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Hinokitiol as a modulator of TLR4 signaling and apoptotic pathways in atopic dermatitis

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ABSTRACT

Atopic dermatitis (AD) poses a significant global health challenge, characterized by dysregulated inflammation and apoptotic processes. This study explores the therapeutic efficacy of hinokitiol, employing a comprehensive in vivo and in vitro approach. Assessment of inflammation-related markers in the animal model included observation of physical appearance, Western blotting, ELISA, and H&E staining. Additionally, the cell culture model enabled the evaluation of apoptosis and ROS levels using MTT assay, crystal violet staining, Western blot, and DCFDA assays. The results revealed hinokitiol's proficiency in ameliorating ear and skin morphology in the DNCB-induced AD model, mediated through the TLR4/MyD88 pathway. Notably, hinokitiol intervention led to a reduction in both M1 and M2 macrophage phenotypes. In vitro investigations demonstrated hinokitiol's ability to enhance cell viability and morphology under TNF-α and IFN-γ induction. Mechanistically, hinokitiol exhibited regulatory effects on apoptosis-related proteins, including Bax, Cytochrome c, Caspase-3, and PARP, thereby averting cellular damage. These findings suggest that hinokitiol is a promising natural compound with significant potential for alleviating inflammation and apoptosis in AD, indicating potential avenues for future therapeutic developments.

1. Introduction

Atopic dermatitis is a chronic inflammatory skin condition characterized by severe itching and recurrent eczema. Its prevalence is approximately 20% in children and 1–3% in adults in developed countries. In developing and underdeveloped regions, there is an observed increasing trend in incidence [1]. Among children with atopic dermatitis, severe nocturnal itching and subsequent scratching can lead to disrupted sleep patterns, negatively impacting their overall quality of life [2].

In clinical settings, the primary approach to treating Atopic Dermatitis (AD) involves the use of topical immunosuppressive therapy, with the drug crisaborole serving as a first-line treatment [3]. In cases where topical treatments prove ineffective in managing moderate or severe conditions, the implementation of systemic immunosuppressive therapies becomes necessary. These may include corticosteroids, methotrexate, glucocorticoids, and cyclosporine [4,5]. However, it is crucial to acknowledge that prolonged use of immunosuppressants can lead to significant adverse effects, such as hypo-immunity and hypothalamic-pituitary-adrenal suppression [6]. Therefore, there is an urgent need to develop effective and safe therapeutic interventions for managing or providing supplementary treatment for AD.

Abbreviations: AD, Atopic dermatitis; Bax, B-cell lymphoma 2-associated X protein; COX-2, Cyclooxygenase-2; DCFH2–DA, 2,7-dichlorodihydrofluorescin diacetate; DNCB, 2,4-Dinitrochlorobenzene; IFN-γ, Interferon-gamma; IL-1β, Interleukin-1 beta; MyD88, Myeloid differentiation primary response protein 88; PARP, Poly ADP-ribose polymerase; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; TNF-α, Tumor necrosis factor- alpha.

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In the pathogenesis of atopic dermatitis, inflammation and apoptosis play pivotal roles. In normal skin tissue, the epidermal layer expresses low levels of Fas cell surface death receptor (Fas) and Fas ligand (FasL) [7]. Notably, atopic dermatitis patients exhibit elevated levels of Fas and FasL [8], which are integral components of the apoptosis pathway [9]. Research has revealed that keratinocyte apoptosis initiates the progression of atopic dermatitis [10,11], leading to the disruption of the epidermal and vesicular structure. Furthermore, proinflammatory factors Tumor Necrosis Factor-alpha (TNF-α) and Interferon-gamma (IFN-γ) promote the activation of the Fas/FasL system, inducing keratinocyte apoptosis [10].

The Toll-like Receptor 4/Myeloid Differentiation Primary Response 88 (TLR4/MyD88) signaling pathway orchestrates the release of cytokines and has been implicated in the progression of atopic dermatitis [12]. Within this pathway, proinflammatory factors and cytokines, such as Cyclooxygenase-2 (COX-2), Interleukin-6 (IL-6), and IL-1β, are released [13]. COX-2 is an enzyme that catalyzes the conversion of arachidonic acid into various substances, including prostaglandins, prostacyclin, and thromboxane. It is associated with inflammation and infection. [14]. IL-1β and IL-6, pro-inflammatory cytokines, are the major cytokines secreted by keratinocytes [15]. Previous studies indicate that IL-6 and IL-1β are linked to the Th2-type immune response and disease progression in atopic dermatitis [16].

Therapies for atopic dermatitis patients can potentially result in adverse side effects [17,18]. Consequently, numerous studies are focusing on the effects of natural compounds in managing atopic dermatitis [19]. Hinokitiol, also known as β-thujaplicin, is an essential oil derived from Cupressaceae known for its anti-inflammatory, antibacterial, and apoptosis-regulating properties [20,21]. Hinokitiol has been shown to enhance antioxidant-related enzymes and reduce inflammation-related indicators [22]. Its apoptotic regulatory effect has also been extensively documented [23,24]. Furthermore, when incorporated into shampoo and applied as an ointment, hinokitiol exhibits antibacterial effects in atopic dermatitis patients [25]. Despite these recognized effects, the impact of hinokitiol on inflammatory and apoptotic regulation in atopic dermatitis remains unclear. Therefore, our hypothesis in this study is to investigate whether hinokitiol exerts an anti-inflammatory effect in vivo and an anti-apoptotic effect in vitro.

2. Materials and methods

2.1. Reagent

Hinokitiol and 2,4-Dinitrochlorobenzene (DNCB) were purchased from Sigma (St Louis, MO, USA). Recombinant Human TNF-α and Recombinant Human IFN-γ were purchased from Peprotech (London, UK).

2.2. Animals treatment

All animal care and experimental procedures strictly adhered to guidelines and regulations approved by the Institutional Animal Care and Use Committee at Taipei Medical University (No. LAC2023–0082). Five-week-old female Balb/c mice were procured from BioLasco Co., Ltd. The animals were housed in a temperature-controlled room (22 ± 1 °C) with a 12-hour light–dark cycle, provided ad libitum access to water, and given a regular chow diet under specific-pathogen-free conditions. After a one-week acclimation period, the mice were anesthetized with Zoletil 50 and Ropum via intraperitoneal route. Dorsal skin hair was removed during anesthesia using both hair remover cream and an electronic hair remover device. The study protocol was referenced from a previous study. [26,27]. The dorsal skin underwent a challenge with 100 μL of 0.5% DNCB (acetone: olive oil (3:1)) for three consecutive days during the sensitization phase in the first week of the experiment. Subsequently, 100 μL of 1% DNCB was applied to the skin three times weekly for the following four weeks. In addition, both ears were challenged with 20 μL of 1% DNCB throughout the four-week duration, and the mice's ear thickness was monitored on a weekly basis. The mice received hinokitiol (50 μL) via oral gavage five times per week for four weeks. The hinokitiol preparation involved using 0.5% sodium carboxymethyl cellulose (CMC-Na) as the vehicle, which was gently mixed before gavage (Fig. 1).

2.3. Modified SCORAD evaluation

We conducted a modified SCORAD (scoring dermatitis) assessment following the methodology outlined in a previous study [26]. Modified SCORAD scores were employed to evaluate changes in skin severity during hinokitiol intervention. Under group-blinding conditions, we comprehensively assessed skin and ear severity, evaluating six symptoms: redness (0−3), swelling (0−3), oozing or crusting (0−3), scratching marks (0−3), skin thickening (0−3), and dryness (0−3). The severity of each symptom was scored, with higher scores indicating more severe symptoms.

2.4. Histological analysis

The spleen, ear and skin sample were fixed in 4% paraformaldehyde, which embedded in paraffin was outsourced to BIO-CHECK LABORATORIES LTD (New Taipei City, Taiwan) for Hematoxylin and Eosin (H&E) staining process.

2.5. ELISA

The quantification of serum IgE (Biolegend, San Diego, California, USA) and IL-6 (Abcam, Cambridge, UK) levels was conducted using a commercially available ELISA kit in strict adherence to the manufacturer's provided protocol. Absorbance readings were recorded at both 450 nm and 570 nm using a microplate reader (Molecular Devices, San Jose, CA, USA).

2.6. Western blot analysis

The cells and animal tissue were lysed in RIPA lysis buffer composed of protease and phosphatase inhibitors (Roche, Mannheim, Baden-Württemberg, Germany). Then, quantify the protein by bicinchoninic acid (BCA) assay, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 95 voltages and 2 h transfer to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 5% bovine serum albumin (BSA) solution for 1 h. Afterwards, the membranes were incubated with the primary antibodies TLR4 (1:1000; Abcam, Cambridge, UK), MyD88 (1:1000; Cell Signaling, Boston, MA, USA), COX-2 (1:1000; BD bio), IL-1β (1:1000, Genetex), CD80/B7–1 (1:1000, Proteintech, Rosemont, IL, USA), CD206/MRC1 (E6T5J) (1:1000, Proteintech, Rosemont, IL, USA), Bax (1:1000,Cell Signaling), Bcl-2 (1:500; Santacruz), Cytochrome-c (1:1000, Cell Signailing) Caspase-3 (1:1000, Cell Signaling), PARP (1:1000; Cell Signaling), and β-actin (1: 1000; Santacruz) at 4 °C overnight and then incubate with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 10,000) at room temperature for 2 h. The signals were captured by an e-Blot Touch Imager (eBlot Photoelectric Technology, Shanghai, China). The band densities were determined using the Image J software program (NIH, Bethesda, MD, USA).

2.7. Cell cultures

The Human Immortalized Keratinocyte Cell Line (HaCaT) were purchased from ATCC. HaCaT were cultured in DMEM/ F12 supplemented with 10% FBS and antibiotics composed of 100 U/ML penicillin, amphotericin B and streptomycin in a humidified incubator at 37 °C with 5% CO_2. After the cells attached to the cell culture dish, the cells were pretreated different concentration of hinokitiol (1, 5, 7.5, 10 μM) for 24 h, then change the medium to 20 ng/ML of human recombinant

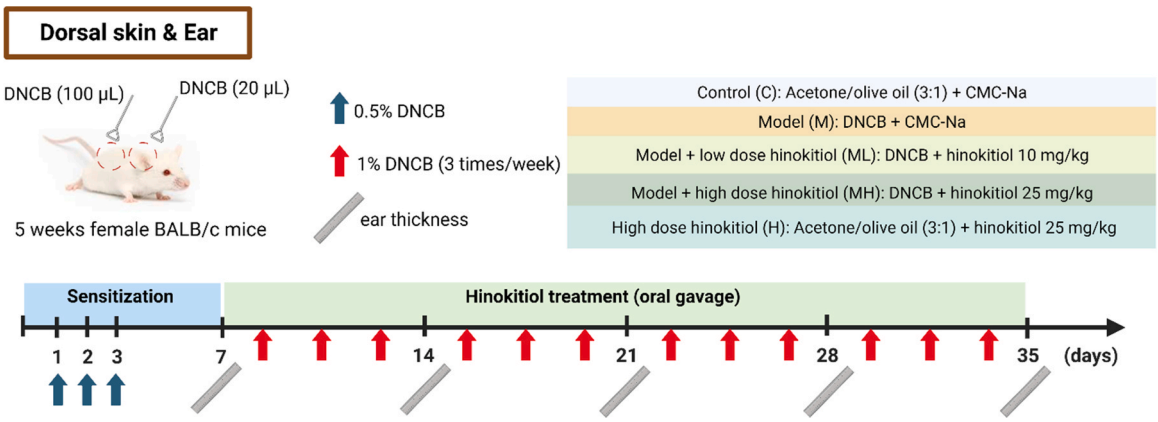

Fig. 1. The flow chart of animal experiment.

TNF-α (Peprotech) and human recombinant IFN-γ (Peprotech) for 24 h.

2.8. Cell viability

The cell viability of hinokitiol under TNF-α and IFN-γ induction (AD model group) were measured by (3-[4,5-dimethyl-2-thiazolyl]−2,5-diphenyl-2 H-tetrazolium bromide) (MTT) assays. The medium were removed and replaced by culture medium supplemented with 1 mg/mL MTT, and incubate for 1.5 h. Then remove the medium and dissolve the crystal formazan in 100 μL dimethyl sulfoxide (DMSO; ECHO Chemical Co. Ltd., Taipei, Taiwan). The absorbance at 570 nm and 630 nm were measured by microplate reader (Molecular Devices, San Jose, CA, USA).

2.9. Crystal violet staining

We also employed crystal violet staining for cell viability analysis. Firstly, the medium was removed, and the cells were fixed with methanol for 10 min. After fixing the cells, we incubated them with a crystal violet solution (0.5%; Sigma-Aldrich, St Louis, MO, USA) for 20 min. Finally, we rinsed the plate to remove any excess staining residues. The area density was measured using the Image J software program (NIH, Bethesda, MD, USA).

2.10. ROS assay

We used 2,7-dichlorodihydrofluorescin diacetate ($DCFH_2$–DA) staining assay to determine ROS production. After remove the medium, we added 20 μM $DCFH_2$–DA and incubated it for 30 min. We measured the excitation wavelength at 492 nm and emission at 515 nm.

2.11. Data and statistical analysis

Using Graphpad Prism 8 software to analysis the data, data represent as mean ± standard error of the mean (SEM) in animal study, and mean ± standard deviation (SD) in cell culture study. One- way ANOVA follow by Tukey post-hoc test to analyze the difference among different animal groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Hinokitiol alleviates the skin abnormality in atopic dermatitis mice model

We assessed the modified SCORAD scores based on ear and skin severity before sacrifice, with scores ranging from 0 to 3; higher scores indicated more severe skin conditions. Fig. 2A illustrates ear morphology, while Fig. 2B depicts skin morphology at week 4. In the M

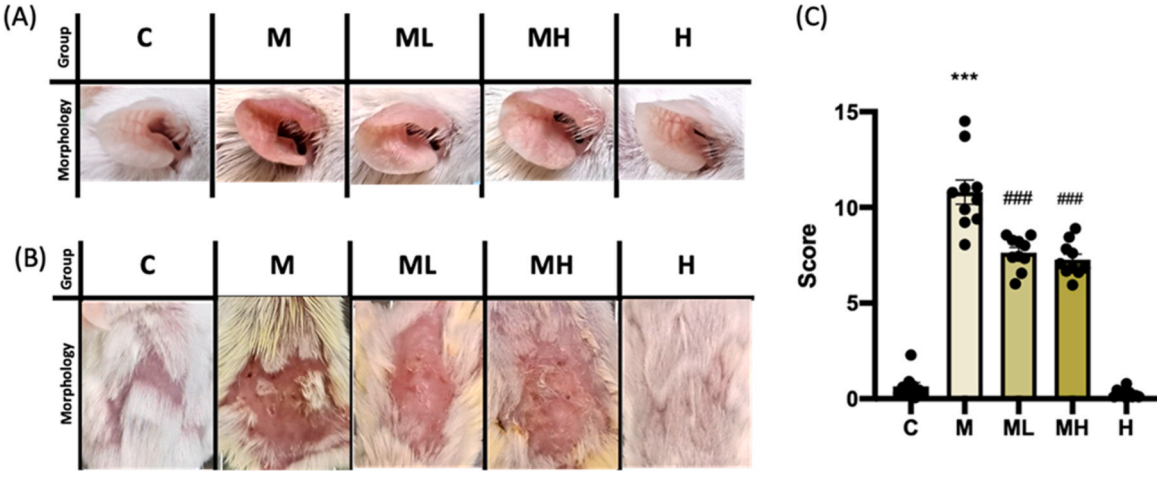

Fig. 2. Effect of Hinokitiol on SCORAD (Scoring atopic dermatitis) evaluation in atopic dermatitis mice. (A) The ear and (B) skin photos were captured. (C) The SCORAD was evaluated, wherein the dorsal skin and ear were scored for redness, swelling, oozing (or crusting), scratching marks, skin thickening, and dryness, with grades ranging from 0 to 3. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Data were presented as mean ± SEM, with one-way ANOVA employed to compare the differences between groups. *** $p < 0.001$ compared with control, ### $p < 0.001$ compared with the model ($n = 10$).

group, both skin and ear exhibited characteristics of redness, swelling, crusting, scarring, and increased thickness compared to the control group (C). However, in the ML and MH groups treated with hinokitiol, there was a noticeable alleviation of these skin abnormalities (Fig. 2A, B). To quantify the severity differences between groups, we utilized the Modified SCORAD, incorporating assessments from nine different observers. The scores demonstrated a significant increase in the M group, while there was a notable decrease in both ML and MH groups (Fig. 2C). In summary, the M group exhibited more pronounced severity in both ear and skin conditions, and treatment with hinokitiol led to a discernible improvement in skin severity.

3.2. Potent immune regulation ability of hinokitiol

After sacrifice, we conducted organ weight measurements normalized to body weight. The heart, lung, and kidney weight indices showed no significant differences between the groups (Fig. 3B-D). However, the liver index exhibited a noteworthy increase in the M group and a significant decrease in the ML group (Fig. 3A). Considering the liver's pivotal role in immune regulation [28], these results suggest that the AD group may have activated the immune system, with alleviation observed in response to hinokitiol treatment. The spleen, acting as a primary filter for blood-borne pathogens and antigens, plays a crucial role linked to the immune system [29]. Morphological changes in the spleen were further examined. Fig. 3E and F illustrate spleen weight changes and morphology. Both spleen size and weight showed a significant increase in the M group, while in both ML and MH groups, there was a noteworthy decrease. Subsequently, HE staining was employed to scrutinize spleen morphology. Fig. 3G depicts the results, revealing irregular spleen organization, diminished white pulp, and augmented red pulp in the M group compared to the C group. In contrast, spleen morphology in both ML and MH groups more closely resembled that of the C group. Serum IgE serves as a crucial biomarker in atopic dermatitis, acknowledged in clinical criteria for the condition [30]. We observed a significant increase in serum IgE levels in the M group, which notably decreased with hinokitiol treatment (Fig. 3H). IL-6, recognized as a pro-inflammatory cytokine, plays a pivotal role in recruiting and activating immune cells, especially T-helper 2 (Th2) cells implicated in Atopic Dermatitis (AD) pathogenesis and influencing the systemic inflammatory response [31]. Elevated IL-6 levels were observed in the M group, while both ML and MH groups effectively reduced IL-6 release. Collectively, the findings related to the liver, spleen, IgE, and IL-6 levels suggest that hinokitiol may indeed play a critical role in immune regulation.

3.3. Effect of hinokitiol on DNCB induced ear morphology change

Application of DNCB to the ear affects its thickness, which we monitored weekly to track changes. At the study endpoint, the ears of animals in group M exhibited a red, edematous, and scarred appearance, which was alleviated by hinokitiol treatment (Fig. 4A). The baseline of thickness of Ear is not significantly different (Fig. 4C). Throughout the experiment, there was a significant increase in ear thickness in the M group at weeks 3, 4, and at the endpoint, while the thickness notably decreased in both ML and MH groups (Fig. 4B,D). Correspondingly, ear weight and size displayed congruent patterns with thickness measurements (Fig. 4E,F). Notably, the epidermal and dermal thickness demonstrated a significant increase in the ML and MH groups, while exhibiting a significant decrease in the ML and MH groups (Fig. 4G,H). To further investigate alterations in skin layer thickness, we employed HE staining. The epidermal and dermal thickness demonstrated a significant increase in group M, whereas both ML and MH groups exhibited a significant decrease in epidermal thickness (Fig. 4I).

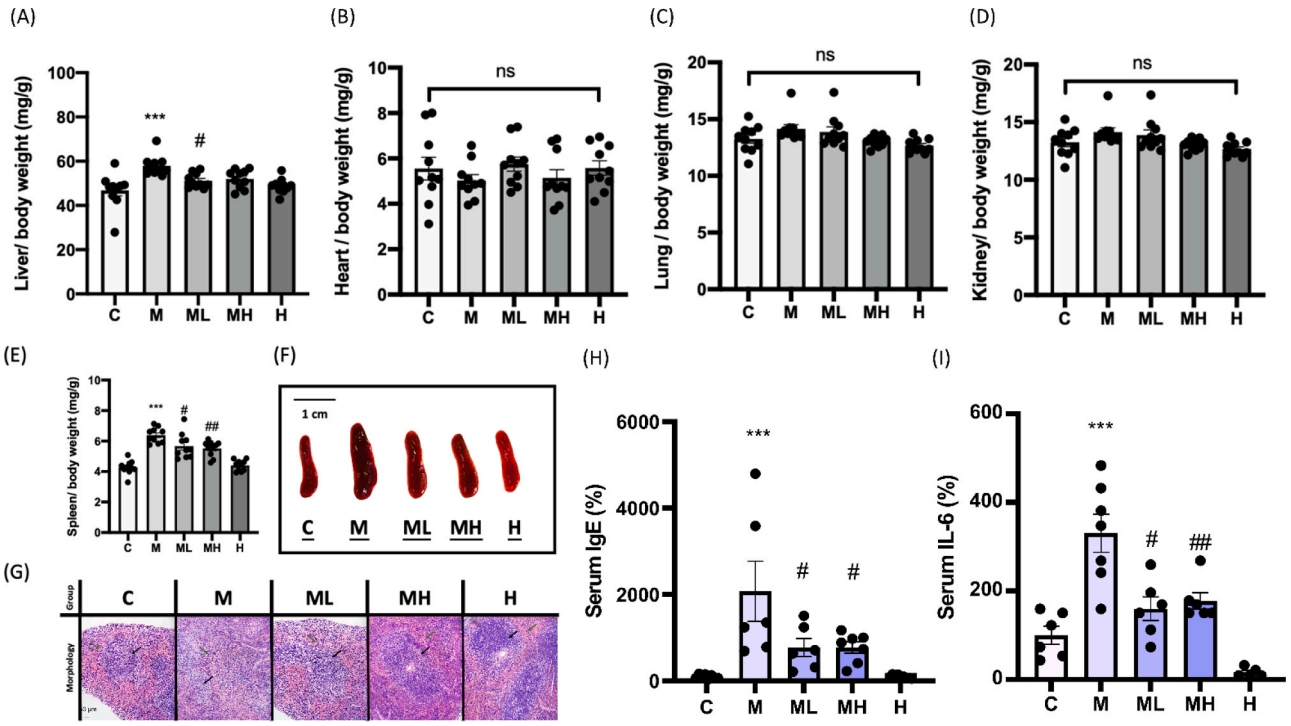

Fig. 3. Effect of Hinokitiol on organ weight, spleen morphology change and serum IgE in atopic dermatitis mice. (A) Liver, (B) heart, (C) lung, (D) kidney and (E) spleen weight was measured by digital scale and normalized to their body weight. (F)The spleen appearance and (G) H&E staining showed the histology change in spleen. (H) Serum IgE and (I) IL-6 were measured by ELISA kit. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Black arrows: white pulp. Green arrows: red pulp. Data were represented as mean ± SEM, using one- way ANOVA to compare the difference between groups. *** $p < 0.001$ compared with control group, and #$p < 0.05$ compared with model group (n = 6–8).

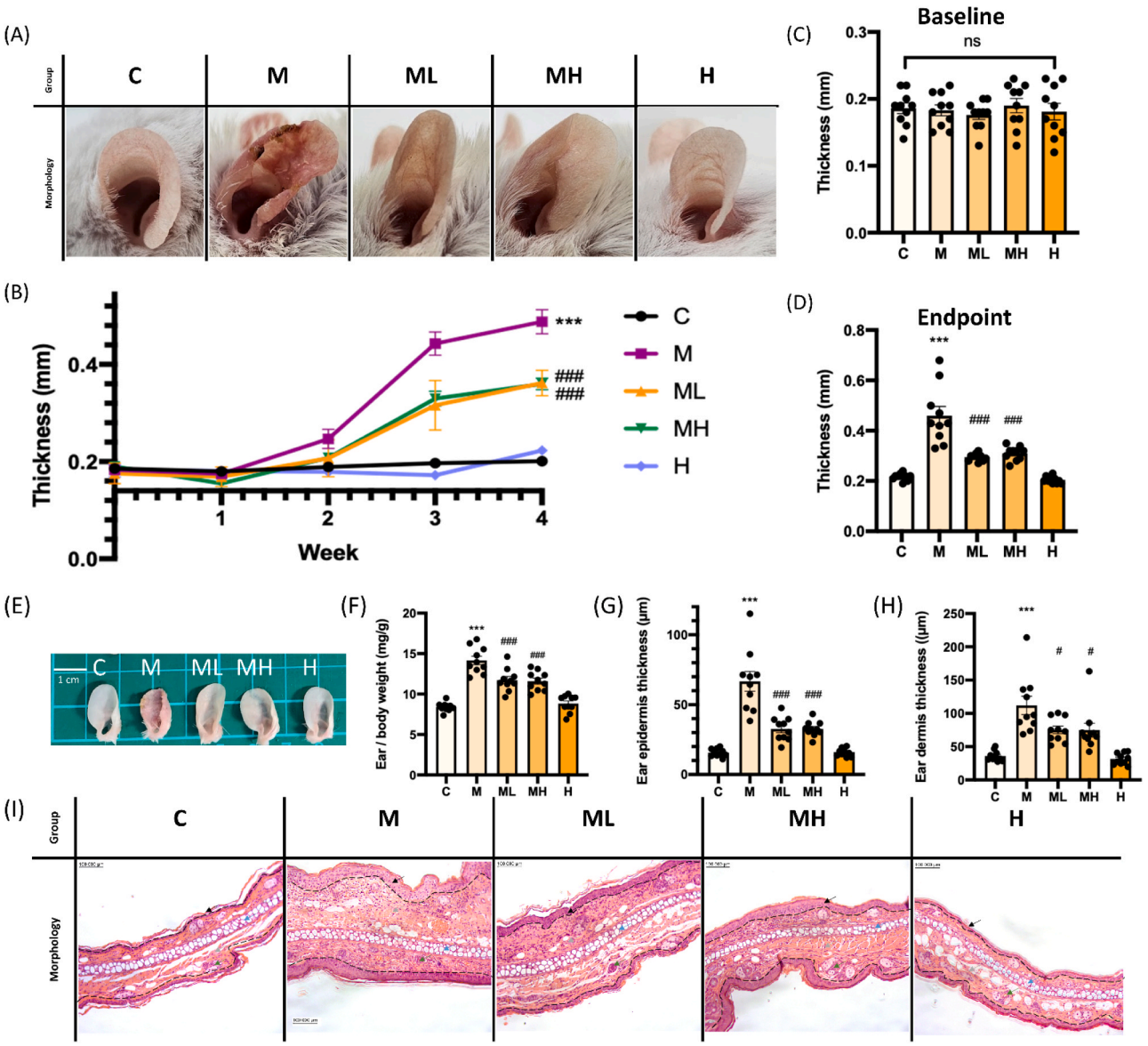

Fig. 4. Effect of Hinokitiol in atopic dermatitis mice on ear morphology and histology change. (A) The ear morphology was photographed. (B) The ear thickness was measured by digital caliper weekly, (C) the baseline and (D) endpoint ear thickness was shown. The (E, F) ear weight was measured by digital scale, and normalized to their body weight. (G) The ear epidermis and (H) dermis thickness was measured by Image J. (I) H&E staining showed the histology change in ear epidermal and dermis thickness. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Black arrows: epidermal. Gray arrows: dermis. Green arrows: vessels. Blue arrows: cartilage. Data were represented as mean ± SEM, using one- way ANOVA to compare the difference between groups. *** $p < 0.001$ compared with control group, and ###$p < 0.001$ compared with model group (n = 10).

3.4. Effect of hinokitiol on DNCB induced skin morphology change

Similar to the observations in the ear, notable changes in skin morphology and thickness were evident in this model. Group M displayed a rash, while in both ML and MH groups, signs of recovery were observed due to hinokitiol treatment (Fig. 5A). To further investigate alterations in skin layer thickness, we employed HE staining. The epidermal and dermal thickness showed a significant increase in group M, whereas both ML and MH groups exhibited a significant decrease in epidermal thickness (Fig. 5B, D). Additionally, in the MH group, there was a significant reduction in dermal thickness (Fig. 5C, D), aligning with the observed changes in ear morphology.

3.5. Effect hinokitiol of macrophage specific marker

CD80 is well-established as a marker for the M1 macrophage phenotype [32], while CD206 is recognized as indicative of the M2 macrophage phenotype [33]. In our study, CD206 expression in the ear tissue notably increased, but this increase was significantly reduced in both the ML and MH groups (Fig. 6B). Conversely, CD80 expression displayed an upward trend in the M group but significantly decreased with hinokitiol treatment (Fig. 6A). Intriguingly, both CD206 and CD80 showed a significant increase in the M group, which was markedly attenuated by hinokitiol treatment (Fig. 6C, D). These findings highlight an elevation in both CD80 and CD206 protein expressions in the ear and skin of the M group, with hinokitiol demonstrating a suppressive effect on their expression levels.

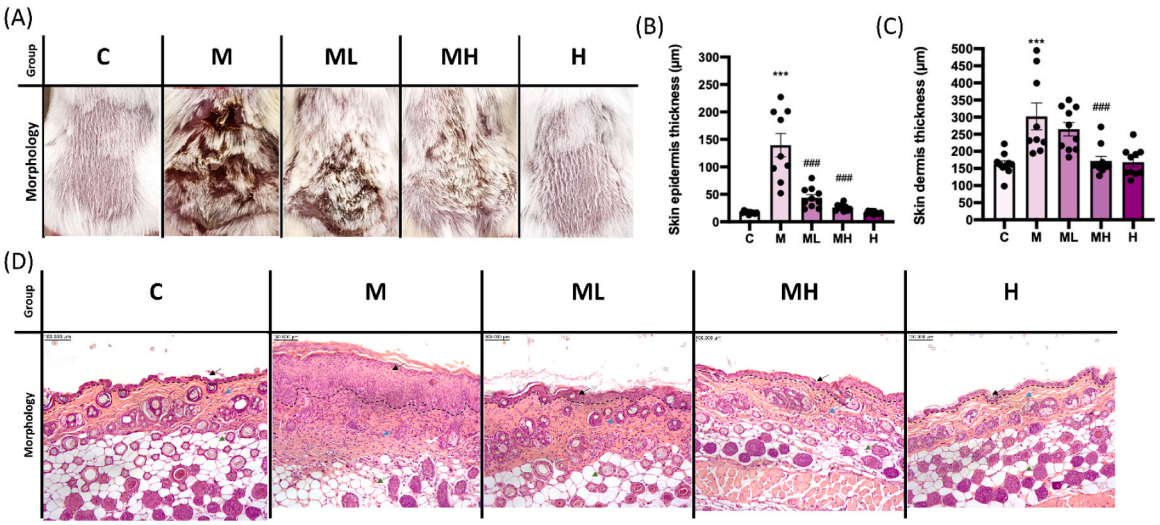

Fig. 5. Effect of Hinokitiol on skin morphology and histology change in atopic dermatitis mice. (A) The skin morphology was photographed. (B) The skin epidermis and (C) dermis thickness was measured by Image J. (D) H&E staining showed the histology change in skin epidermal and dermis thickness. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Black arrows: epidermis. Blue arrows: dermis. Green arrows: subcutaneous tissue. Data were represented as mean ± SEM, using one- way ANOVA to compare the difference between groups. *** $p < 0.001$ compared with control group, and ###$p < 0.001$ compared with model group (n = 9–10).

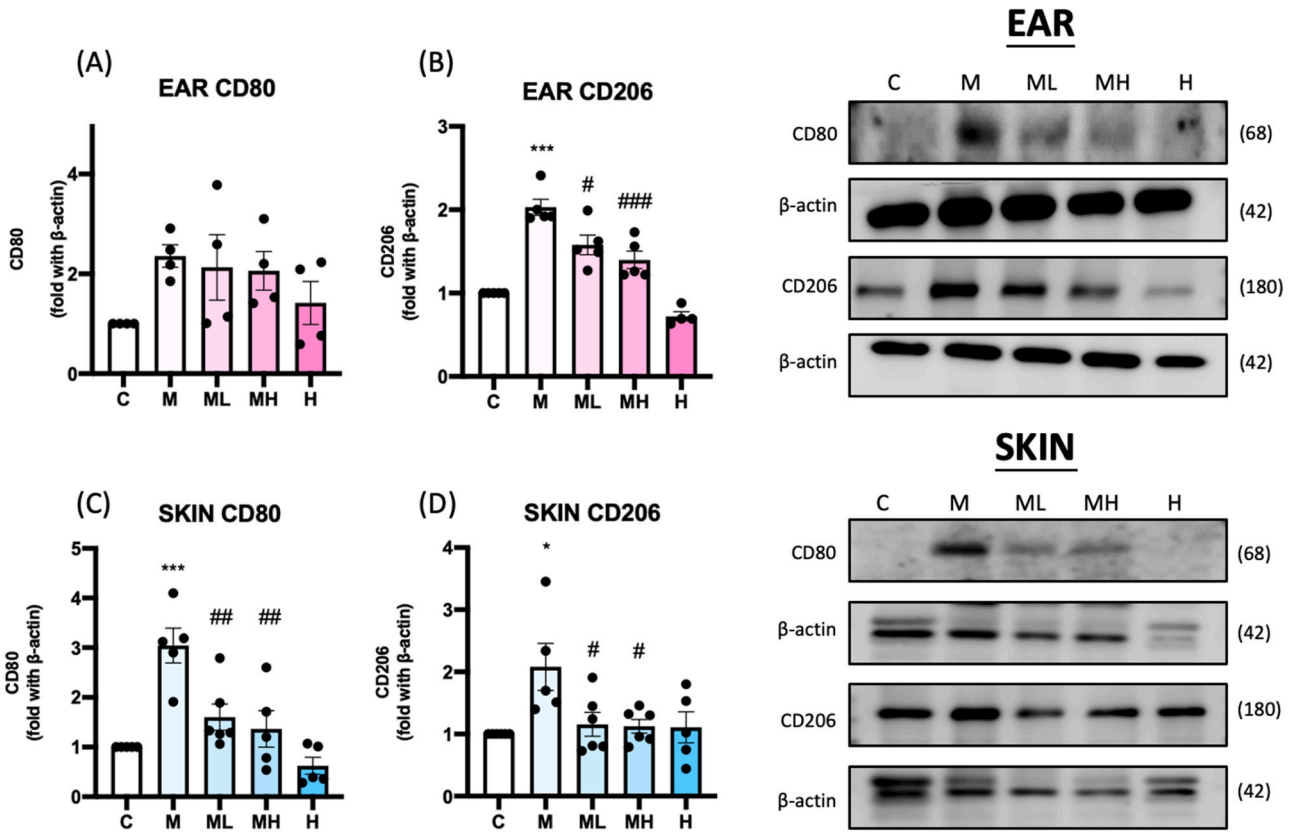

Fig. 6. Effect of Hinokitiol on CD80 and CD206 protein expression in atopic dermatitis mice. (A) The protein expression of CD80, (B) CD206 in ear; (C) CD80, (D) CD206 in skin were determined by Western blot and normalized to β-actin. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Data were represented as mean ± SEM, using one- way ANOVA to compare the difference between groups. * $p < 0.05$, *** $p < 0.001$ compared with control group, and # $p < 0.05$, ## $p < 0.01$ and ###$p < 0.001$ compared with model group.

3.6. Potent meachanism of hinokitiol on immune regulation

To investigate immune regulation in ear and skin tissues, we analyzed the TLR4/MyD88 pathway. In the ear tissue, the M group displayed a significant increase in TLR4 protein expression. Additionally, both MyD88 and COX-2 expressions exhibited a notable elevation

in the M group, which was markedly attenuated in both the ML and MH groups (Fig. 7A-D). Similarly, in the skin tissue, we observed an upward trend in TLR4 expression within the M group. Moreover, MyD88 and COX-2 expressions demonstrated a significant increase in the M group but were notably reduced in both the ML and MH groups (Fig. 7E-H). These findings collectively indicate that hinokitiol possesses the capacity to mitigate the inflammatory response through its regulatory influence on the TLR4/MyD88 pathway.

3.7. Cell viability of hinoktiol on HaCaT cell

In our cell culture experiment, various concentrations of hinokitiol were applied during a 24-hour incubation period to assess its impact on cell viability. The in vitro study revealed a marginal decrease in cell viability at higher dosages of hinokitiol, while it exhibited no discernible effect on cell viability at lower dosages of intervention (Fig. 8). Notably, we selected 10 μM as the highest pretreatment dosage for our study.

3.8. Protective effects of hinokitiol under TNF- α and IFN-γ induced model

Through meticulous microscopy, we scrutinized the morphological alterations induced by hinokitiol in the presence of TNF-α and IFN-γ (AD model). In the AD model group, distinct indications of cellular damage were evident. Conversely, the hinokitiol pretreatment group exhibited a notable mitigation of these deleterious effects (Fig. 9A). This visual

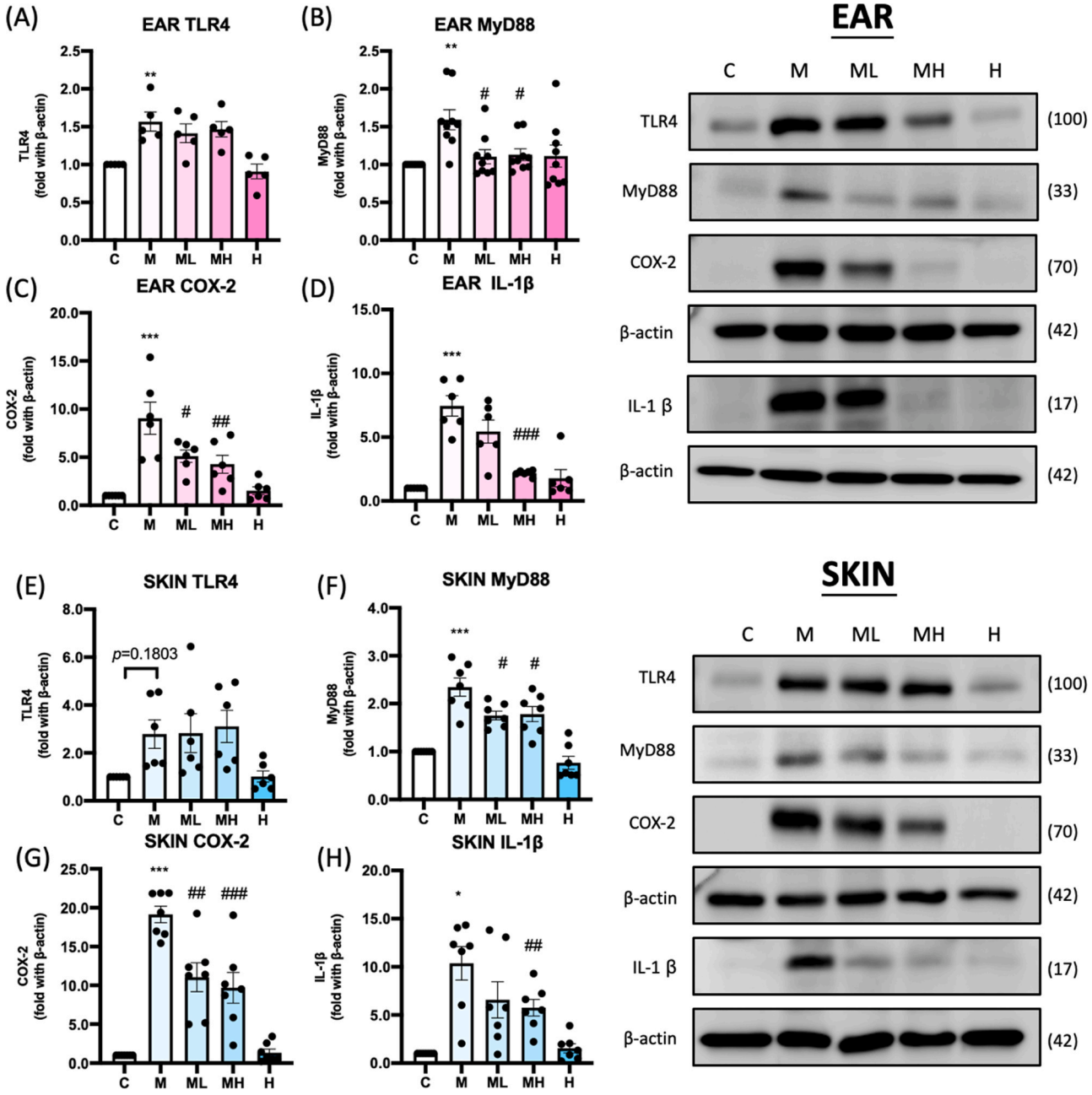

Fig. 7. Effect of Hinokitiol on TLR4/ MyD88 pathway in atopic dermatitis mice. (A) The protein expression of TLR4, (B) MyD88, (C) COX-2 and (D) IL-1β in ear; (E) TLR4, (F) MyD88, (G) COX-2, and (H) IL-1β in skin were determined by Western blot and normalized to β-actin. C: control, M: DNCB-induced model, ML: DNCB-induced model combined with 10 mg/kg of hinokitiol, MH: DNCB-induced model combined with 25 mg/kg of hinokitiol, H: 25 mg/kg of hinokitiol treated group. Data were represented as mean ± SEM, using one- way ANOVA to compare the difference between groups. ** $p < 0.01$, *** $p < 0.001$ compared with control group, and# $p < 0.05$, ## $p < 0.01$ and ###$p < 0.001$ compared with model group.

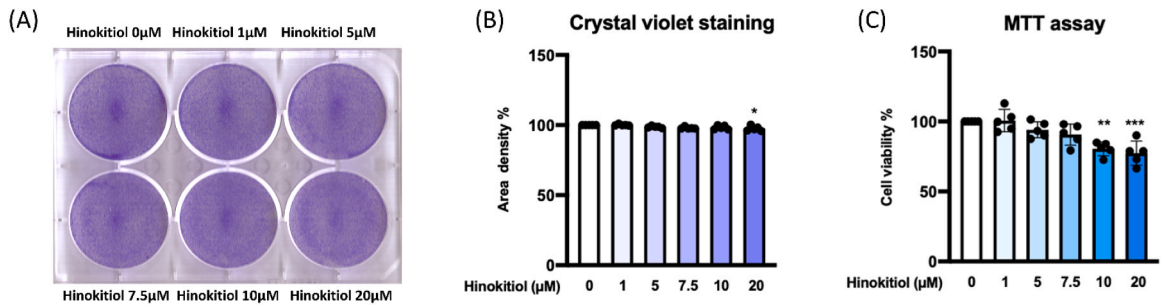

Fig. 8. Effect of Hinokitiol on HaCaT cell viability. (A, B) 2×10^5 HaCaT cells were seeding in 6 well plate and treated with hinokitiol for 24 h and evaluate by crystal violet staining (n = 5). (C) 5000 cells/well were seeded in 96 well plate and evaluated by MTT assay (n = 5) to access the cell viability. Data were represented as mean ± SD, using one- way ANOVA to compare the difference between groups. * $p < 0.05$ compare with hinokitiol 0 μM.

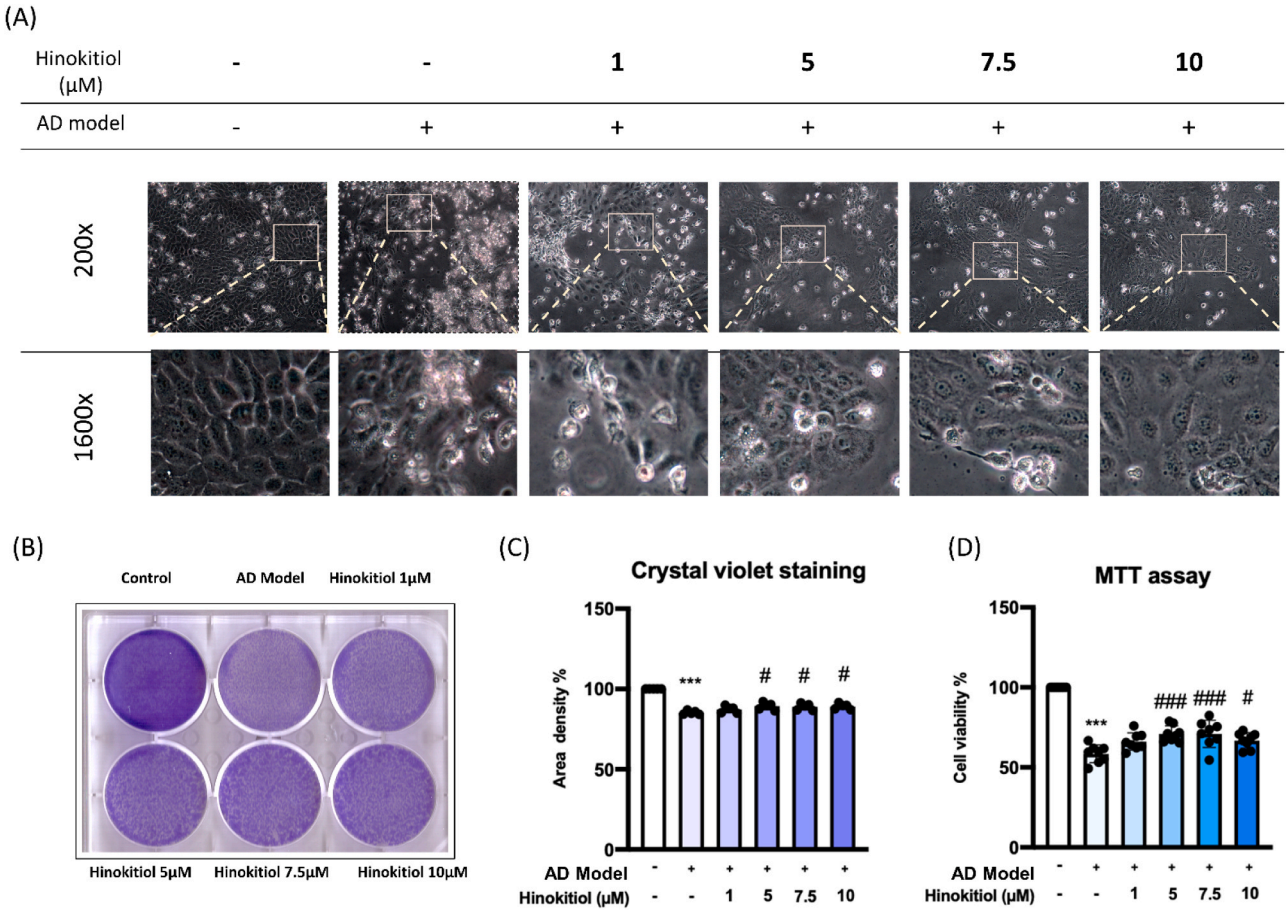

Fig. 9. Protective effect of Hinokitiol on TNF- α and IFN-γ-induced cell damage in HaCaT cell. 2×10^5 HaCaT cells were seeding in 6 well plate, pretreated with hinokitiol for 24 h, then using 20 ng/ML TNF-α and IFN-γ (AD model) induced for 24 h, (B, C) and the crystal violet staining was shown (n = 5). (D) 5000 cells were seeding in 96 well plate, pretreated with hinokitiol for 24 h, then using 20 ng/ML TNF-α and IFN-γ induced for 24 h used MTT assay (n = 8) to access the cell viability. Data were represented as mean ± SD, using one- way ANOVA to compare the difference between groups. ***p < 0.001 compared with control group, #p < 0.05 and ###p < 0.001 compared with model group. AD Model: 20 ng/ML of TNF-α and IFN-γ induced for 24 h.

appraisal is substantiated by the quantitative assessment of cell viability. Specifically, the AD model group demonstrated a noteworthy reduction in cell viability, while the hinokitiol pretreatment group manifested a marked increase (Fig. 9B-D). These concurrent observations substantiate the protective efficacy of hinokitiol against TNF-α and IFN-γ-induced cell damage.

3.9. Potent mechanism of hinokitiol on increase cell viability

To elucidate the underlying mechanisms governing cell death and its protective effects, we conducted an analysis of ROS levels and probed the apoptosis-related pathway. ROS levels exhibited a noteworthy increase in the AD model group but underwent a significant decrease in the hinokitiol pretreatment group (Fig. 10A). Furthermore, the protein expression levels of caspase-3, PARP, Bax, and cytochrome c displayed a substantial increase in the AD model group but saw a marked reduction in the hinokitiol pretreatment group. Bcl-2, however, did not exhibit significant variations between the groups (Fig. 10B-F). These findings collectively reveal hinokitiol's capacity to modulate ROS levels and evoke anti-apoptotic effects within the TNF-α and IFN-γ-induced AD

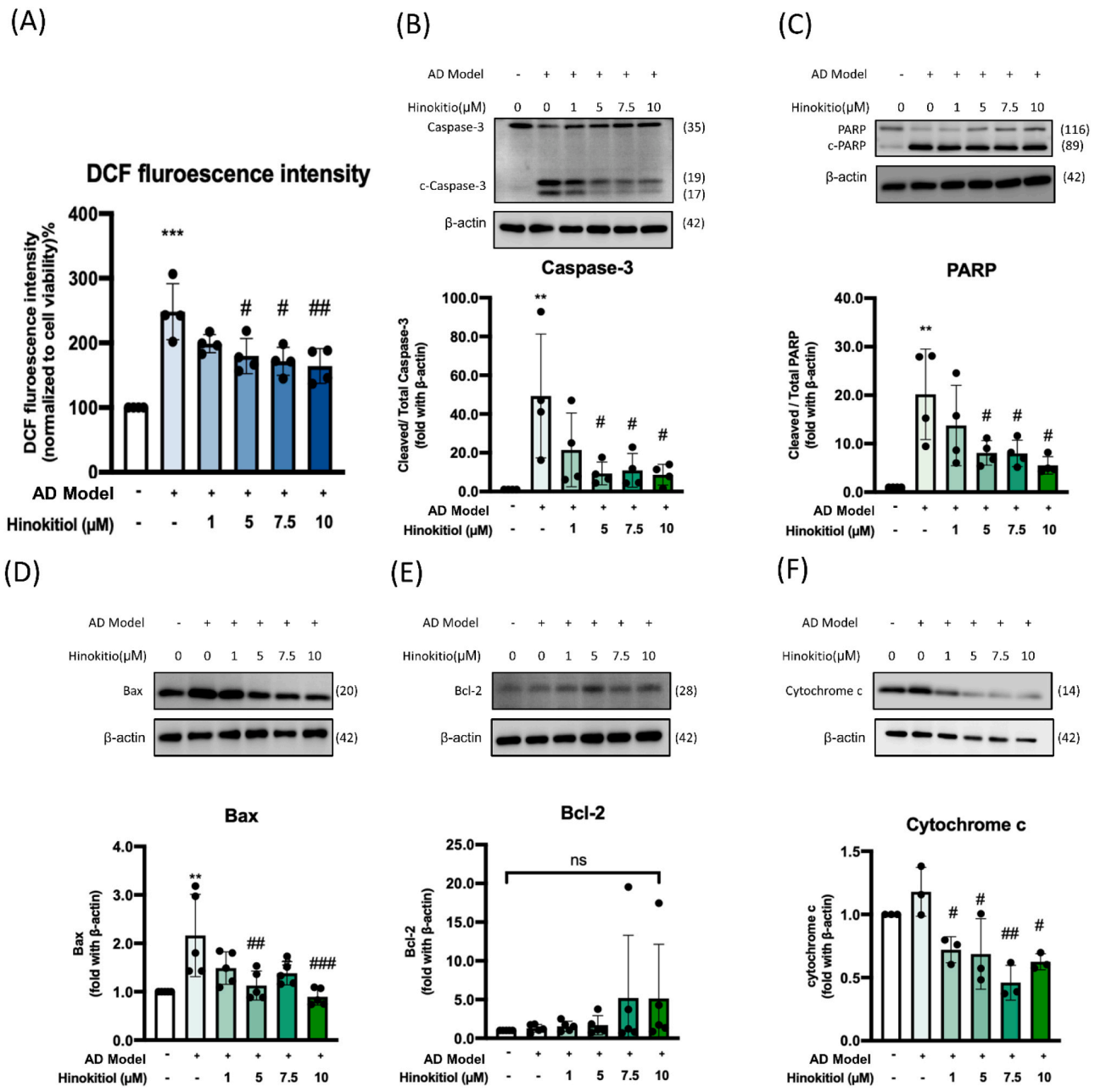

Fig. 10. Effect of Hinokitiol on ROS and apoptosis related protein expression in HaCaT cell induced by TNF-α and IFN-γ. 3.5×10^5 HaCaT cells were seeding on 6 cm dish, pretreated with hinokitiol for 24 h, then using 20 ng/ML TNF-α and IFN- γ (AD model) induced for 24 h. (A) The DCF fluorescence intensity and the protein expression of (B)Caspase-3, (C)PARP, (D)Bax, (E)Bcl-2 and (F) Cytochrome c were determined by western blot, and the protein expression were normalized by β-actin. Data were represented as mean ± SD, using one- way ANOVA to compare the difference between groups. $^{**}p < 0.01$ compared with control group, $\#p < 0.05$ compared with AD model group. c-PARP: cleaved PARP. c-Caspase-3: Cleaved Caspase-3.AD Model: 20 ng/ML of TNF-α and IFN- γ induced for 24 h.

model.

4. Discussion

This study aims to scrutinize the anti-inflammatory and apoptosis-regulatory properties of hinokitiol on atopic dermatitis through a combination of in vitro and in vivo investigations. Initially, our animal model experiment demonstrated a notable enhancement in both appearance and a reduction in skin thickness among subjects treated with hinokitiol in the atopic dermatitis mouse model. Subsequently, hinokitiol exhibited a discernible mitigation of inflammation provoked by the hapten DNCB. Additionally, in vitro analyses indicated that hinokitiol displayed significant anti-apoptotic effects in conditions characterized by immune infiltration.

Atopic dermatitis is typified by a Th2-driven immune response, marked by a compromised skin barrier and persistent scratching, leading to the release of chemokines promoting type 2 inflammation [34]. This sequence of events initiates the activation of effector cells, resulting in the release of Th2 cytokines and subsequent B cell activation, ultimately leading to the release of IgE [30]. Elevated IgE levels have been closely correlated with the severity of atopic dermatitis [35]. Additionally, immune activation may trigger the elevation of Th2 cytokines such as IL-6 [36]. To establish our atopic dermatitis model, we applied DNCB to the dorsal skin and ears of mice, a well-established hapten known to incite immune responses upon binding with proteins [37]. Our findings manifest that hinokitiol ameliorated a range of indicators

reflecting skin condition and notably reduced IgE and IL-6 levels in the intervention group.

In atopic dermatitis patients, macrophages are known to accumulate in inflamed skin tissue [38]. Both M1 and M2 macrophage phenotypes have demonstrated an increase in atopic dermatitis conditions [39,40]. While M1 macrophages are instrumental in orchestrating inflammatory responses, M2 macrophages are primarily involved in anti-inflammatory processes. An aberrant elevation in the M1/M2 ratio can precipitate severe inflammation [41]. Our scrutiny of M1 and M2 macrophage phenotypes unveiled an augmentation in CD206 and CD80 within the model group, which subsequently subsided with hinokitiol administration, aligning with antecedent research findings [39,40]. Additionally, MyD88 and its downstream inflammatory factors witnessed a reduction in response to hinokitiol treatment. Collectively, our animal study substantiates hinokitiol's potential in immune regulation.

Apoptosis, a pivotal mechanism in atopic dermatitis progression, has been well-documented in previous studies [42]. Antecedent investigations have demonstrated hinokitiol's proficiency in shielding cells against H_2O_2-induced apoptosis in cardiomyocytes [43]. In our study, we induced an immune-infiltrated milieu within a keratinocyte cell line using TNF-α and IFN-γ. Hinokitiol unequivocally exhibited antioxidant properties, concomitant with a reduction in the expression of apoptosis-related proteins.

To synopsize, we proffer a working model that delineates the anti-inflammatory and anti-apoptotic effects of hinokitiol in atopic dermatitis (Fig. 11). Notably, our study pioneers the investigation of hinokitiol's oral administration in atopic dermatitis, unraveling its potential in mitigating inflammation via the TLR4/MyD88 pathway. Furthermore, in vitro analyses revealed hinokitiol's ability to augment cell viability and morphology under TNF-α and IFN-γ induction. The modulation of apoptosis-related proteins (bax, cytochrome-c, caspase-3, and PARP) demonstrated hinokitiol's potential as an adjunctive treatment for atopic dermatitis.

The limitations of our study are twofold. Firstly, the absence of a positive control group hinders our ability to establish a benchmark for comparison, limiting the contextual interpretation of the observed effects. Secondly, we acknowledge that liver weight changes may play a crucial role in the Atopic Dermatitis (AD) model and could potentially impact liver function [44,45]. Unfortunately, we did not conduct a thorough examination of changes in the liver within the AD model, restricting our capacity to comprehensively assess potential systemic effects and associated hepatic alterations resulting from the experimental interventions. To address these limitations, future studies should consider incorporating positive controls and conducting a detailed examination of liver changes, thereby enhancing the robustness and interpretability of our findings.

CRediT authorship contribution statement

Chiang Yi-Fen: Investigation, Writing – original draft. **Tai Ling-Ray:** Investigation, Methodology, Writing – original draft. **Ali Mohamed:** Formal analysis, Methodology. **Chen Hsin-Yuan:** Formal analysis, Methodology. **Hsia Shih-Min:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Huang Ko-Chieh:** Formal analysis, Methodology.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant

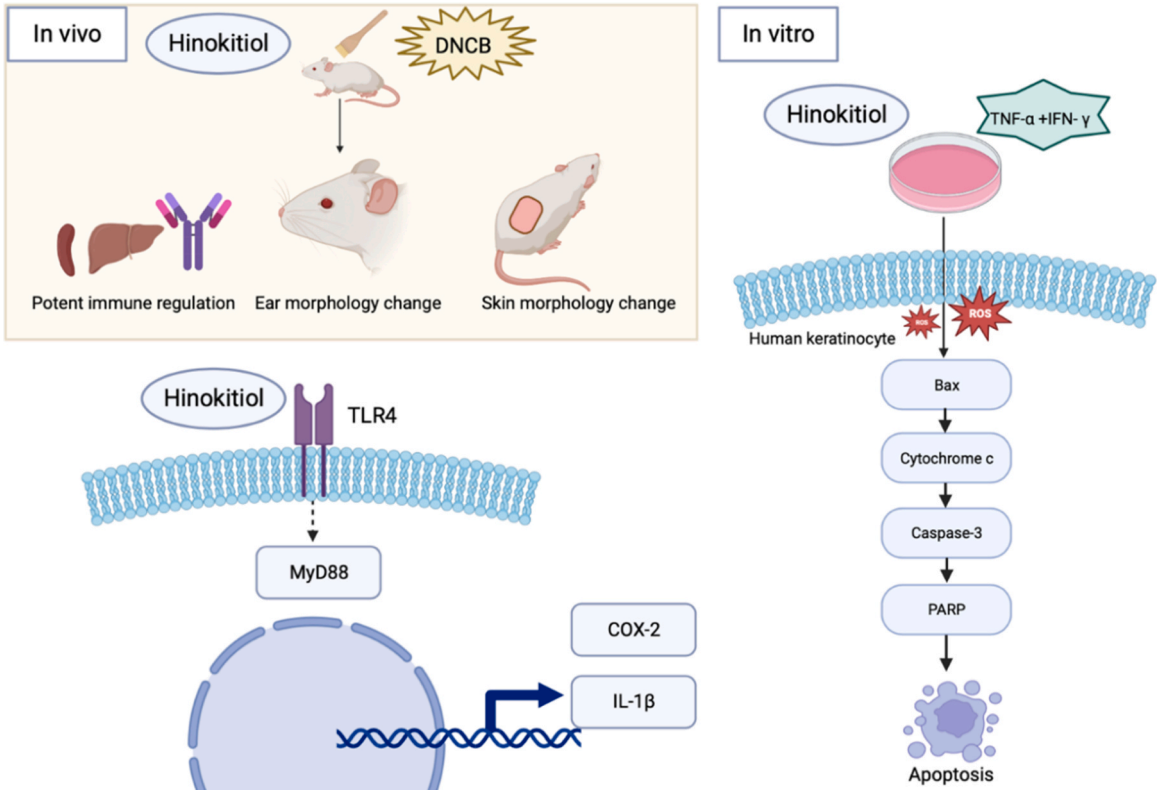

Fig. 11. Summary. In in vivo study, the DNCB was used to establish atopic dermatitis mice model. Treating with 10, 25 mg/kg of hinokitiol could improve the ear and skin morphology, and regulate the TLR4/ MyD88 pathway, reduce inflammation reaction. In in vitro study, the TNF-α and IFN- γ was used to establish chronic atopic dermatitis model. Hinokitiol could decrease ROS and protect the cell from apoptosis. It speculated that hinokitiol could be an adjuvant therapy to atopic dermatitis. DNCB: 2,4-dinitrochlorobenzene. TLR4: Toll-like receptor 4. MyD88: Myeloid differentiation primary response protein 88. COX-2: Cyclooxygenase 2. IL-1β: Interleukin-1 beta. TNF-α: Tumor necrosis factor- alpha. IFN-γ: Interferon-gamma. ROS: reactive oxygen species. Bax: B-cell lymphoma 2-associated X protein. PARP: Poly ADP-ribose polymerase.

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Declaration of Competing Interest

The authors declare no conflict of interest.

References

[1] S.M. Langan, A.D. Irvine, S. Weidinger, Atopic dermatitis, Lancet 396 (10247) (2020) 345–360.
[2] D. Camfferman, et al., Eczema and sleep and its relationship to daytime functioning in children, Sleep. Med Rev. 14 (6) (2010) 359–369.
[3] A. Wollenberg, et al., Consensus-based European guidelines for treatment of atopic eczema (atopic dermatitis) in adults and children: part II, J. Eur. Acad. Dermatol. Venereol. 32 (6) (2018) 850–878.
[4] L.F. Eichenfield, et al., Guidelines of care for the management of atopic dermatitis: section 1. Diagnosis and assessment of atopic dermatitis, J. Am. Acad. Dermatol. 70 (2) (2014) 338–351.
[5] C. Bangert, et al., Persistence of mature dendritic cells, T(H)2A, and Tc2 cells characterize clinically resolved atopic dermatitis under IL-4Rα blockade, Sci. Immunol. 6 (55) (2021).
[6] S. Akhtar, et al., Epigenetic control of inflammation in atopic dermatitis, Semin Cell Dev. Biol. 154 (Pt C) (2024) 199–207.
[7] E. Houben, K. De Paepe, V. Rogiers, A keratinocyte's course of life, Ski. Pharm. Physiol. 20 (3) (2007) 122–132.
[8] S.H. Lee, et al., Fas ligand is expressed in normal skin and in some cutaneous malignancies, Br. J. Dermatol. 139 (2) (1998) 186–191.
[9] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (4) (2007) 495–516.
[10] A. Trautmann, et al., T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis, J. Clin. Invest 106 (1) (2000) 25–35.
[11] U. Szymański, et al., Cytokines and apoptosis in atopic dermatitis, Post. Dermatol. Alergol. 38 (2) (2021) 1–13.
[12] J. Shi, et al., TLR4 polymorphisms as potential predictors of atopic dermatitis in Chinese Han children, J. Clin. Lab Anal. 36 (5) (2022), e24385.
[13] E.K. Grishman, P.C. White, R.C. Savani, Toll-like receptors, the NLRP3 inflammasome, and interleukin-1β in the development and progression of type 1 diabetes, Pedia Res 71 (6) (2012) 626–632.
[14] N.S. Kirkby, et al., Systematic study of constitutive cyclooxygenase-2 expression: role of NF-κB and NFAT transcriptional pathways, Proc. Natl. Acad. Sci. USA 113 (2) (2016) 434–439.
[15] K. Zepter, et al., Induction of biologically active IL-1 beta-converting enzyme and mature IL-1 beta in human keratinocytes by inflammatory and immunologic stimuli, J. Immunol. 159 (12) (1997) 6203–6208.
[16] M. Bernard, et al., IL-1β induces thymic stromal lymphopoietin and an atopic dermatitis-like phenotype in reconstructed healthy human epidermis, J. Pathol. 242 (2) (2017) 234–245.
[17] H. Saeki, et al., Clinical practice guidelines for the management of atopic dermatitis 2016, J. Dermatol. 43 (10) (2016) 1117–1145.
[18] J.B. Rice, et al., Long-term systemic corticosteroid exposure: a systematic literature review, Clin. Ther. 39 (11) (2017) 2216–2229.
[19] S. Wu, et al., A comprehensive review of natural products against atopic dermatitis: flavonoids, alkaloids, terpenes, glycosides and other compounds, Biomed. Pharm. 140 (2021), 111741.
[20] Y.H. Shih, et al., In vitro antimicrobial and anticancer potential of hinokitiol against oral pathogens and oral cancer cell lines, Microbiol Res 168 (5) (2013) 254–262.
[21] T. Isono, et al., Treatment of severe pneumonia by hinokitiol in a murine antimicrobial-resistant pneumococcal pneumonia model, PLoS One 15 (10) (2020), e0240329.
[22] Y. Chelpuri, et al., Tropolone derivative hinokitiol ameliorates cerulein-induced acute pancreatitis in mice, Int Immunopharmacol. 109 (2022), 108915.
[23] Y.S. Lee, et al., Hinokitiol inhibits cell growth through induction of S-phase arrest and apoptosis in human colon cancer cells and suppresses tumor growth in a mouse xenograft experiment, J. Nat. Prod. 76 (12) (2013) 2195–2202.
[24] H.Y. Chen, et al., Hinokitiol Exhibits Antitumor Properties through Induction of ROS-Mediated Apoptosis and p53-Driven Cell-Cycle Arrest in Endometrial Cancer Cell Lines (Ishikawa, HEC-1A, KLE), Int J. Mol. Sci. 22 (15) (2021).
[25] Y. Arima, et al., Antibacterial effect of beta-thujaplicin on staphylococci isolated from atopic dermatitis: relationship between changes in the number of viable bacterial cells and clinical improvement in an eczematous lesion of atopic dermatitis, J. Antimicrob. Chemother. 51 (1) (2003) 113–122.
[26] B. Kwon, et al., Effect of cone of pinus densiflora on dncb-induced allergic contact dermatitis-like skin lesion in Balb/c mice, Nutrients 13 (3) (2021).
[27] D.H. Hwang, et al., Rosa davurica Pall. improves DNCB-induced atopic dermatitis in mice and regulated TNF-Alpa/IFN-gamma-induced skin inflammatory responses in HaCaT cells, Phytomedicine 91 (2021), 153708.
[28] T.R. Abrahamsson, et al., Low diversity of the gut microbiota in infants with atopic eczema, J. Allergy Clin. Immunol. 129 (2) (2012) 434–440, 440.e1-2.
[29] E.J. An, et al., Ophiopogonin D ameliorates DNCB-induced atopic dermatitis-like lesions in BALB/c mice and TNF-α- inflamed HaCaT cell, Biochem Biophys. Res Commun. 522 (1) (2020) 40–46.
[30] D.B. Corry, F. Kheradmand, Induction and regulation of the IgE response, Nature 402 (6760 Suppl) (1999) B18–B23.
[31] S. Huang, et al., Association between IL-6 polymorphisms and Atopic Dermatitis in Chinese Han children, Front Pedia 11 (2023) 1156659.
[32] S.E. Burastero, et al., Increased expression of the CD80 accessory molecule by alveolar macrophages in asthmatic subjects and its functional involvement in allergen presentation to autologous TH2 lymphocytes, J. Allergy Clin. Immunol. 103 (6) (1999) 1136–1142.
[33] T. Rőszer, Understanding the mysterious M2 macrophage through activation markers and effector mechanisms, Mediat. Inflamm. 2015 (2015), 816460.
[34] R. Tanei, Y. Hasegawa, Immunological pathomechanisms of spongiotic dermatitis in skin lesions of atopic dermatitis, Int J. Mol. Sci. 23 (12) (2022).
[35] M. Suárez-Fariñas, et al., Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis, J. Allergy Clin. Immunol. 132 (2) (2013) 361–370.
[36] L. Tang, et al., Topical application with conjugated linoleic acid ameliorates 2, 4-dinitrofluorobenzene-induced atopic dermatitis-like lesions in BALB/c mice, Exp. Dermatol. 30 (2) (2021) 237–248.
[37] E.Y. Zhang, A.Y. Chen, B.T. Zhu, Mechanism of dinitrochlorobenzene-induced dermatitis in mice: role of specific antibodies in pathogenesis, PLoS One 4 (11) (2009), e7703.
[38] R.C. Kiekens, et al., Heterogeneity within tissue-specific macrophage and dendritic cell populations during cutaneous inflammation in atopic dermatitis, Br. J. Dermatol. 145 (6) (2001) 957–965.
[39] D. Zeng, et al., TRPA1 deficiency alleviates inflammation of atopic dermatitis by reducing macrophage infiltration, Life Sci. 266 (2021), 118906.
[40] K. Nakai, et al., IL-17A induces heterogeneous macrophages, and it does not alter the effects of lipopolysaccharides on macrophage activation in the skin of mice, Sci. Rep. 7 (1) (2017), 12473.
[41] S. Kasraie, T. Werfel, Role of macrophages in the pathogenesis of atopic dermatitis, Mediat. Inflamm. 2013 (2013), 942375.
[42] M. Akdis, et al., Cytokine network and dysregulated apoptosis in atopic dermatitis, Acta Odontol. Scand. 59 (3) (2001) 178–182.
[43] H. Xiao, et al., Hinokitiol protects cardiomyocyte from oxidative damage by inhibiting GSK3β-mediated autophagy, Oxid. Med Cell Longev. 2022 (2022) 2700000.
[44] S. Hong, et al., Solanum nigrum Linne improves DNCB-induced atopic dermatitis-like skin disease in BALB/c mice. Mol. Med Rep. 22 (4) (2020) 2878–2886.
[45] S. Seino, et al., Atopic dermatitis causes lipid accumulation in the liver of NC/Nga mouse, J. Clin. Biochem Nutr. 50 (2) (2012) 152–157.