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ORIGINAL ARTICLE

Amelioration of atopic-like skin conditions in NC/Tnd mice by topical application with distilled *Alpinia intermedia Gagnep* extracts

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ABSTRACT

***Alpinia intermedia*, a perennial plant that belongs to the Zingiberaceae family, has been used in folk medicine for a long time in the southern region of Japan. Because skin care is an effective approach that enables patients to manage their atopic dermatitis (AD), various herbal ingredients with few adverse effects have been evaluated for use in AD patients in recent years. In this study, we examined whether distilled extracts obtained from *A. intermedia* were beneficial for AD-like skin conditions in NC/Tnd mice. Topical application with the *A. intermedia* extracts significantly reduced the severity of AD, transepidermal water loss and scratching behavior in the mice. Supplementation of the extracts to cell cultures suppressed the expression of *Tslp* mRNA in PAM212 keratinocytes, degranulation in bone marrow-derived cultured mast cells (BMCMC), and neurite outgrowth in PC12 cells and dorsal root ganglia. In addition, the component analysis revealed that β-pinene was a major constituent of the *A. intermedia* extracts. The inhibitory effects of β-pinene both *in vivo* and *in vitro* were also demonstrated. These results indicate that topical application with the *A. intermedia* extract to the skin of NC/Tnd mice improved the condition of the skin by suppressing multiple inflammatory responses. The extracts may become novel skin-care remedies for AD patients.**

Key words: *Alpinia intermedia*, atopic dermatitis, NC/Tnd mouse, skin care, β-pinene.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease that is accompanied by pruritus.[1] Multiple factors, including those of genetic, immunological and physiological origins, are involved in the onset or augmentation of the disease.[1–3] Because the impairment of the barrier function allows the penetration of irritants or allergens into the body, the skin barrier is an important predisposing element in AD.[4,5] The findings from recent studies have shown that skin care ameliorates the symptoms experienced by AD patients.[1,2] While the exact etiology and the pathogenesis of the disease still remain unclear, palliative care to restore the skin's barrier function is certainly effective at improving the quality of life of AD patients.

Traditionally, a wide variety of herbal remedies have been used to alleviate the symptoms of fatigue, cold and other illnesses.[6,7] *Alpinia intermedia*, a plant that belongs to the genus *Alpinia* in the Zingiberaceae family, has been used for these purposes in Japan's southern region,[8] and residents have used this plant to produce Japanese *kampo* medicine, and to preserve food and protect clothes from pests.[8] Since the anti-inflammatory effects of different strain of *Alpinia* on house dust mite-induced dermatitis in NC/Nga mice were reported,[9] we hypothesized that extracts from *A. intermedia* could have a suppressive effect on AD and improve the condition of the skin of NC/Tnd mice.

In this study, we evaluated the clinical outcomes from NC/Tnd mice to which extracts from a plant of the Zingiberaceae family were applied. The results showed that extracts from *A. intermedia* and their main component, β-pinene, helped to prevent AD development by suppressing neurite outgrowth and the activation of mast cells and keratinocytes.

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METHODS

Reagents

Distilled extracts from *A. intermedia* were provided by Gray Art (Tokyo, Japan). Briefly, *A. intermedia Gagnep* plants were chopped and boiled, and the evaporated fractions were retrieved and diluted in water. β-pinene was purchased from Tokyo Chemical Industry (Tokyo, Japan). All of the other reagents were purchased from Sigma-Aldrich (Tokyo, Japan).

Mice

The NC/Tnd mice were maintained under conventional conditions as previously described.[10] All of the experiments involving the animals complied with the standards specified in the guidelines provided by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology, as well as with those in the Science Council of Japan's guidelines for the use of laboratory animals (approval no. 26-61).

Topical applications (100 μL) of the *A. intermedia* extracts, β-pinene diluted with distilled water or distilled water alone were administrated every day to the dorsal skin of conventional 8–10-week-old male NC/Tnd mice for the indicated period.

Clinical skin severity score, scratching analysis and transepidermal water loss (TEWL) measurements

The severity of the dermatitis was scored once a week by using previously described criteria[10] that are based on the five major clinical signs and symptoms of AD, namely, itching, erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness. The scratching behavior of the mice was quantified using a double-blind evaluation approach that was based on its frequency and duration per 30 min or 5 h using a SCLABA-Real® system (Noveltec, Kobe, Japan).[11] TEWL, which is indicative of the skin's barrier function,[12] was measured using a Multi Probe Adaptor System (Courage + Khazaka Electronic, Cologne, Germany) after the mice had acclimatized for 30 min to the controlled conditions that comprised a temperature of 23 ± 2°C and 40 ± 20% humidity. The measurements for each mouse were carried out three times, and the average measurements were determined.

Histological analysis

Histological samples were obtained after 6-week application of either vehicle or *A. intermedia* extracts. The skin samples were fixed in formalin, embedded in paraffin, and cut into 5-μm sections. The hematoxylin–eosin and toluidine blue staining procedures were performed as described previously.[13] The numbers of mast cells in five randomly selected microscopic fields were counted on each slide. For the immunohistochemical analysis, the tissues were incubated with an anti-PGP9.5 antibody, followed by incubation with the Alexa Fluor 488-conjugated antirabbit immunoglobulin (Ig) G antibody. The tissues were then mounted in the Prolong Gold antifading reagent with 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) and photographs were taken using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Intensity analysis

Images of PGP9.5-stained and isotype control-stained samples were used for analysis. To determine changes in distribution of PGP9.5 neurites, the mean grey intensity of three 100-μm square fields (avoiding hair follicles), which were selected at random, was measured in each sample by using Image J version 1.51 software (National Institutes of Health, Bethesda, MD, USA). The relative intensity was calculated by comparing the mean intensity of PGP9.5- and isotype control-stained sections in each mouse.

Cell culture

The PAM212 keratinocytes were cultured as previously reported,[14] and they were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) that was supplemented with 10% fetal bovine serum (FBS) (Filtron, Brooklyn, Vic., Australia) and antibiotics. The rat pheochromocytoma-derived PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and they were maintained in RPMI-1640 (Thermo Fisher Scientific) that was supplemented with 5% FBS, 10% horse serum and antibiotics. The bone marrow-derived cultured mast cells (BMCMC) were obtained from the bone marrow cells of male NC/Tnd and C57BL/6J mice, and they were maintained in α-minimum essential medium that contained 10% FBS and 10% pokeweed mitogen-stimulated spleen cell-conditioned medium, as described previously.[15]

Real-time reverse transcription polymerase chain reaction

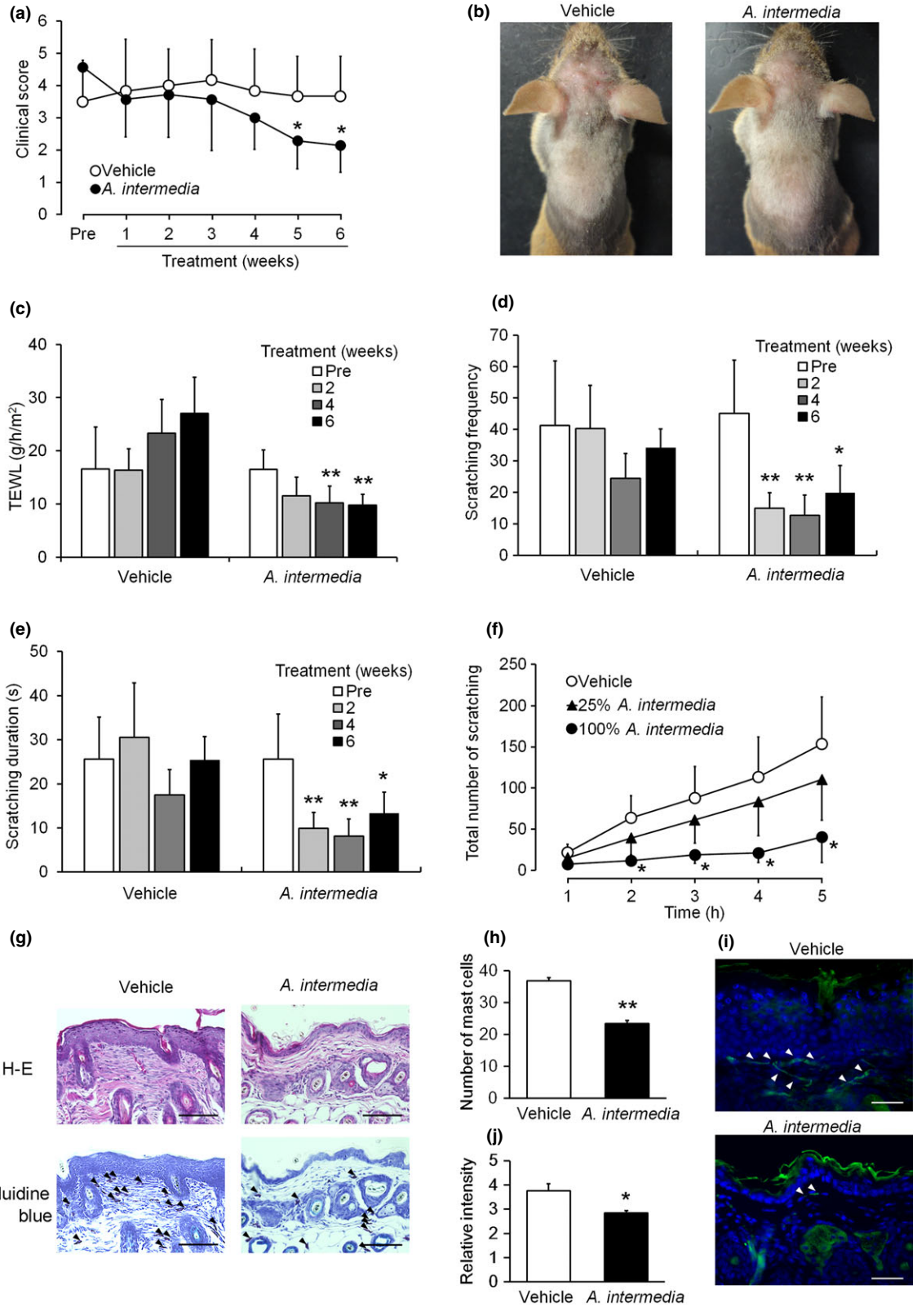
After serum-starvation for 12 h, the PAM212 cells were stimulated with 1 μg/mL of lipoteichoic acid (LTA) in the presence or absence of the *A. intermedia* extracts or β-pinene for 12 h. The real-time reverse transcription polymerase chain reaction was performed using a QuantStudio 3 system (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The following primers were used: a thymic stromal lymphopoietin (*Tslp*)-specific forward primer (5′-CGAGCAAATCGA GGACTGTGAG-3′), a *Tslp*-specific reverse primer (5′-CTTCG GGAGTTACTGGTGACG-3′), a β-actin (*Actb*)-specific forward primer (5′-CATCCGTAAAGACCTCTATGCCAAC-3′) and an *Actb*-specific reverse primer (5′-ATGGAGCCACCGATCCACA-3′).

β-Hexosaminidase (β-HEX) assay

A β-HEX assay was performed according to the method described by Ortega *et al.*[16] After the BMCMC had been treated with either the *A. intermedia* extracts or the β-pinene for 2 h, the cells were sensitized with 1 μg monoclonal anti-dinitrophenyl (DNP) IgE (clone SPE7; Sigma-Aldrich) for 1 h and stimulated with 100 ng/mL DNP bovine serum albumin (Sigma-Aldrich) for 1 h. The absorbance was measured using an ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan).

Neurite outgrowth assay

The neurite outgrowth assay was carried out as previously described.[17] Briefly, PC12 cells were primed with 50 ng/mL murine nerve growth factor (NGF) for 7 days in 10-cm



collagen-coated dishes (Thermo Fisher Scientific); then, they were restimulated with fresh culture medium containing 50 ng/mL murine NGF in the presence or absence of the *A. intermedia* extracts or β-pinene for 2 days. After the incubation, the number of neurites on each cell in five randomly selected microscopic fields, and on at least 100 cells, was counted. For culturing dorsal root ganglia (DRG) from NC/Tnd mice, DRG were obtained according to the method as previously described.[18] Briefly, single cell cultures from DRG were carried out on the laminin-coated glass bottom dish in F-12 medium supplemented with FBS and antibiotics for 3 days. Then the number of neurites on each cell in five randomly selected microscopic fields, and on at least 100 cells, was counted. The processes that were longer than the cell body diameter were regarded as neurites.

Immunocytochemistry

Dorsal root ganglia cultured for 3 days were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, and then stained with the anti-PGP9.5 antibody followed by Alexa Fluor 488-conjugated antirabbit IgG antibody as described above.

Western blot analysis

The immunoblot analysis was conducted as described previously.[19] All of the antibodies used were purchased from Cell Signaling Technology (Beverly, MA, USA), and the catalog numbers of the antibodies used were as follows: anti-phospho-Stat3, #9134; anti-Stat3, #9132; anti-phospho-Stat6, #9361; anti-Stat6, #9362, and anti-β-actin: #4970.

Analysis of the *A. intermedia* extract components

The leaves from *A. intermedia* plants were homogenized in ice-cold 50% ethanol. After centrifugation, the pellet was mixed with 50% ethanol and shaken for 60 h at 4°C. The supernatants obtained were used in the analyses. Headspace (HS) gas chromatography mass spectrometry (GC-MS) was carried out to analyze the compositions of the samples; 0.5 mL of HS gas of a sample was incubated at 40°C for 1 h. One thousand microliters of HS gas of a sample were removed with a 5 mL SGE 5MDR-HSV syringe and injected into a GC-MS QP5050 spectrophotometer equipped with a Shimadzu GC 17A, EP5MS (5% phenyl methylsilane) capillary column (30 m × 250 μm × 0.25 μm). Helium was the carrier gas. The GC-MS operating conditions are described next. The gas chromatography oven temperature was adjusted from 50°C to 150°C with a temperature rise of 3°C/min, which was held for 10 min; then, the temperature was raised to 200°C with an elevation of 10°C/min. The compounds were identified using mass spectra from the National Institute of Standards and Technology (NIST)/National Bureau of Standards. The mass spectra were recorded at 70 eV with a mass range of 50–400 *m/z*, compared against in-house mass spectral libraries at the NIST and Wiley, and they were confirmed against spectra of authenticated standards.

Statistical analyses

The statistical analyses were performed using Welch's *t*-test and Dunnett's test. $P < 0.05$ was considered statistically significant.

RESULTS

To examine the suppressive effects of the *A. intermedia* extracts, they were topically applied to the dorsal skin of NC/Tnd mice every day for 6 weeks. As shown in Figure 1(a,b), the clinical skin scores gradually declined in the *A. intermedia* extract-treated group than in the control group, which correlated with the reduction in the TEWL (Fig. 1c). Significant reductions were observed over a short period of time in relation to both the frequency and duration of the scratching behavior of the mice that had been treated with the

Figure 1. Effects of *Alpinia intermedia* extracts on the development of atopic dermatitis and scratching behavior in NC/Tnd mice. (a) The clinical skin severity scores of the mice. Data represent the means and the standard errors of the means (SE) from the results from seven mice in each group. $*P < 0.05$ compared with the vehicle group by using Welch's *t*-test. (b) Representative photographs of a mouse in each treatment group. (c) Transepidermal water loss (TEWL) analysis. Data represent the means and SE of the results from seven mice in each group. $**P < 0.01$ compared with the vehicle group by using Welch's *t*-test. (d) The scratching frequencies and (e) the total scratching durations are represented. Both sets of data were based on 30-min evaluations of the mice that were treated with the vehicle or the *A. intermedia* extracts for 2–6 weeks. $*P < 0.05$ for the scratching frequency and $**P < 0.01$ for the total scratching durations compared with the vehicle group by using Welch's *t*-test. (f) The effects of diluted extracts application on the development of atopic dermatitis (AD) and scratching behavior in NC/Tnd mice. Either undiluted (100%) or diluted (25%) extracts were applied to the skin of NC/Tnd mice for 2 weeks, and the number of scratching behavior was counted. Each point represents the mean and SE of the accumulated number of scratching behaviors of four mice in each group. $*P < 0.05$ compared with the control by using Dunnett's test. (g,h) Microscopic features of the skin after treatment with either vehicle or *A. intermedia* extracts for 6 weeks (original magnification ×200; scale bar, 100 μm. The arrowheads indicate the mast cells. (h) Each column represents the means ± SE of the mast cell numbers from five randomly selected microscopic fields. $**P < 0.01$ compared with the vehicle group by using Welch's *t*-test. (i) Immunohistochemical analysis of the skin after treatment with either vehicle or *A. intermedia* extracts for 6 weeks. The tissues were incubated with an anti-PGP9.5 antibody, followed by incubation with the Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (Ig)G antibody and 4′,6-diamidino-2-phenylindole (DNP) (original magnification ×200; scale bar, 50 μm). The arrowheads indicate the PGP9.5-positive neurites. (j) The average fold change ± SE of PGP9.5 staining intensity from isotype control-stained sections is indicated ($n = 7$, respectively). $*P < 0.05$ compared with the vehicle group by using Welch's *t*-test. H-E, hematoxylin–eosin.

A. intermedia extracts (Fig. 1d,e). Treatment-related adverse effects were not observed during the experiment (data not shown). To further confirm whether the ameliorative effects on scratching behavior were derived from components contained in the extracts, diluted extracts were also applied to the skin of NC/Tnd mice. In this experiment, we extended the duration of

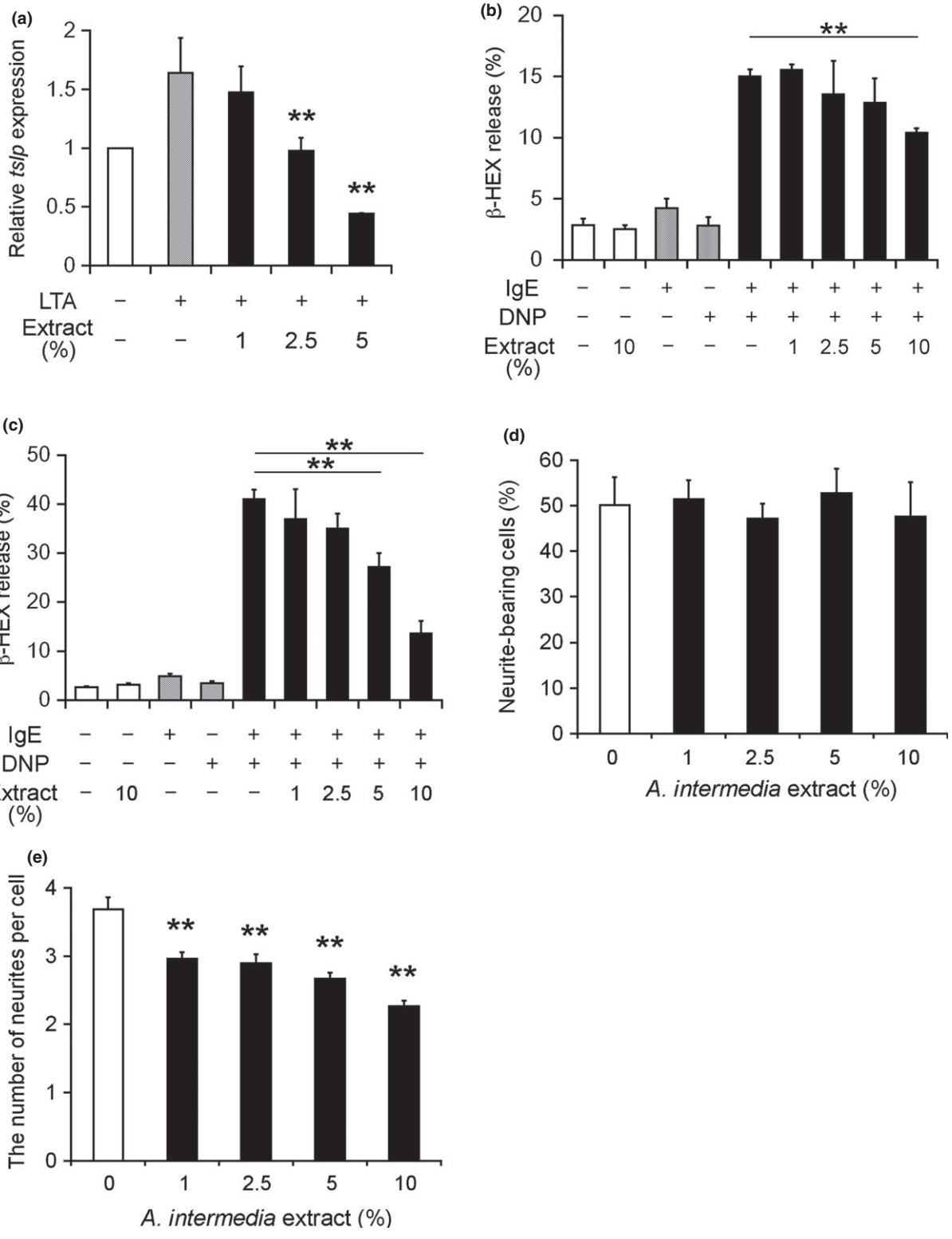


Figure 2. Inhibitory effects of the *Alpinia intermedia* extracts on the inflammatory responses. (a) *Tslp* mRNA production in PAM212 keratinocytes. The cells were serum-starved for 12 h, stimulated with 1 mg/mL lipoteichoic acid (LTA) for 12 h in the presence or absence of the *A. intermedia* extracts, and the real-time reverse transcription polymerase chain reaction was carried out. Data represent the means and the standard errors (SE) from three independent experiments. $^{**}P < 0.01$ compared with the LTA treatment group by using Dunnett's test. (b,c) Degranulation responses of the bone marrow-derived cultured mast cells (BMCMC) from (b) an NC/Tnd mouse and (c) a C57BL/6J mouse. The cells were treated with anti-dinitrophenyl (DNP) immunoglobulin (Ig)E and DNP-bovine serum albumin following treatment with the *A. intermedia* extracts for 2 h. Data represent the means and the SE from three independent experiments. $^{**}P < 0.01$ compared with the control by using Dunnett's test. (d,e) Effects of the *A. intermedia* extracts on neurite outgrowth in PC12 cells. The nerve growth factor (NGF)-primed cells were incubated with 50 ng/mL NGF and the *A. intermedia* extracts for 48 h, and the (d) number of neurite-bearing cells as well as the (e) number of neurites per cell were counted. Data represent the means and SE from three independent experiments. $^{**}P < 0.01$ compared with the control by using Dunnett's test.

the measurement up to 5 h to strengthen the conclusion. As expected, the dose-dependent suppressive effects of the extracts on scratching behavior were observed (Fig. 1f). To check the suppressive effects of the *A. intermedia* extracts, histological analyses were also undertaken. As shown in Figure 1(g,h), the topical application of the *A. intermedia* extracts slightly reduced the skin's hyperplasia, parakeratosis and dermal edema, and the infiltration of the mast cells into the skin. In addition, the neurite density declined in the dermis after the *A. intermedia* extracts were applied (Fig. 1i,j).

These results indicated that the *A. intermedia* extracts abrogated the cutaneous inflammation as well as the itch sensation. Because keratinocytes and mast cells play key roles in the production of inflammatory cytokines in the skin,[20,21] we examined whether the *A. intermedia* extracts inhibited mast cell degranulation and the expression of cytokines in keratinocytes. In response to LTA, which is a Toll-like receptor 2 ligand, the PAM212 keratinocytes upregulated the expression of the mRNA for the cytokine, thymic stromal lymphopoietin (TSLP), which is a well-known trigger of allergic inflammation that is produced by epithelial cells (Fig. 2a).[22,23] When the cells were stimulated with LTA in the presence of the *A. intermedia* extracts, the *Tslp* mRNA expression was suppressed in a dose-dependent manner without any cell toxicity (Figs 2a,S1a). To explore whether the *A. intermedia* extracts inhibited mast cell degranulation, a β-HEX assay was carried out. As shown in Figure 2(b), the IgE cross-linking-mediated degranulation from NC/Tnd-derived BMCMC was clearly abrogated by *A. intermedia* extracts in a dose-dependent manner, and cell toxicity was not observed even at the highest dose (Fig. S1b). This notion was also supported by the data that the extract inhibited the degranulation of C57BL/6J-derived BMCMC (Fig. 2c). We subsequently performed a neurite outgrowth assay using PC12 cells to evaluate the effects of the *A. intermedia* extracts on the differentiation of nerve cells, because the neurite density declined after treatment with the *A. intermedia* extracts (Fig. 1i) and the itch sensation is associated with the elongation of the nerve fibers.[24,25] As the PC12 cells differentiated and formed neurites in response to NGF, the proportions of the neurite-bearing cells were compared. As shown in Figure 2(d), adding the *A. intermedia* extracts did not alter the proportions of the neurite-bearing cells, but the number of neurites that formed on the surface of each cell was significantly reduced in the presence of the *A. intermedia* extracts, without affecting cell viability (Figs 2e,S1c).

These results revealed that the *A. intermedia* extract contained ingredients that suppressed AD development by inhibiting the inflammatory responses. Hence, GC-MS was carried out to determine the main components within the *A. intermedia* extracts, and several terpenes, including camphene, β-pinene, *m*-cymene and limonene, were detected (Fig. 3). On the basis of three separate analyses, the mean ± standard error of the mean concentrations of the components in the *A. intermedia* extracts were determined as follows: camphene, 8.4 ± 0.2 μg/mL; β-pinene, 44.0 ± 0.3 μg/mL; *m*-cymene, 2.0 ± 0.0 μg/mL; and limonene, 0.6 ± 0.1 μg/mL. The dry weights of camphene, β-pinene, *m*-cymene and limonene were 38.4, 201, 9.1 and 2.7 μg/g, respectively. Because β-pinene had the highest concentration and the anti-inflammatory effects of its isomer, α-pinene, had been reported,[26] we hypothesized that the ameliorative effects of the *A. intermedia* extracts that were applied to NC/Tnd mice were partly attributable to β-pinene. To confirm this, β-pinene was applied to the skin of NC/Tnd mice and the suppressive effects were evaluated. As shown in Figure 4(a–c), β-pinene significantly suppressed the elevation of the clinical

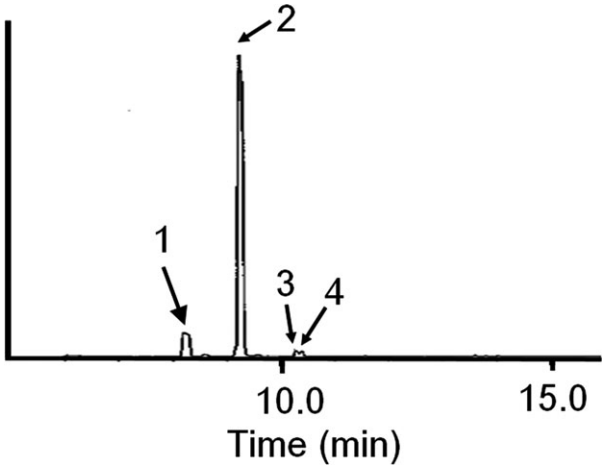


Figure 3. Gas chromatography mass spectrometry (GC-MS) chromatograms of the *Alpinia intermedia* extracts. Peak 1, camphene; peak 2, β-pinene; peak 3, *m*-cymene; and peak 4, limonene.

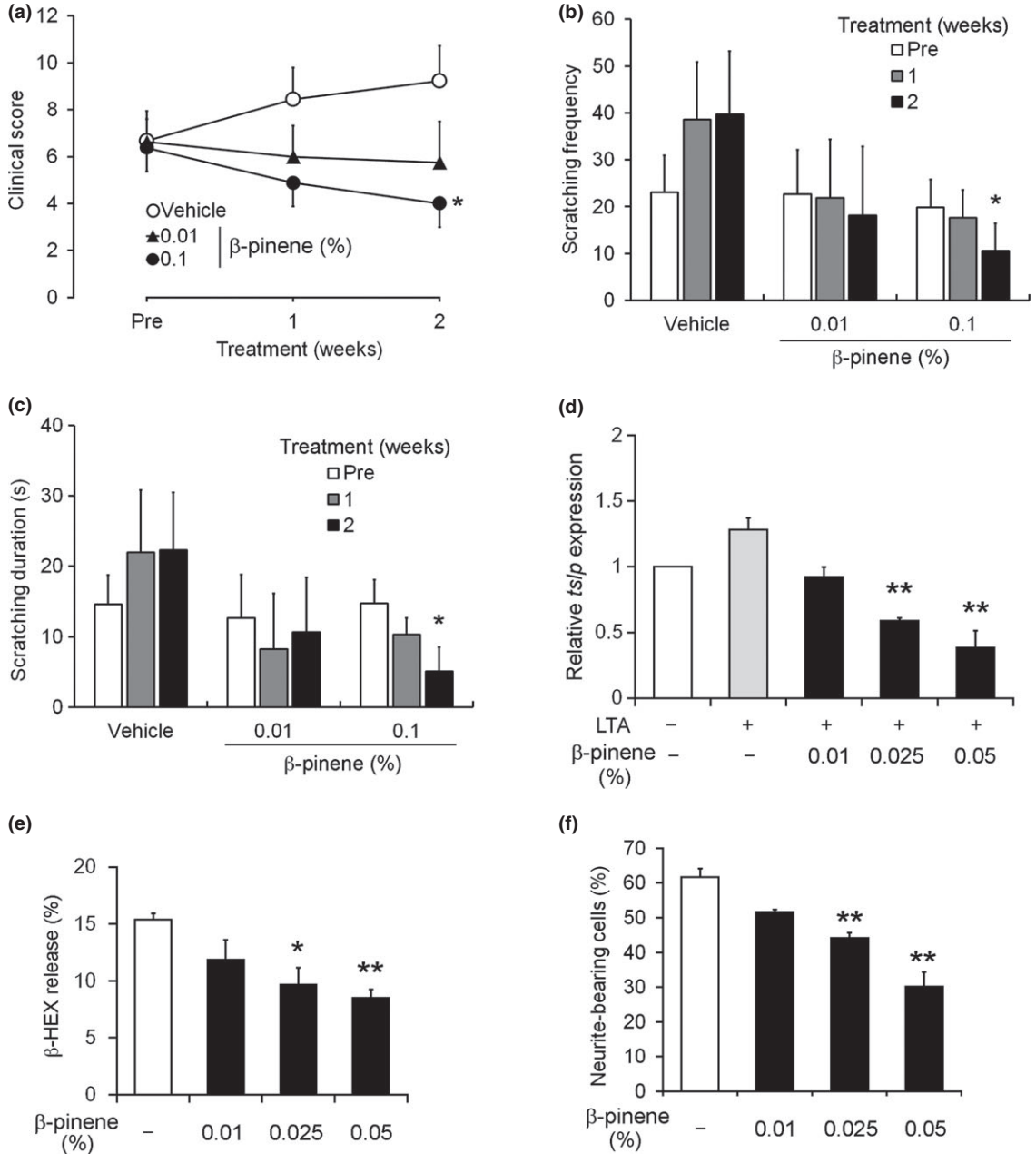


Figure 4. Effects of β-pinene on the development of atopic dermatitis and the scratching behavior in NC/Tnd mice. (a) The clinical skin severity score data from the mice. Data represent the means and standard errors (SE) from the results from seven mice in each group. $*P < 0.05$ compared with the control mice by using Dunnett's test. (b) The scratching frequencies and (c) the total scratching durations are presented. Both sets of data were based on 30-min evaluation of the mice that were treated with the vehicle or β-pinene extracts for 1–2 weeks. $*P < 0.05$ compared with the control group by using Dunnett's test. (d) *Tslp* mRNA production in the PAM212 keratinocytes. The cells were serum-starved for 12 h, stimulated with 1 mg/mL lipoteichoic acid (LTA) for 12 h in the presence or absence of β-pinene, and then the real-time reverse transcription polymerase chain reaction was carried out. Data represent the means and SE from three independent experiments. $**P < 0.01$ compared with the LTA treatment group by using Dunnett's test. (e) Degranulation responses of bone marrow-derived cultured mast cells (BMCMC) derived from NC/Tnd mice. The cells were treated with anti-dinitrophenyl (DNP) immunoglobulin (Ig)E and DNP-bovine serum albumin after being treated with β-pinene for 2 h. Data represent the means and SE from three independent experiments. $**P < 0.01$ compared with the control group by using Dunnett's test. (f) Effects of β-pinene on neurite outgrowth in PC12 cells. Nerve growth factor (NGF)-primed cells were incubated with 50 ng/mL NGF and β-pinene for 48 h, and the number of neurite-bearing cells was counted. Data represent the means and SE from three independent experiments. $**P < 0.01$ compared with the control by using Dunnett's test.

score in NC/Tnd mice and reduced the numbers of scratching episodes and the durations of the scratching behavior.

To further examine whether β-pinene had inhibitory effects on keratinocytes, its effect on the expression of *Tslp* mRNA in the PAM212 cells was evaluated. β-pinene significantly inhibited the expression of *Tslp* mRNA in a dose-dependent manner (Fig. 4d). In addition, β-pinene suppressed mast cell degranulation and neurite outgrowth in PC12 cells (Fig. 4e,f). In these experiments, adding β-pinene did not affect cell viability at all (data not shown).

Inhibitory effects of both *A. intermedia* extracts and β-pinene on neurite outgrowth were observed in both *in vitro* and *in vivo* (Figs 1i,2d,e,4f). To further ascertain these effects, DRG from NC/Tnd mice were isolated and suppressive effects on neurite growth were evaluated. As expected, supplementation of *A. intermedia* extracts and β-pinene significantly suppressed the neurite outgrowth of DRG (Fig. 5a). In addition, neurites formed in the presence of either the extracts or β-pinene were less conspicuous than the DRG cultured in medium alone (Fig. 5b).

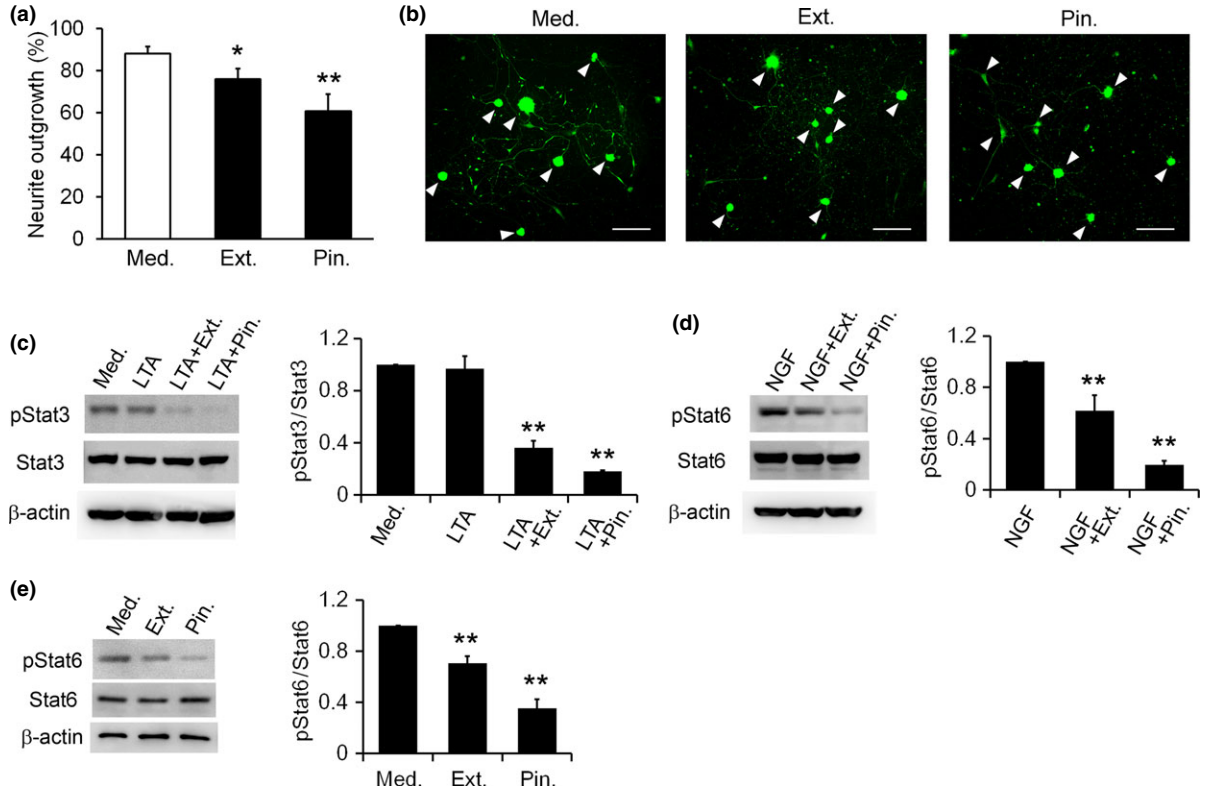


Figure 5. Effects of *Alpinia intermedia* extracts and β-pinene on neurite outgrowth in NC/Tnd mice-derived dorsal root ganglia (DRG). (a) Cells were incubated with either the extracts or β-pinene for 72 h, and the number of neurite-bearing cells was counted. Data represent the means and standard errors (SE) from three independent experiments. $*P < 0.05$ and $**P < 0.01$ compared with the control by using Dunnett's test, respectively. (b) Representative photographs of the neurites cultured for 3 days. Cells were stained with anti-PGP9.5 antibody and Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (Ig)G antibody (original magnification ×200; scale bar, 100 μm). The arrowheads indicate ganglion cells. Possible signaling pathways involved in the suppressive effects of *A. intermedia* extracts and β-pinene. (c) Western blot analysis of PAM212 cells and the quantification of the ratio of the phosphorylated : total amounts of Stat3. The cells were serum-starved for 12 h, stimulated with 1 mg/mL lipoteichoic acid (LTA) for 12 h in the presence or absence of the *A. intermedia* extracts or β-pinene. The relative mean pStat3 : Stat3 ratios ± SE from three independent experiments are shown. $**P < 0.01$ compared with treatment with LTA treatment by using Dunnett's test. (d) Western blot analysis of the PC12 cells and the quantification of the ratio of the phosphorylated : total amounts of Stat6. NGF-primed PC12 cells were incubated with 50 ng/mL NGF and either extracts of *A. intermedia* or β-pinene for 48 h. The relative mean pStat6 : Stat6 ratios ± SE from three independent experiments are shown. $*P < 0.05$ and $**P < 0.01$, compared with treatment with NGF alone by using Dunnett's test. Med, medium alone; Ext., 5% *A. intermedia* extracts; Pin., 0.025% β-pinene. (e) Western blot analysis of DRG and the quantification of the ratio of the phosphorylated : total amounts of Stat6. DRG were incubated with either extracts of *A. intermedia* or β-pinene for 24 h. The relative mean pStat6 : Stat6 ratios ± SE from three independent experiments are shown. $**P < 0.01$ compared with treatment with medium alone by using Dunnett's test. Med, medium alone; Ext., 5% *A. intermedia* extracts; Pin., 0.025% β-pinene.

These results indicated that both the *A. intermedia* extracts and β-pinene abrogated the development of dermatitis in NC/Tnd mice by suppressing the pro-inflammatory processes. Western blot analyses were carried out to gain an insight into the signaling pathways that were being targeted by the *A. intermedia* extracts and β-pinene in the PAM212 cells, PC12 cells and BMCMC. As shown in Figure 5(c,d), treating the cells with either the *A. intermedia* extracts or β-pinene suppressed the activation of signal transducer and activator of transcription (Stat)3 in the PAM212 and they suppressed the activation of Stat6 in the PC12 cells. The activation statuses of the principal signaling molecules, for example, the p38 mitogen-activated protein kinases, the extracellular signal-regulated kinases and Akt, were not altered after the cells had been treated with the *A. intermedia* extracts or β-pinene (data not shown). Because the data above indicated that Stat6 is one of the targets in ganglia, further analysis was performed using DRG. As expected, β-pinene suppressed the activation of Stat6, corresponding to the results obtained in PC12 cells (Fig. 5e).

DISCUSSION

In this study, we showed that *A. intermedia* extracts ameliorated AD development in NC/Tnd mice by suppressing inflammatory responses in the skin. The preventive effects of β-pinene both *in vivo* and *in vitro* reflected those observed when the *A. intermedia* extracts were used, indicating that the β-pinene within the extracts plays a key role in suppressing inflammatory processes. Compared with conventional chemotherapies and steroids, herbal medicines generally have mild effects and are less toxic. Indeed, the anti-inflammatory effects of the *A. intermedia* extracts in NC/Tnd mice were less potent than the anti-inflammatory effects of FK506, which dramatically improved the clinical scores within 2 weeks.[13] Therefore, it is unlikely that herbal medicines, including *A. intermedia* extracts, will become alternative therapies for AD, but they may be useful for reducing the doses and toxicities of conventional therapies when they are combined with these agents. Compared with extraction using organic solvents, distilled extraction yields a small range of chemicals, including terpenes.[27] In fact, the *A. intermedia* extracts obtained using *n*-hexane and chloroform did not contain detectable amounts of β-pinene,[28] which suggests that distilled extraction may be effective for obtaining medicinal ingredients for cutaneous inflammatory symptoms.

The results from the immunoblot analyses indicated that neither the *A. intermedia* extracts nor β-pinene broadly suppressed the principal signaling pathways, thereby supporting the notion that they are less toxic than conventional anti-inflammatory agents, including steroids. The molecules targeted by the *A. intermedia* extracts and β-pinene remain unclear, but there seemed to be a target molecule that was situated upstream of the Stat proteins (Fig. 5). It has been reported that Stat3 activation occurs right after the cell injury in peripheral nerves.[29] Nerve cell injury-derived Stat3 activations in the skin, resulting from pro-inflammatory immune responses, may be one of the targets of the *A. intermedia* extract and β-pinene *in vivo*. Among the molecules analyzed, not all of the activations of all proteins were clearly canceled in BMCMC while degranulation was suppressed, which suggests that some of the molecules that are directly associated with the degranulation process, including the vesicle-associated membrane protein family and the syntaxin protein family,[30] may be targeted by the *A. intermedia* extracts or β-pinene in mast cells.

In conclusion, the results from this study showed the anti-inflammatory effects of *A. intermedia* extracts. Therefore, the extracts or β-pinene itself may be novel options for controlling the condition of the skin of patients with AD.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Fig. S1. Little toxicities of *Alpinia intermedia* extracts on various cells.