

Epimedin A ameliorates DNFB-induced allergic contact dermatitis in mice: Role of NF- κ B/NLRP3-driven pyroptosis, Nrf2/HO-1 pathway, and inflammation modulation

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ABSTRACT

Aims: The present study aimed to investigate the potential of epimedin A to ameliorate DNFB-induced allergic contact dermatitis (CD) and reveal its potential underlying mechanisms of action, emphasizing its role in modulating NF- κ B/NLRP3, Nrf2/HO-1 pathways, and inflammation.

Main methods: Seven-week-old BALB/c mice received epimedin A orally for 11 days at doses of 5, 10, or 20 mg/kg/day, starting from the seventh day of DNFB-inducing CD.

Key findings: Epimedin A dose-dependently ameliorated DNFB-induced CD, as revealed by the repression of the mice's scratching behavior, dermatitis score, ear thickness and weight, and ear tissue's histopathological changes, and area percent of collagen fibers induced by DNFB. These potentials were due to the NF- κ B/NLRP3 pathway suppression and the Nrf2 pathway enhancement, as demonstrated by the reduction of NF- κ B, NLRP3, ASC, caspase-1, and 8 mRNA expression, and NF- κ Bp65, IL-1 β , MDA levels, and NF- κ Bp65 binding activity, along with the enhancement of the Nrf2, HO-1, I κ B- α , GSH levels, SOD activity, and Nrf2 binding activity. Besides, it suppressed ear tissues' NLRP3 and caspase-8 induced pyroptosis by suppressing the ear tissues' caspase-1, 8, GSDMD upregulation, and LDH activity. Additionally, it repressed the local inflammatory reaction of ear tissue, as evidenced by the reduction of the elevated inflammatory cytokines (IL-1 β , IL-6, IL-4, TNF- α , and IFN- γ), the serum level of t-IgE, DNFB s-IgE, s-IgE/t-IgE ratio, and the abrogation of the ear tissues histopathological changes.

Significance: Epimedin A is a novel, hopeful, natural therapeutic agent for CD by modulating NF- κ B/NLRP3, Nrf2 pathways, and inflammation.

1. Introduction

Allergic contact dermatitis (CD) is a common multifactorial, pruritic, inflammatory skin disease with complex etiology [1]. It affects approximately 5–20% of children and 7–14% of adults worldwide, with a significant global social and economic burden [2]. Clinically, the CD develops eczematous lesions, with itching, erythema, edema, dry skin, and recurrent skin infections that affect sleep, quality of life, and patient's physical and mental health [3]. However, therapeutic agents available for CD treatment provide temporary relief of symptoms;

nevertheless, they are associated with many side effects over long-term therapy, such as skin atrophy, immunosuppression, loss of efficacy, and insufficient effect over time [3]. Therefore, the search for potential therapeutic targets or effective agents against CD remains an urge.

The NOD-like receptor containing protein 3 (NLRP3) is widely identified in epithelial cells such as skin keratinocytes, which construct an important inflammasome together with an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1 [4]. Furthermore, NLRP3 inflammasome activation by the nuclear factor kappa B (NF- κ B) signal pathway is a crucial regulator of

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allergic contact dermatitis development, as it triggers NLRP3 inflammasome assembly, caspase-1 activation, release, and maturation of interleukin (IL)-1 β and IL-18, with cleavage of gasdermin D (GSDMD), and augmentation of pro-inflammatory cytokines production with pyroptosis induction [5–7]. Therefore, NLRP3 inflammasome regulation could be a potential therapeutic strategy for treating CD.

Moreover, the nuclear factor-erythroid factor 2-related factor 2 (Nrf2) signaling pathway plays a critical role in maintaining the skin barrier and preventing the development of CD [8]. Nrf2 is a transcription factor that remains inactive in the cytosol, but is activated during inflammatory states, migrates to the nuclei, and interacts with the genes involved to promote the production of antioxidant enzymes, which in turn facilitate the synthesis of the antioxidant molecules, and counteracts the oxidative stress molecules [9,10]. Furthermore, activation of the Nrf2 signaling pathway inhibits the production of pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α , thus dampening skin inflammation [11].

Epimedin A, a prenylated flavanol glycoside, is one of the main active ingredients in *Herba Epimedii*, which are popular Chinese herbs that have been used for centuries to strengthen bones, muscles, and tendons, and to treat rheumatism, cardiovascular disease, diabetes, infertility, and amnesia [12,13]. Recent studies documented that *Herba Epimedii* amends various disease models by suppressing of NF- κ B signaling pathway, NLRP3 inflammasome assembly, pro-inflammatory cytokines secretion, and enhancing the antioxidant system [14–16]. Therefore, in the present study, we aimed to investigate the potential of epimedin A to treat CD using a DNFB mouse model, emphasizing its potential modulating activities on NF- κ B/NLRP3 and Nrf2/HO-1 signaling pathways.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were bought commercially, 2,4-dinitrofluorobenzene (DNFB), acetone, prednisone, epimedin A, bovine serum albumin (BSA), mouse monoclonal anti-dinitrophenyl (DNP)-IgE and Masson trichrome stain from Sigma, St. Louis, MO, USA, carboxymethylcellulose (CMC), phosphate-buffered saline (PBS), formalin-buffered saline, and hematoxylin & eosin stains from El Gomhuria Co., Tanta, Egypt, thiopental sodium from EIPICO, Tenth of Ramadan City, Egypt, and rat monoclonal anti-mouse IgE, Tween 20, biotinylated DNP-BSA and peroxidase-conjugated streptavidin from Thermo Fisher Scientific, Waltham, MA, USA.

2.2. Animals

Seven-week-old BALB/c mice obtained from the Animal Care Unit of Prince Sattam bin Abdulaziz University, College of Pharmacy, Saudi Arabia, were used in the present study. Mice weighing 20–25 g were housed in ventilated polypropylene cages at room temperature under a 12-h (hrs) light / dark cycle and accessed water and standard laboratory diet ad libitum. The mice were allowed to acclimate for seven days before the experimental procedure. All experiments have been approved by the Research Ethics Committee for the experimental animal care and use at Prince Sattam bin Abdulaziz University, Al-Kharj, Saudi Arabia (Approval Reference No. REC-HSD-126-2022), which followed the guidelines of the National Institutes of Health Guide for the care and use of laboratory animals and the ARRIVE guidelines. All animal handlings were carried out between 8:00 am and 5:00 pm.

2.3. Induction of CD

CD induction was performed as designated by Lee et al. (2018). Briefly, mice's backs were shaved on day 0 (24 h before the sensitization) by an electrical clipper, confirming the total removal of the back hairs.

Then, the shaved back of each animal was sensitized on days 1, 2, and 3 by applying 50 μ L 0.2% DNFB in acetone: olive oil (4:1). Starting from the seventh day, each mouse was challenged by painting the dorsum of both ears with 30 μ L of 0.2% DNFB in acetone: olive oil (4:1) on days 7, 9, 11, 13, 15, and 17. However, the animals in the control group were sensitized and challenged with acetone: olive oil (4:1) in an amount corresponding to the volume of DNFB [17] (Fig. 1).

2.4. Experimental protocol

The present study was carried out on 70 mice, randomly allocated into seven groups of 10 mice each. Group I (CON), naïve mice were sensitized and challenged with the acetone: olive oil (4:1) in the corresponding amount to the volume of the DNFB. Group II (CD) was CD-induced mice by DNFB [17]. Group III (CMC) was CD-induced mice treated with 0.5% CMC in the corresponding volume to prednisone and epimedin A. Group IV (PD) was CD-induced mice treated orally with 5 mg/kg/day prednisone suspended in 0.5% CMC (reference group) [18]. Group V (EAL) was CD-induced mice treated orally with 5 mg/kg/day of epimedin A suspended in 0.5% CMC. Group VI (EAM) was CD-induced mice treated orally with 10 mg/kg/day of epimedin A suspended in 0.5% CMC. Group VII (EAH) was CD-induced mice treated orally with 20 mg/kg/day of epimedin A suspended in 0.5% CMC [12]. All drugs used in the present study were freshly prepared daily, administered from day 7 to day 17 of the experiment, and on the challenging days, treatments were administered 1 h before the challenge. Twenty-four hrs after the last challenge (day 18), blood was collected from the retro-orbital sinus, centrifuged at 5000 rpm for 10 min, sera harvested, and stored at -20°C for the assessment of the total immunoglobulin E (t-IgE) and DNFB specific-IgE (s-IgE) levels. Then, mice were euthanized by cervical dislocation under anesthesia with 50 mg/kg thiopental sodium (i.p.), the ears were obtained, and the left ears were preserved in 10% formalin-buffered saline for further histopathological evaluations. However, the right ear was divided into two halves, the first half was homogenized in ice-cold PBS (pH = 7.4) to get a 10% w / v homogenate, centrifuged, and the supernatant collected. Meanwhile, the pellet was resuspended in nuclear lysis buffer, the suspension was centrifuged, and the supernatant was collected. The remaining half of the ear was homogenized in lysis buffer to obtain a 10% w / v homogenate. Ear tissue homogenates and supernatants were stored at -80°C and further used to assess the ear tissue biochemical parameters and mRNA expressions.

2.5. Evaluation of mice's scratching behavior and dermatitis score

The scratching behavior of the mice was evaluated immediately after each challenge for 30 min, as demonstrated by Fei et al. (2021). In short, animals were acclimatized to the testing environment for 30 min on the test day. Each mouse was placed in a polypropylene cage and video recorded. The video recorder recorded the scratching behavior for 30 min; then, the video was played offline with a double-blind count. Scratching behavior was counted when the mouse scratched its ears with its hind paw and put it back on the floor or mouth [19]. Mice's dermatitis score was recorded 1 h after each challenge according to the Eczema Area and Severity Index scoring system, which was built on four clinical symptoms, including erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion, where severity was scored as follows, 0, none, 1, mild, 2, moderate and 3, severe. The dermatitis score was expressed arbitrarily as the sum of the four parameters [20].

2.6. Evaluation of the mice's ear thicknesses and weights

Mice's ear thicknesses and weights were evaluated as Lee et al. (2018) described. In brief, 24 h after each challenge, the thicknesses of both ears were determined under anesthesia using a digital caliper (Mitutoyo, Tokyo, Japan). Then 24 h after the last challenge, the mice

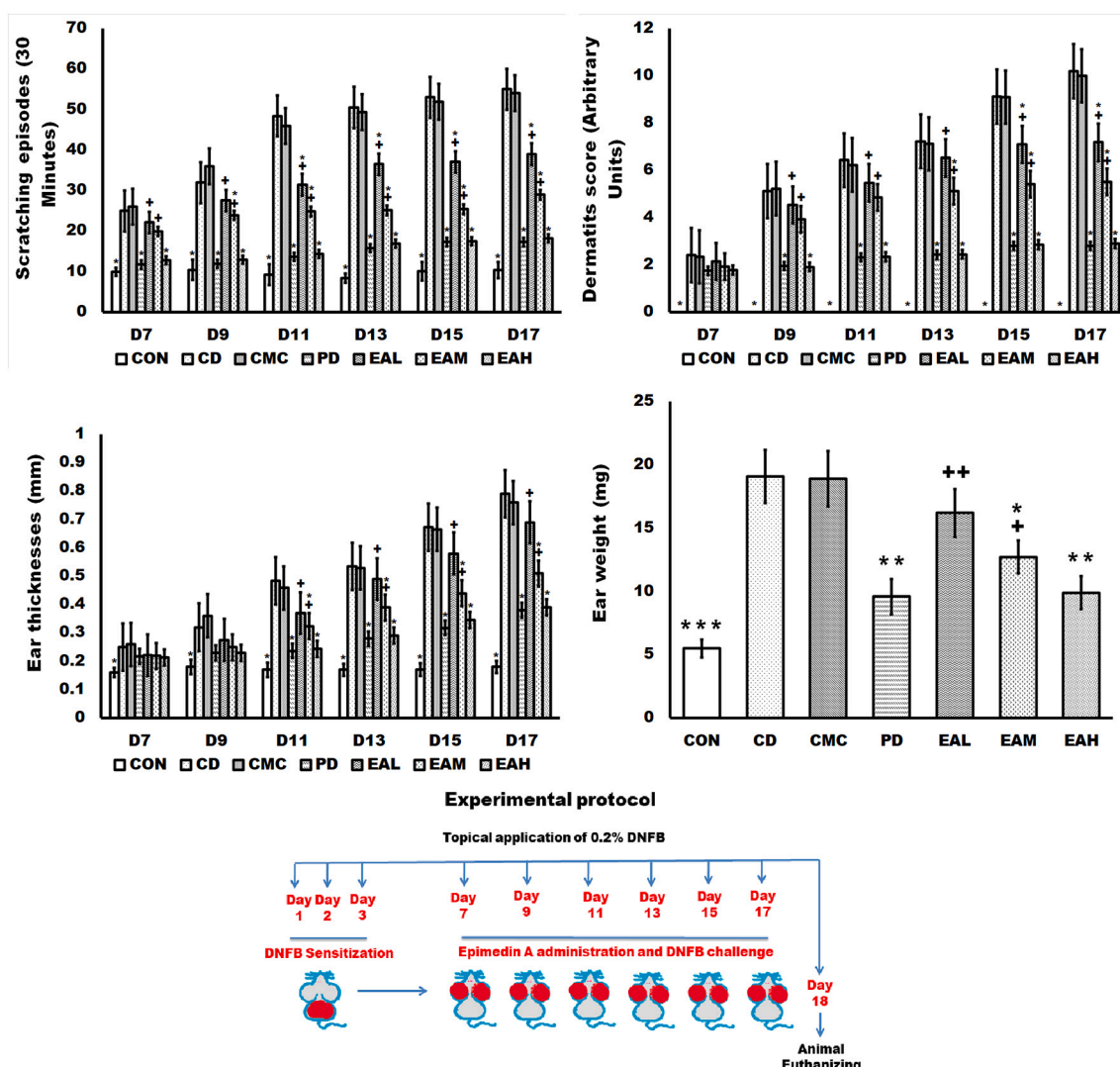


Fig. 1. Epimedin A alleviates DNFB-induced mice's scratching behavior, dermatitis score, ear thicknesses and weights, and the experimental protocol. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. D, day, * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$ & ++ $P < 0.01$ (Vs. EAH group).

were sacrificed by cervical dislocation, ears were obtained, and the weights of both ears were measured with an ADAM balance (EAB 314e, Adam Equipment Co. Ltd. Milton Keynes, U.K.) [17]. Average thickness and weights were expressed in mm and mg, respectively.

2.7. Evaluation of ear tissue's total protein content

The total protein content of the ear tissue was evaluated as described by Gornall et al. (1949) using a colorimetric assay kit purchased from Bio-Diagnostic Co., Giza, Egypt, where 0.025 ml of 10% ear tissue homogenate in PBS was added to 1 ml of Biuret reagent, mixed well, and incubated for 10 min at 37 °C. The optical densities were evaluated at 550 nm. The result was expressed as mg protein/ml ear tissue homogenate [21].

2.8. Evaluation of ear tissues Nrf2/HO-1 pathway and oxidative stress markers

The Nrf2/HO-1 pathway in ear tissue was assessed by the measurement of Nrf2 and HO-1 levels using the manufacturer's instructions of

sandwich enzyme-linked immunosorbent assay (sELISA) kits bought from Antibodies-Online GmbH, Aachen, Germany (Cat. # . ABIN5074537 and ABIN6956450, respectively), where the minimum detection limit for the Nrf2 kit was 9.38 pg/ml and the HO-1 kit was 11.6 pg/ml. Additionally, Nrf2 binding activity to an immobilized oligonucleotide containing the antioxidant response elements (ARE) was measured using an ELISA kit (TransAM® Nrf2, Active Motif, California, USA, Cat. # . 50,296), with 0.6 µg/ml minimum detection limit. A plate reader, Stat Fax 2100, Fisher Bioblock Scientific, BP, Illkirch Cedex, France, was used to detect Nrf2, HO-1 levels, and Nrf2 binding activity expressed as ng and µg/mg tissue protein, respectively. In addition, oxidative stress markers in ear tissue were assessed by measuring MDA, GSH levels, and SOD activity. MDA, GSH levels, and SOD activity in ear tissue were assessed using the manufacturer's instructions of colorimetric assay kits bought from MyBioSource Inc., San Diego, CA, USA (Cat. # MBS2540407, MBS2540412, and MBS2540402). The MDA and GSH levels in ear tissue were expressed as nmol/mg tissue protein and µmol/mg tissue protein, respectively, but the SOD activity in ear tissue was expressed as U/mg tissue protein. Ear tissue oxidative stress markers' absorbances were analyzed using Biosystems

semiautomated analyzer, BTS-350, Barcelona, Spain.

2.9. Evaluation of serum IgE and ear tissues' inflammatory signals

Serum t-IgE was evaluated using the manufacturer's instructions of sELISA kits, bought from MyBioSource Inc., San Deigo, CA, USA (Cat. #. MBS8801594), with a minimum detection limit of 0.37 µg/ml. Moreover, serum DNFB s-IgE was assessed as Nagai et al. (1997) described. Briefly, ELISA microplates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with rat Monoclonal anti-mouse IgE using overnight incubation at 4 °C. Then blocked for 1 h at room temperature with 1% BSA in PBS and washed with 0.2% Tween 20 in PBS (TPBS) for three times. DNP-IgE was sequentially diluted as the standard. After that, 100 µl of the standard and the serum samples were added to each well, incubated at room temperature for 1 h, washed with TPBS, 100 µl of biotinylated DNP-BSA was added to each well, incubated at room temperature for 1 h, and washed for six times with TPBS. Later, 100 µl of peroxidase-conjugated streptavidin was added to each well; the reaction stopped by adding 100 µl of stop solution after incubation at room temperature for 1 h, and absorbance was measured at 450 nm [22]. However, the ear tissue inflammatory signals were evaluated by assessment of IL-1β, IL-6, tumor necrosis factor-α (TNF-α), IL-4, interferon-γ (IFN-γ), IkappaB-α (IκB-α), and nuclear factor kappa B p65 (NF-κBp65) levels, using the manufacturer's instructions for sELISA kits, purchased from MyBioSource Inc., San Deigo, CA, USA, Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China, CUSABIO, Wuhan, Hubei, China, and Abcam, Cambridge, UK (Cat. #. MBS8800273, MBS8800159, MBS2568056, EM0119, CSB-E04578m, ab206308, and MBS009329, respectively). The minimum detection limit of the IL-1β kit was 6.4 pg/ml, IL-6 kit was 3.2 pg/ml, TNF-α kit was 4.96 pg/ml, IL-4 kit was 9.37 pg/ml, IFN-γ kit was 3.9 pg/ml, IκB-α kit was 14.7 pg/ml, and NF-κBp65 kit was 10 pg/ml. Furthermore, the NF-κB binding activity to an immobilized oligonucleotide containing the NF-κB consensus site was measured using an ELISA kit (TransAM® NF-κBp65, Active Motif, California, USA, Cat. #. 40,096), with 0.1 ng/ml minimum detection limit. A Stat Fax 2100 automated plate reader, Fisher Bioblock Scientific, BP., Illkirch Cedex, France, was used to detect the inflammatory signals levels, which were expressed as pg and ng/mg tissue protein, respectively.

2.10. Evaluation of ear tissue NF-κB, NLRP3, ASC, caspase-1, 8, and 11 mRNA expressions

According to the manufacturer's instructions, total RNA was isolated from the ear tissues using a total RNA extraction kit (RNAsimple Total RNA Kit, Tiangen Biotech, Beijing, China, Cat. #. 4,992,858). Reverse transcription was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, Cat. #. 1,708,891). The cDNA obtained was amplified with a real-time PCR system (qTOWER, Analytik Jena GmbH, Jena, Germany). The thermal cycling conditions were as follows, the initial denaturation and enzyme activation at 95 °C for 2 min (1 cycle) and denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s (40 cycles) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, Cat. #. 1,708,880). After the cycle, the melting curve was done to ensure no nonspecific products were present. Relative mRNA expression was normalized to GAPDH as an internal control to standardize the difference, and the primers' sequences are shown in Table 1.

2.11. Evaluation of ear tissues' caspases, GSDMD levels, and lactic dehydrogenase (LDH) activity

The ear tissues' caspases levels were assessed by measuring caspase-1, 8, and 11 using the manufacturer's instructions for the sELISA kits purchased from Wuhan Fine Biotech Co., Ltd., Wuhan, Hubei, China (Cat. #. EM0897, EM0711 and EM0899 respectively), where the minimum detection limits were 18.75 pg/ml, 0.094, and 0.234 ng/ml,

Table 1

Primers used for quantitative real-time PCR.

mRNA	Forward	Reverse
NF-κB	AGAGAAGCACAGATACCACTAAG	CAGCCTCATAGAAGCCATCC
NLRP3	TCCTGGTGACTTTGTATATGCGT	TTCTCGGGCGGGTAATCTTC
ASC	CAGCAACACTCCGGTCAG	AGCTGGCTTTTCGTATATTGTG
Caspase-1	CTTGCCCTCAAGAGGTAACC	AATGCCCAAGACGTGGATCC
Caspase-8	TACTACCGAACTTGGACC	GTGAAAGTAGGTTGTGGC
Caspase-11	AGGAGCCCACTCTACAGAG	AAGGTTGCCCGATCAATGGT
GAPDH	CCCT CC GTGT TCCTA CCCC	GCCCAAGATGCCCTTCAGT

respectively. Moreover, the ear tissue' GSDMD was evaluated using the manufacturer's instructions for the sELISA kits, purchased from Antibodies-Online Inc., PA, USA (Cat. #. ABIN6956056), where the minimum detection limit was 0.063 ng/ml. A Stat Fax 2100 automated plate reader, Fisher Bioblock Scientific, BP., Illkirch Cedex, France, was used to detect the caspases and GSDMD levels expressed as pg/mg and ng/mg tissue protein, respectively. However, the ear tissue's LDH activity was evaluated using a colorimetric assay kit purchased from Bio-Diagnostic Co., Dokki, Giza, Egypt, according to the manufacturer's protocol, using the method described by Pesce (1987). The absorbance of the samples was measured at a one-minute interval for 3 min using a Biosystems semiautomated analyzer (BTS-350, Barcelona, Spain) at 340nm. The ear tissue's LDH activity is expressed as U/mg tissue protein.

2.12. Histopathological evaluation

The ear tissues were processed as Suvarna et al. (2018) described. Briefly, specimens were immersed in 10% formalin-buffered saline, embedded in paraffin, cut into 5-µm sections, and stained with hematoxylin and eosin (H&E) and Masson trichrome stains for further evaluation of the histopathological changes and the collagen fibers deposition [23]. Sections were analyzed by a blinded pathologist using a light microscope (Leica Universal Microscope, Wetzlar, Germany). Histopathological changes of ten randomly selected, non-overlapping H&E sections per slide, arbitrary scored for epidermal hyperplasia, hyperkeratosis, congested blood vessels, and inflammatory cell infiltration, using a 0–3 scale, where 0 = none, 1 = mild, 2 = moderate, and 3 = severe changes. Furthermore, five randomly selected, non-overlapping Masson's trichrome sections per slide were analyzed using Leica Q500 MCO Image Analyzer (Leica, Wetzlar, Germany) to quantify collagen fiber deposition.

2.13. Statistical analysis

The present study's data were assessed using the Statistical Package for the Social Sciences Statistics Software for Windows (IBM-SPSS, version 25, Armonk, New York, USA), and the results were expressed as mean ± SD. The control and CD groups, the CD and CMC groups, and the PD and EAH groups were assessed with the F-test, then compared with either the two-sample Student's *t*-test or the Mann-Whitney's *U* test. However, the CD, EAL, EAM, and EAH groups were assessed with the Bartlett test and then compared with either one-way ANOVA (followed by Tukey's test as a post hoc test) or Kruskal-Wallis' test (followed by Mann-Whitney's *U* test as a post hoc test). *P*-value <0.05 considered significant.

3. Results

3.1. Epimedin A alleviates DNFB-induced mice's scratching behavior and dermatitis score

To understand the therapeutic potential of epimedin A in the context of CD, we administered sequentially increasing doses of epimedin A to a

mouse model of DNFB-induced CD. We assessed the disease severity by measuring animal scratching behavior and dermatitis scores. The untreated CD group had significantly higher scratching episodes and dermatitis scores than the CON group. Additionally, the groups of animals treated with epimedin A showed a dose-dependent significant reduction of scratching episodes and dermatitis score compared to the untreated CD group from the ninth and seventh days, respectively. Since the EAH group showed a more significant reduction in scratching episodes and dermatitis score than the EAL and EAM groups. Interestingly, the EAH group showed a nonsignificant difference in scratching episodes and dermatitis score compared to the PD group. Overall, these results show that epimedin A treatments have an alleviating potential on the allergic contact dermatitis' clinical symptoms (Fig. 1).

3.2. Epimedin A relieves the increased mice's ear thicknesses and weights in DNFB-induced CD

Consistent with the alleviation of the mice's scratching behavior and dermatitis score with epimedin A treatment, epimedin A significantly

reduced the increased mice's ear thicknesses from the eleventh day and ear weights compared to the CD group in a dose-dependent manner, as the EAH group showed a more significant reduction of the mice's ear thicknesses and weights compared to the EAM and EAL groups. Further, there were no significant differences between the EAH and PD groups regarding the thicknesses and weights of the mice's ears. These results imply that epimedin A treatment mitigates the development of inflammatory ear swelling in CD induced by DNFB (Fig. 1).

3.3. Epimedin A inhibits oxidative stress and activates the Nrf2/HO-1 cascade of the mice's ear tissues

The repeated DNFB application triggered an augmented ear tissue oxidative stress state, as revealed by the increased MDA level and the decreased GSH level and SOD activity compared to the CON group. In the same vein, the Nrf2/HO-1 cascade was suppressed, as evinced by reducing the Nrf2, HO-1 levels, and Nrf2 binding activity. Administration of epimedin A boosted the antioxidant system of the ear tissues, as proved by the decrease in MDA level along with the enhancement in

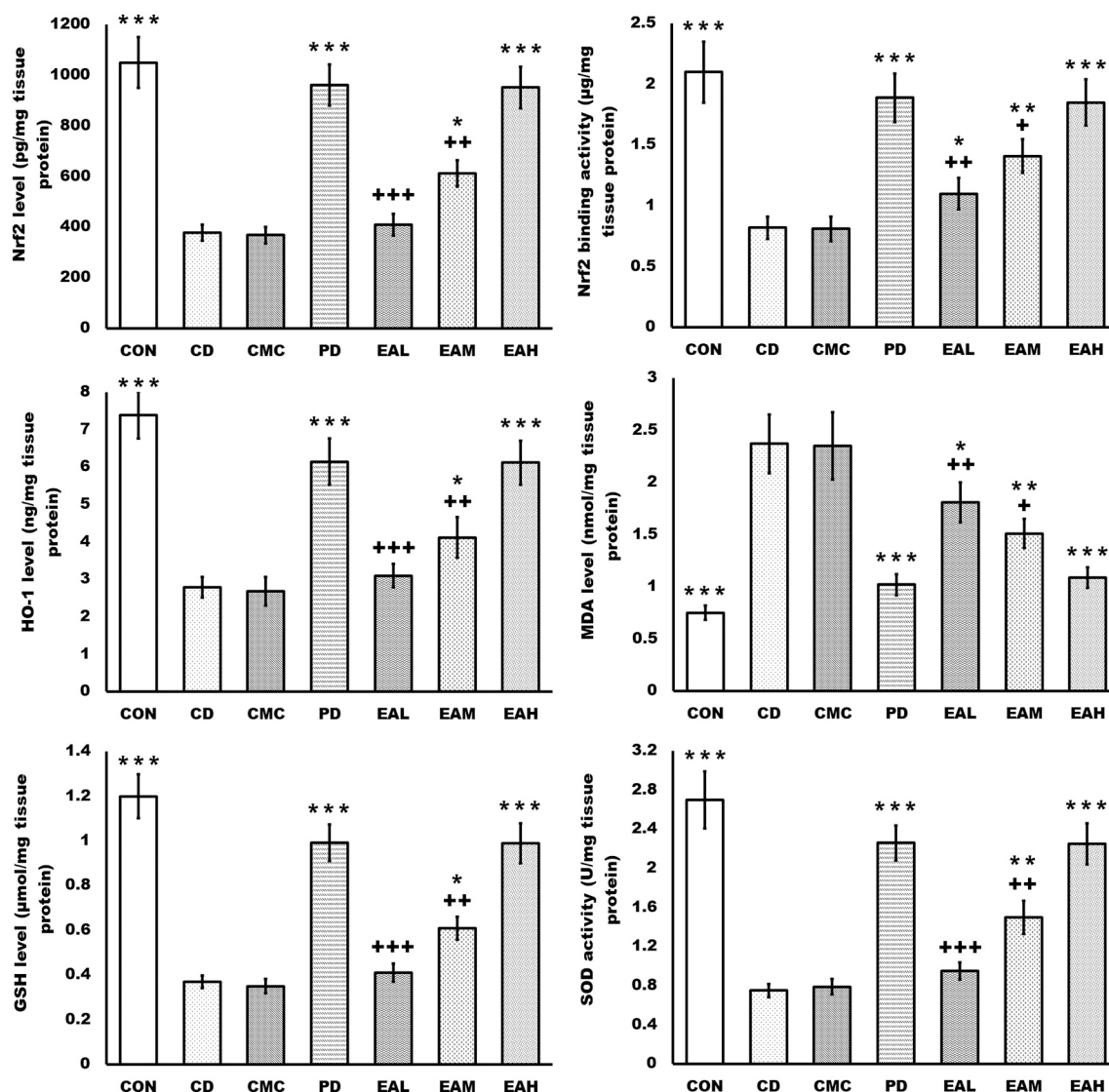


Fig. 2. Epimedin A inhibits the oxidative stress and activates the Nrf2/HO-1 cascade of the mice' ear tissues. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$, ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

GSH, Nrf2, and HO-1 levels, SOD activity, and Nrf2 binding activity compared to the CD group in a dose-dependent manner since the EAH group showed a more effective restoration of the ear tissues' antioxidant status than the EAL and EAM groups. Furthermore, there were no significant differences between the EAH group and the PD group regarding MDA, GSH, Nrf2, HO-1 levels, SOD activity, and Nrf2 binding activity (Fig. 2).

3.4. Epimedin A diminishes serum IgE level and ear tissue's inflammatory signals

Parallel to the intensification of clinical symptoms of allergic contact dermatitis with the repeated DNFB challenge, the serum t-IgE, DNFB s-IgE levels, the s-IgE/t-IgE ratio, the local ear tissues' cytokines (IL-1 β , IL-6, TNF- α , IL-4, and IFN- γ), NF- κ Bp65 levels, and NF- κ Bp65 binding activity significantly increased, the I κ B- α level meanwhile significantly decreased compared to the CON group. On the contrary, treatment with Epimedin A significantly reduced the increase in serum t-IgE, DNFB s-IgE levels, the s-IgE/t-IgE ratio, the ear tissues IL-1 β , IL-6, TNF- α , IL-4,

IFN- γ , NF- κ Bp65 levels, and NF- κ Bp65 binding activity, and significantly increased the reduced I κ B- α level compared to the CD group in a dose-dependent manner. The EAH group had more efficient suppression of the serum IgE and the local ear tissue inflammatory signals than the EAL and EAM groups. Amazingly, the anti-inflammatory potency presented by epimedin A in the EAH group was quietly like that of the reference drug, prednisone; however, the EAH group showed a more significant reduction of the serum DNFB s-IgE level and s-IgE/t-IgE ratio than the PD group. Furthermore, the PD group showed a nonsignificant difference from the CD group regarding the s-IgE/t-IgE ratio (Figs. 3 & 4).

3.5. Epimedin A treatment downregulates NF- κ B, caspase-8 mRNA expression and suppresses the activation of the NLRP3 inflammasome in DNFB-induced CD

The NLRP3/ASC/caspase-1 cascade is involved in the pathogenesis of allergic contact dermatitis by forming the NLRP3 inflammasome, which is mediated by the activation of the NF- κ B pathway. Furthermore,

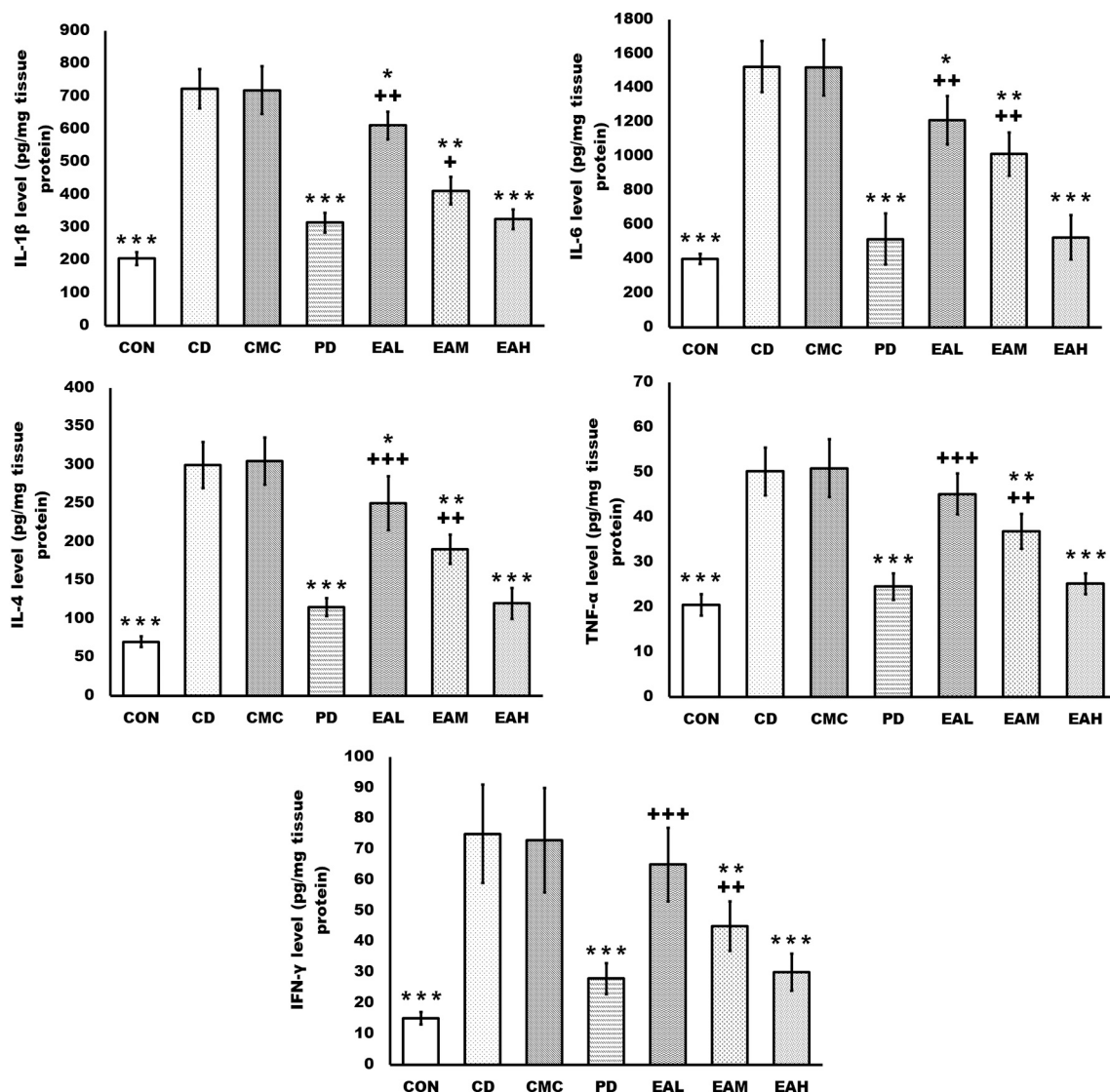


Fig. 3. Epimedin A diminishes ear tissues' cytokine levels. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$, ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

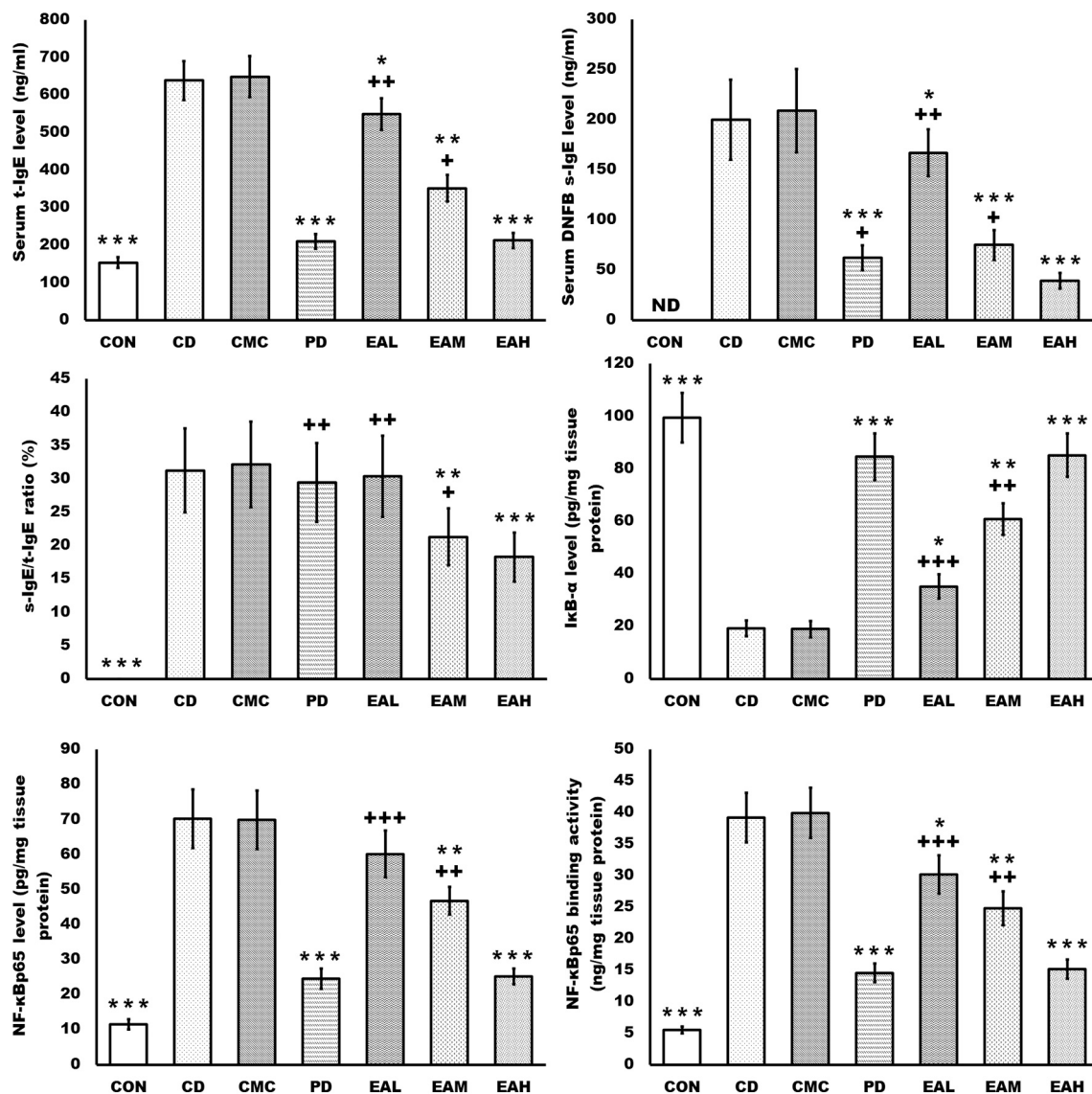


Fig. 4. Epimedin A diminishes serum IgE levels and ear tissues' NF-κB signals. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. ND, not detected, * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$, ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

the expression of caspase-8 and 11 is associated with pyroptosis induction. Therefore, the mRNA expressions of NF-κB, NLRP3, ASC, caspase-1, 8, and 11 were examined. Repeated DNFB application activates the NF-κB, caspase-8 pathways, and the NLRP3/ASC/caspase-1 cascade, as shown by the significant elevation of the relative mRNA expressions of NF-κB, caspase-8, NLRP3, ASC, and caspase-1 compared to the CON group; however, the caspase-11 mRNA was not significantly expressed. Furthermore, epimedin A treatment significantly reduced elevated mRNA expressions of NF-κB, caspase-8, NLRP3, ASC, and caspase-1 compared to the CD group in a dose-dependent manner since the EAH group had a more efficient reduction in elevated expressions of NF-κB, caspase-8, NLRP3, ASC, and caspase-1 mRNA than the EAL and EAM groups. Furthermore, treatment with epimedin A and prednisone had a similar potential to reduce the DNFB-induced elevation in mRNA expression of NF-κB, NLRP3, ASC, and caspase-1, as there were no significant differences between the EAH and PD groups in terms of the mRNA expression of NF-κB, NLRP3, ASC, and caspase-1. However, there was a significant reduction in caspase-8 mRNA expression in the EAH

group more than in the PD group (Fig. 5).

3.6. Epimedin A lessens the ear tissues' caspase-1, 8, GSDMD levels, and LDH activity

In line with the boost of the IL-1 β level, caspase-one and eight mRNA expression with the repeated DNFB challenge, the local ear tissues' caspase-1, 8, GSDMD levels, and LDH activity significantly increased compared to the CON group; however, caspase-11 level showed no significant difference from the CON group. On the other hand, treatment with epimedin A significantly reduced the increase in ear tissues' caspase-1, 8, GSDMD levels, and LDH activity compared to the CD group in a dose-dependent manner since the EAH group had a more efficient suppression of the ear tissues' caspase-1, 8, GSDMD levels and LDH activity than the EAL and EAM groups. Remarkably, the suppression potency presented by epimedin A in the EAH group was like that of prednisone. However, the EAH group showed a more significant caspase-8 level reduction than the PD group (Fig. 6).

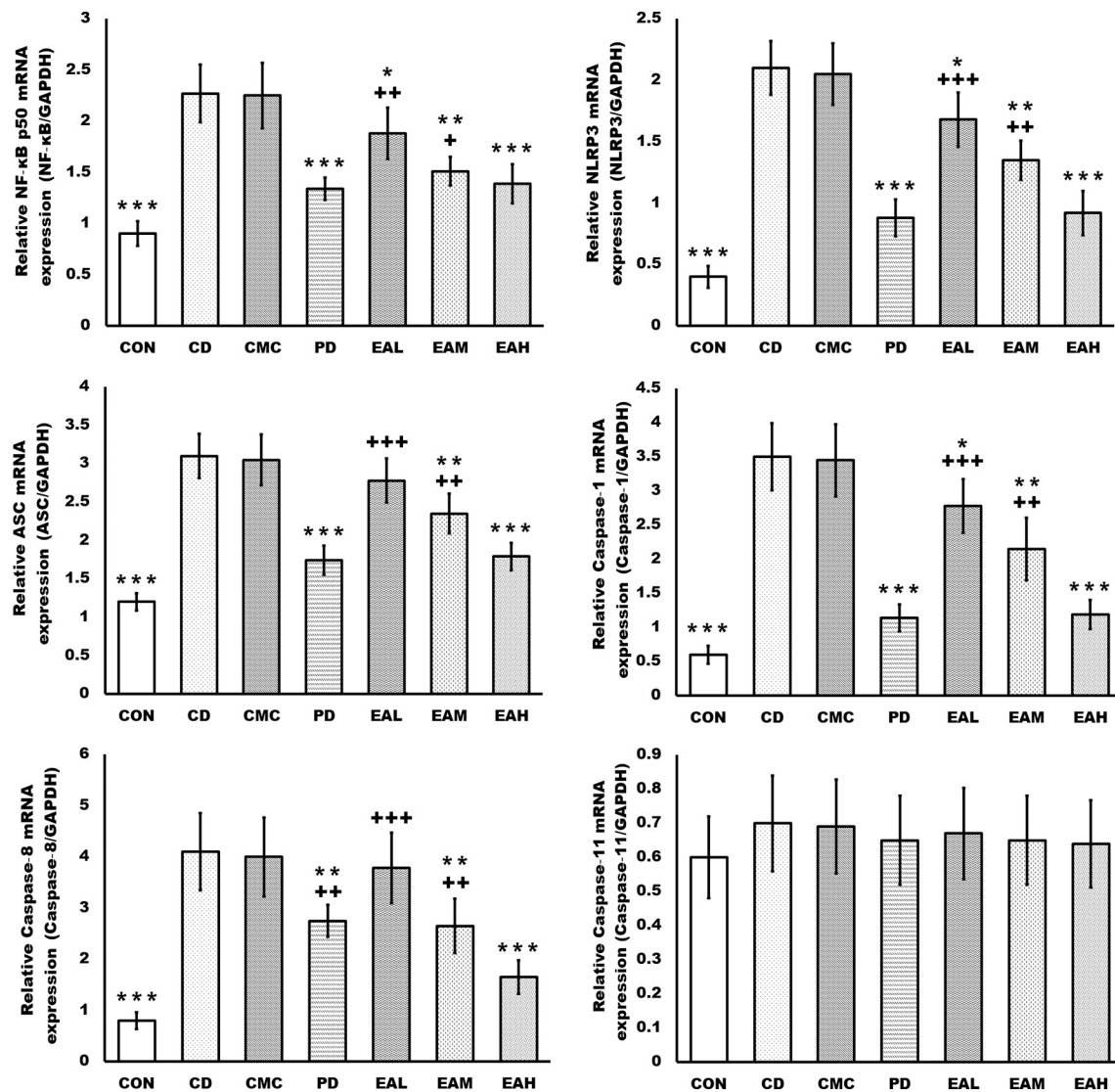


Fig. 5. Epimedinin A treatment downregulates NF-κB and caspase-8 mRNA expressions, and suppresses the activation of the NLRP3 inflammasome in DNFB-induced CD. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedinin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedinin A 5 mg/kg/day orally, EAM, CD group treated by epimedinin A 10 mg/kg/day orally, EAH, CD group treated by epimedinin A 20 mg/kg/day orally. * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$, ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

3.7. Epimedinin A demotes the ear tissue's histopathologic changes, collagen deposition, the pathological score, and the collagen fibers' area percent in DNFB-induced CD

Ear tissue's skin in the CON group stained with H&E was normal intact skin with thin epidermis, dermis, and hair follicles. However, the skin of the CD group showed epidermal hyperplasia, hyperkeratosis, dermal congested blood vessels, and inflammatory cell infiltration; meanwhile, some sections showed ulceration and separation of the epidermis. Epimedinin A treatment demotes these changes dose-dependently, to be like the CON group with the EAH group. Statistically, there was a significant increase in the pathological score of the CD group compared to the CON group. However, epimedinin A treatment significantly reduced these changes compared to the CD group in a dose-dependent manner, as the EAH group had a more efficient reduction in the elevated pathological score than the EAL and EAM groups. Moreover, there was no significant difference between EAH and PD groups regarding the pathological score (Figs. 7 & 9).

Masson trichrome-stained section of the CON group showed intact,

thin, and regular collagen fibers. However, the CD group had thick, dense, disorganized, and more abundant collagen fibers than the CON group. On the other hand, the collagen fibers of epimedinin A treated groups were dose-dependently less, thinner, and more regularly arranged. As a result, the area percent of Masson trichrome-stained collagen fibers increased significantly in the CD group compared to the CON group. Conversely, treatment with epimedinin A dose-dependently and significantly reduced the area percent of Masson trichrome-stained collagen fibers, as the EAH group showed a more efficient reduction than the EAM and EAL groups. Additionally, there were no significant differences between the EAH and PD groups regarding the area percent of Masson trichrome-stained collagen fibers (Figs. 8 & 9).

4. Discussion

Allergic contact dermatitis (CD) is a common multifactorial, pruritic, inflammatory skin disease with complex etiology [1]. However, therapeutic agents available for its treatment provide temporary relief of

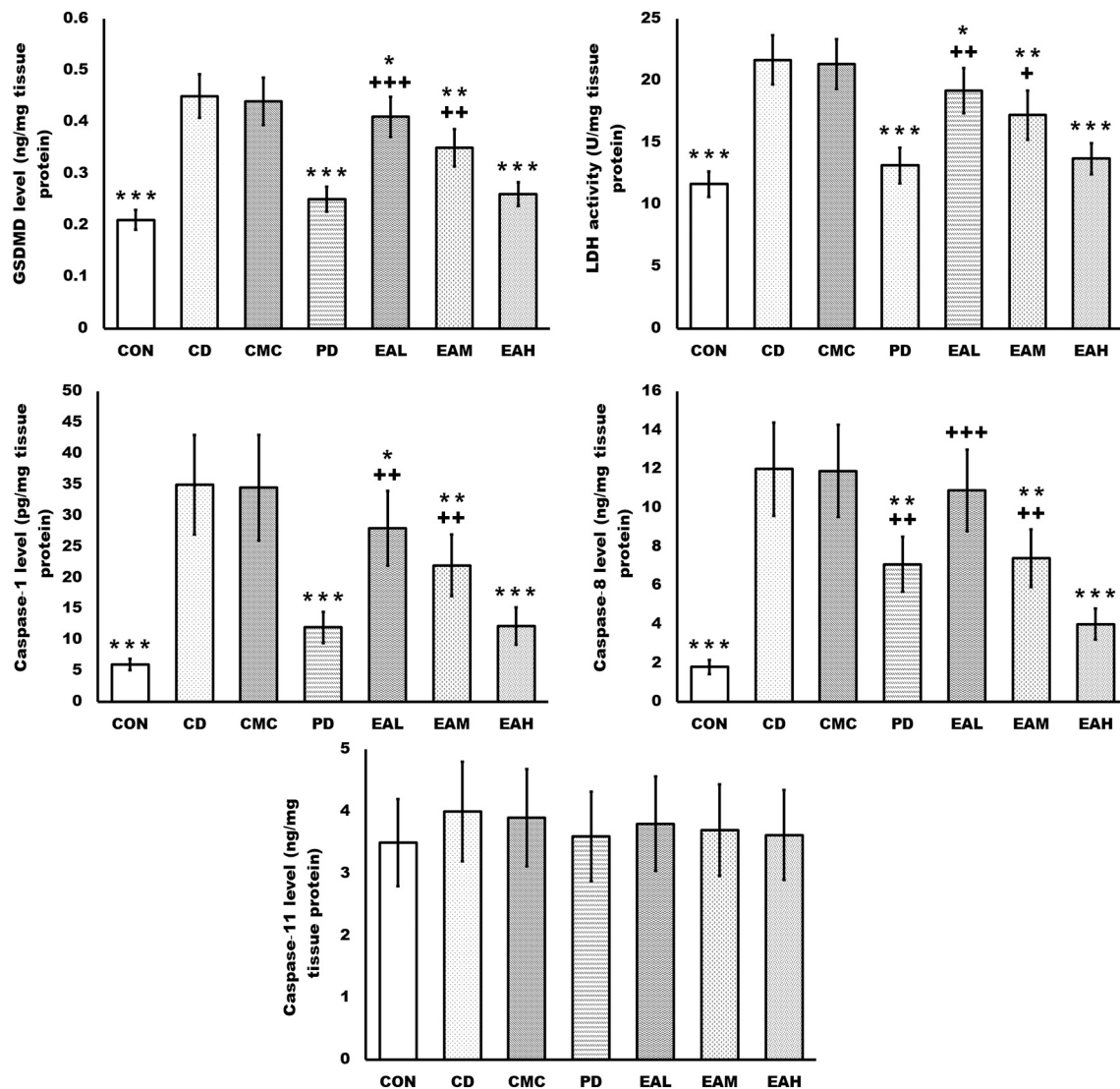


Fig. 6. Epimedin A lessens the ear tissues' caspases, GSDMD levels, and LDH activity. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), + $P < 0.05$, ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

symptoms, are associated with many side effects over long-term therapy, and have insufficient effect over time [3]. Therefore, finding potential therapeutic targets or effective agents against CD remains urgent.

In the present study, we investigated the potential of epimedin A to treat DNFB-induced CD, emphasizing its potential modulating activities on NF- κ B/NLRP3 and Nrf2/HO-1 signaling pathways. The present study showed that epimedin A significantly suppressed NLRP3 inflammasome and pyroptosis in ear tissues by blocking the NF- κ B signal pathway, as revealed by the reduction of the elevated NF- κ B, NLRP3, ASC, caspase-1, eight mRNA expression, NF- κ Bp65, IL-1 β , caspase-1, 8, GSDMD, LDH levels and NF- κ Bp65 binding activity, and the elevation of the reduced level of I κ B- α . Furthermore, conveyed with the repression of the NLRP3 inflammasome and pyroptosis, epimedin A efficiently reduced the clinical symptoms of CD and the histopathological changes in ear tissues, as evidenced by the alleviation of the DNFB-induced increase in the mice's scratching behavior, dermatitis score, mice's ear thicknesses, and weights, serum t-IgE, DNFB s-IgE levels, s-IgE/t-IgE ratio, ear tissues' histopathological score, and area percent of collagen fibers. Moreover, epimedin A restored the normal antioxidant system of the ear tissues,

disturbed by repeated DNFB applications, as it enhanced the Nrf2/HO-1 cascade with a reduction in the MDA level and an elevation in the GSH level and SOD activity. Additionally, epimedin A repressed the local inflammatory reaction of the ear tissue, as evidenced by the reduction of the elevated ear tissues' inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-4, and IFN- γ) and abrogation of the histopathological changes of ear tissue. Therefore, epimedin A could be a novel and hopeful therapeutic agent for CD.

Skin is the immune system's first line that defends against the invasion of microorganisms and allergens into the human body. Terminally differentiated keratinocytes are functional reside in this barrier [24]. The disruption of this barrier by the application of superantigens such as DNFB leads to dysregulation of the immune system with allergic sensitization, IgE secretion and infiltration of inflammatory cells, secretion of pro-inflammatory cytokines, alteration of the antioxidant system, and enormous inflammatory reactions with subsequent induction of CD; however, the precise pathogenesis of CD remains unclear [11,17]. Inflammasomes have recently been identified as critical players in the development of CD, among which the most prominent signaling

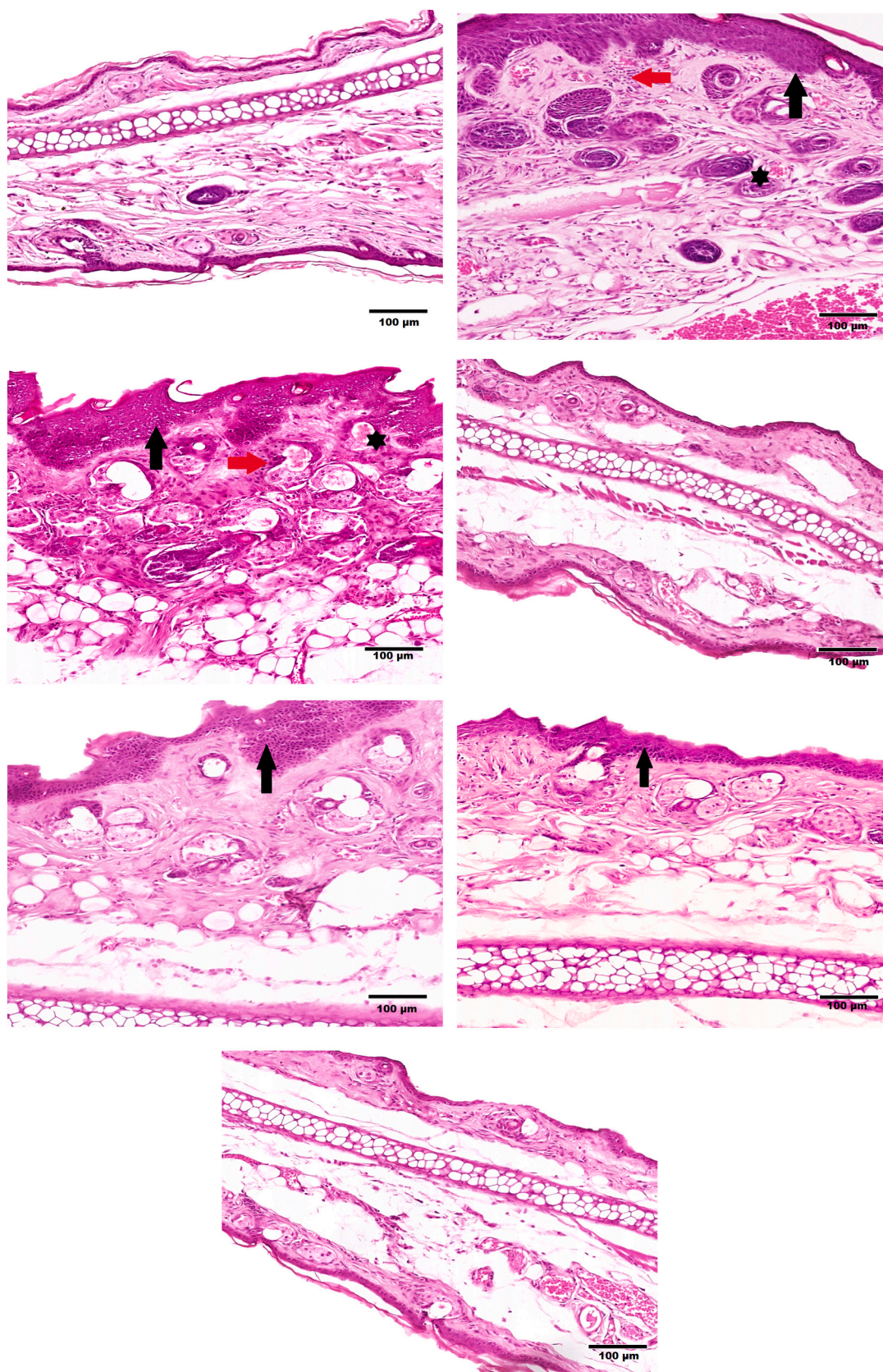


Fig. 7. Epimedin A demotes the ear tissue's histopathologic changes in DNFB-induced CD. A, control group, B, DNFB-induced atopic contact dermatitis (CD) group, C, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, D, CD group treated by prednisone 5 mg/kg/day orally, E, CD group treated by epimedin A 5 mg/kg/day orally, F, CD group treated by epimedin A 10 mg/kg/day orally, G, CD group treated by epimedin A 20 mg/kg/day orally, black arrow, epidermal hyperplasia and hyperkeratosis, red arrow, cellular infiltration, and black star congested blood vessel (H & E, X100). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

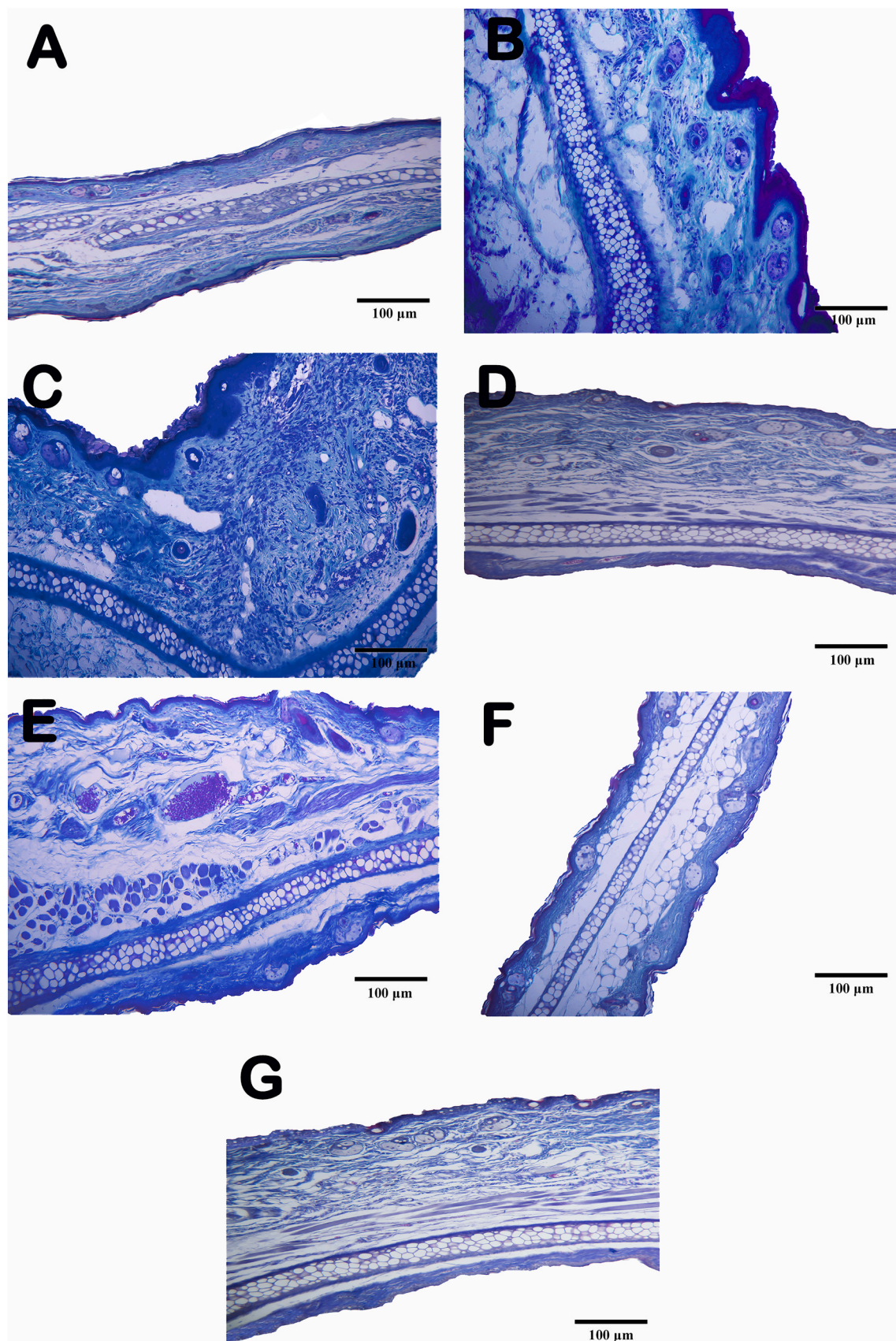


Fig. 8. Epimedin A reduces the ear tissue's collagen deposition in DNFB-induced CD. A, control group, B, DNFB-induced atopic contact dermatitis (CD) group, C, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, D, CD group treated by prednisone 5 mg/kg/day orally, E, CD group treated by epimedin A 5 mg/kg/day orally, F, CD group treated by epimedin A 10 mg/kg/day orally, G, CD group treated by epimedin A 20 mg/kg/day orally (Masson trichrome, X100).

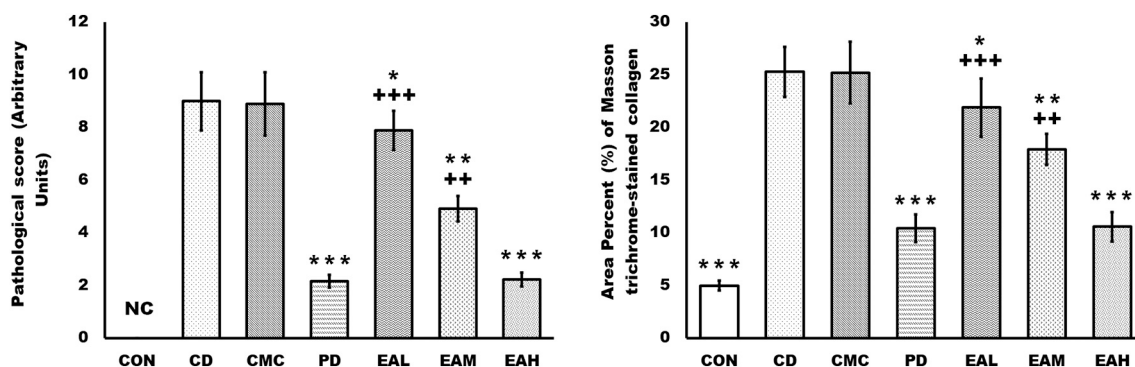


Fig. 9. Epimedin A demotes the ear tissue's pathological score and collagen fiber area percent in DNFB-induced CD. The result was expressed as mean $\pm$ SD of 10 mice per group. CON, control group, CD, DNFB-induced atopic contact dermatitis (CD) group, CMC, CD group treated orally by carboxymethylcellulose in the corresponding amount to epimedin A, PD, CD group treated by prednisone 5 mg/kg/day orally, EAL, CD group treated by epimedin A 5 mg/kg/day orally, EAM, CD group treated by epimedin A 10 mg/kg/day orally, EAH, CD group treated by epimedin A 20 mg/kg/day orally. NC, no change, * $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$ (Vs. CD group), ++ $P < 0.01$ & +++ $P < 0.001$ (Vs. EAH group).

pathway is the NLRP3 inflammasome [20]. NLRP3 activation leads to the assembly of NLRP3, ASC, and caspase-1, forming NLRP3 inflammasome, which enhances the release and maturation of the pro-inflammatory cytokines such as IL-1 β , with the recruitment of inflammatory cells, initiation, and augmentation of CD [25,26]. Epimedin A in the present study repressed the NLRP3 inflammasome pathway by suppressing NLRP3, ASC, caspase-1 mRNA expression, and caspase-1 level, thereby reducing IL-1 β secretion and inflammatory cell infiltration.

In addition, the activation of NLRP3 leads to cleavage of caspase-1 and secretion of IL-1 β , thereby initiating pyroptosis, an inflammatory lytic programmed cell death. GSDMD is the crucial mediator of pyroptosis; once cleaved by the activated caspase-1 and IL-1 β , it induces the plasma membrane pore formation, with induction of pyroptotic cell death [27,28]. Moreover, the GSDMD-dependent pyroptosis promotes IL-1 β and IL-18 release by unconventional secretion, leading to further escalation of CD inflammation [6]. Epimedin A in the present study suppressed pyroptotic cell death induced by repeated DNFB application by inhibiting GSDMD upregulation, and hence it reduced the LDH activity of ear tissues, the cell death indicator.

Furthermore, pyroptosis is induced by inflammatory caspases, such as caspase-1, 8, and 11 in mouse cells, which induce cell death through proteolytic processing of GSDMD and produce an N-terminal fragment that opens pores in the plasma membrane [29–31]. Caspase-1 is activated by canonical inflammasome assemblies such as NLRP3, and the extrinsic apoptosis signaling pathway and TNF- α activate caspase-8; however, caspase-11 is activated by a non-canonical inflammasome cascade that triggers by intracellular lipopolysaccharide [29–32]. Moreover, caspase-8 enhances NF- κ B/NLRP3 signaling pathways, with subsequent further induction of pyroptosis [33]. In the present study, the repeated DNFB challenge enhanced caspase-one and eight mRNA expressions and protein levels, but not caspase-11. Epimedin A treatment dose-dependently repressed the enhanced caspase-one and eight mRNA expressions, protein levels, and GSDMD levels, suggesting that the potential of epimedin A to suppress pyroptosis stimulated by the canonical inflammasome and the extrinsic apoptosis signaling pathway, mediated by repeated challenge with DNFB.

Furthermore, we evaluated the potential of epimedin A to alleviate clinical symptoms of DNFB-stimulated CD-like skin lesions. Epimedin A administration significantly lessened the CD clinical symptoms of mice by reducing t-IgE, DNFB s-IgE, IL-1 β , IL-6, TNF- α production, and s-IgE/t-IgE ratio. It also abrogated the histopathological changes in CD responses, indicating the therapeutic ability of epimedin A on CD. In addition, epimedin A suppressed NLRP3 inflammasome and pyroptosis, as evidenced by the reduction in NLRP3, ASC, caspase-one, eight mRNA expression, caspase-1, 8, GSDMD, and IL-1 β levels, through modulation

of the NF- κ B signaling pathway in a mouse model of DNFB-induced CD. The available evidence suggests that *Herba Epimedii*, in which epimedin A is one of its major bioactive compounds, treats the other disease models by suppressing NLRP3 inflammasome signaling pathways and pyroptosis through modulation of the NF- κ B signaling pathway, reflecting our results support [14–16]. Moreover, epimedin A treatment showed a more significant reduction of s-IgE, caspase-8 levels, caspase-8 mRNA expression, and s-IgE/t-IgE ratio than prednisone treatment, indicating a more powerful potential of epimedin A in suppression of allergic reaction and extrinsic pyroptosis than prednisone in CD induced by DNFB.

In addition, oxidative stress is an essential factor in developing CD [34]. Here, epimedin A effectively restored the normal antioxidant system of the ear tissues in the DNFB model of CD. Besides, oxidative stress has been stated to be necessary and sufficient to activate the NF- κ B signal pathway [35]. Activation of the NF- κ B signal pathway is likewise a crucial pathway for initiating the transcription of NLRP3 [28]. Herein, epimedin A reduced the mRNA expression of NF- κ B, NF- κ Bp65 levels, and NF- κ B binding activity and increased the I κ B- α level; this is believed to be in part due to the antioxidant activity of epimedin A through its enhancement of the Nrf2/HO-1 signal pathway with the reduction of the lipid peroxidation product, MDA, and the replenishment of the antioxidant agents, GSH and SOD.

Furthermore, the Nrf2 pathway is essential in maintaining the epidermal barrier function due to its protective potential against oxidative damage [36]. Since Nrf2 knockout mice are more likely to develop CD than normal mice, confirming the belief that the presence of Nrf2 in keratinocytes constraints inflammation [37]. Moreover, activation of Nrf2 and the upregulation of its downstream antioxidant HO-1 improves the clinical symptoms in patients with CD [38]. Also, activation of the Nrf2 signal pathway can attenuate the expression of IL-1 β , IL-6, TNF- α , IL-4, and IFN- γ in CD, which decreases the cutaneous inflammatory reactions, and prevents their progression [39]. Hence, Nrf2 signal pathway activation has the power to reduce epidermal inflammation in CD [40]. Therefore, it is concluded that epimedin A exerted a therapeutic potential in DNFB-induced CD by enhancing the Nrf2/HO-1 pathway.

Additionally, the CD is due to the response of T-cells to highly reactive, safe, low-molecular-weight agents called haptens, which upon contact with the skin, bind to a variety of proteins, peptides, and structures, and become highly antigenic [41]. The binding of haptens to epidermal Langerhans cells provokes intracellular stress, releasing damage-associated molecular patterns, such as reactive oxygen species. Thus, it activates the epidermal Langerhans cells through NF- κ B/NLRP3 signaling pathways, with subsequent up-regulation of IL-1 β mRNA expression, protein production, and secretion [42–47]. In addition, the

release of IL-1 β from epidermal Langerhans cells activates other epidermal cells such as keratinocytes to secrete other pro-inflammatory cytokines such as IL-6 and TNF- α . The released IL-1 β , together with IL-6 and TNF- α , boosts the maturation of epidermal Langerhans cells, with further release of the pro-inflammatory cytokines and transformation of epidermal Langerhans cells from antigen-processing cells into antigen-presenting cells, resulting in the onset of CD inflammatory reactions [45,47–49].

Furthermore, the augmented pro-inflammatory cytokine secretion stimulates differentiation and maturation of Th2 cells that secrete a massive amount of Th2 inflammatory cytokine such as IL-4, thus, inducing CD within twenty-four hrs of allergen exposure. During this process, Langerhans epidermal cells produce chemoattractants that induce the recruitment of monocytes. As the disease progresses, recruited monocytes release IL-6 and TNF- α , which promote a switch from the initial Th2 response to a Th1 response with the secretion of a vast amount of Th1 cytokines such as IFN- γ [6]. Therefore, Th1, Th2, and keratinocyte activation are essential for CD development, progression, and maintenance; therefore, an ideal therapeutic approach to CD may be a treatment that suppresses T cells and keratinocyte activation [46,50]. Moreover, TNF- α promotes the caspase-8 pyroptosis pathway [32]. In the present study, the inflammatory pro-inflammatory cytokines IL-1 β , IL-6, TNF- α , IL-4, and IFN- γ were up-regulated by repeated challenges with DNFB. Treatment with epimedin A significantly reduced DNFB-induced increases in the inflammatory pro-inflammatory cytokines IL-1 β , IL-6, TNF- α , IL-4, and IFN- γ levels. Consequently, epimedin A believed to exert a therapeutic potential in DNFB-induced CD through its anti-inflammatory potential by suppressing inflammatory and pro-inflammatory cytokines secretion.

5. Conclusions

In conclusion, the present study demonstrated the efficacy of oral administration of epimedin A against DNFB-induced CD, in a dose-dependent manner, by suppressing ear tissue inflammatory responses and cellular pyroptosis and enhancing the ear tissue antioxidant system. Additionally, the potential of epimedin A in CD is partially mediated by inhibiting NF- κ B, NLRP3, and caspase-8 signal pathways and potentiating the Nrf2/HO-1 signal pathway and suppressing inflammatory cytokine production. Therefore, epimedin A is considered a natural, novel, and hopeful therapeutic agent for CD treatment. However, more studies are still essential to outline the prices of molecular and cellular mechanisms underlying the beneficial effects of epimedin A in CD.

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CRedit authorship contribution statement

Mohamed F. Balaha: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Nehad J. Ahmed:** Methodology, Investigation, Formal analysis, Writing – original draft. **Ziyad S. Almalki:** Methodology, Investigation, Formal analysis, Writing – original draft. **Abdullah K. Alahmari:** Methodology, Investigation, Formal analysis, Writing – original draft. **Ahmed M. Alshehri:** Methodology, Investigation, Formal analysis, Writing – original draft. **Gamal A. Soliman:** Methodology, Investigation, Formal analysis, Writing – original draft. **Abubaker M. Hamad:** Methodology, Investigation, Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

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