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Constituents from the Branches of *Sambucus sieboldiana* var. pendula with the Properties of Collagen Synthesis Activation

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ABSTRACT

For the purpose of developing anti-wrinkle cosmetic ingredients, the extracts from branches of a woody plant *Sambucus sieboldiana* var. pendula were examined on collagen synthesis activities using fibroblast HDFn cells. As a result, the S. sieboldiana ethanol extract (SSE) proved to activate the production of type I procollagen in a dose-dependent manner without showing cell toxicity. Phytochemical study was conducted to isolate the active constituents in the extracts by solvent fractionation followed by chromatographic purifications. From this procedure, two known compounds, kaempferol 3-O-sophoroside (1) and daucosterol (2), were identified by spectroscopic studies. From the isolates, the flavonoid glycoside 1 was verified to induce the synthesis of the type I procollagen dose-dependently. These results suggested that S. sieboldiana extract containing the flavonoid 1 could be useful as an active ingredient in wrinkle-care cosmetics.

INTRODUCTION

Collagen, a fibrous protein, is a major component in the extracellular matrix (ECM) of dermis and responsible for the tensile strength of human skin. Collagen is actually a complex family of 18 proteins, and type I collagen comprises 80-85% of the overall collagen fiber in the matrix. The amount of collagen I in photo-aged skin is lower than that in normal skin. It is therefore likely that collagen I is the most important type in the skin aging (Baumann, 2002). The wrinkle in the skin is caused by the lack of balance between production and degradation of collagen fiber. The breakdown of the collagen is primarily mediated by the enzyme matrix metalloproteinase-1 (MMP-1) in the dermis. Collagen synthesis is accomplished through a precursor, procollagen, inside the fibroblast cells in ECM. The generated procollagen is secreted into the ECM after hydroxylation of its proline and lysine residues, and finally transformed to collagen fiber by a series of polymerization processes. For the development of the skin anti-aging ingredients in cosmetic applications, one of the major strategies is to find materials which up-regulate the collagen contents in the dermis (Shuster *et al*., 1975). In this regard, retinoid compounds are widely used to cure the symptoms of the aged skin by the activation of collagen synthesis as well as by inhibition of MMP-1 expression (Brigstock, 2003). Since the reduced collagen content is recognized as the main cause of the skin wrinkles, much attention is focused on finding natural products with collagen synthesis activating properties for use in anti-aging cosmetic formulations (Lee *et al*., 2013; Ko *et al*., 2013). The plant, *Sambucus sieboldiana* var. pendula (Korean local name: malozum namu), is a deciduous shrub belonging to the Caprifoliaceae family. This tree native to East Asia grows up to 4 m tall, and its leaves have been used as foods of culinary vegetables. The water extract of S. sieboldiana has been reported to possess anti-inflammatory activities (Cho *et al*., 1994). In this study, the collagen synthesis activation properties were examined for the ethanol extract of S. sieboldiana branches. In addition, the active constituents were verified by phytochemical investigations.

MATERIALS AND METHODS

Plant material

The branches of S. sieboldiana were collected in February of 2013 from Jeju, the island located at the southernmost part of Korea.

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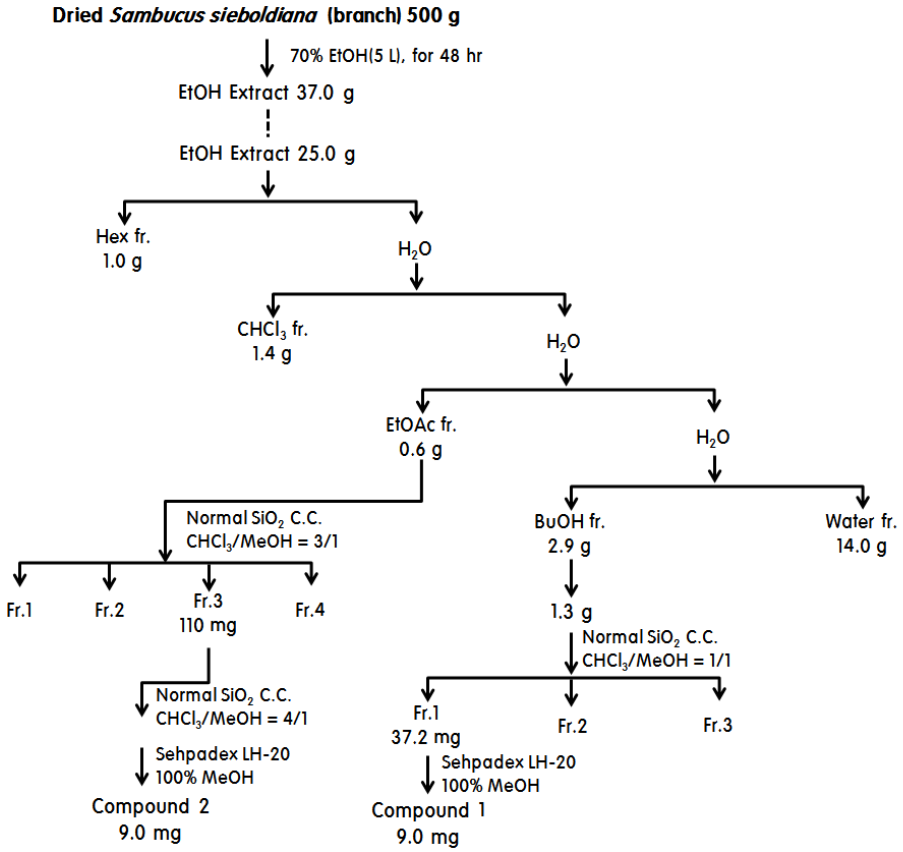


Fig. 1: Isolation scheme of the compounds **1** and **2** from the extract of S. sieboldiana.

Extraction and isolation

The air-dried branches of S. sieboldiana (500 g) were extracted with 70% aq. ethanol (5 L x 2) under stirring at room temperature for 48 hr. The combined resulting ethanol solutions were filtered, and the filtrate was concentrated using a rotary evaporator at a temperature 40℃. Part of the ethanol extract (25.0 g) was suspended in water and fractionated into *n*-hexane (1.0 g), chloroform (1.4 g), ethyl acetate (0.6 g), *n*-butanol (2.9 g) and water (14.0 g) portions. The ethyl acetate (EtOAc) layer was subjected to normal silica gel column chromatography (CC) eluting with chloroform and methanol, which produced four fractions (S1 to S4). The S3 fraction (110 mg) was purified by first passing it through a silica gel CC using chloroform/MeOH (4:1) as eluent, then through Sephadex LH-20 with MeOH to afford compound **2** (9 mg). The *n*-butanol layer was also purified by silica gel CC using chloroform/MeOH (1:1) and subsequently with Sephadex LH-20 on MeOH to give the compound **1** (9 mg) (Figure 1).

Kaempferol 3-O-sophoroside (1)

yellow powder; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.08 (2H, d, *J* = 7.7 Hz), δ 6.89 (2H, d, *J* = 7.7 Hz), δ 6.37 (1H, d, *J* = 1.8 Hz), δ 6.18 (1H, d, *J* = 1.8 Hz), δ 5.32 (1H, d, *J* = 6.8 Hz), δ 4.74 (1H, d, *J* = 6.4 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 158.6 (C-2), 135.0 (C-3), 179.9 (C-4), 163.2 (C-5), 100.1 (C-6), 166.5 (C-7), 94.9 (C-8), 158.9 (C-9), 105.7 (C-10), 122.8 (C-1'), 132.5 (C-2'), 116.4 (C-3'), 161.7 (C-4'), 116.4 (C-5'), 132.5 (C-6'), 101.6 (C-1"), 80.4 (C-2"), 77.1 (C-3"), 70.2 (C-4"), 75.0 (C-5"), 62.0 (C-6"), 104.9 (C-1'"), 75.6 (C-2'"), 78.0 (C-3'"), 71.4 (C-4'"), 78.3 (C-5'") , 62.7 (C-6'").

Daucosterol (2)

white powder; ^{1}H NMR(C_6D_5N, 400 MHz) δ 5.37 (1H, d, 4.5 Hz), δ5.09 (1H, d, *J* = 7.7 Hz), δ 4.10 (1H, m), δ 1.00 (3H, d, *J* = 6.4 Hz), δ 0.95 (3H, s), δ 0.91 (3H, d, *J* = 7.7 Hz), δ 0.87 (3H, d, *J* = 7.7 Hz), δ 0.67 (3H, s); ^{13}C NMR (C_6D_5N, 100 MHz) δ 37.8 (C-1), 30.6 (C-2), 72.0 (C-3), 40.3 (C-4), 141.2 (C-5), 122.3 (C-6), 32.5 (C-7), 32.4 (C-8), 50.7 (C-9), 37.3 (C-10), 21.6 (C-11), 39.7 (C-12), 42.8 (C-13), 57.1 (C-14), 24.8 (C-15), 28.9 (C-16), 56.6 (C-17), 12.5 (C-18), 19.5 (C-19), 36.7 (C-20), 19.3 (C-21), 34.5 (C-22), 23.7 (C-23), 46.4 (C-24), 29.8 (C-25), 20.3 (C-26), 19.8 (C-27), 26.7 (C-28), 12.3 (C-29), 102.9 (C-1'), 75.7 (C-2'), 79.0 (C-3'), 78.4 (C-4'), 78.9 (C-5'), 63.2 (C-6').

Cell culture

Primary human foreskin dermal fibroblasts (HDFn cells) were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with glutamine (2mM), penicillin (400 U/ml), streptomycin (50 mg/l) and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37 ℃.

Procollagen type I protein synthesis test

HDFn cells (5 x 10^4 cells) were seeded in a 24 well plate with DMEM (containing 10% FBS and 100 unit/ml penicillin-streptomycin), and were incubated overnight at 37°C under 5% CO_2. Test compounds were added to the plate in serum free media, which was incubated 24 hour at 37°C under 5% CO_2. The culture supernatant was collected by centrifugation 13,000 rpm, 4°C, 20 min. The procollagen type I C-peptide (PIP) was measured according to the supplier's instructions (Takara, MK101).

Cell viability assay

The cell viability was determined by the MTT assay which is based on the reduction of the soluble yellow MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its insoluble blue MTT formazan product. This biosynthetic reduction is mediated by mitochondrial succinic dehydrogenase. After treatment with samples, the cells ($1x10^5$ cells/well) were cultured for 3 days. After washing with PBS, the cells were treated with 200 µL of MTT solution (0.05 mg/ml) and incubated for 4h at 37 °C. The supernatant was then removed and 200 µL of dimethyl sulfoxide was added to each well to dissolve the formazan product. Wells without cells were used as blanks. Absorbance was determined at 570 nm using an ELISA reader. The results were expressed as the percentage of control cells.

Statistical analysis

All data were obtained in triplicate and are represented as means ± standard error (SE). Significant differences between treatments were determined by the Student's *t* test in one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Collagen is the major protein in human skin, and is produced from fibroblast cells in the dermal layer via procollagen protein. The generated procollagen in fibroblast is secreted into the extracellular matrix, and thereafter its C-peptide is dissociated by the procollagen peptidase present in the cell surface. The subsequent polymerization procedures led to the formation of collagen fiber. In this study, human fibroblast cell (HDFn) is treated with S. sieboldiana ethanol extract (SSE), and the amount of collagen production is measured by the procollagen type-I C-peptide (PIP). The amount of PIP produced was analyzed by ELISA kit.

The experimental result of PIP productions on the treatment of the SSE (10, 50, 100 µg/mL) to HDFn was summarized in Figure 2. This figure indicates that the PIP content increases in a dose-dependent manner. Compared to the control, the SSE at 100 µg/mL increased the PIP production by 130%. As the C-peptide (PIP) dissociated from the procollagen is a measure to determine the collagen production, this data indicated that SSE possesses collagen synthesis activation activities. Cell toxicities were measured by MTT assay. As shown in Figure 2, there was no change of cell viabilities by SSE with the concentrations as high as 100 µg/mL. This clearly indicated that SSE's activities are not associated with cell toxicities. In order to identify the active component from the SSE, a phytochemical study was conducted. As a procedure of solvent fractionation, the ethanol extract (SSE) was partitioned into *n*-hexane, chloroform, ethyl acetate (EtOAc) and *n*-butanol portions. When the PIP contents in the solvent fractions were measured, EtOAc and *n*-butanol fractions were observed to possess collagen synthesis activation properties (data not shown). Phytochemical studies were therefore conducted for these two fractions to isolate their active constituents. As shown in Figure 1, a series of chromatographic purification procedures led to isolation of the compounds **1** and **2**.

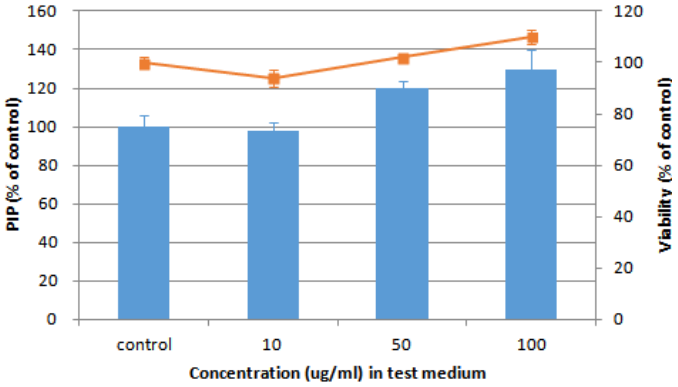
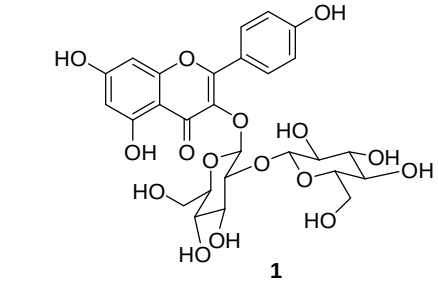


Fig. 2: Effect of the S. sieboldiana ethanol extract (SSE) on procollagen type I C-peptide (PIP) synthesis and cell toxicities. HDFn cells (5 x 104 cells) were incubated overnight, and procollagen type I C-peptide (PIP) was assayed after treatment of the extract (SSE) according to the supplier's instructions (Takara, MK101). The cell viability was determined by the MTT assay.



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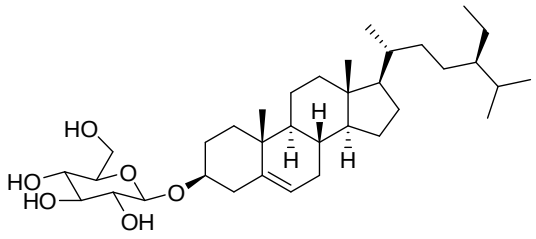

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Fig. 3: Chemical structures of compounds **1** and **2** isolated from S. sieboldiana.

Chemical structures for compounds **1** and **2** were identified primarily by using nuclear magnetic resonance (NMR) spectroscopy (Figure 3). Compound **1** showed *ortho*-coupled aromatic proton signals at δ 8.08 (2H, d) and 6.89 (2H, d) with coupling constant of 7.7 Hz in ^{1}H NMR spectrum. In addition,

signals for *meta*-coupled aromatic protons appeared at δ 6.37 (1H, d, *J* = 1.8 Hz) and 6.18 (1H, d, *J* = 1.8 Hz). Two anomeric proton signals were observed at δ 5.32 (1H, d, *J* = 6.8 Hz) and 4.74 (1H, d, *J* = 6.4 Hz), which suggest the presence of two glucose units in β-configurations in **1**. ^{13}C NMR spectrum exhibited 15 sp^2 carbon peaks (δ 179.9 – 94.9 ppm) for the flavonoid skeleton and 12 sp^3 carbon peaks for two molecules of sugar. Further examination of the NMR data verified that **1** has a flavonol kaempferol as an aglycon and a glucose dimer where glucoside bond was formed at C-2''. Compound **1** was therefore identified as kaempferol 3-*O*-sophoroside, and this structure was confirmed by comparing the observed data to the literature values (Tung *et al.*, 2010). Compound **2** showed five methyl peaks at δ 1.00, 0.95, 0.91, 0.87 and 0.67 in the ^{1}H NMR spectrum. ^{13}C NMR spectrum also indicated the presence of five methyl carbons showing signals at δ 12.3, 12.5, 19.3, 19.5 and 19.8 ppm. Carbon signals at δ_C 122.3 and 141.2 ppm suggested the presence of a C=C bond. A glucose unit was identified as a β-form by a coupling constant 7.7 Hz at δ 5.09 in the ^{1}H NMR spectrum. Based on all of these data, compound 2 was identified as a sterol glycoside, daucosterol (Yayli *et al.*, 2003). Compounds **1** and **2** were isolated for the first time from S. sieboldiana.

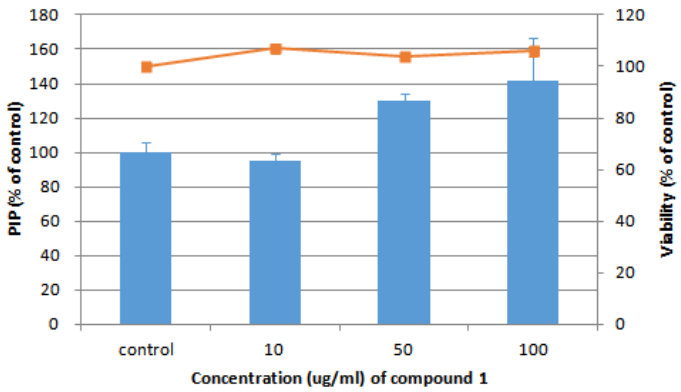

Fig. 4: Effect of the compound **1** on collagen type I synthesis and cytotoxicities. HDFn cells (5 x 104 cells) were incubated overnight, and procollagen type I C-peptide (PIP) was assayed after treatment of the compound **1** according to the supplier's instructions (Takara, MK101). The cell viability was determined by the MTT assay.

The production of procollagen by the isolated compounds **1** and **2** was monitored using the human fibroblast cells (HDFn). In this assay, the flavonoid **1** was verified to increase the PIP production dose-dependently as shown in Figure 4. Compound **1** was examined at concentrations of 10, 50 and 100 µg/mL and was observed to activate the PIP production by 142% at 100 µg/mL. Compound **1** was subjected to the MTT assay to determine whether it would induce any change in the cell viabilities and no cytotoxicities were observed at concentrations up to 100 µg/mL (Figure 4). The daucosterol (**2**) did not exhibit any activities on PIP production in the fibroblast cell culture test.

In summary, ethanol extracts were prepared from branches of a plant *Sambucus sieboldiana* var. pendula, and were examined for collagen synthesis activities using fibroblast HDFn cells. In this assay, the extract (SSE) proved to activate the production of type I procollagen by 130% at 100 µg/mL while maintaining cell viabilities. The active constituents in the extracts were isolated by chromatographic purifications and identified as kaempferol 3-*O*-sophoroside (**1**) and daucosterol (**2**). Among the isolates, the flavonoid glycoside **1** was verified to increase collagen production dose-dependently. These results suggested that S. sieboldiana extract containing the flavonoid **1** has a potential for use as an anti-aging ingredient in cosmetic formulations.

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