



Inhibitory effects of cultured *Dendrobium tosaense* on atopic dermatitis murine model

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ABSTRACT

Dendrobium tosaense is one of the most valuable Chinese medicines and well developed health food. Atopic dermatitis (AD) is a chronic skin disease that occurs mainly in childhood. The pathogenesis of atopic dermatitis had been studied in BALB/c mice modeling by skin-inoculated ovalbumin (OVA) with 2,4,6-trinitro-1-chlorobenzene (TNCl). These mice exhibit features of chronic dermatitis, including skin rash, mast cells infiltration, and elevated serum anti-OVA specific IgE and cytokines modulation. In this study, a standardized ethyl acetate extract of *D. tosaense* (DTE) was used to protect these mice from the OVA/TNCl-induced skin lesions of atopic dermatitis. The results indicated an increased population of natural T regulatory cell was accompanied by immunosuppression in cytokine profiles and anti-OVA IgE level to significantly reduce Th2 polarization. Finally, toluidine blue staining indicated mast cell infiltration and degranulation was reduced in skin lesion. Our results were shed light on the usage of *D. tosaense* in AD.

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1. Introduction

According to the World Health Organization survey, about 70–80% of the world's population depends on alternative medicine for their primary healthcare. (Cordeiro and Oliveira, 2005; Rolim et al., 2006) The herbal extractions contain anti-inflammatory, antioxidant, antiallergic and anti-diabetic activities (Ribnický et al., 2009). *Dendrobium* species are one of the leading cut and potted orchids grown in the tropics. The genus *Dendrobium* (Orchidaceae)

includes about 1600 species (Zhou, 1993), 15 of which are found in Taiwan (Su, 1978). Dried or fresh stems of several *Dendrobium* species are using to prepare “Shi-hu” (*Dendrobium* spp. or Herba *Dendrobii*), which is widely used in both traditional Chinese and folk remedies for antipyretic, ophthalmic, and tonic purposes (Li et al., 2008). *Dendrobium* species are known to contain a number of substances, such as flavonoids (Chang et al., 2010; Yang et al., 2006), phenanthrenes (Honda and Yamaki, 2000; Lin et al., 2001), bibenzyls (Majumder et al., 1999; Zhang et al., 2006a, 2007), fluorenone and sesquiterpenes (Yamaki and Honda, 1996; Zhang et al., 2007), alkaloids (Wang et al., 2010), and polysaccharides (Chen et al., 2012), which are responsible for their wide variety of medicinal properties. In recent years several reports have published on these compounds possess anti-inflammatory (Li et al., 2009a), antioxidant (Miyazawa et al., 1999), anti-cancer (Tsai et al., 2010), anti-aging, anti-diabetic properties (Chang et al., 2010) and antiallergic activities (Majumder et al., 1999). The species *Dendrobium tosaense* MAKINO used as one of important ingredient of “Shi-hu” and also consumed as functional health food in Taiwan. In the help of the Council of Agriculture, farmers used tissue culture propagation and large-scale cultivation of *Dendrobium tosaense* in green house. It is domestically cultivated and has free radical scavenging activity

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due to its quercetin content (Lo et al., 2004a). Quercetin, 3,3',4',5,7-pentahydroxyflavone, is a typical flavonoid ubiquitously present in vegetables and fruits, and its antioxidant effect is implied to be helpful for human health. It occurs mainly in leaves and in the outer parts of the plants as aglycones and glycosides, in which one or more sugar groups is bound to phenolic groups by glycosidic bond (Casagrande et al., 2007; Montenegro et al., 2007). Recently, many research has been focused on the application use of flavonoids to treat skin disease (Gregoris et al., 2011).

Atopic dermatitis (AD) is an allergic disease of unknown origin that usually starts in early infancy; it is characterized by pruritus, eczematous lesions, xerosis, and lichenification. AD was associated with other atopic diseases such as asthma, allergic rhinitis, urticaria, acute allergic reactions to foods and is a pervasive health problem worldwide (Li et al., 2009b; Pople and Singh, 2010). The incidence of AD is increasing, and about 10–20% of children are affected. Though the pathogenesis of this allergic disease is still being researched, its primary causes are associated with genetic factors, environmental interactions, skin barrier disorders or immunological reactions (Abramovits, 2005; Wuthrich et al., 2007). Mast cells are key effector cells in IgE-mediated allergic disorders and are activated by cross-linking of a high affinity IgE receptor (FcεRI) on their surface. Mast cells undergo degranulation on activation and release variety of biologically active substances including platelet activating factor (PAF), histamine, leukotriene C4, leukotriene D4 and prostaglandin E2 (PGE2), they play important role in allergic reactions (Zhang et al., 2007). Vascular endothelial growth factor (VEGF) is a potent agent that causes hyperpermeability of blood vessels and endothelial cell proliferation. The production of VEGF is enhanced in AD, and might be involved in the persisting erythema and edema (Jung et al., 2010; Zhang et al., 2006b). AD is commonly associated with allergen (dust mite, ovalbumin, sea food or fungus) related IgE mechanisms, and the vast majority of patients with AD exhibit over-expression of cytokines and increase IgE production. These cytokines like interleukin-4 (IL-4), IL-5 and IL-13, are produced by type 2 T helper cells (Th2). In addition to increased Th2 cells and type-2 cytokines, most AD patients have decreased levels of interferon gamma (IFN-γ) and IL-12, cytokines that produce a type 1 environment that is capable of counteracting Th2 responses. It is well established that a Th1/Th2 imbalance skewed toward Th2 plays an important role in the development of AD (Choi et al., 2008; Kang et al., 2010; Lee et al., 2006).

In this study, we tested the ability of ethyl acetate extract of *D. tosaense* (DtE) to prevent the IgE-related inflammatory response seen in AD. We simulated AD by using the ovalbumin (OVA) with 2,4,6-trinitrochlorobenzene (TNCB)-sensitized dermatitis murine model and studied the *in vivo* effects of DtE on the production of specific anti-OVA antibodies, Th1/Th2 cytokines, Treg modulation, and mast cell degranulation.

2. Materials and methods

2.1. Preparation of *D. tosaense* extract

Cultured *D. tosaense* (Dt) plants were propagation by tissue culture and grown in the green house of Chaoyang University of Technology to one year (Lo et al., 2004b,c). This plant was identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University (plant specimen number: CMC DT 0303) and genetic identification by internal transcribed spacer (GenBank accession number: HM590367). Dried stems of Dt was grinded into fine powder, about 30 g of powdered Dt was dissolved in 300 ml of methanol and sonicated at an amplitude of 15 for 10 min. The methanolic extract was partitioned for three times with n-hexane followed by

ethyl acetate (EA), chloroform and finally with water. All the fractions were filtered and evaporated under reduced pressure to yield a crude residue. The quercetin content was confirmed by LC/MS (data not shown) and maximum quercetin content was found in EA fraction (DtE), hence EA fraction was further used for this study. The average quercetin content in EA fraction of DtE was 650 μg/g dry weight of the sample.

2.2. Animals and 2,4,6-trinitrochlorobenzene (TNCB) sensitization and challenge

Female BALB/c mice weighing 18–22 g each obtained from National Laboratory Animal Center (Taiwan) and maintained for 2 weeks to acclimatized laboratory conditions before the start of the experiments. They were 8 weeks old at the time of experiments. The animals were housed in plastic cages and maintained at 21 °C to 24 °C with a 12/12 h dark/light cycle. The animals were supplemented with standard sterile rodent chow diet (Test-Diet 5010, Ralston-Purina, St. Louis, MO) and distilled water *ad libitum*. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the Tunghai University.

Allergic modeling mice were modified by Dai et al., and Kigasawa et al. (Dai et al., 2002; Kigasawa et al., 2010) briefly, mice were sensitized to produce allergic skin lesions by applying 7% TNCB dissolved in acetone:olive oil (4:1) and 25 μg ovalbumin (OVA, grade V, Sigma-Aldrich, San Diego, USA) on topically to the dorsal skin (100 μl) for 7 days. After 7 days modeling, DtE at a dose of 30, 100 and 300 mg/kg or quercetin (1.6 mg/kg) dissolved in CMC (carboxymethyl cellulose) gel and orally administered via gastro-gavage throughout the experimental period (Senthilkumar et al., 2008). Sterilized CMC gel (10 ml/kg) was orally administered after OVA/TNCB treatment as control group, and without OVA/TNCB or drug treatment was given as “naïve” group. After the 7 days of DtE administration period, blood sampled from axillary plexus of mouse was used for immunologic or hematologic analysis, and skin tissue was sampled and fixed with 10% neutral buffered formalin solution for histopathological examinations, splenocyte was harvest and stimulated by mitogen (ConA, 5 μg/ml) for 24–72 h and the supernatant was used to analyzed cytokines (IL-4, IL-6 and IFN-γ) by ELISA.

2.3. Ovalbumin-specific antibodies in serum

Ovalbumin-specific antibodies were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) method, as previously described (Hsieh et al., 2010). In brief, different dilutions of serum 1:20–1:100 were added in duplicate into ELISA plates coated with OVA (20 μg/ml in 0.05 M carbonated buffer, pH 9.5) and incubated overnight at 4 °C. The plates were washed and incubated with HRP-conjugated goat anti-mouse polyclonal IgE and IgG1 (Bethyl Laboratories, Inc. Montgomery, TX, USA) for 1 h, followed by washing and developed with SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). The colorimetric reaction was measured at 450 nm and level of OVA-specific IgE and IgG1 were calculated with high-titer of reference serum by our previously study (Hsieh et al., 2010). The calculated equation is list below:

$$\text{ELISA units, EU} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{reference}} - A_{\text{blank}})}$$

Table 1

Serum level of IgG1 and IgE in mice with OVA/TNCB induced AD in the treatment of DtE and quercetin.

Treatment	Anti-OVA antibodies (ELISA unit, EU)	
	IgG1	IgE
Naïve	0.16 ± 0.02	0.31 ± 0.01
OVA/TNCB modeled AD		
Control	0.56 ± 0.10 ^{###}	0.45 ± 0.04 ^{###}
DtE, 30 mg/kg	0.36 ± 0.03 ^{**}	0.34 ± 0.02 ^{**}
DtE, 100 mg/kg	0.43 ± 0.04	0.32 ± 0.02 ^{**}
DtE, 300 mg/kg	0.43 ± 0.03	0.32 ± 0.02 ^{***}
Quercetin, 1.6 mg/kg	0.38 ± 0.04 [*]	0.27 ± 0.01 ^{***}

Data present as mean ± SE ($n = 5$). The allergic antibodies secretion in OVA/TNCB modeled AD mice could significantly increase the IgG1 and IgE levels (^{###}, $P < 0.001$). When mice administered with DtE (30 and 100 mg/kg) for 1 week, IgE levels could significantly decrease (^{**}, $P < 0.01$). And mice administered with DtE (300 mg/kg), IgE level could significantly decrease (^{***}, $P < 0.001$) indicated DtE might be play the anti-allergic potential. Naïve: without atopic dermatitis induction, Control: CMC administration after atopic dermatitis induction, Quercetin: quercetin treatment after atopic dermatitis induction, Experiment: different doses of DtE treatment after atopic dermatitis induction.

2.4. T cell subpopulation evaluation in splenocytes, skin lesion-draining lymph nodes

Leukocytes from spleen and skin lesion-draining lymph nodes were determined by the detection of surface markers with specific binding of monoclonal antibodies (mAb) as recommended by the Becton-Dickinson Monoclonal Center (FACScan, BD Biosciences, Mountain View, CA, USA) and described previously (Hsieh et al., 2010). In brief, 50 μ l of cell suspension (5×10^5 cells) were incubated in the presence of saturating concentrations of fluorescein (FITC), phycoerythrin (PE) or PE-Cy5 conjugated mAb (eBioscience, San Diego, CA USA) on ice for 30 min in the dark. Cells were washed with Dulbecco's modified phosphate buffered saline (DPBS) containing 0.1% sodium azide. After centrifugation ($300 \times g$) at 4 °C for 10 min, the cell pellet was resuspended in 200 μ l of analysis buffer (DPBS containing 2% FBS and 0.1% sodium azide). Cytofluorometric analysis was performed on the lymphocyte fraction as determined by side scatter (SCC) and forward scatter (FSC) with laser excitation at 488 nm. A computer system (CellQuest, BD Biosciences, Mountain View, CA, USA) was used for data acquisition and analysis. List mode data for at least 1×10^4 events of lymphocytes were stored. Percentages were calculated on the basis of the number of lymphocytes found in each quadrant. T cells were determined by the presence of the surface marker CD3 ϵ (clone 145-2C11)/CD45 (clone 30-F11), T helper (Th) cells by CD3 ϵ /CD4 (clone RM4-5), cytotoxic T cells by CD3 ϵ /CD8 (clone 53-6.7), Th1 cells by Tim-3 (clone 8B.2C12)/CD4, Th2 cells by CD278 (clone 7E.17G9)/CD4 and natural Treg cells by CD4/CD25 (clone PC61, IL-2R α).

Table 2

Changes in cytokines expression levels in ConA stimulated splenocytes of the mice modeling with OVA/TNCB followed by oral administered DtE and quercetin.

Treatment	IL-4	IFN- γ	IL-6
Naïve	12.55 ± 8.38	3104.37 ± 1270.20	478.16 ± 76
OVA/TNCB modeled AD			
Control	33.23 ± 15.53	1124.96 ± 496.15 ^{##}	445.34 ± 78.44
DtE, 30 mg/kg	11.30 ± 7.52	2429.18 ± 849.03 [*]	584.09 ± 74.95
DtE, 100 mg/kg	6.61 ± 5.31 [*]	2405.91 ± 602.50 [*]	516.08 ± 121.74
DtE, 300 mg/kg	4.40 ± 2.93 [*]	2599.39 ± 989.98 [*]	391.34 ± 73.69
Quercetin, 1.6 mg/kg	6.50 ± 3.14 [*]	2082.66 ± 545.70	494.33 ± 74.13

Data present as mean ± SE ($n = 5$). The cytokines expression in the OVA/TNCB modeled AD mice could significantly decrease in IFN- γ (^{##}, $P < 0.01$) and administration with DtE (30, 100 and 300 mg/kg) could significantly increase (^{*}, $P < 0.05$). When mice administered with DtE (100 and 300 mg/kg) and quercetin (1.6 mg/kg) for 1 week, IL-4 could significantly decrease (^{*}, $P < 0.05$). Naïve: without atopic dermatitis induction, Control: CMC administration after atopic dermatitis induction, Quercetin: quercetin treatment after atopic dermatitis induction, Experiment: different doses of DtE treatment after atopic dermatitis induction.

2.5. Estimation cytokines in concanavalin A (ConA) stimulated splenocytes

Effect of *D. tosaense* on cytokines in OVA/TNCB-challenged mice was studied to detect IL-4, IL-6 and IFN- γ by double-antibody sandwich ELISA (ELISA Ready-SET-Go! eBioscience, San Diego, CA USA). Capture antibodies were incubated overnight at 4 °C, washed with wash buffer (DPBS containing 0.05% Tween-20) and blocked for 60 min at room temperature with assay diluents. Standards and samples were added and incubated for 2 h at room temperature followed by washing non-specific binding. Detection antibodies were added and incubated for 1 h followed by washing. Substrate solution (TMB) was added to each well and incubated in dark and the enzyme activity was terminated by stop solution (2 N H₂SO₄). Absorbance was measured at 450 nm with reference at 570 nm by ELISA reader (Multiskan Spectrum, Thermo Electron Corporation, San Jose, CA, USA).

2.6. Histopathological examination and mast cell determination

Dorsal skin of the sacrificed mice was embedded in paraffin wax for histopathological examination as described previously (Hsieh et al., 2010). Microtome sections measuring 5 μ m were prepared and bathed in Ehrlich's hematoxylin for 5 min and rinsed for 5 to 10 min in running water. The sections were subsequently dipped for 3–5 s in 70% alcohol containing 1% hydrochloric acid and washed for 5–10 min in running water. The slides were stained for 5 min in 1% eosin and placed under running water until the nuclei appeared blue. The sections were dehydrated by the addition of serially concentrated alcohol (70%, 80%, 90%, and 100%), cleared and mounted. They were then histological examined.

Mast cell infiltration was determined by toluidine blue staining as described previously (Kang et al., 2008). In brief, microtome sections measuring 5- μ m were deparaffinized, hydrated in distilled water and stained in toluidine blue working solution (1% toluidine blue and 1% aluminum potassium sulfate) for 2–3 min, washed again with distilled water, dehydrated gradually with ethanol and examined microscopically.

2.7. Statistical analysis

Results are expressed as mean ± SE. One-way ANOVA was used for multiple group comparisons and Duncan's test for *post hoc* examination. A significant value was defined as $P < 0.05$.

3. Results

3.1. Serum anti-OVA antibody levels

Suppression of atopic dermatitis by oral administration of ethyl acetate extract of *Dendrobium tosaense* in BALB/c mice was exam-

Table 3

Splenocytes distribution in DtE administered OVA/TCNB modeled AD mice.

Treatment	B cells (CD19+, CD45+)	T cytotoxic cells (CD8+, CD3+)	T helper cells (CD4+, CD3+)	Treg cells (CD25+, CD4+)
Naïve	58.40 ± 1.31	16.11 ± 0.99	18.95 ± 0.68	4.77 ± 0.18
OVA/TCNB modeled AD				
Control	60.85 ± 0.98	15.47 ± 1.04	18.05 ± 1.32	3.66 ± 0.33 ^{##}
DtE, 30 mg/kg	60.63 ± 2.14	16.59 ± 0.60	18.33 ± 0.45	4.69 ± 0.24 ^{**}
DtE, 100 mg/kg	61.29 ± 2.02	15.16 ± 0.48	18.87 ± 1.59	4.62 ± 0.12 ^{**}
DtE, 300 mg/kg	61.20 ± 1.80	16.49 ± 1.39	18.53 ± 0.80	4.68 ± 0.26 ^{**}
Quercetin, 1.6 mg/kg	58.12 ± 1.66	17.25 ± 1.20	18.34 ± 0.51	4.61 ± 0.16 ^{**}

Data present as mean ± SE (*n* = 5). The splenocytes subpopulation in the OVA/TCNB modeled AD mice could significantly decrease the Treg cells (^{##}, *P* < 0.01). When mice administered DtE (30 mg/kg) for 1 week, Treg can significantly increase (^{**}, *P* < 0.01), DtE might be play the anti-allergic property. Administration DtE did not interfere B, T helper and T cytotoxic cells population but modulated the Treg cells activity. Naive: without atopic dermatitis induction, Control: CMC administration after atopic dermatitis induction, Quercetin: quercetin treatment after atopic dermatitis induction, Experiment: different doses of DtE treatment after atopic dermatitis induction.

ined. The DtE contain quercetin, a typical flavonoid play pivotal role to suppress AD. Mice at the completion of OVA/TNCB challenge demonstrated higher level of serum IgE than the naïve group (Table 1). Serum anti-OVA IgE and IgG1 levels were significantly increased in the control group compared to the naïve group (*P* < 0.001). When mice were administered with DtE (30 and 100 mg/kg) for 1 week, the anti-OVA IgE level was significantly decreased (*P* < 0.01), and in mice administered with DtE (300 mg/kg), the anti-OVA IgE level was also significantly decreased (*P* < 0.001). Serum anti-OVA IgG1 levels were significantly increased in the control group compared to the naïve group (*P* < 0.001). When mice were administered with DtE (30 mg/kg), the anti-OVA IgG1 level was significantly decreased (*P* < 0.01). When mice were administered with quercetin (1.6 mg/kg) for one week, both anti-OVA IgG1 (*P* < 0.05) and anti-OVA IgE (*P* < 0.001) were significantly decreased. The DtE-mediated decreases anti-OVA antibody levels indicated that DtE might have anti-allergic potential (Table 1).

3.2. Splenocyte cytokine IL-4, IFN- γ and IL-6 levels

The IL-4 level was significantly decreased in the DtE (100 and 300 mg/kg) groups and the quercetin group (*P* < 0.05). OVA/TNCB modeled AD mice could significantly decrease IFN- γ expression (*P* < 0.01) and IFN- γ levels were higher in mice administered DtE (30, 100 and 300 mg/kg, *P* < 0.05) for one week (Table 2). No significant difference in IL-6 expression was observed in all three treatment including control, DtE and quercetin. The above results indicated that DtE might be downregulate Th2 and enhanced Th1 environment.

3.3. Flow cytometry analysis of T-cell subpopulation

The effect of DtE on the different T-cell subsets were determined by flow cytometry analysis of monoclonal antibody staining of CD3, CD4, CD8, CD25, CD19 and CD45 molecules in splenocytes for B, Th, Tc and Treg cells (Table 3). And in the skin lesion of draining lymph nodes was monitored CD3, CD4, CD278, Tim-3

for distinguishing Th, Th1 and Th2 cells subpopulation (Table 4). T cell differentiation in OVA/TNCB-modeled mice can significantly decrease the Treg cell level (*P* < 0.01) in splenocytes. When mice were administered DtE (30 mg/kg), the Treg level was significantly increased in splenocyte (*P* < 0.01). This finding suggests DtE might act in allergic dermatitis through enhanced immunosuppression. Although administration of DtE was increased Treg cell levels, it did not change B, T helper and T cytotoxic cell population. On the other hand, CD278+, CD4+ indicated Th2 subpopulation was significantly reduced in skin lesion draining lymph nodes (*P* < 0.01). That indicated the polarization of Th2 population in local immune modulation.

3.4. Histological analysis

Numerous leukocytes were infiltrated into the dermis and epidermis was thickened in the control mouse skin compared to the naïve. In this study it was found that DtE acts in dose dependent manner. There were no significant difference in cell morphology noted in control (Fig. 1B) and lower concentration of DtE (30 mg/kg; Fig. 1C). Skin specimens were focal, moderate and showed hyperkeratosis of the epidermis and mononuclear cell infiltration of the dermis. While higher doses of DtE (100 mg/kg; Fig. 1D and 300 mg/kg; Fig. 1E) and quercetin (1.6 mg/kg; Fig. 1F) groups focal, slight to moderate, hyperkeratosis of the epidermis and mononuclear cell infiltration of the dermis were found in the. To further investigate, we assessed the effect of DtE on the infiltration of mast cells, an important effector cell involved in AD, into the dorsal skin. As shown in Fig. 2 and Fig. 3, treatment with OVA/TNCB increased the infiltration of mast cells. This infiltration was significantly suppressed by DtE treatment.

4. Discussion

The present study was undertaken to evaluate the efficacy of DtE alleviating atopic dermatitis-like immune alterations in mice. Herba Dendrobii, also known as “Shi-hu” in Chinese, has been used

Table 4

Skin lesion draining lymph node cells distribution in DtE administered OVA/TCNB modeled AD mice.

Treatment	T helper cells (CD4+, CD3+)	Th1 (Tim-3+, CD4+)	Th2 (CD278+, CD4+)
Naïve	47.76 ± 2.12	8.47 ± 0.29	3.09 ± 0.12
OVA/TCNB modeled AD			
Control	45.26 ± 0.88	6.57 ± 0.20 ^{###}	5.27 ± 0.33 ^{###}
DtE, 30 mg/kg	41.83 ± 1.92	7.21 ± 0.21	4.21 ± 0.27 ^{**}
DtE, 100 mg/kg	41.11 ± 1.41 [*]	6.75 ± 0.26	4.33 ± 0.24 ^{**}
DtE, 300 mg/kg	40.99 ± 0.45 [*]	6.91 ± 0.15	4.30 ± 0.19 ^{**}
Quercetin, 1.6 mg/kg	42.95 ± 1.00	7.50 ± 0.23 ^{**}	4.38 ± 0.14 ^{**}

Data present as mean ± SE (*n* = 5). The Th1/Th2 cells subpopulation in the OVA/TNCB modeled mice can significantly increase the Th2 response (^{###}, *P* < 0.001) in skin lesion draining lymph node. When mice administered DtE for 1 week, Th2 response can significantly decrease (^{**}, *P* < 0.01). Naive: without atopic dermatitis induction, Control: CMC administration after atopic dermatitis induction, Quercetin: quercetin treatment after atopic dermatitis induction, Experiment: different doses of DtE treatment after atopic dermatitis induction.

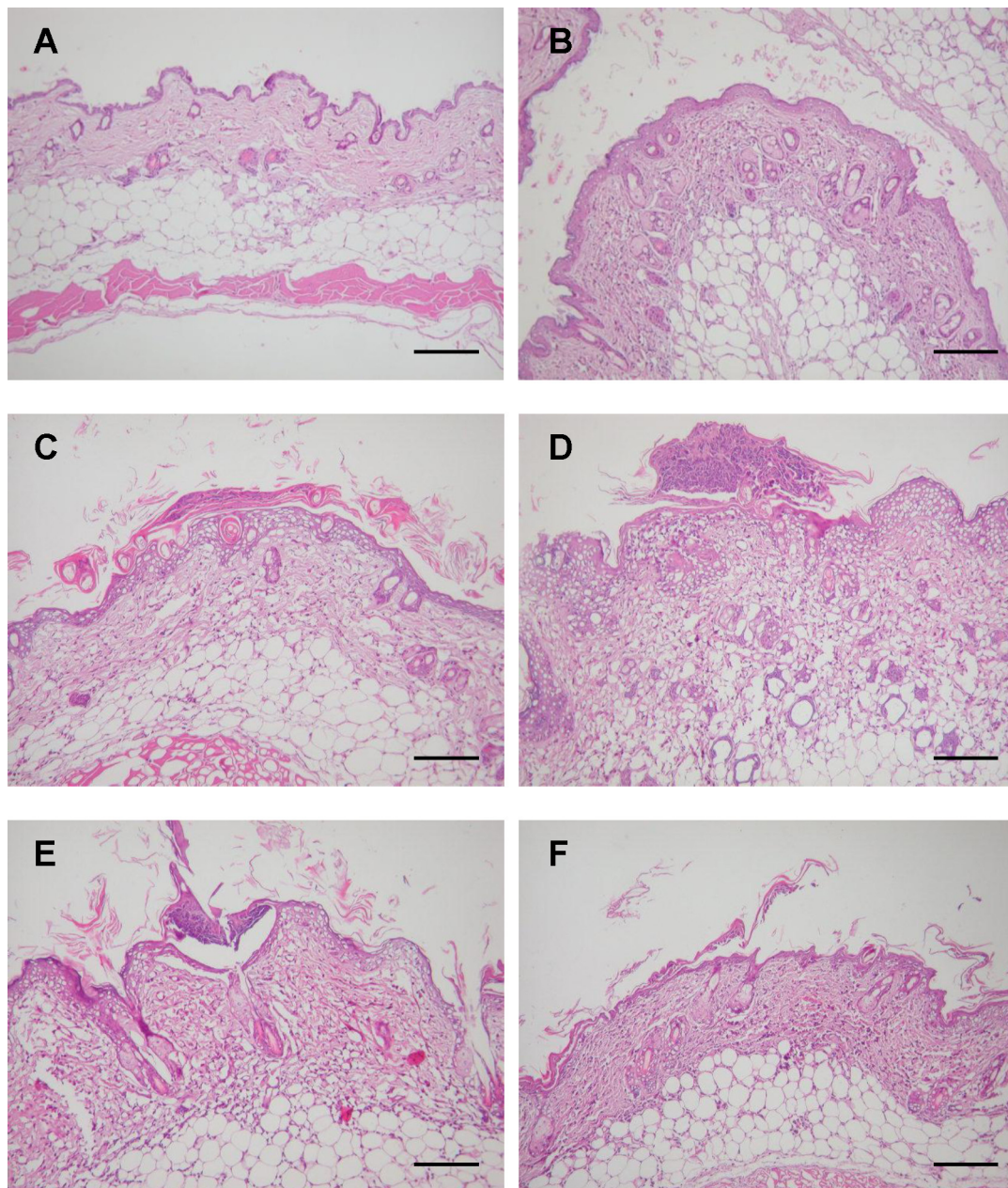


Fig. 1. Histological features of dorsal skin lesions in HE stain. (A) naïve, (B) control, (C) 30 mg/kg DtE, (D) 100 mg/kg DtE, (E) 300 mg/kg DtE and (F) 1.6 mg/kg quercetin. Focal, moderate, hyperkeratosis of epidermis and mononuclear cell infiltration of dermis were found in the control and 30 mg/kg. Focal, slight to moderate, hyperkeratosis of epidermis and mononuclear cell infiltration of dermis were found in the 100 mg/kg, 300 mg/kg and quercetin 1.6 mg/kg. Scale bar indicate 100 μ m.

in traditional Chinese medicine as a tonic and as a therapeutic agent for curing cataracts and throat inflammation. *D. tosaense* contains flavonoids, including quercetin, as pharmacologically active components. Recently, it has been reported that these flavonoids are able to suppress inflammatory reactions *in vivo* (Cheng et al., 2010). The anti-inflammatory effect of these flavonoids suggests the possibility of their therapeutic efficacy in various inflammatory diseases (Kojima et al., 2000; Morikawa et al., 2003). Our study is the first to demonstrate DtE immunoregulatory potential for alleviating atopic dermatitis through immunologic examinations. We had applied US patent in 2012 (Pub. No.: US 2012/0315289 A1) (Tsay et al., 2012). That might be the part of bioactivity from quercetin. Overall, we found that DtE possessed an ability to influence Th2 cell activation involved with the onset or progression of atopic dermatitis. BALB/c

mice are useful as an animal model for AD (Harada et al., 2005). In this study, we investigated the effect of DtE on AD skin lesions using an OVA/TNCB-induced AD model in BALB/c mouse. Skin lesions in this model show increased numbers of mast cells and CD4⁺ T cells, and are clinically and histologically similar to those seen in AD. BALB/c mice with AD skin lesions showed significantly increased specific anti-OVA IgE ($P < 0.001$) in serum and ConA stimulated IL-4 ($P < 0.05$) in splenocyte. Microscopic analysis shows edema in the dorsal skin and infiltration of leukocytes, especially mast cells. DtE significantly reduced the dorsal skin damage and mast cell degranulation. Oral administration of DtE resulted in the up-regulation of IFN- γ production, down-regulation of IL-4 production, suppression of IgE or IgG1 over-production, and demonstrated reduced degranulation of mast cells. But the cellular modulated mechanism was

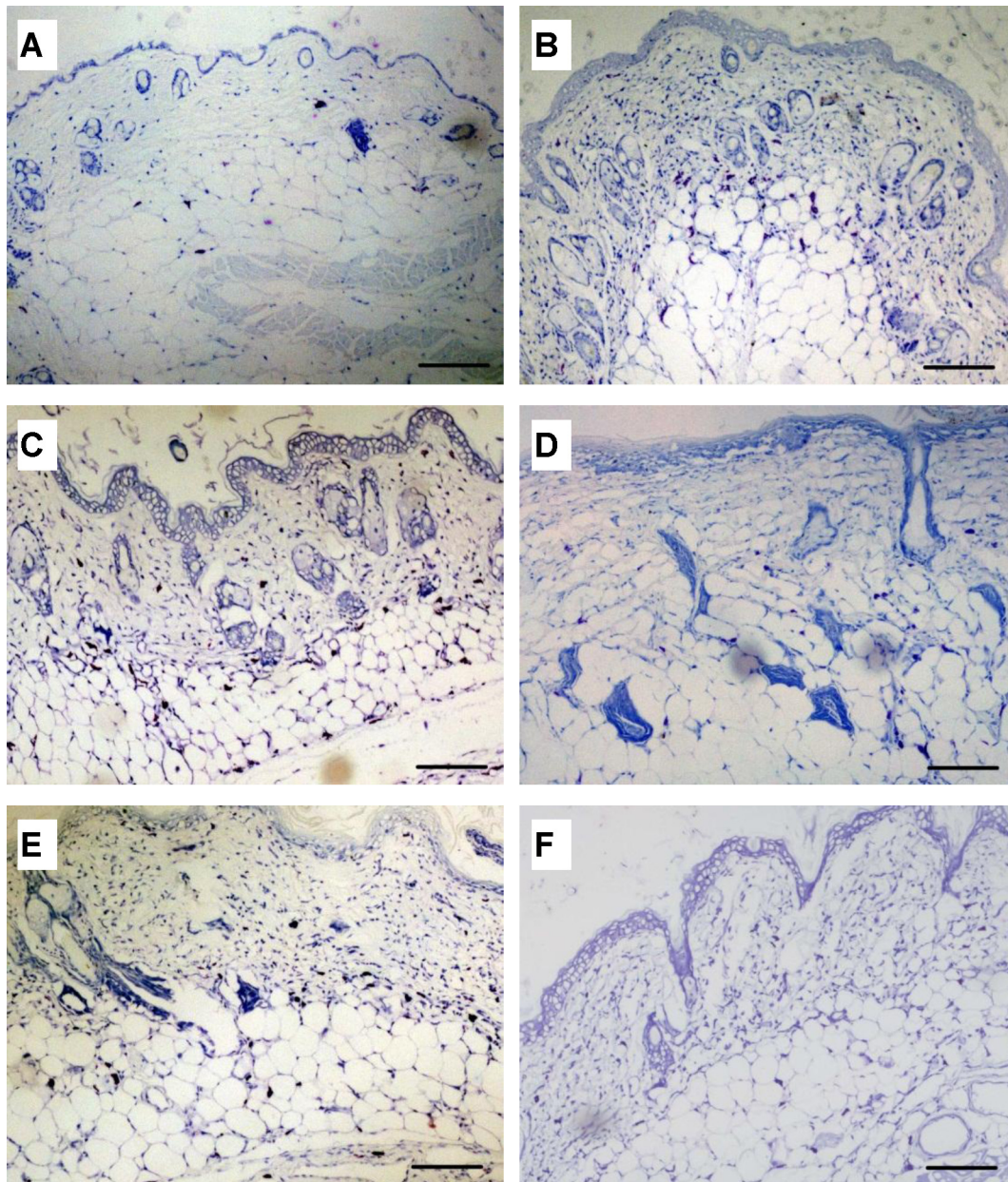


Fig. 2. Histological features of dorsal skin lesions in Toluidine blue stain. (A) naïve (B) control (C) 30 mg/kg DtE (D) 100 mg/kg DtE (E) 300 mg/kg DtE (F) 1.6 mg/kg quercetin. Scale bar indicate 100 μ m.

still not figure out. The subpopulation of splenocyte and AD skin lesion draining lymph node might provide the regulated information in T cell subsets differentiation.

Natural T regulatory cells (Tregs) were T lymphocytes distinguished by CD4 and CD25 surface markers that played an immunosuppressive role in regulating the immune response. This subpopulation of T suppressor cells inhibits Th1 and Th2 cell proliferation and secretion of cytokines (IL-2) (Suto et al., 2001; Xu et al., 2003). IgE synthesis by B cells class switch was primarily regulated by the type 2 cytokines including IL-4 and IL-5. In this study, significant reduced IL-4 in ConA stimulated splenocyte ($P < 0.05$, Table 2) indicated the downregulated Th2 environment. And IFN- γ , in contrast, is produced by Th1 cell and modulated the environment to inhibit Th2 cell proliferation also significantly increased in ConA stimulated splenocyte ($P < 0.05$, Table 2). Reducing Th2 cell proliferation can decrease allergic-B cell class switch and IgE expression, our data indicate AD skin lesion draining lymph

node cells reduced the Th2 cell differentiation (CD278+, CD4+ cells, $P < 0.01$ Table 4). CD278 also known as inducible co-stimulator (ICOS) had been described in the reduced IFN- γ expression in Th cell subset (CD278+, CD4+) (Rojo et al., 2008). In our OVA/TNCB modeling BALB/c mice also present the significant increased Th2 polarization (increase IL-4 and reduced IFN- γ) and increased specific anti-OVA IgE. This model also provided an idea progression in allergen induced Th1/Th2 imbalance finally produced AD skin lesion. In our previous and studies from other researchers have reported that natural substances from plants or herbal therapy can modulate Th1 and/or Th2 cell responses (Hon et al., 2007, 2005; Hsieh et al., 2010; Mainardi et al., 2009). In this study, natural Treg significantly increased in splenocytes ($P < 0.01$, Table 3) indicated immunosuppressive status and significantly reduced Th2 cell differentiation ($P < 0.01$, Table 4) in AD skin lesion draining lymph node subpopulation after DtE oral administration. These results might provide a mechanism in the modulation of Th1/Th2

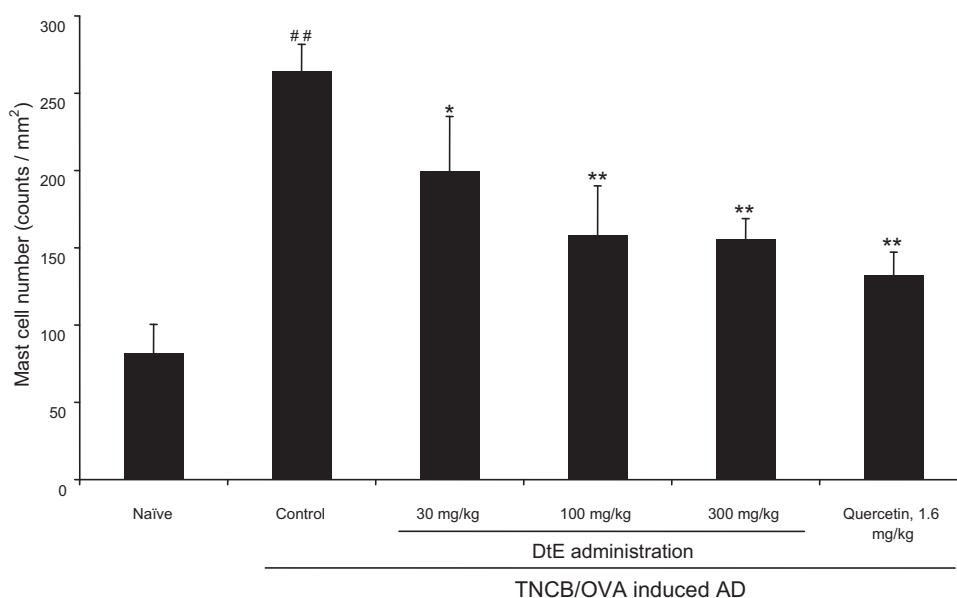


Fig. 3. Effect of DtE on TNCB/OVA-induced infiltration of mast cells into the skin lesions of BALB/c mice. The number of mast cells in three sites chosen at random was counted. Data are presented as mean $\pm$ SE. * $P < 0.05$, ** $P < 0.01$ indicated treatment group vs. control group; ## $P < 0.01$ indicated control group vs. naïve group.

imbalance finally reduced allergic IgE production. The histological examination in skin lesion indicate the reduced inflammatory cell infiltration (Fig. 1C, D and E) and edema lesion that might be the reduced IgE associated mast cell degranulation (Fig. 2D and E) and infiltration (Fig. 3) in administration of DtE. Jung et al. (2010) reported that quercetin reduced CD31 expression and lead to the inhibition of angiogenesis level, which may suppress chronic inflammation in AD like skin lesions. Tsai et al. (2010) reported that moscatilin is one of the bioactive compounds that have been extracted from *Dendrobium loddigesii*. It significantly decreased angiogenesis both *in vitro* and *in vivo*. Our findings might shed light on the immunomodulated effects of *D. tosaense* in the therapy of AD or other allergic disorder.

5. Conclusion

Dendrobium species are widely used in both traditional Chinese and folk remedies for antipyretic, ophthalmic, and tonic purposes. In the paper, we report that *D. tosaense* regulated atopic dermatitis in mouse model. We used ethyl acetate extraction of *D. tosaense* (DtE) that was used to protect these mice from the TNCB-induced skin lesions simulating atopic dermatitis. Our results indicate that the administration of DtE can modulate cytokine expression and T cell subpopulations by regulating mast cell infiltration and thus alleviate the atopic dermatitis.

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