Topical Application of *Rosa multiflora* Root Extract Improves Atopic Dermatitis-Like Skin Lesions Induced by Mite Antigen in NC/Nga Mice

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Received August 6, 2013; accepted October 20, 2013

The roots of *Rosa multiflora* THUNB. (RM) has been used in oriental traditional medicines as remedies for scabies, rheumatic arthralgia and stomatitis which were practicably related with today's inflammatory and allergic diseases. In the present study, we evaluated whether RM root extract (RME) and its major constituent, 2-(3,4-dihydroxyphenyl)-6-(4-hydroxyphenyl)-8-(2,4-dihydroxyphenyl)-2,3-*trans*-6,7-*cis*-7,8-*trans*-3,4,7,8-tetrahydro-2H,6H-pyrano[2,3-*f*] chromene-3,7,9-triol (RM-3) belongs to condensed tannins, improve atopic dermatitis (AD)-like skin lesions in NC/Nga mice induced by mite antigen. Topical application of RME as well as RM-3 improved skin severity and suppressed mRNA levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) on skin tissues, in addition, significantly reduced T helper 2 (Th2) immune responses *via* interleukin 10 (IL-10) up-regulation. Thus, RME, contains lots of condensed tannins such as RM-3 which possesses potent anti-inflammatory and immune-modulatory effects, may be useful for treatment of skin allergies and can be developed as new alternative herbal therapy against AD.

Key words *Rosa multiflora*; Rosaceae; condensed tannin; proanthocyanidin; phlobatannin; atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesion. This disease primarily occurs in infants and children, and the incidence is gradually increasing in developing as well as developed countries. Although the cause of AD has been still unclear, it has been understood as immune disorder characterized by increased immunoglobulin E (IgE) and T helper 2 (Th2) cytokine levels as well as presence of blood eosinophilia.¹⁾ Meanwhile, topical corticosteroids have been widely used as the main therapeutic strategy against AD, however they could only be used for short periods of time and in limited skin regions because of serious adverse effects such as aggravation and recurrence.²⁾ Therefore, the demands of alternative therapies for AD have been increasing.

Rosa multiflora THUNB. (RM), called as multiflora rose, baby rose, many-flowered rose and seven-sisters rose, is native to Eastern Asia. While RM has been used as rootstock for ornamental roses in Western, all parts of RM has been known as folk medicine in Eastern. Especially, hips have been widely used as diuretics and also registered on Korean and Japanese Pharmacopoeia. Meanwhile, root of RM have been rarely researched in recent year although it has been traditionally used for rheumatism, scabies and stomatitis.³⁾ In our previous works, condensed tannins including novel stereoisomers (RM-1, 2 and 3) as well as catechin (CAT), and procyanidin B-3 (ProB3) were isolated from the roots of RM, and RM-1 and ProB3 showed to improve AD-like skin lesion *in vivo*.^{4,5)} As part of the research for development of herbal new drug, the present study was conducted to evaluate therapeutic effects of RM root extract (RME) as well as additional main compound, RM-3, belongs to phlobatannin which is a kind of distinctive condensed tannin on the mite antigen-induced AD-like skin lesion in NC/Nga mice.

MATERIALS AND METHODS

Isolation and Preparation RM-3 and RME were prepared as described earlier.⁴⁾ Briefly, the roots of RM were purchased on Gyeongdong Market (Seoul, Korea) in March 2008, and identified by Prof. Min Won Lee from Chung-Ang University. The voucher specimen (RMR2008) was deposited at the herbarium of the College of Pharmacy, Chung-Ang University, Korea. The roots of RM (2.5 kg) were extracted three times with 80% acetone (18 L, 2 d each) at room temperature. After removing the acetone under vacuum, the residual aqueous solution was filtered and the filtrate was then concentrated (RME, 215 g). This extract was subjected to Sephadex LH-20 (10–25 μ m, GE Healthcare Bio-Science AB, Uppsala, Sweden) (2 kg, 10 $\times$ 80 cm) and eluted with H₂O/MeOH gradient system to afford 3 sub-fractions. Next, fraction 2 (14 g) was subjected to MCI-gel CHP20P (75–150 μ m, Mitsubishi Chemical, Tokyo, Japan) (600 g, 5 $\times$ 60 cm) with H₂O/MeOH gradient system and yielded RM-3 (1.5 g) (Fig. 1).

Quantitative Analysis of Condensed Tannins Quantification of condensed tannins including RM-3 previously isolated from RME⁴⁾ was performed using Waters 600 high-

Fig. 1. Structure of RM-3, Isolated from the Roots of *Rosa multiflora*

The authors declare no conflict of interest.

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performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, U.S.A.). Samples were separated using a Kromasil 100-5C18 column (250×4.6 mm i.d., 5 μ m) (Akzo-Nobel, Bohus, Sweden), with the water-methanol gradient elution (10%→40% MeOH) at 280 nm UV wavelengths during 30 min. The flow rate was 1 mL/min and the injection volume was 20 μ L. The amount of each compound was calculated by peak area of 4 mg/mL RME based on calibration curve of serially diluted-standard samples (50, 100, 200, 300 and 400 μ g/mL). The concentrations of ProB3, CAT, RM-1, RM-2 and RM-3 were 30.23 ± 1.15 , 54.76 ± 2.32 , 85.12 ± 4.13 , 26.79 ± 0.98 and 17.38 ± 0.56 μ g/mg dry extract, respectively (data not shown). The sum of them was over 20 w/w %, thus condensed tannins must be contained mainly from RME.

Animals Twenty female 5 week-old NC/Nga mice (Charles River Co., Ltd., Yokohama, Japan) were maintained under conventional condition on a 12 h light/12 h dark cycle with food and water *ad libitum*. The temperature of the colony room was maintained at 22–23°C and humidity 55±15%. Animal treatment and maintenance were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Animal Care and Use Committee of the Chung-Ang University of Korea.

Induction of AD-Like Skin Lesion After shaving the animals' back hair by clipper, 100 mg of mite cream (Biostir Inc., Kobe, Japan) impregnated with *Dermatophagoides farinae* crude extract was applied to dorsal skin twice a week until 18 weeks of age. Each gram of this cream contained 234 μ g of Der f 1, 7 μ g of Der f 2 and 136.4 mg of proteins.

Topical Application of RM-3 and RME After induction of AD-like skin lesion, the animals were divided into 4 groups, and each group contained 5 mice, respectively. Each group was then topically treated with 1% RM-3, RME (experimental group), 0.1% hydrocortisone (positive control) in base cream, or only base cream (negative control) every day during 4 weeks. The base cream was prepared as o/w emulsion which contained suitable combination of oil phase (5% stearic acid, 2% cetyl alcohol, and 7% mineral oil) and aqueous phase (10% glycerin, 3% polyglyceryl-3-methyl glucose distearate and water).

Evaluation of Skin Severity The severity of dermatitis was assessed macroscopically by the following scoring procedure. The total scores of the skin severity were defined as the sum of the individual score (0, no symptoms; 1, mild; 2, moderate; 3, severe) for each of the following seven signs like erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness.⁶⁾ And, the severity was monitored by taken photographs once a week.

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Skin tissue biopsies were collected from the AD-like skin lesion in NC/Nga mice, and total RNA was isolated using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). After 0.2 mL of chloroform was added and shaken vigorously by hands for 15 s, the mixtures were centrifuged with 12000 rpm at 4°C for 15 min. The upper aqueous phase was transferred to a fresh tube, and the same amount of 2-propanol was added. After mixtures were incubated at 4°C for 15 min, it was centrifuged with 12000 rpm at 4°C for 15 min. The supernatant was removed, and then washed 500 μ L of 70% ethanol with 12000 rpm at 4°C for 5 min. The RNA pellet

was briefly dried. The purified RNA was dissolved in diethyl pyrocarbonate-distilled water (DEPC-DW). The total RNA was reverse transcribed at 42°C for 30 min in a containing reverse transcriptase (TaKaRa, Shiga, Japan), 10X buffer, 10 mM deoxyribonucleotide triphosphate (dNTP) (dNTP mix), oligo dT primer, RNase inhibitor, 25 mM MgCl₂. The 2 μ L of each cDNA sample from the RT-PCR was amplified by PCR in 25 μ L containing 10X buffer 2.5 μ L, 25 mM MgCl₂ 2.5 μ L and 10 pmol 0.75 μ L primer. PCR was experimented 10×buffer for Taq polymerase (100 mM Tris-Cl pH 8.5, 400 mM KCl), 1 mM dNTP each, 50 mM MgCl₂, upstream primer (5 μ m), downstream primer (5 μ m), DNA template (less than 200 ng) SyBr Green I. The specific primer sequences of each gene were as follows: inducible nitric oxide synthase (iNOS) (65 bp): 5'-CTG ATG CCT CTT CCA GGT GT-3' (sense), 5'-GAG GGA GCC CTT TCT GAA TC-3' (anti-sense)/cyclooxygenase (COX)-2 (80 bp): 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense), 5'-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3' (anti-sense)/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (93 bp): 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' (sense), 5'-CCC TGT TGC TGT AGC CGT AT-3' (anti-sense).

Measurement of Eosinophil Ratio Thirty microliter of each capillary blood six fold diluted with 150 μ L of saline. And, eosinophil ratio was measured by Sysmex XE-2100 hematology analyzer.

Measurement of IgE Levels The amount of total IgE in the serum was determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates for ELISA were coated with *anti-mouse* IgE antibody (Ab) by incubation overnight at 4°C, and then were treated further with 2% (w/v) bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) for 2 h at room temperature to block any nonspecific binding. Next, serial dilutions of the serum samples were incubated in the wells in duplicate for 1 h at room temperature. After being washed three times with PBST, biotin-conjugated rat *anti-mouse* IgE Ab (LO-ME-2) was added to the wells, followed by the addition of streptavidin-peroxidase (Seikagaku Corp., Tokyo, Japan). After washing three times, the plates were developed using a substrate solution containing 0.04% *O*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0). The reactions were terminated by the addition of sulfuric acid. The plates were read in a microplate reader at 490 nm, and the amount of the Ig isotype was calculated by comparing it with the mouse IgE standard (Pharmingen, CA, U.S.A.).

Measurement of Th2-Related Cytokine Levels The serum Th2 cytokines (interleukin (IL)-4, 5 and 13) levels were measured with mouse cytokine enzyme immunoassay kit (R&D Systems, Minneapolis, MN, U.S.A.).

Statistical Analysis All data were expressed as the means±S.D. The statistical differences in skin severity score were analyzed by Mann-Whitney *U*-test in order to compare negative control with experimental groups on each week, and assessed by Wilcoxon's signed-ranks test for time-course comparison on each topical treatment. The differences of mRNA levels and Th2 cytokines were assessed by one-way ANOVA followed by Student-Newman-Keuls (S-N-K) test for multiple comparison. In addition, the difference of eosinophil ratio and IgE levels were assessed by paired *t*-test in order to compare before and after each topical treatment. The *p* values were less

Fig. 2. Effect of RM-3 and RME on the Total Clinical Severity Scores

The application of mite cream to the back of mice induced AD-like skin lesions, and ProB3 and RM-1 treated topically for 4 weeks. The total clinical severity score was defined as the sum of scores for seven clinical criteria: erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness. Results are expressed as mean $\pm$ S.D. ($n=5$). * $p<0.05$ versus negative control of each week. # $p<0.05$ versus each group before topical treatment. NC, negative control; HC, hydrocortisone.

than 0.05 were considered to be significantly different.

RESULTS

Effects on Clinical Skin Severity The clinical skin severity of AD-like skin lesion was evaluated by modified SCORAD method, on scoring seven major clinical symptoms of AD: erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness.⁶⁾ AD-like skin lesions were induced by the treatment of mite antigen on the back of NC/Nga mice twice a week and the clinical symptoms of AD got worsen until 18 weeks of age. Topical application of RM-3 and RME dramatically improve AD-like skin lesion, and significantly lowered clinical skin severity scores during experimental period ($p<0.05$) (Fig. 2). In the final week of the experiment, any clinical signs were not observed in the topical application of both RME and RM-3, as almost same as that of positive control (Fig. 2). These results indicated that RME as well as RM-3 could effectively improve skin severity of mite-induced AD-like skin lesions in NC/Nga mice.

Effects on mRNA Levels of iNOS and COX-2 Because AD has been characterized as skin inflammatory disease, we determined mRNA levels of iNOS and COX-2, well-known to involve inflammatory response, in skin tissue of AD-like skin lesion *via* Real-time PCR.⁷⁾ Consequently, RM-3 and RME significantly suppressed respective mRNA levels of iNOS and COX-2 compared to negative control ($p<0.05$) (Fig. 3). These results indicated that RM-3 and RME should improve skin inflammation of AD-like skin lesions induced by mite on NC/Nga mice.

Effects on Eosinophil Ratio and IgE Level Elevation of eosinophil and abnormal high IgE level are representative indicator on allergic disease such as AD.^{8,9)} Thus, we measured changes of eosinophil ratio and IgE level before and after treatment of RM-3 and RME using haematology automated analyser and sandwich ELISA, respectively. Topical application of RM-3 and RME for 4 weeks significantly lowered both blood eosinophil ratio and serum IgE levels, while any significant changes were observed in negative control ($p<0.05$) (Fig. 4). These results indicated that RM-3 and RME could decrease allergic response in AD-like skin lesions induced by

Fig. 3. Effects of RM-3 and RME on mRNA Levels of iNOS and COX-2 in Skin Tissue of AD-like Skin Lesion Induced by Mite in NC/Nga Mice

Results are expressed as mean $\pm$ S.D. ($n=5$). Values bearing different superscripts are significantly different ($p<0.05$). NC, negative control; HC, hydrocortisone.

Fig. 4. Changes in Eosinophil Ratio and IgE Level before and after Topical Application of RM-3 and RME

Results are expressed as mean $\pm$ S.D. ($n=5$). * $p<0.05$ versus each group before treatment with sample. NC, negative control; HC, hydrocortisone.

Fig. 5. Effects of RM-3 and RME on Serum Th2 Cytokine Levels in NC/Nga Mice

Results are expressed as mean $\pm$ S.D. ($n=5$). Values bearing different superscripts are significantly different ($p<0.05$). NC, negative control; HC, hydrocortisone.

mite on NC/Nga mice.

Effects on Th2 Related-Cytokine Production Next, Th2 cytokines that has been known to regulate AD were also quantified in the serum of NC/Nga mice using ELISA.¹⁰ Both RM-3 and RME reduced the serum levels of Th2 cytokines (IL-4, 5 and 13) compared with negative control, significantly ($p<0.05$) (Fig. 5). In contrary, serum IL-10 level was significantly elevated by the topically treatment of RM-3 and RME as same degree of positive control, hydrocortisone ($p<0.05$) (Fig. 5). These results suggested that RM-3 and RME might effectively regulate Th2-immune response like hydrocortisone on mite-stimulated NC/Nga mice.

DISCUSSION

RME has been used as folk medicine on the treatment of scabies, rheumatism and stomatitis. It indicated that RME might possess anti-mite, anti-inflammatory and anti-allergic effects, related with major feature of remedy for today's AD.¹¹ Previously, several condensed tannins were isolated from RME, and a few of them (RM-1 and ProB3) improved AD-like skin lesion induced by mite on NC/Nga mice.^{4,5} RM-3, phlobatannin which has been reported to rare form of condensed tannins, can be simply explained to originate from condensation of *ent*-guibourtinidol-(4 β →6)-catechin (RM-1) on the processing of recyclization involving 5-OH in catechin moiety and C-2 in guibourtinidol moiety.¹² This molecule showed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and inhibitory activity on nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW264.7 cells.⁴ Therefore, RME, together with RM-3, were evaluated on AD-like skin lesions induced by mite on NC/Nga mice in this study.

NC/Nga mice, well-known as AD mouse model, reported to show clinical signs resemble to allergic eczema under conventional conditions, and possess similar traits to human AD such as increased serum IgE levels, chronic dryness and severe pruritis. It was reported that the IgE levels start to increase at 8 weeks of ages, the appearance of skin lesion peaks around 17 weeks in NC/Nga mice under conventional conditions. However, low incidence of AD-like skin lesion or curing it by itself has been problem of this mouse model and resulted to a lack of reproducibility.¹³ Thus, we attempted to treat mite antigen on the back of each mouse until 18 weeks of age for overcoming this obstacle.¹⁴ From this way, AD-like skin lesions begun to appear after 1–2 weeks later from the treatment of mite antigen to 5 week-old NC/Nga mice and got worse gradually until 18 weeks of age (data not shown). Several AD symptoms were rarely observed after 1–2 weeks from the topical application of both RM-3 and RME, and any signs were not observed at the end of experimental period as same degree of positive control, hydrocortisone. These results demonstrated that RM-3 and RME might be helpful to improve the AD symptoms, effectively.

iNOS and COX-2, play a role to produce nitric oxide and prostaglandin respectively, are well-known to mediate inflammatory or immune reactions. They markedly expressed in response to stimulation by several antigens or pro-inflammatory cytokines, thus measurement of suppression iNOS and COX-2 is considered the universal method for examining anti-inflammatory effects. In this study, both RM-3 and RME also decreased mRNA levels of iNOS and COX-2 in the skin tissue compared to negative control. And, these results demonstrated that topical application of RM-3 and RME might effectively reduce inflammatory response induced by mite antigen in AD-like skin lesion of NC/Nga mice.

AD is characterized by abnormalities between Th1 and Th2 cytokines. That is, cases in which the balance of Th1/Th2 is slanted to Th2 correspond to Type 1 allergy in association with the occurrence of acute atopy.¹⁰⁾ These Th2 cytokines then promptly induce the allergic reaction in AD. IL-4 triggers the initial differentiation of naïve T-helper type 0 (Th0) lymphocytes toward a Th2 phenotype, and induces the immunoglobulin isotype switch from IgM to IgE.^{15,16)} In addition, IL-4 induces expression of VCAM-1 on endothelial cells in allergic inflammation, resulting to enhance adhesiveness of endothelium for T cells, eosinophils, basophils, and monocytes, as is characteristic of allergic reactions.¹⁷⁾ IL-13, a homologous to IL-4, is also reported to induce the IgE isotype switch and VCAM-1 expression.¹⁸⁾ In addition, IL-5 is the most important in induction of eosinophils; activates mature eosinophil, induces eosinophil secretion and prolongs eosinophil survival.¹⁹⁾

Meanwhile, IL-10 has been reported to derive from Th2 lymphocyte to inhibit Th1 differentiation in classical paradigm. However, recent studies suggested the primary T-cell source for IL-10 is Th3 (also referred to as Tr1), and IL-10 suppress Th1 as well as Th2 immune responses.²⁰⁾ Especially, IL-10 is reported to inhibit eosinophil survival and IL-4-induced IgE synthesis.²¹⁾ In addition to above immune-suppressive role, IL-10 is well-known to typical anti-inflammatory cytokine. IL-10 induces 'suppressors of cytokine signaling 1 (SOCS1)' can directly inhibit interferon (IFN)- γ signaling pathways via activation Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, and also inhibits nuclear factor- κ B (NF- κ B), transcription factor that controls the expression of many inflammatory proteins such as iNOS and COX-2.^{22,23)} Therefore, our results suggested that elevation of serum IL-10 level by topical application of RM-3 and RME on AD-like skin lesion induced by mite in NC/Nga mice might down-regulate Th2 cytokines (IL-4, 5 and 13)-mediated hyper-productions of IgE and eosinophils, and lower inflammatory response to expression of iNOS and COX-2.

There have been a lot of reports about anti-inflammatory and anti-allergic activities of condensed tannins (refer to proanthocyanidins), which are polymers of flavan 3-ols, and can be found in many plants, most notably apples, cocoa beans, grapes cranberry, red wine, black tea and green tea.²⁴⁾ For example, they inhibited phosphorylation of mitogen-activated protein kinase (MAPK) proteins and the DNA binding of NF- κ B in various experimental inflammatory conditions.²⁵⁾ And, they suppressed the expression of IgE receptors on human mast cells, and also reduced serum IgE levels in mice immunized with ovalbumin.^{26,27)} Especially, more recent study explained that condensed tannins induced Foxp3 and IL-10 expression in Jurkat T cells and increased Tr1 frequencies and numbers *in vivo*.²⁸⁾ Therefore, RM-3 might reduce oxidative stress and pro-inflammatory signaling pathway in skin allergic inflammation, and activate Treg, leads to down-regulate Th2 cytokines-mediated immune system in this study.

Several condensed tannins such as CAT, ProB3, RM-1, RM-2, and RM-3 were isolated from RME in our previous work, and those condensed tannins were contained as 54.8, 30.2, 26.8, 85.1 and 17.4 μ g in 1 mg of RME, respectively and sum of them was over 20 w/w % on quantification by HPLC. Thus, the potent immune-regulatory effect of RME might be derived from rich accumulated condensed tannins.

Topical application of RM-3 as well as RME exhibited to improve mite-induced AD-like skin lesions in NC/Nga mice. They also lowered skin inflammatory response reducing mRNA levels of iNOS and COX-2, and normalized mite antigen-stimulated Th2-mediated immune systems by suppressing Th2 cytokines (IL-4, 5 and 13), blood eosinophil ratio and serum IgE level via activation of Treg such as up-regulation of IL-10. And, these anti-inflammatory and immune regulatory effects of RME might be contributed from several condensed tannins including RM-3 which were major constituents from RME. These results demonstrate that RME, natural source containing rich condensed tannins, may be useful for treatment of skin allergies, and can be developed as an alternative herbal medicine against AD.

Acknowledgments This research was supported by the Chung-Ang University Research Scholarship Grants in 2012.

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