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Triterpenoic Acids of *Prunella vulgaris* var. *lilacina* and Their Cytotoxic Activities *In Vitro*

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The column chromatographic separation of the MeOH extract from the aerial parts of *Prunella vulgaris* var. *lilacina* Nakai led to the isolation of fifteen triterpenoic acids (**2-6**, **9-13**, **16-20**), four flavonoids (**14**, **21-23**), four phenolics (**7**, **8**, **15**, **24**), and a diterpene (**1**). Their structures were determined by spectroscopic methods to be *trans*-phytol (**1**), oleanic acid (**2**) ursolic acid (**3**), 2α,3α,19α-trihydroxyurs-12en-28oic acid (**4**), 2α,3α-dihydroxyurs-12en-28oic acid (**5**), maslinic acid (**6**), caffeic acid (**7**), *p*-hydroxy cinnamic acid (**8**), 2α,3α,19α,23-tetrahydroxyurs-12en-28oic acid (**9**), 2α,3α,23-trihydroxyurs-12en-28oic acid (**10**), 2α,3β-dihydroxyurs-12en-28oic acid (**11**), 2α,3β,24-trihydroxyolea-12en-28oic acid (**12**), (12*R*, 13*S*)-2α,3α,24,trihydroxy-12,13-cyclo-taraxer-14-en-28oic acid (**13**), quercertin 3-*O*-β-D-glucopyranoside (**14**), rosmarinic acid (**15**), 2α,3α,24-trihydroxyurs-12,20(30)-dien-28oic acid (**16**), 2α,3α,24-trihydroxyolea-12en-28oic acid (**17**), 2α,3β,19α,24-tetrahydroxyurs-12en-28oic acid 28-*O*-D-glucopyranoside (**18**), 2α,3α,19α,24-tetrahydroxyurs-12en-28oic acid 28-*O*-D-glucopyranoside (**19**), prunvuloside A (**20**), kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopranoside (**21**), kaempferol 3-*O*-β-D-glucopyranoside (**22**), quercertin 3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**23**), and 2-hydroxy-3-(3',4'-dihydroxyphenly)propanoic acid (**24**). Compounds **1**, **8-12**, **17**, **21**, **23,** and **24** were isolated from this plant source for the first time. The isolated compounds were evaluated for their cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT15 cells *in vitro* using the sulforhodamin B bioassay (SRB) method. Compound **3** exhibited moderate cytotoxic activity against A549, SK-OV-3, SK-MEL-2, and HCT15 cells, with ED_{50} values of 3.71, 3.65, 13.62, and 5.44 μM, respectively.

Key words: *Prunella vulgaris* var. *lilacina*, Triterpenoic acid, Cytotoxicity

INTRODUCTION

Prunella vulgaris var. *lilacina* Nakai, commonly known as 'Ha Go Cho' in Korea, belongs to the Labiatae family and is a perennial herb that is widely distributed in Korea, Japan, and China. *P. vulgaris* var. *lilacina* has anti-allergic, anti-inflammatory, anti-oxidant, and free radical scavenging activities (Ryu et al., 2000; Liu et al., 2000). Previous phytochemical studies isolated pentacyclic triterpenoids (Hisashi et al., 1987) and sterol glucosides (Hisashi et al., 1990). In this study, we report the isolation of fifteen triterpenoic acids (**2-6**, **9-13**, **16-20**), four flavonoids (**14**, **21- 23**), four phenolics (**7**, **8**, **15**, **24**), and a terpene (**1**). Their structures were determined by spectroscopic methods. Compounds **1**, **8-12**, **17**, **21**, **23**, and **24** were isolated for the first time from this plant. The isolated compounds were tested for their cytotoxicity against four human tumor cell lines *in vitro* using the SRB assay. This paper describes the isolation, structural elucidation, and cytotoxic activity of these compounds.

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MATERIALS AND METHODS

General experimental procedures

Melting points were determined on a Gallenkamp

melting point apparatus and uncorrected. Optical rotations were measured on a JASCO P-1020 Polarimeter. UV spectra were obtained using a Shimadzu UV-1601 UV/Visible (Shimadzu Co.). NMR spectra were recorded on a Varian UNITY INOVA 500 NMR spectrometer. FAB-MS data were obtained on a JEOL JMS700 mass spectrometer. Preparative HPLC used a Gilson 306 pump, Shodex refractive index detector, and either an Apollo Silica 5 μ column (250×22 mm) or Econosil® RP-18 10 μ column (250×22 mm). Silica gel 60 (Merck, 70~230 mesh and 230~400 mesh) was used for column chromatography. TLC was performed using Merck pre-coated Silica gel F_{254} plates and RP-18 F_{254s} plates. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co.). Low-pressure liquid chromatography was carried out over Merck LiChroprep Lobar®-A Si 60 (240×10 mm) or LiChroprep Lobar®-A RP-18 (240×10 mm) column with a FMI QSY pump (ISCO).

Plant materials

The aerial parts of *Prunella vulgaris* var. *lilacina* were collected at Uiseong-Gun in Gyeongsangbuk-Do province, Korea, in March 2007. A voucher specimen of the plant (SKK-02-007) was deposited at the College of Pharmacy in Sungkyunkwan University.

***In vitro* cytotoxicity assay**

A sulforhodamin B bioassay (SRB) was used to determine the cytotoxicity of the compounds. The cytotoxic activity of each compound against four cultured human tumor cell lines was examined *in vitro* at the Korea Research Institute of Chemical Technology. The tumor cell lines were A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells) (Skehan et al., 1990).

Extraction and isolation

Half dried aerial parts of *P.vulgaris* var. *lilacina* (2.0 kg) were extracted with 80% MeOH at room temperature and evaporated under reduced pressure to give a residue (120 g), which was dissolved in water (800 mL) and solvent-partitioned to give n-hexane (8.0 g), ethyl acetate (20.0 g), and BuOH fractions (16.0 g). The n-hexane fraction (8.0 g) was separated over a silica gel column using a gradient solvent system of n-hexane:EtOAc = 7:1-1:1 to give five fractions (PH1-PH5). Fraction PH3 (1.4 g) was purified with a silica gel prep. HPLC (n-hexane:EtOAc = 15:1) was performed to yield compound **1** (88 mg). The ethyl acetate fraction (20.0 g) was separated over a silica gel column using a gradient solvent system of $CHCl_3$:EtOAc:MeOH = 8:3:1-1:1:1 to give five fractions (PE1-PE5). Fraction PE1 (4.0 g) was subjected to Sephadex LH-20 (CH_2Cl_2:MeOH = 1:1), a RP-C_{18} silica gel column with 90% MeOH as the eluent, and purified by RP-C_{18} prep. HPLC was performed with 90% MeOH as the eluent to yield compounds **2** (1.0 g) and **3** (1.0 g). Fraction PE2 (3.3 g) was purified by RP-C_{18} prep. HPLC was performed with 85% MeOH as the eluent to yield compounds **4** (32 mg), **5** (42 mg), and **6** (7 mg). Fraction PE3 (3.3 g) was subjected to a RP-C_{18} silica gel column with 90% MeOH as the eluent to give six fractions (PE31-PE36). Fraction PE32 (310 mg) yielded compounds **7** (250 mg) and **8** (21 mg), fraction PE34 (340 mg) compound **9** (21 mg), and fraction PE36 (140 mg) compounds **10** (32 mg), **11** (9 mg), and **12** (6 mg). Fraction PE4 (2.1 g) was subjected to a RP-C_{18} silica gel column with 60% MeOH as the eluent to give two fractions (PE41-PE42). Fraction PE41 (110 mg) was purified by RP-C_{18} prep. HPLC was performed with 100% MeOH as the eluent to yield compound **13** (33 mg). Fraction PE42 (1.3 g) was purified by RP-C_{18} prep. HPLC was performed with 30% MeCN as the eluent to yield compounds **14** (22 mg) and **15** (500 mg). Fraction PE5 (2.5 g) was subjected to a RP-C_{18} silica gel column with 60% MeOH as the eluent to give five fractions (PE51-PE55). Fraction PE52 (150 mg) was purified by RP-C_{18} prep. HPLC was performed with 100% MeOH as the eluent to yield compounds **16** (17 mg) and **17** (12 mg), and fraction PE55 (1.0 g) yielded compounds **18** (12 mg) and **19** (22 mg). The BuOH fraction (16.0 g) was separated over a silica gel column with a solvent system of $CHCl_3$:MeOH:H_2O (9:4:0.5-3:5:0.5) as the eluent to give six fractions (PB1-PB6). Fraction PB3 (2.0 g) was purified by RP-C_{18} prep. HPLC was performed with 25% MeCN as the eluent to yield compounds **20** (10 mg), **21** (80 mg), and **22** (7 mg). Fraction PB4 (0.9 g) was purified by RP-C_{18} prep. HPLC was performed with 25% MeCN to yield compound **23** (131 mg) and fraction PB6 (5 g) yielded compound **24** (16 mg).

2α,3α,19α,23-Tetrahydroxyurs-12en-28oic acid (9)

White powder; mp 255-257°C; $[\alpha]_D^{20}$ = +19.1 (c 1.1 MeOH); FAM-MS *m/z*: 503.3 $[M-H]^-$; ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.31 (1H, br s, H-12), 3.89 (1H, ddd, *J* = 12.0, 5.0, 3.0 Hz, H-2), 3.62 (1H, d, *J* = 3.0 Hz, H-3), 3.55 (1H, d, *J* = 11.0 Hz, H-23a), 3.41 (1H, d, *J* = 11.0 Hz, H-23b), 2.58 (1H, td, *J* = 13.0, 4.0 Hz, H-16), 2.53 (1H, s, H-18), 1.37 (3H, s, H-27), 1.21 (3H, s, H-26), 1.04 (3H, s, H-24), 0.94 (3H, s, H-25), 0.94 (3H, d, *J* = 7.0 Hz, H-30), 0.88 (3H, s, H-29); ^{13}C-NMR data see Table I.

2α,3α,23-Trihydroxyurs-12en-28oic acid (10)

White powder; mp 270-272°C; $[\alpha]_D^{20}$ = +21.7 (c 0.25, MeOH); FAM-MS *m/z*: 487.30 $[M-H]^-$; ^{1}H-NMR (CD_3OD,

Table I. ^{13}C NMR spectral data (δ) for compounds **9-12**, and **17**

Position	9[b]	10[b]	11[a]	12[b]	17[a]
1	41.5	42.2	46.6	46.7	42.5
2	66.0	66.0	68.9	68.1	66.5
3	77.5	77.5	83.9	84.5	74.6
4	41.9	41.1	39.2	39.1	43.3
5	43.0	43.0	55.3	55.7	49.8
6	18.0	17.9	18.9	18.4	19.2
7	32.5	32.6	32.8	32.2	33.5
8	39.9	41.3	39.5	48.1	40.3
9	48.2	48.5	47.5	47.3	48.5
10	37.9	38.0	38.2	37.7	39.0
11	24.7	23.2	23.3	22.6	24.2
12	128.0	125.4	125.3	121.6	122.8
13	138.9	138.7	138.4	144.2	145.1
14	41.3	41.8	42.2	46.0	45.5
15	28.3	28.0	28.0	24.9	28.6
16	25.4	23.4	24.2	22.3	24.1
17	49.5	48.2	48.1	46.4	46.9
18	53.9	53.2	52.8	41.4	42.3
19	72.2	39.2	40.0	42.9	46.7
20	41.0	39.6	38.8	30.2	31.2
21	25.9	30.6	30.6	33.6	34.5
22	37.8	37.9	36.6	32.8	33.5
23	70.1	70.1	28.6	27.4	24.0
24	16.0	16.2	16.8	64.7	65.5
25	17.2	16.3	16.7	16.0	17.4
26	16.3	16.4	16.9	16.3	17.7
27	23.7	23.3	23.6	23.4	26.6
28	182.8	180.8	178.0	180.2	180.5
29	26.1	16.7	17.0	32.5	34.0
30	15.3	20.4	21.1	22.7	24.4

[a]Measured in pyridine-d_5; [b]Measured in CD_3OD

500 MHz): δ 5.26 (1H, br s, H-12), 3.89 (1H, ddd, *J* = 12.0, 5.0, 3.0 Hz, H-2), 3.62 (1H, d, *J* = 3.0 Hz, H-3), 3.54 (1H, d, *J* = 11.0 Hz, H-23a), 3.40 (1H, d, *J* = 11.0 Hz, H-23b), 2.23 (1H, d, *J* = 11.0 Hz, H-18), 1.15 (3H, s, H-27), 1.04 (3H, d, *J* = 6.0 Hz, H-29), 0.98 (3H, s, H-24), 0.90 (3H, d, *J* = 6.0 Hz, H-30), 0.87 (3H, s, H-25), 0.80 (3H, s, H-26); ^{13}C-NMR data see Table I.

2α,3β-Dihydroxyurs-12en-28oic acid (11)

White powder; mp 274-276°C; $[\alpha]_D^{20}$ = +45.6 (c 0.1 MeOH); FAM-MS *m/z*: 471.15 $[M-H]^-$; ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 5.42 (1H, br s, H-12), 4.04 (1H, ddd, *J* = 12.0, 9.0, 5.0 Hz, H-2), 3.35 (1H, d, *J* = 9.0 Hz, H-3), 2.60 (1H, d, 11.0 Hz, H-18), 1.23 (3H, s, H-23), 1.17 (3H, s, H-27), 1.03 (3H, s, H-26), 1.02 (3H, s, H-24), 0.95 (3H, d, *J* = 6.0 Hz, H-29), 0.94 (3H, s, H-25), 0.91 (3H, *J* = 6.0 Hz, H-30); ^{13}C-NMR data see Table I.

2α,3β,24-Trihydroxyolea-12en-28oic acid (12)

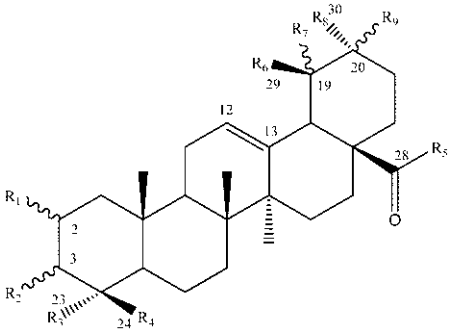
White powder; mp 298-304°C; FAM-MS *m/z*: 511.31 $[M+Na]^+$; ^{1}H-NMR (CD_3OD, 500 MHz): δ 5.25 (1H, br.s, H-12), 4.04 (1H, d, *J* = 11.0 Hz, H-24a), 3.79 (1H, ddd, *J* = 12.0, 10.0, 5.0 Hz, H-2), 3.39 (1H, d, 10.0 Hz, H-3), 3.06 (1H, d, *J* = 11.0 Hz, H-24b), 2.88 (1H, dd, *J* = 12.0, 5.0 Hz, H-18), 1.24 (3H, s, H-23), 1.17 (3H, s, H-27), 1.00 (3H, s, H-26), 0.95 (3H, s, H-29), 0.91 (3H, s, H-30), 0.82 (3H, s, H-25); ^{13}C-NMR data see Table I.

2α,3α,24-Trihydroxyolea-12en-28oic acid (17)

White powder; $[\alpha]_D^{20}$ = +108.4 (c 0.2, MeOH); FAB-MS *m/z*: 487 $[M-H]^-$; ^{1}H-NMR (pyridine-d_5, 500 MHz): δ 5.48 (1H, br.s, H-12), 4.60 (1H, d, *J* = 3.0 Hz, H-3), 4.47 (1H, m, H-2), 4.13 (1H, d, *J* = 11.0 Hz, H-24a), 3.84 (1H, d, *J* = 11.0 Hz, H-24b), 3.31 (1H, dd, *J* = 14.0, 4.0 Hz, H-18), 1.67 (3H, s, H-23), 1.12 (3H, s, H-27), 1.02 (3H, s, H-29), 1.01 (3H, s, H-25), 0.99 (3H, s H-26), 0.91 (3H, s, H-30); ^{13}C-NMR data see Table I.

RESULTS AND DISCUSSION

The structures of compounds **1-8**, **13-16**, **18-23**, and **24** were identified by comparing their spectral data (^{1}H-, and ^{13}C-NMR, MS) with literature reports as *trans*-phytol (**1**) (Brown et al., 1971), oleanic acid (**2**) (Tori et al., 1974), ursolic acid (**3**) (Bhanda et al., 1990), 2α,3α,19α-trihydroxyurs-12en-28oic acid (**4**) (Muhammad et al., 2007), 2α,3α-dihydroxyurs-12en-28oic acid (**5**) (Hisashi et al., 2007), maslinic acid (**6**) (Shoko et al., 2002), caffeic acid (**7**) (Ihsan et al., 1988), *p*-hydroxy cinnamic acid (**8**) (Kim et al., 2000), (12*R*, 13*S*)-2α,3α,24,trihydroxy-12,13-cyclo-taraxer-14-en-28oic acid (**13**) (Hisashi et al., 1988), quercertin 3-*O*-β-D-glucopyranoside (**14**) (Markham et al., 1976), rosmarinic acid (**15**) (Chamila et al., 2003), 2α,3α,24-trihydroxyurs-12,20(30)-dien-28oic acid (**16**) (Hisanshi et al., 1987), 2α,3β,19α,24-tetrahydroxyurs-12en-28oic acid 28-*O*-D-glucopyranoside (**18**) (Um et al., 2001; Choi et al., 2003), 2α,3α,19α,24-tetrahydroxyurs-12en-28oic acid 28-*O*-D-glucopyranoside (**19**) (Um et al., 2001), prunvuloside A (**20**) (Zhang et al., 1995), kaempferol 3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopranoside (**21**) (Mei et al., 2007), kaempferol 3-*O*-β-D-glucopyranoside (**22**) (Markham et al., 1976; Mei et al., 2007), quercetin 3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**23**) (Lee et al., 2004), and danshensu (**24**) (Hao et al., 1996; Ping et al., 2005). Compounds **1**, **8-12**, **17**, **21**, **23,** and **24** were isolated for the first time from this plant. The following described the structural elucidation of compounds **9-12** and **17**, which were not often isolated from natural sources.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
2	H	β-OH	CH_3	CH_3	H	H	H	CH_3	CH_3
3	H	β-OH	CH_3	CH_3	H	CH_3	H	CH_3	H
4	α-OH	α-OH	CH_3	CH_3	H	CH_3	α-OH	CH_3	H
5	α-OH	α-OH	CH_3	CH_3	H	CH_3	H	CH_3	H
6	α-OH	β-OH	CH_3	CH_3	H	H	H	CH_3	CH_3
9	α-OH	α-OH	CH_2OH	CH_3	H	CH_3	α-OH	CH_3	H
10	α-OH	α-OH	CH_2OH	CH_3	H	CH_3	H	CH_3	H
11	α-OH	β-OH	CH_3	CH_3	H	CH_3	H	CH_3	H
12	α-OH	β-OH	CH_3	CH_2OH	H	H	H	CH_3	CH_3
16	α-OH	α-OH	CH_3	CH_2OH	H	CH_3	H		CH_2
17	α-OH	α-OH	CH_3	CH_2OH	H	H	H	CH_3	CH_3
18	α-OH	β-OH	CH_2OH	CH_3	Glc	CH_3	α-OH	CH_3	H
19	α-OH	α-OH	CH_2OH	CH_3	Glc	CH_3	α-OH	CH_3	H
20	α-OH	α-OH	CH_2OH	CH_3	Glc→Glc	CH_3	α-OH	CH_3	H

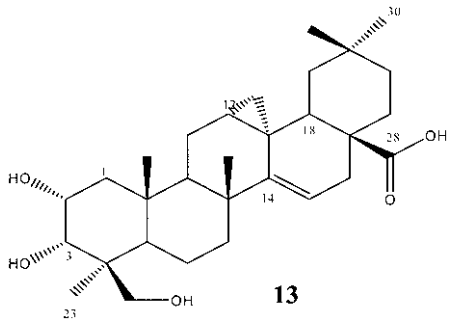


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Fig. 1. The structures of isolated compounds (**2**-**13** and **16**-**20)** from *Prunella vulgaris var. lilacina* Nakai

Compound **9** was obtained as a white powder. The FAB-MS spectrum of **9** showed a molecular ion peak at *m/z* 503.3 $[M-H]^-$. The ^{1}H-NMR spectrum showed a olefinic proton signal at δ 5.31 (1H, br s, H-12), four oxygenated proton signals at δ 3.89 (1H, ddd, *J* = 12.0, 5.0, 3.0 Hz, H-2), 3.62 (1H, d, *J* = 3.0 Hz, H-3), 3.55 (1H, d, *J* = 11.0 Hz, H-23a), and 3.41 (1H, d, *J* = 11.0 Hz, H-23b), and a methine proton signal at δ 2.53 (1H, s, H-18). The ^{13}C-NMR spectrum showed a carbonyl carbon signal at δ 182.8 (C-28), two olefinic carbon signals at δ 138.9 (C-13) and 128.0 (C-12), and four oxygenated carbon signals at δ 77.5 (C-3), 72.2 (C-19), 70.1 (C-23), and 66.0 (C-2). These spectral data suggested that **9** was an ursane skeleton-type triterpenoic acid (Hassane et al., 1999). Based also on literature data (Li et al., 2003), **9** was identified as 2α,3α,19α,23-tetrahydroxyurs-12en-28oic acid. The ^{1}H- and ^{13}C-NMR spectra of **10** were similar to those of **9**, except for the absence of an oxygenated carbon signal at δ 72.2 (C-19). Based on literature data (Yutaka et al., 1992), **10** was determined to be 2α,3α,23-trihydroxyurs-12en-28oic acid. The ^{1}H- and ^{13}C-NMR spectra of **11** were similar to those of **3**, except for an additional oxygenated carbon signal at δ 68.9 (C-2). The ^{1}H- and ^{13}C-NMR spectra of **11** were identical to those of 2α,3β-dihydroxyurs-12en-28oic acid (Shoko et al., 2002). The ^{1}H-NMR spectrum of compound **12** showed an olefinic proton signal at δ 5.25 (1H, br.s, H-12), four oxygenated proton signals at 4.04 (1H, d, *J* = 11.0 Hz, H-

Table II. Cytotoxic activities of compounds (**1**, **3**, **5**, **10-11**, **17**) isolated from *Prunella vulgaris* var. *lilacina* Nakai

Compounds	ED_{50} (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT15
1	12.15	13.77	14.29	12.23
3	3.71	3.65	3.62	5.44
5	12.67	13.14	12.93	12.90
10	28.43	>30.0	13.29	12.85
11	11.84	12.72	12.44	10.57
17	12.64	15.28	18.03	12.81
Doxorubicin	0.003	0.094	0.018	0.107

24a), 3.79 (1H, ddd, J = 12.0, 10.0, 5.0 Hz, H-2), 3.39 (1H, d, 10.0 Hz, H-3), and 3.06 (1H, d, J = 11.0 Hz, H-24b), and a methine proton signal at δ 2.88 (1H, dd, J = 12.0, 5.0 Hz, H-18). The ^{13}C-NMR spectrum showed a carbonyl carbon signal at δ 180.2 (C-28), two olefinic carbon signals that show an oleane skeleton at δ 144.2 (C-13) and 121.6 (C-12), and three oxygenated carbon signals at δ 84.5 (C-3), 68.1 (C-2), and 64.7 (C-24). These spectral data suggested that **12** was an oleane skeleton-type triterpenoic acid (Fourneau et al., 1996). Based also on literature data (Takashi et al., 1988), **12** was identified as 2α,3β,24-trihydroxyolea-12en-28oic acid. The ^{1}H- and ^{13}C-NMR spectra of **17** were similar to those of **12**, except for a different J value at H-3 (**17**: J = 3.0 Hz; **3**: J = 10.0 Hz). Based on the comparison of the ^{1}H- and ^{13}C-NMR spectra of **17** with the literature (Hisanshi et al., 1987), **17** was determined to be 2α,3α,24-trihydroxyolea-12en-28oic acid.

The isolated compounds were tested for cytotoxicity against four human tumor cell lines *in vitro* by the SRB assay. Ursolic acid was reported to show cytotoxic activity against various cancer cell lines (Toshihiro et al., 2006, Ioanna et al., 2007). In this study, compound **3** (ursolic acid) also exhibited moderate cytotoxic activity against A549, SK-OV-3, SK-MEL-2, and HCT15 cells (ED_{50}: 3.71, 3.65, 3.62, and 5.44 μM, respectively). Compounds **1**, **5**, **10**, **11**, and **17** showed weak cytotoxicity against four human tumor cell lines (Table II). The other compounds revealed little activity (ED_{50} values >30 μM).

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