Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.112121

Screening and characterization of active herbal extracts and components stimulating human beta defensing (hBD-3) expression from HaCaT cells

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Abstract

Various antimicrobial peptides play important roles as barrier function against bacteria and as innate host immunity. Several major studies have shown reduced expression of human beta defensin (hBD-3) in atopic dermatitis, highlighting the importance of hBD-3. In course of our search for hBD-3 stimulatory herbal souces and biologically active components, we screened 400 oriental herbal extracts using a range of real-time PCRs in the human keratinocyte cell line, HaCaT cells. We found that only six herbal extracts from Rubi Fructus, Lysimachiae Foenumgraeci Herba, Ailanthi Radicis Cortex, Galla Rhois, Peucedani Radix and Albizziae Cortex showed significant hBD-3 stimulatory effects. Although it did not have antimicrobial activity due to the low cytotoxic effect of Ailanthi Radicis Cortex, we further identified hBD-3-stimulating active ingredient in this extract. Efficient activity profiling based on HPLC was performed to identify active components from the extract of Ailanthi Radicis Cortex, and an hBD-3 stimulatory quassinoid component, ailanthone was identified by spectroscopic analysis. The present results show that Ailanthi Radicis Cortex and ailanthone have the potential to stimulate the expression of hBD-3 in HaCaT cells. These findings suggest new possibilities to control bacterial over-colonization found in atopic dermatitis using hBD-3 stimulatory herbal sources and ailanthone.

Keywords: human beta defensin 3; antimicrobial peptide; atopic dermatitis; ailanthi radicis cortex; ailanthone.

Practical Application: Research about atopic dermatitis activities and functional products from Ailanthi Radicis Cortex.

1 Introduction

Human beta defensins (hBD) are small, cationic, and amphiphilic peptides of around 50 to 70 amino acids with a distinctive three sets of disulfide bonds (Nehls et al., 2020). Among them, human beta defensin 3 (hBD-3) has been suggested as a key molecule, with a broad anti-microbial spectrum and physiological working range on salt in human keratinocytes (García et al., 2001; Silva et al., 2021). Depending on the species, beta-defensins are found in epithelial tissues, skin, certain mucosal surfaces such as the respiratory passage and urinogenital tract, and many bodily fluids. These are produced and secreted by phagocytic white blood cells and intestinal Paneth cells and are later processed to form a mature, secreted peptide. The beta-defensins have six conserved cystein residues with specific disulfide connectivity such as Cys 1 – Cys 5, Cys 2 – Cys 4, and Cys 3 – Cys 6 that are distinct from the human alpha-defensins (García et al., 2001; Klüver et al., 2006; Wu et al., 2003). Some human beta defensins such as hBD-1 are constitutively expressed, whereas others (hBD-2 and hBD-3) are induced by microbial products, inflammatory cytokines, or epidermal growth factor (Harder et al., 2001; Liu et al., 2002). Atopic dermatitis (AD) is a chronic inflammatory skin disease that is associated with an abnormal immune balance between type 1 T cells and type 2 T cells and is also associated with skin infection or colonization by several epidermal pathogenic microorganisms such as *Staphylococcus aureus* (Leung & Bieber, 2003; Leung et al., 2004). Studies described two subsets of AD: 70% - 80% have elevated serum IgE and allergic sensitization, whereas 20% - 30% of patients exhibit normal serum IgE levels and lack allergen-specific sensitization (Novak & Bieber, 2003). These latter patients are classified as having non-atopic eczema, non-IgE mediated, or intrinsic AD (IAD). Recent studies have demonstrated that the Th2 cytokines, interleukin (IL)-4 and IL-13, down-regulate anti-microbial peptide (AMP) expression in atopic eczema or extrinsic AD (EAD) skin, and this may account for their propensity toward recurrent skin infections (Nomura et al., 2003; Ong et al., 2002). In contrast, patients with IAD, as compared with EAD, express significantly lower levels of IL-4 and IL-13 in their skin and peripheral blood (Akdis et al., 1999; Jeong et al., 2003; Kägi et al., 1994). Although there are distinguished differences between IAD and EAD, both groups of AD patients suffer from recurrent skin infections, indicating that more complex mechanisms may account for increased skin infection in AD.

Recently, a reduced expression rate of human beta-defensins in the skin of patients with AD was identified as a key biological factor causing the abnormal colonization of AD-related microorganisms such as *S. aureus* (Ong et al., 2002). Although

Received 12 Nov., 2021
Accepted 27 Dec., 2021
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reduced expression of various human beta defensin isoforms have been related to AD, hBD-3 has a significant interest in AD because it plays a major role in the pathology of AD. Also, hBD-3 shows a broad spectrum of anti-microbial activity and has strong activity at physiological concentrations against *S. aureus* singularly, whereas hBD-2 requires the presence of human cathelicidin (LL-37) for its activity against *S.aureus* (García et al., 2001; Maisetta et al., 2005; Midorikawa et al., 2003).

Interestingly, several previous studies have been published describing human beta defensin stimulatory herbal extracts that improve natural skin barrier function under physiological and pathological conditions. Since herbal extracts has been shown to stimulate natural skin antimicrobial peptides and to have beneficial effects for skin disorders with barrier abnormality, these extracts could be used as skin care ingredients. Consequently, various approaches to screen for candidate molecules and herbal sources have been undertaken (Kippenberger et al., 2005; Pernet et al., 2005). However, exact identification and how the herbal extracts exert their benefits on antimicrobial peptides are unclear. One recent study using normal mouse skin showed that topical treatment of apigenin (4′,5,7-trihydroxyflavone) improves epidermal permeability barrier homoeostasis in normal murine skin by divergent mechanisms (Hou et al., 2013). Apigenin, found extensively in many plants, is a natural product belonging to the flavone class that is one of the well-known aglycones of naturally occurring flavonoid glycosides. Another recent study also using murine skin showed that a topical Chinese herbal mixture (CHM) increased mRNA expression of antimicrobial peptides both *in vivo* and *in vitro* (Man et al., 2011). They suggested that the topical CHM could be an alternative therapeutic regimen against inflammatory dermatoses accompanied by epidermal barrier abnormalities.

This current study was conducted to investigate which possible herbal extracts can stimulate the expression of hBD-3 in HaCaT cells and further identifiy possible single components stimulating this expression. In this paper, we present the screening procedure and activity profiling results of an active herbal extract from Ailanthi Radicis Cortex as well as the isolation, structural elucidation, and genetic expression changes of a corresponding active compound ailanthone, in a search for agents stimulating hBD-3 expression in HaCaT cells.

2 Materials and methods

2.1 Reagents

Dulbecco's modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotics and TRIZOL were purchased from Invitrogen–Gibco (Life Technologies, Carlsbad, CA 92008, USA). iScript cDNA synthesis kit was purchased from BIO-RAD (Hercules, CA, USA), and Taqman Master Mix was obtained from Invitrogen–Applied Biosystems (Life Technologies, Part No.: 4369016, USA). Protease inhibitor (complete, Mini) was obtained from Roche (EDTA-free, Roche Diagnostics, Mannheim, Germany). Primary antibody for beta-defensin 3 was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc. Texas, USA) and Goat anti-rabbit IgG conjugated with horseradish peroxidase and Western Lightning Chemiluminescence Regent Plus were obtained from Dako (Dako A/S, Glostrup, Denmark) and Perkin Elmer (Perkin Elmer, Boston, Massachusetts), respectively. Highly pure (more than 98%) ailanthone was purchased from STANFORD Chemicals (Stanford Chemicals Company, CA, USA).

2.2 Preparation of herbal extracts for screening

A total of 400 oriental medicines (data not show) for screening were obtained from the Plant Extract Bank (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The herbal medicines were fully dried for 3 days in dark and dry places, which were ground by a general plant grinder. All the ground herbal drugs were extracted with hot water by the following extraction method. Each material (100 g of dry weight) was extracted in 2 L of distilled water for 3 h (100 °C for 2 h and 80 °C for 1 h). The extract were cooled down to room temperature, and filtered through gauze, then centrifuged at 4 °C, 6,000 rpm, for 15 minutes. The resulting supernatant was frozen at -70 °C for 12 h and then freeze-dried for over 48 h to remove all water content. To evaluate the human beta defensin 3 (hBD3)-stimulating effects, herbal extracts were re-solubilized in water at concentration of 2 mg/mL, then sterilized by filtration with a 0.22 μm syringe filter.

2.3 Cell preparation

HaCaT cells were seeded into 75 T-culture flasks using DMEM supplemented with 10% FBS and antibiotics (100 U/mL Penicillin, 100 μg/mL Streptomycin, 250 ng/mL Amphotericin B). The cells were incubated at 5% CO_2 and 37 °C with the culture media until confluence (% ratio of growth surface to plate surface) was reached (~90% density). In order to screen herbal extracts with hBD-3 stimulatory effects, HaCaT cells were cultured in 6-well plates (1×10^5 cells/well). The prepared extract solution (2 mg/mL) was added to each well at 90 to 95% culture confluence levels with a 10% ratio to culture media (300 μL for 3 mL culture media), and the cells were further incubated for 48 h.

2.4 Western blotting

HaCaT cells were dissolved in 1 mL of 1% Triton X-100 in PBS buffer containing protease inhibitor. 5 μL of 6X sodium dodecyl sulfate (SDS) loading buffer containing 10% 2-mercaptoethanol was added to 25 μL of the supernatants from each sample. Total cell proteins were separated using 15% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was transferred to a nitrocellulose (NC) membrane (0.45 μm) electrically. The membrane was treated with blocking solution [3% non-fat dry milk, 0.5% bovine serum albumin (BSA), 0.87% sodium chloride (NaCl), and 0.3% Tween-20 in Tris buffer saline (TBS)] overnight at 4 °C and then detected with a rabbit anti-hBD-3 polyclonal antibody (1:5000 in blocking solution) for 4 h at °C After three times washing with 0.1% TBS for 15 min, the signal was detected using a goat anti-rabbit IgG conjugated with horseradish peroxidase and developed by using Western Lightning Chemiluminescence Regent Plus.

2.5 Real-time PCR analysis of hBD-3 expression

Total RNA was isolated from cells with TRIZOL according to manufacturer recommendations. RNA was precipitated with ethanol and re-suspended in diethyl pyrocarbonate (DEPC)-treated H_2O. RNA from HaCaT cells was re-purified one more time to obtain high purity and its concentration was determined by spectrophotometer BioSpec-mini (Fisher Scientific). Total RNA obtained was converted to total cDNA only after the integrity of the RNA was confirmed by electrophoresis on an agarose gel. cDNA was synthesized from 200 ng purified RNA using iScript cDNA synthesis kit according to the manufacturer instructions. hBD-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression were analyzed using AB Taqman Master Mix. The primers for human hBD-3 and GAPDH were inventoried primers for Taqman Gene Expression Assays. Amplification of cDNA was performed at 40 cycles (95 °C, 30 s) followed by additional cycles (58 °C, 30 s) in ABI Real-time PCR 7500 (Applied Biosystems). Data was analyzed by real-time PCR system software. The relative expression of cell treated by each extract was calculated by adjusting with real-time PCR of GAPDH expression (GAPDH normalization).

2.6 MTT assay

HaCaT cell viability after treatment with six hBD-3 stimulating herbal extracts (see, Results and discussion) at 0.2 mg/mL was determined by MTT assay [reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is yellow, to a purple formazan product]. A volume of 10 µL of 12 mM MTT was taken for cell incubation performed at 37 °C for 4 hours in the darkness. The media were then decanted and washed with phosphate-buffered saline solution (PBS). The produced formazan salts were dissolved with dimethylsulphoxide (DMSO, Sigma-Aldrich, USA), and the absorbance was measured at 570 nm to estimate the formazan concentration.

2.7 Anti-microbial analysis

T Paper diffusion anti-microbial assay was used to screen herbal extracts for their anti-microbial activity against *S. aureus* and *E. coli*. A suspension (1×10^7 cells/mL) of tested microorganisms was spread on Luria-Bertani media (LB) medium. The filter paper discs (5 mm in diameter) were individually impregnated with different extracts (2 mg/mL, 25 µL), then placed into the agar plates, which had previously been inoculated with the tested microorganisms. The plates were subsequently incubated at 37 °C for 24 h. After incubation, the growth inhibition rings were quantified by measuring the diameter of the zone of inhibition in mm. All tests were performed in triplicate. Ampicillin (0.5 µg/mL, 5 µg/mL, and 50 µg/mL) served as a positive control.

2.8 Solvents-based fractionations of Ailanthi Radicis Cortex extract

A standardized Ailanthi Racicis Cortex was supplied by Hansol Pharm. (Gwangju, Korea) in 2012. As the first stage to investigate hBD-3 stimulatory components from Ailanthi Radicis Cortex, three types of extract were prepared from hot water extract of the dried Ailanthi Radicis Cortex (700 g) by liquid-liquid extraction method. The freeze-dried product (49 g) was suspended in water (1 L), and successively partitioned with the same volume of ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). Both organic layers were concentrated thoroughly *in vacuo*, and the residual water-soluble layer was freeze-dried to afford EtOAc (13.2 g), *n*-BuOH (12.8 g) and aqueous (15.4 g) extracts. Three extracts obtained were dissolved in water (for aqueous extract) and DMSO (for EtOAc and *n*-BuOH extracts) at the concentration of 10 mg/mL. 10 uL of each extract was treated to HaCaT cell culture in 6-well plates, and incubated for 48 h. The changes in hBD-3 mRNA were compared with water or DMSO control groups by means of real-time PCR analysis.

2.9 HPLC-based activity profiling of Ailanthi Radicis Cortex extract

HPLC was performed on an Agilent HP1100 series, comprised of a degasser, a binary mixing pump, a column oven, and a DAD detector, using a YMC-PAC Pro C18 (10 mm × 250 mm, 5 µm) columns in conjunction with a gradient system of MeOH and H_2O containing 0.1% HCOOH. The active extract of *n*-BuOH (2.1 g) was stored at -20 °C. For activity profiling, a portion (177 mg) of the extract was fractionated using a semi-preparative HPLC at regular intervals of 2 min, based on the following condition: initiation with 90% H_2O containing 0.1% HCOOH/10% MeOH, followed by a gradient to 40% H_2O containing 0.1% HCOOH/60% MeOH for 50 min, at a flow rate of 4.0 mL/min and UV detection under 254 nm. A total of 25 fractions were collected and concentrated. All the fractions were tested for hBD-3 stimulatory effects by PCR analysis.

2.10 Isolation of the hBD-3 stimulatory compound ailanthone

To isolate active compounds from the *n*-BuOH extract, an aliquot (1.07 g) of the extract was repeatedly separated under the same HPLC condition, with the above activity profiling, to give an active fraction containing the hBD-3 stimulating compound, ailanthone. This compound was further purified in a gradient solvent system, 80% H_2O containing 0.1% HCOOH/20% MeOH to 70% H_2O containing 0.1% HCOOH/30% MeOH for 70 min (t_R: 24.70 min, 2.2 mg).

2.11 Spectroscopic analysis of ailanthone

H, ^{13}C, and 2D NMR experiments were recorded using either a Varian VNMRS 600 MHz NMR spectrometer. NMR data was observed in MeOH-d_4 (in ppm, *J* in Hz). ^{13}C NMR data was deduced by the 2D NMR experiments including COSY, HSQC and HMBC. High resolution mass (ESI-MS) and LC-MS/MS spectra were measured on a Waters Synapt HDMS TOF mass spectrometer operating in negative electrospray ion mode.

Ailanthone ^1H NMR (MeOH-d_4): 6.06 (1H, br s, H-3), 5.22 (2H, s, H-21), 4.62 (1H, t, 2.4, H-7), 4.26 (1H, s, H-1), 3.99 (1H, d, 8.1, H-20α), 3.89 (1H, s, H-12), 3.49 (1H, d, 8.1, H-20β), 3.11 (1H, dd, 13.8, 18.6, H-15β), 2.95 (1H, s, H-9), 2.91 (1H, br d, 12.3, H-5), 2.84 (1H, dd, 13.8, 5.4, H-14), 2.65 (1H, dd, 5.4, 18.6, H-15α), 2.27 (1H, br d, 15.0, H-6α), 2.14 (1H, ddd, 15.0, 12.3, 2.4, H-6β), 2.02 (3H, s, H-18), 1.20 (3H, s, H-19);

^{13}C NMR (MeOH-d_4): 197.6 (C-2), 170.6 (C-16), 163.5 (C-4), 145.1 C-13), 124.7 (C-3), 118.9 (C-21), 108.6 (C-11), 82.8 (C-1), 79.4 (C-12), 78.4 (C-7), 71.6 (C-20), 47.0 (C-8), 46.5 (C-14), 44.4 (C-10), 43.8 (C-9), 41.8 (C-5), 33.9 C-15), 25.3 (C-6), 21.2 (C-18), 8.3 (C-19); HRESIMS: *m*/*z* 375.2 $[M–H]^-$ (calcd for $C_{20}H_{24}O_7$ 376.4).

3 Results and discussion

This study aimed to screen herbal drugs stimulating human beta defensin 3 (hBD-3) expressions *in vitro*, and to further identify active compounds responsible for these effects. A total of 400 herbal extracts from traditional medicines were screened for the ability to increase hBD-3 mRNA expression, as shown (data not show) (Supplementary Material). Of the tested herbal extracts, only six extracts showed potent stimulating effects on human beta defensin 3 (hBD-3) expression in HaCaT cells, in relation to GAPDH normalization by real-time PCR analysis. Basal expression without the treatment of extracts was compared as a control. The remaining 394 herbal extracts showed no effects on hBD-3 expression in HaCaT cells. The summarized results and statistical values of the six herbal extracts stimulating hBD-3 expression are listed in Table 1. These herbal extracts were tested in triplicate and the statistical mean values calculated. Stimulation of hBD-3 gene expression with 573 ± 48% and 693 ± 182% were shown in both extracts of Rubi Fructus, which grow in China (No. 136) and in Korea (No. 137), respectively. HaCaT cells treated with herbal extracts from Lysimachiae Foenumgraeci Herba (No. 171), Ailanthi Radicis Cortex (No. 194), Galla Rhois (No. 232), Peucedani Radix (No. 246), and Albizziae Cortex (No. 362) exhibited significantly elevated stimulatory effects on hBD-3 expression.

To confirm the hBD-3 stimulatory effects of these six identified herbal extracts at the protein level, hBD-3 expression was analyzed by Western blotting. The hBD-3 expression from HaCaT cells treated with Ailanthi Radicis Cortex and Galla Rhois extracts showed the most significantly increases; up to 783% and 816%, respectively. The increase (%) of hBD-3 expression from HaCaT cells treated with Rubi Fructus (China), Rubi Fructus (Korea), Lysimachiae foenumgraeci Herba, Peucedani Radix, and Albizziae Cortex were 216%, 143%, 189%, 426%, and 567%, respectively. The elevated expressions of hBD-3 were well matched with the mRNA expression evaluated by real-time PCR analysis (Figure 1A).

To choose an extract with high hBD-3 stimulatory effect as well as high safety for potential skin treatment, *in vitro* cell toxicity for HaCaT cells was measured using the MTT assay. When compared with the untreated control, all tested herbal extracts, except Ailanthi Radicis Cortex, showed cell toxicity at the tested concentration of 0.2 mg/mL. Interestingly, Ailanthi Radicis Cortex increased cell viability up to 141 ± 21% (Figure 1B). In consideration of the hBD-3 expression assay and *in vitro* cell toxicity assay, we chose Ailanthi Radicis Cortex for further studies to identify and structurally characterize possible hBD-3 stimulatory components, using real-time PCR.

Traditionally, Ailanthi Radicis Cortex (stem and root bark of *Ailanthus altissima* or *A. glandulosa* belonging to Simaroubaceae) has been used to treat cold, gastric diseases, and cancer. In relation to typical traditional uses, there are also several previous reports showing various other biological effects of Ailanthi Radicis Cortex, such as herbicide activity, antiplasmodial activity, anti-viral activity, and anti-tumor activity (Kato et al., 1988; Kubota et al., 1997; Okunade et al., 2003; Pedersini et al., 2011). It was known that Ailanthi Radicis Cortex contains quassinoids, indole, sterol and β-carboline alkaloids, and these compounds have various reported biological effects in Ailanthi Radicis Cortex. Considering previous scientific results suggesting the toxic effect of Ailanthi Radicis Cortex, we further checked the direct anti-microbial activities of six active herbal extracts against *S. aureus* and *E. coli* to characterize a possible relationship between antimicrobial peptide, hBD-3, stimulatory effect and the direct anti-microbial effect. However, only Galla Rhois displayed direct anti-microbial effects against *S. aureus* and *E. coli.* Other tested herbal extracts, including Ailanthi Radicis Cortex did not show antimicrobial activity against *S. aureus* or *E. coli* (Figure 2).

Isolation and structure elucidation were carried out to identify hBD-3 active components in Ailanthi Rdadicis Cortex. As a first step of separation, we partitioned successively the water-suspended Ailanthi Radicis Cortex extract with two immiscible solvents, EtOAc and *n*-BuOH. Stimulatory effects on hBD-3 expression were studied by real-time PCR from HaCaT cells, as described in the methods section above, and the relative expression changes produced by EtOAc and *n*-BuOH fractions were measured. The highest changes were up to 30-fold from the *n*-BuOH fraction (data not shown), in comparison with those of the crude water extract.

Table 1. Herbal extracts showing stimulatory effects on hBD-3 expression.

No.	Herbal drugs	Stimulatory activity (%)			
		1st	2nd	3rd	Mean ± SD
136[a]	Rubi Fructus	524	575	619	573 ± 48
137[a]	Rubi Fructus	900	559	619	693 ± 182
171	Lysimachiae Foenumgraeci Herba	831	702	1082	872 ± 193
194	Ailanthi Radicis Cortex	868	1042	1420	1110 ± 282
232	Galla Rhois	1744	1203	1502	1483 ± 271
246	Peucedani Radix	2912	1859	1903	2225 ± 596
362	Albizziae Cortex	853	933	792	859 ± 71

[a]Herbal drug No. 136 and 137 originated from the same species which grown in China and in Korea, respectively. All others were grown in Korea.

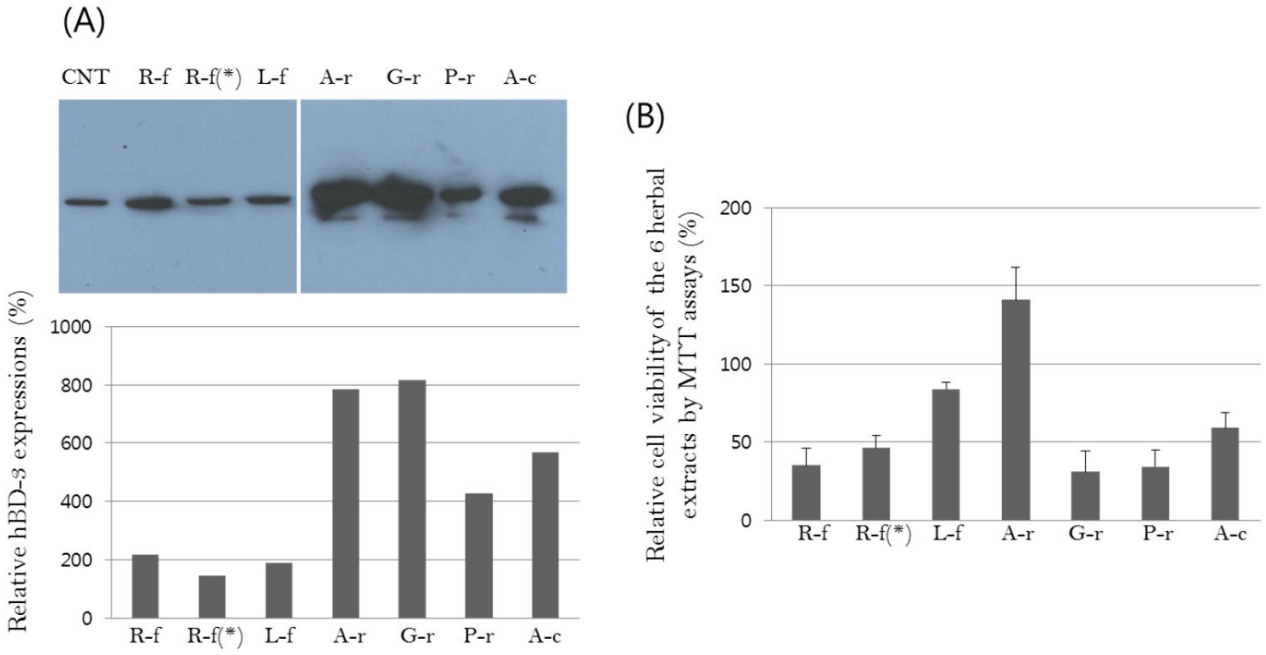

Figure 1. Western blotting of hBD-3expression (A) and *in vitro* cell toxicity (B) by six herbal extracts. (A) Six identified herbal extracts with hBD-3 stimulatory effects were tested for stimulatory effects at the protein level by Western blotting. R-f (Rubi Fructus from China), R-f(*) (Rubi Fructus from Korea), L-f (Lysimachiae Foenumgraeci Herba), A-r (Ailanthi Radicis Cortex), G-r (Galla Rhois), P-r (Peucedani Radix), and A-c (Albizziae Cortex) extracts showed hBD-3 stimulatory effects from HaCaT cells up to 783%, 816%, 216%, 143%, 189%, 426%, and 567%, respectively at the protein level. (B) Among the tested six hBD-3 stimulatory herbal extracts, only A-r showed no cell toxicity, as measured by the MTT assay.

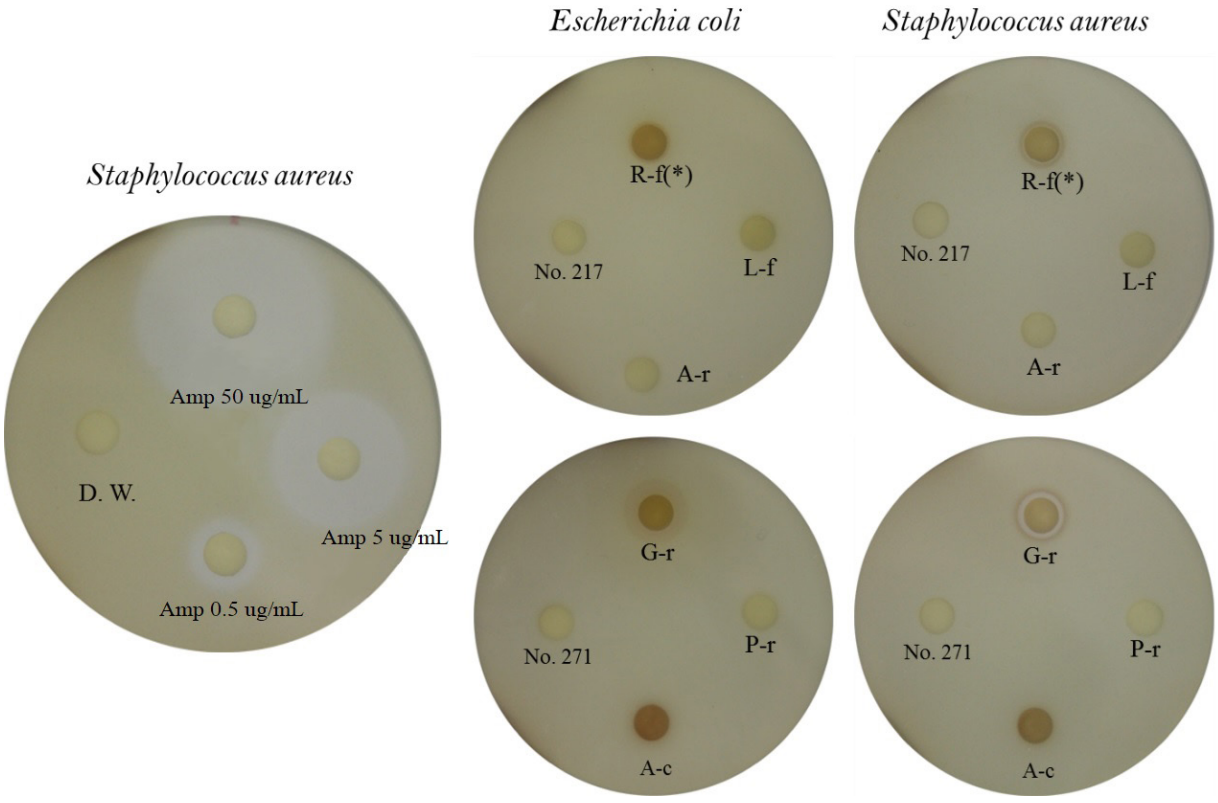

Figure 2. Anti-microbial effects of the six identified hBD-3 stimulatory herbal extracts using a paper diffusion assay. The paper diffusion assay was conducted to find out direct anti-microbial effects against *S. coccus* and *E. coli* from the six identified hBD-3 stimulatory herbal extracts. Only G-r (Galla Rhois) showed minor anti-microbial activity against *S. coccus,* but not against *E. coli*. The other extracts R-f (Rubi Fructus from China), R-f(*) (Rubi Fructus from Korea), L-f (Lysimachiae Foenumgraeci Herba), A-r (Ailanthi Radicis Cortex), P-r (Peucedani Radix), A-c (Albizziae Cortex), and No. 271 (Meliae Fructus) did not show anti-microbial activity.

For HPLC activity profiling of subfractions, a portion (177 mg) from *n*-BuOH extract was microfractionated using a semi-preparative HPLC at regular intervals, using a gradient eluent system with 90% H_2O containing 0.1% HCOOH and 10% MeOH, a total of 25 fractions was collected. As shown in the Figure 3, major LC peaks appeared at fraction number 4, 5, and 11. Each of the 25 fractions was concentrated, and dissolved in DMSO at the concentration of 10 mg/mL. 10 uL of each fraction was added to cell culture media at the amount of 100 μg per each well. After incubation for 48 h with tested subfractions, the changes in hBD-3 mRNA expression were compared with water or DMSO control samples (Figure 4). When compared with relative expression changes between GAPDH and hBD-3, fraction number 11 dramatically elevated the expression of hBD-3. A major compound in fraction 11 was purified by HPLC separation to afford 2.2 mg of ailanthone.

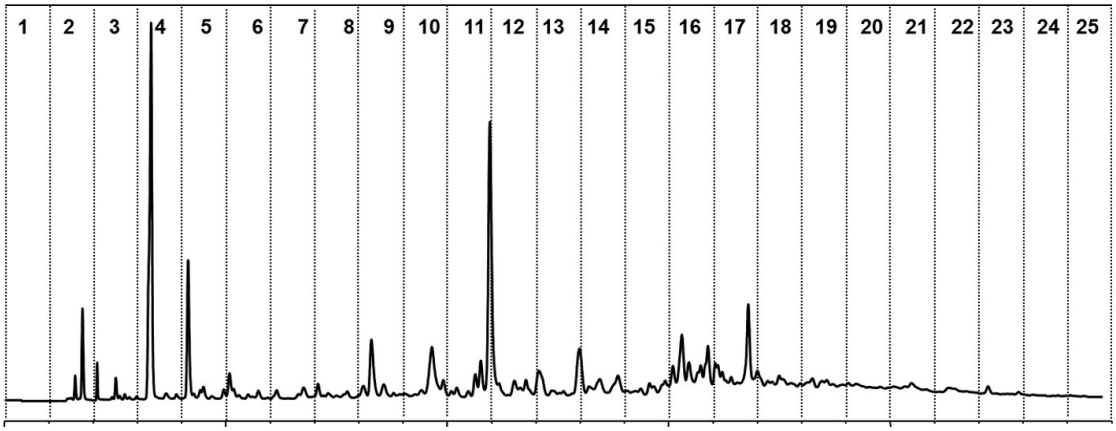

Figure 3. Chromatogram and subfraction profiles obtained by gradient HPLC from the *n*-BuOH extract of Ailanthi radicis Cortex. The extract was fractionated using a HPLC (Agilent 1100 Series, USA) at regular intervals of 2 min, based on the following condition: initiation with 90% H_2O containing 0.1% HCOOH/10% MeOH, followed by a gradient to 40% H_2O containing 0.1% HCOOH/60% MeOH for 50 min, at a flow rate of 4.0 mL/min and a UV detection under 254 nm. A total of 25 fractions were collected, and three fractions containing major peaks were shown (fraction No. 4, No., 5, and No. 11).

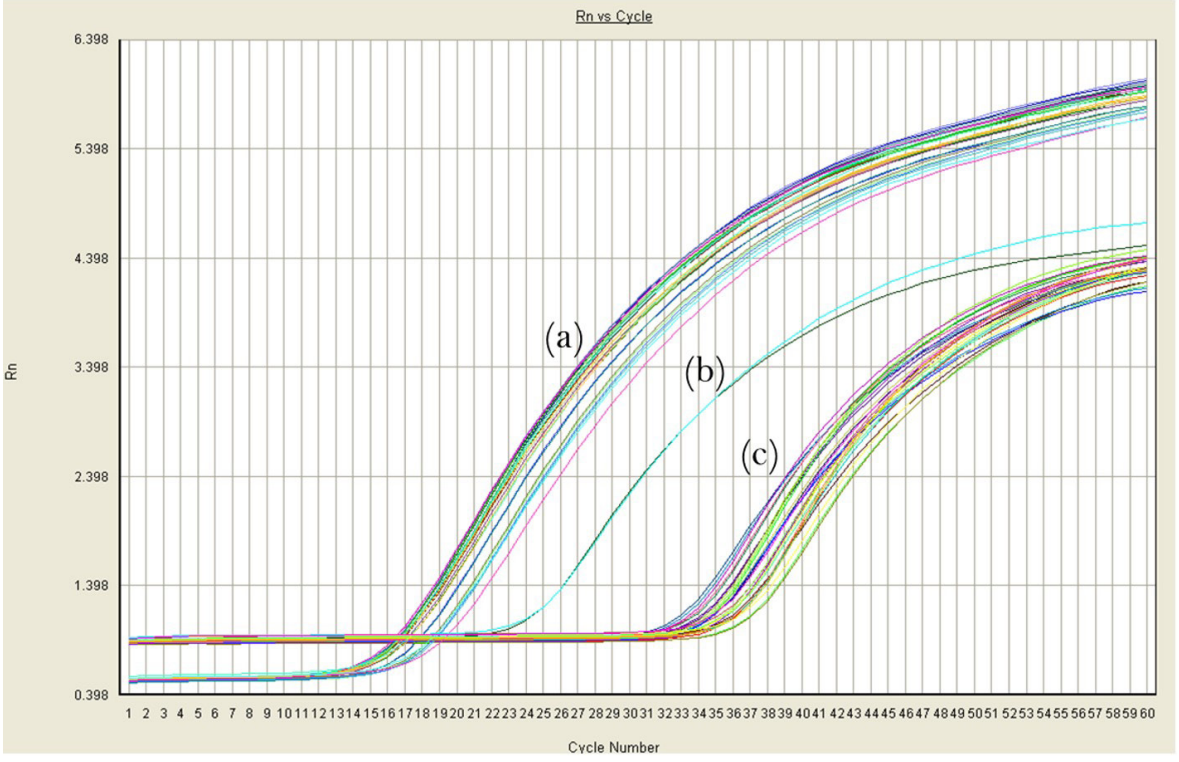

Figure 4. Real-time PCR analysis of hBD-3 expression in HaCaT cells following treatment with 25 subfractions from the *n*-BuOH extract of Ailanthi Radicis Cortex. (a) GAPDH expression was used as an internal control, (b) hBD-3 expression in HaCaT cells treated with subfraction 11, (c) hBD-3 expression in HaCaT cells treated with the other fractions.

The unambiguous structure of ailanthone was elucidated by the comparison of their NMR and MS data with the corresponding data in the literature (Carter et al., 1993; Wang et al., 2013). The ^{1}H NMR spectra showed two methyl proton signals of H-18 and H-19 (each 3H, s) at δ 1.20 and 2.02, and also exhibited an olefinic proton H-3 (1H, br s) at δ 6.06 and an exomethylene proton H-21 (2H, s) signals. Three oxygenated methine proton signals, H-1, H-7 and H-12 was respectively observed at δ 4.26 (1H, s), 4.62 (1H, t, 2.4) and δ 3.89 (1H, s). In addition, three methine proton signals at δ 2.91 (H-5), 2.95 (H-9), and δ 2.84 (H-14) were shown in the spectrum together with three methylene proton signals at δ 2.27, 2.14 (H-6α and H-6β), 2.65, 3.11 (H-15α and H-15β), and δ 3.99, 3.49 (H-20α and H-20β). The γ-lactone and α,β-unsaturated carbonyl signals were detected at δ 170.6 (H-16) and 197.6 (H-2), respectively. Its HMBC spectrum revealed that an isolated methyl of H-19 was correlated with three carbonyl carbons of C-1, C-5, and C-10, and the methine proton signal H-14 showed long-range correlation with carbon signals of C-10, C-12, C-13, C-15, and C-21. Based on NMR structure analysis, the hBD-3 -stimulating compound from Ailanthi Radicis Cortex was identified as ailanthone (Figure 5).

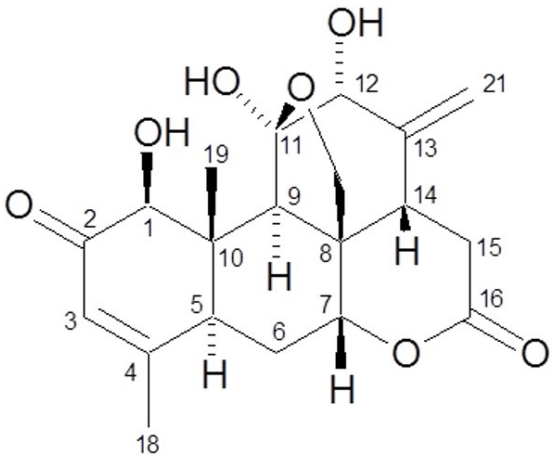

Figure 5. Chemical structure of hBD-3-stimulating quassinoid, ailanthone identified from Ailanthi Radicis Cortex.

To confirm the hBD-3 stimulatory activity of ailanthone identified from Ailanthi Radicis Cortex, we conducted further stimulatory assays with the single pure compound, ailanthone (more than 98% purity). As shown in Figure 6, hBD-3 expression

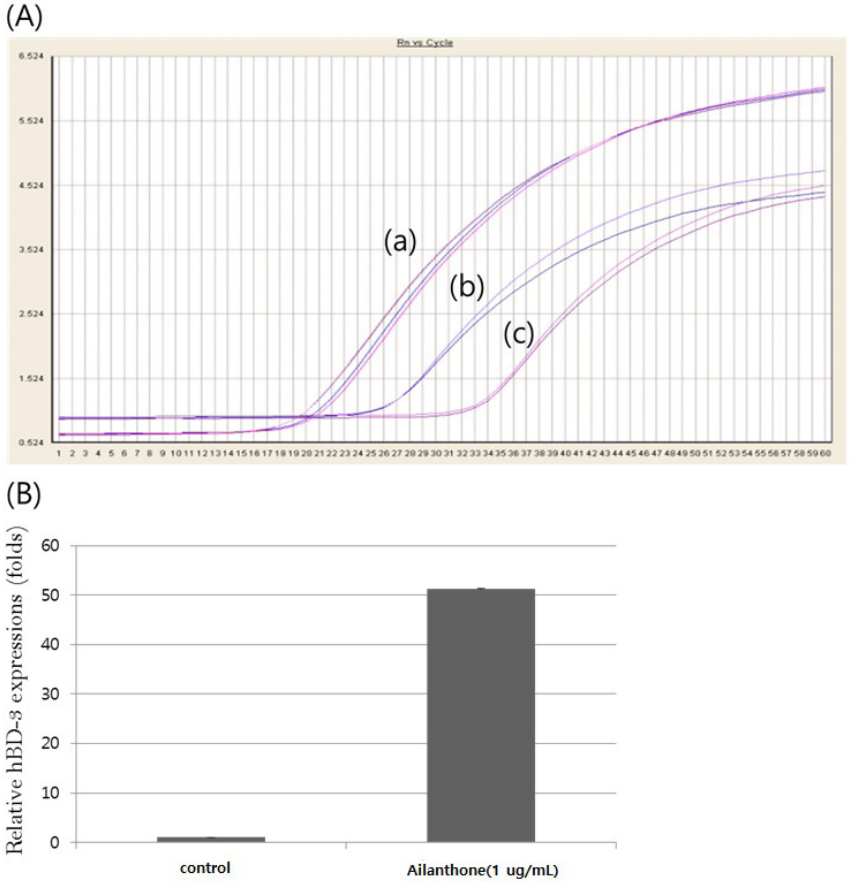

Figure 6. Real-time PCR profiles (A) and summarized graph (B) of hBD-3 expression from HaCaT cells following treatment with highly pure (>98%) single ailanthone. (a) GAPDH expression was used as an internal control, (b) hBD-3 expression in HaCaT cells treated with highly pure (>98%) single ailanthone, (c) hBD-3 expression in untreated HaCaT cells.

from HaCaT cells were dramatically increased up to about 50-fold after treatment with purified ailanthone at 1 μg/mL for 2 days.

4 Conclusions

Together with these works, our present study shows for the first time that Ailanthi Radicis Cortex and its active single component ailanthone possessed hBD-3 stimulatory effect in HaCaT cells. To understand cellular processes that produce increased hBD-3 expression, further studies will focus on the signaling and transcriptional regulation of hBD-3 by the Ailanthi Radicis Cortex extract and ailanthone (Takahashi et al., 2021).

Conflict of interest

The authors declare that they have no conflict of interest.

References

Akdis, C. A., Akdis, M., Simon, D., Dibbert, B., Weber, M., Gratzl, S., Kreyden, O., Disch, R., Wüthrich, B., Blaser, K., & Simon, H. U. (1999). T cells and T cell-derived cytokines as pathogenic factors in the nonallergic form of atopic dermatitis. *The Journal of Investigative Dermatology*, 113(4), 628-634. http://dx.doi.org/10.1046/j.1523-1747.1999.00720.x. PMid:10504452.

Carter, C. G., Tinto, W. F., Reynolds, W. F., & Mclean, S. (1993). Quassinoids from *Quassia multiflora*: structural assignment by 2D nmr spectroscopy. *Journal of Natural Products*, 56(1), 130-133. http://dx.doi.org/10.1021/np50091a019.

García, J. R., Jaumann, F., Schulz, S., Krause, A., Rodríguez-Jiménez, J., Forssmann, U., Adermann, K., Klüver, E., Vogelmeier, C., Becker, D., Hedrich, R., Forssmann, W. G., & Bals, R. (2001). Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. *Cell and Tissue Research*, 306(2), 257-264. http://dx.doi.org/10.1007/s004410100433. PMid:11702237.

Harder, J., Bartels, J., Christophers, E., & Schroder, J. M. (2001). Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *The Journal of Biological Chemistry*, 276(8), 5707-5713. http://dx.doi.org/10.1074/jbc.M008557200. PMid:11085990.

Hou, M., Sun, R., Hupe, M., Kim, P. L., Park, K., Crumrine, D., Lin, T. K., Santiago, J. L., Mauro, T. M., Elias, P. M., & Man, M. Q. (2013). Topical apigenin improves epidermal permeability barrier homoeostasis in normal murine skin by divergent mechanisms. *Experimental Dermatology*, 22(3), 210-215. http://dx.doi.org/10.1111/exd.12102. PMid:23489424.

Jeong, C. W., Ahn, K. S., Rho, N. K., Park, Y. D., Lee, D. Y., Lee, J. H., Lee, E. S., & Yang, J. M. (2003). Differential in vivo cytokine mRNA expression in lesional skin of intrinsic vs. extrinsic atopic dermatitis patients using semiquantitative RT-PCR. *Clinical and Experimental Allergy*, 33(12), 1717-1724. http://dx.doi.org/10.1111/j.1365-2222.2003.01782.x. PMid:14656360.

Kägi, M. K., Wuthrich, B., Montana, E., Barandun, J., Blaser, K., & Walker, C. (1994). Differential cytokine profiles in peripheral blood lymphocyte supernatants and skin biopsies from patients with different forms of atopic dermatitis, psoriasis and normal individuals. *International Archives of Allergy and Immunology*, 103(4), 332-340. http://dx.doi.org/10.1159/000236651. PMid:8130648.

Kato, T., Suzumura, Y., Fukushima, M., Honda, T., Nakanishi, T., & Noguchi, T. (1988). Antitumor activity of novel ailanthone derivatives in vitro and in vivo. *Anticancer Research*, 8(4), 573-579. PMid:3178149.

Kippenberger, S., Loitsch, S., Thaci, D., Kaufmann, R., & Bernd, A. (2005). A detection of human beta defensin-1 and -2 by RT-competitive multiplex PCR. *Archives of Dermatological Research*, 296(11), 539-542. http://dx.doi.org/10.1007/s00403-005-0553-0. PMid:15821924.

Klüver, E., Adermann, K., & Schulz, A. (2006). Synthesis and structure-activity relationship of beta-defensins, multi-functional peptides of the immune system. *Journal of Peptide Science*, 12(4), 243-257. http://dx.doi.org/10.1002/psc.749. PMid:16491514.

Kubota, K., Fukamiya, N., Tokuda, H., Nishino, H., Tagahara, K., Lee, K. H., & Okano, M. (1997). Quassinoids as inhibitors of Epstein-Barr virus early antigen activation. *Cancer Letters*, 113(1-2), 165-168. http://dx.doi.org/10.1016/S0304-3835(97)04607-7. PMid:9065817.

Leung, D. Y., & Bieber, T. (2003). Atopic dermatitis. *Lancet*, 361(9352), 151-160. http://dx.doi.org/10.1016/S0140-6736(03)12193-9. PMid:12531593.

Leung, D. Y., Boguniewicz, M., Howell, M. D., Nomura, I., & Hamid, Q. A. (2004). New insights into atopic dermatitis. *The Journal of Clinical Investigation*, 113(5), 651-657. http://dx.doi.org/10.1172/JCI21060. PMid:14991059.

Liu, A. Y., Destoumieux, D., Wong, A. V., Park, C. H., Valore, E. V., Liu, L., & Ganz, T. (2002). Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *The Journal of Investigative Dermatology*, 118(2), 275-281. http://dx.doi.org/10.1046/j.0022-202x.2001.01651.x. PMid:11841544.

Maisetta, G., Batoni, G., Esin, S., Raco, G., Bottai, D., Favilli, F., Florio, W., & Campa, M. (2005). Susceptibility of Streptococcus mutans and Actinobacillus actinomycetemcomitans to bactericidal activity of human beta-defensin 3 in biological fluids. *Antimicrobial Agents and Chemotherapy*, 49(3), 1245-1248. http://dx.doi.org/10.1128/AAC.49.3.1245-1248.2005. PMid:15728941.

Man, M., Hupe, M., Mackenzie, D., Kim, H., Oda, Y., Crumrine, D., Lee, S. H., Martin-Ezquerra, G., Trullas, C., Mauro, T. M., Feingold, K. R., Elias, P. M., & Man, M. Q. (2011). A topical Chinese herbal mixture improves epidermal permeability barrier function in normal murine skin. *Experimental Dermatology*, 20(3), 285-288. http://dx.doi.org/10.1111/j.1600-0625.2010.01205.x. PMid:21323748.

Midorikawa, K., Ouhara, K., Komatsuzawa, H., Kawai, T., Yamada, S., Fujiwara, T., Yamazaki, K., Sayama, K., Taubman, M. A., Kurihara, H., Hashimoto, K., & Sugai, M. (2003). Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. *Infection and Immunity*, 71(7), 3730-3739. http://dx.doi.org/10.1128/IAI.71.7.3730-3739.2003. PMid:12819054.

Nehls, C., Böhling, A., Podschun, R., Schubert, S., Grötzinger, J., Schromm, A., Fedders, H., Leippe, M., Harder, J., Kaconis, Y., Gronow, S., & Gutsmann, T. (2020). Influence of disulfide bonds in human beta defensin-3 on its strain specific activity against Gram-negative bacteria. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1862(8), 183273. http://dx.doi.org/10.1016/j.bbamem.2020.183273. PMid:32171739.

Nomura, I., Goleva, E., Howell, M. D., Hamid, Q. A., Ong, P. Y., Hall, C. F., Darst, M. A., Gao, B., Boguniewicz, M., Travers, J. B., & Leung, D. Y. (2003). Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *Journal of Immunology*, 171(6), 3262-3269. http://dx.doi.org/10.4049/jimmunol.171.6.3262. PMid:12960356.

Novak, N., & Bieber, T. (2003). Allergic and nonallergic forms of atopic diseases. *The Journal of Allergy and Clinical Immunology*, 112(2), 252-262. http://dx.doi.org/10.1067/mai.2003.1595. PMid:12897728.

Okunade, A. L., Bikoff, R. E., Casper, S. J., Oksman, A., Goldberg, D. E., & Lewis, W. H. (2003). Antiplasmodial activity of extracts and quassinoids isolated from seedlings of Ailanthus altissima (Simaroubaceae). *Phytotherapy Research*, 17(6), 675-677. http://dx.doi.org/10.1002/ptr.1336. PMid:12820239.

Ong, P. Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R. L., & Leung, D. Y. (2002). Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *The New England Journal of Medicine*, 347(15), 1151-1160. http://dx.doi.org/10.1056/NEJMoa021481. PMid:12374875.

Pedersini, C., Bergamin, M., Aroulmoji, V., Baldini, S., Picchio, R., Pesce, P. G., Ballarin, L., & Murano, E. (2011). Herbicide activity of extracts from Ailanthus altissima (Simaroubaceae). *Natural Product Communications*, 6(5), 593-596. http://dx.doi.org/10.1177/1934578X1100600504. PMid:21615014.

Pernet, I., Reymermier, C., Guezennec, A., Viac, J., Guesnet, J., & Perrier, E. (2005). An optimized method for intensive screening of molecules that stimulate beta-defensin 2 or 3 (hBD2 or hBD3) expression in cultured normal human keratinocytes. *International Journal of Cosmetic Science*, 27(3), 161-170. http://dx.doi.org/10.1111/j.1467-2494.2005.00262.x. PMid:18492183.

Silva, A. R. N., Perera, H. E. H., Maddumage, R. S., Sandarenu, K. D. S., Fernando, R. S. P., Sandharenu, K. L. K. T. D., & Dulshika, G. D. S. (2021). Enhancement of phagocytosis of macrophages in the presence of human beta defensin 2. *International Journal of Innovative Science and Research Technology*, 6(2), 930-936.

Takahashi, M., Umehara, Y., Yue, H., Trujillo-Paez, J. V., Peng, G., Niyonsaba, H. L. T., Ikutama, R., Okumura, K., Ogawa, H., Ikeda, S., & Niyonsaba, F. (2021). The antimicrobial peptide human b-defensin-3 accelerates wound healing by promoting angiogenesis, cell migration, and proliferation through the FGFR/JAK2/STAT3 signaling pathwaY. *Frontiers in Immunology*, 12, 712781. http://dx.doi.org/10.3389/fimmu.2021.712781. PMid:34594328.

Wang, Y., Wang, W. J., Su, S., Zhang, D. M., Xu, L. P., He, R. R., Wang, L., Zhang, J., Zhang, X. Q., & Ye, W. C. (2013). Cytotoxic quassinoids from *Ailanthus altissima*. *Bioorganic & Medicinal Chemistry Letters*, 23(3), 654-657. http://dx.doi.org/10.1016/j.bmcl.2012.11.116. PMid:23290052.

Wu, Z., Hoover, D. M., Yang, D., Boulègue, C., Santamaria, F., Oppenheim, J. J., Lubkowski, J., & Lu, W. (2003). Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. *Proceedings of the National Academy of Sciences of the United States of America*, 100(15), 8880-8885. http://dx.doi.org/10.1073/pnas.1533186100. PMid:12840147.

Supplementary Material

Supplementary material accompanies this paper.

Supplement 1-1 400 oriental medicines for possible hBD3 stimulatory effect

Supplement 1-2 400 oriental medicines for possible hBD3 stimulatory effect (continued)

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