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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 ED_{50} 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 phamacological studies on this plant has been reported in the literature. We monitored the cytotoxic effects of this plant and found that the CH_2Cl_2 soluble portion of MeOH Ex. exhibited an inhibitory effect on the cultured human tumor cells, *in vitro*. Hence, the repeated chromatographic separation of the CH_2Cl_2 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 ^{13}C-NMR spectra were recorded with Bruker AM-300 and Varian Gemini 300 spectrometer in $CDCl_3$ 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 C_{18} 10u). TLC chromatography was performed on precoated Kieselgel 60 F_{254} 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

Compound I

Compound II

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 vaccuo. The MeOH extract (90 g) was suspended in H_2O (800 ml) and successively partitioned with CH_2Cl_2 (800 ml), EtOAc (800 ml) and *n*-BuOH (800 ml). The CH_2Cl_2 phase was evaporated under vacuum to yield the CH_2Cl_2 extract (40 g).

The CH_2Cl_2 extract was chromatographed on silica gel with gradient *n*-Hexane-CH_2Cl_2 (1:8~) and CH_2Cl_2-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 SiO_2 column using *n*-hexane:CH_2Cl_2: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 (6), 248 (100), 233 (14), 203 (87), 190 (67), 175 (32), 133 (41), 105 (28), 81 (26), 69 (40), 55 (46); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.72, 0.80, 0.88, 0.90 (×2), 0.92 (each 3H, s, CH_3× 6), 1.11 (3H, s, 29-CH_3), 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); ^{13}C-NMR (75.5 MHz, $CDCl_3$): 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); ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.81, 0.90, 0.93, 1.03, 1. 05, 1.09 (each 3H, s, CH_3×6), 1.14 (3H, s, 29-CH_3), 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); ^{13}C-NMR (75.5 MHz, $CDCl_3$): Table I

Table I. ^{13}C-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

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 temperature. 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, ^{1}H-NMR and ^{13}C-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 ^{1}H-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 amorphorous powder. EIMS and DEPT data established molecular formular of $C_{30}H_{48}O_3$. In the mass spectrum the base peak at *m/z* 248 ($C_{16}H_{24}O_2$) besides other peaks at *m/z* 203 ($C_{15}H_{23}$) and 133 ($C_{10}H_{13}$) 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 (3300~3500 cm^{-1}) and carboxyl group (1700 cm^{-1}) absorption bands. The ^1H-NMR spectrum clearly showed an olefinic proton at δ 5.25 (1H, t, J=3.40 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 Hz, H-3), and seven methyl functionalities all attached to saturated carbons in the δ 0.72-1.11. Its broad-band-decoupled ^{13}C-NMR and DEPT spectrum exhibited the presence of 30 carbon signals ($CH_3\times7$, $CH_2\times10$, $CH\times5$, $C\times8$). 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}C-NMR data of oleanolic acid were similar with compound **I**; the major difference between oleanolic acid and compound **I** was in the ^{13}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 ^1H-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 amorphorous powder and showed many spectral features in common with compound **I**. EIMS and DEPT data established molecular formular of $C_{30}H_{46}O_3$. The IR spectrum exhibited carboxyl group (1700 cm^{-1}) absorption band. The ^1H-NMR spectrum is consistent with the triterpene sturcture and clearly showed an olefinic proton at δ 5.31 (1H, br.s, H-12), a methine 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}C-NMR and DEPT spectra of **II** were very similar to compound **I**, but the difference was that a new peak apeared 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.

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

ED_{50} value of compound against each cancer cell line, which was defined as a concentration (μg/ml) that caused 50% inhibition of cell growth *in vitro*

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 cacer cells) (ED_{50}: 0.7156 μg/ml).

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