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Isolation of *epi*-Oleanolic Acid from Korean Mistletoe and Its Apoptosis-Inducing Activity in Tumor Cells

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A triterpene was isolated as a cytotoxic principle from the dichloromethane extract of Korean mistletoe (KM; *Viscum album coloratum)* by repeated silica gel chromatography and recrystallization. In *in vitro* analysis of cytotoxic activity using various human and murine tumor cell lines, the dichloromethane extract of KM was highly cytotoxic against these cells. We isolated the most active compound, referred to VD-3, from the dichloromethane extract of KM. The VD-3 was shown to be less cytotoxic to normal cells (murine splenocytes). From the identification of the chemical structure of VD-3 by spectral data and chemical synthesis, the compound was proven to be *epi*-oleanolic acid. Tumor cells treated with VD-3 showed a typical pattern of apoptotic cell death, such as apparent morphological changes and DNA fragmentation. These results indicate that *epi*-oleanolic acid is an important compound responsible for antitumor activity of KM.

Key words: Korean mistletoe, *epi*-Oleanolic acid, Antitumor activity, Apoptosis

INTRODUCTION

Mistletoe (*Viscum album*) plant is a semi-parasite growing on various deciduous trees all over the world. It is well known that European mistletoe (EM; *Viscum album loranthaceae*) possesses a variety of biological functions such as antitumor (Kuttan *et al.*, 1990) and immunomodulating activity (Hajto *et al.*, 1990). Among various bioactive components of EM, lectins (Franz, 1986), alkaloids (Khwaja *et al.*, 1986), viscotoxins (Schrader and Apel, 1991), and polysaccharides (Jordan and Wagner, 1986) were considered to be responsible for the antitumor activity of EM.

On the other hand, Korean mistletoe (KM), a different subspecies of *Viscum album* from EM, was shown to be more cytotoxic against tumor cells *in vitro* than EM (Khwaja *et al.*, 1980). We also reported that the water extract of KM had antitumor activity to inhibit tumor metastasis (Yoon *et al.*, 1995), and enhanced natural killer (NK) cell activity in mice (Yoon *et al.*, 1998). In a series of studies on the biological activities of KM and its components, we demonstrated that the lectins of KM were cytotoxic to tumor cells, and its cytotoxicity was related to induction of apoptotic cell death (Yoon *et al.*, 1999). The lectins of KM were also shown to possess an immunomodulating activity to augment antigen-specific cellular and humoral immune responses (Yoon *et al.*, 2001). Furthermore, we found that the lectins of KM inhibited experimental lung metastasis of tumor cells in mice, and its antimetastatic activity was partly due to activation of macrophages and NK cells (Yoon *et al.*, 2003). Thus the lectins of KM were thought to be an important constituent associated with antitumor and immunomodulating activity of this plant. In addition to the lectins, Park *et al.* (1999) recently isolated alkaloids and viscotoxins from heat-treated KM, and demonstrated that these compounds had a strong cytotoxic effect on human tumor cell lines. Even though the lectins, alkaloids and viscotoxins were proven as active molecules related to cytotoxicity of KM, bioactive components responsible for its antitumor activity have not been fully understood yet.

In this study, we isolated a triterpene from the dichloromethane extract of KM, and determined its chemical

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structure and cytotoxic activity against tumor cells.

MATERIALS AND METHODS

General

The melting point was taken on a Gallenkamp melting point apparatus (Sanyo, Japan) and uncorrected. The EIMS spectrum was measured on QP-1000A (Shimadzu, Japan). The ^{1}H-and ^{13}C-NMR spectra were recorded with Bruker Avance Digital 400 spectrometer in $CDCl_3$ and chemical shifts were given as δ (ppm) from TMS (tetramethyl silane). The HPLC was Waters 515 system (U.S.A.) equipped with a refractive index detector. TLC was performed on pre-coated plate (Kiesel gel 60 F254, Merck). Silica gel for column chromatography was Kiesel gel 60 (70~230 mesh, Merck Art. 7734). KMs were collected from Mt. Jiri, Korea during June to July 1999. A voucher specimen was deposited in Department of Biotechnology and Food Science, Handong University.

Extraction and purification

KMs were air-dried in a well-ventilated fume hood. The dried and chopped whole plant (940 g) was extracted thrice with 10 L of dichloromethane in water bath for 3 h, and the resulting extract was concentrated *in vacuo*. The residue (38.9 g) was chromatographed on a silica gel column [(1.7×44.5 cm, hexane-ethyl acetate (10:1 to 1:1)] to give eleven fractions (I to XI). The most cytotoxic fraction VII (275 mg) was further purified by silica gel column chromatography [1.7×32 cm, benzene-ethyl acetate (20:1 to 15:1)]. Final purification using HPLC [column; Econosphere Silica 10u (250×10 mm, Alltech, U.S.A.), detector; RI (Waters model 415), mobile phase; benzene-ethyl acetate-acetic acid (15:1:0.3), flow rate; 0.6 mL min^{-1}] afforded an active compound (12 mg, t_R 12.37 min).

Analytical data

The active compound (designated as VD-3) was obtained as a white powder; melting point: 280±1 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.68, 0.76, 0.83, 0.86 (×s2), 0.88 (each 3H, s, CH_3×6), 1.07 (3H, s, 27-CH_3), 2.75 (1H, dd, *J*=4.0, 13.6 Hz, H-18), 3.34 (1H, brs, H-3), 5.21 (1H, t, *J*=3.4 Hz, H-12); ^{13}C-NMR (100 MHz, $CDCl_3$): Table I.

Synthesis of *oxo*-oleanolic acid

Oxo-oleanolic acid was synthesized by the method described previously (Cainelli and Cardillo, 1984; Huneck, 1953; Yagi *et al.*, 1978). In brief, PCC (pyridinium chlorochromate, 600 mg, 2.8 mmol) was added to a solution of oleanolic acid (400 mg, 0.88 mmol) dissolved in benzene (140 mL), and the reaction mixture was stirred for 8 h at room temperature. The product was filtered and evaporated to dryness. The residue was chromatographed on a silica gel with hexane-ethyl acetate (3:1) as a mobile phase. *Oxo*-oleanolic acid was re-crystallized from methanol as needles (100 mg). The structure of the final product was confirmed by the comparison of its TLC, ^{1}H-NMR, and ^{13}C-NMR data with those of the reported ones (Kwon *et al.*, 1997).

Synthesis of *epi*-oleanolic acid

Epi-oleanoloc acid was synthesized by the method described by Huneck (1953). The synthetic *oxo*-oleanolic acid (100 mg, 0.22 mmol) and aluminum *iso*-propoxide (300 mg, 1.47 mmol) were dissolved in *iso*-propanol (10 mL). The reaction mixture was stirred for overnight at 70°C. The reaction mixture was evaporated and the residue was suspended in 80 mL of 1N HCl. The suspension was extracted thrice with ether. The ether layer was finally purified by a silica gel column with hexane-ethyl acetate (3:1) to yield *epi*-oleanolic acid as a white powder (10 mg).

Assay of cytotoxic activity

Human and murine tumor cells (0.5-1×10^5/well) in 96-well plates were incubated with various doses of the indicated specimens at 37°C for 24 h. The cultures were added by 10 μL/well of Cell Counting Kit (Allexis, MA) solution, and incubated for 2 h before termination. Cytotoxic activity was determined using absorbance value of each well at 450 nm. The 50% inhibitory concentration (IC_{50}) values for cell growth were expressed as the dose resulting in 50% reduction of tumor cell growth.

Apoptosis analysis

Apoptosis of tumor cells treated with VD-3 was examined by DNA fragmentation assay described by Yoo *et al.* (1997). Briefly, VD-3-treated Colon 26 cells (1×10^6) were digested in 50 mL of sodium-*N*-lauroyl-sarcosinate, 10 mM EDTA and 50 mM Tris-HCl (pH 7.8) containing RNAse-A (20 μg/mL) at 50°C for 30 min. Digestion was continued for 60 min after addition of proteinase K (20 μg/mL). After centrifugation at 5,000 rpm for 1 min, DNA samples were loaded on 0.5% agarose gel containing 0.1 μg/mL ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The dichloromethane extract of KM was highly cytotoxic against RAW 264.7 cells in a dose-dependent manner (Fig. 1A). In a silica gel chromatography of the dichloromethane extract of KM, the fraction VII (F-7) was shown to be most active among 11 fractions (Fig. 1B). The further activity-guided purification led to the isolation of an active compound (designated as VD-3), which was obtained as

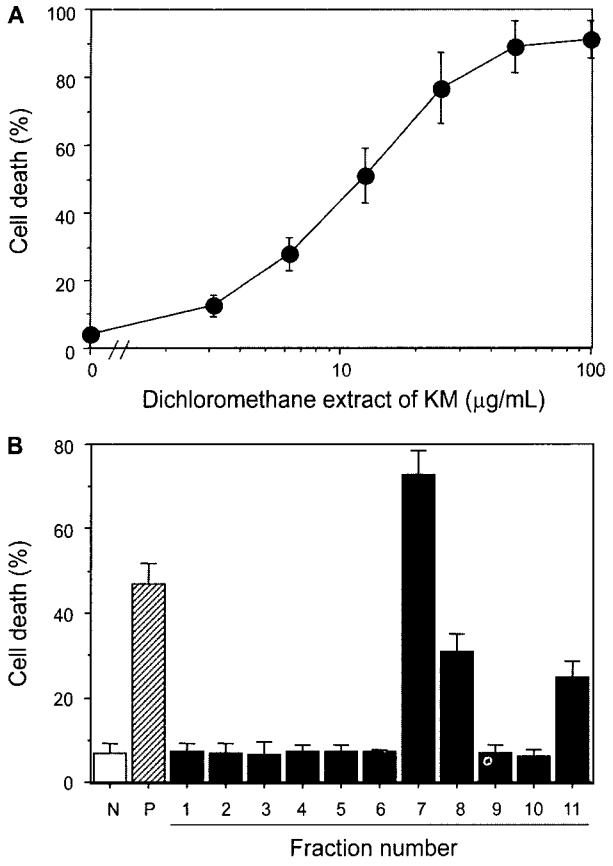

Fig. 1. Cytotoxic acticity of the dichloromethane extract of KM and its fractions. RAW 264.7 cells were treated with the indicated doses of the dichloromethane extract of KM (A), or with various fractions (10 μg/mL) obtained from a silica gel chromatography of the dichloromethane extract of KM (B) for 24 h. N: non-treated, P: treated with dichloromethane extract of KM (10 μg/mL).

a colorless amorphous powder. The molecular formula of $C_{30}H_{48}O_3$ was determined from Electron Impact Mass Spectroscopy (EIMS) and Distortionless Enhancement by Polarization Transfer (DEPT) data. In the mass spectrum, the base peak at *m/z* 248 ($C_{16}H_{24}O_2$) besides two peaks at *m/z* 203 ($C_{15}H_{23}$) and 133 ($C_{10}H_{13}$) strongly suggested a D^{12}-amyrin skeleton with a carboxyl group in ring D/E and hydroxyl groups in ring A and/or B (Budzikiewicz *et al.*, 1963; Akhtar and Malik, 1993). The presence of seven singlet methyl groups indicated the olean-12-ene skeleton (Budzikiewicz *et al.*, 1963; Seo *et al.*, 1975). The ^{1}H-NMR spectrum clearly showed an olefinic proton at δ 5.21 (1H, t, *J*=3.4 Hz, H-12), a methine proton at δ 2.75 (1H, dd., *J* = 4.0, 13.6 Hz, H-18), an α-carbinol proton at 3.34 (1H, brs, H-3), and seven methyl groups which were appeared at δ 0.76 to 1.07. The proton-decoupled ^{13}C-NMR and DEPT spectrum exhibited the presence of 30 carbon signals (CH_3x7, CH_2x10, CHx5, Cx8) including two olefinic carbon signal at 122.9 (CH) and 143.8 ppm (C) and a carbonyl carbon at 183.6 ppm (Table I). These data were

Table I. ^{13}C-NMR and DEPT data of VD-3

No	^{13}C(δ)	DEPT
1	33.0	CH_2
2	26.3	CH_2
3	76.4	CH
4	37.5	C
5	48.1	CH
6	18.4	CH_2
7	32.6	CH_2
8	39.6	C
9	47.6	CH
10	37.3	C
11	23.1	CH_2
12	122.9	CH
13	143.8	C
14	41.8	C
15	27.8	CH_2
16	23.5	CH_2
17	46.7	C
18	41.1	CH
19	46.0	CH_2
20	30.9	C
21	34.0	CH_2
22	33.3	CH_2
23	28.5	CH_3
24	22.4	CH_3
25	15.3	CH_3
26	17.4	CH_3
27	25.4	CH_3
28	183.6	C
29	32.6	CH_3
30	23.8	CH_3

consistent with oleanane type triterpenes having an OH group at C-3 position.

The ^{1}H- and ^{13}C-NMR data of an oleanolic acid were similar with those of VD-3. The major difference between oleanolic acid and VD-3 was the ^{13}C-NMR chemical shifts of the C-3 and C-24 carbon. The C-3 (76.4 ppm) and C-24 carbon (22.4 ppm) of VD-3 were downfield-shifted to 2.9 and 7.0 ppm, respectively, compared to those of oleanolic acid (C-3, 73.5 and C-24, 15.4 ppm). Also, the stereochemistry of the hydroxyl group at C-3 was deduced as a-type since the coupling constant of H-3 was almost zero (Akhtar and Malik, 1993; Chem *et al.*, 1983; Hylands *et al.*, 1980; Mahato and Kudu, 1994). Thus, VD-3 was postulated as *epi*-oleanolic acid (Fig. 2). This was confirmed

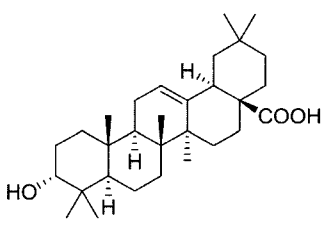

Fig. 2. Chemical structure of *epi*-oleanolic acid

by the comparison of ^{1}H-NMR and ^{13}C-NMR data with those in the previous report (Kwon *et al.*, 1997). In addition, when we synthesized *epi*-oleanolic acid and compared its cytotoxicity against RAW 264.7 cells with that of VD-3, we found that the cytotoxic activity of these two compounds was absolutely identical (Fig. 3).

In order to further characterize antitumor activity of *epi*-oleanolic acid, we carried out some experiments using VD-3. As described in Table II, VD-3 was highly cytotoxic against various types of murine as well as human tumor cells. Interestingly, however, the compound showed just a low cytotoxic activity against normal cells (Fig. 4). Treatment with VD-3 caused apparent typical apoptotic bodies in tumor cells (Fig. 5A), and the cells undergoing cell death exhibited fragmented DNA (Fig. 5B). These results suggest that *epi*-oleanolic acid has antitumor activity to induce apoptosis in tumor cells, and this compound is one of the major components responsible for the cytotoxic activity of KM.

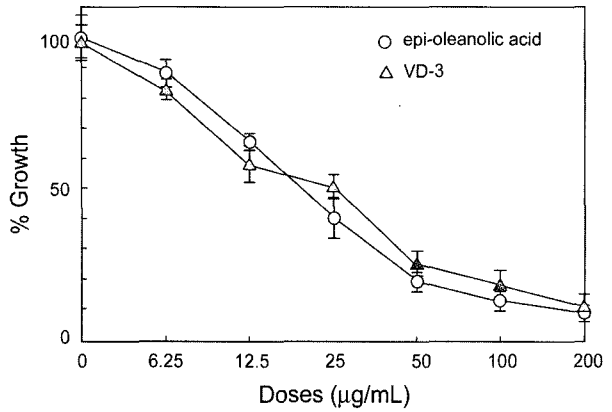

Fig. 3. Comparison of cytotoxic activity between VD-3 and *epi*-oleanolic acid in tumor cells. RAW 264.7 cells were treated with various doses of VD-3 or *epi*-oleanolic acid for 24 h.

Table II. Cytotoxic effect of VD-3 on various tumor cell lines

Cell lines	Origin	IC_{50} (μg/mL)
B16-BL6	murine melanoma	16.5
RAW 264.7	murine leukemia	18.7
Colon 26	murine carcinoma	28.3
P388	murine carcinoma	12.7
NIH-3T3	murine fibroblast	16.5
THP-1	human leukemia	94.5
MDA-435S	human carcinoma	7.5

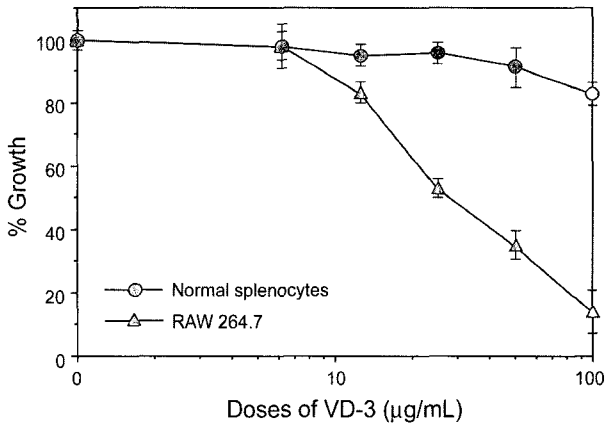

Fig. 4. Effect of VD-3 on the growth of normal cells. Murine splenocytes or RAW 264.7 cells were treated with the indicated doses of VD-3 for 24 h. Murine splenocytes were prepared with single cell suspension of the spleens of normal mice.

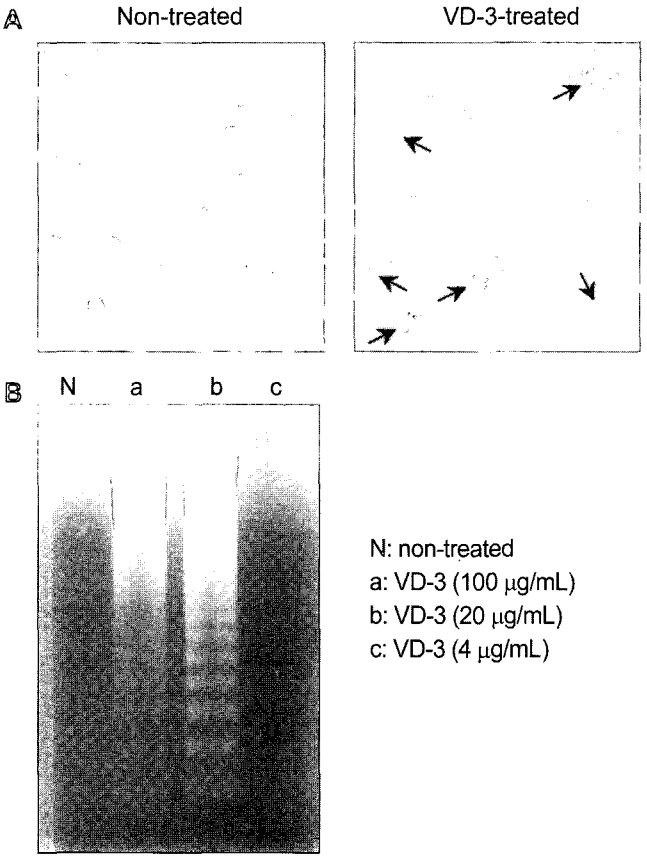

Fig. 5. Characterization of VD-3-induced cell death in tumor cells. Colon 26 cells were incubated with various doses of VD-3 for the indicated times. (A) Morphological alterations of the cells incubated for 3 hr in the presence of VD-3 (50 μg/mL) were observed under a phase-contrast microscope. Arrows indicate cells showing apparent apoptotic bodies. (B) DNA fragmentation of Colon 26 cells undergoing apoptosis was examined 24 h after VD-3 treatment.

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