

Effects of topical application of a tri-herb formula on inflammatory dry-skin condition in mice with oxazolone-induced atopic dermatitis

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ABSTRACT

Background: Atopic dermatitis (AD) is a chronic relapsing inflammatory and pruritic skin disease, affecting 10–20% of the population worldwide. *Paeonia suffruticosa* Andrews (Paeoniaceae) (Cortex Moutan) and *Mentha haplocalyx* Briq. (Labiatae) (Herba Menthae) have shown beneficial effects on AD. *Calendula officinalis* L. (Asteraceae) is commonly used for treating skin rashes and wounds.

Purpose: In the present study, a three-herbs formula including Cortex Moutan and Herba Menthae, together with *C. officinalis* at 1:1:1 weight ratio was used as a topical agent and its therapeutic effects on AD was investigated.

Methods: *In vitro* effects of individual herbs and three-herbs formula (0.125–1 mg/ml) were examined using cytokine release assay on human mast HMC-1 cells, inflammation test on murine macrophage RAW cells and human keratinocyte (HaCaT) cells, and migration scratch assay on human umbilical vein endothelial cells (HUVEC). The contributing functional pathway of three-herbs formula in AD was explored using Western Blot assay in HMC-1 cells. Oxazolone-induced AD-like mice model was also used to investigate the *in vivo* therapeutic effect of the topical application of the three-herbs formula.

Results: Herba Menthae, Cortex Moutan, and three-herbs formula significantly reduced the production of IL-6 and tumor necrosis factor (TNF)- α in HMC-1 cells, inhibited the expression of IL-6, IL-8 and CCL2 in TNF- α /IFN- γ stimulated HaCaT cells, and suppressed the lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells. Moreover, Herba Menthae and three-herbs formula significantly suppressed CCL2 and TNF- α production in LPS-induced RAW 264.7 cells. *C. officinalis* and three-herbs formula promoted wound healing in HUVEC. For intracellular mechanisms, three-herbs formula inhibited the expressions of molecules in STAT1 and STAT3-dependent pathways. *In vivo* model showed that topical application of three-herbs formula on challenged ear reduced ear swelling and mice scratching frequencies. H&E and toluidine blue staining of the challenged ear tissue demonstrated that three-herbs formula reduced the epidermal thickness and mast cell infiltration, respectively.

Conclusion: The three-herbs formula of Cortex Moutan, Herba Menthae and *C. officinalis* at 1:1:1 (w/w) exhibited anti-inflammatory effect and promotion of cell migration *in vitro*. It also alleviated ear redness, swelling,

Abbreviations: AD, atopic dermatitis; AQP3, aquaporins 3; FBS, fetal bovine serum; HaCaT, human keratinocyte cells; HDF, human dermal fibroblasts; HMC-1, human mast cell line-1; HUVEC, human umbilical vein endothelial cells; KC, keratinocytes chemoattractants; LCMS, liquid chromatography mass spectrometry; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein 2; MRSA, Methicillin resistant *Staphylococcus aureus*; NO, nitric oxide; OXA, oxazolone; PBMC, peripheral blood mononuclear cells; PHF, Pentaherbs formula; PMA, phorbol-12 myristate 13-acetate; PS, penicillin-streptomycin; SA, *Staphylococcus aureus*; TNF, tumor necrosis factor; wt, weight; 3H, three-herbs formula/tri-herb formula.

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epidermal thickness and inflammation of the OXA-induced AD mice. These findings suggest a potential beneficial role of the topical application of the three-herbs formula for treatment of AD.

Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a chronic relapsing inflammatory and pruritic skin disease. It affects 10–20% of the population worldwide and results in itchy, red, dry and cracked skin (Kamer et al., 2013). AD usually starts during infancy or early childhood and is the most common skin disease in children, affecting up to 10% of adults in high-income countries (Langan et al., 2020). Furthermore, AD can occasionally cause significant morbidity, as its signs and symptoms can significantly diminish the quality of life of affected children and families by disrupting family life and causing sleep deprivation (Watson and Kapur, 2011). However, the causes of AD are still poorly understood, thereby causing clinicians a great challenge for its management. It has been shown that the skin barrier function was reduced in patients with AD, and inflammation and trauma from scratching will lead to further barrier dysfunction (McPherson, 2016). Presently, the available therapeutic approaches of AD often focus on symptomatic relief and control rather than cure, and the long-term treatment for AD are confined to reducing the clinical manifestations, minimizing relapses, and improving life quality. Current approaches for alleviating AD symptoms, include hydration, avoidance of typical AD triggers, specific behavioral approaches to reduce scratching, and antibacterial measures (Guttman-Yassky et al., 2017). Conventional medication for AD involves the use of topical or systematic corticosteroids which may cause serious adverse effects including striae or hypertension for AD children (Wang et al., 2016). Therefore, the development of a safe and non-steroid immunomodulatory herbal formula for the treatment of AD has attracted much attention (Hon et al., 2011).

In our previous randomized, double-blinded and placebo-controlled study, we have demonstrated that the oral application of Pentaherbs formula (PHF), a traditional Chinese herb formula containing Cortex Moutan, root bark of *Paeonia suffruticosa* Andr. (Paeoniaceae), Cortex Phellodendri, bark of *Phellodendron chinensis* Schneid. (Rutaceae), Flos Lonicerae, flower of *Lonicera japonica* Thunb. (Caprifoliaceae), Herba Menthae, aerial part of *Mentha haplocalyx* Briq. (Labiatae) and Rhizoma Atractylodis, rhizome of *Atractylodes lancea* (Thunb.) DC. (Compositae) in the ratio of 2:2:2:1:2 (w/w) could not only reduce the use of topical corticosteroids but also improve the quality of life in children with moderate-to-severe AD (Leung et al., 2008). Moreover, *in vitro* mechanistic study suggested that PHF could suppress the release of inflammatory mediators from mast cells, in which Cortex Moutan and Herba Menthae were the two most effective herbs (Chan et al., 2008). Clinically, patients with AD tend to scratch the AD affected areas repeatedly, leading to bacterial infections and open wounds (Hong et al., 2011). Previous studies have demonstrated that *Calendula officinalis* L. (Asteraceae) exerted excellent antifungal and antimicrobial activities over a broad spectrum of gram-positive and negative bacteria (Efstratiou et al., 2012). Also, *in vitro* and *in vivo* studies suggested that *C. officinalis* possessed angiogenic and fibroblastic properties acting positively on the proliferative phases of the healing process (Parente et al., 2012). Therefore, we have prepared a three-herbs formula containing Cortex Moutan and Herba Menthae, along with *C. officinalis* in the weight ratio of 1:1:1 for the treatment of AD. Due to the fact that orally administered agents for AD might have increased risk of toxicity and require monitoring (Frantz et al., 2019), the tri-herb formula has been used topically in this study.

The present study aimed to investigate the effects of three-herbs formula topically applied on oxazolone-induced AD mice, as well as *in vitro* cell experiments on the medication. *In vitro* experiments were used to assess anti-cytokine production, anti-inflammation, and promotion of cell migration. Specifically, Cortex Moutan and Herba Menthae were

tested in both the anti-cytokine production and anti-inflammation assay for their potential immunosuppressive and anti-inflammatory effects; *C. officinalis* was tested in scratch wound healing assay for its promotion of wound healing effects. The effects of three-herbs formula were tested in all assays, also mechanistic study was performed using Western blot assay, and skin toxicity of the three-herbs formula investigated.

Material and methods

Herbal material authentication and extraction

Herba Menthae and Cortex Moutan were purchased from ZiXin Company, a renowned supplier in Guangzhou, China. *C. officinalis* was kindly provided by the Venture Tycoon Limited (Batch no.: STP-QCP-JHR670-003). The five herbal components contained in the original PHF were purchased and authenticated as previously described (Chan et al., 2008). Chemical authentication of Herba Menthae and Cortex Moutan were performed in accordance with Chinese Pharmacopoeia 2015. Water solvent of root bark of Cortex Moutan, aerial part of Herba Menthae and whole plant of *C. officinalis* were used for chemical analysis. Liquid chromatography-mass spectrometry (LCMS) System (Agilent, CA, USA) was used for the chemical authentication of each extract and the mixture formula. Sample solution was injected onto a Waters Agilent ZORBAX Eclipse Plus C18 Column (150 × 2.1 mm internal diameter (i.d.), particle size 1.8 µm) with Agilent ZORBAX Eclipse Plus C18 1.8µm Guard Column (10 × 2.1 mm i.d., particle size 1.8 µm). All solvents were pre-filtered with 0.45 µm Millipore filter disk (Millipore) and de-gassed. A gradient elution was carried out using the following solvent systems: mobile phase A - double distilled water/formic acid (99.9/0.1; v/v); mobile phase B - acetonitrile (99.9/0.1; v/v). A detailed description of the gradient elution system used is shown in Table S1. The flow rate used was 0.5 ml/min. Each sample (5 µl) was injected into the column after filtration through a 0.2 µm filter disk. Identification of the chemical markers was carried out by comparing the retention times of the unknown peaks to those of the standards, markers of Herba Menthae and Cortex Moutan were selected according to Chinese Pharmacopoeia 2015, while *C. officinalis* was identified following a previous protocol (Nicolaus et al., 2016). The system was monitored by a computer equipped with the Agilent Mass Hunter Workstation Software for data collection, 2018 integration and analysis.

Cell culture

Human mast cell line-1 (HMC-1) was provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). Mouse RAW 264.7 macrophages and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human dermal fibroblasts (HDF) were purchased from Cell Applications Inc. (San Diego, CA, USA). HMC-1 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, 100 U/ml penicillin-streptomycin (PS) and 100 µg/ml streptomycin (Gibco, Grand Island, NY, USA), and 100 µM α-thioglycerol. RAW 264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) FBS, 1% (v/v) PS, and 100 µg/ml streptomycin. HUVEC were grown in Nutrient Mixture F-12 (DMEM/F12; Life Technologies, Carlsbad, CA, USA) supplemented with 0.1 mg/ml heparin and 0.03 mg/ml endothelial cell growth supplement. HDF were maintained in the all-in-one ready-to-use HDF Growth Medium (Cell Applications Inc., San Diego, CA, USA). Human keratinocyte cells (HaCaT) was maintained in Dulbecco's Modified Eagle's Medium

(DMEM, ATCC, Manassas, VA, USA) supplemented with 10% (v/v) FBS and 1% (v/v) PS. All cells were kept in a 5% CO₂ humidified atmosphere at 37 °C.

Cell viability assay

Cells (5×10^4 /ml RAW, HMC-1, HUVEC or HaCaT cells) were seeded in a 96-well flat-bottom culture plate in 100 µl of medium and incubated at 37 °C overnight. They were then treated with or without individual herbal extracts or three-herbs formula (0–2 mg/ml) for 24 h. Relative number of viable cells were determined according to the procedures described previously (Wu et al., 2018).

Assay of cytokine release from A23187 plus PMA activated HMC-1 cells

HMC-1 cells (1×10^5 /ml) were pre-incubated for 30 min with Herba Menthae, Cortex Moutan or tri-herb formula (0.2 mg/ml) or dexamethasone (1 µM), followed by a 24 h incubation with a mixture of calcium ionophore A23187 (1 µM) and phorbol-12 myristate 13-acetate (50 nM) (A23187 + PMA, Sigma-Aldrich, St. Louis, Missouri, USA) as previously described (Chan et al., 2008). Then, cell free supernatants were assayed for IL-6, IL-10 and TNF-α production by commercially available human cytokine ELISA kits (BD OptEIA, San Diego, CA, USA). Furthermore, the effects of three-herbs formula on the expression of histamine and leukotrienes B₄ were tested using histamine ELISA kit (Abcam, Cambridge, UK) and leukotrienes B₄ ELISA kit (Cayman Chemical, Michigan, USA), respectively.

In vitro inflammation assay using LPS-activated-RAW 264.7 cells and TNF-α/IFN-γ-stimulated HaCaT cells

RAW 264.7 cells (8×10^5 cells/ml) in 500 µl medium were seeded in a 24-well plate overnight, followed by treatment with different concentrations of Herba Menthae, Cortex Moutan, three-herbs formula (0.2 mg/ml), or dexamethasone (1 µM) for 24 h with or without 1 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich; Cheung et al., 2013). The production of nitric oxide (NO) was determined by assaying the accumulation of nitrite, which is the primary stable breakdown product of NO, in the culture medium with Griess reagent (Sigma-Aldrich). The level of nitrite was quantified using a microplate reader (BioTek Instruments, Winooski, VT, USA). The production of prostaglandin E₂ (PGE₂) was measured using a Parameter™ Prostaglandin E₂ Assay Kit (R&D Systems, Minneapolis, MN, USA), while the production of IL-1β, IL-6, TNF-α and CCL2 were measured using BD OptEIA ELISA kits (BD Biosciences; Cheung et al., 2013). HaCaT cells (5×10^5 cells/ml in a 96-well plate) were treated with medium alone or TNF-α/IFN-γ (20 ng/ml) in the presence or absence of Herba Menthae, Cortex Moutan, three-herbs formula (0.2 mg/ml), or dexamethasone (1 µM) for 24 h for the detection of IL-6, IL-8 and MCP-1. Supernatant were collected and measured using BD OptEIA ELISA kits.

Migration scratch assay

The wound healing property of *C. officinalis* and three-herbs formula was examined using the scratch wound healing assay. HUVEC (1×10^5 /ml) were seeded in a 24-well plate and incubated with 1 ml complete medium overnight. Cells were then starved in medium with 1% (v/v) FBS for 24 h. After starvation, HUVEC were scrapped horizontally and vertically in the middle of the plate with a pipette tip. Crosses of each well were photographed under Olympus IX71 microscope at 0 h and after incubation with *C. officinalis*, three-herbs formula, or dexamethasone at non-toxic dosages for 9 h. Migration of cells was measured by comparing the increase in the percentage of cells migrated to the crossed area using the Tscratch software (CSELab, Zurich, Switzerland).

In vitro toxicity and irritation test of cream/lotion on skin cells

In order to determine the most suitable and dermatologically safe vehicle as carrier for the herbal formula extract, we performed further *in vitro* experiments to compare the safety and toxicity of two different vehicles, lotion and cream. Cytotoxicity effects of lotion and cream (0.1 mg/ml) on HDF, HaCaT and human peripheral blood mononuclear cells (PBMC) were investigated using MTT assay. PBMCs were isolated from buffy coat. Cell irritation assay was performed using HDF cells (Lee et al., 2019). HDF (5×10^5 /well) were seeded in 96-well flat-bottom culture plates overnight, followed by treatment with or without various doses of lotion or cream (0.1 mg/ml) for 24 h. For positive control, cells were treated with TNF-α/IFN-γ (50 ng/ml each). Following the 24 h treatment, cell-free supernatants were collected for the determination of IL-6 concentration to confirm whether treatment of cells with lotion or cream would lead to inflammatory cytokine release.

Epidermal skin toxicity test

The toxic effect of three-herbs formula and lotion on skin was performed using the commercially available EpiDerm EPI-200 kit (MatTek Corporation, Ashland, MA, USA) since the general morphology of this model system mimicked that of normal human epidermis. These EpiDerm cultures comprised human-derived epidermal keratinocytes, which was cultured on standing cell culture inserts at the air-liquid interface to form a multilayered, differentiated model of the human epidermis. Briefly, upon kit arrival, the epidermal cultures were placed in a 6-well plate and pre-conditioned overnight at 37 °C and 5% CO₂. Three-herbs formula (0.25–2 mg/ml), lotion (0.25–1 mg/ml) and positive control dexamethasone (1 µM) were added to the skin inserts the next day and transferred to fresh medium for 24 h. All skin inserts were then transferred to fresh plates with 300 µl MTT solution and incubated at 37 °C for 3 h. After the incubation was completed, 2 ml of the extraction solution per well was used for the EpiDerm sample extraction, with the determination of the optical density of the extracted samples at 570 nm by a microplate reader (BioTek, USA) (Kandárová et al., 2009).

Western blot analysis

HMC-1 cells (1×10^6 /well) seeded in a 24-well plate were pre-incubated for 30 min with three-herbs formula (0.2 mg/ml) or dexamethasone (1 µM), followed by a 24 h incubation with or without A23187 (1 µM) + PMA (50 nM) as previously described. Cells were harvested and lysed in lysis buffer and subjected to electrophoresis with 10% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane for protein expression analysis according to the previously described protocol (Wu et al., 2018). Briefly, blots were incubated overnight with primary antibodies against human β-actin, JAK1, JAK2, p-STAT1, p-STAT3, p-STAT5, STAT1, STAT3 and STAT5. After incubation with the secondary antibodies HRP-goat anti-rabbit IgG or HRP goat anti-mouse IgG for 1 h, detection was performed using a molecular imager, ChemiDoc XRSC (Bio-Rad Laboratory, Hercules, CA, USA).

Atopic dermatitis-like mouse model

To determine the *in vivo* effect of three-herbs formula on atopic dermatitis, atopic dermatitis-like animal model was established (Tsang et al., 2018). Balb/c female mice were supplied and maintained by the Laboratory Animal Service Center, the Chinese University of Hong Kong. All experimental procedures were approved by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong [Ref. No. (19-173-ITF)]. Female Balb/c mice were firstly shaved on the dorsal side and sensitized with 200 µl of 1% oxazolone in olive oil-acetone solution (1:4 v/v) or acetone on day 1. After a week, mice were topically challenged with 20 µL of 1% oxazolone on the right ear,

while the left ear was challenged with vehicle alone to serve as control every other day for ten times till day 26. Mice were divided into group of healthy control, induce only, lotion vehicle, three-herbs formula in lotion and dexamethasone positive control (10 µg/ear). Three-herbs formula was given as lotion by dissolving the mixture of herb extracts into a lotion at 5% w/w, each mouse was given 50 mg lotion per ear per day. Treatment was given every other day for ten times, 30 min before oxazolone challenged. During treatments, the body weight of each mouse was measured as a parameter of herb toxicity. As a parameter of cutaneous inflammation, ear thickness was also measured at the same site using a dial micrometer before challenge with oxazolone or vehicle (Mitsutoyo Co., Tokyo, Japan). Scratching behavior was recorded for 15 min in an unattended environment, 2 h after OXA sensitization in the observation chamber using a digital video camera as preciously described (Kitamura et al., 2018). The scratching numbers were counted as the number of times a mouse scratched the ears within a period of 15 min when viewing the videotape. On day 27, the mice were anesthetized for blood collection. They were then euthanized immediately and ear samples were harvested,

Quantification of mouse serum cytokines and chemokines concentrations

Plasma concentrations of IgE, IL-12, keratinocytes chemoattractants (KC), IL-6, macrophage inflammatory protein 2 (MIP-2), IL-1β and TNF-α were measured with a Mouse Cytokine Milliplex MAP assay kit (Millipore Corporation, Billerica, MA, USA) by using a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA).

Histological analysis

Formalin embedded tissue sections of ear (5 µm thickness) were stained with hematoxylin and eosin for histological examination of epidermal thickness or toluidine blue for identification of mast cells, and then examined under light microscopy (Olympus, Tokyo, Japan). Epidermal hyperplasia was evaluated as epidermal thickness. Epidermal thickness at three different locations on each image was measured as the distance from the basement membrane to the outer edge of the

epidermis using ImageJ software, mast cells were counted manually.

Immunohistochemistry

Paraffin sections (5 µm thickness) of mice ears tissue were deparaffinized and immunohistochemically stained for aquaporins 3 (AQP3) and involucrin. Immunoperoxidase staining was carried out using the VectaStain Elite ABC staining kit (Vector Laboratories, CA, USA) with an appropriate secondary antibody. Sections were developed with DAB substrate (Vector Laboratories). Images were taken using an Olympus IX71 microscope and positive immunostaining cells were counted manually.

Statistical Analysis

Data were expressed as mean + SD (*in vitro*) or mean + SEM (*in vivo*). Statistical analyses and significance were analyzed by one-way ANOVA with Tukey's post hoc test using GraphPad PRISM software version 6.0 (GraphPad Software, CA, USA). * $p < 0.05$ was considered as statistically significant.

Results

LCMS profiles of herbal extract and formula

Each extract and three-herbs formula were mixed in water and analyzed by LCMS. Fig. 1 shows that the LCMS profiles of Herba Menthae (Fig. 1A), Cortex Moutan (Fig. 1B), *C. officinalis* (Fig. 1C) and three-herbs formula (Fig. 1D), and the quantification of marker compounds is presented in Fig. 1.

Effects of herbs on the surface expression of the functional markers on human mast cell line

The effects of individual herbs and mixed herbs on the expression levels of inflammatory cytokines IL-6, IL-10 and TNF-α of A23187 and PMA-activated HMC-1 cells were investigated at non-toxic doses

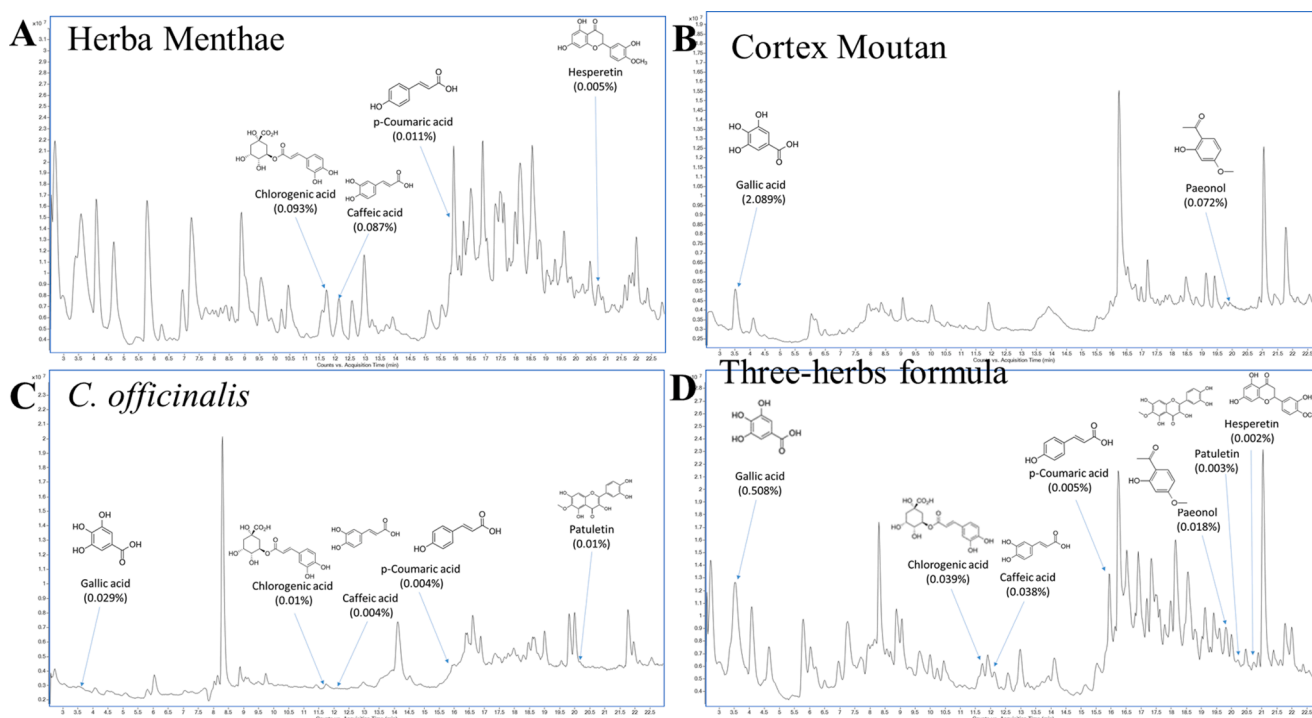


Fig. 1. LCMS profile of (A) Herba Menthae, (B) Cortex Moutan, (C) *C. officinalis* and (D) Three-herbs formula dissolved in distilled water.

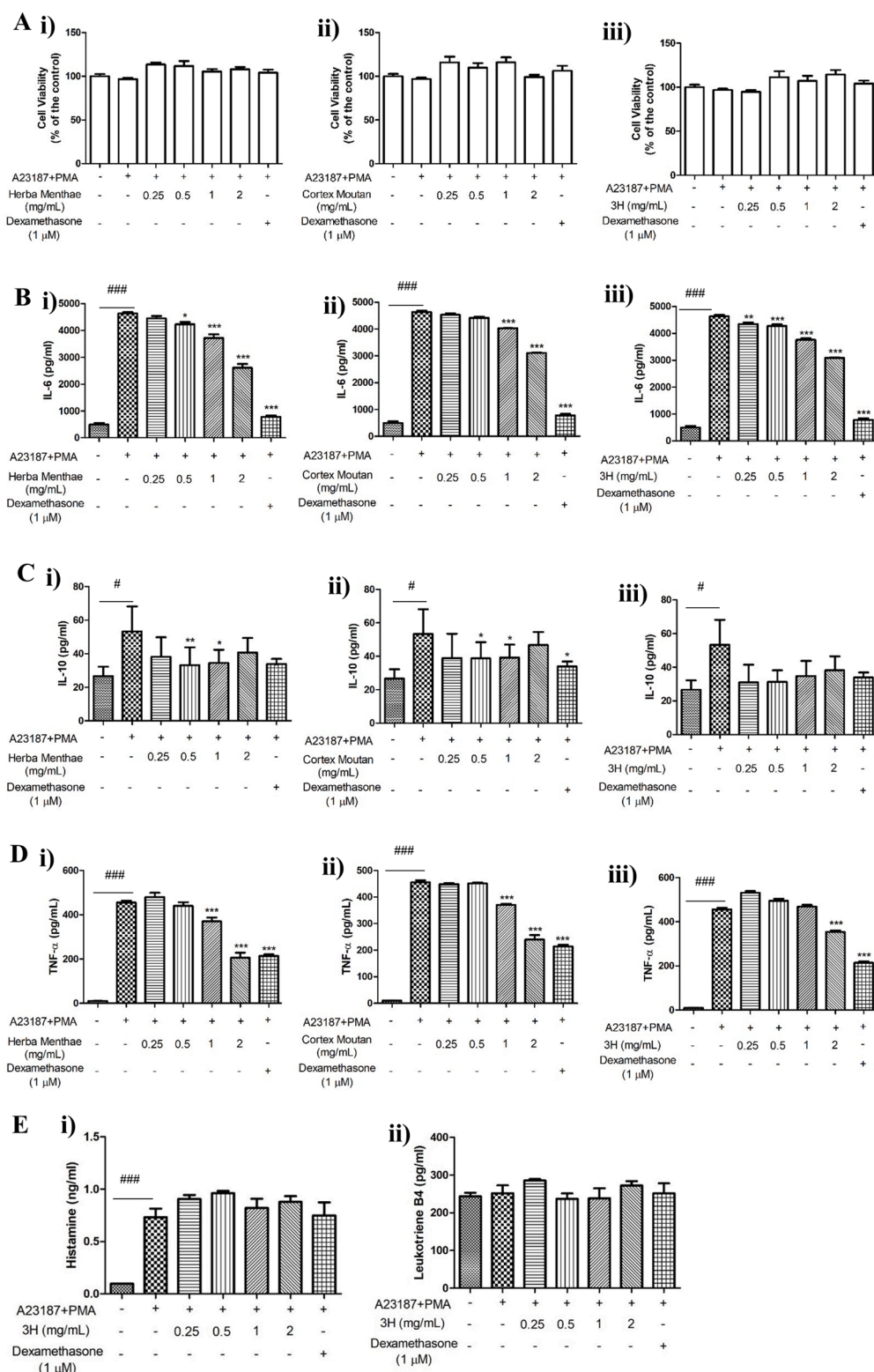


Fig. 2. Effects of Herba Menthae, Cortex Moutan and tri-herb formula (3H) on A23187 + PMA activated HMC-1 viability and cytokine release. (A) Effects of (i) Herba Menthae, (ii) Cortex Moutan and (iii) 3H (0.25–2 mg/ml) on the viability of HMC-1 cells upon 24 h treatment using MTT assay. (B) Effect of different concentrations (0.25–2 mg/ml) of (i) Herba Menthae, (ii) Cortex Moutan and (iii) 3H on IL-6 release from A23187 + PMA activated HMC-1 cells. (C) Effects of different concentrations (0.25–2 mg/ml) of (i) Herba Menthae (ii) Cortex Moutan and (iii) 3H on A23187 + PMA induced IL-10 release from HMC-1 cells. (D) Effects of different concentrations (0.25–2 mg/ml) of (i) Herba Menthae, (ii) Cortex Moutan and (iii) 3H on A23187 + PMA induced TNF-α release from HMC-1 cells. (E) Effects of three-herbs formula on the levels of (i) histamine and (ii) leukotriene B4 on A23187 + PMA induced HMC-1 cells. Dexamethasone (1 μM) was used as the positive control. Values represent means ± SD ($n = 3-6$). Significant difference between medium control and control group using Student's t -test. # $p < 0.05$, ### $p < 0.001$. Significant difference among control and all treatment groups was assessed by one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

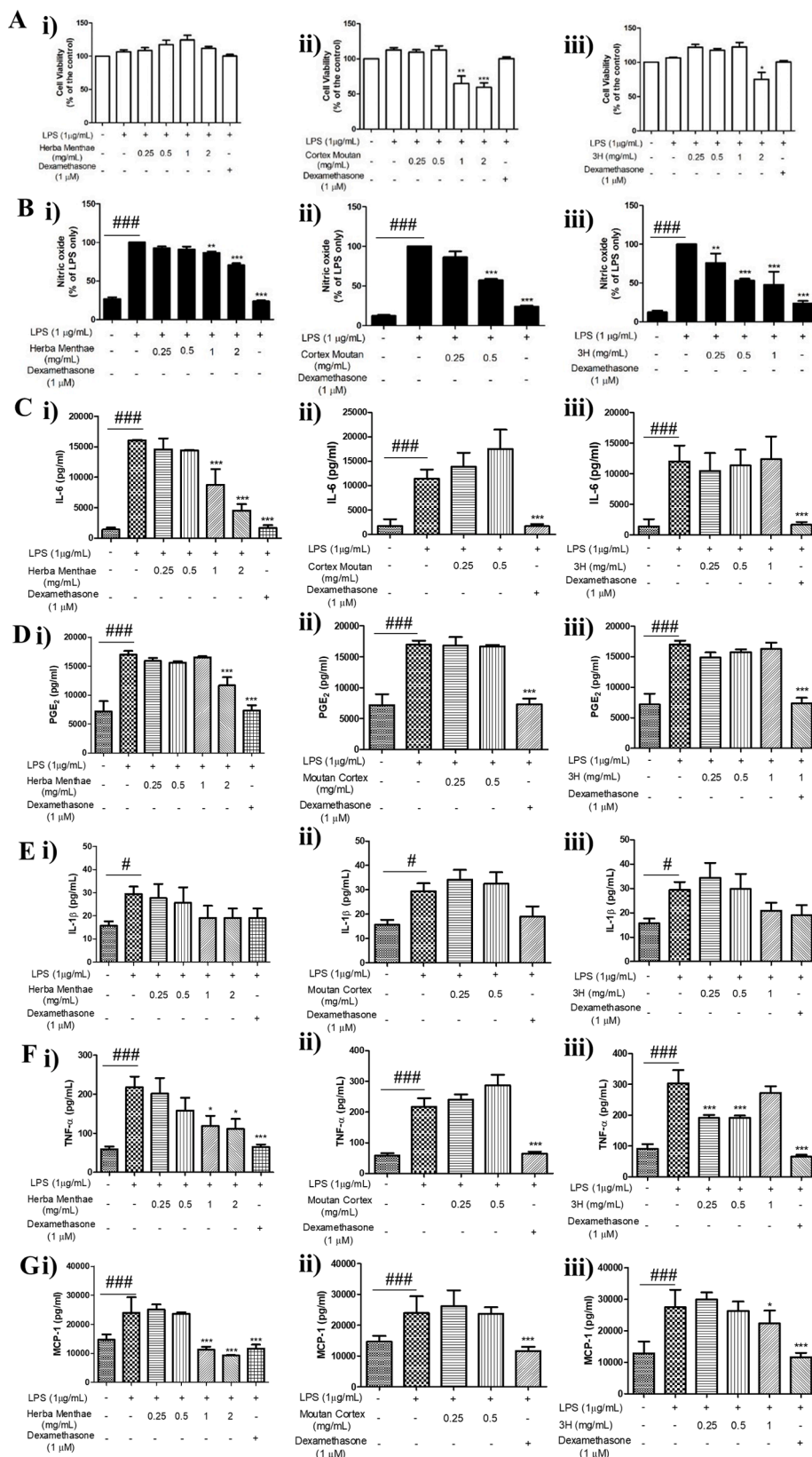


Fig. 3. Effects of Herba Menthae, Cortex Moutan and tri-herb formula (3H) on LPS-activated viability, nitrite oxide production and cytokine expression of RAW 264.7 cells. (A) Effects of (i) Herba Menthae, (ii) Cortex Moutan and (iii) 3H on the viability of RAW 264.7 cells upon 24 h treatment using MTT assay. (B) Effects of different concentrations of (i) Herba Menthae (0.25–2 mg/ml), (ii) Cortex Moutan (0.25–0.5 mg/ml) and (iii) 3H (0.25–1 mg/ml) on LPS-activated nitrite oxide production from RAW 264.7 cells. (C) Effects of different concentrations of (i) Herba Menthae (0.25–2 mg/ml), (ii) Cortex Moutan (0.25–0.5 mg/ml) and (iii) 3H (0.25–1 mg/ml) on LPS-induced IL-6 production from RAW 264.7 cells; (D) Effects of different concentrations of (i) Herba Menthae (0.25–2 mg/ml), (ii) Cortex Moutan (0.25–0.5 mg/ml) and (iii) 3H (0.25–1 mg/ml) on LPS-induced PGE-2 production from RAW 264.7 cells; (E) Effects of different concentrations of (i) Herba Menthae (0.25–2 mg/ml), (ii) Cortex Moutan (0.25–0.5 mg/ml) and (iii) 3H (0.25–1 mg/ml) on LPS-activated TNF-α production; (F) Effects of different concentrations of (i) Herba Menthae (0.25–2 mg/ml), (ii) Cortex Moutan (0.25–0.5 mg/ml) and (iii) 3H (0.25–1 mg/ml) on LPS-induced CCL2/MCP-1 production. Dexamethasone (1 μM) was used as the positive control. Values represent means ± SD ($n=3-6$). Significant difference between medium control and control group was assessed by Student's *t*-test. ### $p < 0.001$. Significant difference among control and all treatment groups using one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(Fig. 2A). Dexamethasone (1 μM) was used as positive control. Results showed that IL-6 production was significantly decreased by the treatment of Herba Menthae at 0.5–2 mg/ml (Fig. 2Bi), while Cortex Moutan inhibited the production of IL-6 at the dosage of 0.5–2 mg/ml (Fig. 2Bii). Moreover, three-herbs formula dose-dependently inhibited IL-6

production at the tested dosages from 0.25–2 mg/ml (Fig. 2Biii). The positive control dexamethasone also significantly inhibited the production of IL-6. For IL-10 production, only Herba Menthae and Cortex Moutan exhibited the inhibitory effect (Fig. 2Ci, ii). Herba Menthae and Cortex Moutan significantly inhibited the expression of TNF-α at the

dosage of 1 and 2 mg/ml (Fig. 2Di, ii). When mixed together, only the dose of 2 mg/ml showed significant inhibitory effect on TNF- α production (Fig. 2Diii). Histamine and leukotrienes are important pro-inflammatory mediators in allergic inflammation. However, the three-herbs formula and dexamethasone showed no significant effect on expression of histamine and leukotriene B4 in activated human mast cells (Fig. 2Ei, ii).

Anti-inflammatory effects of herbs on RAW 264.7 cells and HaCaT cells

Cell viability results showed that there was no significant effect of the *Herba Menthae* extract (0.25–2 μ g/ml) and dexamethasone (1 μ M) on the viability of RAW 264.7 cells (Fig. 3Ai). *Cortex Moutan* exerted no significant toxicity at concentrations up to 0.5 mg/ml (Fig. 3Aii) and three-herbs formula exerted no toxicity on RAW 264.7 cells (up to 1 mg/ml, Fig. 3Aiii). Individual herbs, three-herbs formula and

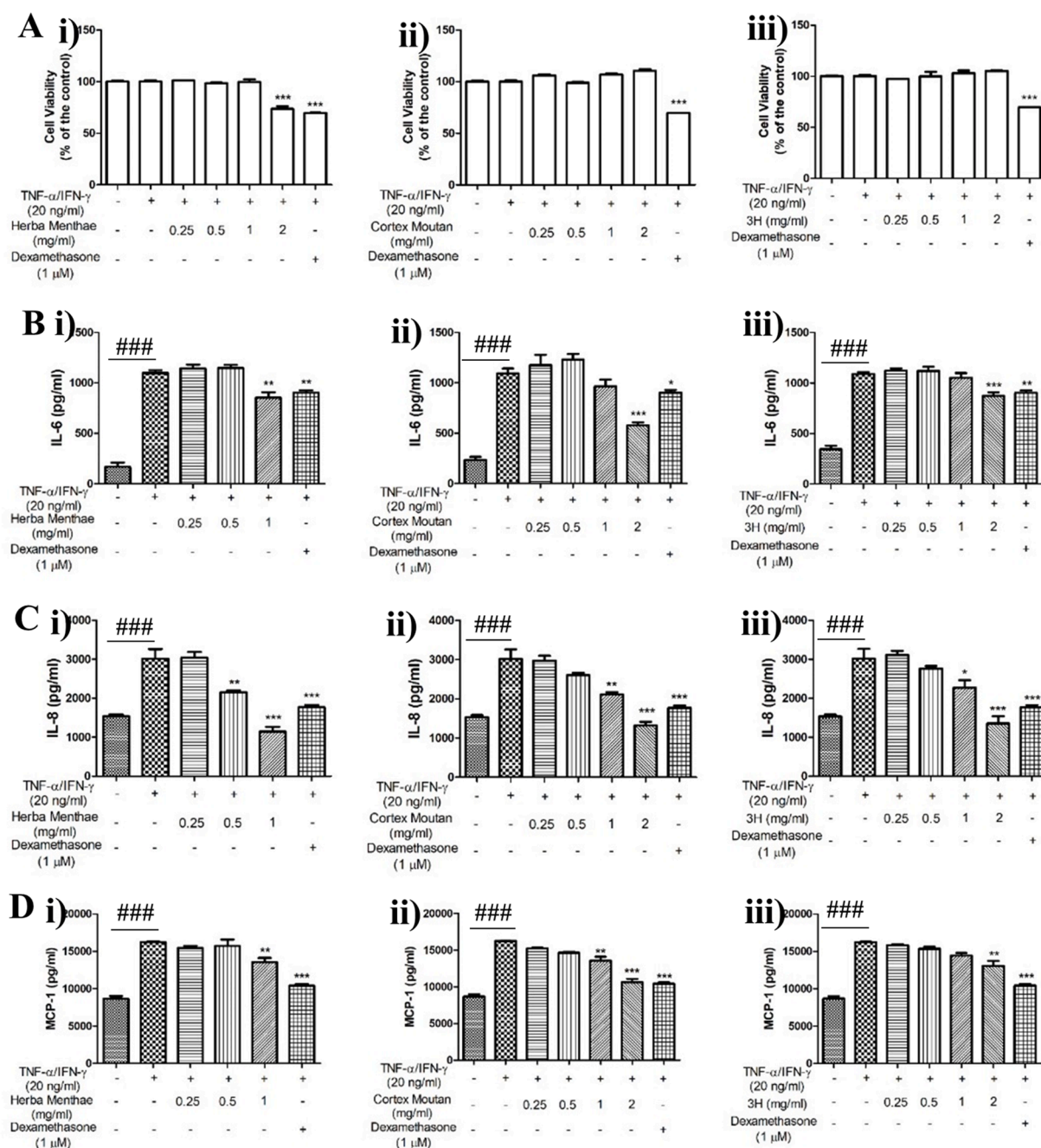


Fig. 4. Effects of *Herba Menthae*, *Cortex Moutan* and tri-herb formula (3H) on TNF- α /IFN- γ activated HaCaT viability and cytokines release. (A) Effects of (i) *Herba Menthae*, (ii) *Cortex Moutan* and (iii) 3H (0.25–2 mg/ml) on the viability of HaCaT cells upon 24 h treatment using MTT assay. (B) Effects of different concentrations of (i) *Herba Menthae*, (ii) *Cortex Moutan* and (iii) 3H on IL-6 release from TNF- α /IFN- γ activated HaCaT cells. (C) Effects of different concentrations of (i) *Herba Menthae* (ii) *Cortex Moutan* and (iii) 3H on IL-8 release from TNF- α /IFN- γ activated HaCaT cells. (D) Effects of different concentrations of (i) *Herba Menthae*, (ii) *Cortex Moutan* and (iii) 3H on MCP-1 release from TNF- α /IFN- γ activated HaCaT cells. Dexamethasone (1 μ M) was used as the positive control. Values represent means $\pm$ SD ($n=3-6$). Significant difference between medium control and control group using Student's t -test: ### $p < 0.001$. Significant difference among control and all treatment groups using one-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

dexamethasone at the non-toxic dosages were further tested on LPS-induced nitrite oxide production in RAW 264.7 cells. Results demonstrated that LPS (1 $\mu\text{g/ml}$) induced significant increases in NO production in RAW 264.7 cells, which were dose-dependently reduced by Herba Menthae (Fig. 3Bi), Cortex Moutan (Fig. 3Bii), and the three-herbs formula extracts (Fig. 3Biii). Herba Menthae (1 and 2 mg/ml) inhibited the NO production by 14% and 30%, respectively. Cortex Moutan (2 mg/ml) decreased NO production by 44%. Moreover, the three-herbs formula (0.5 and 1 mg/ml) significantly inhibited the NO production by 47% and 52%, respectively. The positive control dexamethasone significantly inhibited NO production by 73% at 1 μM . Production of IL-6, PGE2, IL-1 β , TNF- α and CCL2/MCP-1 in LPS-induced RAW 264.7 cells upon the treatment with Herba Menthae, Cortex Moutan, three-herbs formula, and dexamethasone. Herba Menthae and dexamethasone (1 μM) significantly reduced IL-6 (Fig. 3Ci), PGE2 (Fig. 3Di), TNF- α (Fig. 3Fi) and CCL2/MCP-1 (Fig. 3Gi) production in LPS-induced RAW 264.7 cells. There were no significant inhibitory effects of Cortex Moutan or three-herbs formula on IL-6 (Fig. 3Cii, iii) or PGE2 (Fig. 3Dii, iii) production. Fig. 3E shows effects of the herbal extracts and herbal formula extracts on IL-1 β production. While Herba Menthae and three-herbs formula extracts exerted a trend to reduce the LPS-induced increase in IL-1 β release (Fig. 3Ei, iii), Cortex Moutan and dexamethasone exerted no significant effect on reducing the LPS-induced increase in IL-1 β release (Fig. 3Eii). Herba Menthae extract exerted dose-dependent reduction on TNF- α production (Fig. 3Fi). There was however no significant effect of Cortex Moutan on TNF- α

production (Fig. 3Fii). Three-herbs formula (up to 0.5 mg/ml) exerted significant reduction on TNF- α production (Fig. 3Fiii). For CCL2/MCP-1 production, both Herba Menthae and three-herbs formula extracts significantly reduced CCL2/MCP-1 release in a dose-dependent manner (Fig. 3Gi, iii). Cortex Moutan however exerted no significant effects on CCL2/MCP-1 release (Fig. 3Gii). In TNF- α /IFN- γ stimulated HaCaT cells, Herba Menthae and dexamethasone showed toxicity at 2 mg/ml and 1 μM , respectively (Fig. 4Ai), Cortex Moutan and three-herbs formula has no cytotoxicity at up to 2 mg/ml (Fig. 4Aii, iii). Therefore, Herba Menthae at up to 1 mg/ml, Cortex Moutan and three-herbs formula at up to 2 mg/ml were used at the ELISA assay. Results showed that Herba Menthae significantly inhibited the levels of IL-6 (Fig. 4Bi), IL-8 (Fig. 4Ci) and MCP-1 (Fig. 4Di), Cortex Moutan (2 mg/ml) and three-herbs formula (2 mg/ml) also significantly reduced the levels of IL-6 (Fig. 4Bii, iii), IL-8 (Fig. 4Cii, iii) and MCP-1 (Fig. 4Dii, iii). The inhibition effect of positive control dexamethasone in cytokine release may be due to the cytotoxicity in HaCaT cells.

C. officinalis and three-herbs formula increase the HUVEC migration

Effect of *C. officinalis* and three-herbs formula on the migration of HUVEC cells were determined using scratch wound healing assay. Cell viability assay showed that *C. officinalis* and three-herbs formula had no toxicity on HUVEC cells at the dosage up to 0.25 mg/ml (Fig. 5Ai, ii). *C. officinalis* (0.125 mg/ml) was found to significantly increased closed wound area after 8 h incubation compared to the control group

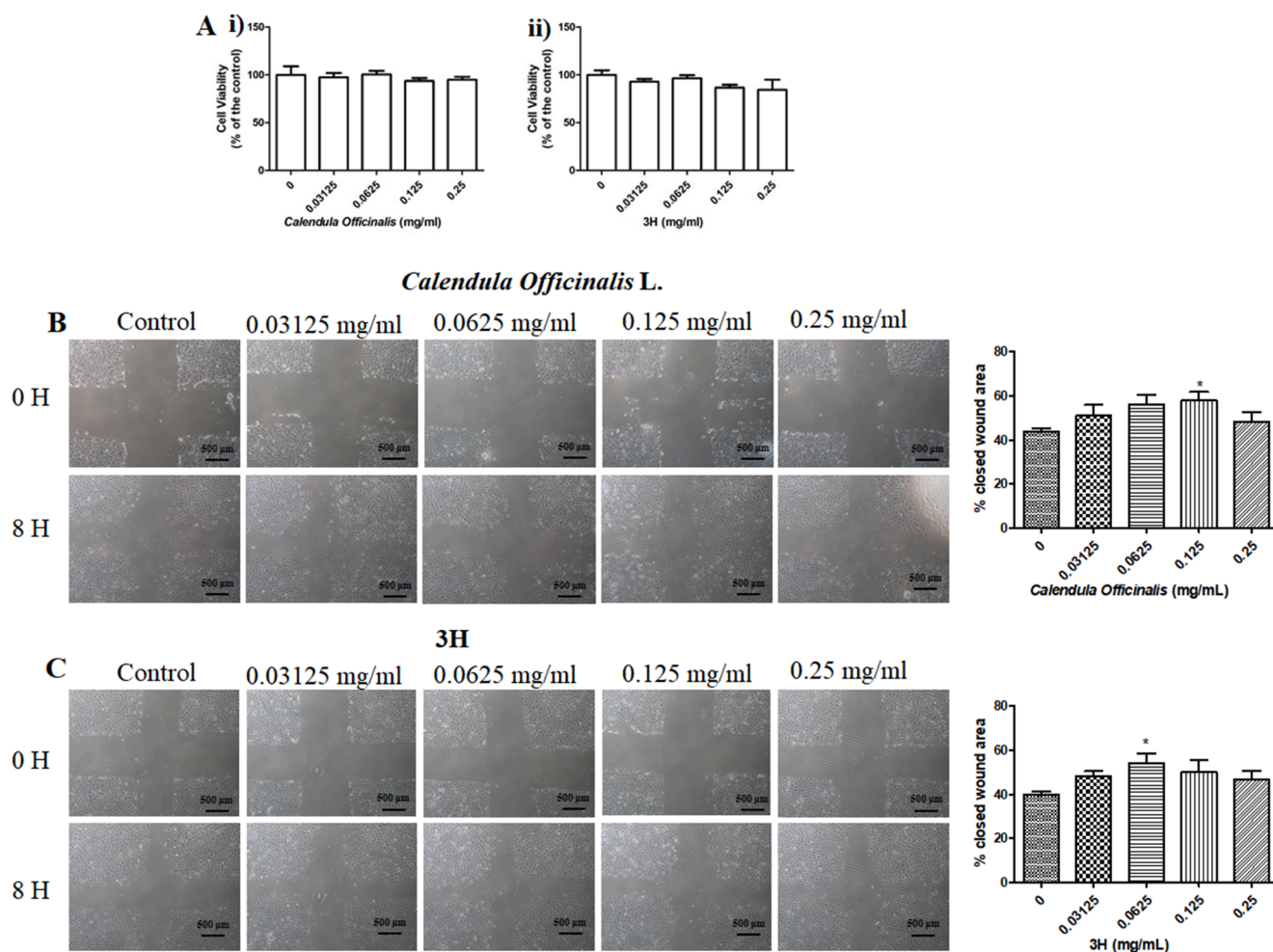


Fig. 5. Effects of different concentrations of *C. officinalis* and tri-herb formula (3H) on the migration of HUVEC. (A) Effects of (i) *C. officinalis* and (ii) 3H on the viability of HUVEC cells upon 24 h treatment using MTT assay. (B) Effects of *C. officinalis* and 3H on the migration of HUVEC cells. Values represent means $\pm$ SD ($n=3-6$). Significant difference among control and all treatment groups was assessed by one-way ANOVA. * $p < 0.05$.

Table 1
List of ingredients in lotion and cream.

Lotion	Cream
Water	Water purified
Cetomacrogol 1000	Glycerine (Vegetable)
Cetyl-stearyl Alcohol (50:50)	Lanette 22 (Behenyl Alcohol)
paraffin liquid light	Cosmowax D (Cetearyl Alcohol (and) Ceteareth-20)
petroleum Jelly	Avocado Oil
Chlorocresol	Tocopheryl Acetate 1250
Propylene glycol	D Panthenol
Glycerine	Dow Corning 200/350CST Fluid (Dimethicone)
	Xiameter PMX 200/50 (Dimethicone)
	Palmerol 1899 (Stearyl alcohol)
	Pemulen TR2 (acrylates /C10-30 alkyl acrylate crosspolymer)
	Citric Acid Anhydrous (BP)
	Geogard 221 (Dehydroacetic Acid and Benzyl Alcohol)
	Triethanolamine 99
	Tretan 50
	Kobo MSS-500W

(Fig. 5B). Similarly, three-herbs formula (0.0625 mg/ml) also significantly increased the closed area (Fig. 5C) which indicated that it helped wound healing.

Effects of different vehicles on cell viability and LPS-induced IL-6 production

Lotion and cream were prepared as carrier for the herbal formula extract. Table 1 shows the composition of the lotion and cream, respectively. Cell viability results showed that lotion exerted no significant toxicity on human PBMCs (Fig. 6Ai), HaCaT (Fig. 6Bi) or HDF cells (Fig. 6Ci). However, cream significantly reduced cell viability of PBMC at 1 mg/ml (Fig. 6Ai), and HDF cells at 0.5 and 1 mg/ml (Fig. 6Cii). To determine whether lotion and cream could cause any irritations on skin cells, cell irritation tests were performed on HDF cells. Positive control (TNF α /IFN- γ —induced cells) had significantly increased levels of IL-6. Lotion or cream had no significant effect on inducing IL-6 release from HDF (Fig. 6Di, ii).

Effects of three-herbs formula and lotion on Epidermal skin

A commercially available human epidermal skin model was used to test the toxicity of three-herbs formula and lotion, 5% SDS was used as a positive control. Fig. 6E shows that three-herbs formula (0.25–2 μ M) and lotion (0.25–1 μ M) had no toxicity on epidermal cells while the positive control 5% SDS significantly inhibited epidermal cell viability.

Effects of three-herbs formula on the expression of proteins in Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway

Recent studies have shown that inhibition of JAK/STAT pathway may ameliorate chronic dermatitis by improving skin barrier function (Rodrigues and Torres, 2020). Our above results indicated the anti-inflammatory activity of three-herbs formula in HMC-1, RAW 264.7 and HaCaT cells. We further investigated whether the three-herbs formula exerted its anti-inflammatory effect through the modulation of JAK/STAT pathway. Western blot assay was performed for HMC-1 cells (Fig. 7A). Three-herbs formula significantly inhibited STAT-1, p-STAT1 and p-STAT3 expression without significant effect on STAT5 and p-STAT5 (Fig. 7B–D). Taken together, three-herbs formula might exert its anti-inflammatory activity through inhibiting STAT1 and STAT3-dependent mechanisms.

Effects of topical application of three-herbs formula on inflammatory responses in mice with oxazolone-induced atopic dermatitis

The *in vivo* effect of three-herbs formula in AD was investigated by topical application of the formula to oxazolone-induced atopic dermatitis like mice. As shown in Fig. 8A, the OXA-mediated mice ear was red and swollen, comparing with the healthy mice on the sacrifice day. After treating the OXA-challenged mice ear with lotion, three-herbs formula or dexamethasone, the redness of ear was relieved (Fig. 8A). Ear thickness and scratching behavior, the features of atopic dermatitis, were also observed. Fig. 8B shows that repeated oxazolone challenges resulted in a marked increase in ear swelling from about 0 to 0.4 mm in mice (day 8–24). Since day 21, mice ear thickness was significantly inhibited by tri-herb formula and dexamethasone, while in the lotion treated group, ear thickness was significantly reduced until day 23. Scratching times of mice in 15 mins were also counted, tri-herb formula significantly inhibited mice scratching times from day 19, while lotion had no significant effect on mice scratching (Fig. 8C). Dexamethasone inhibited mice scratching since day 17. Although dexamethasone exhibited the best effect in reducing mice scratching and ear swelling, it significantly reduced mice weight since day 19 compared to the healthy mice (Fig. 8D). Moreover, levels of IL-6 and KC in mouse serum were significantly reduced by tri-herb formula and dexamethasone (Fig. 8E). Only dexamethasone inhibited the mouse serum level of TNF- α (Fig. 8Eii). Epidermal thickness can be one of the factors that contributes to the ear swelling as mentioned above. In the oxazolone-induced atopic dermatitis mice model, there was increased epidermal thickness (Fig. 9A). A layer of deep red or purple color on the skin surface indicated the thickened epidermis. As shown in Fig. 9B, repeated application of oxazolone resulted in more than 3-times thicker epidermal layer comparing with that observed in the normal control group. This increase in epidermal thickness was inhibited by 28.2% with topical application of tri-herb formula. However, topical treatment with lotion had no significant effect in reducing the epidermal thickness, implying that three-herbs formula contributed to the anti-inflammatory action. Dexamethasone also markedly reduced epidermal thickness by 39.3% (Fig. 9B). These findings were also consistent with the histology of the challenged ear tissue as observed in H&E staining (Fig. 9A). Mast cells contribute to skin inflammation; they were visualized in the dermis of oxazolone-induced mice by toluidine blue staining (Fig. 9A). Quantitative result showed that mast cells were significantly increased in oxazolone induced mice compared to healthy mice (Fig. 9C). Moreover, all lotion, tri-herb formula and dexamethasone treatments exhibited inhibition effect on the mast cells in mice ear. Among them, tri and dexamethasone have better inhibition effect, while comparing to lotion (Fig. 9C). AQP3 is important for water transport and hydration in skin epidermis (Boury-Jamot et al., 2009). To investigate the effect of three-herbs formula on AQP3 following repeated oxazolone challenge, AQP3 was measured by immunohistochemistry. Results showed that repeated oxazolone challenge induced the downregulation of AQP3 (Fig. 9D). Expression of AQP3 per section was doubled in three-herbs formula-treated mice compared with the oxazolone-induced group while lotion had no effect on the downregulation of AQP3 by repeated oxazolone challenge (Fig. 9D). Moreover, abnormalities in the differentiation of keratinocyte result in the damage of skin barrier and antimicrobial function (Goleva et al., 2019). We have investigated the expression of common keratinocyte differentiation marker involucrin in mice ear skin by immunohistochemical staining. However, the results did not show any significant differences of involucrin (data not shown) among all treatments (Fig. 9A).

Discussion

The present study demonstrated that a novel herbal formula containing Cortex Moutan, Herba Menthae and *C. officinalis* in the weight ratio of 1:1:1 at non-toxic dose exhibited immunosuppressive and anti-

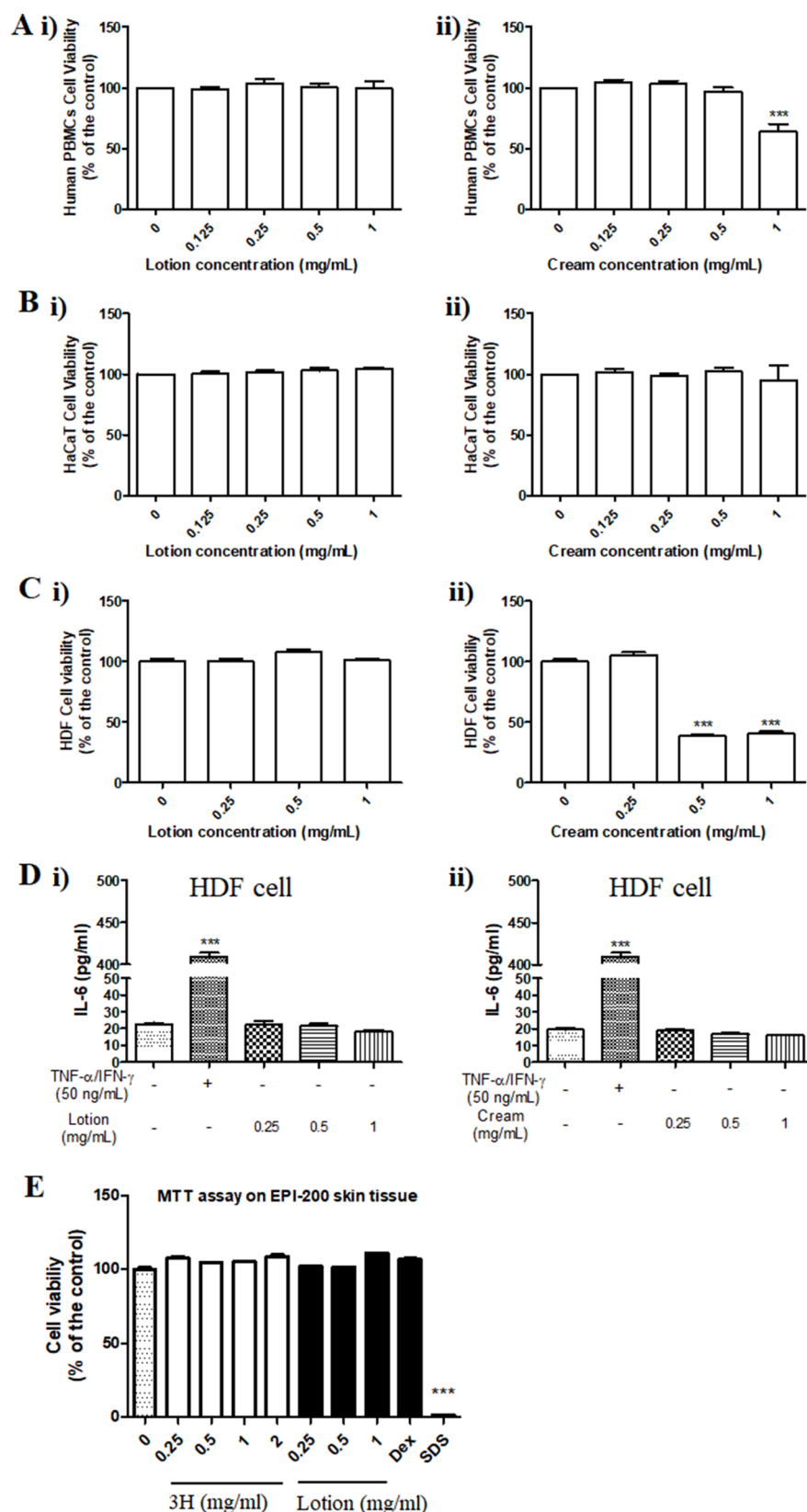


Fig. 6. Effects of lotion and cream on cell viability and LPS-induced IL-6 production. Different concentrations of lotion and cream on the viability of (A) PBMCs, (B) HaCaT cells and (C) HDF, (D) Different concentrations of (i) lotion and (ii) cream on LPS-induced IL-6 production in HDF cells, and (E) Cell viability of 3H and lotion on Epidermal skin tissue. Values represent means $\pm$ SD ($n=3-6$). Significant difference among control and all treatment groups was assessed by one-way ANOVA. *** $p < 0.001$.

inflammatory activities and promoted cell migration *in vitro*. Moreover, the three-herbs formula significantly inhibited the expression of proteins in JAK/STAT signaling pathways of HMC-1 cells. Moreover, *in vitro* skin toxicity tests proved that the formula exhibited no toxic effects on skin in the tested dosages. *In vivo* oxazolone-induced atopic dermatitis-like mice

model confirmed the inhibitory effect of the formula on the ear redness, swelling and itching behavior of OXA-induced dermatitis mice without any toxicity. H&E and toluidine blue staining of the challenged ear tissue demonstrated that the three-herbs formula decreased the epidermal thickness and mast cells, respectively. AQP3, a water transporter, was

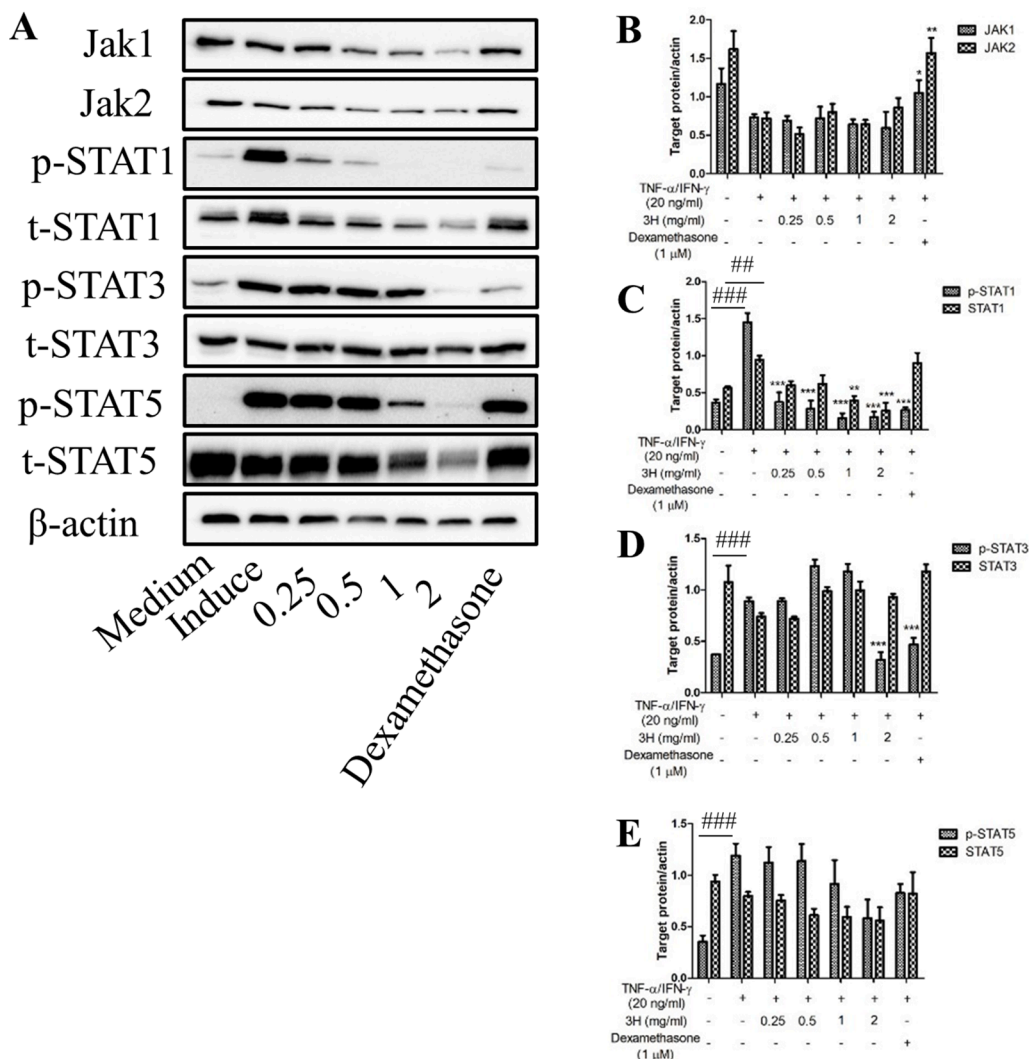


Fig. 7. Effects of tri-herb formula (3H) on the expression of proteins in JAK-STAT signaling pathways in HMC-1 cells. (A) Representative Western blots showing the expressions of JAK/STAT signaling pathways related proteins in HMC-1 cells. (B) Bar charts showing the results of the protein expression of JAK1, JAK2, p-STAT1, STAT1, p-STAT3, STAT3, p-STAT5 and STAT5 using Western blot analysis, which were normalized with corresponding β-actin protein expression and expressed as fold of control (mean + SD of 4 independent experiments). Significant difference between medium alone and TNF-α/IFN-γ induced group was assessed by Student's *t*-test: ## *p* < 0.01, ### *p* < 0.001. Significant difference among TNF-α/IFN-γ induced group and all treatment groups was assessed by one-way ANOVA: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

significantly increased in oxazolone challenged ear tissue upon the treatment with three-herbs formula. Furthermore, transdermal experiments showed that three-herbs formula could successfully penetrate through porcine ear skin (data not shown).

Inflammation is implicated in the pathogenesis of AD (McPherson, 2016), and the inflammatory process involves both signaling that initiate and maintain inflammation and signals that shut down the process. An imbalance between these two signals causes the dysregulation of inflammation, leading to cellular and tissue damage (Fujiwara and Kobayashi, 2005). Macrophages and keratinocytes are the major cellular component and chief effector cells in response to inflammatory stimuli (Cheung et al., 2013). Deactivation or removal of mediators and inflammatory effector cells leads to the suppression of inflammation, enabling the host to repair the damaged tissues. Therapeutic interventions targeted macrophages and keratinocytes and their products may open new avenues for treating inflammatory diseases since macrophages participated in both beneficial and detrimental outcomes in inflammation. The present study clearly demonstrated that Herba Menthae could significantly suppress the production of NO, PGE₂, TNF-α and CCL2 in LPS-stimulated RAW 264.7 macrophages. Moutan Cortex also exhibited significant inhibition effect on the NO production. Most importantly, the three-herbs formula significantly decreased NO production as well as CCL2 and TNF-α in RAW cells. High concentration of NO can mediate inflammation and cause cell death by inducing

apoptosis (Aktan, 2004). Macrophages could synthesize CCL2 which can cause accumulation and activation of immune cells in the affected sites to promote the inflammation process, in response to pro-inflammatory mediators, such as LPS and TNF-α (Charo and Taub, 2011). In HaCaT cells, Herba Menthae, Coetex Moutan and three-herbs formula all significantly inhibited the levels of IL-6, IL-8 and CCL-2. Results from our study implicated that three-herbs formula possessed the anti-inflammatory activity by inhibiting the production of NO and CCL2 from macrophages as well as inhibition levels of IL-6, IL-8 and CCL-2 in keratinocytes.

Mast cells are key mediators in allergic diseases which attributes to their potent capability to produce multiple allergic inflammation-related cytokines including IL-6, IL-10 and TNF-α upon activation (Wang and Lau, 2006). TNF-α and IL-6 play a key role in triggering and sustaining the allergic inflammation in mast cells, when overexpressed, they could trigger pathological conditions (Kim et al., 2006). TNF-α is considered to be an initiator of cytokine related inflammation by stimulating cytokine production and promoting inflammation, leukocyte infiltration, granuloma formation and tissue fibrosis (Jeong et al., 2002). Inflammatory IL-6 is also produced from mast cells and its local accumulation is associated with passive cutaneous anaphylaxis reaction. The main function of anti-inflammatory IL-10 appears to be the prevention of extensive tissue damage upon inflammation and infection (Ouyang et al., 2011). Therefore, the reduction of TNF-α and IL-6 from mast cell is

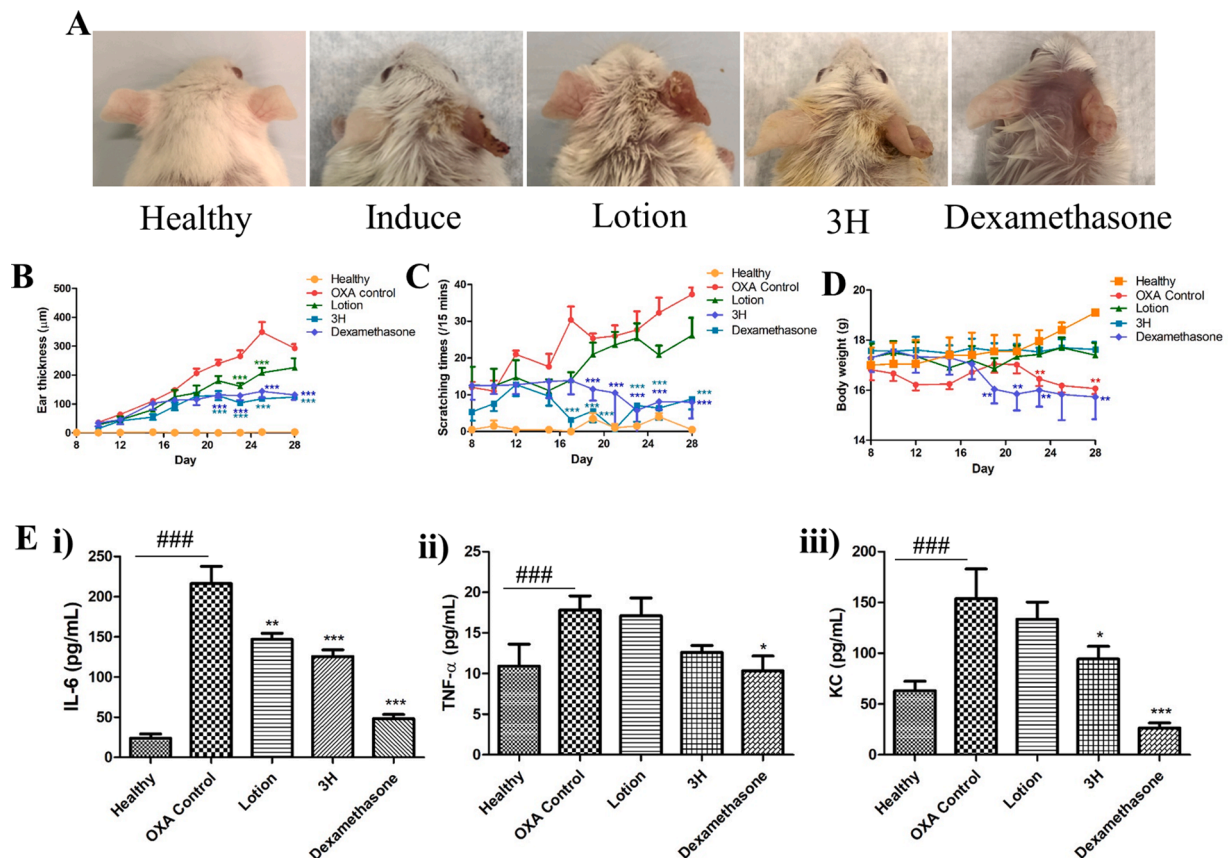


Fig. 8. Effects of topical application of tri-herb formula (3H) on inflammatory responses in mice with oxazolone-induced atopic dermatitis. (A) Representative photographs of ear redness of mice with different treatments were taken on Day 27 before mice were sacrificed ($n=4-6$). (B) The ear thickness was measured and shown with mean $\pm$ SEM upon different treatments. (C) Scratching times of mice in 15 min were counted and shown as mean $\pm$ SEM, $**p < 0.01$, $***p < 0.001$ when compared with the OXA control group. (D) Body weight was measured and shown as mean $\pm$ SEM, $**p < 0.01$ when compared with the healthy group ($n=4-6$). (E) Bar charts show the concentrations of i) IL-6, ii) TNF- α and iii) KC in serum ($n = 4-6$). Significant difference between healthy and OXA control group was assessed by Student's t -test: $###p < 0.001$. Significant difference among OXA control and all treatment groups was assessed by one-way ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

a feature of reduced allergic symptoms and the increase of IL-10 may alleviate skin damage in AD patients. Our results verified that Moutan Cortex, Herba Menthae and three-herbs formula significantly inhibited the production of IL-6 and TNF- α in PMA plus A23187-stimulated HMC-1 cells. Although IL-10 production was decreased when treated with individual herb Moutan Cortex and Herba Menthae, three-herbs formula showed a slight increase on IL-10 production in HMC-1 cells, thereby implying it may help for the healing of skin damage in AD. These results are consistent with our scratch wound healing assay, showing three-herbs formula significantly increased wound healing area in HUVEC. Furthermore, in our AD-like mice model, toluidine blue staining of OXA-challenged mice ear tissue showed that the number of mast cells was significantly decreased, upon the treatment with lotion, three-herbs formula or dexamethasone. Taken together, three-herbs formula could not only reduce the number of mast cells and the productions of inflammatory IL-6 and TNF- α but also accelerated wound healing in AD. JAK/STAT pathway is a vital regulator of immune function, involved in the downstream signaling of inflammatory cytokines, including interleukins (ILs), interferons (IFNs) and multiple growth factors (Blauvelt et al., 2017). Previous evidence indicated that the dysregulation of JAK-STAT signaling is a potential driver of many dermatologic conditions, including AD (Rodríguez and Torres, 2020), suggesting that targeted inhibition of individual components in this pathway, including STAT1, p-STAT1, STAT3 and p-STAT3 may be a potential strategy for AD therapy. Western blots results showed that three-herbs formula

significantly inhibited the expressions of p-STAT1, p-STAT3 and STAT1. Taken together, efficacy of three-herbs formula on inflammation may work through inhibiting STAT1 and STAT3-dependent mechanisms.

There are a variety of moisturizers used in the treatment of AD, including gels, oils, creams, ointments, and lotions (Giam et al., 2016). Each of them has pros and cons, depending on the needs of patients. There is no clinical classification system to determine objectively which types of moisturizers are most appropriate for specific AD phenotype. In our present study, lotion was chosen as the vehicle and herbs mixed well in the lotion. In oxazolone-induced atopic dermatitis like mice model, dexamethasone mixed in lotion (10 μ g in 30 mg lotion per mice) was applied in mice and served as a positive control. Although results showed that dexamethasone exhibited the most potent anti-swelling, anti-itching and anti-cytokine release effect in mice; it significantly decreased mice weight compared to the healthy mice. Previous studies have shown that weight loss in mice after treatment with dexamethasone was also observed (Kim et al., 2015). The metabolic effects of glucocorticoids (include dexamethasone) are dose-dependent, with low doses exhibiting anabolic effects and high doses producing catabolic effects, associated with decreased body weight and food intake (El Haggan et al., 2002).

Ear swelling consequent to increased scratching and skin thickness is often the first hallmark of skin irritation and local inflammation, which are the indications of a number of processes during skin inflammation, such as increased vascular permeability, edema within the dermis, and

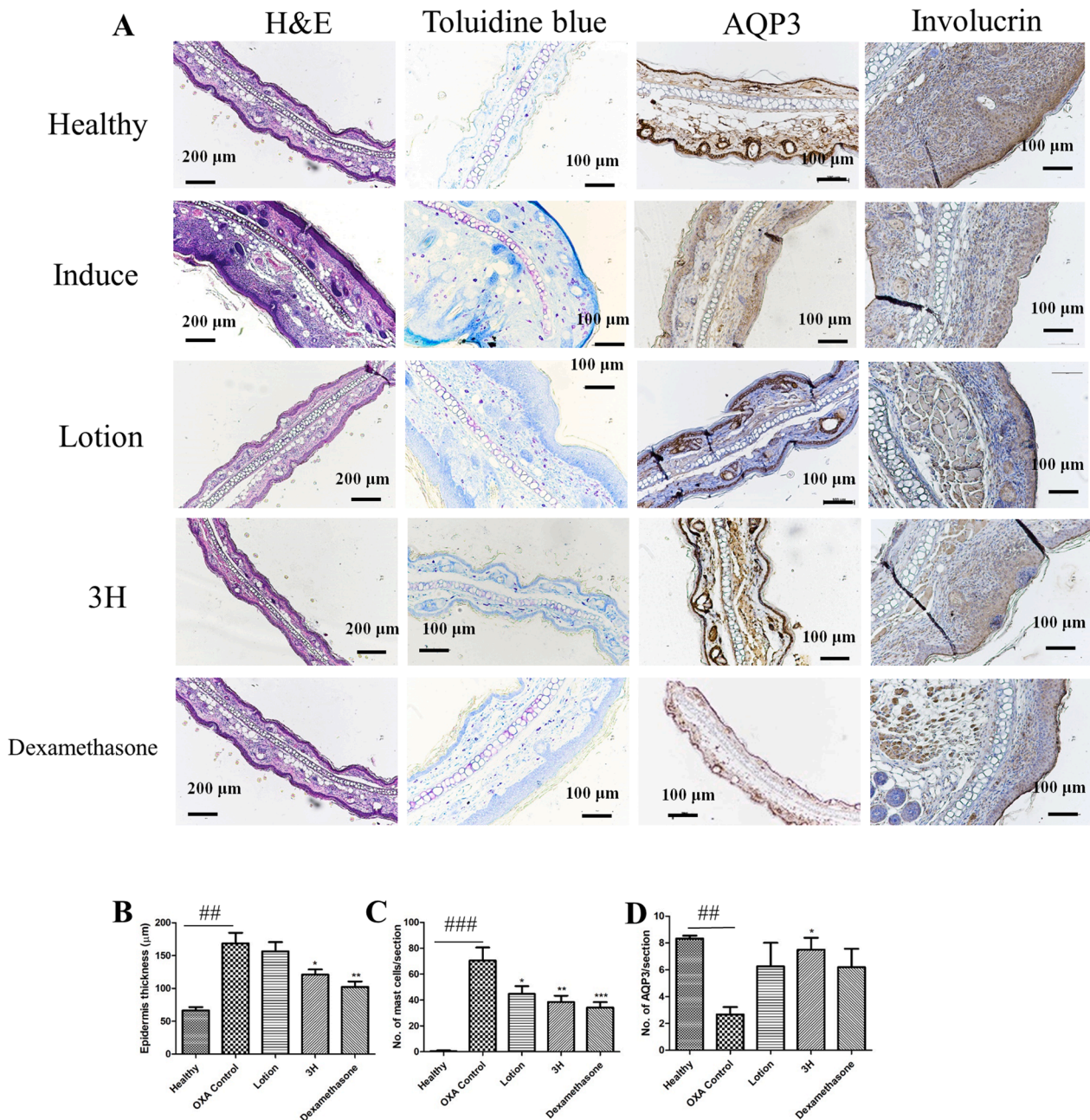


Fig. 9. Various staining of oxazolone-induced AD like mice ear tissue. (A) Representative photographs of H&E, toluidine blue, AQP3 and involucrin staining of challenged ear (right ear) upon different treatments (100 ×); (B) The ear epidermis thickness was measured using ImageJ and shown in bar chart with means $\pm$ SD ($n=4-6$); number of (C) mast cells and (D) AQP3 in per section was counted and shown in bar chart with mean $\pm$ SD ($n=4-6$). Significant difference between healthy and OXA control group was assessed by Student's *t*-test: ^{##} $p < 0.01$, ^{###} $p < 0.001$. Significant difference among OXA control and all treatment groups was assessed by one-way ANOVA: ^{*} $p < 0.05$, ^{**} $p < 0.01$, ^{***} $p < 0.001$.

epidermal hyperplasia (Yeom et al., 2012). Our results showed that the three-herbs formula significantly reduced ear swelling, epidermal thickness and mice scratching behavior. These findings suggested that three-herbs formula exhibited the suppressive effect on skin irritation and anti-inflammatory activity *in vivo*. Furthermore, AD could cause a dry and cracked skin. The key factor in skin hydration and integrity is the presence of AQP (Boury-Jamot et al., 2009), which act mainly as water-selective pores. AQP3 is the predominant AQP in human skin, allowing glycerol to move into more superficial layers of the epidermis. Inhibition of AQP3 activity significantly decreased epidermal water transport. In contrast, several other studies reported that increased AQP3 was found in skin biopsies from AD patients in response to skin

stress (Hara-Chikuma and Verkman, 2008). There are other reports related to the increased expression of AQP3 in human atopic skin with dryness (Olsson et al., 2006). These reports confirmed the importance of AQP3 in skin physiology. In the present study, repeated oxazolone challenge downregulated AQP3 in mice ear tissue. Number of AQP3 was significantly increased in the three-herbs formula treated group, thereby indicating that three-herbs formula could elevate epidermal water transport and skin repair. These findings further proved the beneficial effect of topically use of three-herbs formula on cracked inflammatory skin.

Conclusion

In conclusion, this study has provided evidence that the topical application of the formula of Cortex Moutan, Herba Menthae, and *C. officinalis* in the weight ratio of 1:1:1 exhibited anti-inflammatory and anti-itching activity in atopic dermatitis like mice. This three-herbs formula markedly reduced cutaneous inflammation via down-regulation of inflammatory IL-1 β , IL-6 and IL-8, and suppressed an increase in stratum corneum dehydration through up-regulation of AQP3, also promoted wound healing in AD. These results suggest that three-herbs formula could be applied as a topical formula for the alleviation of inflammatory skin diseases atopic dermatitis.

Declaration of Competing Interest

The study is partially financially supported by Venture Tycoon Ltd.. Venture Tycoon Ltd. acts as our Industry Co-Applicant for the Innovation and Technology Fund, Innovation and Technology Commission, Hong Kong.

Authors' contributions

X-XW designed the study, carried out animal studies, analyzed the data and wrote the manuscript. W-SS and C-LW participated in the design of the study and revised the manuscript. XL and WC carried out the *in vitro* and animal studies. HM, C-WL participated in manuscript revision. M-ST participated in data analysis. C-LW, C-LC, C-MK, B-SL and P-CL participated in the design of the study. C-KW conceived of the study, participated in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript. All data were generated in-house, and no paper mill was used. All authors agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153691.

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Supplementary Materials

Effect of the topical application of a tri-herb formula on inflammatory dry-skin condition in mice with oxazolone-induced atopic dermatitis

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Material and methods

Herbal material authentication and extraction

A detailed description of the gradient elution system used for LCMS analyses is shown in **Table S1**.

Upon chemical authentication, herbarium voucher specimens of all tested herbs were deposited at the museum of the Institute of Chinese Medicine at CUHK, with voucher specimen numbers as listed in **Table S2**.

Table S1. Gradient elution system used for LCMS analyses

Time (mins)	Mobile phase A	Mobile phase B
0	90	10
5	90	10
6	85	15
10	77	23
26.5	0	100
30	0	100

Table S2. Herbarium voucher specimen numbers of all tested herbs

Herbs	Voucher Specimen Numbers
Cortex Moutan	3591
Herba Menthae	3592