

Topical Application of Two Condensed Tannins from the Root of *Rosa multiflora* Thunberg for the Treatment of Atopic Dermatitis (AD) in NC/Nga Mice

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Recently, the isolation of several condensed tannins from the roots of *Rosa multiflora* Thunberg, a traditional herbal therapy in oriental medicine for rheumatoid arthritis and scabies, was described. Two of the major condensed tannins – procyanidin B-3 (ProB3) and *ent*-guibourtinidol-(4 β →6)-catechin (RM-1) – were then applied topically to atopic dermatitis-like skin lesions on NC/Nga mice in order to assess their immunomodulatory properties. Both ProB3 and RM-1 significantly reduced the serum levels of eosinophils, IgE and certain Th2 cytokines (IL-4, 5 and 13) ($p < 0.05$ or 0.01). Additionally, ProB3 and RM-1 significantly reduced both the mRNA and protein expression of COX-2 and iNOS in mouse skin tissues ($p < 0.01$). Such results strongly suggest that ProB3 and RM-1 may be useful in the treatment allergic skin conditions, most notably atopic dermatitis. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: *Rosa multiflora*; condensed tannins; atopic dermatitis; procyanidin; proguibourtinidin.

INTRODUCTION

Atopic dermatitis (AD) is a skin disease clinically characterized by pruritic and eczematous skin lesions, which usually develops in infants and has the potential seriously to impact the quality of life (Beattie and Lewis-Jones, 2006). Although the etiology of AD remains unclear, gene–environment interactions are now believed to contribute to the underlying pathophysiology. Accordingly, AD is now believed to be an immune inflammatory skin disorder and is associated with increased serum IgE levels, increased Th2 cytokines in lesional skin, increased serum eosinophils and the increased expression of pro-inflammatory enzymes such as iNOS and COX-2 (Van Bever, 1992). Although topical steroids are frequently used to treat AD, the associated side effects, such as aggravation and recurrence, result in suboptimal treatment (Leung and Barber, 2003). Accordingly, a wide variety of natural products are currently being evaluated for alternative AD therapies, with the ideal agent possessing potent efficacy and a minimal side effect profile (Lee *et al.*, 2006; Kim *et al.*, 2008; Ahn *et al.*, 2010).

Rosa multiflora Thunberg (RM) – commonly known as the multiflora, baby or rambler rose – is native to China, Japan and Korea, where the hips of this plant have been used widely as a treatment for edema and beriberi in folk

medicine (Kim *et al.*, 1998) and are currently registered in both the Korean Pharmacopoeia (KP) and Japanese Pharmacopoeia (JP). However, as the underground parts of RM have been used traditionally in the treatment of rheumatism and scabies, the efficacy of these parts was evaluated previously as a potential AD therapy (Kim *et al.*, 1998). Specifically, several condensed tannins were identified derived from the roots of RM, which were found to possess potent antioxidative and antiinflammatory activities *in vitro* in a previous study (Park *et al.*, 2010). To further clarify which specific compounds have this anti-atopic effect, the main condensed tannins of the RM roots – procyanidin B-3 (ProB3) and *ent*-guibourtinidol-(4 β →6)-catechin (RM-1) (Fig. 1) – are evaluated *in vivo* herein.

MATERIALS AND METHODS

Animals. Twenty female 5-week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan), and maintained under conventional conditions: a 12 h light/12 h dark cycle with freely available food and water. The colony room temperature was kept at 22–23 °C with a humidity of 55 ± 15%.

Induction of AD-like skin lesion. Mice were anesthetized with ether, after which the animal's back hair was shaved by clipper 1 day prior to sensitization. Next, 100 mg of mite cream (Biostir Inc., Kobe, Japan) impregnated with *Dermatophagoides farina* crude extract was applied to the dorsal skin of these animals and

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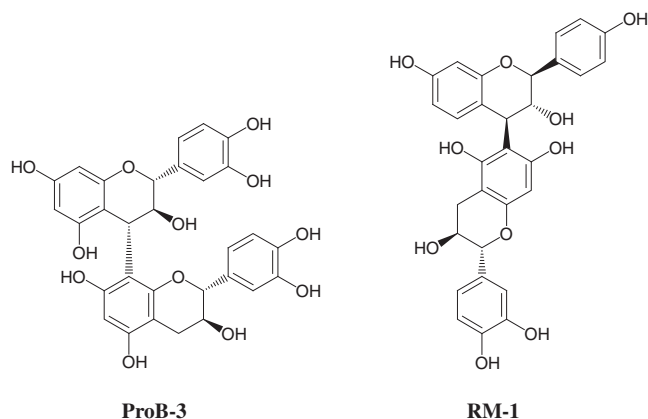


Figure 1. Structure of ProB3 and RM-1, isolated from the roots of *Rosa multiflora*. ProB3, catechin-(4a→8)-catechin (procyanidin B-3); RM-1, *ent*-guibourtinidol-(4β→6)-catechin.

secured by a patch twice a week for 20 weeks. Each gram of mite cream contained 234 µg of Der f 1, 7 µg of Der f 2 and 136.4 mg of proteins.

Topical application of ProB3 and RM-1. After the induction of the AD-like skin lesions, the animals were divided into four groups, each containing five mice. These groups were then treated topically for 4 weeks with one of four agents: 1% ProB3/RM-1 (experimental), hydrocortisone in base cream (positive control), or base cream only (normal control).

Evaluation of skin severity. The relative dermatitis severity was assessed macroscopically using the following scoring procedure. The total skin severity score was defined as the sum of the individual scores for each of the following seven signs: erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness (Kunz *et al.*, 1997). In this system, 0 was defined as exhibiting no symptoms, 1 as mild symptoms, 2 as moderate symptoms, and 3 as severe symptoms. Additionally, the mice were photographed once per week.

Measurement of serum eosinophils. Blood samples were collected after the induction of AD-like skin lesions and at the end of experiment. Whole blood cell (WBC) counts were performed using 30 µL of capillary blood diluted six-fold by 150 µL of saline. Microscopic differential cell counts (200 cells) were also performed on Wright-Giemsa stained blood smears. The total eosinophil count was calculated using the differential count.

Measurement of serum IgE levels. The total serum IgE was quantified by sandwich ELISA. Briefly, 96-well ELISA plates were coated with anti-mouse IgE Ab by overnight incubation at 4 °C, then treated with 2% (w/v) BSA dissolved in PBS with 0.05% Tween 20 (PBS-T) for 2 h at room temperature, so as to block any nonspecific binding. Next, serial dilutions of the serum samples were incubated in duplicate wells for 1 h at room temperature. After the wells were washed three times with PBS-T, biotin-conjugated rat anti-mouse IgE Ab (LO-ME-2) was added to each well, followed by streptavidin-peroxidase (Seikagaku Corp., Tokyo, Japan). After an additional three washes, the plates were developed using a substrate solution of 0.04% *O*-phenylenediamine dissolved in a phosphate citrate

buffer (pH 5.0). All reactions were then terminated by sulfuric acid. Plate reading was accomplished using a microplate reader at 490 nm, with the amount of the Ig isotype calculated by comparison with the mouse IgE standard (Pharmingen, CA, USA).

Measurement of Th2 (IL-2, 5 and 13) cytokine levels.

The serum and splenocyte concentrations of the Th2 cytokines (IL-4, 5 and 13) were quantified using a mouse cytokine enzyme immunoassay kit (R & D Systems, Minneapolis, MN, USA). Briefly, splenocytes were first isolated from mouse spleen tissue, while red blood cells (RBC) were lysed in a RBC lysis buffer (Sigma, St Louis, MO, USA). After lysate centrifugation, the resulting supernatant was removed and the cells were seeded at 10⁶/well in 24-well plates for culture. The supernatants from these cultured splenocytes were then analysed for respective cytokine levels.

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

Total RNA was quantified by tissue isolation using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA): 1 mL of TRIzol reagent was added to each specimen of cells in the culture dishes. After a period of 5 min at room temperature, chloroform was added to each specimen at a ratio of 0.2 mL per 1 mL of TRIzol reagent. The tubes were then shaken vigorously by hand for 15 s, then subsequently incubated at temperatures between 15 °C and 30 °C for 3 min. Next, the mixtures were centrifuged at a speed of 12000 rpm for 15 min at 4 °C, after which the resulting upper aqueous phase was transferred to a fresh tube containing the same amount of 2-propanol. This mixture was incubated again for 15 min at 4 °C, then centrifuged again at 12000 rpm for 15 min at 4 °C. After removal of the resulting supernatant, the RNA pellet was washed in 500 µL of 70% ethanol with 12000 rpm at 4 °C for 5 min, briefly dried, and then dissolved in diethyl pyrocarbonate-treated distilled water (DEPC-DW). The total cellular RNA was reverse transcribed at 42 °C for 30 min in a reaction mixture containing reverse transcriptase (Takara, Shiga, Japan), 10X buffer, 10 mM dNTP (dNTP mix), oligo dT primer, RNase inhibitor, 25 mM MgCl₂. Next, 2 µL of each cDNA sample produced from RT-PCR was PCR amplified in 25 µL of a solution containing 10X buffer (2.5 µL), 25 mM MgCl₂ (2.5 µL) and 10 pmol primer (0.75 µL). PCR was conducted in a 10X buffer for Taq polymerase (100 mM Tris-Cl pH 8.5, 400 mM KCl), 1 mM dNTP each, 50 mM MgCl₂, upstream primer (5 µM), downstream primer (5 µM), DNA template (less than 200 ng) SyBr Green I. The specific primer sequences used for each of the respective genes are as follows. iNOS (65 bp): 5'-CTG ATG CCT CTT CCA GGT GT-3' [sense]; 5'-GAG GGA GCC CTT TCT GAA TC-3' [antisense]. 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' [antisense]. GAPDH (93 bp): 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' [sense], 5'-CCC TGT TGC TGT AGC CGT AT-3' [antisense].

Western blotting. Tissues were first lysed in a buffer solution composed of 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/mL phenylmethanesulfonyl fluoride (PMSF), 1 µg/mL aprotinin and 1% Triton X-100. This mixture was centrifuged at 12000 rpm

for 30 min at 4 °C, at which time the resulting supernatant was transferred into a new tube. Next, 30 µg of soluble protein was loaded for 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a sample buffer containing 1 M Tris and 50% glycerol. All samples were heated at 95 °C for 5 min prior to gel loading. For the detection of iNOS and COX-2, the gel electrophoresis-separated proteins were transferred onto nitrocellulose membranes (Osmonics, Milwaukee, WI, USA) for 1 h at 0.16 A. These membranes were then washed three times with Tris-buffered saline Tween 20 (TBST), before being blocked with 5% skim milk for 1 h at room temperature, incubated overnight at 4 °C in a solution containing a goat anti-human iNOS and COX-2 polyclonal antibody (1:1000 in 5% bovine serum albumin) (Chemicon, CA, USA), and rewashed another three times with TBST. The membranes were then incubated with a secondary mouse anti-rabbit peroxidase-conjugated antibody (1:2000 in blocking solution) (Chemicon, CA, USA) for another hour at room temperature.

Statistical analysis. All results are expressed in the format mean ± SD. Data were analysed using the Student's *t*-test, and values were considered significantly significant when $p < 0.05$.

RESULTS

Clinical severity. To determine the clinical severity of each group, seven major clinical signs and symptoms of AD – erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness – were scored using the

criteria defined above (Kunz *et al.*, 1997). These major clinical signs and symptoms developed shortly after the mite antigen was applied to the backs of the mice, and the AD-like skin lesions progressively worsened until 20 weeks after the initial treatment. However, animals receiving topical treatment with ProB3 and RM-1 exhibited a significantly lower degree of clinical skin severity comparable to those treated with hydrocortisone (the positive control). Conversely, no change was observed in the normal control group during the experimental period ($p < 0.01$ or 0.005) (Fig. 2, Table 1). By the final week of the experiment, few of these clinical signs and symptoms were observed in any of the groups treated with ProB3 and RM-1 (Fig. 2). Such results indicate that both ProB3 and RM-1 are able to effectively decrease the skin severity of mite-induced AD-like skin lesions in NC/Nga mice.

Table 1. Effects of ProB3 and RM-1 on total clinical severity scores

Week	Sample			
	NC	ProB3	RM-1	HC
0	8.50 ± 0.50	8.50 ± 0.50	7.83 ± 0.29	8.50 ± 0.50
1	8.50 ± 0.50	7.00 ± 0.50 ^a	6.50 ± 0.50 ^a	6.83 ± 0.29 ^a
2	8.17 ± 0.76	4.17 ± 1.04 ^a	5.17 ± 0.76 ^a	4.17 ± 1.04 ^a
3	8.67 ± 0.58	3.17 ± 1.04 ^b	3.50 ± 0.50 ^b	3.17 ± 0.76 ^b
4	8.33 ± 0.58	2.00 ± 1.00 ^b	1.50 ± 0.50 ^b	1.00 ± 1.00 ^b

Results are expressed as mean ± SD ($n = 5$). ^a $p < 0.01$ and ^b $p < 0.005$ versus normal control each week. NC, normal control; HC, hydrocortisone.

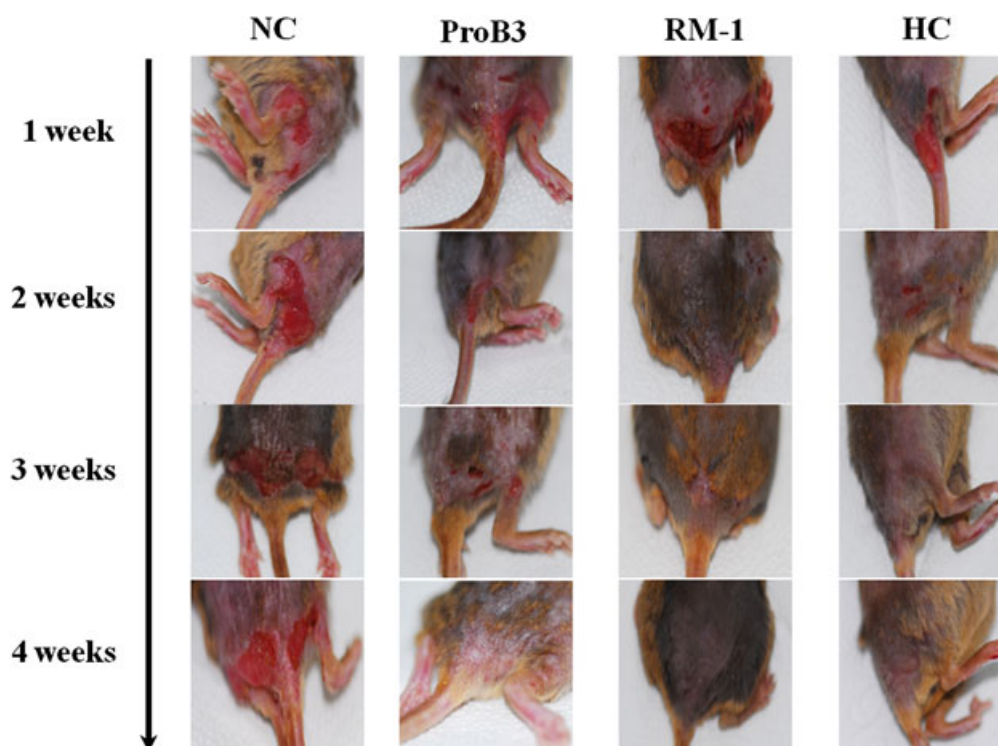


Figure 2. Effect of ProB3 and RM-1 on clinical observations. AD-like skin lesions induced by attachment of mite patches on the backs of mice for 20 weeks, and ProB3 and RM-1 treated topically for 4 weeks. Results are expressed as mean ± SD ($n = 5$). * $p < 0.01$ versus normal control each week. NC, normal control; HC, hydrocortisone. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

Eosinophil counts and IgE levels. Serum eosinophil count, a well-recognized and characteristic indicator of allergic diseases such as AD (Akdis *et al.*, 2000), was quantified by Wright-Giemsa-stained blood smears. As shown in Fig. 3, the topical application of ProB3 and RM-1 significantly decreased the number of eosinophils in blood, while no such change was observed among the normal controls ($p < 0.01$). ProB3 and RM-1 treatment also effectively reduced the absolute percentage of eosinophils among all WBCs (around 8% $\rightarrow$ 2% each, data not shown). All serum IgE levels, considered a representative indicator in AD patients, were measured by ELISA (Rousset *et al.*, 1991) to evaluate the therapeutic effects of ProB3 and RM-1 in NC/Nga mice. As seen in Fig. 3, IgE levels decrease significantly after topical application of ProB3 and RM-1, while no

such change occurred among the normal controls ($p < 0.01$). These results indicate that ProB3 and RM-1 may diminish the allergic response in mite-induced AD-like skin lesions among NC/Nga mice.

Th2 cytokine levels (IL-4, 5 and 13). Three Th2 cytokines (IL-4, 5 and 13) that are believed to regulate AD were also quantified in the serum and splenocytes of NC/Nga mice using ELISA. Animals treated topically with ProB3 and RM-1 exhibited significantly reduced serum levels of IL-4, 5 and 13, when compared with normal controls ($p < 0.05$ or 0.01) (Table 2). Levels of IL-4, 5 and 13 in splenocytes were also reduced significantly by the topical application of these experimental agents ($p < 0.05$ or 0.01) (Table 2). These results indicate that ProB3 and RM-1 have a suppressive effect on the hyper-production of Th2 cytokines in mite antigen-induced AD-like skin lesions among NC/Nga mice.

Expressions of iNOS and COX-2 proteins and mRNAs.

Here, the expression of iNOS and COX-2, two pro-inflammatory proteins (Vane *et al.*, 1994) was quantified by western blot, while the corresponding mRNA levels in NC/Nga mouse skin tissue was by RT-PCR. The data show that ProB3 and RM-1 significantly suppressed iNOS and COX-2 protein expression, while protein expression remained high among normal controls ($p < 0.01$) (Fig. 4). ProB3 and RM-1 also significantly reduced the expression of the corresponding mRNA ($p < 0.01$) (Fig. 4). These results indicate that ProB3 and RM-1 exerted an antiinflammatory effect against AD-like skin lesions in NC/Nga mice.

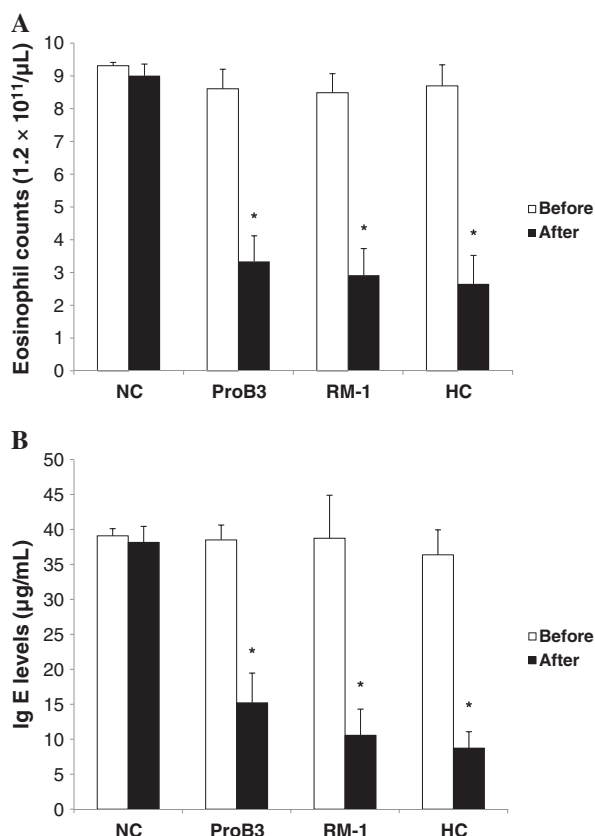


Figure 3. Changes in serum eosinophil and IgE levels before and after topical application of ProB3 and RM-1. Results are expressed as mean $\pm$ SD ($n = 5$). * $p < 0.01$ versus each group before treatment with sample. NC, normal control; HC, hydrocortisone.

DISCUSSION

Previously, we described the isolation of several condensed tannins from the roots of RM, with ProB3 and RM-1 identified as the major components. In LPS-stimulated RAW 264.7 cells, these two compounds have been shown to exhibit both a potent DPPH radical scavenging activity as well as an inhibitory effect on nitric oxide production (Park *et al.*, 2010). The present study evaluated the therapeutic effect of ProB3 and RM-1 on AD-like skin lesions induced in NC/Nga mice.

In order to better evaluate the biological parameters of AD, NC/Nga mice were used as an experimental animal model. NC/Nga mice have been used widely in

Table 2. Effects of ProB3 and RM-1 on serum and splenocyte Th2 cytokine levels in NC/Nga mice

Th2 cytokines (pg/mL)	NC	ProB3	RM-1	HC
Serum				
IL-4	1.17 $\pm$ 0.11	0.53 $\pm$ 0.29 ^b	0.31 $\pm$ 0.05 ^b	0.17 $\pm$ 0.01 ^b
IL-5	2.32 $\pm$ 0.20	1.43 $\pm$ 0.21 ^a	1.21 $\pm$ 0.22 ^b	0.29 $\pm$ 0.05 ^b
IL-13	0.77 $\pm$ 0.15	0.44 $\pm$ 0.08 ^b	0.37 $\pm$ 0.12 ^b	0.27 $\pm$ 0.17 ^b
Splenocyte				
IL-4	0.36 $\pm$ 0.08	0.27 $\pm$ 0.03 ^a	0.22 $\pm$ 0.03 ^b	0.22 $\pm$ 0.01 ^a
IL-5	0.62 $\pm$ 0.10	0.45 $\pm$ 0.07 ^a	0.37 $\pm$ 0.16 ^a	0.28 $\pm$ 0.01 ^b
IL-13	0.35 $\pm$ 0.05	0.26 $\pm$ 0.04 ^a	0.24 $\pm$ 0.04 ^b	0.15 $\pm$ 0.05 ^b

Results are expressed as mean $\pm$ SD ($n = 5$). ^a $p < 0.05$ and ^b $p < 0.01$ versus normal control. NC, normal control; HC, hydrocortisone.

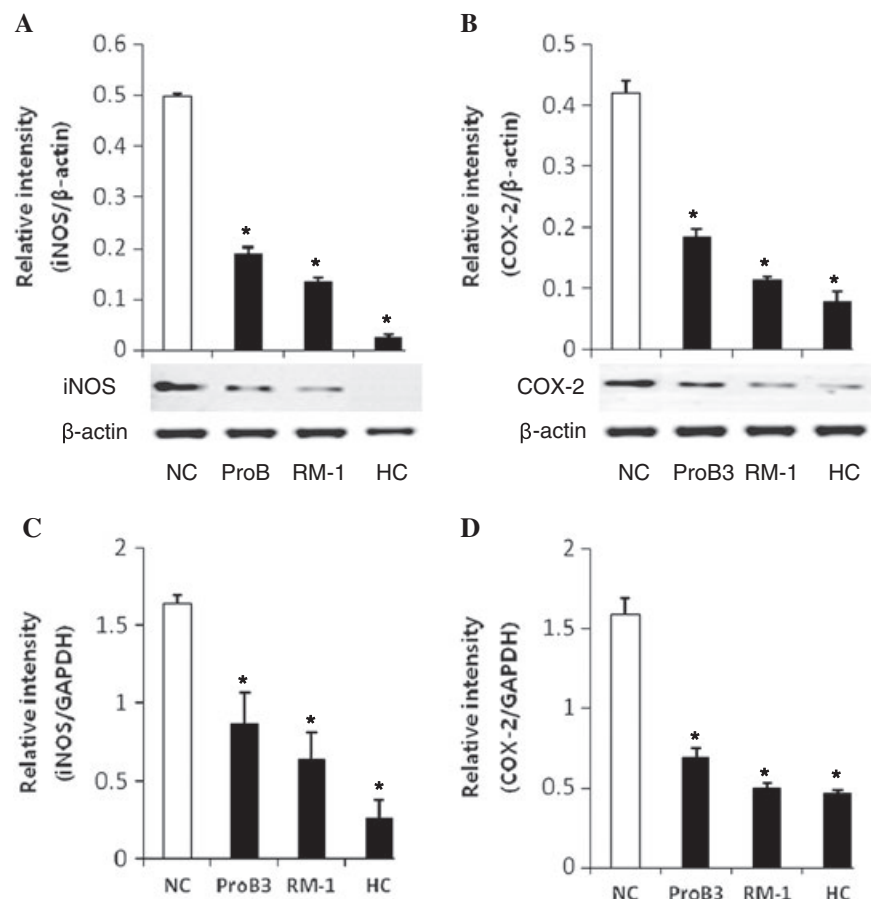


Figure 4. Effects of ProB3 and RM-1 on the expression of iNOS and COX-2 in skin of NC/Nga mice. Protein and mRNA expressions determined by western blot (A and B) and real-time RT-PCR (C and D), respectively. Results are expressed as mean $\pm$ SD ($n = 5$). * $p < 0.01$ versus normal control. NC, normal control; HC, hydrocortisone.

previous studies pertaining to AD, as this animal model manifests many clinical traits characteristic of AD, including increased serum IgE levels, chronic skin dryness and severe pruritis. Although some evidence suggests that under conventional conditions IgE levels in NC/Nga mice begin increasing at 8 weeks of age and skin lesion severity peaks at 17 weeks, the greatest challenge with this animal model is the low incidence of AD-like skin lesions resulting in limited reproducibility (Shiohara *et al.*, 2004). As such, the mite antigen was applied to the backs of all the mice for 20 weeks (Sasagawa *et al.*, 2001), allowing the effective induction of AD-like skin lesions. Increased serum levels of eosinophils, IgE and Th2 cytokines, were all also observed following the application of mite antigen, as was the hyper-expression of iNOS and COX-2.

All skin severity scoring was performed as described above. As shown in Fig. 2 and Table 1, AD-like skin lesions were effectively induced by the application of the mite antigen for 20 weeks (around 8–9 points). Once topical treatment with ProB3 and RM-1 began, several AD symptoms began immediately to improve, and, by the end of experimental period, had almost resolved completely. These results demonstrate that ProB3 and RM-1 may prove effective in treating the symptoms of AD.

High levels of serum eosinophils are common in many allergic diseases, such as AD. These cells infiltrate the dermis in response to allergens and subsequently secrete eosinophil cationic protein (EPC), which mediates the migration of other immune cells into the

forming skin lesions (Simon *et al.*, 2004). Moreover, eosinophils are now also believed to play a crucial role in aggravating AD symptoms (Magnarin *et al.*, 1995). Here, the relative eosinophilia induced by mite antigen was effectively decreased by the topical application of ProB3 and RM-1 (Fig. 3).

IgE production is one of the most important therapeutic targets in AD, as high serum IgE levels mediate the critical clinical characteristics of atopic diseases. Specifically, IgE binds mast cells causing the release of inflammatory mediators that correlates with the AD severity (Leung, 2000). In our study, ProB3 and RM-1 reduced both serum IgE levels as well as the severity of the skin disease (Fig. 3). Accordingly, these results suggest that the topical application of ProB3 and RM-1 may down-regulate IgE levels in AD-like lesions.

Abnormalities between Th1 and Th2 cytokines are one of the pathophysiological characteristics attributed to AD, as cases of acute atopy are now associated with a dysregulation of the Th1/Th2 axis towards Th2 that corresponds with Type I allergies (Grewe *et al.*, 1998). These Th2 cytokines also promptly induce an allergic-type reaction in AD, as IL-4 and IL-13 induce IgE secretion from activated B cells (Grewe *et al.*, 1998), while IL-5 stimulates the differentiation of eosinophils and prolongs eosinophil survival (Baatjes *et al.*, 2002). Here, ProB3 and RM-1 significantly reduced the mite-induced hyperproduction of Th2 cytokines in NC/Nga mice (Table 2). If ProB3 and RM-1 do exert such an immunoregulatory function, evidenced by decreases in

mite-induced eosinophil counts and IgE levels in treated NC/Nga mice, these molecules may also be able to palliate the severity of AD skin disease.

iNOS and COX-2 expression is markedly upregulated in proinflammatory cells by cytokine or mitogen stimulation from inflammatory reactions or other states of heightened immune response. As such, quantifying iNOS and COX-2 synthesis is considered the universal method for evaluating the antiinflammatory effect of pharmacological agents. In this study, iNOS and COX-2 were both overexpressed in the skin of normal control NC/Nga mice, as was the corresponding RNA, thus indicating that an inflammatory response was induced by mite antigen. Conversely, ProB3 and RM-1 significantly decreased the expression of both these markers (Fig. 4), effectively demonstrating that ProB3 and RM-1 may possess potent antiinflammatory properties in AD.

Significant evidence now exists to suggest that condensed tannins, including flavan 3-ols, possess both antiinflammatory and antiallergic activities. Specifically, these compounds have been shown to inhibit the phosphorylation of MAPK proteins and NF- κ B DNA binding in various experimental inflammatory conditions (Zhang *et al.*, 2006), suppress serum IgE and IgG1 levels in mice immunized with ovalbumin (Takano *et al.*, 2007), and inhibit the expression of IgE receptors on human mast cells (Tamura *et al.*, 2010). Additionally, oxidative stress generated by reactive oxygen species (ROS) from eosinophils and other leukocytes also is believed to play an essential role in allergic inflammation (Jung *et al.*, 2008).

Accordingly, the potent antioxidative activity of condensed tannins such as ProB3 and RM-1 may be able to reduce allergic inflammation by decreasing intracellular oxidative stress.

In summary, ProB3 and RM-1, two condensed tannins isolated from the roots of RM, possess potent free radical scavenging activities. Here, these agents exhibited a potent anti-atopic effect on mite-induced AD-like skin lesions in NC/Nga mice. Not only were ProB3 and RM-1 able to improve the clinical severity of AD lesions, they also reduced the serum levels of eosinophils, IgE, and several Th2 cytokines (IL-4, 5 and 13). Furthermore, these two compounds also suppressed iNOS and COX-2 expression, as well as their precursor mRNA. Such results strongly suggest that ProB3 and RM-1 may prove useful for the treatment of allergic skin conditions, including atopic dermatitis.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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