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Triterpenes from *Perilla frutescens* var. *acuta* and Their Cytotoxic Activity†

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Abstract – Nine triterpenes were isolated from the petroleum ether and MeOH extract of *Perilla frutescens* var. *acuta* leaves. Their structures were determined to be arjunic acid (**1**), maslinic acid (**2**), oleanolic acid (**3**), euscaphic acid (**4**), tormentic acid (**5**), 3-*O*-*trans*-*p*-coumaroyltormentic acid (**6**), 28-formyloxy-3β-hydroxy-urs-12-ene (**7**), ursolic acid (**8**), and corosolic acid (**9**) by spectroscopic methods. The compounds **1**, **2**, **4**, **6**, and **7** were isolated for the first time from this plant and the Genus *Labiatae*. The isolated compounds (**1**-**9**) were tested for cytotoxicity against four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15) *in vitro* using a Sulforhodamin B bioassay.
Keywords – *Perilla frutescens* var. *acuta*, Labiatae, triterpene, cytotoxicity

Introduction

The leaves of *Perilla frutescens* var. *acuta* (Labiatae), known as 'Ja So Yeop', have been used as a food, and also as a medicinal source for the treatment of various disorders such as sore throat, dyspersia, and diabetes in Korea.[1,2] There have been several reports on the isolation of triterpenes and phenoilc derivatives.[3-5] Anti-allergic and anti-bacterial effects of an EtOH extract of *P. frutescens* var. *acuta* have also been reported.[6,7] In the course of our continuing search for biologically active compounds from natural Korean medicinal sources, we investigated the petroleum ether and methanol extract of *P. frutescens* var. *acuta* leaves. Column chromatographic purification of this plant led to the isolation of nine triterpenes (**1 - 9**) (Fig. 1). Their structures were determined by spectroscopic methods such as 1D-NMR and MS. The isolated compounds **1-9** were evaluated for their cytotoxic activities against the four human tumor cell lines, A549, SK-OV-3, SK-MEL-2, and HCT15 by using the SRB bioassay. We describe here the isolation, structures, and biological activities of these triterpenes.

Experimental

General experimental procedures – Silica gel F_{254} plates (Merck) and RP-C_{18} F_{254s} plates (Merck) were used for TLC. Spots on TLC were detected by UV light and/or by heating after spraying with 10% H_2SO_4 in EtOH (v/v). Si gel 60 (Merck, 70 - 230 and 230 - 400 mesh) and RP-C_{18} silica gel (Merck, 230 - 400 mesh) was used for open column chromatography. Low pressure liquid chromatography was carried out over a Merck Lichroprep Lobar® - A Si 60 (240 × 10 mm) columns with a FMI QSY-0 pump (ISCO). Semi-preparative HPLC used a Gilson 306 pump with Shodex refractive index detector. NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). FAB MS spectra were obtained on a JEOL JMS 700 mass spectrometer.

Plant materials – The leaves of *P. frutescens* var. *acuta* (25 kg) were collected from Namwon in Jeollanam-do, Korea, in April 2012. The plants were authenticated by one of the authors (K.R.Lee). A voucher specimen (SKKU-NPL-1207) of the plant was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation – The dried leaves of *P. frutescens* var. *acuta* (25 kg) were extracted with petroleum ether and evaporated under reduced pressure to give residues (264 g). The petroleum ether soluble extract (130 g) was fractionated by column chromatography (CC) with hexane to obtain three fractions (A-C). Fraction C (32 g) was separated over a silica gel column (Hexane : EtOAc = 40 : 1) to obtain five subfractions (C1-C5). Subfraction

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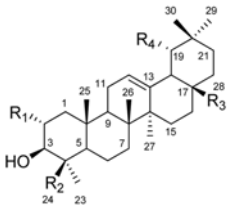
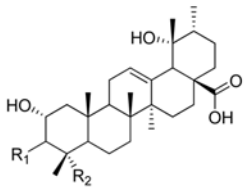
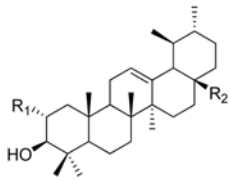


1 R_1=α-OH, R_2=CH_3, R_3=COOH, R_4=α-OH
2 R_1=α-OH, R_2=CH_3, R_3=COOH, R_4=H
3 R_1=H, R_2=CH_3, R_3= COOH, R_4=H



4 R_1=α-OH, R_2=CH_3
5 R_1=α-OH, R_2=CH_3
6 R_1=α-*O*-*trans*-*p*-coumaroyl, R_2 = CH_3



7 R_1=H, R_2=CH_2OCOH
8 R_1=H, R_2=COOH
9 R_1=α-OH, R_2=COOH

Fig. 1. The structures of **1 - 9** from *P. frutescens* var. *acuta.*

C5 (3 g) was further separated over a silica gel column (Hexane : EtOAc = 3 : 1) and purified by a silica gel semi-prep. HPLC (Hexane : EtOAc = 3 : 1) to yield compound **7** (3 mg). Its leaves were further extracted with methanol and evaporated under reduced pressure to give residues (2 kg). The MeOH extracts (1 kg) were suspended in distilled water (2.4 L) and then successively partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding residues of 190 g, 144 g, 60 g, and 95 g, respectively. The $CHCl_3$ soluble extract (25 g) was separated over a silica gel column ($CHCl_3$: MeOH = 40 : 1) to obtain five fractions (A-E). Fraction B (9 g) was separated over an RP-C_{18} silica gel column with a gradient solvent of 80~100% MeOH to afford four subfractions (B1-B4). Subfraction B4 (280 mg) was further separated over an silica Lobar A®-column ($CHCl_3$: MeOH = 30 : 1) and purified by an RP-C_{18} semi-prep. HPLC (80% MeOH) to yield compound **1** (23 mg). The EtOAc soluble extract (33 g) was fractionated by CC ($CHCl_3$: MeOH = 40 : 1 – 1 : 1) to obtain seven fractions (A-G). Fraction B (2.6 g) was separated over an RP-C_{18} silica gel column with a gradient solvent of 30~100% MeOH to afford fifteen subfractions (B1-B15). Subfraction B12 (30 mg) was purified by an RP-C_{18} semi-prep. HPLC (30% MeOH) to yield compound **4** (12 mg). Subfraction B15 (450 mg) was further separated over a silica gel column ($CHCl_3$: MeOH = 40 : 1 – 1 : 1) and purified by an RP-C_{18} semi-prep. HPLC (95% MeOH) to yield compounds **3** (9 mg) and **8** (13 mg). Fraction C (2.6 g) was separated over an RP-C_{18} silica gel column with a gradient solvent of 30~100% MeOH to afford ten subfractions (C1-C1O). Subfraction C8 (50 mg) was purified by an RP-C_{18} semi-prep. HPLC (87% MeOH) to yield compound **5** (25 mg). Subfraction C9 (25 mg) was purified by an RP-C_{18} semi-prep. HPLC (87% MeOH) to yield compound **6** (4 mg). Subfraction C10 (80 mg) was purified by an RP-C_{18} semi-prep. HPLC (87% MeOH) to yield compounds **2** (15 mg) and **9** (10 mg).

Arjunic acid (**1**) – White powder, FAB-MS *m/z* 489 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.53 (1H, brs, H-12), 4.09 (1H, ddd, *J* = 11.0, 9.0, 4.5 Hz, H-2), 3.60 (1H, brs, H-18), 3.58 (1H, d, *J* = 6.0 Hz, H-19), 3.37 (1H, d, *J* = 9.0 Hz, H-3), 2.81 (1H, ddd, *J* = 13.5, 12.5, 5.0 Hz, H-16), 2.22 (1H, ddd, *J* = 13.0, 12.5, 4.0 Hz, H-1), 1.62 (3H, s, Me-27), 1.25 (3H, s, Me-23), 1.17 (3H, s, Me-29), 1.09 (3H, s, Me-30), 1.06 (3H, s, Me-24), 1.05 (3H, s, Me-25), 1.00 (3H, s, Me-26);^{13}C-NMR data, see Table 1.

Maslinic acid (**2**) – White powder, FAB-MS *m/z* 473 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ 5.27 (1H, t, *J* = 3.5 Hz, H-12), 3.62 (1H, m, H-2), 2.91 (1H, d, *J* = 10.0 Hz, H-3), 1.17 (3H, s, Me-23), 1.02 (3H, s, Me-27), 1.01 (3H, s, Me-24), 0.95 (3H, s, Me-30), 0.91 (3H, s, Me-25), 0.82 (3H, s, Me-26), 0.81 (3H, s, Me-29); ^{13}C-NMR data, see Table 1.

Oleanolic acid (**3**) – White powder, FAB-MS *m/z* 457 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.49 (1H, s, H-12), 3.44 (1H, dd, *J* = 10.0, 6.5 Hz, H-3), 3.30 (1H, dd, *J* = 13.0, 3.5 Hz, H-18), 1.27 (3H, s, Me-27), 1.23 (3H, s, Me-23), 1.02 (3H, s, Me-24, 30), 1.00 (3H, s, Me-26), 0.94 (3H, s, Me-29), 0.89 (3H, s, Me-25); ^{13}C-NMR data, see Table 1.

Euscaphic acid (**4**) – White powder, FAB-MS *m/z* 489 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.55 (1H, brs, H-12), 4.27 (1H, dt, *J* = 10.0, 3.5 Hz, H-2), 3.72 (1H, d, *J* = 2.5 Hz, H-3), 3.11 (1H, ddd, *J* = 13.5, 13.0, 4.5 Hz, H-16), 3.01 (1H, s, H-18), 2.29 (1H, ddd, *J* = 13.5, 13.0, 4.0 Hz, H-15), 1.59 (3H, s, Me-27), 1.38 (3H, s, Me-29), 1.22 (3H, s, Me-23), 1.07 (3H, d, *J* = 6.0 Hz, Me-30), 1.05 (3H, s, Me-26), 0.94 (3H, s, Me-25), 0.85 (3H, s, Me-24); ^{13}C-NMR data, see Table 1.

Tormentic acid (**5**) – White powder, FAB-MS *m/z* 489 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.53 (1H, brs, H-12), 4.05 (1H, ddd, *J* = 10.5, 9.5, 4.5 Hz, H-2), 3.32 (1H, d, *J* = 9.5 Hz, H-3), 3.06 (1H, ddd, *J* = 13.5, 12.5, 4.5 Hz, H-16), 3.00 (1H, s, H-18), 1.65 (3H, s, Me-

Table 1. ^{13}C NMR spectral data for compounds **1 - 9**

Position	**1**[a]	**2**[b]	**3**[a]	**4**[a]	**5**[a]	**6**[a]	**7**[c]	**8**[a]	**9**[a]
1	47.0	47.3	38.7	42.0	48.0	47.9	38.8	40.3	47.7
2	68.1	68.0	27.8	66.7	68.3	65.9	27.2	29.4	68.3
3	83.4	83.2	77.8	79.6	83.6	84.5	79.0	79.4	83.5
4	39.4	39.4	39.1	39.0	39.8	39.4	38.7	40.6	39.6
5	55.5	55.4	55.5	48.8	55.7	55.0	55.1	57.1	55.6
6	18.5	18.4	18.7	18.8	18.9	18.2	18.2	19.9	18.6
7	33.2	32.8	33.0	33.6	33.2	32.8	32.7	34.8	33.2
8	39.6	39.3	39.5	40.8	40.1	39.8	40.0	41.2	39.7
9	47.9	47.7	49.4	47.8	48.1	47.2	47.6	49.3	47.8
10	38.2	38.0	37.1	39.4	38.5	37.8	36.8	38.5	38.2
11	23.1	23.5	23.6	24.2	24.5	23.5	23.4	24.9	23.4
12	122.8	121.7	122.3	128.6	127.7	127.2	125.8	126.9	125.3
13	144.5	144.4	144.6	138.9	144.6	139.5	138.0	140.5	139.0
14	41.7	41.7	41.9	42.2	42.1	41.6	41.9	43.7	42.3
15	28.6	27.8	28.1	29.2	29.1	28.7	25.9	29.9	28.4
16	23.8	23.3	23.6	26.9	26.1	25.8	23.3	26.2	24.6
17	45.6	46.2	47.9	48.7	48.5	47.7	37.2	49.3	47.8
18	44.3	41.5	41.8	54.8	54.3	54.1	54.2	54.8	53.2
19	80.7	46.0	46.2	73.3	72.4	72.1	39.2	40.7	39.1
20	35.2	30.5	30.7	42.6	42.6	41.9	39.3	40.6	39.2
21	27.9	33.8	34.0	26.3	26.7	26.4	30.4	32.3	30.8
22	28.7	32.7	32.9	38.7	38.5	38.0	35.6	38.7	37.2
23	28.3	28.9	28.5	28.7	29.2	28.5	28.1	30.1	29.1
24	16.3	17.1	16.6	16.4	17.5	17.7	15.5	17.5	17.4
25	17.1	16.4	15.4	16.4	16.7	16.3	15.3	16.4	16.7
26	17.1	17.2	17.7	17.2	17.1	16.7	17.4	18.5	17.2
27	24.3	25.7	25.9	24.5	24.6	24.2	23.5	25.2	23.6
28	180.4	179.0	180.0	183.7	180.4	179.5	70.7	181.2	179.1
29	28.8	32.8	33.0	26.7	26.8	26.6	17.2	18.6	21.1
30	24.3	23.3	23.6	16.2	16.4	16.3	21.1	22.3	17.2
CHO							161.3		
1'						167.4			
2'						115.5			
3'						144.3			
1"						125.7			
2"						129.5			
3"						116.2			
4"						160.8			
5"						116.2			
6"						130.0			

[a] Measured in pyridine-d_5; [b] Measured in CD_3OD; [c] Measured in $CDCl_3$

27), 1.38 (3H, s, Me-29), 1.21 (3H, s, Me-23), 1.17 (3H, d, J = 6.0 Hz, Me-30), 1.07 (3H, s, Me-26), 1.02 (3H, s, Me-24), 0.93 (3H, s, Me-25);^{13}C-NMR data, see Table 1.

3-*O*-*Trans*-*p*-coumaroyltormentic acid (**6**) – White powder, FAB-MS *m/z* 657 $[M + Na]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 7.93 (1H, d, J = 16.0 Hz, H-3'), 7.55 (2H, d, J = 8.0 Hz, H-3", 5"), 7.15 (2H, d, J = 8.0 Hz, H-2", 6"), 6.65 (1H, d, J = 16.0 Hz, H-2'), 5.50 (1H, t, J = 3.0 Hz, H-12), 5.24 (1H, d, J = 9.0 Hz, H-3), 4.32 (1H, m, H-2), 3.14 (1H, td, J = 12.0, 5.0 Hz, H-16), 3.00

Table 2. Cytotoxic activities of compounds (**1** - **9**) isolated from *P. frutescens* var. *acuta*

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	> 30	> 30	29.05	> 30
2	> 30	> 30	> 30	> 30
3	12.81	13.59	11.96	12.27
4	> 30	> 30	> 30	> 30
5	> 30	> 30	> 30	> 30
6	13.72	14.29	14.61	14.04
7	> 30	> 30	> 30	> 30
8	4.16	3.82	4.20	5.44
9	10.86	12.33	11.65	10.73
Doxorubicin	0.0010	0.0083	0.0012	0.0207

[a]IC_{50} value of compounds against each cancer cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth *in vitro*.

(1H, s, H-18), 1.70 (3H, s, Me-27), 1.41 (3H, s, Me-29), 1.10 (3H, d, J = 6.5 Hz, H-30), 1.07 (3H, s, Me-26), 1.02 (3H, s, Me-23), 1.01 (3H, s, Me-24), 0.99 (3H, s, Me-25); ^{13}C-NMR data, see Table 1.

28-Formyloxy-3β-hydroxy-urs-12-ene (**7**) – Colorless gum, FAB-MS *m/z* 470 $[M]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.08 (1H, s, CHO), 5.16 (1H, brs, H-12), 4.15 (1H, d, J = 11.0 Hz, H-28a), 3.73 (1H, d, J = 11.5 Hz, H-28b), 3,22 (1H, d, J = 11.0, 5.0 Hz, H-3), 1.10 (3H, s, Me-27), 0.99 (3H, s, Me-26), 0.97 (3H, s, Me-23). 0.93 (3H, s, Me-25), 0.93 (3H, d, J = 6.0, Me-30), 0.82 (3H, d, J = 6.5 Hz, Me-29), 0.79 (3H, s, Me-24); ^{13}C-NMR data, see Table 1.

Ursolic acid (**8**) – White powder, FAB-MS *m/z* 457 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.47 (1H, brs, H-12), 3,44 (1H, dd, J = 10.0, 5.5 Hz, H-3), 2.62 (1H, d, J = 11.0 Hz, H-18), 2.31 (1H, td, J = 13.0, 3.5 Hz, H-15), 2.10 (1H, td, J = 13.5, 4.0 Hz, H-16), 1.23 (3H, s, Me-23), 1.21 (3H, s, Me-27), 1.03 (3H, s, Me-26), 1.01 (3H, s, Me-24), 0.98 (3H, d, J = 6.0 Hz, Me-29), 0.95 (3H, d, J = 6.0 Hz, Me-30), 0.87 (3H, s, Me-25); ^{13}C-NMR data, see Table 1.

Corosolic acid (**9**) – White powder, FAB-MS *m/z* 473 $[M + H]^+$; ^{1}H-NMR (500 MHz, Pyridine-d_5): δ 5.40 (1H, brs, H-12), 4.05 (1H, ddd, J = 11.0, 9.5, 4.0 Hz, H-2), 3.34 (1H, d, J = 9.0 Hz, H-3), 2.57 (1H, d, J = 10.5 Hz, H-18), 2.31(1H, ddd, J = 13.5, 13.0, 4.5 Hz, H-16), 2.13 (1H, ddd, J = 13.0, 12.5, 3.5 Hz, H-1), 1.21 (3H, s, Me-23), 1.15 (3H, s, Me-27), 1.02 (3H, s, Me-26), 0.98 (3H, s, Me-24), 0.92 (3H, s, Me-25), 0.91 (3H, d, J = 6.0 Hz, Me-30), 0.85 (3H, d, J = 6.5 Hz, Me-29); ^{13}C-NMR data, see Table 1.

Cytotoxicity assay – A sulforhodamine B bioassay (SRB) was used to determine the cytotoxicity of each compound against four cultured human cancer cell lines.[8] The assays were performed at the Korea Research Institute of Chemical Technology. The cell lines used were A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells). Doxorubicin (Sigma Chemical Co., ≥ 98%) was used as a positive control.

Results and Discussion

Compounds **1** - **6**, **8** and **9** were identified as arjunic acid (**1**),[9] maslinic acid (**2**),[10] oleanolic acid (**3**),[11] euscaphic acid (**4**),[12] tormentic acid (**5**),[13] 3-*O*-*trans*-*p*-coumaroyl-tormentic acid (**6**),[14] ursolic acid (**8**),[11] and corosolic acid (**9**)[10] by comparing the ^{1}H-, ^{13}C-NMR, and MS spectral data with the literature values. The compounds **1**, **2**, **4**, **6**, and **7** were isolated for the first time from this plant and the genus *Labiatae*. The following describes the structural elucidation of compound **7**, which was isolated for the second time from natural sources.

Compound **7** was obtained as a colorless gum. Based on FAB-MS (*m/z* 470 $[M + H]^+$) and ^{1}H- and ^{13}C-NMR spectral data, the molecular formula of **7** was deduced to be $C_{31}H_{50}O_3$. The ^{1}H-NMR spectrum showed the presence of five tertiary methyl protons at δ 1.10 (3H, s, Me-27), 0.99 (3H, s, Me-26), 0.97 (3H, s, Me-23). 0.93 (3H, s, Me-25), and 0.79 (3H, s, Me-24), two secondary methyl protons at δ 0.93 (3H, d, J = 6.0, Me-30) and 0.82 (3H, d, J = 6.5 Hz, Me-29), one oxygenated methine at δ 3.22 (1H, dd, J = 11.0, 5.0 Hz, H-3), one oxygenated methylene proton at δ 4.15 (1H, d, J = 11.0 Hz, H-28a) and 3.73 (1H, d, J = 11.5 Hz, H-28b), one olefinic proton at δ 5.16

(1H, brs, H-12), and one formyl proton at δ 8.08 (1H, s, CHO). The ^{13}C-NMR spectrum exhibited 31 carbon signals composed of carbonyl carbon signal at δ 167.4, two olefinic carbon signals at δ 138.0 and 125.8, and two oxygenated carbon signals at δ 79.0 and 70.7, seven methyl, nine methylene, five methine, and five quaternary carbons (Table 1). These data indicated that compound 7 is to be triterpene derivative. By comparison with the physical and NMR spectral data in the literatures,[15,16] the structure of **7** was identified as 28-formyloxy-3β-hydroxy-urs-12-ene.

The isolated compounds (**1 - 9**) were tested *in vitro* for cytotoxicity against the A549, SK-OV-3, SK-MEL-2, and HCT15 human tumor cells using the SRB assay. The compounds **3**, **6**, **8**, and **9** showed considerable cytotoxicity against the four human cell lines as shown in Table 2. The presence of a formyl group linked at C-28 in compound **7** could reduce the cytotoxic activity, compared with that of compounds **8** and **9**.

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