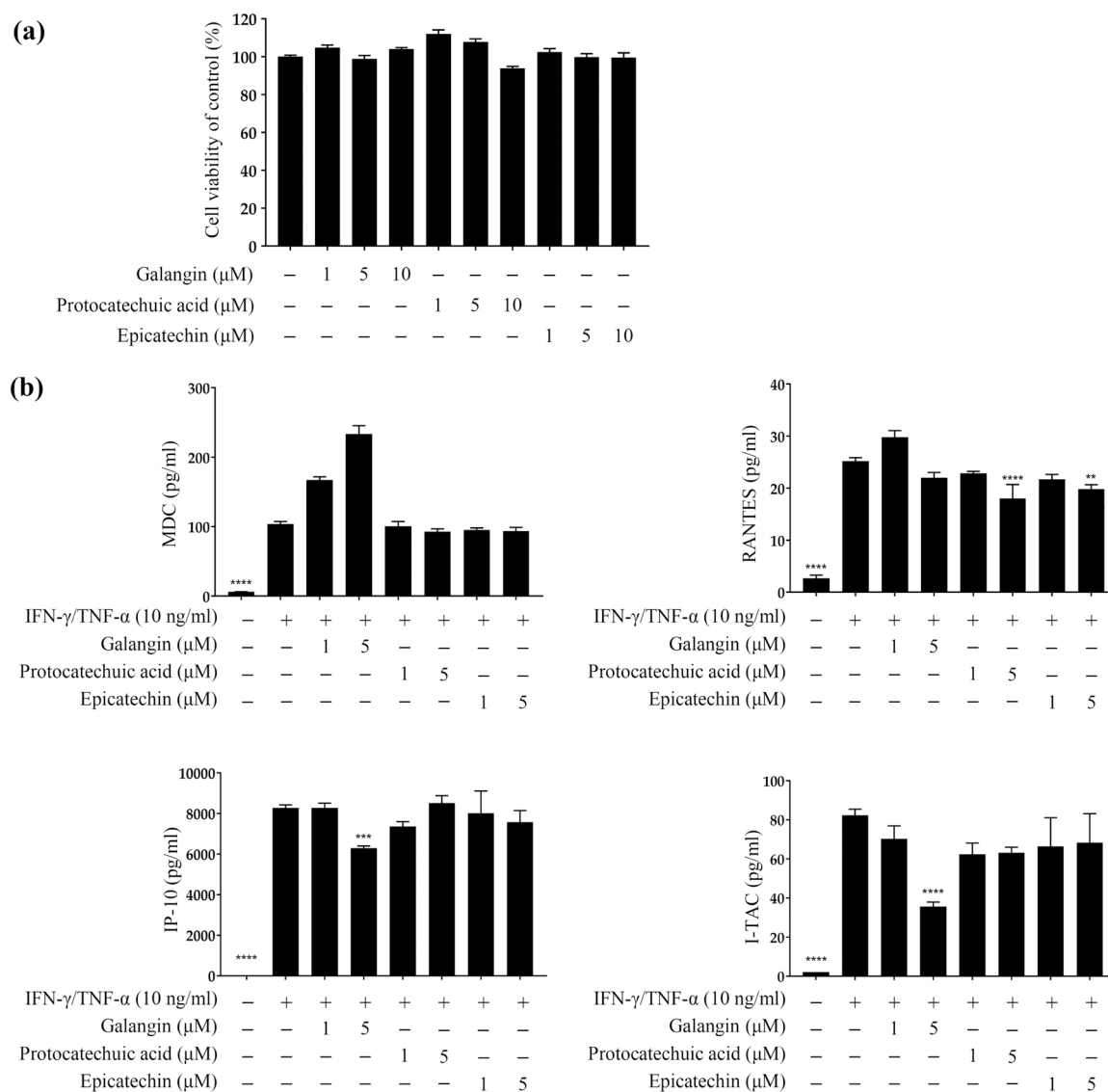


Fig. 7. Inhibitory effects of AOWex standard compounds, namely galangin, protocatechuic acid and epicatechin, on IFN- γ /TNF- α -induced proinflammatory chemokines in HaCaT cells. (a) To determine the effects of the compounds on the viability of HaCaT cells, the cells were seeded in 96-well plates and treated with different concentrations of each compound (0, 1, 5 and 10 μ M). Cytotoxicity of compounds was investigated after treatment for 24 h. (b) Secreted MDC was detected using ELISA. RANTES, IP-10 and I-TAC were measured via a bead-based immunoassay. Cells were pre-incubated with 1 and 5 μ M of compounds or 300 μ g/mL of AOWex for 1 h and stimulated with IFN- γ /TNF- α for 24 h. Data shown as the means $\pm$ SEM of three separate experiments. * p < 0.05, ** p < 0.005, *** p < 0.0005, **** p < 0.0001 vs. IFN- γ /TNF- α .

3.6. Effects of galangin, protocatechuic acid, and epicatechin on IFN- γ /TNF- α -induced proinflammatory chemokine production in HaCaT cells

Galangin is known to possess several pharmacological activities, such as anti-mutagenesis, anti-oxidation and radical scavenging [52–54]. In particular, it has been reported that galangin exerts anti-inflammatory effects on collagen-induced arthritis, mast cell-derived allergic inflammation, and AD in vivo and in vitro models [55–58]. Protocatechuic acid, a phenolic compound of anthocyanin, has been demonstrated to exert various pharmacological effects, including cardiac protective, anti-tumorigenic, anti-hyperglycemic, and anti-inflammatory effects [59–62]. Recently, several studies have indicated that epicatechin exerts anti-oxidant and anti-inflammatory effects and reduces obesity-associated insulin sensitivity and sustained inflammation [63–65]. Thus, to determine the active compounds of AOWex, we investigated the effects of galangin, protocatechuic acid, and epicatechin on IFN- γ /TNF- α -induced production of proinflammatory chemokines in HaCaT cells.

The cell viability assay showed that the three compounds were not cytotoxic to HaCaT cells after 24 h of treatment (Fig. 7a). Based on these results, the following experiment was conducted with 1 and 5 μ M of the three compounds. The most abundant component, galangin, inhibited IFN- γ /TNF- α -induced IP-10 and I-TAC. In addition, in HaCaT cells stimulated with IFN- γ /TNF- α , protocatechuic acid and epicatechin inhibited the production of RANTES (Fig. 7b). However, the reduction in MDC production in AOWex was not shown by the three compounds; rather, galangin increased the production of MDC. Further experiments on these results are needed. Galangin suppressed the IFN- γ /TNF- α -induced STAT-1 activation in the HaCaT cell nucleus but did not affect NF- κ B activation (Fig. 8). Jaruga et al. [66] reported that the expression of IP-10 and I-TAC is regulated via STAT-1 activation. These reports suggest that galangin inhibits IFN- γ /TNF- α -induced IP-10 and I-TAC expression by suppressing the activation of STAT-1 in HaCaT cells. The expression of the RANTES gene has been demonstrated to be mediated by the NF- κ B pathway [67]. Our results confirmed that protocatechuic acid and epicatechin inhibit the production of RANTES by regulating

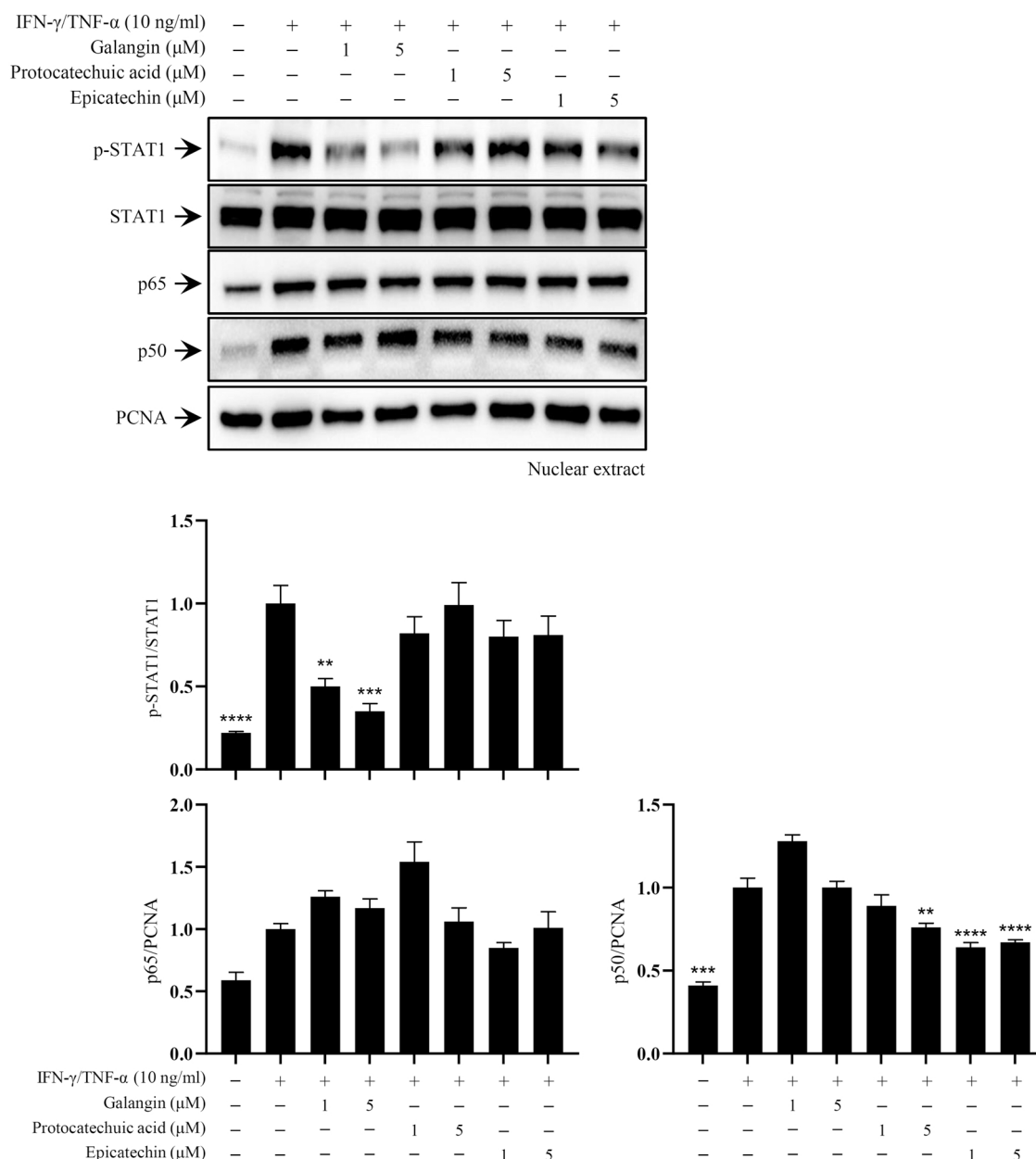


Fig. 8. Inhibitory effects of AOWex standard compounds (galangin, protocatechuic acid and epicatechin) on IFN- γ /TNF- α -induced STAT1 and NF- κ B nuclear activation in HaCaT cells. The cells were pretreated with the compounds or AOWex for 1 h and then stimulated with IFN- γ /TNF- α for 1 h. The activated levels of p-STAT1 and NF- κ B in nuclear extracts were detected by western blot analysis. PCNA was used as a loading control for nuclear extracts. Quantified western blot data shown as the means $\pm$ SEM of three separate experiments. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 vs. IFN- γ /TNF- α .

p50 NF- κ B nuclear activation in IFN- γ /TNF- α -stimulated HaCaT cells (Fig. 8). Therefore, the inhibitory effects of galangin, protocatechuic acid and epicatechin on the nuclear activation of STAT1 and p50 in IFN- γ /TNF- α -stimulated HaCaT cells were determined using nuclear fractionation and western blot analysis (Fig. 8). These results showed that the standard compounds of AOWex exert synergistic anti-AD effects.

4. Conclusion

In this study, we demonstrated the anti-inflammatory activity of AOWex in NC/Nga mice with DfE-induced AD-like symptoms. Our data showed that AOWex alleviated AD-like skin lesion symptoms by reducing skin thickness and mast cell infiltration. The extracts also reduced serum MDC, RANTES, and IgE levels in DfE-induced AD mice.

Moreover, we found that AOWex inhibited IFN- γ /TNF- α -induced proinflammatory chemokine production in HaCaT cells. Our results suggest that these inhibitory effects are exerted by suppressing p50 NF- κ B and STAT1 activation via the JNK and ERK MAPK signaling pathways. Although further research is needed to clarify the effect on reducing MDC production, the experiments with the standard compounds of AOWex confirmed that these compounds exhibit a synergistic anti-AD effect with AOWex. Therefore, we expect AOWex to be a potential preventive agent for AD.

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Institutional Review Board Statement

All animal experiments were conducted under approval (No. 20-069) of the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine (KIOM).

CRedit authorship contribution statement

Hyun-Kyung Song: Investigation, Methodology, Validation, Formal analysis, Data curation, Writing, Visualization. **Sun Haeng Park:** Investigation, Methodology, Validation, Formal analysis. **Hye Jin Kim:** Investigation, Validation, Formal analysis. **Seol Jang:** Investigation, Validation, Formal analysis. **Taesoo Kim:** Conceptualization, Methodology, Data curation, Supervision, Project administration.

Author contributions

Conceptualization and supervision, T.K.; investigation, H.-K.S., S.H.P., H.J.K and S.J.; formal analysis and writing, H.-K.S. and T.K.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Data Availability

The data presented in this study are available on request from the corresponding author.

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