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Original article

Total sesquiterpene lactones isolated from *Inula helenium* L. attenuates 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in mice

Wang Qun[a,1], Gao Shuang[b,1], Wu Guo-zhen[b], Yang Niao[b], Zu Xian-peng[b], Li Wen-cai[b], Xie Ning[c], Zhang Rong-rong[d], Li Chen-wei[e], Hu Zhen-lin[b,*], Zhang Wei-dong[a,b,*]

[a] Institute of Interdisciplinary Research Complex, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
[b] School of Pharmacy, Second Military Medical University, Shanghai 200433, China
[c] State Key Laboratory of Innovative Natural Medicines and TCM Injections, Jiangxi Qingfeng Pharmaceutical Co., Ltd., Ganzhou, Jiangxi 341000, China
[d] Department of Gynecology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200092, China
[e] Shanghai Sunstem Biotechnology Co., Ltd, Shanghai 200439, China

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ABSTRACT

Background: *Inula helenium* L. is an herb whose anti-inflammatory properties are attributed to its active components, the sesquiterpene lactones (SLs). Our previous study demonstrated that the total sesquiterpene lactones isolated from *Inula helenium* L. (TSL-IHL), consisting mainly of alantolactone (AL) and isoalantolactone (IAL), may have potential in the prevention and treatment of rheumatoid arthritis (RA). However, the effect of TSL-IHL on atopic dermatitis (AD) has not been studied yet.

Aim of the study: The present study evaluates the potential of TSL-IHL as a treatment for AD.

Methods/Study designs: The effects of TSL-IHL on the expression of inflammatory genes and the activation of NF-κB signaling pathway in HaCat cells were examined by quantitative real-time PCR and western blotting, respectively, and compared with those of AL and IAL. The protective effect of TSL-IHL against AD was tested in a mouse model induced by 2,4-dinitrochlorobenzene (DNCB), in which AD-like skin lesions were induced in ICR mice by sensitizing once with 100 μl of 7% DNCB painted on their shaved back skin and then challenging with 20 μl of 0.2% DNCB five times on their right ears at 3 day intervals starting on day 5 post-sensitization.

Results: TSL-IHL, as well as AL and IAL, could all inhibit TNF-α-induced activation of NF-κB and the expression of TNF-α, IL-1, and IL-4 in HaCat cells in a dose-dependent manner in the range of 0.6–2.4 μg/ml. The topical application of TSL-IHL (1% W/W in emollient cream) attenuated DNCB-induced dermatitis severity and right ear swelling. The serum levels of IgE, TNF-α and IFN-γ in TSL-IHL-treated mice were reduced by 81.39%, 89.69%, and 87.85%, respectively, while the mRNA levels of IL-4, IL-5 and IL-13, in the back-skin lesions of TSL-IHL-treated mice were reduced by 39.21%, 40.62% and 48.12%, respectively, compared with the untreated controls. Histopathological examination showed that TSL-IHL treatment reduced epidermis/dermis thickening and dermal inflammatory infiltration in both ear and back skins.

Conclusions: We suggest that TSL-IHL inhibited the development of AD-like skin symptoms by regulating cytokine expression and may be an effective alternative therapy for AD.

Introduction

Atopic dermatitis (AD) is a chronic, relapsing and inflammatory skin disease with a clinical presentation of erythematous rashes, lichenification, and intense pruritus. AD is also a multifactorial skin disease, involving complex interactions of the innate and adaptive immune responses. It is based on a strong genetic predisposition and is triggered by various environmental, psychological, allergenic, and pharmacological factors (Spergel and Paller., 2003; Beltrani., 1999; Leung et al., 2004; Leung et al., 2003). Although the pathogenesis of AD is not yet fully defined, a recent study reports that the development of AD involves deregulated expression of the Th2 and Th1 cytokines. In the

Abbreviations: SLs, Sesquiterpene lactones; TSL-IHL, total sesquiterpene lactones; AL, alantolactone; IAL, isoalantolactone; RA, rheumatoid arthritis; AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; MFC, Momestasone furoate cream; CCK-8, Cell Counting Kit-8

* Corresponding author at: School of Pharmacy, Second Military Medical University, 325 Guohe Road, Yangpu District, Shanghai 200433, China.
E-mail address: wdzhangy@hotmail.com (W.-d. Zhang).

[1] These authors contributed equally to this work.

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acute stage of AD, Th2-dominant allergic inflammation predominates, leading to the increased expression of IL-4 and IgE; in the chronic stage of AD, Th1-dominant allergic inflammation predominates, leading to the increased expression of TNF-α and IFN-γ (Kim et al., 2016; Bieber., 2008; Cesare et al., 2008). In addition to immune cells, epidermal keratinocytes are also an important source of cytokines, which play key roles in the pathogenesis of AD. Activated keratinocytes in the epidermal lesions of AD are capable of producing a variety of chemokines and pro-inflammatory cytokines, which can further affect Th cell balance (Novak et al., 2003; Mu et al., 2014).

The standard therapy for AD has long been the application of local or systemic steroids and immunosuppressive agents. However, various side effects have prevented the long-term use of these agents (Ingber., 2002; Furue et al., 2003; Hengge et al., 2006). Recently, the use of natural herbs as an alternative therapeutic in inflammatory disorders has attracted much interest due to their proven safety and potent anti-inflammatory activities (Elias., 2016; Tan and Lenon., 2016). Sesquiterpene lactones (SLs) comprise a large group of secondary metabolites that possess diverse biological activities, and more than 5000 structural variants have been characterized, mostly from *Asteraceae* spp. They have become an important source of compounds for the development of novel anti-inflammatory agents (Mamedov et al., 2005; Merfort., 2011; Jarić et al., 2015; Kim et al., 2014). The dried roots of *Inula helenium* L., a plant of the Asteraceae family, have long been used in traditional Chinese medicine for the treatment of inflammatory diseases such as enterogastritis, tuberculotic enterorrhea, and bronchitis (Seca et al., 2014; Chinese Pharmacopoeia Commission, 2015). Previous research has demonstrated that the major active components in *Inula helenium* L. are SLs, mainly alantolactone (AL) and isoalantolactone (IAL) (Fig. 1). Pharmacological investigations have shown that AL and IAL possess a wide range of activities such as anti-inflammatory, anti-bacterial, and anti-tumor activities (Rasul et al., 2013). Our previous study has demonstrated that total sesquiterpene lactones extracted from *Inula helenium* L. (TSL-IHL), which consists mainly of AL and IAL, could effectively suppress the expression of inflammatory genes in activated synovial fibroblasts and RAW 264.7 macrophages, which are associated with the amelioration of adjuvant-induced arthritis and collagen-induced arthritis in rats (Gao et al., 2016). In the present study, we investigated the potential of TSL-IHL in the treatment of AD.

Materials and methods

Reagent

2,4-dinitrochlorobenzene (DNCB) was purchased from Sigma-Aldrich (St. Louis, MO). Momestasone furoate cream (MFC) was obtained from Shenyang Pharmaceutical Company (Shenyang, China). Recombinant human TNF-α was purchased from Peprotech (Rocky Hill, NJ). All chemicals and solvents were of the highest grade commercially available.

Plant material

AL (95% purity), IAL (95% purity), and TSL-IHL were isolated from the roots of *Inula helenium* L. in our laboratory using methods as previously described (Gao et al., 2016). TSL-IHL consists mainly of AL and IAL and the consistent of AL and IAL account for 35.29 ± 0.63 and 54.70 ± 0.42 (%, m/m) of the extract, respectively. The roots of *Inula helenium* L. (Asteraceae) were obtained from Anguo county, Hebei province, China, in September 2013. The identification and authentication of the plant material were carried out by Prof. Bao-Kang Huang from the department of pharmacognosy of Second Military Medical University (Shanghai, China). The voucher specimen (No. 2013.09.22) was deposited in the herbarium of Second Military Medical University.

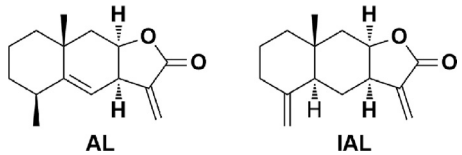

Fig. 1. Chemical structures of AL and IAL.

Cell culture

The human keratinocyte line, HaCat cells, were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, BRL) containing 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, BRL) and 1% penicillin and streptomycin at 37 °C in 5% CO_2.

Western blotting

The cells were washed with PBS and lysed with lysis buffer (50 mM of Tris–HCl pH 8, 150 mM of NaCl, 1 mM of EDTA, 0.5% NP40, and 0.1% SDS) containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Total cell protein was extracted, and equal amounts of protein were electrophoresed on SDS-PAGE. The separated proteins were transferred to a PVDF membrane and probed with primary antibodies against inhibitor of kappa (IκBα), phosphorylated IκBα (p-IκBα) and p65 (p-p65). Blots were visualized using IRDye 800CW goat anti-human secondary antibody (Li-Cor Biotechnology, Lincoln, NE). Detection was performed with an Odyssey infrared imaging system (Li-Cor Biotechnology, Lincoln, NE).

Cell viability assay

Cell viability was determined using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions (Dojindo Laboratories).

RNA isolation and quantitative real-time RT-PCR (qRT-PCR) analysis

RNA from cultured cells was extracted with the RNA isoreagent (Takara, Dalian, China) following the manufacturer's instructions. Isolated total RNA (1 μg) was then reverse transcribed using the Prime Script RT reagent Kit (Takara, Dalian, China). PCR amplification was performed on a StepOne Plus™ real-time PCR system (Applied Biosystems, Waltham, MA) using the SYBR Green Master Mix PCR Kit (Takara, Dalian, China). The sequences of the primers are indicated in Table 1. The relative levels of assayed mRNAs were calculated with the comparative CT method using GAPDH expressions as endogenous

Table 1
Primer sequences used in this study.

Gene name	Primer sequences
Human GAPDH	F:5′-TTCGACAGTCAGCCGCATCTTCTT-3′ R:5′-CAGGCGCCCAATACGACCAAATC-3′
IL-1	F: 5′-TGGCAATGAGGATGACTTGT-3′ R: 5′-GTGGTGGTCGGAGATTCGTA-3′
IL-4	F:5′-AACAGCCTCACAGAGCAGAAGAC-3′ R: 5′-GCCCTGCAGAAGGTTTCCTT-3′
TNF-α	F: 5′-TCTGGGCAGGTCTACTTTGG-3′ R: 5′-GGTTGAGGGTGTCTGAAGGA-3′
Mouse GAPDH	F: 5′-AAC TTT GGC ATT GTG GAA GG-3′ R: 5′-GGA TGC AGG GAT GTT CT-3′
IL-4	F: 5′-GAA TGT ACC AGG AGC CAT ATC-3′ R: 5′-CTC AGT ACT ACG AGT AAT CCA-3′
IL-5	F: 5′-CACCGAGCTCTGTTGACAAG-3′ R: 5′- TCCTCGCCACACTTCTCTTT-3′
IL-13	F: 5′- TGAGGAGCTGAGCAACATCA-3′ R: 5′- ATTTTGGTATCGGGGAGGCT-3′

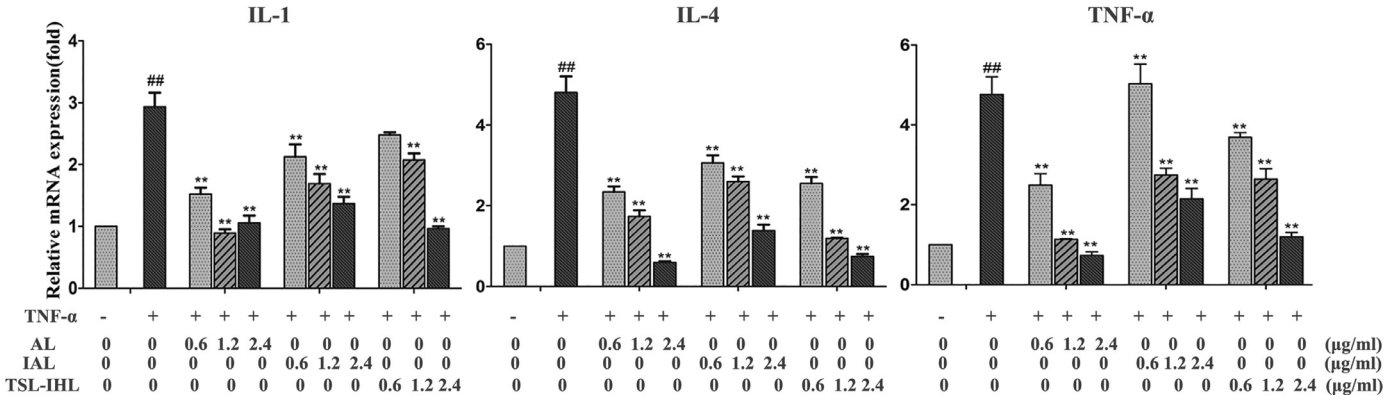

Fig. 2. TSL-IHL, AL, and IAL suppressed the expression of inflammatory cytokines in TNF-α-stimulated HaCat cells. The HaCat cells were pretreated with AL, IAL, or TSL-IHL (0.6, 1.2, 2.4 μg/ml) for 2 h at indicated concentration. The HaCat cells were stimulated with 20 ng/ml of TNF-α for 6 h, and then the mRNA levels of IL-1, IL-4 and TNF-α were measured by qPCR. Data were obtained from three independent experiments, and the values are means ± SEM. $^{\#\#}p < 0.01$ *vs.* negative control and $^{**}p < 0.01$ *vs.* TNF-α alone control.

control and were normalized to the non-treated control.

Animals

ICR mice (female, 5–8 weeks old, 18–22 g body weight) were purchased from Laboratory Animal Company (Shanghai, China). The animals were housed (five mice per cage) at 22 ± 1 °C with a relative humidity of 55 ± 5% throughout the study. All animal experiments were performed in accordance with Institutional Animal Research Committee guidelines (SMMU, License No. 2013061).

Induction of AD-like skin lesions in ICR mice

After a 7-day adaptation period, the hair on the backs of mice was shaved. AD-like skin lesions in mice were induced by DNCB as previously described with small modification (Kim et al., 2014, Lin et al., 2016). Briefly, DNCB was dissolved in AOO (acetone: olive oil = 4:1). ICR mice were sensitized once on day 1 by topically applying 100 μl of 7% DNCB on their shaved back skin. Four days after sensitization, the mice were challenged 5 times every 3 days by painting 20 μl of 0.2% DNCB solution on the inner and outer surfaces of the right ears. The normal control animals were painted with solvent alone.

Groups and treatment

The experimental mice were randomly divided into four groups ($n = 5$) as follows: control group (normal mice without any treatment), DNFB group (DNCB rechallenge without drug treatment), DNCB + TSL-IHL group (DNCB rechallenge plus 1% TSL-IHL treatment), and MFC group (DNCB rechallenge plus 0.1% MFC treatment). TSL-IHL (1%, W/W) was mixed in the emollient cream vehicle for animal treatment. In DNCB + TSL-IHL group, 300 μl of emollient cream containing TSL-IHL was applied topically on the dorsal skin and ears once per day for 17 days starting from day 5 after DNCB sensitization. Mice in the control and DNCB groups received topically applied 300 μl of emulsions without TSL-IHL on the dorsal skin and ears at the same time. Moreover, 0.1% MFC was used as a positive control. Mice were sacrificed on day 22 of the experiment. The right ears and dorsal skin were removed and subjected to histological examination, and blood was collected from the vena cava.

Evaluation of skin dermatitis severity

The severity of dermatitis in the dorsal skin was evaluated 48 h after each challenge according to the criteria described previously (Leung et al., 1990). The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 4 (severe). The average scores were defined as the dermatitis score.

Evaluation of ear swelling

The extent of ear swelling was evaluated by measuring the thickness difference between the right and left ear after each DNCB challenge and evaluated by measuring the weight difference between the right and left ear pieces after mice were sacrificed, and a small round piece of each ear was cut.

Statistical analysis

The data are presented as the mean ± SEM. Comparison of more than two groups was made with a one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was determined as $P < 0.05$.

Results

TSL-IHL, AL, and IAL could all similarly suppress the expression of inflammatory cytokines and inhibit NF-kB activation in TNF-α stimulated HaCat cells

In a previous study, we had determined the chemical constituents of TSL-IHL and evaluated its potential in RA treatment. Our previous results showed that AL and IAL represent major constituents of TSL-IHL, accounting for 35.29 ± 0.63 and 54.70 ± 0.42 (%, m/m) of TSL-IHL, respectively, and the inhibitory activity of TSL-IHL on the inflammatory gene expressions in TNF-α-stimulated synovial fibroblasts and RAW 264.7 cell is similar to those of purified AI and IAL (Gao et al., 2016). To investigate the potential of TSL-IHL in the treatment of AD, we further tested its effect on inflammatory cytokine expression in HaCat cells, a human keratinocyte line. The effects of AL and IAL were also simultaneously studied for comparison. The results reveal that TSL-IHL inhibited the expression of IL-1, IL-4 and TNF-α in TNFα-stimulated HaCat cells dose-dependently in the dose range of 0.6–2.4 μg/ml, and purified AI and IAL also showed similar dose-dependent inhibitory activities on the expression of these inflammatory genes (Fig. 2). To further probe into the related intracellular mechanisms, we next investigated their effects on the activation of NF-κB pathway, the most important signaling pathway triggered by TNF-α that controls the expression of inflammatory genes. The results revealed that TSL-IHL, AL and IAL could all inhibit TNF-α-induced IκBα phosphorylation and degradation, as well as the phosphorylation of p65 NF-κB subunit in

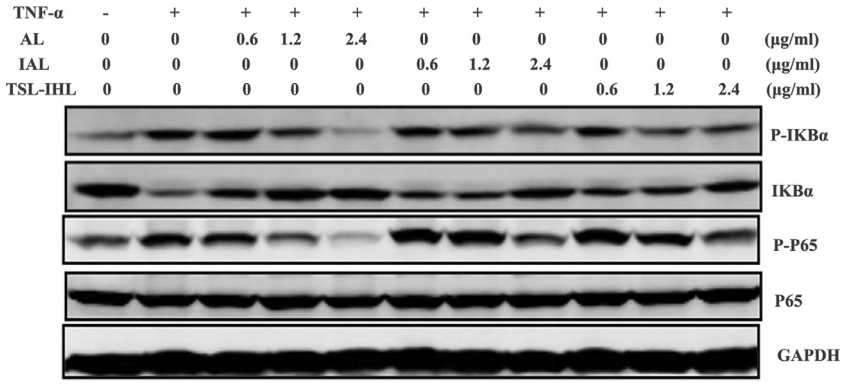

Fig. 3. TSL-IHL, AL, and IAL inhibited NF-*k*B pathway activation in TNF-*α*-stimulated HaCat cells. The HaCat cells were stimulated with 20 ng/ml of TNF-*α* for 20 min, and then the total proteins were extracted, and the level of I*κ*B*α*, p65, p-I*κ*B*α*, p-p65 were evaluated by western blot.

HaCat cells, in a dose-dependent manner in the dose range of 0.6–2.4 μg/ml (Fig. 3). These results suggest that TSL-IHL, as well as purified AL and IAL, could inhibit TNF-*α*-induced activation of NF-*κ*B pathways by suppressing the expression of pro-inflammatory genes in keratinocytes.

TSL-IHL alleviates DNCB-induced inflammatory reactions in dorsal skin and ear

Our *in vitro* experiment on HaCat keratinocytes suggests that TSL-IHL may have anti-dermatitis potential. Thus, we next performed *in vivo* experiments to assess the effectiveness of TSL-IHL against AD-like skin lesions. As shown in Fig. 4, AD-like clinical symptoms were induced by sensitizing mice once by the topical application of 100 μl of 7% DNCB on shaved back skin followed by five consecutive challenges with 20 μl of 0.2% DNCB on the right ear. Repeated challenges with DNCB resulted in aggravating dermatitis symptoms, including erythema, hemorrhage, excoriation, erosion, and scaling in the sensitized dorsal skin of mice. However, all these dermatitis symptoms were significantly attenuated by the topical application of TSL-IHL, as well as by the topical application of MFC, a positive control drug (Fig. 4(A)). Repeated DNCB challenges also significantly increased dermatitis scores and right ear swelling, and the topical application of TSL-IHL and MFC resulted in lower dermatitis scores and markedly decreased ear swelling, compared with the untreated DNCB-challenged control (Fig. 4(B)–(D)). Histopathological analysis showed that DNCB exposure caused apparent inflammatory changes such as epidermal thickening and excessive inflammatory infiltration, which were all markedly alleviated by TSL-IHL and MFC treatment (Fig. 5).

TSL-IHL reduces serum levels of IgE, TNF-α, and IFN-γ in DNCB-challenged mice

Repeated DNCB challenges resulted in a significant increase in the serum levels of IgE, TNF-*α*, and IFN-γ. In the TSL-IHL treated group, the serum levels of IgE, IFN-γ, and TNF-*α* were reduced by 81.39%, 89.69%, and 87.85%, respectively, compared with untreated control (Fig. 6).

TSL-IHL suppresses DNCB-induced expression of cytokines in back skins

To gain a better understanding of the mechanisms underlying the anti-inflammatory effects of TSL-IHL, we assessed the mRNA levels of inflammatory cytokines in skin lesions by qRT-PCR. As shown in Fig. 7, the repeated topical application of DNCB significantly increased the expression of IL-4, IL-5, and IL-13 in back-skin lesions. In TSL-IHL-treated mice, the mRNA levels of IL-4, IL-5, and IL-13 were reduced by 39.21%, 40.62% and 48.12%, respectively, compared with the untreated control. These results demonstrate that TSL-IHL suppressed DNCB-induced inflammatory cytokine expression, thereby leading to the inhibition of the skin inflammation.

Discussion

Traditional Chinese medicines contain many ingredients, some of which may act synergistically on the same signaling pathway or act on multiple pharmacological targets simultaneously; thus, they may exert better clinical efficacy than a single component. However, some components may just as well act antagonistically to each other, leading to a compromised pharmacological activity. In the present study, we found that TSL-IHL, a SL-enriched extract of *Inula helenium* L. with AL and IAL as the main constituents, exhibited similar pharmacological activity as purified AL and IAL in inhibiting the expression of inflammatory genes and NF-*κ*B activation in cultured HaCat cells. This result suggests that AL and IAL in TSL-IHL act neither synergistically nor antagonistically to each other but most possibly addictively. Both AL and IAL are abundant in *Inula helenium* L., and while the preparation of TSL-IHL is quite easy, the further purification of AL and IAL is difficult, complicated, low-yielding and expensive; there is currently no effective and economic way to synthesize these compounds. Here, our *in vitro* data indicate that TSL-IHL has anti-inflammatory activity that is not inferior to purified AL and IAL. Therefore, we propose that TSL-IHL is more suitable to be developed as a therapeutic material.

Since the expression of pro-inflammatory cytokines in epidermal keratinocytes is associated with atopic skin inflammation, the finding that TSL-IHL effectively inhibits the expressions of pro-inflammatory cytokines in TNF-*α*-activated HaCat cells prompted us to investigate the therapeutic potential of TSL-IHL for AD. We tested its protective effects on DNCB-induced AD-like skin lesion in a mouse model. Our *in vivo* data indicated that the DNCB-induced AD-like pathological changes in the back skins, including severe edema, hemorrhage, excoriation, erosion, and scaling, were all prevented by topical treatment of TSL-IHL. Accordingly, the symptom severity scores were reduced by TSL-IHL treatment. TSL-IHL treatment also led to a significant inhibition of ear swelling induced by DNCB challenge, reflecting a profound inhibition of the edema and the cell infiltration in ears. Histologically, TSL-IHL treatment decreased hypertrophy and infiltration of inflammatory cells in both ear and back-skin tissues. These results indicate that TSL-IHL treatment was able to resolve AD-like skin lesions.

The elevated serum IgE level is one of the most important immunological hallmarks of AD (Ott et al., 2009; Novak., 2009; Liu et al., 2011). IgE is known to cause both acute and chronic phase skin symptoms of AD. IgE synthesis by B cells is up-regulated by Th2 cytokines, especially IL-4. In addition to IgE and Th2 cytokines, Th1-type cytokines such as IFN-γ and TNF-*α* also play key roles in the pathogenesis of AD. (Brandt and Sivaprasad., 2011; Turner et al., 2012).

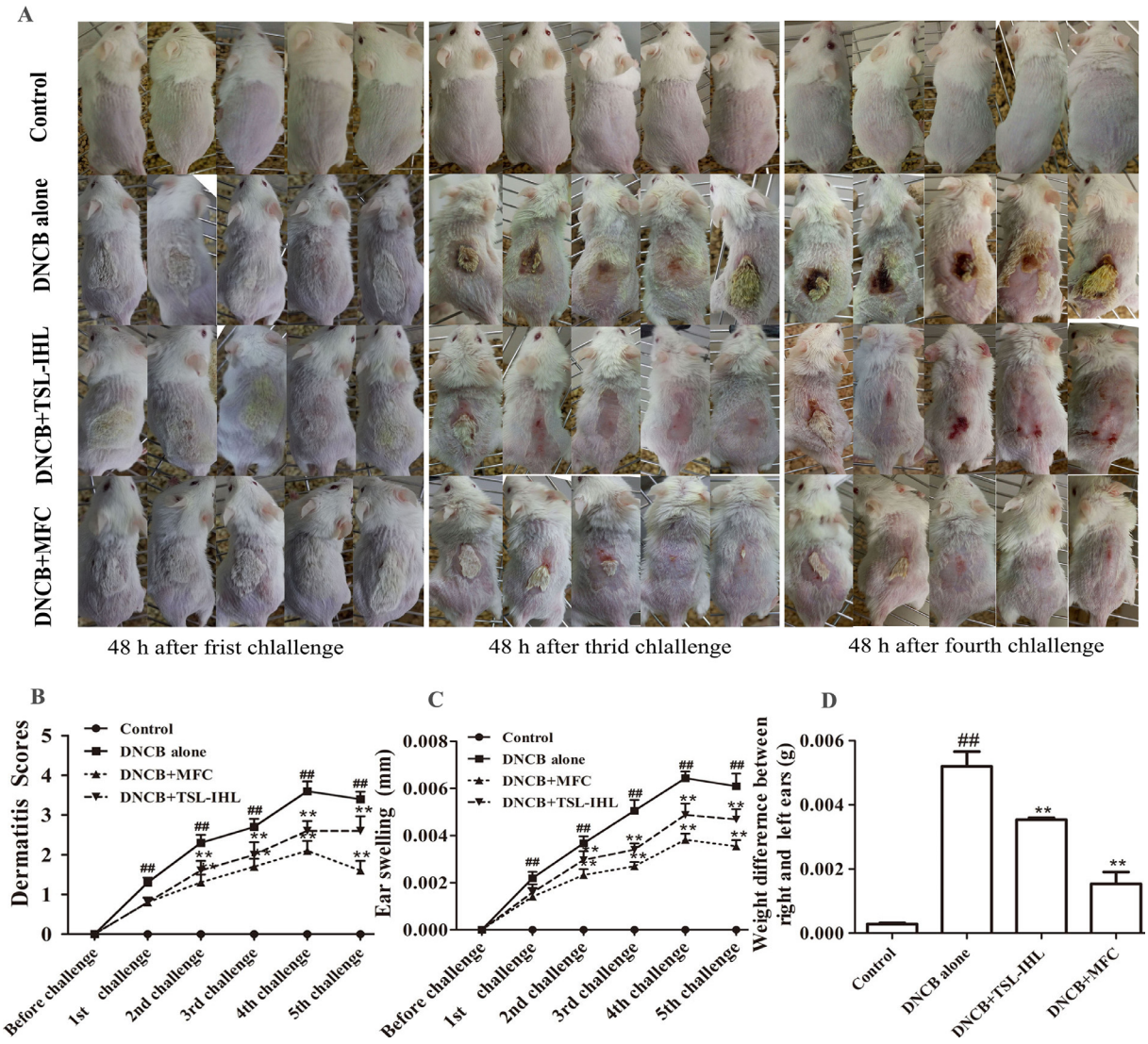

Fig. 4. TSL-IHL alleviates DNCB-induced inflammatory reactions in dorsal skin and ear. Dermatitis were induced by painting 100 μl of 7% DNCB on shaved back skin of ICR and then challenging them with DNCB 5 times on their right ears at 3 days interval. TSL-IHL was applied topically on the dorsal skin and ears once per day for 17 days starting from day 5 after DNCB sensitization. (A) Photos were taken 48 h after the first, third and fourth challenge. (B) The dermatitis score was evaluated 48 h after each challenge based on the following symptoms: erythema, edema, hemorrhage, excoriation, and escharosis. (C) Ear swelling was evaluated after each DNCB challenge by measuring the thickness difference between the right and left ears. (D) Ear swelling was also evaluated by measuring the weight difference between the right and left ear pieces after mice were sacrificed and a small round piece in each ear were cut. The results are means ± SEM of 5 mice per group. Statistical difference is indicated as $^{\#\#}p < 0.01$ *vs.* control group, $^{**}p < 0.01$ *vs.* DNCB group.

Although a Th2-biased immune response is closely linked to the acute phase of AD, atopic dermatitis is a biphasic inflammation with a Th1-predominant paradigm in the chronic phase (Grewe et al., 1998). IFN-γ, a characteristic Th1 type cytokine, exerts a vital function in immune response. In chronic AD lesions, there is a switch from a Th2 phenotype toward a Th1 phenotype, in which IFN-γ plays a critical role. IFN-γ also acts as a promoter of chronic inflammation in AD. TNF-*α* is an important pro-inflammatory cytokine involved in the maintenance of chronic inflammatory processes in the skin. TNF-*α* can activate NF-*κ*B, which is responsible for the expression of other pro-inflammatory cytokines (Aggarwal., 2003). In this study, we found that repeated DNCB challenges resulted in elevated serum levels of IgE, IFN-γ and TNF-*α*, which were all significantly reduced by TSL-IHL treatment. Furthermore, mRNA expression of IL-4, IL-5, and IL-13 in the back-skin lesions were elevated in the DNCB-challenged mice, and the expression of these cytokines were significantly suppressed by TSL-IHL treatment. Consistent with the *in vivo* results, our *in vitro* study demonstrated that TSL-IHL (0.6–2.4 μg/ml) dose-dependently suppressed the expression of TNF-*α*, IL-1, and IL-4 in TNF-*α*-stimulated HaCat cells. Collectively, these results suggest that the protective effects of TSL-IHL against DNCB-induced mouse models are associated with the down-regulation of these cytokines. Further studies are required to determine the exact molecular mechanism and to evaluate the therapeutic potential of TSL-IHL in the prevention and treatment of human AD.

Conclusion

The present study revealed that TSL-IHL, a SL-enriched extract of *Inula helenium* L. with AL and IAL as the main constituents, could effectively suppress the inflammatory gene expression and inhibit NF-*κ*B activation in activated HaCat cells. More importantly, topical treatment of TSL-IHL could inhibit the development of AD-like skin symptoms possibly by down-regulating Th1/Th2 cytokines. Our results indicate that TSL-IHL could provide an effective alternative therapy for the treatment of AD.

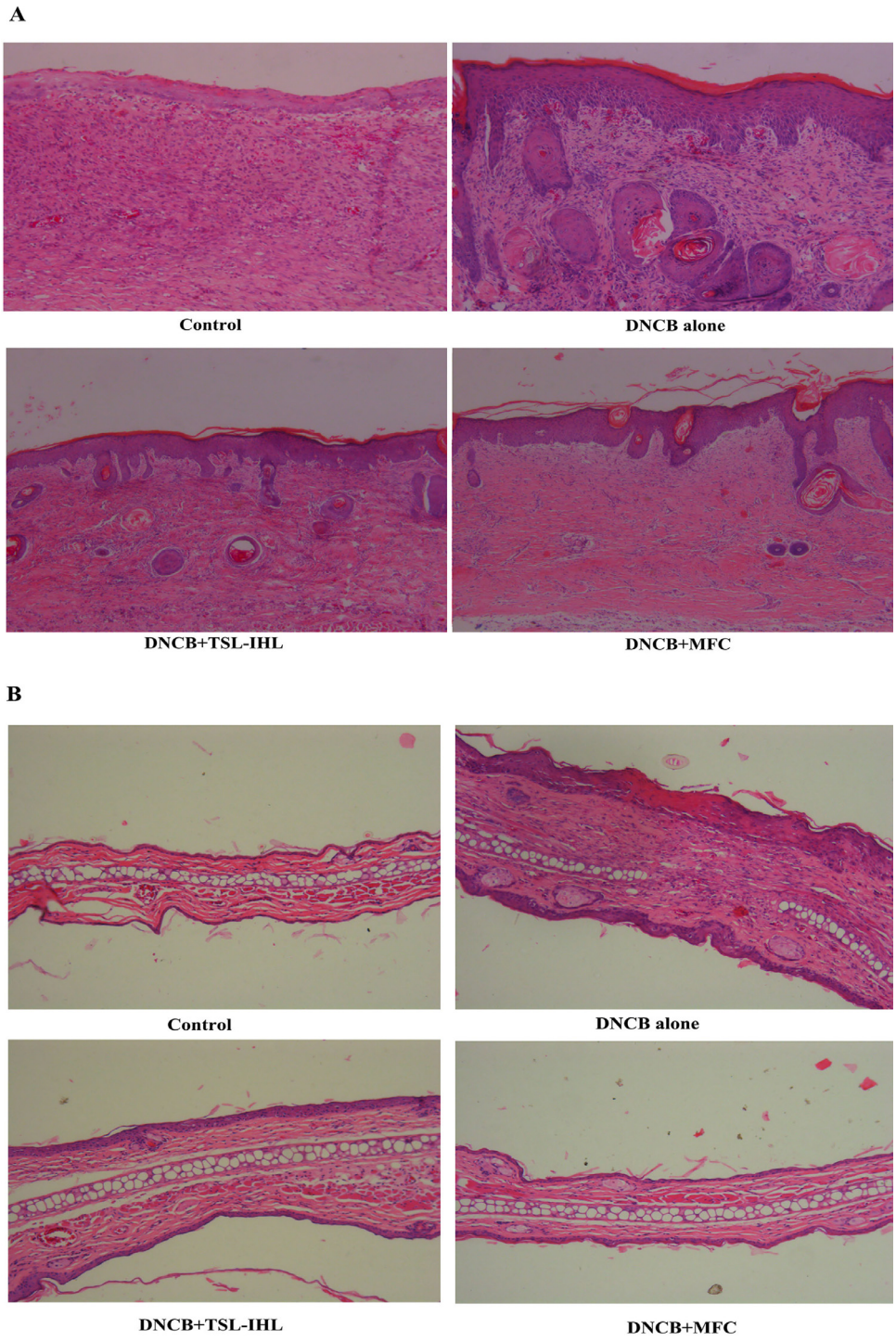

Fig. 5. The effect of TSL-IHL on histological changes in the (A) back and (B) ears skins of DNCB challenged mice. The right ear tissues and the back skin were harvested at 48 h after fifth DNCB challenged. The skin sections were prepared and stained with hematoxylin-eosin.

Conflict of interest

The authors report that they have no conflict of interest.

Author contributions

Z.W.D. and H.Z.L. designed the experiments, analyzed the experimental data and wrote the paper. W.Q and G.S. conducted the *in vitro* experiments. W.G.Z. and Y.N collected the plant material and prepared total sesquiterpene lactones from Inula helenium (TSL-IHL). Z.X.P identified and quantified the chemical constituents of TSL-IHL. L.W.C., X.N., Z.R.R. and L.C.W. conducted the *in vivo* experiments.

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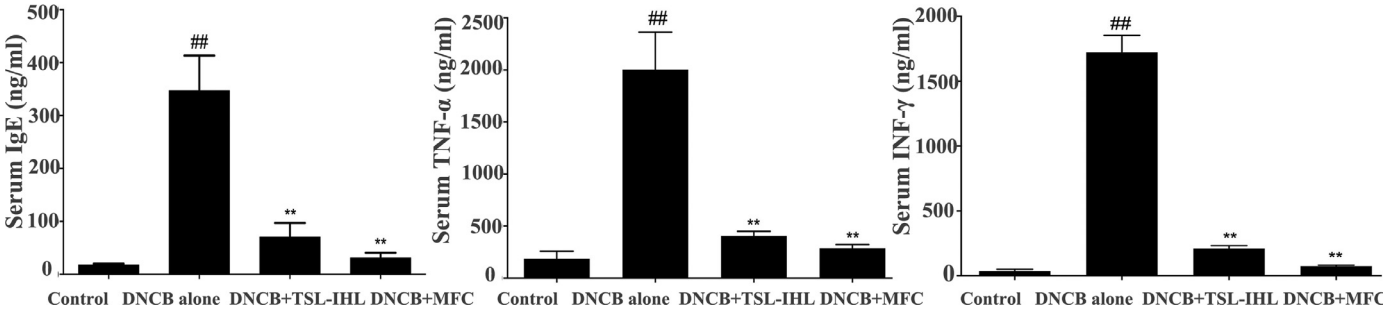

Fig. 6. The effect of TSL-IHL on serum levels of IgE, TNF-*α*, and IFN-γ in DNCB-challenged mice. The blood samples were collected at 48 h after the fifth DNCB challenge, and serum levels of IgE, TNF-*α*, and IFN-γ were assayed by ELISA. The results are means ± SEM of 5 mice per group. Statistical difference is indicated as $^{\#\#}p < 0.01$ *vs.* control group, $^{**}p < 0.01$ *vs.* DNCB group.

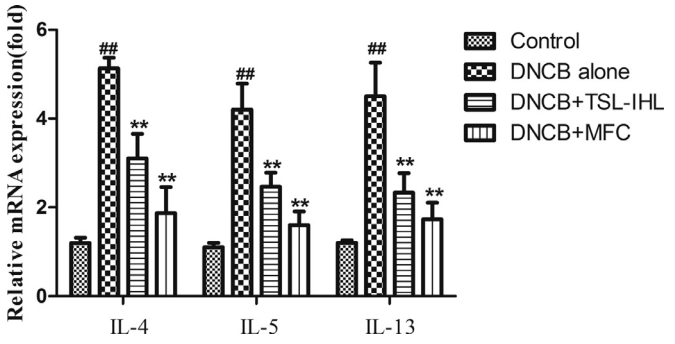

Fig. 7. The effect of TSL-IHL on DNCB-induced expression of cytokines in back skins. The skin specimens were harvested at 48 h after the fifth DNCB challenge, and the mRNA levels of indicated cytokines were assessed by qRT-PCR. Data were obtained from three independent experiments. Statistical difference is indicated as $^{\#\#}p < 0.01$ *vs.* control group, $^{**}p < 0.01$ *vs.* DNCB group.

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