



Azuki bean (*Vigna angularis*) extract inhibits the development of experimentally induced atopic dermatitis-like skin lesions in NC/Nga mice

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ABSTRACT

The present study investigated the effects of azuki bean (*Vigna angularis*) extract (VAE) on the progress of atopic dermatitis (AD)-like skin lesions in NC/Nga mice induced by 1-chloro-2,4-dinitrobenzene. The efficacy of VAE in NC/Nga mice was determined by measuring gross and histological skin lesions, serum IgE levels, eosinophil ratio in peripheral leucocytes, and mRNA expression levels of interleukin (IL)-4, tumour necrosis factor (TNF)- α and interferon (IFN)- γ in splenocytes. Continuous ingestion of VAE inhibited the development of the AD-like skin lesions in a dose-dependent manner. In the VAE-treated mice, the numbers of mast cells in the skin, eosinophil ratio in peripheral leucocytes, relative mRNA expression of inflammatory cytokines in the spleen, and serum IgE levels were significantly reduced. Results suggest that VAE can inhibit the development of AD-like skin lesions in NC/Nga mice by regulating immune mediators and cells, and may be an effective alternative therapy for AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic, relapsing, and non-infectious disease in humans and animals, caused by a complex interrelationship among genetic, environmental, pharmacologic, psychological, immunological, and skin barrier dysfunction factors (Ginn, Mansell, & Rakich, 2007). In humans, AD has a characteristic morphology and age-related distribution pattern of the affected skin and is associated with pruritus (Liu, Goodarzi, & Chen, 2011). The acute lesions of AD include erythematous, edematous papules, and plaques with scaling, whereas chronic lesions appear as lichenified plaques with increased skin lesions on the face and flexural areas (Tong & Mihm, 1986). Specific immune and inflammatory mechanisms culminate into a complex series of cellular interactions leading to the signs and symptoms of AD (Liu et al., 2011). Acute AD skin lesions exhibit the important role of Th2 type inflammation such as the infiltration of CD4⁺ T cells and the secretion of interleukin (IL)-4, IL-5, and IL-13 (Jin, He, Oyoshi, & Geha, 2009; Leung & Bieber, 2003), whereas in chronic AD lesions, Th1 type immune responses such as the expression of interferon (IFN)- γ produced by Th1 cells, and delayed-type hypersensitivity reactions are responsible (Werfel et al., 1996). Most

AD patients showed peripheral blood eosinophilia and high serum IgE levels by the switching of B cells from IgM to IgE through the mediation of IL-4 upregulation (Carmi-Levy, Homey, & Soumelis, 2011; Leung & Bieber, 2003).

The prevalence of AD has been growing progressively at 2 to 3-fold increases during the past three decades in industrialised countries, with a lifetime prevalence in children of 10–20%, and a prevalence of 1–3% in adults (Leung & Bieber, 2003; Schultz-Larsen & Hanifin, 2002). As a result, topical glucocorticoids are used for treatment of AD as important and effective remedies. However, they can only be used for short periods of time and in limited skin regions, because prolonged use of high dose glucocorticoids causes a variety of potential side-effects (Barnetson & White, 1992). Therefore, complementary or alternative medicine (CAM), the main alternative resource being phytotherapy (natural extracts as medicine), has become increasingly popular for the treatment of AD, and has been used to satisfy the demands not found in orthodox medicine (Boneberger, Rupec, & Ruzicka, 2010). Consequently, the high prevalence of using CAM in AD patients necessitates further scientific research on their efficacy, safety, and use (Boneberger et al., 2010).

The azuki bean (*Vigna angularis*) is one of the most important crops in the Far East, and has been used in traditional Chinese medicine for various purposes; e.g., as an antidote, a diuretic, and a remedy for dropsy and beriberi (Itoh, Umekawa & Furuichi,

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2005). It has been reported that azuki bean extracts possess tumour-suppressive (Itoh et al., 2005), renal-protective (Sato et al., 2005), and anti-diabetes (Itoh et al., 2004) effects. Recently, azuki bean rich in polyphenol was reported to display anti-oxidative stress and anti-inflammation effects in spontaneously hypertensive rats (Mukai & Sato, 2011). However, little is known about the beneficial effects of azuki bean extract on AD.

The objective of this study was to evaluate the effects of *V. angularis* extract (VAE) administration on the progress of AD-like skin lesions in NC/Nga mice by continuous application of the hapten DNCB. VAE has shown excellent anti-AD activity and could, thus, be possibly applied to treat and prevent AD. This study may further provide insights on the potential of VAE as an integral part of a novel therapeutic modality for AD in humans or animals.

2. Materials and methods

2.1. Animals

Specific pathogen-free (SPF) 6-week-old female NC/Nga mice from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan) were randomized into four groups (Supplementary Table 1). Mice were acclimated for one week and fed a commercially prepared dry mouse feed. The mice were housed in polypropylene cages placed in an air-controlled room, with free access to their diet and tap water from drinking bottles with stainless steel sipper tubes. All animal procedures were conducted in accordance with the university guidelines of the local ethical committee at Chonnam National University [CNU IACUC-YB-2009-15].

2.2. Drug preparation and treatment

Azuki bean (*V. angularis*) was purchased from an herbal medicine store in Jeongeup, Korea. Dried azuki bean was pulverised to a fine powder, and extracted with 95% methanol (MeOH) for one week at room temperature. The VAE extracted by MeOH was concentrated on a rotary evaporator. VAE was dissolved in distilled water and was administered orally using an animal feeding needle to respective groups of mice. The experiment group received VAE in suspensions (50 mg/kg and 250 mg/kg doses) daily with a pre-treatment period of 2 weeks before induction of AD (Supplementary Table 1).

2.3. Food compositions analysis

The food composition analysis of VAE was commissioned to Scientec Lab Center Co., LTD in Daejeon, Korea. Crude ash, crude protein, crude fat, moisture, and total nitrogen contents were determined according to food ingredients test methods of Korean Food Standards Codex (KFSC, 2011). The carbohydrate content was determined by calculating the percent remaining after all the other components have been measured: % carbohydrates = 100 – (% moisture – % crude protein – % crude fat – % crude ash).

2.4. Induction of atopic dermatitis

1-Chloro-2,4-dinitrobenzene (DNCB) (Sigma–Aldrich, St. Louis, MO, USA) was used to induce AD-like dermatitis in the positive control group and treatment groups of NC/Nga mice (Supplementary Table 1) through repeated topical application as described elsewhere (Jung, Cho, Koh, Han, & Lee, 2010). Briefly, mice were anesthetized and the dorsal skin regions from the neck to the base of the tail were shaved with electric razors. DNCB was dissolved in an acetone/olive oil mixture (3:1) and spread on the dorsal skin

and ear of mice. Initially, 1% DNCB solution was used to sensitise the ear and skin, and 4 days later, 0.2% DNCB was applied twice a week for 21 days. Peripheral blood, skin, and spleen samples were collected for further analysis at the end of the experiment.

2.5. Evaluation of clinical skin severity

Clinical skin severity was evaluated by scoring clinical signs on the face, ears, nose, neck, and dorsal skin twice a week starting from day 1 of DNCB treatment (Jung et al., 2010). A severity index with four criteria was utilised: erythema (redness, haemorrhage), edema (swelling), excoriation (erosion), and dryness (scaling, crusting). The grading for each individual criteria ranged from 0–3 (none = 0, mild = 1, moderate = 2, and severe = 3). The clinical skin severity score was the sum of these individual criteria.

2.6. Histological analysis of skin lesions

Upon euthanasia, skin samples from the dorsal area and ears were collected. The samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 µm, and stained with haematoxylin and eosin (H&E). Pathologic changes (epidermal and dermal hyperplasia, hyperkeratosis, acanthosis, spongiosis, vesicular formation, and inflammation) were evaluated and compared between groups. Selected sections were stained with toluidine blue for evaluation of mast cell infiltration. Mast cell numbers were counted at five random sites per 400× field.

2.7. Measurement of eosinophil ratios in peripheral leucocytes

Peripheral blood was collected in BD Microtainer® tubes coated with ethylene di-aminetetraacetic acid (BD, Franklin Lakes, NJ, USA) at the end of the experiment. Blood leucocyte and differential counts were determined using an automated Veterinary Hematology Analyzer (Oxford Science, Oxford, CT, USA).

2.8. Measurement of IgE concentration in serum

Blood from the orbital sinus was collected from each mouse at the end of the experiment. Serum was obtained by centrifugation at 1000g for 10 min and stored at –80 °C until used. Total serum IgE levels were measured using a BD OptEIA™ Mouse IgE ELISA Set (BD, Sparks, MD, USA) according to the manufacturer's instructions.

2.9. Measurement of relative mRNA expression levels of IL-4, IFN-γ and TNF-α in spleen

Splenocytes were isolated under sterile conditions as described elsewhere (Cho, Baik, Jung, & Park, 2000). Total RNA extraction was performed using Tri Reagent® RNA Isolation Reagent (Molecular Research Center, Cincinnati, OH, USA). Target RNA was reverse transcribed using QuantiTect® Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. All samples were transcribed concurrently to reduce discrepancies in reverse transcriptase efficiency. Real-time PCR primers and probes for murine IL-4, IFN-γ, TNF-α and β-actin were designed as described elsewhere (Dijkmans et al., 1985; Overbergh et al., 2003; Sideras et al., 1987; Tokunaga, Taniguchi, Yoda, Shimizu, & Sakiyama, 1986). The sequences of primers used are shown in Supplementary Table 3. The probes were dual labelled with the reporter dye 6-carboxyfluorescein at the 5' end and 6-carboxytetramethylrhodamine at the 3' end. IFN-γ, IL-4 and TNF-α mRNA expression levels were quantified by a real-time PCR assay using Rotor-gene 6000 (Corbett Research, Sydney, NSW, Australia) with 1 µg of cDNA. The threshold cycle (Ct; the number of cycles required for the

fluorescent signal to cross a fixed threshold) was determined. Relative quantitation of target genes of IL-4, IFN- γ and TNF- α was normalised to an internal control gene, β -actin, relative to a calibrator. It was expressed as $2^{-\Delta\Delta Ct}$ (fold), where $\Delta Ct = Ct$ of target gene (IL-4, IFN- γ or TNF- α)- Ct of internal control gene (β -actin). $\Delta\Delta Ct = \Delta Ct$ of samples for target gene- ΔCt of the calibrator for the target gene.

2.10. Statistical analysis

The data gathered from this experiment were written as mean $\pm$ standard deviation (SD). To compare between groups, means of the different parameters were analysed for variance. In order to evaluate the significant differences between the groups, data was analysed with Tukey's multiple comparison tests using Minitab Statistical Software 13.20 (Minitab, State College, PA, USA), and the level of significance was selected at $P < 0.05$.

3. Results

3.1. Food composition of VAE

Food composition of VAE was determined according to KFSC methods (2011). As shown in [Supplementary Table 2](#), the VAE was rich in carbohydrate (37.09%) and crude fat (38.06%). In addition, the crude ash and crude protein contents were 6.20% and 6.02%, respectively.

3.2. VAE inhibited development of AD-like skin lesions in the NC/Nga mice

To evaluate preventive and curative effectiveness of VAE against AD-like skin lesions, mice received 50 mg/kg or 250 mg/kg VAE per day before induction of AD-like skin lesions by DNCB treatment. At regular 3–4 day intervals, all mice were treated with DNCB for 21 days to induce AD-like skin lesions. Clinical skin lesions and mean clinical severity score of DNCB-treated positive control, and DNCB and 50 mg/kg VAE-treated groups showed increasing clinical skin lesions and severity scores ([Figs. 1 and 2](#)). Mice treated with DNCB and 250 mg/kg VAE revealed only mild dryness and redness, and low clinical severity scores, compared to the positive control, and DNCB and 50 mg/kg VAE-treated groups ([Figs. 1 and 2](#)). Negative control showed no clinical skin lesions. Statistically, the

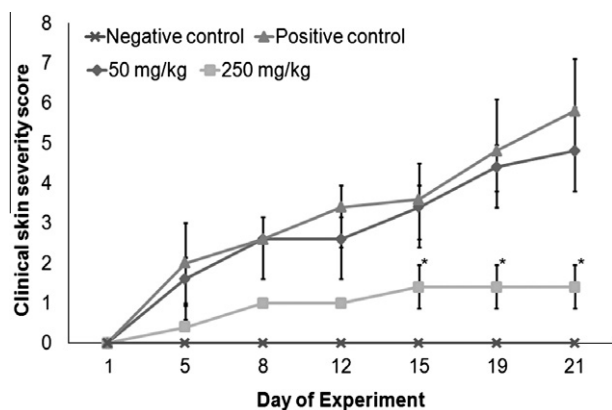


Fig. 1. Effects of azuki bean extract on clinical skin features during 21-day atopic dermatitis-like lesion induction. Clinical skin severity of dermatitis was defined as the sum of individual scores graded as 0 (none), 1 (mild), 2 (moderate), 3 (severe) for 4 criteria observed (erthyema, edema, excoriation, dryness). Atopic dermatitis was induced by topical application of DNCB, at 1% on day 1 and 0.2% (in acetone 3:olive oil 1) on subsequent days at 3–4 day intervals. 250 mg/kg treated groups showed significant lower clinical severity scores compared to the positive control and 50 mg/kg treated groups (* $P < 0.05$).

250 mg/kg group showed significant differences from day 15–21 of the experiment ($P < 0.05$), in contrast to the 50 mg/kg, which did not show any statistically significant differences when compared to the positive control group.

3.3. VAE decreased histological skin lesions and mast cell infiltration in the NC/Nga mice with AD-like skin lesions

[Supplementary Table 4](#) shows epidermal thickness in ear and dorsal skins sampled from non-treated negative, DNCB-treated positive, and DNCB and VAE-treated groups. Compared to the non-treated negative control group, skin lesions of the positive control group showed marked hyperkeratosis, acanthosis, spongiosis, intra-epidermal vesicle formation, elongation of rete ridges, dermal infiltration of lymphoid cells, and dermal fibrosis ([Supplementary Table 4](#) and [Fig. 3](#)). With increasing VAE, these skin lesions gradually decreased, particularly in the ear; marked decrease of skin lesions in the mice treated with 250 mg/kg VAE was evident, in comparison with those of the positive control group ([Supplementary Table 4](#) and [Fig. 3](#)).

To compare the mast cell numbers in the skin between the positive control and VAE-treated groups, selected skin sections were stained with toluidine blue. Mice treated with only DNCB had marked infiltration of mast cells in the dermis and subcutis, whereas VAE-treated groups showed a decreasing number of mast cells ([Fig. 4](#)). As shown in [Supplementary Table 4](#), mast cell numbers were significantly lower in the treatment groups compared to the positive control group ($P < 0.05$).

3.4. VAE reduces the peripheral blood eosinophil ratio in the NC/Nga mice with AD-like skin lesions

To compare the peripheral eosinophil ratios among each group, EDTA-treated blood samples were measured using a Veterinary Hematology Analyzer. Mean eosinophil counts of each group showed that VAE-treated groups had decreasing levels of circulating eosinophils ([Fig. 5](#)). The 250 mg/kg group had significantly lower eosinophil ratios (%) compared to the positive control and 50 mg/kg VAE-treated groups ($P < 0.05$).

3.5. VAE diminished the IL-4, IFN- γ and TNF- α mRNA expression levels in the splenocytes from the NC/Nga mice with AD-like skin lesions

To compare the IL-4, IFN- γ and TNF- α mRNA levels expressed in the splenocytes among each group, relative mRNA expression levels with β -actin were evaluated by real-time PCR. The positive control group had higher mRNA levels of IL-4, IFN- γ and TNF- α , compared to the treatment groups ([Fig. 6](#)). The 250 mg/kg VAE-treated group showed significantly lower IL-4, IFN- γ and TNF- α mRNA expression levels compared to the positive control and 50 mg/kg VAE-treated groups ($P < 0.05$).

3.6. VAE declined the serum IgE levels in the NC/Nga mice with AD-like skin lesions

To evaluate the statistical significance of the serum IgE levels between the positive control and VAE-treated groups, sera sampled from each group at the end of the experiment served to measure total serum IgE levels. [Fig. 6D](#) shows serum IgE levels in the positive control and VAE-treated groups. With increasing VAE treatment, the serum IgE levels in the VAE-treated groups decreased in comparison with those of the control group; comparatively marked decrease of serum IgE levels in 250 mg/kg VAE-treated group, compared with the control and 50 mg/kg VAE-treated groups ($P < 0.05$).

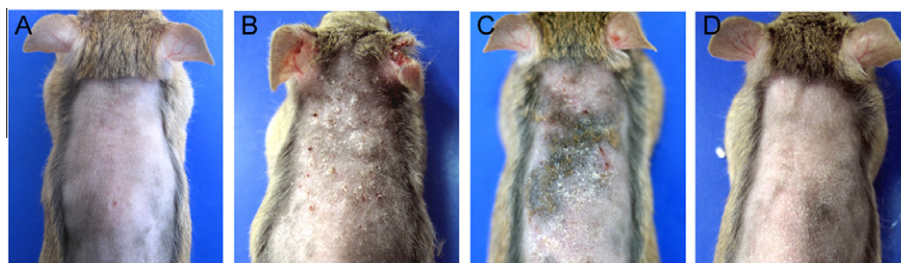


Fig. 2. Comparison of gross atopic dermatitis-like skin lesions between the control and treated groups. (A) Negative control group, (B) Positive control group, (C) 50 mg/kg treated group, (D) 250 mg/kg treated group. Severity of dermatitis was markedly inhibited in the 250 mg/kg group compared to the positive control and 50 mg/kg treated groups after 21 days of treatment.

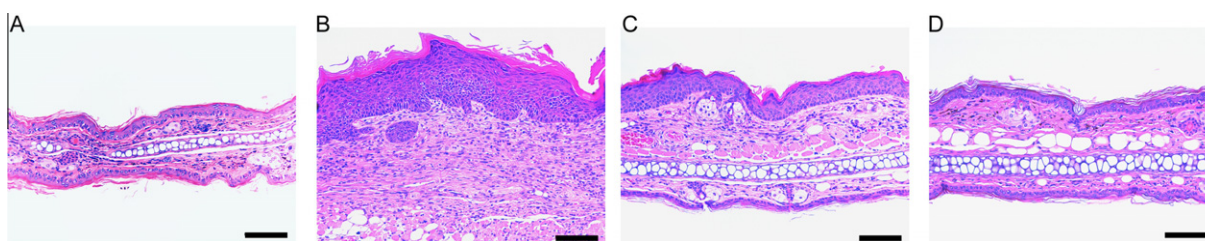


Fig. 3. Comparison of histological atopic dermatitis-like skin lesions between the control and treated groups. (A) Negative control group, (B) Positive control group, (C) 50 mg/kg treated group, (D) 250 mg/kg treated group. Hyperkeratosis and elongation of rete ridges were observed in the control group but were decreased in drug treated groups. Decreasing epidermal and dermal thickness was also observed in drug treated groups. H&E stain. Bar = 100 μ m.

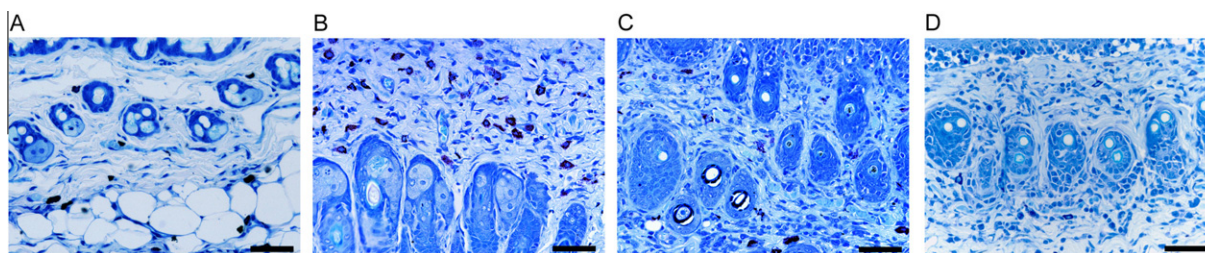


Fig. 4. Comparison of mast cell numbers in the dermis and subcutis between the control and treated groups. (A) Negative control group, (B) Positive control group, (C) 50 mg/kg treated group, (D) 250 mg/kg treated group. Drug-treated groups showed less mast cell infiltration compared to the control group. Toluidine blue stain. Bar = 50 μ m.

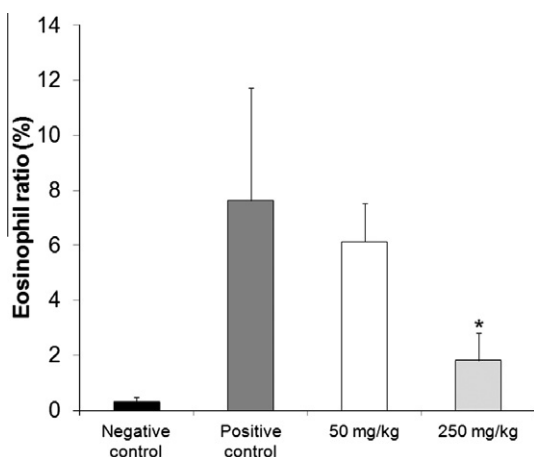


Fig. 5. Comparison of eosinophil ratios in peripheral leucocytes between the control and treated groups. Eosinophilia was observed in the positive control group but was significantly decreased in the 250 mg/kg group (* $P < 0.05$).

4. Discussion

Since AD-like skin lesions in NC/Nga mice are very similar to those of human and canine AD (Jin et al., 2009), NC/Nga mice are frequently used to study the mechanisms of AD development, and for evaluating the effects of various drug candidates against AD (Jin et al., 2009). Therefore, this animal model was used to evaluate the effects of VAE on the progress of AD-like skin lesions by continuous application of the hapten derivative, DNCB. Positive control mice showed high grades of clinical features such as severe erythema, haemorrhage, superficial erosion, deep excoriation and dryness, which are consistent with another report (Jin et al., 2009).

Presently, oral administration of VAE to NC/Nga mice treated with DNCB tended to suppress the development of AD-like skin lesions. Although mice treated with 50 mg/kg VAE showed similar clinical features like the positive control group, mice treated with 250 mg/kg VAE were able to maintain skin integrity throughout the course of induction with only minimal symptoms of dryness. Since an epidermal barrier defect occurring in AD leads to transepidermal water loss, decreased irritancy threshold and dryness, maintenance of epidermal skin barrier condition is an important

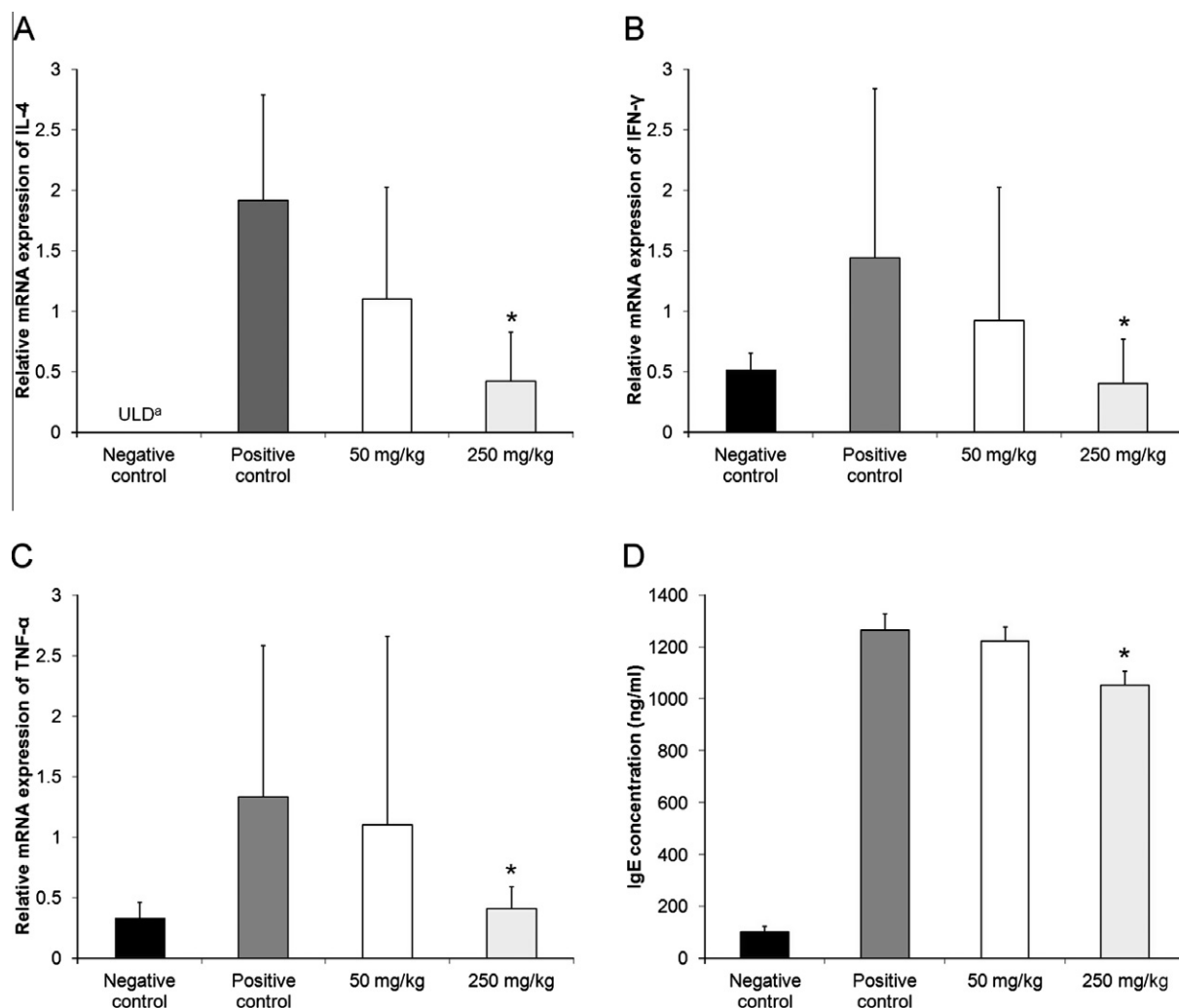


Fig. 6. Relative mRNA expression of IL-4, IFN- γ and TNF- α in the spleen, and IgE concentration in the serum. (A) Relative mRNA expression of IL-4 in the spleen, (B) Relative mRNA expression of IFN- γ in the spleen, (C) Relative mRNA expression of TNF- α in the spleen, (D) IgE concentration in the serum. Relative expression of murine cytokines IL-4, IFN- γ and TNF- α in the spleen was evaluated using real-time PCR with Taqman probes relative to a housekeeping gene, β -actin. Results showed significantly reduced expression of IL-4 (A), IFN- γ (B), and TNF- α (C) in the 250 mg/kg group as compared to the control group (* $P < 0.05$). ^aULD: under limit of detection. (D) Serum IgE concentration was measured by ELISA. Higher levels of IgE were found in the positive control group but was significantly decreased in the 50 mg/kg and 250 mg/kg group (* $P < 0.05$).

aspect of AD therapy (De Benedetto, Agnihothri, McGirt, Bankova, & Beck, 2009). Therefore, the ability of VAE to maintain skin integrity may possibly denote that it helps in maintaining skin barrier condition by preventing enhanced transepidermal water loss and dryness, and by decreasing irritancy threshold. Histological examination of skin lesions illustrated that most skin areas of positive control group were thickened because of epidermal and dermal hyperplasia, and consequent infiltration of immunocytes such as leucocytes, mast cells and eosinophils. In contrast, these histological skin changes were gradually decreased with increasing VAE; the 250 mg/kg treatment group had less histopathological skin lesions, with minimal dermal and epidermal thickness, as well as decreased manifestation of acanthosis and spongiosis. Like other oriental and herbal therapies to AD (Boneberger et al., 2010), these results prove the inhibitive effects of VAE on the progress of AD-like skin lesions in the NC/Nga mice.

Patients with AD and NC/Nga mice with AD-like skin lesions have strong expression of Th2-type cytokines, such as IL-4 in the blood and T cells in the regional lymph node and spleen (Leung & Bieber, 2003). Consequently, increased IL-4 upregulates the total serum IgE, a hallmark of AD, by the switching of B cells from IgM to

IgE (Carmi-Levy et al., 2011). In the present study, total IL-4 mRNA levels along with TNF- α in the splenocytes from the 250 mg/kg treatment group were significantly lower than those in the positive control and 50 mg/kg treatment groups. Moreover, total serum IgE levels were markedly lower in the 250 mg/kg treatment group than those in the positive control and 50 mg/kg treatment groups. Like natural substances from plants or herbal therapy to AD (Jung et al., 2010; Lee et al., 2007), these results indicate that VAE can decrease the secretion of Th2-type cytokines such as IL-4 and TNF- α , and subsequently reduce the IgE production by the inhibition of the switching of B cells from IgM to IgE.

In chronic AD lesions, numbers of mast cells are significantly increased, especially in areas of lymphocytic infiltration in the papillary dermis (Irani, Sampson, & Schwartz, 1989). These AD skin lesions usually show intensive degranulation of mast cells so that mast cells may serve as a marker of AD severity (Irani et al., 1989). Previous studies have shown that natural extracts such as from *Cordyceps bassiana* can suppress activation and migration of mast cells in the AD-like skin lesions in NC/Nga mice (Jung et al., 2010; Lee et al., 2006; Wu et al., 2011). In the present study, the number of mast cells in the skin was also significantly diminished in the

250 mg/kg treatment group in comparison with those in the positive control and 50 mg/kg treatment groups. It is well known that increased IgE facilitates the development of AD through mediating activation of mast cells localised in the skin induced by various allergens (Liu et al., 2011). Therefore, it is conceivable that VAE may regulate the downstream pathway of mast cell activation by inhibition of mast cell IgE sensitisation and release of inflammatory mediators.

Eosinophils are most commonly known for their role in defense against parasites and mediators of allergy and asthma along with mast cells and basophils (Liu et al., 2011). Eosinophilia has been shown to be present in most AD patients and NC/Nga mice with AD-like skin lesions and correlates with the disease activity (Matsuda et al., 1997). The present group treated with 250 mg/kg VAE showed lower eosinophil ratio in peripheral leucocytes than that of positive control and 50 mg/kg VAE-treated groups. This result implies that VAE can alleviate AD-like skin lesions by inhibition of eosinophil recruitment.

Generally, Th1 cytokines, especially IFN- γ , are strong inhibitors of IgE synthesis, Th2 cell proliferation and IL-4 receptor expression on T cells (Leung & Bieber, 2003). In the present study, however, VAE administration unexpectedly decreased the mRNA expression levels of IFN- γ along with the IL-4 and TNF- α in the splenocytes compared to the positive control group. It has also been reported that, depending on the different oriental or herbal treatment, expression levels of Th1 and Th2 cytokines are different in the mice with AD-like skin lesions (Jung et al., 2010; Lee et al., 2006, 2007). Although the precise role of VAE on IFN- γ expression is unknown, the inhibitory effect on IFN- γ expression by VAE administration may ameliorate DNCB induced AD-like skin lesions in NC/Nga mice (Lee et al., 2007).

Azuki bean is known to have plentiful polyphenols such as proanthocyanidins and quercetin glycoside (Yoshida, Knodo, Ito & Kondo, 2005). The polyphenol-containing azuki bean (*V. angularis*) extracts inhibit the elevation of blood pressure and macrophage infiltration in the heart and kidney of spontaneously hypertensive rats (Sato et al., 2008). Moreover, polyphenol-enriched apple extract decreases food allergy in mice (Zuercher, Holvoet, Weiss, & Mercenier, 2010). It is plausible that polyphenols rich in azuki beans may attenuate the development of AD. However, it remains unclear which component(s) would be effective for AD because VAE contains a complex array of compounds. Therefore, precise knowledge of the major component(s) of azuki bean extract responsible for its inhibitory effects on AD-like skin lesions is needed and studies are currently in progress.

In conclusion, oral administration of VAE at 250 mg/kg dose inhibited the development of AD-like skin lesions in NC/Nga mice. On the basis of our results, VAE can suppress inflammatory responses occurring in AD by modulating Th1 and Th2 responses and diminishing the mast cell infiltration in the skin lesions. This study also furthers knowledge of the therapeutic effects of remedies from natural resources, and that VAE may further be considered as an integral part of numerous therapeutic options in humans and animals with AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.11.100.

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Supplementary Table 1.

Group	No. of mice	Age/sex	DNCB treatment*	VAE treatment*
1	5	7 weeks old/female	-	-
2	5	7 weeks old/female	+	-
3	5	7 weeks old/female	+	50 mg/kg
4	5	7 weeks old/female	+	250 mg/kg

*-: not treated, +: treated.

Supplementary Table 2.

Food component	Percentage (% dry weight)
Carbohydrate	37.09
Moisture	12.63
Crude ash	6.20
Crude protein	6.02
Crude fat	38.06
Total nitrogen	0.97

Supplementary Table 3.

Cytokine*		Primer sequence (5'-3')	Length	Reference
IL-4	Forward	ACAGGAGAAGGGACGCCAT	92 bp	Dijkmans et al., 1985
	Reverse	GAAGCCCTACAGACGAGCTCA		
	TaqMan probe	TCCTCACAGCAACGAAGAACACCACA		
IFN-γ	Forward	TCAAGTGGCATAGATGTGGAAGAA	92 bp	Sideras et al., 1987
	Reverse	TGGCTCTGCAGGATTTTCATG		
	TaqMan probe	TCACCATCCTTTTGCCAGTTCCTCCAG		
TNF-α	Forward	CATCTTCTCAAAATTCGAGTGACAA	175 bp	Overbergh et al., 2003
	Reverse	TGGGAGTAGACAAGGTACAACCC		
	TaqMan probe	CACGTCGTAGCAAACCACCAAGTGGA		
β- actin	Forward	AGAGGGAAATCGTGCGTGAC	148 bp	Tokunaga et al., 1986
	Reverse	CAATAGTGATGACCTGGCCGT		
	TaqMan probe	CACTGCCGCATCCTCTTCCTCCC		

TaqMan probe dual-labeled with 5' FAM (reporter dye) and 3' TAMRA (quencher dye)

*IL-4, interleukin- 4; IFN- γ , interferon- γ ; TNF- α , tumor necrosis factor α .

Supplementary Table 4.

	Negative control	Positive control	50 mg/kg	250 mg/kg
Epidermal thickness (µm)				
Dorsal skin	11.7 ± 1.99	69.72 ± 9.59	65.64 ± 1.73	31.56 ± 6.9*
Ear skin	18.3 ± 2.25	62.3 ± 6.20	42.16 ± 8.26*	29.5 ± 5.56*
Mast cell number	8.4 ± 0.96	19.27 ± 3.10	9.70 ± 2.30*	8.70 ± 2.58*

*P<0.05 versus control. Data are presented as mean ± SD.