Kaempferol Glycosides and Cardenolide Glycosides, Cytotoxic Constituents from the Seeds of *Draba nemorosa* (Brassicaceae)

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Bioassay-directed fractionation of a methanolic extract from the seeds of *Draba nemorosa* (Brassicaceae) led to isolation of a new flavonol glycoside, drabanemoroside (5, kaempferol 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranose) along with four known flavonoid derivatives (1-4), four cardenolide glycosides (6-9). Kaempferol glycosides 2 and 5 showed strong cytotoxicity against human small lung cancer cell line A549 and melanoma SK-Mel-2 with an IC$_{50}$ of 0.5 µg/mL and 1.9 µg/mL, respectively. Cardenolide glycosides 6-9 showed potent cytotoxicity (A549) in the range of 0.01-0.032 µg/mL. Their structures were characterized based on spectroscopic data (2D NMR, HRTOFMS, IR, and UV) and comparison of literature values. The carbohydrate units were also confirmed by comparing the hydrolysate of 5 with authentic monosaccharides.

**Key words:** *Draba nemorosa*, Drabanemoroside, Kaempferol rhamnopyranosyl-arbinopyranoside, Cytotoxicity, Cardenolides

INTRODUCTION

An annual plant *Draba nemorosa* L. is widely distributed in Korea and found in sunny fields or mountainous area. It blossoms in March to May. Its seeds in June (Kim, 1996). The seeds of this plant have been traditionally used for anti-asthmatic, demulcent, diuretic, expectorant and pleural anti-inflammatory effect (Kung and Huang, 1949; Graham et al., 2000). However, a few chemical works have been reported. In search for bioactive compounds from medicinal plants (Moon et al., 2008; Sultan et al., 2008; Song et al., 2009), *D. nemorosa* was chosen because the seed extract showed strong cytotoxic activity against human small lung cancer cell line A549 (IC$_{50}$~25 µg/mL). Here, we describe the isolation and structure determination of kaempferol glycosides (1-5) and cardenolide glycosides (6-9) from the seed extract of *D. nemorosa*. Cytotoxic activities of isolated compounds were also investigated.

MATERIALS AND METHODS

**Plant material**

The seeds of *D. nemorosa* were purchased from the local market at Geumsan, Daejeon, Korea in August, 2005 and identified by Dr. Eunkyu Lim at the Busong Clinic of Medicinal Herbs. A voucher specimen has been deposited at the Natural Product Chemistry Lab, Department of Chemistry, Kongju National University, Korea (SM1372).

**General experimental procedures**

The melting points were measured on a Fisher melting point apparatus and are reported uncorrected. High resolution TOF mass spectra were measured on a Waters LCT Premier mass spectrometer coupled with a Waters AQUITY HPLC system and data acquisition was achieved using MassLynx software, version 4.0. Optical rotations were measured on a Perkin Elmer 341-LC polarimeter. UV and IR spectra were measured on a Shimadzu UV-2401 PCR spectrometer and a Perkin-Elmer BXFT-IR spectrometer, respectively. NMR spectra were recorded on a Varian Mercury 400 spectrometer with standard pulse sequences operating at 400 MHz in $^1$H NMR and 100 MHz in $^{13}$C NMR. The chemical shifts given in ppm ($^1$H NMR) or ppm ($^{13}$C NMR) were referenced to...
solvent peaks: δ_H 3.30 and δ_c 49.15 for CD3OD, and δ_H 7.24 and δ_c 77.0 for CDCl3. Flash column chromatography was carried out on C18 column (40-63 µm, 90 id x 70 mm, Merck). Medium-pressure liquid chromatography (MPLC) was carried out on a FMI lab pump system using silica gel 60 (25-40 µm, 30 id x 300 mm, 85:14.5:0.5 to 80:18.2 CH2Cl2-MeOH-H2O) at a flow rate of 8 mL/min. Thin-layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel 60, F254, 20 x 20 cm, 0.25 mm thick, Merck). Chiral TLC was performed on Chiralplate (10 x 20 cm, Alltech). Spots were detected under UV light at 254 nm or by staining with a solution of p-anisaldehyde-sulfuric acid in methanol followed by heating. Reverse phased HPLC was performed on a Waters 600 model system with a photodiode array UV detector 996 using mobile phase: 25% CH3CN in H2O; flow rate: 1.4 mL/min. Thin-layer chromatography (TLC) was performed on precoated silica gel 60, Kieselgel 60, F254, 20 x 20 cm, 0.25 mm thick, Merck). Medium-pressure liquid chromatography (MPLC) was carried out on a FMI lab pump system using silica gel 60 (25-40 µm, Diasogel, 30 id x 250 mm) with a gradient elution of 30 to 51% aqueous MeOH for 10 min and 51 to 65% aqueous MeOH for 80 min at a flow rate of 7 mL/min. Optical density for a 96-well microplate was measured on a Tecan Sunrise A-5080 microplate reader at 520 nm. L-rhamnose and L-arabinose were purchased from Sigma-Aldrich.

**Extraction and isolation**

The seeds (6 kg) of *D. nemorosa* were pulverized and soaked with a series of extraction solvents: 80% aqueous MeOH (8 L) at room temperature for one week, MeOH (8 L) for one week, and CH2Cl2 (7 L) for 5 days. The extracts were pooled and evaporated under reduced pressure to yield brownish oily syrup (402 g), which was suspended in 30% aqueous MeOH (1.4 L) and was extracted with hexane (900 mL x 5). Aqueous methanol layer was concentrated to give brownish residue (190 g) and partitioned between H2O (1.2 L) and butanol (800 mL x 4). The butanol fraction (50 g, cytotoxicity: IC50=10 µg/mL against lung cancer cell line A549) was chromatographed on C18 flash column eluting with H2O to MeOH to give twelve fractions. Fractions 5 to 8 were found to be cytotoxic (IC50 range of 1.0-2.0 µg/mL).

Fraction 5 was chromatographed on a silica gel column and purified by using C18 HPLC to give 3 (170 mg) as pale yellowish powder. Fractions 6 (4.5 g), 7 (768 mg), and 8 (600 mg) were chromatographed independently by silica MPLC to give thirteen subfractions (Fr. 6.1-6.13), twelve subfractions (Fr. 7.1-7.12), and eleven subfractions (Fr. 8.1-8.11), respectively. C18 HPLC of Fr. 6.4 gave 6 (6.0 mg) and 7 (60 mg) as white amorphous powder. Fr. 6.7, 6.8, and 6.10 were further purified by C18 HPLC to give 4 (50 mg) as yellowish powder, 8 (17 mg) as white amorphous powder, and 5 (45 mg) as light yellowish powder, respectively. Fr. 7.5 and 7.10 were purified by using C18 HPLC to give 2 (5.2 mg) as brownish powder and 9 (28 mg) as light yellowish amorphous powder, respectively. Fr. 8.3 (31 mg, pale brown powder) was triturated in a mixture of MeOH and CH2Cl2 (7:3) and the filtrate was chromatographed by C18 HPLC to give 1 (11 mg) as yellowish powder.

**Drabanemoroside (Kaempferol 3-O-α-L-rhamnopyranosyl-(1-2)-α-L-arabinopyranoside) (5)**

Amorphous yellow powder, Rf 0.22 (TLC, silica gel, CH2Cl2-MeOH-H2O, 80:19.5:0.5); mp 189-191°C; [α]20D -1.18 (c 0.72, CH3OH); UV λmax (MeOH) nm (log ε): 346 (4.21), 265 (4.27), 203 (4.50); IR (KBr) νmax cm⁻¹: 3386 (OH), 1654 (carbonyl), 1609 (aromatic ring), 1508, 1458; 1H-NMR (CD3OD, 400 MHz) δ_H: 8.03 (2H, d, J = 8.8 Hz, H-2' and H-6'), 6.90 (2H, d, J = 8.8 Hz, H-3' and H-5'), 6.38 (1H, d, J = 2.4 Hz, H-8), 6.18 (1H, d, J = 2.4 Hz, H-8), 5.52 (1H, d, J = 5.2 Hz, H-1''), 5.09 (1H, d, J = 1.6 Hz, H-1''), 4.10 (1H, dd, J = 6.4, 5.2 Hz, H-2''), 3.91 (1H, dd, J = 3.2, 1.6 Hz, H-2''), 3.87 (1H, dq, J = 9.6, 6.0 Hz, H-5''), 3.82 (1H, dd, J = 6.4, 2.8 Hz, H-3''), 3.78 (1H, m, H-4''), 3.76 (1H, dd, J = 11.2, 5.2 Hz, H-5''), 3.70 (1H, dd, J = 9.6, 3.2 Hz, H-3''), 3.35 (1H, t, J = 9.6 Hz, H-4''), 3.34 (1H, m, H-5''), 1.09 (3H, d, J = 6.0 Hz, H-6'); 13C-NMR (CD3OD, 100 MHz): δ_C: 179.3 (C-4), 165.6 (C-7), 162.9 (C-5), 161.3 (C-4'), 158.4 (C-9), 158.3 (C-2), 135.0 (C-3), 132.1 (C-2' and C-6'), 122.7 (C-1'), 116.3 (C-3' and C-5'), 105.8 (C-10), 102.2 (C-1''), 101.0 (C-1''), 99.8 (C-6), 94.7 (C-8), 77.2 (C-2''), 74.0 (C-4''), 72.8 (C-3''), 72.5 (C-2''), 72.3 (C-3''), 70.2 (C-5''), 68.4 (C-4''), 65.2 (C-5'), 17.9 (C-6''), HR-TOF-MS (+ESI) m/z: 565.1542 [M+H]+ (calcd. for C39H39O14+H: 565.1557).

**Hydrolysis and sugar analysis of drabanemoro-**

The glycoside 5 (2.5 mg) was added to a solution (1 mL) of 2 M HCl in water and stirred at 100°C for 4 h. The hydrolysate was passed through a short column of C18 (40-63 µm) with stepwise gradient elution of a mixture of H2O and MeOH. The H2O eluate was analyzed on Waters Alliance HPLC system connected to evaporative light scattering detector (Waters ELSL 2420; column: Waters high performance carbohydrate column, 3.9 id x 300 mm; column temperature: 35°C; mobile phase: 25% CH3CN in H2O; flow rate: 1.4 mL/min; injection volume: 20 µL; nebulizer: 30%; drift tube: 50°C; pressure: 50 psi). Rhamnose (tR = 3.09 and 4.03 min) and arabinose (tR = 3.55 min) were identified by comparison with authentic samples of L-rhamnose and L-arabinose. Absolute configuration of the rhamnose and arabinose were determined by compar-
ing with authentic standards using chiral TLC ($R_f$ value of L-rhamnose, 0.60 and 0.52; L-arabinose, 0.37; 6:1 ethyl acetate-methanol).

**Cytotoxicity measurements**

Cytotoxicity was measured by previously reported SRB assay method (Moon et al., 2008; Song et al., 2009). In brief, seeding density for A-549, SK-MEL-2 and B16-F1 was $1 \times 10^5$, $1 \times 10^5$ and $2 \times 10^4$ cells/mL, respectively, in the culture medium (RPMI-1640) in a 96-well plate. After 24 h incubation at 37°C under humidified 5% CO$_2$, test solutions in RPMI medium were added and incubated for additional 48 h. The cells were fixed and stained with a sulforhodamine B solution. After unbound dye was removed by 1% acetic acid, protein-bound dye was extracted with 10mM tris base (pH 10.5). Optical density of the released dye was measured at 520 nm in a microplate reader. The results were expressed in terms of the concentrations of 50% inhibition (IC$_{50}$). Cisplatin was used as a positive control.

**RESULTS AND DISCUSSION**

The powdered seeds were successively extracted with 80% aqueous MeOH, MeOH, and CH$_2$Cl$_2$. The combined extracts were concentrated and suspended in a mixture of hexane and aqueous MeOH. The aqueous MeOH layer was partitioned between butanol and H$_2$O. The butanol layer (cytotoxicity: IC$_{50}$~10 µg/mL against lung cancer cell line A549) was fractionated on a C18 flash column. The bioactive fractions were further subjected to silica gel MPLC and reversed phase C18 HPLC to yield nine compounds (1-9) (Fig. 1).

Compound 5 was obtained as amorphous yellow powder with mp. 189-191°C. The mass spectrum (HR-TOF-MS, positive ESI mode) displayed a protonated molecular ion [M+H]$^+$ at $m/z$ 565.1542, indicating a molecular formula of C$_{26}$H$_{28}$O$_{14}$. The UV spectrum showed the absorption bands at $\lambda_{max}$ 346 and 265 nm, typical of a flavonol structure (Rosch et al., 2004). The IR spectrum (KBr) showed the presence of a hydroxyl (3386 cm$^{-1}$), carbonyl (1654 cm$^{-1}$) and aromatic group (1609 cm$^{-1}$). The $^1$H-NMR spectrum displayed characteristic signals for a kaempferol moiety (Ho et al., 2005) at $\delta$ 6.18 (d, $J$ = 2.4 Hz, H-6) and 6.38 (d, $J$ = 2.4 Hz, H-8) in the ring A and at $\delta$ 8.03 (d, $J$ = 8.8 Hz, H-2'/6') and 6.90 (d, $J$ = 8.8 Hz, H-3'/5') in the ring B. The $^1$H NMR spectrum also displayed fourteen nonexchangeable protons coupled with eleven carbon signals extracted from HSQC experiment ($\delta_C$): nine methine protons at $\delta$ 5.52 (101.0), 5.09 (102.2), 4.10 (77.2), 3.91 (72.5), 3.87 (70.2), 3.82 (72.8), 3.78 (68.4), 3.70 (73.7), 3.70 (72.3), 3.70 (72.3),

![Fig. 1. Structures of compounds 1-9 isolated from the seeds of D. nemorosa](image-url)
and 3.35 (74.0); two methylene protons at δ 3.76 and 3.34 (65.2); and three methyl protons at δ 1.09 (17.9). This suggested the presence of a disaccharide unit. Two anomeric proton signals at δ 5.52 (1H, d, J = 5.2 Hz) and 5.09 (1H, d, J = 1.6 Hz) were used as the starting point for the sequential assignment of the disaccharide resonances. The 1H-1H COSY of 5 revealed contiguous couplings between δ 5.52 (H-1'') and 4.10 (H-2'', dd, J = 6.4, 5.2 Hz); H-2'' and 3.82 (H-3'', dd, J = 6.4, 2.8 Hz); H-3'' and 3.78 (H-4''); H-4'' and 3.76 (H-5'', dd, J = 11.2, 5.2 Hz); H-4'' and 3.34 (H-5''); 3.76 (H-4'', dd, J = 11.2, 5.2 Hz); H-4'' and 3.34 (H-5''); 5.09 (H-1'''') and 3.91 (H-2''', dd, J = 3.2, 1.6 Hz); H-2''' and 3.70 (H-3''', dd, J = 9.6, 3.2 Hz); H-3'' and 3.35 (H-4''', t, J = 9.6 Hz); H-4''' and 3.87 (H-5''', dq, J = 9.6, 6.0 Hz); and H-5''' and 1.09 (H-6'''', d, J = 6.0 Hz). These COSY correlations were supported by 1D TOCSY experiments, which suggested the presence of monosaccharide moieties of arabinopyranose (δ 5.52, 4.10, 3.82, 3.78, 3.76, and 3.34) and rhamnopyranose (δ 5.09, 3.91, 3.70, 3.35, 3.87, and 1.09). They were confirmed by HPLC analysis of the acid-hydrolysate of 5. Their absolute configurations were determined to be both L by chiral TLC analysis. The connectivities between three partial structures (kaempferol, rhamnopyranose, and arabinopyranose moieties) were established by HMBC (Fig. 2). Long range 1H-13C correlations [H-1'' (δ 5.52, Ara) to C-3 (δ 135.0, kaempferol) and H-1''' (δ 5.09, Rha) to C-2'' (δ 77.2, Ara)] indicated that the rhamnopyranose moiety was attached to the C-2 of arabinopyranose which was in turn attached to the kaempferol aglycone. A downfield shift of C-2'' (δ 77.2, Ara) also supported the attachment of rhamnopyranosyl unit. The 1H- and 13C-NMR data of the disaccharide part [α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl] were in good accordance with those of the sugar unit of a quercetin derivative, calabricoside A (Calis et al., 2001). Thus, the structure of 5 was determined to be kaempferol 3-O-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside], named drabanemoro side.

Compounds 1-4 were identified as isorhamnetin (1) (Park and Lee, 1996), quercetin (2) (Ly et al., 2005), quercetin-3-O-β-D-glucopyranoside (3) (Sanbongi et al., 1998), and isorhamnetin-3-O-β-D-glucoside (4) (Lee et al., 2005) on comparison with literature values. These were isolated for the first time from D. nemorosa. Compounds 6-8 (Fig. 1) were obtained as white amorphous powders. From spectral comparison with literature values, corchoroside A (6), helveticoside (7), and erysimoside (8) were identified as strophanthidin glycosides (Nakamura et al., 1998). Compound 9 was obtained as light yellowish amorphous powder. Spectroscopic data displayed the characteristic features of digitoxigenin glucoside and was identified as cellobiosyldigigulomethyloside, which was reported from Cheiranthus allioni Hort. (Makarevich et al., 1996).

Strophanthidin glycosides have been reported in various plants, e.g. Adonis aestivalis L. (Ranunculaceae), Strophanthus speciosus (Apocynaceae), Convallaria majalis L. (Liliaceae), Corchorus olitorius L. (Malvaceae), and Erysimum cheiranthoides L. (Brassicaceae).

### Table I. Cytotoxicity data (IC50 in µg/mL) of isolates 1-9

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<th>Compounds</th>
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<tr>
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<td>A549</td>
<td>SK-Mel-2</td>
<td>B16F1</td>
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<tr>
<td>2</td>
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<td>5</td>
<td>0.69</td>
<td>1.9</td>
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a The assay was done in duplicate and (-) signs indicate they were not active below a concentration of 40 µg/mL.

b A549, human small lung cancer cell line; SK-Mel-2, human melanoma cell line; B16F1, mouse melanoma cell line.

Fig. 2. Important HMBC correlations of compound 5 (arrows point from H to C)
(Melero et al., 2000; Filigenzi et al., 2004). Recently, antiproliferative cardenolide glycosides against human ovarian cell line have been reported from the Madagascar rainforests, which were different in sugar moieties from 6-8 (Karkare et al., 2007; Hou et al., 2009). To our knowledge, these are the first reports of cardenolide glycosides from the Draba genus and there have been no report on their cytotoxic activities.

Compounds 1-9 were tested against human lung cancer cell lines A549, human melanoma SK-Mel-2, and mouse melanoma B16F1 by SRB assay (Table I). Compounds 2 and 5 were moderately cytotoxic against A549 and SK-Mel-2 cell lines in the range of IC_{50} 0.50 -0.69 and 1.1-1.9 µg/mL, respectively, whereas other flavonoids (1, 3, and 4) were not active below 40 µg/mL concentration. All cardenolides (6-9) were highly cytotoxic at the range of IC_{50} 0.01-0.90 µg/mL. Although an isomer of 5, kaempferol 3-O-[α-L-rhamnopyranosyl-(1→2)-α-L-arabinofuranoside], was reported from Artabotrys hexapetalus (Li et al., 1997), no bioactivity data were available. The strong cytotoxic activities of the seed extract of D. nemorosa seem to be from cardenolide derivatives.

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REFERENCES


