

RESEARCH ARTICLE

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Ameliorative effects of total coumarins from the fructus of *Cnidium monnieri* (L.) Cuss. on 2,4-dinitrochlorobenzene-induced atopic dermatitis in rats

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Funding information

National Major Public Health Project (Chinese Medicine Academic Genre Inheritance Studio Construction Project, Grant/Award Number: 210049; Technology Huimin Project of Chengdu Science and Technology Bureau, Grant/Award Number: 2016-HM01-00356-SF

Atopic dermatitis (AD), which is characterized by intense pruritus and serious inflammation, is a chronic skin disease. Modern studies have testified that the total coumarins from the fructus of *Cnidium monnieri* (TCFC) possess evident biological activities based on their coumarin compounds. The purpose of this manuscript is to investigate the effects of topical use of TCFC on immune response, inflammation, and skin barrier function in rats with 2,4-dinitrochlorobenzene (DNCB)-induced AD. Results indicated that the skin lesion scores of rats were obviously reduced after the management of TCFC, and the spleen and thymus indices also were markedly repressed. TCFC significantly inhibited the overproduction of TNF- α , interferon- γ , interleukin (IL)-4, IL-13, thymic stromal lymphopoietin, and immunoglobulin E; the epidermal thickness and number of mast cells were notably decreased. The western blot experiment was conducted to determine the effects of TCFC on the mitogen-activated protein kinases signaling pathway. Results indicated that phosphorylation of extracellular signal-regulated kinases, p38, and c-Jun amino-terminal kinases was significantly blocked by TCFC. In addition, TCFC could upregulate the expression of filaggrin in dorsal skin, which means that TCFC showed a protective effect on skin barrier disruption. Furthermore, TCFC downregulated the levels of IL-1 β , IL-4, IL-31, and TSLP mRNA and upregulated the expression of filaggrin mRNA in the dorsal skin of rats. Our research demonstrated the ameliorative effects of TCFC on AD-like rats by inhibiting immune response and inflammation and recovering skin barrier function.

KEYWORDS

atopic dermatitis, *Cnidium monnieri*, immune response, MAPKs pathway, skin barrier function, topical use

Abbreviations: AD, atopic dermatitis; *C. monnieri*, *Cnidium monnieri* (L.) Cuss.; DC, dendritic cells; DNCB, 2,4-dinitrochlorobenzene; ERK1/2, extracellular signal-regulated kinases 1 and 2; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IL-2, interleukin-2; IL-31RA, IL-31 receptor α ; JNK, c-Jun amino-terminal kinases; *m/z*, mass/charge ratio; MAPKs, mitogen-activated protein kinases; STAT3, signal transducer and activator of transcription 3; TCFC, the total coumarins from the fructus of *C. monnieri*; TSLP, thymic stromal lymphopoietin.

[†]These authors contributed equally to this work.

1 | INTRODUCTION

Atopic dermatitis (AD) is an allergic chronic inflammatory dermatosis with characteristics of relapsing pruritus and severe erythema, occurring frequently in children and adults (Fan et al., 2018). About 18% of children and 5% of adults worldwide suffer from AD (Kim et al., 2014). AD patients are commonly affected by eczema and show similar clinical symptoms, such as erythema, edema, excoriation,

lichenification, and pruritus (Ruff et al., 2018). Thus, AD has a serious impact on the quality of life of both infants and adults.

Currently, extensive evidence has demonstrated that the pathogenesis of AD is mainly associated with T-helper cell (Th)-driven immune response and skin barrier disruption (Furue et al., 2017). After the repeated challenge of allergens, CD4⁺ T cells gradually differentiate into Th1 cells, which mainly release the proinflammatory cytokines interleukin (IL)-2, interferon (IFN)- γ , TNF- α , and Th2 cells capable of producing IL-4, IL-10, and IL-13 to initiate the immune responses (Böhm & Bauer, 1997). In addition, IL-4 can activate B cells to secrete immunoglobulin E (IgE), inducing type I hypersensitivity reaction. Furthermore, the polarization of Th2 cells is considered responsible for the acute phase of AD and induces the infiltration of mast cells and eosinophils. However, what is interesting is that the predominant Th2-driven phenotype switches to Th1 cells in the chronic phase. Meanwhile, Th17 and Th22 cells also participate in the development of AD. Th22 cells of marked activation and its associated products principally emerge in the acute stage of AD, whereas Th17-mediated products show no significant differences between acute and chronic lesions. In the acute AD lesions, IL-22 released by Th22 and IL-17 derived from Th17 cells synergistically induce an abrupt increase in a subset of terminal differentiation proteins genes (S100A7, S100A8, and S100A9 proteins), resulting in epidermal hyperplasia. Those increased proteins are responsible for inflammation and recurrent skin infections (Gittler et al., 2012; Weidinger & Novak, 2016). In addition, the decreased expression of filaggrin in the epidermis is frequently

associated with the skin barrier disruption in AD patients, and T cells stimulated by thymic stromal lymphopoietin (TSLP) migrate to the filaggrin-deficient skin even in the absence of dendritic cells (DC) (Cabanillas & Novak, 2016; Wallmeyer et al., 2017). Protection and defense functions of the skin barrier are attributed to the filaggrin and tight junction proteins within the granular layer of the epidermis. Thereby, filaggrin deficiency is mostly responsible for the disruption of the skin barrier evoked by AD (Zaniboni, Samorano, Orfali, & Aoki, 2016). Meanwhile, other targets involved in the reduction of the skin barrier in AD have also been successively found such as loricrin, hornerin, and occluding (Lee, 2020; Yang et al., 2020).

To the best of our knowledge, *Cnidium monnieri* (L.) Cuss. (Umbelliferae family, Figure 1), which is widespread and found around field edges and ditches, is distributed in many countries, including China, Vietnam, Korea, Russia, Mongolia, India, Europe, and North America (Lee et al., 2014). Meanwhile, the fructus of *C. monnieri* has a long history of use as a traditional Chinese medicine for many diseases (e.g., genitalia pruritus, eczema impotence in the form of external use, or oral administration) (Chinese Pharmacopoeia Commission, 2015). Currently, plenty of active compounds have been isolated and identified from the fructus of *C. monnieri*, mainly involving coumarins, chromones, benzofurans, and monoterpenoids. Furthermore, increasing in vivo and in vitro research has proven that the fructus of *C. monnieri* possesses multiple biological activities, for instance, antiinflammatory effects, antiallergic effects, antipruritic effects, antifungal effects, vasodilative effects, antitumor effects, and



FIGURE 1 Flower and fructus of *C. monnieri* [Colour figure can be viewed at wileyonlinelibrary.com]

so on (Li et al., 2015). High content of coumarins extracted from the fructus of *C. monnieri* has also been found, which exhibit a diverse set of pharmacological functions, such as neuroprotective activities, immunomodulatory activities, osteogenic activities, hepatoprotective activities, and anticancer activities (Zhang, Leung, Cheung, & Chan, 2015). However, osthole might have a therapeutic application in the treatment or chemoprevention of human cancers, but the molecular mechanism of anticancer effect of osthole remains unclear.

Until now, the coumarin compounds from *C. monnieri* have been confirmed to exert improved effects on allergic asthma and contact dermatitis (Chiang et al., 2017; Matsuda, Tomohiro, Ido, & Kubo, 2002). Only a few pharmacological studies of the effects of coumarins from *C. monnieri* on AD have been reported, no profound research on AD has been found to date (Sun, Yang, & Lenon, 2020). Therefore, the purpose of this manuscript is to investigate the effect of total coumarins from the fructus of *C. monnieri* (TCFC) on the potential molecular mechanisms via inflammation and allergy in rats with 2,4-dinitrochlorobenzene (DNCB)-induced AD.

2 | MATERIALS AND METHODS

2.1 | Preparation of TCFC

A total of 100 g of powder of *C. monnieri* fructus (provided from Xinhehua Company, Sichuan province, China) was added to the 75% ethanol of 500 mL, using the method of hot reflux extraction (three times, 1 hour each) to extract the total coumarins. Then, the filtrate was separated and merged after standing for 24 hours. Next, a rotary evaporator was used to heat and recycle ethanol in filtrate at 60°C. The concentrate remained in the water bath (60°C) to obtain the dry extracts (18.3 g) and calculate the yield (18.3%). Finally, the TCFC was dissolved in ethylene glycol to obtain the preparation of 20 and 80 mg/mL, respectively.

2.2 | Ultra-performance liquid chromatography–tandem mass spectrometry analysis

The aforesaid dry extracts were dissolved in methanol, from which was acquired the sample solution (50.00 µg/mL). The coumarin compounds from the sample solution were identified and quantified by the conventional triple quadrupole ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS)/MS with multiple reaction monitoring on an AB SCIEX QTRAP 5500 mass spectrometer. Initially, the coumarin compound separation was administered with a UPLC HSS C18 analytical column (2.1 mm × 100 mm, 1.7 µm; Waters, MA) with a column temperature of 30°C and flow rate of 10 µL/min. A 2-µL sample solution was injected, and elution was performed using the gradient mobile phase A (0.1% formic acid aqueous solution) and B (methanol) (Shi et al., 2017; Song et al., 2015). The gradient elution of the mobile phase was as follows:

0 to 3.5 minutes, 55% to 65% B; 3.5 to 4.0 minutes, 65% to 90% B; 4.0 to 6.0 minutes, 90% B. In addition, the parameters of the mass spectra were set as follows: positive ion mode for a mass range of m/z 200 to 1,000, capillary voltage 2.8 kV, cone voltage 25 V, source temperature 100°C, desolvation temperature 250°C, cone gas flow 40 L/h, and desolvation gas flow 500 L/h. After the corresponding coumarin compounds were identified by literature contrast, quantification of those compounds was performed by standard curve.

2.3 | Animals

Sprague Dawley rats (8 weeks old, male, 180–220 g) were acquired from Chengdu Dossy Experimental Animals Co. Ltd. (SCXK: 2015-030). The rats were housed in a standard animal laboratory with a temperature of 21 to 27°C and 50% to 60% humidity under a 12-hour light–dark cycle, and were allowed to food and water ad libitum. All animals were acclimated for 1 week before the experiment. The experiment was conducted in accordance with the principles of the Committee for Animal Care and Use of Laboratory Animals, College of Pharmacy, Chengdu University of Traditional Chinese Medicine.

2.4 | Therapy

According to a previous investigation, the topical application of 7% and 2% DNCB (100 µL) was aimed to evoke the AD model in the dorsal skin of rats (Xiao, Xiao, Zhang, Pan, & Ouyang, 2018). First, approximately 4 × 4 cm² areas of dorsal skin of rats were shaved by a harmless shaver before the experiment. Then, 100 µL of 7% DNCB solution (acetone/olive oil, 3:1, v/v) was used to induce sensitization in rats besides the control group. Next, the animals were repeatedly challenged by 100 µL of 2% DNCB solution seven times besides the control group, which was to trigger AD-like skin lesions such as erythema, edema, erosion, scab, and so on. The experiment was randomly carried out in five groups composed of 10 rats each, including the control group, DNCB group, halcinonide group, and TCFC (20 and 80 mg/mL) groups. The halcinonide was regarded as the positive control due to its accepted clinical treatment for AD. Rats in the control group were topically challenged by a solvent of the same volume containing acetone and olive oil, without DNCB. Ethylene glycol and 0.1% halcinonide was topically applied to the dorsal skin of rats in the DNCB and halcinonide groups, respectively. Rats in the TCFC groups received topical administration of 20 and 80 mg/mL TCFC, respectively. Topical administration of all drugs continued for 10 days (100 µL twice per day), which was to ensure that the drugs could be adequately absorbed on dorsal skin (Chen et al., 2020; Nair & Jacob, 2016). Details of the therapeutic protocol are described in Figure 2. Additionally, relevant requirements in recent guidelines for best practice in natural products pharmacological research had been considered according to previous literature (Heinrich et al., 2018; Izzo et al., 2020).

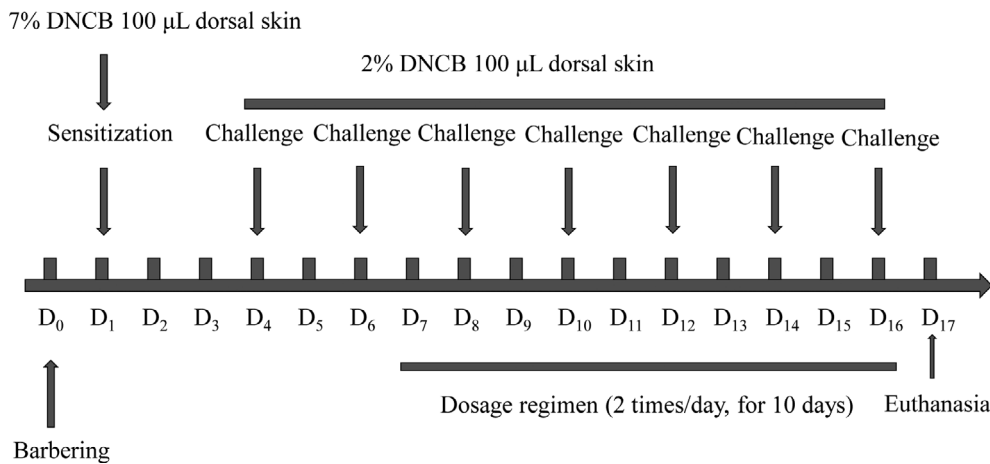


FIGURE 2 Diagram of the therapeutic protocol

2.5 | Skin lesion score

The lesion severity degree of the dorsal skin of the rats was scored at 4, 7, 10, 13, and 16 days. On the basis of the clinical skin lesion score, erythema, edema, excoriation, scab, and lichenification were used to evaluate the degree of the skin lesions of the rats in this study. The evaluation criteria for each symptom were as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = severe (Liang, 2016). The total skin lesion score was dependent on the sum of all individual dermatitis scores, and the investigator was blind to the entire experiment process to minimize subjective error.

2.6 | Immune organ indices

After the rats were euthanized, the spleen and thymus of all animals were immediately obtained, and the wet weight was calculated using a precision electronic scale. The spleen and thymus indices were calculated to investigate the inhibitory effect of TCFC on the immune responses evoked by DNCB. The calculation method of immune organ index was given as follow:

$$\text{spleen index (mg/g)} = \text{spleen weight (mg)} / \text{body weight (g)}$$

$$\text{thymus index (mg/g)} = \text{thymus weight (mg)} / \text{body weight (g)}.$$

2.7 | Enzyme-linked immunosorbent assay analysis

Blood was collected from the abdominal aorta after the rats were narcotized and centrifuged under the condition of 3,000 rpm, 4°C, for 10 minutes. The serum was then acquired to quantify the level of TNF- α , IFN- γ , IL-4, IL-13, IgE, and TSLP according to the manufacturer's instructions of the enzyme-linked immunosorbent assay (ELISA) kits (TNF- α , IFN- γ , IL-4, and IL-13 ELISA kits: ExCell Bio, Shanghai, China; IgE ELISA kits: Neobioscience, Shenzhen, China; TSLP ELISA kits: Elabscience, Wuhan, China).

2.8 | Histopathological analysis

The dorsal skin of the rats was obtained after euthanization. The dorsal skins were then fixed by 4% paraformaldehyde for 24 hours, embedded in paraffin, and cut into 3- μ m-thick slices for hematoxylin and eosin (H&E) and toluidine blue staining, which was to observe the skin lesion in the epidermis of the skin and to count the number of mast cells in the dermis, respectively. The four consecutive fields of the microscope (100 $\times$ magnification) were randomly selected to calculate the epidermal thickness of the rats' skin (scale bar = 400 μ m). Meanwhile, the number of mast cells under six continuous fields of the microscope per 0.24 mm² was counted at 400 $\times$ magnification. All histopathological analyses were performed using light microscopy (Olympus, Tokyo, Japan, BX53) and CCD (DP73, Olympus, Tokyo, Japan).

2.9 | Immunohistochemical analysis

The dorsal skin of the rats was fixed with 4% paraformaldehyde for 24 hours, and embedded in paraffin block sections of 3- μ m thickness to assess the expression of filaggrin in the epidermal skin. Five random fields (400 $\times$ magnification) were chosen to measure the relative integral optical density of filaggrin. Filaggrin antibody was provided by Gene Tex.

2.10 | Western blot analysis

Proteins were obtained from the dorsal skin frozen in liquid nitrogen using radioimmunoprecipitation assay lysis buffer. Then, the aforesaid lysates were centrifuged at 12,000g for 10 minutes at 4°C, and the protein concentration was measured by the BCA Protein Assay Kit (Thermo Scientific). A total of 40 μ g of protein was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes then were incubated by blocking with 5% nonfat milk dissolved in Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature

for 1 hour. After blocking, the membranes were incubated at 4°C overnight with different primary antibodies phosphor-extracellular signal-regulated kinase (ERK), ERK, phospho-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, phospho-c-Jun amino terminal kinases (JNK), JNK, and GAPDH (all 1:1,000 dilution in 5% bovine serum albumin [BSA]). Following incubation, the membranes were washed and incubated with TBST and horseradish peroxidase-conjugated goat anti-rabbit (1:1,000 dilution in 5% BSA) at 37°C for 1 hour, respectively. Finally, the protein bands were detected with the enhanced chemiluminescence reagents and were quantified by densitometric quantification with Tanon-5200 image system software (Tanon, Shanghai, China).

2.11 | Real-time quantitative polymerase chain reaction analysis

Total RNA was extracted from the dorsal skin tissues of rats with Trizol reagent (Invitrogen, USA). The complementary DNA (cDNA) was synthesized from total RNA using the First Strand cDNA Synthesis kit (Fermentas, USA). After that, gene expressions were undertaken using real-time quantitative PCR with an SYBR Green PCR kit (Thermo Scientific, USA) on an ABI Prism 7,300 system (ABI, USA). The primer sequences of interest genes were as follows: filaggrin (F:5'-ACAGCCACAAGCATTGACAG-3', R:5'-ACTCGTCCTCGGTTTCTTCTAC-3'); TSLP (F:5'-ATGATTGGCTTCGGCTTCTG-3', R:5'-TGAAGTGCCTGAACATTTCC-3'); IL-31 (F:5'-TATTCTGCCCGACCTCAGTTC-3', R:5'-TGC TCAAGGACTCGCTACTAC-3'); IL-1 β (F:5'-ATCTCACAGCAGCATCTC-3', R:5'-TAGCAGGTCGTCATCATC-3'); IL-4 (F:5'-CCTTGCTGTACACCTGTT C-3', R:5'-CTCGTTCTCCGTGGTGTTTC-3'); GAPDH (F:5'-TGGCCTTCC GTGTTCTTAC-3', R:5'-GAGTTGCTGTTGAAGTCGCA-3'). The relative gene expressions of target genes were normalized by GAPDH.

2.12 | Statistical analysis

All results are presented as the mean $\pm$ SD using SPSS software version 19.0 (SPSS Inc., Chicago, IL). One-way analysis of variance was conducted to obtain the statistics and data analyses; the significant difference in multiple comparisons was set as $p < .05$ by the Fisher's protected least-significant difference post hoc test or Dunnett's t -test.

3 | RESULTS

3.1 | Identification and quantitative determination of TCFC

The TCFC was isolated and identified via UPLC-MS/MS at the positive mode, and the chromatogram is shown in Figure 3. Five coumarin compounds were detected. In brief, the five coumarin compounds identified from the TCFC were, respectively, xanthotoxin (compound 1), isopimpinellin (compound 2), bergapten (compound 3), imperatorin (compound 4), and osthole (compound 5). Finally, the contents of xanthotoxin,

isopimpinellin, bergapten, imperatorin, and osthole were determined to evaluate the total coumarin contents from the fructus of *C. monnieri* (TCFC) by standard substances, and the corresponding percentage content was, respectively, 0.55%, 0.41%, 0.53%, 4.07%, and 7.02%. Obviously, the main coumarin compounds in TCFC were osthole and imperatorin.

3.2 | Inhibited effects of the TCFC on DNCB-induced skin lesion in rats

The effects of topical use of TCFC on DNCB-induced skin lesion were investigated via observing and scoring according to the severity of clinical skin lesions on 4, 7, 10, 13, and 16 days. The erythema was first present at 4 days in the dorsal skin of rats. Then, on day 7, the exacerbation of the skin lesion became most severe with the appearance of edema and scab. The skin lesion scores were significantly different among the DNCB, halcinonide, and TCFC 20 and 80 mg/mL groups at day 7 compared to the control groups, which confirmed that the replicated challenge of DNCB could provoke AD-like clinical symptoms. It was also obvious that the dorsal skin developed lichenification in the DNCB group at 16 days. As presented in Figure 4, the severity of the skin lesion was significantly alleviated after external application of TCFC (20 and 80 mg/mL) and halcinonide as compared with the DNCB group. Especially on days 7 to 10, the skin lesion scores markedly decreased for the halcinonide and TCFC 20 and 80 mg/mL groups.

3.3 | Effects of the TCFC on the immune organ indices

To investigate the effects of TCFC on the immunoreaction ignited by the replicated challenge of DNCB, the spleen and thymus indices were performed (Figure 5). The spleen and thymus indices in the DNCB group were significantly elevated after only DNCB was applied as compared with the control group. Nevertheless, the exorbitant spleen and thymus indices were pronouncedly reversed with the treatment of 20 and 80 mg/mL concentration of TCFC as compared with the DNCB group, except the spleen indices with the treatment of 20 mg/mL of TCFC. Thus, the topical application of TCFC could markedly alleviate the immunoreaction of the proliferation and differentiation of T and B lymphocytes in spleen and thymus of AD rats. In addition, the spleen and thymus indices of the rats in the halcinonide group were significantly inhibited and even displayed an inhibited immune response.

3.4 | Effects of TCFC on DNCB-induced cytokine production in serum

The elevated cytokine levels produced by Th2/Th1 cells in serum were evidently decreased by TCFC. As can be seen from Figure 6, the expression levels of TNF- α , IFN- γ , IL-4, IL-13, TSLP, and IgE in the DNCB group were significantly increased compared with the control group. Meanwhile, the aforementioned cytokines were notably

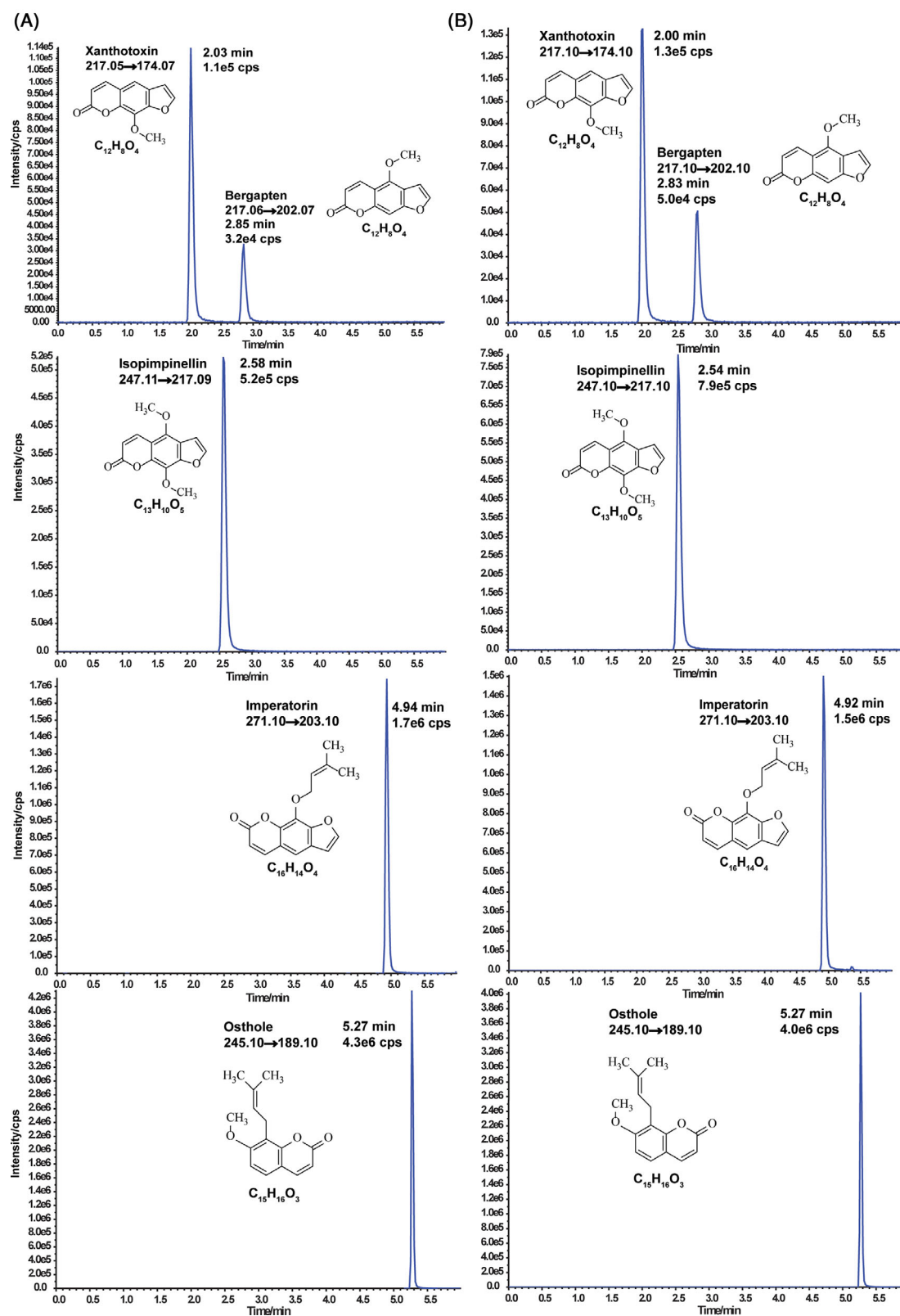


FIGURE 3 Chromatogram and chemical structures of (a) standards and (b) sample of TCFC by UPLC-MS/MS with multiple reaction monitoring [Colour figure can be viewed at wileyonlinelibrary.com]

reduced after the treatment of halcinonide. In addition, the topical use of TCFC (20 and 80 mg/mL) could significantly downregulate the excessive levels TNF- α , IFN- γ , IL-4, IL-13, TSLP, and IgE in comparison

with the DNCB group. Thus, external treatment with TCFC was able to balance the differentiation of Th2/Th1 cells in the serum of DNCB-evoked AD rats.

(A)

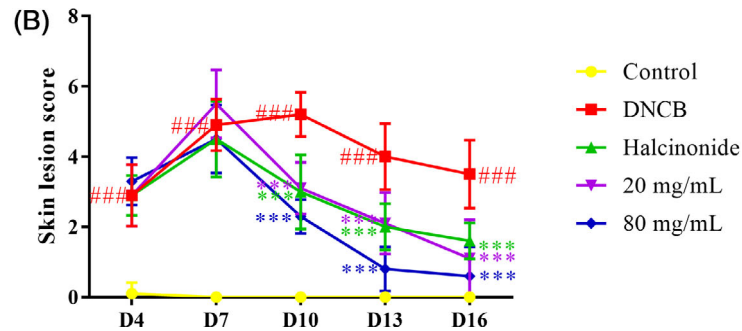
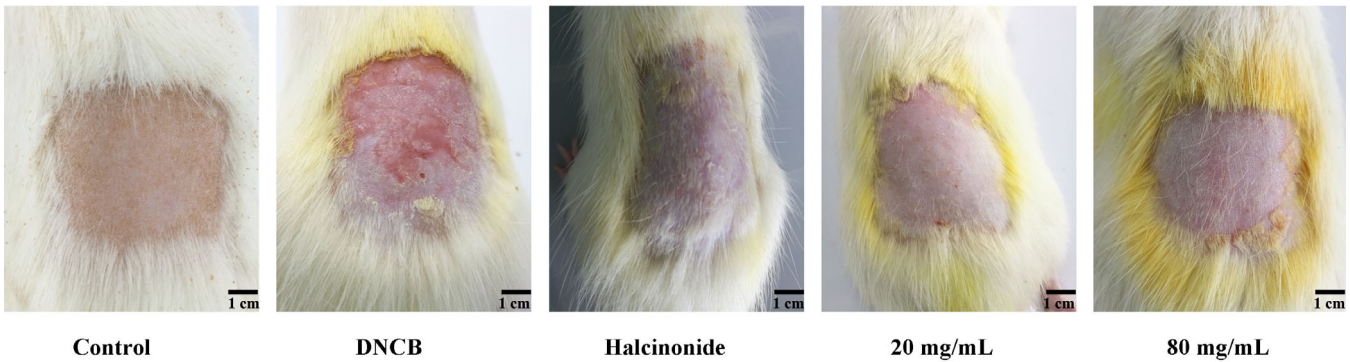


FIGURE 4 (a) Changes in topical use of TCFC on DNCB-induced skin lesions in rats at 16 days. The corresponding representative pictures of the dorsal skin of rats from the different groups are presented. (b) The skin lesion scores of the rats were based on the severity of the erythema, edema, excoriation, scab, and lichenification at 4, 7, 10, 13, and 16 days. The results are shown as mean $\pm$ SD ($n = 10$). Compared with the control group, $###p < .001$ and compared with the DNCB group, $***p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

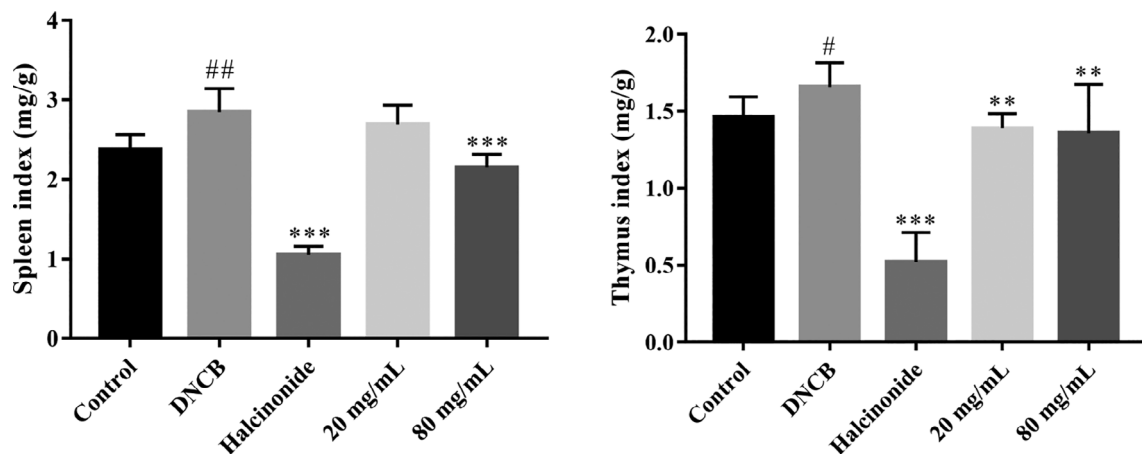


FIGURE 5 (a) Effects of topical use of TCFC on the immune responses evoked by DNCB. The all data are presented as mean $\pm$ SD ($n = 10$). Compared with the control group, $#p < .05$, $##p < .01$ and compared with the DNCB group, $**p < .01$, $***p < .001$

3.5 | Protective effects of the TCFC on DNCB-induced histopathological changes in rats

The dorsal skin sections of rats were stained with the H&E and toluidine blue to further observe the histopathological changes in AD rats after the treatment of TCFC. As shown in Figure 7a,b, topical use of DNCB was responsible for the AD skin lesions, that is, hyperkeratosis, stratum spinosum thickening, and inflammatory cell infiltration in the

skin epidermis and excessive mast cells in the skin dermis, respectively. The epidermal thickness and number of mast cells in the DNCB group were significantly increased compared with the control group. After the treatment of TCFC (20 and 80 mg/mL), the epidermal thickness and number of mast cells of rats were significantly dose-dependently inhibited in comparison with the DNCB group. In addition, the halcinonide group also showed a similar inhibitory effect on the epidermal thickness and number of mast cells (Figure 7c).

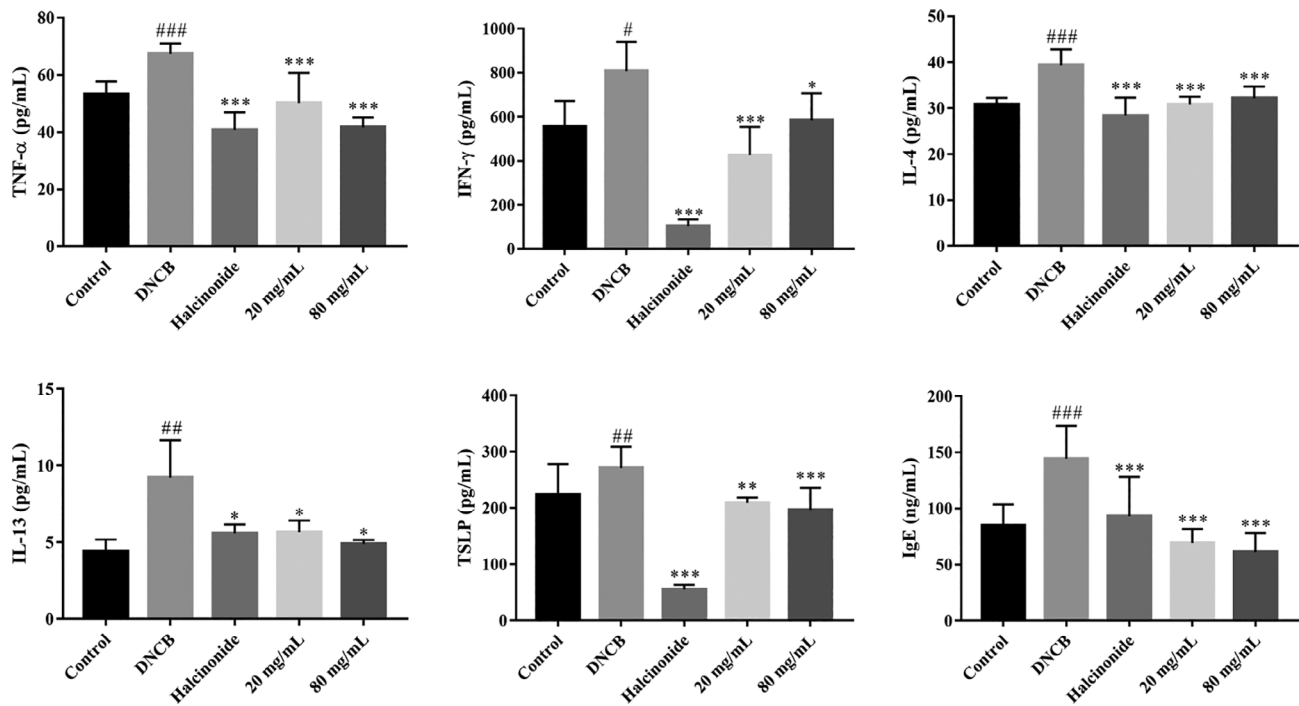


FIGURE 6 The effects of topical use of TCFC on the serum levels of TNF- α , IFN- γ , IL-4, IL-13, TSLP, and IgE in AD rats induced by DNCB. The results are presented as mean $\pm$ SD ($n = 8-9$). Compared with the control group, * $p < .05$, ** $p < .01$, *** $p < .001$ and compared with the DNCB group, # $p < .05$, ## $p < .01$, ### $p < .001$

3.6 | Effects of the TCFC on the skin barrier function in DNCB-induced AD rats by immunohistochemistry

Because the skin barrier function was highly associated with the filaggrin expression in the stratum corneum and granular layer of skin, the expressions of filaggrin in the dorsal skin were determined by yellow color on immunohistochemical staining. The study had demonstrated that filaggrin monomers were derived from polymer profilaggrin by the effect of proteases during the process of keratinocyte cornification (Matsui & Amagai, 2015). The expressions of filaggrin in the outer layers of the epidermis were significantly downregulated after the challenge of DNCB, resulting in the disruption of skin barrier function in the dorsal skin of rats. However, TCFC with a concentration of 20 and 80 mg/mL significantly enhanced the filaggrin expression as compared with the DNCB group. Thus, topical treatment with TCFC notably ameliorated the destroyed skin barrier function in DNCB-induced AD rats. Halcinonide also exerted a similar protective effect on the skin barrier function in AD rats. All results and data are presented in Figure 8.

3.7 | Effects of the TCFC on the expression of MAPKs (ERK, JNK, p38) in the dorsal skin of rats

To investigate the effects of TCFC on inflammation caused by DNCB in the dorsal skin of rats, the levels of p-ERK, ERK, p-JNK, JNK, p-p38 MAPK, and p38 MAPK, were determined by western blot. As shown in Figure 9, the expression of p-ERK and p-JNK were significantly

upregulated in the DNCB group as compared with the control group, but the change was evidently mitigated after the topical use of 20 and 80 mg/mL TCFC, respectively. Meanwhile, the expression of p-p38 was markedly decreased after the administration of TCFC, compared to the DNCB group. However, what strange is that the p-p38 levels no significant increased between the DNCB group and control group. In brief, the results of the western blot suggested that topical treatment with TCFC suppressed the inflammatory response induced by DNCB in AD rats via blocking MAPK signaling pathway.

3.8 | Effects of the TCFC on IL-1 β , IL-4, IL-31, TSLP, and filaggrin mRNA expression in dorsal skin tissues

Expression levels of TCFC on the proinflammatory and pruritic cytokines mRNA in skin tissues were carried out via real-time quantitative PCR. After the challenge of DNCB, the IL-1 β , IL-4, IL-31, and TSLP mRNA levels were prominently increased and the expression of filaggrin mRNA was significantly reduced. However, the intervention of TCFC with 20 and 80 mg/mL doses successfully reversed those mRNA levels produced by DNCB. Results are shown in Figure 10.

4 | DISCUSSION

As is known, AD was considered to be a common chronic disease with specific genetic and immunological mechanisms. Development of AD

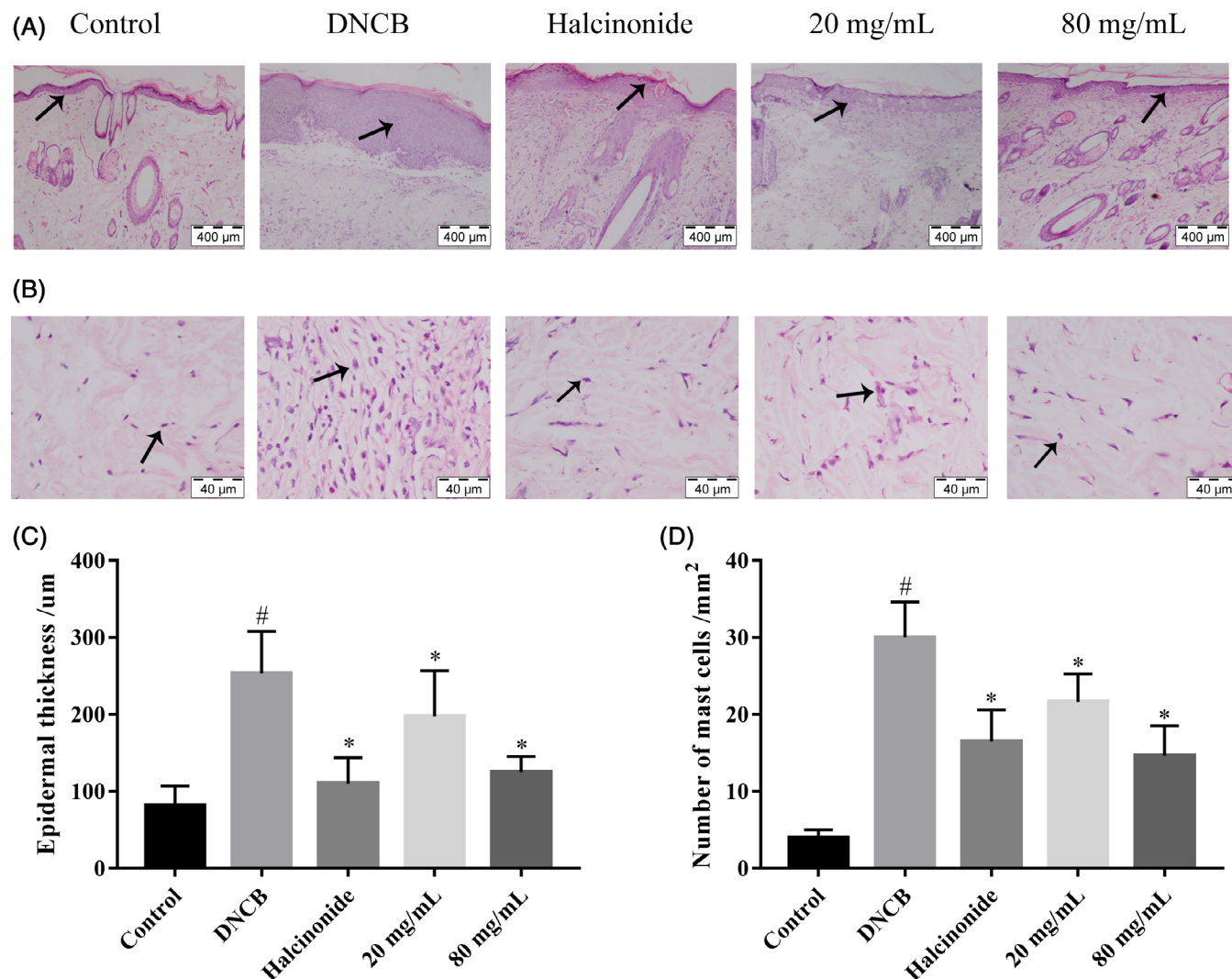


FIGURE 7 The effects of topical use of TCFC on DNCB-induced skin lesion in rats on day 17 and the representative pathological pictures of the dorsal skin of rats in different groups. (a) The dorsal skins of rats were subjected to H&E staining at 100× magnification to measure the epidermal thickness in four consecutive microscope fields (scale bar = 400 μm). (b) To count the number of mast cells, the dorsal skins underwent toluidine blue staining at 400× magnification in six continuous microscope fields of 0.24 mm². (c) All statistical results are shown as mean ± SD (n = 6–8). Compared with the control group, [#]*p* < .05 and compared with the DNCB group, ^{*}*p* < .05 [Colour figure can be viewed at wileyonlinelibrary.com]

consists of the chronic and acute phase, presenting the typical characteristics of erythema, edema, excoriation, scab, lichenification, and relapse. The overall patient quality of life is heavily influenced by AD, especially in infants (Mu, Zhao, Liu, Chang, & Zhang, 2014). Currently, topical corticosteroids constitute the main treatment of AD, but they can result in side effects such as skin atrophy and telangiectasia (Dhossche, Simpson, & Hajar, 2017). Hence, it is of great importance to seek new alternative therapeutics without those adverse effects. Nowadays, an increasing body of research has indicated that herbal medicines are promising resources for finding novel candidate constituents for treating various diseases and also for a feasible approach to AD treatment (Gu, Zhang, Coyle, Chen, & Xue, 2016; Peng et al., 2018). Furthermore, *C. monnieri*, a traditional Chinese native medicine, has been frequently used in China for 2000 years to treat

eczema and pruritus. In accordance with previous mentioned studies, the ethanol extract of the fructus of *C. monnieri* has shown a significant inhibitory effect on AD, which is attributed to the coumarin compounds from the fructus of *C. monnieri* (Matsuda et al., 2002). However, its potential molecule mechanisms remain elusive. Thus, the aim of this study was to further investigate the underlying mechanism of action of TCFC on AD in DNCB-induced rats. Results showed that TCFC (20 and 80 mg/mL) could significantly inhibit the inflammatory response triggered by DNCB and the excessive immune reaction produced by lymphocytes, respectively.

First, the process of AD exhibited different clinical symptoms. In the acute phase, patients mainly showed erythema and edema and then presented excoriation, scab, and even lichenification skin lesions in the chronic phase. In addition, dry skin and intense pruritus were

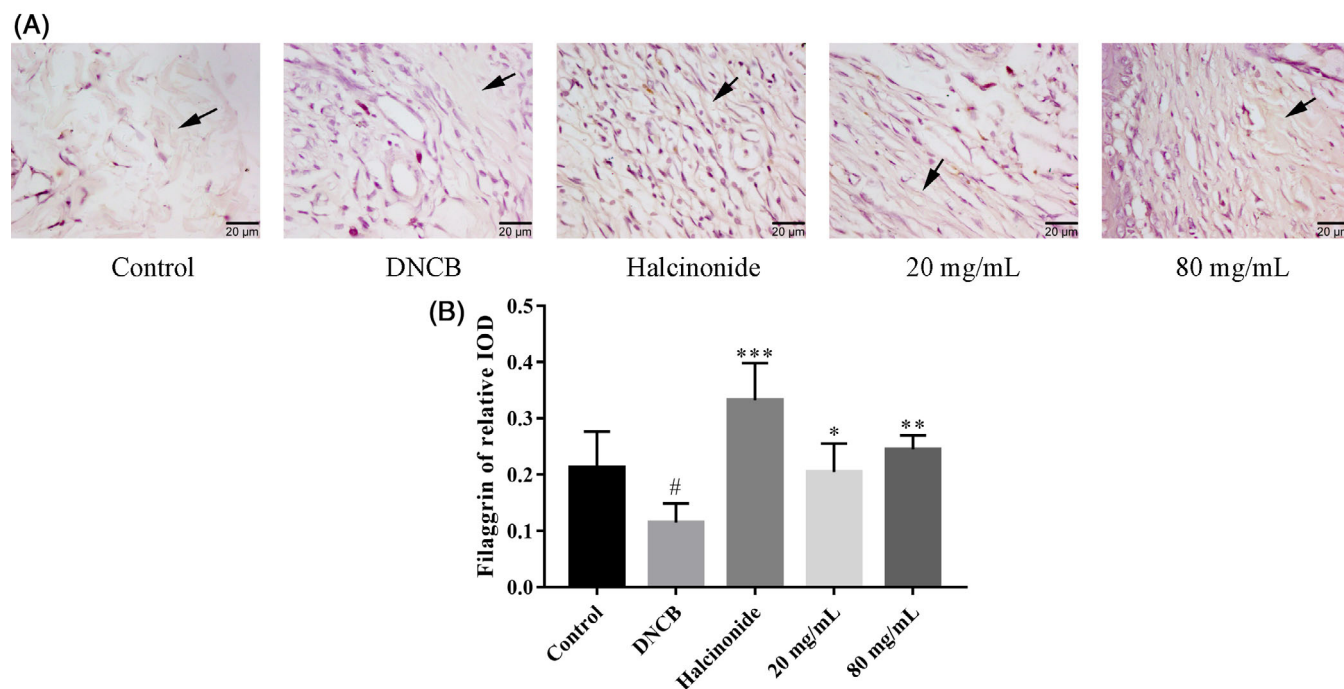


FIGURE 8 The effects of topical use of TCFC on the expression of filaggrin in the skin of AD rats by immunohistochemical staining. The data were presented as mean $\pm$ SD ($n = 4$). Compared with the control group, # $p < .05$ and compared with the DNCB group, * $p < .05$, ** $p < .01$, *** $p < .001$ [Colour figure can be viewed at wileyonlinelibrary.com]

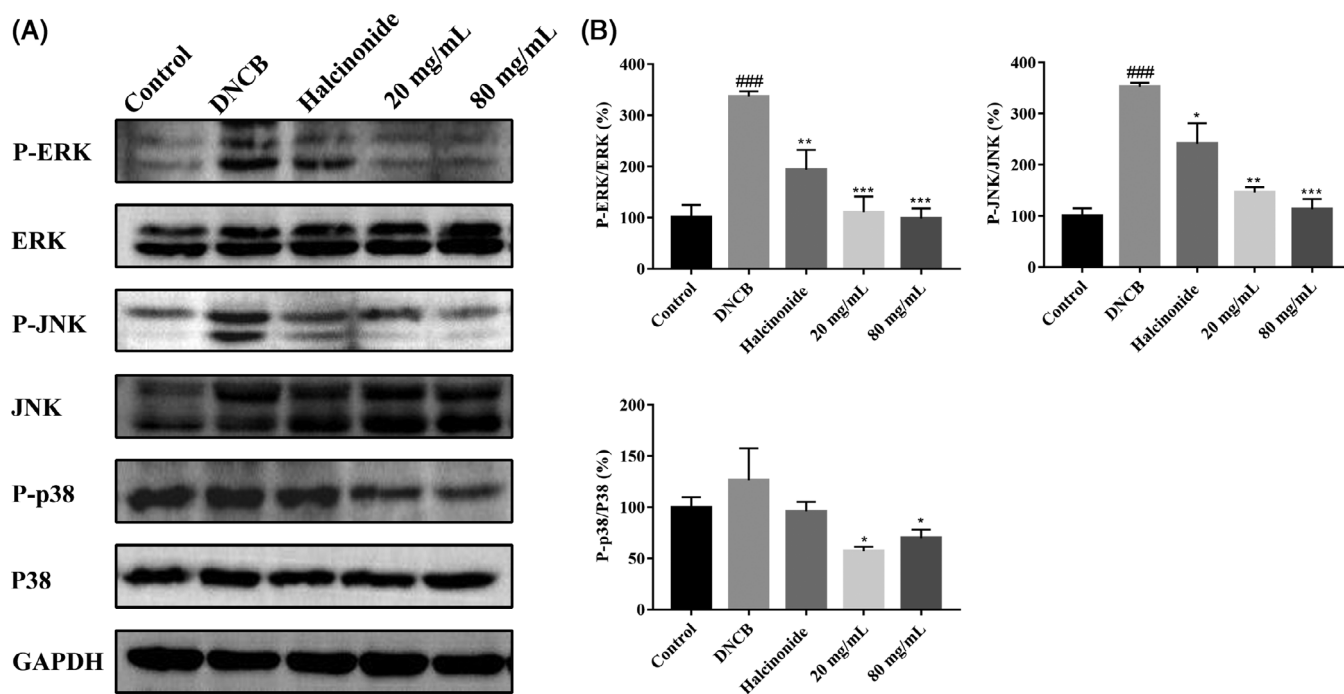


FIGURE 9 The effects of topical use of TCFC on the expression of MAPKs protein. The relative expression of p-ERK, p-JNK, and p-p38 was assessed by western blot. GAPDH was used to normalize. Data are presented as mean $\pm$ SEM ($n = 3-4$). Compared with the control group, ## $p < 0.01$, ### $p < 0.001$ and compared with the DNCB group, * $p < 0.05$, ** $p < 0.01$, *** $p < .001$

also the prominent properties of this disease, particularly at night (Mu et al., 2014; Ng, Lew, & Sin, 2014). Our results indicated that erythema was first present at day 4 in the dorsal skin of rats after

sensitization with high-concentration DNCB, along with intense itch. On day 7, skin lesions of rats were at the highest severity, which demonstrated that the AD-like model was successfully established.

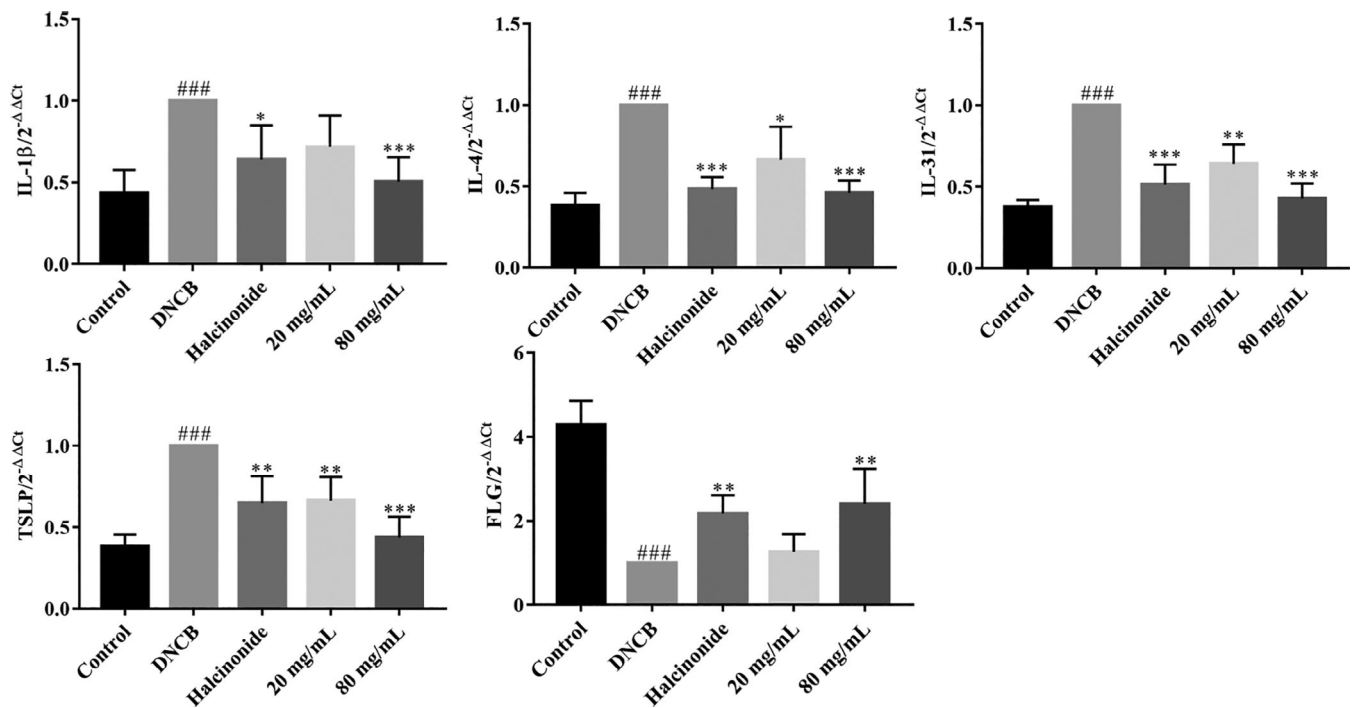


FIGURE 10 The effects of topical use of TCFC on IL-1 β , IL-4, IL-31, TSLP, and filaggrin mRNA expression in DNCB-induced skin lesion tissues. The results are expressed as mean $\pm$ SD ($n = 7-8$). Compared with the control group, $^{\#}p < .01$, $^{\#\#}p < .001$ and compared with the DNCB group, $^*p < 0.05$, $^{**}p < .01$, $^{***}p < .001$

Subsequently, the skin lesions of rats were evidently improved by TCFC (Figure 4). Further, it has been confirmed that AD-like symptoms were closely bound to the proliferation and differentiation of eosinophils, mastocytes, T lymphocytes, and cytotoxic cells in the epidermis (Frings, Böer-Auer, & Breuer, 2018; Kim et al., 2013). Thus, the histopathological changes of the dorsal skin of rats were analyzed with H&E and toluidine blue staining. As presented in Figure 7, hyperkeratosis and stratum spinosum thickening in the skin epidermis were obviously observed in rats of the DNCB group while showing inflammatory cell infiltration and mast cell proliferation in the skin dermis. Nevertheless, topical treatment with TCFC could significantly improve these effects.

Abundant CD4 $^{+}$ and CD8 $^{+}$ T cells in the skin of AD patients has been proven by numerous studies (Kang & Choung, 2016). The production of proinflammatory cytokines secreted by Th cells and non-Th cells was also associated with the pathogenesis of AD, including Th1, Th2, Th17, and Th22 cells; B lymphocytes; macrophages; and DC (Zaniboni et al., 2016). After the repetitive challenge of allergen, CD4 $^{+}$ T cells differentiated Th1, Th2, and Th17 cells, respectively. In the early stage of AD, Th2 cytokines were abundantly produced, displaying a predominant Th2 phenotype (e.g., IL-4, IL-5, IL-13, etc.) (Kang et al., 2016; Nomura & Kabashima, 2016). In addition, fewer TNF- α , IFN- γ , and IL-1 β caused by macrophages and eosinophils further aggravated tissue damage evoked by inflammation (Di, 2015). But what is interesting is that the imbalance of the Th cytokine secretion was diverted to the Th1-driven immune response in the chronic phase, and TNF- α and IL-2 inflammatory cytokines were

largely secreted by Th1 cells (Werfel et al., 2016). TSLP is a potential promising target for AD that is primarily expressed by keratinocytes and DC in the skin. TSLP play crucial roles in numerous immune diseases (asthma, AD) and possess function that activate the MAPK signaling pathway and downregulates the expression of filaggrin mRNA (Tsilingiri, Fornasa, & Rescigno, 2017; Wallmeyer et al., 2017). Furthermore, the IgE-mediated type I allergic reaction also participated in the AD process (Lee, Wu, Jang, & Choung, 2018). Our study also suggested that the levels of TNF- α , IFN- γ , IL-4, IL-13, TSLP, and IgE in serum were significantly reduced by the topical use of TCFC, which conformed to previous research (Lee et al., 2016; Ryu et al., 2018). Thus, treatment with TCFC 20 and 80 mg/mL could suppress inflammation induced by a series of proinflammatory cytokines.

For the purpose of elucidating the anti-inflammatory mechanisms of TCFC in depth, a western blot study was undertaken to determine the TCFC of influence on the MAPK signaling pathway. It is well known that MAPKs are primarily composed of ERK1/2, JNK, p38, and ERK5 and regulate inflammation, apoptosis, and differentiation (Peti & Page, 2013). Protein phosphorylation is the key to MAPK activation. A previous study also verified that activation of MAPK signaling was implicated in AD pathogenesis (Choi et al., 2016). From the results shows in Figure 9, TCFC markedly blocked the phosphorylation of ERK, JNK, and p38 MAPK in the dorsal skin of rats, resulting in a balance of the hyperactive immune response caused by proinflammatory cytokines. Thereby, TCFC could weaken the inflammation and mitigate skin lesions via blocking the MAPK signaling pathway.

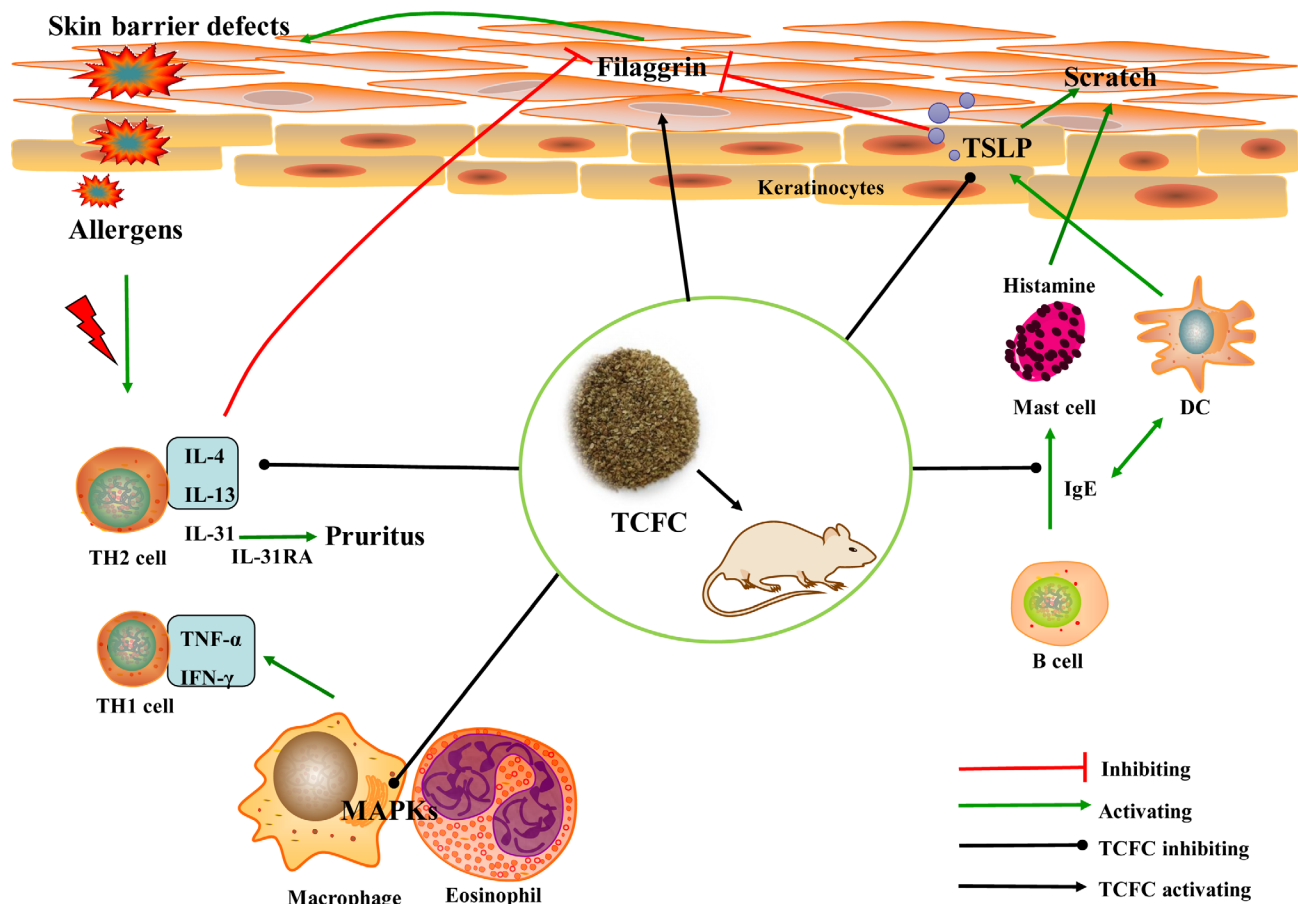


FIGURE 11 The ameliorative effects of topical use of TCFC on AD induced by DNCB in rats by inhibiting proinflammatory cytokines and upregulating filaggrin. After the rats' dorsal skin was repeatedly challenged by allergen DNCB, the naive T cells were stimulated and differentiated to various immune T cells. Among those, Th1 and Th2 cells secreting cytokines IL-4, IL-13, IL-31, TNF- α , and IFN- γ played an important role in aggravating the inflammation in acute AD lesions. IL-31 is mostly bound with IL-31 receptor α (IL-31RA), leading to intense pruritus. Furthermore, those cytokines, in particular IL-4 and IL-13, negatively modulated the expression of filaggrin in keratinocytes, causing defective skin barrier function. In addition, activated macrophages and eosinophils also largely produced TNF- α and IFN- γ . Fortunately, TCFC could inhibit the production of Th1 and Th2 cell cytokines possibly via blocking the MAPKs signaling pathway for alleviating skin lesions induced by inflammation. Further, scratch caused by histamine was indirectly suppressed by TCFC through blocking the B cell-stimulated mast cell, and DC excited by IgE-facilitated TSLP yield also were weakened by TCFC. Meanwhile, TSLP was directly responsible for the defective filaggrin [Colour figure can be viewed at wileyonlinelibrary.com]

Currently, filaggrin deficiency is largely responsible for AD pathogenesis. Expression of filaggrin produced by epidermal keratinocytes involves multiple factors, including cytokine environment, environmental influences, and genetic mutations of filaggrin (Cabanillas & Novak, 2016). Plenty of proinflammatory cytokines have important modulatory effects on filaggrin expression, such as IL-4, IL-13, IL-17, IL-22, IL-33, and TSLP. But among these, IL-4 and TSLP have greater negative modulating effects on the expression of filaggrin than the others (Jenny, Roesner, Hesler, Wittmann, & Werfel, 2015). Another study proved that TSLP secreted by keratinocytes downregulated filaggrin expression via the signal transducer and activator of transcription 3 (STAT3)-dependent pathways and ERK-dependent pathways (Jin et al., 2015). Meanwhile, Th cells directly stimulated by TSLP migrated to the filaggrin-deficient skin, leading to exacerbation of skin damage (Wallmeyer et al., 2017). To study the effect of TCFC on filaggrin expression in dorsal skin, the levels of filaggrin in the dorsal skin of rats were determined by

immunohistochemistry. It was clear that filaggrin expression was significantly increased by the topical treatment of TCFC (Figure 8).

Furthermore, IL-1 β , IL-4, IL-31, TSLP, and filaggrin mRNA levels in skin were quantified by real-time quantitative PCR. As shown in Figure 10, TCTC (20 and 80 mg/mL) significantly decreased the expression of IL-1 β , IL-4, IL-31, and TSLP and increased defective filaggrin expression, respectively. It was notable that IL-31, which was released in large amounts by eosinophils and CD4⁺ T cells, was directly responsible for pruritus in AD (Bağcı & Ruzicka, 2018; Takamori et al., 2018). In addition, 20 and 80 mg/mL of TCTC notably suppressed the thymus index of rats, but only 80 mg/mL of TCTC exerted an inhibitory effect on the spleen index (Figure 5). The over inhibition of the spleen and thymus indices in the positive group might be ascribed to the excessive use of halcinonide.

In brief, our work suggests that the topical use of TCFC (20 and 80 mg/mL) is capable of improving skin lesions caused by

inflammation and the immune response in DNCB-induced AD rats. Further, TCFC not only can inhibit the detrimental immune response trigger by proinflammatory cytokines and pruritogenic cytokines via blocking the activation of the inflammation-related signaling pathway but also can recover skin barrier function through upregulating the expression of filaggrin. Hence, TCFC possesses anti-inflammatory, antiallergic effects and protects skin barrier function. However, the TCFC how to regulate the specific inflammation MAPKs pathway still need to further study. In this study, because TCFC mainly consists of osthole and imperatorin, the coumarin compounds from *C. monnieri* have potential as a therapeutic agent against AD, in particular osthole with low toxicity and high activity (Yuan et al., 2016). Meanwhile, previous study also indicated that the coumarins compounds could not cause the skin irritation in vitro and in vivo experiment (Pan et al., 2014). Under the dose of 30 mg/cm², topical usage of the extract of *C. monnieri* had without acute skin toxicity, and the 3.75 mg/cm² of dose had also no any evident skin irritation in guinea-pig (Wang, Zhou, Li, & Zhang, 2007). In our manuscript, the TCFC (80 mg/mL, 100 μ L twice per day, yield 18.3%) is topical administrated on the dorsal skin of rats (4 $\times$ 4 cm² areas), which is equal to the 5.4 mg/cm² of dose. Thus, our high dose is also safe to rats. Further, investigation had revealed that osthole exerted inhibitory effect to Th2 cell response and could directly down-regulate the expression of TSLP (Fu & Hong, 2019). Thus, future in vitro and clinical studies should be conducted on the effects of topical treatment with osthole.

5 | CONCLUSION

In brief, our work suggested that TCFC had a significant inhibitory effect on AD-like rats induced by DNCB. As presented in Figure 11, AD symptoms of rats were evidently improved after the topical treatment of TCFC via reducing the production of IL-4, IL-13, TNF- α , IFN- γ , TSLP, and IgE; elevating the expression of filaggrin; decreasing the phosphorylated MAPKs (ERK, JNK, p38); and suppressing the pruritus ignited by IL-31, TSLP, and histamine. Hence, the coumarin compounds from *C. monnieri* are already deemed a potential natural therapeutic agent for AD. Subsequent research should emphasize on in vitro and clinical studies of bioactive compounds separated and identified from TCFC. Furthermore, this manuscript may provide brand new insights into seeking AD therapeutic agents.

ACKNOWLEDGMENTS

The authors would like to thank all of our colleagues who contributed to this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Zhijie Yu designed the research; Ting Deng performed the detailed experiments; Ping Wang performed the statistical analysis; Zhijie Yu and Ting Deng wrote the original manuscript; Tao Sun and Ying Xu

revised the manuscript. All authors approved and submitted the final version.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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How to cite this article: Yu Z, Deng T, Wang P, Sun T, Xu Y. Ameliorative effects of total coumarins from the fructus of *Cnidium monnieri* (L.) Cuss. on 2,4-dinitrochlorobenzene-induced atopic dermatitis in rats. *Phytotherapy Research*. 2021; 1–15. <https://doi.org/10.1002/ptr.7052>