A Glycosidic Isoflavonoid from Viola hondoensis W. BECKER et H. BOISSIEU (Violaceae), and Its Effect on the Expression of Matrix Metalloproteinase-1 Caused by Ultraviolet Irradiation in Cultured Human Skin Fibroblasts

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Isolation of the ethyl acetate soluble fraction from aerial parts of Viola hondoensis W. BECKER et H. BOISSIEU yielded one major isoflavonoid glycoside, tectoridin-4′-O-β-D-glucoside. The structure of the compound was certainly determined by chemical analyses, as well as 1D- and 2D-NMR spectroscopy. The compound exhibited potent inhibitory activity against the expression of matrix metalloproteinase-1 caused by UV-irradiation in cultured human skin fibroblasts.

Key words Viola hondoensis; tectoridin-4′-O-β-D-glucoside; matrix metalloproteinase; human skin fibroblasts

Viola hondoensis W. BECKER et H. BOISSIEU (Violaceae) is distributed in the southern part of Korea.1) In traditional medicine, the herb has been used as an expectorant and a remedy for skin eruptions.2) Previous pharmacological and phytochemical studies on Viola species have revealed to be a rich source of cyclotides,3) and several flavone glycosides.4) Antiphotoactivity of V. hondoensis was also reported.5)

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endoproteinases that play pivotal roles in the dynamic remodeling of extracellular matrix. Based on substrate preference and structural homology, MMPs are sub-classified into functional groups: collagenases, gelatinases, stromelysins, matrixysins, membrane type-MMPs (MT-MMPs) and other non-classified MMPs.6) Naturally aged skin is smooth, pale, and finely wrinkled. In contrast, photoaged skin is definitely determined by chemical analyses, as well as 1D- and 2D-NMR spectroscopy. The compound exhibited potency against a MMP-1 expression; the MMP-1 expression level decreased to 84.6% at 10 μg/ml compared with the control by subfraction E4 human MMP-1 promoter luciferase assay. The EtOAc extract (6 g) was subjected to column chromatography on silica gel 60 (Merck, 40–63 μm, 350 g, 4.5×68 cm) eluting with n-hexane (22 g), CHCl3 (3 g), EtOAc (7 g), and n-BuOH (13 g). Among these fractions, the MeOH extract had inhibitory effect against a MMP-1 expression; the MMP-1 expression level decreased to 82.6% at 10 μg/ml and 73.2% at 100 μg/ml compared with the control by human MMP-1 promoter luciferase assay, and the EtOAc extract had potent inhibitory effect against a MMP-1 expression; the MMP-1 expression level decreased to 72.8% at 10 μg/ml and 53.7% at 100 μg/ml compared with the control by human MMP-1 promoter luciferase assay. The EtOAc extract (6 g) was subjected to column chromatography on silica gel (63—200 μm, 310 g, 4.5×68 cm) eluting with n-hexane followed by n-hexane–EtOAc (20:1, 4:1, 3:2, 2:3, 1:4, 1:20, each 5 l) and finally with CHCl3–MeOH (1:1, 2.5 l). The fractions were pooled into 6 major subfractions (E1 (1.3 g, 1 l), E2 (310 mg, 4.5 l), E3 (128 mg, 1.5 l), E4 (325 mg, 1 l), E5 (103.2 mg, 1 l), E6 (2.6 g, 1.5 l)) based on their TLC profiles. Among these subfractions the MMP-1 expression level decreased to 82.6% at 1 μg/ml and 93.2% at 10 μg/ml compared with the control by human MMP-1 promoter luciferase assay.

MATERIAL AND METHODS

General Experimental Procedures The melting points were determined using by Fisher Scientific melting point apparatus (Fisher Scientific) and were uncorrected. UV spectra were obtained on a Shimadzu UV/Visible Spectrophotometer. The IR spectra were measured in KBr pellets using an apparatus (Fisher Scientific) and were uncorrected. UV spectra, as well as 1D- and 2D-NMR spectroscopy. The compound exhibited potency against a MMP-1 expression; the MMP-1 expression level decreased to 84.6% at 10 μg/ml and 73.2% at 100 μg/ml compared with the control by human MMP-1 promoter luciferase assay. The EtOAc extract (6 g) was subjected to column chromatography on silica gel (63—200 μm, 310 g, 4.5×68 cm) eluting with n-hexane followed by n-hexane–EtOAc (20:1, 4:1, 3:2, 2:3, 1:4, 1:20, each 5 l) and finally with CHCl3–MeOH (1:1, 2.5 l). The fractions were pooled into 6 major subfractions (E1 (1.3 g, 1 l), E2 (310 mg, 4.5 l), E3 (128 mg, 1.5 l), E4 (325 mg, 1 l), E5 (103.2 mg, 1 l), E6 (2.6 g, 1.5 l)) based on their TLC profiles. Among these subfractions the MMP-1 expression level decreased to 82.6% at 1 μg/ml and 93.2% at 10 μg/ml compared with the control by human MMP-1 promoter luciferase assay.

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preparative TLC (RP-18 F254S, 0.5 mm, MeOH/CH2O:H2O 1:1), and finally with TOYOPEARL HW-40 (40 g, 2.8×70 cm, MeOH/H2O: 1:1 (500 ml)) to give the compound 1 (15 mg).

**Tectoridin-4′-O-β-D-glucoside** White plate crystals, IR (KBr) νmax (cm−1): 3600–3200 (OH), 1755 (C=O), 1595, 1510 (aromatic ring); UV λmax (EtOH) nm (log e): 219 (4.21), 271 (3.81) 333 (1.72); +AlCl3, 276, 329 (sh); +AlCl3/HCl, 278, 321 (sh); +NaOAc 269, 328. FAB-MS (positive); [M+1] + m/z 625 (Calc. for C28H32O16); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+); HR-FAB-MS m/z 625.4566 (Calc. for C28H32O16 [M+H]+). The UV light source was a F75/85W/UV21 fluorescent sun lamps, having an emission spectrum between 285–350 nm (peak at 310–315 nm) as previously described.5) A Kodacel filter (TA401/407; Kodak, Rochester, NY, U.S.A.) was mounted 2 cm in front of the UV tubes to remove wavelengths <290 nm (UV-C). The fibroblasts were grown in 10 cm culture dishes (Falcon, Lincoln Park, NJ, U.S.A.) until subconfluent. Subsequently, the cells were cultured in serum-free medium for 24 h, and the medium was replaced by 2 ml of phosphate-buffered saline. Then the cells were exposed to UV (0–100 ml/cm²) light. After irradiation (1 min 20 s), the cells were washed with phosphate-buffered saline, and 72 h cultured in the serum free media with or without compounds.

### Western Blot and Statistical Analysis
Supernatant extract were centrifuged at 12000×g for 10 min, and used for western blot analysis. A monoclonal anti-MMP-1 antibody (Oncogen, Co., Boston, MA, U.S.A.) was used as primary antibodies. Anti-mouse IgG-HRP conjugates was used as secondary antibodies. The antibody-antigen complexes were detected using the ECL system (Amersham Pharmacia Biotech; Little Chalfont, U.K.). Signal strengths were quantified using a densitometric program (TINA; Raytest Isotopen Gerate, Germany). Statistical significance was determined using the Student t-tests. Results are presented by means±S.E.M. All p values quoted are two-tailed and were accepted as significant when p was<0.05 (n=10).

### RESULTS AND DISCUSSION
Column chromatographic isolation of the ethyl acetate soluble fraction from aerial parts of *V. hondoensis* yielded an isoflavonoid glycoside (1) which inhibited expression of MMP-1 by UV-irradiation in cultured human skin fibroblast. (Fig. 1). It has been reported that new isoflavonoid glycoside from *Iris spuria* L.12) Compound (1) was obtained as white plate crystals. The molecular formula of 1 was found to be C28H32O16 by HR-FAB-MS spectrometry (m/z 625.4566 [M+H]+). The UV spectrum of 1 exhibited absorption maxima at 219, 271 and 333 nm, which are characteristic absorption bands of isoflavonoid, and showed bathochromic shift characteristic in positive reaction with AlCl3 reagents. A positive reaction in AlCl3 reagent same suggested the substitutions in this compound.13) In the IR spectrum of 1, absorptions due to a hydroxyl (3600–3200 cm⁻¹), unsaturated carbonyl (1755 cm⁻¹), and aromatic ring (1595, 1510 cm⁻¹) were observed. On acid hydrolysis of 1, compound gave the D-glucose as the sugar component by GC analysis after diastereomeric derivatization. Spectral data for obtained the recommendations (Applied Biosystems).

### Human Skin Fibroblast Cell Culture and Cell Proliferation Assay
Primary cultures of human skin fibroblasts were established from human adult foreskin in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin (100 U/ml), streptomycin (100 μg/ml) in a 37°C humidified incubator containing 5% CO2. The fibroblasts were cultured until 90% confluency and then, subcultivated. Cells cultured after 5 passages were used for the experiments. Cell proliferation of human skin fibroblasts (5×10⁵/well) was determined by the MTT assay.11)
aglycone from 1 were identical to those of tectorigenin.\textsuperscript{[13]}

The \(^1\)H-NMR spectrum of 1 showed six aromatic protons at \(\delta 6.91\) (H-8), \(\delta 7.13\) (J=8.9 Hz, H-3‘, 5’), \(\delta 7.53\) (J=8.8 Hz, H-2‘, 6’), \(\delta 8.51\) (H-2), one methoxy group at \(\delta 3.78\) (OMe). In addition, the two anomeric proton at \(\delta 4.94\) (J=7.7 Hz, H\(_{\text{aglycone}}^\text{1}’\)) and \(\delta 5.12\) (J=7.6 Hz, H\(_{\text{aglycone}}^\text{1}’\)) and several other protons at \(\delta 3.78\)—3.30 (6H) suggest the presence of a glucosyl moiety. Based on the coupling constant value of the anemic proton of 1, the configuration of the glucosidic linkage was determined to be a \(\beta\)-linkage.\textsuperscript{[14]}

The \(^{13}\)C-NMR spectral data of 1 was in good agreement with those of the literature values in tectoridin glucoside.\textsuperscript{[15]}

All proton and carbon resonances were assigned according to the data obtained from DEPT and HMQC experiments of 1. \(^{13}\)C-NMR chemical shifts of the sugar carbons in 1 were consistent with two glucose units.\textsuperscript{[16]}

The \(^1\)H-NMR spectrum of 1 showed the presence of two interglucosidic linkage signals at \(\delta 60.2\) (C\(_{\text{glucoside}}^\text{6}’\)-6”) and \(\delta 61.0\) (C\(_{\text{glucoside}}^\text{6}’\)-6”), one methoxy group at \(\delta 60.8\) (O–CH\(_3\)), benzene ring signals and glucosyl moiety. In the HMBC spectrum of 1, \(\delta 4.94\) (J=7.7 Hz, H\(_{\text{aglycone}}^\text{1}’\)) was correlated to a \(\delta 156.0\) (C-7) and the \(\delta 5.12\) (d, J=7.6 Hz, H\(_{\text{aglycone}}^\text{1}’\)) was correlated to a \(\delta 157.1\) (C-4’). In addition, the \(^1\)H–\(^{13}\)C long-range correlation between the anomeric proton and the benzene ring carbon of 1 suggest that the sites of attachment of sugars to aglycone moiety at C-7 and C-4’ position. The position of the glucosyl group of 1 was confirmed by the 2D-NOESY spectrum. The 2D-NOESY spectrum of 1 showed correlations between H-3’, 5’ (\(\delta 7.13\)) and H\(_{\text{aglycone}}^\text{1}’\) (\(\delta 5.12\)), and between H-8 (\(\delta 6.91\)) and H\(_{\text{aglycone}}^\text{1}’\) (\(\delta 4.94\)). Based on these observations, the compound (1) was determined to be a tectoridin-4’-O-\(\beta\)-D-glucoside, and reported the first isolation from the viola species.

To examine the effect of compound 1 on the expression of MMP-1 in primary human skin fibroblasts, the compound (1) did not show cytotoxicity against human skin fibroblast in test dose (0.1—1 \(\mu\)M, \(p<0.001\)) as compared to control. Human skin fibroblasts were treated with 0.01—1 \(\mu\)M of 1 for 72 h after UV irradiation and then, the expression levels of MMP-1 were determined in the culture media by Western blot analysis. The decreased MMP-1 protein expression significantly in a dose-dependent manner; by an average of 73.1±9.3% (\(p<0.05\), \(n=10\)) at 0.01 \(\mu\)M, 60.8±11.4% (\(p<0.05\), \(n=10\)) at 0.1 \(\mu\)M, and 58.4±10.2% (\(p<0.05\), \(n=10\)) at 1 \(\mu\)M, compared with UV-treated cells. Treatment of positive control (all trans retinoic acid), by an average of 69.5±8.3% (\(p<0.05\), \(n=10\)) at 0.01 \(\mu\)M, 58.4±9.9% (\(p<0.05\), \(n=10\)) at 0.1 \(\mu\)M, and 54.3±8.7% (\(p<0.05\), \(n=10\)) at 1 \(\mu\)M, compared with UV-treated cells. Compound 1 markedly inhibited UV-induced MMP-1 expression to that of control (Fig. 2). These results demonstrate that compound 1 reduces the expression of MMP-1 in protein levels. It is well established that the UV irradiation of cultured human skin fibroblasts in vitro or human skin in vivo induces the expression of MMPs, which play important roles in the degradation of extracellular matrix components during premature skin aging or photoaging. In conclusion, tectoridin-4’-O-\(\beta\)-D-glucoside may be used for the treatment and prevention of skin aging processes by UV irradiation.

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REFERENCES