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Two New Phenolic Constituents of *Humulus japonicus* and their Cytotoxicity Test *In Vitro*

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Two new phenolic constituents (**4** and **6**), together with four known constituents, methyl ferulate (**1**), eugenyl-β-D-glucopyranoside (**2**), apigenin-7-*O*-β-D-glucopyranoside (**3**), and (*E*)-resveratrol-3-O-β-D-glucopyranoside (**5**) were isolated from the MeOH extract of the aerial part sof *Humulus japonicus*. The structures of the new compounds were determined by spectroscopic methods to be divarin-3-*O*-β-glucopyranoside (**4**), and lariciresinol-9-*O*-β-xylopyranoside (**6**). Compounds **1** and **3** exhibited moderate cytotoxicity against two human cancer cell lines (SK-OV-3 and HCT15) with ED_{50} values ranging from 8.84 to 8.79 μM.

Key words: *Humulus japonicus*, Lignan, Phenyl propanoid, Cytotoxicity

INTRODUCTION

The perennial herb *Humulus japonicus* Sieboid & Zucc (Cannabaceae) has been used in the treatment of pulmonary tuberculosis, tuberculous cervical lymphadenitis and hypertension in Korean traditional medicine and found to possess antioxidant and antibacterial activities (Park *et al.*, 1994) and antimutagenic effects (Park *et al.*, 1995). Terpenes, lupulones and flavonoids have been reported to be constituents of this plant (Naya *et al.*, 1970; Aritomi, 1962). In continuation of our search for biological active compounds from Korean medicinal sources, we investigated the constituents of the aerial parts of *H. japonicus*. Chromatographic separation of the MeOH extract of this plant led to the isolation of two new phenolic compounds, as well as four known phenolic constituents. The isolated compounds were tested for cytotoxicity against four human tumor cells (A549, SK-OV-3, SK-MEL-2, and HCT) *in vitro* by SRB assay. This paper deals with the isolation, structure determination and cytotoxic activity of these six phenolic constituents.

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MATERIALS AND METHODS

General experimental procedures

All melting points were determined on a Gallenkamp melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco P-1020 Polarimeter. UV spectra were recorded with a Shimadzu UV 1601 and Varian Cary 5000 spectrophotometer. IR spectra were recorded with a Bruker IFS-66/S. NMR spectra were recorded on Bruker (Avance-300 and Avance-600) and Varian (Unity Inova 300Nb, Unity Inova 500Nb) spectrometers. MS spectra were recorded on JEOL JMS-700 mass spectrometer. Preparative HPLC used a Knauer K1001 instrument with a refractive index detector, a UV detector and an Alltech Econosil silica 5 μm (length: 250 mm, I.D 22 mm) column, and an Alltech Econosil C18 5 μm (length: 250 mm, I.D 4.6 mm) column. For open column chromatography, silica gel (Merck, 70-230), ODS (Cosmosil 140 C_{18}) and Sephadex LH-20 (Pharmacia) were used. Low pressure liquid chromatography was carried out over Merck LiChroprep Lobar-A Si 60 or Merck LiChroprep Lobar-A RP-C18 columns with an FMI QSY pump (Isco).

Plant materials

The aerial parts of *H. japonicus* (3.4 kg) were collected at Yangyang, Gang Won province, Korea in June 2005. A voucher specimen (SKKU-2005-07) was deposited at the College of Pharmacy, Sungkyunkwan University, Korea.

Test for cytotoxicity *in vitro*

A sulforhodamin B bioassay (SRB) was used to determine the cytotoxicity of each compound (Skehan *et al.*, 1990) against four cultured human cancer cell lines. The assays were performed examined at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non small cell lungcarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma) and HCT (colon adenocarcinoma).

Extraction and Isolation

The dried and chopped aerial parts of *H. japonicus* (3.2 kg) were extracted three times with 80% methyl alcohol. The MeOH extract (430 g) was suspended in distilled water (1.6 L) and successively partitioned with n-hexane, methylene chloride, ethyl acetate and n-butanol followed by evaporation to yield 30 g, 30 g, 10 g and 45 g, respectively.

The hexane fraction (30 g) was chromatographed over a silica gel column using a gradient solvent system of hexane:EtOAc (10:1 - 1:1) to give nine subfractions (H1-H9). The H9 fraction (0.8 g) was chromatographed over silica gel (hexane:methylene chloride:EtOAc = 20:10:3), Sephadex LH-20 gel filtration (methylene chloride:MeOH = 1:1) and purified using HPLC (hexane:methylene chloride: EtOAc = 20:10:3) to yield **1** (8 mg). The ethyl acetate fraction (10 g) was chromatographed over a silica gel column eluted with a gradient solvent system of chloroform:acetone (10:1) to afford four subfractions (E1-E4). The E1 fraction (1.0 g) was column chromatographed over silica gel column (methylene chloride:methanol = 40:1) and purified using HPLC (methylene chloride:methanol = 20:1) to afford **2** (8 mg). E3 (3.8 g) was recrystallized (80% MeOH) and further purified by HPLC (ODS column 30% MeOH to 40% MeOH, 50 min, 2.5 mL/min, 320 nm) to yield **3** (20 mg). The E3 subfraction (3.0 g) was chromatographed on ODS silica gel column eluting by a gradient solvent system (30% MeOH to 60% MeOH) to afford six subfractions (E31-E36). The E33 subfraction (120 mg) was further purified by HPLC using 14% methanol in methylene chloride to afford **4** (30 mg). The subfraction E35 (40 mg) was purified by HPLC using methylene chloride:methanol (10:1) to yield **5** (10 mg). The E2 fraction (1.2 g) was repeatedly chromatographed on silica gel (methylene chloride:methanol = 30:1 - 5:1), Sephadex LH-20 with 100% MeOH and purified by HPLC using methylene chloride:methanol:water = 25:2:0.06 to yield **6** (8 mg).

Methyl ferulate (1)

White powder; mp: 70°C; UV (MeOH) λ_{max} nm (log ε): 285 (4.37); ESI-MS(*m/z*): 231.0 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 3.81 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 6.31 ('H, d, *J* = 15.5 Hz, H-8), 6.94 (H, d, *J* = 8.0 Hz, H-6) 7.05 ('H, d, *J* = 2.0 Hz, H-2), 7.09 ('H, dd, *J* = 2.0, 8.0 Hz, H-6) 7.64 ('H, d, *J* = 15.5 Hz, H-7); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 51.79 (OCH_3), 56.19 (OCH_3), 109.65 (C-2), 114.97 (C-8), 115.47 (C-5), 123.25 (C-6), 127.24 (C-1), 145.15 (C-7), 147.02 (C-3), 148.23 (C-4), 167.91 (C-9)

Eugenyl-β-D-glucopyranoside (2)

Yellowish gum; UV (MeOH) λ_{max} nm (log ε): 206 (4.37), 275 (3.54); ESI-MS (*m/z*): 349.1 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 3.34 (2H, d, *J* = 7.0 Hz, H-7), 3.40 (1H, dd, *J* = 2.0, 5.0 Hz), 3.48 (3H, m), 3.70 (1H, dd, *J* = 5.0, 12.5 Hz, H-6'a), 3.86 (3H, s, OMe), 3.88 (1H, d, *J* = 12.5 Hz, H-6'b), 4.86 (1H, d, *J* = 7.5 Hz, H-1'), 5.05 (1H, dd, *J* = 2.0, 12.0 Hz, H-9a), 5.07 (1H, dd, *J* = 2.0, 8.0 Hz, H-9b), 5.98 (1H, m, H-8), 6.74 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 6.84 (1H, d, *J* = 2.0 Hz, H-2), 7.10 (1H, d, *J* = 8.0 Hz, H-5); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 39.56 (C-7), 55.57 (3-OMe), 61.39 (C-6'), 70.23 (C-4'), 73.81 (C-2'), 76.70 (C-5'), 77.02 (C-3'), 101.98 (C-1'), 113.09 (C-5), 114.67 (C-9), 117.23 (C-2), 120.96 (C-6), 135.36 (C-8), 137.81 (C-1), 145.21 (C-4), 149.68 (C-3)

Apigenin-7-*O*-β-D-glucopyranoside (3)

Yellow powder; mp.: 236°C; UV (MeOH) λ_{max} nm (log ε): 264 (4.66), 364 (4.26); FAB-MS (*m/z*): 455.1 $[M+Na]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz): δ 3.18 (1H, t, *J* = 8.0 Hz, H-4"), 3.21-3.58 (4H, m, H-2, 3, 5, 6"b), 3.71 (1H, d, *J* = 10.0 Hz, H-6"a), 5.07 (1H, d, *J* = 7.5 Hz, H-1"), 6.44 (1H, d, *J* = 2.0 Hz, H-6), 6.83 (1H, d, *J* = 2.0 Hz, H-8), 6.87 (1H, s, H-3), 6.93 (2H, d, *J* = 9.5 Hz, H-3',5'), 7.95 (2H, d, *J* = 9.5 Hz, H-2',6'), 12.98 (br s, OH); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ 61.34 (C-6"), 70.30 (C-4"), 73.82 (C-2"), 77.16 (C-3"), 77.89 (C-5"), 95.57 (C-8), 100.24 (C-6), 100.65 (C-1"), 103.79 (C-3), 106.05 (C-10), 116.73 (C-3'), 116.73 (C-5'), 121.69 (C-1'), 129.31 (C-2'), 129.31 (C-6'), 157.65 (C-9), 161.82 (C-4'), 162.15 (C-5), 163.67 (C-7), 164.99 (C-2), 182.69 (C=O).

Divarin-3-*O*-β-glucopyranoside (4)

White powder; mp.: 128°C; $[\alpha]_D^{25}$: -32.3° (*c*=0.06, MeOH); UV (MeOH) λ_{max} nm (log ε): 208 (5.37), 280 (4.57); IR ν_{max} (neat) cm^{-1}: 3354 (OH), 1596 (C=C), 1172 (C-O); FAB-MS (*m/z*): 337.15 $[M+Na]^+$; HR FAB-MS: 337.12601 $[M+Na]^+$ (calcd. for 337.12633); ^{1}H-NMR (CD_3OD, 300 MHz): δ 0.92 (3H, t, *J* = 7.8 Hz, H-9), 1.60 (2H, sext. *J* = 7.8 Hz, H-8) 2.47 (2H, t, *J* = 7.8 Hz, H-7), 3.38-3.46 (4H, m, H-2', 3', 4', 5'), 3.70 (1H, dd, *J* = 4.5, 12.5 Hz, H-6'a), 3.88 (1H, dd, *J* = 4.5, 12.5 Hz, H-6'b), 4.84 (1H, d, *J* = 7.5 Hz, H-1'), 6.29 (1H, d, *J* = 1.5 Hz, H-6), 6.39 (1H, dd, *J* = 1.5, 1.8 Hz, H-4), 6.40 (1H, d, *J* = 1.5 Hz, H-2); ^{13}C-NMR (CD_3OD, 125 MHz): δ 12.89 (C-9), 24.24 (C-8), 37.97 (C-7), 61.39 (C-6'), 70.26 (C-4'), 73.77 (C-2'), 76.90 (C-5'), 77.95 (C-3'), 101.07 (C-1'), 101.30 (C-4), 108.07 (C-2), 109.48 (C-6),

145.01 (C-1), 158.08 (C-5), 158.93 (C-3).

(*E*)-Resveratrol-3-*O*-β-D-glucopyranoside (5)
Yellow gum; UV (MeOH) λ_{max} nm (log ε): 215 (4.49), 304 (4.42); FAB-MS(*m/z*): 413.3 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 3.35-3.51 (4H, m), 3.71 (1H, dd, *J* = 5.8, 11.7 Hz, H-6"b), 3.94 (1H, dd, *J* = 5.8, 11.7 Hz, H-6"a), 4.91 (1H, d, *J* = 6.8 Hz, H-1"), 6.46 (1H, dd, *J* = 2.0, 2.0 Hz, H-4), 6.63 (1H, s, H-6), 6.78 (1H, d, *J* = 9.0 Hz, H-5'), 6.78 (1H, d, *J* = 9.0 Hz, H-3'), 6.79 (1H, br s, H-2), 6.86 (1H, d, *J* = 16.5 Hz, H-α), 7.03 (1H, d, *J* = 16.5 Hz, H-β), 7.38 (1H, d, *J* = 9.0 Hz, H-6'), 7.38 (1H, d, *J* = 9.0 Hz, H-2'); ^{13}C-NMR ($CDCl_3$, 125 MHz): δ 61.44 (C-6"), 70.32 (C-4"), 73.79 (C-2"), 76.89 (C-3"), 77.08 (C-5"), 101.25 (C-1"), 102.95 (C-4), 105.86 (C-2), 107.20 (C-6), 115.33 (C-3'), 115.33 (C-5'), 125.51 (C-β), 127.74 (C-2'), 127.74 (C-6'), 128.82 (C-1'), 129.16 (C-α), 140.26 (C-1), 157.30 (C-4'), 158.41 (C-5), 159.30 (C-3).

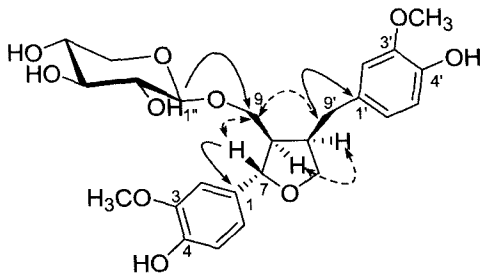
Lariciresinol-9-*O*-β-xylopyranoside (6)
Yellowish gum; $[\alpha]_D^{25}$: +21.3° (*c*=0.33, MeOH); UV (MeOH) λ_{max} nm (log ε): 206 (4.26), 228 (3.84), 281 (3.48); IR ν_{max} (neat)cm^{-1}: 3353 (OH), 1604 (C=C), 1035 (C-O); ESI-MS (*m/z*): 515.59 $[M+Na]^+$; HR-ESI-MS: 515.1888 $[M+Na]^+$ (calcd. for 515.1893); ^{1}H-NMR (CD_3OD, 600 MHz): δ 2.46 (1H, q, *J* = 6.6 Hz, H-8), 2.52 (1H, dd, *J* = 11.4, 13.8 Hz, H-7'a), 2.76 (1H, m, H-8'), 2.93 (1H, dd, *J* = 5.4, 13.8 Hz, H-7'b), 3.20 (1H, m, H-5"a), 3.21 (1H, dd, *J* =, 1.8, 8.4 Hz, H-2"), 3.34 (1H, m, H-3"), 3.49 (1H, dt, *J* = 5.4, 9.0 Hz, H-4"), 3.72 (1H, dd, *J* = 7.2, 10.8 Hz, H-9'a), 3.75 (1H, dd, *J* = 7.2, 9.6 Hz, H-9a), 3.83 (3H, s, H-OMe), 3.84 (3H, s, H-OMe), 3.87 (1H, dd, *J* = 5.4, 11.4 Hz, H-5"b), 3.97 (1H, dd, *J* = 6.6, 9.6 Hz, H-9b), 3.99 (1H, dd, *J* = 6.6, 7.8 Hz, H-9'b), 4.32 (1H, d, *J* = 7.8 Hz, H-1"), 4.86 (1H, d, *J* = 7.2 Hz, H-7), 6.65 (1H, dd, *J* = 1.8, 7.8 Hz, H-6"), 6.71 (1H, d, *J* = 7.8 Hz, H-5'), 6.75 (1H, d, *J* = 7.8 Hz, H-5), 6.78 (1H, d, *J* = 1.8 Hz, H-2'), 6.79 (1H, dd, *J* = 1.8, 7.8 Hz, H-6), 6.92 (1H, d, *J* = 1.8 Hz, H-2); ^{13}C-NMR (CD_3OD, 75 MHz): δ 32.70 (C-7'), 42.76 (C-8'), 50.49 (C-8), 55.29 (2C, OMe), 62.33 (C-9), 65.86 (C-5"), 70.07 (C-4"), 72.56 (C-9'), 73.85 (C-2"), 76.77 (C-3"), 83.18 (C-7), 104.24 (C-1"), 109.60 (C-2), 112.36 (C-2'), 114.83 (C-5), 115.01 (C-5'), 118.59 (C-6), 121.04 (C-6'), 132.45 (C-1'), 134.46 (C-1), 144.62 (C-4'), 145.85 (C-4), 147.81 (C-3'), 147.83 (C-3); ^{1}H,^{1}H-COSY (CD_3OD, 600 MHz), HSQC (CD_3OD, 600 MHz); HMBC and NOESY (CD_3OD, 600 MHz): Fig. 1.

RESULTS AND DISCUSSION

The structures of compounds **1**-**3** and **5** were identified as methyl ferulate (**1**) (Helm *et al.*, 1992), eugenyl-β-D-glucopyranoside (**2**) (Takeda *et al.*, 1998; Fujita *et al.*, 1992), apigenin-7-*O*-β-D-glucopyranoside (**3**) (Bannini *et al.*, 1992; Oyama *et al.*, 2004; Chaudhuri *et al.*, 1986) and (*E*)-resveratrol-3-*O*-β-D-glucopyranoside (**5**) (Kanchanapoom *et al.*, 2002; Hanawa *et al.*, 1992; Miyaichi *et al.*, 2006; Jayatilake *et al.*, 1993). These compounds were isolated for the first time from this plant source.

Compound **4** was obtained as a white powder. Its molecular formula $C_{15}H_{22}O_7$ was determined by HR-FABMS [*m/z* 337.12601 $[M+Na]^+$, (calcd. for 337.12633)]. The ^{1}H-NMR spectrum showed n-propyl signals at δ 0.92 (3H, t, *J* = 7.8 Hz), 1.60 (2H, sept, *J* = 7.8 Hz), and 2.47 (2H, t, *J* = 7.8 Hz) and aromatic signals at δ 6.29 (1H, d, *J* = 1.5 Hz), 6.39 (1H, dd, *J* = 1.5, 1.8 Hz), 6.40 (1H, d, *J* = 1.5 Hz). The ^{1}H- and ^{13}C-NMR signals were very similar to those of divarinol, which was isolated from *Protusnea malacea* (Chamy *et al.*, 1985), except for the glucose moiety (Nikas *et al.*, 2002). The ^{1}H-NMR signals at δ 3.38-3.46 (4H, m), 3.70 (1H, dd, *J* = 4.5, 12.5 Hz, H-6'a), 3.88 (1H, dd, *J* = 4.5, 12.5 Hz, H-6'b), 4.84 (1H, d, *J* = 7.5 Hz, H-1') and ^{13}C-NMR signals at δ 61.39 (C-6'), 70.26 (C-4'), 73.77 (C-2'), 76.90 (C-5'), 77.95 (C-3') and 101.07 (C-1') suggested it to be glucose (Saracoglu *et al.*, 2004). The coupling constant (*J* = 7.5 Hz) of anomeric signal of glucose at 4.84 (d, *J* = 7.5 Hz) suggested that it was β-oriented. In HMBC spectrum, the correlation of H-1' (δ 4.84) to C-3 (δ 158.93) indicated the connection of glucose at C-3. Thus, the structure of **4** was determined to be divarin-3-*O*-β-glucopyranoside, which was isolated for the first time from natural sources.

Compound **6** was obtained as a yellowish gum ($[\alpha]_D^{25}$ +21.3°, (*c*=0.33, MeOH)). The molecular formula of $C_{25}H_{32}O_{10}$ was determined by HR-ESIMS (*m/z* 515.1888 $[M+Na]^+$, calcd. for 515.1893). The ^{1}H-NMR spectrum of **6** showed 21 protons, which consisted of two methoxyl protons (δ 3.83, 3.84), three methine protons (δ 2.46, 2.76, 4.86), three sets of methylene protons (δ 2.52/2.93, 3.72/3.99 and 3.75/3.97), and six aromatic protons (δ 6.65, 6.71, 6.75, 6.79, 6.79 and 6.92), in addition to six protons of sugar at δ 3.20, 3.21, 3.34, 3.49, 3.87 and 4.32. The ^{1}H- and ^{13}C-NMR spectral data were very similar to those of lariciresinol 9-O-β-D-glucopyranoside, except for the sugar



HMBC : ^{1}H → ^{13}C
NOESY : ^{1}H ‹ › ^{1}H

Fig. 1. Key HMBC and NOESY correlations of **6**

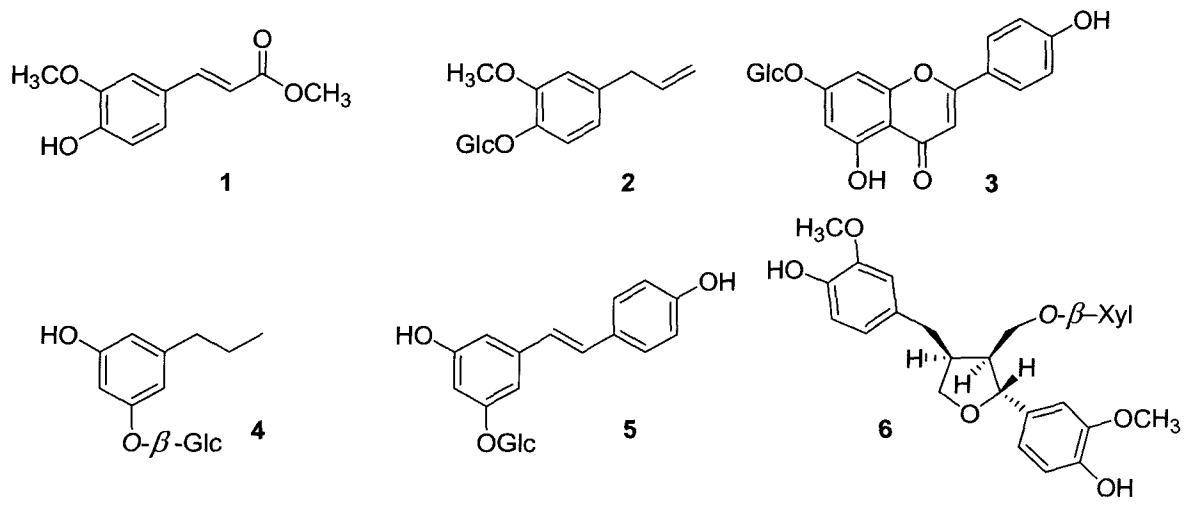


Fig. 2. The structures of the isolated compounds (**1-6**) from *H. japonicus*

moiety, which was isolated from *Arum italicum* (Erdemoglu *et al.*, 2004; Roy *et al.*, 2002; Kitajima *et al.*, 2003; Della *et al.*, 1993). These observations indicated that the glucose moiety was replaced with the another sugar group. The ^{1}H-NMR signals at δ 3.20 (m), 3.21 (dd, *J* = 1.8, 8.4 Hz), 3.34 (m), 3.49 (dt, *J* = 5.4, 9.0 Hz), 3.87 (dd, *J* = 5.4, 11.4 Hz), 4.32 (d, *J* = 7.8 Hz) and ^{13}C-NMR signals at δ 65.86 (C-5"), 70.09 (C-4"), 73.85 (C-2"), 76.77 (C-3") and 104.24 (C-1") were assigned to xylose (Drew *et al.*, 1998; Wu *et al.*, 2006). The coupling constant of the anomeric signal of sugar at δ 4.32 (d, *J* = 7.8 Hz) suggested it to be in the β-form. The position of xylose was confirmed by HMBC experiment (Fig. 1), which showed the correlations of H-1" to C-9, H-9' to C-7, H-7' to C-8, H-7 to C-1 and H-7' to C-1'. The stereochemistry of **6** was assumed to be same to that of lariciresinol 9-*O*-β-D-glucopyranoside on the basis of the ^{1}H-NMR chemical shifts and the coupling constant values, which were reconfirmed by NOESY experiment (Fig. 1). Thus, the structure of compound **6** was determined to be lariciresinol 9-*O*-β-xylopyranoside, which was isolated for the first time from natural sources.

The isolated compounds were tested for cytotoxicity against four human tumor cells using the *in vitro* SRB assay. Compound **1** exhibited moderate cytotoxic activity against SK-OV-3 and HCT15 with ED_{50} values of 8.84 and 8.62 μM, respectively, and the compound **3** was also moderately cytotoxic against SK-OV-3 with an ED_{50} value of 8.79 μM. The other compounds however showed little cytotoxic activity against the tested cancer cell lines (ED_{50} > 30 μM).

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