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Cytotoxic Compounds from the Roots of *Pulsatilla koreana*

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Abstract – Seven compounds including hederagenin 3-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl) (**1**), 3*β*-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid (**2**), caffeic acid methyl ester (**3**), ferulic acid (**4**), orebiusin A (**5**), latifonicinin C (**6**) and 5-(hydroxymethyl)-2-furfuraldehyde (**7**) were isolated from the ethyl acetate fraction of the roots of *Pulsatilla koreana*. Their chemical structures were established based on physicochemical and spectroscopic data analyses. All isolates were investigated for their cytotoxic activity against cancer cell lines. Among them, compound **1** showed inhibitory activity against A549, COLO 205, and L1210 cancer cell lines with IC_{50} values of 15.8, 36.5, and 22.8 μg/mL, respectively.
Keywords – *Pulsatilla koreana*, Ranunculaceae, Cytotoxic activity, Hederagenin glucoside

Introduction

Pulsatilla koreana N. belongs to the family Ranunculaceae and is an endemic species in Korea. The roots of this species have been widely used in traditional medicine for the treatment of several diseases, such as particular malaria and amoebic dysentery (Bae, 1999). This plant has been investigated extensively, ranunculin, anemonin, protoanemonin and lupane-type triterpenes saponins have been reported as constituents (Zhang *et al.*, 1990; Ye *et al.*, 1995; Ye *et al.*, 1996) which posses antimicrobial (Lee *et al.*, 2001) and cytotoxic activity (Mimaki *et al.*, 1999; Bang *et al.*, 2005a, 2005b). In our study, extraction and fractionation of the roots of *P. koreana* resulted in the isolation of seven compounds (**1** - **7**). This paper describes the isolation, structural elucidation of the isolated compounds and their cytotoxic effects against various cancer cell lines.

Experimental

General experimental procedures – Column chromatography was carried out on Kieselgel 60; particle size 0.0063 - 0.200 mm and 0.040 - 0.063 mm (Merck Germany), RP-18; particle size 40 - 63 mm (Merck. Germany). TLC was performed on pre-coated Kieselgel 60 F_{254} (Merck Germany), detection was achieved by spraying with 10% H_2SO_4 followed by heating. Solvent used special and first grade without purification. Optical rotation was measured with a JASCO DIP-370 digital polarimeter (JASCO, Japan). The NMR spectrum was measured with a Varian Unity INOVA-400 spectrometer (Varian, USA). Chemical shifts of NMR spectra are given on a δ (ppm) scale with TMS (Tetramethylsilane) as an internal standard. HPLC was carried out using a YMC-Pac ODS-A (250 × 20 mm I.D., YMC, Japan) column with a WATERS 515 HPLC pump and WATER 486 Tunable Absorbance detector. UV spectrum was obtained on a Thermo 9423AQA2200E (Thermo, England).

Plant material – The dried roots of *P. koreana* were purchased in May 2008 from a market 'Yak-ryoung-si' in Daegu, Korea. Botanical identification was performed by Prof. Byung-Sun Min, and the voucher specimen CUD-1384 was deposited at the herbarium of the college of Pharmacy, Catholic University of Daegu, Korea.

Extraction and Isolation – The dried roots of *P. koreana* (5 kg) were extracted three times with MeOH at room temperature for seven days. The MeOH solution was concentrated under reduced pressure to give a residue (737.2 g). The residue was suspended in water (3 L) and extracted with EtOAc (3 L × 3 times), and *n*-BuOH (3 L × 3 times), successively. The resulting fractions were concentrated *in vacuo* to give the EtOAc-soluble fraction (108.8 g), and BuOH-soluble fraction (534.2 g). The EtOAc-soluble fraction (108.8 g) was chromatographed on a silica gel column eluting with a gradient of *n*-hexane-acetone (9 : 1 → 2 : 1) and $CHCl_3$-MeOH (9 : 1 → 0 : 1)

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to give eleven fractions (Fr.1 to Fr.11) according to their TLC profiles. Re-chromatography of fraction 5 (2.77 g) on a silica gel column eluting with a gradient of *n*-hexane–acetone (9 :1 → 2 : 1) and $CHCl_3$-MeOH (10 : 1 → 0 : 1) afforded nine subfractions (Fr.5-1 ~ 5-9). The subfraction 5-5 (112.0 mg) was recrystallized to give **4** (24.0 mg). Fraction 7 (1.5 g) was subjected to a silica gel column eluting with a gradient of $CHCl_3$-acetone (20 : 1 → 1 : 1) to afford six subfractions (Fr.7-1 ~ 7-6). Further purification of fraction Fr.7-1 (110 mg) using silica gel column with a gradient of $CHCl_3$-MeOH (10 : 1 → 0 : 1) resulted in the isolation of **3** (21.0 mg). Fraction Fr.7-2 (120.0 mg) was purified by silica gel column using a gradient of $CHCl_3$–acetone (10 : 1 → 0 : 1) resulted in the isolation of **5** (23.0 mg) and **6** (20.6 mg). The fraction 8 (1.90 g) was chromatographed over a RP-18 column chromatography eluting with a gradient of MeOH-H_2O (1 : 3 → 1 : 0) to afford seven subfractions (Fr.8-1 ~ 8-7). The subfraction 8-1 (546.0 mg) was subjected to silica gel column chromatography eluting with a gradient of $CHCl_3$-MeOH (20 : 1 → 2 : 1) to afford **1** (36.0 mg) and **2** (64.0 mg). Furthermore, subfraction 8-2 (95.0 mg) was subjected to silica gel column chromatography eluting with a gradient of $CHCl_3$-MeOH (10 : 1 → 0 : 1) to afford **7** (28.3 mg).

Hederagenin 3-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl) (1) – white amorphous powder; mp. 264 - 265 °C; $[\alpha]_D^{25}$ = –16.9 (*c* 0.13, MeOH); FABMS *m/z*: 773 $[M + Na]^-$, HRFABMS (positive mode) *m/z*: 773.9598 (calcd for $C_{41}H_{66}O_{12}Na$, 773.9576); ^{1}H NMR (400 MHz, CD_3OD) *δ*: 1.15 (3H, s, Me-27), 0.94 (3H, s, Me-24), 0.91 (3H, s, Me-26), 0.88 (3H, s, Me-30), 0.78 (3H, s, Me-25), 0.67 (3H, s, Me-29), 3.59 (1H, dd, *J* = 3.6, 18.0 Hz, H-3), 3.79 (1H, m, H-24a) and 3.47 (1H, br d, *J* = 11.6 Hz, H-24b), 5.21 (1H, brs, H-12), 5.13 (1H, brs, rha-1''), 4.52 (1H, d, *J* = 5.6 Hz, ara-1'), 1.21 (3H, d, *J* = 6.0 Hz, rha-6''); ^{13}C NMR (100 MHz, CD_3OD) *δ*: 39.7 (C-1), 26.6 (C-2), 82.3 (C-3), 37.7 (C-4), 55.3 (C-5), 18.9 (C-6), 33.7 (C-7), 42.8 (C-8), 48.5 (C-9), 37.7 (C-10), 24.2 (C-11), 123.7 (C-12), 145.4 (C-13), 43.1 (C-14), 28.9 (C-15), 24.6 (C-16), 47.3 (C-17), 40.6 (C-18), 47.7 (C-19), 31.7 (C-20), 35.0 (C-21), 33.5 (C-22), 64.7 (C-23), 13.8 (C-24), 16.5 (C-25), 17.9 (C-26), 26.6 (C-27), 182.0 (C-28), 33.9 (C-29), 24.1 (C-30), 104.4 (ara C-1'), 76.7 (ara C-2'), 72.1 (ara C-3'), 69.2 (ara C-4'), 64.9 (ara C-5'), 102.0 (rha C-1''), 73.8 (rha C-2''), 72.1 (rha C-3''), 70.2 (rha C-4''), 74.0 (rha C-5''), 18.1 (rha C-6'').

3β-[(*O*-α-L-Rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl) oxy]olean-12-en-28-oic acid (2) – white powder; mp. 260 - 262 °C; $[\alpha]_D^{25}$ = –6.0 (*c* 0.1, MeOH); UV ($CHCl_3$) λ_{max}^{EtOH} nm: 228.3, 280.5; IR (KBr) ν_{max} cm^{-1}: 3400 (OH), 2930, 1680 (C = O), 1640 (C = C), 1050 (C-O-C); HRESIMS *m/z* 779.4618 $[M + COOH]^-$ (calcd for $C_{41}H_{66}O_{11}$ + COOH, 779.4606); ^{1}H NMR (400 MHz, C_5D_5N) *δ*: 1.32 (3H, s, Me-27), 1.20 (3H, s, Me-23), 1.09 (3H, s, Me-24), 1.03 (3H, s, Me-26), 1.00 (3H, s, Me-30), 0.98 (3H, s, Me-25), 0.86 (3H, s, Me-29), 3.28 (1H, dd, *J* = 3.6, 18.0 Hz, H-3), 5.49 (1H, brs, H-12), 6.16 (1H, brs, rha-1''), 4.91 (1H, d, *J* = 5.6 Hz, ara-1'), 1.64 (3H, d, *J* = 6.0 Hz, rha-6''). ^{13}C NMR (100 MHz, C_5D_5N) *δ*: 39.3 (C-1), 27.0 (C-2), 89.2 (C-3), 40.2 (C-4), 56.4 (C-5), 19.0 (C-6), 33.7 (C-7), 42.0 (C-8), 48.5 (C-9), 37.5 (C-10), 24.2 (C-11), 123.0 (C-12), 145.3 (C-13), 42.6 (C-14), 28.8 (C-15), 24.3 (C-16), 47.1 (C-17), 42.5 (C-18), 46.9 (C-19), 31.5 (C-20), 34.7 (C-21), 30.5 (C-22), 28.6 (C-23), 17.5 (C-24), 16.0 (C-25), 17.9 (C-26), 26.7 (C-27), 180.7 (C-28), 33.8 (C-29), 24.3 (C-30), 105.4 (ara C-1'), 76.4 (ara C-2'), 74.5 (ara C-3'), 73.1 (ara C-4'), 65.3 (ara C-5'), 102.3 (rha C-1''), 72.9 (rha C-2''), 69.3 (rha C-3''), 74.5 (rha C-4''), 74.4 (rha C-5''), 19.2 (rha C-6'').

Caffeic acid methyl ester (3) – pale yellow needles; mp. 150 - 152 °C; IR (KBr) ν_{max} cm^{-1}: 3478 (OH), 1676 (ester C = O), 1607, 1537, 1439 (aromatic C = C), 1308, 1281, 1242, 1190, 972 (*trans* C=C); EIMS *m/z* 194 $[M]^+$; ^{1}H NMR (CD_3OD, 400 MHz) *δ*: 7.05 (1H, s, H-2), 6.79 (1H, d, *J* = 8.0 Hz, H-5), 6.95 (1H, d, *J* = 8.0 Hz, H-6), 7.56 (1H, d, *J* = 16.0 Hz, H-7), 6.27 (1H, d, *J* = 16.0 Hz, H-8), 3.75 (3H, s, 9-OCH_3); ^{13}C NMR (100 MHz, CD_3OD) *δ*: 127.8 (C-1), 115.2 (C-2), 149.7 (C-3), 146.9 (C-4), 116.6 (C-5), 123.0 (C-6), 147.0 (C-7), 114.9 (C-8), 169.9 (C-9), 52.0 (9-O<u>C</u>H_3).

Ferulic acid (4) – pale yellow needles; IR (KBr) ν_{max} cm^{-1}: 1622, 1592, 1516, 1465, 1325, 1033; UV (MeOH) λ_{max} : 311, 287 nm; ESIMS *m/z*: 195 $[M + H]^+$; ^{1}H NMR (CD_3OD, 400 MHz) *δ*: 7.13 (1H, s, H-2), 6.77 (1H, d, *J* = 8.0 Hz, H-5), 7.02 (1H, d, *J* = 8.0 Hz, H-6), 7.54 (1H, d, *J* = 16.0 Hz, H-7), 6.27 (1H, d, *J* = 16.0 Hz, H-8), 3.86 (3H, s, 3-OCH_3); ^{13}C NMR (100 MHz, CD_3OD) *δ*: 127.9 (C-1), 111.7 (C-2), 150.6 (C-3), 149.4 (C-4), 116.0 (C-5), 124.1 (C-6), 147.0 (C-7), 116.5 (C-8), 171.1 (C-9), 56.5 (3-O<u>C</u>H_3).

Orebiusin A (5) – brown amorphous powder; IR (KBr) ν_{max} cm^{-1}: 3464 (OH), 1679 (ester C = O), 1605, 1537, 1432 (aromatic C = C); EIMS *m/z* 212 $[M]^+$; ^{1}H NMR (CD_3OD, 400 MHz) *δ*: 6.65 (1H, d, *J* = 2.0 Hz, H-2), 6.66 (1H, d, *J* = 8.0 Hz, H-5), 6.51 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), 2.76 (1H, dd, *J* = 7.6, 14.0 Hz, H-7a), 2.96 (1H, dd, *J* = 5.2, 14.0 Hz, H-7b), 4.27 (1H, dd, *J* = 5.2, 7.6 Hz, H-8), 3.67 (3H, s, 9-OCH_3); ^{13}C NMR (100 MHz, CD_3OD) *δ*: 129.9 (C-1), 116.3 (C-2), 146.1 (C-3), 145.1

(C-4), 117.6 (C-5), 121.9 (C-6), 41.2 (C-7), 73.5 (C-8), 175.9 (C-9), 52.4 (9-O<u>C</u>H_3).

Latifonicinin C (6) – colorless amorphous powder; IR (KBr) ν_{max} cm^{-1}: 3468 (OH), 1673 (ester C = O), 1602, 1536, 1435 (aromatic C=C); EIMS *m/z*: 196 $[M]^+$; ^1H NMR (CD_3OD, 400 MHz) δ: 7.04 (1H, dd, J = 2.0, 8.4 Hz, H-2), 6.71 (1H, dd, J = 2.0, 8.4 Hz, H-3), 6.71 (1H, dd, J = 2.0, 8.4 Hz, H-5), 7.04 (1H, dd, J = 2.0, 8.4 Hz, H-6), 2.84 (1H, dd, J = 7.6, 14.0 Hz, H-7a), 2.95 (1H, dd, J = 5.2, 14.0 Hz, H-7b), 4.31 (1H, dd, J = 5.2, 7.6 Hz, H-8), 3.68 (3H, s, 9-OC<u>H</u>$_3$); ^{13}C NMR (100 MHz, CD_3OD) δ: 129.3 (C-1), 131.6 (C-2), 116.1 (C-3), 157.3 (C-4), 116.1 (C-5), 131.6 (C-6), 41.0 (C-7), 73.5 (C-8), 175.9 (C-9), 52.4 (9-O<u>C</u>H_3).

5-(Hydroxymethyl)-2-furfuraldehyde (7) – yellow oil; UV (EtOH) λ_{max} 278 nm; IR (KBr) ν_{max} cm^{-1}: 3390, 2932, 2871, 1680 (α,β-unsaturated CO), 1023 (C-O); EIMS *m/z*: 126 $[M]^+$; ^1H NMR (400 MHz, CD_3OD) δ: 4.64 (2H, s, H-6), 6.61 (1H, d, J = 3.6 Hz, H-4), 7.41 (1H, d, J = 3.6 Hz, H-3), 9.55 (1H, s, H-1); ^{13}C NMR (100 MHz, CD_3OD) δ: 179.5 (C-1), 153.9 (C-2), 125.0 (C-3), 111.0 (C-4), 163.3 (C-5), 57.8 (C-6).

General Acid Hydrolysis of Compounds 1 and 2 – A solution of each compound (15 mg) in 0.4 M HCl (dioxane-H_2O, 1 : 1, 10 mL) was heated to 90 °C for 2 h under an Ar atmosphere. After cooling, the reaction mixture was extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ extract was evaporated and the residue was purified on a Si gel using a discontinuous gradient of $CHCl_3$-MeOH (99 : 1 to 1 : 1) to give an aglycon (compound **1**: hederagenin (5.6 mg) and compound **2**: oleanolic acid (5.0 mg) which were identified by comparison of the spectroscopic data with literature values). Each of the H_2O layers was neutralized with Ag_2CO_3 and analyzed by TLC (Kieselgel, eluting solvent *n*-BuOH-AcOH-H_2O, 4 : 1 : 1, sprayed with 10% H_2SO_4 and heated) to reveal the presence of rhamnose (R_f = 0.14) and arabinose (R_f = 0.20) for compounds **1** and **2**. All the R_f values were coincident with those of authentic samples.

Cytotoxic assay – The cancer cell lines (MCF-7 and LLC) were maintained in RPMI 1640 that included L-glutamine with 10% FBS and 2% penicillin–streptomycin. Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified MTT assay (Van *et al*., 2009). Viable cells were seeded in the growth medium (100 μL) into 96 well microtiter plates (1×10^4 cells per well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 100 μg/mL by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 24 h, 10 μL of the test sample was added to each well. The same volume of medium with < 0.5% DMSO was added to the control wells. Removing medium after 48 h of the test samples treatment, MTT 10 μL (5 mg/mL) was added to the each well. After 4 h in incubator, the plates were removed, and the resulting formazan crystals were dissolved with DMSO 100 μL. The optical density (O.D.) was measured at 570 nm. The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.

Results and discussion

The dried roots of *P. koreana* were successively partitioned with EtOAc and *n*-BuOH solvents to give EtOAc and *n*-BuOH-soluble fraction, respectively. The subsequent isolation of the EtOAc-soluble fraction resulted in the isolation of seven compounds (**1** - **7**) (Fig. 1).

Compound **1** was obtained as white amorphous powder with a negative optical rotation –16.9. The FABMS of **1** displayed a $[M + Na]^+$ peak at *m/z* 773, consistent with a molecular formula of $C_{41}H_{66}O_{12}$, which was supported by the ^{13}C NMR and DEPT spectroscopic data. The ^1H and ^{13}C NMR spectra of **1** clearly showed the presence of a triterpene bearing an olefinic group and carbonyl group. The DEPT spectra of **1** revealed signals for seven methyls, twelve methylenes, fourteen methines, and eight quaternary carbons. The ^1H NMR spectra of **1** exhibited signals due to seven tertiary methyl groups at δ 1.21 (3H, d, J = 6.0 Hz, H-6" of rhamnose), 1.15, 0.94, 0.91, 0.88, 0.78, and 0.67, and an olefinic proton at δ 5.21 (1H, br, s, H-12). Also, the ^1H NMR spectrum of **1** indicated the presence of two anomeric proton signals at δ 5.13 (1H, br, s, H-1" of rhamnose) and 4.52 (1H, d, J = 5.6 Hz, H-1' of arabinose) which were correlated with the ^{13}C NMR signals for anomeric carbons at δ 104.4 (C-1' of arabinose), and 102.0 (C-1" of rhamnose) in the ^1H-^1H COSY and HMQC spectra. The corresponding six tertiary methyl carbons at δ 24.1 (C-30), 18.1 (C-6" of rhamnose), 16.5 (C-25), 17.9 (C-26), 26.6 (C- 27), and 13.8 (C-24) and two olefinic carbons at δ 145.4 (C-13) and 123.7 (C-12) appeared in the ^{13}C NMR spectum. In the comparison of the ^{13}C NMR spectrum of **1** with oleanolic acid 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (Mimaki *et al.*, 1999), the chemical shifts of the carbohydrate moieties were very similar, except that the chemical shift

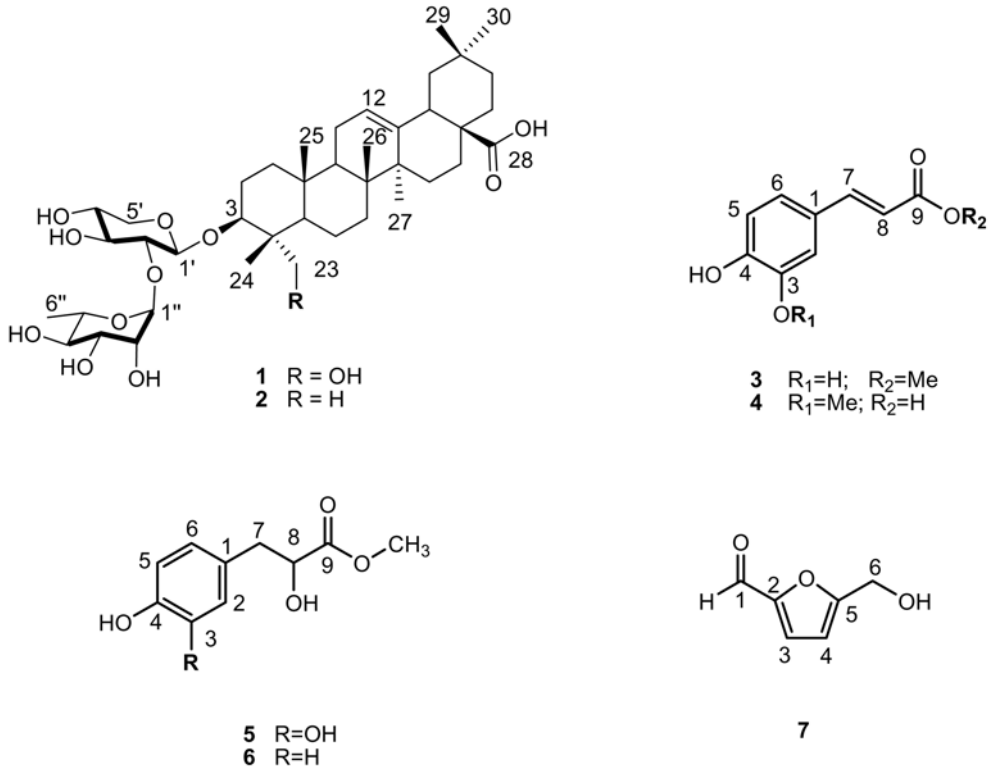

Fig. 1. Chemical structures of isolated compounds (**1 - 7**).

of C-23 of the aglycon was displaced downfield 36.1 ppm, due to a hydroxyl group, to δ 64.7, suggesting that the aglycon of **1** is hederagenin (Bang *et al.,* 2005a, 2005b). By acid hydrolysis of **1**, hederagenin could be confirmed as the aglycon (Ye *et al.,* 1995). In addition, L-arabinose, and L-rhamnose were observed as the sugar moieties by TLC and HPLC. Thus, the structure of **1** was elucidated as hederagenin 3-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl) (Bang *et al.,* 2005a, 2005b).

The ESIMS of **2** displayed a $[M + COOH]^+$ peak at *m/z* 779, consistent with a molecular formula of $C_{41}H_{66}O_{11}$. Analysis of the ^{13}C NMR spectrum of **2** revealed that the diglycoside structure was identical to that of **1**, but differed slightly from **1** in terms of the aglycon structure (Ikuta *et al.*, 1989). Acid hydrolysis of **2** liberated the known triterpenoid, oleanolic acid (Kiru *et al.,* 1985) as well as L-arabinose, and L-rhamnose. Thus, **2** was assigned as 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]olean-12-en-28-oic acid (Ikuta *et al.*, 1989).

Compound **3** was isolated as pale yellow needles with the molecular formula $C_{10}H_{10}O_4$, as determined by the EIMS ($[M]^+$ *m/z* 194). The IR spectrum of compound **3** suggested the presence of OH functional group at 3478, 1676 (ester C = O), 1607, 1537, 1439 (aromatic C = C), 1308, 1281, 1242, 1190, 972 (*trans* C = C) cm^{-1}. The ^1H NMR spectra of **3** showed three aromatic protons at δ 7.05 (1H, s, H-2), 6.79 (1H, d, J = 8.0 Hz, H-5), 6.95 (1H, d, J = 8.0 Hz, H-6), two olefinic protons at δ 7.56 (1H, d, J = 16.0 Hz, H-7), 6.27 (1H, d, J = 16.0 Hz, H-8), and one methoxy δ 3.75 (3H, s, 9-OCH_3). The ^{13}C NMR spectrum of **3** showed ten carbon signals including six carbon signals of benzen rings at δ 127.8 (C-1), 115.2 (C-2), 149.7 (C-3), 146.9 (C-4), 116.6 (C-5), 123.0 (C-6), two olefinic carbons at δ 147.0 (C-7), 114.9 (C-8), one methoxy group at δ 52.0 (9-O<u>C</u>H_3), and one carbonyl group at δ 169.9 (C-9). The structure of compound **3** was determined as caffeic acid methyl ester by comparison its physicochemical and spectral data with those of literature (Pyo *et al.,* 2002).

Compound **4** was isolated as pale yellow needles with the molecular formula $C_{10}H_{10}O_4$, as determined by the ESIMS ($[M + H]^+$ *m/z* 195). The IR spectrum of compound **4** suggested the presence of ester functional (C = O) group at 1622, 1592, 1516 (aromatic C = C), 1325, 1033 cm^{-1}. Its UV spectrum showed absorption maxima at 311, 287 nm. The ^1H NMR spectra of **4** showed three aromatic protons at δ 7.13 (1H, s, H-2), 6.77 (1H, d, J = 8.0 Hz, H-5), 7.02 (1H, d, J = 8.0 Hz, H-6), two olefinic protons at δ 7.54 (1H, d, J = 16.0 Hz, H-7), 6.27 (1H, d, J = 16.0 Hz, H-8) and one methoxy at δ

3.86 (3H, s, 3-OCH_3). The ^{13}C NMR spectrum of **4** showed ten carbon signals including six carbon signals of benzen rings at δ 127.9 (C-1), 111.7 (C-2), 150.6 (C-3), 149.4 (C-4), 116.0 (C-5), 124.1 (C-6), two olefinic carbons at δ 147.0 (C-7), 116.5 (C-8), one methoxy group at δ 56.5 (3-O$\underline{C}H_3$), and one carbonyl group at δ 171.1 (C-9). The structure of compound **4** was determined as ferulic acid by comparison its physicochemical and spectral data with those of literature (Abd-Alla *et al.,* 2009).

Compound **5** was isolated as brown amorphous powder with the molecular formula $C_{10}H_{12}O_5$, as determined by the EIMS ($[M]^+$ *m/z* 212). The IR spectrum of compound **5** suggested the presence of OH functional group at 3464, 1679 (ester C = O), 1605, 1537, 1432 (aromatic C = C) cm^{-1}. The ^1H NMR spectra of **5** showed three aromatic protons at δ 6.65 (1H, d, *J* = 2.0 Hz, H-2), 6.66 (1H, d, *J* = 8.0 Hz, H-5), 6.51 (1H, dd, *J* = 2.0, 8.0 Hz, H-6), one methylene proton at δ 2.76 (1H, dd, *J* = 7.6, 14.0 Hz, H-7a), 2.96 (1H, dd, *J* = 5.2, 14.0 Hz, H-7b), one oxygenated methine proton at δ 4.27 (1H, dd, *J* = 5.2, 7.6 Hz, H-8), and one methoxy at δ 3.67 (3H, s, 9-OCH_3). The ^{13}C NMR spectrum of **5** showed ten carbon signals including six carbon signals of benzen rings, one methylene carbon, one oxygenated methine carbon, one methoxy, and one carbonyl group. The structure of compound **5** was determined as orebiusin A by comparison its physicochemical and spectral data with those of literature (Sun *et al.,* 1996).

Compound **6** was isolated as colorless amorphous powder with the molecular formula $C_{10}H_{12}O_4$, as determined by the EIMS ($[M]^+$ *m/z* 196). The IR spectrum of compound 6 suggested the presence of OH functional group at 3468, 1673 (ester C = O), 1602, 1536, 1435 (aromatic C = C) cm^{-1}. The ^1H- NMR spectra of 6 showed four aromatic protons at δ 7.04 (2H, dd, *J* = 2.0, 8.4 Hz, H-2,6), 6.71 (1H, dd, *J* = 2.0, 8.4 Hz, H-3,5), one methylene proton at δ 2.84 (1H, dd, *J* = 7.6, 14.0 Hz, H-7a), 2.95 (1H, dd, *J* = 5.2, 14.0 Hz, H-7b), one oxygenated methine proton at δ 4.31 (1H, dd, *J* = 5.2, 7.6 Hz, H-8), and one methoxy at δ 3.68 (3H, s, 9-OCH_3). The ^{13}C NMR spectrum of 6 showed ten carbon signals including six carbon signals of benzen rings at δ 129.3 (C-1), 131.6 (C-2), 116.1 (C-3), 157.3 (C-4), 116.1 (C-5), 131.6 (C-6), one methylene carbon at δ 41.0 (C-7), one oxygenated methine carbon at δ 73.5 (C-8), one methoxy group at δ 52.4 (9-O$\underline{C}H_3$), and one carbonyl group at δ 175.9 (C-9). Thus, the structure of compound **6** was determined as latifonicinin C by comparison its physicochemical and spectral data with those of literature (Siddiqui *et al.,* 2006).

Table 1. Inhibitory effects of isolated compounds **1 - 7** on the cytotoxic activity

Compounds	IC_{50} (μg/mL)		
	A549	COLO-205	L1210
1	15.8	36.5	22.8
2	22.7	> 100	13.7
3	>100	> 100	31.5
4	> 100	> 100	> 100
5	> 100	> 100	68.7
6	> 100	> 100	> 100
7	58.8	> 100	> 100
Adriamycine[a]	0.41	0.87	0.23

[a] Used as a positive control

Compound **7** was isolated as yellow oil with the molecular formula $C_6H_6O_3$, as determined by the EIMS ($[M]^+$ *m/z* 126). The IR spectrum of compound **7** suggested the presence of OH functional group at 3468, and 1680 (α,β-unsaturated aldehyde) cm^{-1}. The ^1H NMR spectra of **7** showed one aldehyde proton at δ 9.55 (1H, s, H-1), two methine protons of furan skeleton at δ 6.61 (1H, d, *J* = 3.6 Hz, H-4), 7.41 (1H, d, *J* = 3.6 Hz, H-3), and one methylenehydroxy at δ 4.64 (2H, s, H-6). The ^{13}C NMR spectrum of **7** showed six carbon signals including one aldehyde carbon signal at δ 179.5 (C-1), four carbon signals of furan skeleton at δ 153.9 (C-2), 125.0 (C-3), 111.0 (C-4), 163.3 (C-5), and one methylenehydroxy carbon signal at δ 57.8 (C-6). Thus, the structure of compound **7** was determined as 5-(hydroxymethyl)-2-furfuraldehyde by comparison its physicochemical and spectral data with those of literature (Mineo *et al.,* 1993).

Seven isolated compounds (**1-7**) were tested for their cytotoxic activity against A549, COLO-205 and L1210 cancer cell lines *in vitro* assay by the method as described in the Material and Methods section. The results are summarized in Table 1. Among them, compound **1** showed inhibition against three cancer cell lines, A549, COLO 205, and L1210 with IC_{50} values of 15.8, 36.5, and 22.8 μg/mL, respectively. Compound **2** also showed cytotoxic activity against A549 and L1210 with IC_{50} values of 22.7 and 13.7 μg/mL, respectively. Compounds **3** and **6** displayed activity against L1210 cells with IC_{50} values as 31.5 and 68.7 μg/mL, respectively. Compound **7** showed weak inhibitory effect with IC_{50} value as 58.8 μg/mL, meanwhile, the others are inactive.

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