

Cytotoxic Constituents of *Pilea mongolica*

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Bioassay-guided fractionation of the aerial parts of *Pilea mongolica* (Urticaceae) afforded two cytotoxic triterpenoids, epi-oleanolic acid (I) and oxo-oleanolic acid (II). The structures of the compounds were confirmed by spectral and synthetic evidences. Compound I and compound II exhibited cytotoxicity against cultured human tumor cell lines, A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (CNS) and HCT15 (colon) with ED₅₀ values of 3.2~8.1 µg/ml and 0.7~6.8 µg/ml, respectively.

Key words : *Pilea mongolica*, Urticaceae, Cytotoxicity, Epi-oleanolic acid, Oxo-oleanolic acid

INTRODUCTION

Pilea mongolica (Urticaceae) is a perennial herb which is common in the swamp in Korea. It has been used in traditional Chinese medicine for the treatment of acute gastritis, diabetes, urethritis, endometritis and leukorrhea (Lee, T. B., 1989). No phytochemical and pharmacological studies on this plant has been reported in the literature. We monitored the cytotoxic effects of this plant and found that the CH₂Cl₂ soluble portion of MeOH Ex. exhibited an inhibitory effect on the cultured human tumor cells, *in vitro*. Hence, the repeated chromatographic separation of the CH₂Cl₂ fraction has been carried out and finally resulted in the isolation of I and II as active principles (Fig. 1). The present paper describes isolation, structural characterization, synthesis, and cytotoxicity of these compounds.

MATERIALS AND METHODS

General experimental procedures

The mps were taken on a Gallenkamp melting point apparatus and were uncorrected. The EIMS spectrum was measured on VG70-VSEQ (VG ANALITICAL, UK). The IR spectrum was measured with a shimadzu IR-435. The ¹H- and ¹³C-NMR spectra were recorded with Bruker AM-300 and Varian Gemini 300 spectrometer in CDCl₃ and chemical shifts are given as δ (ppm). The Column of LPLC used was Lobar-A Lichroprep Si-60 (Merck) and pump was DURAMAT 80 pump (Germany). HPLC was equipped with 112 UV/

VIS detector set at 254 nm, 306 pump with 10SC head (Gilson) and 10×250 mm stainless steel column prepacked with octadecylsilane chemically bonded on totally porous silica (Alltech Econosil C₁₈ 10µ). TLC chromatography was performed on precoated Kiesel-gel 60 F₂₅₄ plates (Merck). Silica gel used for column chromatography was Kiesel gel 60 (70~230 mesh, 230~400 mesh, Merck) and sephadex was lipophilic sephadex LH-20 (Sigma).

Plant materials

Pilea mongolica was collected from the Chuk Ryong Mountain in Kyunggido in September 1996. A voucher specimen is deposited in College of Pharmacy, Sungkyunkwan University.

Test for cytotoxicity *in vitro*

Sulforhodamin B Bioassay(SRB) was used as cytotoxicity screening method. Activities of fractions were monitored in several concentration level against five kinds of cultured human tumor cells, *i.e.*, A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (CNS) and HCT15 (colon) *in vitro* (Detailed experimental procedures are

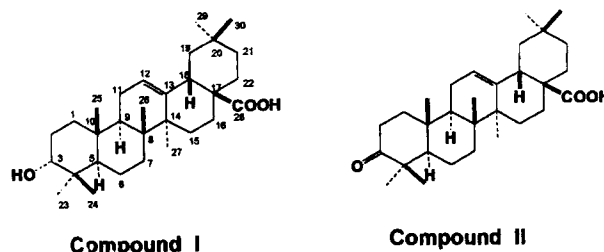


Fig. 1. Structures of compounds I and II.

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in reference, Ryu *et al.*, 1992).

Extraction, separation, purification and synthesis of compounds

The half-dried and chopped whole plant (1.5 kg) was extracted with MeOH for 7 days at room temperature (2 times) and 5 hours in 50°C (1 time), then concentrated in vacuo. The MeOH extract (90 g) was suspended in H₂O (800 ml) and successively partitioned with CH₂Cl₂ (800 ml), EtOAc (800 ml) and *n*-BuOH (800 ml). The CH₂Cl₂ phase was evaporated under vacuum to yield the CH₂Cl₂ extract (40 g).

The CH₂Cl₂ extract was chromatographed on silica gel with gradient *n*-Hexane-CH₂Cl₂ (1:8~) and CH₂Cl₂-MeOH (~5:1) to give nine subfractions (PM1~9), which were assayed by SRB method. The PM7 fraction, which was exhibited cytotoxic activity, was rechromatographed with SiO₂ column using *n*-hexane:CH₂Cl₂:MeOH (15:30:1) and sephadex LH-20 column using acetone:MeOH (1:1) to give two subfraction. The first and second subfraction was further purified by preparative HPLC (Econosil C-18, 10×250 mm; 3.0 ml/min) with MeOH and MeCN:MeOH (1:1) to afford compound I (21 mg) and compound II (10 mg), respectively.

Compound I

white powder; mp. 280°C; IR ν_{\max} cm⁻¹: 3300~3500 (OH), 2900, 1700 (C=O), 1450, 1380, 1250; EIMS m/z (rel. int., %): 456 (M⁺, 3), 438^q(6), 248 (100), 233 (14), 203 (87), 190 (67), 175 (32), 133 (41), 105 (28), 81 (26), 69 (40), 55 (46); ¹H-NMR (300 MHz, CDCl₃) δ : 0.72, 0.80, 0.88, 0.90 (×2), 0.92 (each 3H, s, CH₃×6), 1.11 (3H, s, 29-CH₃), 2.78 (1H, br.dd, H-18), 3.38 (1H, t, J=2.48 Hz, H-3), 5.25 (1H, t, J=3.40 Hz, H-12); ¹³C-NMR (75.5 MHz, CDCl₃): Table I

Compound II

white powder; mp. 154°C; ν_{\max} cm⁻¹: 2900, 1700 (C=O), 1550, 1450, 1390, 1250; EIMS m/z (rel. int., %): 454 (M⁺, 33), 408 (33), 248 (100), 233 (47), 203 (97), 189 (55), 133 (62), 105 (42), 95 (35), 55 (42); ¹H-NMR (300 MHz, CDCl₃) δ : 0.81, 0.90, 0.93, 1.03, 1.05, 1.09 (each 3H, s, CH₃×6), 1.14 (3H, s, 29-CH₃), 2.40 (1H, m, H-2), 2.52 (1H, m, H-2), 2.84 (1H, br. dd, H-18), 5.31 (1H, br.s, H-12); ¹³C-NMR (75.5 MHz, CDCl₃): Table I

Synthesis of oxo-oleanolic acid (Cainelli, G. *et al.*, 1984; Huneck, S., 1953; Yagi, A. *et al.*, 1978)

To a solution of oleanolic acid (20 mg, 0.044 mmol) dissolved in benzene (7 ml), PCC (pyridinium chlorochromate, 30 mg, 0.14 mmol) was added and the reaction mixture was stirred for 8 hours at room tem-

Table I. ¹³C-NMR and DEPT data of compounds I and II

C	Compound I		Compound II	
	¹³ C (δ)	DEPT	¹³ C (δ)	DEPT
1	32.48	CH ₂	39.15	CH ₂
2	25.18	CH ₂	34.16	CH ₂
3	76.18	CH	217.60	C
4	37.34	C	47.44	C
5	48.98	CH	55.37	CH
6	18.24	CH ₂	19.61	CH ₂
7	32.48	CH ₂	32.23	CH ₂
8	39.45	C	39.36	C
9	47.41	CH	46.64	CH
10	37.17	C	36.83	C
11	22.93	CH ₂	23.02	CH ₂
12	122.70	CH	122.37	CH
13	143.60	C	143.75	C
14	41.67	C	41.82	C
15	27.63	CH ₂	27.74	CH ₂
16	23.32	CH ₂	23.59	CH ₂
17	46.52	C	46.64	C
18	40.95	CH	41.16	CH
19	45.89	CH ₂	45.92	CH ₂
20	30.67	C	30.70	C
21	33.81	CH ₂	33.89	CH ₂
22	32.86	CH ₂	32.49	CH ₂
23	28.27	CH ₃	26.51	CH ₃
24	22.25	CH ₃	21.46	CH ₃
25	15.10	CH ₃	15.04	CH ₃
26	17.18	CH ₃	17.00	CH ₃
27	26.09	CH ₃	25.84	CH ₃
28	183.28	C	183.30	C
29	33.06	CH ₃	33.07	CH ₃
30	23.59	CH ₃	23.59	CH ₃

perature. The product was filtered and evaporated to dryness. The residue was chromatographed on silica gel with *n*-hexane:EtOAc (3:1). The main fraction was evaporated to dryness and was recrystallized from MeOH to give white powder (16 mg). The mp., TLC, ¹H-NMR and ¹³C-NMR spectra of this product were compared with those of compound II

Synthesis of epi-oleanolic acid (Huneck, S., 1953)

The synthetic oxo-oleanolic acid (10 mg, 0.022 mmol) and aluminiumisopropoxide (30 mg, 0.147 mmol) were dissolved in isopropanol (1 ml). The reaction mixture was stirred for overnight at 70°C. The reaction mixture was evaporated to dryness and 1N HCl (8 ml) was added. The reaction mixture was extracted three times with ether. The ether layer was dried over magnesium sulfate and concentrated under reduced pressure. The resulting mixture was chromatographed on silica gel with *n*-Hexane:EtOAc (3:1) to give white powder (3 mg). The mp., TLC and ¹H-NMR spectrum of this product were compared with those of compound I

RESULTS AND DISCUSSION

The CH_2Cl_2 soluble fraction of *Pilea mongolica* has been found to exhibit the cytotoxic activity against several human cancer cell lines. Bioassay-guided fractionation led to isolation of two oleanane type triterpene as active principles.

Compound **I** was obtained as colorless amorphous powder. EIMS and DEPT data established molecular formula of $\text{C}_{30}\text{H}_{48}\text{O}_3$. In the mass spectrum the base peak at m/z 248 ($\text{C}_{16}\text{H}_{24}\text{O}_2$) besides other peaks at m/z 203 ($\text{C}_{15}\text{H}_{23}$) and 133 ($\text{C}_{10}\text{H}_{13}$) were characteristic of a Δ^{12} -amyrin skeleton with a carboxyl group in ring D/E and hydroxy groups in ring A and/or B. (Budzikiewicz, H. *et al.*, 1963; Akhtar, N. *et al.*, 1993) The presence of only tertiary methyl groups indicated the olean-12-ene skeleton (Budzikiewicz, H. *et al.*, 1963; Seo, S. *et al.*, 1981). The IR spectrum exhibited hydroxyl ($3300\sim 3500\text{ cm}^{-1}$) and carboxyl group (1700 cm^{-1}) absorption bands. The $^1\text{H-NMR}$ spectrum clearly showed an olefinic proton at δ 5.25 (1H, t, $J=3.40\text{ Hz}$, H-12), a methine proton at δ 2.78 (1H, br.dd, H-18), an α -carbinol proton at 3.38 (1H, t, $J=2.48\text{ Hz}$, H-3), and seven methyl functionalities all attached to saturated carbons in the δ 0.72-1.11. Its broad-band-decoupled $^{13}\text{C-NMR}$ and DEPT spectrum exhibited the presence of 30 carbon signals ($\text{CH}_3\times 7$, $\text{CH}_2\times 10$, $\text{CH}\times 5$, $\text{C}\times 8$). These data showed two olefinic carbon signal at 122.7 (CH) and 143.6 ppm (C), and a carbonyl carbon at 183.3 ppm (C). These data were consistent with having a OH group at C-3 of oleanane type triterpene. ^1H and $^{13}\text{C-NMR}$ data of oleanolic acid were similar with compound **I**; the major difference between oleanolic acid and compound **I** was in the $^{13}\text{C-NMR}$ chemical shift of the C-24 carbon. C-24 methyl carbon (22.25 ppm) in compound **I** showed at downfield to 7 ppm than C-24 peak (15.6 ppm) of oleanolic acid. Also, the stereochemistry of the α -OH at C-3 was deduced from the comparison of the coupling constant with those reported ones (Akhtar, N. *et al.*, 1993; Chem, T. K. *et al.*, 1983; Hylands, P. J. *et al.*, 1980; Mahato, S. B. *et al.*, 1994). Consequently, we suggested that compound **I** was epi-oleanolic acid. The structure was further confirmed by comparison of $^1\text{H-NMR}$ data of epi-oleanolic acid methyl ester in previous papers (Ikuta, A., 1989; Konishi, T. *et al.*, 1981) and synthesis of epi-oleanolic acid from oxo-oleanolic acid.

Compound **II** was obtained as colorless amorphous powder and showed many spectral features in common with compound **I**. EIMS and DEPT data established molecular formula of $\text{C}_{30}\text{H}_{46}\text{O}_3$. The IR spectrum exhibited carboxyl group (1700 cm^{-1}) absorption band. The $^1\text{H-NMR}$ spectrum is consistent with the triterpene structure and clearly showed an olefinic proton at δ 5.31 (1H, br.s, H-12), a methine

Table II. Cytotoxicity of compounds **I** and **II**

ED ₅₀ values					
cancer cell line	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
compound I	3.2523	5.3536	8.1379	3.6646	3.8027
compound II	2.3578	6.5829	6.8531	4.1675	0.7156

ED₅₀ value of compound against each cancer cell line, which was defined as a concentration ($\mu\text{g/ml}$) that caused 50% inhibition of cell growth *in vitro*

proton at δ 2.84 (1H, br.dd, H-18), and seven methyl functionalities all attached to saturated carbons in the δ 0.81-1.14. $^{13}\text{C-NMR}$ and DEPT spectra of **II** were very similar to compound **I**, but the difference was that a new peak appeared at 217.60 ppm (C) in compound **II** instead of peak at 76.18 ppm (CH) in **I**. Consequently, we supposed that a hydroxyl group of **I** was reduced to ketone in to give **II**. On the basis of above mentioned evidence and previous papers, (Seo, S. *et al.*, 1975) compound **I** was suggested as oxo-oleanolic acid. It's structure was further confirmed by synthesis of oxo-oleanolic acid from oleanolic acid.

As shown in Table **II**, compound **I** and **II** showed significant cytotoxicity against cancer cells. Especially, compound **II** showed strong cytotoxic activity against HCT15 (colon cancer cells) (ED₅₀: 0.7156 $\mu\text{g/ml}$).

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