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Ameliorative effects of *Juniperus rigida* fruit on oxazolone- and 2,4-dinitrochlorobenzene-induced atopic dermatitis in mice

Sullim Lee[a], No-June Park[a], Sim-Kyu Bong[a], Jonghwan Jegal[b], Sang-a Park[b], Su-Nam Kim[a,*], Min Hye Yang[b,*]

[a] Natural Products Research Institute, Korea Institute of Science and Technology, Gangneung 25451, Republic of Korea
[b] College of Pharmacy, Pusan National University, Busan 46241, Republic of Korea

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ABSTRACT

Ethnopharmacological relevance: The fruits of *Juniperus rigida* have been used in Korean traditional medicine for the treatment of inflammatory diseases in humans such as rheumatoid arthritis.

Aim of the study: This study aimed to investigate the anti-atopic properties of *J. rigida* fruit in *in vivo* murine atopic dermatitis (AD) models.

Methods and results: BALB/c mouse ears ad SKH-1 hairless mice stimulated with oxazolone (4 weeks) and DNCB (3 weeks), respectively, were treated with the 1% *Juniperus rigida* fruit EtOH extract (JFE). The JFE improved AD symptoms in both oxazolone- and DNCB-induced AD mice by accelerating skin barrier recovery function and suppressing the overproduction of serum immunoglobulin E (IgE) and interleukin 4 (IL-4). The JFE was found to contain isoscutellarein-7-*O*-β-xylopyranoside, cupressuflavone, podocarpusflavone A, and hinokiflavone as major components based on phytochemical analysis. Eight flavonoids were isolated from JFE, and of those, cupressuflavone and isoscutellarein-7-*O*-β-xylopyranoside strongly down-regulated IL-4 expression and β-hexosaminidase release in RBL-2H3 cells.

Conclusion: Therapeutic attempts with *J. rigida* fruit and its active components might be useful in treating AD and related skin inflammatory diseases.

1. Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common multifactorial chronic inflammatory skin disease. Worldwide, AD occurs with a prevalence of 2–10% in adults and up to 15–30% in children (Kapoor et al., 2008). Atopic dermatitis arises most frequently in childhood, and its prevalence has increased over the last three decades (Kapoor et al., 2008). The symptoms of AD include intense pruritus, dry skin, and skin hypersensitivity (Guttman-Yassky et al., 2011). Both extrinsic (environmental or allergic) and intrinsic (genetic or non-allergic) factors are involved in the pathophysiology of AD (Jonathan and Amy, 2003). Although the major risk factor for AD is still unknown, allergic sensitization by extrinsic agents appears to more precisely define the onset of AD (Jonathan and Amy, 2003; Misery, 2011). Extrinsic or environmental triggers enhance the IgE-mediated sensitization and allergic reaction, thus further contributing to severe forms of skin inflammation in AD (Darlenski et al., 2014).

Diverse inflammatory cytokines orchestrate atopic skin inflammation. According to previous studies, mononuclear cells from AD patients produced higher amounts of IL-4, IL-5, and IL-13 compared to those from healthy individuals (Poulsen et al., 1995; Leung, 2000). In addition, the ratio of IL-4-producing cells to IFN-γ was distinctly increased in acute AD lesions (Poulsen et al., 1995; Leung, 2000). Current treatments for AD include a topical ointment and oral medication to target a reduction in inflammation damage and itching (Cury Martins et al., 2015). Corticosteroid creams are usually used for the treatment of chronic AD but have side effects such as acne induction and skin atrophy (Hengge et al., 2006). Pimecrolimus (Elidel, SDZ ASM 981) and tacrolimus, which are immunosuppressive calcineurin inhibitors, are also known to be effective in the treatment of severe AD (Cury Martins et al., 2015; Huang and Xu, 2015). However, these topical ointments can lead to increased risks of transient localized inflammation and burning pain (Tatlican et al., 2009). For this reason, attention has continuously focused on new anti-atopic agents derived from natural

Abbreviations: JFE, *Juniperus rigida* fruit EtOH extract; AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; IL-4, interleukin-4; IgE, immunoglobulin E; Th2, T-helper cell type-2; TEWL, transepidermal water loss; PMA, phorbol 12-myristate 13-acetate

* Corresponding authors.
E-mail addresses: sullimlee@kist.re.kr (S. Lee), 115519@kist.re.kr (N.-J. Park), 115044@kist.re.kr (S.-K. Bong), jhjegal@pusan.ac.kr (J. Jegal), sapark@live.co.kr (S.-a. Park), snkim@kist.re.kr (S.-N. Kim), mhyang@pusan.ac.kr (M.H. Yang).

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resources to minimize the possibility of adverse drug reactions.

Juniperus rigida belongs to the Cupressaceae family and is an evergreen conifer that mainly grows in China, Korea, and Japan. The fruits of *J. rigida* have been used in traditional Korean medicine to treat inflammatory diseases such as rheumatoid arthritis and edema (Lee et al., 2010). *Juniperus rigida* branches and leaves were recommended as a traditional cure for rheumatoid arthritis, nephritis, and skin diseases in Tibetan and Mongolian medicines (Luo, 2004; Ministry of Health Institution for Biological Products Control, 1984). *J. rigida* contains diverse phenolics and lignans known to have anti-inflammatory, anticancer, and antiviral activities (Jeong et al., 2012; Cho et al., 2001). The biological activity of *J. rigida* extract against inflammatory disorders has been already documented (Han et al., 2016). However, there have not been any attempts to reveal the anti-atopic effect of *J. rigida* fruit. In this study, we investigated the potential therapeutic effects of *J. rigida* fruit EtOH extract (JFE) on oxazolone- and 2,4-dinitrochlorobenzene (DNCB)-induced AD animal models. Histological analysis was performed to observe the cutaneous changes in JFE-treated mice. The anti-inflammatory properties of isolates from JFE and the modulation of IL-4 expression were also evaluated in RBL-2H3 cells to identify the active constituents.

2. Material and methods

2.1. Plant material and extraction

The fruits of *J. rigida* were purchased at Kyung-Dong Herb market (Seoul, Korea) and identified by Prof. Eun Ju Jeong of the Department of Agronomy and Medicinal Plant Resources, Gyeongnam National University of Science and Technology. A voucher specimen (PNU-0021) has been deposited in the Medicinal Herb Garden, Pusan National University. Dried fruits of *J. rigida* (900 g) were extracted with 95% EtOH (20 L) and evaporated under reduced pressure to yield *J. rigida* fruit extract (JFE) (39 g).

2.2. Animals

Six-week-old female BALB/c and SKH-1 hairless mice were purchased from the animal facility of Orient Bio Inc. (Seongnam, Republic of Korea) and housed in an air-conditioned animal room at a temperature of 25 ± 5 °C and 55 ± 5% humidity. Mice were given access to a standard laboratory diet and water *ad libitum*. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication no. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the KIST (Certification no. KIST-2016-011).

2.3. Evaluation of ear swelling and erythema in the oxazolone-induced AD mouse model

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) (1%) dissolved in vehicle (propylene glycol: EtOH = 7:3) was used to induce atopic dermatitis in BALB/c mouse ears as described previously (Blaylock et al., 1995); the experimental scheme is shown in Fig. 1A. Briefly, the ears of BALB/c mice in both the oxazolone (OX)-treated group and the oxazolone and 1% JFE-treated (OX-JFE) group were sensitized with 20 μL of 1% oxazolone on the first day. After the first challenge, 20 μL of 0.1% oxazolone was repeatedly applied to the ears for an additional 3 weeks at 2-day intervals. At the same time, 20 μL of JFE was applied daily to the ears of the BALB/c mice in the OX-JFE group for 3 weeks, and the application of JFE was separated by 4 h from that of oxazolone. The control (CON) group was treated with distilled water instead of oxazolone. No substances were applied to the skin surface on the last day of the experiment. On the last day, measurements of skin inflammation signs, including ear swelling and erythema, were carried out. The mice were then sacrificed, and skin was collected for further analysis.

2.4. Evaluation of skin severity in the DNCB-induced AD mice model

DNCB dissolved in acetone was used to induce atopic dermatitis in SKH-1 hairless mice as described previously (Matsumoto et al., 2005, 2004). The experimental scheme is shown in Fig. 2A. Briefly, the dorsal skin of hairless mice in both the DNCB-treated group and the DNCB and 1% JFE-treated (DNCB-JFE) group were sensitized with 100 μL of 1% DNCB daily for 7 days. After the first challenge, 100 μL of 0.1% DNCB was repeatedly applied to the dorsal skin for an additional 2 weeks at 3-day intervals. At the same time, 100 μL of JFE was applied daily to the entire dorsal skin of the hairless mice in the DNCB-JFE group for 2 weeks, and the application of JFE was separated by 4 h from that of DNCB. The control (CON) group was treated with distilled water instead of DNCB. No substances were applied to the skin surface on the last day of the experiment. Every week, characteristics of skin barrier function, including transepidermal water loss (TEWL), hydration and erythema were evaluated. On the last day, mice were sacrificed, and skin and blood samples were collected for further analysis.

2.5. Measurement of TEWL and skin hydration

A Tewameter TM 210 device (Courage and Khazaka, Cologne, Germany) was used to evaluate the skin surface of the hairless mice at the end of the experiment according to the manufacturer's instructions. A SKIN-O-MAT (Cosmomed, Ruhr, Germany) was used to evaluate the skin surface of the hairless mice at the end of the experiment according to the manufacturer's instruction. TEWL and hydration were measured under standard conditions.

2.6. Measurement of total serum IgE and IL-4 levels by ELISA

Blood samples were centrifuged at 10,000 rpm for 15 min at 4 °C, and then serum was collected and stored at − 80 °C for further investigations. Total IgE and IL-4 concentration in mouse serum were measured *via* enzyme-linked immunosorbent assay (eBioscience, San Diego, USA) according to the manufacturer's instructions.

2.7. Histopathological examination

Tissue specimens from the ear or dorsal skin of mice were removed, fixed in 10% formalin, embedded in paraffin, and serially sectioned at 2–3 mm. Tissue sections were then stained with hematoxylin and eosin. Histopathological changes were examined by light microscopy (Olympus CX31/BX51, Olympus Optical Co., Tokyo, Japan) and photographed (TE-2000U, Nikon Instruments Inc., Melville, USA)

2.8. Phytochemical analysis by HPLC-Time of Flight (TOF)-MS

The JFE was analyzed with an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies) for phytochemical characterization. A Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 μm, Agilent) was used for the analysis at a flow rate of 0.3 ml/min. The mobile phase consisted of acetonitrile (solvent A) and water (solvent B) with a linear gradient elution: 5–95% A (0–10 min); 10% A (10–20 min). All acquisitions were performed under positive ionization mode. Mass spectra were recorded across the range m/z = 100–1500 with accurate mass measurement of all mass peaks.

2.9. General methods for compound isolation

^{1}H and ^{13}C NMR, COSY, HMQC, HMBC, and NOESY spectral data were run on an Agilent Superconducting FT NMR 400–500 MHz Spectrometer System. HR-ESI mass spectra were recorded on an Agilent