



Anti-atopic effect of *Viola yedoensis* ethanol extract against 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin dysfunction

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ABSTRACT

Ethnopharmacological relevance: *Viola yedoensis* Makiho (VY, Violaceae) is a well-known medicinal herb in Chinese medicine, which is traditionally used to treat inflammation-related disorders, including allergic skin reactions. Although studies have uncovered its anti-inflammatory effects and corresponding bioactive constituents, the exact mechanism of action is still unclear in treating allergic skin reactions.

Objective: Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by severe pruritus, dry, edema and inflamed skin. It affects people's quality of life seriously and causes huge economic losses to society. This study proposes VY as a possible remedy for atopic dermatitis since its traditional usage and superior anti-inflammatory effects.

Materials and methods: Atopic dermatitis-like skin lesion was induced by topical application of 2,4-dinitrochlorobenzene (DNCB) in ICR mice. After treatment with *Viola yedoensis* Makiho ethanol extract (VYE) or dexamethasone (positive control) for 3 weeks, skin pathological observation and the molecular biological index were performed for therapeutic evaluation, including visual inspection in the change of the stimulated skin, scar formation, pathological morphology by hematoxylin and eosin (HE) staining, the measurement of interleukin IL-1 β , IL-6 and tumor necrosis factor- α (TNF- α) levels in serum as well as spleen index. The expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) were analyzed by western blot. The ratio of CD4+/CD8+ T lymphocyte in the spleen was detected by flow cytometry. Meanwhile, immunohistochemistry staining for CD68 identified the number of activated macrophages in skin lesions. Additionally, a reliable ultrahigh-performance liquid chromatography coupled with a Q exactive hybrid quadrupole-orbitrap mass spectrometry (UHPLC-Q-Orbitrap-MS) method was established for the systematic identification and characterization of main components in VYE.

Results: VYE alleviated DNCB-stimulated AD-like lesions symptoms as evidenced by a significant decrease in hypertrophy, hyperkeratosis, and infiltration of inflammatory cells in dorsal skin. The levels of IL-1 β , IL-6, and TNF- α in serum were suppressed in mice treated with VYE as compared to the DNCB-induced model group. Also, the administration of VYE reduced the ratio of CD4+/CD8+ T lymphocyte in the spleen and the number of activated macrophages stimulated by DNCB. Besides, the expression of iNOS and COX-2 were down-regulated in the dorsal skin.

Conclusions: VYE showed therapeutic effects on atopic dermatitis in DNCB-induced AD-like lesion mouse models by inhibiting the T cell-mediated allergic immune response. Our results indicated that VY could act as a potential remedy for atopic dermatitis.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory cutaneous disease

with high morbidity, characterized by epidermal hyperplasia and relapsing eczema with pruritus (Katoh et al., 2019). The specific pathogenesis of AD is complex and involves genetic factors (Apfelbacher et al., 2011),

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epidermal barrier dysfunction (Jungersted et al., 2010), disordered microbiome, immunological dysregulation and inflammation (Langan et al., 2020). It was estimated that AD occurs in approximately 10% of adults and 20% of children worldwide (Odhiambo et al., 2009), making the major contributor to the global burden of skin disease. Studies on quality of life in children and young adults reported that atopic dermatitis negatively impacted numerous aspects of patients in physical, emotional, and social functioning (Langan et al., 2020). The management of atopic dermatitis remains a challenge in clinical practice, with most approaches limited to symptomatic, unspecific anti-inflammatory, or immunosuppressive treatments (Simpson et al., 2017). Topical corticosteroids are widely endorsed as the first-line anti-inflammatory therapy. However, their continuous use or long-term use can cause local side-effects, including skin atrophy, telangiectasias, purpura, striae, and facial acneiform changes (Barnes et al., 2015). For these reasons, more researches have focused on identifying remedies for atopic dermatitis from Chinese medicine with few unpleasant adverse effects.

Cutaneous inflammation is a hallmark in atopic dermatitis, which is regulated by diverse inflammatory cytokines (Park et al., 2015; Yoon-Young and HoYoungKim, 2018). The production of first-line cytokines (IL-1 β , IL-6, and TNF- α) by epithelial cells is now regarded as a key initiatory event for the induction of allergic responses at epithelial interfaces (Olivry et al., 2016; Danso et al., 2014). High levels of IL-1 β , IL-6, and TNF- α were recorded in the serum of AD patients, with a direct correlation with AD severity (M Ee Phansan et al., 2012; Nutan et al., 2012). Besides, CD4⁺ T cells and macrophages in the skin lesions are involved in the regulation of AD pathogenesis. Over-expression of CD4⁺ T cells and activation of macrophages have been found in atopic dermatitis (Langan et al., 2020). A large number of studies have demonstrated that macrophages are accumulated in acutely or chronically inflamed skin and promote the production of cytokines and expression of inflammatory protein (COX-2 and iNOS), thereby leading to the aggressive progression of severe AD (Kasraie and Werfel, 2013).

Viola yedoensis Makiho (VY) is one of the commonly used Traditional Chinese Medicines and belongs to the violet family of Violaceae with its dried whole plant (including the roots) called “Zi Hua Di Ding” in China (Chinese Pharmacopoeia Commission, 2020). “Zi Hua Di Ding” decoction was traditionally used to treat inflammation-related disorders, including snake bites, allergic skin reactions, and hepatitis by topical or oral administration (Li and Wei, 2008). The chemical compositions of VY are diverse and complex, mainly including flavonoids and its glycosides, organic acids, phenols, alkaloids, peptides, saponins, sesquiterpenes, and coumarins (Chen et al., 2010). Recent studies have suggested that VY extract exerts various biological activities and pharmacological functions, such as anti-HIV, anti-coagulant activities, and protective effects against LPS-induced acute lung injury in mice (Wang et al., 2008; Zhou et al., 2009; Li et al., 2012). Furthermore, VY was capable of inhibiting iNOS and COX-2 activities and down-regulating the expression of inflammatory cytokines (such as IL-1 β , TNF- α , IL-6, and iNOS) to block the inflammatory development in various disease processes (Jeong et al., 2016). Nevertheless, the anti-atopic effect of VY in atopic dermatitis-like skin dysfunction has not been investigated. In this study, we aimed to investigate the therapeutic effect of VY against DNCB-induced AD-skin dysfunction in mice and uncover the anti-inflammatory and immune-regulatory mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

The whole herb of *Viola yedoensis* Makiho was provided by the Zisun Herbal Pharmaceutical Co., Ltd. (Guangzhou, China) and identified by Prof. Qilai Cheng. A voucher specimen (voucher number CM-VY-20200315) was deposited in the Herbarium of Traditional Chinese Medicine, Gannan Medical University. Compound dexamethasone acetate gel (DEX) containing 0.075% dexamethasone was obtained by

Renhe Pharmacy Co., Ltd. (Jiujiang, China). 2, 4-dinitrochlorobenzene (DNCB) and other organic solvents were purchased from Reagent Company (Shanghai, China). CD68 antibody (Cambridge, MA, USA) was utilized to identify macrophages. Mouse IL-6 Valukine ELISA kit, Mouse IL-1 β Valukine ELISA kit, and Mouse TNF- α Valukine ELISA kit were purchased from Novus Biologicals (Littleton, CO, USA). For western analysis, rabbit polyclonal anti-COX2 antibody and rabbit polyclonal anti-GAPDH were purchased from Servicebio technology Co., Ltd. (Wuhan, China), and rabbit anti-iNOS polyclonal antibody was purchased from Abcam (Cambridge, UK). The Mouse T Lymphocyte Subset Antibody Cocktail for flow cytometry was purchased from eBioscience (San Diego, CA, USA), including anti-mouse CD4-PE, anti-mouse CD8a-APC, and anti-mouse CD3e-FITC.

2.2. Preparation and quality evaluation of the blank and VYE gels

2.2.1. Extraction and concentration of *Viola yedoensis*

Air-dried whole plant of *Viola yedoensis* (300g) was powdered and extracted with 75% (v/v) ethanol (1:10) in a round-bottom flask with a temperature-controlled electric jacket for reflux extraction twice with the following conditions: 60 °C as the extraction temperature, and 120 min as the extraction time. Then, the extract was filtrated through Whatman filter paper (Grade 1, 11 μ m) and vacuum-dried in a rotary evaporator at 40 °C until the excess solvent was completely removed to obtain the concentrated extract. It was followed by freezing at –80 °C and freeze-drying via vacuum freeze drier (Lichen Instrument Technology, Shanghai, China). The lyophilized extract was deposited at –20 °C until use.

2.2.2. Preparation of gels

The gel formulation was based on previous method with minor modification (Sun et al., 2021). The blank gel was prepared by dissolving 1 wt% of carbomer 940, 5 wt% of glycerin, 0.2 wt% of polysorbate 80, 5 wt% of ethanol, and 0.05 wt% of ethylparaben in distilled water, followed by adjusting the pH to 6.5 using triethanolamine. Thereafter, blank gel and concentrated extract of *Viola yedoensis* were mixed at a ratio of 97.5:2.5 (w/w) to formulate the VYE-L gel, and at a ratio of 95:5 (w/w) to formulate the VYE-H gel, respectively.

2.2.3. Quality evaluation of gels

Throughout the study period, the following tests were performed to evaluate the quality of the ready-prepared gel: organoleptic inspection to detect homogeneity, color or odor changes and pH measurements. Besides, the blank and VYE gels were subjected to centrifugation at 300 $\times$ g for 10 min at 20 °C to observe stratification.

2.3. UHPLC-Q-orbitrap-MS analysis of VYE

2.3.1. Liquid chromatography

Chromatographic separation was performed using the UltiMate 3000 Rapid Separation LC system (Thermo Fisher Scientific, Bremen, Germany) by RP-C₁₈ column (2.1 $\times$ 150 mm, 1.8 μ m, Welch Materials, Maryland, USA). The gradient elution was composed of two solvents: Solvent A (water containing 0.1% formic acid) and Solvent B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.3 mL/min with the column temperature of 35 °C, and injection volume of 5 μ L. The binary gradient elution using the following gradients: 0–1min (2% B), 1–5 min (2–20% B), 5–10 min (20–50% B), 10–15 min (50–80% B), 15–20 min (80–95% B), 20–25 min (95% B), 25–26 min (95–2% B), and 26–30 min (2% B).

2.3.2. Mass spectrometry

The MS analysis was conducted with Q-Exactive Orbitrap (Thermo Fisher Scientific, Bremen, Germany) in Full Scan/Data Dependent (full MS/dd-MS₂) acquisition mode. An electrospray ionization (ESI) probe was used to nebulize and ionize samples in both positive and negative

ion modes. The following ESI parameters were used: scan range 150–2000 m/z, full mass 70000 FWHM, dd-MS₂ 17500 FWHM, spray Voltage 3.8 kV, capillary temperature 300 °C, auxiliary gas heater temperature 350 °C, sheath gas flow 40 Arb, data collection time 30.0 min.

2.4. Animals

Male mice of the strain ICR (8 weeks old ; 20–24 g; purchased from Hunan SJA Laboratory Animal Co, Ltd.) were housed in an environment with a temperature of 24 ± 2 °C, the humidity of $50 \pm 10\%$ and 12 h/12 h light/dark cycle according to the Institutional Animal Care and the Institutional Animal Ethical Committee (IAEC) of Gannan Medical University (IAEC Code Number: 11/2013). All animals were allowed free access to tap water and standard chow and were acclimatized under normal environmental conditions for 7 days before experimentation.

2.5. Skin irritation test of VYE

Skin irritation over a period of 14 days was assessed in accordance with the CFDA technical research guidelines for toxicity studies (China Food and Drug Administration, 2014). Mice were randomly divided into three groups (control, VYE-L, and VYE-H). After the dorsal fur of mice was shaved and depilated with Veet depilatory paste (Reckitt Benckiser, Massy, France), a 2×2 cm² area of skin was treated with 100 μ L blank gel, 100 μ L VYE-L gel, and 100 μ L VYE-H gel, respectively. The gel was administrated twice a day (namely 200 μ L VYE-L gel equal to 5 mg VYE per day and 200 μ L VYE-L gel equal to 10 mg VYE per day, respectively). Abnormality in skin was monitored daily for 14 days. Mortality, changes in the skin, presence or absence of piloerection and exophthalmos, abnormal posture were observed. Also, body weight changes were recorded every 7 days. At the end of the experiment, all mice were sacrificed and skins were excised for H&E staining to observe the topical tissue damage and inflammation.

2.6. Induction of AD-like skin lesions in ICR mice by DNCB and VYE intervention

Adult male mice were allocated into five groups (n = 10 per group) randomly, including control, DNCB, DEX-0.1 mg/day, VYE-5 mg/day, and VYE-10 mg/day group. AD-like skin dysfunction was induced by DNCB dissolved in a vehicle containing olive oil and acetone (1:4) in the hairless dorsal skin of ICR mice except for the control group (Fan et al., 2019). Briefly, the dorsal of mice was shaved and depilated with Veet depilatory paste to remove all hair from the area of approximately 2 cm $\times$ 2 cm in size 24 h before treatment with 100 μ L of 7% (w/v) DNCB. After 5 days, 5 μ L of 0.5% (w/v) DNCB was applied to both sides of the right ear of the test group and the control group received the vehicle

(olive oil/acetone) every three days. On the dorsal skin and both sides of the right ear, the control and DNCB groups were treated with 100 μ L blank gel twice a day, whereas the DNCB-DEX and DNCB-VYE groups were treated with 100 μ L of DEX gel (compound dexamethasone acetate gel mixed with the blank gel at a ratio of 2:1, equal to 0.1 mg dexamethasone per day) or VYE gels twice a day, respectively (200 μ L VYE-L gel equal to 5 mg VYE per day; 200 μ L VYE-H gel equal to 10 mg VYE per day). We measured the right ear thickness 24 h before the initial DNCB applied treatment. AD progression was assessed by measuring the right ear thickness using a micrometer 24 h after each DNCB treatment. Blood samples were collected from the experimental mice by retro-orbital puncture 2 h after the last dose of treatment, then centrifuged at 3000 rpm for 15 min to obtain serum, which was stored at -80 °C for additional experiments. Next, all mice were humanely killed, and their dorsal skins were collected from the mice for further processing. The experimental scheme was summarized in Fig. 1.

2.7. Histopathological evaluation of atopic dermatitis-like skin lesions

To evaluate histopathological characteristics, dorsal skin from ICR mice was fixed with 4% (w/v) paraformaldehyde for 24 h at room temperature and embedded in paraffin blocks. Sections (5 μ m) were cut using a microtome, then stained with hematoxylin and eosin (H&E). CD68 immunohistochemistry staining was performed to detect the accumulation of macrophages in the skin. Images were then captured under a microscope (Nikon TE2000-S; Nikon) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

2.8. Determination of serum IL-1 β , IL-6, and TNF- α concentrations

Serum IL-1 β , IL-6, and TNF- α were determined by mouse IL-1 β , IL-6, and TNF- α ELISA kits (Novus Biologicals, Littleton, CO, USA), respectively. All procedures were performed following the manufacturer's instructions.

2.9. Spleen index and flow cytometry

After the mice were sacrificed, each spleen was weighed to calculate the spleen index: spleen index (g/g) = spleen weight (g)/body weight $\times$ 100% (g). To analyze the CD4⁺ and CD8⁺ lymphocytes, spleen single-cell suspensions were prepared by grounding in incomplete Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA), followed by red blood cell lysis. After washing by phosphate-buffered saline (PBS), lymphocytes were collected by filtering through a 200-gauge mesh. Splenocytes (1×10^6 cells) were stained with the Mouse T Lymphocyte Subset Antibody Cocktail (anti-CD3, anti-CD4, and anti-CD8, BD Biosciences), and different T cell subsets were analyzed in BD Accuri C6 flow cytometer followed by data analysis using FlowJo

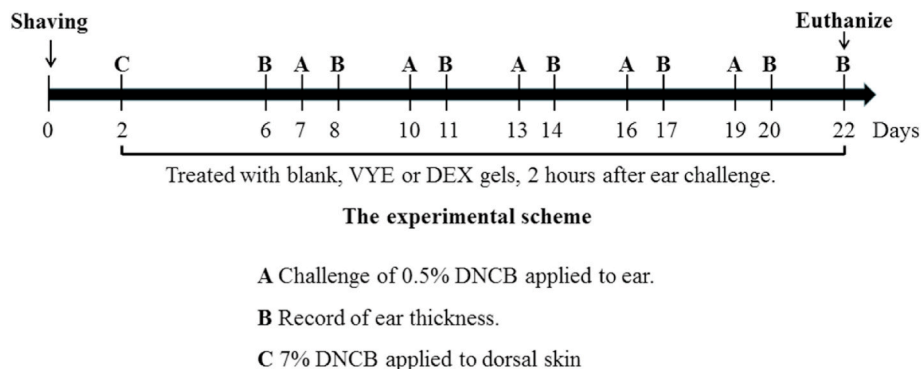


Fig. 1. Experimental scheme. AD-like skin dysfunction mouse model was induced by 7% (w/v) DNCB on the dorsal skin on day 2. Additional applications of 0.5% (w/v) DNCB were administered on days 7, 10, 13, 16, and 19. Ear thicknesses were also recorded. VYE, *Viola yedoensis* ethanol extract; DEX, dexamethasone; DNCB, 2,4-dinitrochlorobenzene.

analytical software.

2.10. Determination of tissue COX-2 and iNOS expression

The expressions of COX-2 and iNOS protein in the dorsal skin were detected by Western blot analysis as described previously. To determine the levels of COX-2 and iNOS, tissues were homogenized by RIPA buffer with protease inhibitor (Servicebio, Wuhan, China) and centrifuged for 10 min at 12000 rpm at 4 °C. Then a BCA protein assay Kit (Servicebio, Wuhan, China) was used to measure the total protein concentration in the collected supernatant. Equal amounts of protein (40 µg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. After transferring, membranes were blocked with 5% nonfat milk in tris-buffered saline-Tween 20 (T-BST) at 25 °C for 60 min and then incubating overnight at 4 °C with primary antibodies against COX-2, iNOS, and GAPDH (all diluted 1:3000). Primary antibodies were removed and blots were washed 3 times with T-BST for 5 min and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution in 5% nonfat milk) at room temperature for 1 h. Quantification of the western blot signals was performed using computerized densitometry (KODAK Image Station 4000 MM Digital Imaging System, Rochester, NY).

2.11. Statistical analysis

Statistical analysis was conducted with SPSS 21.0 software. Data were presented as mean $\pm$ standard errors of the means, and one-way analysis of variance was used to examine the differences and least

significant difference post hoc test for multiple comparisons. A value of $P < 0.05$ is considered statistically significant and $P < 0.01$ considered highly significant.

3. Results

3.1. VYE gels showed no observable toxicity in mice

Throughout the experimental period of acute skin irritation test, no significant body weight changes (Day 7, Control: 25.3 ± 0.48 g, VYE-L: 25.1 ± 0.56 g, VYE-H: 25.4 ± 0.51 g; Day 14, Control: 30.6 ± 0.78 g, VYE-L: 29.7 ± 0.66 g, VYE-H: 31.1 ± 0.73 g), abnormal skin signs (Fig. 2A) or mortality were occurred in mice treated with different dosage of VYE (VYE-5 mg/day and VYE-10 mg/day). The histomorphological analysis by H&E staining also revealed no significant structural changes in skin tissue as evidenced by integrated epidermis, dermis and hair follicle or inflammatory responses (Fig. 2B). According to the previous references, 5% (w/w) was a frequently-used dose for testing the pharmacological effects of herbal extract in skin illness-related animal experiments (Balekar et al., 2012). Meanwhile, based on the results of skin irritation test, dose response studies of VYE against DNCB-induced AD-like skin dysfunction were conducted with administration dosage of 2.5%, 5% (w/w).

3.2. VYE alleviated DNCB-induced AD-like symptoms in ICR mice

AD-like symptoms mouse model were introduced by the epicutaneous application of DNCB on shaved dorsal skin and ears (Krupp et al., 2014). To investigate the therapeutic effects of VYE

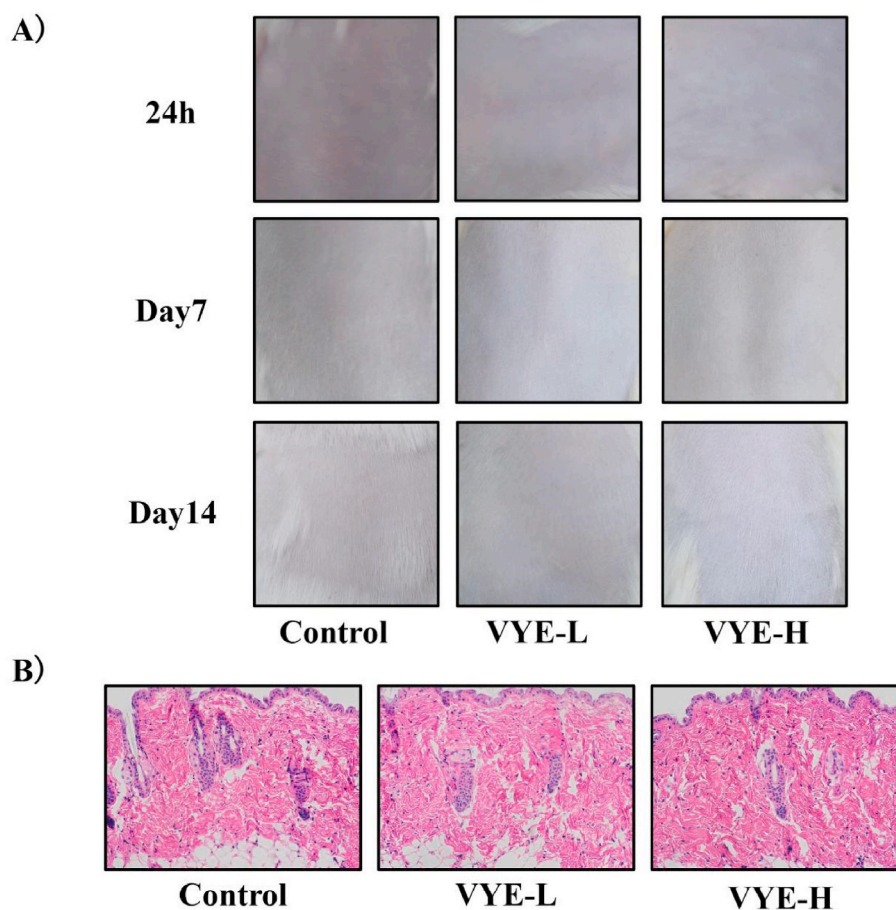


Fig. 2. VYE gels showed no observable toxicity in ICR mice. Representative skin pictures were captured after topical treatment for 7 and 14 days. (A). Hematoxylin and eosin (H&E) staining (magnification: $200 \times$), (B). VYE-L, *Viola yedoensis* ethanol extract gel (2.5%, w/w); VYE-H, *Viola yedoensis* ethanol extract gel (5%, w/w).

on the DNCB-induced AD-like symptoms in the ICR mice, the dorsal skin tissues were collected for H&E and CD68 immunohistochemistry staining, as well as the measurement of ear thickness. The DNCB-induced AD mouse model had previously shown typical allergic responses including increased ear and epidermal thickness, swelling, dryness, and erythema (Jin et al., 2009). Pathological observations showed that VYE and DEX gels application improved AD-like skin lesions, such as swelling, dryness, and erythema (Fig. 3A). Dermal infiltration with macrophages and epidermal hyperplasia were hallmark features of changes in AD animal models (Jin et al., 2009), whereas VYE and DEX reversed these

alterations (Epidermal thickness, Control: $23.61 \pm 0.76 \mu\text{m}$, DNCB: $252.89 \pm 7.41 \mu\text{m}$, VYE-5 mg/day: $172.05 \pm 5.44 \mu\text{m}$, VYE-10 mg/day: $123.24 \pm 5.86 \mu\text{m}$, DEX-0.1 mg/day: $87.69 \pm 2.54 \mu\text{m}$, Fig. 3B, E; Number of macrophage, Control: 56.13 ± 3.13 , DNCB: 171.63 ± 6.07 , VYE-5 mg/day: 146.38 ± 6.24 , VYE-10 mg/day: 90.63 ± 4.50 , DEX-0.1 mg/day: 74.38 ± 4.68 , Fig. 3C and D). The DNCB-treated group demonstrated increased ear thickness, which was suppressed in the VYE-treated group and DEX-treated group significantly. Further, the contrast of ear thickness became more obvious with the passage of time (Ear thickness on day 22, Control: $0.351 \pm 0.014 \text{ mm}$, DNCB: $0.529 \pm$

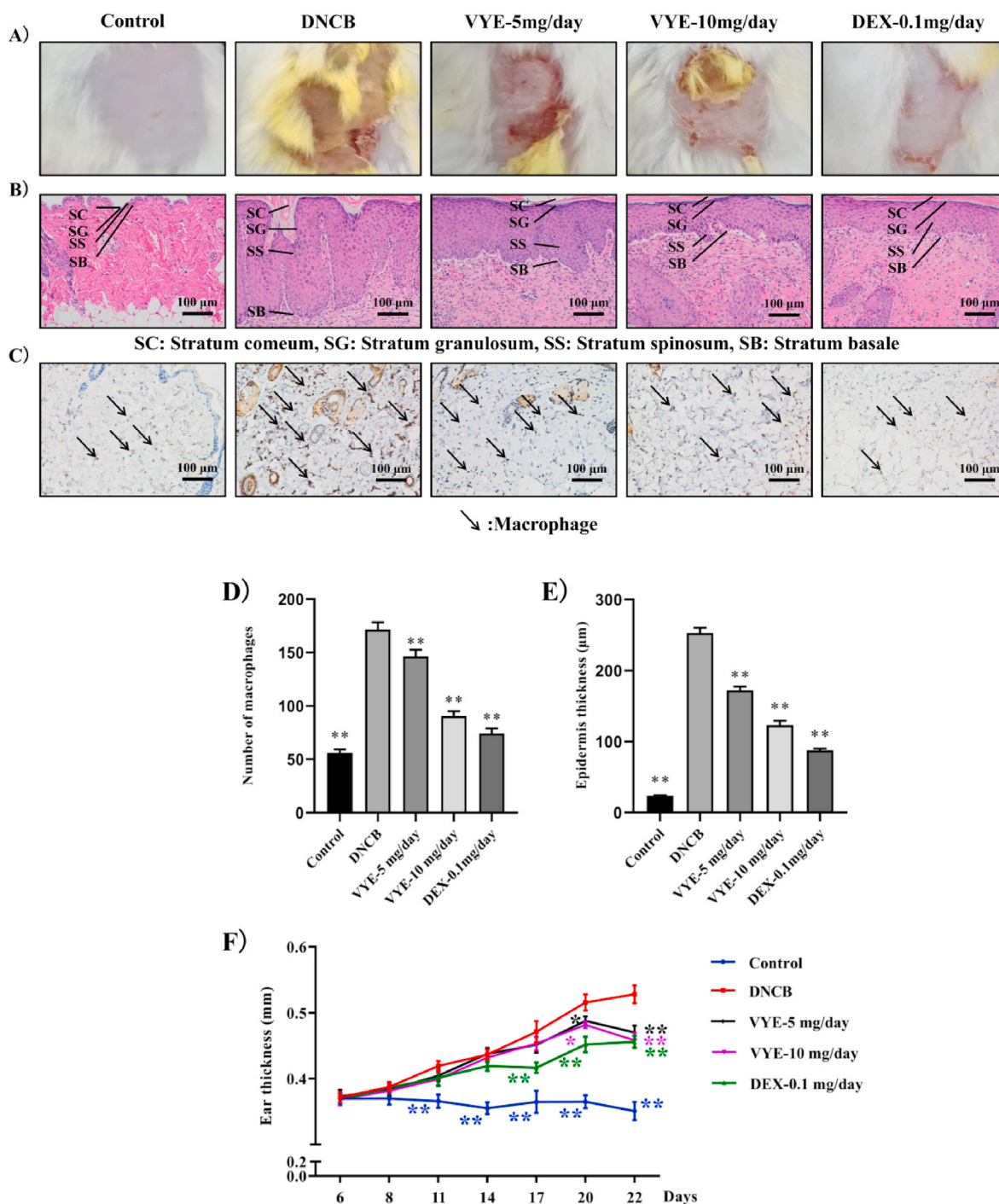


Fig. 3. VYE improved DNCB-induced AD-like skin lesions and reduced dermal infiltration of macrophages in a dose-dependent manner. Clinical features of mouse skin (A). Hematoxylin and eosin (H&E) staining (magnification: $200\times$, B). CD68 immunohistochemistry staining (C). The number of macrophages (D). The epidermal thickness of dorsal skin tissues (E). Ear thickness (F). Data are presented as the means $\pm$ SEM, * $P < 0.05$ vs DNCB, ** $P < 0.01$ vs DNCB. VYE, *Viola yedoensis* ethanol extract; AD, atopic dermatitis. DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone.

0.135 mm, VYE-5mg/day: 0.470 ± 0.011 mm, VYE-10 mg/day: 0.459 ± 0.011 mm, DEX: 0.456 ± 0.009 mm, Fig. 3F). These results indicated that the application of VYE and DEX had significant attenuating effects on AD.

3.3. Effects of VYE on the IL-1 β , IL-6, and TNF- α concentrations in serum

Data with respects to concentrations of IL-1 β , IL-6, and TNF- α in the serum showed that mouse with AD-like skin dysfunction had greater concentrations of IL-1 β , IL-6, and TNF- α compared with that of normal mice in this study. Moreover, DEX-0.1 mg/day, VYE-5 mg/day, and VYE-10 mg/day significantly decreased the serum IL-1 β , IL-6, and TNF- α concentrations compared with that of the DNCB group (IL-1 β , Control: 144.74 ± 20.85 pg/mL, DNCB: 325.64 ± 20.63 pg/mL, VYE-5 mg/day: 190.82 ± 15.23 pg/mL, VYE-10 mg/day: 153.35 ± 5.19 pg/mL, DEX: 148.57 ± 9.27 pg/mL; IL-6, Control: 113.66 ± 31.02 pg/mL, DNCB: 252.64 ± 20.58 pg/mL, VYE-5 mg/day: 161.55 ± 17.04 pg/mL, VYE-10 mg/day: 131.79 ± 38.65 pg/mL, DEX: 121.20 ± 12.23 pg/mL; TNF- α , Control: 102.45 ± 22.18 pg/mL, DNCB: 223.39 ± 20.02 pg/mL, VYE-5 mg/day: 151.85 ± 15.67 pg/mL, VYE-10 mg/day: 132.15 ± 12.36 pg/mL, DEX: 121.73 ± 11.78 pg/mL) (Fig. 4). These results showed that VYE had anti-inflammatory activity.

3.4. VYE decreased iNOS and COX-2 expression in the skin lesions of DNCB-treated ICR mice

Representative pro-inflammatory enzymes, such as iNOS and COX-2, were overexpressed in atopic dermatitis, and amplified the inflammatory response. Thus, western blot analysis was performed to examine whether VYE affected iNOS and COX-2 protein expressions. Stimulation with DNCB in ICR mice by topical applications, resulted in accumulation of iNOS and COX-2 proteins (COX-2/GAPDH, Control: 0.218 ± 0.075 , DNCB: 0.715 ± 0.128 ; iNOS/GAPDH, Control: 0.173 ± 0.050 , DNCB: 0.795 ± 0.096), as determined by Western blot analysis. VYE significantly reduced the level of iNOS protein (iNOS/GAPDH, DNCB: 0.795 ± 0.096 , VYE-5 mg/day: 0.298 ± 0.091 , VYE-10 mg/day: 0.188 ± 0.088). Especially, the high dose of VYE (10 mg/day) showed a remarkable effect on the inhibition of COX-2 and iNOS expression (COX-2/GAPDH, DNCB: 0.715 ± 0.128 , VYE-5 mg/day: 0.665 ± 0.128 , VYE-10 mg/day: 0.283 ± 0.123) (Fig. 5).

3.5. VYE restored the enhanced CD4 + /CD8 + ratio in the spleen and spleen index

In this study, the DNCB-treated group demonstrated an increase in spleen index and the ratios of CD4 + /CD8 + in the spleen. After 21 days

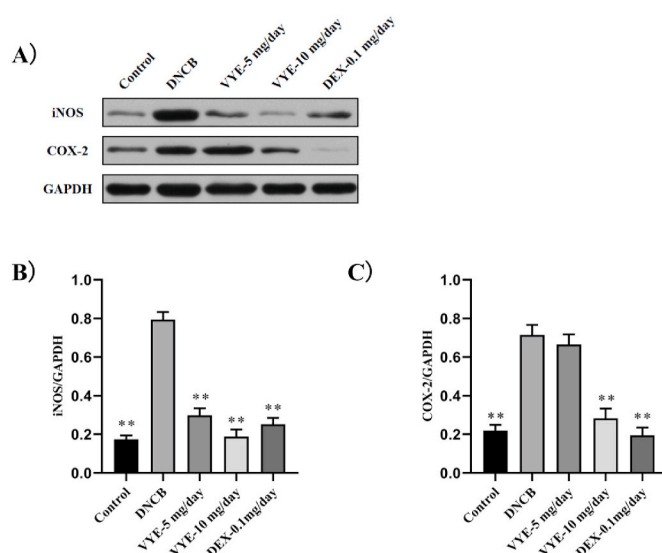


Fig. 5. Effects of topical VYE on protein expression of iNOS and COX-2 in dorsal skin tissues. The protein expression of COX-2 and iNOS were detected by Western blotting, and quantified by densitometry (A). Relative expressions of COX-2 and iNOS were normalized to GAPDH internal control (B and C). Data were presented as the means $\pm$ SEM, **P < 0.01 vs DNCB. VYE, *Viola yedoensis* ethanol extract; DNCB, 2,4-dinitrochlorobenzene; COX, cyclooxygenase; iNOS, inducible NO synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

of treatment, the ratios of CD4 + /CD8 + in the spleen was increased in the DNCB group (3.88 ± 0.17) as compared to the control group (2.27 ± 0.04). Whereas, VYE-5 mg/day application suppressed the ratios to 3.21 ± 0.07 and VYE-10 mg/day application suppressed the ratios to 2.84 ± 0.07 (Fig. 6A and B). Spleen index was increased in the DNCB group (0.31 ± 0.02) compared with the control group (0.23 ± 0.01). VYE-5 mg/day (0.29 ± 0.05) had no significant action on spleen index compared with the DNCB group. But VYE-10 mg/day application decreased spleen index to 0.26 ± 0.03 (Fig. 6C). Moreover, the DEX-treated group had an over-inhibited immune response, with the spleen CD4 + /CD8 + T lymphocyte ratio and spleen index (1.72 ± 0.13 and 0.13 ± 0.02 , respectively, P < 0.01) being even lower than those of the control group. Thus, DNCB was expected to excessively up-regulate immune responses. However, VYE dose-dependently inhibited the immune response induced by DNCB at least in part.

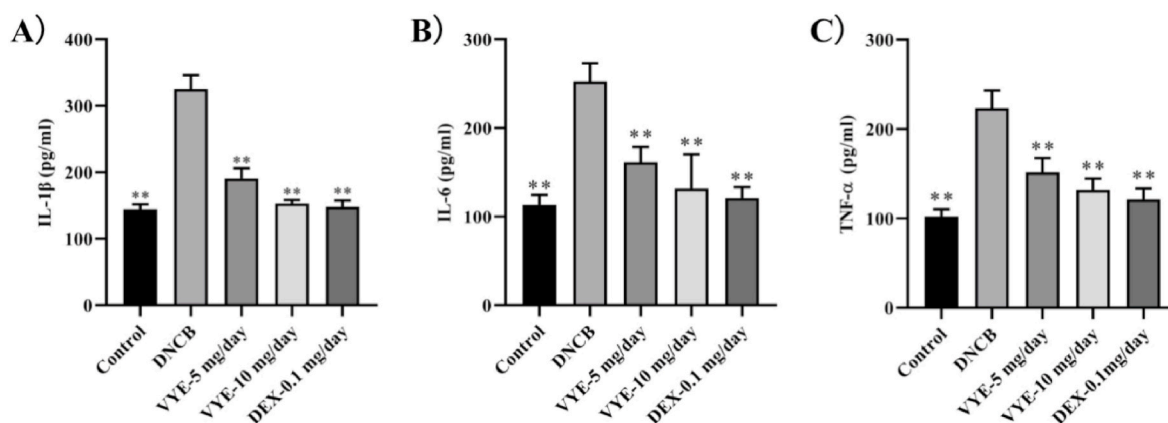


Fig. 4. VYE dose-dependently reduced IL-1 β , IL-6, and TNF- α levels in the serum as presented in figure A, figure B, and figure C, respectively. Data were presented as the means $\pm$ SEM, **P < 0.01 vs DNCB. VYE, *Viola yedoensis* ethanol extract; DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone; IL, interleukin; TNF, tumor necrosis factor.

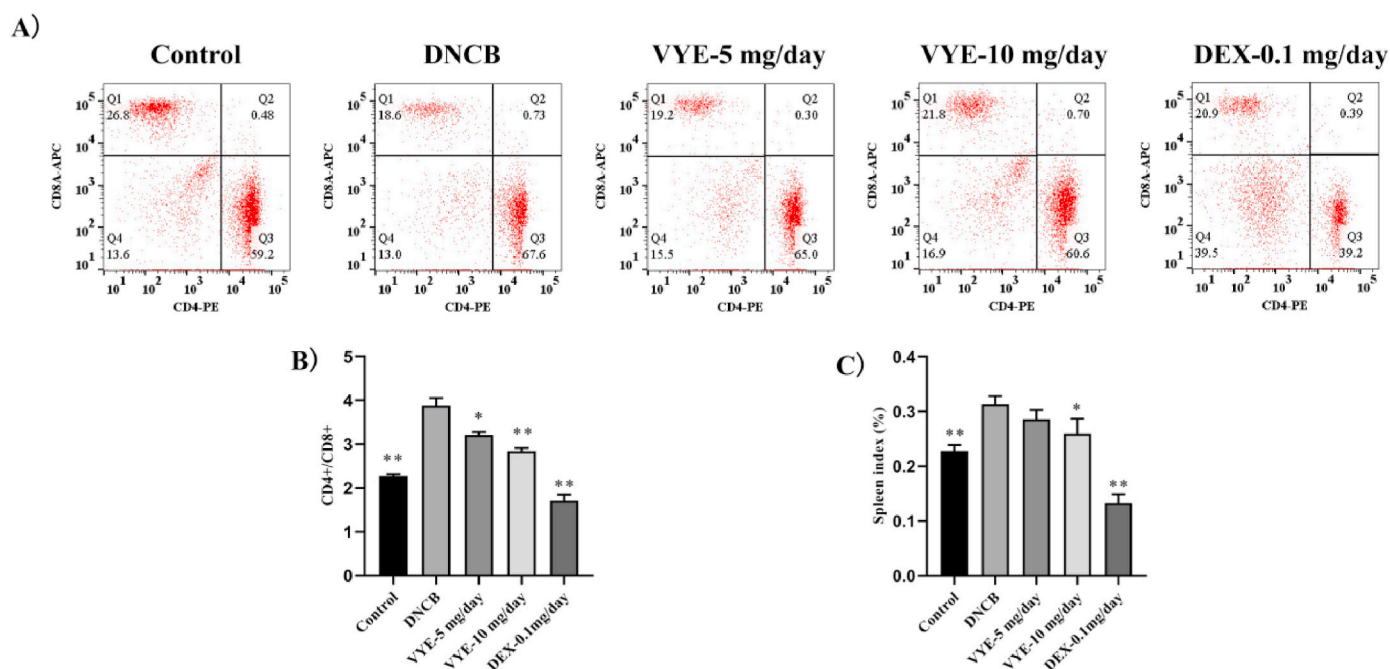


Fig. 6. VYE reduced the enhanced CD4 + /CD8 + ratio in the spleen and spleen index. Subsets of splenic CD4 and CD8 T cells assessed by flow cytometry (A). The spleen CD4⁺/CD8⁺ T lymphocyte ratio (B). Spleen index: spleen index (g/g) = spleen weight (g)/body weight (g) × 100% (C). Data were presented as the means ± SEM, *P < 0.05 vs DNCB, **P < 0.01 vs DNCB. VYE, *Viola yedoensis* ethanol extract; DNCB, 2,4-dinitrochlorobenzene; DEX, dexamethasone.

3.6. Chemical profiling and quality evaluation of the blank and VYE gels

According to retention time, precise molecular weight and characteristic fragmentation ions, 23 chemical components were tentatively characterized and identified from VYE by comparing with published references (Wang et al., 2008; Wang et al., 2008; Li et al., 2013), including 9 flavonoids, 5 coumarins, 5 organic phenolic acids and 4 triterpenes. Phytochemistry had revealed that VY mainly contained flavonoids, coumarins, and organic phenolic acids, all of which were reported to possess significant anti-inflammatory and immuno-regulatory activities (Jeong et al., 2016; Zeng et al., 2016). Along with our results that topical application of VYE could improve AD-like skin lesions in ICR mice by inhibiting inflammatory factors and regulating immune function, we speculated these chemical components (flavonoids, coumarins, and organic phenolic acids identified by high resolution LC-MS/MS) might be the potential active material basis in VY

responsible for anti-atopic effects against DNCB-induced atopic dermatitis-like skin dysfunction. Total ion current (TIC) chromatogram and chemical structures were presented in Fig. 7. The MS data and identification results were given in Table 1.

In addition, quality evaluation of the blank and VYE gels (2.5% and 5%, w/w) were conducted by character observation (homogeneity, color or odor changes), pH measure and centrifugal experiments. Results suggested that the blank gel was a homogeneous transparent gels and VYE gels (2.5% and 5%, w/w) was a homogeneous brown gel (shown in Fig. 6C) without bubble in appearance. The pH values for the blank gel, VYE-L gel and VYE-H gel were 6.50, 6.48 and 6.46, respectively. No stratification was observed under centrifugation at 300×g for 10 min, suggesting the high stability and good uniformity.

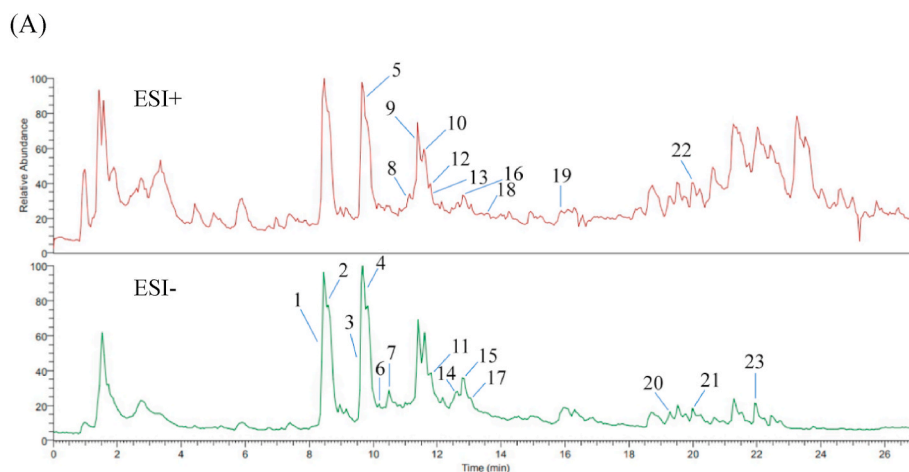
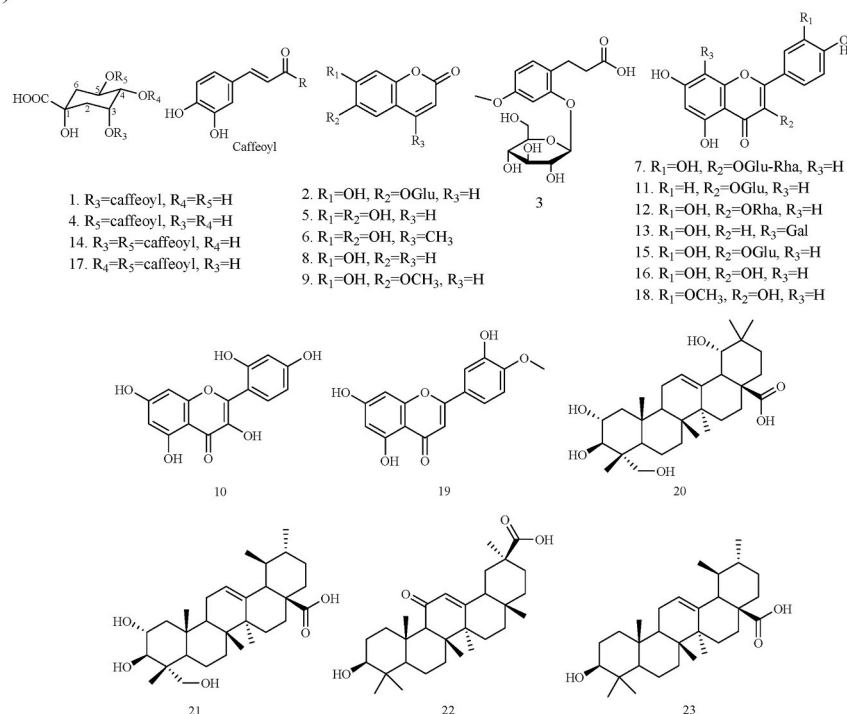


Fig. 7. (A) TIC chromatogram of VY extract in the positive/negative mode; (B) Chemical ingredients identified from VY extract; (C) Appearance of the blank and VYE gels (2.5% and 5%, w/w).

(B)



(C)

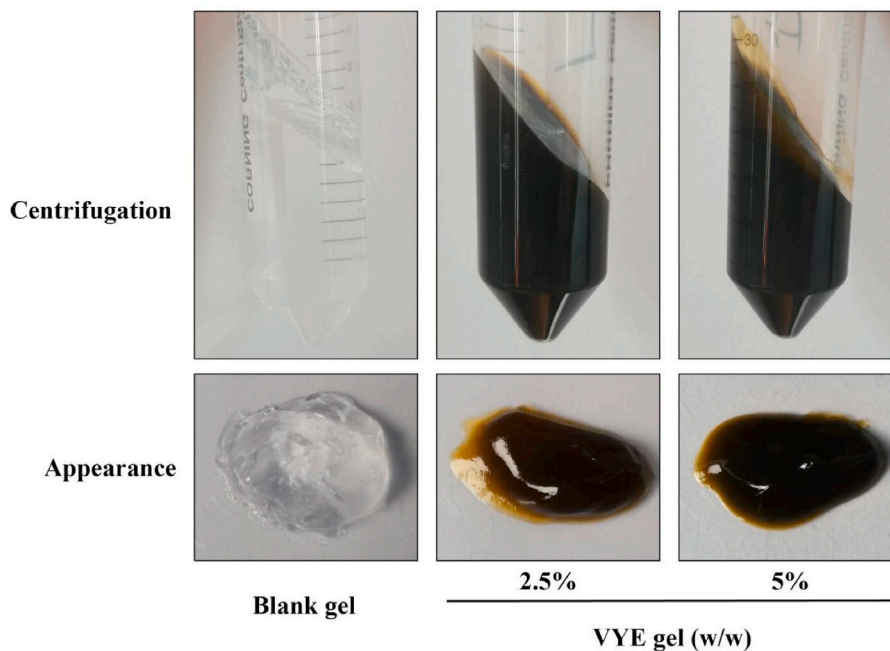


Fig. 7. (continued).

4. Discussion

AD is one of the most common inflammatory disorders which is characterized by pruritus, flushes, recurrent eczematous, and depression (Charlesworth and Ernest, 2001). It affects many aspects of patient's lives, including psychological stress, low self-esteem, and sleep deprivation, and is exacerbated by visible disease, stigma, and itch. These lead to adverse psychosocial consequences and health outcomes

(Deckert et al., 2014). AD can be a considerable high global burden in healthcare costs, including direct costs of treatment and indirect costs, such as negatively affecting the work performances of individuals. Topical corticosteroids are widely recommended as the first-line anti-inflammatory treatment for AD, and their long-term use results in many side effects, such as skin atrophy, purpura, striae, dyspigmentation, and declined immune function (Barnes et al., 2015). Therefore, the attempts to develop new effective remedies with low adverse drug reactions

Table 1
Identification of main components in *Viola yedoensis* by high resolution LC-MS/MS.

Peak	t _R (min)	Molecular formula	Quasimolecular ion (m/z)		Product ions (m/z)	Identification
			Measured	ppm		
1	8.13	C ₁₆ H ₁₈ O ₉	[M-H] ⁻ 353.08795	-0.41	191, 179, 161, 135	Neochlorogenic acid
2	8.59	C ₁₅ H ₁₆ O ₉	[M-H] ⁻ 339.0716	1.63	177, 133	Esculin
3	9.46	C ₁₆ H ₂₂ O ₉	[M-H] ⁻ 357.11948	-1.04	195	3-[2-(β-D-Glucopyranosyloxy)-4- methoxyphenyl]propanoic acid
4	9.63	C ₁₆ H ₁₈ O ₉	[M-H] ⁻ 353.088	-0.55	191, 179, 161, 135	Chlorogenic acid
5	9.65	C ₉ H ₆ O ₄	[M+H] ⁺ 179.0335	2.16	151, 133, 123, 105	Esculetin
6	10.19	C ₁₀ H ₈ O ₄	[M-H] ⁻ 191.03421	4.02	176	6,7-Dihydroxy-4-methylcoumarin
7	10.39	C ₂₇ H ₃₀ O ₁₆	[M-H] ⁻ 609.14594	0.28	301, 300, 271, 255, 151	Rutin
8	11.09	C ₉ H ₆ O ₃	[M+H] ⁺ 163.03876	1.3	135, 133, 105	7-Hydroxycoumarine
9	11.29	C ₁₀ H ₈ O ₄	[M+H] ⁺ 193.04942	0.6	178, 133	Scopoletin
10	11.53	C ₁₅ H ₁₀ O ₇	[M+H] ⁺ 303.04947	1.52	257, 229, 153, 137, 121	Morin
11	11.78	C ₂₁ H ₂₀ O ₁₁	[M-H] ⁻ 447.09369	-0.9	285, 284, 255, 227	Astragalin
12	11.79	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺ 449.10748	0.8	303	Quercitrin
13	11.81	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺ 449.107	1.87	357, 327, 299	1,5-Anhydro-1-[2-(3,4- dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-8-yl]-D-galactitol
14	12.41	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻ 515.11975	-0.48	353, 191, 179, 173, 161, 135	3,5-Dicaffeoylquinic acid
15	12.81	C ₂₁ H ₂₀ O ₁₂	[M-H] ⁻ 463.08838	-0.39	301, 300, 271, 255, 151	Quercetin-3β-D-glucoside
16	12.83	C ₁₅ H ₁₀ O ₇	[M+H] ⁺ 303.04953	1.32	257, 229, 165, 153, 137	Quercetin
17	13.09	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻ 515.11969	-0.37	353, 191, 179, 173, 151	4,5-Dicaffeoylquinic acid
18	13.67	C ₁₆ H ₁₂ O ₇	[M+H] ⁺ 317.06552	0.19	302, 153	Isorhamnetin
19	15.71	C ₁₆ H ₁₂ O ₆	[M+H] ⁺ 301.07047	0.65	286, 258	Diosmetin
20	19.17	C ₃₀ H ₄₈ O ₆	[M-H] ⁻ 503.33786	-0.09	485, 459, 441	Arjungenin
21	20.14	C ₃₀ H ₄₈ O ₅	[M-H] ⁻ 487.3428	0.2	469, 441, 423	Asiatic acid
22	20.17	C ₃₀ H ₄₆ O ₄	[M+H] ⁺ 471.34637	1.1	453, 427, 217, 203, 191, 121	18-β-Glycyrrhetic acid
23	21.98	C ₃₀ H ₄₈ O ₃	[M-H] ⁻ 455.35287	0.44		Ursolic acid

Glu:β-D-glucose; Rha: β-D-rhamnose; Gal:β-D-galactose.

remain an important priority.

VY is a traditional Chinese medicinal herb with remarkable medical and economic value that is widely used to treat inflammation-related diseases, including erysipelas, eczema, urticarial, acute lung injury, and acne vulgaris (Liao et al., 2010). Besides, previous studies had revealed that VY exerted anti-HIV effects, anticoagulant, antibacterial activities, and anti-inflammatory effect *in vitro* (Li et al., 2012; Zhou et al., 2009). However, its anti-atopic properties remained to be evaluated. In this study, a series of studies were conducted to demonstrate that VYE significantly alleviated the DNCB-induced AD-like disease in mice.

In this study, a series of studies were conducted to demonstrate that VYE significantly alleviated the DNCB-induced AD-like disease in mice. Our results suggested that topical application of VYE alleviated the DNCB-induced AD-like symptoms, such as diminished ear swelling, suppressed epidermal thickness, inhibited inflammatory cytokine production, and regulated the immune response in a dose-dependent manner. Recent studies had demonstrated that over-expression of TNF-α, IL-1β, and IL-6 were responsible for atopic dermatitis (Fan et al., 2019; Lee et al., 2020). We investigated the inhibitory effect of VYE against the expression of proinflammatory cytokines in the DNCB-induced AD mouse model. VYE reduced the production of TNF-α, IL-1β, and IL-6 in serum, which potentially contributed to the remission

of inflammatory response and the amelioration of AD-like skin dysfunction. These findings were consistent with previous reports, which showed that VY could downregulate inflammatory cytokines, such as that the petroleum ether fraction of VY had reduced TNF-α, IL-1β, and IL-6 in lipopolysaccharide-induced acute lung injury (Li et al., 2012), and that VYE (150 μg/mL) suppressed TNF-α and IL-1β production by lipopolysaccharide stimulation in mouse peritoneal macrophages.

Macrophages play a critical role in the immune system and contribute to a broad spectrum of pathologies processes. It is also crucial in host defense in the acute inflammatory phase by producing nitric oxide (NO) and numerous cytokines in response (Park et al., 2015), as well as associated with the development of various chronic inflammatory diseases (Hirayama et al., 2018). A study by optical reporter-gene-based molecular imaging to evaluate the effects of anti-AD therapeutic agents in immunocompetence, showed that the accumulation of macrophages was positively correlated with the severity of AD-like skin lesions (Lee et al., 2020). NO was a highly reactive free radical, which played a role in mediating numerous biological functions such as vasodilation, neurotransmission, blood coagulation, and immune regulation, etc. An excessive NO in inflammatory response aggravates localized tissue injury and led to chronic inflammation. iNOS was a major producer of NO when it was stimulated by bacterial lipopolysaccharides or various cytokines, such as IL-1β, IL-6,

and TNF- α (Orita et al., 2011). COX-2 was an inducible isoform of cyclooxygenase whose expression was enhanced in inflammation-related cells in response to stimulations by cytokines (Huang et al., 2012). Accordingly, evaluation of the anti-inflammatory effects of medicine through quantifying iNOS and COX-2 synthesis were considered the universal method. In the present study, long-term topical VYE showed considerable effects in remission of macrophages and suppressing protein expression levels, including iNOS, and COX-2 on dorsal skin tissues in DNCB-induced AD mice.

Cutaneous inflammation is central to the pathogenesis of AD. The complex interactions between cytokines, immune cells, and affected tissues result in sustained inflammation in AD-like skin dysfunction. Lesional skin of patients with AD shows a dysregulation of T-lymphocytes characterized by CD4⁺ over-expression (Verhagen et al., 2006). CD4⁺ T cells act as co-stimulators for macrophage activation via antigen presentation and the release of proinflammatory cytokines. Moreover, the CD4⁺/CD8⁺ T-cell ratio reflects the immune status of the body with a higher ratio being indicative of enhanced immune status. In confirmation of previous studies (Correa et al., 2014; Fan et al., 2019), we found that the ratio of CD4⁺/CD8⁺ was significantly increased in DNCB-treated mice compared to control group mice. Also, it was observed that topical administration of DEX and VYE significantly reduced CD4⁺/CD8⁺ ratio of the spleen in DNCB-induced AD-like mice, which was consistent with the suppression in spleen index. Meanwhile, CD4⁺/CD8⁺ ratio and spleen index was exhibited very low levels in the DEX-treated group, suggesting that long-term topical DEX (21 days in this study) might increase the risk of over-immunosuppression. Thus, VYE was a potential novel therapeutic remedy for AD by improving skin dysfunction without the risk of over-immunosuppression and treatment-related side effects.

5. Conclusions

We uncovered the therapeutic effects of VYE on atopic dermatitis by DNCB-induced AD-like lesion mouse models. Topical application of VYE improved the progression of AD-like lesions, including ear swelling, epidermal hyperplasia inflammatory cell infiltration, and skin lesion severity. Additionally, it was demonstrated that the anti-atopic effects were exerted through down-regulating the expression of inflammatory cytokines in the serum, reducing iNOS and COX-2 levels in the skin tissues, and decreasing the CD4⁺/CD8⁺ ratio in spleen lymphocytes. These effects suggested that VYE could be a valuable herbal remedy for atopic dermatitis.

Author's contributions

Pinglong Fan, Ying Kuang and Lei Chen take charge of the experimental design and article writing. Pinglong Fan, Ying Kuang and Yanling Yang are responsible for finishing related experiments.

Lei Chen, Tao Liu, Xiaolu Lu and Hao Huang are responsible for revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- Apfelbacher, C.J., Diepgen, T.L., Schmitt, J., 2011. Determinants of eczema: population-based cross-sectional study in Germany. *Allergy* 66, 206–213.
- Barnes, L., Kaya, G., Rollason, V., 2015. Topical corticosteroid-induced skin atrophy: a comprehensive review. *Drug Saf.* 38, 493–509.
- Balekar, N., Katkam, N.G., Nakpheng, T., Jehtae, K., Srichana, T., 2012. Evaluation of the wound healing potential of *Wedelia trilobata* (L.) leaves. *J. Ethnopharmacol.* 141, 817–824.
- Chinese Pharmacopoeia Commission, 2020. Pharmacopoeia of the People's Republic of China. China Medical Science Publisher, Beijing, p. 352.
- China Food and Drug Administration, 2014. Technical Research Guidelines for Acute Dermal Toxicity Study, Beijing.
- Charlesworth, Ernest, N., 2001. Atopic dermatitis: the epidemiology, causes and prevention of atopic eczema. *Ann. Allergy. Asthma. Immunol.* 86, 349, 349.
- Chen, H.L., Dong, X.P., Zhang, M., Pei, J., Tang, P.R., Zhang, Y., 2010. Chemical constituents of *Viola yedoensis*. *Chin. Tradit. Herb Drugs* 41, 874–880.
- Correa, B.L., Ornaghi, A.P., Muller, G.C., Engroff, P., Lopes, R.P., Irênio, G., Bosch, J.A., Bonorino, C., Bauer, M.E., 2014. The inverted CD4:CD8 ratio is associated with cytomegalovirus, poor cognitive and functional states in older adults. *Neuroimmunomodulation* 21, 206–212.
- Danso, M.O., Drongelen, V.V., Mulder, A., Esch, J.V., Scott, H., Smeden, J.V., Ghalbzouri, A.E., Bouwstra, J.A., 2014. TNF- α and Th2 cytokines induce atopic dermatitis-like features on epidermal differentiation proteins and stratum corneum lipids in human skin equivalents. *J. Invest. Dermatol.* 134, 1941–1950.
- Deckert, S., Kopkow, C., Schmitt, J., 2014. Nonallergic comorbidities of atopic eczema: an overview of systematic reviews. *Allergy* 69, 37–45.
- Fan, H.J., Zhao, X.S., Tan, Z.B., Liu, B., Xu, H.L., Wu, Y.T., Xie, L.P., Bi, Y.M., Lai, Y.G., Liang, H.F., Zhou, Y.C., 2019. Effects and mechanism of action of Huang-Lian-Jie-Du-Tang in atopic dermatitis-like skin dysfunction *in vivo* and *in vitro*. *J. Ethnopharmacol.* 240, 111937, 111937.
- Hirayama, D., Iida, T., Nakase, H., 2018. The phagocytic function of macrophage-enforcing innate immunity and tissue homeostasis. *Int. J. Mol. Sci.* 19, 92–105.
- Huang, G.J., Reddy, M., Kuo, P.C., Huang, C.H., Shih, H.C., Lee, E.J., Yang, M.L., Leu, Y. L., Wu, T.S., 2012. A concise synthesis of viscolin, and its anti-inflammatory effects through the suppression of iNOS, COX-2, ERK phosphorylation and proinflammatory cytokines expressions. *Eur. J. Med. Chem.* 48, 371–378.
- Jeong, Y.H., Oh, Y.C., Cho, W.K., Shin, H., Lee, K.Y., Ma, J.Y., 2016. Anti-inflammatory effects of *Viola yedoensis* and the application of cell extraction methods for investigating bioactive constituents in macrophages. *BMC. Complement. Altern. Med* 16, 180.
- Jin, H., He, R., Oyoshi, M., Geha, R.S., 2009. Animal models of atopic dermatitis. *J. Invest. Dermatol.* 129, 31–40.
- Jungersted, J.M., Scheer, H., Mempel, M., Baurecht, H., Cifuentes, L., Hgh, J.K., Hellgren, L.L., Jemec, G., Agner, T., Weidinger, S., 2010. Stratum corneum lipids, skin barrier function and filaggrin mutations in patients with atopic eczema. *Allergy* 65, 911–918.
- Karuppagounder, V., Arumugam, S., Thandavarayan, R.A., Pitchaimani, V., Watanabe, K., 2014. Resveratrol attenuates HMGB1 signaling and inflammation in house dust mite-induced atopic dermatitis in mice. *Int. Immunopharm.* 23, 617–623.
- Kasraie, S., Werfel, T., 2013. Role of macrophages in the pathogenesis of atopic dermatitis. *mediators. Inflamm* 2013, 1–15.
- Katoh, N., Ohya, Y., Ikeda, M., Ebihara, T., Katayama, I., 2019. Clinical practice guidelines for the management of atopic dermatitis 2018. *J. Dermatol.* 46, 1053–1101.
- Langan, S.M., Irvine, A.D., Weidinger, S., 2020. Atopic dermatitis. *Lancet* 396, 345–360.
- Lee, S., Jegal, H., Bong, S.K., Yoon, K.N., Park, N.J., Shin, M.S., Min, H.Y., Yong, K.K., Kim, S.N., 2020. Anti-atopic effect of acorn shell extract on atopic dermatitis-like lesions in mice and its active phytochemicals. *Biomolecules* 10, 57.
- Li, J.Y., Wei, Z.M., 2008. Research progress in *Viola yedoensis* makino. *Mod. Chin. Med.* 10, 27–29.
- Li, W., Xie, J.Y., Li, H., Zhang, Y.Y., Cao, J., Cheng, Z.H., Chen, D.F., 2012. *Viola yedoensis* liposoluble fraction ameliorates lipopolysaccharide-induced acute lung injury in mice. *Am. J. Chin. Med.* 40, 1007–1018.
- Liao, H.F., Lu, M.C., Chang, H.C., Wei, C.C., Kao, C.H., Chen, Z.H., Huang, C.C., Li, C., 2010. Effects of herbal medicinal formulas on suppressing viral replication and modulating immune responses. *Am. J. Chin. Med.* 38, 173–190.
- M Ee Phansan, J., Tsuda, H., Komine, M., Tominaga, S.I., Ohtsuki, M., 2012. Regulation of IL-33 expression by IFN- and tumor necrosis factor- in normal human epidermal keratinocytes. *J. Invest. Dermatol.* 132, 2593–2600.
- Nutan, F., Kanwar, A.J., Parsad, D., 2012. The effect of topically applied corticosteroids on interleukin 1 β levels in patients with atopic dermatitis. *J. Eur. Acad. Dermatol. Venereol.* 26, 1020–1022.
- Odhiambo, J.A., Williams, H.C., Clayton, T.O., Robertson, C.F., Asher, M.I., 2009. Global variations in prevalence of eczema symptoms in children from ISAAC phase three. *J. Allergy Clin. Immunol.* 124, 1251–1258.
- Olivry, T., Mayhew, D., Paps, J.S., Linder, K.E., Peredo, C., Rajpal, D., Hofland, H., Cote-Sierra, J., 2016. Early activation of Th2/Th22 inflammatory and pruritogenic pathways in acute canine atopic dermatitis skin lesions. *J. Invest. Dermatol.* 136, 1961–1969.
- Orita, K., Hiramoto, K., Kobayashi, H., Ishii, M., Sekiyama, A., Inoue, M., 2011. Inducible nitric oxide synthase (iNOS) and -melanocyte-stimulating hormones of iNOS origin play important roles in the allergic reactions of atopic dermatitis in mice. *Exp. Dermatol.* 20, 911–914.
- Park, E.J., Kim, J.Y., Jeong, M.S., Park, K.Y., Park, K.H., Lee, M.W., Joo, S.S., Seo, S.J., 2015. Effect of topical application of quercetin-3-O-(2'-gallate)- α -l-

- rhampopyranoside on atopic dermatitis in NC/Nga mice. *J. Dermatol. Sci.* 77, 166–172.
- Simpson, E.L., Bruin-Weller, M., Flohr, C., Arderm-Jones, M.R., Barbarot, S., Deleuran, M., Bieber, T., Vestergaard, C., Brown, S.J., Cork, M.J., 2017. When does atopic dermatitis warrant systemic therapy? Recommendations from an expert panel of the International Eczema Council. *J. Am. Acad. Dermatol.* 77, 623.
- Sun, X., Yang, Y., Liu, T., Huang, H., Chen, L., 2021. Evaluation of the wound healing potential of *Sophora alopecuroides* in SD rat's skin. *J. Ethnopharmacol.* 113998.
- Verhagen, J., Akdis, M., Traidloffmann, C., Schmidgrendelmeier, P., Hijnen, D., Knol, E., Behrendt, H., Blaser, K., Akdis, C., 2006. Absence of T-regulatory cell expression and function in atopic dermatitis skin. *J. Allergy Clin. Immunol.* 117, 176–183.
- Wang, C., Colgrave, M.L., Gustafson, K.R., Ireland, D., Craik, D.J., 2008. Anti-HIV cyclotides from the Chinese medicinal herb *Viola yedoensis*. *J. Nat. Prod.* 71, 47–52.
- Yoon-Young, Sung, 2018. Crocin ameliorates atopic dermatitis symptoms by down regulation of Th2 response via blocking of NF- κ B/STAT6 signaling pathways in mice. Ho, Kyoung, Kim *Nutrients* 10, 1625.
- Zeng, H.R., Wang, B., Zhao, Z., Zhang, Q., Liang, M.Y., 2016. Effects of *Viola yedoensis* Makino anti-itching compound on degranulation and cytokine generation in RBL-2H3 mast cells. *J. Ethnopharmacol.* 189, 132–139.
- Zhou, H.Y., Hong, J.L., Pan, S., Yue, J.N., Min, J.Q., 2009. A new dicoumarin and anticoagulant activity from *Viola yedoensis* Makino. *Fitoterapia* 80, 283–285.