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Enzyme-treated date plum leave extract ameliorates atopic dermatitis-like skin lesion in hairless mice

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ABSTRACT

Objective: To evaluate the effect of different extracts of *Diospyros lotus* leaves in atopic dermatitis

Methods: *Diospyros lotus* leaves were extracted in ethanol and treated with or without hydrochloric acid or α-rhamnosidase to obtain three different extracts-ethanol, acid-hydrolyzed, and enzyme-hydrolyzed leaf extracts of date plum. The myricitrin content in all samples was measured using HPLC analysis. *In vitro* antioxidant and anti-inflammatory activities of the extracts were determined by measuring DPPH radical scavenging activities and nitric oxide production in RAW264.7 cells, respectively. Seven-week-old male hairless mice were used to evaluate the anti-atopic dermatitis effects of three extracts *in vivo*. Splenocytes and mast cells were used to further determine the anti-atopic dermatitis effects of the major compound in the ethanol leaf extract.

Results: Enzyme-hydrolyzed leaf extract showed significant *in vitro* antioxidant and anti-inflammatory activities, and attenuated atopic dermatitis-like skin symptoms and clinical signs more significantly than ethanol and acid-hydrolyzed leaf extracts in 1-fluoro-2,4-dinitrobenzene and house dust mite antigen-treated hairless mice. Enzyme-hydrolyzed leaf extract also suppressed the serum level of immunoglobulin E, interleukin (IL)-4, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, thymic stromal lymphopoietin, and thymus and activation-regulated chemokine in mice with atopic dermatitis. Furthermore, histological analysis revealed that enzyme-hydrolyzed leaf extract suppressed the increased epidermal thickness, dermal infiltration of inflammatory cells, and infiltration and degranulation of mast cells more markedly than the other two extracts in atopic dermatitis-like skin lesions. In addition, this extract effectively inhibited the production of IFN-γ, IL-4, and thymus and activation-regulated chemokine compared with the other two extracts in concanavalin A-stimulated splenocytes. Myricitrin, a major compound of enzyme-hydrolyzed leaf extract, suppressed atopic dermatitis biomarkers in stimulated mouse splenocytes and HMC-1 human mast cells.

Conclusions: These results suggest that enzyme-hydrolyzed leaf extract might be a potential candidate to treat atopic dermatitis.

KEYWORDS: Date plum leaves; Enzyme; Atopic dermatitis; Inflammation; Cytokine

1. Introduction

Atopic dermatitis is a chronic disease that affects the health and quality of life in many children and some adults in developed countries. Reports show that the spread of atopic dermatitis has increased by a factor of two or three[1]. This has led to an increasing interest in the discovery of new drugs or remedies for atopic dermatitis. Typical symptoms of atopic dermatitis are intense itch, rash, swelling, thickened skin, dry skin, blisters, and erythema. These symptoms are common in flexion areas such as neck folds, elbow creases, and back of the knee[2]. Another outcome of atopic dermatitis, which decreases the quality of life of atopic dermatitis patients is itch, which especially at night interferes with sleep, and the unconscious scratching during sleep worsens atopic dermatitis symptoms on the skin[3]. In addition, allergens exposure through the damaged epidermis in atopic dermatitis can cause a systemic

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allergic reaction that can lead to asthma, rhinitis, and food allergy in a phenomenon called atopic march[4]. The pathophysiology of atopic dermatitis involves an imbalance between Th1 and Th2 immune response. The overexpression of interleukin (IL)-4 drives the differentiation of T cells towards the Th2 cell lineage, thus generating the Th1 and Th2 imbalance in atopic dermatitis. The Th2 response then results in an increase in the production of IL-4, IL-5, IL-13, and IL-31 that promotes atopic dermatitis symptoms[5]. Besides, the Th2 response, other cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, thymus and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP) have been described in the pathophysiology of atopic dermatitis[6–8]. Therefore, modulating the expression of these cytokines in atopic dermatitis will be necessary for the amelioration of the disease.

Date plum, also known as *Diospyros lotus* (*D. lotus*) L. is a deciduous plant distributed in Asia, including Korea and China, where the matured fruits are eaten. Date plum has been used in traditional medicine as a sedative, astringent, anti-tumor, anti-diabetic, antipyretic and febrifuge and a laxative[9]. Recently, fatty acids, sugars, flavonoids, and nonvolatile components of *D. lotus* have been reported to have brain cell protection, anticoagulant, antioxidant, and anticancer effects[10,11]. Besides, recent studies have reported anti-atopic dermatitis, anti-allergic and anti-inflammatory effects of date plum leave extract[12,13]. In the current study, using hairless mice, *D. lotus* extracts obtained *via* different methods were used to determine their effects on atopic dermatitis.

2. Materials and methods

2.1. Chemicals

Myricitrin and house dust mite antigen were purchased from ChemFaces (Wuhan, China) and Biostir (Hyogo, Japan), respectively. DPPH, lipopolysaccharide (LPS), Greiss reagent, concanavalin A (ConA), 1-fluoro-2,4-dinitrobenzene (DNFB), prednisolone, hematoxylin, eosin, toluidine blue, and ImmunoHistoMount™ were purchased from Sigma-Aldrich (St, Louis, MO, USA). IL-4, IgE, TNF-α, IFN-γ, TSLP, IL-6, and TARC enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). The Histamine ELISA kit was purchased from Biolegend (San Diego, CA, USA). ImmPRESS™ HRP and 3-amino-9-ethylcarbazole (AEC) peroxidase substrates were purchased from Vector laboratory (Burlingame, CA, USA). The tryptase antibody was purchased from Abcam (Cambridge, UK). All other chemicals used in the present study were of reagent grade.

2.2. Plant materials and extract preparation

Date plum (*D. lotus* L.) leaves were collected on 22 June 2018 from Cheonjam Mountain, Jeonju-si, Jeonbuk, Republic of Korea. The plant was identified and authenticated by Prof. Hong-Jun Kim (College of Oriental Medicine, Woosuk University, Jeonbuk, Republic of Korea). A voucher specimen (#2018-06-22) was deposited in the Department of Health Management, College of Medical Science, Jeonju University. The leaves were washed five times with distilled water and dried at 50℃ for 16 h. After drying, the leaves (100 g) were extracted in 50% (V/V) ethanol (2 L) at room temperature for 24 h. The extracted sample was filtered using 0.45 μm filter paper; concentrated under reduced pressure and lyophilized to obtain the ethanol leaf extract of date plum (DLE) in powder form. To produce acid-hydrolyzed extract of date plum (HDLE), the first ethanol extract was obtained as above. Immediately after ethanol extraction, 1 mol/L HCl was added to the extract until the pH was 2.7. At this pH, the extract was kept in a water bath at 80℃ for 2 h. After 2 h, the extract was cooled at room temperature and 1 mol/L NaOH was added to restore the initial pH of 4.5. The hydrolyzed extract (HDLE) was then filtered using 0.45 μm filter paper; concentrated under reduced pressure and lyophilized to obtain the powder. To produce enzyme-hydrolyzed extract of date plum (EDLE), ethanol extract was obtained as above. The extract was adjusted to the pH of 6.5, α-rhamnosidase (Megazyme, Chicago, IL, USA) was added, and then the extract was kept in a water bath at 80℃ for 24 h. After the reaction, α-rhamnosidase enzyme was inactivated, filtered using 0.45 μm filter paper, concentrated under reduced pressure, lyophilized to obtain the powder and stored at −80℃ for subsequent experiments.

2.3. High performance liquid chromatography (HPLC) analysis

HPLC was performed using a Waters e2695 Alliance HPLC system (Waters, MA, USA). The separation was performed using Xbridge™ C18 column (250 mm×4.6 mm, 5 μm, Waters, Dublin, Ireland) through the gradient elution with acetonitrile (A) and 1% aqueous acetic acid (B): 0 min, 15% A; 10 min, 15% A; 20 min, 40% A; 30 min, 90% A; 35 min, 90% A; 40 min, 15% A; 45 min, 15% A. The mobile phase was retained at a flow rate of 0.8 mL/min and the column oven was set at a temperature of 35℃. Ten microliters of the extracts were injected and UV detection was monitored at 354 nm. The myricitrin was identified based on the retention time of the authentic standard compound.

2.4. DPPH radical scavenging activity assay

The DPPH radical scavenging activity of the extract was analyzed as described previously[14]. In brief, each extract (0.1 mL) was mixed with 0.3 mM DPPH solution (0.1 mL) for 30 min in the dark at room temperature. The absorbance was then measured at 517 nm. The DPPH radical scavenging activity was determined and expressed as a percentage, using the following equation:

DPPH radical scavenging activity (%)= [1 – (sample absorbance / absorbance of blank)] × 100

2.5. Cell culture and nitric oxide (NO) assay

Raw264.7 macrophage cells were cultured using DMEM (HyClone, Logan, UT, USA) at 37℃ with 5% CO_2 in a humidified incubator. The culture media were supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Waltham, MA, USA) and 1% penicillin-streptomycin antibiotics (Invitrogen, Carlsbad, CA, USA). Raw264.7 cells were seeded in 96-well plates at a density of 2×10^5 cells/mL and incubated overnight for 16 h. The cells were then treated with the DLE (100 μg/mL), HDLE (100

μg/mL), EDLE (100 μg/mL), and prednisolone (5 μg/mL) for 1 h. After 1 h, the cells were stimulated with 1 μg/mL of LPS for 16 h. After stimulation, 100 μL of supernatants were mixed with 100 μL of Griess reagent (Sigma) in 96-well plates and incubated at room temperature for 15 min. The absorbance at 540 nm was measured using a spectrophotometer (TECAN, Mannedorf, Switzerland). Diluted $NaNO_2$ standards were used to determine the concentrations of NO.

2.6. Animals and treatments

Seven-week-old male hairless mice used in this study were purchased from the animal facility of Orient Bio Inc. (Gwangju, Republic of Korea). The mice were maintained in plastic cages (with five animals per cage) in an air-conditioned room at a temperature of (22±2)℃, humidity of 50%–60%, and12/12 h constant light/dark cycles. The mice were given a commercial standard diet and normal water *ad libitum*. After a week of acclimatization, the mice were randomly divided into six groups (n=5 per groups) as follows: (1) Normal control group; (2) atopic dermatitis model (DNFB + house dust mite antigen challenged group); (3) DLE group (atopic dermatitis model with 200 mg/kg of DLE); (4) HDLE group (atopic dermatitis model with 200 mg/kg of HDLE); (5) EDLE group (atopic dermatitis model with 200 mg/kg of EDLE); (6) prednisolone group (atopic dermatitis model with 10 mg/kg of prednisolone). The atopic dermatitis model was generated using methods described previously by Cho *et al.*[15]. The normal control and atopic dermatitis model were orally administered with saline while experimental groups were administered with extracts or prednisolone throughout the experimental period. All mice received an equal volume of the extract, saline or prednisolone. The mice were sacrificed on day 16 approximately 5 h after the last treatment.

2.7. Ethical statement

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Jeonju University (Approved No. JJU-IACUC-2018-3).

2.8. Dermatitis scores and skin thickness

The clinical severity scores and skin thickness of the mice were measured as described previously[15]. The degree of excoriation, dryness, erosion, and scaling was investigated and scored in a double-blinded manner. The degree of each symptom was graded from 0 (none), 1 (mild), 2 (moderate) and 3 (severe). The skin thickness was measured with the help of a digital caliper (Mitutoyo Corporation, Japan).

2.9. ELISA assay

On day 16, blood samples were collected shortly before the mice were sacrificed. The blood samples were centrifuged at 12000 rpm for 15 min and the serums were separated and stored at −80℃ for subsequent experiments. The levels of IL-4, IL-6, IgE, TNF-α, IFN-γ, TARC, and TSLP in the serum were measured using an ELISA kit (R&D Systems) according to the manufacturer's instruction.

2.10. Histopathological analysis

Histopathological analysis was performed using methods described previously by Cho *et al.*[15]. The hematoxylin-eosin stain was used for the evaluation of epidermal thickness. Toluidine blue stain was used for mast cell quantification. Immunohistochemistry was performed using anti-tryptase for characterizing mast cell degranulation. Histopathological changes were examined under the light microscope (Leica, Wetzlar, Germany).

2.11. Mouse splenocytes preparation

Mouse splenocytes were prepared as described previously[16]. Briefly, four-week-old male BALB/c mice were purchased from Orient Bio Inc. (Gwangju). The spleens were aseptically removed from mice. After washing with cold PBS, the spleen was then crushed with sterilized glass slides, filtered using a 40 μm cell strainer to obtain the single-cell suspension, and centrifuged. After removing the red blood cells, the splenocytes were suspended and counted.

2.12. Determination of inflammatory mediators

To analyze the inflammatory mediators, the splenocytes were seeded in 6-well plates at a density of 5×10^6 cells/mL and incubated overnight for 16 h. The cells were treated with the DLE (100 μg/mL), HDLE (100 μg/mL), EDLE (100 μg/mL), prednisolone (5 μg/mL), and myricitrin (20 μM) for 1 h. After 1 h treatment, the cells were stimulated with 2 μg/mL of ConA for 48 h. After stimulation, the supernatants were harvested and analyzed by the ELISA method mentioned above. Next, HMC-1 human mast cells were cultured in 6-well plates at a density of 5×10^5 cells/mL and incubated overnight. The cells were treated with 20 μM of myricitrin for 1 h and then stimulated with 50 nM of phorbol-12-myristate 13-acetate and 1 μM of calcium ionophore A23187 (PI) for 12 h. The supernatants were assayed by the ELISA method mentioned above.

2.13. Statistical analysis

Data were presented as the mean±SD (n=5). Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple comparison tests. The P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Analysis of major flavonoid myricitrin of DLE, HDLE, and EDLE

HPLC analysis was used to investigate the myricitrin, the major flavonoid component of date plum leaves extracts. The representative HPLC chromatogram of each extract was indicated in Figure 1. The content of myricitrin was (39.58±0.63) mg/g in DLE, (37.21±0.20) mg/g in HDLE, and (42.73±0.42) mg/g in EDLE, respectively.

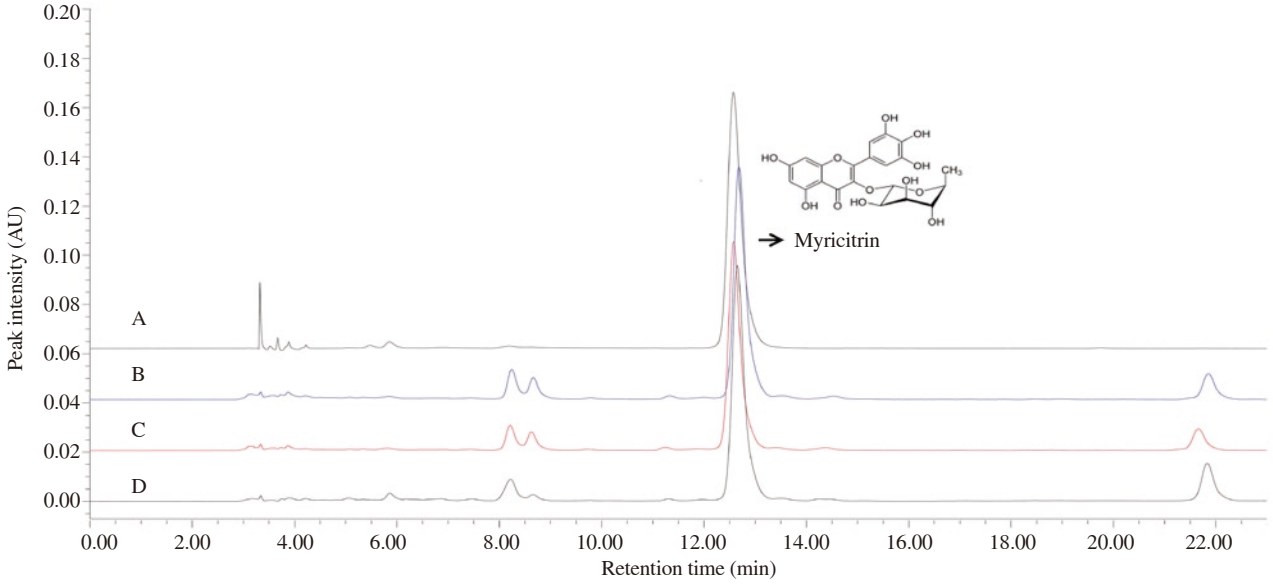

Figure 1. HPLC chromatograms of various extracts of date plum. Samples were eluted using Xbridge™ C18 column (250 mm×4.6 mm, 5 μm, Waters, Dublin, Ireland) and UV detection was monitored at 354 nm. Standard compound (A), HDLE (B), EDLE (C), DLE (D). DLE, HDLE, EDLE: ethanol, hydrochloric acid-hydrolyzed, and enzyme-hydrolyzed leaf extracts of date plum.

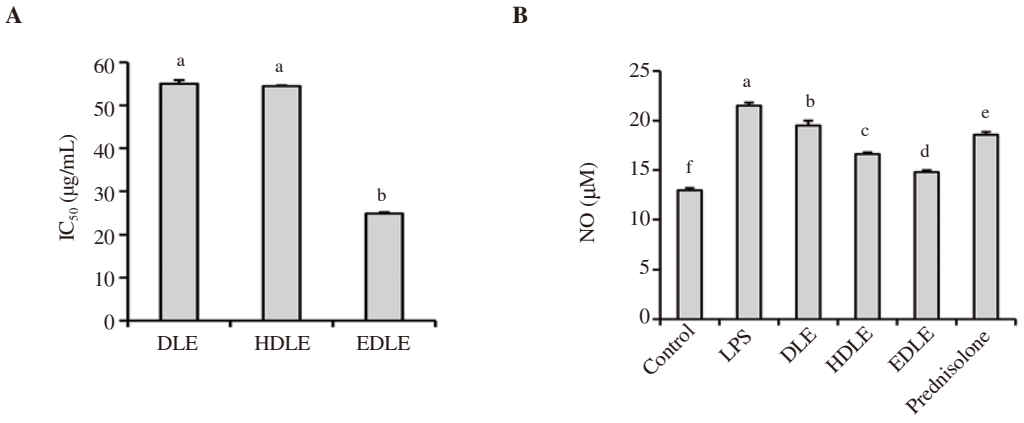

Figure 2. DPPH radical scavenging activity (A) and inhibition of NO production (B) in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Data are presented as mean ± SD. Bars with different letters indicate statistically significant differences at $P<0.05$.

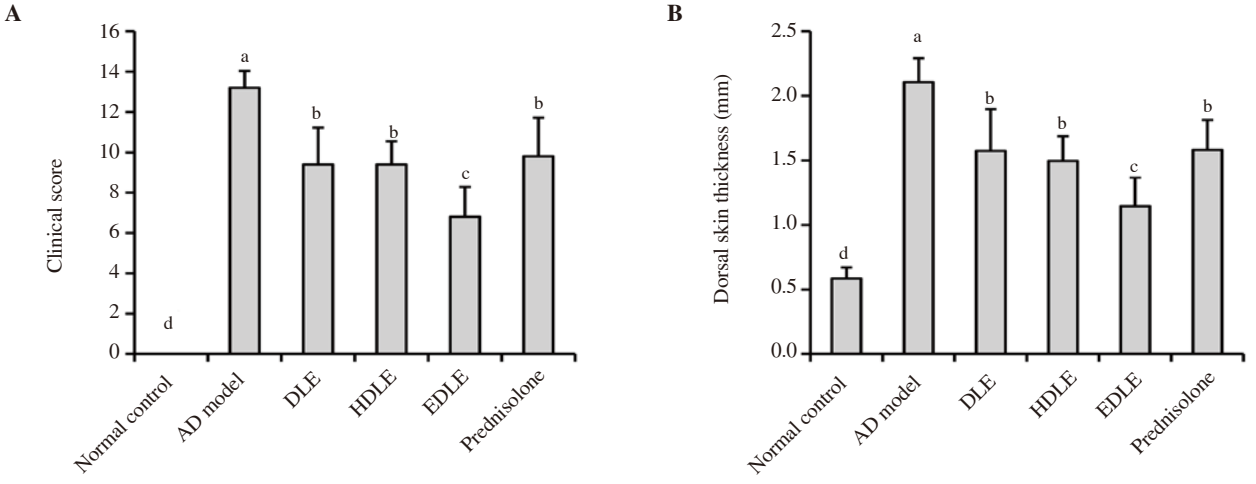

Figure 3. Atopic dermatitis-like clinical symptoms in hairless mice. (A) Clinical score of atopic dermatitis-like skin lesions in hairless mice evaluated macroscopically. (B) Dorsal skin thickness measured with the help of a digital calliper. Data are presented as mean±SD (n=5). Bars with different letters indicate statistically significant difference at $P<0.05$. AD: atopic dermatitis.

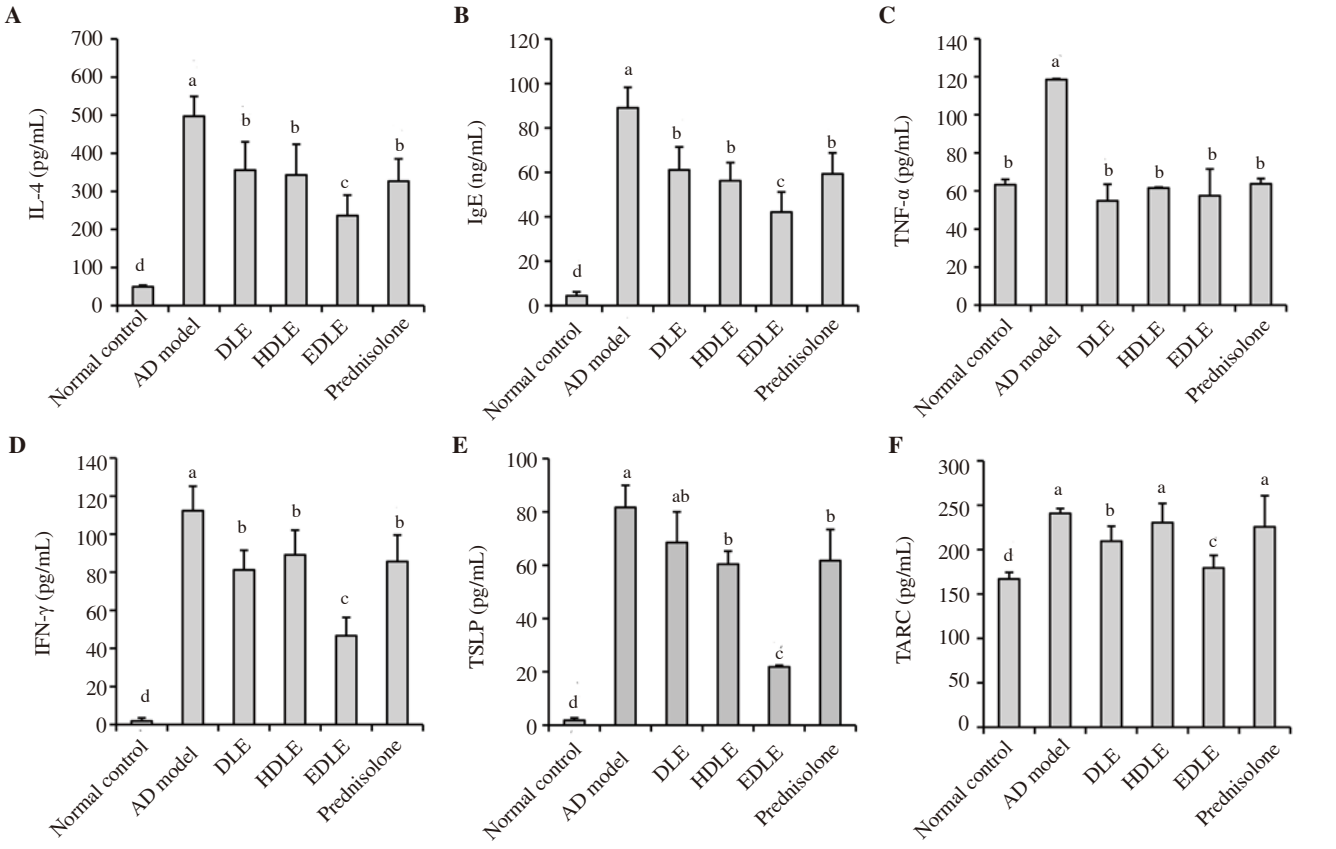

Figure 4. Effect of extracts of date plum on atopic dermatitis-related biomarkers. IL-4 (A), IgE (B), TNF-α (C), IFN-γ (D), TSLP (E), and TARC (F) were measured in serum of the mice in each group. Data are presented as the mean±SD (n=5). Bars with different letters indicate statistically significant difference at P<0.05. TSLP: thymic stromal lymphopoietin; TARC: thymus and activation-regulated chemokine.

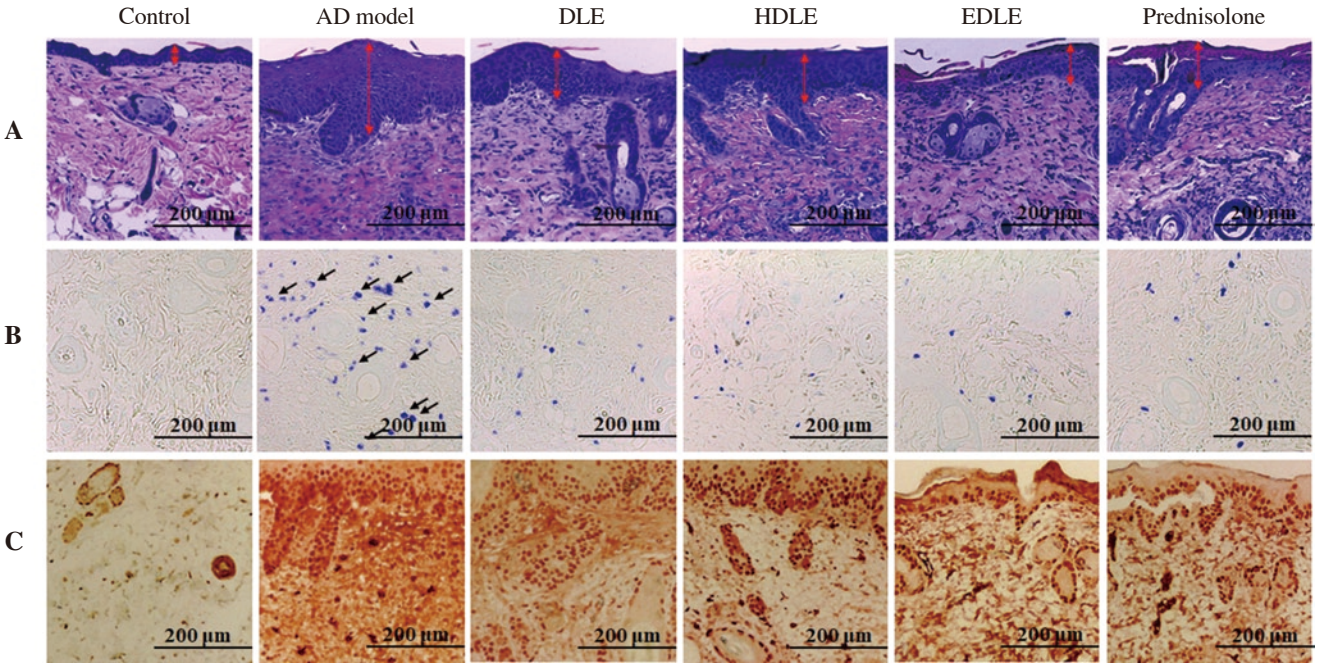

Figure 5. Effect of extracts of date plum on histopathology of mast cells in atopic dermatitis mice. (A) Morphological changes of dorsal skin stained *via* hematoxylin/eosin staining showing epithelial hyperplasia. (B) Toluidine blue stained tissues showing mast cell infiltration into the dermis. (C) Immunohistochemically stained tissues showing tryptase, a marker of mast cell degranulations. Images were taken at 20 × magnification.

3.2. Effect of DLE, HDLE, and EDLE on DPPH radical scavenging activity and NO production

We tested the antioxidant effects of DLE, HDLE, and EDLE before the *in vivo* experiment. The antioxidant activities of DLE, HDLE, and EDLE measured by DPPH assay showed IC_{50} values of 55.00, 54.45, and 24.80 μg/mL, respectively (Figure 2A). The inhibitory effects of DLE, HDLE, and EDLE on NO production were also investigated in LPS-stimulated RAW264.7 macrophage cells. As shown in Figure 2B, the production of NO was increased in the LPS-stimulated RAW264.7 cells. However, treatment with DLE, HDLE, and EDLE significantly inhibited NO production in the LPS-stimulated RAW264.7 cells. EDLE treatment had significantly lower levels of NO compared to the DLE and HDLE treatment.

3.3. Effect of DLE, HDLE, and EDLE on atopic dermatitis-like clinical symptoms

To investigate the effect of DLE, HDLE, and EDLE on the clinical symptoms in a mice model with atopic dermatitis, we evaluated the SCORAD scores of atopic dermatitis and thickness of the dorsal skin. As shown in Figure 3, the skin of model mice with atopic dermatitis exhibited atopic dermatitis-like symptoms such as dryness, erythema, excoriations, and crusts as well as an increase of dorsal skin thickness compared with the control group. However, these symptoms were ameliorated in mice treated with DLE, HDLE,

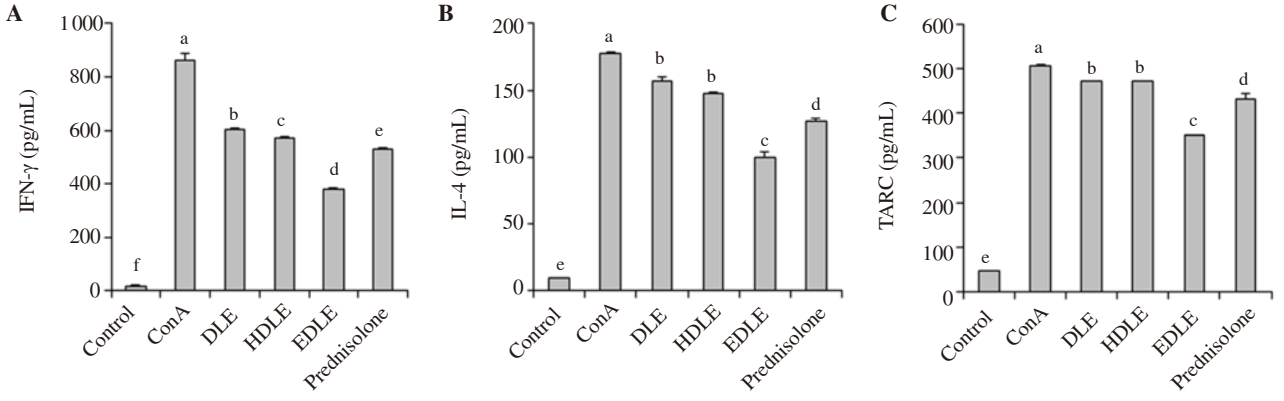

Figure 6. Effect of extracts of date plum on the production of IFN-γ (A), IL-4 (B), and TARC (C) in concanavalin A (ConA)-treated splenocytes. Data are presented as the mean±SD. Bars with different letters indicate statistically significant difference at $P<0.05$. TARC: thymus and activation-regulated chemokine.

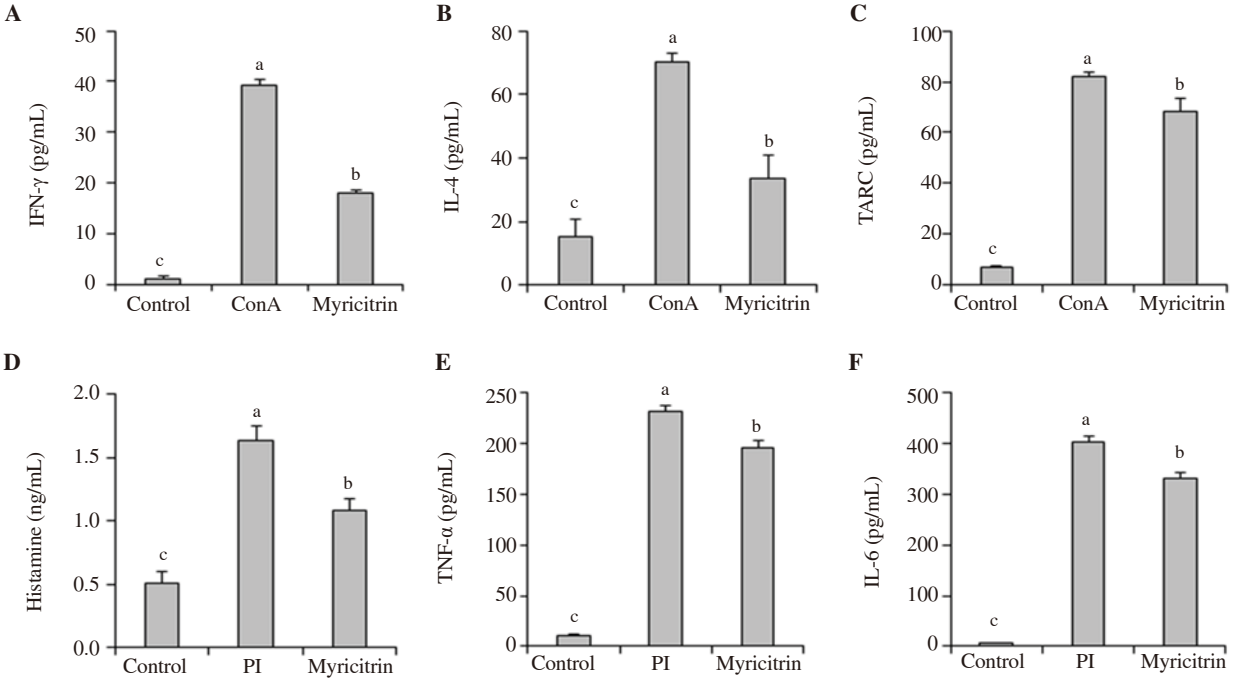

Figure 7. Anti-inflammatory effects of myricitrin (major active compound of enzyme-hydrolyzed leaf extracts of date plum) in mouse splenocytes and HMC-1 human mast cells. (A, B, C) Effects of myricitrin on IFN-γ, IL-4, and TARC production in concanavalin A (ConA)-treated splenocytes. (D, E, F) Effects of myricitrin on histamine, TNF-α, and IL-6 production in phorbol-12-myristate 13-acetate and calcium ionophore (PI)-stimulated HMC-1 cells. Data are presented as mean±SD. Bars with different letters indicate statistically significant difference at $P<0.05$. TARC: thymus and activation-regulated chemokine.

and EDLE. Prednisolone demonstrated similar results and had no significant difference with the DLE and HDLE treatment. EDLE treatment group indicated better effects than the other treatment groups.

3.4. Effect of DLE, HDLE, and EDLE on production of IgE, IL-4, TNF-α, IFN-γ, TSLP, and TARC in a mice model with atopic dermatitis

To evaluate the effect of DLE, HDLE, and EDLE on the inflammatory response in the serum of atopic dermatitis-induced mice, we analysed the atopic dermatitis-related biomarkers including IgE, IL-4, TNF-α, IFN-γ, TSLP, and TARC using an ELISA assay. As shown in Figure 4, the levels of IgE, IL-4, TNF-α, IFN-γ, TSLP, and TARC were significantly elevated in the atopic dermatitis mice model group when compared with the control group. However, the levels of IgE, IL-4, TNF-α, and IFN-γ were significantly decreased in mice treated with DLE, HDLE, EDLE, and prednisolone when compared with the atopic dermatitis mice model group (Figure 4A-D). There was no significant difference in the TNF-α level between the normal control group and mice treated with DLE, HDLE, EDLE, and prednisolone. The level of TSLP was also lowered in HDLE, EDLE, and prednisolone groups, but not in DLE group (Figure 4E). Besides, the level of TARC was lowered in DLE and EDLE group except the HDLE and prednisolone group (Figure 4F). Mice in the atopic dermatitis model group that were treated with EDLE had significantly lower levels of IgE, IL-4, IFN-γ, TSLP, and TARC except TNF-α compared to those treated with DLE, HDLE, and prednisolone (Figure 4).

3.5. Effect of DLE, HDLE, and EDLE on epidermal hyperplasia, infiltration, and degranulation of mast cell in a mice model with atopic dermatitis

As shown in Figure 5, epidermal hyperplasia (Figure 5A), massive infiltration (Figure 5B), and degranulation of mast cells (Figure 5C) were markedly increased in the atopic dermatitis model group. However, mice in the atopic dermatitis model group treated with DLE, HDLE, EDLE or prednisolone reduced epidermal hyperplasia, infiltration, and degranulation of mast cells (Figure 5). Of these, mice in the atopic dermatitis model group that were treated with EDLE showed the best effect.

3.6. Effect of DLE, HDLE, and EDLE on cytokines production in ConA-stimulated splenocytes

As shown in Figure 6, IFN-γ, IL-4, and TARC levels were significantly enhanced in splenocytes treated with ConA. However, treatment with DLE, HDLE, EDLE, and prednisolone markedly reduced the levels of IFN-γ, IL-4, and TARC. EDLE treatment had significantly lower levels of IFN-γ, IL-4, and TARC compared to those treated with DLE, HDLE, and prednisolone.

3.7. Effect of myricitrin on inflammatory response

The stimulation of splenocytes with ConA resulted in a significant increase in the production of IFN-γ, IL-4, and TARC cytokine. However, treatment with myricitrin at 20 μM before stimulation with ConA significantly decreased the production of all these cytokines (Figure 7A-C). We also investigated the effect of myricitrin on histamine release and inflammatory cytokines (TNF-α and IL-6) in phorbol-12-myristate 13-acetate and calcium ionophore A23187 (PI)-stimulated HMC-1 human mast cells. As expected, treatment with myricitrin reduced the histamine release and the production of TNF-α and IL-6 (Figure 7D-F).

4. Discussion

Diospyros species have been well studied for its dermatological and cosmetic benefits. The plant has been used to treat several skin conditions such as pimples, skin eruptions, and eczema in Chinese traditional medicine. Several studies have revealed that different parts of the plant possess anti-inflammatory, photo-protective, antiallergic, and anti-wrinkle effects[17]. Previous studies demonstrated that date plum has anti-atopic dermatitis, anti-obesity as well as UV-protective effects[12,18-21]. In this study, we sought to investigate whether hydrolysing date plum leaves extract will enhance the anti-atopic dermatitis properties of date plum. Hydrolysis has been used in several studies for the conversion of less biologically active phytochemicals to more active compounds. For example, acid hydrolysis of saponin-rich extracts yielded sapogenin-rich extracts and other bioactive compounds with better biological activities[22]. We recently demonstrated that hydrolysing celery leaves extracts with an acid increased their bioactive composition with improved anti-proliferative and anti-inflammatory activities[16]. In this light, we investigated and compared the anti-atopic dermatitis activities of three leave extracts of date plum obtained *via* different extraction methods. Firstly, through HPLC analysis, we found that the main compound present in all extracts was myricitrin. The result was in accordance with those of other studies that identified myricitrin in date plum leaves and fruit peels[17,23]. Rhamnosidase-treated extract had higher amounts of myricitrin, followed by DLE and HDLE. Myricetin, as a derivative of myricitrin, was not detected in any of the extract. Although we expected an increase in the formation of new bioactive compounds like myricetin from myricitrin after enzyme or acid treatment, the increase in myricitrin concentration meant that enzymes had increased the yield of myricitrin and hence can be employed during the extraction of active compounds from date plum to improve process performance and better yield. The result of this study is in line with previous studies that employed enzyme assisted methods to improve the extraction efficiencies of bioactive compounds from medicinal plants[24,25].

Next, we investigated the *in vitro* antioxidant activity and anti-inflammatory activity of DLE, HDLE, and EDLE. EDLE demonstrated stronger DPPH radical scavenging activities compared to DLE and HDLE. EDLE also significantly inhibited LPS-induced

NO production in RAW264.7 cells. The result suggests that enzyme-assisted extraction methods can be used to improve the antioxidant and anti-inflammatory activities of date plum leaves. Previous studies had also reported that enzyme-assisted extraction of medicinal plants improved their antioxidant and anti-inflammatory activities[26,27].

Following the antioxidant and anti-inflammatory effects of DLE, HDLE, and EDLE, we then evaluated the ameliorative effects of DLE, HDLE, and EDLE on DNFB + house dust mite antigen-induced atopic dermatitis signs and symptoms. Epidermal and dermal hyperplasia, skin erythema, dried and eroded skins with excoriations and crusts are frequently seen in atopic dermatitis[28]. In this study, all these parameters of atopic dermatitis were noticed in the developed atopic dermatitis mice model. The parameters were summarised in the SCORAD clinical scores of atopic dermatitis, which revealed that EDLE was most effective in reducing the clinical scores including skin thickness due to epidermal and dermal hyperplasia. EDLE ameliorated symptoms of atopic dermatitis more significantly than the prednisolone, a commercial drug used for the treatment of atopic dermatitis in patients. The findings here suggest that enzyme-assisted extraction of date plum leaves may be employed for improving the anti-atopic dermatitis effects of date plum, which are comparable to the approved atopic dermatitis drug-prednisolone. In addition, the fact that all the extracts decreased the SCORAD clinical scores of atopic dermatitis, with no death and no significant differences in weight loss or abnormal behaviour among the various groups indicated that all extracts especially EDLE were well tolerated in the mice with no noticeable toxicity.

To understand the mechanism of action of the various extracts in ameliorating the clinical signs of atopic dermatitis, we investigated biomarkers that are constantly expressed in atopic dermatitis. In atopic dermatitis, there is an increase in mast cell infiltration and degranulation which was also noticed in this study. All extracts in this study prevented this increase in mast cell infiltration as demonstrated by the toluidine blue staining of mast cells in the skin tissue. Mast cell degranulation was also prevented as demonstrated by immunohistochemical staining of tryptase, a marker of mast cell degranulation. The pathogenesis of atopic dermatitis involves predominant IL-4, which causes the class switching of activated B cells to IgE-producing cells to produce IgE. The cross-linking of IgE by allergens then causes the release of histamine, the cause of itch in atopic dermatitis[29]. IFN-γ and TNF-α present in the skin are also known to stimulate keratinocytes to proliferate, causing epidermal hyperplasia and infiltration of inflammatory cells into inflamed tissues[30,31]. TSLP stimulates the activation and migration of dendritic cells to the dermis of atopic dermatitis to produce TARC which contributes to inflammation in atopic dermatitis by attracting Th2 cells to the skin secreting Th2 cytokines[32]. Reducing the secretions of these cytokines in atopic dermatitis is thus deemed necessary in the management of the disease. Our results showed that EDLE was most effective in reducing IgE, IL-4, TNF-α, IFN-γ, TARC, and TSLP in the serum of mice. In addition, EDLE showed better efficacy then prednisolone. To confirm the above results, we studied the effects of DLE, HDLE, and EDLE on ConA-stimulated primary splenocytes. We confirmed that DLE, HDLE, and EDLE prevented ConA-stimulated production of IFN-γ, IL-4, and TARC. EDLE had the overall best results and effectively inhibited these cytokines compared with prednisolone. Therefore, it can be concluded that date plum extract exerts pharmacological effects on the pathogenesis of atopic dermatitis by reducing the infiltration and degranulation of mast cells; reducing inflammatory cytokines to ameliorate atopic dermatitis in mice. Enzyme-assisted extraction can also be used to improve the biological activities of date plum.

To determine if the major bioactive compound detected in all the extracts was responsible for the anti-atopic dermatitis effects found in the study, we studied the effect of myricitrin on ConA-stimulated primary splenocytes and phorbol 12-myristate 13-acetate and ionomycin-stimulated mast cells. We demonstrated that myricitrin inhibited the production of IFN-γ, IL-4, and TARC in stimulated splenocytes as well as histamine, TNF-α, and IL-6 in stimulated mast cells. Previous studies demonstrated that myricitrin exhibits antioxidant, anti-inflammatory, anti-allergic, and antifibrotic activities in mice[33,34]. Thus we concluded that myricitrin among other actives compounds in date plum leaves might have been responsible for the amelioration of atopic dermatitis in the mice.

In summary, our results demonstrated that administration of EDLE was protective against DNFB and HDM antigen-induced atopic dermatitis-like lesions more than DLE and HDLE in hairless mice. EDLE effectively alleviated clinical signs, epidermal hyperplasia and inflammatory mast cell infiltration in the skin of atopic dermatitis induced mice model. EDLE inhibited the over-production of IgE, IL-4, TNF-α, IFN-γ, TSLP, and TARC in atopic dermatitis mice. Myricitrin was confirmed to be one of the major active compounds of EDLE. These results imply that EDLE might be a potential candidate to treat atopic dermatitis.

Conflict of interest statement

The authors have declared no conflict of interest.

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Authors' contributions

BOC and SIJ designed the current study and analyzed the data. JYS, JSK, DNC, HJK, and HJK performed the experiments. JYS and BOC wrote the manuscript. BOC, DNC, HO, YSK, and SIJ revised the manuscript. SIJ managed the research project.

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