Natural Product Sciences
23(3) : 217-221 (2017)
https://doi.org/10.20307/nps.2017.23.3.217

Phytochemical Constituents from the Rhizomes of *Osmunda japonica* Thunb and Their Anti-oxidant Activity

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Abstract – Eleven compounds (**1**–**11**) were isolated from the rhizomes of *Osmunda japonica*, and their structures were elucidated based on ^{1}H, ^{13}C-NMR and LC-IT-TOF MS data. Of these compounds, all compounds (**1** – **11**) have been previously reported, although five (**6** – **9**, **11**) have not previously been isolated from this plant. The antioxidant activities of isolated compounds (**1** – **11**) were measured by DPPH and ABTS assays, and compound **10** showed the high antioxidant activity.
Keywords – *Osmunda japonica*, Osmundaceae, Chemical constituents, Anti-oxidant

Introduction

Osmunda japonica (Osmundaceae) is a perennial herb, widely distributed throughout Taiwan, Japan, and Korea.[1] Its young leaves are edible, consumed as a vegetable in Korea, and its rhizomes have long been used in traditional Korean medicine for treating hemostasis and fever.[2] Previous phytochemical investigations of this plant resulted in the isolation of flavonoids and phenolic constituents.[3,4] Anti-oxidant, anti-microbial and herbicidal effects of an MeOH extract of *O. japonica* have also been reported.[5-8] As part of our ongoing research for bioactive compounds from indigenous plant in Korea, we investigated the MeOH extract of *O. japonica* and isolated eleven known compounds (**1** - **11**) (Fig. 1). Their structures were characterized by spectroscopic data and identified by comparing these data with those in the literature. All isolated compounds (**1** - **11**) were tested for antioxidant activity using DPPH and ABTS assays.

Experimental

General experimental procedures – TLC was performed using Merck pre-coated silica gel F_{254} plates. Spots were visualized on TLC under UV light or by spraying with 10% H_2SO_4 in C_2H_5OH (v/v), and heating. Silica gel 60 (Merck, 70 – 230 mesh and 230 – 400 mesh) and RP-C_{18} silica gel (YMC GEL ODS-A, 12 nm, S-75 μm) were used for column chromatography. All the compounds were purified on an Agilent A1200 series HPLC (Agilent Technologies) using a Phenomenex Luna C_{18}-100A column (25 cm × 3 mm, 5 μm particle size). NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C); chemical shifts are given in ppm (δ). ESI-mass spectra were obtained on a Schimadzu LCMS-IT-TOF mass spectrometer. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich and used as supplied. Fluorescence analyses were performed using ELISA microplate reader (Infinite 200 pro, TECAN, Austria).

Plant materials – The rhizomes of *O. japonica* (2 kg) were collected at Wando-gun in Jeollanam-Do province in July 2015. This plant was identified by Professor Hui Kim (Mokpo National University, Korea). A voucher specimen (TKM-2081) of the plant was deposited in the herbarium at NIKOM, Jangheung, Korea.

Extraction and isolation – The rhizomes of *O. japonica* (2 kg) were extracted with 100% MeOH under reflux, and filtered. The filtrate was concentrated *in vacuo* to give a

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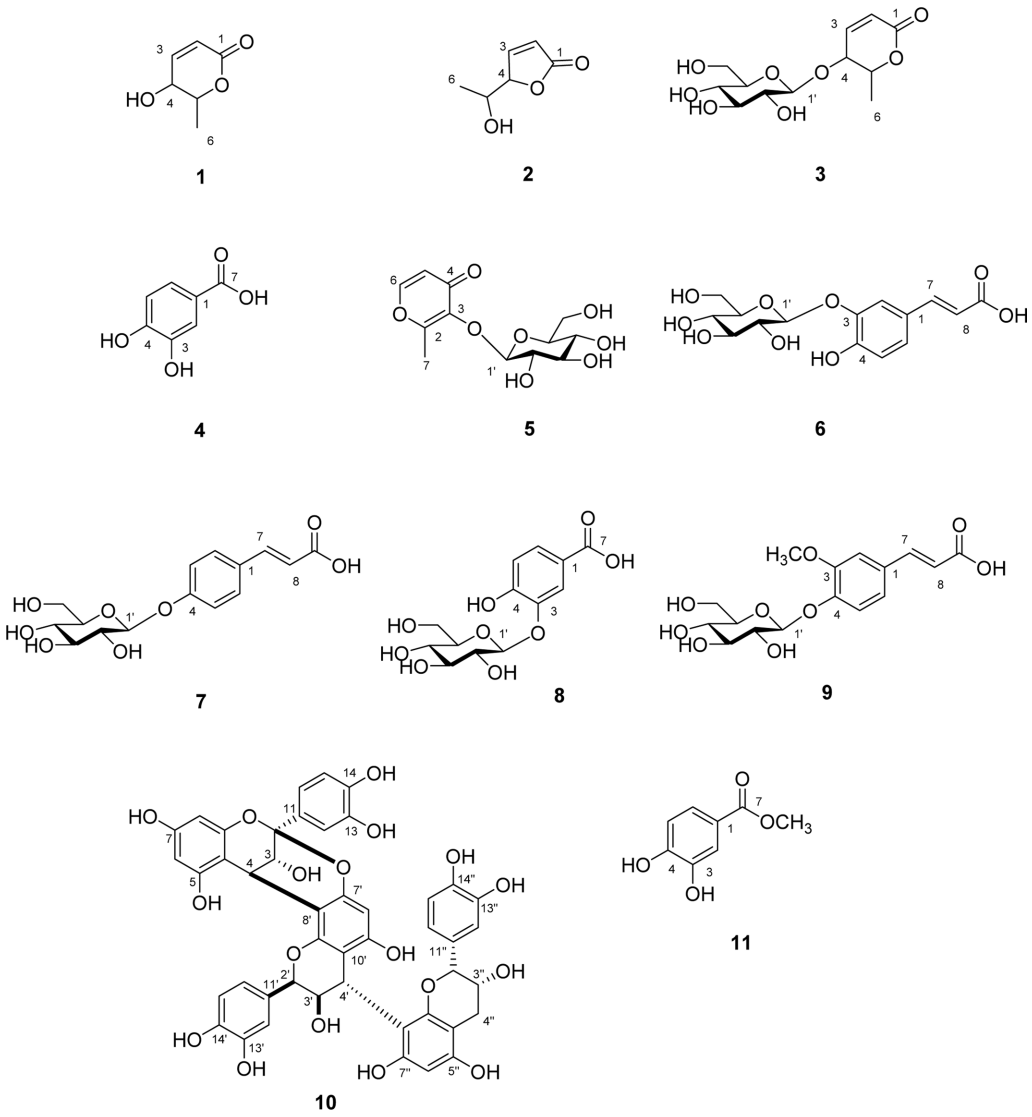

Fig. 1. The structures of **1** - **11** isolated from *O. japonica*.

MeOH extract (137 g), which was suspended in water (800 mL) and successively partitioned with *n*-hexane and H_2O to give 20 and 100 g, respectively. The H_2O soluble fraction (100 g) was chromatographed on a diaion HP-20 column, eluting with a gradient solvent system consisting of 100% water and 50% MeOH to give two sub-fractions (A and B). Fraction A (80 g) was separated over an RP-C_{18} silica gel column with 0 – 25% MeOH as the eluent to give ten fractions (A1–A10). Fraction A5 (7 g) was separated over a RP-C_{18} silica gel column with 0 – 20% MeOH as the eluent to give four fractions (A5-1 – A5-4). Subfraction A5-1 (300 mg) was purified with a RP-C_{18} prep HPLC (0 – 5% MeOH, gradient, 40 min, 240 nm, 25 mL/min) to yield **2** (14 mg, t_R = 22 min) and **1** (8 mg, t_R = 27 min). Sub-fraction A5-2 (1.3 g) was subjected to column chromatography (CC) on silica gel (230 - 400 mesh, 20 g), eluting with a solvent system of ethyl acetate/MeOH/H_2O(10:1:0~2:1:0.2) to give four sub-fractions (A5-2-1 – A5-2-4). Sub-fraction A5-2-3 (800 mg) was purified with a RP-C_{18} prep HPLC (3% MeOH, isocratic, 35 min, 254 nm, 25 mL/min) to yield **3** (520 mg, t_R = 25 min). Sub-fraction A7 (60 mg) and A8 (40 mg) were purified with a RP-C_{18} prep HPLC (8~11% MeOH, gradient, 35 min, 220 nm, 20 mL/min) to yield **4** (10 mg, t_R = 21 min). Fraction B (10 g) was separated over a RP-C_{18} silica gel column with 0 – 20% MeOH as the eluent to give eleven fractions (B1 – B11). Sub-fraction B1 (1.0 g) and B2 (400 mg) were purified with a RP-C_{18} prep HPLC (5% MeOH, isocratic, 40 min, 254 nm, 25 mL/min) to yield **5** (758 mg, t_R = 30 min). Sub-fraction B3 (60 mg) was purified with a RP-C_{18} prep HPLC (10% MeOH, isocratic, 40 min, 289 nm, 22 mL/min) to yield **6** (11 mg, t_R = 28 min). Sub-

fraction B4 (1.3 g) and B5 (500 mg) were subjected to column chromatography (CC) over a silica gel (230 - 400 mesh, 20 g) eluted with a solvent system of $CHCl_3$/MeOH (20:1~1:1) to give seven sub-fractions (B4-5-1–B4-5-7). Sub-fraction B4-5-3 (560 mg) was purified with a RP-C_{18} prep HPLC (8% MeOH, isocratic, 40 min, 290 nm, 25 mL/min) to yield **7** (14 mg, t_R = 26 min) and **8** (265 mg, t_R = 33 min), respectively. Sub-fraction B4-5-4 (250 mg) was purified with a RP-C_{18} prep HPLC (8% MeOH, isocratic, 40 min, 290 nm, 25 mL/min) to yield **9** (10 mg, t_R = 27 min). Sub-fraction B7 (2.0 g) was purified with a RP-C_{18} prep HPLC (14% MeOH, isocratic, 40 min, 277 nm, 25 mL/min) to yield **10** (675 mg, t_R = 28 min). Sub-fraction B10 (123 mg) was purified with a RP-C_{18} prep HPLC (12~20% MeOH, gradient, 35 min, 260 nm, 21 mL/min) to yield **11** (8 mg, t_R = 30 min).

Osmundalactone (**1**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 6.91 (1H, dd, *J* = 10.0, 2.5 Hz, H-3), 5.94 (1H, dd, *J* = 9.5, 1.5 Hz, H-2), 4.31 (1H, m, H-5), 4.16 (1H, m, H-4), 1.44 (1H, d, *J* = 6.5 Hz, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 164.7 (C-1), 150.3 (C-3), 119.0 (C-2), 77.3 (C-5), 66.7 (C-4), 16.9 (C-6); LC ESI IT-TOF MS: m/z 127 $[M-H]^-$.

3,5-Dihydroxy-γ-caprolactone (**2**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.75 (1H, dd, *J* = 5.0, 2.0 Hz, H-3), 6.20 (1H, dd, *J* = 5.5, 2.0 Hz, H-2), 5.50 (1H, dt, *J* = 5.0, 2.0 Hz, H-4), 3.93 (1H, m, H-5), 1.25 (1H, d, *J* = 6.5 Hz, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 174.1 (C-1), 155.0 (C-3), 121.5 (C-2), 87.6 (C-4), 67.0 (C-5), 17.7 (C-6); LC ESI IT-TOF MS: m/z 127 $[M-H]^-$.

Osmudarin (**3**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.09 (1H, dd, *J* = 10.0, 2.5 Hz, H-3), 6.02 (1H, dd, *J* = 9.5, 1.5 Hz, H-2), 4.59 (1H, m, H-5), 4.50 (1H, m, H-4), 4.49 (1H, d, *J* = 7.5 Hz, H-1'), 3.90 (1H, dd, *J* = 12.0, 5.5 Hz, H-6b), 3.68 (1H, dd, *J* = 12.0, 2.0 Hz, H-6a), 1.47 (1H, d, *J* = 6.5 Hz, H-6); ^{13}C NMR (CD_3OD, 125 MHz) δ 163.7 (C-1), 146.3 (C-3), 120.2 (C-2), 101.5 (C-1'), 77.9 (C-5), 76.8 (C-5'), 76.6 (C-3'), 73.5 (C-2'), 72.0 (C-4'), 70.1 (C-4), 61.4 (C-6'), 17.2 (C-6); LC ESI IT-TOF MS: m/z 289 $[M-H]^-$.

Protocatechuic acid (**4**) – White amorphous powder; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.43 (1H, d, *J* = 2.0 Hz, H-2), 7.40 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.79 (1H, d, *J* = 8.0 Hz, H-5); ^{13}C NMR (CD_3OD, 125 MHz) δ 169.1 (C-7), 150.3 (C-4), 144.9 (C-3), 122.7 (C-6), 122.1 (C-1), 116.6 (C-2), 114.6 (C-5); LC ESI IT-TOF MS: m/z 153 $[M-H]^-$.

Maltol-*β*-D-glucopyranoside (**5**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 8.01 (1H, d, *J* = 6.0 Hz, H-6), 6.45 (1H, d, *J* = 5.5 Hz, H-5), 4.81 (1H, d, *J* = 7.5 Hz, H-1'), 3.83 (1H, dd, *J* = 12.0, 5.5 Hz, H-6b), 3.67 (1H, dd, *J* = 12.0, 2.0 Hz, H-6a), 2.47 (3H, s, H-7); ^{13}C NMR (CD_3OD, 125 MHz) δ 175.8 (C-4), 163.2 (C-2), 155.7 (C-6), 142.2 (C-3), 115.9 (C-5), 104.0 (C-1'), 77.1 (C-5'), 76.6 (C-2'), 74.0 (C-3'), 69.7 (C-4'), 62.1 (C-6'), 14.4 (C-7); LC ESI IT-TOF MS: m/z 287 $[M-H]^-$.

Caffeic aicd 3-*O*-*β*-D-glucopyranoside (**6**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.43 (1H, d, *J* = 16.0 Hz, H-7), 7.12 (1H, d, *J* = 2.0 Hz, H-2), 7.10 (1H, d, *J* = 8.0 Hz, H-5), 7.06 (1H, dd, *J* = 8.5 , 1.5Hz, H-6), 6.31 (1H, d, *J* = 16.0 Hz, H-8), 4.77 (1H, d, *J* = 7.5 Hz, H-1'), 3.45 (1H, dd, *J* = 12.0, 5.5 Hz, H-6b), 3.44 (1H, dd, *J* = 12.0, 2.0 Hz, H-6a); ^{13}C NMR (CD_3OD, 125 MHz) δ 168.3 (C-9), 147.6 (C-3), 147.2 (C-4), 144.0 (C-7), 129.3 (C-1), 121.0 (C-6), 118.0 (C-8), 116.5 (C-2), 115.2 (C-5), 102.0 (C-1'), 77.7 (C-5'), 76.2 (C-3'), 73.7 (C-2'), 70.2 (C-4'), 61.7 (C-6'); LC ESI IT-TOF MS: m/z 341 $[M-H]^-$.

Coumaric acid 4-*O*-*β*-D-glucopyranoside (**7**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.60 (1H, d, *J* = 8.0 Hz, H-3, 5), 7.51 (1H, d, *J* = 16.0 Hz, H-2), 7.04 (1H, d, *J* = 8.0 Hz, H-2, 6), 6.39 (1H, d, *J* = 16.0 Hz, H-8), 4.91 (1H, d, *J* = 7.5 Hz, H-1'); ^{13}C NMR (CD_3OD, 125 MHz) δ 176.0 (C-9), 159.7 (C-4), 141.2 (C-7), 131.5 (C-1), 130.1 (C-2, 6), 124.5 (C-8), 118.0 (C-3, 5), 102.0 (C-1'), 78.1 (C-5'), 77.8 (C-3'), 74.8 (C-2'), 71.3 (C-4'), 62.4 (C-6'); LC ESI IT-TOF MS: m/z 325 $[M-H]^-$.

Protocatechuic acid 3-*O*-*β*-D-glucopyranoside (**8**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.37 (1H, d, *J* = 2.0 Hz, H-2), 7.35 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 7.10 (1H, d, *J* = 8.0 Hz, H-5), 4.90 (1H, d, *J* = 7.5 Hz, H-1'), 3.60 (1H, dd, *J* = 12.0, 5.5 Hz, H-6b), 3.42 (1H, dd, *J* = 12.0, 2.0 Hz, H-6a); ^{13}C NMR (CD_3OD, 125 MHz) δ 167.4 (C-7), 149.3 (C-4), 146.6 (C-3), 125.3 (C-6), 121.7 (C-1), 117.0 (C-2), 115.4 (C-5), 101.0 (C-1'), 76.2 (C-5'), 75.8 (C-3'), 73.5 (C-2'), 72.0 (C-4'), 59.9 (C-6'); LC ESI IT-TOF MS: m/z 315 $[M-H]^-$.

Ferulic acid 4-*O*-*β*-D-glucopyranoside (**9**) – Colorless gum; ^{1}H NMR (CD_3OD, 500 MHz) δ 7.52 (1H, d, *J* = 16.0 Hz, H-7), 7.33 (1H, d, *J* = 2.0 Hz, H-2), 7.17 (1H, dd, *J* = 8.5, 1.5Hz, H-6), 7.08 (1H, d, *J* = 8.0 Hz, H-5), 6.45 (1H, d, *J* = 16.0 Hz, H-8), 4.97 (1H, d, *J* = 7.5 Hz, H-1'), 3.81 (3H, s, 3-OCH_3), 3.65 (1H, dd, *J* = 12.0, 5.5 Hz, H-6b), 3.44 (1H, dd, *J* = 12.0, 2.0 Hz, H-6a); ^{13}C NMR (CD3OD, 125 MHz) δ 168.3 (C-9), 149.5 (C-3), 148.8 (C-4), 144.4 (C-7), 128.6 (C-1), 122.7 (C-6), 117.7 (C-8), 111.6 (C-2), 115.4 (C-5), 100.1 (C-1'), 77.5 (C-5'), 77.3 (C-3'), 73.6 (C-2'), 70.0 (C-4'), 61.1 (C-6'), 56.2 (3-OCH_3); LC ESI IT-TOF MS: m/z 355 $[M-H]^-$.

Cinnamtannin B-1 (**10**) – Brown powder; ^{1}H NMR

(CD_3OD, 500 MHz) δ 7.31 (1H, d, J = 2.0 Hz, H-12'), 7.19 (1H, dd, J = 8.5, 2.0 Hz, H-16'), 7.03 (1H, d, J = 2.0 Hz, H-12), 6.85 (1H, d, J = 1.5 Hz, H-15''), 6.83 (3H, m, H-16, 15', 12''), 6.75 (2H, m, H-15, 16''), 6.10 (1H, s, H-6''), 6.01 (1H, d, J = 2.5 Hz, H-6), 5.97 (1H, d, J = 2.5 Hz, H-8), 5.80 (1H, s, H-6'), 5.70 (1H, s, H-2'), 4.56 (1H, t, J = 1.5 Hz, H-4'), 4.39 (1H, s, H-2''), 4.15 (1H, d, J = 3.5 Hz, H-4), 4.12 (1H, d, J = 2.0 Hz, H-3'), 3.86 (1H, dd, J = 4.0, 3.0 Hz, H-3''), 3.29 (1H, d, J = 3.5 Hz, H-3), 2.83 (2H, t, J = 4.0 Hz, H-4''); ^{13}C NMR (CD_3OD, 125 MHz) δ 156.4 (C-7), 155.4 (C-9), 154.6 (C-7''), 154.4 (C-5', 5''), 154.2 (C-9''), 152.7 (C-5), 150.4 (C-9'), 149.7 (C-7'), 145.3 (C-14), 144.9 (C-14'), 144.5 (C-13'), 144.3 (C-13''), 144.1 (C-13), 143.9 (C-14''), 131.8 (C-11''), 131.1 (C-11), 130.4 (C-11'), 119.9 (C-16'), 118.5 (C-16), 118.0 (C-16''), 115.3 (C-15), 114.7 (C-12'), 114.6 (C-12), 114.3 (C-15', 15''), 114.1 (C-12''), 107.4 (C-8''), 105.3 (C-10'), 105.0 (C-8'), 103.5 (C-10), 98.6 (C-10''), 98.5 (C-2), 96.9 (C-8), 95.1 (C-6''), 95.0 (C-6), 94.6 (C-6'), 78.9 (C-2''), 77.5 (C-2'), 71.2 (C-3'), 66.1 (C-3''), 65.8 (C-3), 36.9 (C-4'), 28.5 (C-4''), 27.5 (C-4); LC ESI IT-TOF MS: *m/z* 863 $[M-H]^-$.

Protocatechuic acid methyl ester (**11**) Colorless oil; ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.43 (1H, d, J = 2.0 Hz, H-6), 7.41 (1H, dd, J = 8.5, 2.0 Hz, H-2), 6.81 (1H, d, J = 8.5 Hz, H-5), 3.84 (3H, s, 7-OCH_3); ^{13}C-NMR (CD_3OD, 125 MHz): δ 167.6 (C-7), 150.5 (C-3), 145.0 (C-4), 122.4 (C-1), 121.4 (C-6), 116.2 (C-2), 114.6 (C-5), 51.0 (7-OCH_3); LC ESI IT-TOF MS: m/z 167 $[M-H]^-$.

DPPH radical scavenging activity – Antioxidant activities of all compounds (**1 - 11**) were evaluated by DPPH and ABTS methods. Free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (100 μM) was incubated with each test compound (100 μL) for 30 min at room temperature, and absorbance was monitored at 517 nm using a Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland) microplate reader. DPPH radical scavenging activities of all compounds (**1 - 11**) were calculated as following formula: DPPH radical scavenging activity (%) = (1–absorbance of sample/absorbance of control) × 100.

ABTS radical scavenging activity – A solution of ABTS[2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (7.4 mM) and of $K_2S_2O_8$ (2.45 mM) were mixed and incubated for 16 h in the dark. ABTS/ $K_2S_2O_8$ solution was diluted in 50% EtOH, mixed with each compounds (**1 - 11**) in different concentrations and distributed into 96-well plates. The stock solution was incubated for 20 min to an absorbance of 0.75 ± 0.05 at 734 nm (UVmini-1240 spectrophotometer, Shimazu, Japan). ABTS radical scavenging activities (%) of all compounds (**1 - 11**) were expressed as a percentage using the following formula: ABTS radical scavenging activity (%) = (1–absorbance of sample / absorbance of control)×100.

Result and Discussion

The rhizomes of *O. japonica* (2.0 kg) were extracted with 100% MeOH under reflux and evaporated under reduced pressure to give a residue (137.0 g), which was dissolved in water (800 ml) and partitioned with solvent to give *n*-hexane (20.0 g) and H_2O (100.0 g) soluble portions. Purification of the H_2O-soluble fractions by multiple chromatographic steps led to the isolation of eleven known compounds (**1 - 11**), identified as osmundalactone (**1**),[9] 3,5-dihydroxy-γ-caprolactone (**2**),[9] osmundalin (**3**),[10] protocatechuic acid (**4**),[11] maltol-β-D-glucopyranoside (**5**),[10] caffeic acid 3-*O*-β-D-glucopyranoside (**6**),[12] coumaric acid 4-*O*-β-D-glucopyranoside (**7**),[13] protocatechuic acid 3-*O*-β-D-glucopyranoside (**8**),[14] ferulic acid 4-*O*-β-D-glucopyranoside (**9**),[15] cinnamtannin B-1 (**10**),[16] and protocatechuic acid methyl ester (**11**)[17] by comparison of their spectroscopic data with previously reported values.

The imbalance between pro-oxidant and antioxidant homeostasis causes multiple diseases.[18] Free radical scavenging is effective for disease prevention, and an important factor in antioxidant defense system.[19] This study was designed to increase the practical value of Korean indigenous plant *O. japonica* by investigating the anti-oxidant activity of isolated compounds (**1 - 11**). Total antioxidant capacity was measured by DPPH and ABTS assays. DPPH is a stable nitrogen synthetic radical, commonly used to measure the hydrogen donating and free radical scavenging activities of sulfur-containing amino acids, aromatic amines. The ABTS assay can be applied to both water-soluble and lipid-soluble compounds, and is used for measuring the antioxidant activities in foods.[20] Compounds isolated from *O. japonica* showed comparable results on DPPH and ABTS assays. The SAR (Structure-activity relationship) analysis suggests that compound **11** possessing methoxy group at C-7 showed a better antioxidant effect than **4** and **8** (Table 1). Two phenylpropanoid glycosides, compounds **7** and **8** have similar chemical structures, except that methoxy moiety at C-3, but there was no significant difference shown in the anti-oxidant activity.

Compound **10** exhibited high scavenging activities in both assays. Recent studies have reported that cinnamtannin B-1 reduces endogenous ROS production stimulated by thrombin, modulates Ca^{2+} mobilization in human platelets,[21] and significantly reduces the oxidant

Table 1. The antioxidant activities of *O. japonica* compounds (**1 - 11**)*

Compound	IC_{50} (μM)	
	DPPH	ABTS
1	>1000	>1000
2	>1000	>1000
3	>1000	>1000
4	153.6 ± 4.21	227.7 ± 2.45
5	>1000	>1000
6	>1000	75.6 ± 1.54
7	237.8 ± 6.31	>1000
8	>1000	120 ± 3.72
9	256.3 ± 5.19	>1000
10	9.1 ± 0.14	16.1 ± 1.45
11	92.4 ± 4.52	107.2 ± 6.23
Ascorbic acid	3.9 ± 0.21	14.1 ± 2.8

*Each value in the tables is represented as mean ± S.D. (n = 3).

action of H_2O_2 by the decrease in the oxidation of the redox sensitive dye CM-H_2DCFDA on CCK-8-evoked responses in mouse pancreatic acinar cells.[22] In addition, our previous study have determined that cinnamatannin B-1 (**10**) is a major compound of *O. japonica*.[2] Therefore, a Korean indigenous plant *O. japonica* may be considered to be functional and effective material for treatment of various diseases.

Acknowledgments

This study was conducted as a part of a basic standardization project for herbal medicinal materials of Korean indigenous resources, supported by a grant from the Ministry of Health and Welfare Korea and Jellanamdo, in 2012-2016. We are thankful to the Korea Basic Science Institute (KBSI) for the measurements of NMR and mass spectra.

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Received May 29, 2017
Revised July 5, 2017
Accepted July 7, 2017