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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.

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 CD_4 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-1α/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; CD_4, cluster of differentiation 4; NF-κB, 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-κB 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 μm 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 Baekgumyeon 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 μm 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 μg/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. Group1- 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 μm thin sections were cut and stained with hematoxylin-eosin stain and toluidine blue stains. Histopathological changes were examined under the light microscope (Leica, wetzla, 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