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Protective effect of *Rhododendron weyrichii* flower extract against UVB-induced proinflammatory cytokine production in human keratinocytes

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ABSTRACT

Ultraviolet B (UVB) radiation is harmful to the skin and induces cytokine release from keratinocytes leading to inflammatory skin disorders. Previous studies have shown that chronic exposure to UVB radiation increases tumor necrosis factor (TNF)-α and interleukin (IL)-6 secretion through various signaling pathways, resulting in skin inflammation and increased risk of skin cancer. The present study was undertaken to investigate the protective effects of *Rhododendron weyrichii* flower (RWF) extracts against UVB damage of immortalized human keratinocytes (HaCaT). To determine the anti-inflammatory effects of RWF, we examined UVB-induced proinflammatory cytokine production in HaCaT cells in the presence or absence of RWF extract, using enzyme-linked immunosorbent assay (ELISA). The results indicated that the RWF extract inhibited the production of proinflammatory molecules such as IL-6 and TNF-α, but not IL-8, in UVB-irradiated HaCaT cells. These results demonstrate that RWF potentially protects against UVB-induced skin inflammation. In addition, using high-performance liquid chromatography (HPLC) fingerprinting, kaempferol (0.335 ppm) and astragalin (2.569 ppm) were identified and quantified as RWF extract constituents. Moreover, we tested the potential application of RWF extracts as a cosmetic treatment by performing human skin primary irritation tests. In these tests, the RWF extracts did not induce adverse reactions. Based on these results, we suggest that RWF extracts be considered anti-inflammatory candidates for pharmaceutical and/or cosmetic applications.

INTRODUCTION

The skin is the largest organ in the body and serves as a primary defense against a harmful environment that includes pathogens and ultraviolet (UV) light. Although UV radiation is necessary for the biosynthesis of vitamin D_3 to promote bone formation, the induction of melanogenesis for photoprotection, and as a treatment for vitiligo and psoriasis, its acute exposure is a major risk factor for skin inflammation, skin aging, and skin cancer (Kim *et al.*, 2013; Kim *et al.*, 2015). Human keratinocytes are found in the epidermal layer of the skin and play an important role in the inflammatory response to a variety of stress stimuli such as UV radiation. There are three types of UV rays, UVA, UVB, and UVC. Of these, UVC cannot pass through the ozone layer of the earth and has little effect on the skin, but humans are exposed to UVA and UVB, inducing melanin pigmentation that causes skin tanning and spotting, and potentially leading to skin damage, wrinkling, and cancer (Ha *et al.,* 2016; Park *et al.,* 2017). Among the UV wavelengths, UVB (290–315 nm) radiation also increases the production of some proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-8, from human keratinocytes, leading to skin inflammation. Because TNF-α, IL-6, and IL-8 are specifically upregulated in the epidermis as a result of chronic exposure to UVB radiation, the levels of proinflammatory cytokines may serve as early markers of acute skin inflammation (Kim *et al.*, 2018; Janda *et al.*, 2016; Lembo *et al.*, 2014; Krolikiewicz-Reniml *et al.*, 2013).

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There are 11 species and 2 varieties of *Rhododendron weyrichii* Maxim on the Korea Peninsula including North Korea. It is known that *R. weyrichii* is native to Jeju in Korea and is distributed in the southern part of Japan. *R. weyrichii* is a deciduous shrub that grows 3–6 m high on the mountainside of Mt. Halla up to 1.200 m above sea level (Yoichi *et al.*, 2016). The leaves at the ends of the branches are trilobed with dark green and brown hairs on their surface. The antioxidant, anti-inflammatory, and skin-whitening effects of the leaves of *R. weyrichii* have been noted (Kim, 2015). Recently, our laboratory has also reported that extracts of the *R. weyrichii* flower can be used as skin-whitening agents because they inhibit melanin formation by suppressing the proteins involved in melanogenesis (Kim *et al.*, 2016). In addition, there was a study promoting the whitening effect of rhododendrol, a natural substance that is commonly found in *Rhododendron* plants and has a similar structure to that of arbutin. However, some patients using brightening/lightening cosmetics containing rhododendrol unexpectedly developed leukoderma. Most of the patients developed leukoderma at the site of repeated rhododendrol application, but some patients also showed white patches at remote sites (Ito and Wakamatsu, 2018; Yoshikawa *et al.*, 2016; Inoue *et al.*, 2016).

Although published evidence suggests that *R. weyrichii* has promising biological effects against various diseases, its effect on UVB-induced skin inflammation and the mechanisms responsible remain unclear. Therefore, this study was designed to explore the anti-inflammatory activity of *R. weyrichii* extracts by measuring their effects on proinflammatory cytokine secretion from UVB-irradiated HaCaT keratinocytes. In addition, potential active components of the extracts were identified by HPLC analysis, and tolerability was confirmed using skin irritation tests. To the best of our knowledge, this is the first report of the anti-inflammatory biological activity of *R. weyrichii* in UVB-irradiated HaCaT keratinocytes.

MATERIALS AND METHODS

Materials and solvent extraction

R. weyrichii flowers (RWFs) were collected from Daejung (a region on Jeju Island, Korea) in May 2017. The materials for extraction were air-dried and then ground into a fine powder using a blender. The dried powder (20 g) was extracted with 70% ethanol (EtOH; 600 mL) at room temperature for 24 h and then evaporated under vacuum. The yield and recovery of the RWF extracts were 5.78 g and 9.63%, respectively.

Cell culture and UVB irradiation

Immortalized human keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) in a humidified incubator at 37°C and 5% CO_2. HaCaT cells were irradiated by UVB at 25 mJ/cm^2 using a UV lamp with a peak emission of 312 nm (Vilber Lourmat, BLX-312, Marne-la-Vallée, France). The HaCaT cells were rinsed with phosphate-buffered saline (PBS), and irradiation was performed with the cells under PBS. After UVB irradiation, the cells were incubated in fresh DMEM for 24 h.

Cell viability assay

Cell viability in the presence of RWF was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. HaCaT cells were seeded at 5 × 10^4 cells per well in 24-well plates and were treated with various concentrations of RWF extract (50, 100, 200, or 400 µg/mL) for 24 h. Then, the media in all wells was replaced with MTT solution (5 mg/mL) and the plates were incubated for 4 h at 37°C. Finally, the MTT-containing medium was removed by aspiration and 1 mL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 540 nm using a Tecan sunrise microplate reader (Tecan Trading AG, GrÖdlg, Austria).

Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were seeded in 24-well plates at a density of 5 × 10^4 cells per well for the determination of TNF-α, IL-8, and IL-6 levels. After 24 h, the cells were irradiated with 25 mJ/cm^2 UVB (as described in the cell culture and UVB irradiation section above) and treated with various concentrations of RWF extract (100, 200, or 400 µg/mL) in DMEM medium for 24 h. The culture supernatants were harvested, and the levels of the cytokines were measured using ELISA kits (R&D Systems, MN, USA).

HPLC fingerprinting of RWF extracts

The RWF extracts were brought to a concentration of 1 mg/mL and filtered through a 0.22-µm membrane filter (Millipore, Bedford, MA, USA). The kaempferol and astragalin analyses were carried out using HPLC (Waters e2695 and 2489 UV Detector). The analysis was performed on a YMC-Triart C18 (5 µm, 250 × 4.6 mm ID) column (YMC Europe GmbH, Dinslaken, Germany). Kaempferol was eluted with a mobile phase of 40% A (acetonitrile) and 60% B (20 mM phosphoric acid); the wavelength of the UV detector was 370 nm. Astragalin was eluted with 30% A and 70% B; the wavelength of the UV detector was 264 nm. For both compounds, the flow rate was 1.0 mL/min, column temperature was 37°C, and the injection volume was 10 µL. Calibration curves of known concentrations were used for quantification of the compounds in the extracts.

Human skin primary irritation test

This study was conducted by adopting a 48 h single patch test using the guidelines proposed by the Personal Care Products Council (PCPC). This test was conducted in accordance with the PCPC Guidelines for the purpose of contributing to the development of safe cosmetics by anticipating side effects that may be caused by cosmetics. The subjects were healthy, nonsmoking women of Korean origin. Thirty-one subjects participated in the entire course of the test. The mean age of the subjects was 40.32 ± 7.60 years, the highest age was 50, and the lowest age was 23. The test site was washed with 70% ethanol and then dried. The test substance was diluted to 200 µg/mL in squalane. Sixteen microliters of the diluted test substance were placed into the Finn chamber (SmartPractice, Denmark), situated on the back of the test site, and fixed with Micropore™ tape (3M/Medical-Surgical Division, USA). After 48 h, removing the patches, the test area was marked with a skin marker (Chemotechnique Diagnostics AB, Sweden), and the test sites were observed after at 30 min and 24 h. The skin response was evaluated in accordance with the Frosch

and Kligman rules. The evaluation results were calculated after 48 h and 72 h using the equation below. The average reactivity for 400 μg/mL RWF extract was assessed according to the criteria in Table 1.

$$\text{Response} = \frac{\Sigma(\text{Grade} \times \text{No. of Responders})}{4\ (\text{Maximum grade}) \times n\ (\text{Total Subjects})} \times 100 \times 1/2$$

Table 1: Human primary irritation index for cosmetic products.

Range of Response	Criteria
0.00 ≤ R < 0.87	Slight
0.87 ≤ R < 2.42	Mild
2.42 ≤ R < 3.44	Moderate
3.44 ≤ R	Severe

R: response range.

RESULTS AND DISCUSSION

Numerous studies have indicated that proinflammatory cytokines such as IL-6, TNF-α, and IL-8 play a role in the immunological regulation of human skin, including skin inflammation. In order to validate the use of RWF extracts as anti-inflammatory agents, we investigated the effects of RWF extracts on the production of IL-6, TNF-α, and IL-8 in UVB-irradiated HaCaT keratinocyte cells. First, we evaluated the effect of RWF on HaCaT cell viability after 24 h treatment using an MTT assay. Treatment with RWF extracts at a concentration of 100 to 400 μg/mL for 24 h, rather than reduce cell viability, significantly increased it instead (Fig. 1). UVB irradiation alone dose-dependently reduced the viability of the HaCaT keratinocyte cells (data not shown). As shown in Fig. 2, UVB irradiation of 25 mJ/cm^2 significantly reduced the viability of the HaCaT keratinocyte cells. However, following treatment with various concentrations of RWF extracts (from 100 to 400 μg/mL), the cell viability was increased in a concentration-dependent manner. Therefore, RWF extracts at a concentration of 400 μg/mL normalized the cell growth inhibition of UVB-treated HaCaT keratinocyte cells, with the cell viability closely approaching that of non-UVB-treated control cells.

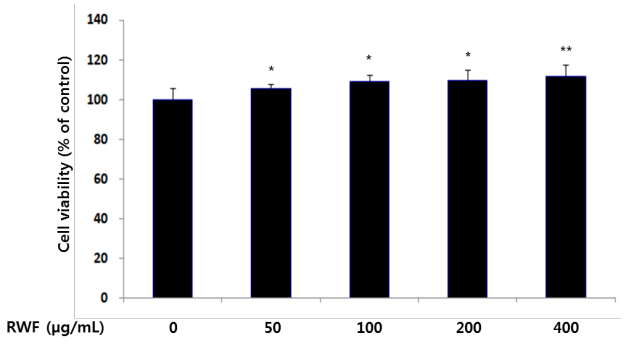

Fig. 1: Cytotoxicity of various concentrations of *Rhododendron weyrichii flower* (RWF) extracts on HaCaT cells. The cells were treated with 50, 100, 200, or 400 μg/mL of RWF extract for 24 h, and then the cell viability was measured by MTT assay. The data represent the means ± SD of quadruplicate experiments. *p < 0.05, **p < 0.01 compared to 0 μg/mL RWF concentration.

As aforementioned, IL-6, TNF-α, and IL-8 undoubtedly play pivotal roles in immunologic regulation in human skin and are involved in skin inflammation. Therefore, the inhibition of IL-6, TNF-α, and IL-8 would be expected to have favorable anti-inflammatory effects on skin diseases. Thus, we determined the inhibitory effect of RWF extract on IL-6, TNF-α, and IL-8 production in UVB-irradiated HaCaT keratinocyte cells. The cells were pretreated with RWF extracts (from 100 to 400 μg/mL) prior to irradiation with UVB (25 mJ/cm^2) for 24 h and then analyzed by ELISA. As shown in Fig. 3, the levels of IL-6 (Fig. 3A) and TNF-α (Fig. 3B) were considerably increased in HaCaT cells after UV-B irradiation. Pretreatment of the cells with RWF extracts (from 100 to 400 μg/mL) inhibited these upregulations in a concentration-dependent and statistically significant manner. In contrast, the RWF extracts did not inhibit IL-8 production in UVB-irradiated HaCaT keratinocyte cells (Fig. 3C). These results provide direct evidence to show that RWF extracts act as inhibitory agents against IL-6 and TNF-α production.

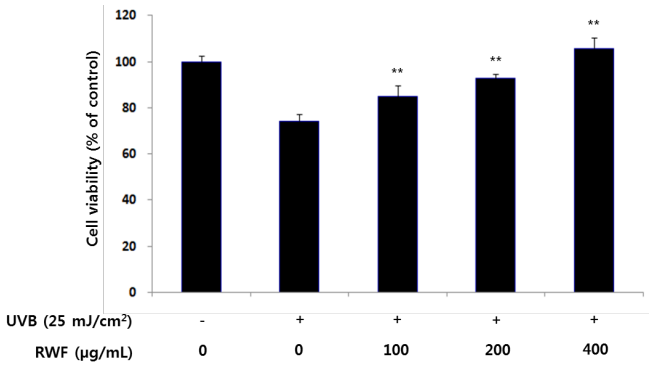

Fig. 2: Effect of *Rhododendron weyrichii flower* (RWF) extract on cell viability in UVB-irradiated HaCaT cells. The cells were treated with or without RWF extract and then irradiated with UVB (25 mJ/cm^2). After 24 h, the cell viability was measured by MTT assay. The data represent the means ± SD of quadruplicate experiments. **p < 0.01 compared to UVB alone.

The identification of isolated compounds is very important for the development of functional materials using natural extracts. With the development of analytical technology, chromatographic methods are highly recommended for developing fingerprints of natural raw materials (Kim *et al.*, 2014; Yoon *et al.*, 2009) Therefore, a simple HPLC fingerprint of the plant extract was explored in this study. Since kaempferol and astragalin have been reported as constituents of the *Rhododendron* plant with effective anti-inflammatory activities, their content in the extracts was measured (Wang *et al.*, 2015; Mok *et al.*, 2013). Using the conditions described in the Materials and Methods section, both kaempferol (Fig. 4A) and astragalin (Fig. 4B) were well resolved from the RWF extract with excellent peak shapes. The contents of kaempferol and astragalin in the ethanol extract were 0.335 and 2.569 ppm, respectively (Fig. 4).

Finally, we tested the potential application of RWF extract as a cosmetic material by performing human skin primary irritation tests to exclude adverse skin reactions. As shown in Table 2, all of the 31 volunteers experienced no severe adverse reactions after RWF extract treatment. Considering these results, we suggest that RWF extracts be considered anti-inflammatory candidates for topical applications. The inhibition of pro-inflammatory cytokines in keratinocytes offers new therapeutic strategies for the treatment of skin inflammation. Further research is required to purify and identify novel active compounds and to determine other biomarkers of UVB-induced skin-inflammation.

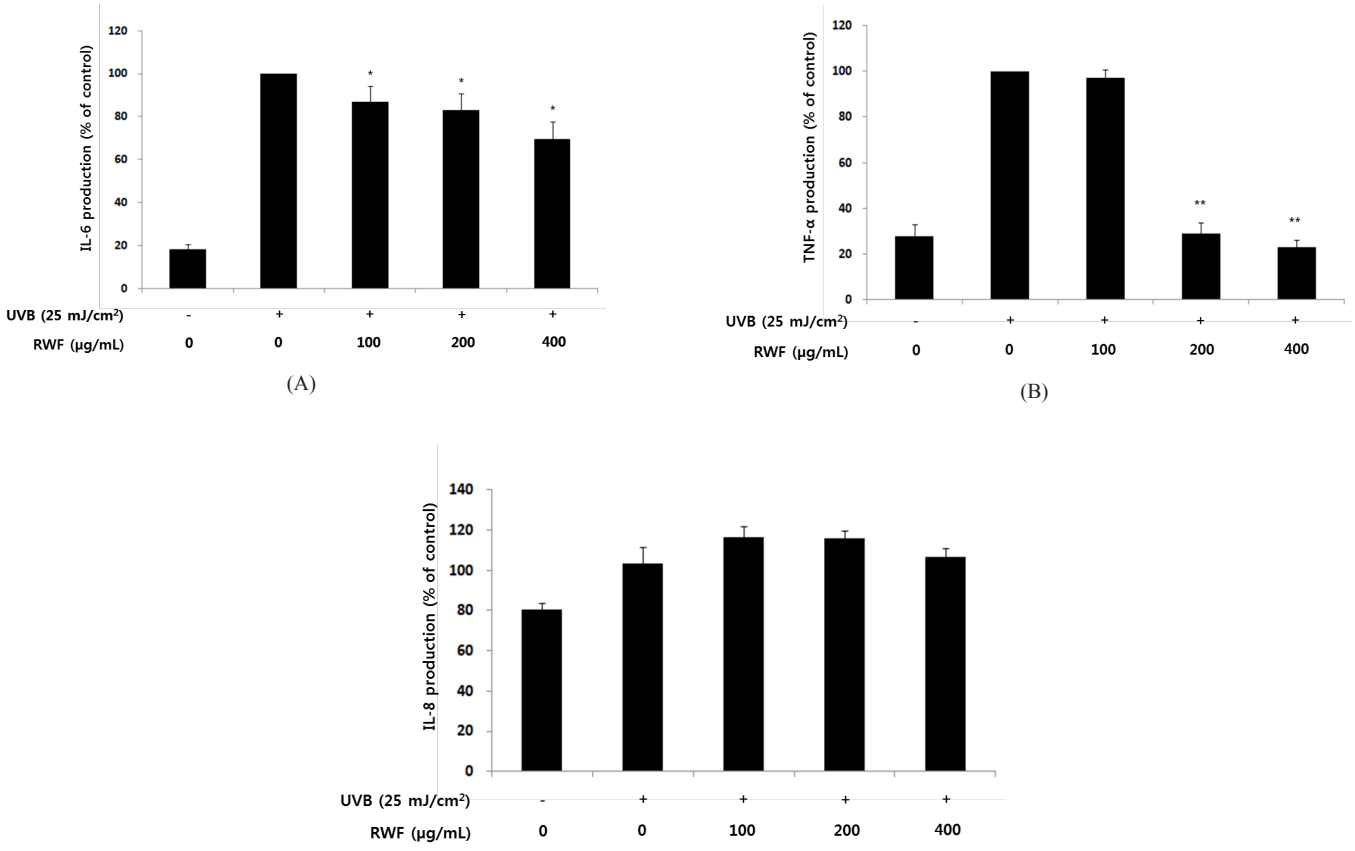

Fig. 3: Effect of *Rhododendron weyrichii flower* (RWF) extract on IL-6, TNF-α, and IL-8 production in UVB-irradiated HaCaT cells. The cells were irradiated with 25 mJ/cm^2 of UVB only or with UVB plus various concentrations (100, 200, or 400 μg/mL) of RWF extract for 24 h. IL-6 (A), TNF-α (B), and IL-8 (C) production was determined by ELISA. The data represent the means ± SD of quadruplicate experiments. *p < 0.05, **p < 0.01 compared to UVB alone.

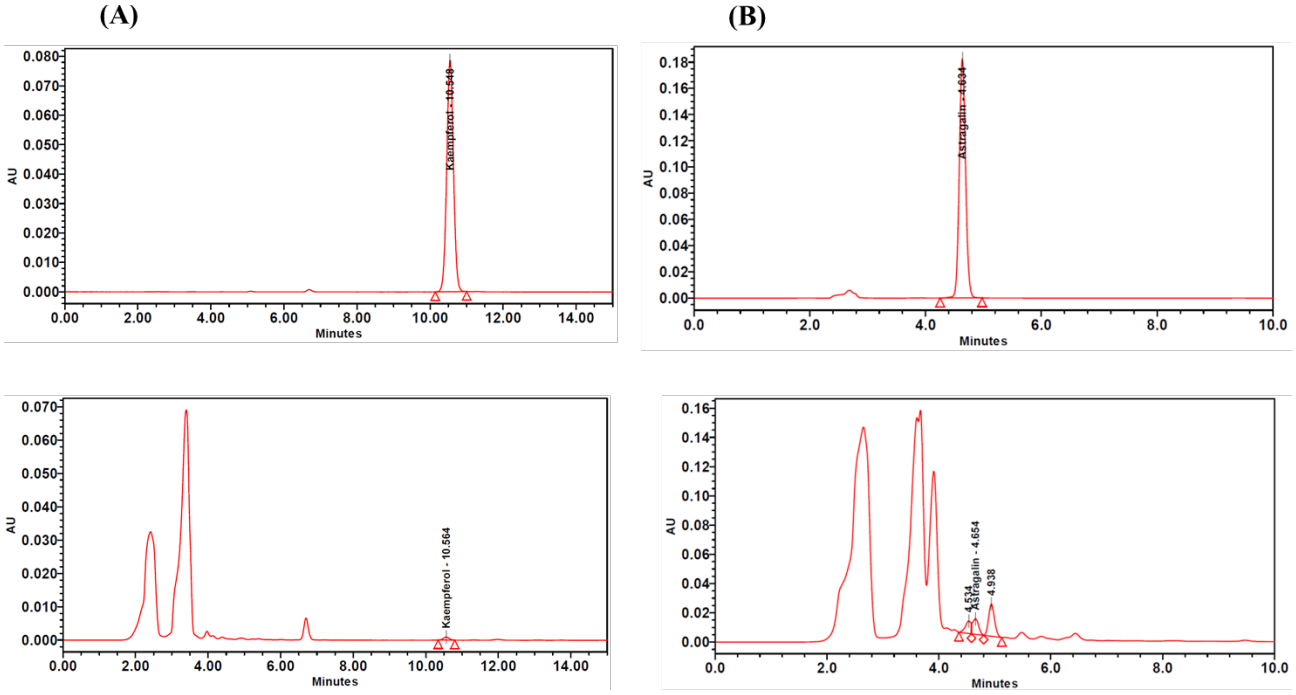

Fig. 4: HPLC chromatograms of kaempferol and astragalin of *Rhododendron weyrichii flower* (RWF) extract. (A) Kaempferol HPLC analysis of kaempferol standard (upper) and RWF extract (lower). (B) Astragalin HPLC analysis of astragalin standard (upper) and RWF extract (lower).

Table 2: Results of human skin primary irritation test.

No.	Test material	No. of responders	48 h				72 h				Reaction Grade		
			1+	2+	3+	4+	1+	2+	3+	4+	48 h	72 h	Mean
1	RWF extract (400 μg/mL)	0	-	-	-	-	-	-	-	-	0.0	0.0	0.0
2	Squalane (Negative control)	0	-	-	-	-	-	-	-	-	0.0	0.0	0.0

-: Not detected.

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