



The bark of *Betula platyphylla* var. *japonica* inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice

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Abstract

The bark of *Betula platyphylla* Sukat. var. *japonica* Hara (Asian White Birch, AWB) is one of the herbs used in Eastern countries for the treatment of various inflammatory diseases including atopic dermatitis (AD). The present study was performed to examine if and how the bark of AWB inhibits the development of AD-like skin lesions in NC/Nga mice induced by repeated application of picryl chloride (PC). With this aim, we examined the skin symptom severity, itching behavior, serum immunoglobulin (Ig) E level and mRNA expression of cytokines at iliac and cervical lymph nodes in the mice. Oral administration of AWB extracts (25, 100 and 250 mg/kg) to the PC-treated mice inhibited the development of AD-like skin lesions as exemplified by a significant decrease in the total skin severity scores, itching behavior and a decrease in hypertrophy and infiltration of inflammatory cells into dermis. The serum IgE level was also significantly reduced by AWB extract. In the RT-PCR results, the expression of interleukin-4 mRNA was reduced by AWB extract, whereas the expression of interferon- γ mRNA was not changed. These results suggest that AWB inhibits the development of AD-like skin lesions in NC/Nga mice through the suppression of the T-helper 2 cell response.
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1. Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disease that is characterized by pruritic and eczematoid skin lesions. AD is commonly present in early infancy and childhood, although it can be present later in life and persist in adulthood (Johansson et al., 2001). This disease results from interactions between susceptibility genes, the host's environment, skin barrier defects, bacterial and viral skin infections and

immunological factors (Leung et al., 2003). Recent immunological analyses of the pathogenesis of this disease have revealed that activated mast cells and an excess of differentiated T-helper (Th) 2 cells through chemical mediators and cytokines might play major roles in the development of dermatitis and immunoglobulin (Ig) E synthesis (Grewe et al., 1998). In contrast, it was also suggested that the T-helper (Th) 1-type cytokine interferon (IFN)- γ is involved in the chronicity of AD lesions (Grewe et al., 1994).

In the treatment of AD, topical glucocorticoids are frequently used to control acute exacerbation of AD (Van Der Meer et al., 1999). However, it is well known that prolonged use of high dose glucocorticoids frequently causes a variety of adverse effects (Leung and Barber, 2003). In recent years, therefore, alternative approaches have been used to target specific defects in AD

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(Panhans-Gross et al., 2001). Results from several studies indicate that patients with AD may benefit from herbal Oriental medicine therapy (Gao et al., 2005; Lee et al., 2006). Some medicinal plants in Oriental medicine have been used for treating AD. *Betula platyphylla* Sukat. var. *japonica* Hara (Asian White Birch, AWB) is one of these plants and is found in Korea and Japan (Alakurtti et al., 2006). The bark of AWB contains betulin, several triterpenes and phenolic compounds, such as diarylheptanoids and arylbutanoids, as active compounds. It has been reported that lupane triterpens from the bark of AWB have antiviral activity and that the extract of the bark of AWB has antioxidant, anticancer, hepatoprotective, chondroprotective and superoxide scavenging activities (Matsuda et al., 1998; Baltina et al., 2003; Zhang et al., 2003; Ju et al., 2004; Cho et al., 2006). However, it has not yet been studied if and how AWB suppresses the development of AD.

The present study was designed to investigate if and how the extract of the bark of AWB inhibits the development of atopic dermatitis like skin lesions in NC/Nga mice, which is a recently recognized murine model of AD (Matsuda et al., 1997). To this aim, we examined the skin symptom severity, itching behavior, serum IgE level and mRNA expression of the cytokines at lymph node in the mice. The hapten-induced dermatitis model was used in this study since repeated hapten (picryl chloride; PC) treatment causes apparent dermatitis in 100% of NC/Nga mice (Shiohara et al., 2004).

2. Materials and methods

2.1. Experimental animals

Female 4-week-old NC/Nga mice (25 ± 2 g) were purchased from SLC (Japan) and were maintained for 1 week before the start of the experiments. They were housed in an air-conditioned animal room with a 12-h light/dark cycle (08:00–20:00 light, 20:00–08:00 dark), a temperature of 23 ± 2 °C and a humidity of $50 \pm 10\%$. Mice were provided with a laboratory diet and water *ad libitum*. These mice were randomly divided into four groups: (1) control group (oral administration of normal saline, $n=7$); (2) AWB25 group; (3) AWB100 group; (4) AWB250 group oral administration of AWB extract at dose of 25, 100 and 250 mg/kg, $n=8$, respectively). The study was approved by the Institutional Animal Care and Use Committee of Kyung-Hee University and all procedures were conducted in accordance with the U.S. National Institutes of Health guidelines.

2.2. Reagents & quantitative chromatographic analysis of AWB

1-Chloro-2,4,6-trinitrobenzene (Picryl Chloride, purchased by Tocris, Japan) was used after recrystallization with ethanol. Picryl Chloride (PC)-induced dermatitis was developed in NC/Nga mice according to standard instructions provided by Charles River Japan. Briefly, the abdomen and ears of mice were sensitized epicutaneously with 150 μ l of 5% PC dissolved in an ethanol and acetone mixture (4:1). On day 7 after the sensitization, the dorsal skin of mice was challenged with 150 μ l of

1% PC dissolved in olive oil. After the first challenge, 1% PC solution was repeatedly applied to the dorsal skin of mice for further 8 times at 1-week intervals (Taniguchi et al., 2003).

HPLC analysis were conducted using Waters system (Waters Co., Milford, MA, USA) with a 717+ autosampler, 2996 photodiode array detector (PDA) 2487 dual λ absorbance detector and 1525 binary HPLC pump and Waters Millennium³² System (Waters Co., Milford, MA, USA) were used for data acquisition and integration. HPLC grade and other reagents (J.T. Baker Co. Ltd., Phillipsburg, NJ, USA) were used for HPLC analysis. All solvents were filtered and degassed before be used.

The cortex of *Betula platyphylla* var. *japonica* Hara was accurately weighed to 0.490 g and dissolved in 50 ml of methanol. The sample was ultrasonic for 15 min and centrifuged at 15,000 rpm for 10 min. The supernatant of the sample was filtered through a 0.45 μ m syringe filter (PVDF, GELMAN, USA). For the quantitative analysis to *Betula platyphylla*, betulin was purchased from Sigma–Aldrich, as the standard material.

The separation was carried out on a reverse phase system (XTerraTM C₁₈, 4.6 mm $\times$ 150 mm i.d., 5 μ m, Waters Co., USA). A gradient mobile phase consisting of A (acetonitrile) and B (water) was used to run the separation. The elution program was set as follows: from 30%A–70%B to 100% A in 30 min; temperature, 35 °C; flow rate, 1.0 ml/min; injection volume, 25 μ l; UV detection, 210 nm. Betulin was weighed to 1 mg and dissolved in 1 ml in methanol. The dissolved standard solution was diluted to 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/ml, respectively.

2.3. Preparation and administration of AWB

The cortex of *Betula platyphylla* Sukat. var. *japonica* Hara as a dried herb was purchased from Nanumherb Inc. (Youngchun, South Korea) and authenticated by H. Bae, College of Oriental Medicine, Kyung Hee University. A voucher specimen was deposited at the Herbarium of the College of Oriental Medicine, Kyung Hee University. The bark of AWB was prepared by decocting the dried herb (100 g) with boiling distilled water (500 ml). The duration of decoction was about 3 h and filtered through a nylon mesh. After centrifugation at $5000 \times g$ for 20 min at 4 °C, the supernatant of the AWB was lyophilized and kept at 4 °C. The yield of dried extract from starting materials was about 8%. Dilutions were made in normal saline then filtered through a 0.4 μ m syringe filter. Suspension of AWB extract (25, 100 and 250 mg/kg, 0.2 ml/day) was orally administered ($n=8$) by gastric intubation with an animal-feeding needle, 5 days per week for 8 weeks starting from 1 week after the sensitization. Oral administration of normal saline (0.2 ml/day, $n=7$) served as a control.

2.4. Evaluation of skin severity

The severity of dermatitis was assessed macroscopically in a blinded fashion using 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 four signs and symptoms: erythema (hem-

orrhage), edema, excoriation (erosion) and scaling (dryness), on the ear, back, neck and facial regions (Matsuda et al., 1997).

2.5. Evaluation of itching behavior

Mice were placed individually in a clear plastic cage and their behavior was videotaped for 30 min 9 weeks after sensitization. Scratching of the rostral back and biting of the caudal back were observed during playback; scratching movements by the hind paw was defined as a scratching bout which ended when the rat either licked its hind paw or placed its hind paw back on the floor and a series of one or more biting movements were counted as one episode which ended when the rat returned to the straight-forward position (Maekawa et al., 2002).

2.6. Histological analysis

Mice were sacrificed at the end of the experiment. The auricular skin was removed, fixed in 10% formalin, embedded in paraffin, sectioned at 10 μ m and then stained with hematoxylin and eosin solution.

2.7. Measurement of serum IgE level

Blood was collected from the retro-orbital plexus of NC/Nga mice on the day of sacrifice. Serum samples were obtained by centrifugation and stored at -70°C until required. Total IgE levels in the serum were measured using a sandwich enzyme-linked immunosorbent assay kits (R&D systems Inc., Minneapolis, MN, USA) following the manufacturer's instructions.

2.8. Detection of IL-4 and IFN- γ mRNA in lymph node

Expression of interleukin (IL)-4 and interferon (IFN)- γ mRNA in whole iliac and cervical lymph nodes of each mouse was examined respectively. The whole excised lymph nodes were homogenized for 90 s (30×3 s) in Isogen (Nippon Gene, Tokyo, Japan) using HG 30 Homogenizer (Hitachi, Tokyo, Japan). Excised lymph nodes were homogenized in Isogen (Nippon Gene, Tokyo, Japan) using HG 30 Homogenizer (Hitachi, Tokyo, Japan). One milliliter of homogenate was mixed with 200 μ l of chloroform (Nacalai Tesque, Kyoto, Japan) vigorously, and centrifuged at $13,000 \times g$ for 15 min at 4°C using a microcentrifuge (Laboratory Centrifuge 1900, Kubota Co., Tokyo, Japan). The aqueous phase was separated and RNA in the phase was precipitated by mixing 0.5 ml of 2-propanol (Nacalai Tesque). The precipitate was washed with 75% ethanol (Nacalai Tesque) and dried, and then dissolved in diethyl pyrocarbonate (DEPC)-treated water (Nacalai Tesque). The total RNA content was calculated based on the absorbance at 260 nm and the quality was confirmed by electrophoresis.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was employed for the detection of IL-4 and IFN- γ mRNA. The primer sequence from 5' to 3' for β -actin was, forward primer, GTGGGCCGCTAGGCACCA and reverse primer, CGGTTCGCCTTAGGGTTCAGGGGGG. The primer sequence from 5' to 3' for IL-4 was, forward primer, ACGGAGATGGATGT-

GCCAAACGTC and reverse primer, CGAGTAATCCATTG-CATGATGC. The primer sequence from 5' to 3' for IFN- γ was forward primer, TACTGCCACGGCAGTCATTGAA and reverse primer, GCAGCGACTCCCTTTTCCGCTTCCT. A mixture of 11 μ l containing 1 μ g RNA, DEPC-treated water and random primer (GIBCO BRL, Grand Island, NY, USA) was heated at 70°C for 10 min and then mixed with 4 μ l of $5 \times$ First Strand Buffer (GIBCO), 1 μ l of 10 mM deoxynucleoside triphosphate (dNTP, GIBCO) and 2 μ l of 0.1 M dithiothreitol (GIBCO). After 5 min at 25°C , 1 μ l of reverse transcriptase (Superscript II, GIBCO) was added and RT, at 25°C for 10 min, 42°C for 50 min and then 70°C for 15 min, was performed on Trio-Thermoblock (Biometra, Goettingen, Germany). Then, cDNA at a volume of 1 μ l was mixed with 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 10 mM dNTP, 5 U/ml ampliTaQ DNA polymerase (TaKaRa Taq, Takara, Kyoto, Japan) and 1 μ M primers (Stratagene, La Jolla, CA, USA) and PCR (denaturation at 94°C for 1.5 min, annealing at 62°C for 1.5 min and extension at 72°C for 1.5 min, 35 cycles) was performed on Trio-Thermoblock. Products were electrophoresed on 2% agarose gel containing ethidium bromide. The bands were recorded by Polaroid camera (Polaroid 665 film, Nippon Polaroid, Tokyo, Japan) and densitometrically scanned for semiquantitative evaluation. Results were normalized by β -actin expression.

2.9. Liver function tests

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured to assess hepatic dysfunction in mouse serum. ALT activity was determined with an ALT/GPT kit (Sigma, St Louis, MO, USA). Briefly, 10 μ l of each sample was added to a well of a 96-well plate, in duplicate, followed by 50 μ l GPT substrate solution. After 1 h incubation at 37°C , 50 μ l of color reagent was added to each well and the plate was incubated for 20 min at room temperature. After incubation, 200 μ l 0.5 mol/l NaOH was added and 5 min later absorbance was read at 492 nm using a plate reader. AST was determined with an AST/GOT kit (Sigma). Briefly, 10 μ l of each sample was added to a well of a 96-well plate, in duplicate, followed by 50 μ l GOT substrate solution. The remainder of the procedure was as described for the GPT assay (Ma et al., 2003).

2.10. Statistical analysis

All data are presented as mean $\pm$ S.E.M. One-way ANOVA and post hoc by Tukey's test were used for the statistical analysis. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. Effects of oral administration of AWB extract on skin symptom severity and itching behavior

The development of dermatitis was observed in the control group of mice: mild eczema and hemorrhage of the rostral lesions: alopecia and scaling of the neck and dorsal skin 9 weeks

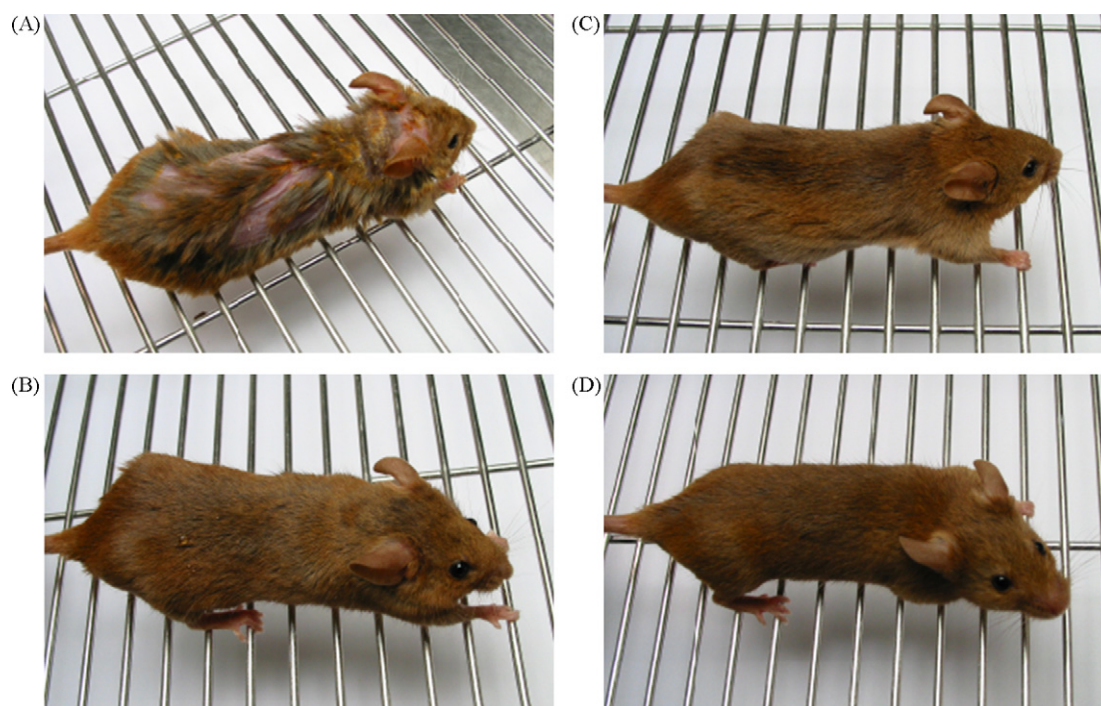


Fig. 1. Clinical features of NC/Nga mice. The photograph was taken on the 9 week after sensitization. (A) Control group (normal saline administered, $n = 7$). (B) AWB25 group (25 mg/kg AWB extract-administered, $n = 8$). (C) AWB100 group (100 mg/kg AWB extract administered, $n = 8$). (D) AWB250 group (250 mg/kg AWB extract administered, $n = 8$).

after sensitization (Fig. 1A.). However, oral administration of AWB extracts (25, 100 and 250 mg/kg) inhibited the appearance of these skin symptoms (Fig. 1B–D.). The skin conditions were evaluated 9 weeks after sensitization by skin severity score, and the results are shown in Fig. 2. Oral administration of AWB extract significantly lowered the skin severity score when compared with that in the control group (AWB25 vs. control, $P < 0.01$; AWB100 or AWB250 vs. control, $P < 0.001$). Itching behaviors in the AWB extract-administered groups were

significantly lower than those in the control group (Fig. 3, $P < 0.001$).

3.2. Histological analysis

The histological study revealed that hypertrophy and hyperkeratosis of the epidermis, intracellular edema and infiltration of inflammatory cells were observed in the control group (Fig. 4A.). Whereas, treatment with AWB extract inhibited these inflammatory changes (Fig. 4B.).

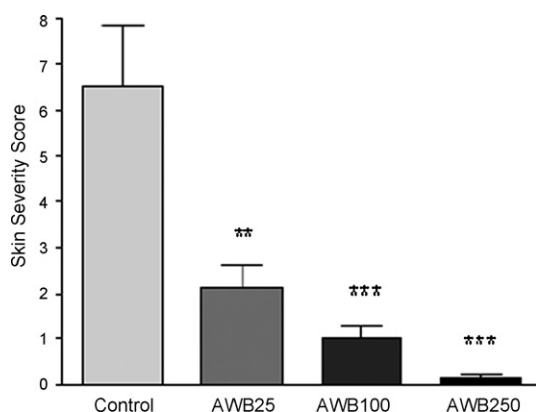


Fig. 2. Effect of AWB extract on clinical scores of skin symptoms. The total scores of the skin severity were defined as the sum of the individual score (0, no symptoms; 1, mild; 2, moderate; 3, severe) for each of the following four signs and symptoms: erythema (hemorrhage), edema, excoriation (erosion) and scaling (dryness). Values represent the means $\pm$ S.E.M. of the total score from mice for each group. (** $P < 0.01$, *** $P < 0.001$; compare to control group ($n = 7$). $n = 8$ in other groups).

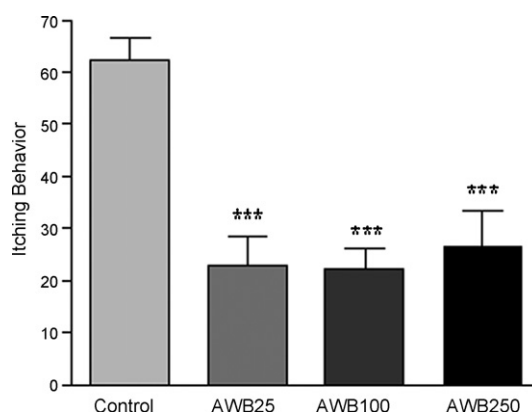


Fig. 3. Comparison of itching behavior. Itching behavior was observed for 30 min on the 9 week after sensitization by measuring the number of scratching of rostral back and biting of caudal back. Values represent the means $\pm$ S.E.M. of each group (** $P < 0.01$, *** $P < 0.001$; compare to control group ($n = 7$). $n = 8$ in other groups).

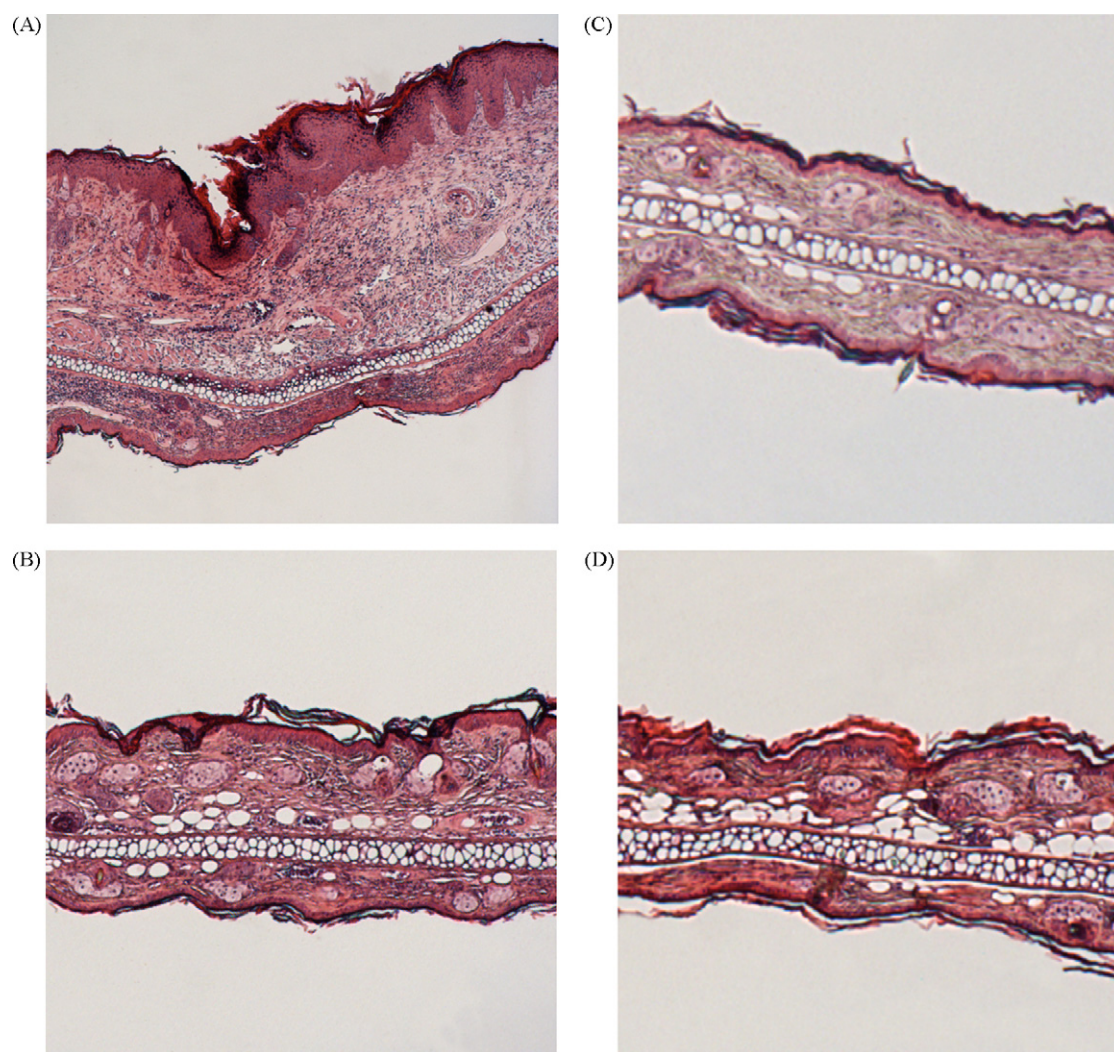


Fig. 4. Histologic features of auricular skin in the control NC/Nga mice (A) and in the AWB25 group (B), AWB100 group (C) and AWB250 group (D) (hematoxylin and eosin; original magnification 40 $\times$).

3.3. Effects of oral administration of AWB extract on serum IgE levels and mRNA expression of IL-4 and IFN- γ

The serum levels of IgE were significantly decreased in the AWB extract-administered groups, compared to the control group. However, there was no significant difference in the serum levels of IgE among the three AWB groups (Fig. 5, AWB25 or AWB250 vs. control, $P < 0.05$; AWB100 vs. control, $P < 0.01$). The IL-4 and IFN- γ mRNA expression in the whole body lymph node was examined, using RT-PCR (Fig. 6). IL-4 mRNA expression was undetectable in the lymph node of AWB extract-administered mice, whereas an apparent IL-4 mRNA expression was induced in the lymph node of the control mice (Fig. 6A.). IFN- γ mRNA expression was undetectable in both the AWB extract-administered and control mice (Fig. 6B.).

3.4. Effects of AWB extract on alanine aminotransferase and aspartate aminotransferase levels

There was no elevation of plasma ALT and AST levels in the groups given AWB, which indicates that AWB did

not cause liver injury. Mean $\pm$ S.E.M. ALT levels in the control, AWB25, AWB100 and AWB250 groups were 10.0 ± 0.5 , 9.9 ± 1.4 , 9.4 ± 1.8 and 10.0 ± 0.6 IU/l, respectively ($P > 0.05$). Mean $\pm$ S.E.M. AST levels in the control, AWB25, AWB100

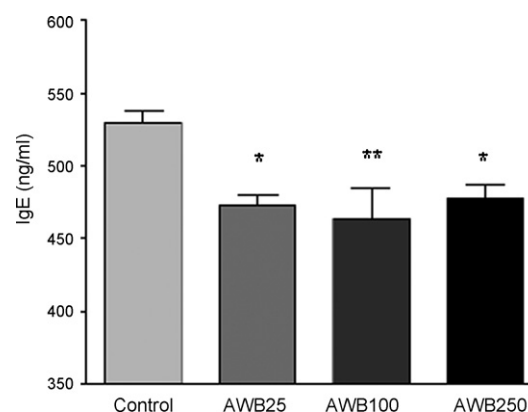


Fig. 5. Effect of AWB extract on serum IgE level. The serum level of IgE was measured by ELISA. The serum level of IgE was significantly decreased in AWB administered group compare to control group ($n = 7$). $n = 8$ in other groups. Values are means $\pm$ S.E.M. (* $P < 0.05$ ** $P < 0.01$).

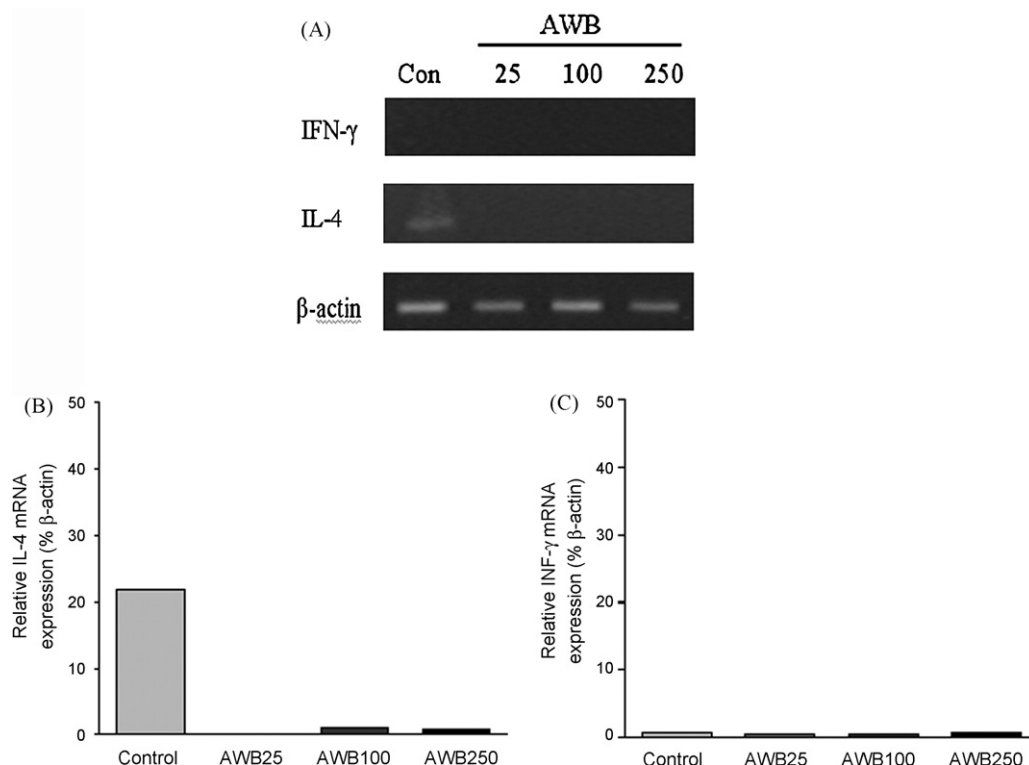


Fig. 6. Comparison of IFN- γ and IL-4 in lymph nodes. IFN- γ and IL-4 mRNA expression in lymph nodes were measured to examine the effect of AWB administration on cytokines by RT-PCR. Resultant electrophoretic bands (A) were measured by Image analyzer (Lab Work, USA). Experiments were repeated at least twice and a representative experiment is indicated. Results are expressed as a ratio of (B) IL-4 and (C) IFN- γ to β -actin mRNA expression. $n = 7$ in control group and $n = 8$ in other groups.

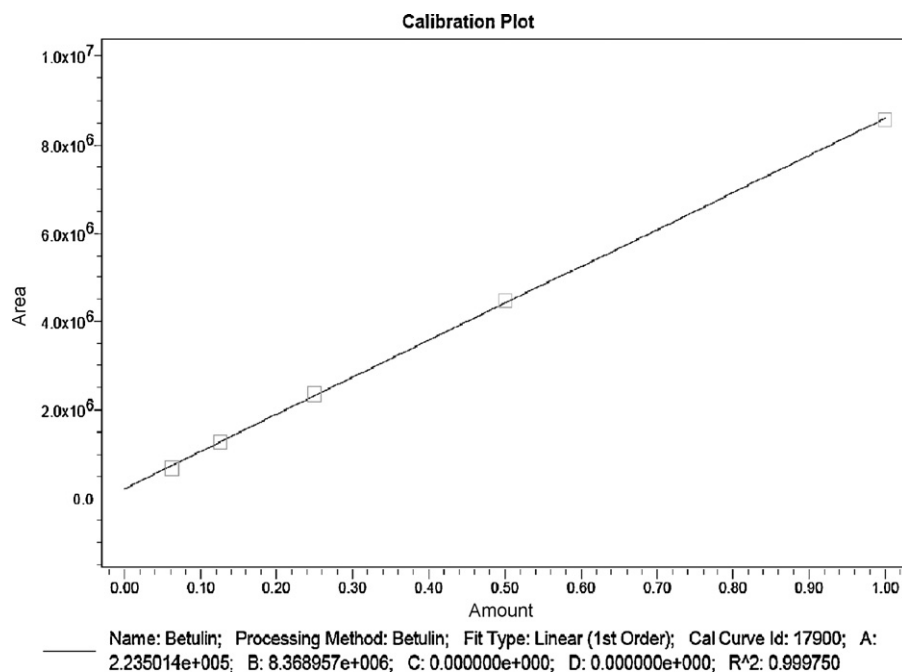


Fig. 7. Standard calibration curve of betulin.

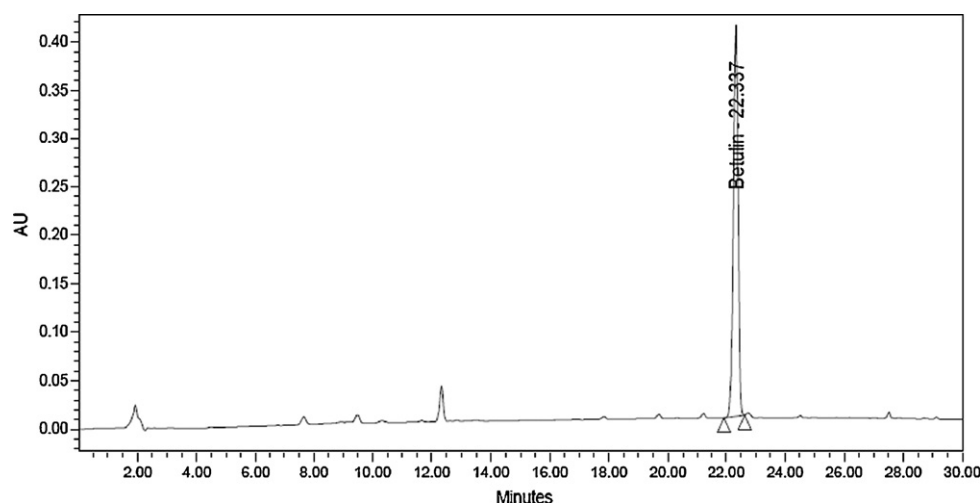


Fig. 8. Auto-scaled chromatogram of betulin.

and AWB250 groups were 14.1 ± 0.5 , 13.8 ± 1.6 , 14.2 ± 1.0 and 13.8 ± 0.7 IU/l, respectively ($P > 0.05$).

3.5. Quantitative chromatographic analysis of AWB

The relationship between the concentration and the peak-area was measured by the minimum square method (R^2 value). The standard calibration curve of betulin $Y = 8368957 X + 223501.4$ ($R^2 = 0.999$) as shown in Fig. 7. Betulin was shown at the retention time 22.361 min (Fig. 8). The average content of betulin in *Betula platyphylla* was calculated 59.0 ± 0.35 mg/g ($n = 3$) by the above formula (Fig. 9).

4. Discussion

Betula platyphylla var. *japonica* (Asian White Birch, AWB) is one of these plants and is found in Korea and Japan. The bark of AWB contains betulin, several triterpenes and phenolic compounds, such as diarylheptanoids and arylbutanoids, as active compounds. It has been reported that betulin is a triter-

pene forming the principal extractive (up to 30% of dry weight) of the bark of birch trees. Betulin can be easily converted to betulinic acid, which possesses a wide spectrum of biological and pharmacological activities. Betulinic acid has antimalarial and anti-inflammatory activities. Betulinic acid and its derivatives have shown anti-HIV activity and cytotoxicity against a variety of tumor cell lines. Moreover, it is non-toxic up to 500 mg/kg body weight in mice. (Alakurtti et al., 2006). In this study, AWB has been suppressed the development of AD and there was no elevation of plasma ALT and AST levels in the groups given AWB extract, which indicates that AWB extract did not cause liver injury. In addition, the weight gain in NC/Nga mice during the experiment was not disturbed by AWB administration (data not shown).

NC/Nga mice have been recently recognized as atopic dermatitis model mice (Matsuda et al., 1997). AD-like skin lesions in NC/Nga mice highly resemble those of human AD that show various grades of signs and symptoms like erythema and hemorrhage, followed by edema, superficial erosion, deep excoriation, alopecia and dryness in the skin. In addition, NC/Nga mice with

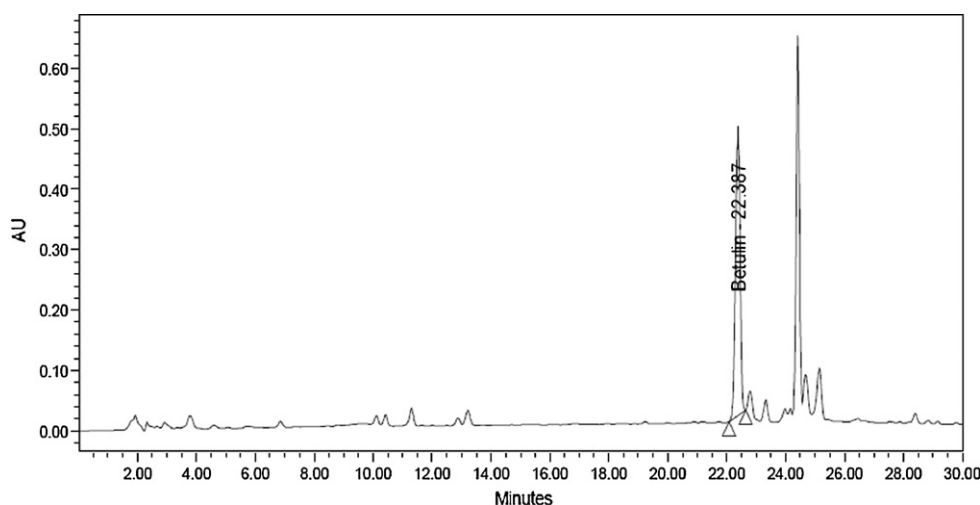


Fig. 9. Auto-scaled chromatogram of *Betula platyphylla* var. *japonica* Hara.

AD-like skin lesions show IgE hyperproduction in serum, severe itching behavior and invasion of inflammatory cells into the skin (Matsuda et al., 1997; Maekawa et al., 2002; Shiohara et al., 2004). In the present study, the control group of mice also developed skin symptoms as described above.

There were significant differences among the control and AWB groups in skin severity score. The control group showed the development of dermatitis; eczema and hemorrhage of the rostral lesions: alopecia and scaling of the neck and dorsal skin. On the other hand, the administration of AWB extract at all doses prevented these skin changes, and the total skin scores were reduced by AWB extract administration in a dose-dependent fashion. In addition, histological analysis clearly showed that oral administration of AWB extract inhibited skin inflammations in NC/Nga mice. The thickening of the epidermis and infiltration of inflammatory cells were observed in control mice, whereas little changes were seen in AWB administered groups. These results indicate the therapeutic efficacy of AWB extract in NC/Nga AD model mice.

Itching is a characteristic symptom in various forms of dermatosis, especially atopic dermatitis; consequently, it constitutes major diagnostic criteria (Williams et al., 1994). Scratching their skin with toenails seemed to be the most important factor leading to dermatitis through increasing various immunological responses such as the elevation of serum IgE concentration and number of mast cells (Hashimoto et al., 2004). Therefore effective control of itching is believed to be one of the fundamental approaches in controlling the skin lesions and decreasing various immunological responses. In this study, oral administration of AWB extract (25, 100 and 250 mg/kg) significantly inhibited the itching behavior and decreased the elevation of serum IgE levels in NC/Nga AD model mice.

Many studies reported that serum IgE level increased in NC/Nga mice with AD-like skin lesions (Matsuda et al., 1997; Kotani et al., 2000; Aioi et al., 2001). It has been shown that IgE synthesis by B cells is primarily regulated by cytokines; Th2-derived cytokines, including IL-4 and IL-13; IgE class-switching factors, upregulated IgE synthesis; and Th1-derived cytokines, especially IFN- γ , are strong inhibitors of IgE synthesis (Romagnani, 1994). In the present study, IL-4 and IFN- γ mRNA expression in whole body lymph node was examined by RT-PCR to identify the immunological mechanism through which IgE elevation is suppressed in the AWB extract-administered groups. As a result, oral administration of AWB extract inhibits the increase in IL-4 mRNA expression. Whereas IFN- γ mRNA expression was minimal in lymph node of both the AWB administered and control mice. These results imply that the AWB treatment can reduce the serum levels of total IgE by suppression of Th2 cell response, similar with the results of a previous study on *Rumex japonicus* Houtt (Lee et al., 2006).

In conclusion, we demonstrated that the oral administration of AWB inhibited the development of AD-like symptoms in NC/Nga mice. The results from the present study suggest that AWB could inhibit the development of AD-like skin lesions in NC/Nga mice through the suppression of Th2 cell response.

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