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# ANTI-ATOPIC DERMATITIS OF PURIFIED BEE VENOM ON KERATINOCYTES VIA SUPPRESSION OF PAR2, ICAM-1, AND IL-6 EXPRESSION

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**Abstract**

**Emerging evidence indicates that inflammation in atopic dermatitis (AD) is associated with immune-mediated abnormalities in the skin. The history and severity of AD are risk factors for dust mite allergy. Bee venom (BV) is used in a complementary medicine to treat various diseases and skin disorders. Purified BV is obtained through electric stunning with a BV collector, without the harming of honeybees, followed by the removal of impurities from the collected BV and lyophilization of the final product. To evaluate the therapeutic potential of purified BV for AD, we investigated the anti-inflammatory effects of BV on house dust mite (*Dermatophagoides farinae*) antigen-stimulated HaCaT keratinocytes. The results showed that *D. farinae* induced significant increased levels of protease-activated receptor 2 (PAR2), intercellular adhesion molecule-1 (ICAM-1), and interleukin-6 (IL-6) compared to those in the normal control. However, purified BV inhibited the elevated expression of PAR2, ICAM-1 and IL-6 at the gene and protein levels. Thus, purified BV may have a therapeutic potential for the treatment and management of AD.**

Keywords: atopic dermatitis, bee venom, *Dermatophagoides farina*

## INTRODUCTION

Atopic dermatitis (AD) is a chronic skin disorder with complex interactions of innate and adaptive immune responses. As an intensely pruritic dermatosis, AD is characterized by a dry skin, impaired skin epidermal barrier, eczematous lesions, abnormal immune responses and an IgE-mediated allergy to various exogenous antigens (Patel, D'Ambra, & Feldman, 2017). In response to antigen cross-linking of the IgE receptor, mast cells, the key player in an IgE-mediated allergy, undergo degranulation and release inflammatory mediators, including histamine, proteases, chemokines, and cytokines (Wu et al., 2014). Proteases present in the skin are essential for the homeostasis of the epidermal permeability barrier. A number of endogenous proteases located in the stratum corneum mediate various cellular responses, including inflammation.

Meanwhile, exogenous proteases from such allergens as dust mites, ovalbumin, fungi and bacteria signal cells by activating protease-activated receptors (PARs), a subfamily of G protein-coupled receptors (Lee, Jeong, & Lee, 2010). In particular, house dust mites are the main allergens responsible for the household dust allergy. They are members of the family Pyroglyphidae, which includes *Dermatophagoides pteronyssinus*, *D. farinae*, and *Euroglyphus maynei* (Hossny, El-Sayed, & Abdul-Rahman, 2014). *D. farinae* is the most important environmental allergen associated with human AD. The expression of PAR2 on human skin as a sensor for endogenous as well as exogenous proteases and its role in numerous physiological and pathophysiological reactions in the skin have been demonstrated (Rattenholl & Steinhoff, 2008). The activation of PAR2 on keratinocytes stimulates inhibitory κB kinases

α and β, c-Jun N-terminal kinase, and p38 mito-gen-activated protein kinase activity (Kanke et al., 2001), including nuclear factor (NF)-κB DNA binding (Kanke et al., 2001; Buddenkotte et al., 2005). Moreover, PAR2 is directly involved in the NF-κB-mediated upregulation of intercellu-lar adhesion molecule-1 (ICAM-1) in human ke-ratinocytes, which increases the inflammation and release of such skin cytokines as interleu-kin (IL)-6 (Buddenkotte et al., 2005). Thus, the activation of PAR2 and its relevant signaling pathways plays a pivotal role in inflammatory processes in the skin.

An effective strategy to prevent the devel-opment of AD could potentially mitigate both the disease and its downstream effects. As such, bee venom (BV) from the honeybee (*Apis mellifera* L.) has been used as both an alterna-tive medicine to treat skin diseases (Kim et al., 2015) and an integrative medicine for chronic inflammatory disorders (Lee et al., 2017). Purified BV is generally obtained by electric stunning using a BV collector, without harming honeybees, followed by removal of impurities from the collected BV and lyophilization of the final product. BV contains a number of such bioactive substances as melittin, apamin, adolapin and mast cell degranulating peptide (Han et al., 2011).

In this study, we aimed to evaluate the anti-inflammatory effects of purified BV on house dust mite antigen-stimulated HaCaT keratino-cytes. HaCaT cells are commonly used to mimic AD symptoms in response to allergens such as dust mites by inducing immunological responses.

## MATERIAL AND METHODS

### Preparation of purified BV
BV was collected from colonies of natural honey bees using a bee venom collector (Chungjin Biotech, Ansan, Korea) under sterile conditions and then purified under strict laboratory conditions. The collected venom was diluted in cold sterile water and centrifuged at 10,000 × *g* for 5 min at 4°C. The supernatant containing residues was discarded. The purified BV was lyophilized through freeze-drying and refriger-ated at 4°C for later use.

### Ultra-performance liquid chromatography (UPLC) analysis
The purified BV (1 mg/mL) was dissolved in ionized water and then filtered through a 0.2-µm polytetrafluoroethylene membrane filter. It was analyzed using a UPLC-diode array detection I class model (Waters Corp., Milford, MA, USA). The separation of apamin, phospho-lipase A2, and melittin from BV was conducted on a Halo ES-C18 column (Advanced Materials Technology, Wilmington, DE, USA) using a mobile phase consisting of 20 mM trifluoroacetic acid (TFA)/acetonitrile (MeCN) and 20 mM TFA/H$_2$O. The analytical conditions are shown in Tab. 1.

Table 1.

UPLC conditions for purified bee venom

| Item | Conditions | | |
|---|---|---|---|
| Column | Halo ES-C18 (2.1 × 100 mm, 2.7 µm) | | |
| Column temperature | 50°C | | |
| Flow rate | 0.8 mL/min | | |
| Injection volume | 2 µL | | |
| Wavelength | 220 nm | | |
| Mobile phase | Time (min) | A (%) | B (%) |
| (A) 20 mM TFA/MeCN | 0 | 10 | 90 |
| (B) 20 mM TFA/H$_2$O | 3 | 31 | 69 |
| | 5 | 40 | 60 |
| | 10 | 45 | 55 |

## Purity evaluation of purified BV using scanning electron microscopy

For scanning electron microscopy (SEM) experiments, purified and crude BV were fixed overnight at 4°C in Karnovsky's fixative (25% glutaraldehyde and 8% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.2). The samples were then washed three times in distilled water and fixed in 1% osmium tetroxide for 2 h at 4°C. All specimens were washed again in distilled water and dehydrated in a graded ethanol series (50, 75, 90, 95, and 100%) for 20 min. After dehydration, the samples were dried in a critical point dryer, mounted on SEM stubs, and coated with gold. A Hitachi S3000N scanning electron microscope (Tokyo, Japan) was used for SEM.

## Cell culture

HaCaT cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (10,000 U/mL; Gibco BRL) at 37°C under 5% $CO_2$ in air. Cells were treated with a house dust mite (*D. farinae*) extract (0.3 and 3 μg/mL for HM-0.3 and HM-3, respectively) for 24 h. The house dust mite extract was purchased from the Arthropods of Medical Importance Resource Bank (Seoul, Korea).

## Cell viability assay

Effects of the purified BV and house dust mite extract on the viability of HaCaT cells were determined using a Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). The CCK-8 assay is based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon the reduction by dehydrogenases in the presence of an electron carrier (Han et al., 2011). Cells (5.0 × 10³ cells/well) were seeded into 96-well plates and preincubated for 24 h. After preincubation, the cells were treated with various concentrations (0.1, 1, 5, and 10 μg/mL) of the purified BV or house dust mite extract for 24 h. Then, 10 μL

of WST-8 was added into each well, and the cells were incubated for an additional 4 h at 37°C. Cell viability was measured by absorbance at 450 nm using a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

## Levels of PAR2, ICAM-1, and IL-6 measured by enzyme-linked immunosorbent assays

Culture supernatants of HaCaT cells treated with the house dust mite extract and purified BV were collected at 24 h. Enzyme-linked immunosorbent assay (ELISA) kits were used to quantitatively measure the concentrations of secreted PAR2 (Abbexa, Ltd., Cambridge, UK), ICAM-1 (R&D Systems, Minneapolis, MN, USA), and IL-6 (R&D Systems) in the supernatants.

## Quantitative real-time polymerase chain reaction

RNA was extracted from cultured cells with the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. A reverse transcription reaction was performed using the AccuPower RT premix and oligo (dT)$_{18}$ primer (Bioneer, Daejeon, Korea) according to the manufacturer's recommendations. Real-time polymerase chain reaction (PCR) was performed in a LightCycler Nano system (Roche Applied Science, Mannheim, Germany) using the LightCycler DNA SYBR Green I master (Roche Applied Science). PCR mixtures contained 100 ng of cDNA and 0.5 μM each forward and reverse primers. The samples were denatured at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s. Expression values were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The specificity of the reaction was further confirmed by melting curve analysis. The primer sequences were as follows: PAR2 forward, 5'-CCT GGC CAT GTA CCT GAT CT-3'; PAR2 reverse, 5'-TTT ACA GTG CGG ACA CTT CG-3'; ICAM-1 forward, 5'-GGC TGG AGC TGT TTG AGA AC-3'; ICAM-1 reverse, 5'-ACT GTG GGG TTC AAC CTC TG-3'; IL-6 forward, 5'-TAC CCC CAG GAG AAG ATT CC-3'; IL-6 reverse, 5'-TTT TCT GCC AGT GCC TCT TT-3'; GAPDH forward,

Table 2.

Main components of the purified bee venom, based on UPLC analysis

| Component (%) | | |
|---|---|---|
| Apamin | Phospholipase A2 | Melittin |
| 2.3 ± 0.3 | 10.9 ± 1.6 | 63.9 ± 5.4 |

The values are expressed as the mean ± SEM (n=15).

5′-GTG AAG GTC GGA GTC AAC G-3′; and GAPDH reverse, 5′-TGA GGT CAA TGA AGG GGT C-3′.

## Western blotting
Cells were lysed using CelLytic M (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 30 min on ice, the lysed cells were centrifuged at 12,000 × *g*, 4°C for 10 min. The supernatants were collected, and the protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein extracts were separated on 8% to 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) using a standard SDS-polyacrylamide gel electrophoresis procedure. The membrane was blocked in 5% (w/v) skim milk in TBS-T (25 mM Tris, pH 7.4, 3 mM KCl, 140 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. After blocking, the membrane was probed with a primary antibody for 4 h at room temperature and then washed three times with TBS-T for 10 min on a shaker. Subsequently, the membrane was probed with a horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. After washing, the membrane was incubated with enhanced chemiluminescence detection reagents (Thermo Fisher Scientific) for 1 min. The signal intensity was quantified by an image analyzer (LAS-3000; Fuji, Tokyo, Japan). Primary antibodies used in the present study were purchased from Abcam,

Cambridgeshire, UK (anti-PAR2, ab138479, and anti-IL-6, ab1672) and Santa Cruz Biotechnology, Dallas, TX, USA (GAPDH, sc-32233).

## Statistical analysis
All data are expressed as the mean ± standard error of the mean (SEM). Statistical differences among groups were calculated by analysis of variance, followed by Duncan's multiple range test (SPSS version 18.0; SPSS, Inc., Chicago, IL, USA). P-values of less than 0.05 were considered significant.

## RESULTS

### Composition of purified BV
The UPLC analysis of the purified BV revealed three peaks matching those of the commercial standards (data not shown), apamin, phospholipase A and melittin, with retention times of approximately 1.8, 4.3, and 8.8 min, respectively (Fig. 1). The purified BV contained 63.9 ± 5.4% (n=15) melittin, 10.9 ± 1.6% (n=15) phospholipase A2, and 2.3 ± 0.3% (n=15) apamin (Tab. 2).
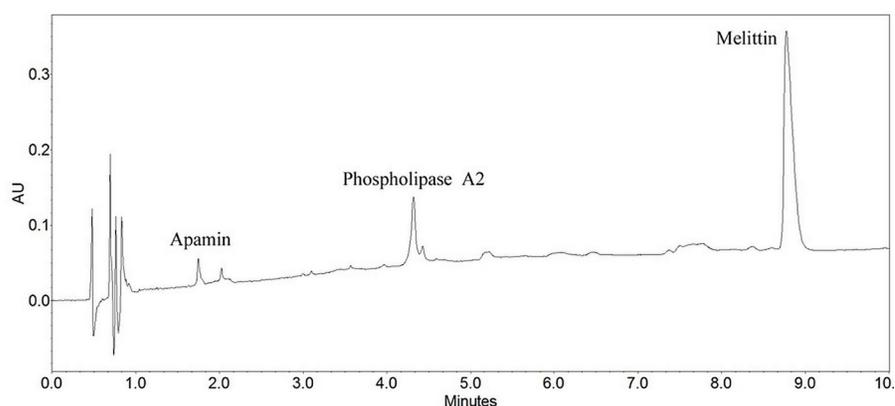


Fig. 1. UPLC spectrum of the purified bee venom.

### Purity of purified BV

Fig. 2 depicts the SEM images of the naturally harvested BV and purified BV. The crude BV had some dust, honey, pollen, and bee parts, while the purified BV contained particles of a uniform shape and dimensions.
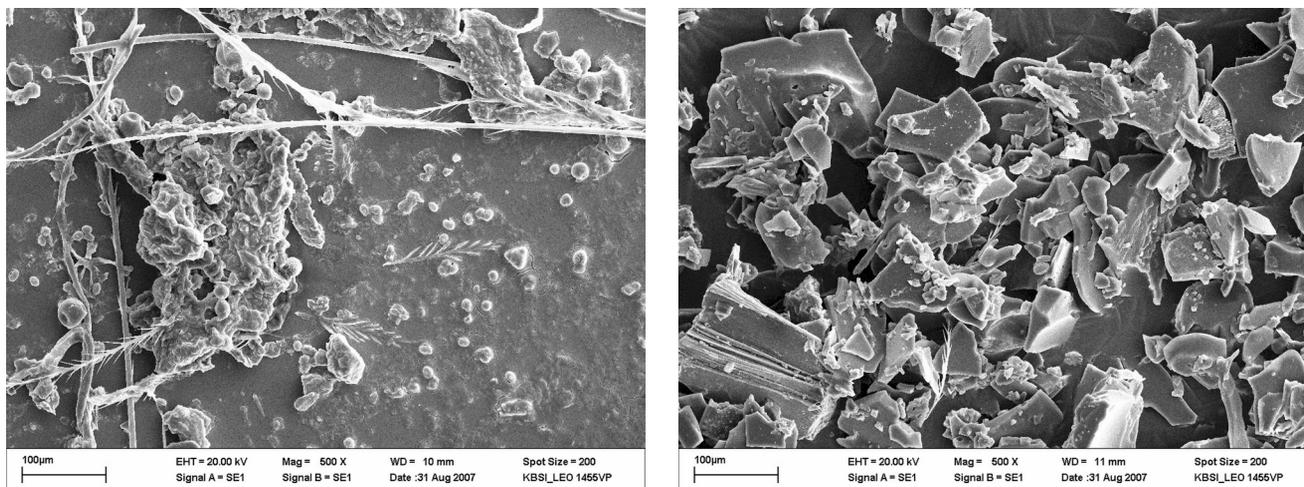


Fig. 2. Scanning electron micrographs of collected crude (A) and purified (B) bee venom.

### Cell viability test

The CCK-8 assay demonstrated dose-dependent toxic effects on HaCaT cells at increasing concentrations of the house dust mite extract and purified BV under starvation conditions (Fig. 3). No significant toxicity was observed at concentrations of the house dust mite extract and purified BV up to 1 µg/mL. However, at concentrations of the house dust mite extract and BV of 10 µg/mL, a significant toxicity for HaCaT cells was observed.
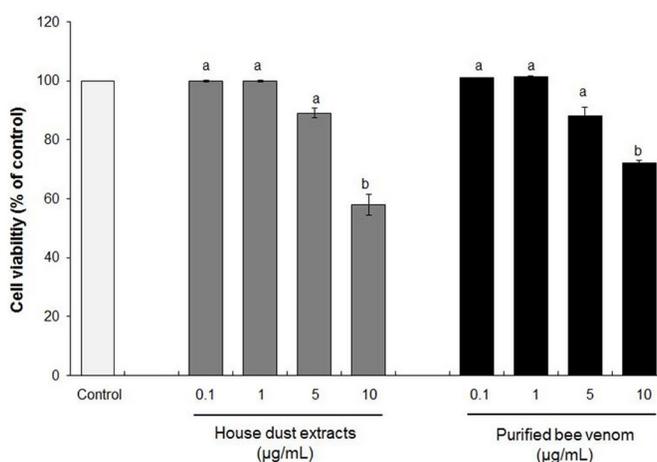


Fig. 3. CCK-8 assay data showing a dose-dependent toxic effect of the house dust mite extract and purified bee venom on HaCaT cells. After 24 h of incubation, the house dust mite extract and bee venom at concentrations of 10 ug/mL led to a significant reduction of cell viability. The values are expressed as the means SEM (n=9) of three independent experiments. Different letters indicate significant differences between groups (p<0.05).

**Effects of purified BV on PAR2, ICAM-1, and IL-6 secretion levels from *D. farinae* extract-stimulated HaCaT cells**

To investigate the effects of the purified BV on the secretion levels of PAR2, ICAM-1, and IL-6, HaCaT cells were stimulated with the *D. farinae* extract in the presence or absence of BV for 24 h. The levels of PAR2, ICAM-1, and IL-6 increased in response to *D. farinae* treatment relative to those in the normal control. However, treatment with purified BV markedly decreased the PAR2, ICAM-1, and IL-6 levels in the *D. farinae* extract-stimulated HaCaT cells (Fig. 4).
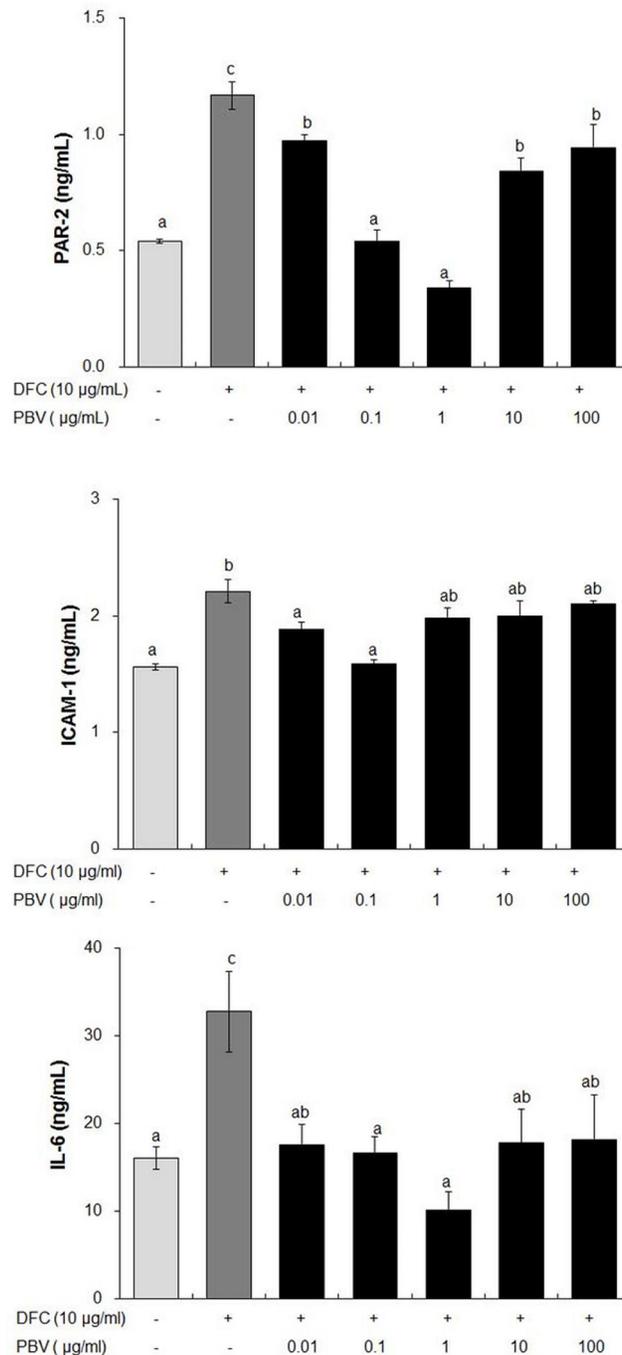


Fig. 4. Effects of purified bee venom (PBV) on the levels of PAR2 (A), ICAM-1 (B), and IL-6 (C) in HaCaT cells stimulated with the *Dermatophagoides farinae* extract (DFC). The values are expressed as the means SEM (n=9) of three independent experiments. Different letters indicate significant differences between groups (p<0.05).

### Effects of purified BV on the expression of PAR2, ICAM-1, and IL-6 in *D. farinae* extract-stimulated HaCaT cells

*D. farinae* treatment significantly induced the mRNA expression of PAR2, ICAM-1, and IL-6, which was significantly inhibited by treatment with the purified BV (Fig. 5). Furthermore, the protein levels of PAR2, ICAM-1, and IL-6 increased in response to *D. farinae* treatment relative to those in the normal control. However, treatment with the purified BV decreased the PAR2, ICAM-1, and IL-6 protein levels (Fig. 6).
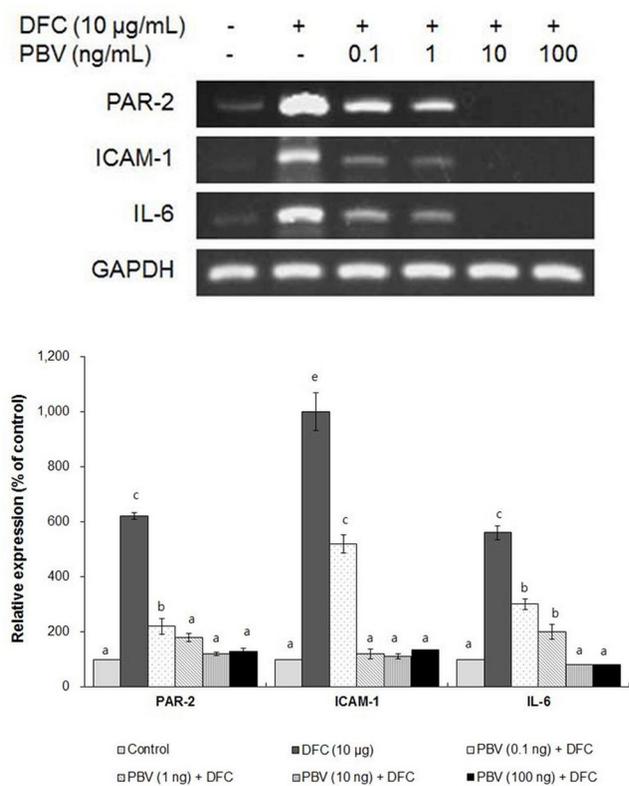


Fig. 5. Effects of purified bee venom (PBV) on mRNA expression levels of PAR2, ICAM-1, and IL-6 in HaCaT cells stimulated with the Dermatophagoides farinae extract (DFC), determined by RT-PCR. The values are expressed as the means SEM 9n=9) of three independent experiments. Different letters indicate significant differences between groups (p<0.05).



Fig. 6. Effects of purified bee venom (PBV) on mRNA expression levels of PAR2, ICAM-1, and IL-6 in HaCaT cells stimulated with the Dermatophagoides farinae extract (DFC), determined by RT-PCR. The values are expressed as the means SEM 9n=9) of three independent experiments. Different letters indicate significant differences between groups (p<0.05).

## DISCUSSION

AD is the most common allergic inflammatory skin disease. Although many potential mechanisms of AD have been investigated and proposed, its etiology still remains unclear. In addition, despite many reports on BV, the therapeutic potency of BV against AD-like symptoms and its relevant mechanisms remain unknown. As evidenced by our in vitro findings, purified BV possessed potent anti-atopic dermatitis activities. In the current study, u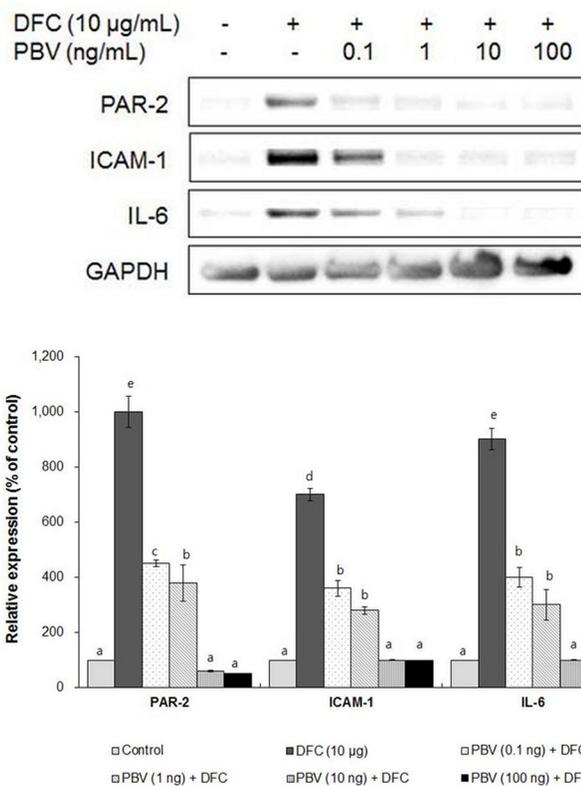sing *D. farinae* extract-stimulated human keratinocytes, we demonstrated that purified BV inhibited the elevated expression of PAR2, ICAM-1, and the proinflammatory cytokine IL-6 at the gene and protein levels. Consequently, this study revealed that purified BV had immunomodulatory activity, which was associated with the regulation of T helper cell differentiation.

The role of PAR2 in the regulation of inflammation has been extensively studied. It has been demonstrated in a recent report that a PAR2 antagonist peptide suppressed the initiation of inflammatory signals and disturbance of tight junction proteins, which were induced by

trypsin, a major serine protease (Han et al., 2011). Another study has reported that the stimulation of PAR2 by its agonist peptide augmented the expression of inflammatory cytokines and chemokines in canine keratinocytes (Maeda et al., 2013). In *Propionibacterium acnes*-stimulated human keratinocytes, a selective PAR2-specific antagonist caused a decrease in the expression of inflammatory cytokines and antimicrobial peptides (Lee et al., 2010). An observation that PAR2-deficient mice exhibited reduced ear swelling because their induced ear edema and infiltration of inflammatory cells were attenuated compared to those in the wild-type counterparts suggested a crucial role for PAR2 in AD (Kawagoe et al., 2002). During skin inflammation, another endogenous serine protease, tryptase from mast cells, activates PAR2, similar to trypsin from keratinocytes. It has been shown that tryptase induced a significant increase in mast cell numbers in the mouse peritoneum, which confirmed the role of tryptase in eliciting mast cell accumulation (Liu et al., 2016). In the same study, a PAR2 antagonist suppressed the tryptase-induced mast cell recruitment, suggesting that the action of tryptase was PAR2-dependent. Furthermore, an anti-ICAM-1 antibody suppressed the tryptase-provoked mast cell accumulation, confirming that tryptase-induced mast cell recruitment was also dependent upon ICAM-1 activity. Tryptase and proinflammatory cytokines such as IL-18 have been reported to correlate well in the plasma of moderate and severe asthmatic patients (Wang et al., 2016). Moreover, tryptase and a PAR2 agonist peptide induced a substantial amount of IL-18 release in mice. Another study has demonstrated that house dust mites induced the tryptase secretion from a human mast cell line by activating PAR2 receptors on the cell surface, suggesting the role of serine protease/PAR2 signaling in allergic inflammation (Wang et al., 2016). Overall, serine protease inhibitors or PAR2 antagonist peptides may be promising therapeutic tools for the management of AD.

We demonstrated that treatment with purified BV significantly lowered the mRNA and protein levels of PAR2, ICAM-1, and IL-6 in house dust mite antigen-stimulated HaCaT keratinocytes. These results suggest that the anti-atopic dermatitis effects of purified BV in keratinocytes might be mediated in part by the inhibition of proinflammatory cytokines secreted upon activation of PAR2 and ICAM-1 signaling. In conclusion, purified BV may have a therapeutic potential in the treatment of AD.

*D. farinae* house dust mites are the most important environmental allergen associated with human AD, which is the most common allergic inflammatory skin disease. The anti-inflammatory effects of purified BV were evaluated for the first time using house dust mite antigen-stimulated HaCaT keratinocytes. The study revealed that purified BV contained compounds corresponding to the commercial standards of apamin, phospholipase A and melittin and was free of dust, honey and pollen. Purified BV significantly lowered the mRNA and protein levels of PAR2, ICAM-1 and IL-6 in house dust mite antigen-stimulated HaCaT keratinocytes. These results suggest that the anti-atopic dermatitis effects of purified BV in keratinocytes might be mediated in part by the inhibition of proinflammatory cytokines secreted upon the activation of PAR2 and ICAM-1 signalling. In conclusion, purified BV may have a therapeutic potential in the treatment of AD.

## ACKNOWLEDGEMENTS

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