Korean J. Medicinal Crop Sci. 14(5) : 299 - 302 (2006)

Cytotoxic Constituents from the Aerial Part of *Clematis apiifolia* L.

UiJung Youn*, WenYi Jin*, KyungSik Song, Yeon Hee Seong***, and KiHwan Bae*†**

**College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea.*
***College of Agriculture and life Scinece, Kyungpook Natinonal University, Daegu 702-701, Korea.*
****College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea.*

ABSTRACT : Three known triterpenoids were isolated from MeOH extract of *C. apiifolia* (Ranunculaceae). Their structures were identified as oleanolic acid (1), ursolic acid (2), hederagenic acid (3) by comparison of their physicochemical and spectral data with the literature values. Among them, 2 was isolated for the first time from this plant. The isolated compounds were evaluated for their cytotoxicity against L1210, HL-60, SK-OV-3 tumor cell lines. All compounds 1-3 were shown good activities with IC_{50} values ranging from 7.7 to 25.6 ㎍/㎖. This result suggests that triterpenoids 1-3 are main cytotoxic principles of this plant.

Key words : *Clematis apiifolia*, Ranunculaceae, triterpenoids, cytotoxicity

INTRODUCTION

The genus *Clematis* L., which comprises of 106 species worldwide, belong to Ranunculaceae. Among them, *Clematis apiifolia* L. is a species distributed all over Korea. Its aerial part has been used for a long time to treat asthma, beriberi, urination, and travail (Bae, 1999). Although it is widely used in Korean traditional medicine, it has been poorly investigated on phytochemical constituents and biological acitivity. A previous phytochemical study of *C. apiifolia* led to the isolation of four compounds stigmasterol, b-sitosterol, oleanolic acid, hederagenic acid (Woo *et al.*, 1976). This paper describes the isolation, structure elucidation, and cytotoxic activity of isolated compounds from aerial part of this plant.

MATERIALS AND METHODS

General procedure

Melting points were measured by using an Electrothermal apparatus. FT-IR spectra were recorded on a Jasco Report-100 infrared spectrometer. NMR spectra (^1H, 300 MHz, ^{13}C, 75 MHz) were measured by using a JEOL 400 or 600 FT-NMR spectrometers. For column chromatography, silica gel (Kieselgel 60, 70~230 mesh and 230~400 mesh, Merck) was used. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 (0.25 ㎜, Merck).

Plant material

The aerial part of *Clematis apiifolia* was collected at Yanggu, Kangwon, Korea in Aug. 15, 2002. The plant was identified by one of the authors, K. Bae. The voucher specimen (CNU-651) was deposited at the herbarium of the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and isolation

The dried aerial part (3 ㎏) was extracted with methanol (MeOH) three times under reflux for 4 h. The MeOH extracts were combined, filtered and concentrated to yield a dry MeOH extract (260 g). The MeOH extract (260 g) was suspended in distilled water and extracted with hexane, ethyl acetate (EtOAc), butanol (BuOH) to yield a hexane soluble fraction (35 g), an EtOAc soluble fraction (30 g) and a BuOH soluble fraction (34 g), respectively. As bioassay-guided fractionation shown in Table 1, the hexane fraction and EtOAc fraction showed moderate cytotoxic activity with inhibition values of 79.5% and 83.5% against L1210 tumor cell line, respectively. Therefore, we isolated compounds from hexane and EtOAc fractions.

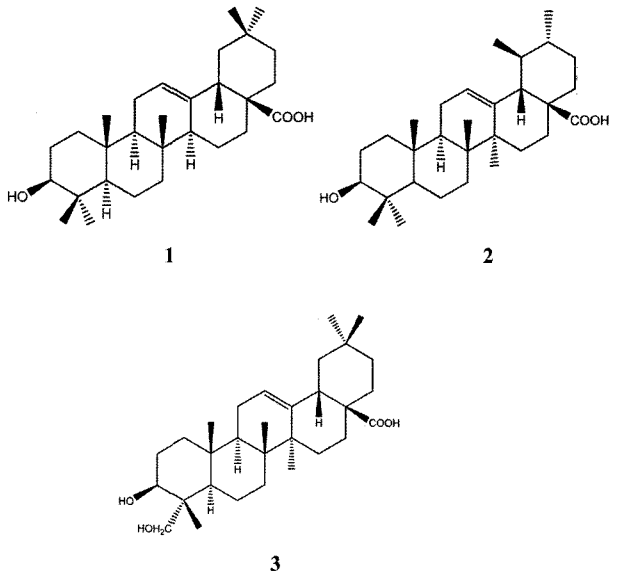
The hexane soluble fraction was chromatographed over a silica gel column, eluting with hexane-EtOAc (50 : 1~0 : 1) to afford four fractions (Fr. H1-H4). Fr. H3 was chromatographed on a silica gel column eluting with hexane-EtOAc (10 : 1~2 : 1) affording four subfractions (Fr. E31-Fr. H34). Among the subfractions, only subfraction Fr. H33 showed a high cytotoxicity (72.0% at 30 ㎍/㎖). This fraction was further chromatographed on a silica gel column eluted with $CHCl_3$-MeOH (5 : 1) to give compound **1** (16.5 ㎎).

† Corresponding author: (Phone) +82-42-821-5925 (E-mail) beakh@cnu.ac.kr
Received August 3, 2006 / Accepted September 28, 2006

Table 1. The cytotoxicity of the MeOH extract and the fractions against L1210 cell.

Fractions	Cytotoxicity[1] (%)
MeOH ex.	50.6 ± 0.8
Hexane fr.	79.5 ± 2.2
EtOAc fr.	83.5 ± 0.2
BuOH fr.	–[2]
Water fr.	–[2]
Fr.H33	72.0 ± 0.4
Fr.E2	83.4 ± 0.6
Fr.E3	82.1 ± 0.3

1 The inhibitory effect of fractions was examined at 30 ㎍/㎖.
2 Inactive.



1 2 3

Fig. 1. Structures of compounds (**1-3**) from *C. apiifolia*.

The EtOAc fraction was chromatographed over a silica gel column, eluting with $CHCl_3$-MeOH (50 : 1~1 : 1) to afford five fractions (Fr. E1-E5). Fr. E2 and Fr. E3 showed a high cytotoxicity (83.4 and 82.1 % at 30 ㎍/㎖). Fr. E2 was chromatographed on a silica gel column using $CHCl_3$-MeOH (10 : 1~5 : 1) as eluted solvent to give compound **2** (12.0 ㎎). Compound **3** (7.3 ㎎) was crystallized from Fr. E3 by using MeOH solvent.

Compound 1 : White amorpous powder; mp 296-298 ℃; IR $(KBr)_{max}$ cm^{-1}: 3420, 2930, 1720, 1678; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 5.14 (1H, m, H-12), 3.33 ((1H, t, J = 5.5, 11.4 Hz, H-3), 2.86 (3H, dd, J = 3.9, 13.5 Hz, H-18), 1.27 (3H, s, H-29), 1.15 (3H, s, H-30), 1.00 (3H, s, H-26), 0.93 (3H, br s, H-23), 0.93 (3H, br s, H-25), 0.79 (3H, s, H-27), 0.78 (3H, br s, H-24); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 15.5 (C-25), 15.9 (C-24), 17.4 (C-26), 18.7 (C-6), 23.3 (C-11), 23.8 (C-16), 23.9 (C-30), 26.3 (C-27), 27.5 (C-2), 28.0 (C-15), 28.4 (C-23), 31.0 (C-20), 32.8 (C-7), 33.0 (C-22), 33.4 (C-29), 34.2 (C-21), 37.4 (C-1), 37.8 (C-10), 39.1 (C-4), 39.6 (C-8), 41.4 (C-18), 42.0 (C-14), 46.2 (C-19), 46.9 (C-17), 48.0 (C-9), 55.6 (C-5), 79.4 (C-3), 121.5 (C-12), 143.8 (C-13), 178.6 (C-28).

Compound 2 : White powder; mp 287-288 ℃; IR $(KBr)_{max}$ cm^{-1}: 3430, 2924, 2855, 1728, 1694, 1458, 1387; ^{1}H-NMR (300 MHz, pyridine-d_5) δ: 0.78, 0.85 (3H, each, s, H-24, 25), 0.94 (3H, s, H-27), 0.96 (3H, d, J = 6.5 Hz, H-29), 0.97 (3H, s, H-23), 1.10 (3H, d, J = 6.5 Hz, H-30), 1.12 (3H, s, H-26), 2.20 (1H, br s, H-18), 3.16 (1H, dd, J = 10.4, 5.9 Hz, H-3), 5.22 (1H, t, J = 3.6 Hz, H-12); ^{13}C-NMR (75 MHz, pyridine-d_5) δ: 15.6 (C-24), 15.8 (C-25), 17.0 (C-26), 17.1 (C-29), 18.4 (C-6), 21.0 (C-30), 23.3 (C-11), 23.5 (C-27), 24.5 (C-16), 28.2 (C-2), 28.4 (C-23), 29.2 (C-15), 30.5 (C-21), 33.1 (C-7), 36.8 (C-22), 37.0 (C-10), 38.6 (C-1), 38.9 (C-20), 39.0 (C-19), 39.0 (C-4), 39.5 (C-8), 42.0 (C-14), 47.6 (C-9), 53.0 (C-18), 55.3 (C-5), 77.6 (C-3), 125.0 (C-12), 138.6 (C-13), 179.2 (C-28).

Compound 3 : Colorless crystal; mp > 300 ℃; IR $(KBr)_{max}$ cm^{-1}: 3455, 2944, 1725, 1699, 1466, 1389; ^{1}H-NMR (300 MHz, pyridine-d_5) δ: 5.48 (1H, s, H-12), 4.18 (1H, br t, J = 9.6 Hz, H-3a), 4.17 and 3.70 (1H each, d, J = 10.5 Hz, H-23), 3.29 (1H, dd, J = 3.6, 13.5 Hz, H-18), 1.22 (3H, s, H-27), 1.04 (3H, s, H-24), 1.03 (3H, s, H-26), 0.99 (3H, s, H-30), 0.96 (3H, s, H-25), 0.91(3H, s, H-29); ^{13}C-NMR (75 MHz, pyridine-d_5) δ: 13.1 (C-24), 15.9 (C-25), 17.4 (26), 18.6 (C-6), 23.7 (C-16), 23.7 (C-11), 23.8 (C-30), 26.1 (C-27), 27.6 (C-2), 28.3 (C-15), 30.9 (C-20), 32.9 (C-7), 33.2 (C-22), 33.2 (C-29), 34.2 (C-21), 37.2 (C-10), 38.8 (C-1), 39.7 (C-8), 42.1 (C-14), 42.8 (C-4), 42.9 (C-18), 46.4 (C-19), 46.6 (C-17), 48.1 (C-9), 48.6 (C-5), 67.9 (C-23), 73.4 (C-3), 122.5 (C-12), 144.8 (C-13), 180.1 (C-28).

Cytotoxic assay

Cells were maintained in RPMI 1640 including L-glutamine (JBI), 10% FBS (JBI), and 2% penicillin-streptomicin (GIBCO). Trypsin-EDTA was used to separate cell from culture flask. All cell lines were cultured at 37 ℃ in a 5% CO_2 incubator. Cytotoxicity was measured by a modified Microculture Tetrazolium (MTT) assay. Viable cells were seeded in the growth medium (180 ㎍/㎖) into 96 well microtiter plates (1×10^4 cells per each well) and incubated at 37 ℃, 5% CO_2. A test sample was dissolved in DMSO and adjusted to the final sample concentrations ranging from 1.875 ㎍/㎖ to 30 ㎍/㎖ by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to < 0.1%. After standing for 2 h, 20 ㎕ of the test sample was added to each well. The same volume of DMSO

was added to the control group well. Forty-eight hours after the test sample was added, 20 ㎕ MTT was added to each well (final concentration of 5 ㎍/㎖). Two hours later, the plate was centrifuged for 5 minutes at 1500 rpm, then the medium was removed and the resulting formazan crystals were dissolved in 150 ㎕ DMSO. The optical density (O.D.) was measured at 570 ㎚ using a Titertek microplate reader (Multiskan MCC/340, Flow). The IC_{50} value was defined as the needed concentration of sample to reduce 50% of absorbance relative to the vehicle-treated control.

RESULTS AND DISCUSSION

In our search of the biological constituents from the plants, the MeOH extract of *C. apiifolia* showed moderate cytotoxicity with inhibitory value of 50.6% against L1210 tumor cell line at 30 ㎍/㎖. Further bioassay-guided fractionation and isolation led to yield three compounds (**1-3**) from the hexane and EtOAc fractions.

Compound **1** was obtained from hexane soluble fraction as an amorphous powder and was deduced to be a triterpenoid based on positive Liebermann-Burchard test. In the IR spectrum, absorption bands at 3420 (OH), 2930 (CH), 1720 (C = O), 1678 (C = C) were apparent. The ^{1}H-NMR spectrum of **1** was characterized by signals for seven tertiary methyl groups and a triplet at δ 5.14 due to H-12. In addition, the carbinolic region revealed a double doublet at δ 3.33 (J = 5.5 and 11.4 Hz) whose chemical shift and coupling constants were in accordance with a 3β-OH substitution pattern. All these features were typical for an olean-12en-28-oic acid. The ^{13}C-NMR spectrum confirmed the above data and the presence of the 28-carboxyl function at δ 178.6 and the oleane skeleton by the chemical shifts of the sp^2 hybridezed carbons C-12 at δ 121.5 and C-13 at δ 143.8 respectively, that are useful to distinguish between urs-12-ene and olean-12-ene derivatives. Thus, **1** was identified as oleanolic acid by comparison of its spectral data with the literature values (Bax *et al.*, 1985).

Compound **2** was obtained from EtOAc soluble fraction as an amorphous powder and was deduced to be triterpenoid based on positive Liebermann-Burchard test. In the IR spectrum, absorption bands at 3430 (OH), 2924 (CH), 1728 (C = O), 1694 (C = C) were observed. The ^{1}H-NMR spectrum of compound **2** showed a triplet at δ 5.22 (J = 3.6 Hz) due to H-12, and a double of doublet at δ 3.16 (J = 5.9, 10.4 Hz) due to the axial H-3. Additionally, among seven methyl groups, five were singlet signals, and two were doublets (δ 0.96 and 1.10, 3H each, d, J = 6.5 Hz). In the ^{13}C-NMR, the chemical shifts of C-12, C-13, and C-28 were observed at δ 125.0, 138.6, and 179.2, respectively. These indicated that **2** could be an ursane-type triterpenoid. Accordingly, **2** was identified as ursolic acid by comparison of its spectral data with the literature values (Choi *et al.*, 1991).

Table 2. The cytotoxicity of compounds **1-3**.

Compounds	IC_{50} (㎍/㎖) [a]		
	L1210	HL-60	SK-OV-3
1	25.6 ± 0.3	21.2 ± 0.4	15.4 ± 0.3
2	8.3 ± 0.5	12.8 ± 0.4	7.7 ± 0.2
3	19.4 ± 0.7	16.7 ± 0.5	18.0 ± 0.4
AM [b]	0.8 ± 0.4	2.8 ± 0.2	2.5 ± 0.2

[a] IC_{50} values mean the 50% inhibition concentration and were calculated from regression lines using five different concentrations in triplicate experiments.
[b] Adriamycin was used as a positive control.

Compound **3** was obtained from EtOAc soluble fraction as a colorless crystal and was deduced to be triterpenoid based on positive Liebermann-Burchard test. In the IR spectrum, absorption bands at 3455 (OH), 2944 (CH), 1725 (C = O), 1699 (C = C) were observed. The ^{1}H-NMR spectrum of compound **3** showed six tertiary methyl groups at δ 0.91~1.22, a double doublet at δ 3.29 (J = 3.6 and 13.5 Hz, H-18), and two doublets protons at δ 4.17 and δ 3.70 with J = 10.5 Hz were assignable to two hydroxylated vicinal protons. ^{13}C-NMR spectrum showed two olefinic signals at δ 144.8 (C-13) and 122.5 (C-12), an acid signal at δ 180.1 (C-28), two oxygenated carbons at δ 73.4 (C-3) and 67.9 (C-23). All these evidence suggested an olean-type triterpenoid. Thus, compound **3** was identified as hederagenic acid (Jung *et al.*, 1993; Kizu and Tomimori, 1982). Accordingly, two compounds **1** and **3** have been isolated previously from this plant (Woo *et al.*, 1976). However, compound **2** was isolated for the first time from the plant *C. apiifolia*.

The isolated compounds were evaluated for their cytotoxicity against L1210, HL-60 and SK-OV-3 tumor cell lines. As shown in Table 2, compounds **1-3** were shown good activity against the tested cell lines with IC_{50} values ranging from 7.7 to 25.6 ㎍/㎖. Among them, compound **2** exhibited the strongest activity against all these cell lines with IC_{50} values of 8.3, 12.8, 2.5 ㎍/㎖, respectively.

The result suggests that triterpenoids **1-3** are main cytotoxic principles of this plant.

ACKNOWLEDGEMENT

This work was supported by the grant (PF0320302-00) from the Plant Diversity Research Center of the 21st century Frontier Program (2006) funded by the Ministry of Science and Technology of Korea.

LITERATURE CITED

Bae K (1999) The Medicinal Plants of Korea. Kyo-Hak Publishing Co., Seoul. p. 133.

Woo WS, Kang SS, Yoon MH (1976) Phytochemical study on *Clematis apiifolia*. Seoul Taehakkyo Saengyak Yonguso Opjukjip. 15:1-4.

Ju KH, Hwang BY, Ahn BT, Kim MJ, Choi WH (2000) Chemical compounents of *Evodia, daniellii* Hems. Korean Journal of Pharmacognosy. 31:300-305.

Bax A, Ferretti JA, Nashed N, Jerina DM (1985) Complete proton and carbon-13 NMR assignment of complex polycyclic aromatic hydrocarbons. J. Org. Chem. 50:3029.

Jung KY, Son KH, Do JC (1993) Triterpeniods from the roots of *Dipsacus asper*. Arch. Pharm. Res. 16:32-35.

Kizu H, Tomimori T (1982) Studies on the constituents of *Clematis* species. V. On the saponins of the root of *Clematis chinensis* Osbeck. Chem. Pharm. Bull. 30:3340-3346.

Choi YS, Son KH, Do JC (1991) Triterpenoids from the roots of *Rubus parvifolius*. Arch. Pharm. Res. 14:225-230.