Natural Product Sciences
21(2) : 122-127 (2015)

Gastrodia elata Blume Attenuates 2, 4-Dinitrochlorobenzene-induced Atopic Dermatitis-like Skin Lesions in Balb/c Mice and SD Rats

Na-Hyung Kim, Myung Ro Lee, and Young-Mi Lee*

Department of Oriental Pharmacy, Wonkwang University, Iksan 570-749, South Korea

Abstract – *Gastrodia elata* Blume is a well-known kind of natural products used as a folk medicine for thousands of years. However, anti-atopic dermatitis-like effects of *G. elata* Blume had not been evaluated until now. The aim of the present study is to investigate the protective effects of water extract from the roots of *G. elata* Blume (GE) on 2, 4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions using balb/c mice. Combination treatment of GE (at a dose of 12.5 mg/kg body weight by administrated per os + 0.5 mg/cm^2 as ointment to apply on ear and dorsal skin) was significantly inhibited spleen weight, ear thickness, levels of serum immunoglobulin E and number of mast cells, compared with that of 2, 4-dinitrochlorobenzene-included groups without GE. Furthermore, combination application by oral administration plus by ointment of GE significantly inhibited the histamine release from rat peritoneal mast cells. These results suggest that combination treatment of oral administration plus ointment form of GE could be helpful as a potentially natural pharmaceutical treatment on atopic-like dermatitis.
Keywords – Atopic dermatitis, *Gastrodia elata* Blume, Skin lesion, 2, 4-Dinitrochlorobenzene

Introduction

Atopic dermatitis (AD), defined as a chronic or chronically relapsing inflammatory skin disease, represents various aspects, such as immune dysregulation or skin barrier disruption, accompanied by infiltrating lymphocytes, macrophages and granulated mast cells.[1] The incidence of AD has dramatically increased for the past three decades in whole countries.[2] In the pathogenesis of AD, inflammatory immune dysregulation that cause immunoglobulin (Ig) E-mediated sensitization, including an imbalance between T-helper (Th) 1 and Th2 immune response, and skin barrier dysfunctions are likely to play important roles in the development of AD, although the pathogenesis of AD remains incompletely understood.[1,3] AD patients show the aspects like erythema, dryness, epidermal hyperplasia, accumulation of mast cells in both the epidermis and the dermis, Th2 phenotypes such as interleukin (IL)-4, 5, and 13 secretion, and Th2 type cytokine-mediated IgE production.[4,5] Representative animal models with AD-like skin lesions that were similar to human AD aspects, have been induced by the repeated application of 2, 4-Dinitrochlorobenzene (DNCB), oxazolone, trinitrochlorobebzene, or picryl chloride in balb/c mice, the repeated epicutaneous treatments with house dust mite allergen or staphylococcal enterotoxin B in mice, and spontaneous mutants or genetically engineered mutants such as Nc/Nga mice or IL-4, 18-overexpressing mice.[3,6] These animal models with AD-like skin lesions represent increasing of serum IgE levels and use to investigate the effect of a drug candidate for anti-AD agents. Conventional drugs for treatment of AD are topical therapy with emollients or anti-inflammatory agents, and cyclosporine which is the only substance that has been approved for systemic therapy of AD for a limited time.[7] However, typically topical steroid with significant systemic unexpected effects.[7] Therefore, it is important to develop more safer and effective therapeutic agents for AD. Especially, the researchers of many countries make an effort to develop the drug for the prevention and treatment of AD from natural resources.[8]

Gastrodia elata Blume is a well-known kind of herbs widely distributed in Asia and it has traditionally used as a folk medicine for thousands of years in Korea, China, and Japan. *G. elata* Blume contains phenolic compounds including gastrodin, vanillin, 1, 3-bis (4-hydroxybenzyl) citrate, 1-(4-beta-D-glucopyranosyloxybenzyl) citrate, parishin B, 4-hydroxybenzyl alcohol (4-HBA), and 4-hydroxybenzaldehyde (4-HBZ).[9,10] It has been known that *G. elata* Blume and its constituents have anti-convulsant,

*Author for correspondence
Dr. Young-Mi Lee, Department of Oriental Pharmacy, Wonkwang University, 460 Iksandae-ro, Iksan 570-749, South Korea.
Tel: +82-63-850-6807; E-mail: ymlee@wku.ac.kr

analgesic, sedative, free radical scavenging, antianxiety functions alleviating of headaches, epilepsy, dizziness, rheumatism, neuralgia, paralysis, hypertension and other neuralgic disorders.[11,12] Additionally, previous study has shown that 4-HBA, one of the representative components of *G. elata* Blume, ameliorated ischemic injury induced by transient focal cerebral ischemia in rats, via increasing the expression of bcl-2 and inhibition the activation of Caspase-3.[13] However, it has not been reported about the physiologic effects of water extract of *G. elata* Blume in 2, 4-dinitrochlorobenzene-induced atopic dermatitis-like animal models. Therefore, in the present study we evaluated whether water extract of *G. elata* Blume treatment has anti atopic like dermatitis activity in 2, 4-dinitrochlorobenzene induced animal model.

Experimental

Reagents – Compound 48/80, alcian blue solution, o-phthaldialdehyde, trypan blue solution, phosphate buffered saline (PBS) and metrizamide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DNCB was purchased from Junsei Chemicals Co., Ltd. (Tokyo, Japan) and dissolved in acetone:olive oil (ratio, 4:1). Hydrophilic petrolatum was obtained from Maruishi pharmaceutical Co., Ltd. (Osaka, Japan).

Plant sample preparation – The roots of *G. elata* Blume were purchased from Jinan market (Jeonbuk, South Korea) in June 2013. Professor Dong-Yeul Kwon of Wonkwang University identified the plants. A voucher specimen of roots from *G. elata* Blume was deposited at the Department of Oriental Pharmacy, Wonkwang University, Iksan, South Korea. The roots of *G. elata* Blume (200 g) were extracted twice with 2 L water for 2 h in a reflux apparatus. After reflux and filtration, the water extract was evaporated *in vacuo*, and lyophilized at −55 ºC in a freeze dryer. The dried extract yield from staring crude plants was about 7.2% (w/w). For combination application by oral administration plus by ointment of water extract of roots of *G. elata* Blume (GE), we prepared two type forms of GE, as a drink and ointment. To administrate by oral, the samples were dissolved in saline and then filtered through 0.45-μm syringe filter. To apply as an ointment on skin, the sample mixed with hydrophilic petrolatum of 0.5 mg, according to experimental dose. Preparation of sample for rat peritoneal mast cells were dissolved in saline, filtered through 0.45-μm syringe, and treated with at concentration of 0.1, 1, and 10 μg/mL.

Animals – Male balb/c mice (8-week-old, 23 - 26 g) and male Sprague-Dawley rats (7-week-old, 200 - 230 g) were purchased from the Da-Mul Experimental Animal Center (Daejeon, Korea), and then maintained at Wonkwang University. The animals were housed five to ten per cage in a laminar air-flow room maintained at a temperature of 22 ± 1 ºC and relative humidity of 55 ± 10% throughout the study. All protocols were approved by the Institutional Animal Care and Use Committee of Wonkwang University (No. WKU15-8). The experimental animals were divided into three groups (n = 3 for each group) as follows: i) the normal (Control) group, ii) the DNCB-induced atopic dermatitis-like (AD) group, administrated per os with saline of 10 μL/g body weight + applied on skin with hydrophilic petrolatum of 0.5 mg/cm^2 on ear and dorsal skin in each mice and iii) the combination treatment of GE (ADGE) group, at a dose of 12.5 mg/kg body weight by administrated per os + 0.5 mg/cm^2 as ointment to apply on ear and dorsal skin in each mice. DNCB was used for sensitization. After hair shaving on dorsal skin of each mouse in the AD group and ADGE group, they were sensitization with 100 μL of 1% DNCB on days 1 to 4 and with 100 μL of 0.5% DNCB on 8th, 10th, 12th, and 14th day.[3,18,19] Considering the DNCB-induced AD-like symptoms of animals, the experiment continued for 2 weeks. The experimental flow chart is shown in Fig. 1.

Body weight, spleen weight and ear thickness – The body weight in all experimental mice was measured on initial and final days. On 15 days, ear thickness was measured with a vernier caliper (Mitutoyo, Japan) under mild anesthesia and spleen weight was measured after the mice were sacrificed.

Measurement of serum IgE levels – Serum were obtained from blood collected by centrifugation at 5000 rpm for 15 min at 4 ºC and stored at −70 ºC until analysis. The levels of serum IgE was measured using murine IgE ELISA kit (Shibayagi Co., Ltd., Gunma, Japan) according to the manufacturer's instructions. The optical density was determined by using a microplate reader at 450 nm.

Mast cell numbers of skin lesions – The number of mast cells in dorsal lesion of mice was performed in sections stained with alcian blue for evaluation of mast cell degranulation. The dorsal lesion of mice was fixed in 85% methanol-10% formalin-5% glacial acetic acid, embedded in paraffin, sectioned into 5 μm slices and stained with alcian blue solution. Values are reported as mean ± S.E.M. of the number of cells per mm^2.

Preparation of rat peritoneal mast cells – Rats were anesthetized by diethyl ether, and injected with 20 mL of Tyrode buffer B (NaCl, glucose, $NaHCO_3$, KCl, NaH_2PO_4) containing 0.1% gelatin into the peritoneal cavity;

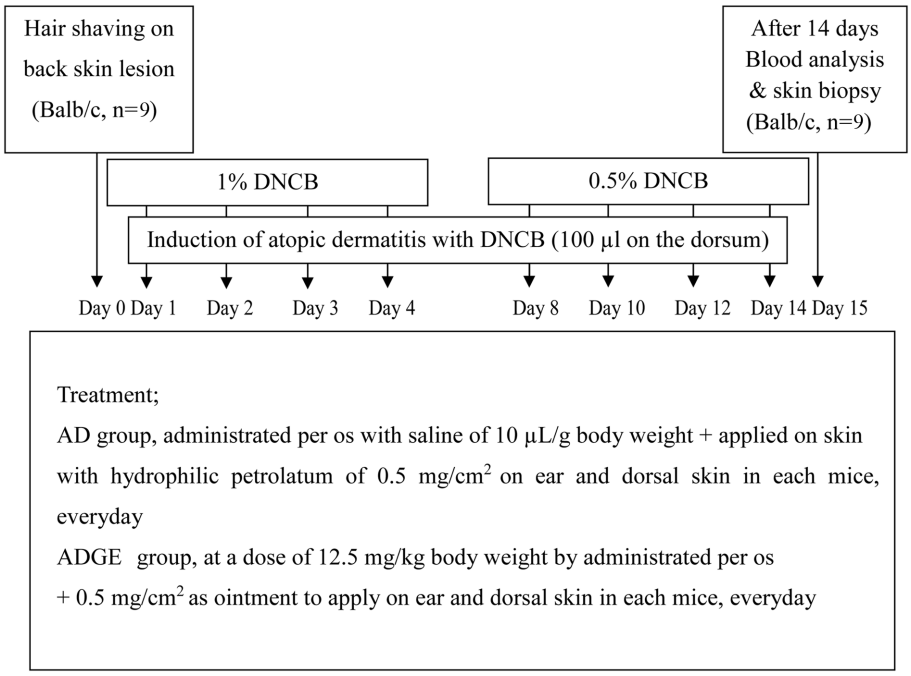

Fig. 1. Study flow chart. The DNCB was used for sensitization and was treated for 2 weeks. After hair shaving on dorsal skin of each mouse in the AD group and ADGE group, they were sensitization with 100 μL of 1% DNCB on days 1 to 4 and with 100 μL of 0.5% DNCB on 8th, 10th, 12th, and 14th day. Total number of animals sacrificed is 9. DNCB, 2, 4-dinitrochlorobenzene.

the abdomen was gently massaged for about 90 s, according as previous methods.[14] Mast cells were separated from the major components of rat peritoneal cells. Peritoneal cells suspended in 1 mL of Tyrode buffer B were layered onto 2 mL of 0.225 mg/L metrizamide and centrifuged at room temperature for 15 min at 400 × g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Rat peritoneal mast cell preparations were about 95% pure as assessed by alcian blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Histamine assay – Purified rat peritoneal mast cells were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. Respective suspensions of rat peritoneal mast cells were preincubated for 10 min at 37 ºC before the addition of compound 48/80 for stabilization. The cells were preincubated with the sample for 30 min and then incubated for 15 min with compound 48/80 (5 mg/L). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at 400 × g for 5 min at 4°C. The histamine content in the cell-free supernatant was measured by the o-phthalaldehyde spectrofluorometric method. The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in spectrofluorometer.

Statistical analysis – All results were statistically analyzed by SPSS 19.0 analysis program and presented as mean ± S.E.M. of three dependent experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with Dunnett's t-tests.

Result and Discussion

To determine anti-AD-like activity of GE treatment, we evaluated the body weight, spleen weight, ear thickness, serum IgE levels, and number of infiltrated mast cells on skin lesions in DNCB induced animal model. Animal models with AD-like skin lesions that were similar to human AD aspects, have been induced by the repeated application of various sensitizers such as ovalbumin and chemical haptens including DNCB, oxazolone, trinitrochlorobebzene, or picryl chloride in balb/c mice, and by the repeated epicutaneous treatments with house dust mite allergen or staphylococcal enterotoxin B in mice.[3,6] These animal models with AD-like skin lesions represent increasing of serum IgE levels and use to investigate the effect of a drug candidate for anti-AD agents. Additionally, in this study, we selected treatment method of the

Table 1. Weight gain, spleen weight, ear thickness, serum IgE levels and number of mast cells in DNCB-induced balb/c mice.

Groups	Body weight (g)		Spleen weight (g)	Ear thickness (mm)		Serum IgE (ng/ml)	Number of mast cells
	Initial (0 day)	Final (15th day)		Initial (0 day)	Final (15th day)		
Control	24.65 ± 0.59	29.58 ± 0.11	0.09 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	346.14 ± 180.30	17.22 ± 1.35
AD; Saline+hydrophilic petrolaturn	24.10 ± 0.90	21.95 ± 1.53*	0.29 ± 0.08*	0.18 ± 0.02	0.25 ± 0.01*	1602.38 ± 302.61*	150.11 ± 22.05*
ADGE; Drink (12.5 mg/kg BW)+ Ointment (0.5 mg/cm^2)	24.01 ± 0.35	21.81 ± 0.47	0.20 ± 0.02#	0.18 ± 0.01	0.19 ± 0.01#	1017.25 ± 235.63#	25.67 ± 8.11#

*$p < 0.05$, significant difference compared to the control group. #$p < 0.05$, significant difference compared to the DNCB-induced atopic dermatitis-like group (AD). Data are presented as mean ± S.E.M. of three dependent experiments. DNCB, 2, 4-dinitrochlorobenzene; IgE, immunoglobulin E; BW, body weight; GE, water extract from the roots of *G. elata* Blume.

combination application by oral administration plus by ointment of GE, to enhance effectiveness for alleviating AD aspects as a potentially natural pharmaceutical treatment on atopic-like dermatitis, not the same administration to animals.

Commonly, locally applied skin-sensitizing materials such as DNCB or ovalbumin affect the proliferative responses of organ or tissue related immune function including lymph node, thymus or spleen.[15] As shown Table 1., the change of spleen weight by sensitization of DNCB can be an indicator for reflecting roughly elevated immune states of AD-induced mice. The ear swelling response was determined from any increment in thickness measured on initial day and final day of experiment. DNCB was applied (100 μg/site) on the dorsal aspect of the mouse ear. After 15 day, the ear thickness of mice in the AD group increased than the value measured on the first day of experiments, although the ear thickness of mice in the Control group had no difference between first day and last day of experiment. Combination treatment of oral administration (12.5 mg/kg body weight) plus ointment form (0.5 mg/cm^2) of GE suppressed the ear thickness compared to the ear thickness of mice in the AD group on final day of experiment.

AD patients show the aspects like erythema, dryness, epidermal hyperplasia, accumulation of mast cells in both the epidermis and the dermis, Th2 phenotypes such as interleukin (IL)-4, 5, and 13 secretion, and Th2 type cytokine-mediated IgE production.[4,5] As shown Fig. 2A., clinical lesions of mice skin in DNCB-induced AD group showed the appearance such as erythema, edema, scale, eschar and lichenification compared to skin of mice in Control group. However, AD-like appearances in DNCB-induced skin lesions of mice tended to alleviate by treatment of GE, compared to that of mice in the AD group. To assess alleviate effects on AD-like lesions from the treatment of GE, we examined degranulation of mast cell and the number of mast cells in dorsal lesion of mice after staining with alcian blue solution (Fig. 2B) (Table 1). Degranulation of mast cells was observed in the AD group, while that were not appeared in the Control group. However, degranulation of mast cells from mice skin in ADGE group tended to reduce as compared to that of mice skin in the AD group. Mast cells are increased in number in lesional versus nonlesional samples in all dermatosis including AD.[16] Number of mast cells of mice skin in the AD group increased nonuple compared to that of mice in the Control group. Number of mast cells infiltrated on dorsal lesions of mice in ADGE group decreased significantly compared to that of mice in the AD group. Mast cells are broadly viewed in the progress of immune reaction and release histamine, prostaglandins D_2, leukotriene C_4, chemokines, and cytokines when activation through the high affinity receptor for IgE (FcεRI).[8,17,18] Previous study showed that IgE hyperproduction in the serum, severe itching behavior, and infiltration of inflammatory cells including mast cell into the skin has been observed in mice with AD-like skin lesions.[19] Thus, we evaluated whether suppression of the progression of AD-like skin lesions by GE is related to the serum IgE levels from blood of experiment mice of each group (Table 1). DNCB-induced mouse skin of the AD group showed the elevated levels of the serum IgE, as compared to the Control group. Combination treatment of oral administration (12.5 mg/kg body weight) plus ointment form (0.5 mg/cm^2) of GE significantly inhibited serum IgE levels as compared to that of mice in the AD group. Our data, along with that from previous studies, showed that mice in DNCB-induced AD group increased the spleen weight, the value of serum IgE and number of infiltrated mast cells as compared to the Control group in mice. The changes of these elevated levels improved by

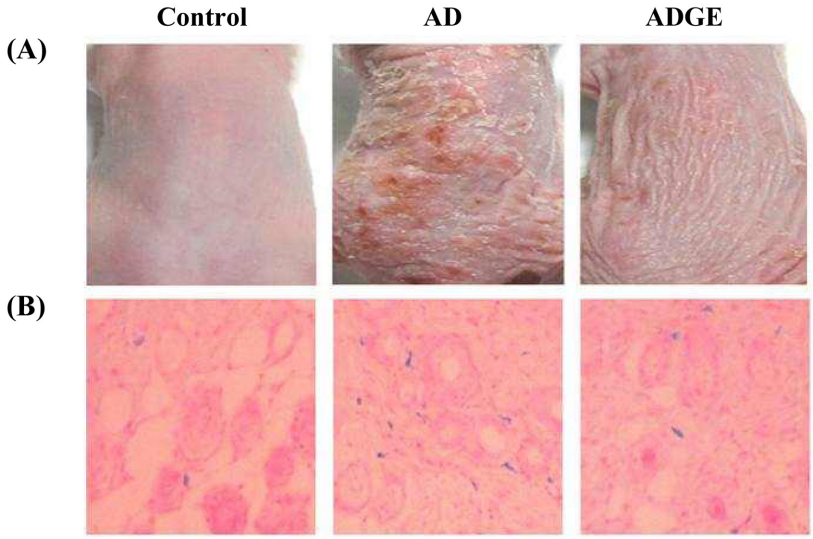

Fig. 2. Effect of GE on skin features and mast cell degranulation in DNCB-induced atopic dermatitis like dorsal skin of mice. (A) Representative image of skin features; (B) Representative image of alcian blue stained mast cell in dorsal lesions of balb/c mice. Mast cells were photographed by microscopy (magnification: ×100). DNCB, 2, 4-dinitrochlorobenzene; GE, water extract from the roots of *G. elata* Blume; AD, the DNCB-induced atopic dermatitis like group administrated per os with saline of 10 μL/g body weight + applied on skin with hydrophilic petrolatum of 0.5 mg/cm^2 on ear and dorsal skin in each mice; ADGE, combination treatment of GE at a dose of 12.5 mg/kg body weight by administrated per os + 0.5 mg/cm^2 as ointment to apply on ear and dorsal skin in each mice.

combination treatment of GE. These data imply that GE may have anti-AD like effects, although we did not examine how to regulate on AD-like aspects by GE.

Activated mast cells can produce histamine, as well as various inflammatory mediators such as eicosanoids, prodeoglycans, proteases, and several cytokines like tumor necrosis factor-α, IL-1, 6, 8, 13, and transforming growth factors-β1.[14] Elevated concentrations of histamine have been observed in the skin and plasma of AD patients.[16] Thus, we also examined the influence of GE on histamine release using rat peritoneal mast cells. Histamine release from mast cells treated with GE tended to decrease a concentration-dependent manner when compared to vehicle-treated control cells. In particular, the treatment of higher concentration of GE (10 μg/mL) significantly inhibited compound 48/80 induced histamine release using rat peritoneal mast cells as compared to vehicle cells (Fig. 3). Structural features of some of the molecules such as various proteases, phospholipase C, and sialidase on mast cell membranes are involved in the initiation of histamine release.[20] Thus, we assumed that treatment of GE inhibited histamine release from rat peritoneal mast cells induced by compound 48/80, via suppression of IgE-mediated inflammation. However, further studies will be conducted to examine IgE-mediated or Th2 related cytokines secreted from activated mast cells for supporting anti-AD effects from the combination treatment of GE. Gschwandtner *et al*. showed that histamine significantly suppressed the differentiation of epidermal keratinocytes by reducing the expression of tight junction proteins and desmosomal proteins, resulting in a thinning of the epidermis and stratum corneum in a human skin lesion.[21] Additionally, we supposed that GE can have the possibility to affect against impaired skin barrier function, together with inhibition of IgE-mediated reactions.

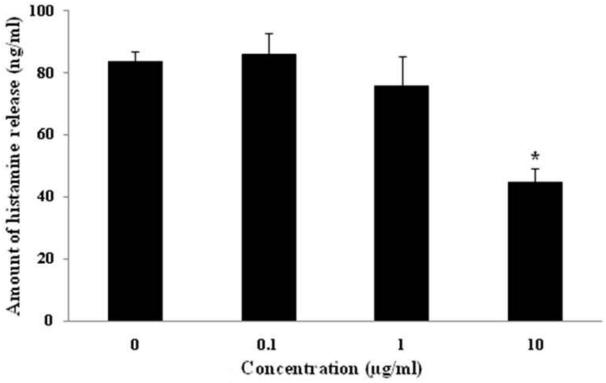

Fig. 3. Effect of GE on compound 48/80-mediated histamine release from rat peritoneal mast cells. Cells (2×10^5 cells/mL) were stimulated with compound 48/80 for 30 min at 37 ºC in presence of GE. *$p < 0.05$, significant difference compared to the saline value. Data are presented as mean ± S.E.M. of three dependent experiments. GE, water extract from the roots of *G. elata* Blume.

In conclusion, the present study revealed that combination treatment by orally administered and rubbed in

ointment form of GE were significantly inhibited spleen weight, ear thickness, levels of serum IgE and number of mast cells, compared with that of DNCB-induced groups without GE. Furthermore, combination application by oral administration plus by ointment of GE significantly inhibited the histamine release from rat peritoneal mast cells. The results demonstrate that combination treatment of oral administration plus ointment form of GE might have a therapeutic potential on atopic-like dermatitis.

Acknowledgments

This research was financially supported by the Ministry of Trade, Industry & Energy (MOTIE) & Korea Institute for Advancement of Technology (KIAT) through the Inter-ER Cooperation Projects (R0002020).

References

(1) Bieber, T. *N. Engl. J. Med.* **2008**, *358*, 1483-1494.
(2) Silverberg, J. I. *Am. J. Clin. Dermatol*. **2014**, *15*, 149-164.
(3) Jin, H.; He, R.; Oyoshi, M.; Geha, R. S. *J. Invest. Dermatol.* **2009**, *129*, 31-40.
(4) Navi, D.; Saegusa, J.; Liu, F. T. *Clin. Rev. Allergy Immunol.* **2007**, *33*, 144-155.
(5) Wollenberg, A.; Klein, E. *Clin. Rev. Allergy Immunol.* **2007**, *33*, 35-44.
(6) Marsella, R.; Olivry, T. *Clin. Dermatol*. **2003**, *21*, 122-133.
(7) Schlapbach, C.; Simon, D. *Allergy.* **2014**, *69*, 1571-1581.
(8) Kim, N. H.; Kang, I. C.; Kim, H. M.; Jeong, H. J. *Immunopharmacol. Immunotoxicol.* **2011**, *33*, 384-390.
(9) Tang, W.; Eisennbrand, G. Chinsese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine; Springer-Verlag; Germany, **1992**, pp 545-548.
(10) Duan, X. H.; Li, Z. L.; Yang, D. S.; Zhang, F. L.; Lin, Q.; Dai, R. *Zhong. Yao. Cai.* **2013**, *36*, 1608-1611.
(11) Huang, N. K.; Lin, Y. L.; Cheng, J. J.; Lai, W. L. *Life Sci.***2004**, *13*, 1649-1657.
(12) Sarris, J.; Mclntyre, E.; Camfield, D. A. *CNS Drugs.* **2013**, *27*, 207-219.
(13) Yu, S. S.; Zhao, J.; Zheng, W. P.; Zhao, Y. *Brain Res.* **2010**, 1308, 167-175.
(14) Kim, N. H.; Jeong, H. J.; Kim, H. M. *Amino Acids.* **2012**, *42*, 1609-1618.
(15) Kimber, I.; Basketter, D. A.; Berthold, K.; Butler, M.; Garrigue, J. L.; Lea, L.; Newsome, C.; Roggeband, R.; Steiling, W.; Stropp, G.; Waterman, S.; Wiemann, C. *Toxicol. Sci.* **2001**, *59*, 198-208.
(16) Järvikallio, A.; Harvima, I. T.; Naukkarinen, A. *Arch. Dermatol. Res.* **2003**, *295*, 2-7.
(17) Takeshita, S.; Tokunaga, T.; Tanabe, Y.; Arinami, T.; Ichinose, T.; Noguchi, E. *Allergy Asthma Clin. Immunol.* **2015**, *11*, 3.
(18) Kawakami, Y.; Kawakami, T. *Methods Mol. Biol.* **2015**, *1220*, 497-502.
(19) Shiohara, T.; Hayakawa, J.; Mizukawa, Y. *J. Dermatol. Sci.* **2004**, *36*, 1-9.
(20) Bach, M. K.; Brashler, J. R. *Immunology* **1975**, *29*, 371-386.
(21) Gschwandtner, M.; Mildner, M.; Mlitz, V.; Gruber, F.; Eckhart, L.; Werfel, T.; Gutzmer, R.; Elias, P. M.; Tschachler, E. *Allergy.* **2013**, *68*, 37-47.

Received February 11, 2015
Revised March 17, 2015
Accepted March 17, 2015