Cytotoxic Triterpenes from *Crataegus pinnatifida*

Byung Sun Min[1], Young Ho Kim[1], Sang Myung Lee[1], Hyun Ju Jung[1], Jun Sung Lee[1], Min Kyun Na[1], Chong Ock Lee[2], Jong Pill Lee[3], and KiHwan Bae[1]

[1]College of Pharmacy, Chungnam National University, Taejon 305-764, [2]Korea Research Institute of Chemical and Technology, Taejon 305-606, and [3]Natural Medicinal Product Division, Korea Food and Drug Administration, Seoul, 122-704, Korea

(Received November 4, 1999)

Bioassay-guided fractionation of *Crataegus pinnatifida* (Rosaceae) gave two cytotoxic ursane-type triterpenes which were identified as uvaol (**1**) and ursolic acid (**2**) by physicochemical and spectroscopic methods. 3-Oxo-ursolic acid (**3**) was synthesized from ursolic acid (**2**) by Jones method. The cytotoxic activities of these compounds were tested against murine L1210 and human cancer cell lines (A549, SK-OV-3, SK-MEL-2, XF498, and HCT15) *in vitro*. Compounds **1** and **2** showed moderate cytotoxicities against L1210, whereas they showed weak activities against human cancer cell lines. However, compound **3** exhibited potent cytotoxic activities both in murine and in human cancer cell lines.

Key words: *Crataegus pinnatifida*, Rosaceae, Uvaol, Ursolic acid, 3-Oxo-ursolic acid, Cytotoxicity

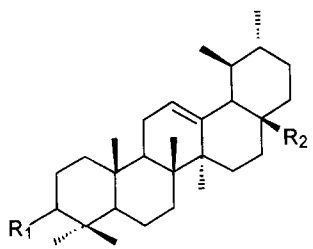
INTRODUCTION

Crataegus pinnatifida Bunge (Rosaceae) is a perennial tree which grows widely in Korea. The fruits of *C. pinnatifida* have been used for gastric malignancy and diarrhea (Perry, 1980). The leaf of *C. pinnatifida* has been recognized to possess antihyperlipemic, antiarrhythmic, and antioxidative activity (Jeong *et al.*, 1999). Several triterpenes (Oh *et al.*, 1993; Park *et al.*, 1994) and flavonoids (Kim *et al.*, 1993; Oh *et al.*, 1994) were reported from the fruits and leaves of *C. pinnatifida* var. *psilosa*. In continuation of our study on anticancer agents from natural products, we found that the dichloromethane soluble fraction of the leaves of *C. pinnatifida* displayed cytotoxic activity against L1210 murine leukemia cell. Repeated column chromatography of the cytotoxic fraction resulted in two ursane-type triterpenes. We report here the isolation, and structure identification of compounds **1** and **2**, along with the synthesis of **3** from **2**. Cytotoxic activities of these compounds **1-3** were also evaluated with several cancer cell lines *in vitro*.

MATERIALS AND METHODS

General procedures

Correspondence to: KiHwan Bae, College of Pharmacy, Chungnam National University, Taejon 305-764, Korea
E-mail: baekh@hanbat.chungnam.ac.kr



	R_1	R_2
1	– OH	CH_2OH
2	– OH	COOH
3	= O	COOH

Fig. 1. Structures of Compounds **1-3**

Melting points were obtained with an Electrothermal Series IA9100 apparatus and are uncorrected. Optical rotations were measured with a DIP-370 automatic polarimeter (JASCO Co.). IR spectra were recorded on a JASCO Infrared spectrophotometer IR Report-100 (JASCO Co.). ^1H and ^{13}C NMR experiments were run in $CDCl_3$ and DMSO-d_6 containing TMS as an internal standard using Bruker DRX 300 spectrometers. Si gel 60 (70-230 mesh; Merck) was used for chromatography. TLC was performed on pre-coated Si gel 60 (F254, Merck). Fischer's and RPMI medium were purchased from Hyclone and fetal calf serums from GIBCO-BRL (Grand Island, NY). Other supplement materials for cell

culture were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant material

The leaves of *C. pinnatifida* were collected at Kongju, Chungnam province, Korea in October, 1996. The voucher specimens (CNU0954) are deposited at the herbarium in College of Pharmacy, Chungnam National University.

Extraction and isolation

The leaf of *C. pinnatifida* was extracted with MeOH three times to give MeOH extract (401 g) on removal of solvent *in vacuo*. The MeOH extract was fractionated with dichloromethane and H_2O. The dichloromethane fraction was chromatographed on silica gel eluted with gradiently hexane-ethyl acetate (10:1→2:1). Repeated column chromatography with DOWEX-50W and recrystallization in MeOH gave two triterpenoids, compound 1 (307 mg) and compound 2 (977 mg).

Compound 1 (uvaol): colorless needle (MeOH); mp. 214-216°C; $[\alpha]_D^{25}$: +56 (*c*=1.0, EtOH), IR ν_{max}(KBr) cm^{-1}: 3350, 2910, 1450, 1360; ^1H NMR (300 MHz, $CDCl_3$+DMSO-*d*6): 5.09 (1H, t, *J*=3.5 Hz, H-12), 3.84 (2H, s, H-28), 3.09 (1H, dd, *J*=10.8, 4.8 Hz, H-3), 1.08, 0.98, 0.96, 0.93 (each 3H, s, H-23, 27, 26, 24), 0.92 (3H, d, *J*=5.3 Hz, H-30), 0.79 (3H, d, *J*=5.8 Hz, H-29) and 0.75 (3H, s, H-25); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-*d*6): see Table I.

Compound 2 (ursolic acid): white amorphous powder (MeOH); mp. 261-263°C; $[\alpha]_D^{25}$: +63 (*c*=1.0, EtOH); IR ν_{max}(KBr) cm^{-1}: 3400, 2920, 1680, 1450, 1380; ^1H NMR (300 MHz, $CDCl_3$+DMSO-*d*6): 5.17 (1H, t, *J*=3.5 Hz, H-12), 3.04 (1H, dd, *J*=10.9, 4.8 Hz, H-3), 1.07 (3H, s, H-23), 0.94 (6H, s, H-26, 27), 0.90 (3H, s, H-24), 0.84 (3H, d, *J*=6.4 Hz, H-30), 0.77 (3H, d, *J*=9.6 Hz, H-29) and 0.72 (3H, s, H-25); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-*d*6): see Table I.

Synthesis of 3-oxo-ursolic acid (Compound 3)

Jones reagent (1 ml) was added to a solution of compound **2** (100 mg, 0.22 mM) dissolved in dichloromethane-acetone (1:1, 1 ml), and reaction mixture was stirred for 45 min at 5°C (Fieser *et al.*, 1967). Then, *i*-PrOH (0.1 ml) and H_2O (0.4 ml) were added to the reaction mixture and it was stirred for 10 min at room temperature. The reaction product was extracted with dichloromethane and washed with saturated NaCl solution (10 ml). The dichloromethane layer was evaporated to dryness and chromatographed on Si gel with *n*-hexane-acetone (5:2). Compound **3** (65 mg) was obtained by recrystallization in MeOH.

Table I. ^{13}C NMR Spectral Data of Compounds 1-3 ($CDCl_3$ +DMSO-d_6, 75 MHz)

Carbon No.	1	2	3
1	37.4	38.1	39.3
2	25.4	26.9	34.2
3	76.8	76.8	217.8
4	37.4	36.5	47.4
5	54.6	54.7	55.3
6	17.8	17.8	19.6
7	32.3	32.6	32.5
8	38.3	38.7	39.5
9	47.0	46.7	46.6
10	36.3	36.4	36.7
11	22.8	22.7	23.4
12	123.9	124.5	125.6
13	138.5	137.9	138.1
14	41.4	41.5	42.1
15	26.8	27.4	28.0
16	22.9	23.7	24.1
17	47.0	46.9	48.0
18	53.6	52.2	52.6
19	38.2	38.4	39.1
20	38.2	38.2	38.8
21	30.2	30.1	30.6
22	35.1	36.2	36.7
23	28.0	28.1	26.6
24	15.7	15.1	17.0
25	15.3	15.8	15.2
26	16.3	16.8	17.0
27	22.5	23.1	23.5
28	67.9	178.3	183.4
29	17.1	16.7	21.5
30	21.1	20.9	21.2

White amorphous powder (MeOH); mp. 238-241°C; $[\alpha]_D^{25}$:+87 (*c*=1.0, EtOH); IR ν_{max}(KBr) cm^{-1}: 3400, 2920, 1695, 1670, 1450, 1380; ^1H NMR (300 MHz, $CDCl_3$+DMSO-d_6): 5.27 (1H, t, *J*=3.5 Hz, H-12), 1.09, 1.08, 1.05, 1.03 (each 3H, s, H-23, 27, 26, 24), 0.94 (3H, d, *J*=7.8 Hz, H-30), 0.87 (3H, d, *J*=6.4 Hz, H-29) and 0.84 (3H, s, H-25); ^{13}C NMR (75 MHz, $CDCl_3$+DMSO-d_6): see Table I.

In vitro cytotoxicity

In vitro cytotoxicity was measured with murine (L1210, leukemia) and human cancer cells. Five different human cancer lines, A549 (lung adenocarcinoma), SK-OV-3 (ovarian), SK-MEL-2 (skin melanoma), XF498 (CNS) and HCT15 (colon) were purchased from National Cancer Institute (NCI) in U. S. A. The cytotoxic activities were carried out previously before (Kwon *et al.*, 1998) using sulforhodamine B (SRB) method (Skehan *et al.*, 1990). ED_{50} value was determined graphically by plotting the viability versus the concentration of the test sample.

RESULTS AND DISCUSSION

For the isolation of cytotoxic compounds from the leaves of *C. pinnatifida*, the methanol extract was

fractionated with dichloromethane, ethyl acetate and buthanol, successively. The dichloromethane-soluble fraction showed a moderate cytotoxic activity against L1210 cells with ED_{50} value of 9.5 μg/ml, while the ethyl acetate and buthanol-soluble fraction displayed no cytotoxic activity (>20 μg/ml). Bioassay-guided isolation of dichloromethane fraction afforded two triterpenoids as active principles. Their structures were determined by physico-chemical and spectral data (Nakanishi *et al.*, 1983). They were identified as uvaol (**1**) and ursolic acid (**2**) in comparison with authentic samples. Compound **3** was synthesized from compound **2** by Jones reagent with 65% yield. The spectral data of ^1H and ^{13}C NMR of compound **3** were similar to those of compound **2**. However, compound **3** showed a carbonyl carbon at δ 216.2 instead of carbinol carbon at δ 76.8 and downfield shift of C-2 (δ26.9→ δ34.2) and C-4 (δ36.5→ δ47.4) in the ^{13}C NMR spectrum. Therefore, the structure of compound **3,** was confirmed to be 3-oxo-urs-12-en-28-oic acid (3-oxo-ursolic acid).

The cytotoxicities of **1-3** were evaluated *in vitro* against six cancer cells which were originated from murine (L1210) and human (A549, SK-OV-3, SK-MEL-2, XF498, and HCT15) cells. As indicated in Table II, **1** and **2** showed moderate cytotoxicities against L1210 with ED_{50} values of 2.97 and 3.70 μg/ml, respectively, while they showed weak cytotoxicities against five human cancer cell lines. Compound **3** exhibited a significant cytotoxic activities against all tested cancer cells. ED_{50} values were 3.15, 0.91, 3.27, 3.46, 3.89, and 0.56 μg/ml, against L1210, A549, SK-OV-3, SK-MEL-2, XF498, and HCT15, respectively. Especially, compound **3** showed more potent cytotoxic activity against HCT15 than cisplatin. The oxo-oleanolic acid, isolated from *Pilea mongolica*, also showed a significant potent cytotoxicity against HCT15 cancer cell lines with ED_{50} value of 0.72 μg/ml (Kwon *et al.*, 1997). It is possible that carbonyl group at C-3 position in triterpene (ursane and oleanane type) might be a pharmacophore for cytotoxicity expression against HCT15. Ursolic acid (**2**) was previously reported to possess various biological activities such as antiviral (Kashiwada *et al.*, 1998, Min *et al.*, 1999, Tommasi *et al.*, 1992), antitumor promotion (Trumbull *et al.*, 1976), protein kinase C inhibition (Ahn *et al.*, 1998), and anti-angiogenic (Sohn *et al.*, 1995) activities. Moreover, this compound have been reported from many kinds of plants with relatively low toxicity (Jeong *et al.*, 1999). These facts suggested that compound **2** or its analogue would be an important target for developing new antitumor agents.

Table II. Cytotoxicities data of Compounds **1-3**

Compound	ED_{50} values (μg/ml)					
	L1210	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	2.97	11.46	13.17	>10	9.54	9.32
2	3.70	>10	>10	9.27	>10	>10
3	3.15	0.91	3.27	3.46	3.89	0.56
Cisplatin[a)]	3.25	0.91	1.32	0.89	0.72	3.17

a) Positive Control

ACKNOWLEDGEMENTS

This work was partly supported by Institute for New Drug Developmet, College of Pharmacy, Chungnam National University. We are grateful to KBSI for ^1H and ^{13}C NMR measurements.

REFERENCES

Ahn, K. S., Hahm, M. S., Park, E. J., Lee, H. K. and Kim, I. H., Corosolic acid, isolated from the fruit of *Crataegus pinnatifida* var. *psilosa* is a protein kinase C inhibitors as well as a cytotoxic agent. *Planta Med.*, 64, 468-470 (1998).

Fieser, L. F. and Fieser, M., Reagent for organic synthesis, John Wiley and Sons, INC., Vol. 1, p. 142 (1967).

Jeong, T. S., Hwang, E. I., Lee, H. B., Lee, E. S., Kim, Y. K., Min, B. S., Bae, K. H., Bok, S. H. and Kim, S. U., Chitin synthase II inhibitory activity of ursolic acid, isolated from *Crataegus pinnatifida, Planta Med.*, 65, 261-263 (1999).

Kashiwada, Y., Wang, H. K., Nagao, T., Kitanaka, S., Yasuda, I., Fyjioka, T., Yamagishi, T., Cosentino, L. M., Kozuka, M., Okabe, H., Ikeshiro, Y., Hu, C. Q., Yeh, E. and Lee, K. H., Anti-HIV Activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *J. Nat. Prod.*, 61, 1090-1095 (1998).

Kim, J. S. and Kim, I. H., Pharmaco-constituents of *Crataegus pinnatifida* var. *pubescens* leaves. *Yakhak Hoeji*, 37, 193-197 (1993).

Kwon, B. M., Lee, S. H., Choi, S. U., Park, S. H., Lee, C. O., Cho, Y. K., Sing, N. D., and Bok S. H., Synthesis and in vitro cytotoxicity of cinnamaldehydes to human solid tumor cells. Arch. Pharm. Res., 21, 147-152 (1998).

Kwon, H. C., Lee, K. R. and Zee, O. P., Cytotoxic constituents of *Pilea mongolica*. *Arch. Pharm. Res.*, 20, 180-183 (1997).

Min, B. S., Jung, H. J., Lee, J. S., Kim, Y. H., Bok, S. H., Ma, C. M., Nakamura, N., Hattori, M. and Bae, K. H., Inhibitory effect of triterpenes from *Crataegus innatifida* on HIV-1 Protease. *Planta Med.*, 65, 374-375 (1999).

Nakanishi, K., Goto, T., Ito, S., Natori, S., Nozoe, S., *Natural Products Chemistry Vol. 3*, Kodansa, Tokyo, (1983).

Oh, I. S. and Kim, I. H., Pharmaco-constituents of *Craetagus pinnatifida* var. *psilosa* leaves (I). *Yakhak Heoji*, 37, 476-482 (1993).

Oh, I. S., Whang, W. K. and Kim, I. H., Constituents of *Crataegus pinnatifida* var. *psilosa* leaves (II). *Arch. Pharm.*

Res., 17, 314-317 (1994).

Park, S. W., Yook, C. S. and Lee, H. K., Chemical components from the fruits of *Crataeguss pinnatifida* var. *psilosa*. *Kor. J. Pharmacog.* 25, 328-335 (1994).

Perry, L. M., *Medicinal Plants of East & Southeast Asia : Attributed Properties and Uses*. The MIT Press, Massachusetts, pp 342 (1980).

Skehan, P., Storeng, R., Schudiero, D. A., Monks, A., McMahan, J., Vistica, D., Warren, J. T., Bokesch, H., Kenny, S. and Boyd, M. R., New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.*, 82, 1107-1112 (1990).

Sohn, K. H., Lee, H. Y., Chung, H. Y., Young, H. S., Yi, S. Y. and Kim, K. W., Anti-angiogenic activity of triterpene acids. *Cancer Letters*, 94, 213-218 (1995).

Tommasi, N. D., Simone, F. D., Pizza, C., Mahmood, N., Moore, P. S., Conti, C., Orsi, N. and Srein, M. L., Constituents of *Eriobotrya japonica*. A study of their antiviral properties. *J. Nat. Prod.*, 55, 1067-1073 (1992).

Trumbull, E. R., Bianchi, E., Eckert, D. J., Wiedhopf, R. M. and Cole, J. R., Tumor inhibitory agents from *Vauquelinia corymbosa*. *J. Pharm. Sci.*, 65, 1407-1408 (1976).