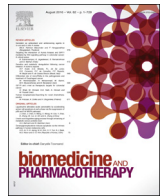




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Diospyros lotus leaf and grapefruit stem extract synergistically ameliorate atopic dermatitis-like skin lesion in mice by suppressing infiltration of mast cells in skin lesions



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ABSTRACT

Atopic dermatitis, a chronic relapsing and pruritic inflammation of the skin also thought to be involved in, or caused by immune system destruction is an upsetting health problem due to its continuously increasing incidence especially in developed countries. Mast cell infiltration in atopic dermatitis skin lesions and its IgE-mediated activation releases various cytokines and chemokines that have been implicated in the pathogenesis of atopic dermatitis. This study was aimed at investigating synergistic anti-inflammatory, anti-pruritic and anti-atopic dermatitis effects of *Diospyros lotus* leaf extract (DLE) and Muscat bailey A grapefruit stem extract (GFSE) in atopic dermatitis-like induced skin lesions in mice. Combinations of DLE and GFSE inhibited TNF- α and IL-6 production more than DLE or GFSE in PMA plus calcium ionophore A23187-activated HMC-1 cells. DLE and GFSE synergistically inhibited compound 48/80-induced dermal infiltration of mast cells and reduced scratching behavior than DLE or GFSE. Furthermore, DLE and GFSE synergistically showed a stronger ameliorative effect in skin lesions by reducing clinical scores; dermal infiltration of mast cells; ear and dorsal skin thickness; serum IgE and IL-4 production in atopic dermatitis-like mice. Collectively, these results suggest that DLE and GFSE synergistically exhibit anti-atopic dermatitis effects in atopic dermatitis-like skin lesions in mice.

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1. Introduction

Atopic dermatitis (AD), a chronic relapsing and pruritic inflammation of the skin also thought to be involved in or caused by immune system destruction is an upsetting health problem due to its continuously increasing incidence especially in developed countries [1]. Although great attention is still focused on knowing

the exact pathogenesis and etiology of AD, this must go on simultaneously with developing new approaches to AD therapy using the currently available knowledge we have of the pathogenesis and etiology of this disturbing health problem especially at a time when topical steroids for treatment of AD has proven to have more adverse effects on patients. Many authors do accept that inflammatory cytokines, mast cells, and CD₄ T cells in skin lesions are involved in the pathogenesis of AD [2–7]. IL-4 signals on B cells to switch from IgG production to IgE synthesis [8]. IgE activates mast cells to release various chemokines that cause infiltration of inflammatory cells into the skin lesions and pro-inflammatory mediators like TNF- α , IL-6, IL-1 α/β , IL-2, IL-4 etc; chemicals and protein mediators such as histamine, serotonin that mediates itch in AD [9,10]. Structural abnormalities in the epidermis is also a sector that is involved in the pathophysiology of AD and often leads to the development of Asthma, allergies and an increased susceptibility to infections and cutaneous colonization [11,12].

Diospyros lotus belongs to Ebenaceae family, a deciduous tree native in China and Asia with its fruits widely eaten for its medical

Abbreviations: AD, atopic dermatitis; IgE, immunoglobulin E; DLE, *Diospyros lotus* leaf extract; GFSE, Muscat bailey A grapefruit stem extract; TNF- α , tumor necrosis factor- α ; IL, interleukin; CD₄, cluster of differentiation 4; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; STAT1, signal transducer and activator of transcription 1; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; HO-1, Heme oxygenase 1; LPS, lipopolysaccharides; ROS, reactive oxygen species; DNFB, dinitrofluorobenzene; PMA, phorbol 12-myristate 13-acetate; SCORAD, scoring atopic dermatitis; TSLP, thymic stromal lymphopoietin; Th2, type 2 helper T cells.

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importance such as sedative, antiseptics, antidiabetic and antitumor [13,14]. The roots of *Diospyros lotus* have marked antinociceptive and anti-inflammatory activities [15]. The fruits are widely regarded as potential sources of natural anti-oxidants owing to the dominant gallic acid and myricetin contents [16]. We have recently reported that myricetin from *Diospyros lotus* inhibits the productions of pro-inflammatory mediators by suppressing NF- κ B and STAT1 activation and induction of Nrf2 mediated HO-1 expression in lipopolysaccharides (LPS)-stimulated RAW264.7 macrophages [17]. On the other hand, grape stem, the most disposed part of the grape tree has been shown to be a rich source of natural antioxidants based on their polyphenol and flavonoid contents [18]. It is rich in bioactive polyphenol-*trans*-resveratrol [19]; display antioxidant and protective activities against ROS-induced DNA damage [20] and also inhibits vascular endothelial growth factor expression thereby posing anti-angiogenic potentials [21]. However, very little is known about the grapefruit stem itself. Due to the continuous search for better therapies for the treatment of AD and inflammation, there is a growing interest in the synergistic effects of phytochemicals for the treatment of such diseases. In this context, the aim of the present study is to access the synergistic effects of *Diospyros lotus* leaf and grapefruit stem extracts in inhibiting the activation of mast cells and for improving AD-like skin lesion in mice.

2. Materials and methods

2.1. Chemicals

Dinitrofluorobenzene (DNFB), phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 and compound 48/80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). House dust mite antigen came from Biostir (Hyogo, Japan).

2.2. Plant extraction

Fresh *Diospyros lotus* leaves (DLE) were harvested from Bugwi-Myeon, Jinan-Gun, Jeonbuk, Korea. The plant was identified and authenticated by Prof Kim Hong-Jun at the college of oriental medicine, Woosuk University and thereafter a voucher specimen was brought to our laboratory in the department of healthcare science, college of medical science, Jeonju University. The leaves were washed with several changes of distilled water and dried at 60 °C for 16 h. After drying, 100 g of dried leaves were extracted in 50% (V/V) ethanol (2000 mL) for 72 h. The extracted sample was filtered with 0.45 μ m filter paper (purchased from ADVANTEC, Togo, Japan), lyophilized and stored at –20 °C until used. An exact concentration of 5 mg/mL was made with distilled water.

Muscat bailey A grapefruit stem (GFSE) came from Baekgu-myeon Gimje-si, Jeonbuk, Korea. The fruit stems were washed with several changes of distilled water, dried at 40 °C for 16 h, extracted (50 g) in 80% (V/V) ethanol (1000 mL) for 3 days and the resulting sample filtered with 0.45 μ m filter paper, concentrated and lyophilized to obtain the dry extract which was stored at –20 °C until used. An exact concentration of 1.25 mg/mL was made with distilled water.

In order to prepare a final concentration containing 2.5 mg/mL of DLE and 0.625 mg/mL of GFSE, equal volume of 5 mg/mL DLE and 1.25 mg/mL of GFSE were mixed.

2.3. Animals and experimental design

Seven weeks old male hairless mice were obtained from Orient Bio Inc. (Iksan, Korea), housed under standard environmental conditions and fed with commercial standard laboratory diet and water ad libitum. The mice were maintained in an air-conditioned

room with temperature -22 ± 2 °C, humidity 50–60%, and 12/12 h light-dark cycle. All experimental procedures were performed following Jeonju University Institutional Animal Care and Used Committee guidelines. After one week of acclimatization, mice were randomly divided into 6 groups (n = 5) i.e. Group 1-Normal control; Group 2-AD model (DNFB + house dust mite antigen); Group 3-AD + DLE; Group 4-GFSE; Group 5-DLE + GFSE and Group 6-prednisolone. To induce AD-like symptoms and skin lesions, the dorsal skin and ears of the mice in each group except the normal control groups were sensitized by repeated application of 0.1 mL of 0.15% DNFB (prepared with acetone/olive oil in the ratio of 3:1) once on days 1 and 4. On days 7, 10 and 13, the sensitized mice were challenged with 0.1 mL of 0.2% DNFB and 100 mg of dust mite antigen on the dorsal skin and ear. The control mice were treated with the same volume of vehicle. Group 1 and 2 mice were treated with saline while Group 3, 4, 5, and 6 were treated with DLE 40 mg/kg, GFSE 20 mg/kg, DLE 20 mg/kg + GFSE 10 mg/kg and prednisolone 10 mg/kg respectively. All mice received an equal volume of 0.1 mL.

Four weeks old male ICR mice were purchased at Central laboratory Animal (Seoul, Korea). The mice were housed and fed with similar conditions as above. After one week of acclimatization, they were randomly divided into 6 Groups (n = 5) i.e. Group 1-normal control; Group 2-compound 48/80 treated group; Group 3-DLE; Group 4-GFSE; Group 5-DLE + GFSE; Group 6-prednisolone. 60 min before injection of 50 μ g/site (0.1 mL each) of compound 48/80 to the left site of the shoulder, the mice were administered with 0.1 mL each of the various extracts, i.e. Group 1- saline, Group 2-saline, Group 3-DLE 40 mg/kg, Group 4-GFSE 20 mg/kg, Group 5-DLE 20 mg/kg + GFSE 10 mg/kg and Group 6-prednisolone 10 mg/kg. Thereafter, according to Mihara et al's method [22], scratching behavior was monitored with micro-cameras (ONCCTV, Seoul, Korea) for 60 min and the number of scratches on the site of the compound 48/80 injection was counted in a double-blinded manner.

2.4. Dermatitis score

The severities of AD-like symptoms were evaluated macroscopically by SCORAD method [23]. SCORAD's method evaluates the severity of dryness, erosion, excoriation and scaling. The overall dermatitis score was determined from the sum of all individual scores in a double-blinded manner. The degree of each symptom was graded from 0 (none), 1 (mild), 2 (moderate) and 3 (severe). Skin thickness was measured with the help of a digital caliper (Mitutoyo, Kawasaki, Japan).

2.5. Histopathology

On day 13 after the mice were sacrificed, the right ears and a section of dorsal skins of the hairless mice were removed. The dorsal skin sections of the ICR mice injected with compound 48/80 were also removed. All tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) for 24 h. The tissues were washed in PBS for 24 h with 5 times PBS changes. The tissues were then dehydrated in a series of graduated ethanol (60–100%), cleared in xylene and embedded in paraffin. 5 μ m thin sections were cut and stained with hematoxylin-eosin stain and toluidine blue stains. Histopathological changes were examined under the light microscope (Leica, Wetzlar, Germany).

2.6. Cell culture

HMC-1 cells were obtained from Jeonju AgroBio-Material Institute. The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal

bovine serum, 1% penicillin G (100 IU/mL)/streptomycin (100 mg/mL) and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.7. ELISA assay

On day 13 after the hairless mice were sacrificed, serum samples were collected and stored at –20 °C. These samples were used to measure the level of IgE and IL-4 (R&D systems, USA). Also, HMC-1 (3×10^5 cells/mL) were grown in 6 well plates for 24 h, with either DLE, GFSE, DLE and GFSE or nothing at all and after 1 h, the cells except for the negative control were stimulated with 50 nM of PMA (phorbol 12-myristate 13-acetate) + 0.001 mM of calcium ionophore A23187 for 16 h. The cell culture supernatants were used to measure TNF- α and IL-6 (R&D systems, Minneapolis, MN, USA). The measurements were performed according to the manufacturer's protocol.

2.8. Statistical analysis

All data are presented as the mean $\pm$ SD of 3 different experiments. Student's *t*-test was used to determine significant differences between the various groups. P-values of < 0.05, < 0.01, and < 0.001 were considered statistically significant.

3. Results

3.1. Combined DLE and GFSE significantly reduced pruritus in ICR mice by suppressing the infiltration of mast cells

In our past study, we reported that *Diospyros lotus* reduced pruritus, in Balb/c mice and have potent antioxidant activity [23]. Also, Cho et al. [24] reported that grapefruit stems possess antioxidants activity due to its high resveratrol contents. Resveratrol can be used as an anti-inflammatory therapy [25] and also ameliorates AD-like lesions in Balb/c mice [26]. Thus we investigated the synergistic effects of DLE and GFSE on the relief of itch in ICR mice. Injection of compound 48/80 on the dorsal skin of mice induced scratching in mice which were significantly reduced in mice treated with both DLE and GFSE compared to mice treated with DLE or GFSE alone. Of note, both extracts combined or not reduced scratching in mice more than the control compound prednisolone. Further histopathological examinations of tissue-stained sections showed a massive reduction of mast cells in the skin of mice treated with DLE and GFSE after compound 48/80 caused massive infiltration of those cells in the skin. (Fig. 1).

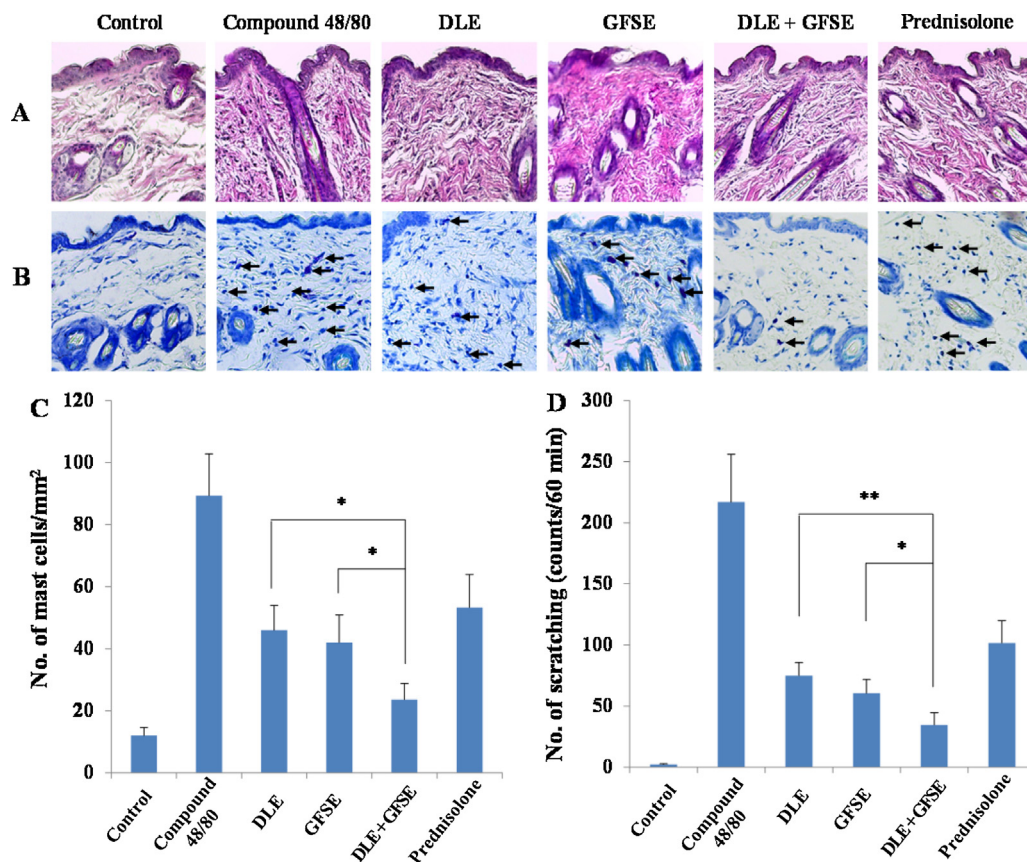


Fig. 1. Synergistic effects of co-treatment of DLE and GFSE combination on the reduction of pruritus in ICR mice. Five mice each were placed into six groups: compound 48/80 (–); compound 48/80 (+); compound 48/80 with DLE 40 mg/kg; compound 48/80 with GFSE 20 mg/kg; compound 48/80 with DLE 20 mg/kg + GFSE 10 mg/kg; compound 48/80 with prednisolone 10 mg/kg. 60 min before injection of 50 μ g/site (0.1 mL each) of compound 48/80 to the left site of the shoulder, the mice were administered orally with 0.1 mL each of the various extracts. Compound 48/80 (–) and compound 48/80 (+) groups were instead treated with the same volume of saline. The number of scratches were monitored with micro- cameras for 60 min and counted in a double-blinded manner. (A) Morphological changes of skin stained with hematoxylin/eosin stain. (B) Toluidine blue stained tissues showing mast cells degranulation. (C) The number of mast cells. (D) The number of scratches. Images were taken at 20 $\times$ magnification. Data are presented as the mean $\pm$ SD of five determinations. * *p* < 0.05, ** *p* < 0.01 vs. DLE or GFSE groups.

3.2. Combined DLE and GFSE significantly ameliorates DNFB + house dust mite antigen- induced AD-like clinical symptoms in the ear and dorsal skin of hairless mice

In this study, we also investigated the ameliorative effects of DNFB and house dust mite antigen-induced AD-like symptoms in the dorsal skin and ear of hairless mice. DNFB can induce epidermal hyperplasia and massive infiltrations of mononuclear cells and eosinophils in the skin of AD-like mice [27]. House dust mite antigen have been showed to induce eczema in skin lesions of atopic dermatitis and also induce the expression of thymic stromal lymphopoietin (TSLP) gene and proteins, a key biomarker, and an indicator of AD severity and even IL-33 [28]. In this light, we investigated the synergistic effects of combined DLE and GFSE on the relief of DNFB and house dust mite antigen-induced AD-like symptoms in hairless mice. Repeated application of DNFB + house dust mite antigens induced AD-like symptoms which were significantly reduced after oral administration of DLE and GFSE than DLE or GFSE alone. Combined DLE and GFSE significantly reduced the severity of the skin lesion (Fig. 2). The SCORAD clinical scoring of atopic dermatitis (dryness, erosion excoriation oozing, and crust) was thrillingly reduced. To further analyze the efficacy of our combined DLE and GFSE, we histologically examined the ear

and dorsal skin sections of the mice. DNFB + house dust mite antigen challenge markedly induced ear swelling as well as epidermal hyperplasia. Also, there was a marked infiltration of mast cells in the ear and skin lesions of the mice meanwhile oral administration of both DLE and GFSE significantly reduce epidermal thickness in the ear and skin, ear swelling and massive infiltration of mast cells than DLE or GFSE alone. (Figs. 3 and 4)

3.3. Combined DLE and GFSE significantly suppresses DNFB + house dust mite antigens-induced serum IgE and IL-4 in hairless mice

One of the hallmarks of atopic dermatitis is the elevation of total serum IgE. Controlled challenges show that allergens that trigger acute IgE-mediated mast-cell-dependent exacerbation of eczema can also lead to an expansion of allergen-specific T helper 2 cells that produce IL-4 and 5 [29,30]. To investigate whether our combined DLE and GFSE inhibit IgE production, serum samples were prepared and IgE was measured by ELISA method. Repeated administration of DNFB and house dust mite significantly induced IgE and IL-4 production which was also significantly reduced after oral administration of both DLE and GFSE compared with DLE or GFSE alone (Fig. 5).

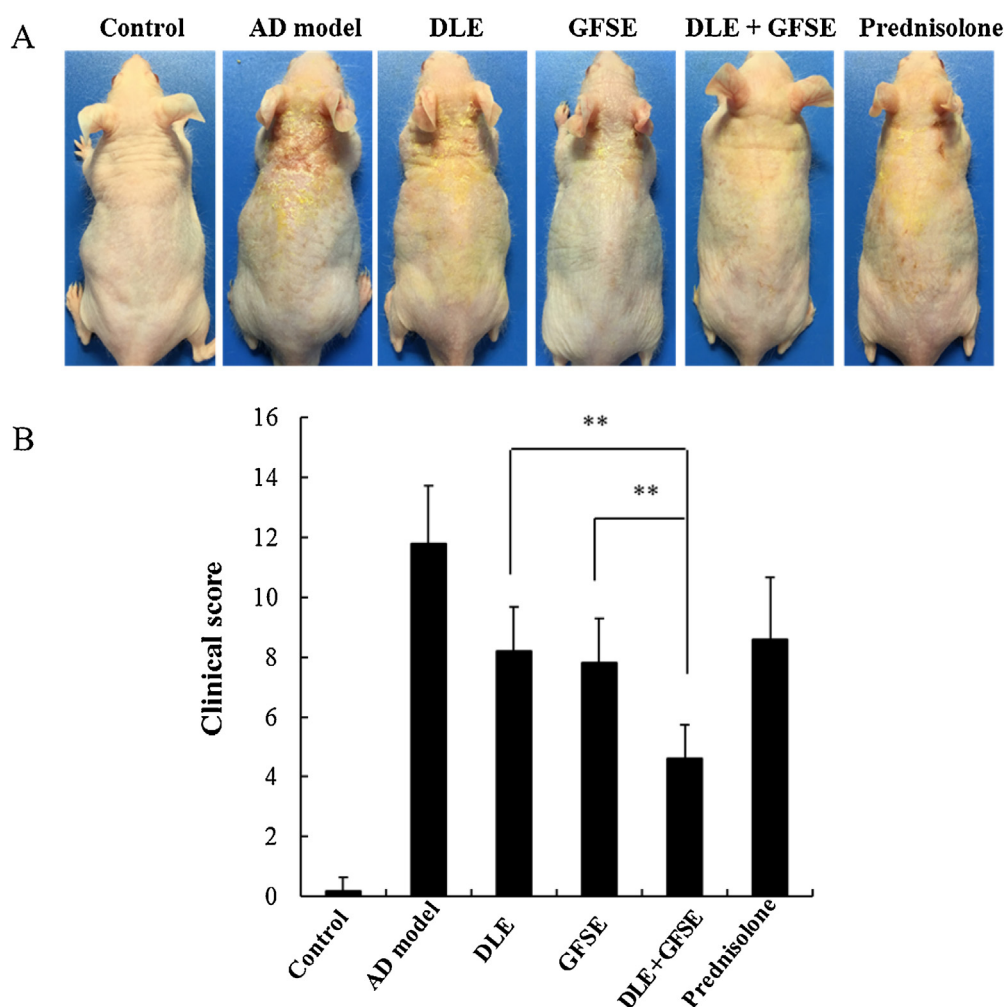


Fig. 2. Synergistic effects of co-treatment of DLE and GFSE combination on the severity of clinical scores in hairless mice. (A) Representative dorsal skin photographs captured at the end of experiments showing a comparison of AD-like skin lesions (20× magnification). Five mice were placed into six groups: DNFB + house dust mite (–); DNFB + house dust mites (+); DNFB + house dust mites with DLE 40 mg/kg; DNFB + house dust mites with GFSE 20 mg/kg; DNFB + house dust mites with DLE 20 mg/kg + GFSE 10 mg/kg. The extracts were administered orally. (B) Clinical score of AD-like skin lesions in hairless mice evaluated macroscopically. The dermatitis score was determined from the sum of all individual scores. Data are presented as the mean ± SD of five determinations. **p < 0.01 vs. DLE or GFSE groups.

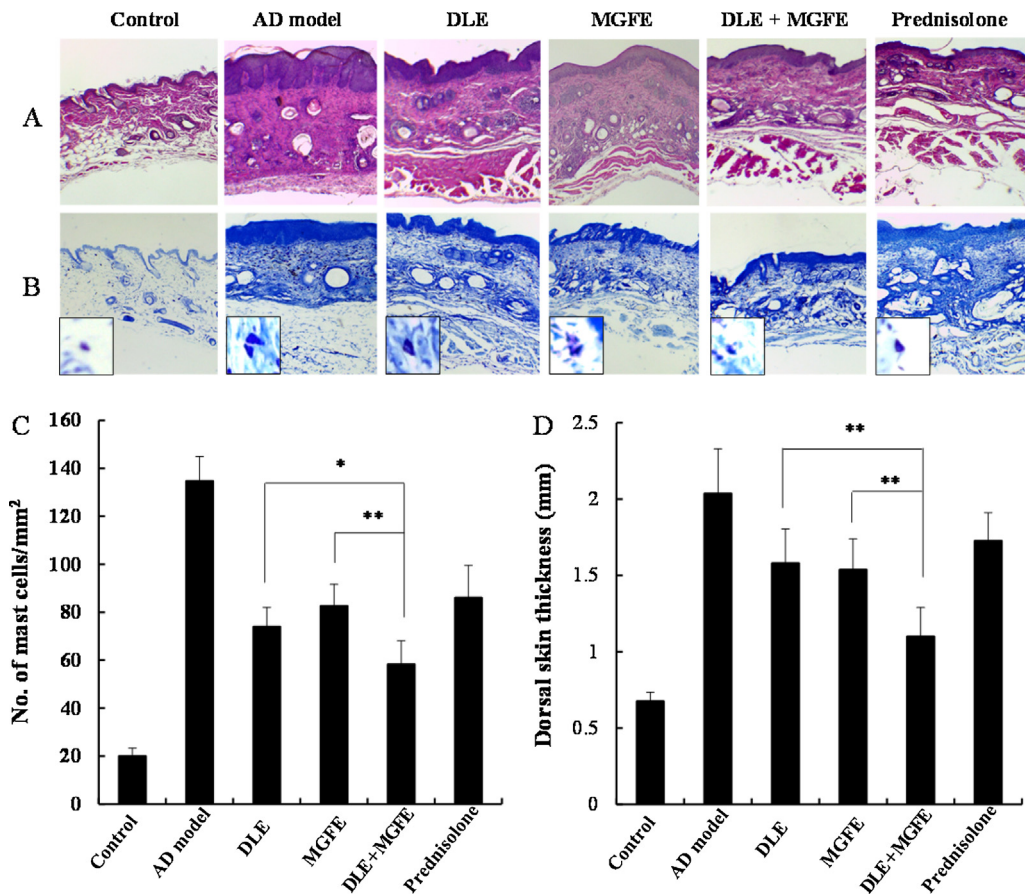


Fig. 3. Synergistic effects of co-treatment of DLE and GFSE combination on amelioration of atopic dermatitis-like skin lesions in the dorsal skin hairless mice. Five mice each were divided into six groups: DNFB + house dust mice (–); DNFB + house dust mites (+); DNFB + house dust mites with DLE 40 mg/kg; DNFB + house dust mites with GFSE 20 mg/kg; DNFB + house dust mites with DLE 20 mg/kg + GFSE 10 mg/kg. The extracts were administered orally. (A) Morphological changes of dorsal skin stained with hematoxylin/eosin stain. (B and C) Toluidine blue stained tissues showing mast cells infiltration in dorsal skin. (D) Dorsal skin thickness measured with a thickness gauge. Images were taken at 20× magnification. Data are presented as the mean ± SD (n = 5). *p < 0.05, **p < 0.01 vs. DLE or GFSE groups.

3.4. Combined DLE and GFSE significantly inhibits TNF- α and IL-6 production in HMC-1 cells

To further investigate the efficiency of combined DLE and GFSE as an anti-inflammatory agent, we studied the inhibition of pro-inflammatory cytokines like TNF- α and IL-6 production in the HMC-1 cell. Results showed that combined DLE and GFSE treatment of PMA + calcium ionophore A23187 stimulated HMC-1 cells strongly inhibited these cytokine productions than when the cells were treated with DLE or GFSE alone (Fig. 6).

4. Discussion

Over the past years, interest has been focused on the development of alternative therapies for the treatment of atopic dermatitis, a chronically relapsing skin inflammatory disease characterized by pruritus, erythema, edema and scaling that constitutes a form of a nuisance to humans [1]. This is so because, topical steroid therapy, though proven effective for treatment of atopic dermatitis has its own side effects which are detrimental to human health. Repetitive long-term applications of steroids produce side effects such as skin atrophy or thinning. Natural products contain rich amounts of polyphenols and flavonoids that possess biological activities ranging from antioxidant, antiviral, anti-tumor, anti-allergic to anti-inflammatory activities. For instance, *Diospyros lotus* has been shown to have antinociceptive and anti-inflammatory activities [15]. The polyphenol (trans-resveratrol) obtained from grape stem possess antioxidant

activities [19] and displays biological activities by inhibiting vascular endothelial growth factors [21]. Even though drug development in phytomedicine is based on the discovery of the single active compound in the plant that possesses therapeutic effects, traditional systems of medicine like the Chinese traditional medicine and European phytotherapy assures that synergy of all ingredients of plants could bring maximum therapeutic effects [31]. Researchers have demonstrated the potential synergistic effects of various phytochemicals in the treatment of human disease. Wang et al. [32] demonstrated that isoflavone and curcumin combination elicited a greater inhibition of pancreatic cancer cell growth and induced apoptosis more than the single compounds alone. Different combinations of dietary natural products including curcumin, quercetin, soybean isoflavone, silibinin have potentials for the treatment of prostate cancer [33]. In this light, we hypothesized that a combination of DLE and GFSE could increase the therapeutic effects of these plants for the treatment of atopic dermatitis. We investigated if combined DLE and GFSE could significantly reduce pruritus more than the single extracts alone. Our results proved that DLE and GFSE in synergy greatly reduce pruritus in ICR mice. This observation was mediated by the combined extract's suppression of inflammatory mast cells infiltration. Further serological examination of the mice showed that serum IgE and IL-4 was significantly reduced in mice treated with combined DLE and GFSE more than in mice treated with the single extracts alone. The Th2 cytokine, IL-4 mediates VDJ recombination that rearranges the genes responsible for the production of IgE, by joining the variable diversity joining gene

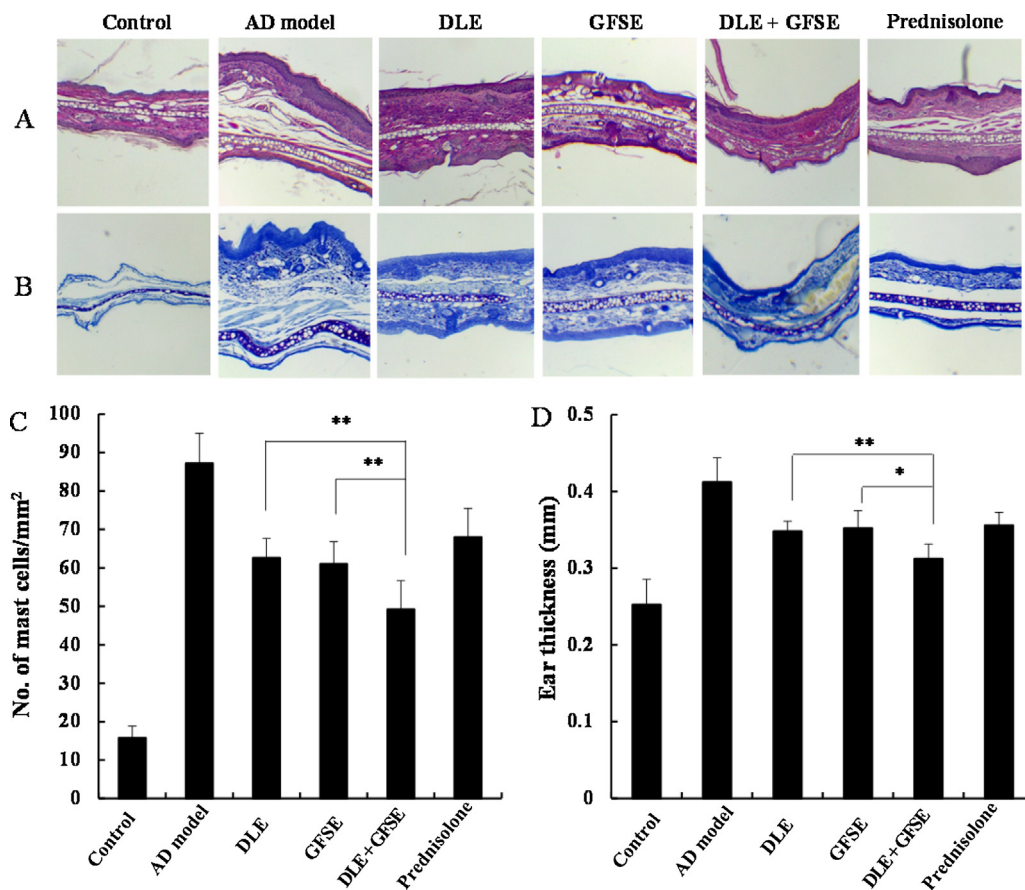


Fig. 4. Synergistic effects of co-treatment of DLE and GFSE combination on ear thickness and mast cells infiltration in the ears of hairless mice. Five mice each were divided into six groups: DNFB + house dust mice (–); DNFB + house dust mites (+); DNFB + house dust mites with DLE 40 mg/kg; DNFB + house dust mites with GFSE 20 mg/kg; DNFB + house dust mites with DLE 20 mg/kg + GFSE 10 mg/kg. The extracts were administered orally. (A) Morphological changes of ear sections stained with hematoxylin/eosin stain. (B and C) Toluidine blue stained tissues showing mast cells infiltration in the ears. (D) Ear thickness measured with a thickness gauge. Images were taken at 20× magnification. Data are presented as the mean ± SD (n = 5). *p < 0.05, **p < 0.01 vs. DLE or GFSE groups.

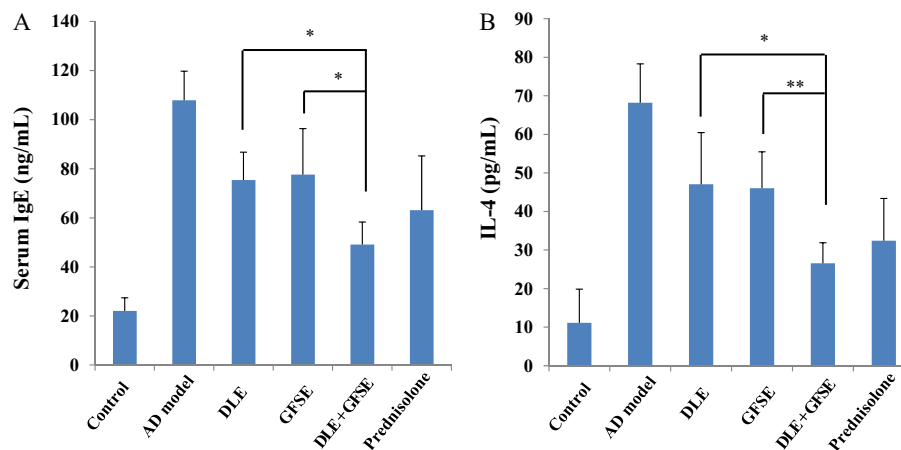


Fig. 5. Synergistic effects of co-treatment of DLE and GFSE combinations on IgE (A) and IL-4 (B) inhibition in hairless mice. On day 13 after the mice were sacrificed, serum samples were collected and stored at –20 °C. The production of IgE and IL-4 in serum samples was quantified by ELISA. Results are based on duplicate experiments. Data are presented as the mean ± SD (n = 5). *p < 0.05, **p < 0.01 vs. DLE or GFSE groups.

segment to C γ 4 or C ϵ in B cells during maturation. IgE then mediates mast cell degranulation that causes the infiltration of other inflammatory cells and release of substance responsible for itch in AD [8]. These observations made us conclude that the combined effects of our extracts in the reduction of pruritus were mediated by decreased production of IL-4 that inhibited the

synthesis of IgE and infiltration/activation of mast cells. To further elaborate on the role of our combined extracts in mast cells activation and cytokine production, we investigated the production of inflammation cytokines TNF- α and IL-6 in PMA + calcium ionophore A23187-activated mast cells treated with combined DLE and GFSE. Our results showed that combined DLE and GFSE

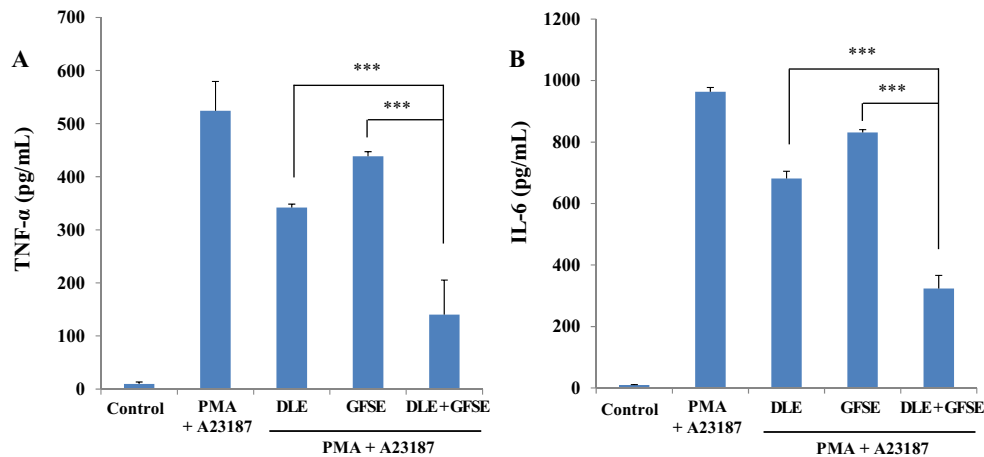


Fig. 6. Synergistic effects of co-treatment of DLE and GFSE on TNF- α (A) and IL-6 secretion (B) in PMA + calcium ionophore A23187-stimulated HMC-1 cells. Human mast cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) in 6 well plates for 24 h with DLE, GFSE, DLE, and GFSE or nothing at all. The cells were then stimulated with 50 nm of PMA (phorbol 12-myristate 13-acetate) + 0.001 mM of calcium ionophore A23187 for 16 h. The culture supernatant was used to quantify IL-6 and TNF- α by ELISA. Data are presented as the mean $\pm$ SD of duplicate measures. ***p < 0.001 vs DLE or GFSE alone.

strongly inhibited production of these cytokines. These observations show that combined DLE and GFSE possess anti-inflammatory effects partly by strongly regulating the production of inflammatory cytokines in mast cell. We then went ahead to investigate if our combined extract could also ameliorate DNFB + house dust mites-induced AD-like symptoms in mice. Continual DNFB + house dust mite antigen application will disturb epidermal barrier and induce adhesion molecules such as intracellular adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM), thymic stromal lymphopoietin (TSLP) expression and Fc ϵ R1 on eosinophil or mast cells and the secretion of immune mediators such as IL-5, IL-13 [28] that ultimately increases the severity of atopic dermatitis. We observed that repeated treatment of mice with combined doses of DLE and GFSE significantly relieved edema and excoriation much better than the single extracts alone. More so, increased skin and ear swelling were inhibited. Further research is needed to investigate the molecular mechanism of combined DLE and GFSE that inhibits production of inflammatory mediators and amelioration of AD.

5. Conclusion

In conclusion, our data, to the best of our knowledge showed for the first time that the combined treatment of an atopic dermatitis-like lesion in mice with DLE and GFSE significantly ameliorates atopic dermatitis in mice suggesting that this synergistic approach of a combination of 2 or more extracts could be an effective approach for the treatment of AD.

Authors' contributions

Byoung Ok Cho and Seon Il Jang designed the research. Denis Nchang Che, Hong Hua Yin, and Jae Young Shin performed the research. Byoung Ok Cho and Seon Il Jang analyzed the research data. Byoung Ok Cho, Denis Nchang Che, and Seon Il Jang wrote the manuscript.

Conflicts of interests

The authors declare that there is no conflict of interests.

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