

Original Article

Anti-atopic dermatitis effect of a modified Huang-Lian-Jie-Du decoction and its active fraction on 2,4-dinitrobenzene and MC903-induced mouse models

Lan Wang^a, Zhen Hu^a, Wen Yang^a, Steven King Fan Loo^{a,b}, Siu Po Ip^a, Yan-Fang Xian^{a,*}, Zhi-Xiu Lin^{a,b,c,*}

^a School of Chinese Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Room 101-J, 1/F, Li Wai Chun Building, Shatin, NT, China Hong Kong Special Administrative Region

^b Hong Kong Institute of Integrative Medicine, The Chinese University of Hong Kong, China Hong Kong Special Administrative Region

^c Li Dak Sum Yip Yio Chin R&D Centre for Chinese Medicine, The Chinese University of Hong Kong, China Hong Kong Special Administrative Region



ARTICLE INFO

Keywords:

Atopic dermatitis
Modified Huang-Lian-Jie-Du decoction
DNCB-sensitized mouse
MC903-sensitized mouse
NF-κB and MAPK pathways
Gut microbiota

ABSTRACT

Background: Huang-Lian-Jie-Du Decoction is a traditional Chinese medicine formula which has long been used to treat inflammatory skin disease including AD. However, *Gardeniae Fructus*, a component herb of HLJDD, has noticeable toxicity in liver and kidney. We therefore replaced *Gardeniae Fructus* with *Dictamni Cortex* with a hope to derive a modified HLJDD (MHLJDD) with better safety profile.

Purpose: The present study aimed to develop MHLJDD and identify its active fraction as innovative therapeutic agents for AD using 2,4-dinitrobenzene (DNCB) and calcipotriol (MC903)-sensitized mouse models of AD.

Methods: MHLJDD and the combination of the 1-butanol-soluble-fraction and the water-soluble-fraction (MHLJDD-F) were given intragastrically to the DNCB-induced mice and MC903-induced mice for two weeks. The body weight, dorsal skin/ear thickness and severity of AD symptoms of the mice were measured throughout the study. Scratching behaviors were observed after drug treatment. The blood and dorsal skin/ear tissues of mice were harvested for histopathological examination and biochemical analyses.

Results: The results revealed that DNCB- and MC903-induced AD symptoms, including skin thickening, dryness, erythema and excoriations, in the dorsal skin and ears were significantly alleviated in the MHLJDD and MHLJDD-F-treated mice. Ceramides content and protein expressions of filaggrin and loricrin were also up-regulated after treatment with MHLJDD and MHLJDD-F. In addition, skin inflammation induced by DNCB and MC903 were markedly suppressed in the MHLJDD and MHLJDD-F-treated mice, and the action mechanisms involve suppression of the release of inflammatory cytokines, as well as downregulation of the activation of NF-κB and MAPKs pathways. Besides, MHLJDD and MHLJDD-F could reverse the abundance of gut microbiota induced by DNCB in mice.

Conclusions: MHLJDD and MHLJDD-F could markedly relieve AD-like symptoms induced by DNCB and MC903 in mice through, at least in part, improving the epidermal barrier function and inhibiting skin inflammation via suppressing the activation of NF-κB and MAPKs pathways and regulation of the gut microflora dysbiosis. This study reported for the first time that MHLJDD and its active fraction could be used as innovative therapeutic agents for AD.

Abbreviations: AD, atopic dermatitis; CMC-Na, sodium carboxymethyl cellulose; DC, Dictamni Cortex; DNCB, 1-chloro-2,4-dinitrochlorobenzene; ECL, enhanced chemiluminescence; ELISA, enzyme linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FLG, filaggrin; GF, *Gardenia Fructus*; H&E, hematoxylin and eosin; IFN-γ, interferon gamma; IL, interleukin; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; JNK, c-Jun N-terminal kinase; LOR, loricrin; MAPK, mitogen-activated protein kinase; MC903, calcipotriol; NF-κB, nuclear factor-κB; SDS, sodium dodecyl sulfate; TCM, traditional Chinese medicine.

* Corresponding authors at: School of Chinese Medicine, Faculty of Medicine, The Chinese University of Hong Kong, Room 101-J, 1/F, Li Wai Chun Building, Shatin, NT, China Hong Kong Special Administrative Region.

E-mail addresses: lisaxian@cuhk.edu.hk (Y.-F. Xian), linzx@cuhk.edu.hk (Z.-X. Lin).

<https://doi.org/10.1016/j.phymed.2022.154346>

Received 28 February 2022; Received in revised form 5 June 2022; Accepted 17 July 2022

Available online 18 July 2022

0944-7113/© 2022 Elsevier GmbH. All rights reserved.

Introduction

Atopic dermatitis (AD) is a common skin condition characterized by allergic inflammation, and usually runs a chronic relapsing course (Chen et al., 2016; Leung et al., 2004). AD affects 15–30% of children and 2–10% of adults all over the world. It is well-known that AD can adversely affect the quality of life of the sufferers and renders a substantial financial burden to the patients and healthcare system as a whole. Nowadays, topical anti-inflammatory agents such as corticosteroids, calcineurin inhibitors, and systemic anti-histamine are commonly used for AD treatment, while phototherapy and systemic immune suppressants are sometimes used as adjunct treatments for severe AD. However, the long-term injudicious application of corticosteroids and calcineurin inhibitors can give rise to side effects, including cutaneous atrophy, pruritus, burning sensation, and tachyphylaxis. Since the undesirable side effects and drug tolerance are associated with long-term application of corticosteroids and calcineurin inhibitors, there clearly exists an unmet need for AD management (Tan et al., 2013).

Many AD patients, especially those in mainland China and Hong Kong, hence seek help from Chinese herbal medicine as an alternative treatment method. Chinese medicine has been used to treat various skin conditions such as eczema for hundreds of years in clinical practice. The efficacy of Chinese herbal medicine for AD is not solely anecdotal. In fact, several well-designed randomized controlled clinical trials have demonstrated the effectiveness of Chinese herbal medicine for AD (Errahali et al., 2013; Sheehan and Atherton, 1992).

Huang-Lian-Jie-Du Decoction (HLJDD, meaning *Coptidis Rhizoma* Detoxification Decoction) is one of the most frequently written prescriptions for inflammatory conditions. This formula, first recorded in a famous Chinese medicine book named “Arcane Essentials from the Imperial Library” published in AD 752 of the Tang dynasty, is composed of four herbs, i.e. *Coptidis Rhizoma* (Huanglian), *Phellodendri Chinensis Cortex* (Huangbai), *Scutellariae Radix* (Huangqin) and *Gardeniae Fructus* (Zhizi), at a ratio of 3:2:2:3. In Chinese medicine, HLJDD has the therapeutic function of purging fire and removing toxicity. A number of clinical studies have demonstrated that HLJDD could significantly ameliorate the clinical symptoms of AD (Chen and Wang, 2010; Ko and Baek, 2012; Sheehan et al., 1992). Our previous studies showed that HLJDD possessed potent anti-inflammatory and anti-allergic effects in AD experimental models, indicating that HLJDD is a promising therapy for AD (Chen et al., 2020).

Besides skin inflammation, allergy is also another important pathogenesis of AD (Leung et al., 2004). *Dictamnii Cortex* (DC, Baixianpi), originated from the dried root barks of *Dictamnus dasycarpus* Turcz., is traditionally used as a dampness eliminating and wind dispelling herb. DC is widely used in Chinese medicine clinic to treat various inflammatory diseases of the skin. Our recent study has revealed that DC has significant anti-allergic effect in AD animal models (Chen et al., 2021). Since *Gardenia Fructus* (GF) has been reported to possess noticeable toxicity in the liver and kidney after long-term treatment (Li et al., 2021), we intended to replace GF with DC in HLJDD to form a modified HLJDD (MHLJDD) with a hope to develop a safer and more effective anti-AD formula. We hypothesize that MHLJDD may be a better formula with more potent anti-AD efficacy and better safety profile than that of the original HLJDD. To further simplify the MHLJDD, bioassay-guided fractionation approach was also used to identify the fraction of MHLJDD with the most potent anti-AD effect. With an aim to develop this MHLJDD as an innovative therapeutic agent for AD, the current study aimed to test this hypothesis using two preclinical experimental models, i.e. the DNCB- and MC903-induced AD mouse models.

Methods and materials

Extraction and fractionation of MHLJDD

Coptidis Rhizoma, *Phellodendri Chinensis Cortex*, *Scutellariae Radix* and

Dictamnii Cortex were purchased from a reliable Chinese herbal supplier with GMP accreditation. The herbs were ground to powder or pieces and mixed in the ratio 3:2:2:3. The mixture was then extracted with 80% aqueous ethanol in an ultrasonic bath for 30 min. The extract was filtered, and the residue further extracted twice as before. All of the three filtrates were combined, and concentrated in a rotary evaporator under negative pressure, and finally dried in a lyophilizer to get a freeze-dried power. The resultant MHLJDD extract was stored at -20°C for subsequent study. To further simplify the MHLJDD, bioassay-guided fractionation approach was used to identify the fraction of MHLJDD with the most potent anti-AD effect. Briefly, the MHLJDD extract was resuspended in water and then transferred into a separatory funnel. The solution was partitioned with 10-fold ethyl acetate and 1-butanol, respectively, for three times to successively obtain the ethyl acetate-soluble-fraction (MHLJDD-EA), the 1-butanol-soluble-fraction (MHLJDD-B) and the water-soluble-fraction (MHLJDD-W), respectively. Afterwards, these solutions were concentrated in a rotary evaporator under negative pressure, and finally dried in a lyophilizer to get a freeze-dried powders. The yields of MHLJDD, MHLJDD-W, MHLJDD-B and MHLJDD-EA were 16.45%, 5.69%, 5.98% and 1.83%, respectively.

Based on the results of our pilot study (see Supplementary data Fig. S1), we found that MHLJDD-B and MHLJDD-W could markedly reduce the dorsal skin thickness, the severity of AD symptoms and scratching behaviors in the DNCB-induced mouse model, indicating that MHLJDD-B and MHLJDD-W fractions are the active fractions of MHLJDD. Therefore, we considered that the combination of MHLJDD-B and MHLJDD-W was the active fraction of MHLJDD (MHLJDD-F) with the most potent anti-AD effect. MHLJDD-F was a combination of MHLJDD-B and MHLJDD-W (1:1, v/v). In this study, we aimed to determine the anti-AD effects of MHLJDD-F using MC903- and DNCB-treated mouse models of AD and to illuminate its underlying molecular mechanisms.

Quality control of MHLJDD and MHLJDD-F

HPLC fingerprinting was constructed as a mean for the quality control of MHLJDD and MHLJDD-F. To construct the HPLC fingerprinting, MHLJDD, MHLJDD-F and four standard references including berberine, baicalin, baicalein and wogonin were dissolved in methanol to prepare the stock solution, and then filtered through a $0.45\ \mu\text{m}$ filter before injection. The final concentration of standard references was at 1 mg/ml. Analysis was carried out on an ACQUITY UPLC system (Waters, USA). An Inertsil ODS-4 analytical C18 column ($4.6 \times 250\ \text{mm}$, GL Sciences, Japan) was used for chromatographic separation. For determination of berberine, the mobile phase of isocratic elution was acetonitrile and 0.1% phosphoric acid (1:1) containing 0.1% sodium dodecyl sulfate (SDS). For determination of baicalin, baicalein and wogonin, the mobile phase was composed of acetonitrile (solvent A) and 12 mM ammonium acetate in 0.5% acetic acid (solvent B). The gradient elution progress was as follow: 10 to 60% solvent A in 0 to 40 min. The flow rate was at 1 ml/min. The separation was conducted at room temperature and the detection wavelengths were at 264 nm (for berberine) and 278 nm (for others), respectively. The injection volume was 10 μl . The identification was carried out through comparing the relative retention time and UV characteristics between standards and MHLJDD/MHLJDD-F. The contents of the berberine, baicalin, baicalein and wogonin were calculated based on pre-constructed standard curves and the results were listed in Table 1 and Fig. 1. MHLJDD-F was found to contain 18.37% berberine, 0.24% baicalin, 0.30% wogonin and 0.05% baicalein, while MHLJDD contained 39.88% berberine, 3.27% baicalin, 0.48% wogonin and 0.12% baicalein.

Animals

Six-week-old BALB/c mice of both sexes were obtained from Laboratory Animal Services Center, The Chinese University of Hong Kong

Table 1

The standard curves and contents of the four chemical standards of MHLJDD and MHLJDD-F.

Standard	Standard curve	Content (w/w) in MHLJDD	Content (w/w) in MHLJDD-F
Berberine	$y = 73802x - 34,686$ ($R^2 = 0.9971$)	39.88%	18.37%
Baicalin	$y = 15913x - 7041.2$ ($R^2 = 0.9948$)	3.27%	0.24%
Baicalein	$y = 36004x - 2895.1$ ($R^2 = 0.9884$)	0.12%	0.05%
Wogonin	$y = 69852x - 1113.5$ ($R^2 = 0.9811$)	0.48%	0.30%

(CUHK). The animals were kept on a 12 h light/dark cycle under thermo-regulated (22 ± 2 °C), and humidity-controlled ($50 \pm 10\%$) condition and provided with standard diet and water *ad libitum*. The experimental procedures of this study were approved by the Animal Experimentation Ethics Committee of CUHK on 24 May 2021 (Reference no. 20/015/ITF).

Evaluation of the anti-AD effects of MHLJDD and MHLJDD-F using the MC903-treated mice

MC903 is the low-calcemic analog of vitamin D3 and has been used

as an inducer for establishment of AD model. To establish MC903-induced AD model, on day 1, 12.5 μ l of MC903 (1 nM dissolved in 99% ethanol) was applied to the left ear of the mice once daily for five days, followed by a two-day interruption (16 days in total) (Moosbrugger-Martinz et al., 2017). For the naïve control mice, the same procedure was followed except ethanol was applied to the left ear instead of MC903. For drug treatment, the MC903-induced AD mice were randomly divided into 7 groups of 8 mice each: (a) normal control group (NC, no AD symptoms developed); (b) MC903 model control group (MC); (c) MC903 plus dexamethasone group (5 mg/kg, DXM, positive control); (d) MC903 plus MHLJDD group (1.48 g/kg, MD); (e) MC903 plus MHLJDD-F (0.26 g/kg, MHLJDD-F-L) group; (f) MC903 plus MHLJDD-F (0.52 g/kg, MHLJDD-F-M) group and (g) MC903 plus MHLJDD-F (1.04 g/kg, MHLJDD-F-H) group. One week after challenge with the first MC903 (from day 8) application, DXM, MHLJDD and MHLJDD-F were suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) and administered by gavage once a day for 14 consecutive days, while the mice of naïve and model groups were treated with the same volume of vehicle for the same duration. Upon MC903 application, inflammation developed as hyper-vascularization and subsequent redness became visible in the left ears. The time course of inflammation could be monitored by measuring ear thickness with a vernier caliper under light anesthesia, and the severity of dermatitis was monitored on days 1, 8, 15 and 22. After the last dosing, the scratching behaviors on

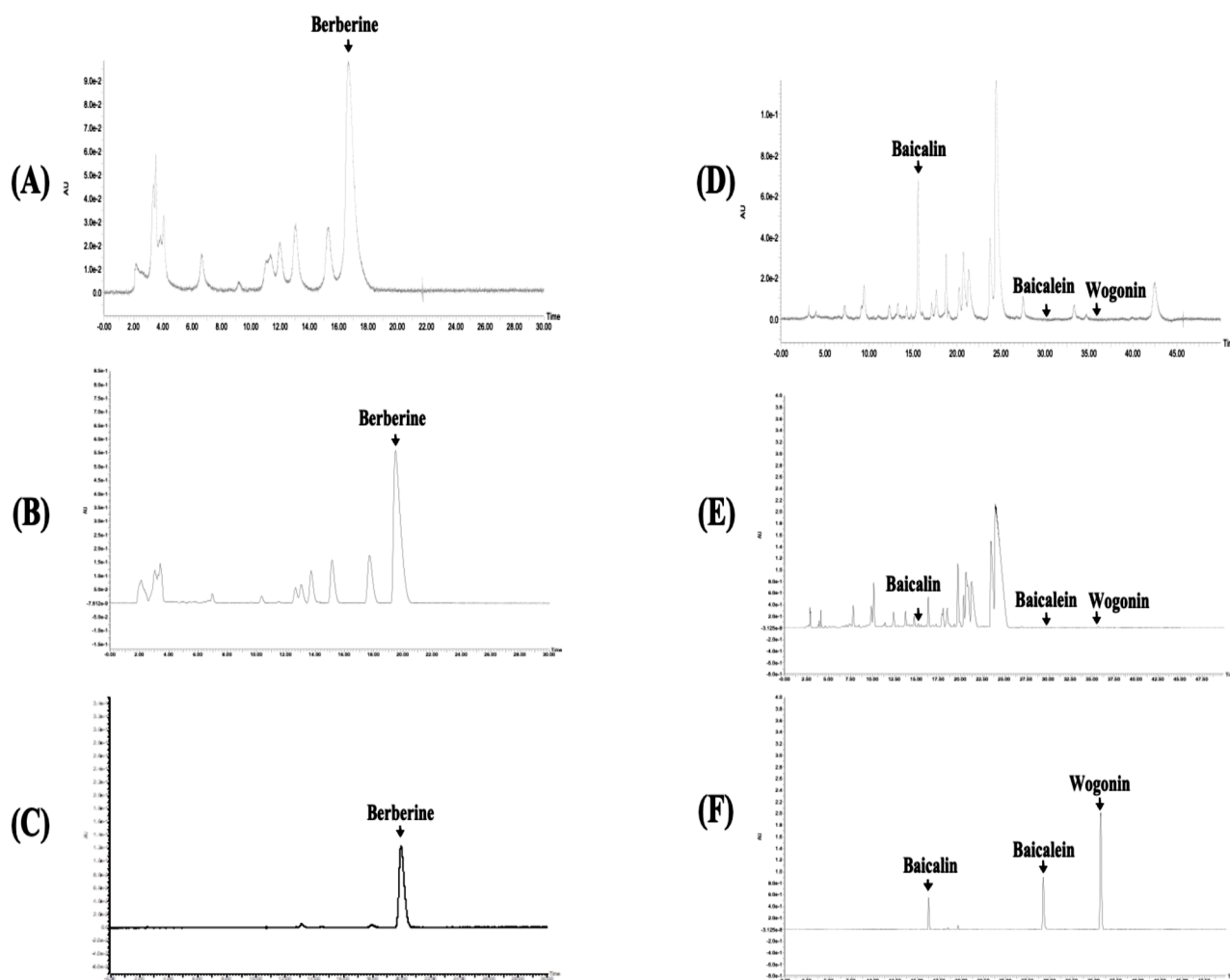


Fig. 1. The high performance liquid chromatography (HPLC) fingerprinting of MHLJDD and MHLJDD-F. (A) Chromatogram of MHLJDD detected at 264 nm. (B) Chromatogram of MHLJDD-F detected at 264 nm. (C) Chromatogram of berberine detected at 264 nm. (D) Chromatogram of MHLJDD detected at 278 nm. (E) Chromatogram of MHLJDD-F detected at 278 nm. (F) Chromatogram of baicalin, baicalein and wogonin detected at 278 nm.

the left ears of the mice were observed. The mice were subsequently sacrificed by cervical dislocation under anesthesia, and the blood and ear tissues were collected for the following biological analyses and histopathological examinations.

On days 1, 8, 15, 22, the severity of AD symptoms, including scoring of dermatitis and ear thickness, were measured. The symptoms of AD include (1) hemorrhage/erythema, (2) itching/crusting, (3) edema, (4) excoriation/erosion, and their severity was scored from 0 to 3, denoting none, mild, moderate and severe, respectively in the severity (Matsuda et al., 1997). The ear thickness (mm) was evaluated in the same region of the ear using a vernier caliper.

Evaluation of the anti-AD effects of MHLJDD and MHLJDD-F using the DNCB-treated mice

The procedure for establishing the DNCB-induced AD model was described in our previous publication (Wang et al., 2021). Female six-week-old BALB/c mice were used in the DNCB induced mice model. Briefly, 0.5% DNCB dissolved in 3:1 mixture of acetone and olive oil was applied onto the dorsal skin once daily in all groups except for the normal control group for three days. After one week, 200 μ l 1% DNCB was applied onto the same dorsal skin once every 3 days, and this procedure was repeated for 7 times. Mice were randomly assigned to 7 groups of 8 animals each on day 15 as that described in Section 2.1. From day 15, drugs were suspended in 0.5% CMC-Na and administered by gavage once a day for 14 consecutive days, while the mice of naïve and model groups were treated with the same volume of vehicle for the same duration.

The body weight, dorsal skin thickness and severity of AD symptoms of the mice were determined on the grouping day, at week one after treatment and at week two after treatment. After the last treatment, scratching behaviors were evaluated. Blood samples were collected from orbit under anesthesia by inhalation of isopropanol. After mice were sacrificed by cervical dislocation under anesthesia, the dorsal skin tissues were harvested for histopathological examination and biochemical analyses. The detailed methods of histopathological examination and biochemical analyses could be found in our previous publication (Wang et al., 2022).

Analysis of inflammatory cytokines in the dorsal skin of the DNCB-treated mice by real time-polymerase chain reaction (RT-PCR)

After drug treatment, skin tissues were homogenized, and RNA extraction was performed using TRIzol (Invitrogen, NY, USA) as described in our previous publication (Wang et al., 2022). We performed RT-PCR with reagents and protocols from TaqMan fast universal PCR master mix kit (2 $\times$) (Applied Biosystems) and mouse TaqMan gene expression assays (Applied Biosystems, assay ID: IL-4, Mm00445259_m1; TNF- α , Mm00443258_m1; IL-6, Mm99999064_m1; IL-1 β , Mm00434228_m1; IL-13, Mm00434206_g1; IFN- γ , Mm01950217_u1 and β -actin, Mn02619580_g1). The mRNA levels of TNF- α , IL-4, IL-6, IL-1 β , IL-13 and IFN- γ of different treatment groups were quantified and statistically compared (ANOVA).

Western blot (WB) analysis

We examined the protein expression levels of filaggrin (FLG), loricrin (LOR), NF- κ B and MAPKs in the skin tissues of the DNCB-treated mice and the ears of the MC903-treated mice using WB analysis as per our previous study (Wang et al., 2021). Total protein was extracted from the skin or the ear tissues using RIPA reagent. Separation of nucleus and cytoplasm was achieved using a nuclear extraction kit (Abcam, USA). Primary antibodies against β -actin, FLG, LOR, p65, p-p65, IKB- α , p-IBK- α , p38, p-p38, ERK1/2, p-ERK1/2, JNK and p-JNK were purchased from Cell Signaling Technology, Inc. The protein bands were developed by the ECL method (Biorad, USA). The chemiluminescent signal was

detected and recorded by exposure of the membrane using Azure c300 Chemiluminescent Western Blot Imaging System. The band intensity was quantified using Image J software (NIH, USA).

Gut microbiota analysis on the effects of MHLJDD and MHLJDD-F on the DNCB-treated mice

Feces of the DNCB-treated mice were collected and stored at -80°C after frozen in liquid nitrogen. Fecal genomic DNA was extracted with OMGA-soil DNA kit following the manufacturer's instruction as per our previous study (Wang et al., 2022).

Evaluation of the acute toxicity of MHLJDD-F

Eight-week old Balb/c mice were randomly assigned to each of five groups (8 mice of both sexes (1:1) per group). They were fasted overnight (12 h) with free access only to water prior to administration of a single dose (1.04, 2.08, 4.16, 8.32 and 16.64 g/kg) of MHLJDD-F. MHLJDD-F was suspended in 0.5% CMC-Na solution and given intragastrically to mice in the volume of 10 ml/kg. The general behaviors of the mice were continuously monitored for 4 h after the treatment, intermittently during a 24-h period, and thereafter daily until day 7.

Statistical analysis

All data were expressed as mean $\pm$ SEM. Multiple group comparisons were performed using one-way ANOVA followed by Bonferroni test to detect differences between experimental groups. The statistical analysis was performed on GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA) and statistical significance was considered when $p < 0.05$.

Results

Effects of MHLJDD and MHLJDD-F on the ear thickness, ear dermatitis score and scratching behaviors of the MC903-treated mice

As shown in Fig. 2A, obvious increases in the ear thickness could be observed from day 8 in the MC903-treated groups, as compared with the normal control group. On day 15, MC903 induced significant increases in the ear thickness in the MC group ($p < 0.01$), as compared with the NC groups. Besides, the ears of the MC903-treated mice gradually developed dryness, swelling and hyper-vascularization, as shown in Fig. S2A and B. However, after 2-week treatment with MHLJDD and MHLJDD-F, the MC903-induced ear dryness and hyper-vascularization were markedly attenuated, especially in the middle dose and high dose of MHLJDD-F ($p < 0.01$ for both), as compared with the MC group (Fig. 2B). The therapeutic effects of middle and high dose of MHLJDD-F were more potent than MHLJDD in reducing the severity of the ear dermatitis in the mice (Fig. 2B). However, after 2-week treatment, both MHLJDD and MHLJDD-F did not effectively inhibit the ear swelling induced by MC903 in the mice. The positive control medicine DXM showed potent therapeutic effect on the MC903-induced ear dermatitis in mice, as evidenced by reduction in the ear thickness and relief of the ear swelling and redness (Fig. S2A and B).

As shown in Fig. 2C, repeated MC903 applications significantly increased the frequency and duration of scratching of the mice. The total score of the MC mice was significantly higher than the normal control mice ($p < 0.01$). Administration of DXM, MHLJDD and MHLJDD-F-M could significantly inhibit the MC903-induced scratching behaviors ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively).

As shown in Fig. S3, after two-week application of MC903, slight body weight loss could be observed in the MC903-treated groups, as compared with the non-treated NC mice. Noticeably, marked decrease in body weight could be observed in the DXM group and MHLJDD groups. However, after two-week treatment, the body weight of MHLJDD-F-treated mice maintained at the normal level, indicating that

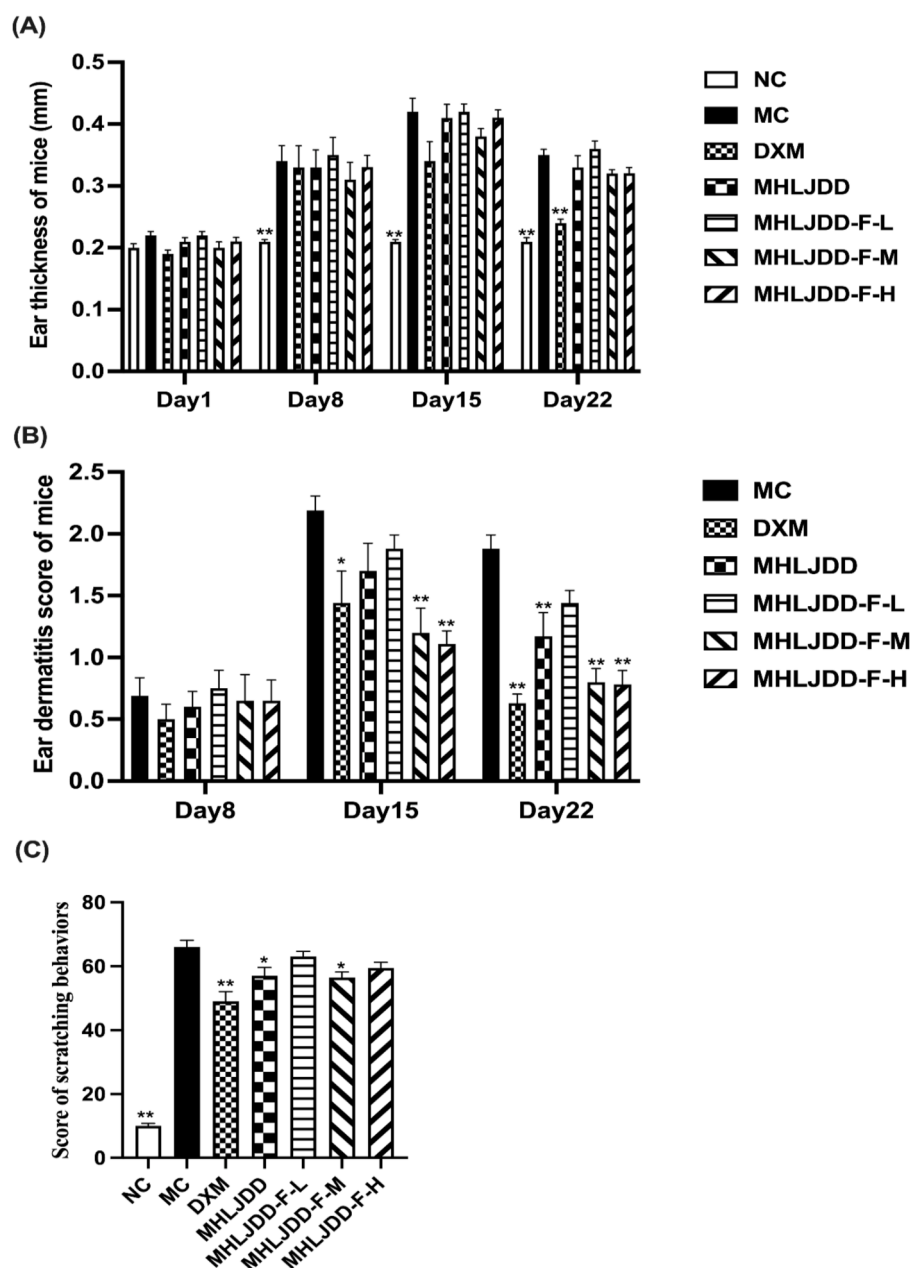


Fig. 2. Effects of MHLJDD and MHLJDD-F on the ear thickness (A), ear dermatitis score (B), and scratching behaviors (C) in the MC903-treated mice. Data were presented as mean $\pm$ SEM ($n = 8-10$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

application of MHLJDD-F exerted no significant side effect on mice. Moreover, the results of acute toxicity study revealed that the LD_{50} of MHLJDD-F was 8.25 g/kg (5.62~12.1 g/kg, 95% CI) calculated using Horn's method (Toxicological test methods for pesticides registration Part 2, GB/T 15,670.2-2017, China), and MHLJDD-F below the dose of 4.16 g/kg did not show any overt acute toxicity on mice.

Effects of MHLJDD and MHLJDD-F on the epidermal thickness and mast cells of the MC903-treated mice

As shown in Fig. 3A and B, abnormal histological alterations could be observed in the dorsal skin of the MC903-treated mice. The H & E staining showed that repeated MC903 applications evidently stimulated epidermal thickening in the MC group, with increase by 2.52-fold in the ears ($p < 0.01$), when compared with the NC group (Fig. 3A). Moreover, the epidermis of the ears of the MC mice displayed dense infiltration of

inflammatory cells, as compared with the NC mice. However, treatment with MHLJDD and MHLJDD-F (middle and high dose) significantly improved these pathologic changes ($p < 0.01$ for all), as compared with the MC group. The epidermal thickness in the ears of MHLJDD- and MHLJDD-F (middle and high dose)-treated mice decreased by 49.29%, 51.64%, and 46.24%, respectively, as compared with the MC group. Their inhibitory effects on the MC903-induced epidermal thickening were on a par with that of the positive control DXM, which decreased by 50.75% as compared with the MC group. As for the mast cell infiltration, MC903 application significantly increased the number of mast cells in the ears of the MC903-treated groups ($p < 0.01$), as compared with the NC group (Fig. 3B). Treatment of DXM, MHLJDD and MHLJDD-F (middle and high dose) could significantly suppress the infiltration of mast cells in the ears ($p < 0.01$ for all), as compared with the MC group (Fig. 3B). The numbers of mast cells were significantly decreased by 46.51%, 42.79%, 28.83% and 17.20% after treatment with DXM,

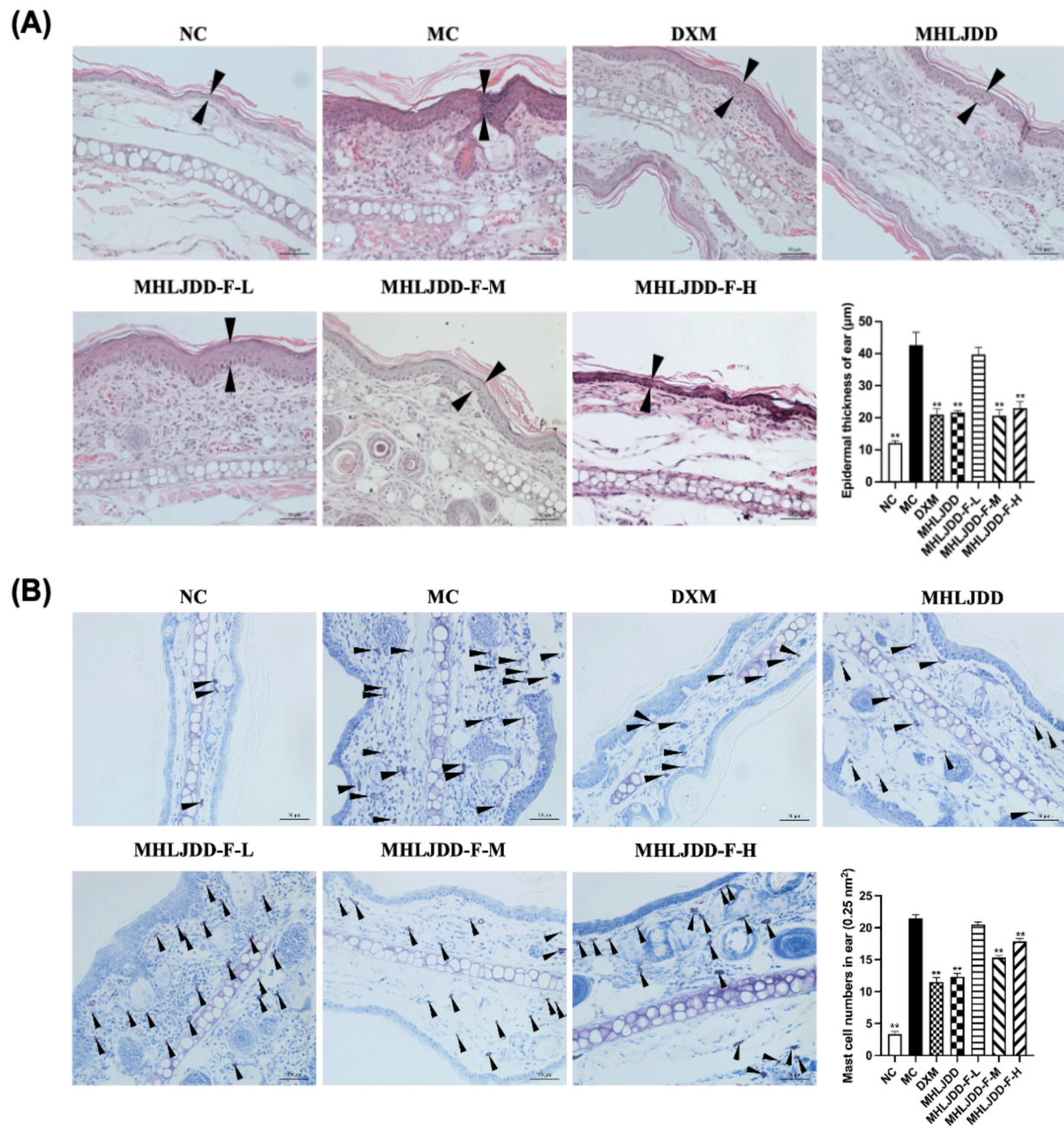


Fig. 3. Effects of MHLJDD and MHLJDD-F on the score of epidermal thickness of the ears (A), and the mast cell numbers of the ears (B) in the MC903-treated mice. Data were presented as mean $\pm$ SEM ($n = 7-8$). ** $p < 0.01$ compared with the MC group.

MHLJDD, MHLJDD-F-M and MHLJDD-F-H ($p < 0.01$ for all), respectively, as compared with the MC group. However, the inhibitory effects of MHLJDD at low dose on epidermal thickness and mast cells infiltration were insignificant.

Effects of MHLJDD and MHLJDD-F on the serum biomarker levels and ceramides content in the ears of the MC903-treated mice

In the biochemical analysis, we examined whether the levels of serum IgE, TNF- α and IL-4 in the MC903-treated mice could be modulated by MHLJDD-F. As shown in Fig. 4A–C, the serum levels of IgE, TNF- α and IL-4 were significantly increased in the MC mice ($p < 0.01$ for all), as compared with the NC group. However, after treatment with MHLJDD-F, serum levels of IgE and TNF- α were significantly decreased in the MHLJDD-F-M- and MHLJDD-F-H-treated mice ($p < 0.01$ for all),

as compared with the MC mice. The serum level of IL-4 in the MHLJDD-F-treated mice also reduced, as compared with the MC mice, although the reduction is not statistically significant. The inhibitory effect of MHLJDD-F on these serum biomarkers was dose-dependent. In addition, the inhibitory effects of middle and high doses of MHLJDD-F on suppressing the serum IgE and TNF- α levels were comparable to that of MHLJDD.

The content of ceramides was reported to be positively related to the skin barrier function (Di Nardo et al., 1998). In this study, we found that repeated MC903 application significantly reduced the ceramides content of the ear in the MC group ($p < 0.01$), as compared with the NC mice. After two-week treatment, the ceramides content was markedly recovered in the DXM group ($p < 0.01$), as compared with the MC group, while MHLJDD and MHLJDD-F exerted less marked recovery on this protein (Fig. 4D).

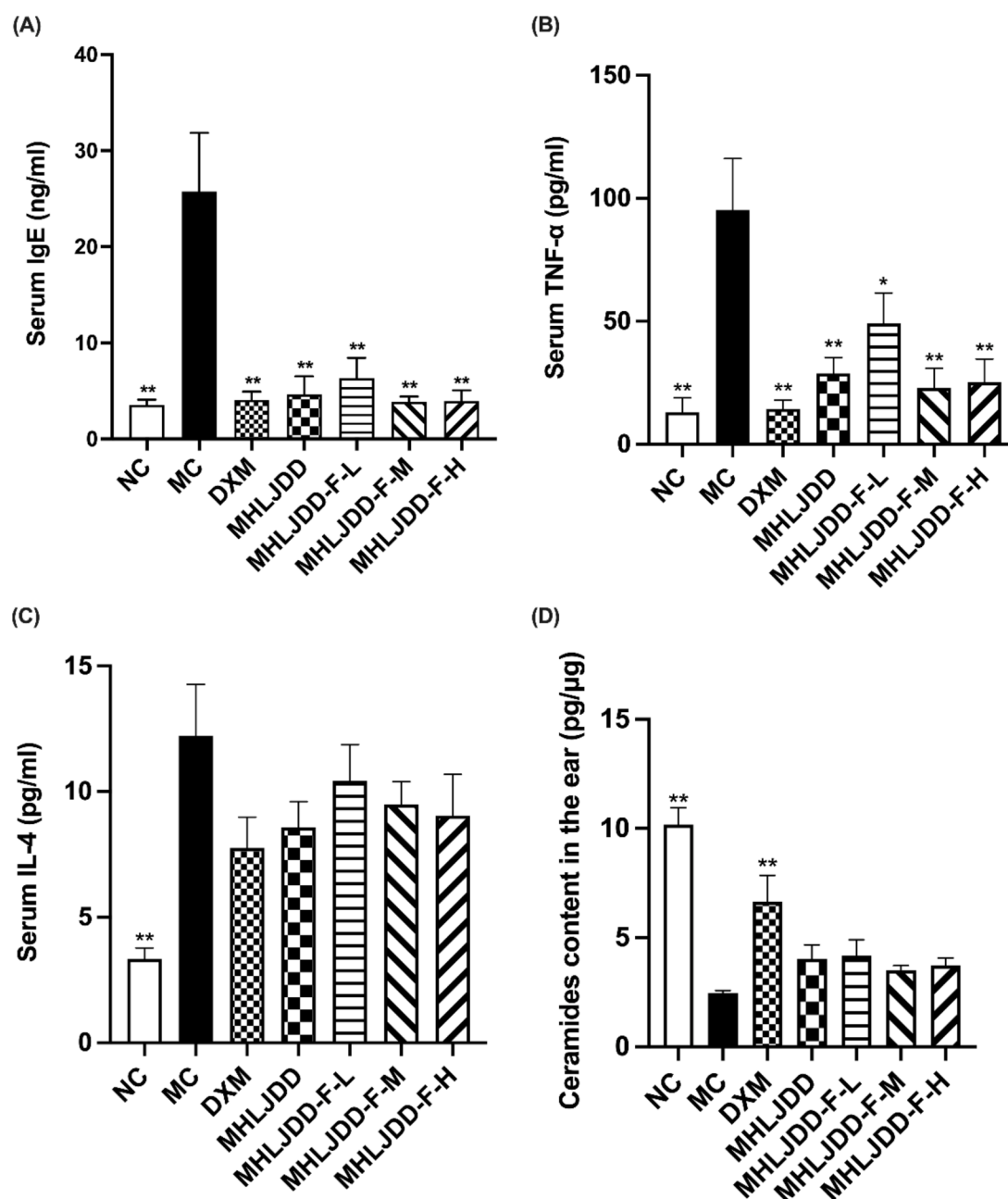


Fig. 4. Effects of MHLJDD and MHLJDD-F on the levels of IgE (A), TNF-α (B), IL-4 (C) in the serum, and ceramides content (D) in the ear of the MC903-treated mice using ELISA kits. Data were presented as mean $\pm$ SEM ($n = 7-8$). ** $p < 0.01$ compared with the MC group.

Effects of MHLJDD and MHLJDD-F on the protein expressions of FLG and LOR in the ear of the MC903-treated mice

FLG is a filament-associated protein that binds to keratin fibers in the epidermis. Defect in FLG induces skin barrier dysfunction leading to increased water loss from the skin. Besides FLG, LOR is also the essential protein in maintaining epidermal barrier function. We investigated whether MHLJDD and MHLJDD-F could regulate protein expression levels of these essential epidermal components in the MC903-treated mouse skin by WB analyses. As shown in Fig. 5, the protein expression levels of FLG and LOR in the MC group were decreased after DNCB challenge, as compared with the NC group. However, the expression levels of FLG and LOR significantly increased by 36.58% and 33.66% ($p < 0.01$ for both), respectively, in the dorsal skin of the DNCB-treated mice after treatment with the high dose MHLJDD-F, as compared with the MC group. The effect of MHLJDD on FLG expression level was

comparable to that of the high dose of MHLJDD-F, with an increase of 42.70%, while it only slightly accentuated the LOR expression level (by 14.63%).

Effects of MHLJDD and MHLJDD-F on the dorsal skin thickness, the AD-like skin lesions and score of scratching behavior of the DNCB-treated mice

As shown in Fig. 6, significant increases in the dorsal skin thickness could be observed in the DNCB-treated groups on day 15 ($p < 0.0$), as compared with the normal control mice. However, the DNCB-induced dorsal skin thickening was significantly reduced in the DXM-treated group on days 22 and 29 ($p < 0.01$ for both). Two-week treatment with MHLJDD-F showed the inhibitory effect on the DNCB-induced dorsal skin thickening. The dorsal skin thickness of the MHLJDD-F-M and MHLJDD-F-H-treated groups was reduced to the normal level.

As shown in Fig. S4, the body weight of the mice were recorded on

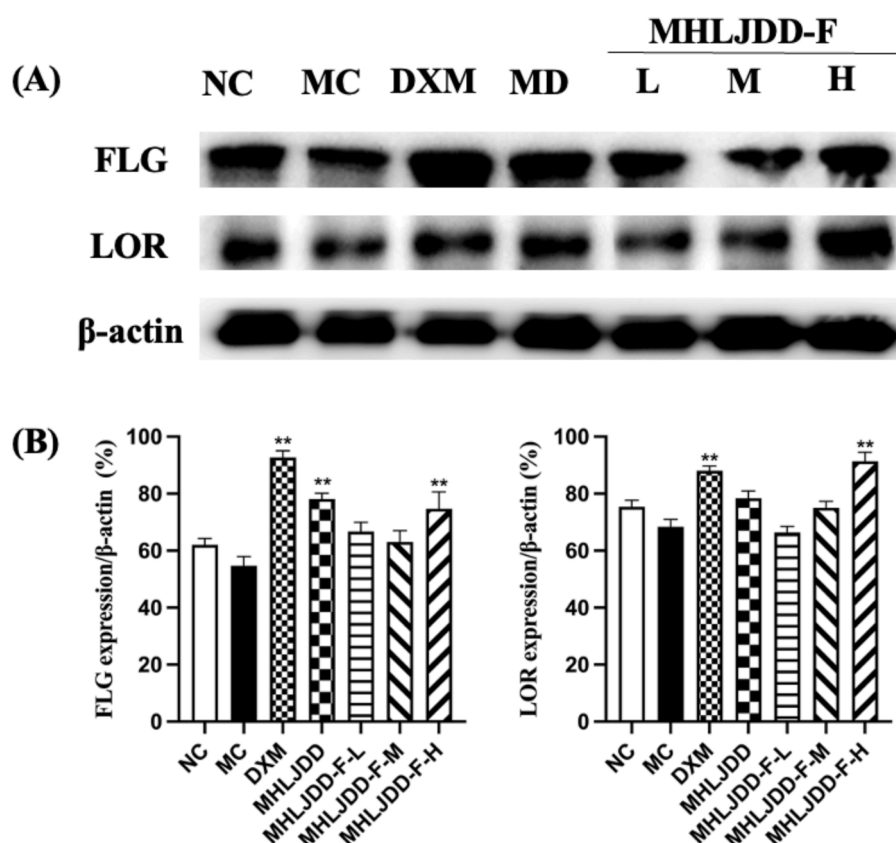


Fig. 5. Effects of MHLJDD and MHLJDD-F on the protein expressions of FLG and LOR in the ear of the MC903-treated mice. (A) Representative immunoblot bands of FLG and LOR in the ear of the MC903-treated mice. (B) Quantitative analysis of the immunoblot bands. Data were presented as mean $\pm$ SEM ($n = 3$). ** $p < 0.01$ compared with the MC group.

days 15 (on the grouping day), 22 (after 1-week treatment) and 29 (after 2-week treatment). After application of DNCB, the loss of the mouse body weight could be observed in the DNCB-treated groups, as compared with non-treated NC mice on day 29. However, after two-week treatment, the body weight of MHLJDD-treated mice showed a marked recovery, when compared with the DNCB-treated mice. The body weight of MHLJDD-F-treated mice also showed some visible recovery although without statistical differences, as compared with the MC group. On the contrary, DXM treatment caused a marked decrease in the body weight, when compared with mice in the MC group on days 22 and 29.

Application of MHLJDD, the middle dose and high dose of MHLJDD-F showed significant inhibitory effects on the DNCB-induced AD symptoms, while the inhibitory effect of low dose MHLJDD-F was only marginal (Fig. 6B). Based on the clinical score evaluated using EASI scoring system on day 29, administration of the MHLJDD, DXM, MHLJDD-F-M and MHLJDD-F-H significantly reduced the EASI score on day 29 ($p < 0.05$, $p < 0.05$, $p < 0.05$ and $p < 0.01$, respectively), as compared with the MC group (Fig. 6C). The therapeutic effects of MHLJDD-F on the skin EASI score of the DNCB-treated mice were dose-dependent. However, treatment with MHLJDD-F-L did not reduce the EASI score on day 29, as compared with the MC group.

As shown in Fig. 6D, repeated DNCB applications significantly increased the frequency and duration of scratching in mice. The total score of the MC mice was significantly higher than the NC mice ($p < 0.01$). Application of DXM, MHLJDD-F-L, MHLJDD-F-M and MHLJDD-F-H could significantly inhibit the DNCB-induced scratching behaviors ($p < 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively) and the inhibitory effects of the middle dose and high dose MHLJDD-F were much more evident than the low dose. However, the effect of MHLJDD on inhibiting DNCB-induced skin itchiness was unremarkable.

Effects of MHLJDD and MHLJDD-F on the serum biomarker levels and ceramides content in the dorsal skin of the DNCB-treated mice

In the biochemical analysis, we examined whether the levels of serum IgE, histamine and TNF- α in the DNCB-treated mice could be modulated by MHLJDD-F. As shown in Fig. 7A–C, repeated DNCB applications significantly increased the levels of TNF- α , IgE and histamine in the serum of the mice in the MC group ($p < 0.01$ for all), as compared with the NC group. However, treatment with MHLJDD-F-L, MHLJDD-F-M and MHLJDD-F-H could significantly decrease the level of TNF- α in the serum of the DNCB-treated mice ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively), as compared with the MC group. The inhibitory effect of MHLJDD-F on serum TNF- α level was dose-dependent. Treatment with MHLJDD-F-L, MHLJDD-F-M and MHLJDD-F-H could also markedly decrease the level of IgE in the serum of the DNCB-treated mice ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively), as compared with the MC group. The inhibitory effect of MHLJDD-F-H on serum IgE level was significantly more potent than that of MHLJDD-F-L. In addition, treatment with MHLJDD-F-L, MHLJDD-F-M and MHLJDD-F-H could decrease the level of histamine in the serum of the DNCB-treated mice ($p < 0.01$ for all), as compared with the MC group. The inhibitory effect of MHLJDD-F on serum histamine level was dose-dependent. MHLJDD treatment also conspicuously decreased the levels of IgE, histamine and TNF- α in the serum of the DNCB-treated mice ($p < 0.01$ for all), as compared with the MC group. Moreover, MHLJDD-F-M exerted more potent inhibitory effect than MHLJDD on the level of histamine in the serum of the DNCB-treated mice.

In this study, we found that repeated DNCB applications could significantly reduce the ceramides content of the dorsal skin in the MC group ($p < 0.01$), as compared with the NC mice (Fig. 7D). After two-week treatment, the ceramides content in the DXM, MHLJDD and

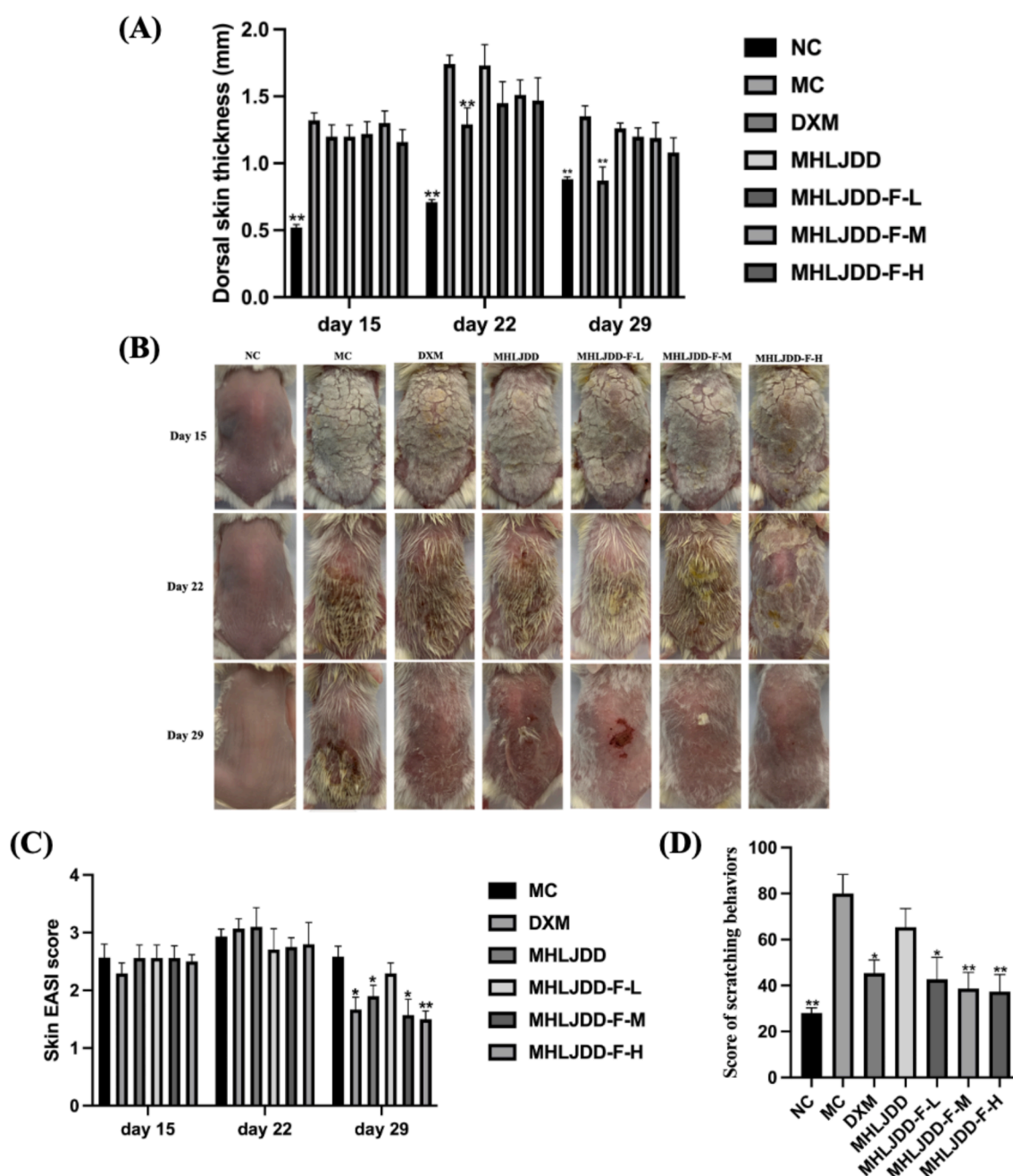


Fig. 6. Effects of MHLJDD and MHLJDD-F on the dorsal skin thickness (A), EASI scores of dorsal skin (B), and the score of scratching behaviors (C) in the DNCB-treated mice. (B) Representative photos of the dorsal skin of mice taken on grouping (day 15), after 1-week treatment (day 22) and 2-week treatment (day 29). Data were presented as mean $\pm$ SEM ($n = 7-8$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

MHLJDD-F groups was only slightly increased, as compared with the MC group. The up-regulatory effects of MHLJDD-F-M and MHLJDD-F-H on ceramides were superior to that of MHLJDD and DXM.

Effects of MHLJDD and MHLJDD-F on the epidermal thickness and mast cell infiltration of the dorsal skin of the DNCB-treated mice

Abnormal histological alterations could be observed in the dorsal skin of the DNCB-treated mice. As shown in Fig. 8A, the H & E staining showed that repeated DNCB applications evidently stimulated epidermal thickening in the MC group ($p < 0.01$), with increases by 8.39-fold in the dorsal skin, when compared with the NC group. Moreover, the dorsal skin of the MC mice displayed denser infiltration of inflammatory cells, as compared with the NC mice. However, MHLJDD

and MHLJDD-F treatment significantly improved these pathologic changes ($p < 0.01$ for all), as compared with the MC group. The epidermal thickness in the dorsal skin of the mice in MHLJDD and the low, medium and high doses of MHLJDD-F markedly decreased by 69.54%, 59.09%, 62.23% and 80.13%, respectively. The inhibitory effect of the high dose MHLJDD-F (80.13%) were more potent than the positive control DXM (69.46%), indicating that MHLJDD-F could effectively inhibit the DNCB-induced epidermal thickening in mouse dorsal skin.

As for the mast cell infiltration (Fig. 8B), DNCB application significantly augmented the number of mast cells in the dorsal skin of the mice ($p < 0.01$), as compared with the NC group. Treatment with DXM, MHLJDD and MHLJDD-F could significantly inhibit the infiltration of mast cells in the ear tissues of the DNCB-treated mice ($p < 0.01$ for all),

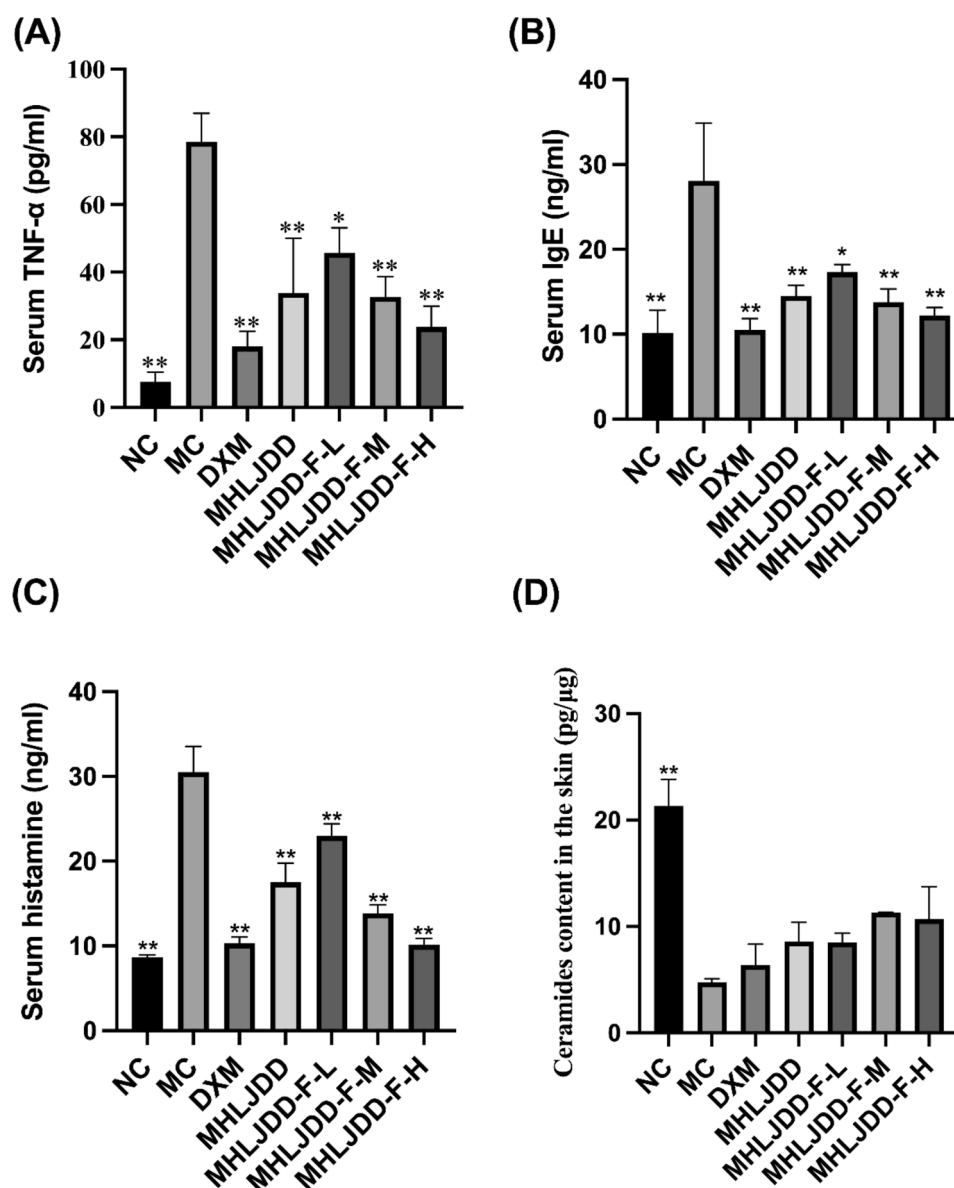


Fig. 7. Effects of MHLJDD and MHLJDD-F on the levels of TNF- α (A), IgE (B) and histamine (C) in the serum and ceramides content (D) of the skin in the DNCB-treated mice using ELISA kits. Data were presented as mean $\pm$ SEM ($n = 7-8$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

as compared with the MC group. The numbers of mast cells were decreased by 48.14%, 28.51%, 15.55%, 54.44% and 51.48% in the DXM, MHLJDD, MHLJDD-F-L, MHLJDD-F-M, and MHLJDD-F-H groups, respectively, as compared with the MC group. The inhibitory effect of MHLJDD on mast cell infiltration was inferior to that of MHLJDD-F-M/H.

Effects of MHLJDD and MHLJDD-F on the mRNA expressions of inflammatory cytokines in the dorsal skin of the DNCB-treated mice

As shown in Fig. 9, the mRNA expression levels of inflammatory cytokines such as TNF- α , IL-1 β , and Th2 cytokines including IL-4 in the dorsal skin of the mice markedly increased after repeated applications of DNCB ($p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively), when compared with the NC group. The mRNA expression level of IFN- γ did not change after DNCB application. However, treatment with MHLJDD-F effectively attenuated the mRNA expression levels of these inflammatory cytokines. High dose of MHLJDD-F significantly decreased the mRNA expressions of TNF- α , IL-1 β and IL-4 by 90.41%, 98.09% and 99.29% ($p < 0.05$ for

all), respectively, in the dorsal skin of the DNCB-treated mice, as compared with the MC group. Besides, the mRNA expression of IFN- γ in the high dose of MHLJDD-F treatment group was increased by 5.84-fold, as compared with the MC group. These results amply suggested that MHLJDD-F inhibited the inflammatory response by modulating the mRNA expression of inflammatory cytokines.

Effects of MHLJDD and MHLJDD-F on the epidermal barrier function, NF- κ B and MAPK pathways in the dorsal skin of the DNCB-treated mice

We investigated whether MHLJDD and MHLJDD-F could regulate the protein expression levels of these essential epidermal components in the DNCB-treated mouse skin by WB analyses. As shown in Fig. 10A and B, the results showed that the protein expressions of FLG and LOR were significantly decreased by 31.60% and 41.68% ($p < 0.01$ for all), respectively, after the DNCB challenge, as compared with the NC group. However, the protein levels of FLG and LOR were significantly increased after treatment with MHLJDD and MHLJDD-F ($p < 0.01$ for all), as compared with the MC group. Noticeably, the enhancing effect of the

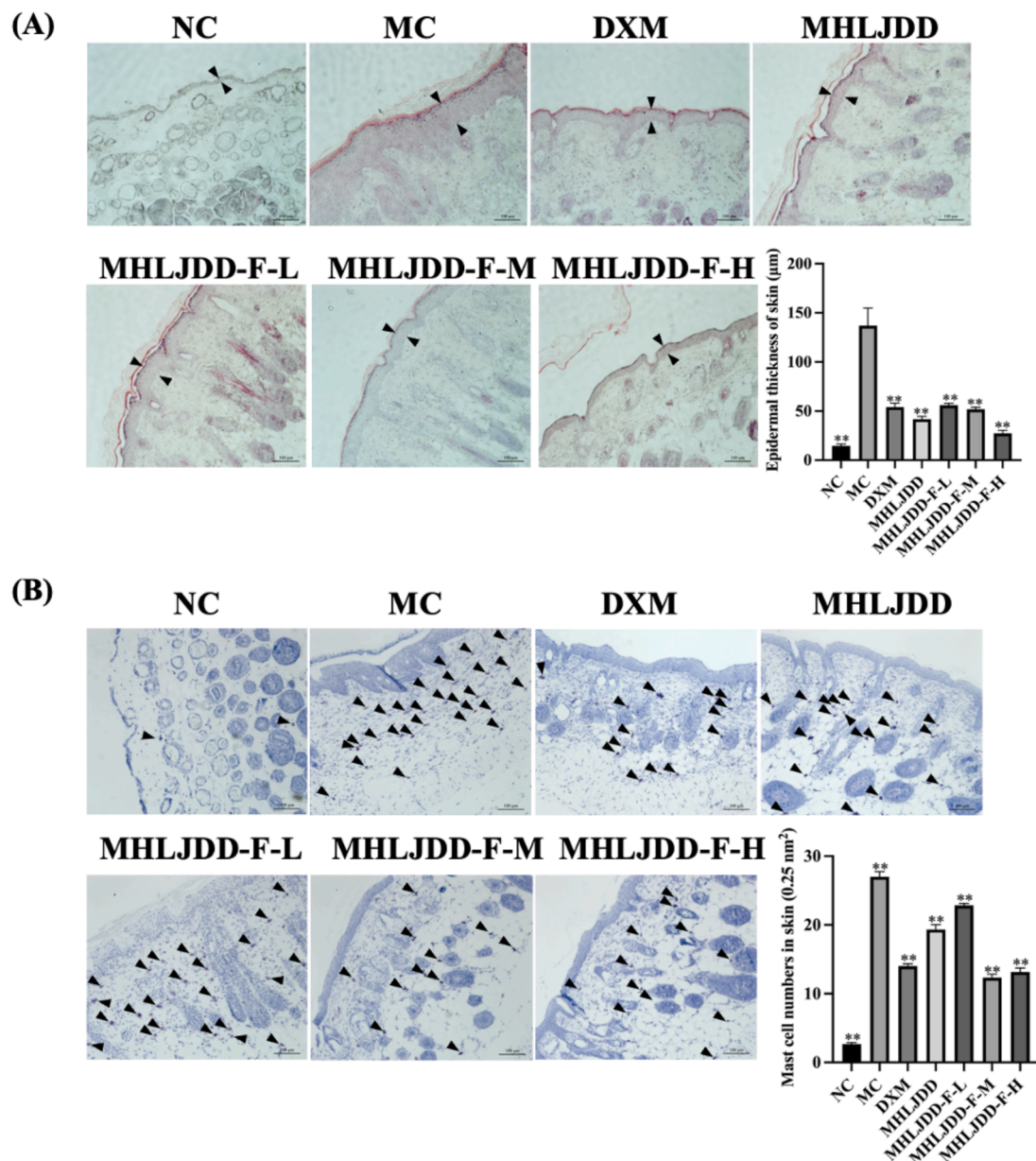


Fig. 8. Effects of MHLJDD and MHLJDD-F on the epidermal thickness of the dorsal skin and the mast cell numbers of the dorsal skin in the DNCB-treated mice. (A) Representative photos of the histopathological features of the dorsal skin with H & E staining and epidermal thickness of the dorsal skin. (B) Representative photos of the mast cells infiltration in the ears with toluidine blue staining and the mast cell numbers of the dorsal skin. Magnification at 100 $\times$, black arrows indicate epidermal thickness in the DNCB-treated mice. Data were presented as mean $\pm$ SEM ($n = 6$). ** $p < 0.01$ compared with the MC group.

high dose of MHLJDD-F was the most potent, with 4.26-fold increase in FLG and 2.96-fold increase in LOR expressions, as compared with the MC group.

To explore the action mechanisms of the anti-AD effects of MHLJDD and MHLJDD-F on the DNCB-induced skin inflammation in mice, we detected their effects on NF- κ B and MAPK pathways by WB analysis. As shown in Fig. 10C and D, repeated DNCB applications significantly increased the ratios of p-p65/p65, p-IK β /IK β , p-p38/p38 and p-JNK/JNK by 10.98-fold, 4.88-fold, 1.31-fold and 2.21-fold, respectively, in the dorsal skin of the DNCB-treated mice ($p < 0.01$ for all), as compared with the NC group. However, treatment with MHLJDD and MHLJDD-F-M/H significantly reduced the ratios of p-p65/p65, p-IK β /IK β , p-p38/p38 and p-JNK/JNK ($p < 0.01$ for all), as compared with the MC group. However, DNCB treatment did not significantly affect the ratio of p-ERK1/2/ERK1/2. These results unequivocally indicated that both MHLJDD and MHLJDD-F could significantly inhibit the activation

of NF- κ B and MAPK pathways.

Effects of MHLJDD and MHLJDD-F on the gut microbiota in the DNCB-treated mice

Emerging evidence has shown that dysbiosis of gut microbiota is associated with AD development. We investigated whether MHLJDD and MHLJDD-F could affect the abundance and diversity of the gut microbiota in the DNCB-treated mice. Firstly, as shown in Fig. 11A, application of DNCB significantly decreased the total number of operational taxonomic units (OTU), and the species richness of gut microbiota was significantly reduced in the DNCB-treated mice through assessing alpha diversity by Shannon index and Chao1 index. After administration of MHLJDD and MHLJDD-F, the species richness of gut microbiota showed significant differences, as compared with the MC groups, indicating that MHLJDD and MHLJDD-F could alter the species

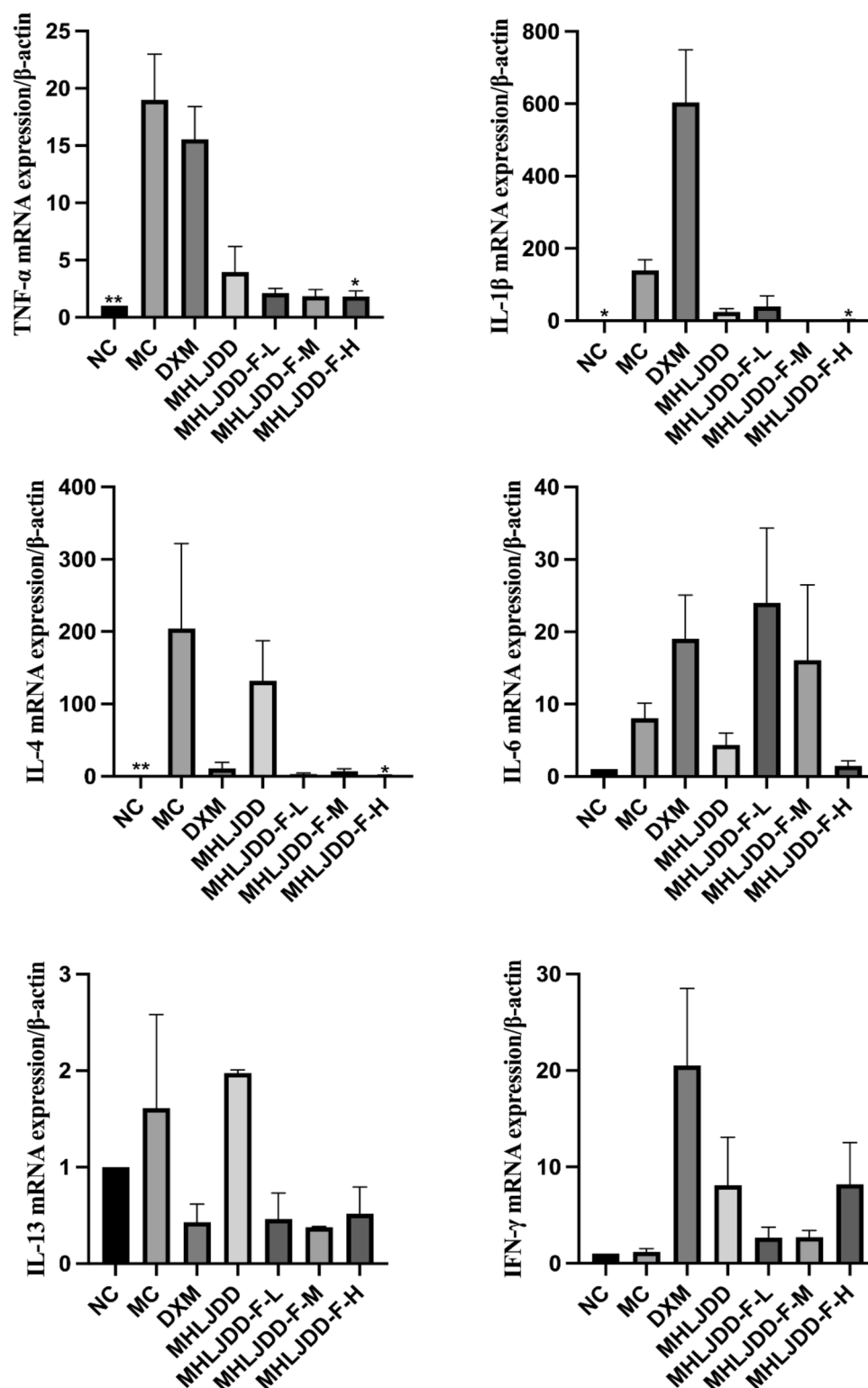


Fig. 9. Effects of MHLJDD and MHLJDD-F on the mRNA expressions of inflammatory cytokines in the dorsal skin of the DNCB-treated mice. Data were presented as mean $\pm$ SEM ($n = 4$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

richness of gut microbiota.

Besides, assessing from the Weighted UniFrac beta diversity between groups analysis and PCoA analysis (Fig. 11B), the diversity of the gut microbiota significantly changed after DNCB application. In PCoA analysis, spots with the same color represent samples from the same group, and the distance between any two spots represents the difference

in the composition of gut microbiota between the two samples. It could be observed that samples of the MC group were significantly distant from the samples from MHLJDD/MHLJDD-F-treated groups. In addition, there were significant differences among the NC group, the MC group and MHLJDD/MHLJDD-F-treated groups based on ANOSIM analysis ($p < 0.01$ for all). The results further demonstrated that

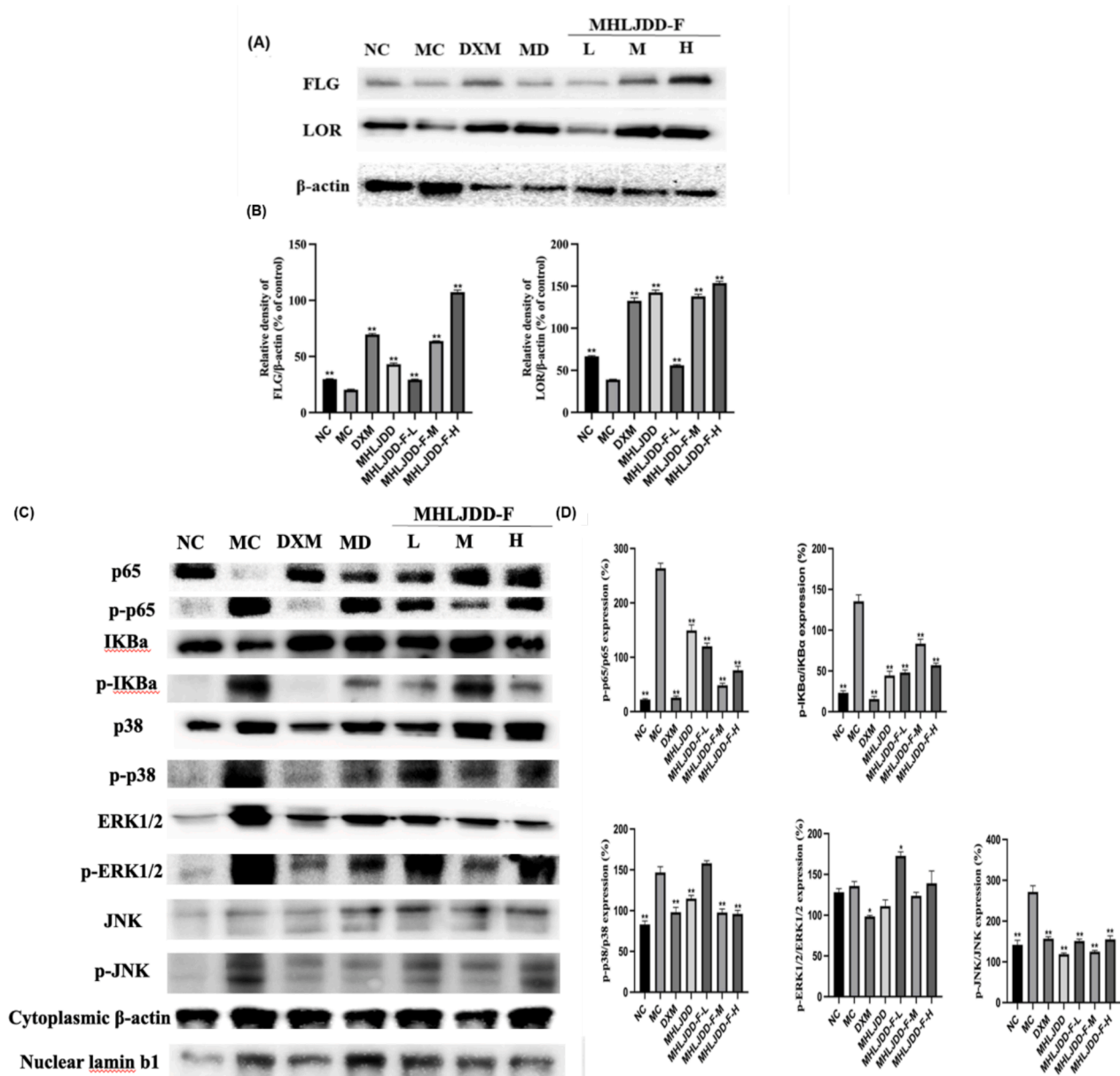


Fig. 10. Effects of MHLJDD and MHLJDD-F on the epidermal barrier function, NF- κ B and MAPK pathways in the dorsal skin of the DNCB-treated mice. (A) Representative immunoblot bands of FLG and LOR in the dorsal skin of the DNCB-treated mice. (B) Quantitative analysis of the immunoblot bands of FLG and LOR. (C) Representative immunoblot bands of NF- κ B and MAPK pathways. (D) Quantitative analysis of immunoblot bands of NF- κ B and MAPK pathways. Data were presented as mean $\pm$ SEM ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

application of MHLJDD or MHLJDD-F significantly changed the species diversity of the gut microbiota in the DNCB-treated mice.

Moreover, as shown in Fig. 12A and B, MHLJDD and MHLJDD-F markedly modulated the gut microbiota both at the phylum and genus levels. In the taxonomic analysis (Fig. 12C), there were also statistically significant differences in the MHLJDD and MHLJDD-F-treated groups, as compared with MC group. The abundance of *Alistipes*, which was positively related with atopic diseases, was significantly decreased in the MHLJDD and MHLJDD-F-treated groups. As for *Lactobacillus*, which is beneficial for AD patients, its abundance did not change in the MHLJDD and MHLJDD-F-treated groups, while DXM treatment significantly increased at the genus level of *Lactobacillus*. These results amply demonstrated that MHLJDD and MHLJDD-F might work through the inhibition on the genus level of *Alistipes* to alleviate the AD symptoms in the DNCB-treated mice. Besides, MHLJDD and MHLJDD-F also significantly attenuated the abundance of *Ruminococcaceae* UCG-014 and

Lachnospiraceae NK4A136, as well as accentuated the abundance of *Bacteroides*, *Akkermansia*, *Parabacteroides* and *Enterococcus*.

Discussion

Chinese medicine has been regarded as a popular treatment method for AD. The safety and efficacy of Chinese medicine on relieving AD symptoms have been well documented (Lin et al., 2014). Ancient classical formulae such as HLJDD and Xiao-Feng-San are commonly prescribed by Chinese medicine doctors to treat AD (Lopez Carrera et al., 2019). In this study, we modified HLJDD by replacing *Gardeniae Fructus* with *Dictamni Cortex* to form a safer and more effective anti-AD formula, and further fractionated MHLJDD to the ethyl acetate-soluble-, 1-butanol-soluble- and the water-soluble-fractions. Upon the identification of the fractions with the most potent anti-AD effect, we compared the anti-AD effects of MHLJDD and MHLJDD-F using the MC903 and the

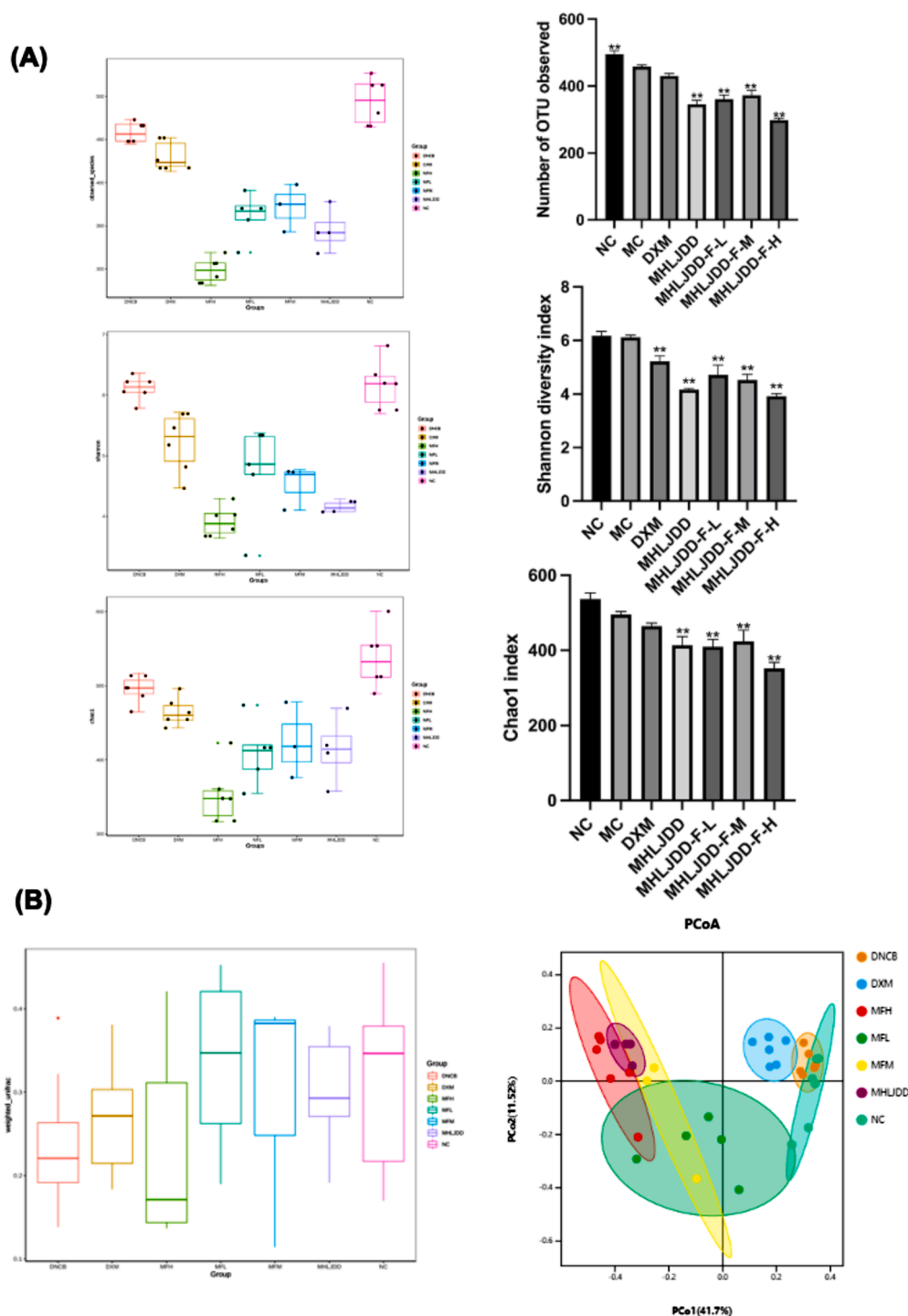


Fig. 11. Effects of MHLJDD and MHLJDD-F on the alpha diversity (A) and beta diversity (B) of gut microbiota in the DNCB-treated mice. Data were presented as mean $\pm$ SEM ($n = 6$). ** $p < 0.01$ compared with the MC group.

DNCB-induced mouse AD models and explored the underlying molecular mechanisms.

Phytochemical and pharmacological studies have found alkaloids such as coptisine, berberine and baicalin are the major active compounds of *Coptidis Rhizoma*, *Scutellariae Radix* and *Phellodendri Chinensis Cortex*, respectively. Both *Coptidis Rhizoma* and its alkaloids, especially berberine, have strong anti-inflammatory, anti-bacterial, anti-tumor, anti-oxidative and neuroprotective activities (Wang et al., 2019). In addition, berberine also exhibited anti-AD effects in NC/Nga mice with atopy-like dermatitis through downregulation of cutaneous EIF3F and

MALT1 (Andoh et al., 2021). *Phellodendri Chinensis Cortex* was able to downregulate the inflammatory response in the experimental models (Xian et al., 2011). The prominent anti-inflammatory effect was thought to be associated with their major chemical components, such as berberine and phillyrin (Pirillo and Catapano, 2015). *Scutellariae Radix* was reported to modulate the immunological response in the NC/Nga AD mice (Kim et al., 2010), indicating that *Scutellariae Radix* could be used to treat inflammatory skin diseases. The main active compounds of *Scutellariae Radix* are baicalin and wogonoside, which have been reported to possess strong antioxidant, anti-inflammatory, and

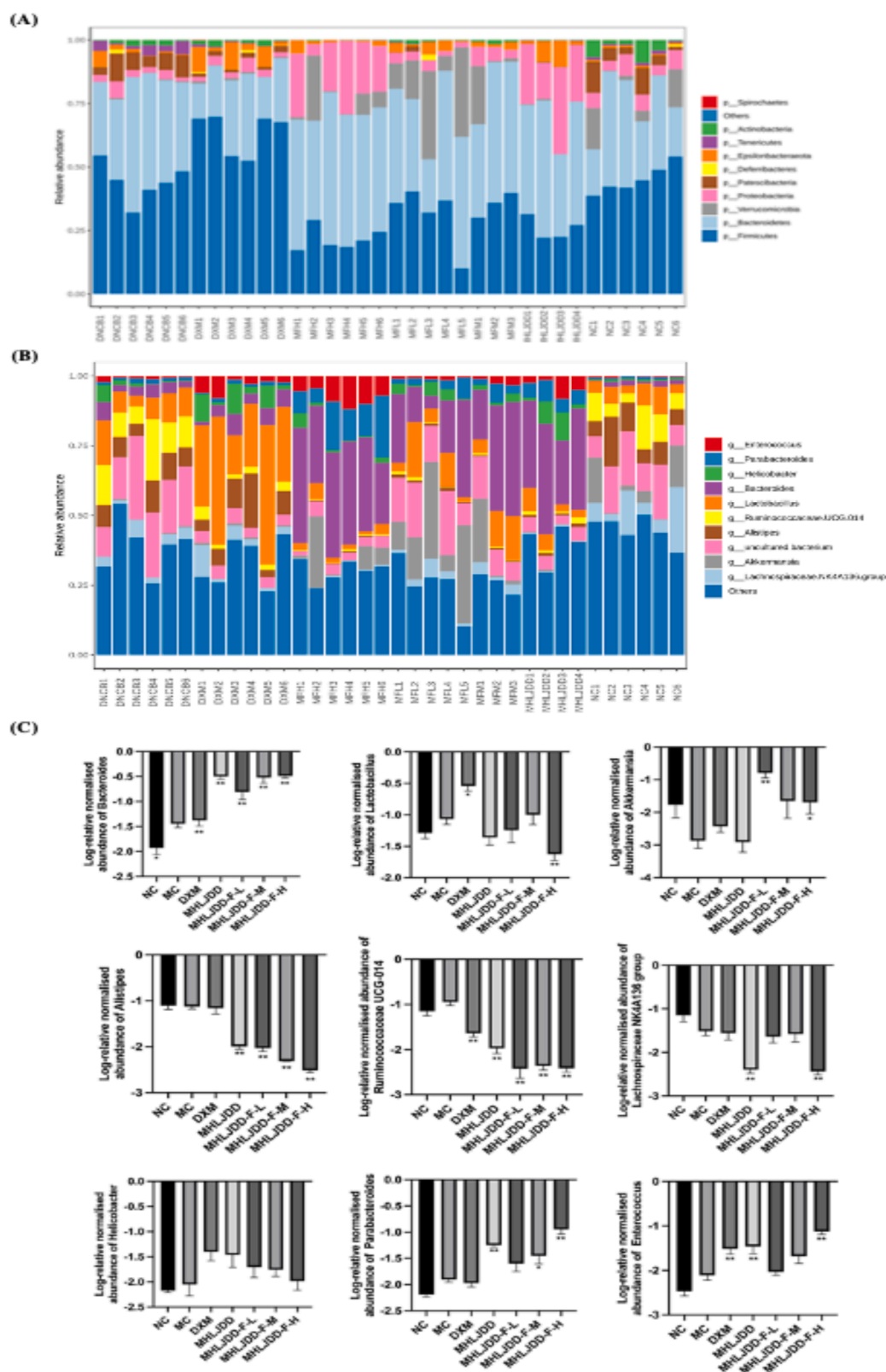


Fig. 12. Effects of MHLJDD and MHLJDD-F on the relative abundance at the phylum level (A), the genus level (B) and log relative normalized abundance of different taxa at the genus level of the gut microbiota in the DNCB-treated mice. The color of each cell indicates the relative abundance. Data were presented as mean $\pm$ SEM ($n = 6$). * $p < 0.05$ and ** $p < 0.01$ compared with the MC group.

anti-bacterial properties (Wang et al., 2018). Our previous study indicated that baicalin could effectively relieve AD-like skin lesions induced by DNCB in mice via inhibiting the activation of NF- κ B and JAK1/STAT3 pathways (Wang et al., 2022). The major constituents of DC are flavonoids (obakunone), terpenoids (fraxinellone) and limonins (Yang et al., 2019). DC showed potent anti-AD effects in the DNCB-induced mouse model via suppressing the activation of MAPKs and NF- κ B pathways (Chen et al., 2021). The above findings provide sufficient evidence regarding the rationality and efficacy of MHLJDD.

HPLC analysis showed that berberine, baicalin and wogonin were abundant in MHLJDD and MHLJDD-F. Especially, berberine accounts for 39.88% and 18.37% in MHLJDD and MHLJDD-F, respectively. However, in our present study, MHLJDD-F exerted more potential anti-AD effects than MHLJDD in DNCB- and MC903-treated mice, indicating that there are other constituents in MHLJDD-F which exhibit better anti-AD effects than berberine and baicalin. Further studies are needed to find out the constituents with better anti-AD effects in MHLJDD-F in future investigations.

In this study, a chemical hapten DNCB and vitamin D3 analog MC903 were used to induce AD-like skin lesions in mice to mimic human AD symptoms. When DNCB and MC903 applied on the skin, allergens were internalized by skin Langerhans cells, immature dermal dendritic cells and macrophages, processed and presented to T cells for activation. Immature dendritic cells could take up allergens and become mature dendritic cells to induce immune hyper-activation in the skin. Mature dendritic cells could initiate naïve Th0 cells differentiate into Th2 subgroup and induce downstream NF- κ B and MAPK pathways. The increasing Th2-mediated cytokines IL-4 and IL-13 could not only induce barrier dysfunction by down-regulating the protein expression of FLG and LOR and ceramides content, but also promoting B cells to produce allergen-specific IgE. IgE binds to mast cells to make them degranulate and release histamine into the bloodstream, thereby facilitating the initiation of anaphylactic reactions.

Compared with the DNCB-induced mice model, the MC903-induced mice model is much quicker and easier to establish. MC903 was only applied to the left ears and DNCB was applied to the whole dorsal skin, so that the symptoms of AD were usually more severe in the DNCB-induced model than that of the MC903-induced model. MC903-induced mouse model enabled us to rapidly assess the effects of MHLJDD and MHLJDD-F on AD mice, while the DNCB-induced mouse model could provide abundant tissue samples for us to assess the pharmacodynamics and to elucidate the action of mechanisms and signaling pathways. Therefore, we firstly established the MC903-induced model to evaluate the anti-AD effects of MHLJDD and MHLJDD-F. After application of MC903 for five times, AD-like symptoms such as skin thickening, apparent redness and dryness in the left ears of mice could be observed, and the symptoms were more severe in female than male mice. At the last day, the body weight of the mice in the MC and MHLJDD-F treated groups did not change, while that in the DXM and MHLJDD-treated groups decreased significantly, when compared with the normal mice, especially in the female animals. These results suggested that MHLJDD may have some side effects in the female mice. After two-week treatment with DXM, MHLJDD and MHLJDD-F, AD-like symptoms were more markedly alleviated in male mice than that in the female mice. Compared with DXM, MHLJDD and MHLJDD-F showed modest effects on reducing ear thickness. However, MHLJDD-F was superior to MHLJDD in relieving MC903-induced ear redness and dryness, especially at middle dose and high dose. As for MC903-induced pruritus, the anti-pruritic effect of MHLJDD-F was inferior to MHLJDD. In addition, MC903-induced epidermal thickening and mast cell infiltration in the ears were significantly reduced in all treatment groups, except MHLJDD-F-L group. These results suggested that the anti-AD effects of MHLJDD-F was comparable to MHLJDD, while MHLJDD-F had fewer side effects.

Both MHLJDD and MHLJDD-F could significantly decrease the serum levels of IgE and TNF- α , while serum IL-4 level was also reduced to some

extent. The anti-inflammatory effect of MHLJDD and MHLJDD-F might be the underlying mechanism of the anti-AD effects, and the action mechanism were further investigated in the DNCB-induced mice model. As for epidermal barrier function, the content of ceramides and protein expressions of FLG and LOR are positively correlated with the skin hydration, which is important for preventing the trans-epidermal water loss and maintaining epidermal barrier function (Di Nardo et al., 1998; Flohr et al., 2010). MHLJDD and MHLJDD-F could increase the ceramides content and FLG protein expression in the MC903-induced mice, especially the high dose of MHLJDD-F could significantly enhance LOR protein expression. These results unambiguously indicated that MHLJDD and MHLJDD-F could restore the epidermal barrier function which was disrupted by MC903 application.

Repeated applications of DNCB to the skin have been well known to induce typical AD symptoms in mice, including edema, excoriations, lichenification, frequent scratching behaviors and excessive thickening of epidermis (Zhang et al., 2009). Besides, abnormal immune response is activated and over-production of serum IgE and histamine could be found, and these changes were correlated with increased infiltration of inflammatory cells and mast cells in the dorsal skin. After two-week treatment, the DNCB induced dorsal skin lesions were significantly inhibited in all treatment groups. Noticeably, MHLJDD-F at high dose exerted more potent effects than MHLJDD with regards the reduction in dorsal skin thickness, EASI score and itchiness. In the biochemical analysis, both MHLJDD and MHLJDD-F showed significant inhibitory effects on the serum levels of TNF- α , IgE and histamine, but the histamine level in MHLJDD-F-M/H groups were much lower than that in the MHLJDD group. These results could explain why the frequency and duration of scratching behaviors were significantly decreased in the MHLJDD-F-M/H groups, as compared to the MHLJDD group. In addition, the epidermal thickness and mast cell infiltration in the dorsal skin of the mice were markedly suppressed in both MHLJDD and MHLJDD-F treatment groups. The high dose of MHLJDD-F exhibited better suppressive effect than MHLJDD.

As for restoring the epidermal barrier function, the content of ceramides was reported to be positively related with FLG (Pacha et al., 2012). Both FLG and LOR are essential proteins in the epidermis, which are responsible for keeping skin hydration and maintaining the epidermal barrier function. The ceramides content was modestly increased in MHLJDD and MHLJDD-F treatment groups, compared with the MC group. The protein expressions of FLG and LOR were significantly higher in the MHLJDD and MHLJDD groups than in the MC group, suggesting that MHLJDD and MHLJDD-F could help restore the skin barrier function. With regard to restriction on skin inflammation, the effect of MHLJDD-F-H was superior to MHLJDD on down-regulating the mRNA expression levels of the inflammatory cytokines such as TNF- α , IL-4 and IL-1 β , as well as the protein expression levels of the ratios of p-p65/p65, p-IK β /IK β , p-JNK/JNK and p-p38/p38. These results strongly indicated that inhibition of the activation of NF- κ B and MAPK pathways might be the action mechanisms of the anti-inflammatory effects of MHLJDD and MHLJDD-F.

To conclude, MHLJDD and MHLJDD-F could significantly inhibit DNCB and MC903-induced Th2 predominant skin inflammation and immune hyper-activation through inhibiting the activation of NF- κ B and MAPK pathways. In addition, MHLJDD and MHLJDD-F also significantly regulated the gut microbiota in the DNCB-treated mice by restoring the richness and the relative abundance of probiotics in the gut microbiota. The alteration in gut microbiota identified in this study would help elucidate the role of the gut-skin axis in the treatment of AD with MHLJDD and MHLJDD-F. Therefore, AD-like skin lesions of the mice such as skin dryness and itchiness were alleviated by MHLJDD and MHLJDD-F (Fig. 13). This study for the first time reported that MHLJDD could be a better formula with more potent anti-AD efficacy and better safety profile than that of the original HLJDD through restoring skin barrier function, inhibiting skin inflammation and regulating gut microbiota. Its active fraction MHLJDD-F has the potential to be further

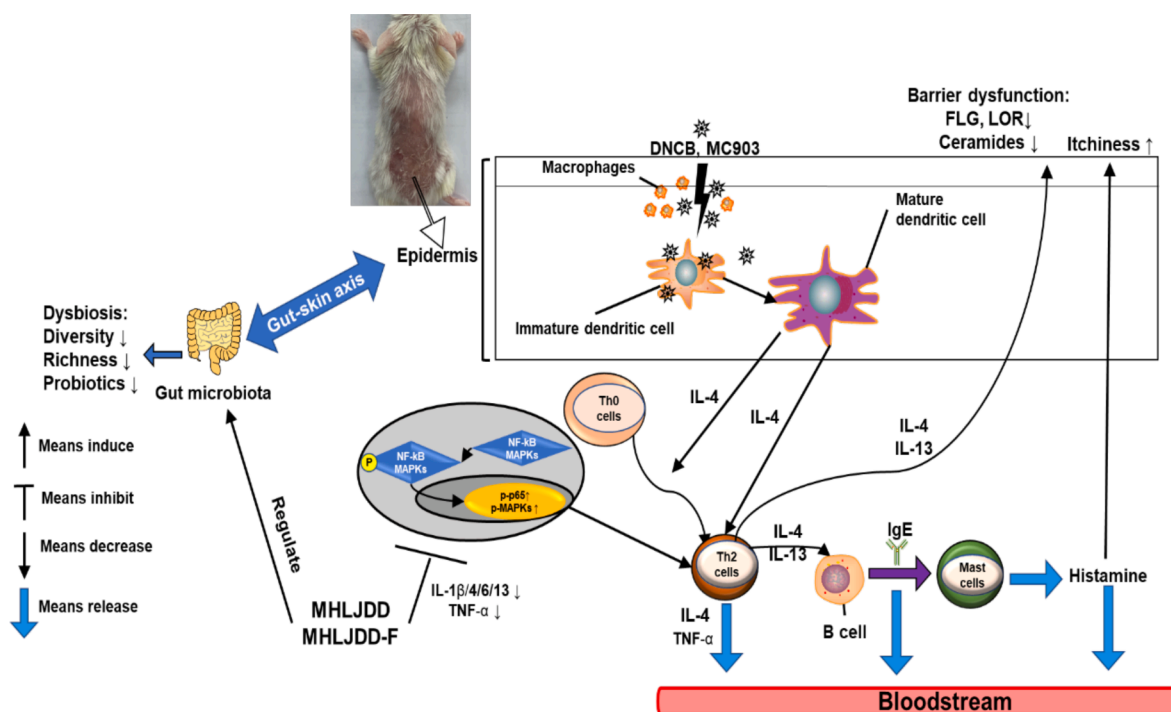


Fig. 13. A schematic drawing summarizing the cellular and molecular mechanisms underlying the anti-AD effects of MHLJDD and MHLJDD-F in the MC903- and DNCB-induced AD mouse models. The action mechanisms involve alleviation of inflammation and regulation of epidermal barrier function via inhibiting the activation of NF- κ B and MAPK pathways, as well as modulation of gut microbiota.

developed into an innovative therapeutic agent for AD.

Data availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Lan Wang: Methodology, Visualization, Investigation, Data curation, Writing – original draft, Software, Validation. **Zhen Hu:** Project administration. **Wen Yang:** Data curation, Software, Validation. **Steven King Fan Loo:** Data curation, Software, Validation. **Siu Po Ip:** Data curation, Software, Validation. **Yan-Fang Xian:** Conceptualization, Supervision, Writing – review & editing. **Zhi-Xiu Lin:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests related to this study.

Acknowledgment

This work was supported by the Innovation and Technology Commission Funding Administrative System of Hong Kong (Project No: ITS/122/19FP).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.phymed.2022.154346](https://doi.org/10.1016/j.phymed.2022.154346).

References

Andoh, T., Yoshihisa, Y., Rehman, M.U., Tabuchi, Y., Shimizu, T., 2021. Berberine induces anti-atopic dermatitis effects through the downregulation of cutaneous

- EIF3F and MALT1 in NC/Nga mice with atopy-like dermatitis. *Biochem. Pharmacol.* 185, 114439.
- Chen, J., Wang, X., 2010. Experience of professor XUAN Guo-wei's Pifu Jiedu Tang on eczema hard to be cured. *J. Liaoning Univ. Tradit. Chin. Med.* 12, 131–132.
- Chen, Y., Xian, Y., Lai, Z., Loo, S., Chan, W.Y., Lin, Z.X., 2016. Anti-inflammatory and anti-allergic effects and underlying mechanisms of Huang-Lian-Jie-Du extract: implication for atopic dermatitis treatment. *J. Ethnopharmacol.* 185, 41–52.
- Chen, Y., Xian, Y.F., Loo, S., Chan, W.Y., Liu, L., Lin, Z.X., 2021. Anti-atopic dermatitis effects of dictamnii cortex: studies on *in vitro* and *in vivo* experimental models. *Phytomedicine* 82, 153453.
- Chen, Y., Xian, Y.F., Loo, S., Lai, Z., Chan, W.Y., Liu, L., Lin, Z.X., 2020. Huang-Lian-Jie-Du extract ameliorates atopic dermatitis-like skin lesions induced by 2,4-dinitrobenzene in mice via suppression of MAPKs and NF- κ B pathways. *J. Ethnopharmacol.* 249, 112367.
- Di Nardo, A., Wertz, P., Giannetti, A., Seidenari, S., 1998. Ceramide and cholesterol composition of the skin of patients with atopic dermatitis. *Acta Derm. Venereol.* 78, 27–30.
- Errahali, Y.J., Thomas, L.D., Keller, T.C., Lee, H.J., 2013. Inhibition by new glucocorticoid antedugs [16 α , 17 α -d] isoxazoline and [16 α , 17 α -d]-3'-hydroxy-iminoformyl isoxazoline derivatives of chemotaxis and CCL26, CCL11, IL-8, and RANTES secretion. *J. Interf. Cytokine Res.* 33, 493–507.
- Flohr, C., England, K., Radulovic, S., McLean, W.H., Campbell, L.E., Barker, J., Perkin, M., Lack, G., 2010. Filaggrin loss-of-function mutations are associated with early-onset eczema, eczema severity and transepidermal water loss at 3 months of age. *Br. J. Dermatol.* 163, 1333–1336.
- Kim, J., Lee, I., Park, S., Choue, R., 2010. Effects of Scutellariae radix and aloe vera gel extracts on immunoglobulin E and cytokine levels in atopic dermatitis NC/Nga mice. *J. Ethnopharmacol.* 132, 529–532.
- Ko, M.J., Baek, J.H., 2012. A clinical study on the effect of Hwangryunhaedock-tang on atopic dermatitis. *J. Pediatr. Korean Med.* 26, 51–60.
- Leung, D.Y., Boguniewicz, M., Howell, M.D., Nomura, I., Hamid, Q.A., 2004. New insights into atopic dermatitis. *J. Clin. Invest.* 113, 651–657.
- Li, C., Gao, X., Gao, X., Lv, J., Bian, X., Lv, J., Sun, J., Luo, G., Zhang, H., 2021. Effects of medicine food Fructus Gardeniae on liver and kidney functions after oral administration to rats for 12 weeks. *J. Food Biochem.* 45, e13752.
- Lin, J.F., Liu, P.H., Huang, T.P., Lien, A.S., Ou, L.S., Yu, C.H., Yang, S.L., Chang, H.H., Yen, H.R., 2014. Characteristics and prescription patterns of traditional Chinese medicine in atopic dermatitis patients: ten-year experiences at a medical center in Taiwan. *Complement. Ther. Med.* 22, 141–147.
- Lopez Carrera, Y.I., Al Hammadi, A., Huang, Y.H., Llamado, L.J., Mahgoub, E., Tallman, A.M., 2019. Epidemiology, diagnosis, and treatment of atopic dermatitis in the developing countries of Asia, Africa, Latin America, and the middle east: a review. *Dermatol. Ther.* 9, 685–705. Heidelberg.
- Matsuda, H., Watanabe, N., Geba, G.P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P.W., Ra, C., 1997. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *Int. Immunol.* 9, 461–466.

- Moosbrugger-Martinz, V., Schmuth, M., Dubrac, S., 2017. A mouse model for atopic dermatitis using topical application of vitamin D3 or of its analog MC903. *Methods Mol. Biol.* 1559, 91–106.
- Pacha, O., Sambrano, B.L., Hebert, A.A., 2012. Skin barrier repair in eczema: a review of current understanding of pathophysiology and treatment. *Curr. Dermatol. Rep.* 1, 115–122.
- Pirillo, A., Catapano, A.L., 2015. Berberine, a plant alkaloid with lipid- and glucose-lowering properties: from *in vitro* evidence to clinical studies. *Atherosclerosis* 243, 449–461.
- Sheehan, M.P., Atherton, D.J., 1992. A controlled trial of traditional Chinese medicinal plants in widespread non-exudative atopic eczema. *Br. J. Dermatol.* 126, 179–184.
- Sheehan, M.P., Rustin, M.H., Atherton, D.J., Buckley, C., Harris, D.W., Brostoff, J., Ostlere, L., Dawson, A., 1992. Efficacy of traditional Chinese herbal therapy in adult atopic dermatitis. *Lancet* 340, 13–17.
- Tan, H.Y., Zhang, A.L., Chen, D., Xue, C.C., Lenon, G.B., 2013. Chinese herbal medicine for atopic dermatitis: a systematic review. *J. Am. Acad. Dermatol.* 69, 295–304.
- Wang, J., Wang, L., Lou, G.H., Zeng, H.R., Hu, J., Huang, Q.W., Peng, W., Yang, X.B., 2019. *Coptidis Rhizoma*: a comprehensive review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. *Pharm. Biol.* 57, 193–225.
- Wang, L., Xian, Y.F., Hu, Z., Loo, S.K.F., Ip, S.P., Chan, W.Y., Lin, Z.X., Wu, J.C.Y., 2021. Efficacy and action mechanisms of a Chinese herbal formula on experimental models of atopic dermatitis. *J. Ethnopharmacol.* 274, 114021.
- Wang, L., Xian, Y.F., Loo, S.K.F., Ip, S.P., Yang, W., Chan, W.Y., Lin, Z.X., Wu, J.C.Y., 2022. Baicalin ameliorates 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in mice through modulating skin barrier function, gut microbiota and JAK/STAT pathway. *Bioorg. Chem.* 119, 105538.
- Wang, Y.S., Cho, J.G., Hwang, E.S., Yang, J.E., Gao, W., Fang, M.Z., Zheng, S.D., Yi, T.H., 2018. Enhancement of protective effects of radix scutellariae on UVB-induced photo damage in human HaCaT keratinocytes. *Appl. Biochem. Biotechnol.* 184, 1073–1093.
- Xian, Y.F., Mao, Q.Q., Ip, S.P., Lin, Z.X., Che, C.T., 2011. Comparison on the anti-inflammatory effect of Cortex Phellodendri Chinensis and cortex phellodendri amurensis in 12-O-tetradecanoyl-phorbol-13-acetate-induced ear edema in mice. *J. Ethnopharmacol.* 137, 1425–1430.
- Yang, S., Sun, F., Ruan, J., Yan, J., Huang, P., Wang, J., Han, L., Zhang, Y., Wang, T., 2019. Anti-inflammatory constituents from Cortex Dictamni. *Fitoterapia* 134, 465–473.
- Zhang, E.Y., Chen, A.Y., Zhu, B.T., 2009. Mechanism of dinitrochlorobenzene-induced dermatitis in mice: role of specific antibodies in pathogenesis. *PLoS One* 4, e7703.