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Effect of *Alnus japonica* extract on a model of atopic dermatitis in NC/Nga mice

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ABSTRACT

Aim of the study: The bark of *Alnus* species has long been used in traditional oriental medicine in the treatment of many pathological conditions, including fever, hemorrhage, diarrhea, alcoholism, various skin diseases (e.g. chronic herpes, eczema and prurigo), and inflammation. In order to assess the immunomodulatory efficacy of a novel herbal medicine in treating atopic dermatitis, we measured serum levels of several allergic and inflammatory biomarkers in NC/Nga mice before and after treatment with this experimental agent.

Materials and methods: Gene and protein expression analyses of iNOS and COX-2 were quantified by real time PCR and Western blot analysis and serum levels of IL-4, -5 and -13 were also measured by ELISA, all of which were reduced after treatment with the experimental agent. Additionally, serum concentrations of IgE and blood eosinophil counts were reduced in treated mice.

Results: The topical application of leaf and bark extract from *Alnus japonica* suppressed the development of AD-like skin lesions. The percent of blood eosinophils was decreased after treatment with leaf and bark extract from *Alnus japonica*. The serum IgE and Th2-related cytokine levels were decreased after treatment with leaf and bark extract from *Alnus japonica* compared with those treated with base cream (vehicle treated AD group). The IL-4, IL-5 and IL-13 were lower than those of vehicle treated AD group.

Conclusions: We contend that leaf and bark extract from *Alnus japonica* may prove useful in the treatment of atopic dermatitis and other allergic skin diseases, although more in-depth clinical studies are necessary before clinical implementation.

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1. Introduction

Atopic dermatitis is a chronic, incurable immune disease, characterized by relapsing course. Although the etiology of atopic dermatitis has not been fully described, both genetic and environmental factors are believed to contribute to the underlying pathogenic mechanism, resulting in an imbalance between the Th1- and Th2-classes of cytokines. In atopic dermatitis, increased concentrations of cytokines secreted by activated Th2 cells – including interleukin-4 (IL-4), IL-5 and IL-13 – activate host allergic response, ultimately upregulating B cell synthesis of IgE. Conversely, serum levels Th1-associated cytokines, such as IL-

2 and interferon-gamma, are significantly decreased (Thepen et al., 1996; Grewe et al., 1998; Homey et al., 2006). Additionally, atopic dermatitis is characterized serologically by increased eosinophil counts and elevations in free IgE. Such increases in serum eosinophil concentration have been shown to exacerbate symptoms of atopic dermatitis, and result in an increase of eosinophilic infiltration into the dermis 2–6 h after peak serum levels. Approximately 24–48 h later, these cells secrete eosinophil cationic protein (EPC), which promotes the infiltration of other immune cells into the dermis, eventually resulting in new lesion formation (Bruijnzeel, 1993; Simon et al., 2004). The characteristic increases of serum IgE concentration are also believed to not only indicate disease activity but also contribute to atopic dermatitis pathogenesis (Rousset et al., 1991; Bos et al., 1992).

NC/Nga mice are a well-described animal model for atopic dermatitis, as they exhibit many of the characteristic clinical traits of the disease, including increased serum concentrations of IgE, chronic cutaneous xerosis, and severe pruritis (Vestergaard et al., 1999, 2000; Hashimoto et al., 2004). Notably, NC/Nga

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mice do not display these atopic dermatitis-like symptoms in specific-pathogen-free (SPF) conditions, and instead require antigen exposure to develop cutaneous immunologic hypersensitivity reactions. Given these conditions, we used a specific species of mite to induce cutaneous symptoms in the animals, as documented in several previous reports (Jung et al., 2010).

To date, atopic dermatitis has been considered an incurable dermatologic disease, with therapy mainly consisting of topical steroids, and systemic anti-histamines and immunosuppressives. As most of these treatment modalities are associated with side effects, rebound phenomenon, or intermittent recurrences, the therapeutic options for are imperfect and limited. Many alternative therapies are also used to treat atopic dermatitis, however, such modalities typically are not well grounded in scientific evidence and often have a higher side effect incidence. Nonetheless, one recent trend in drug development has been the application of natural, raw materials in the treatment of atopic dermatitis. Presumably, these efforts are aimed at scientifically and effectively developing medications that derive from natural raw materials, which can be used either as monotherapy agents or in concomitant therapy to synergistically enhance the overall effectiveness of the regimen.

Alnus species plant is a member of the Family Betulaceae, and is colloquially referred to as *Jeok Yang* among practitioners of Oriental medicine, who believe that it “clears body heat” and “reduces body heat”. In Oriental medicine, it has been believed that the body heat was closely related to skin diseases (Yang et al., 1999). Accordingly, *Alnus* species plant has been used in America as a traditional medicine for various skin affections (e.g. chronic herpes, eczema and prurigo) (Boericke, 1927). Recently, we isolated several diarylheptanoids such as 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-O-β-D-glucopyranosyl(1 → 3)-β-D-xylopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-O-β-D-apiofuranosyl(1 → 6)-β-D-glucopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-O-β-D-glucopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane, 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-O-β-D-glucopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane-3-O-β-D-xylopyranoside, platyphylloside, hirsutanonol, hirsutenone, and oregonin contained mainly in the *Alnus* genus (Jeong et al., 2000; Lee et al., 2000c). In our previous study, oregonin effectively scavenged free radicals (Lee et al., 2000d), inhibited production of nitric oxide and prostaglandin E₂ synthesis (Lee et al., 2000b), downregulated cyclooxygenase-2 expression (Lee et al., 2000a), suppressed the synthesis of oxygen radicals, reactive nitrogen species (RNS) and multiple proinflammatory molecules (e.g. IL-12, p70 and TNF-α), and also reduced elevations in calcium level (Choi et al., 2008) *in vitro*. In addition, oregonin reduced the atopic dermatitis-like state in NC/Nga mice *in vivo* (Choi et al., 2009).

Based on above various biological effects of oregonin, we evaluated the anti-atopic effects of *Alnus japonica* bark and leaf extracts. After a cream formulation containing a 60% edible ethanol extract of *Alnus japonica* bark and leaves was compounded, it was subsequently applied at a concentration of 1% to symptomatic NC/Nga mice for four weeks. And we examined clinical observation as well as additional laboratory assessments were also obtained for serum eosinophils, IgE, IL-4, IL-5, IL-13, iNOS and COX-2.

2. Materials and methods

2.1. Preparation of *Alnus japonica* extract

Alnus japonica was collected (4 kg barks and 1.5 kg leaves) from Mt. Sudal, Seoul, Republic of Korea, on August 2010, certificated by Prof. Lee (Pharmacognosy Lab, College of Pharmacy, Chung-Ang

University). And, a voucher specimen (AJB2010-08 and AJL2010-08) was deposited at the herbarium of the College of Pharmacy, Chung-Ang University. The 4 kg of AJB and 1.5 kg AJL were extracted twice with 60% edible ethanol extract at room temperature. Concentration that is removing the edible ethanol under vacuum afforded to 154.73 g and 163.5 g of extraction.

2.2. Animals

Female 5 week-old NC/Nga mice were purchased from Charles River Japan (Yokohama, Japan) and kept under conventional conditions, including a 12 h light/12 h dark cycle and freely available food and water. The temperature of the colony room was maintained at 22–23 °C and humidity 55 ± 15% (Hajime et al., 1998; Norikazu et al., 2003; Takano et al., 2006). To induce atopic lesion formation, house dust mite allergens (Biostar AD, Japan) were applied to the mice for 12 weeks after removing the animals' back hair. The animals were then divided into 5 groups of 5 mice: group I was treated with the vehicle (baseline), group II was treated with a formulation containing a 1% *Alnus japonica* bark extract (experimental), group III was treated with a 1% *Alnus japonica* leaf extract (experimental), group IV was treated with 1% plancol (positive control; Choongwae Pharma Co., Seoul, South Korea). All animal care and handling, and experimental procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of the Chung Ang University of Korea.

2.3. Clinical observation of AD

The backs of the NC/Nga mice were observed clinically for 4 weeks, with the areas exposed to dust mite antigens serially photographed. To compare the skin lesions between mice, the clinical severity of the dermatitis was scored using previously described macroscopic diagnostic criteria, normally applied to human AD. Briefly, dermatitis severity was evaluated once per week, by assessing for four specific criteria: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion. Each criterion was then scored on a three point scale: 0 (none), 1 (mild), 2 (moderate) and 3 (severe), with the sum of the individual scores used to denote overall dermatitis severity (Tomomi et al., 2001; Noh et al., 2008).

2.4. *Alnus japonica* extract treatment

Two separate base creams were compounded, one containing a 1% *Alnus japonica* leaf extract and the other containing a 1% *Alnus japonica* bark extract. Both formulations were applied topically to exposed mouse back skin on an indicated day for 4 weeks.

2.5. Measurement of eosinophil count

Blood samples were obtained at 20 weeks of age, from which whole blood cell counts were conducted using 30 μL of capillary blood that had been diluted six fold with 150-μL saline. Microscopic differential counts were performed using Wright–Giemsa stained blood smears, with serum eosinophil counts calculated from these differentials.

2.6. Serum IgE quantification

Serum IgE was quantified by a previously described sandwich ELISA method (Hiroshi et al., 1997; Eishin et al., 1999; Parente and Perretti, 2003). Briefly, 96-well plates were coated with monoclonal anti-mouse IgE, incubated overnight at 4 °C, and then treated for 2 h at room temperature with 2% (w/v) BSA (bovine serum albumin) dissolved in PBS containing 0.05% Tween 20 (PBS-T) to prevent any

nonspecific binding. Next, duplicate serial dilutions of the serum samples were incubated in the wells for 1 h at room temperature. After rinsing three times with PBS-T, biotin-conjugated rat anti-mouse IgE Ab (LO-ME-2) and streptavidin-peroxidase (Seikagaku Corp., Tokyo, Japan) were added sequentially to each well. After an additional three-rinse cycle, the plates were developed using a substrate solution containing 0.04% O-phenylenediamine dissolved in a phosphate citrate buffer (pH 5.0). The reactions were then terminated by the addition of sulfuric acid. The resulting absorbance was read using a microplate reader set at 490 nm, with the Ig isotype amount calculated by comparison to the mouse IgE standard (Pharmingen, CA, USA) (Nobuyuki et al., 2001).

2.7. Lymphocyte culture

Lymphocytes were isolated from spleen tissue. Red blood cells (RBC) were lysed using RBC lysis buffer (Sigma, MS, USA), and the resulting lysate was centrifuged at 1200 rpm for 15 min at a temperature of 4 °C. The supernatant was then removed and the cells were seeded at a ratio of 1×10^6 /well, before being cultured in 24-well plates. The supernatant containing cultured lymphocytes was harvested and measured for the presence of cytokines.

2.8. Cytokine measurement

Blood samples were collected at 20 weeks of age. The supernatants of cultured lymphocytes were analyzed for cytokine levels using a mouse cytokine enzyme immunoassay kit (R&D system, USA, MN) to detect for the presence of IL-4, IL-5 and IL-13 (Yoshiki et al., 2001).

2.9. Tissue biopsy

Tissue biopsies were obtained from the AD-like skin lesions to quantify iNOS and COX-2 expression.

2.10. Real-time PCR

2.10.1. Preparation of primer

PCR primers (Genotech, YG, KOR) were chemically synthesized with a DNA synthesizer (Pharmacia, Björkgatan, Uppsala, Sweden), using data from the Gene Bank. Their resulting sequences 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' (anti-sense)

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)

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)

RNA was isolated from tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) using the following procedure. Initially, 1 ml of TRIzol reagent was added to cells grown in the culture dishes. After 5 min at room temperature, 0.2 ml of chloroform was added for each ml of reagent, and the tubes were shaken vigorously by hand for 15 s. These were then incubated at temperatures between 15 °C and 30 °C for 3 min. Next, the mixture was centrifuged at 12,000 rpm for 15 min at 4 °C, after which the resulting upper aqueous phase was transferred to a fresh tube into which the same amount of 2-propanol was also added. After incubation for 15 min at 4 °C, the mixture was centrifuged again at 12,000 rpm for 15 min at 4 °C. After separation, the supernatant was removed, washed with 500 μ l of 70% ethanol and centrifuged again at 12,000 rpm for 5 min at 4 °C. The resulting RNA pellet was then briefly dried, before the purified RNA was dissolved in diethyl pyrocarbonate-distilled water (DEPC-DW). The total cellular RNA was reverse transcribed

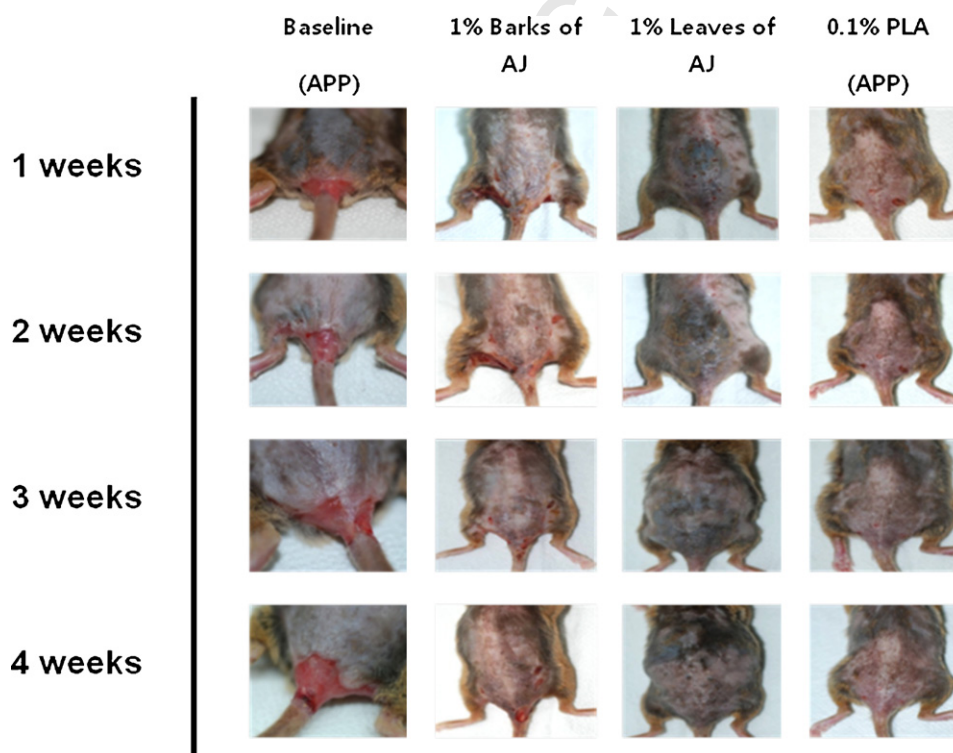


Fig. 1. Clinical observations of AD-like skin lesions induced in NC/Nga mice during experimental periods. Treatment with *Alnus* extract significantly reduced the severity of AD-like lesions induced in NC/Nga mice. PLA: hydrocortisone cream, AJ: *Alnus japonica*, APP: application.

at 42 °C for 30 min using a reverse transcriptase (TaKaRa, Shiga, Japan), 10× buffer, 10 mM dNTP (dNTP mix), oligo dT primer, RNase inhibitor and 25 mM MgCl₂.

2 μl of each cDNA sample from the RT-PCR (LightCycler® Carousel-Based System model, Roche-Applied Science Co. Ltd., IN, USA) reaction was amplified by PCR in 25 μl of total volume containing 10× buffer 2.5 μl, 25 mM MgCl₂ 2.5 μl and 10 pmol (0.75 μl) primer. PCR was conducted with 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 μM), downstream primer (5 μM), DNA template (less than 200 ng) and SyBr Green I.

2.11. Quantitative analysis of iNOS and COX-2

A densitometer was used for a Western blot analysis of the protein products from iNOS, COX-2 and Actin on DIG chemiluminescent film (volume of iNOS, COX-2/volume of Actin X100).

2.12. Western blot analysis

Tissue was lysed in a buffer solution containing 50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethanesulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% Triton X100. This mixture was then centrifuged at 12,000 rpm for 30 min at 4 °C. The resulting supernatant transferred into a new tube and 30 μg of soluble protein was loaded into a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) system with a sample buffer containing 1 M Tris, glycerol 50%. Before being loaded into the gel, the samples were heated at 95 °C for 5 min.

To quantify iNOS and COX-2, the electrophoretically resolved proteins were transferred onto nitrocellulose membranes (Osmonics, Milwaukee, WI, USA) at 0.16 A for 1 h. These membranes were washed 3 times with Tris-buffered saline Tween 20 (TBS–T), before being blocked with a solution of 5% skim milk for 1 hour at room temperature.

Next, the membranes were incubated overnight at 4 °C with goat anti-human iNOS and COX-2 polyclonal antibodies (1:1000 in 5% bovine serum albumin, Chemicon, CA, USA), before being washed 3 times with TBS–T the following morning. The membranes were then incubated in a secondary murine anti-rabbit peroxidase conjugated antibody (1:2000 in blocking solution, Chemicon, CA, USA) for 1 h at room temperature.

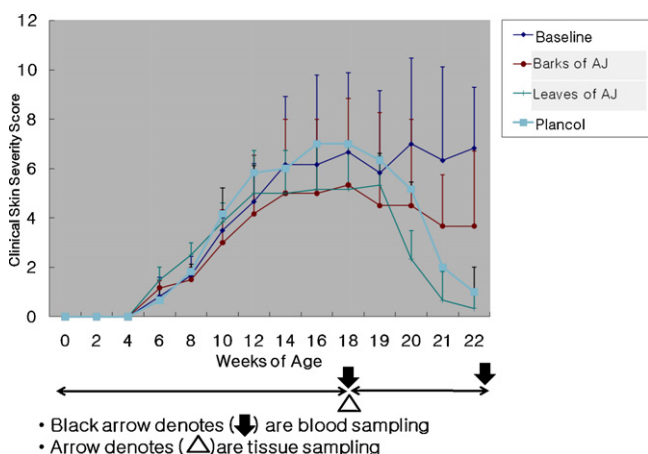


Fig. 2. Changes of the total clinical severity score after topical application of *Alnus* extract. The application of mite cream to the back of mice induced AD-like skin lesions. The total clinical severity score was defined as the sum of scores for seven clinical criteria: erythema, hemorrhage, edema, excoriation, erosion, scaling and dryness. Each category was graded using the following four-point scale: 0 (none), 1 (mild), 2 (moderate), and 3 (severe). All results are expressed as means $\pm$ S.D. ($n = 5$). PLA: hydrocortisone cream, AJ: *Alnus japonica*.

2.13. Quantitative chromatographic analysis of *Alnus japonica*

HPLC analysis were conducted using Waters system (Waters Co., Milford, MA, USA) with a Waters 600 pump, Waters 600 controller, Waters 112 UV/VIS (280 nm), Kromasil C₁₈ HPLC column (Varian, 5 μm, 250 mm $\times$ 4.6 mm) and Autochro-Win 3.0 plus (Young-lin Co., Korea) were used for data acquisition and integration. HPLC grade reagents (J.T. Baker Co. Ltd., USA) were used for HPLC analysis. All solvents were filtered and degassed before be used. The barks and leaves of *Alnus japonica* were accurately 2 g and dissolved in 50 ml of methanol. The sample was ultrasonic for 15 min and filtered through a No. 20 filter paper (600 mm $\times$ 600 mm, HYUNDAI Micro, KOREA). The filtrate layer of the sample was filtered through a 0.45 μm syringe filter (PVDF, GELMAN, USA). For the quantitative analysis to *Alnus japonica*, oregonin was previously isolated from *Alnus japonica*, as the standard material. The separation was carried out on a reverse phase system (Kromasil 5 μm C₁₈ HPLC column, Varian, 5 μm, 250 mm $\times$ 4.6 mm). A gradient mobile phase consisting of CH₃CN (A) and water (B) was used to run the separation. The elution program was set as follows: from 20% CH₃CN (A) and 80% water (B) to 40% CH₃CN (A) and 60% water (B) in 12 min;

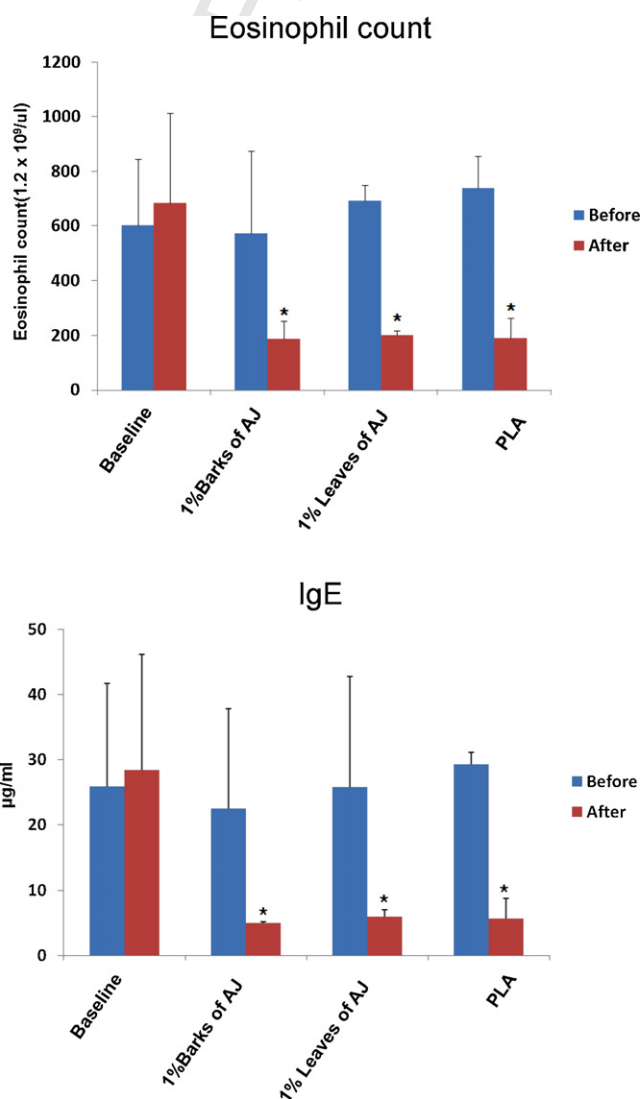


Fig. 3. Changes in eosinophil counts and IgE levels before and after *Alnus* extract treatment: eosinophil number and plasma IgE concentration were both reduced after *Alnus* extract treatment. All data are shown as mean $\pm$ S.D. PLA: hydrocortisone cream, AJ: *Alnus japonica*. * $p < 0.05$ compared with the base group, $n = 5$ /group.

temperature, room temperature; flow rate, 1.0 ml/min; injection volume, 25 μ l; UV detection, 280 nm. Oregonin was weighed to 1 mg and dissolved in 1 ml in methanol. The dissolved standard solution was diluted to 1000, 500, 250, 125 and 62.5 ppm, respectively.

2.14. Statistical analysis

All data are expressed in the format: mean $\pm$ SE. Statistical differences between groups were first calculated by factorial analysis of variance (ANOVA), then unpaired *t*-test.

3. Results and discussion

3.1. Clinical severity score reduced by *Alnus japonica* extract

Many of the classic cutaneous histopathological findings of atopic dermatitis – including erythema, bleeding, edema, desquamation, dryness, abrasions and erosions – were also observed in the NC/Nga mice after the atopic dermatitis-like syndrome was

induced by exposure to dust mite antigens. Modified SCORAD scores (Hajime et al., 1998; Tomomi et al., 2001; Takano et al., 2006) were recorded weekly, starting during the initial exposure phase and continuing through treatment with the topical agent formulations containing *Alnus japonica* extract. Based on these data, no improvements were observed in the negative control group. Besides, in both the positive control group and the groups treated with topical formulations of *Alnus japonica* extract the symptoms associated with atopic dermatitis improved significantly.

3.2. Eosinophil and IgE levels were decreased by *Alnus japonica* extract

Furthermore, the serum eosinophil count and IgE concentration (Rousset et al., 1991; Bos et al., 1992; Bruijnzeel, 1993; Simon et al., 2004), both recognized as the most characteristic histopathological indicators of disease activity, were compared among all the treatment groups where the occurrence of atopic dermatitis was induced using dust mite allergen and that where vaseline, cream formulation containing the bark and leaf of *Alnus japonica* (1%

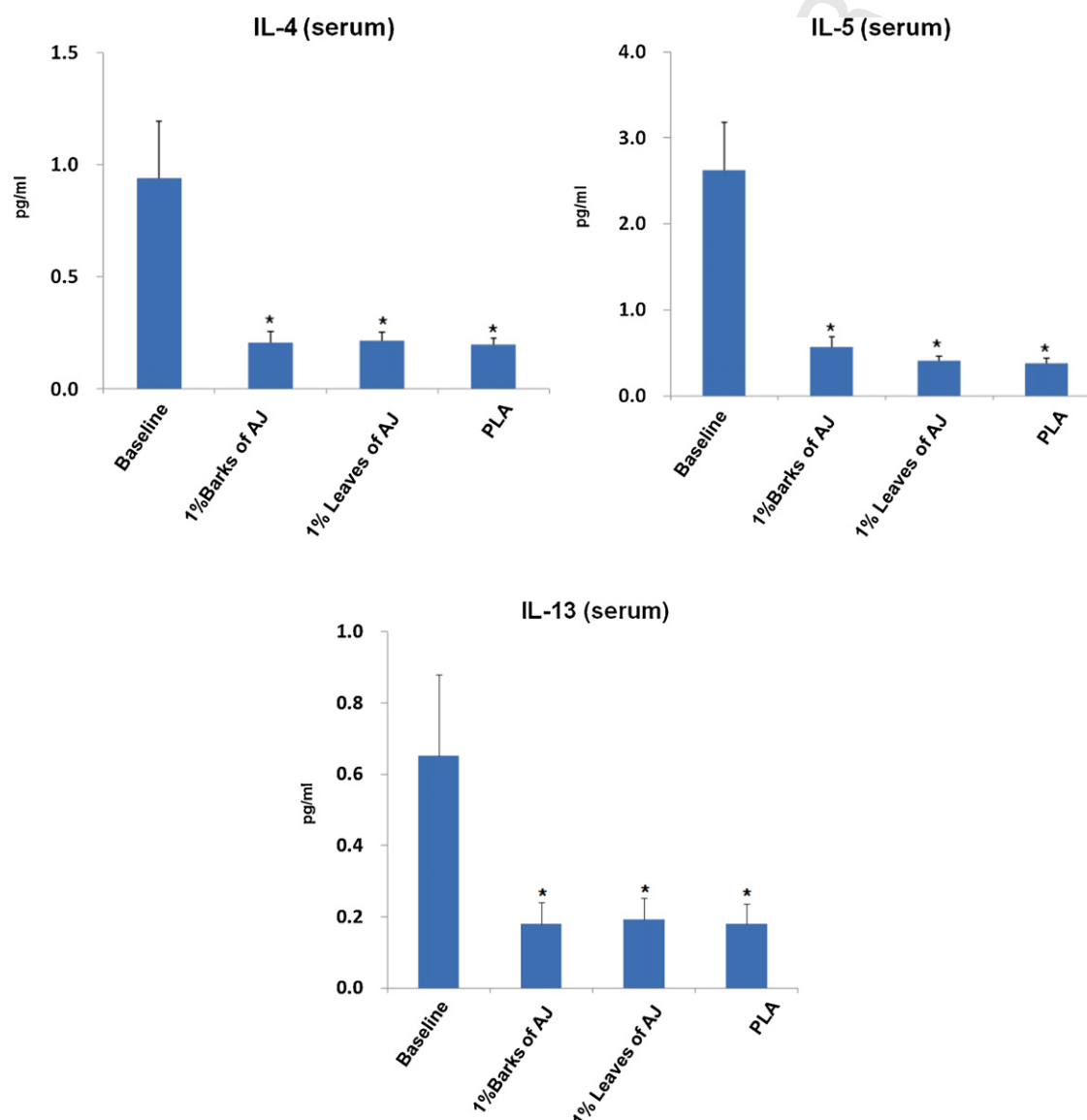


Fig. 4. Changes in serum cytokine concentrations after *Alnus* extract treatment. *Alnus* extract significantly decreased the levels of IL-4, IL-5 and IL-13 in the AD-like skin lesions of NC/Nga mice. Analyses were repeated a minimum of three times. All data are presented in the form: mean $\pm$ S.D. PLA: hydrocortisone cream, AJ: *Alnus japonica*. **p* < 0.05 compared with the base group, *n* = 5/group.

each) and plancol (0.1% hydrocorticosteroid) were topically applied for four weeks. After four weeks of therapy, the atopic dermatitis severity was significantly decreased in the groups treated with the two cream formulation containing the *Alnus japonica* bark and leaf extracts, and plancol (0.1% hydrocorticosteroid). Accordingly, these data indicate that both *Alnus japonica* extracts effectively decrease atopic dermatitis clinical severity.

3.3. Th2-related cytokines were decreased by *Alnus japonica* extract

In the current study, Th2 cytokines were serially measured to directly assess the immunomodulatory effects of the various topical treatments, since most evidence indicates that atopic dermatitis results from an imbalance of Th1 and Th2 responses. Specifically, the levels of three overexpressed Th2-associated (IL-4, IL-5 and IL-13) were measured. In addition to promoting the proliferation

of activated B- and T-cells, IL-4 mediates the T cell differentiation (CD4+ T cells) into the Th2 isotype. Clinically, IL-4 also is secreted at increased concentrations in patients with atopic dermatitis. Here, our data demonstrate that the topical application of extracts from *Alnus japonica* effectively reduces serum IL-4 concentrations, and ultimately suppress cutaneous allergic responses. IL-5 also promotes B cell growth, thereby facilitating immunoglobulin secretion. Additionally, IL-5 also is believed to play a key role in mediating eosinophil activation. Like IL-4, serum levels of IL-5 were significantly reduced after four weeks of topical treatment with *Alnus japonica* extracts. Blood eosinophil counts, a common serologic finding in patients with atopic dermatitis, were also decreased in the experimental groups. While IL-13 is secreted by many various cell types, it is most associated with Th2, as it is critically involved in initiating and mediating allergic inflammatory disease. Here, our results indicate that serum IL-13 concentrations were significantly lower among mice undergoing four weeks of topical treatment with

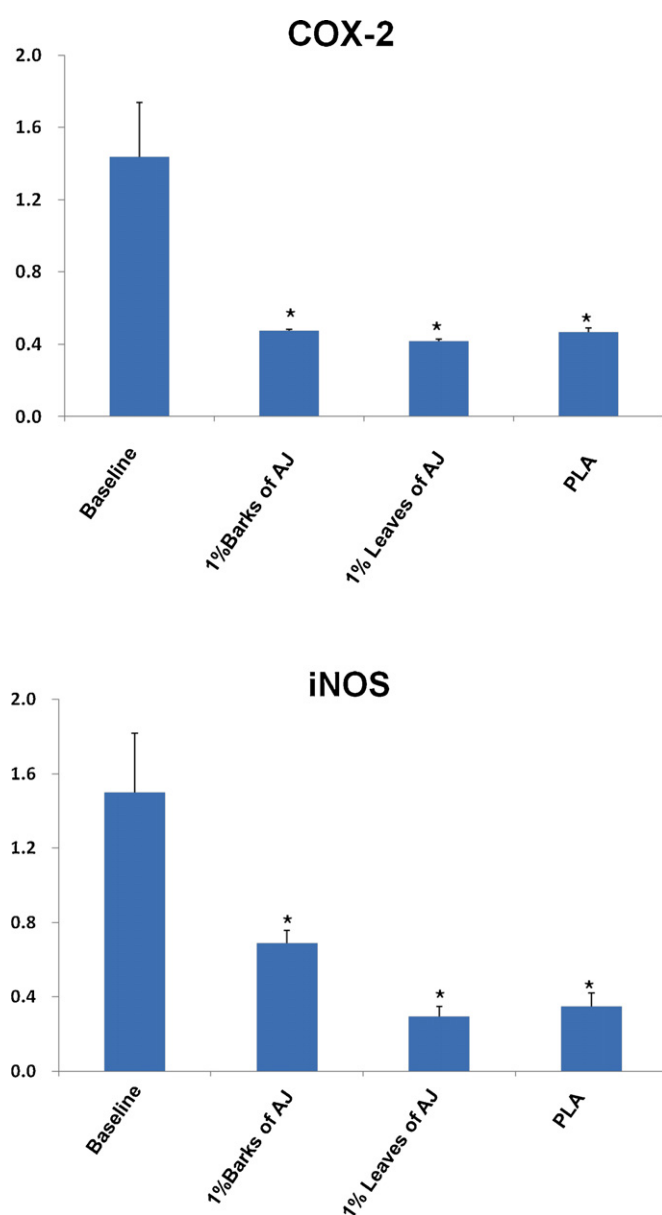


Fig. 5. Effect of topical application of *Alnus* extract on iNOS and COX-2 mRNA expression, as determined by real time RT-PCR. The mRNA expression levels of both iNOS and COX-2 were significantly inhibited by treatment with *Alnus* extract. The results are expressed as mean ± S.D. (* $p < 0.01$) ($n = 5$).

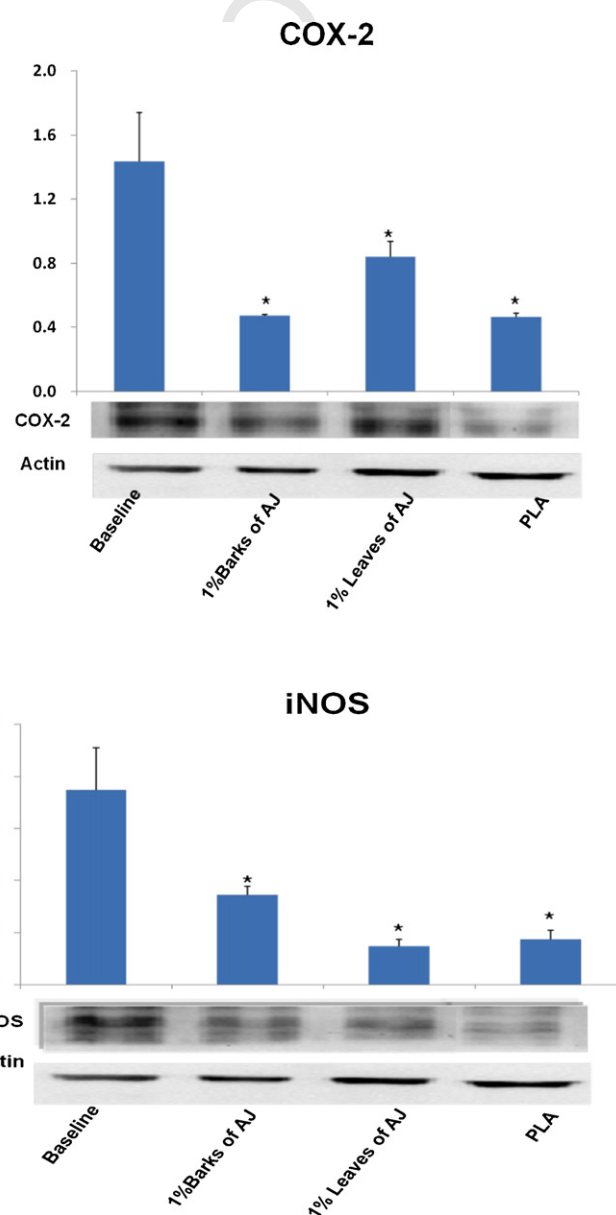


Fig. 6. Effects of topical application of *Alnus* extract on iNOS and COX-2 protein expression, as determined by Western blot. The protein expression levels of both iNOS and COX-2 were inhibited in the groups treated with *Alnus* extracts. The results are expressed as means ± S.D. (* $p < 0.01$) ($n = 5$).

Alnus japonica extract. Accordingly, we contend that this agent may have clinical efficacy against atopic dermatitis and other allergic inflammatory diseases (Thepen et al., 1996; Grewe et al., 1998; Homey et al., 2006).

3.4. Expression of iNOS and COX-2 in the skin lesion were decreased by *Alnus japonica* extract

Finally, as atopic dermatitis is regarded as an inflammatory dermatologic disease, we evaluated whether production of two inflammation-related factors, iNOS and COX-2, and mRNA and protein expressions were inhibited. In antigen-exposed NC/Nga mice, the serum levels of iNOS and COX-2 and its mRNA and protein expressions were significantly reduced after four weeks of topical treatment with the formulations containing the *Alnus japonica* extracts (Figs. 1–6).

3.5. Quantitative chromatographic analysis of *Alnus japonica*

The relationship between the concentration and the peak-area was measured by the minimum square method (R^2 value). The standard calibration curve obtained with concentrations in four

Calibration Plot

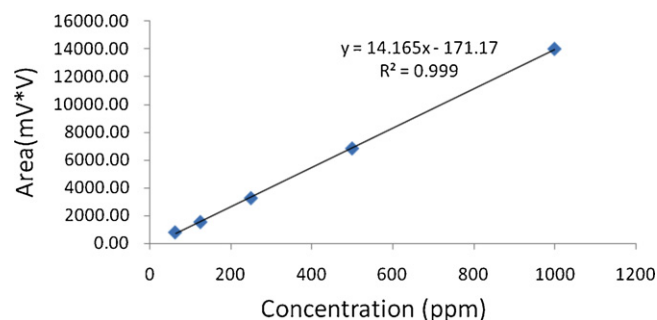


Fig. 7. Calibration curve and equation of oregonin. $Y = 14.165x - 171.17$ ($R^2 = 0.999$).

increments for oregonin, which is $Y = 14.165x - 171.17$ ($R^2 = 0.999$) as shown in Fig. 7. A good linearity (correlation coefficient ≥ 0.999) is shown for a calibration curve. The retention time of oregonin was 10.27 ± 0.01 min (Fig. 8). The average content of oregonin in *Alnus japonica* was calculated $31.00 \pm 0.001\%$ (barks) and $10.17 \pm 0.002\%$ (leaves) ($n = 3$) by the above formula (Fig. 9).

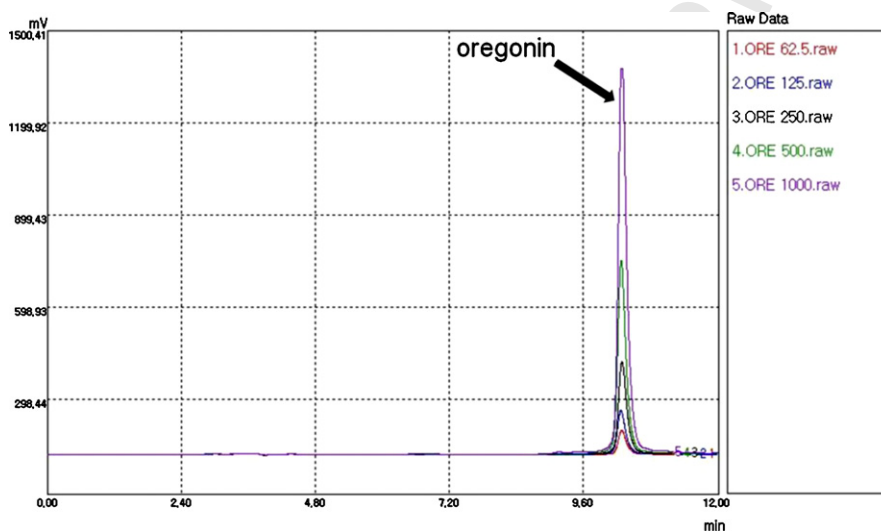


Fig. 8. HPLC chromatogram of oregonin. Sample 1 = oregonin 62.5 ppm, sample 2 = oregonin 125 ppm, sample 3 = oregonin 250 ppm, sample 4 = oregonin 500 ppm and sample 5 = oregonin 1000 ppm.

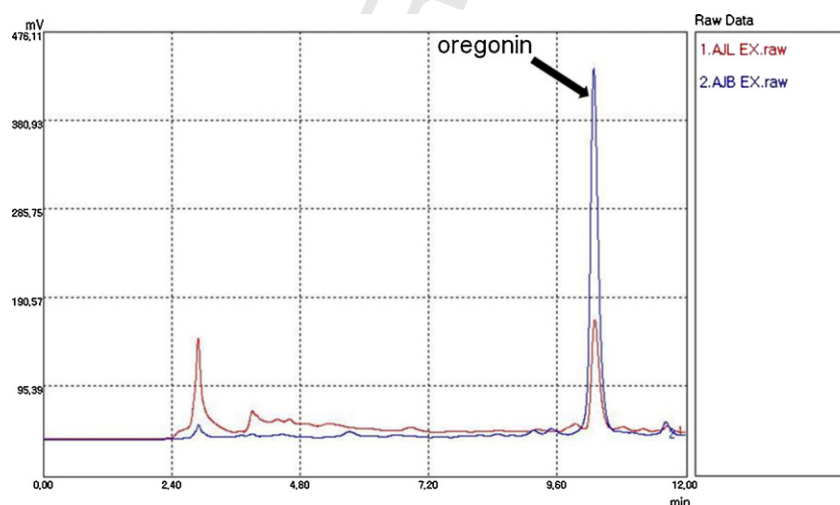


Fig. 9. HPLC chromatogram of extract of *Alnus japonica*. Sample 1 = leaves of *Alnus japonica* and sample 2 = barks of *Alnus japonica*.

4. Conclusion

In conclusion, our results indicate that, in a murine model of atopic dermatitis, topically applied *Alnus japonica* extract reduces Th2 cytokine overexpression, blood eosinophil counts, and serum IgE concentrations. Additionally, this agent was found to significantly suppress iNOS and COX-2. Clinically, we also observed symptom improvement in the experimental groups of NC/Nga mice, in which an atopic dermatitis-like syndrome was induced through exposure to dust mite allergens. Based on these results, *Alnus japonica* extract may represent a natural raw substance, which could be further developed as herbal new drug for the treatment of atopic dermatitis.

Q4 Uncited references

Kharitonov et al. (1994) and Lee (1966).

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