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Effects of Unripe Apple Polyphenols on the Expression of Matrix Metalloproteinase-1 and Type-1 Procollagen in Ultraviolet Irradiated Human Skin Fibroblasts

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Abstract Bioassay-guided isolation of an aqueous ethanolic extract from the unripe fruits of *Malus pumila* cv. Fuji using matrix metalloproteinase-1 (MMP-1) inhibition and type-1 procollagen inducing assays led to isolation of eight phenolic compounds, reynoutrin (1), quercitrin (2), isoquercitrin (3), phloridzin (4), phloretin-2'-*O*-xyloglucoside (5), 5-*O*-*p*-coumaroylquinic acid (6), chlorogenic acid methyl ester (7), and chlorogenic acid (8). The structures were established on the basis of nuclear magnetic resonance and mass spectroscopic data interpretations. All isolates were evaluated for their inhibitory effects on MMP-1, and compounds 1, 2, and 6 exhibited potent inhibitory effects on MMP-1 with IC₅₀ values of 1.3±0.1, 1.4±0.1, and 3.2±0.6 μM, respectively. When evaluated for their effects on type I procollagen synthesis on human fibroblast cells, compounds 1 and 3 were respectively found to display potent activity for induction of type-1 procollagen by 78.5 and 78.6% at the tested concentration of 12.5 M.

Keywords Matrix metalloproteinase-1 · Phenolic compounds · Type-1 procollagen · Unripe *Malus pumila* cv. Fuji · UV

Introduction

UV irradiation induces photodamage, and causes skin cancer and premature skin aging (Fisher et al., 1996). The functional properties of human skin are dependent on the integrity of collagens, which are the most abundant structural proteins in dermis (Kavitha and Thampan, 2008). Type-1 collagen, the most abundant structural protein in skin connective tissue, is synthesized primarily by thermal fibroblasts and is responsible for conferring strength and resiliency to skin. In addition, collagen is tightly controlled by matrix metalloproteinases (MMPs) and their tissue inhibitors (Scharffetter et al., 1991; Chung et al, 2001). MMPs belong to the family of zinc-dependent endoproteinases that play important roles in dynamic remodeling of extracellular matrix. Based on substrate preference and structural homology, MMPs are divided into functional groups, such as collagenases, gelatinases, stromelysins, matrilysins, membrane type-MMPs (MT-MMPs), and other non-classified MMPs (Visse and Nagase, 2003). MMPs are frequently overexpressed by various extracellular stimuli, including UV, cytokines, tumor promoters, and growth factors; these enzymes increase in MMP-related activities and may be involved in the pathogenesis of diseases such as cancer and inflammation as well as in physiologic processes (Sternlicht and Werb, 2001). The production and fibrillar organization of collagen in the skin are reduced by chronological and UV-induced skin aging (Fisher et al., 1996). With increasing age, collagen synthesis decreases and MMP-1 levels increase in naturally aged human skin; these alterations cause changes such as skin wrinkling and loss of elasticity (Varani et al., 2000). Therefore,

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regulation of collagen metabolism might be a useful strategy for prevention and treatment of UV-induced skin aging.

The genus *Malus*, which is comprised of approximately 35 species of small deciduous trees or shrubs in the family Rosaceae, is native to the temperate zones of the northern hemisphere, Europe, Asia, and North America. Apples contain several health-beneficial principles, including dietary fiber, sugars, vitamins, and phenolic compounds. Numerous polyphenols were isolated from dietary apple in order to demonstrate a wide variety of biological properties, such as antiobesity, anti-inflammatory, anticancer, and antiallergic activities (Akiyama et al., 2000; Tokura et al., 2005; Sugiyama et al., 2007; Yoshioka et al., 2008; Miura et al., 2008). Procyanidins, the main constituents of apple polyphenols, have attracted attention particularly due to their positive effects on vascular health (Corder et al., 2006). Despite the various biological evaluations performed on unripe apple extracts and isolated polyphenols, studies on the isolation of anti-wrinkle agents from unripe fruits of *M. pumila* cv. Fuji are limited. The aqueous ethanolic extracts of ripe and unripe apples were dried and tested for the MMP-1 suppressing and type-1 procollagen synthesis effects. The ethanolic extract of unripe apples exhibited a significant activity (IC_{50} , $17.8 \pm 1.3 \mu\text{g/mL}$) in the MMP-1 inhibition assay (Table 1), whereas the ripe apples extract was inactive (IC_{50} , $>100 \mu\text{g/mL}$). In addition, the effects of unripe and unripe apples extracts on anti-wrinkle activity were tested using type-1 procollagen synthesis assay. When assayed at a single concentration ($20 \mu\text{M}$) of unripe apples, the extract exhibited approximately 70% promotion of type-1 procollagen synthesis. As part of our continuing investigation of the biologically active constituents, the ethyl acetate (EtOAc)-soluble extract of unripe apples showed significant MMP-1 suppressing and type-1 procollagen inducing effects; thus the extract was fractionated by activity-guided isolation using these two assays. The present study describes the characterization of the purified compounds **1–8**, as well as biological evaluation of all isolates obtained using MMP-1 suppressing and type-1 procollagen inducing assays.

Materials and Methods

Plant material. Unripe fruits of *M. pumila* cv. Fuji were collected at Youngchun-si, Korea, in May, 2011, and identified by Dr. Tea Hoon Kim. A representative sample was deposited at the natural product chemistry laboratory of Daegu Haany University.

Instrumentation. UV spectra were obtained using a Hitachi U-2000 spectrophotometer (Hitachi, Japan). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured on a Varian VNS600 instrument (Varian, USA) operating at 600 and 150 MHz, respectively. The chemical shifts are given in δ (ppm) values relative to those of the solvent CD_3OD (δ_{H} 3.35; δ_{C} 49.0) on a tetramethylsilane scale. The standard pulse sequences programmed into the instruments were used for each 2D measurement. The J_{CH} value was set at 8 Hz in the heteronuclear

multiple bond connectivity HMBC spectra. Fast atom bombardment (FAB) mass spectroscopic (MS) were obtained on a Micro Mass Auto Spec OA-TOF spectrometer (Micromass, UK). HPLC analysis was performed on a YMC-Pack ODS A-302 column (4.6 mm i.d. \times 150 mm; YMC Co., Japan) and the solvent system consisted of a linear gradient that started with 10% (v/v) MeCN in 0.1% $\text{HCOOH}/\text{H}_2\text{O}$ (detection: UV 280 nm; flow rate: 1.0 mL/min; temperature: 40°C), increased to 90% MeCN over 20 min, and then increased to 100% MeCN over 5 min. Column chromatography was performed using Toyopearl HW-40 (coarse grade; Tosoh Co., Japan), and YMC GEL ODS AQ 120–50S (YMC Co., Japan). Thin-layer chromatography was performed on Kieselgel 60 F₂₅₄ plates (0.25 mm layer thickness, Merck, Germany), and the spots were detected by UV irradiation (254, 365 nm) and by spraying with 10% H_2SO_4 reagent.

Extraction and Isolation. Fresh unripe apples (2.0 kg) were ground and extracted exhaustively using 70% EtOH (7 L \times 3) at room temperature, and the solvent was evaporated *in vacuo*. This concentrated extract (112.9 g) was suspended in 10% MeOH (2.0 L), and then partitioned with *n*-hexane (2 L \times 3), EtOAc (2 L \times 3), and *n*-BuOH (2 L \times 3) to yield dried *n*-hexane- (1.1 g), EtOAc- (18.8 g), *n*-BuOH- (17.6 g) and H_2O -soluble (73.6 g) residues (Fig. 2). In a MMP-1 inhibition assay, the EtOAc-soluble extract was found to be the most active, with an IC_{50} value of $8.8 \pm 1.0 \mu\text{g/mL}$ (Table 1). On performance of chromatographic separation, fractions were monitored with reversed-phase HPLC. A portion (3.5 g) of the EtOAc extract was chromatographed over a Toyopearl HW-40 column (coarse grade; 2.8 cm i.d. \times 52 cm) with H_2O containing increasing amounts of MeOH in a stepwise gradient mode and fractionated into six subfractions MP01–MP06, respectively (Fig. 2). Bioactive subfraction MP02 was subjected to column chromatography over a YMC GEL ODS AQ 120-50S column (1.1 cm i.d. \times 33 cm) with aqueous MeOH to yield pure compounds **6** (3.2 mg, t_{R} 9.8 min), **7** (9.3 mg, t_{R} 10.3 min), and **8** (55.6 mg, t_{R} 9.1 min). In a similar fashion, the subfraction MP04 was chromatographed over a YMC GEL ODS AQ 120-50S column (1.1 cm i.d. \times 33 cm) with aqueous MeOH to yield pure compounds **1** (5.3 mg, t_{R} 10.9 min), **2** (44.3 mg, t_{R} 11.2 min), **3** (29.9 mg, t_{R} 10.6 min), **4** (13.2 mg, t_{R} 11.6 min), and **5** (9.3 mg, t_{R} 10.4 min).

Compound 1 (Reynoutrin). Yellow amorphous powder, FABMS m/z 435 $[\text{M}+\text{H}]^+$. ^1H -NMR (CD_3OD , 600 MHz): δ 7.58 (1H, dd, $J=8.4$, 2.0 Hz, H-6'), 7.56 (1H, d, $J=2.0$ Hz, H-2'), 6.85 (1H, d, $J=8.4$ Hz, H-5'), 6.38 (1H, d, $J=1.8$ Hz, H-8), 6.19 (1H, d, $J=1.8$ Hz, H-6), 5.17 (1H, d, $J=6.6$ Hz, H-1"), 3.77–3.09 (6H, sugar-H). ^{13}C -NMR (CD_3OD , 150 MHz): δ 179.4 (C-4), 166.1 (C-7), 163.1 (C-5), 158.9 (C-2), 158.4 (C-9), 149.9 (C-4'), 146.1 (C-3'), 135.4 (C-3), 123.3 (C-6), 123.0 (C-1'), 117.2 (C-5'), 115.9 (C-2'), 105.6 (C-10), 104.6 (C-1"), 99.9 (C-6), 94.7 (C-8), 77.5 (C-4"), 75.3 (C-2"), 71.0 (C-3"), 67.2 (C-5").

Compound 2 (Quercitrin). Yellow amorphous powder, FABMS m/z 449 $[\text{M}+\text{H}]^+$. ^1H -NMR (CD_3OD , 600 MHz): δ 7.34 (1H, d, $J=1.8$ Hz, H-2'), 7.31 (1H, dd, $J=8.4$, 1.8 Hz, H-6'), 6.91 (1H, d,

$J=8.4$ Hz, H-5'), 6.37 (1H, d, $J=2.4$ Hz, H-8), 6.20 (1H, d, $J=2.4$ Hz, H-6), 5.35 (1H, d, $J=1.8$ Hz, H-1''), 4.22–3.33 (4H, sugar-H), 0.94 (3H, d, $J=6.0$ Hz, H-6''). $^{13}\text{C-NMR}$ (CD_3OD , 150 MHz): δ 179.7 (C-4), 165.9 (C-7), 163.2 (C-5), 159.3 (C-2), 158.5 (C-9), 149.8 (C-4'), 146.5 (C-3'), 136.2 (C-3), 123.2 (C-6'), 123.0 (C-1'), 117.3 (C-5'), 116.8 (C-2'), 105.9 (C-10), 103.8 (C-1''), 100.0 (C-6), 95.1 (C-8), 73.0 (C-4''), 72.8 (C-2''), 72.5 (C-3''), 72.0 (C-5''), 17.8 (C-6'').

Compound 3 (Isoquercitrin). Yellow amorphous powder, FABMS m/z 465 $[\text{M} + \text{H}]^+$. $^1\text{H-NMR}$ (CD_3OD , 600 MHz): δ 7.83 (1H, d, $J=2.4$ Hz, H-2'), 7.58 (1H, dd, $J=8.4$, 2.4 Hz, H-6'), 6.86 (1H, d, $J=8.4$ Hz, H-5'), 6.39 (1H, d, $J=1.8$ Hz, H-8), 6.20 (1H, d, $J=1.8$ Hz, H-6), 5.15 (1H, d, $J=7.8$ Hz, H-5''), 3.84–3.46 (6H, sugar-H). $^{13}\text{C-NMR}$ (CD_3OD , 150 MHz): δ 177.7 (C-4), 165.5 (C-7), 161.4 (C-5), 157.4 (C-9), 156.4 (C-2), 148.7 (C-4'), 144.52 (C-3'), 134.4 (C-3), 121.79 (C-1'), 121.5 (C-6'), 116.3 (C-2'), 114.8 (C-5'), 104.4 (C-10), 103.2 (C-1''), 99.9 (C-6), 94.3 (C-8), 77.0 (C-5''), 75.8 (C-3''), 73.9 (C-2''), 68.7 (C-4''), 60.6 (C-6'').

Compound 4 (Phloridzin). White amorphous powder, FABMS m/z 437 $[\text{M} + \text{H}]^+$. $^1\text{H-NMR}$ (CD_3OD , 600 MHz): δ 7.05 (2H, d, $J=7.8$ Hz, H-2, 6), 6.67 (2H, d, $J=7.8$ Hz, H-3, 5), 6.18 (1H, d, $J=2.4$ Hz, H-3'), 6.13 (1H, d, $J=2.0$ Hz, H-5'), 5.03 (1H, d, $J=7.2$ Hz, H-1''), 3.71–2.87 (6H, sugar-H). $^{13}\text{C-NMR}$ (CD_3OD , 150 MHz): δ 206.5 (C=O), 167.5 (C-4'), 165.9 (C-6'), 162.2 (C-2'), 156.3 (C-4), 133.8 (C-1), 130.3 (C-2, 6), 116.0 (C-3, 5), 106.7 (C-1'), 102.0 (C-1''), 98.3 (C-5'), 95.4 (C-3'), 78.5 (C-5''), 78.4 (C-3''), 74.7 (C-2''), 71.0 (C-4''), 62.4 (C-6''), 46.9 (C-7), 30.8 (C-8).

Compound 5 (Phloretin-2'-*O*-xyloglucoside). White amorphous powder, FABMS m/z 569 $[\text{M} + \text{H}]^+$. $^1\text{H-NMR}$ (CD_3OD , 600 MHz): δ 7.07 (2H, d, $J=8.4$ Hz, H-2, 6), 6.69 (2H, dd, $J=8.4$ Hz, H-3, 5), 6.28 (1H, d, $J=2.0$ Hz, H-3'), 5.97 (1H, d, $J=2.0$ Hz, H-5'), 5.00 (1H, d, $J=7.2$ Hz, H-1''), 4.35 (1H, d, $J=7.8$ Hz, H-1'''), 4.02–2.88 (11H, sugar-H). $^{13}\text{C-NMR}$ (CD_3OD , 150 MHz): δ 206.6 (C=O), 167.5 (C-4'), 165.9 (C-6'), 162.2 (C-2'), 156.4 (C-4), 133.9 (C-1), 130.9 (C-2), 130.2 (C-6), 116.4 (C-5), 116.6 (C-3), 106.9 (C-1'), 105.3 (C-1'''), 102.7 (C-1''), 98.3 (C-5'), 95.4 (C-3'), 78.4 (C-3'''), 77.9 (C-3''), 77.6 (C-5''), 77.2 (C-2'''), 75.2 (C-2''), 74.7 (C-4'''), 71.7 (C-4''), 71.4 (C-5'''), 69.6 (C-6''), 46.9 (C-8), 30.8 (C-7).

Compound 6 (5-*O*-*p*-Coumaroylquinic acid). White amorphous powder. FABMS m/z 339 $[\text{M} + \text{H}]^+$. $^1\text{H-NMR}$ (CD_3OD , 600 MHz): δ 7.62 (1H, d, $J=16.2$ Hz, H-7'), 7.20 (1H, d, $J=2.0$ Hz, H-2'), 6.97 (1H, d, $J=8.0$, 2.0 Hz, H-6'), 6.80 (1H, d, $J=8.0$ Hz, H-5'), 6.32 (1H, d, $J=16.2$ Hz, H-8'), 5.32 (1H, m, H-5), 4.15 (1H, m, H-3), 3.65 (1H, m, H-4), 2.19 (4H, m, H-2, 6).

Compound 7 (Chlorogenic acid methyl ester). Amorphous powder, FABMS m/z 369 $[\text{M} + \text{H}]^+$. $^1\text{H-NMR}$ (CD_3OD , 600 MHz): δ 7.53 (1H, d, $J=16.2$ Hz, H-7'), 7.04 (1H, d, $J=1.8$ Hz, H-2'), 6.95 (1H, dd, $J=7.8$, 1.8 Hz, H-6'), 6.78 (1H, d, $J=7.8$ Hz, H-5'), 6.22 (1H, d, $J=15.6$ Hz, H-8'), 5.28 (1H, m, H-5), 4.14 (1H, m, H-6), 3.73 (1H, dd, $J=9.2$, 2.5 Hz, H-4), 3.69 (3H, s, OCH₃), 2.20 (2H, m, H-6), 2.14 (2H, m, H-2). $^{13}\text{C-NMR}$ (CD_3OD , 150 MHz): δ 175.4 (COOH), 168.3 (C-9'), 149.7 (C-4'), 147.0 (C-3'), 146.9

(C-7'), 127.7 (C-1'), 123.0 (C-6'), 116.2 (C-2'), 115.1 (C-5'), 115.0 (C-8'), 75.8 (C-1), 72.6 (C-5), 72.2 (C-4), 71.6 (C-3), 52.7 (OCH₃), 38.2 (C-6), 37.8 (C-2).

Compound 8 (Chlorogenic acid). White Amorphous powder, $^1\text{H-NMR}$ (CD_3OD , 600MHz): δ 7.60 (1H, d, $J=16.0$ Hz, H-7'), 7.09 (1H, d, $J=2.0$ Hz, H-2'), 6.99 (1H, dd, $J=8.0$, 2.0 Hz, H-6'), 6.82 (1H, d, $J=8.0$ Hz, H-5'), 6.30 (1H, d, $J=16.0$ Hz, H-8'), 5.37 (1H, m, H-5), 4.20 (1H, m, H-3), 3.76 (1H, dd, $J=9.5$, 2.5 Hz, H-4), 2.24 (2H, m, H-6), 2.14 (2H, m, H-2).

Cell culture and MTT assay. Human skin fibroblast cells, CCD-986sk, were purchased from the American Type Culture Collection (ATCC, USA). MTT assay was performed using a previously reported method (Shim et al., 2009) with a minor modification. Cells were cultured in Dulbeccos's Modified Eagle's Medium (Gibco, USA) supplemented with antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin) and 10% heat inactivated fetal bovine serum. Cells were grown at 37°C in a humidified incubator containing 5% CO₂, and the MTT test was performed for determination of the viability of CCD-986sk. The MTT assay is based on capacity of functional mitochondria to catalyze the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan, the concentration of which can be determined using an ELISA reader (Tecan Austria GmbH, Austria) at 540 nm.

MMP-1 inhibition assay. Levels of MMP-1 protein in the medium were measured using commercially available ELISA kits in accordance with the manufacturer's instructions (R&D Systems, USA). The standard solution in the MMP-1 kit, 6.25–100 ng/mL, was prepared using a 1:2 serial dilution. One hundred microliters of the standard and 100 μL of sample were added to the micro plates included in the MMP-1 ELISA kit. Following incubation of the samples and standards, 100 μL horseradish peroxidase conjugate was added to the micro plates. The plates were covered with plastic film and incubated at 37°C for two h. For removal of unbound antigens, the micro plates were washed five times with a buffer solution, followed by addition of 100 μL of chromogenic tetramethyl benzidine agent for 30 min. Subsequently, 100 μL stop solution was added for 15 min. The absorbances of both enzymes were measured at 450 nm using an ELISA plate reader (Tecan Austria GmbH, Austria).

Type I collagen synthesis assay. Human dermal fibroblasts (5×10^4 cells) were seeded into 24-well plates, and the medium was decanted 24 h later. The cells were then washed twice with phosphate-buffered saline, followed by addition of serum-free medium. After 24 h, samples were added and cultivated for 48 h. The culture medium was collected after stimulation and used for assessment of collagen synthesis. The collagen content was determined using the procollagen type I C-peptide assay kit (Takara Bio Inc., Japan).

Statistical analysis. Data are expressed as the means \pm SD of three independent experiments. Statistical significance was accepted at a level of $p < 0.05$.

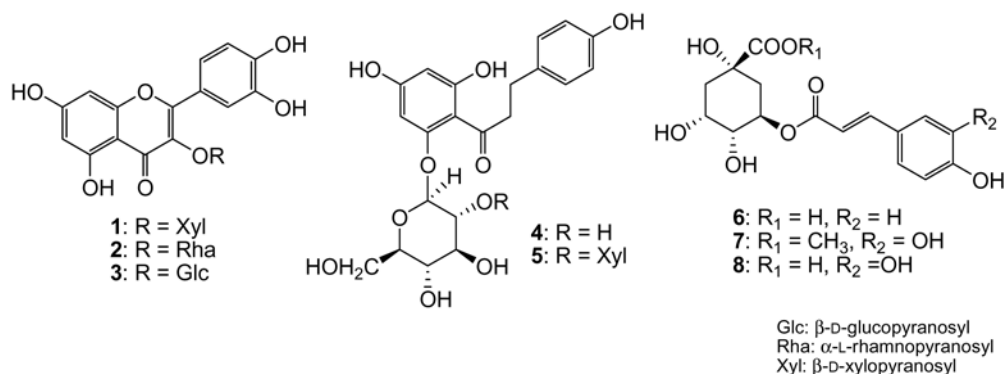


Fig. 1 Structures of isolated compounds **1–8** from unripe fruits of *M. pumila* cv. Fuji.

Results and Discussion

Results of the MMP-1 suppressing and type-1 procollagen inducing assays showed that among the *n*-hexane, EtOAc, *n*-BuOH, and H₂O-soluble fractions, the EtOAc-soluble fraction of unripe fruits of *M. pumila* cv. Fuji was the most potent; therefore, it was selected for further detailed purification. Subfractions MP02 and MP04 of the EtOAc-soluble fraction showed potent activity in the MMP-1 inhibition assay, with IC₅₀ values of 6.2 and 7.1 μg/mL, respectively. In addition, subfractions MP02 and MP04 also exhibited 70.1 and 78.3% promotion of type-1 procollagen-inducing activity at the concentration of 12.5 μg/mL, respectively. Bioassay-guided purification of the EtOAc-soluble portion of unripe apples led to isolation of eight phenolic compounds **18**. On the basis of physical and spectroscopic data measurements ([α]_D, ¹H NMR, ¹³C NMR, DEPT, 2D NMR, and MS) and comparison of data obtained with published values, the structures of the isolated compounds were identified as reynoutrin (**1**) quercitrin (**2**), isoquercitrin (**3**), phloridzin (**4**), phloretin-2'-xyloglucoside (**5**), 5-*O*-*p*-coumaroylquinic acid (**6**), chlorogenic acid methyl ester (**7**), and chlorogenic acid (**8**) (Lu and Foo, 1997; OP de Beck et al., 1998; Zhu et al., 2005; Rodríguez et al., 2006) (Fig. 1).

Compounds **4** and **8** were found to be the major secondary metabolites of the EtOAc-soluble portion of unripe apples.

All of the eight compounds isolated in this investigation were evaluated for MMP-1 inhibition and type-1 procollagen inducing assays, using (–)-epigallocatechin gallate (EGCG) as the positive control. Quercetin derivatives were found to be the major MMP-1 inhibitors from unripe apples (Table 1 and Fig. 2). Eight known flavonoids and quinic acid derivatives **1–8** exhibited pronounced inhibitory activity against MMP-1, showing IC₅₀ values ranging from 1.3±0.1 to 14.1±1.4 mM, with reynoutrin (**1**) and quercitrin (**2**) as the most potent principles (Table 1). On the basis of the analysis of the structure-activity relationship of the evaluated active compounds in the MMP-1 inhibition assay, it could be suggested that the glycosylation pattern of C₃-OH had a slight effect on MMP-1 suppressing activity.

Non-toxic concentrations of the tested compounds were evaluated in human skin fibroblast cells, CCD-986sk, with concentrations of 12.5 and 6.25 μM. Following incubation for 2 days, cell viability was measured by MTT assay, and no cytotoxic effect was detected for all tested compounds **1–8** at concentrations up to 12.5 μM. Thus, the effects of the isolated compounds on induction of type-1 procollagen were tested and fibroblast cells were treated with

Table 1 Inhibitory effects of the Fractions and isolated compounds **1–8** on MMP-1

Fraction	IC ₅₀ value (μg/mL) ^{a)}	Compound	IC ₅₀ value (μM) ^{a)}
70% EtOH ext.	17.8±1.3	1	1.3±0.1
<i>n</i> -Hexane-soluble portion	>200	2	1.4±0.1
EtOAc-soluble portion	8.8±1.0	3	10.6±1.2
<i>n</i> -BuOH-soluble portion	52.1±2.8	4	10.9±1.2
H ₂ O-soluble portion	>200	5	10.8±1.3
MP01	>200	6	3.2±0.6
MP02	7.1±0.8	7	14.1±1.4
MP03	183.3±7.5	8	11.0±1.4
MP04	5.7±0.8	(–)-EGCG ^{b)}	7.9±0.7
MP05	>200		
MP06	>200		

^{a)}All samples were examined in triplicate experiments.

^{b)}Used as a positive control.

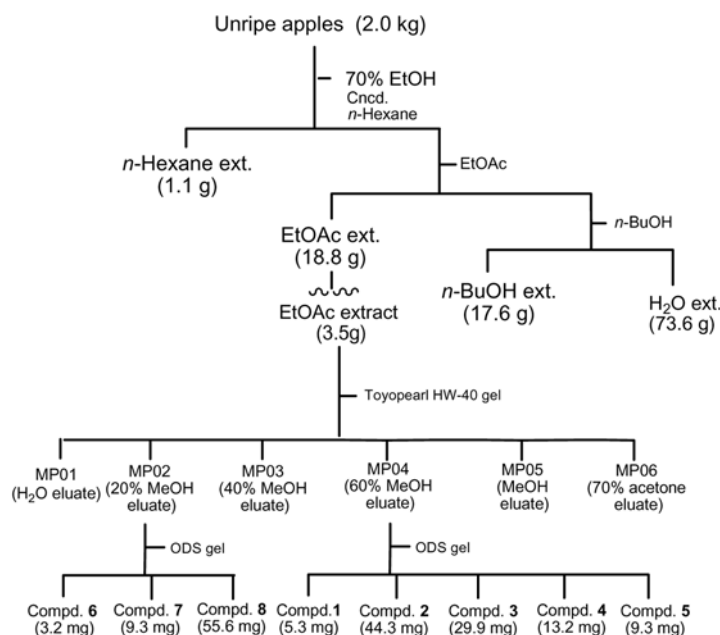


Fig. 2 Isolation procedure of compounds 1–8 from unripe fruits of *M. pumila* cv. Fuji.

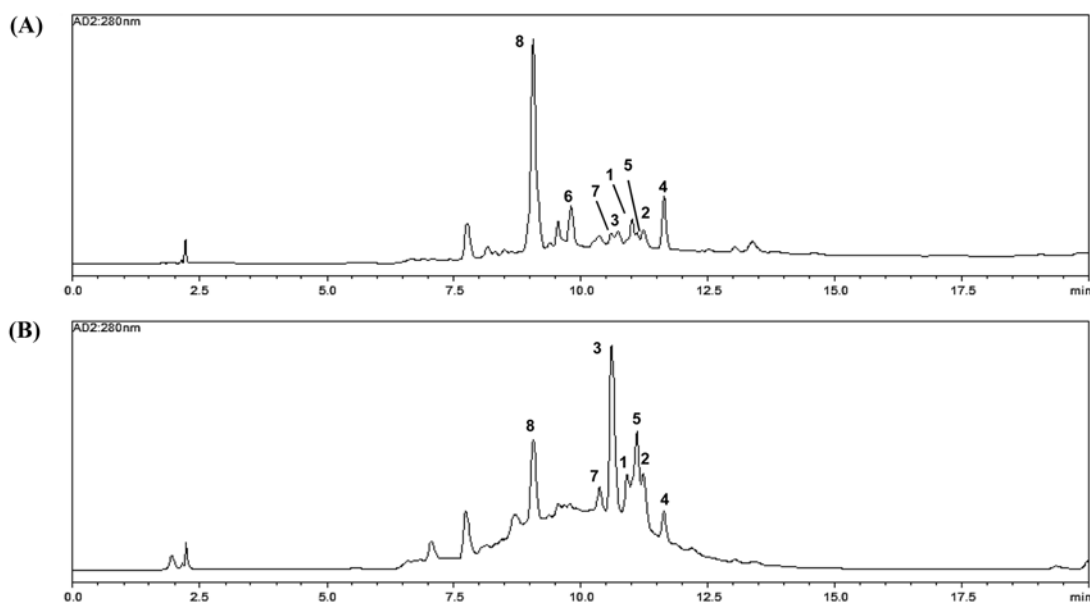


Fig. 3 HPLC comparison of isolated compounds 1–8 in unripe (A) and ripe apple (B) extracts; reynoutrin (quercetin 3-*O*-xyloside) (1), quercitrin (quercetin 3-*O*-rhamnoside) (2), isoquercitrin (quercetin 3-*O*-glucoside) (3), phloridzin (phloretin-2'-*O*-glucoside) (4), phloretin-2'-*O*-xyloglucoside (5), 5-*O*-*p*-coumaroylquinic acid (6), chlorogenic acid methyl ester (7), and chlorogenic acid (8).

the non-toxic concentrations during the evaluation period. Of the eight phenolic constituents isolated from unripe apples, compounds 1, 3, 4, and 6 were found to display potent activity for induction of type-1 procollagen by 78.5, 78.6, 76.6, and 74.0% at the tested concentration of 12.5 μM (Fig. 4), respectively, and exhibited potency comparable to that of the positive control at the same tested concentration, (-)-EGCG (77.1% induction).

Previous phytochemical investigation has resulted in the characterization of bioactive triterpene, diterpene, lignin, and flavonoid with potent inhibition of MMP-1 and type-1 procollagen inducing effects (Tokuda et al., 1986; Kim et al., 2004; Moon et al., 2006; Lee et al., 2008). A number of flavonoids isolated from apples and chemical components in the ripe and unripe apples demonstrated different existence in some species (Lu and Foo,

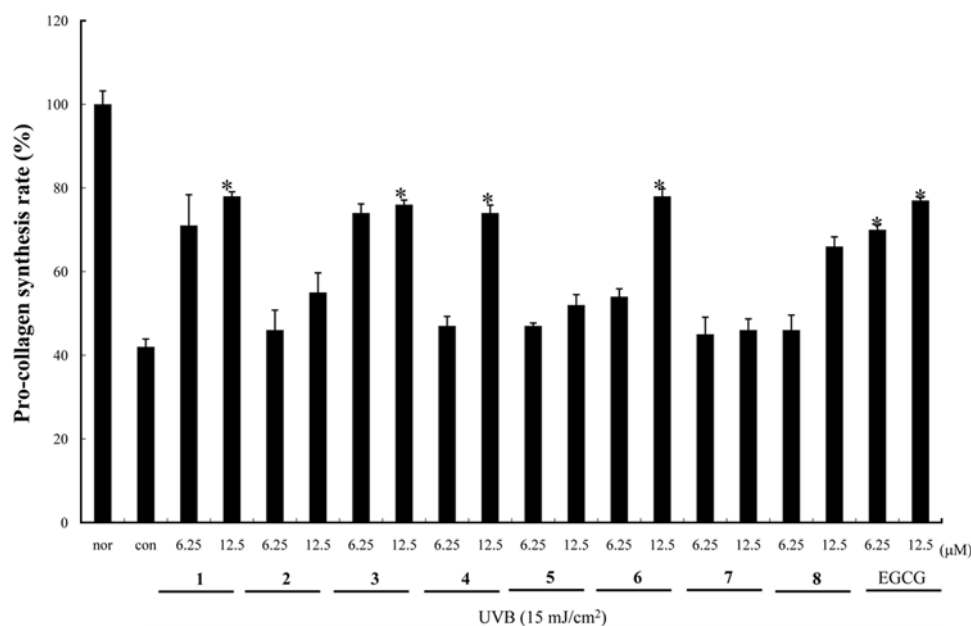


Fig. 4 Effects of the isolated constituents on Type I collagen synthesis in UVB-irradiated human fibroblast cells. UV (20 mJ/cm²)-exposed cells were cultured for 48 h in the presence of compounds. EGCG was used as a positive control. Data are expressed as percent of control and represent the mean value \pm SD ($n=3$) of triplicate experiments. * $p < 0.05$ compared to the non-ultraviolet irradiated control (student's t -test).

1997; Yue et al., 2012). Hence, comparison of chemical constituents of both apples was undertaken in the present study. Eight compounds of known structure were isolated from unripe apples, as described in the Extraction and Isolation section. A larger quantity of phloridzin (phloretin-2'-*O*-glucoside) (4), 5-*O*-*p*-coumaroylquinic acid (6), and chlorogenic acid (8) in 70% EtOH extract of unripe apples was identified relative to 70% EtOH extract of ripe apples by comparison of HPLC analysis results (Fig. 3). Whereas, reynoutrin (quercetin 3-*O*-xyloside) (1), quercitrin (quercetin 3-*O*-rhamnoside) (2), isoquercitrin (quercetin 3-*O*-glucoside) (3), phloridzin (phloretin-2'-*O*-glucoside) (4), phloretin-2'-*O*-xyloglucoside (5), chlorogenic acid methyl ester (7), and chlorogenic acid (8) were common in both samples. Among the isolates, chlorogenic acid methyl ester (7) was obtained from unripe and ripe apples for the first time. These results did not suggest that there was distinct relationship between the inhibition of MMP-1, and type-1 procollagen inducing effects and composition of quercetin glycosides in unripe and ripe apples. Previously studies (Lee et al., 2009; Liu et al., 2010), the concentrations of phenolic constituents were determined and correlation analysis for the significant relationship between matrix metalloproteinases and total phenolic content were performed. In current study, we found that total phenolic content of the unripe apple extract was nearly 4 times higher than that the ripe apple extract (data not shown). These findings suggested that total phenolic contents in unripe and ripe apples may influence MMP-1 inhibition and type-1 procollagen inducing effects. Apart from this relationship, there is evidence to suggest that the quercetin glycosides in unripe apples could be major contributors to anti-wrinkle activity, depending on the

different sugar moieties, may have influenced the bioactivity. Phytochemicals, including phenolic constituents, which are major bioactive secondary metabolites, are ubiquitous in edible fruits. In the search for bioactive natural products, our study also provided additional evidence of the effectiveness of dietary apple polyphenols. In conclusion, in the current investigation, because of the large amount of bioactive constituents present in unripe fruits of *M. pumila* cv. Fuji, unripe apple is an effective source as a potential anti-wrinkle agent and as a possible functional food ingredient of natural origin. Several of the isolated major constituents could also serve as standard markers for unripe apple preparations with regard to anti-wrinkle effects.

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