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# Triterpenoid Saponin from *Viola hondoensis* W. Becker et H Boss. and Their Effect on MMP-1 and Type I Procollagen Expression

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Bioassay-guided fractionation has led to the isolation of triterpenoid saponins such as Acutoside A (3-*O*-[*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl] oleanolic acid) from the whole plants of *Viola hondoensis*. Among them, Saponin 1 exhibited potent inhibitory activity against matrix metalloproteinase (MMP)-1, and prevented the UV-induced changes in the MMP-1 expression. In addition, compound was isolated from this plant for the first time.

**Key words:** *Viola hondoensis*, Violaceae, Triterpenoid saponin, MMP1 inhibitor

## INTRODUCTION

*Viola hondoensis* Becker et H Boiss (Violaceae) distributed in southern area of Korea (Lee, 1996). In traditional medicine, the herb has been used as an expectorant, a diuretic, and an antiinflammatory for bronchitis, rheumatism, skin eruptions, and eczema (Schuele and Lederman, 2004; Tennstedt *et al.*, 1979). Previous phytochemical studies on *Viola* species have revealed them to be a rich source of cyclotides (Lindholm *et al.*, 2002; Svangard *et al.*, 2003), and several flavone glycosides (Carnat *et al.*, 1998; Xie *et al.*, 2003). But, phytochemical and biological studies of *V. hondoensis* have not been reported.

In an attempt to search for natural products with potent matrix metalloproteinases (MMP)-1 expression inhibition effects, we identified that the *n*-BuOH soluble fraction from the whole plants of *V. hondoensis* showed significant MMP-1 expression inhibition effects on the UV-induced damage of cultured human fibroblasts.

The matrix metalloproteinases (MMPs) are a family of >20 zinc-dependent endoproteinases that are capable of degrading almost all of the components of the extracellular matrix (Chambers *et al.*, 1997). MMPs can be divided into four categories based on substrate preference: collagenases, gelatinases, stromelysins, and membrane-associated matrix metalloproteinases (Bernhard *et al.*, 1994). MMPs are necessary for tissue remodeling and the healing cascade under normal physiological condition. The aging process of skin can be divided into intrinsic aging and photoaging. Clinically, naturally aged skin is smooth, pale, and finely wrinkled. In contrast, photoaged skin is coarsely wrinkled (Gilchrest., 1989). Alterations in collagen, the major structural component of skin, have been suggested as a cause of the changes, such as skin wrinkling and loss of elasticity, observed in naturally aged and photoaged skin (Fisher *et al.*, 1997; Varani *et al.*, 2000). With increasing age, collagen synthesis becomes lower and MMP-1 levels become higher in sun-protected human skin *in vivo* (Varani *et al.*, 2000). UV irradiation induces the synthesis of matrix metalloportienases (MMP) in fibroblast cell *in vitro* and MMP-mediated collagen destruction accounts, in large part, for the connective tissue damage that occurs in photoaging (Fisher *et al.*, 1996; Fisher *et al.*, 1997).

In this paper, we report matrix metalloproteinase-1 expression inhibitor from the whole plants of *V. hondoensis* on the UV-induced damage of cultured human dermal fibroblasts. Epigallocatechin-3-gallate (EGCG) was used as a positive control (Fig. 2-4). Antioxidants, such as EGCG, vitamin C, and E inhibit the expression of MMP-1 in human dermal fibroblasts. EGCG is a major constituent

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polyphenol in green tea and commonly used for preventing UV-induced adverse skin reaction such as sunburn and photoaging (Salah *et al.*, 1995).

## MATERIALS AND METHODS

### General procedure

NMR spectra were recorded on a Bruker DMX-300 and 600 spectrometer with TMS as an internal standard. Column chromatography was carried out Silica gel 60 (70-230 and 230-400 mesh, Merck) and YMC-GEL ODS-A (12 nm, S-75 mm, YMC). TLC and preparative TLC were carried out on precoated Silica gel 60 $F_{254}$ (art.5715, Merck) and RP-18 $F_{254}$s (art.15389, Merck) plates.

### Plant materials

The whole plants of *Viola hondoensis* (Violaceae) were collected in October 2003 at Ullung island, Korea.and The botanical identification was made by one of the authors, Dr. Joong-Ku Lee. A voucher specimen of this raw material has been deposited at the herbarium of the Seoul National University (SNU-03-10-01).

### Extraction and isolation

Dried and chopped whole plants of *V. hondoensis* (430 g) were extracted with MeOH (4L×5). The extract was concentrated *in vacuo* to yield a dark green residue (53 g), which was suspended in water and successively partitioned with hexane (22 g), $CHCl_3$ (3 g), EtOAc (7 g), and *n*-BuOH (13 g). and the *n*-BuOH extract (13 g) was subjected to RP-18 column and eluted with water-MeOH mixture (1 : 1 → 0 : 1) yielded five subfractions (Fr. I-V). Acutoside A (7 mg) were isolated from subfraction IV (0.9 g) using silica gel column chromatography ($CHCl_3$-MeOH-water, 7 : 2.5 : 1).

### Acutoside A

Needle crystal (EtOH); mp: 215; $[\alpha]_D^{26}$ + 34° (MeOH; c 1.2); $C_{42}H_{68}O_{13}$ (ESI-MS$^-$: m/z 779 [M-H]$^-$); $^{13}$C-NMR (pyridine-$d_5$, 300 MHz): 180.2 (COOH, C-28), 106.0 (CH, C-1''), 105.0 (CH, C-1'), 89.0 (CH, C-3), 83.3 (CH, C-2'), 78.4 (CH, C-3'), 78.2 (CH, C-5'), 78.1 (CH, C-3''), 77.9 (CH, C-5''), 77.1 (CH, C-2''), 71.7 (CH, C-4'), 71.5 (CH, C-4''), 62.8 ($CH_2$, C-6'), 62.7 ($CH_2$, C-6'').

### Acid hydrolysis of saponins

The Acutoside A (3 mg) was refluxed with 5% HCl in 60% aqueous dioxane for 3 h. The sapogenin mixture was extracted with EtOAc (15 mL, 3 time), washed with $H_2O$, dried over $Na_2SO_4$ and evapd. to dryness. Oleanolic acid was identified from the sapogenin residue with authentic samples by TLC in $CHCl_3$:MeOH (98:2). The acid aq. layer was neutralised with 0.5 M KOH and freeze-dried. Sugar was identied with authentic samples by TLC in MeCOEt:isoPrOH:$Me_2CO$:$H_2O$ (20:10:7:6) as glucose.

### Human dermal fibroblasts culture

Primary cultures of dermal fibroblasts were established from human adult foreskins 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% $CO_2$.

### Cell proliferation assay and UV irradiation

Cell proliferation was determined by the MTT assay. (Mosmann *et al.*, 1983) The UV-B 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 (Seo *et al.*, 2001).

### Western blots

Soluble protein fraction was extracted from the cultured human fibroblast cells with WCE buffer (containing 1% triton X-100, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid, 0.1 mM dithiothreitol, 1 mM PMSF, 1 g/mL aprotinin, and 2 g/mL leupeptin). Supernatants were centrifuged at 12,000×g for 10 min, and the supernatant was used for western blot analysis. A monoclonal anti-type I procollagen aminoterminal extension peptide (SP1.D8) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and monoclonal anti-MMP-1 antibody (Oncogen, Co., Boston, MA, USA) were used as primary antibodies.

### Statistical analysis

Statistical significance was determined using the Student t-tests. Results are presented by means ± SEM. All p values quoted are two-tailed and were accepted as significant when p was ≤ 0.05.

## RESULTS AND DISCUSSION

Activity-guided column chromatographic separation of *n*-BuOH soluble fraction of *Viola hondoensis* led to the isolation of triterpene saponin (Fig. 1).

Acid hydrolysis of the isolated saponin yielded oleanolic acid, thus the genin of the saponins was idnetified as oleanolic acid. The $^{13}$C-NMR spectra of compound showed the signals of C-3 at δ 88.8-89.4 and C-28 at δ 180.1-180.3, which indicated compound was monodesmosides. Compound showed anomeric proton signals at δ 4.88 (1H, d, $J$ = 7.5 Hz, H-1') and 5.27 (1H, d, $J$ = 7.9 Hz, H-1''), respectively. In $^{13}$C-NMR spectrum, Sugar moiety of compound was also consisted of two glucose, however, the linkage between glucose was proved to be (1→2) by

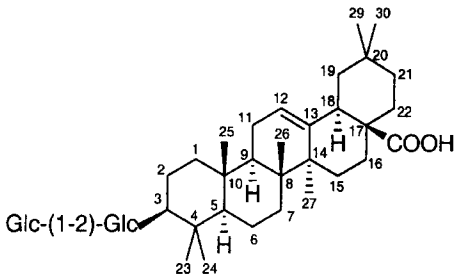


**Fig. 1.** Structures of isolated compound from *Viola hondoensis* Becker et H Boiss

comparison of reported NMR data (Nagao *et al.*, 1991), thus compound was identified as Acutoside a. The *in vitro* cytotoxicity assay was investigated according to reference (Mosmann *et al.*, 1983). Saponin 1 not showed cytotoxicity against human dermal fibroblast in test dose (0.1-10 μM, $p<0.001$) as compared to control. We studied the effects of compound on the expression of type I procollagen, MMP-1 proteins in cultured human dermal fibroblasts. Human dermal fibroblasts were treated with 10 μM for 72 h, and then, the expression levels of type I procollagen, MMP-1 were determined in the culture media by western blot analysis. therefore, human dermal fibroblasts were treated with various doses of compound for 72 h to investigate the dose-dependent effects of compound increased the type I procollagen protein expression by 156.1 ± 11% ($p<0.05$) at 0.1 μM, 192.4 ± 15 % at 1 μmM, and 512.7 ± 18% at 10 μM (Fig. 2), and decreased MMP-1 protein expression significantly in a dose-dependent manner; by an average of 76.2 ± 13% at 0.1 μM, 67.5 ± 9% at 1 μM, and 60.3 ± 12% at 10 μM, compared with vehicle-treated control cells (Fig. 3). Compound has any

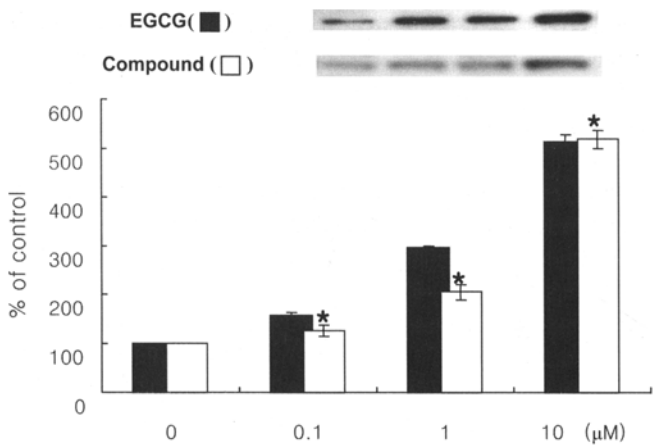


**Fig. 2.** The effect of Acutoside A on the product of type I procollagen. Acutoside A indcreased the type I procollagen expression in the culture human dermal fibroblasts. Epigallocatechin-3-gallate was used as a positive control. Each value represents a mean±SEM (n=5). *$p<0.05$ compared with the control.

inhibitory effects on the UV-induced changes of MMP-1 protein expression, human dermal fibroblast were irradiated with 100 mJ/cm$^2$ of UV light with (0.1, 1, 10 μM) or without treatment of compound. Treatment of compound (0.1, 1, 10 μM) significantly, UV-induced MMP-1 protein expression was inhibited by pretreatment of compound by 63.2% ($p<0.05$, n=5) at 0.1 μM, 38.2% ($p<0.05$, n=5) at 1 μM, 30.9% ($p<0.05$, n=5) at 10 μM in cultured fibroblasts in a dose-dependent manner (Fig. 4). In conclusion, Saponin

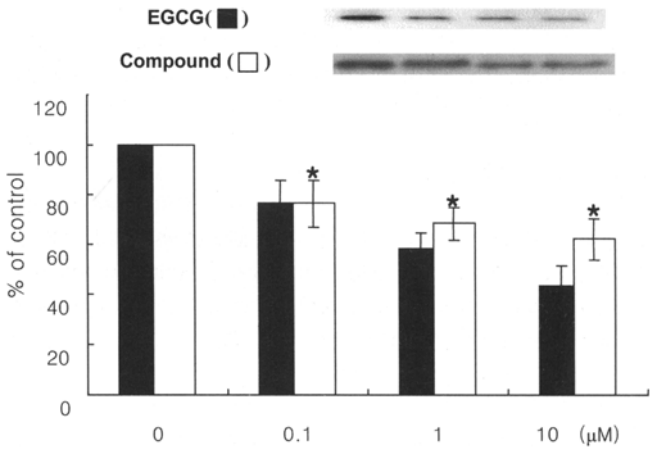


**Fig. 3.** The effect of Acutoside A on the product of MMP-1. Acutoside A decreased the MMP-1 expression in the culture human dermal fibroblasts. Epigallocatechin-3-gallate was used as a positive control. Each value represents a mean±SEM (n=5). * $p<0.05$ compared with the control.

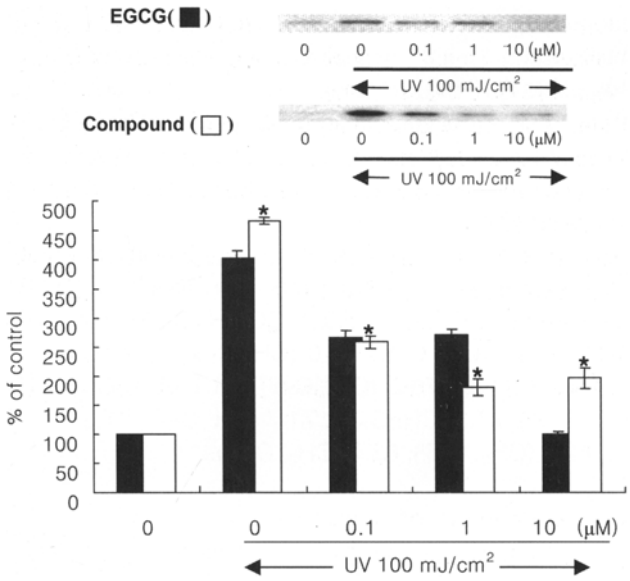


**Fig. 4.** Acutoside A prevent UV-induced increase in the MMP-1 expression in the culture human dermal fibroblasts. The cells were pretreated with Acutoside A prior to UV irradiation (100 mJ/cm$^2$) and harvested 72 h later. Epigallocatechin-3-gallate was used as a positive control. Each value represents a mean±SEM (n=5). * $p<0.05$ compared with the control.

1 can be used for the treatment and prevention of skin aging processes, based on following our results; 1) Topical application of compound in aged human skin increases the procollagen and decreased the MMP-1 expression. 2) Compound prevent UV-induced changes of MMP-1 expression.

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