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Phytochemical Studies of Korean Endangered Plants: A New Flavone from *Rhododendron brachycarpum* G.Don

Wei Zhou, Joonseok Oh, Wei Li,† Dong Woo Kim,‡ Seung Ho Lee,* and MinKyun Na†,*

*College of Pharmacy, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Korea. *E-mail: seungho@ynu.ac.kr*
*†College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea. *E-mail: mkna@cnu.ac.kr*
‡Western District Office, National Forensic Service, Jangseong, Jeonnam 515-822, Korea
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Globally at least 13% of known flora are endangered or threatened and the rate of the global biodiversity decline is not improving.[1] The USDA claims that there are now over 780 endangered or threatened species of plants in the United States and its territories,[2] and the Korea National Arboretum reports that over 500 rare species exist in the Korean peninsula.[3] In spite of the growing number of endangered and rare plant species, there are few studies that address the potential of such species to play a pivotal role in the provision of lucrative drug-prototypes or new chemical entities. According to a phylogenetic study, drug-producing plant families are more likely to be clustered than distributed in the phylogenetic tree.[4] Most of the selected families contain endangered species, which validates the prioritization of chemical investigation of endangered species, as extinction of these species leads to the permanent loss of potential drug leads or novel chemistry.

Rhododendron brachycarpum G. Don (Ericaceae) is an evergreen broad-leaved shrub native to northern Korea and central Japan.[5] Radical climate changes, such as global warming, have caused a significant population reduction of *R. brachycarpum*, which led to the designation of the shrub as a rare species.[3] The leaves of *R. brachycarpum* are traditionally employed in the treatment of diabetes, cardiovascular and hepatitis disorders, hypertension and rheumatoid arthritis.[6,7] Previous investigations regarding biologically active components of this rare species identify an anti-tumor diterpenoid, grayanotoxin I, and a cardioprotective flavonoid, quercetin.[8-10] Additionally, our previous investigation led to the discovery of the anti-diabetic triterpenoids rhododendric acid A, corosolic acid and ursolic acid.[8]

In our continuous efforts to explore potential drug leads or new chemicals from *R. brachycarpum*, antioxidant bioassay-guided fractionation was performed utilizing chromatographic techniques and free radical scavenging assays. Herein, we describe the isolation and structural characterization of flavonoids, including a new flavone (**1**), from the active EtOAc extract and the evaluation of anti-oxidant potential of isolated compounds.

Results and Discussion

Compound **1** (Figure 1) was obtained as a yellowish amorphous powder, the molecular formula was established as $C_{24}H_{26}O_{11}$ based on HR-ESI-MS (obsd. $[M+Na]^+$ at *m/z* 513.1371, theor. $[M+Na]^+$ at *m/z* 513.1373). The ^{1}H-NMR spectrum of **1** (Table 1) exhibited signals that indicate an anomeric proton [δ_H 6.30 (d, 1.5 Hz)] and four oxygenated protons [δ_H 4.69 (dd, 9.2, 3.4 Hz), 4.77 (dd, 3.4, 1.5 Hz), 4.42, 4.31], a disubstituted aromatic ring with an ABX-spin coupling system [δ_H 7.96 (d, 2.1 Hz), 7.84 (dd, 8.4, 2.1 Hz), 7.37 (d, 8.4 Hz)] and an aromatic ring with an AX spin coupling system [δ_H 7.23 (d, 2.1 Hz), 6.77 (d, 2.1 Hz)]. The presence of three methoxy groups were evidenced by downfield methyl proton signals (δ_H 4.05, 3.90, 3.85). The ^{13}C-NMR spectrum displayed a carbonyl signal (δ_C 173.7), three methoxy carbon signals (δ_C 60.1, 56.7, 56.3) and two benzene rings bearing two oxygenated carbons (δ_C 161.86, 97.82, 161.62, 96.68, 159.27, 110.99; 123.10, 112.95, 148.81, 151.23, 117.02, 122.58) (Table 1). The observed NMR data suggest that compound **1** is a glycosylated flavone. The aglycone is identified as quercetin 3,5,3'-trimethyl ether based on the close similarity of the NMR data with those of caryatin-3' methyl ether-7-*O*-β-D-glucoside,[11] and the HMBC correlations between δ_H 4.05 (3-OCH_3) and δ_C 141.7 (C-3),

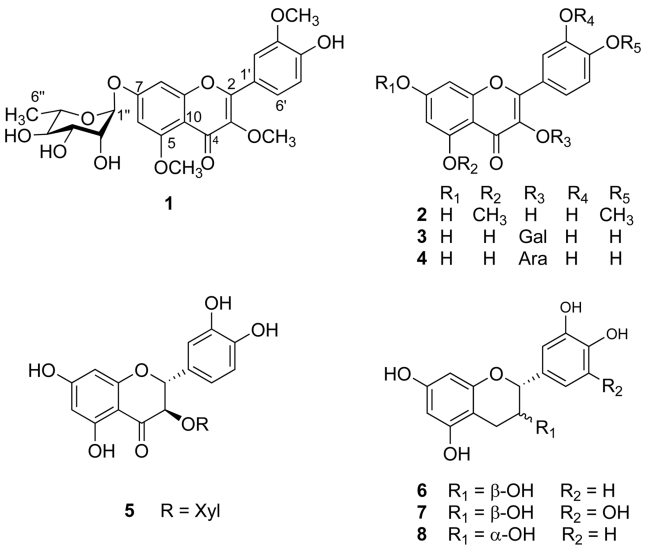

Figure 1. Structures of compounds **1-8** from *R. brachycarpum*

Table 1. ^{1}H and ^{13}C NMR data of compound **1** acquired in pyridine-d_5, 600 MHz and 150 MHz

Position	δ_C	δ_H (*J* in Hz)
2	153.59	
3	141.67	
4	173.68	
5	161.86	
6	97.82	6.77 (1H, d, 2.1)
7	161.62	
8	96.68	7.23 (1H, d, 2.1)
9	159.27	
10	110.99	
1'	123.10	
2'	112.95	7.96 (1H, d, 2.1)
3'	148.81	
4'	151.23	
5'	117.02	7.37 (1H, d, 8.4)
6'	122.58	7.84 (1H, dd, 8.4, 2.1)
1"	100.37	6.30 (1H, d, 1.5)
2"	72.81	4.69 (1H, dd, 9.2, 3.4)
3"	72.06	4.77 (1H, dd, 3.4, 1.5)
4"	73.94	4.42 (1H, m)
5"	71.81	4.31 (1H, m)
6"	19.02	1.66 (3H, d, 6.1)
5-OCH_3	56.33	3.90 (3H, s)
3-OCH_3	60.05	4.05 (3H, s)
3'-OCH_3	56.68	3.85 (3H, s)

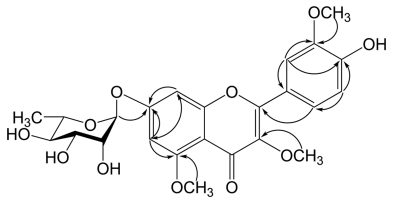

Figure 2. Key HMBC (H → C) correlations of compound **1**.

δ_H 3.90 (5-OCH_3) and δ_C 161.9 (C-5), and δ_H 3.85 (3'-OCH_3) and δ_C 148.8 (C-3') (Figure S4 in Supporting information). To determine the identity of sugar moiety in compound **1**, it was acid-hydrolyzed by 10% HCl. The acquired sugar moiety was identified as L-rhamnose based on comparison of the retention time with that of an authentic L-rhamnose sample up on gas chromatography analysis (retention time is 5.31 min). The glycosidic linkage was established based on an HMBC correlation between the anomeric proton signal (δ_H 6.30) (Figure 2) and the oxygenated aromatic carbon signal (δ_C 161.6). The coupling constant (J = 1.5 Hz) of the anomeric proton signal determined L-rhamnose as the α-anomer. Consequently, the structure of compound **1** was elucidated as caryatin-3' methyl ether-7-*O*-α-L-rhamnoside. The known polyphenols were identified as 3,3',7-trihydroxy-4',5-dimethoxyflavone (**2**),[12] hyperin (**3**),[13] guaijaverin (**4**),[14] taxifolin-3-β-D-xylopyranoside (**5**),[15] (+)-catechin (**6**),[16] (+)-gallocatechin (**7**),[17] and (−)-epicatechin (**8**) (Figure 1) by comparison of the experimental and reported spectroscopic data. Of the known flavonoids, compounds **2** and **5** were isolated from *R. brachycarpum* for the first time.

Table 2. Antioxidant activity of extracts (μg/mL) and compounds **1-8** (μM) from *R. brachycarpum*

	IC_{50}[a]	AAI[a]
MeOH	19.21 ± 1.88	2.88 ± 0.30
n-hexane	550.98 ± 98.63	0.10 ± 0.02
$CHCl_3$	35.08 ± 0.62	1.57 ± 0.03
EtOAc	4.23 ± 0.26	13.00 ± 1.41
BuOH	5.46 ± 0.68	10.15 ± 1.26
H_2O	10.54 ± 0.37	5.22 ± 0.18
1	386.46 ± 12.57	0.29 ± 0.01
2	17.37 ± 0.35	9.59 ± 0.19
3	13.35 ± 0.15	8.87 ± 0.10
4	18.49 ± 0.14	6.85 ± 0.05
5	30.70 ± 0.40	4.11 ± 0.05
6[b]	13.46 ± 0.03	14.08 ± 0.03
7	13.22 ± 0.29	13.58 ± 0.30
8	14.43 ± 0.21	13.13 ± 0.19

[a]Results in terms of mean ± standard deviation. [b]Positive control.

The purified compounds were evaluated for antioxidant potential. IC_{50} and an antioxidant activity index (AAI) were used for the evaluation of the activity.[18] Among of the tested compounds, compounds **2-8** exhibited strong antioxidant activity, whereas compound **1** showed poor activity (Table 2). The phenolic hydroxy groups of flavonoids, especially 3-OH, 5-OH and 3'-OH, are reported to play a pivotal role in DPPH scavenging activity,[19] which is also observed in the present study given that compounds **6-8** exhibited potent activity and compound **1** showed poor activity. Compounds **3** and **4**, which possess an olefinic bond between C-2 and C-3, displayed stronger scavenging activity than compound **5** in agreement with a previous study.[19]

In the present study, one new and seven known polyphenols were purified from a Korean endangered species *R. brachycarpum* and evaluated for the antioxidant activity. This study demonstrates new chemical entities remain undiscovered among endangered plant species and motivates further research in the discovery of a new scaffold from such species.

Experimental

General Procedures. The NMR experiments were conducted on a Bruker DMX 300 (^{1}H-300 MHz, ^{13}C-75 MHz), and a Bruker DMX 600 (^{1}H-600 MHz, ^{13}C-150 MHz) spectrometers and the chemical shifts (δ) were displayed in ppm and referenced by residual pyridine signals. Optical rotations were measured using a JASCO DIP-1000 (Tokyo, Japan). Mass spectra were obtained on a JMS 700 high resolution mass spectrometer (Jeol, Japan). MPLC was carried out employing a Biotage Isolera™ reversed phase C_{18} SNAP Cartridge (KP-C_{18}-HS) and normal phase SNAP Cartridge (KP-Sil; 340 g, Biotage AB, Uppsala, Sweden). Preparative

HPLC was performed using a Gilson system equipped with a UV detector and Luna C_{18} columns (250 × 21.2 mm, 10 μm and 250 × 4.60 mm, 5 μm). TLC was conducted on glass plates precoated with silica gel 60 F_{254} or RP-18 F_{254} (Merck). Silica gel (Merck, 70-230 mesh) was used for large scale column chromatography.

Plant Material. Leaves of *R. brachycarpum* were collected on a farm in Gongju, Korea, in 2011. The plant material was identified by Prof. MinKyun Na (College of Pharmacy, Chungnam National University). A voucher specimen (CNU00195) was deposited at the Pharmacognosy Laboratory of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation. Dried leaves of *R. brachycarpum* (25 kg) were extracted two times with MeOH (250 L for each extraction) at room temperature for one week and filtered to acquire the MeOH extract. The extract was concentrated to afford a brownish slurry (6 kg) and evaluated for antioxidant activity. Half of the extract (3 kg) was suspended in H_2O (10 L) and partitioned with *n*-hexane (10 L × 3), $CHCl_3$ (10 L × 3), EtOAc (10 L × 3) and BuOH (10 L × 3) to obtain four organic extracts (438, 140, 450 and 320 g, respectively). The extracts were re-tested for DPPH free radical scavenging activity. The EtOAc fraction was further subjected to vacuum liquid chromatography employing a silica gel column and eluted with $CHCl_3$:MeOH (90:10 → 0:100) to generate 10 fractions (Fr. E1-E10). Fr. E2 (62.88 g) was chromatographed on a silica gel column (18 × 30 cm) and eluted with $CHCl_3$:EtOAc (9:1 → 2:8) to yield eight fractions (E21-E28). Fr. E28 (13.36 g) was fractionated into seven fractions (E281-E287) employing MPLC (a C_{18} SNAP Cartridge KP-C_{18}-HS column) and eluting with MeOH:H_2O (3:7 → 7:3). Fr. E284 (1.46 g) was purified using preparative HPLC (Luna C_{18}, 250 × 21.2 mm, flow rate 6 mL/min) and eluted with 20% MeCN to afford compound **2** (40 mg, t_R 64.5 min). Fr. E4 (143.77 g) was subjected to silica gel column (18 × 30 cm) chromatography and eluted with CH_2Cl_2:MeOH (9:1 → 8:2) to yield seven fractions (E41-E47). Fr. E43 (29.83 g) was chromatographed on a silica gel column (10 × 50 cm) and eluted with CH_2Cl_2:MeOH:H_2O (8:1:0.1 → 1:1:0.1, 3 L for each step) to obtain six fractions (E431-E436). Fr. E436 (1.8 g) was purified employing an HPLC RP-C_{18} column and eluting with MeOH:H_2O (1:9 → 5:5) to obtain compound **8** (7.6 mg, t_R 52.5 min) and 7 (70 mg, t_R 63.4 min) and eight fractions (E4361-E4368). Compound **6** (270 mg, t_R 15.8 min) was acquired from Fr. E4363 (550 mg) utilizing preparative HPLC (Luna C_{18}, 250 × 4.6 mm, flow rate 1 mL/min) and eluting with MeOH:H_2O (1:9 → 5:5). Fr. E47 (9.97 g) was purified by RP-C_{18} MPLC eluting with MeOH:H_2O (3:7 → 5:5) to afford compound **3** (3.4 g) and six fractions (E471-E476). Fr. E472 (374.8 mg) was purified by HPLC with an RP-C_{18} column eluting with MeOH:H_2O (from 4:6 → 6:4) to afford compound **5** (10 mg, t_R 25.2 min). Fr. E6 (12.74 g) was subjected to an MPLC RP-C_{18} column and eluted with acetone:MeOH:H_2O (0:0:100 → 12:28:60) yielding seven fractions (E61-E67). Fr. E67 (800 mg) was chromatographed on silica gel column and eluted with $CHCl_3$:MeOH:H_2O (7:1:0.1) to yield compounds **1** (7 mg) and **4** (14 mg).

Acid Hydrolysis. Compound **1** (2.0 mg) was hydrolyzed with 10% HCl (1.0 mL) at 85 °C for 3 h and the reaction mixture was partitioned with EtOAc. The aqueous layer was concentrated to yield a residue. The residue was dissolved in anhydrous pyridine (0.1 mL) after the addition of L-cysteine methyl ester hydrochloride (0.1 mL) and heated at 60 °C for 2 h. The trimethylsilylimidazole solution was added followed by heating at 60 °C for 2 h and the mixture was concentrated using rotary evaporation. The dried product was partitioned with EtOAc and H_2O. The aqueous extract was analyzed utilizing gas liquid chromatography analysis and the monosaccharide moiety was confirmed as L-rhamnose based on comparison of the retention time with that of an L-rhamnose standard (t_R 5.31 min).

Antioxidant Assay. The optimized reaction time for each compound was 60 min (Supporting information Fig S21). The DPPH assay displays a color change from purple to yellow upon quenching the secondary nitrogen radical in DPPH through the addition of hydrogen provided by an antioxidant compound.[18] The evaluation of scavenging activity was performed by following the Kassim and Rahmani method with some modifications.[20] Briefly, 20 μL of the sample dissolved in DMSO (3.125-200 μM) was added to 96 well plates and 180 μL DPPH (140 μM) was applied to each well. DMSO was used as a negative control. After an incubation period in the dark, absorbance was measured at 517 nm. The radical scavenging activity (RSA) was calculated as follows: RSA% = %Inhibition = [1 − (optical density (OD_{sample}/OD_{blank})] × 100

The IC_{50} was calculated using a calibration curve in the linear range plotting sample concentration *vs.* the corresponding scavenging effect. The antioxidant activity was evaluated using AAI calculated by the following equation[18]:

$$\text{AAI} = \frac{\text{Final concentration of DPPH } (\mu\text{g mL}^{-1})}{\text{IC}_{50}\ (\mu\text{g mL}^{-1})}$$

Based on the evaluation standard of Scherer and Godoy method,[19] antioxidant potency of tested compounds were classified as poor antioxidant activity (AAI < 0.5), moderate antioxidant activity (AAI 0.5-1.0), strong antioxidant activity (AAI 1.0-2.0) and very strong antioxidant activity (AAI > 2.0).

Caryatin-3' methyl ether-7-*O*-α-L-rhamnoside (1). Yellowish amorphous powder; $[\alpha]_D^{20}$: −87.1 (*c* 0.1, MeOH); HR-ESI-MS: *m/z* 513.1371 $[M+Na]^+$ (calcd for $C_{24}H_{25}O_{11}Na$, 513.1373). ^{1}H-NMR (pyridine-d_5, 600 MHz) and ^{13}C-NMR (pyridine-d_5, 150 MHz) data, see Table 1.

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Supporting Information. HR-ESI-MS, ^{1}H, ^{13}C-NMR, HMQC, HMBC and COSY spectra of compound **1**, ^{1}H and ^{13}C-NMR spectra of compounds **2-8** and details of the optimized reaction time for the DPPH assay are provided.

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