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ORIGINAL ARTICLE

Ethanol Extract of *Thuja orientalis* L. Seeds Ameliorated Skin Lesions in a Dinitrofluorobenzene-Induced Mouse Model of Contact Dermatitis

Kukhwa Kim[#], Daniel Lee[#], Yoonhyoung Kang, Seonkyung Jang, Seung-Jeong Yang[1], Hyungwoo Kim

Division of Pharmacology, School of Korean Medicine, Pusan National University, Yangsan City, Gyeongnam, [1]Department of Korean Medicine Obstetrics and Gynecology , Korean Medicine Hospital, Dongshin University, Naju, Jeonnam, South Korea
[#]These authors contributed equally to this work

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ABSTRACT

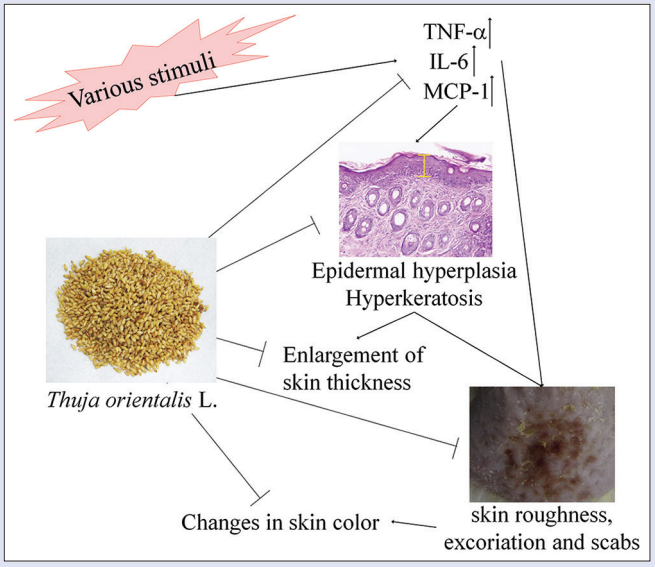
Background: Seed oils like sesame and camellia oils are used in herbal medicine to treat skin diseases and it has been reported that the seeds of *Thuja orientalis* have antioxidant, antibacterial, anti-inflammatory, and anti-allergic effects. **Objectives:** We investigate the anti-inflammatory effects of an ethanol extract of the seeds of *T. orientalis* (EETO) on a dinitrofluorobenzene-induced mouse model of contact dermatitis (CD). **Materials and Methods:** We investigated the effects of EETO on CD lesions and skin thickness, skin color, histopathologic findings, and cytokine production in mouse skins. **Results:** EETO ameliorated skin roughness, excoriation, scabs, and erythema. EETO also reduced erythema, melanin index, and inhibited skin thickness increases in CD mice. In addition, histopathologic examinations showed that EETO decreased epidermal hyperplasia, keratinization, and the productions of inflammatory cytokines (i.e., interleukin [IL]-6, tumor necrosis factor [TNF]-α, and monocyte chemotactic protein [MCP]-1). **Conclusion:** These results suggest that ethanolic extracts of the seeds of *T. orientalis* might provide relatively safe treatments for CD and that its therapeutic effect is exerted through inhibitions of IL-6, TNF-α, and MCP-1.

Key words: Contact dermatitis, herbal medicine, inflammation, seed oil, *Thuja orientalis*

SUMMARY

- Ethanolic extract of the seeds of *Thuja orientalis* (EETO) ameliorated skin lesions of CD such as roughness, excoriation, and scabs
- EETO reduced erythema, melanin index, and inhibited skin thickness increases
- EETO decreased epidermal hyperplasia and keratinization in inflamed tissues
- EETO prevented the productions of inflammatory cytokines such as interleukin-6, tumor necrosis factor-α, and monocyte chemotactic protein-1.

Abbreviations used: CD: Contact dermatitis; IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon; EETO: Ethanolic extract of the seeds of *T. orientalis*; CBA: Cytometric bead array; ED: Experimental day; DNFB: Dinitrofluorobenzene; CTL: CD control; NOR: Normal; DEX: Dexamethasone; MCP: Monocyte chemotactic protein.



Correspondence:
Prof. Hyungwoo Kim,
Division of Pharmacology, School of Korean Medicine, Pusan National University, Yangsan City, Gyeongnam, 50612, South Korea.
E-mail: kronos7@pusan.ac.kr
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INTRODUCTION

Seed oils extracted from plants have long been used on the skin as cosmetics and as medical agents because they have many positive dermatophysiological benefits. Plant seed oils, such as olive oil, coconut oil, argan oil, soybean oil, sesame oil, avocado oil, jojoba oil, almond oil, and shea butter, have anti-inflammatory and antioxidant effects on skin and promote wound healing and skin barrier repair.[1] These plant seed oils are composed of unsaturated and saturated fatty acids such as linoleic (omega 6), oleic, palmitic, and stearic acids, which both prevent and provide treatments for skin microbial infections, inflammation, and barrier disruption.[2] In particular, the skin barrier protective effects of seed oils protect the skin from dehydration.[3]

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Thuja orientalis L. is a member of the *Cupressaceae* (cypress) family and is native to Korea, China, and Japan,[4] and in Korean traditional medicine, its seeds are widely used to treat weakness, insomnia, and constipation. It has been reported that the seeds of *T. orientalis* contain steroids, diterpenoids, sesquiterpenes, and many fatty acids,[4-6] which have been shown to have calming, nourishing, strengthening, and laxative effects.[7,8] In addition, previous studies have shown that the seeds of *T. orientalis* have antioxidant, anti-inflammatory, anti-allergic, anticancer, and melanin inhibitory effects. They are usually used to treat atopy and hair loss.[9-14]

Contact dermatitis (CD) is caused by repeated exposure to various allergens or stimuli.[15] Skin symptoms include itching, erythema, papules, petechiae, and excoriation during the acute period and dryness, hyperkeratosis, and even skin fissures in the chronic phase.[15,16] Melanin often accumulates postinflammation, which causes cosmetic problems.[16,17] In addition, the inflammatory cytokines interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, heavy metals, and chemokines such as IL-8 and interferon (IFN)-γ that induce/prolong inflammation are secreted by stimulated keratinocytes and may cause edema by inducing inflammatory cell infiltration.[18]

Given the above background, we investigated the therapeutic effects of an ethanolic extract of the seeds of *T. orientalis* (EETO) in a dinitrofluorobenzene-induced mouse CD model that exhibited symptoms including itching, erythema, excoriation, and hyperkeratosis. Clinically relevant aspects such as skin surface lesions, cytokine levels, and histopathological changes were observed.

MATERIALS AND METHODS

Preparation of *Thuja orientalis* extract

T. orientalis seeds were obtained from Gwangmyeong dong (Ulsan, South Korea), authenticated by Professor Jung-Hoon Kim (Voucher no. MS2017-007), and extracted with ethanol as previously described.[19]

Animals

Balb/c mice were used in all experiments (male, 6-week-old, Samtaco, Gyeonggi, Republic of Korea). The study protocol was approved beforehand by the Animal Care and Use Committee of our institution (PNU-2016-1301).

Induction of contact dermatitis and experimental design

The experimental CD was produced using a standardized method as previously described.[20] The experimental procedure is schematized in Figure 1.

Evaluation of skin lesion severities

Severity scores of skin lesions (i.e., erosion, scabs, surface roughness, and redness) were evaluated as we previously described[19] using a 4-point scale (score 0 = no symptom, score 1 = light, score 2 = mild, and score 3 = severe).

Measurement of skin thicknesses

Dorsal skin samples were obtained using a biopsy punch (diameter 5 mm). Skin thicknesses were measured using Vernier calipers (Mitutoyo, Tokyo, Japan).

Evaluation of erythema and melanin index

Mouse skin colors of shaved dorsal skins were assessed using a skin colorimeter (DSM II, Cortex Technology, Denmark). Measurements were made at three different points on each mouse.

Histopathological examination

Inflamed tissue was excised, embedded in paraffin, and subsequently, stained with hematoxylin and eosin. Stained tissues were observed under an optical microscope (×100). Epidermal hyperplasia was assessed as previously described.[19]

Measurement of cytokine levels

Cytokine levels in skin samples were evaluated using a cytometric bead array (CBA) Mouse Inflammation Kit (BD, San Jose, CA, USA) according to the manufacturer's instructions.

Calculation of spleen body weight ratios

Body weight changes between experimental day 1 (ED1) and ED16 are presented as percentages of weights on ED1. Spleens were weighed after sacrificing on ED16.

Statistical analysis

One-way ANOVA followed by Dunnett's multiple comparison test was used to determine the significances of intergroup differences. The analysis was performed using Prism 5 (version 5.01) software. Results are presented as means ± standard deviations, and statistical significance was accepted for $P < 0.05$.

RESULTS

Ethanolic extract of the seeds of *Thuja orientalis* ameliorated contact dermatitis symptoms and inhibited skin thickening

Topical dinitrofluorobenzene (DNFB) treatment induced severe skin symptoms such as skin roughness, excoriation, and scabs on the shaved backs of mice in the CD control (CTL) group. These skin symptoms were improved by the topical application of EETO [Figure 2A]. The mean severity score in the CD control group was significantly greater than in the normal (NOR) group. EETO treatment at ≥75 μg/day produced significant improvements. Dexamethasone (DEX) treatment also produced a significant improvement [Figure 2B]. The skin thickness in the CD control group was significantly greater than in the NOR group. Treatment with EETO significantly prevented skin thickening even at the lowest concentration used (25 μg/day). The topical application of DEX also significantly reduced DNFB-induced skin thickening [Figure 2C].

Ethanolic extract of the seeds of *Thuja orientalis* reduced lowered erythema and melanin indices

Erythema indices were significantly higher and melanin indices were slightly higher in the CD group than in the NOR group. Topical treatment with EETO significantly reduced erythema and melanin indices. On the other hand, DEX aggravated melanin and erythema indices [Figure 3].

Ethanolic extract of the seeds of *Thuja orientalis* inhibited epidermal hyperplasia and hyperkeratosis

Topical application of DNFB induced epidermal hyperplasia and hyperkeratosis in the CTL group, but animals treated with EETO showed decreases in both variables [Figure 4A] and at ≥250 μg/day EETO significantly reduced epidermal hyperplasia [Figure 4B].

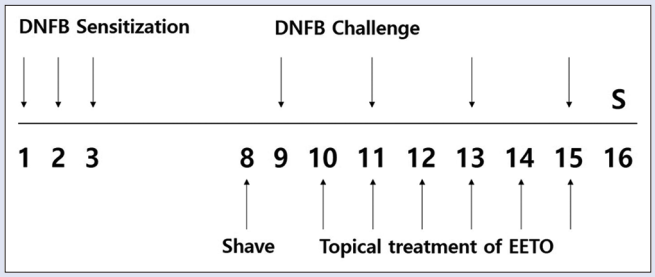

Figure 1: Experimental design. Dried seeds of *Thuja orientalis* were extracted using 70% ethanol (yield, 6.5%). Ethanol extract of *Thuja orientalis* seed and dexamethasone groups were topically treated with EETO or DEX for 6 days. The naive animals (NOR) were treated with vehicle for induction and treatment (n = 7). Control animals (CTL) were painted with DNFB for induction and painted with vehicle (n = 7). EETO-treated animals were painted with 60 μl of EETO(25, 75, and 250 μ50O ted onto the shaved back for 6 days (n = 8). DEX(75 μg/day) was used as a positive control. S means sacrifice. All animals were sacrificed on day 16. CTL: CD control; EETO: Ethanol extract of the seeds of *Thuja orientalis*; NOR: Normal; DEX: Dexamethasone

Ethanolic extract of the seeds of *Thuja orientalis* lowered tumor necrosis factor-αN, interleukin-6, and monocyte chemotactic protein-1 levels in skin tissues

Significant increases in TNF-α, IL-6, and monocyte chemotactic protein (MCP)-1 levels were observed in the CD group and topical application of EETO effectively suppressed these DNFB-induced increases [Figure 5].

Ethanolic extract of the seeds of *Thuja orientalis* did not affect body weights or spleen/body weight ratios

Changes in body weights and spleen body weight ratios between ED1 and ED16 in the EETO treated groups were similar to those observed in the NOR group. On the other hand, DEX treatment significantly reduced weight gain and spleen/body weight ratios [Figure 6].

DISCUSSION

The number of patients with dermatitis is increasing. CD is often associated with the occupational activities and like other inflammatory diseases, manifests symptoms of pain, erythema, redness, and edema. However, in CD, lesions are visually obvious, vary on an individual basis, are uncomfortable, and in many patients have psychological consequences. As a result, corticosteroids are

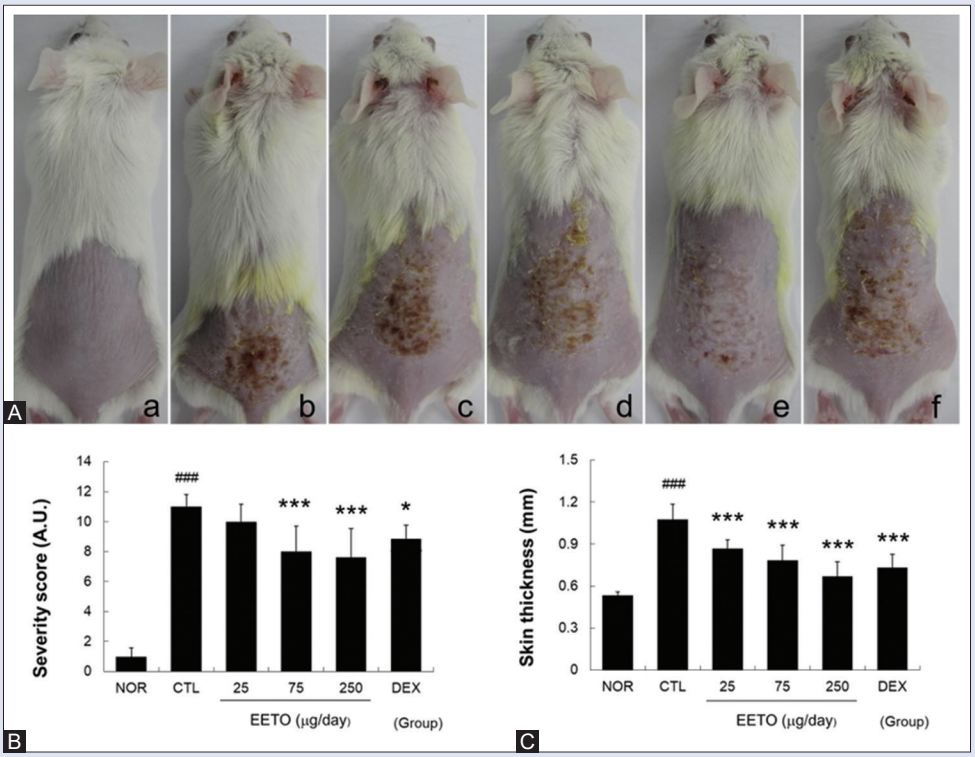

Figure 2: Effects of EETO on skin lesions and thickness in CD mice. The skin lesions of each mouse were observed using a digital camera on day 16. a NOR group; b. CTL group; c. 25 μg/day; EETO group; d. 75 μg/day; EETO group; e. 250 μg/day; EETO group; f. DEX group (A). NOR, Naive group; CTL, nontreated CD mice group; EETO, ethanol extract of *Thuja orientalis* seed treated CD mice groups; DEX, Dexamethasone treated CD mice group. A. U. means arbitrary unit (B). Abbreviations are the same as Figure 2b (C). All values are expressed as the mean ± standard deviation. $^{###}P < 0.001$ versus NOR; $^{*}P < 0.05$ and $^{***}P < 0.001$ versus CTL. CTL: CD control; EETO: Ethanol extract of the seeds of *Thuja orientalis*; NOR: Normal; DEX: Dexamethasone; CD: Contact dermatitis

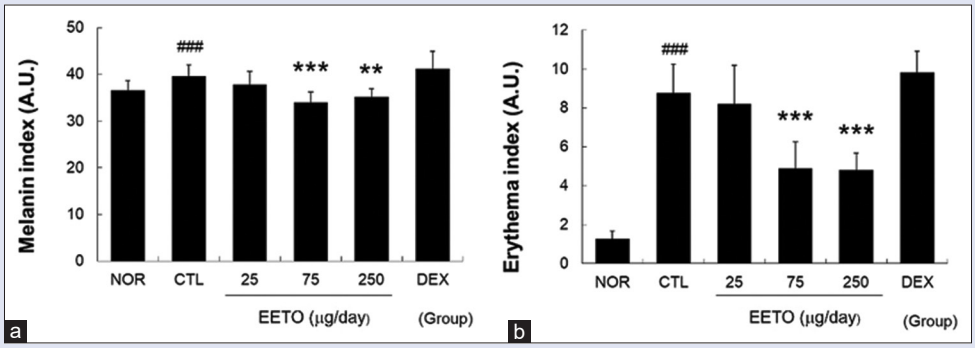

Figure 3: Effects of ethanol extract of the seeds of *Thuja orientalis* on erythema and melanin index. The melanin index (a) and erythema index (b) were measured using a dermospectrophotometer. Abbreviations are the same as Figure 2. All values are expressed as the mean ± standard deviation. ###$P < 0.001$ versus normal; **$P < 0.01$, and ***$P < 0.001$ versus CD control

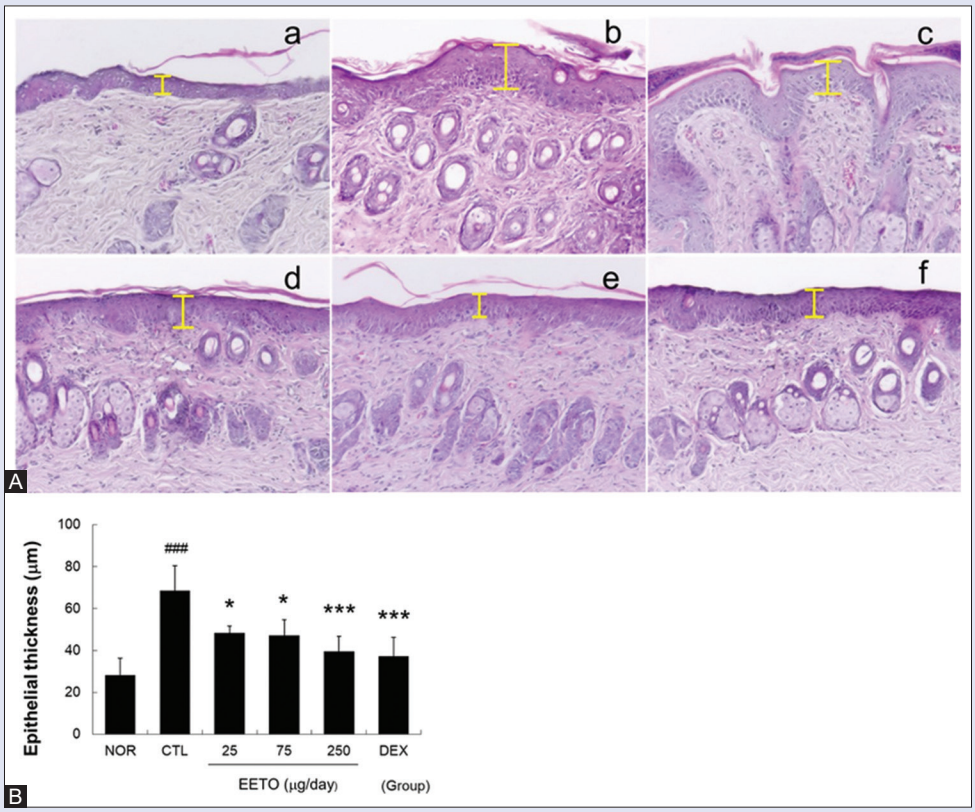

Figure 4: Effects of ethanol extract of the seeds of *Thuja orientalis* on histopathological changes in contact dermatitis mice. The skin tissues were stained with hematoxylin and eosin (H and E) and observed with a light microscope (×100). Yellow bars indicate the vertical length of the epidermis (A). The vertical length between the basal lamina and the top of the stratum granulosum was quantified (B). Abbreviations are the same as Figure 2. All values are expressed as the mean ± standard deviation. ###$P < 0.001$ versus normal; *$P < 0.05$ and ***$P < 0.001$ versus CD control

frequently and repeatedly used to treat inflammatory and allergic disorders. However, corticosteroids have well-known side effects such as immunosuppression, skin atrophy, and Cushing's syndrome.[21] In particular, skin atrophy causes problems such as wrinkles, reduced skin elasticity, and vessel exposure. For this reason, drugs safer than corticosteroids are required, and drugs extracted from herbs are viewed as a possible solution.

In the present study, EETO ameliorated DNFB-induced skin surface symptoms, lowered erythema and melanin indices, and alleviated gross skin thickening [Figure 2] and histopathologically determined epidermal thickening [Figures 3 and 4]. Although the ameliorative effect of EETO on skin surface symptoms was not significant at low concentration [Figures 2B and 3], significant improvements in severity scores, skin thickening, epidermal thickening, and erythema and melanin indices were observed in animals treated with EETO at 75 or 250 μg/day [Figures 3 and 4]. Furthermore, EETO-induced improvements in severity scores and skin thickening occurred in a dose-dependent manner. DEX did not influence melanin or erythema indices [Figure 3].

TNF-α is a typical inflammatory cytokine and is the target of most anti-inflammatory treatments. In CD affected skin, TNF-α levels are elevated and keratinocytes are activated, and these changes result

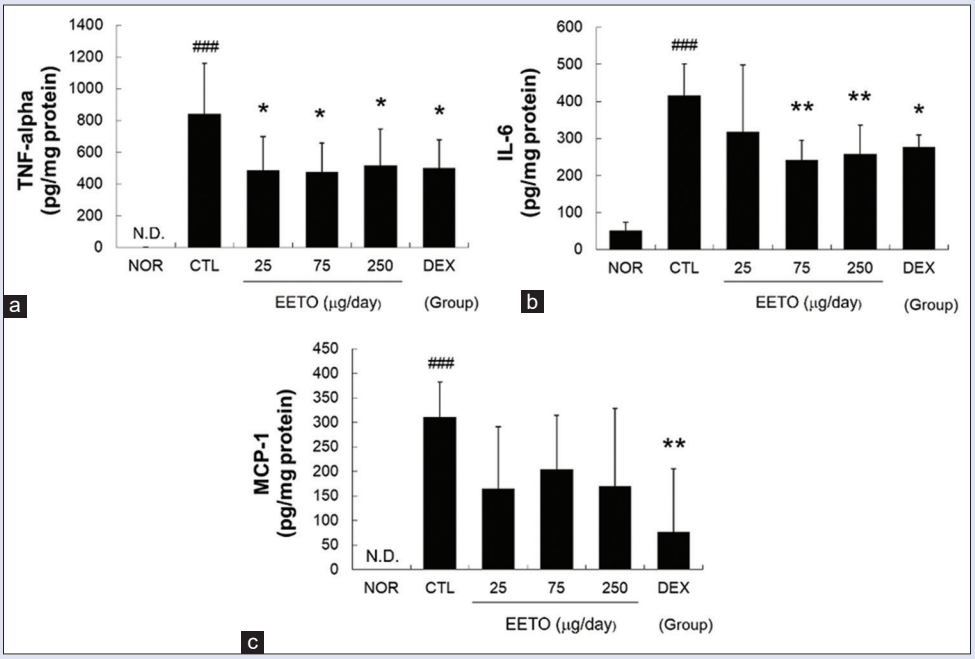

Figure 5: Effects of ethanol extract of the seeds of *Thuja orientalis* on cytokine and chemokine levels in inflamed tissues. The levels of cytokines and chemokine were evaluated using the cytometric bead array method. (a) tumor necrosis factor-NF (b) interleukin-6; (c) monocyte chemotactic protein-1. N. D. means not detectable; Abbreviations are the same as Figure 2. All values are expressed as the mean ± standard deviation. $^{###}P < 0.001$ versus normal; $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CD control

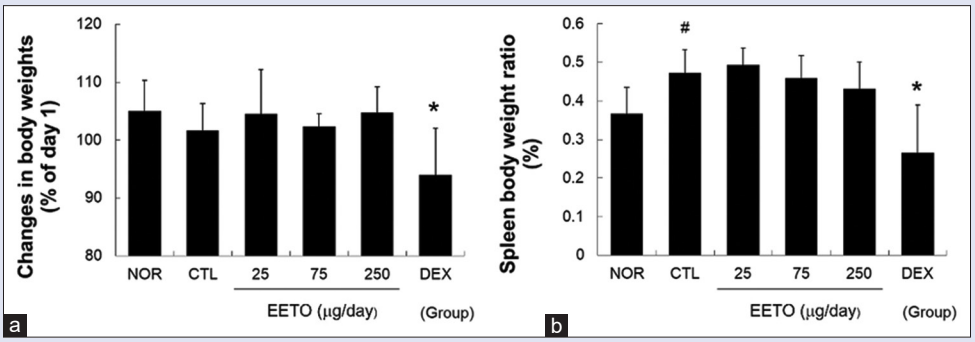

Figure 6: Effects of ethanol extract of the seeds of *Thuja orientalis* on changes in body weight and spleen/body weight ratio in contact dermatitis mice body and spleen weight were measured on day 15 and the spleen/body weight ratio was calculated. (a), changes in body weights; (b), spleen/body weight ratio. Abbreviations are the same as Figure 2. $^{#}P < 0.05$ versus normal; $^{*}P < 0.05$ versus CD control

in epidermal hyperplasia and hyperkeratosis,[22] which lead to skin inflammation and skin thickening. Treatment with EETO was found to reduce TNF-α levels [Figure 5a], which suggests that its inhibition of skin inflammatory responses is due to its targeting of TNF-α.

IL-6 and MCP-1 are chemokines that recruit leukocytes and monocytes and when these cells aggregate, they cause pathologic changes in the skin.[23] EETO reduced IL-6 and MCP-1 levels, though its effect on MCP-1 was not not significant [Figure 5b and c]. These results indicate that EETO reduced skin inflammatory response by inhibiting the chemokines involved in immune cell infiltration.

Characteristically, animals in the DEX group showed a significant reduction in body weight and spleen/body weight ratio as compared with the other groups. Body weight reduction is a known side effect of corticosteroid use. Spleens (a component of the secondary immune system) generally become enlarged in the presence of systemic inflammatory response, but in the DEX group, spleens were reduced in size [Figure 6]. This suggests that DEX reduces inflammatory reactions by systemically suppressing the immune system, which in turn, suggests that DEX might inhibit normal immune responses to infections.

In contrast, EETO treatment improved inflammatory response but did not affect normal body weight change or spleen/body weight ratios. These results suggest that EETO, unlike DEX, does not inhibit the systemic inflammatory response, but rather inhibits the expressions of inflammatory cytokines and chemokines locally in the skin. As a result, DEX and EETO similarly improved dermatitis in our model, but DEX was not as effective as EETO at ameliorating skin symptoms.

Melanin accumulates and pigments skin,[17] however it has been suggested that EETO has an inhibitory effect on melanin production [Figure 3a].[24] Furthermore, it has been reported that herb seed oils reinforce skin barrier properties and prevent dehydration.[25] Therefore, it would appear that the seed oil *T. orientalis* also has a moisturizing effect on the skin.

CONCLUSION

This study shows that EETO reduces the severities of DNFB skin lesions and inhibits inflammatory cytokine production in CD mice and thus, ameliorates CD symptoms (erythema, melanin, and roughness) and prevents epidermal hyperplasia and hyperkeratosis. EETO also attenuated visual symptoms without causing the systemic immune suppression observed after DEX treatment. Our findings suggest that EETO offers a potential means of safely replacing corticosteroids for the treatment of inflammatory skin diseases like CD.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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