

Oral Administration of *Vaccinium uliginosum* L. Extract Alleviates DNCB-Induced Atopic Dermatitis in NC/Nga Mice

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ABSTRACT Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease that responds to the interplay of environmental, immunological, and genetic factors. To explore the effect of *Vaccinium uliginosum* (VU) extract on AD, we orally administrated VU total water extract to AD-induced NC/Nga mice. VU extract reduced AD-like skin lesions, ear thickness, and the frequency of scratching episodes in a time-dependent manner. VU also suppressed the levels of IgE and histamine and the ratio of IgG1/IgG2a in the serum of AD-induced NC/Nga mice. VU administration resulted in the reduction of splenic cytokine production, epidermal thickening, and the infiltration of eosinophils, mast cells, and degranulated mast cells induced by 2,4-dinitrochlorobenzene (DNCB). In addition, VU significantly reduced the mRNA expression of chemokine ligands in dorsal skin. Total water extract and subfractions of VU inhibited interleukin (IL)-4 production in splenocytes, suggesting that VU total extract has a Th2 cytokine modulating effect. These results suggest that the VU total water extract could be a candidate therapeutic agent for the treatment of AD through an immunoregulatory effect.

KEY WORDS: • chemokine ligand • cytokine production inhibition • eosinophil • IgG1 • IgG2a • mast cell

INTRODUCTION

ATOPIC DERMATITIS (AD) is a chronic and relapsing form of dermatitis occurring commonly in children and characterized by symptoms of eczematous inflammatory skin lesions, highly elevated IgE levels, and itching.^{1,2} AD has been reported to develop in two successive phases: an early phase of Th2 cytokine (interleukin [IL]-4, IL-13) production and chemokine (MDC, TARC, and CTACK) synthesis and a chronic phase of IL-5 synthesis, eosinophil infiltration, and interferon-gamma (IFN- γ) synthesis.³ In the early phase of AD, exogenous allergens are taken up and presented by antigen-presenting cells (APCs), including Langerhans cells in the epidermis and dendritic cells (DCs) in the dermis.⁴ APCs secrete Th2 cytokines, IL-16, and chemokines that recruit CD8⁺ Th2 cells to the epidermis.⁵ Toxic mediators (histamine and nitric oxide) released due to Th2 cell activation trigger pruritus, which promotes scratching behavior and subsequent skin damage.⁶ TNF- α and IFN- γ induced by skin damage maintain the Th2 phenotype, but allow Th1 inflammatory response development, which leads to the chronic phase of AD.^{7,8} A recent study reported that inflammatory dendritic epidermal cells (IDECs) and eosinophils are responsible for secreting high amounts of IL-12, IFN- γ , and GM-CSF, which allow Th1

inflammation.⁸ The fruits of *Vaccinium uliginosum* L. (VU), also known as bog bilberry, contain a variety of flavonoid compounds (quercetin, myricetin, and its glycosides) and an anthocyanin compound (cyanidine-3-O-glucoside). Quercetin and myricetin are known to have an inhibitory effect against asthma, which is a similar Th2-mediated allergic disease to AD.^{9–11} Cyanidine-3-O-glucoside, the major anthocyanin compound of VU, has also been reported to have an anti-inflammatory effect.^{12,13} The effects of VU total extract on AD have not been reported. Thus, the purpose of this study was to investigate the therapeutic effect of VU total water extract on AD in an NC/Nga mouse model. We orally administrated VU total extract in distilled water to AD-induced NC/Nga mice for 4 weeks and investigated the changes in AD-like symptoms, including AD-like skin lesions, scratching behavior, ear swelling, serum levels of immunoglobulins and histamine, and splenic cytokine production. We also analyzed the changes in mRNA expression of chemokine ligands, and changes in histological features were observed. Furthermore, we subfractionated VU extracts and investigated the possible effect of total extract or VU subfractions on IL-4 production in ConA-sensitized splenocytes from NC/Nga mice.

MATERIALS AND METHODS

Instruments

¹H-nuclear magnetic resonance (NMR) (400 MHz), ¹³C-NMR (100 MHz), and two-dimensional NMR spectra were

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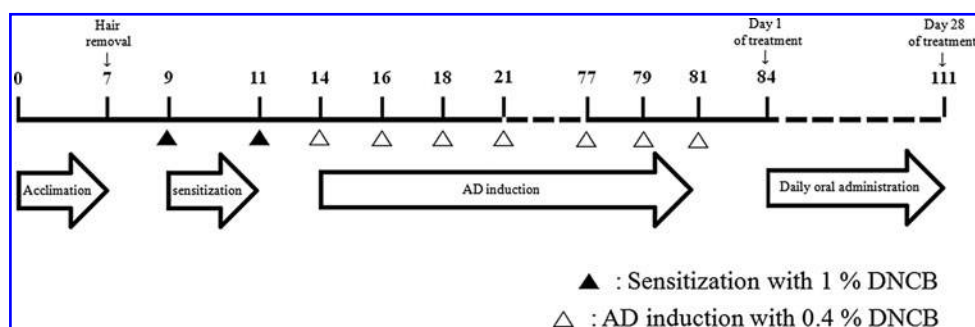


FIG. 1. Scheme of atopic dermatitis (AD) induction and dosing. The hair-removed dorsal skin of mice was sensitized twice a week with 200 μL of 1% 2,4-dinitrochlorobenzene (DNCB) mixture. The dorsal skin of mice was challenged with 150 μL of 0.4% DNCB mixture three times a week for 10 weeks. After AD induction was completed, *Vaccinium uliginosum* (VU) extract (90, 150, 250 mg/kg·body weight [bw]) and prednisolone (3 mg/kg·bw) dissolved in distilled water were orally administrated to NC/Nga mice daily for 4 weeks.

recorded on a Varian (Palo Alto, CA, USA) INOVA 400 MHz NMR spectrometer in CD_3OD . For the two-dimensional heteronuclear correlation spectroscopy, the refocusing delays were optimized for $^1J_{\text{CH}}=145\text{ Hz}$ and $^nJ_{\text{CH}}=10\text{ Hz}$. Electrospray ionization (ESI) mass spectra were acquired using a Finnigan (Thermo Electron, Waltham, MA, USA) Navigator 30086 ESI-mass spectrometer. Preparative high-performance liquid chromatography (HPLC) was carried out using a Hitachi HPLC system equipped with a photodiode array detector using a YMC Co. (Kyoto, Japan) J'sphere ODS-H80 column (150×20 mm; particle size, 4 μm).

Preparation and subfractionation of VU extract

Total water extract of VU was prepared and subfractionated, as previously described, with slight modification.¹⁴ Briefly, fruits of VU were pulverized in a grinder and extracted with 10-fold volume of boiled water and filtered. The aqueous solution was evaporated under low pressure and diluted in distilled water, freeze-dried, and stored at -70°C until use. Two C18 Sep-Pak cartridges were connected and preconditioned by sequentially eluting with ethyl acetate, absolute methanol, and 0.01 N aqueous HCl. A total extract was loaded onto the cartridges, which were washed with 0.01 N aqueous HCl to collect sugar/acid fractions. Cartridges were eluted with ethyl acetate, and then the ethyl acetate was collected. The absorbed anthocyanins were eluted from the cartridges with 10 mL of absolute methanol with 0.1% (v/v) HCl, which was also collected. The fractions of anthocyanin, sugar/acid (dissolved in distilled

water), and polyphenol (dissolved in 50% aqueous ethanol) were stored at -70°C until use.

Animals

Four-week-old male NC/Nga mice were purchased from SLC (Shizuoka, Japan) and housed in individually ventilated cages with a 12-h light/12-h dark cycle (light on 8 a.m.–8 p.m.) at $23^\circ\text{C}\pm 2^\circ\text{C}$ and a relative humidity of $55\%\pm 5\%$. After 1 week acclimation, mice were divided into six groups ($n=5$): (i) normal group (DNCB^- , 0.9% saline treated), (ii) negative control group (DNCB^+ , 0.9% saline treated), (iii) prednisolone 3 group (DNCB^+ , 3 mg/kg·body weight [bw] prednisolone treated), (iv) VU 90 group (DNCB^+ , 90 mg/kg·bw VU extract treated), (v) VU 150 group (DNCB^+ , 150 mg/kg·bw VU extract treated), and (vi) VU 250 group (DNCB^+ , 250 mg/kg·bw VU extract treated). All experimental procedures were conducted according to the Institutional Animal Care and Use Committee guidelines of Kyung Hee University.

Induction of AD and oral administration of VU total water extract

AD was induced in NC/Nga mice by topical application of a 2,4-dinitrochlorobenzene (DNCB) mixture, as previously described,^{15–17} with a slight modification. Briefly, the dorsal hair of the NC/Nga mice was removed by topical application of cream containing thioglycolic acid. The hair-removed dorsal skin of the mice was sensitized twice a week with 1200 μL of 1% DNCB mixture (1% DNCB dissolved in

TABLE 1. PRIMERS OF REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Gene	Quantification method	Sequence (5'-3')	Tm ($^\circ\text{C}$)
Eotaxin-1/CCL11	Forward primer	CACCCTGAAAGCCATAGTGT	57
	Reverse primer	TGTGTACCTGGAAATTAG	53
LARC/CCL20	Forward primer	GCCTCTTCCTTCCAGAGCTATTG	68
	Reverse primer	TGATGTGCAGGTGAAGCCTTC	64
GAPDH	Forward primer	TACATGTTCCAGTATGACTC	56
	Reverse primer	TGTGAGGGAGATGCTCAGTG	61

Sequences and Tm values for each primers.

TABLE 2. ISOLATED COMPOUNDS IN *VACCINIUM ULIGINOSUM* TOTAL EXTRACT

Compound	Compound group	MW	Composition g/g, %)
Cyanidin-3-O-glucoside	Anthocyanin	449	3.272
Myricetin	Polyphenol	318	0.094
Quercetin-3-O- β -galactoside	Polyphenol	464	0.079
Myricetin-3-O- β -galactoside	Polyphenol	480	0.066
Laricitrin-3-O- β -galactoside	Polyphenol	494	0.051
Quercetin	Polyphenol	302	0.050
Myricetin-3-O- α -arabinofuranoside	Polyphenol	450	0.034
Quercetin-3-O- α -arabinofuranoside	Polyphenol	434	0.026
Syringetin	Polyphenol	346	0.014

HPLC-mass spectrometry was used to separate and identify single active compounds of VU total extract. The structures of the purified compounds were determined by NMR and mass spectrometry. The common names, compound groups, molecular weights of isolated single compounds, and their compositions in VU total extract were listed.

HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; VU, *Vaccinium uliginosum*.

acetone and olive oil [3:1 v/v]). The dorsal skin of the mice was challenged with 150 μ L of 0.4% DNCB mixture three times a week for 10 weeks. After AD induction was completed, VU extract (90, 150, 250 mg/kg·bw) and prednisolone (3 mg/kg·bw) dissolved in distilled water were orally administrated to NC/Nga mice daily for 4 weeks. The dosing volume of each mouse was calculated with recently measured body weight (10 mL/kg·bw). Figure 1 illustrates the schedule of AD induction and dosing.

Measurement of skin severity and ear thickness

On days 0, 7, 14, 21, and 28 of treatment, skin severity was scored macroscopically, as previously described.^{18,19} Briefly, the skin severity score was calculated as the sum of the individual score grades of the following five symptoms: itching, edema, hemorrhage, excoriation/erosion, and scaling. For each skin symptom, individual scores were graded as follows: 0 (no symptom), 1 (mild), 2 (moderate), 3 (severe). Skin severity scores were evaluated by a single ex-

perienced person 1 h before treatment. The thickness of the right ear of NC/Nga mice was measured with a dial thickness gage (Mitutoyo, Kawasaki, Japan) after treatment.

Scratching behavior test

The total scratching behavior number was measured thrice a week, as described previously, with a slight modification.^{20,21} Briefly, mice were placed into cages for 1 h for habituation. After habituation, the number of scratching episodes for 30 min was counted macroscopically. A series of scratching movements made only with the hind paw was counted as one scratching episode. Each scratching episode for 30 min was scored from 0 to 4: 0 (no scratching), 2 (scratching shorter than 1.5 sec), 4 (scratching longer than 1.5 sec). The total scratching behavior number was calculated as the sum of individual scratching scores within 30 min.

Measurement of serum immunoglobulin and histamine levels

At the end of the study, mice were sacrificed under diethyl ether anesthesia. Blood was collected from the inferior vena cava and allowed to clot for 30 min at room temperature. The serum was prepared by centrifugation (10,000 g, 10 min) and stored at -70°C until use. The serum levels of IgE, IgG1, IgG2a, and histamine were determined by the appropriate enzyme-linked immunosorbent assay (ELISA) kits (Shibayagi, Gunma, Japan; Assay Designs, Ann Arbor, MI, USA; and DAsource, Nivelles, Belgium) according to each manufacturer's instructions.

Splenic cytokine production assay

The spleens of mice were obtained, and isolated single cells were seeded into a 24-well plate at a concentration of 1×10^6 cells per well in the RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (HyClone). Splenocytes were stimulated with 5 $\mu\text{g/mL}$ of ConA (Sigma, St. Louis, MO, USA) and incubated under 5% CO_2 at 37°C for 72 h. After incubation, the supernatant was collected by centrifugation and stored at -70°C until use. The levels of IL-4, IFN- γ , IL-12, and IL-13 in the supernatant were measured by commercial ELISA kits (Assay Designs; and eBioscience, San Diego, CA, USA) according to each manufacturer's instructions.

TABLE 3. *VACCINIUM ULIGINOSUM* TOTAL EXTRACT REDUCES SKIN SEVERITY IN A TIME-DEPENDENT MANNER

Group	Day 0	Day 7	Day 14	Day 21	Day 28
Normal	1 $\pm$ 0.6	0.8 $\pm$ 0.7	1.4 $\pm$ 0.8	1 $\pm$ 0.6	0.8 $\pm$ 0.4
Negative control	12 $\pm$ 1.3 ^{###}	11.6 $\pm$ 1.9 ^{###}	10.2 $\pm$ 1.5 ^{###}	7.8 $\pm$ 1.1 ^{###}	6.8 $\pm$ 1.3 ^{###}
Prednisolone 3	12.4 $\pm$ 1.0	11 $\pm$ 1.1	9.6 $\pm$ 0.5	7.4 $\pm$ 2.1	5.8 $\pm$ 1.6
VU 90	11.8 $\pm$ 0.4	10.2 $\pm$ 1.9	7.4 $\pm$ 1.2*	4.8 $\pm$ 1.9*	4.4 $\pm$ 1.3*
VU 150	12 $\pm$ 1.1	10 $\pm$ 1.5	8.6 $\pm$ 2.6	6.4 $\pm$ 1.0	3.6 $\pm$ 1.8*
VU 250	12 $\pm$ 1.7	10.4 $\pm$ 1.5	8.8 $\pm$ 1.7	6.6 $\pm$ 1.9	4.2 $\pm$ 1.6*

At day 0, 7, 14, 21, and 28 of treatment, clinical score of experimental mice was measured. Clinical skin score was evaluated as sum of the scores in five clinical symptoms and expressed as mean $\pm$ SEM of five mice per group. ^{###} $P < .001$ when compared with normal group, and * $P < .05$ when compared with negative control group.

TABLE 4. *VACCINIUM ULIGINOSUM* TOTAL EXTRACT REDUCES EAR THICKNESS IN A TIME-DEPENDENT MANNER

Group	Day 0	Day 7	Day 14	Day 21	Day 28
Normal	34.4 ± 6.4	36 ± 2.3	36.4 ± 6.1	35.8 ± 3.0	33 ± 4.7
Negative control	65.8 ± 12.6 ^{##}	56.4 ± 9.6 ^{###}	55.8 ± 5.9 ^{###}	52.4 ± 8.9 ^{##}	49.3 ± 6.0 ^{##}
Prednisolone 3	61.2 ± 15.3	56.6 ± 8.6	54.6 ± 10.5	49 ± 12.6	41.6 ± 13.7
VU 90	59.2 ± 12.0	51.4 ± 8.3	53.2 ± 8.4	45.2 ± 7.4	39.3 ± 2.6*
VU 150	60.4 ± 11.1	53.4 ± 6.6	50 ± 8.1	40.4 ± 6.1	37.8 ± 10.6
VU 250	62.4 ± 12.0	58.2 ± 6.6	54.4 ± 6.3	42 ± 10.1	38.3 ± 2.6*

At day 0, 7, 14, 21, and 28 of treatment, ear thickness of DNCB-treated right ear of experimental mice was measured. Ear thickness was measured by dial thickness gauge and expressed as mean ± SEM of five mice per group. ^{##}*P* < .01, ^{###}*P* < .001 when compared with normal group, and **P* < .05 when compared with negative control group.

DNCB, 2,4-dinitrochlorobenzene.

Histological analysis

Samples of the upper dorsal skin (2 × 2 cm²) of sacrificed mice were obtained and fixed in 10% phosphate-buffered formalin (pH 7.2) for 24 h. The dorsal skin samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E) to investigate the effects of VU on eosinophil infiltration and epidermal thickening or toluidine blue to investigate the effects of VU on infiltration of degranulated mast cells and mast cells. Sections were observed under a microscope at a magnification of ×200, and digital images were captured using an imaging program (DP Controller Software; Olympus, Tokyo, Japan). Degranulation of mast cells was determined by morphological changes of mast cells, and the number of infiltrated cells was counted as average infiltrated cells in areas of 0.0625 mm². The infiltrated cell numbers and the thickness of the epidermis (the average distance from the granular layer to the basal layer) were calculated as means in five randomly selected fields per mouse.

Reverse transcription–polymerase chain reaction analysis

The total RNAs of dorsal skin were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified with a spectrophotometer (MECASYS, Daejeon, Korea). Next, reverse transcription–polymerase chain reaction (RT-PCR) was conducted with 30 ng of total RNA using MaximTM RT-PCR Premix (iNtRON, Sungnam, Korea) with specific primers. The sequences of primers are shown in Table 1. RT-PCR was conducted under the following thermal conditions: 1 cycle for reverse transcription at 45°C for 30 min, 94°C for 5 min, followed by amplification and 45 cycles at 94°C for 30 sec, 58°C for 30 sec, and 72°C for

1 min. The PCR products were size fractionated by 1.5% agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified by densitometry evaluation of digital gel images using the ImageJ program (NIH, Bethesda, MD, USA). The densitometry value of each band was normalized to that of the housekeeping gene, GAPDH.

Ex vivo inhibition of IL-4 production analysis

AD was induced in 5-week-old NC/Nga mice by a 4-week challenge of DNCB 0.4% mixture. After the mice were sacrificed, spleens were obtained, and isolated splenocytes (1 × 10⁶ cells/well) were seeded in the RPMI 1640 medium supplemented with 10% FBS, antibiotics (HyClone), and 5 μg/mL of ConA (Sigma). Splenocytes were treated with 25, 50, 100, and 200 μg/mL of VU total extract and subfractions of VU and incubated under 5% CO₂ at 37°C for 48 h. After incubation, supernatants were collected by centrifugation, and the levels of IL-4 in supernatants were determined by a commercial IL-4 ELISA kit (Assay Designs).

Statistical analysis

Data are expressed as mean ± SEM. Differences between groups were analyzed by means of the Student's *t*-test. A *P*-value < .05 was considered to be statistically significant.

RESULTS

Isolation of single compounds

The structures of the purified VU compounds separated by HPLC were determined by NMR and mass spectrometry.

TABLE 5. *VACCINIUM ULIGINOSUM* TOTAL EXTRACT SUPPRESSED SCRATCHING BEHAVIOR EPISODES

Group	Week 1	Week 2	Week 3	Week 4
Normal	19.4 ± 7.2	19.3 ± 7.3	20.2 ± 3.6	22.1 ± 8.6
Negative control	82.3 ± 24.1 ^{##}	75.5 ± 22.8 [#]	72.7 ± 39.1	66.1 ± 24.5 [#]
Prednisolone 3	69.1 ± 11.6	33.4 ± 1.7*	45.3 ± 22.0	22.3 ± 7.9*
VU 90	66.9 ± 21.0	35.0 ± 7.2*	25.3 ± 20.0	20.2 ± 8.0*
VU 150	59.8 ± 15.1	64.3 ± 28.7	49.6 ± 13.7	37.6 ± 21.9
VU 250	40.7 ± 13.2*	39.0 ± 24.8	24.8 ± 14.7	30.7 ± 7.9

Scratching behavior number for 30 min was counted 1 h after VU extract or prednisolone treatment, three times a week. Weekly data are expressed as mean ± SEM of each scratching number of 3 days in a week. [#] *P* < .05, ^{##} *P* < .01 when compared with normal group, and **P* < .05 when compared with negative control group.

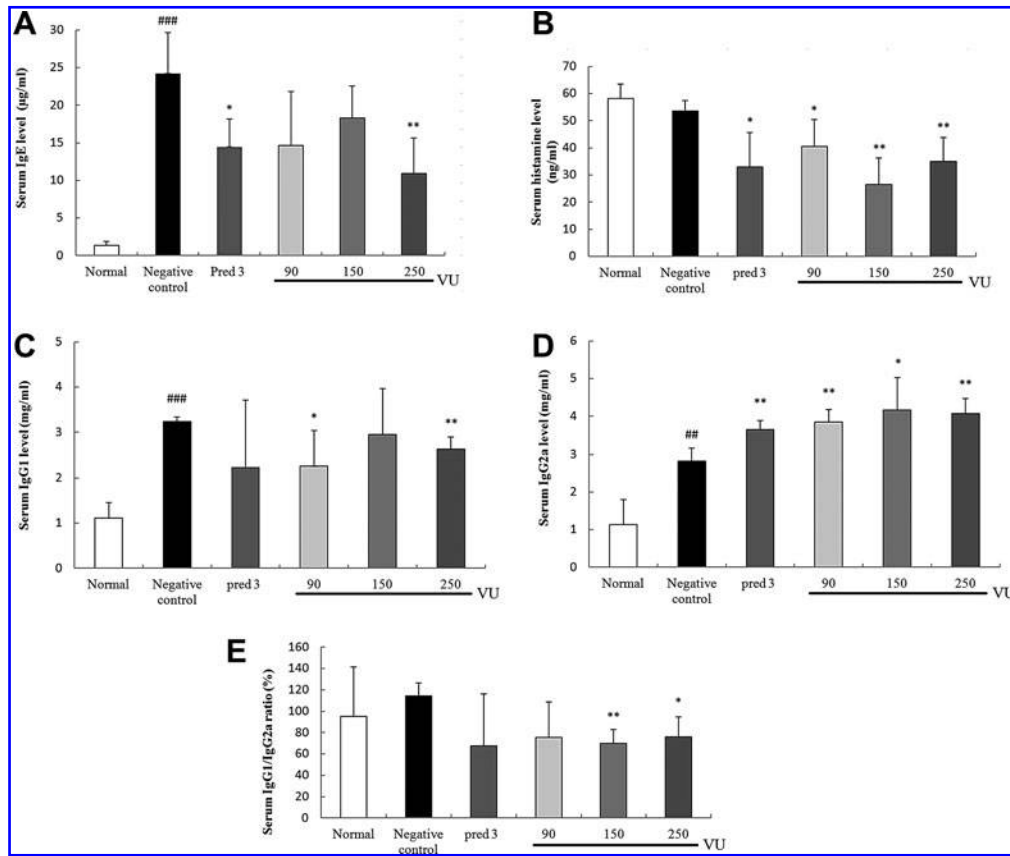


FIG. 2. VU total extract downregulated serum levels of IgE, histamine, and IgG1/IgG2a ratio. At day 28 of treatment, mice were sacrificed and serum of each mouse was collected. Then, serum levels of (A) IgE, (B) histamine, (C) IgG1, and (D) IgG2a were measured by enzyme-linked immunosorbent assay (ELISA) kits and (E) IgG1/IgG2a ratio (%) was calculated as $100 \times \text{IgG1/IgG2a levels of each mouse}$. Data are expressed as mean $\pm$ SEM of each five mice per group. $^{\#}P < .01$, $^{\#\#}P < .001$ when compared with normal group, and $^*P < .05$, $^{**}P < .01$ when compared with negative control group.

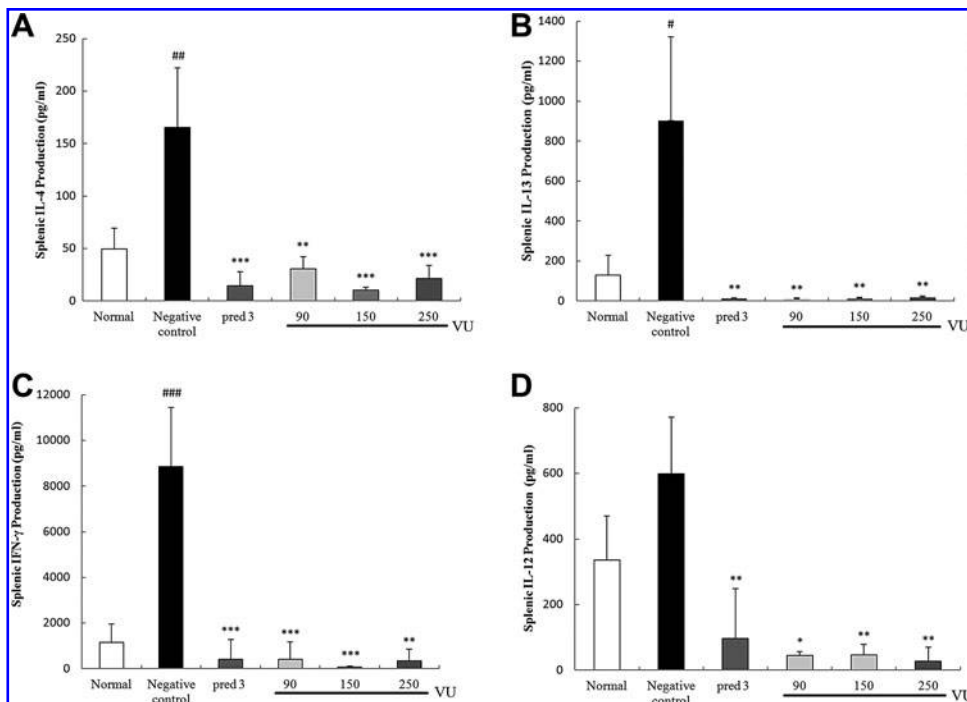


FIG. 3. VU total extract suppressed splenic production of both Th2 or Th1 cytokines. At day 28 of treatment, mice were sacrificed and spleens of each mouse were obtained. Isolated splenocytes were stimulated by $5 \mu\text{g/mL}$ of ConA and incubated under 5% CO_2 at 37°C for 72 h. The levels of (A) interleukin (IL)-4, (B) IL-13, (C) interferon-gamma (IFN- γ), and (D) IL-12 in supernatants were measured by ELISA kits. Data are expressed as mean $\pm$ SEM of each five mice per group. $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ when compared with normal group, and $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ when compared with negative control group.

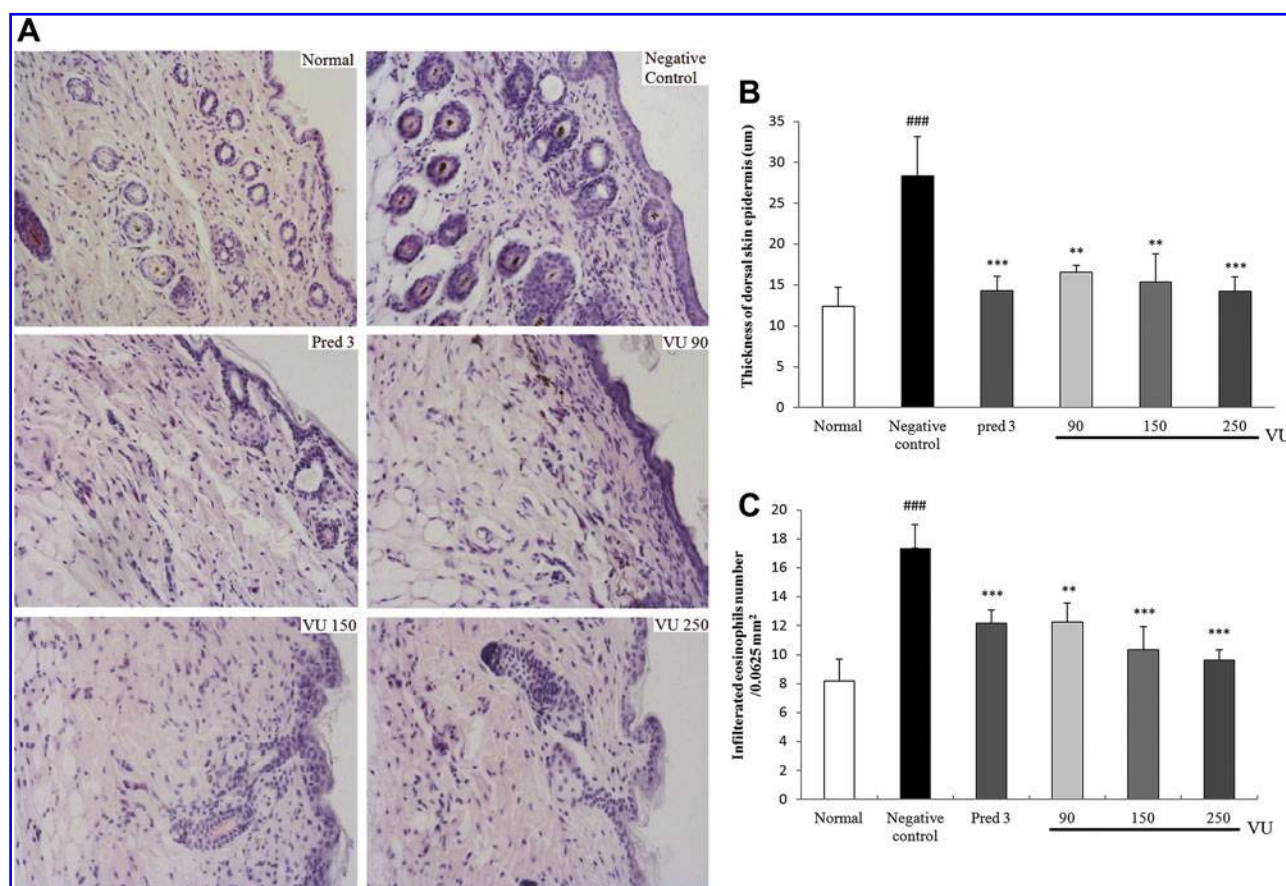


FIG. 4. VU total extract alleviates histopathological symptoms of dorsal skin stained by hematoxylin and eosin (H&E). Dorsal skins of each mouse were biopsied, sectioned, and H&E stained. (A) H&E-stained sections were observed under microscope at a magnification of $\times 200$, (B) epidermal thickness was quantified as means in randomly selected 25 fields per mouse and, (C) infiltration of eosinophil was quantified as means in randomly selected five fields per mouse. Data are expressed as mean $\pm$ SEM of each five mice per group. ### $P < .001$ when compared with normal group, and ** $P < .01$, *** $P < .001$ when compared with negative control group. Color images available online at www.liebertpub.com/jmf

The ^1H and ^{13}NMR spectral data of the compounds were compared with those reported in the literature²² and confirmed by mass spectral data; isolated compounds and their compositions are shown in Table 2.

Oral administration of VU total extract alleviates AD-like skin lesions and ear thickening due to AD

On days 0, 7, 14, 21, and 28 of treatment, skin severity was scored macroscopically, and the thickness of the right ear of NC/Nga mice was measured with a dial thickness gage. VU and prednisolone alleviated AD-like skin lesions in a time-dependent manner. The percent reductions (reduced % compared with negative control group) of the skin severity score in the VU 90, 150, 250, and prednisolone groups were 35.5%, 47.1%, 38.2%, and 14.7%, respectively, on day 28 (Table 3). The VU 90 group on days 14, 21, and 28 and the VU 150 and VU 250 groups on day 28 showed significantly decreased skin severity when compared with the negative control group. The ear thickness of NC/Nga mice was also reduced by VU extract and prednisolone treatment. The percent decrease

in ear thicknesses in the VU 90, 150, 250, and prednisolone groups on day 28 was 20.3%, 23.3%, 22.3%, and 15.6%, respectively (Table 4). On day 28, mice in the VU 90 and 250 groups showed significantly decreased ear thickness.

VU total extract reduced the frequency of scratching episodes within 30 min

VU and prednisolone suppressed the scratching behavior in a time-dependent manner. On day 28 of treatment, the inhibitory effects of the VU 90, 150, 250, and prednisolone on scratching behavior were 69.4%, 43.2%, 53.6%, and 66.2%, respectively, compared to nontreated control (Table 5). In the first week of treatment, only the VU 250 group showed a significant decrease in scratching number when compared with the negative control group. The VU 90 and prednisolone groups showed a significant decrease in the second week, but the significance disappeared in the third and fourth weeks. The VU 90 and prednisolone groups showed significantly reduced scratching episodes when compared with the negative control group.

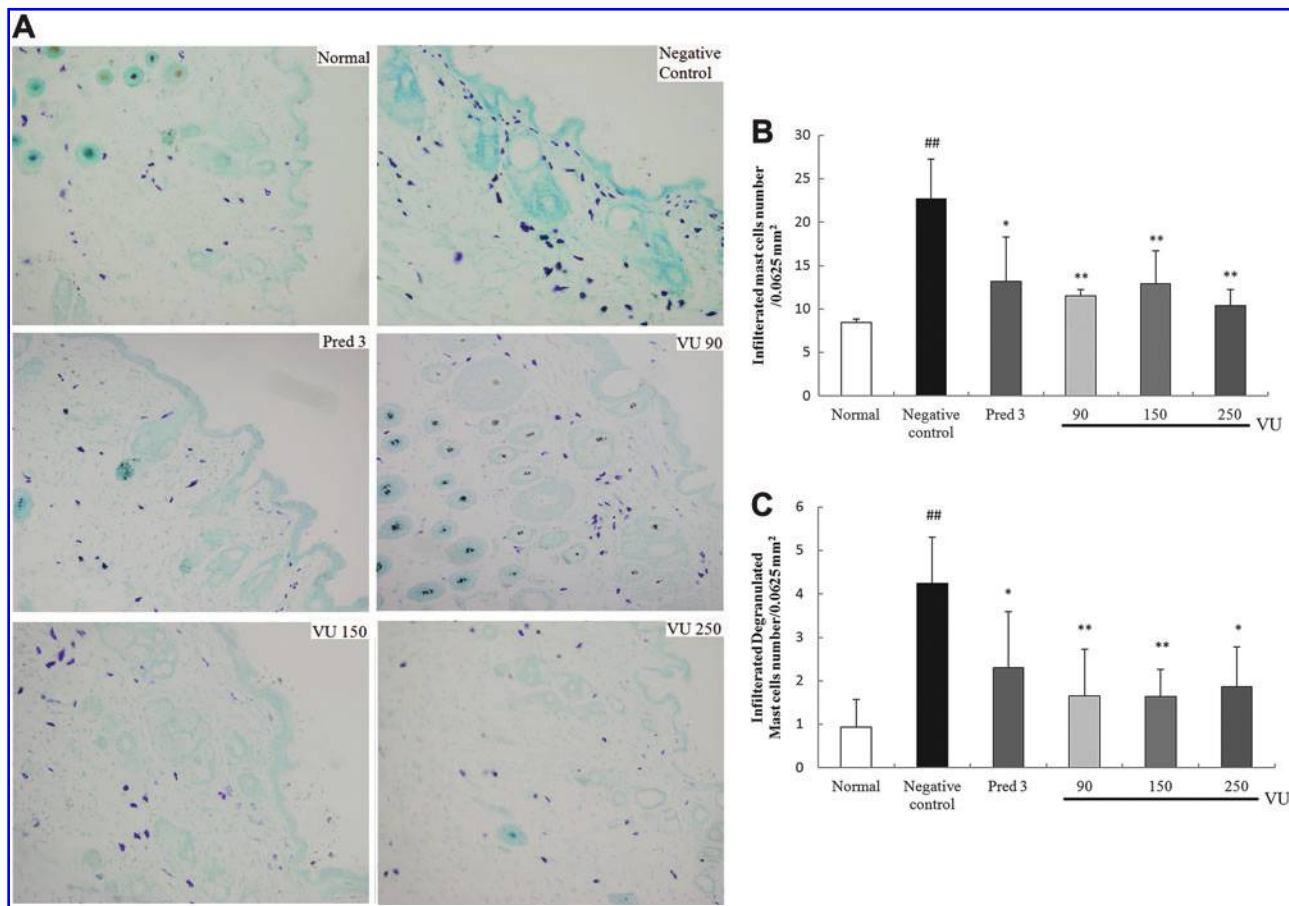


FIG. 5. VU total extract alleviates histopathological symptoms of dorsal skin stained by toluidine blue. Sectioned dorsal skins were toluidine blue stained. (A) Toluidine blue-stained sections were observed under a microscope at a magnification of $\times 200$, (B) infiltration of mast cell, and (C) infiltration of degranulated mast cell were quantified as means in randomly selected five fields per mouse. Data are expressed as mean $\pm$ SEM of each five mice per group. ^{##} $P < .001$ when compared with normal group, and ^{*} $P < .05$, ^{**} $P < .01$ when compared with negative control group. Color images available online at www.liebertpub.com/jmf

VU total extract downregulated the serum IgE and histamine levels and IgG1/IgG2a ratio

VU and prednisolone decreased the serum IgE and histamine levels. The inhibitory effects of VU 90, 150, 250, and prednisolone on IgE and histamine release 39.5%, 24.5%, 55.6%, and 40.3% decrease in the IgE level (Fig. 2A) and 24.4%, 50.5%, 34.9%, and 38.7% decrease for the histamine level (Fig. 2B), respectively. The serum Th2-mediated IgG1 level was decreased (Fig. 2C), whereas the Th1-mediated IgG2a level was increased (Fig. 2D) by VU in a dose-dependent manner. VU lowered the serum IgG1/IgG2a ratio with a 34.2%, 38.9%, 33.6%, and 41% reduction in each group, respectively (Fig. 2E). As a result, both VU and prednisolone at the highest concentration significantly suppressed serum IgE and histamine levels, but the serum IgG1/IgG2a ratio reduction was only significant in the VU-treated group.

VU total extract suppressed both Th1 cytokine and Th2 cytokine production stimulated by ConA in splenocytes from experimental mice

VU and prednisolone dramatically suppressed both Th1 cytokine and Th2 cytokine production stimulated by ConA

in splenocytes from experimental mice, reaching almost the basal level. The inhibitory effects of the VU 90, 150, 250, and prednisolone groups were as follows: 81.5%, 93.7%, 87.1%, and 91.1% for the IL-4 level (Fig. 3A); 98.7%, 98.5%, 98%, and 98.6% for the IL-13 level (Fig. 3B); 95.4%, 99.1%, 96.1%, and 95.3% for the IFN- γ level (Fig. 3C); and 92.5%, 92.1%, 95.4%, and 83.9% for the IL-12 level (Fig. 3D), respectively.

VU total extract reduced epidermal thickness and infiltration of eosinophils, mast cells, and degranulated mast cells

The dorsal skin samples from experimental mice were embedded, sectioned, stained with H&E or toluidine blue, and observed under a microscope. VU and prednisolone significantly alleviated AD-like histological changes in NC/Nga mice in a dose-dependent manner (Fig. 4A). Quantitative analysis revealed that the VU 90, 150, 250, and prednisolone groups had a 41.5%, 45.8%, 49.7%, and 49.5% reduction in epidermal thickness (Fig. 4B); 29.4%, 40.3%, 44.6%, and 29.9% reduction in eosinophil infiltration (Fig. 4C); 49.3%, 43.2%, 54.1%, and 41.8% reduction in mast

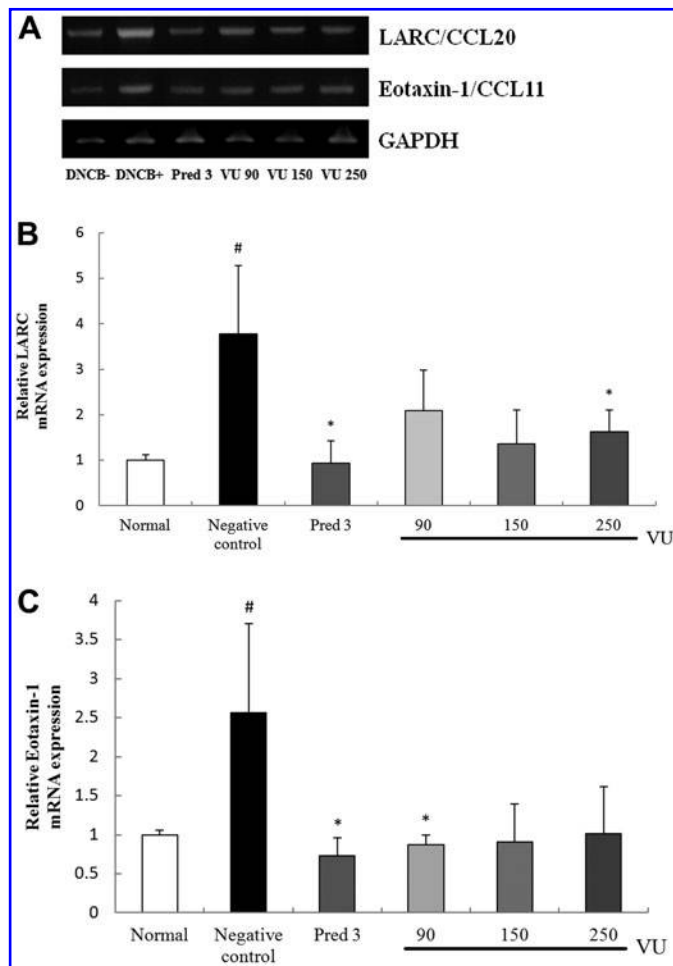


FIG. 6. VU total extract suppressed the expression of LARC/CCL20 and Eotaxin-1/CCL11 mRNA in dorsal skin. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with total RNA of dorsal skin. (A) RT-PCR gel images of LARC/CCL20, Eotaxin-1/CCL11, and GAPDH, (B) relative mRNA expression of LARC/CCL20, (C) relative mRNA expression of Eotaxin-1/CCL11. Data are expressed as mean $\pm$ SEM of each five mice per group. [#] $P < .05$ when compared with normal group, and ^{*} $P < .05$ when compared with negative control group.

cell infiltration (Fig. 5B); and 61.1%, 61.3%, 56%, and 45.8% reduction in degranulated mast cell infiltration, respectively (Fig. 5C).

VU total extract suppressed the expression of LARC/CCL20 and Eotaxin-1/CCL11 mRNA in dorsal skin

RT-PCR was conducted with total RNA using the primers listed in Table 1. VU (90, 150, 250 mg/kg·bw) and prednisolone reduced mRNA expression of LARC/CCL20 by 75.4%, 44.8%, 64%, and 56.83% (Fig. 6B) and Eotaxin/CCL11 by 71.4%, 66%, 64.5%, and 60.4%, respectively (Fig. 6C). The 4-week repeated treatment with prednisolone resulted in a significant reduction of the expression of both LARC/CCL20 and Eotaxin-1/CCL11. Both 90 and 250 mg/kg·bw of VU total extract significantly decreased the expression of LARC/CCL20 and Eotaxin-1/CCL11, respectively.

VU total extract and its subfractions directly suppressed IL-4 production in ConA-stimulated splenocytes isolated from AD-induced NC/Nga mice

ConA-stimulated splenocytes from AD mice were treated with 25, 50, 100, and 200 μ g/mL of VU total extract or subfractions of VU and incubated for 48 h. After incubation, the levels of IL-4 in supernatants were determined by the ELISA kit. VU extract and subfractions suppressed splenic IL-4 production in a dose-dependent manner. The decreases in IL-4 by 200 μ g/mL of the VU total extract or the polyphenol, anthocyanin, and sugar/acid subfractions were 21.9%, 30.3%, 30.3%, and 13.5%, respectively (Fig. 7). The effect of VU on IL-4 production was highest in the polyphenol subfraction followed by the anthocyanin subfraction and the total water extract and sugar/acid subfraction. The effect of the total extract, polyphenol subfraction, and anthocyanin subfraction on the inhibition of IL-4 production was significant. In contrast, the sugar/acid subfraction did not show any significant effect on the IL-4 production in splenocytes isolated from AD-induced NC/Nga mice.

DISCUSSION

AD is a chronic and relapsing form of dermatitis characterized by symptoms of eczematous and inflammatory skin lesions, highly elevated IgE levels, and itching.^{1,2} The previous findings that fruits of VU contain flavonoids and anthocyanins, which are known to have antiallergic or anti-inflammatory effects,⁹⁻¹³ led us to investigate the anti-AD effect of VU total extract. In a preliminary study, 150 mg/kg·bw of VU total extract significantly reduced AD-like symptoms in AD-induced NC/Nga mice, so we administered 90, 150, and 250 mg/kg of VU total extract (common ratio of 1.67) to AD-induced NC/Nga mice to investigate the

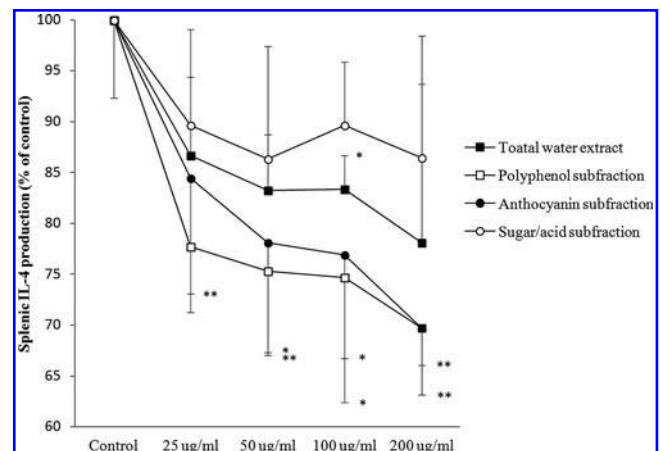


FIG. 7. VU total extract and subfractions of VU inhibit splenic IL-4 production. Splenocytes obtained from AD-induced NC/Nga mice were stimulated with 5 μ g/mL of conA and treated with VU total extract and subfractions (12.5, 25, 50, 100, and 200 μ g/mL). The level of IL-4 in supernatants was measured by the ELISA kit and values are calculated as % of negative control and expressed as mean $\pm$ SEM of each treated group ($n = 4$). ^{*} $P < .05$, ^{**} $P < .01$ when compared with negative control group.

dose–response relationship in the anti-AD effect of VU total extract. Oral administration of VU extract significantly alleviated the AD-like skin lesions and ear swelling in a time-dependent manner (Tables 3 and 4). We demonstrated that VU total extract suppresses inflammation in the chronic phase of AD. Itching and subsequent scratching behaviors play a critical role in AD pathogenesis, causing it to progress from the acute phase of AD to the chronic phase of AD through promoting skin damage in the patient.²³ The treatment with VU suppressed scratching behavior in a time-dependent manner (Table 5). These results demonstrate that VU total water extract alleviates itching and subsequent scratching behavior, which probably subsequently reduce skin damage and inflammatory responses. Highly increased Th2 cytokines, including IL-4 and IL-13, are known to promote the synthesis of IgE and IgG1 in mice.^{24,25} VU total water extract significantly reduced serum IgE and IgG1 levels (Fig. 2A, C). On the other hand, VU total water extract increased the serum level of IgG2a, the immunoglobulin isotype of Th1 immunity, in a dose-dependent manner (Fig. 2D). IgG2a and IgG1 are regarded as markers for Th1 and Th2 immune dominance, respectively,^{26,27} and the alleviating effect of the lowered serum IgG1/IgG2a ratio is currently accepted as modulating the Th2/Th1 imbalance caused by AD.^{28,29} The oral administration of VU significantly decreased the IgG1/IgG2a ratio (Fig. 2E). These results demonstrate that VU total water extract has a Th2/Th1 imbalance modulating effect. Histamine is released by the degranulation of mast cells and promotes allergic reactions.³⁰ VU total extract significantly decreased the serum histamine level (Fig. 2B). Because histamine is known to be the direct cause of itching symptoms in AD,^{31,32} this result is correlated with the above itching-alleviating effect of VU total water extract. High histamine levels of the normal group probably were caused by spontaneous onset of AD in NC/Nga mice due to aging.^{7,19} Th2 cytokines, including IL-4 and IL-13, are responsible for initiating the acute phase of AD through promoting Th2 allergic responses, and they act as chemokines for inflammatory cells.^{3,6} Th1 cytokines, including IL-12 and IFN- γ , are responsible for progression to the chronic phase of AD through promoting inflammation.^{2,5} VU dramatically suppressed the splenic production of both Th2 and Th1 cytokines in splenocytes to even below normal levels (Fig. 3A–D). A recent study reported that both Th1 and Th2 immune reactions are activated during the chronic phase of AD when compared with the healthy state through production of IFN- γ and IL-12 by IDECs.²⁹ Because both Th1 and Th2 cytokines are implicated in AD in the chronic state, a decrease in both Th1 and Th2 cytokines represents a reduction in the hyperimmune responses caused by the chronic phase of AD. The modulating effect of VU on the balance between Th1 and Th2 immune responses seems to have been demonstrated by the above serum IgG1/IgG2a ratio. Highly increased Th2 cytokines stimulate infiltration by inflammatory leucocytes, especially eosinophils and mast cells, in AD patients.^{5,20} Eosinophils play a crucial role in AD symptoms; they secrete eosinophil cationic proteins and thereby induce infiltration of other immune cells into the

skin lesions.^{33,34} In addition, infiltrated mast cells were degranulated to release histamine and nitric oxide, which promote severe itching.^{31,32} Treatment with VU alleviated histopathological AD-like symptoms in dorsal skin, including epidermal thickening (Fig. 4B) and infiltration of eosinophils (Fig. 4C), mast cells (Fig. 5B), and degranulated mast cells (Fig. 5C). The suppressed infiltration of eosinophils was correlated with reduced IL-4 and IL-13 production. This is supported by a recent study suggesting that Th2 cytokines are involved in eosinophil recruitment.^{33,34} Moreover, the suppressed degranulation of mast cells was correlated with the alleviation of the itching and scratching behavior of mice (Fig. 3). Many studies have revealed that the infiltration of inflammatory cells to AD skin lesions is dependent on the local production of various members of the chemokine family.^{35–37} The level of CCR3 expressed by eosinophils is especially increased in the skin lesions of AD patients, and its ligand, CCL11/Eotaxin-1, is considered to mediate the migration of eosinophils.^{35,36} It is also known that CCL20/LARC is a candidate for the recruitment of DC precursors from the circulation to sites of atopic skin inflammation.³⁷ The RT-PCR method was used, and changes in mRNA expression on chemokine ligands were analyzed. VU significantly reduced the mRNA expression of both LARC/CCL20 and Eotaxin-1/CCL11 (Fig. 6B, C). These results demonstrate that VU total extract decreases the mRNA expressions of chemokine ligands, which are responsible for recruiting DC precursors and eosinophils to dorsal skin. These results are coincident to the decreased eosinophil infiltration in dorsal skin (Fig. 4C). The development of AD is known to be caused primarily by the overproduction of Th2-mediated cytokines.³ In particular, IL-4, one of the major Th2 cytokines, acts as a Th2 response amplifier and chemoattractant for other Th2 cells.^{8,20} We previously showed that quercetin and cyanidine-3-O-glucoside are mainly included in the polyphenol and anthocyanin subfractions, respectively, and the mixture of polyphenol and anthocyanin significantly alleviates AD symptoms *in vivo*.³⁸ Thus, we investigated the effect of total water extract and subfractions of VU on IL-4 production in ConA-stimulated splenocytes. Treatment with total water extract and subfractions of VU significantly suppressed splenic IL-4 production in a dose-dependent manner (Fig. 7). Taken together, we concluded that the anti-AD effect of VU might be due to suppressed Th2 cytokines, subsequent downregulated IgE and IgG1 synthesis, and decreased recruitment of inflammatory cells. Throughout the study, dose–response relationships were weak. Possibly, the maximum effect has been already reached by the treatment with 90 mg/kg·bw VU total extract. A more detailed study may help to establish the dose–response relationship. Our study demonstrated that VU total water extract could be developed as an anti-AD agent.

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AUTHOR DISCLOSURE STATEMENT

There are no conflicts of interest by the authors of this article.

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