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Topical treatments of *Saussurea costus* root and *Thuja orientalis* L. synergistically alleviate atopic dermatitis-like skin lesions by inhibiting protease-activated receptor-2 and NF-κB signaling in HaCaT cells and Nc/Nga mice

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ABSTRACT

Ethnopharmacological relevance: The root of *Saussurea costus* (*Aucklandia lappa* Decne, Aucklandiae Radix, SC) and *Thuja orientalis* L. (TOL) have been traditionally used as anti-inflammatory agents in Korea. However, they have not been studied for the efficacy of atopic dermatitis (AD) treatment, a chronic inflammatory skin disease. We investigated the efficacy of topical applications with 1,3-butyleneglycol extracts of SC and TOL to alleviate the symptoms of AD.

Materials and methods: HaCaT cells and the dorsal skin of Nc/Nga mice had a local exposure of house mite extracts and 2,4-dinitrochlorobenzene (DNCB), respectively. After lesions developed, we topically applied 1,3-butylen glycol (vehicle; control), SC (30%), TOL (30%), or SC (15%)+TOL (15%) to the skin lesions for 5 weeks. The normal-control was not exposed to DNCB. The skin thickness, mast cell infiltration, serum immunoglobulin E (IgE) and IgG1 and gene expressions of interleukin (IL)−4, IL-13, and IFN-γ in the dorsal skin and HaCaT cells were measured.

Results: Chlorogenic acid (129.6 ± 10.2 μg/g) for SC and catechin and apigenin (93.4 ± 13.2 and 16.9 ± 1.3 μg/g, respectively) for TOL were used as indicator compounds for the strength of the extracts. SC+TOL decreased the expression of protease-activated receptor-2 and ICAM-1 and the release of TNF-α and IL-6 in HaCaT cells activated by 3 μg/mL house mite extracts in comparison to either of SC or TOL alone. In Nc/Nga mice challenged with DNCB, SC+TOL synergistically attenuated clinical symptoms of AD such as erythema, hemorrhage, edema, excoriation and dryness in the dorsal skin better than either SC or TOL alone. Histological analysis of the dorsal skin also showed that SC+TOL treatment significantly and additively decreased the inflammatory cellular infiltrate, including mast cells and eosinophils in comparison to either of SC or TOL. SC+TOL also decreased serum IgE and IgG1 levels and the expression of IFN-γ, IL-4, and IL-13 mRNA in dorsal skin in DNCB-treated Nc/Nga mice.

Conclusion: SC+TOL relieved the symptoms of AD by reducing pro-inflammatory activity and over-activated immune responses. These data suggest that SC+TOL may be an effective alternative intervention for the management of AD.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease. The prevalence of childhood AD has increased from 9.2% at in 1995 to 20.6% at in 2010, and has the greatest prevalence among chronic diseases especially in low-income countries (Nutten, 2015). Although the reason for its increase is not known, it may be related to allergens that induce inflammation and itching. This process is associated with

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autoimmune disease, and lifestyle changes including sanitation, and dietary patterns increases the prevalence of autoimmune diseases including AD (Darlenski et al., 2014). Since AD negatively influences the mental and physical growth of the children (Misery, 2011), parents are highly motivated to treat AD. Its exact causes remain unknown, but environmental factors lead to immunological abnormalities that contribute to the development of AD (Darlenski et al., 2014; Misery, 2011). Therefore, interventions that treat the fundamental causes of AD are not available. Current treatments mainly attempt reduce inflammation and itching by applying topical ointments and administering oral medications (Cury Martins et al., 2015). Topical corticosteroid cream has adverse effects such as skin atrophy and acne induction (Hengge et al., 2006). Tacrolimus and pimecrolimus, immunosuppressants, are also used as topical ointment and oral administration for the treatment of severe AD (Cury Martins et al., 2015; Huang and Xu, 2015). However, these topical ointments may also produce side effects such as transient local irritations and burning-tingling sensations (Tatlican et al., 2009).

Skin barrier dysfunction is a major characteristic of AD. Protease-activated receptor (PAR-2) is included in the G protein-coupled receptor subfamily and it is involved in epidermal permeability barrier function homeostasis (Steinhoff et al., 2003). PAR-2 is activated by proteases from allergens such as house mites and the activation by mites contributes to the delay of epidermal barrier recovery (Steinhoff et al., 2003). The activation of PAR-2 activates IκB kinase (IKK)→ nuclear factor-kappaB (NF-κB) signaling, which induces inflammation by increasing Th2 cells acutely. As a result, the expression of IL-4, IL-5, and IL-13 mRNA and protein increases in AD lesions (Spergel et al., 1999). The increase of proinflammatory cytokines is associated with the exacerbation of AD. Therefore, increased expression or activity of PAR-2 may be a pathophysiological factor for AD that induces the cytokines in skin tissues.

Since corticosteroids as AD treatment have adverse effects, alternative medicines like herbal extracts need to be developed with potent efficacy and minimal side effects. Several herbal extracts including *Thuja orientalis* L. and *Saussurea lappa* root (*Aucklandia lappa* Decne, Aucklandiae radix) have been traditionally used for alleviating various symptoms related to inflammation in Asian countries (Heo, 1613, 2005). *Thuja orientalis* L. of the family Cupressaceae contains flavonol derivatives such as quercetin-3-O-α-L-rhamnopyranoside and myricetin-3-O-α-L-rhamnopyranoside, as well as small amount of flavan compounds such as (+)-catechin and (+)-gallocatechin and monoterpene constitutes such as α-thujone (Kupeli Akkol et al., 2015). *Thuja orientalis* L. decreases the production of reactive oxygen species and NF-κB activation in HUVEC, along with tumor necrosis factor-α (TNF-α)-induced inflammation (Lee et al., 2010). *Thujae Orientalis* Semen also decreases the production of nitric oxide (NO), prostaglandin (PG)E_2 and IL-1β in lipopolysaccharides (LPS)-stimulated BV-2 microglia (Jung et al., 2013). *Thuja orientalis* L. has been used to treat gout, rheumatism, diarrhea and chronic tracheitis (Fan et al., 2012). The root of *Saussurea lappa* is included in the family Asteraceae, and contains sesquiterpene (costunolide), sesquiterpene lactone (dehydrocostuslactone, alanatolactone, isoalantolactone, saussurealactone), polyene alcohols, lignan, alkaloid and tannin (Jung et al., 1998; Kumar et al., 2014). The major components of *Saussurea lappa* root, costunolide and dehydrocostuslactone, have anti-microbial, ant-oxidant and anti-inflammatory activities (Jung et al., 1998). It has been demonstrated that the extracts of *Saussurea lappa* root alleviate inflammation induced by different inducers such as LPS, collagen, and house dust mites in various cells and tissues (Lim et al., 2014, 2015; Tag et al., 2016; Xu et al., 2016). Although *Saussurea lappa* root does not contain many flavonoids, much less than *Thuja orientalis* L., it does exert anti-inflammatory activities. The two herbs have different major components that suppress inflammation and they may have synergistic effects. However, their possible synergistic anti-inflammatory effects have not been studied.

Therefore, the hypothesis of the present study was that *Saussurea lappa* root and *Thuja orientalis* L. can synergistically alleviate the symptoms of AD in cell-based and animal studies. In this study, we examined the synergistic activity of extracts of *Saussurea lappa* root and *Thuja orientalis* L. against AD induced by butylene glycol in HaCat cells and induced by dust mite extracts and 2,4-dinitrochlorobenzene (DNCB) treatment in Nc/Nga mice, and the mechanisms for alleviating the symptoms were explored. HaCat cells are one of the cell lines used to mimic AD symptoms in response to allergens such as dust mites and DNCB by inducing immunological responses (Huang et al., 2016; Yang et al., 2015). Repeated application of dust mite extract and DNCB in mice have been reported to increase in serum IgE and T-helper (Th) 2 cytokines such as interleukin-4 (IL-4) and IL-13 at the chronic dermatitis site of mice (Kitagaki et al., 1995). These immunological changes are similar to those observed in AD patients (Leung et al., 2004).

2. Materials and method

2.1. Preparation of extracts

The root of *Saussurea lappa* (Aucklandiae radix) and leaves of *Thuja orientalis* L. were purchased in Kyung-Dong Herb market (Seoul, Korea) in 2011 and identified by Dr. Byung Seob Ko (Korean Herbal Medicine Institute, Daejeon, Korea) and Dr. Young Seung Joo (Department of Herbology, Woosuk University, Wanju, Korea). Voucher specimens were deposited at the herbarium of Department of Food & Nutrition, Hoseo University (No. 2014-05 and No. 2014-06, respectively). Since 1,3-butylene glycol is one of major solvents for cosmetic usages, it was used as the solvent to make the herbal treatments. *Saussurea lappa* root and *Thuja orientalis* L. were extracted with 3.3 L of 1,3-butylene glycol (a good solvent for making skin lotion) in an ultrasonic extractor at room temperature for 12 h, and filtered. The filtrates were centrifuged at 450×*g* and the supernatants were used for topical application and each extract was abbreviated as SC and TOL. The final concentration of each extract was 30%. The herbal extracts did not induce skin irritation such as rash without DNCB pretreatment in the preliminary study.

2.2. UPLC-MS/MS analysis

The components of SC and TOL were extracted with methanol and the methanol extracts were lyophilized. The powder was dissolved in methanol and their components were determined using an Acquity UPLC system (Waters, Miliford, MA, USA) with an Acquity UPLC BEH C18 column (2.1 mm×100 mm, 1.7 μm). The mass spectrometer was a Waters Xevo TQ triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) mode. MassLynx 4.1 (Waters) software was used for data processing. The mobile phase included 10 mM ammonium formate and 0.1% formic acid aqueous solution (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a gradient elution program was performed: 0–2 min, 90–90% solvent A; 2–3 min, 90–0% solvent A; 3–5 min, 0–0% solvent A; 5–5.5 min, 0–90% solvent A. The flow rate was set at 0.35 mL/min. Column temperature was kept at 40 °C and the total run time was 7 min. The auto-sampler was conditioned at 4 °C and the injection volume was 5 uL. An LC-MS/MS system was operated in negative ESI mode and scanned using multiple reaction monitoring (MRM) mode. The voltage of the capillary, cone and collision energy was set at 2.5 kV, 20 V and 15 V, respectively. The gas flow for desolvation and cone was 800 and 50 L/h. The source temperature and desolvation gas temperature was 150 and 400 °C, respectively.

2.3. Cell culture

HaCaT cells, a human immortalized keratinocyte cell line, were cultured as described previously (Park et al., 2015). HaCaT cells were