

RESEARCH ARTICLE

Pinus densiflora bark extract ameliorates 2,4-dinitrochlorobenzene-induced atopic dermatitis in NC/Nga mice by regulating Th1/Th2 balance and skin barrier function

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Korean red pine (*Pinus densiflora*) bark has been traditionally used in Korea and other parts of East Asia to relieve inflammatory diseases. Although many studies using *P. densiflora* bark have been reported, its effect on atopic dermatitis (AD) has not been elucidated. Thus, we investigated whether the *P. densiflora* bark extract (PBE) has potential to attenuate AD symptoms and elucidated the molecular mechanism. Oral administration of PBE to mice with 2,4-dinitrochlorobenzene (DNCB)-induced AD lessened dermatitis scores and scratching behavior and significantly reduced measures of epidermal thickness, infiltration of mast cells and eosinophils, levels of immunoglobulin E (IgE), and IgG₁/IgG_{2a} ratio in serum. PBE not only inhibited IL-4, IL-5, and IL-13 but also increased IFN- γ in splenic production. Furthermore, PBE significantly suppressed mRNA expression of thymic stromal lymphopoietin and further downregulated the mRNA expression of Th2 and Th17 cytokines such as IL-4, IL-13, IL-17, IL-31, and TNF- α . In addition, the protein expressions of filaggrin, involucrin, and loricrin in lesional skin were recovered by PBE. These results suggest that PBE attenuates DNCB-induced AD via regulating Th1/Th2 balance and skin barrier function.

KEYWORDS

atopic dermatitis, filaggrin, NC/Nga mice, *Pinus densiflora*, Th1, Th2

1 | INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with environmental and genetic factors that have no influence on normal individual (Leung, Boguniewicz, Howell, Nomura, & Hamid, 2004). The prevalence of AD is estimated to be about 3–10% of adults and up to 20% of children worldwide (Thaçi et al., 2016). Although corticosteroids are commonly prescribed for mild to moderate AD patients, the treatment with corticosteroids in long term and even short term may carry variable side effects, such as dermal atrophy, rebound dermatitis, dyspigmentation, hypertension, osteoporosis, blurred vision, and Cushing's syndrome (Hengge, Ruzicka, Schwartz, & Cork, 2006; Waljee et al., 2017). Calcineurin inhibitors have been shown to be effective and quite safe alternative to corticosteroids (Jensen et al., 2012). However, calcineurin inhibitors were also reported to cause malignancies, such as skin cancer and melanoma (Carr, 2013). To care for AD without adverse effects, alternative therapeutic products are continually sought.

1.1 | The pathogenesis of AD involves T-helper cell (Th) type 2 immune responses

The differentiation of naïve CD4⁺ T cells into Th2 cells leads to secrete IL-4 and IL-13 subsequently inducing IgE synthesis and IL-5 contributing to eosinophilia (Novak & Bieber, 2003). Th2 cytokines also lead epidermal cells to produce a variety of cytokines and chemokines, resulting in infiltration of immune and inflammatory cells such as T cells, mast cells, and eosinophils (Homey, Steinhoff, Ruzicka, & Leung, 2006). Furthermore, the increased Th2 polarization causes abnormalities in the epidermal barrier, which then leads to enhanced allergen penetration through the damaged epidermis (Howell et al., 2007; B. E. Kim, Howell, et al., 2011). 2,4-Dinitrochlorobenzene (DNCB) easily penetrates into the epidermis and induces allergic contact dermatitis with elevated serum IgE level (Proksch & Brasch, 2012). Repeated challenging of DNCB induces reproducible AD-like lesions in NC/Nga mice skin under specific pathogen-free conditions. Although the AD

model is not the same as human AD, this model has been widely used for initial screening of potential anti-AD agents.

Korean red pine (*Pinus densiflora*) belongs to the *Pinaceae* family and is widely distributed in Korea and other parts of East Asia. Various parts of *P. densiflora*, including needles, cones, bark, and pollen, have a long history as an oriental medicine in many diseases (Bae, 2000). Among them, the bark of *P. densiflora* has been shown antioxidant, anti-inflammatory, and antiadipogenic activities in various studies (Ahn & Go, 2017; K. D. Kim, Kim, et al., 2011). We previously demonstrated that the *P. densiflora* bark extract (PBE) is effective in preventing cataract formation with no accompanying toxicity (J. Kim & Choung, 2017). Similar to the French maritime pine (*Pinus maritima*) bark extract, PBE contains similar amounts of polyphenols, such as catechin and taxifolin, and procyanidins (Hwang et al., 2016; Saliou et al., 2001). Many studies indicated that *P. maritima* bark extract is effective in the treatment of allergies and asthma through reduction of histamine release and inhibition of inflammatory cytokines (Choi & Yan, 2009; I.-S. Shin et al., 2013). However, there exist comparatively few studies regarding the anti-AD of PBE and *P. maritima* bark extract. We hypothesized that PBE may attenuate AD symptoms. To verify the effects of PBE on AD symptoms and elucidate the recovery mechanism related to Th2-skewed immunity and skin barrier defects, we used DNCB-induced NC/Nga mice.

2 | MATERIALS AND METHODS

2.1 | Preparation of PBE

P. densiflora bark extract was obtained through our previous study and stored at -70°C until use (J. Kim & Choung, 2017).

2.2 | HPLC-PDA analysis

P. densiflora bark extract was dissolved in 50% methanol to prepare final concentration of 5.0-mg/ml stock solution and then filtered through a 0.2- μm PVDF syringe filter (Whatman International Ltd., Maidstone, Kent, UK) before being injected. Analysis was carried out on Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA, USA) linked with a photodiode array detector (PDA 2996 detector, Waters Corp.) with the Capcell Pak C18-UG120 column (250 $\times$ 4.6 mm id, 5- μm particle size; Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile (solvent A) and acidified water with 0.1% trifluoroacetic acid (solvent B). The gradient condition of the mobile phase was 0–27 min, 8% to 25%; 27–35 min, 25% to 100%; 35–40 min, 100% as percent of solvent A. The injection volume was 10.0 μl . The flow rate was set at 1.0 ml/min, and the column oven temperature was maintained at 30°C . Peak area for taxifolin in the PBE was integrated from the HPLC chromatogram at 300 nm wavelength, and the concentration was calculated using the equation of standard curve.

2.3 | Animals

Four-week-old male NC/Nga mice were purchased from SLC (Shizuoka, Japan) and were housed in individually ventilated cages at

$23 \pm 3^{\circ}\text{C}$ in $55 \pm 5\%$ humidity with 12/12 hr light/dark cycle under specific pathogen-free condition in experimental period. Animals were fed a standard laboratory diet (Central Lab Animal, Seoul, Korea) with water ad libitum. All experimental procedures were carried out in accordance with the protocol approved by the Institutional Animal Care and Use Committee guideline of Kyung Hee University (approval no. KHPASP(SE)-16-069).

2.4 | Treatment

The topical application of DNCB (Sigma-Aldrich, St Louis, Mo, USA) was performed to induce AD-like skin lesion and itch based on the method of our previous study (Shim & Choung, 2014). Briefly, 200 μl of 1% DNCB dissolved in acetone and ethanol (2:3 v/v) was topically applied on the shaved dorsal area twice for sensitization. Following the sensitization, 150 μl of 0.4% DNCB dissolved in acetone and olive oil (3:1 v/v) was repeatedly challenged on the dorsal skin three times a week for 9 weeks. After challenging, the mice were divided into six groups comprising eight mice in each group: naïve group, negative control group, positive control (prednisolone, PD) group, and PBE (12.5, 25, and 50) groups. The mice were orally administered 0.5% carboxymethyl cellulose (vehicle), PD (3 mg/kg), or PBE (12.5, 25, and 50 mg/kg) daily for 4 weeks.

2.5 | Dermatitis score

The severity of dermatitis was macroscopically evaluated three times a week as described (Suto et al., 1999). The degree of each symptom was graded from 0 to 3 (0, none; 1, mild; 2, moderate; 3, severe) for erythema/edema, dryness, erosion, excoriation, and lichenification. The total dermatitis score was determined from the sum of all individual scores.

2.6 | Scratching behavior

The DNCB-induced scratching was measured three times a week as described (Takano, Arai, & Kurachi, 2003). Briefly, mice were placed into acryl cages at least 1 hr of acclimation following vehicle administration, and then scratching behavior was monitored for 30 min. A bout of scratching movements with hind paw was defined as one scratching episode, which was scored from 0 to 4 (0, none; 2, scratching shorter than 1.5 s; 4, scratching longer than 1.5 s). The total number of scratch bouts was quantified as the sum of individual score for 30 min.

2.7 | Histological analysis

Dorsal skin tissues were excised, fixed in 10% neutral formalin, embedded in paraffin, and sectioned. The tissue sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and toluidine blue. Infiltrated eosinophils and mast cells were counted under high-power fields (400 $\times$, DP Controller Software; Olympus, Tokyo, Japan).

2.8 | Serum immunoglobulin analysis

Serum Ig levels were measured using mouse IgE, IgG₁, and IgG_{2a} ELISA kits in accordance with the manufacturers' instructions (IgE, Shibayagi, Gunma, Japan; IgG₁ and IgG_{2a}, Enzo Life Sciences, USA).

2.9 | Splenocyte isolation and splenic cytokine analysis

Splenocytes were isolated from NC/Nga mice after last treatment as described (Yang & Cook, 2003). Briefly, spleens were obtained aseptically and smashed with syringe plunger against cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). The splenocytes were treated with red blood cell lysing buffer Hybri-Max (Sigma-Aldrich, St. Louis, Mo, USA) and washed three times using RPMI-1640 containing with 10% fetal bovine serum supplemented with 100-U/ml penicillin and 50-mg/ml streptomycin. The isolated single cells were seeded into a 24-well plate and then stimulated with 5-μg/ml concanavalin A (Sigma-Aldrich, St. Louis, Mo, USA) in 5% CO₂ incubator for 72 hr at 37 °C. After the incubation, the supernatants were collected and stored at -70 °C for subsequent cytokine analysis. The productions of IL-4, IL-5, IL-13, IL-12, and IFN-γ from the supernatants were measured using mouse ELISA kits in accordance with the manufacturer's instructions (eBioscience, Vienna, Austria).

2.10 | RNA isolation and real-time quantitative PCR analysis

Total RNA from dorsal skin tissues was isolated using the R&A-BLUE total RNA extraction kit (Intron Biotechnology, Seoul, Republic of Korea) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The complementary DNA (cDNA) was synthesized from 5 μg of total RNA with PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). After cDNA synthesis, gene expression was performed by using real-time quantitative PCR with the SYBR Premix EX Taq (Takara Bio, Shiga, Japan) on an ABI StepOnePlus system (Applied Biosystems, Waltham, MA, USA). The primer sequences used for PCR are as follows: TSLP (5'-TGC AAG TAC TAG TAC GGA TGG GGC-3', 5'-GGA CTT CTT GTG CCA TTT CCT GAG-3'); IL-4 (5'-ACA GGA GAA GGG ACG CCA T-3', 5'-GAA GCC GTA CAG ACG AGC TCA-3'); IL-13 (5'-GCA ACG GCA GCA TGG TAT GGA G-3', 5'-TGG TAT AGG GGA GGC TGG AGA C-3'); IL-17A (5'-AAG GCA GCA GCG ATC ATC C-3', 5'-GGA ACG GTT GAG GTA GTC TGA G-3'); IL-31 (5'-TCA GCA GAC GAA TCA ATA CAG C-3', 5'-TCG CTC AAC ACT TTG ACT TTC T-3'); TNF-α (5'-GGG ACA GTG ACC TGG ACT GT-3', 5'-CTC CCT TTG CAG AAC TCA GG-3'); β-actin (5'-CCC AAC TTG ATG TAT GAA GG-3', 5'-TTG TGT AAG GTA AGG TGT GC-3'). The expression levels of interest genes were normalized using β-actin ($\Delta\Delta C_t$).

2.11 | Western blot analysis

Dorsal skins tissues were frozen with liquid nitrogen and grinded using pestle and then lysed with buffer containing 20-mM Tris-HCl (pH 7.4), 0.32-mM sucrose, protease inhibitor, 1-mM PMSF, 0.5 M EDTA (pH 8.0), 1-mM NaF, and 1-mM Na₃VO₄. The lysates were centrifuged at 13,000 rpm for 15 min at 4 °C following brief sonication. The

protein content in the supernatants was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of proteins (20–40 μg) were applied on SDS-polyacrylamide gel (7.5%) for electrophoresis and transferred onto polyvinylidene fluoride membranes. Western blot was conducted using filaggrin rabbit polyclonal antibody, loricine rabbit polyclonal antibody (Abcam, Cambridge, MA, USA), involucrin mouse monoclonal antibody and β-actin mouse monoclonal antibody (Santa Cruz Biotechnology, TX, USA). Antibody bindings were visualized using ChemiDoc™ XRS+ System (Bio-Rad, CA, USA). The band intensity was quantified using Image Lab statistical software (Bio-Rad, CA, USA).

2.12 | Statistical analysis

Data are shown as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance followed by Tukey's honestly significant difference test. Statistically significant differences were assessed with SPSS (Chicago, IL, USA). A *p* value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Identification and qualification of taxifolin in the PBE

The established standard chromatogram of PBE is shown in Figure 1a. The major peak was identified as taxifolin by direct comparison with reference standard. This flavonoid is known to be the major phytochemical in PBE (Hwang et al., 2016). The retention time for the taxifolin were 22.783 min, where the content of taxifolin was 0.7178% of the extract of PBE on the peak area.

3.2 | PBE attenuated DNCB-induced AD-like skin lesions in NC/Nga

We first investigated whether PBE can suppress AD-like skin lesion using NC/Nga mice. As shown in Figure 2a,b, repeated application of DNCB on the dorsal skin developed AD-like skin lesion including severe erythema, dryness, erosion, excoriation, and lichenification compared with the naïve group. Administration of PBE significantly reduced the dermatitis score, resulting in less erythema, dryness, and excoriation in dose-dependent manner over time. The efficacy of all PBE groups on dermatitis score tended to be higher than the PD group, which served as the positive control.

3.3 | PBE alleviated DNCB-induced scratching behavior in NC/Nga

To assess antipruritic effects of PBE, we measured scratching behavior. The robust scratching behavior was observed following repeated DNCB application and reached a peak at nine weeks after induction compared with the naïve group. The PBE-treated mice showed significantly reduced scratching frequency in a dose-dependent manner compared with the control group (Figure 2c). The scratching behavior observed in the PD group was between PBE 25 and 50 group.

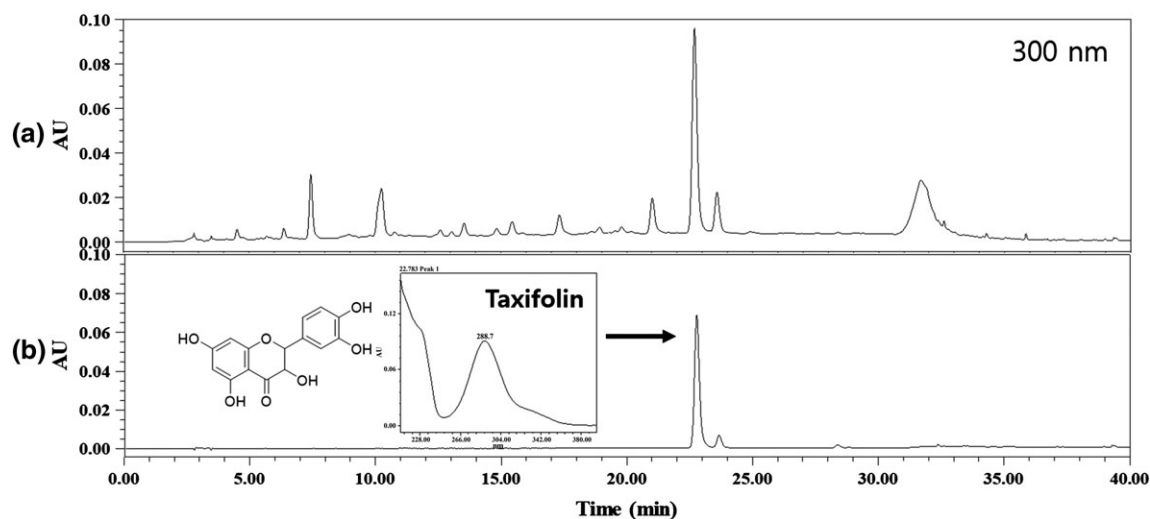


FIGURE 1 HPLC chromatograms of (a) *Pinus densiflora* bark extract and (b) taxifolin. The UV-visible absorption spectra and structure of major standard were represented

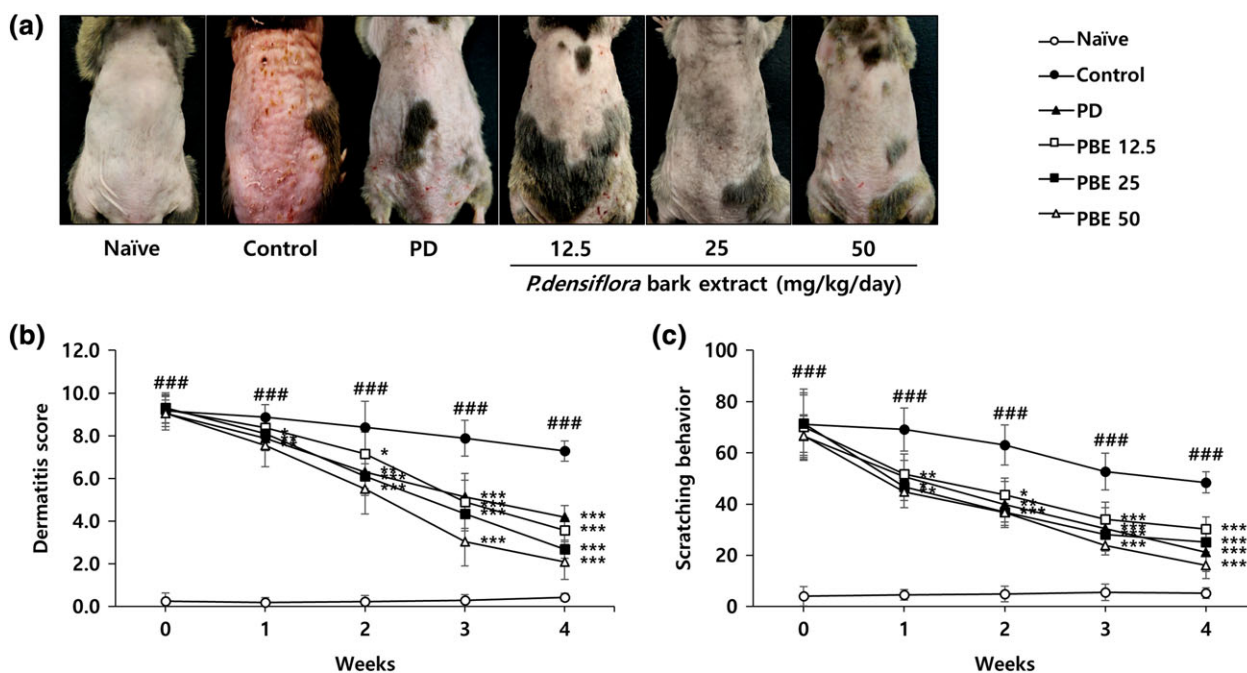


FIGURE 2 Effect of *Pinus densiflora* bark extract (PBE) on dermatitis and scratching behavior in DNCB-treated NC/Nga mice. (a) Skin photographs in NC/Nga mice were taken in the fourth week. (b) Dermatitis score and (c) scratching behavior were assessed three times a week by the criteria described in Section 2. Each value represents mean $\pm$ SEM of six animals and three independent experiments. ### p < .001 significant different from naïve group. * p < .05, ** p < .01, *** p < .001 significant different from control group [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | PBE inhibited thickening of the epidermis and infiltration of inflammatory cells into skin

Histological alterations in the skin are one of hallmarks of AD. H&E staining demonstrated that the skin lesion of the control group displayed marked thickening of the epidermis and dense infiltration of eosinophils into the skin tissue compared with the naïve group (Figure 3a,b). However, PBE reduced those pathologic changes including hyperkeratosis and excoriation at a dose of 50 mg/kg, resulting in a histological presentation very similar to that of the naïve group (Figure 3d). Toluidine blue staining indicated that PBE suppressed DNCB-induced infiltration and degranulation of mast cells in skin.

3.5 | Effects of PBE on DNCB-induced serum Ig level in NC/Nga

We investigated the serum levels of Th2-mediated IgE and IgG₁, and Th1-mediated IgG_{2a} (Table 1). Repeated DNCB application dramatically increased the serum IgE and IgG₁ level in mice, whereas the naïve group showed very low. Administration of PBE (12.5, 25, and 50 mg/kg) dose-dependently decreased in levels of IgE (32%, 42%, and 51%, respectively) and IgG₁ (32%, 47%, and 56%, respectively), whereas serum IgG_{2a} level was not decreased by PBE. Previous study indicated that PD inhibits IgE, IgG₁, and IgG_{2a} in mice (Mimura, Shinzaki, Kawasaki, & Iwamura, 2005). The PD group showed suppressed serum

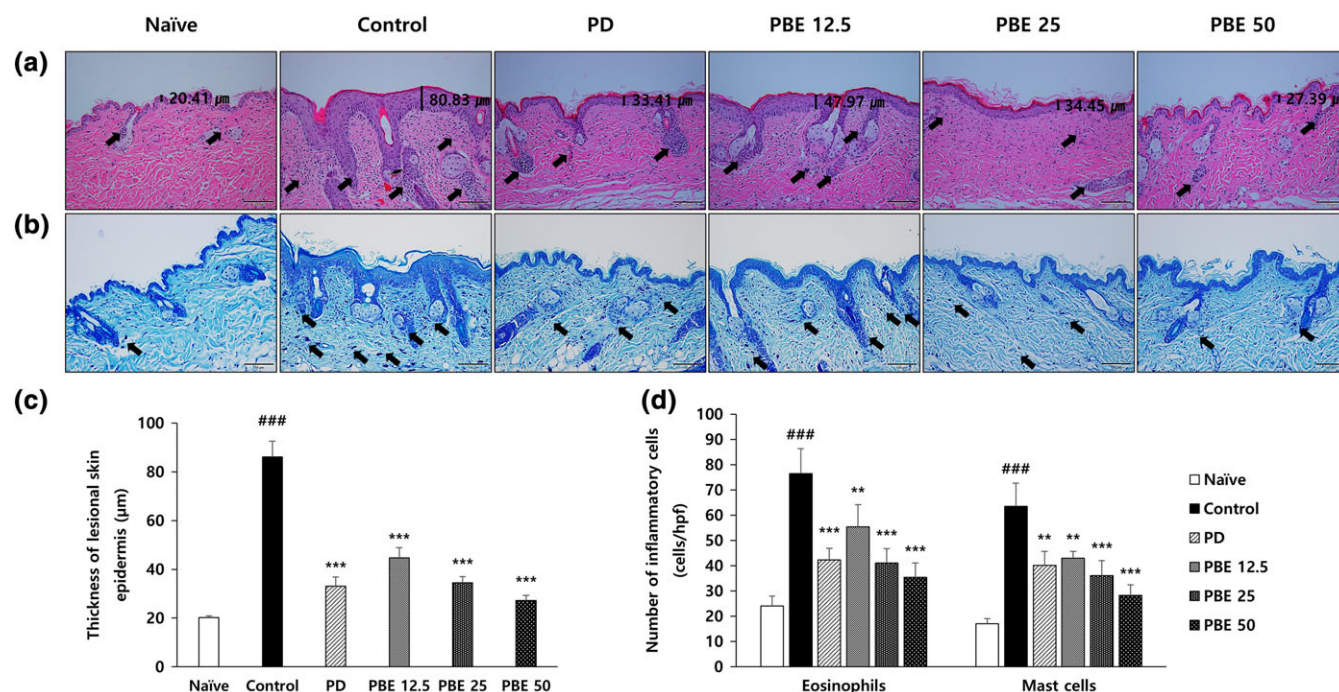


FIGURE 3 Representative picture of the histopathological features of AD-like skin lesions in NC/Nga mice. Dorsal skins were excised, fixed in 10% formaldehyde, embedded in paraffin, and sectioned. The tissue sections were analyzed by (a) H&E staining and (b) toluidine blue staining (original magnification $\times 400$, scale bar = 100 μm). (c) Epidermal thickness was quantified as means in randomly selected four fields per mouse. (d) Number of infiltrated inflammatory cells, eosinophils and mast cells, were quantified as means in randomly selected four fields per mouse. Each value represents mean $\pm$ SD of six animals and three independent experiments. ### $p < .001$ significant different from naïve group. ** $p < .01$, *** $p < .001$ significant different from control group [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Effects of *Pinus densiflora* bark extract on serum IgE, IgG₁, IgG_{2a}, and IgG₁/IgG_{2a} ratio levels in NC/Nga mice

Groups	Concentration (mg/kg b.w.)	Serum level ($\mu\text{g/ml}$)			Normalized to naïve IgG ₁ /IgG _{2a}
		IgE	IgG ₁	IgG _{2a}	
Naïve	–	1.1 $\pm$ 0.1	581.0 $\pm$ 83.0	936.2 $\pm$ 146.6	1.0
Control	–	21.1 $\pm$ 1.3###	2063.0 $\pm$ 432.1###	1915.7 $\pm$ 168.8###	1.9###
PD	3	13.1 $\pm$ 0.3***	1293.2 $\pm$ 229.6**	1350.6 $\pm$ 155.2*	1.7*
<i>P. densiflora</i>	12.5	14.8 $\pm$ 0.7***	1569.1 $\pm$ 325.6	1921.9 $\pm$ 129.2	1.4**
	25	12.7 $\pm$ 0.6***	1339.2 $\pm$ 293.0**	2119.4 $\pm$ 175.5	1.1***
	50	11.0 $\pm$ 0.3***	1208.7 $\pm$ 250.9**	2153.3 $\pm$ 238.4	1.0***

Note. Serum was collected from each group 24 hr after the last sample treatment. Serum levels of IgE, IgG₁, and IgG_{2a} were determined using ELISA. Each value represents mean $\pm$ SD of six animals and three independent experiments.

$p < .001$ significant different from naïve group. * $p < .05$. ** $p < .01$. *** $p < .001$ significant different from control group.

IgE, IgG₁, and IgG_{2a} levels compared with the control group (40%, 50%, and 58%, respectively). IgG₁/IgG_{2a} ratio in the control group was increased approximately two-fold compared with the naïve group. Administration of PBE dose-dependently suppressed IgG₁/IgG_{2a} ratio compared with the control group, and those of PBE 25 and 50 groups were normalized compared to the naïve group.

3.6 | Effects of PBE on DNCB-induced cytokine production in splenocytes

We analyzed cytokine production by splenocytes upon in vitro Con A stimulation. As shown in Figure 4a, splenocytes from the control group produced higher levels of Th2-mediated cytokines (IL-4, IL-5, and IL-13) than those from the naïve group. However, treatment with

PBE significantly decreased the production of IL-4, IL-5, and IL-13 in a dose-dependent manner, and the levels of PBE 50 group were similar to the naïve and PD groups. The levels of Th1-mediated cytokines (IFN- γ and IL-12) were comparable in the naïve and control group in Figure 4b. PBE-treated groups exhibited the increase of IFN- γ and IL-12 compared with the control group, whereas those from the PD group were decreased in Con A-induced splenocytes.

3.7 | PBE suppressed DNCB-induced cytokines expression in skin

We investigated whether PBE would reduce mRNA expressions of proinflammatory cytokines in the skin (Cabanillas & Novak, 2016). The mRNA expressions of TSLP, IL-4, IL-17A, TNF- α , and IL-31 in

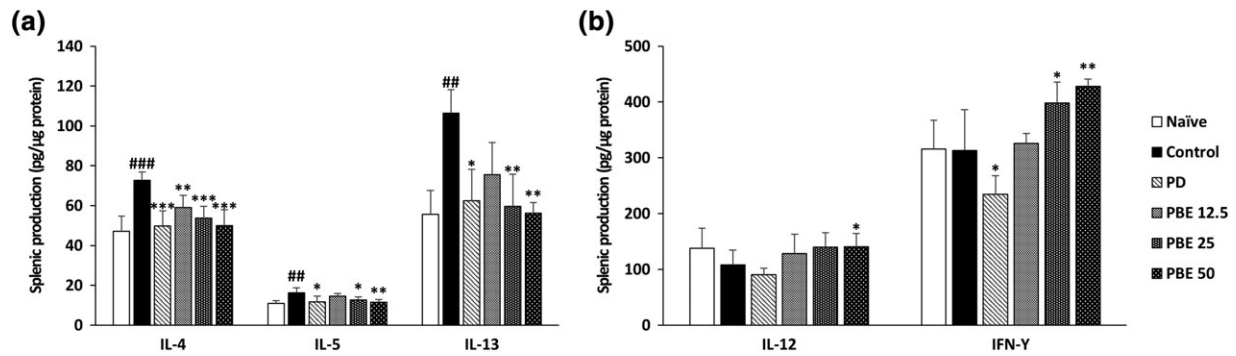


FIGURE 4 Effects of *Pinus densiflora* bark extract (PBE) on cytokine secretion by splenocytes in response to in vitro Con A stimulation. The levels of (a) IL-4, IL-5, IL-13, (b) IL-12, and IFN-γ in supernatant were measured by ELISA. Each value represents mean $\pm$ SD of independent experiments ($n = 5$). ## $p < .01$, ### $p < .001$ significant different from naïve group. * $p < .05$, ** $p < .01$, *** $p < .001$ significant different from control group

dorsal skin were increased by repeated application of DNCB compared with the naïve group. However, treatment with PBE reduced these cytokine expressions in a dose-dependent manner (Figure 5). These results suggest that PBE suppressed expression of TSLP, IL-4, IL-17A, TNF-α, and IL-31, resulting in the inhibition of AD-like lesion caused by inflammatory cells.

3.8 | PBE recovered DNCB-induced defects in skin barrier function

We investigated whether PBE can regulate skin barrier proteins, including filaggrin (FLG), loricrin (LOR), and involucrin (IVL), in DNCB-induced AD-like dorsal skin tissue by western blot analysis (Figure 6).

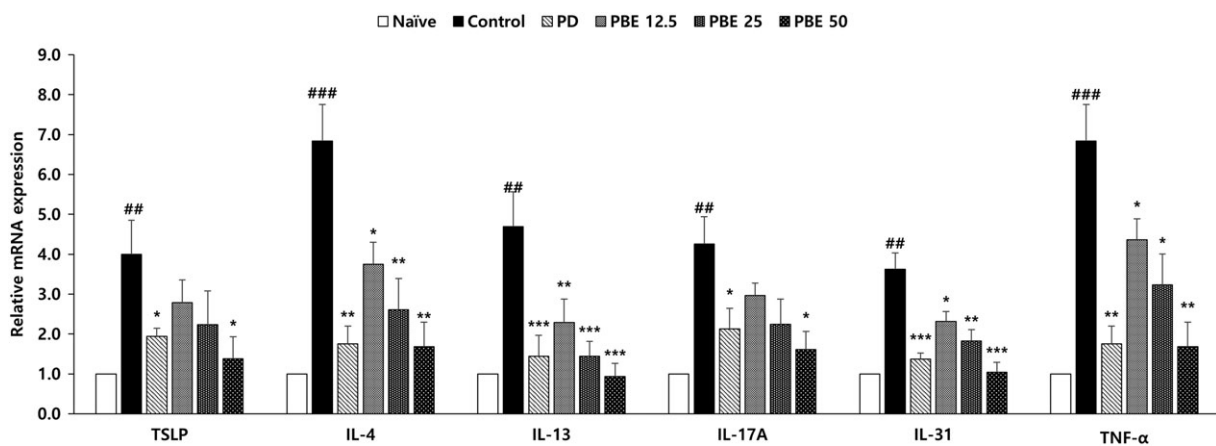


FIGURE 5 Effects of *Pinus densiflora* bark extract (PBE) on DNCB-induced TSLP, IL-4, IL-13, IL-17, IL-31, and TNF-α mRNA expressions in NC/Nga mice. RNA was extracted from dorsal skin. mRNA expression of TSLP, IL-4, IL-13, IL-17, IL-31, and TNF-α was measured by real-time quantitative PCR (normalized to GAPDH). Each value represents mean $\pm$ SD of three independent experiments ($n = 5$). ## $p < .01$, ### $p < .001$ significant different from naïve group. * $p < .05$, ** $p < .01$, *** $p < .001$ significant different from control group

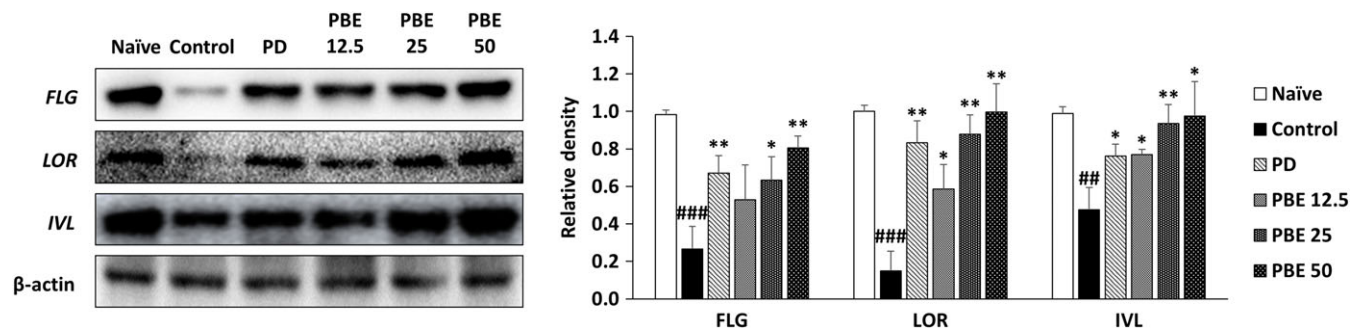


FIGURE 6 Effect of *Pinus densiflora* bark extract (PBE) on recovery of skin barrier functions. Western blotting analysis indicated protein expression of FLG (34 kDa), LOR (26 kDa), and IVL (68 kDa). The protein levels were quantified with band density. The results are presented as the mean $\pm$ SD of three independent experiments ($n = 4$). ## $p < .01$, ### $p < .001$ significant different from naïve group. * $p < .05$, ** $p < .01$ significant different from control group [Colour figure can be viewed at wileyonlinelibrary.com]

FLG expression was significantly decreased in the control group compared with the naïve group, and even repeated challenging with DNCB dramatically decreased the expressions of LOR and IVL. Administration of PBE (12.5, 25, and 50 mg/kg) dose-dependently recovered FLG expression compared with the control group. Restoration of the protein expressions in PBE groups may indicate that PBE protected DNCB-induced abnormal changes in FLG and components of the epidermal barrier.

4 | DISCUSSION

In this study, we aimed to investigate the effects of PBE on AD-like skin lesions in DNCB-induced NC/Nga mice. We found that oral administration of PBE ameliorates the clinical features of AD in NC/Nga mice. In addition, PBE significantly normalized the Th2/Th1 ratio and the expression of skin barrier proteins.

The effect of PBE on AD symptoms was evaluated using dermatitis score and scratching behavior measurement. Dermatitis score showed that extent and severity of AD was suppressed dose-dependently by orally administration of PBE. In addition, the treatment with PBE also decreased scratching behavior. It is important because itch ultimately leads to the disruption of the skin barrier and aggravation of inflammation (Proksch, Fölster-Holst, & Jensen, 2006). Histological analysis showed that PBE inhibited thickness of epidermis, infiltration of inflammatory cells including mast cells and eosinophils.

AD is commonly characterized by immune responses that consist of an increased systemic Th2 response and a combination of Th2 and Th1 responses (Ong & Donald, 2006). Th2 cells differentiate in the presence of IL-4 and produce IL-4 and IL-13, which trigger the generation of IgG₁ and the isotype switching of IgE synthesis by B cells, and IL-5, which plays an important role for the recruitment and survival of eosinophils (Bieber, 2008). Th1 cells develop in the presence of IL-12 and IFN- γ and release IFN- γ known to generate IgG_{2a} and downregulate inflammatory Th2 cytokines, including IL-4, IL-5, and IL-13 (Leung et al., 2004). The production of IL-4, IL-5, and IL-13 were all significantly reduced by PBE in vitro Con-A stimulated splenocytes. In addition, an increase of IFN- γ was observed in the splenocytes, whereas the production of IL-12 was comparable. Consequently, restoring of Th2/Th1 balance in the groups treated with PBE was found through the serum IgG_{2a}/IgG₁ ratio, which is marker for Th1 and Th2 lymphocytes, respectively (Shershakova et al., 2016). In contrast, PD inhibited not only splenic production of Th2 cytokines and serum level of IgG₁ but also splenic production of IFN- γ and serum level of IgG_{2a}. The inhibitory effects of PD were also observed in mouse allergic dermatitis model (Mimura et al., 2005). These results show that PBE on IgG_{2a}/IgG₁ ratio appeared to be more effective rather than PD. Taken together, we can assume that PBE has an effect on Th2-skewed immunity through inhibiting Th2 response and promoting Th1 response.

Recently, TSLP produced by keratinocytes was shown to induces the expression of the OX40 ligand (OX40L) in dendritic cells, promoting naïve CD4⁺ T cells toward Th2 cells and establishing the predominant immune polarization of AD (Leyva-Castillo, Hener, Jiang, & Li, 2013). In the DNCB-induced NC/Nga mice, gene expression of TSLP is expressed by keratinocytes, resulting in Th2 polarization (J.-H. Shin,

Chung, & Seo, 2016). PBE suppressed gene expressions of IL-4 and IL-13 by inhibiting gene expression of TSLP. The elevation of serum IgE levels is frequently exhibited by isotype switching from IgM to IgE through overexpression of IL-4 and IL-13, which play a key role in inducing IgE synthesis (Brandt & Sivaprasad, 2011). Administration of PBE reduced the serum IgE level dose-dependent manner by suppressing Th2 cytokines.

IL-17A is known to be an essential factor in host defense against pathogens and a therapeutic target of psoriasis (Miossec & Kolls, 2012). IL-17A was also reported to have a possible role in establishing Th2 inflammation in AD (Dhingra & Guttman-Yassky, 2014). IL-17A deficiency attenuates cutaneous inflammation and Th2 polarization in both hapten-induced mice and flaky-tailed mice (Nakajima et al., 2014). IL-17A gene expression was found to be high in the DNCB-induced group, similar to those found by (Shim & Choung, 2014). PBE suppressed IL-17A gene expression dose-dependent manner.

IL-31 is deeply involved scratching behavior. IL-31, produced predominantly by Th2 cells, is known to be a pruritogenic cytokine (Grimstad et al., 2009). Several studies demonstrated that cutaneous IL-31 gene expression were increased in NC/Nga mice, and the increase coincided with scratching behavior (Arai, Tsuji, Takeda, Akiyama, & Saito, 2013; Park et al., 2015). PBE suppressed IL-31 gene expression dose-dependent manner, as well as the results of scratching behavior. IL-31 may represent a pathway related to an exacerbation of pruritus, ultimately leading to skin barrier defects (Nygaard et al., 2016). Further skin barrier estimations were also performed.

FLG aggregates the keratin cytoskeleton to promote the flattening of keratinocytes in the outermost skin layer. LOR and IVL, essential components of the epidermal barrier, play a role in the facilitation of terminal differentiation of the epidermis and the formation of the skin barrier with cross linked to FLG. Inflammation of all types, including Th1-, Th2-, and Th17-mediated responses, induces skin barrier dysregulation due to downregulation of FLG and essential components, and the decrease in natural moisturizing factor is dependent on the severity of lesional inflammation (Cabanillas & Novak, 2016). TSLP directly decrease FLG expression in keratinocytes regardless of FLG gene mutation, and the expressions of FLG, LOR, and IVL are also negatively modulated by other inflammatory cytokines, including IL-4, IL-13, and TNF- α (Howell et al., 2007). IL-17A was reported to be able to downregulate not only pro-filaggrin and FLG expression but also proteins associated with cell adhesion between the keratinocytes (Gutowska-Owsiak et al., 2012). In previous studies, FLG was downregulated in DNCB-induced AD, while there exist comparatively few reports indicating that LOR and IVL were downregulated in DNCB-induced AD yet (Cha et al., 2017). In the present study, repeated application of DNCB on the dorsal skin appeared to degradation of FLG, IVL, and LOR. These results indicate that administration of PBE enhanced FLG, IVL, and LOR.

In summary, the results of this study suggest that PBE is effective in preventing AD symptoms by regulation of IgE synthesis, the IgG₁/IgG_{2a} ratio, Th2-mediated cytokine expressions, recruitment of inflammatory cells, and altered skin barrier function. Although it is not clear whether PBE regulates TSLP and further inhibits Th2 polarization directly or indirectly, the extract possibly acts as a modulator of TSLP expression. Therefore, our next study will focus on elucidating the

molecular effects of PBE on TSLP expression, and on identifying the active compounds responsible for these effects.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

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