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Antioxidant Compounds of *Sambucus pendula* Stem

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Abstract – *Sambucus pendula* (Viburnaceae) is an Ulleung-do native plant belonging to the *Sambucus* species, widely known as "Jeob-Gol-Mok". In this study, the antioxidant activity of *S. pendula* methanol extract and its fractions were observed. Seven compounds were isolated from the CH_2Cl_2 fraction, which showed the most potent antioxidant activity among the fractions, using silica gel and Sephadex LH-20 column chromatography, recrystallization, MPLC, and prep-HPLC. The chemical structures of the isolates were determined as (+)-isolariciresinol (**1**), (+)-cycloolivil (**2**), *p*-coumaryl alcohol (**3**), vanillin (**4**), ursolic acid (**5**), guaiacylglycerol (**6**), and ficusol (**7**) based on the spectroscopic data including ^{1}H-NMR, ^{13}C-NMR, DEPT, HMBC, and MS data. All isolated compounds were first reported as components of this plant. Among these compounds, (+)-isolariciresinol (**1**) and (+)-cycloolivil (**2**) showed potent antioxidant activities on DPPH and ABTS radical scavenging assays, and significant inhibitory activity on lipopolysaccharide-induced NO and ROS productions in RAW 264.7 cells. This is the first study to show the antioxidant activity related to the anti-inflammatory activity of *S. pendula*, and these results suggested that the methanol extract, CH_2Cl_2 fraction, and the isolated compounds could be developed as a new natural material for treating oxidative stress-related diseases.
Keywords – *Sambucus pendula,* Antioxidant activity, (+)-Isolariciresinol, (+)-Cycloolivil

Introduction

A free radical contains one or more unpaired electrons, such as nitric oxide (NO) and reactive oxygen species (ROS).[1,2] NO and ROS are produced in various cell systems, such as the plasma membrane, cytoplasm, and mitochondria, and serve as signaling molecules that regulate different physiological functions, such as gene activation, cell growth, and intracellular chemical reactions.[3,4] Moderate free radical concentration plays a beneficial role in the body, but a high concentration of free radicals causes oxidative stress. Oxidative stress is caused by an imbalance between free radicals and protective mechanisms to eliminate them. This imbalance damages biomolecules and organs, causing potential damage to the entire organism. Oxidative stress causes transformation and damage to cells, proteins, lipids, and DNA. As a result, it causes inflammation and various diseases such as nervous system disease, cardiovascular disease, cancer, diabetes, metabolic syndrome, and attention-deficit hyperactivity disorder (ADHD). In addition, it induces cellular senescence and causes aging and age-related diseases.[5,6]

Antioxidants are substances that play a role in suppressing the initiation or progression of oxidation reactions within a cell. They react with free radicals to minimize the loss of nutrients necessary for the body and prevent various diseases, including inflammatory diseases that free radicals can cause.[7] These antioxidants are divided into natural and synthetic antioxidants. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyl anisole (BHA), and *tert*-butylhydroquinone (TBHQ) have been widely used.[8,9] Still, they are currently not in use due to liver toxicity, including disruption of the endocrine system, and DNA damage.[10,11] Accordingly, studies on natural antioxidants that can be used more safely are being actively conducted, and natural antioxidants such as plant-derived phenolic compounds and vitamins are attracting attention.[12]

Sambucus pendula Nakai, known as the elder tree (Viburnaceae family), is a tree native to Ulleung island in Korea. The dried stem and branch of *S. pendula* have been used as "Jeob-Gol-Mok" (接骨木), a traditional folk medicine in Korea, to treat diseases such as bone fracture and arthritis. Several studies on *S. pendula* have reported

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that isolated compounds daucosterol and kaempferol 3-*O*-sophoroside showed whitening and anti-inflammatory activities.[14–19]

This study was accomplished to reveal the antioxidant activities, including intracellular radical scavenging activities, related to the anti-inflammatory activity of *S. pendula* stem extract and the bioactive compounds.

Experimental

General – Extra pure-grade solvents (Daejung, Siheung, Korea) were used for extraction, fractionation, and isolation. The NMR spectra were obtained using DRX-300 and DRX-500 spectrometers (Bruker, Billerica, MA, USA). NMR solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). EI-MS and FAB-MS spectra were recorded on Xevo G2-XS TOF (Waters, Framingham, MA, USA). Victor X5 multilabel plate reader (PerkinElmer, Waltham, MA, USA) was used to measure the absorbance. Column chromatography was performed on silica gel 60 (0.063–0.43 mm, Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Medium-pressure liquid chromatography (MPLC) was performed on SNAP Cartridge KP-SIL 340 g (Biotage, Uppsala, Sweden). TLC was performed on silica gel 60 F_{254} (Merck, Darmstadt, Germany). A CO_2 incubator (Eppendorff, Hamburg, Germany) was used for RAW 264.7 cell line culture. The other reagents were purchased from Sigma (St. Louis, MO, USA).

Plant Materials – The stem of *Sambucus pendula* Nakai was collected from Ulleung-do province in September 2020. The botanical origin of this plant was identified by Dr. Mi-Jeong Ahn, College of Pharmacy, Gyeongsang National University. The voucher specimen (PGSC No. 602) was deposited in the Herbarium of the College of Pharmacy, Gyeongsang National University.

Extraction and Isolation – The dried stem of *S. pendula* (2.3 kg) was ground and extracted with methanol at room temperature. The methanolic extract was concentrated through the rotary evaporator to give a crude extract (90 g). This methanolic extract was suspended in water and partitioned successively with *n*-hexane, methylene chloride, ethyl acetate, and *n*-butanol, respectively, to yield *n*-hexane fr. (12.4 g), CH_2Cl_2 fr. (8.4 g), EtOAc fr. (1.2 g), *n*-BuOH fr. (9.8 g), and aqueous fr. (55 g) fractions, respectively.

The CH_2Cl_2 fraction, which showed potent antioxidant activity, was subjected to open silica column chromatography (CC) with a gradient elution of hexane, methylene chloride, and methanol mixture (100:0:0 → 0:100:0 → 0:0:100), giving 15 subfractions (Fr. 1–Fr. 15). A subfraction, Fr. 6 was applied on the silica gel CC using a mixture of hexane, methylene chloride, and methanol which gave nine subfractions (Fr. 6.1–Fr. 6.9). From Fr. 6.6, compounds **1** (16 mg, t_R 25.3 min) and **2** (1.4 mg, t_R 12.3 min) were isolated by prep-HPLC. The prep-HPLC separation was performed with an Agilent 1260 HPLC system (Hewlett-Packard, Waldbronn, Germany) with a Luna C18 column (250 × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA). The chromatogram was performed by a gradient elution of water (A) and acetonitrile (B) mixture under the following conditions: 0 to 5 min, 15% B; 5 to 30 min, 20% B. The flow rate was 1 mL/min, and the temperature was maintained at 30°C. Compound **3** (9 mg) was isolated from Fr. 10 by medium-pressure liquid chromatography (MPLC) using a mixture of hexane, methylene chloride, and methanol mixture (100:0:0 → 0:100:0 → 0:0:100). Compound **4** (1.4 mg, t_R 12.3 min) was isolated from Fr. 5 by the silica gel CC using prep-HPLC with the same HPLC system and solvent. The gradient elution was accomplished as follows: 0 to 5 min, 20% B; 5 to 15 min, 35% B; 15 to 30 min, 45% B; 30 to 35 min, 100% B. Compound **5** (70 mg) was isolated from Fr. 6.5 by recrystallization. From Fr. 6.4, compounds **6** (3.4 mg, t_R 36.0 min) and **7** (1.4 mg, t_R 12.6 min) were isolated by prep-HPLC with the same HPLC system and solvent. The gradient elution was accomplished as follows: 0 min, 10% B; 0 to 15 min, 25% B; 15 to 50 min, 25% B; 50 to 52 min, 100% B; 52 to 60 min, 100% B.

Isolariciresinol (1) – Yellow oil; QTOF-MS: *m/z* 361.1620 $[M+H]^+$ (calcd. $C_{20}H_{25}O_6$ 399.1652); ^{1}H-NMR (CD_3OD, 500 MHz): δ 6.64 (1H, d, J = 8.0 Hz, H-5′), 6.57 (1H, d, J = 1.9 Hz, H-2′), 6.55 (1H, s, H-5), 6.51 (1H, dd, J = 8.0. 1.9 Hz, H-6′), 6.08 (1H, s, H-2), 3.82 (3H, s, 3-OCH_3), 3.80 (3H, s, 3′-OCH_3), 3.71 (1H, d, J = 10.2 Hz, H-7′), 3.60 (1H, dd, J = 11.0, 3.3 Hz, H-9a), 3.57 (1H, d, J = 4.1 Hz, H-9′b), 3.55 (1H, d, J = 3.3 Hz, H-9b), 3.29 (1H, dd, J = 4.1, 11.0 Hz, H-9′a), 2.67 (2H, d, J = 7.7 Hz, H-7), 1.80 (1H, m, H-8), 1.65 (1H, td, J = 3.3, 10.2 Hz, H-8′); ^{13}C-NMR (CD_3OD, 125 MHz): δ 147.6 (C-3′), 145.8 (C-3), 144.5 (C-4′), 143.8 (C-4), 137.2 (C-1′), 132.7 (C-6), 127.6 (C-1), 121.8 (C-6′), 115.9 (C-2), 114.5 (C-5′), 112.3 (C-2′), 110.97 (C-5), 64.5 (C-9), 60.7 (C-9′), 54.9 (3-OCH_3, 3′-OCH_3), 46.6 (C-7′, 8′), 38.5 (C-8), 32.1 (C-7).

(+)-Cycloolivil (2) – Pellucid oil; QTOF-MS: *m/z* 341.1384 $[M-2H_2O+H]^+$ (calcd. $C_{20}H_{21}O_5$ 341.1390); ^{1}H-NMR (CD_3OD, 300 MHz): δ 6.77 (1H, d, J = 7.9 Hz, H-5′), 6.71 (1H, d, J = 1.8 Hz, H-2′), 6.68 (1H, dd, J = 7.9, 1.8 Hz, H-6′), 6.65 (1H, s, H-2), 6.20 (1H, s, H-5), 4.03 (1H, d, J = 11.6 Hz, H-7′), 3.83 (1H, d, J = 2.6 Hz, H-

9′b), 3.82 (3H, s, 3-OCH_3), 3.80 (3H, s, 3′-OCH_3), 3.79 (1H, d, J = 4.1 Hz, H-9b), 3.61 (1H, d, J = 4.1 Hz, H-9a), 3.58 (1H, dd, J = 4.1, 2.6, Hz, H-9′a), 3.23 (1H, d, J = 16.6 Hz, H-7b), 2.62 (1H, d, J = 16.6 Hz, H-7a), 2.08 (1H, m, J = 11.6, 4.1, 2.6 Hz, H-8′); ^{13}C-NMR (CD_3OD, 125 MHz): δ 147.7 (C-3′), 146.1 (C-3), 144.7 (C-4′), 143.9 (C-4), 137.0 (C-1′), 132.1 (C-1), 125.0 (C-6′), 122.1 (C-6), 115.9 (C-2), 114.6 (C-5′), 112.5 (C-2′), 111.5 (C-5), 73.5 (C-8), 68.0 (C-9), 59.4 (C-9′), 54.9 (3-OCH_3, 3′-OCH_3), 46.1 (C-8′), 43.4 (C-7′), 38.5 (C-7).

***p*-Coumaryl alcohol (3)** – Yellow powder; QTOF-MS: *m/z* 133.0664 $[M+H-H_2O]^+$ (calcd. $C_9H_9O_1$ 133.0654); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 7.25 (2H, d, J = 8.5 Hz, H-2, 6), 6.73 (2H, d, J = 8.5 Hz, H-3, 5), 6.51 (1H, d, J = 15.8 Hz, H-7), 6.20 (1H, dt, J = 15.8, 5.9 Hz, H-8), 4.20 (2H, dd, J = 5.9, 1.4 Hz, H-9); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 156.8 (C-4), 130.5 (C-7), 128.6 (C-1), 127.2 (C-2, 6), 125.2 (C-8), 114.9 (C-3, 5), 62.5 (C-9).

Vanillin (4) – White powder; QTOF-MS: *m/z* 153.0563 $[M+H]^+$ (calcd. $C_8H_9O_3$ 153.0552); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.85 (1H, s, H-1), 7.45 (1H, dd, J = 8.5, 1.8 Hz, H-6), 7.44 (1H, d, J = 1.8 Hz, H-3), 7.06 (1H, d, J = 8.5 Hz, H-7), 6.26 (1H, s, H-5), 3.99 (1H, s, 4-OCH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 190.8 (C-1), 151.6 (C-5), 147.1 (C-4), 129.9 (C-2), 127.7 (C-7), 114.3 (C-6), 108.7 (C-3), 56.15 (4-OCH_3).

Ursolic acid (5) – White powder, QTOF-MS: *m/z* 479.3532 $[M+Na]^+$ (calcd. $C_{30}H_{48}O_3Na$ 479.3503); ^{1}H-NMR (DMSO-d_6, 300 MHz): δ 12.0 (1H, s, H-28), 5.13 (1H, t, 3.1 Hz, H-12), 4.14 (1H, d, J = 5.1 Hz, H-3), 3.00 (1H, m, H-18), 2.50 (1H, m, H-11b), 2.11 (1H, d, 11.13 Hz, H-15a), 1.04 (3H, s, H-27), 0.92 (3H, d, J = 6.4 Hz, H-23), 0.90 (3H, s, H-25), 0.87 (3H, s, H-26), 0.81 (3H, d, J = 6.4 Hz, H-29), 0.75 (3H, d, H-30), 0.68 (3H, s, H-24); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 181.3 (C-28), 137.9 (C-13), 125.7 (C-12), 79.0 (C-3), 55.2 (C-5), 52.6 (C-18), 47.9 (C-17), 47.5 (C-9), 41.9 (C-14), 39.4 (C-8), 39.0 (C-1), 38.8 (C-4), 38.7 (C-19), 38.6 (C-20), 37.0 (C-22), 36.7 (C-10), 32.9 (C-7), 30.6 (C-21), 29.7 (C-23), 28.1 (C-2), 28.0 (C-15), 27.2 (C-16), 24.7 (C-11), 23.5 (C-27), 23.3 (C-30), 18.3 (C-6), 17.1 (C-26), 16.9 (C-29), 15.6 (C-24), 15.4 (C-25).

Guaiacylglycerol (6) – Yellow oil; QTOF-MS: *m/z* 177.0556 $[M-2H_2O-H]^-$ (calcd. $C_{10}H_9O_3$ 177.0552); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 6.83 (1H, s, H-2), 6.81 (1H, d, J = 6.5 Hz, H-5), 6.75 (1H, d, J = 6.5 Hz, H-6), 5.52 (1H, s, H-4), 4.66 (1H, d, J = 4.1 Hz, H-7), 4.17 (1H, dd, J = 9.0, 4.1 Hz, H-8), 3.83 (3H, s, 3-OCH_3), 3.80 (1H, dd, J = 9.2, 3.5 Hz, H-9a), 3.03 (1H, m, J = 3.5 Hz, H-9b); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 146.7 (C-3), 145.2 (C-4), 132.9 (C-1), 118.9 (C-6), 114.2 (C-5), 108.5 (C-2), 85.8 (C-7), 71.6 (C-8), 55.9 (C-9), 54.1 (3-OCH_3).

Ficusol (7) – White powder; QTOF-MS: *m/z* 249.0754 $[M+Na]^+$ (calcd. $C_{11}H_{14}O_5Na$ 249.0739); ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 6.81 (1H, d, J = 8.0 Hz, H-5′), 6.71 (1H, d, J = 1.7 Hz, H-2′), 6.70 (1H, dd, J = 8.0, 1.7 Hz, H-6′), 5.52 (1H, s, H-4′), 4.04 (1H, m, Hz, H-3a), 3.82 (3H, s, 3′-OCH_3), 3.73 (1H, m, H-3b), 3.70 (1H, m, H-2), 3.64 (3H, s, 1-OCH_3); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 173.8 (C-1), 146.7 (C-4′), 145.3 (C-3′), 127.3 (C-1′), 121.2 (C-6′), 114.9 (C-2′), 110.5 (C-5′), 64.7 (C-3), 60.0 (C-3′-OCH_3), 53.5 (C-2), 52.2 (1-OCH_3).

Total phenolic content – Total phenolic content was measured according to our previously reported method.[20,21] One hundred microliter of each extract was reacted with 500 μL of 10% Folin-Ciocalteau phenol reagent and 400 μL of sodium carbonate at room temperature for 10 min and centrifuged 3,000 × g for 5 min. The absorbance was measured at 765 nm after transferring 200 μL of the supernatants to 96 wells. The calibration curve was obtained with gallic acid, and the phenolic content was expressed as gallic acid equivalents per gram (μmol GAE/g).

Antioxidant activity test with DPPH and ABTS radicals – DPPH radical scavenging activity was measured by our previously reported method.[22] Each sample of 10 μL was added to 990 μL of 0.2 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in each well of a 24-well plate. After 10 min, the absorbance was measured at 520 nm. ABTS radical scavenging activity was assessed using the following method.[23] Each sample of 10 μL was added to 990 μL of 7 mM ABTS in 2.45 mM potassium persulfate ethanol solution in each well of a 24-well plate. After 10 min, the absorbance was measured at 405 nm. The antioxidant activity was expressed as trolox equivalents (μmol TE/g for fractions and μmol TE/mol for compounds).

Cell culture – RAW 264.7 cell line was provided by the Korea Cell Line Bank (Seoul, Korea). The cell line was grown and maintained under 100% humidity and 5% CO_2 for 2–3 days at 37°C. Dulbecco's modified eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used.

Cell viability – Cell viability was assessed by MTT assay. The cells were seeded in a 96 well-plate at a density of 1×10^5 cells/well, incubated for 24 h, and treated with 12.5–100 μg/mL concentrations of samples for 24 h. Each well was treated with the MTT solution, and the absorbance was measured at 570 nm.

The measurement of NO and ROS production – The cells were seeded in a 96 well-plate at a density of 1×10^5 cells/well, incubated for 24 h, pretreated with 12.5–100

μg/mL concentrations of samples for 3 h, and stimulated with lipopolysaccharide (LPS) (10 μg/mL) for 21 h. The measurement of NO productions was used by supernatant with Griess reagent for 10 min, and the absorbance was measured at 540 nm. A calibration curve was constructed using $NaNO_2$ as the standard, and the values were expressed as nitric oxide (μM). The residue was reacted with $DCFH_2$-DA solution for 30 min, and the fluorescence was determined with a fluorescent detector (Excitation, 485 nm; Emission, 535 nm) to measure ROS production.

Statistical analysis – All data were expressed as mean ± SD of triple independent experiments. The ANOVA and Pearson's correlation coefficients were evaluated using SPSS 24.0 software (IBM, Armonk, NY, USA).

Results and Discussion

The stems of *Sambucus* species, including *S. pendula*, are called "Jeob-Gol-Mok" and have been used in traditional medicine for fractures and contusions. Studies on *S. nigra* L. and *S. williamsii* Hance have been actively conducted, and their antioxidant and anti-inflammatory activities have been reported. On the other hand, the research on active components related to the antioxidant and anti-inflammatory activities of *S. pendula* is insufficient. Therefore, in this study, we evaluated the antioxidant activity associated with the anti-inflammatory activity of *S. pendula*, and isolated the bioactive compounds. Seven compounds were isolated from CH_2Cl_2 fraction, which showed the most potent inhibitory activities on NO and ROS productions, and the chemical structures of the compounds were determined as two lignans of (+)-isolariciresinol (**1**) and (+)-cycloolivil (**2**), four phenolics of *p*-coumaryl alcohol (**3**), vanillin (**4**), guaiacylglycerol (**6**) and ficusol (**7**), and a triterpene of ursolic acid (**5**) based on spectroscopic data such as ^{1}H-NMR, ^{13}C-NMR, and Q-TOF MS.[24–33] All seven compounds were first reported from this plant (Fig. 1).

The total phenolic content of the methanol extract from the stem of *S. pendula* was 35.1 ± 5.2 mg GAE/g. Among the fractions, EtOAc (242.9 ± 28.0 mg GAE/g) and CH_2Cl_2 (105.9 ± 18.2 mg GAE/g) fractions showed higher amounts of the total phenolic contents than other fractions (Table 1). The total extracts of *S. pendula* showed DPPH and ABTS radical scavenging activities with values of 2.05 ± 0.15 mmol TE/g and 2.56 ± 0.28 mmol TE/g, respectively. The EtOAc fraction showed the highest DPPH (4.97 ± 0.64 mmol TE/g) and ABTS (3.26 ± 0.16 mmol TE/g) radical

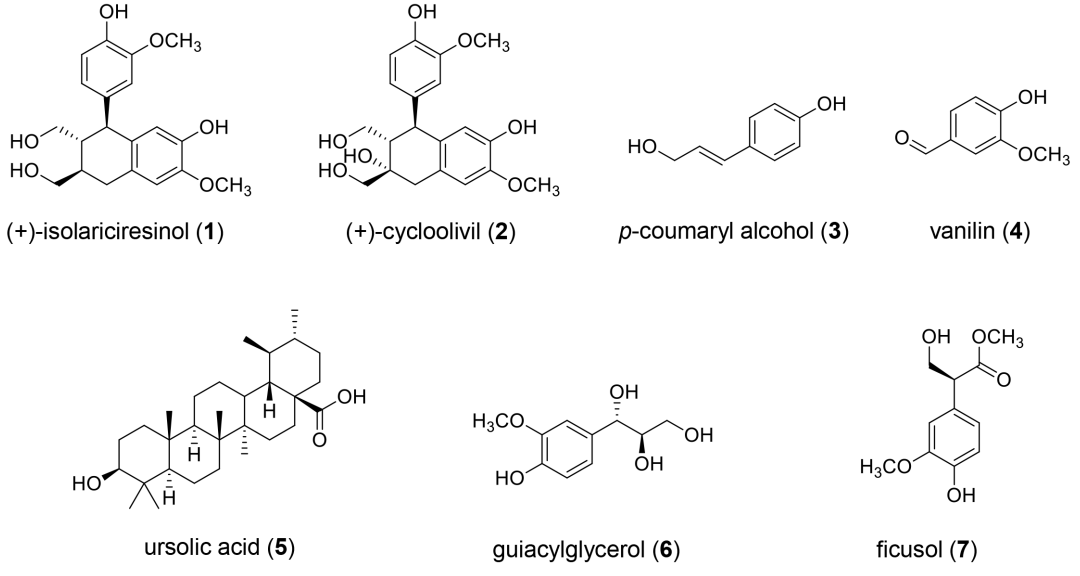

Fig. 1. Chemical structures of compounds **1**–**7** isolated from *S. pendula* stem.

Table 1. Total phenolic contents of *S. pendula* extract and fractions

Total Ex.	Hexane fr.	CH_2Cl_2 fr.	EtOAc fr.	BuOH fr.	Aqua fr.
35.1 ± 5.2^d	20.6 ± 4.1^d	105.9 ± 18.2^b	242.9 ± 28.0^a	80.1 ± 8.4^c	10.6 ± 1.0^d

*All data were expressed as mean ± standard deviation (n = 3) of mg GAE/g values.
**Different letters in the same row mean significantly different by Duncan's multiple range test (p < 0.05).

Table 2. DPPH and ABTS radical scavenging activities of *S. pendula* extract and fractions

	Total Ex.	Hexane fr.	CH_2Cl_2 fr.	EtOAc fr.	BuOH fr.	Aqua fr.
DPPH	2.05 ± 0.15^{d}	1.45 ± 0.16^{de}	3.53 ± 0.21^{b}	4.97 ± 0.64^{a}	2.71 ± 0.32^{c}	1.15 ± 0.29^{e}
ABTS	2.56 ± 0.28^{c}	1.47 ± 0.09^{d}	2.61 ± 0.18^{c}	3.26 ± 0.16^{a}	2.92 ± 0.07^{b}	1.53 ± 0.12^{d}

*All data were expressed as mean ± standard deviation (n = 3) of mmol TE/g values.
**Different letters in the same row mean significantly different by Duncan's multiple range test (p < 0.05).

scavenging activities, followed by the CH_2Cl_2 fraction with the values of 3.53 ± 0.21 and 2.61 ± 0.18 mmol TE/g, respectively (Table 2). Pearson's correlation coefficient was calculated to reveal the correlation between the phenolic contents and antioxidant activities. The phenolic content showed a high positive correlation with a scavenging capacity of DPPH (r = 0.97) and ABTS (r = 0.80) radicals, respectively. Therefore, the free radical scavenging activities of *S. pendula* were associated with high phenolic contents.

The cellular antioxidant capacity was determined as the inhibitory activities against NO and ROS productions in LPS-stimulated RAW 264.7 cells. The total extract and fractions of *S. pendula* stem were treated with RAW 264.7 cells to be final concentrations of 12.5, 25, 50, and 100 μg/mL, respectively, and cell viability was measured using an MTT reagent (Fig. 2A). As a result, the cell viability was over 95% at all concentrations of the samples used in the experiment, showing no cytotoxicity. The CH_2Cl_2 fraction showed the most potent NO production inhibitory activity of 83.8% and 95.1% at 50 and 100 μg/mL concentrations, respectively (Fig. 2B). Hexane fraction showed the second potent intracellular antioxidant activity at 100 μg/mL, followed by EtOAc fraction and total extract. As for the intracellular ROS production, the highest 92.4% inhibitory activity was observed in the CH_2Cl_2 fraction at 100 μg/mL, followed by hexane and EtOAc fractions (Fig. 2C). The EtOAc fraction exhibited the highest phenolic content and showed potent DPPH and ABTS radical scavenging activities. However, this fraction demonstrated weak inhibitory activity on NO production, and its inhibitory activity on ROS production was similar to that of the hexane fraction. These results suggest that other factors including cell membrane permeability could be involved in the cellular antioxidant capacity different from antioxidant assay using DPPH and ABTS radicals. The *n*-hexane fraction with lower phenolic content, and DPPH and ABTS radical scavenging activities than *n*-BuOH fraction showed higher cellular antioxidant capacity than *n*-BuOH fraction.

The antioxidant activity of the seven isolates (**1**−**7**) was measured by DPPH and ABTS radical scavenging assay (Table 3). Two lignans of **1** and **2** showed strong DPPH (529 ± 27 and 469 ± 25 mmol TE/mol) and ABTS (323 ± 14 and 320 ± 21 mmol TE/mol) radical scavenging activity. In the cellular antioxidant assay, the cells were treated with the seven isolates to final concentrations of 12.5, 25, 50, 100, and 200 μM (Fig. 3A). As a result, compound **3** exhibited cytotoxicity at concentrations above 100 μM,

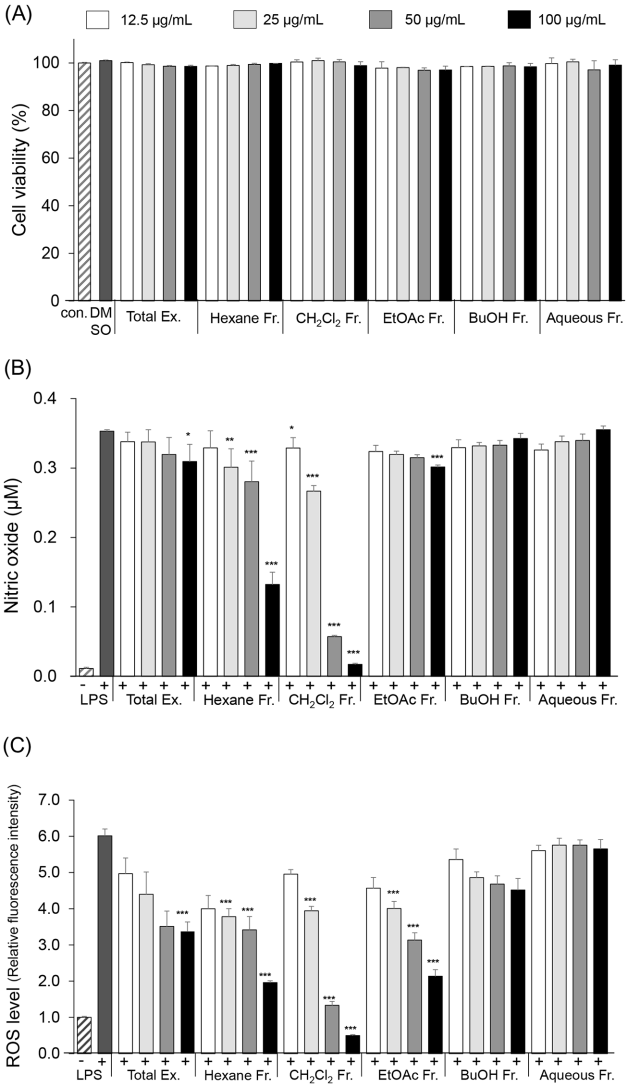

Fig. 2. Cell viability (A), and inhibitory activities on NO (B) and ROS (C) productions of the *S. pendula* extract and fractions in RAW 264.7 cell line. Each value was expressed as mean ± standard deviation (n = 3). Mean value was significantly different (*p < 0.05, **p < 0.01 and ***p < 0.001) from the negative control.

Table 3. DPPH and ABTS radical scavenging activities of isolated compounds **1**–**7** from *S. pendula* stem

	1	2	3	4	5	6	7
DPPH	529 ± 27[a]	469 ± 25[b]	171 ± 7[d]	80 ± 8[e]	53 ± 5[e]	364 ± 19[c]	395 ± 14[c]
ABTS	323 ± 14[a]	320 ± 21[a]	264 ± 15[b]	91 ± 9[d]	25 ± 3[e]	220 ± 14[c]	236 ± 12[c]

*All data were expressed as mean ± standard deviation (n = 3) of mmol TE/mol values.
**Different letters in the same row mean significantly different by Duncan's multiple range test ($p < 0.05$).

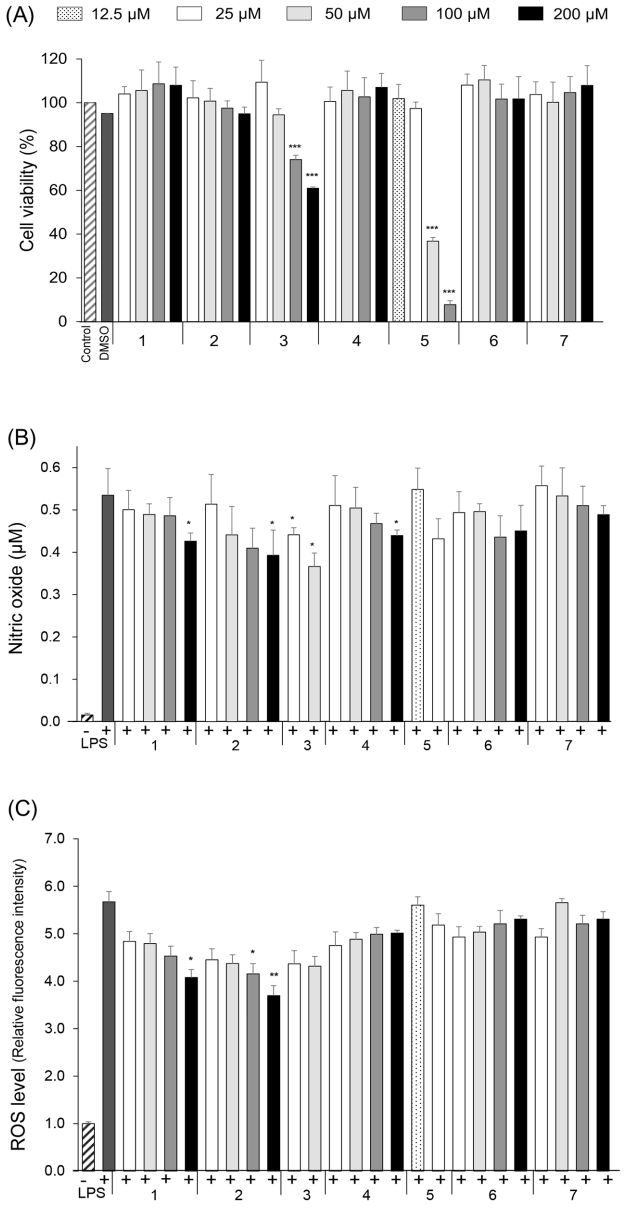

Fig. 3. Cell viability (A), and inhibitory activities against NO (B) and ROS (C) productions of isolated compounds **1**–**7** from *S. pendula* in RAW 264.7 cell line. Each value was expressed as mean ± standard deviation (n = 3). Mean value was significantly different (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) from the negative control.

and compound **5** also showed cytotoxicity at concentrations above 50 μM. Therefore, the NO and ROS assay were conducted for compounds **3** below 100 μM and **5** below 50 μM, respectively, not exhibiting cytotoxicity. It is known that the administration of excessive doses of ursolic acid (**5**) caused hepatic cytotoxicity.[34] While *p*-coumaryl alcohol (**3**) significantly reduced LPS-mediated NO production at concentrations of 25 and 50 μM, three compounds of **1**, **2**, and **4** showed a significant reduction at a higher concentration of 200 μM (Fig. 3B). (+)-Isolariciresinol (**1**) and (+)-cycloolivil (**2**) also showed weak inhibitory activity against ROS production stimulated by LPS in RAW 264.7 cells (Fig. 3C). While two phenolics of guaiacylglycerol (**6**) and ficusol (**7**) showed significant DPPH and ABTS radical scavenging activities, they failed to show inhibitory activity with ursolic acid (**5**) against NO or ROS production. According to previous reports, a lignan, isolariciresinol (**1**) showed antioxidant activity with the IC_{50} value of 53.0 μM against DPPH radical scavenging activity,[35] and anti-inflammatory activity by inhibition of NO and TNF-α productions in LPS-stimulated RAW 264.7 cell line.[35,36] Another lignan, cycloolivil (**2**), has also been reported to have 19.7% DPPH radical scavenging activity at 10 μg/mL.[37] Moreover, cycloolivil (**2**) inhibited TNF-α/IFN-γ by blocking NF-κB and JAK/STAT activation.[38] Among the phenolics, *p*-coumaryl alcohol (**3**) exhibited antioxidative activity against autoxidation of methyl linoleate.[39] Guaiacylglycerol (**6**) also showed antioxidant properties due to its phenolic structure, which can donate hydrogen atoms to neutralize free radicals.[40] Ficusol (**7**) displayed DPPH and ABTS radical scavenging activities similar to other phenolic compounds.[41] These previous results were well consistent with our results.

This study confirmed the antioxidant activity related to the anti-inflammatory activity of the methanol extract from *S. pendula* stem for the first time. The principal bioactive compounds would be (+)-isolariciresinol (**1**) and (+)-cycloolivil (**2**) with potent DPPH and ABTS radical scavenging activity and significant intracellular antioxidant activity. Moreover, the seven compounds of (+)-isolariciresinol (**1**), (+)-cycloolivil (**2**), *p*-coumaryl alcohol (**3**), vanillin (**4**), ursolic acid (**5**), guaiacylglycerol (**6**) and ficusol (**7**) are reported for the first time as isolates from *Sambucus pendula*. Through a further study on the

methanol extract and CH_2Cl_2 fraction, (+)-isolariciresinol, and (+)-cycloolivil, they would be used to develop natural candidates for treating oxidative stress-related diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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