Anti-inflammatory effects of tectroside on UVB-induced HaCaT cells

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Abstract. Ultraviolet B (UVB) irradiation causes skin damage and inflammation by inducing the secretion of various cytokines, which are immune regulators produced by cells. To prevent skin inflammation, keratinocytes that have been irreversibly damaged by UVB must be eliminated through apoptosis. *Ixeris dentata* (*I. dentata*) (family Asteraceae) is a perennial medicinal herb indigenous to Korea. It is used in Korea, China and Japan to treat indigestion, pneumonia, diabetes, hepatitis, contusions and tumors. Guaiane-type sesquiterpene lactones were isolated from the whole extract of *I. dentata*. This led to the isolation of the anti-inflammatory sesquiterpene lactone compound tectroside (TES), which was tested on a human keratinocyte cell line. To determine the anti-inflammatory effects of TES, we examined its influence on UVB-induced pro-inflammatory cytokine production in human keratinocytes (HaCaT cells) by observing these cells in the presence or absence of TES. In the present study, pro-inflammatory cytokine production was determined by performing enzyme-linked immunosorbent assay, reverse transcription-polymerase chain reaction and western blot analysis to evaluate the activation of mitogen-activated protein kinases (MAPKs). TES inhibited UVB-induced production of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 in a dose-dependent manner. In addition, TES inhibited the expression of cyclooxygenase (COX)-2 and the phosphorylation of c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) MAPKs, suggesting that it inhibits the secretion of the pro-inflammatory cytokines IL-6 and IL-8 and COX-2 expression by blocking MAPK phosphorylation. These results suggest that TES can potentially protect against UVB-induced skin inflammation.

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Introduction

The skin is the largest organ of the body and its function in the immune system has attracted the attention of both immunologists and dermatopathologists (1,2). Ultraviolet (UV) radiation is the major environmental cause of skin damage (3,4).

UVA (320.400 nm) and UVB (280.320 nm) radiations reach the earth's surface in amounts sufficient to have important biological consequences for the skin (5). UVB, in particular, has a wide spectrum of biological effects on the skin, and acute exposure causes a variety of adverse skin reactions, including erythema, edema, sunburn, hyperplasia, inflammation and immunosuppression (6). Furthermore, chronic UVB exposure leads to skin carcinogenesis and premature skin aging (6,7).

In addition, exposure of cells to UVB radiation results in the loss of keratinocyte viability, an increase in membrane blebbing (8), cytoskeletal molecular changes (9-12) and apoptosis (13,14). The whole plant of *Ixeris dentata* (*I. dentata*), a typical Oriental herb, has been used for the treatment of indigestion, pneumonia, hepatitis, contusions and tumors (15,16); it is also used in folk medicine in Korea for the treatment of inflammatory diseases.

Recent pharmacological studies of *I. dentata* showed that water or organic solvent extracts of whole herbal medicine lower lipid concentrations and act as an antioxidant (17), antiallergic (18), monamine oxidase (19), anti-inflammatory (20,21) antimutagenic and anticancer (22) activity. Although crude extracts of a single herbal medicine or herbal formula can exhibit striking biological effects, their mechanisms cannot be fully elucidated as they can contain innumerable compounds (23).

Guaiane-type sesquiterpene lactones were isolated from the whole extract of *I. dentata*. Subsequently, the effects of tectroside (TES) isolated from *I. dentata* on UVB-induced pro-inflammatory mediators were evaluated by inhibiting mitogen-activated protein kinases (MAPKs) in a human keratinocyte cell line, HaCaT.

The effect on skin inflammation has yet to be reported; therefore, as part of our ongoing screening program to evaluate anti-inflammatory potential of natural compounds, we investigated the *in vitro* anti-inflammatory activity of TES isolated from *I. dentata*.

Materials and methods

Plant material. Whole plants of *I. dentata* were collected in May 2006 from the herbarium at the Korea Research Institute of Chemical Technology (KRICT) and were authenticated by Dr Young Sup Kim. A voucher specimen (KR0472) was deposited at the herbarium at KRICT (24).

Extraction and isolation. The air-dried whole plants (6 kg) of *I. dentata* were soaked in methanol (MeOH) (2x40 liters) at room temperature for 7 days. The MeOH extract was filtered and evaporated to dryness under reduced pressure. The concentrated extract (840 g) was suspended in 20 liters of water and then extracted successively with an equal volume of dichloromethane (MC), ethyl acetate (EtOAc), and n-butanol (n-BuOH), which yielded 160 g of the MC fraction, 15 g of the EtOAc fraction, and 60 g of the n-BuOH fraction, respectively (24). The detailed purification procedures for TES from the fraction are shown in Fig. 1.

Reagents. RPMI-1640, penicillin, and streptomycin were obtained from HyClone Laboratories, Inc. (Logan, UT, USA). Bovine serum albumin and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for extracellular signal-regulated kinase (ERK)1/2, phosphorylated ERK1/2, c-Jun NH2-terminal kinase (JNK), phosphorylated JNK, and β-actin, and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies for human interleukin (IL)-6 and IL-8 and biotinylated antibodies for human IL-6 and IL-8 were purchased from BD Biosciences (San Jose, CA, USA). The RNeasy Mini Kit and QuantiTect Reverse Transcription kit were purchased from Qiagen (Hilden, Germany). IL-6, IL-8, cyclooxygenase (COX)-2, and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejeon, Korea).

Cell culture. HaCaT cells were grown in RPMI-1640 medium containing 5% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37˚C. To stimulate the cells, the medium was replaced with fresh RPMI-1640 medium, and exposed to UVB in the presence or absence of TES for the indicated periods.

UVB source. UVB irradiation was delivered by a closely spaced array of 5 sunlamps (G9T5E lamps; Sankyo Denki Co., Hiratsuka, Japan). The distance between the sunlamps and the surface of the cell cultures was fixed at 7.5 cm, and the distance between the sunlamps and the surface of the cage was fixed at 30 cm. The energy output of the UVB (290-320 nm) lamps was measured using a UV radiometer (UVX; UVP Inc., Upland, CA, USA).

Cell viability assay. Cell viability was determined by the MTS assay. HaCaT cells were plated at a density of $3x10^4$ cells/well in 96-well plates (Nunc, Copenhagen, Denmark). Each experiment included a non-treated group as the control. To determine the non-toxic concentration for the cells, TES (2.5, 5, 10 and 20 μM) was added to each well. The plates were incubated for 24 h at 37˚C under 5% CO_2. The MTS solution (5 mg/ml) was added to each well, and the cells were cultured for another 2 h, after which the optical density was read at 490 nm. Cytotoxicity was then calculated using the formula: 1 - (mean absorbance value of treated cells/mean absorbance value of untreated cells).

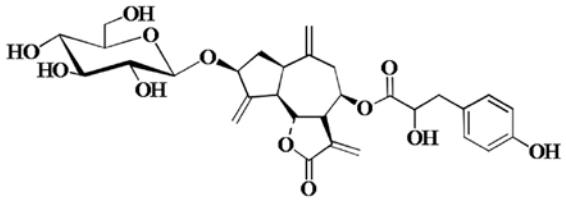

Figure 1. The chemical structure of tectroside.

Enzyme-linked immunosorbent assay (ELISA). Cells were seeded at a density of $3x10^4$ cells/well in 48-well tissue culture plates and pretreated with 2 concentrations of TES (5 and 10 μM) for 24 h prior to UVB (100 mJ/cm^2) stimulation. ELISA plates (Nunc) were coated overnight at 4˚C with anti-human IL-6 and IL-8 antibodies diluted in coating buffer (0.1 M carbonate, pH 9.5), and then washed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Nonspecific protein-binding sites were blocked with an assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. Immediately, each sample or the IL-6 or IL-8 standard was added to the wells. Following incubation for 2 h, a working detector was added and incubated for 1 h. Accordingly, the substrate solution (tetramethylbenzidine) was added to the wells and incubated for 30 min in the dark before the reaction was stopped with 2 N H_3PO_4. Absorbance was read at 450 nm. All subsequent steps were performed at room temperature, and all standards and samples were assayed in duplicate.

Western blot analysis. Protein expression was assessed by western blot analysis according to standard procedures. The HaCaT cells were cultured in 60-mm-diameter culture dishes ($4x10^6$ cells/well) and pretreated with 2 concentrations of TES (5 and 10 μM). After 30 min, 2 or 24 h, the cells were UVB-irradiated (100 mJ/cm^2) and then incubated at 37˚C. Following incubation, the cells were washed twice in ice-cold PBS (pH 7.4). The cell pellets were resuspended in lysis buffer on ice for 20 min, and the cell debris was removed by centrifugation. Protein concentrations were determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene membrane (Millipore, Bedford, MA, USA).

The membrane was blocked with 5% nonfat milk in Tris-buffered saline with Tween-20 buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.05% Tween-20, pH 7.4). After blocking, the membrane was incubated with primary antibodies for 18 h, washed with Tris-buffered saline with Tween-20, and incubated again with anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Milan, Italy).

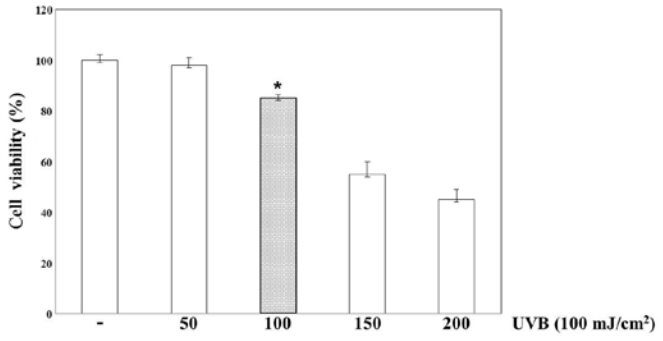

Figure 2. Viability of HaCaT cells under various conditions. HaCaT cells were used for the MTS assay at 24 h after 50, 100, or 150 mJ/cm^2 UVB irradiation and comparison of the viability of irradiated cells with that of the non-irradiated control. Data are the means ± SD values from triplicate experiments. *P<0.05.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). HaCaT cells were cultured in 6-well tissue culture plates (8x10^5 cells/well) and pretreated with 2 concentrations of TES (5 and 10 μM). After 30 min, the cells were irradiated with UVB (100 mJ/cm^2) and incubated at 37˚C. Following incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Total cellular RNA was isolated using the RNA Mini Kit (Qiagen), and 1 μg of total RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The total RNA (2 μg) was converted to cDNA by treating it with 200 units of reverse transcriptase and 500 ng of oligo(dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates at 42˚C for 1 h. The reaction was stopped by heating at 70˚C for 15 min, and the cDNA mixture (3 μl) was used for enzymatic amplification. PCR was performed using 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 units of TaqDNA polymerase, and 0.1 μM each of the IL-6, IL-8, COX-2, or β-actin primers.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's t-test for single comparisons. All data are presented as the means ± standard error (SE), and the number of individual experiments conducted is mentioned in each figure legend.

Results

Cell viability of UVB-irradiated HaCaT cells. The effect of TES on cell viability following UVB irradiation was tested on HaCaT cells. Cell viability was evaluated using an MTS assay (Fig. 2). When the cultures were incubated after UVB irradiation, UVB-induced toxicity increased compared to that in non-irradiated cells. Cell viability declined, depending on the dose of UVB irradiation, and sharply reduced at 24 h after UVB irradiation of 150 mJ/cm^2. Accordingly, we selected an exposure dose of 100 mJ/cm^2 for studying cellular toxicity in HaCaT cells treated with TES 24 h after UVB irradiation (Fig. 3).

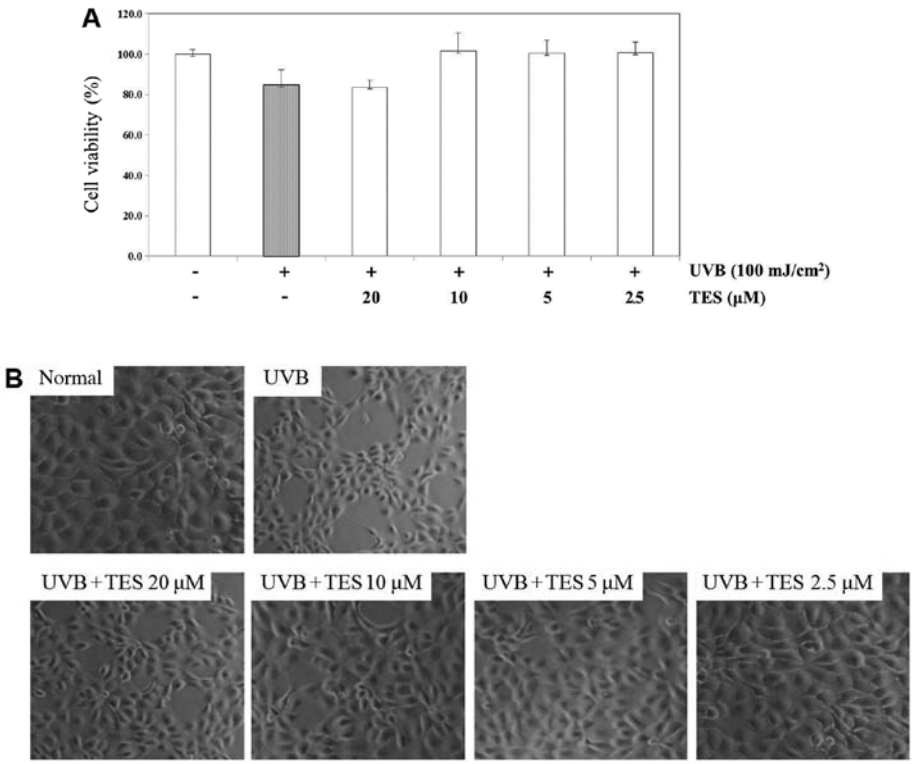

Figure 3. Effect of tectroside (TES) on cell viability and morphological alterations in HaCaT cells. (A) Cell viability was evaluated with the MTS assay. Data represent the means ± SE of duplicate determinations from three separate experiments. (B) For morphological studies, cells were treated with TES (2.5, 5, 10 and 20 μM) for 24 h and then stimulated with UVB (100 mJ/cm^2) for 24 h.

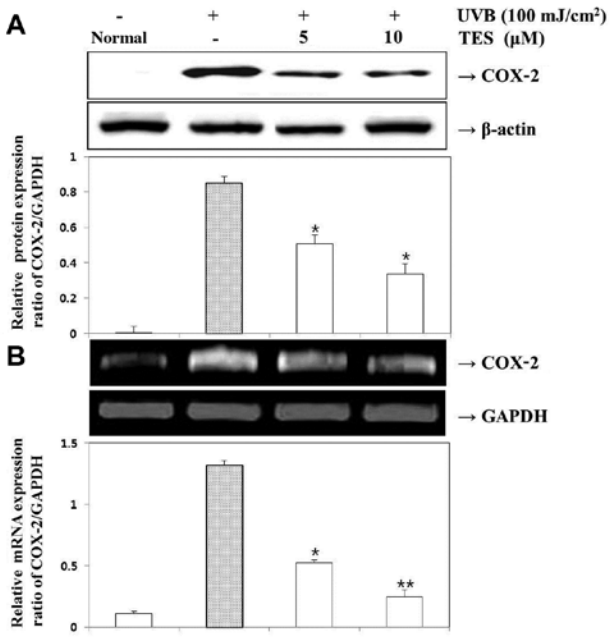

Figure 4. Effect of tectroside (TES) on UVB-induced COX-2 protein and mRNA expression in HaCaT cells. HaCaT cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with COX-2 antibodies. Equal protein loading was verified using β-actin. (A) COX-2 mRNAs were assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of TES for 24 h before being incubated with UVB (100 mJ/cm^2) for 24 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) The experiment was repeated three times, and similar results were obtained. *P<0.05, **P<0.005, when compared to the UVB-treated group. Significant differences between treated groups were determined using the Student's t-test. Values shown are the means ± SE of duplicate determinations from three separate experiments.

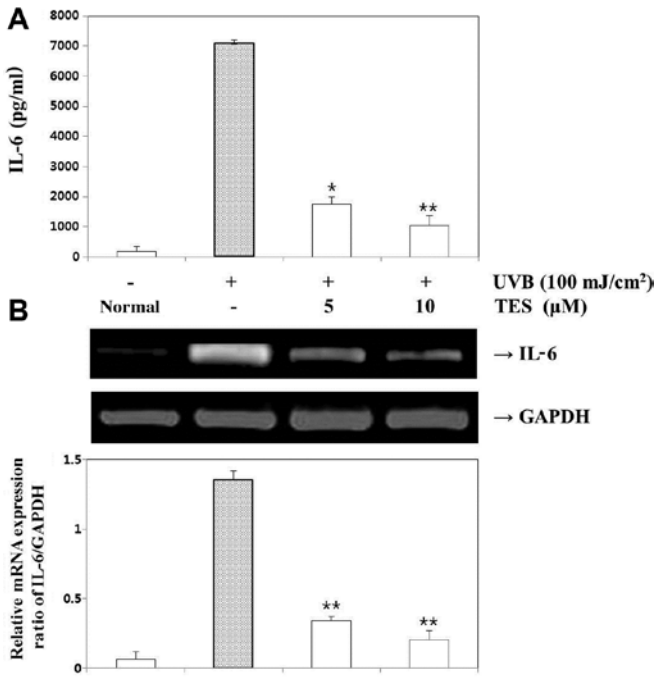

Figure 5. Effect of tectroside (TES) on UVB-induced IL-6 production. HaCaT cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. The production of IL-6 was measured by ELISA. Cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. (A) Effect of TES on UVB-induced IL-6 mRNA expression. IL-6 mRNA was assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 2 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) Data are the means ± SE values of duplicate determinations from three separate experiments. *P<0.05; **P<0.005.

Effect of TES on UVB-induced COX-2 mRNA expression. We next examined the effects of TES on COX-2 expression in UVB-irradiated HaCaT cells. The expression levels of COX-2 protein and COX-2 mRNA were measured in HaCaT cells exposed to UVB (100 mJ/cm^2) for 24 h. TES effectively suppressed UVB-induced COX-2 expression. UVB (100 mJ/cm^2) also increased COX-2 mRNA expression, which was inhibited in the presence of TES (Fig. 4). Hence, TES suppressed the expression of genes that are implicated in the pathogenesis of inflammatory responses.

Effect of TES on IL-6 and IL-8 production in UVB-irradiated cells. Since TES inhibited the production of pro-inflammatory mediators in HaCaT cells, we further investigated its effects on UVB-induced IL-6 and IL-8 production by performing ELISA and RT-PCR. We found that, depending on its concentration, TES inhibited UVB-stimulated IL-6 (Fig. 5) and IL-8 (Fig. 6) expression at both the protein and mRNA levels.

Effect of TES on the phosphorylation of MAPKs in UVB-induced HaCaT cells. MAPKs are essential for UVB-induced inflammation in HaCaT cells; therefore, we evaluated the effects of TES on the activation of MAPKs in these cells. TES markedly inhibited the phosphorylation of JNK1/2 and ERK1/2 MAPK; these results indicate that MAPK phosphorylation was inhibited by TES pretreatment (Fig. 7).

Discussion

I. dentata is a perennial medicinal herb indigenous to Korea. It was reported that intraperitoneal administration of the herb extract resulted in decreased blood glucose in alloxan diabetic mice (25) and prevented neurodegenerative diseases (19,26). Young *I. dentata* sprouts are commonly used as a bitter appetizing in Korea. Chemical components including triterpenes, sesquiterpene glycosides, and flavonoids have been isolated from the genus *Ixeris*, which comprises approximately 20 species (27).

I. dentata is characterized by the presence of guaiane sesquiterpene lactones, which are chemosystematic markers. As a continuation of our effort to purify minor sesquiterpenes from *I. dentata* amino acid-sesquiterpene lactones, ixerisamine A and ixerisamine B were isolated together with 12 related sesquiterpene lactones (8-epi-desacylcynaropicrin glucoside; ixerisoside A; ixerisoside A 6'-O acetate; ixerin N; ixerin N 6'-O acetate; ixerin M; TES; 4,8-epiisolipidiol; 8-epi-isolipidiol; 11βH-11,13-dihydrointegrifolin; 8β-hydroxy-4β,15-dihydrozaluzanin C and integrifolin) (24).

The isolation and structure determination of compounds, as well as the inhibitory effects of isolated sesquiterpenes on the proliferation of 4 cultured human tumor cell lines: MES-SA (human uterine carcinoma cell line), MES-SA/DX5 (multidrug-resistant subline of MES-SA), HCT-15 (human

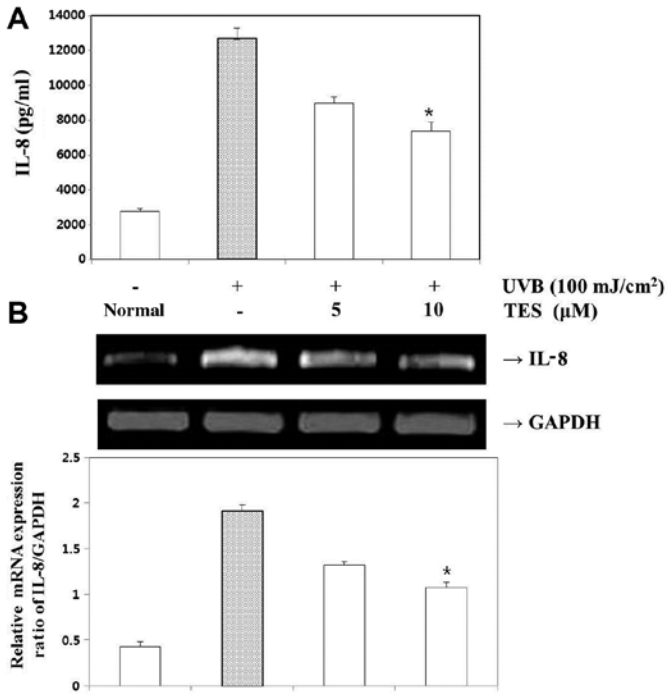

Figure 6. Effect of tectroside (TES) on UVB-induced IL-8 production. HaCaT cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 24 h. The production of IL-8 was measured by ELISA. Cells were pretreated with the indicated concentrations of TES for 24 h before being incubated with UVB (100 mJ/cm^2) for 24 h. (A) Effect of TES on UVB-induced IL-8 mRNA expression. IL-8 mRNA was assessed by RT-PCR in HaCaT cells. Cells were pretreated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for 2 h. β-actin mRNA was assayed in parallel to confirm the equivalency of the cDNA preparations. (B) Data are the means ± SE values of duplicate determinations from three separate experiments. *P<0.05.

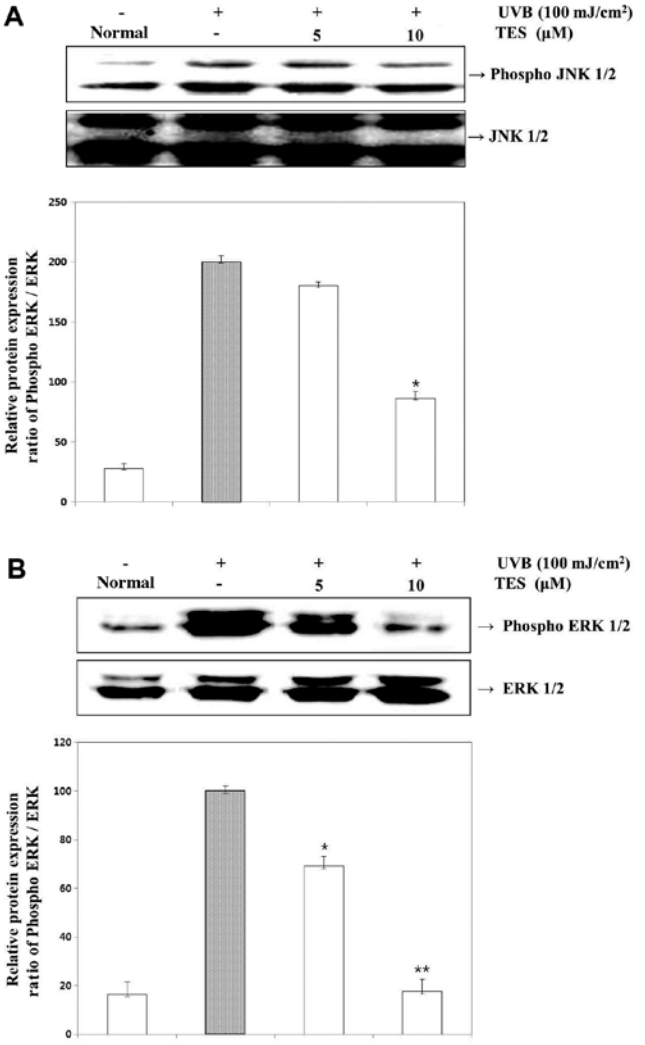

Figure 7. Effect of tectroside (TES) on the phosphorylation (P-) of MAPKs in UVB-stimulated HaCaT cells. HaCaT cells were treated with the indicated concentrations of TES for 24 h before being irradiated with UVB (100 mJ/cm^2) for (A) 30 min and (B) 1 h. Whole-cell lysates were analyzed by western blot analysis. The experiment was repeated three times, and similar results were obtained. *P<0.05; **P<0.005

colorectal adenocarcinoma cell line), and HCT-15/CL02 (multidrug-resistant subline of HCT-15) were evaluated (24).

UVB irradiation induces skin damage and inflammation by causing the secretion of various cytokines, which are immune regulators produced by cells. To prevent the initiation of skin inflammation, keratinocytes that have been irreversibly damaged by UVB must be removed through apoptosis. UVB crosses the epidermis and reaches the upper dermis. Compared with UVA, it is more active in terms of causing cutaneous carcinogenesis and alterations of the cell-cycle-control signaling pathways (5).

Keratinocytes are the major target of UVB and play a central role in inflammatory and immune modulatory changes observed after UV exposure, at least partly through the UV-induced release of cytokines (IL-1, IL-6, IL-8, IL-10, GM-CSF and TNF-α) (28) and cyclooxygenase products (PGE2) (29).

Inappropriate expression and/or activity of COX-2, a rate-limiting enzyme involved in the biosynthesis of prostaglandins, has been implicated in UVB-induced skin carcinogenesis (30). MAPKs are a family of proline-directed Ser/Thr kinases comprising ERK, JNK and p38 MAPK. Recent studies have shown that activation of ERK, JNK and p38 MAPK is strongly correlated with acute inflammation and development of skin cancer through increased expression of COX-2 (31-33).

In the present study, TES was isolated from *I. dentata*. We examined the effect of TES on UVB-induced pro-inflammatory cytokine production in HaCaT cells by evaluating cells that were stimulated with UVB in the presence or absence of TES. Pro-inflammatory cytokine production was measured by ELISA and RT-PCR, and the activation of MAPKs was determined by western blot analysis.

In particular, we investigated whether TES inhibits the UVB-induced production of IL-6 and IL-8 by inhibiting the expression of MAPK and COX-2 at the protein and mRNA levels. We found that the inhibitory effects of TES on the production of inflammatory mediators were accompanied by concentration-dependent decreases in the protein and mRNA expression levels of IL-6, IL-8 and COX-2. These data demonstrate that IL-6, IL-8 and COX-2 expression in HaCaT cells is suppressed by TES; UVB-induced phosphorylation of JNK1/2 and ERK1/2 in HaCaT cells. Therefore, it is likely that TES acts as an antiphotoinflammatory agent by mainly inhibiting COX-2 expression *in vivo*.

Inhibition of COX-2 expression has been shown to be an important anti-inflammatory mechanism of some compounds, including epigallocatechin gallate (34), resveratrol (35) and curcumin (36) similar to TES. Furthermore, these effects are mediated by the inhibition of COX-2 expression and JNK1/2 and ERK1/2 phosphorylation. In practice, the whole *I. dentata* plant, a typical Oriental herb, has been used for the treatment of indigestion, pneumonia, diabetes, hepatitis, contusions and tumors (16). It has also been used in Korean folk medicine for the treatment of inflammatory diseases; therefore, this represents a potent anti-inflammatory effect of TES accomplished by blocking inflammatory mediators. Our data suggest that TES represents a new source of potential drugs for the treatment of inflammatory diseases.

In conclusion, we evaluated the effect of TES on skin inflammation *in vitro* and found that TES has potential to attenuate UVB-induced skin inflammation by suppressing MAPK activation. Our findings provide new insight into the application of TES as well as its nutraceutical value. Further studies on TES are required to confirm its medicinal use.

Acknowledgements

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