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Inhibitory Effects of *Pterocarpus santalinus* Extract against IgE/Antigen-Sensitized Mast Cells and Atopic Dermatitis-Like Skin Lesions

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ABSTRACT

Pterocarpus santalinus has been traditionally used as a medicinal plant owing to its anti-inflammatory, anthelmintic, tonic, antihyperglycemic, and diaphoretic properties. We hypothesized that *P. santalinus* might have therapeutic potential in alleviating allergy and atopic dermatitis. Thus, we investigated the inhibitory effects of *P. santalinus* extract against allergic responses and 2,4-dinitrochlorobenzene-induced atopic dermatitis-like dorsal skin lesions using immunoglobulin E-sensitized rat basophilic leukemia-2H3 mast cells and NC/Nga mice. Degranulation and enzyme-linked immunosorbent assays were conducted to measure degranulation, proinflammatory cytokine levels, and prostaglandin E₂ concentrations in immunoglobulin E/antigen-sensitized RBL-2H3 mast cells. The therapeutic efficacy of *P. santalinus* extract in 2,4-dinitrochlorobenzene-induced atopic dermatitis was evaluated through morphological, physiological, and immunological analysis. *P. santalinus* extract inhibited β -hexosaminidase and histamine release and reduced tumor necrosis factor- α , interleukin-4, and prostaglandin E₂ secretion. Furthermore, *P. santalinus* extract suppressed atopic dermatitis-like skin lesions by regulating the serum levels of immunoglobulin E and immunoglobulin G2a, and messenger ribonucleic acid expression of T helper cell 1- and T helper cell 2-related mediators in the skin lesions. Histopathological analyses showed a decrease in epidermal thickness and intradermal inflammatory cell infiltration. These results suggested that *P. santalinus* extract might have beneficial effects in treating allergic and atopic dermatitis-like skin disorders.

Introduction

The prevalence of atopic dermatitis (AD), a chronic inflammatory skin disorder, is increasing in industrialized countries. Although occurrence during infancy and childhood is common, adults may also be affected [1]. AD is characterized by chronic eczematous and pruritic skin lesions owing to the invasion of inflammatory cells, such as eosinophils, mast cells, and monocytes/macrophages [2].

Mast cells play critical roles in the various biological processes of allergic reactions [3]. In particular, they are involved in the pathogenesis of allergic disorders, including allergic rhinitis, asthma, anaphylaxis, and AD [4]. Mast cells express Fc ϵ R1 receptors with high specificity for immunoglobulin E (IgE) on their membrane surface, where antigen binding activates the cross-linking

* These two authors contributed equally to the present study.

between IgE and FcεR1 receptors [3]. The binding of antigen-bound IgE to FcεR1 receptors induces degranulation and the production of histamine, inflammatory lipid mediators, and several inflammatory cytokines such as TNF-α and interleukin 4 (IL-4) [5]. The rat basophilic leukemia (RBL)-2H3 cell line has the same biological properties as mast cells [6]. Therefore, RBL-2H3 is the primary cell line used in allergy-related studies for finding potential mast cell stabilizers [7].

The development of AD is influenced by several factors, including immunological abnormalities [8]. In most patients, the developmental triggers include elevated levels of serum IgE and circulating eosinophils, owing to the increase in IL-4, IL-5, and IL-13 secretion by T helper (Th) 2 cells [9]. Th1 cells that produce interferon (IFN)-γ and IL-12 are present in large numbers during the chronic phase and are correlated with the pathogenesis of AD [10, 11]. Th1/Th2 immune response is a known pathogenic mechanism for AD; thus, AD is a biphasic chronic skin disorder induced by the Th1/Th2 immune response [12].

The epidermis serves as an important physical barrier to harmful insults from the external environment. Patients with AD exhibit augmented transepidermal water loss (TEWL) and a decrease in skin barrier function, including dye penetration and *stratum corneum* hydration [13]. TEWL is associated with the lipid barrier of the *stratum corneum* and the weakening of corneocyte adhesion that results in a decrease of the water content, which increases TEWL [13]. These changes in skin barrier function are closely related to severity and pruritus in AD [13].

In East Asia, *Pterocarpus santalinus* L. (Leguminosae) (also known as Red Sandalwood) is commonly used as both a pharmaceutical and nutraceutical supplement. The roots, bark, and leaves of *P. santalinus* have been used in traditional Indian, Chinese, and Korean medicine as anti-inflammatory, anthelmintic, tonic, antihemorrhagic, antidiysenteric, antihyperglycemic, and diaphoretic agents [14]. Previous studies have shown that the extracts of *P. santalinus* exhibit antimicrobial [15], antioxidant [16], hepatoprotective [17], antidiabetic [18], gastroprotective [19], and wound healing effects [20] in *in vivo* models. Continuous triggering of inflammation and changes in macrophage functions induce AD [21]. In addition, *Pterocarpus indicus* (also known as Burmese Rosewood, Red Sandalwood) water extract has been shown to inhibit IgE/antigen-sensitized mast cells and AD-like lesions in mouse models [22]. However, the effects of *P. santalinus* extract (PSE) on allergic reactions and AD have not been examined. Therefore, the present study was conducted to investigate whether PSE could alleviate antigen-induced allergic responses and AD in IgE/antigen-sensitized mast cells and a 2,4-dinitrochlorobenzene (DNCB)-induced model of AD.

Results

Six major compounds were found in the HPLC chromatogram of PSE: protocatechuic acid, (–)epicatechin, taxifolin, *cis*-piceid, santalin A, and quercetin (Fig. 1S, Supporting Information). These six major compounds all belong to the family of phenolic acids. The protocatechuic acid is a simple phenolic acid, which is a major metabolite of polyphenols, and three compounds, (–)epicatechin, taxifolin, and quercetin, are flavonoids. *Cis*-piceid is a major res-

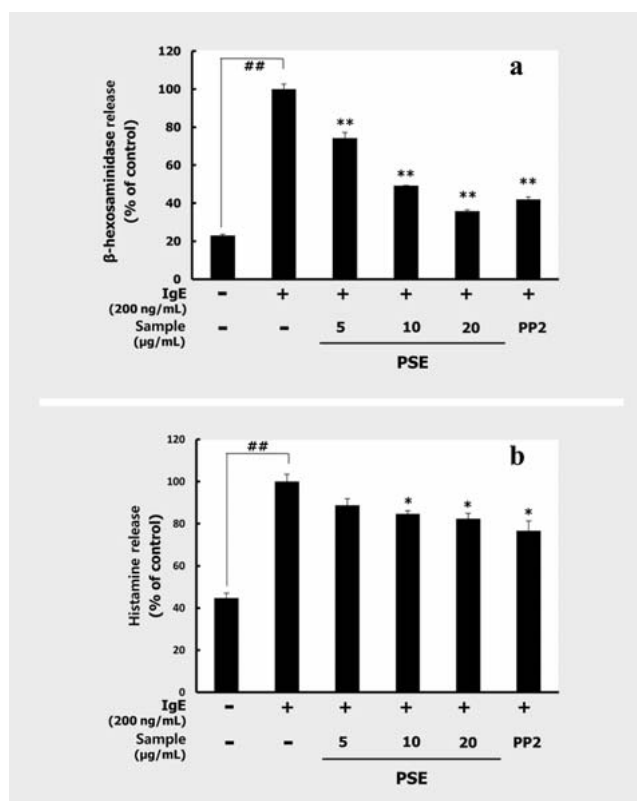


Fig. 1 Effects of *P. santalinus* extract (PSE) on β-hexosaminidase and histamine release. **a** Degranulation was assessed by measuring β-hexosaminidase release in DNP-IgE/antigen-sensitized RBL-2H3 cells. RBL-2H3 cells were treated with or without PSE (5, 10, or 20 μg/mL) for 1 h and then stimulated with DNP-BSA for 1 h. **b** Histamine release was measured using an ELISA kit. RBL-2H3 cells were treated with or without PSE (5, 10, or 20 μg/mL) for 1 h and then stimulated with the antigen for 1 h. PP2 (10 μM) is a general Src-family kinase inhibitor. Results are expressed as the mean ± SD of three independent experiments (##p < 0.01 compared to the control; *p < 0.05 and **p < 0.01 compared to the DNP-BSA-treated cells).

veratrol derivative, abundant in the grape and grape wine [23]. The structure of *cis*-piceid is a glucoside form of *cis*-resveratrol, and *cis*-piceid exhibits highly similar beneficial activities to resveratrol [24]. Santalin A is a characteristic compound of *Pterocarpus* species, such as *P. santalinus*, *Pterocarpus osun*, and *Pterocarpus soyauxii* [25].

The inhibitory effects of PSE against β-hexosaminidase and histamine production were assessed. The cytotoxic effects of PSE were investigated by using an MTT colorimetric assay. At concentrations between 5 and 20 μg/mL, PSE did not affect cell viability (data not shown). A β-hexosaminidase assay was performed to assess the effects of PSE on the degranulation of mast cells. PSE inhibited β-hexosaminidase production in IgE/antigen-sensitized mast cells in a dose-dependent manner (► Fig. 1a). To further determine the effects of PSE on degranulation, we measured histamine release. PSE also suppressed histamine secretion from IgE/antigen-sensitized RBL-2H3 cells (► Fig. 1b).

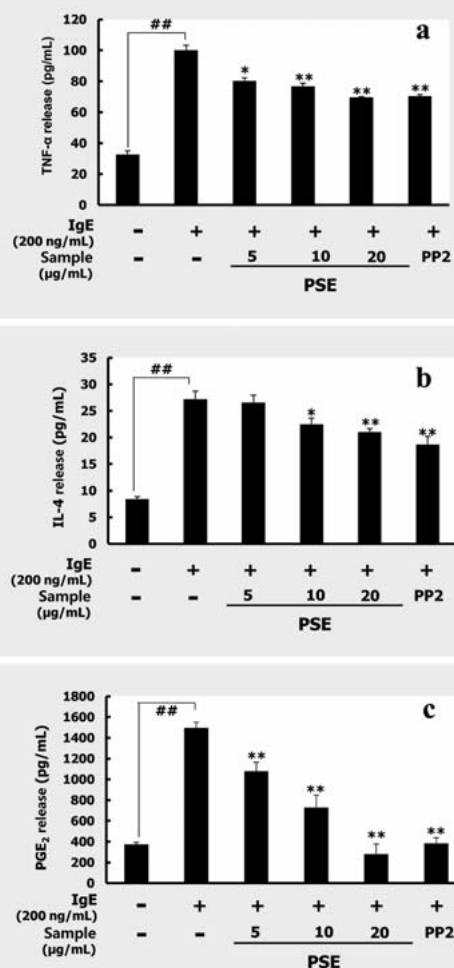


Fig. 2 Effects of *P. santalinus* extract (PSE) on TNF- α , IL-4, and PGE₂ production. RBL-2H3 cells were treated with PSE for 1 h and then stimulated with DNP-BSA for 4 h. a–c TNF- α , IL-4, and PGE₂ production was measured using ELISA kits. PP2 (10 μ M) is a general Src-family kinase inhibitor. Results are expressed as the mean $\pm$ SD of three independent experiments (## p < 0.01 compared to the control; * p < 0.05 and ** p < 0.01 compared to the DNP-BSA-treated cells).

The inhibitory effects of PSE against proinflammatory cytokine and prostaglandin E₂ (PGE₂) production were evaluated. The proinflammatory cytokines, TNF- α and IL-4, are essential in allergy-related diseases [26]. In this study, we investigated the effects of PSE on the production of TNF- α and IL-4 from IgE/antigen-sensitized mast cells. PSE treatment suppressed TNF- α and IL-4 production (► Fig. 2a,b). The arachidonate cascade is initiated via the Fc ϵ RI receptor in antigen-stimulated mast cells [27]. Therefore, the effects of PSE on the production of PGE₂ were investigated. PSE significantly decreased PGE₂ production in antigen-stimulated mast cells in a dose-dependent manner (► Fig. 2c).

The inhibitory effects of PSE against AD-like skin lesions in NC/Nga mice were investigated. An NC/Nga mouse model of AD was generated by a twice weekly application of DNCB solution to the dorsal skin. DNCB initially caused skin dryness, which was followed

by edema, erythema, itching, and thickening. The twice weekly DNCB application was continued during topical treatment with PSE (0.1 or 0.3% w/w emulsion) for 35 days (► Fig. 3). PSE treatment inhibited hyperkeratosis and itching to a greater extent than DNCB alone. Furthermore, hematoxylin & eosin (H&E), Congo red, and toluidine blue staining of the dorsal skin sections illustrated that PSE attenuated the epidermis thickening and inflammatory cell infiltration, particularly mast cells and eosinophils (► Fig. 3a–c). TEWL was also evaluated to determine the effects of PSE on skin barrier function. Although DNCB treatment significantly increased TEWL, mice cotreated with PSE showed reduced TEWL in the dorsal skin lesions (► Fig. 3d). These results indicated that PSE reduced inflammatory cell infiltration, skin hypertrophy, and skin barrier damage in AD skin lesions.

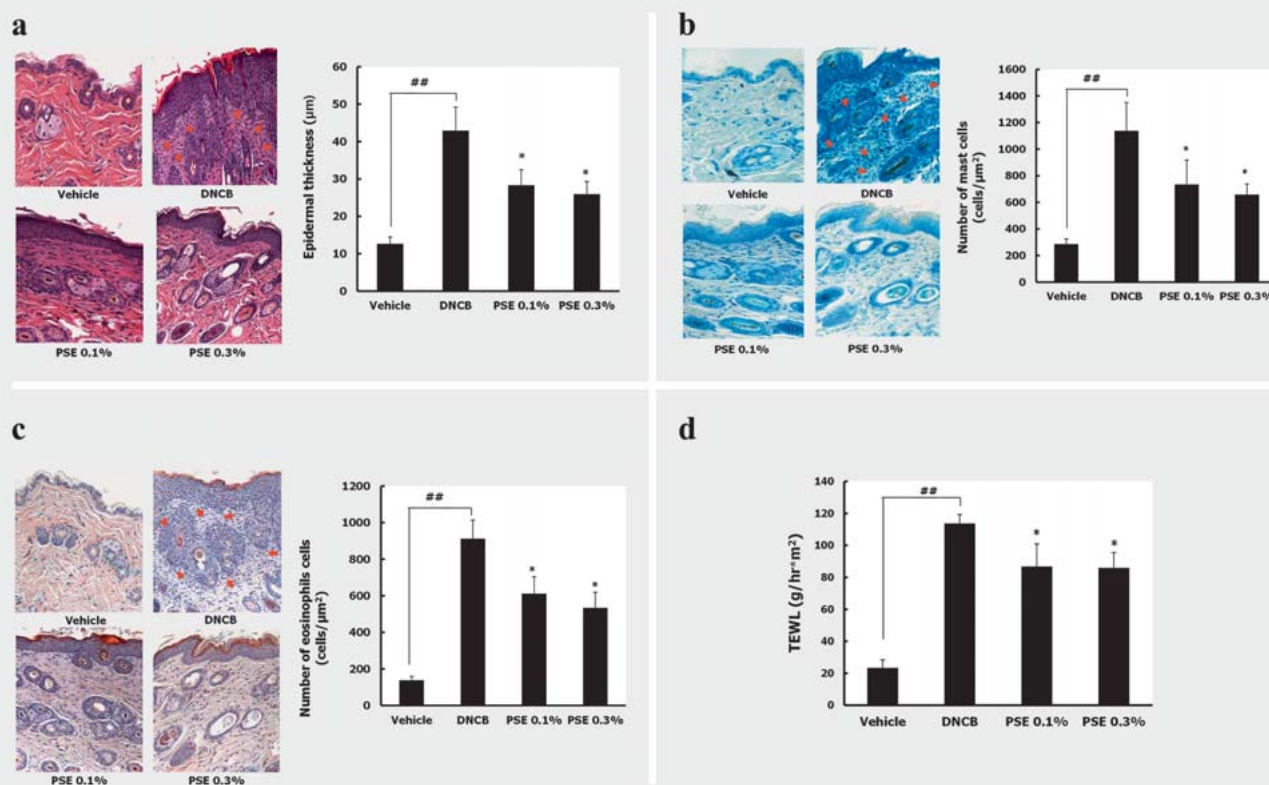
The effects of PSE on IgE and IgG2a levels in serum samples isolated from NC/Nga mice were investigated because IgE and IgG2a are known to play pivotal roles in the progression of AD [28, 29]. The topical administration of DNCB markedly increased serum IgE levels and decreased serum IgG2a levels compared to the vehicle control group. However, treatment with PSE reduced serum IgE levels and increased serum IgG2a levels in DNCB-treated mice (► Fig. 4a,b).

PSE reduces messenger ribonucleic acid (mRNA) expression of Th1- and Th2-associated chemokines and cytokines in skin lesions. We examined whether PSE could affect the mRNA expression of cytokines, chemokines, and other important markers of AD in the skin lesions of NC/Nga mice [9, 10]. PSE inhibited the mRNA expression of cytokines and chemokines related to Th1 and Th2 immune responses (► Table 1). In addition, PSE suppressed the mRNA expression of proinflammatory cytokines in the dorsal skin lesions of NC/Nga mice (► Table 1). These results showed that the topical application of PSE inhibited the mRNA expression of Th1-, Th2-, and inflammation-related cytokines and chemokines in the skin lesions of NC/Nga mice.

Discussion

The 50% ethanol reflux extract of *P. santalinus* has been shown to contain several active compounds (flavonoids), such as taxifolin and quercetin [30]. These flavonoids are well known for their antioxidant and anti-inflammatory effects. The effects of taxifolin and quercetin on the production of β -hexosaminidase, histamine, IL-4, TNF- α , and PGE₂ have also been reported in previous studies [30]. The protocatechuic acid identified from PSE in this study is also known for antioxidant and anti-inflammatory effects [31].

The initiation of an allergic response is followed by associated symptoms, such as AD, anaphylactic shock, asthma, and conjunctivitis [4]. β -Hexosaminidase, which is associated with degranulation, is secreted from mast cells in addition to allergic mediators (histamine and serotonin) upon IgE cross-linking with Fc ϵ RI [32]. We found that PSE treatment inhibited the degranulation of activated mast cells, as evidenced by the reductions in the secretion of β -hexosaminidase (► Fig. 1a) and histamine (► Fig. 1b). Therefore, we suggested that the anti-allergic activities of PSE might be mediated by inhibition of the production of histamine, which causes smooth muscle contraction, vascular permeability, and inflammation.



► Fig. 3 Effects of topical treatment with *P. santalinus* extract (PSE) on AD-like skin lesions in NC/Nga mice. **a–c** Histopathological analysis of skin lesions. Skin lesions were excised, fixed in 10% formalin, embedded in paraffin, and cut into sections. The sections were stained with hematoxylin and eosin (H&E), toluidine blue, and Congo red. Epidermal thickness was observed, and mast cells and eosinophils were counted under a microscope at $\times 200$ magnification. **d** The level of transepidermal water loss (TEWL) in the mouse dorsal skin was measured using a skin evaporative water recorder on the last day of the experiment. Results are expressed as the mean $\pm$ SD (n = 6) (^{##}p < 0.01 compared to the vehicle group; ^{*}p < 0.01 and ^{*}p < 0.05 compared to the DNCB-treated group).

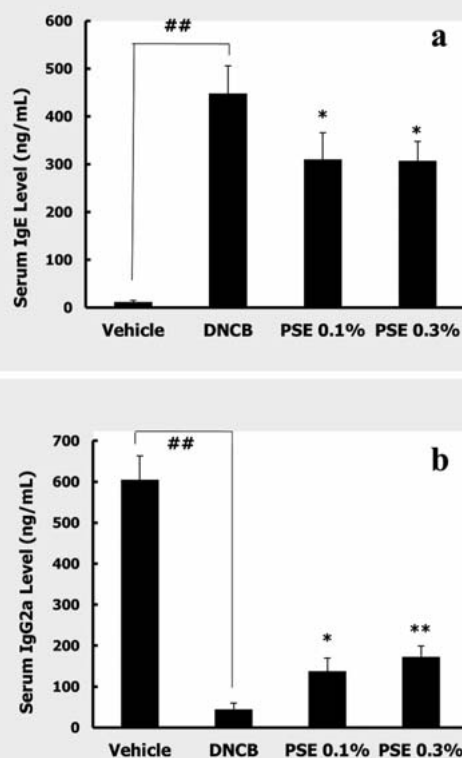
In addition, PSE treatment markedly inhibited the production of the proinflammatory cytokines (TNF- α and IL-4) and the lipid mediator (PGE₂), which is an arachidonic acid metabolite (► Fig. 2a–c). Cyclooxygenase (COX)-2-derived PGE₂ is known to mediate AD [33]. TNF- α and IL-4 are known to play an important role in the triggering of the immune responses in mast cells [34]. IL-4 is involved in the development of T cells and the conversion of B cells to the IgE isotype [34]. These results suggested that PSE could be a beneficial therapeutic agent for the alleviation of allergic responses through the inhibition of the production of proinflammatory cytokines.

Mast cells play critical roles in the allergic response, and release a number of biologically active substances. To identify the therapeutic effects of PSE on AD exerted by the anti-allergy activity, we investigated the effect of DNCB induction in an AD-like NC/Nga mouse [35] and examined the effects of PSE on the skin lesions. Under conventional conditions, NC/Nga mice develop AD that mimics the clinical symptoms observed in patients with AD (erythema, edema, itchiness/scratching, and dry skin) [36]. NC/Nga mice have a mutation on chromosome 9; this is associated with increases in Th2 response and IgE production [36]. In addition to skin changes, NC/Nga mice exhibit an increase in splenic Th2 cell

differentiation, eosinophil, and mast cell infiltration into the skin lesions and serum total IgE levels [37]. This indicated that the immune response of Th2 cells is related to the pathogenesis of AD-like skin lesions in NC/Nga mice. Here, we used DNCB to induce AD because it causes contact dermatitis and a type IV hypersensitivity reaction in almost all people exposed to it [38].

The skin of patients with AD is often characterized by increased TEWL, epidermal thickening, and the infiltration of inflammatory cells [13]. Therefore, we investigated the effects of PSE on TEWL, epidermal thickness, and the infiltration of inflammatory cells in DNCB-sensitized NC/Nga mice. PSE reduced AD-like skin symptoms (hyperkeratosis and erythema) and TEWL (► Fig. 3a–d). The inhibitory effects of PSE against AD in NC/Nga mice were confirmed by reduced epidermal thickening, and eosinophil and mast cell infiltration into the skin lesions (► Fig. 3a–c). In addition, PSE decreased serum IgE levels and increased IgG2a levels in DNCB-induced mice (► Fig. 4a, b). Thus, it was concluded that PSE ameliorated the symptoms of AD through the regulation of IgE and IgG2a levels.

Th2 cytokines, such as IL-4 and IL-5, are involved in the development of AD. IL-4 induces isotype switching from B cells to IgE, which activates mast cells [11, 39]. IL-5 plays an important role in



► **Fig. 4** Effects of *P. santalinus* extract (PSE) on serum levels of total IgE and IgG2a in NC/Nga mice. The concentrations of (a) IgE and (b) IgG2a in serum collected on day 35 were determined by ELISA. Results are expressed as the mean $\pm$ SD ($n = 5$) [## $p < 0.01$ compared to the vehicle group; ** $p < 0.01$ and * $p < 0.05$ compared to the 2,4-dinitrochlorobenzene (DNCB)-treated group].

the development and survival of eosinophils through its action as a potent chemoattractant [11]. In contrast, the expression of T-bet, a transcription factor of Th1 cells, correlates with the expression of IFN- γ in Th1 cells [40]. In addition, IFN- γ upregulates the expression of IL-12, which is an important cytokine for the inhibition of IgE production [41]. The interaction between chemokines and chemokine receptors also induces the recruitment of inflammatory cells. Chemokine ligands (CCL) play pivotal roles in the development of AD [42]. The topical application of PSE induced a significant decrease in Th1-mediated cytokines (IL-12 and IFN- γ) and Th2-mediated cytokines and chemokines (IL-4, IL-5, and CCLs) in the dorsal skin lesions (► **Table 1**). These results indicated that PSE could regulate Th1- and Th2-associated immune responses through the inhibition of Th1/Th2 production.

Pro- or anti-inflammatory cytokines, particularly IL-1 β and IL-10, are important in the initiation of AD [43]. IL-1 β , which is produced early in AD, induces the production of various chemokines that result in infiltration and recruitment of inflammatory cells into skin lesions [43]. IL-10 is a key mediator in the prevention of allergic inflammation, where it plays an important role in the modulation of immune reactions in AD-like skin lesions [44]. In the present study, PSE treatment reduced the mRNA expression of proinflammatory cytokines and increased IL-10 expression in skin lesions (► **Table 1**).

PSE significantly alleviated allergic responses and DNCB-induced AD-like dorsal skin lesions. The inhibitory effects of PSE against degranulation and inflammatory mediators suggest that it may be useful for the treatment of allergic and AD-like skin disorders. However, further investigations are needed to confirm its clinical application.

Materials and Methods

Plant material

The dried bark of *P. santalinus* was purchased from Kyungdong Oriental Market (Seoul, South Korea; <http://www.internet.kyungdong.or.kr/>) and collected from Yecheon, Gyeongbuk Province of South Korea in August 2015 (latitude, N 35°8' 60.118''; longitude, E 128°5' 63.385''). The plant was identified by one of the authors (M. S. Kim) and a voucher specimen (D-1016) was deposited at the Korea Institute of Science and Technology (KIST)-Gangneung Institute, Gangneung, South Korea. One kilogram of the dried bark of *P. santalinus* was refluxed with 1 L of boiling 50% ethanol for 3 h. The extract was filtered using Whatman No. 1 filter paper (GE Healthcare Life Sciences), dried under vacuum, and subsequently freeze-dried to obtain PSE. The active molecules of PSE were characterized by high-performance liquid chromatography and electrospray ionization mass spectrometry as shown below. All samples were dissolved in cell culture media containing DMSO (0.03% v/v) for *in vitro* assays.

High-performance liquid chromatography and quadrupole time-of-flight MS/MS analysis

The HPLC chromatogram of PSE was obtained by using an Agilent infinity series 1260 (Agilent) machine, and a YMC Triat C18 (250 mm $\times$ 4.6 mm, 5 μ m) column (YMC) was used for separation. Two solvents (A: 0.1% formic acid in methanol, B: 0.1% formic acid in water) were used for the mobile phase using a binary pump, and its gradient profile was as follows: initial mobile phase of A and B was 5:95 (v/v); 0–10 min, linear gradient to 10:90; 10–20 min, linear gradient to 20:80; 20–30 min, linear gradient to 30:70; 30–40 min, linear gradient to 40:60; 40–50 min, linear gradient to 5:95, which is equal to the initial ratio. The flow rate of the mobile phase was 1 mL/min. UV-vis spectral detection was conducted at 254 nm, and an injection volume of 10 μ L was used. The sample was prepared by dissolving PSE for 20 mg/mL in 50% EtOH.

For the identification of the chemical compounds in PSE, mass spectrometry was conducted on a Q-TOF 5600 (AB SCIEX). Mass spectrometry was operated in the positive and negative modes, both with an electrospray ionization source. The operating conditions were set as follows: 3.2 kV, capillary voltage; 27 V, sample cone voltage; 100 °C, source temperature; 600 L/h, gas flow; 400 °C, gas temperature. The MS survey mode, which can analyze the three most abundant ions automatically with auto-switching collision-induced dissociation (CID) energies ranging from 20 to 50 eV, was used for MS/MS experiments. The collision gas was argon, and the mass scan range was 50–1000 m/z . Sulfadimethoxine (Sigma-Aldrich), $[M + H]^+$: 311.0814, was used as a reference compound for the determination of accurate mass figures.

► **Table 1** Effects of PSE on the mRNA expression of Th1/Th2-associated cytokines and chemokines in the skin.

Th1/Th2 cytokines	IFN- γ	IL-4	IL-5	IL-12
Vehicle	100 $\pm$ 13	100 $\pm$ 14	100 $\pm$ 19	100 $\pm$ 16
DNCB	178 $\pm$ 15 [#]	172 $\pm$ 23 ^{##}	228 $\pm$ 37 ^{##}	359 $\pm$ 38 ^{##}
0.1 % PSE	129 $\pm$ 12 [*]	105 $\pm$ 18 [*]	145 $\pm$ 25 [*]	184 $\pm$ 23 ^{**}
0.3 % PSE	114 $\pm$ 15 [*]	82 $\pm$ 12 ^{**}	124 $\pm$ 12 ^{**}	176 $\pm$ 22 ^{**}
Th1/Th2 chemokines	CCL5	CCL11	CCL17	
Vehicle	100 $\pm$ 26	100 $\pm$ 18	100 $\pm$ 15	
DNCB	161 $\pm$ 15 [#]	243 $\pm$ 31 ^{##}	257 $\pm$ 23 ^{##}	
0.1 % PSE	127 $\pm$ 9 [*]	163 $\pm$ 18 [*]	177 $\pm$ 12 [*]	
0.3 % PSE	109 $\pm$ 8 ^{**}	129 $\pm$ 22 ^{**}	145 $\pm$ 22 ^{**}	
Inflammatory cytokines	IL-1 β	IL-10		
Vehicle	100 $\pm$ 37	100 $\pm$ 16		
DNCB	237 $\pm$ 38 ^{##}	47 $\pm$ 4 ^{##}		
0.1 % PSE	158 $\pm$ 26 [*]	152 $\pm$ 32 ^{**}		
0.3 % PSE	135 $\pm$ 39 ^{**}	162 $\pm$ 14 ^{**}		

Total RNA was isolated from the skin lesions of NC/Nga mice treated with PSE, and qRT-PCR was performed. β -Actin was used as an internal control. Results are expressed as the means $\pm$ SD (n = 5) [^{##}p < 0.01 compared to the vehicle group; ^{**}p < 0.01 and ^{*}p < 0.05 compared to the 2,4-dinitrochlorobenzene (DNCB)-treated group]

The obtained MS/MS spectra were searched in MS/MS libraries, such as the NIST mass spectral library and HMDB. In conclusion, chemical compound candidates, which were selected by the MS/MS library, were purchased from Sigma-Aldrich and confirmed by HPLC analysis.

Cell culture

RBL-2H3 cells (from the American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum and 100 U/mL penicillin) for 72 h at 5% CO₂ and 37 °C.

Cell viability

Cell viability was determined by using the MTT colorimetric assay (Sigma-Aldrich). RBL-2H3 cells were grown in 24-well plates (2 $\times$ 10⁵ cells/mL) for 24 h. We treated RBL-2H3 cells with several concentrations of PSE for 12 h. After washing, the cells seeded in 24-well plates were incubated for 3 h with MTT solution. After dissolving the insoluble formazan products in 200 μ L of dimethyl sulfoxide, the absorbance was measured at 550 nm by using a Synergy HT Multi-microplate reader (Bio-Tek Instruments).

β -Hexosaminidase and histamine production

RBL-2H3 cells were sensitized with monoclonal anti-dinitrophenol (DNP)-IgE (0.2 μ g/mL; Sigma-Aldrich) overnight and then washed with modified piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2; 25 mM PIPES, 119 mM NaCl, 40 mM NaOH, 5.6 mM glucose, 5 mM KCl, 1 mM CaCl₂, 0.4 mM MgCl₂ · 6H₂O, and 0.1 % bovine serum albumin [BSA]). The cells were treated with various concentrations of PSE at 37 °C for 1 h and then challenged with DNP-BSA (0.2 μ g/mL) for 30 min. PP2 (> 98 % purity; Sigma-Aldrich), a general Scr-family kinase inhibitor, was used as a posi-

tive control. Histamine concentration was then measured using an ELISA kit (Enzo Life Sciences). To measure the degree of degradation of the RBL-2H3 cells, the cell culture medium was transferred to 96-well plates and then used in the β -hexosaminidase assay via reaction with a substrate solution (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosamine in 0.05 M citrate buffer, pH 4.5) at 37 °C for 1 h. After the reaction was terminated by the addition of 0.05 M sodium carbonate buffer (pH 10), the absorbance was measured at 405 nm by using a microplate reader.

Prostaglandin E₂ and cytokine release

RBL-2H3 cells were sensitized with DNP-IgE (0.2 μ g/mL) overnight, treated with PSE for 1 h, and activated with DNP-BSA for 4 h. After the collection of the supernatants of cultured cells, PGE₂ concentration was measured in the cell culture medium by ELISA (Abcam). The concentrations of IL-4 and TNF- α were also estimated using ELISA (Abcam).

Animal experiments

Four-week-old NC/Nga female mice (Central Lab) were housed in individual ventilated cages under specific pathogen free conditions with a 12-h light-dark cycle at 22 $\pm$ 2 °C. After acclimation for 1 week, the dorsal hair of the mice was removed by using an electronic clipper 2 days before DNCB treatment, and 1 % DNCB dissolved in a mixture of acetone:olive oil (3:1 v/v) was applied topically on the skin five times per day. After 5 days, the 24 mice were divided into 4 experimental groups (n = 6): a vehicle group (vehicle without DNCB treatment), DNCB group (vehicle plus DNCB treatment), 0.1 % PSE group (topical application of 0.1 % PSE plus DNCB treatment), and 0.3 % PSE group (topical application of 0.3 % PSE plus DNCB treatment). All mice, except those in the vehicle control group, were topically treated with 0.2 % DNCB

► **Table 2** Primers used for quantitative real-time polymerase chain reaction.

Gene	Primer	Sequence	Product length (bp)
Mouse IFN- γ	Forward	5' - CGG CAC AGT CAT TGA AAG CC - 3'	268
	Reverse	5' - TGC ATC CTT TTT CGC CTT GC - 3'	
Mouse IL-1 β	Forward	5' - TCG GCC AAG ACA GGT CGC TC - 3'	231
	Reverse	5' - CCT CAG TGC GGG CTA TGA CC - 3'	
Mouse IL-4	Forward	5' - CCA TAT CCA CGG ATG CGA CA - 3'	254
	Reverse	5' - AAG CCC GAA AGA GTC TCT GC - 3'	
Mouse IL-5	Forward	5' - GCT GAA GGC CAG CGC TGA AGA - 3'	160
	Reverse	5' - ACA GAG CTC GGT GAG CGG ACA - 3'	
Mouse IL-10	Forward	5' - CCA GTC GGC CAG AGC CAC AT - 3'	208
	Reverse	5' - GGC CAT GCT TCT CTG CCT GGG - 3'	
Mouse IL-12	Forward	5' - TCT CTG GAC CTG CCA GGT GT - 3'	542
	Reverse	5' - CCT GTT GAT GGT CAC GAC GCG - 3'	
Mouse CCL5	Forward	5' - AGG AAC CGC CAA GTG TGT GTG CC - 3'	67
	Reverse	5' - AGT GGC ATC CCC AAG CTG GCT A - 3'	
Mouse CCL11	Forward	5' - ATG CAC CCT GAA AGC CAT AGT - 3'	97
	Reverse	5' - CAG GTG CTT TGT GGC ATC CT - 3'	
Mouse CCL17	Forward	5' - CCG AGA GTG CTC CCT GGA TTA - 3'	123
	Reverse	5' - AGC TTG CCC TGG ACA GTC AGA - 3'	
Mouse β -Actin	Forward	5' - TAA CCA ACT GGG ACG ATA TG - 3'	203
	Reverse	5' - ATA CAG GGA CAG CAC AGC CT - 3'	

three times per week for 5 weeks (15 challenges in total; Fig. 2S, Supporting Information). Mice in the 0.1% PSE and 0.3% PSE groups were concomitantly treated with PSE daily for 5 weeks. At the end of the treatment period, blood was collected via cardiac puncture, and the dorsal skin was removed and stored at -80°C . The experimental protocol was endorsed by the Animal Use and Care Committee of the KIST (2016–023, June 7, 2016), Seoul, South Korea. All experiments were performed in accordance with the relevant guidelines and regulations.

Histopathological analyses

Skin tissues were fixed in 10% formalin. After fixation, the specimens were embedded in paraffin and cut into $4\text{ }\mu\text{m}$ thick sections. To determine the number of mast cells and eosinophils, and epidermal thickness, sections were stained with dyes, including H&E, toluidine blue, and Congo red. The epidermal and dermal images were visualized and captured by using an inverted differential interference contrast fluorescence microscope fitted with twin charge-coupled device cameras ($\times 200$ magnification; Nikon). The epidermal thickness of each H&E-stained section ($10\text{ }\mu\text{M}$) was quantified in five fields per section by using ProgRes CapturePro software (Jenoptik). The number of mast cells or eosinophils in each toluidine blue- or Congo red-stained section was also counted in five fields per section by using ProgRes CapturePro software (Jenoptik).

Measurement of immunoglobulin E and immunoglobulin G2a release

Serum levels (IgE and IgG2a) were estimated by using ELISA kits (Abcam). The Ig concentration in each sample was determined using the linear regression equation of the standard absorbance curve.

Analysis of transepidermal water loss

To evaluate the barrier function of the dorsal skin, TEWL (a measure of the amount of water that passes from the inside of the body to the surrounding atmosphere through dispersion and evaporation from the skin epidermis [45]) was measured on the day before sacrifice by using a Tewameter (TM 300; Courage & Khazaka). TEWL was measured in a controlled environment ($22 \pm 2^{\circ}\text{C}$ and $55 \pm 15\%$ relative humidity) and recorded after the probe was stabilized for approximately 30 s when placed on the dorsal skin of the mice. The data are expressed in the unit of $\text{g}/\text{m}^2/\text{h}$.

Quantitative real-time PCR

Total RNA was isolated from the dorsal skin of the mice using RNAiso reagent (Takara), and its concentration was measured by using a NanoDrop spectrophotometer (Thermo Fisher Scientific) at 260 nm. cDNA was synthesized by using reverse transcriptase premix (Elpis-Biotech). qRT-PCR was performed using SYBR Green Master Mix (Roche) in the LightCycler (LC 480) system (Roche Applied Science). The mRNA expression of specific mouse genes was determined by using the specific primers listed in ► Table 2.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviations (SD). Statistical analyses were computed by using SPSS 12.0 (SPSS Inc.). The differences between groups were assessed by a one-way analysis of variance test followed by the Duncan's multiple range test. A p value ≤ 0.05 was considered statistically significant.

Supporting information

The HPLC chromatogram of *P. santalinus* 50% EtOH reflux extracts (PSE) and the experimental scheme are available in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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