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ent-Kaurane- and *ent*-Pimarane-Type Diterpenoids from *Siegesbeckia pubescens* and Their Cytotoxicity in Caki Cells

Sanghyun Lee*†, Eun Jung Noh, Jung Sook Kim*, Eun Mi Son*, Xu Pan*, Yeong Shik Kim**, Bak-Kwang Kim***, and Burm-Jong Lee***

*Biohealth Products Research Center, Inje University, Gimhae 621-749, Korea
**Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea
***College of Pharmacy, Seoul National University, Seoul 151-742, Korea

ABSTRACT : *ent*-Kaurane- and *ent*-pimarane-type diterpenoids were isolated from the methanol extract of *Siegesbeckia pubescens* by column chromatography. Their structures were elucidated as *ent*-16αH,17-hydroxy-kauran-19-oic acid (1), *ent*-4,17-dihydroxy-16α-methyl-kauran-19-oic acid (2), *ent*-16β,17-dihydroxy-kauran-19-oic acid (3), kirenol (4) and *ent*-16β,17,18-trihydroxy-kauran-19-oic acid (5) by spectral analysis. The cytotoxicity of these compounds in Caki cells was assayed by a cell counting kit. Only one group treated with kirenol (4), an *ent*-pimarane-type diterpenoid, showed the inhibition of the cell growth in Caki cells.

Keywords: *Siegesbeckia pubescens*, compositae, diterpenoid, cytotoxicity, Caki cell

The plants of *Siegesbeckia pubescens* are annual herbs widely distributed in Korea and they have been used as a traditional medicine to treat rheumatic arthritis, hypertension, malaria and neurasthenia.

Investigation on the isolation of sesquiterpenoids, diterpenoids and steroids from *S pubescens* (Han *et al*, 1973a; Han *et al*, 1973b; Han *et al*, 1975; Jiang *et al*, 1992; Kim 1972; Kim & Ahn 1988; Kim & Han 1974; Kim *et al*., 1979; Kim *et al*, 1980; Murakami *et al*, 1973; Oh, 1973), and sesquiterpene lactones, germacranolides, melampolides, geranylnerol derivatives and *ent*-pimarenes from *S orientalis* (Giang *et al*, 2005; Gua *et al*., 1997; Xiang *et al*, 2004; Zdero *et al*, 1991) was investigated. It was reported the various activities of *S pubescens* (Ahn *et al*, 1998; Kang *et al*, 1997; Kim 1997; Kim *et al*, 1997; Kim 1980; Kim *et al*, 1980), *S orientalis* (Hwang *et al*, 2001) and *S glabrescens* (Dong *et al*, 1989; Kim *et al*., 2001). But there is no report on the cytotoxicity of compounds from *S pubescens* grown in Korea as a medicinal crop in Caki cells.

We report the isolation and structure elucidation of compounds from *S pubescens* and describe the evaluation of the cytotoxicity of compounds in Caki cells.

†Corresponding author. (Phone) +82-55-320-3812 (E-mail) jnp@korea.com
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MATERIALS AND METHODS

Plant-material

Siegesbeckia pubescens Makino produced in Korea was purchased from the Kyung Dong Market, Seoul, Korea in March 2001 and verified by Prof. Emeritus D. S. Han, Seoul National University, Korea.

Instruments and Reagents

MS spectrum was measured with a Jeol JMS-AX505WA mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AVANCE 400 NMR spectrometer in pyridine-d_5 using TMS as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (*J*) were expressed in hertz. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by spraying with 20% H_2SO_4 followed by charring at 100 °C. Silica gel (Merck, 200-400 mesh ASTM) was used for column chromatography. All other chemicals and reagents were analytical grade.

Extraction and Isolation

The air-dried and powdered herbs of *S pubescens* (5 kg) were extracted three times with MeOH under reflux. The resultant extracts were combined and concentrated under reduced pressure to afford 1125 g of the residue. The MeOH extract was suspended in water, and then fractionated successively with equal volumes of *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH, leaving residual H_2O soluble fraction. Each fraction was evaporated *in vacuo* to yield the residues of *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. A portion of the $CHCl_3$ fraction was chromatographed on a silica gel column (7 × 60 cm) eluting with a gradient of *n*-hexane-EtOAc to

afford compounds **1** (78 mg, 80:20) and **3** (24 mg, 75:25). A portion of the EtOAc fraction was chromatographed on a silica gel eluting with a gradient of $CHCl_3$-MeOH to afford compounds **2** (3 mg, 90:10), **4** (56 mg, 80:20) and **5** (17 mg, 80:20).

Compound **1**: EI-MS (70 eV, rel. int. %): *m/z* 320 $[M]^+$ (42), 302 (100), 274 (82), 243 (19); IR ν_{max}: 3414, 2919, 1693 cm^{-1}; ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, Pyridine-d_5): see Tables 1 and 2.

Compound **2**: EI-MS (70 eV, rel. int. %): *m/z* 320 $[M-O]^+$ (31), 302 (80), 274 (100), 243 (24); IR ν_{max}: 2923, 2853, 1641 cm^{-1}; ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, Pyridine-d_5): see Tables 1 and 2.

Compound **3**: EI-MS (70 eV, rel. int. %): *m/z* 336 $[M]^+$ (2), 318 (11), 305 (100), 287 (27), 259 (46); IR ν_{max}: 3434, 2943, 1697 cm^{-1}; ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, Pyridine-d_5): see Tables 1 and 2.

Compound **4**: EI-MS (70 eV, rel. int. %): *m/z* 338 $[M]^+$ (3), 307 (2), 277 (100), 259 (97), 241 (19); IR ν_{max}: 3323, 2935, 1636, 1033 cm^{-1}; ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, Pyridine-d_5): see Tables 1 and 2.

Compound **5**: EI-MS (70 eV, rel. int. %): *m/z* 334 $[M-H_2O]^+$ (3), 321 (100), 303 (23), 285 (31), 257 (40); IR ν_{max}: 3430, 2914, 1704, 1034 cm^{-1}; ^1H- and ^{13}C-NMR (400 and 100 MHz, respectively, Pyridine-d_5): see Tables 1 and 2.

Cell culture

Caki cells, renal carcinoma cells, were obtained from the American Type Culture Collection (Rockville, Md). These cells were maintained at subconfluence in 95% air, 5% CO_2 humidified atmosphere at 37 °C. The medium used for routine subculture was Dulbecco's Modified Eagle's Medium (DMED, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were counted with a hemocytometer and the number of viable cells was determined by trypan blue dye exclusion.

Cell viability assay

Cytotoxicity of compounds was evaluated by Cell Counting Kit (CCK-8) purchased from Dojindo Laboratories (Tokyo, Japan). Caki cells were plated at a density of 1.0×10^4 cells/well into 96-well plate and incubated for 24 hr. They were changed with new media and incubated with various concentrations of the compounds for 24 hr. After incubation, 10 ml of CCK-8 solution was added each well and incubated for 3 hr in the incubator. The absorbance was read at 450 nm with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA). Cell viability was expressed as a percentage of the control.

RESULTS AND DISCUSSION

A portion of the *n*-hexane and EtOAc fractions were chromatographed on a silica gel to afford five diterpenoids (**1-5**).

Compounds **1** and **2** were obtained as white crystals from MeOH. The EI-MS of **1** and **2** showed a molecular ion peak at *m/z* 320 and *m/z* 336, respectively. The ^1H- and ^{13}C-NMR spectra of **1** and **2** were similar to each other, while suggesting **1** and **2** to be *ent*-kaurane-type diterpenes (Tables 1 and 2). In the ^1H-NMR spectra, the methylene signals of **1** and **2** were observed at δ 3.66, 3.64 (each 1H, d, J = 8.5 Hz) and δ 3.66, 3.63 (each 1H, d, J = 10.8 Hz), respectively, and the methyl signals at δ 1.34 and 1.15 of **1** and δ 1.23 and 1.35 of **2**. The ^{13}C-NMR spectra revealed that **1** and **2** have a carboxyl carbon (δ 180.2 and 180.1, respectively), a carbon bearing hydroxy (δ 67.0 and 67.1, respectively) and methyl carbons (δ 29.4, 16.0, and 29.4, 16.1, respectively). Therefore, **1** and **2** were elucidated as *ent*-16αH,17-

Table 1. ^1H-NMR data of compounds **1-5***.

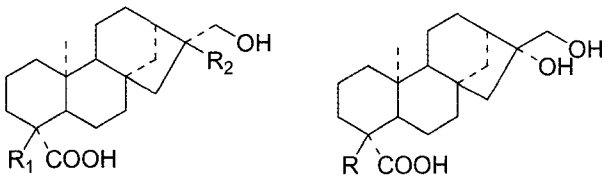
Position	1	2	3	4	5
2α	-	-	-	4 19 *tt* (9.5, 3.8)	-
14	-	-	-	5.43 *br s*	-
17a	3.66 *d* (8.5)	3.66 *d* (10.8)	4.13 *d* (10.9)	-	4.12 *d* (10.9)
17b	3.64 *d* (8.5)	3 63 *d* (10.8)	4 05 *d* (10.9)	-	4 03 *d* (10.9)
18a	-	-	-	-	4.40 *d* (10.1)
18b	-	-	-	-	3.99 *d* (10.1)
19a	-	-	-	4.05 *d* (10.4)	-
19b	-	-	-	3.65 *d* (10.4)	-
Me-16	-	1.23 *s*	-	-	-
Me-17	-	-	-	1.16 *s*	-
Me-18	1.34 *s*	1 35 *s*	1.33 *s*	1.27 *s*	-
Me-20	1.15 *s*	-	1.17 *s*	0.80 *s*	1.26 *s*

*Chemical shifts are given in ppm values (pyridine-d_5).

Table 2. ^{13}C-NMR data of compounds **1-5***.

Position	1	2	3	4	5
1	41 2	41.3	40 9	49.5	40.8
2	19.9	19.9	19.8	63 9	19.5
3	37.4	37.5	37.8	45.8	33.1
4	43.9	43.9	40.0	41.0	50 5
5	57.1	57 3	53.8	55.6	51.3
6	23.2	23.3	22.9	22.7	22 7
7	42.2	42.2	42.7	36.8	42.5
8	45.1	45.1	45.9	138.1	44 8
9	55.7	55.8	56.9	51.4	56.4
10	40.0	40.0	43.9	39.8	39.8
11	19.2	19.3	18.9	19.1	19.0
12	31.9	31.9	26.8	32.9	26.8
13	38.7	38.8	44.9	38.0	45.9
14	37.4	38 8	38.7	129.9	37.8
15	45.8	45.8	56.2	76.7	53.8
16	44.2	44.2	81.6	64.0	81 6
17	67.0	67.1	66.4	23.3	66.4
18	29.4	29.4	29.3	28 3	70.4
19	180.2	180.1	180.1	64.9	179.0
20	16.0	16.1	16 0	17.0	16.2

*Chemical shifts are given in ppm values (pyridine-d_5).



1 R_1: CH_3, R_2: H

2 R_1: OH, R_2: CH_3

3 R: CH_3

5 R: CH_2OH

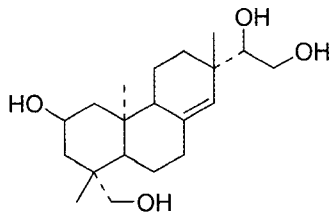


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Fig. 1. Structures of compounds **1-5**

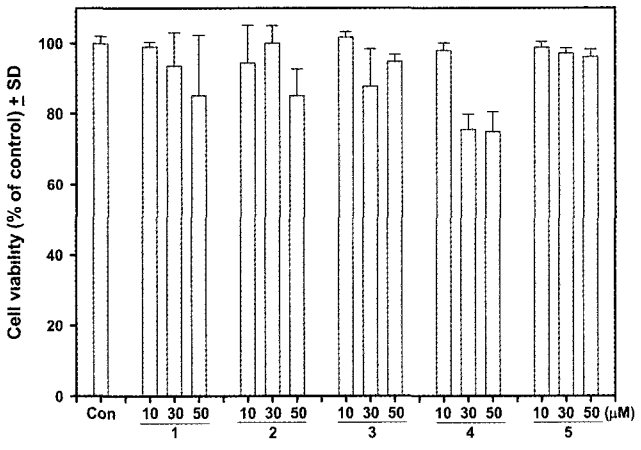

Fig. 2. Cell viability of compounds **1-5** in Caki cells.

hydroxy-kauran-19-oic acid and *ent*-4,17-dihydroxy-16α-methyl-kauran-19-oic acid, respectively, by comparing their spectral data in the literature (Jiang *et al.*, 1992).

Compounds **3** and **5** were obtained as white crystals from MeOH. The EI-MS of **3** and **5** showed a molecular ion peak at *m/z* 336 and *m/z* 352, respectively. The ^{1}H- and ^{13}C-NMR spectra of **3** and **5** were similar to each other, while suggesting **3** and **5** to be *ent*-kaurane-type diterpenes like compounds **1** and **2** (Tables 1 and 2). In the ^{1}H-NMR spectra, the methylene signals were observed at δ 4,13, 4.05 (each 1H, d, J = 10.9 Hz) of **3**, and δ 4.40, 3.99 (each 1H, d, J = 10.1 Hz) and δ 4.12, 4.03 (each 1H, d, J = 10.9 Hz) of **5**, and the methyl signals at δ 1.33 and 1.17 of **3** and δ 1.26 of **5**. The ^{13}C-NMR spectra revealed that **3** and **5** have a carboxyl carbon (δ 180.1 of **3** and 179.0 of **5**), a carbon bearing hydroxy (δ 66.4 of **3** and 66.4, 70.4 of **5**) and methyl carbons (δ 29.3, 16.0 of **3** and 16.2 of **5**). Therefore, **3** and **5** were elucidated as *ent*-16β,17-dihydroxy-kauran-19-oic acid and *ent*-16β,17,18-trihydroxy-kauran-19-oic acid, respectively, by comparing their spectral data in the literature (Jiang *et al*, 1992).

Compound **4** was obtained as yellow-white crystals from MeOH. The EI-MS of **4** showed a molecular ion peak at *m/z* 338. In the ^{1}H-NMR spectrum, the methylene signals of **4** observed at δ 4.05, 3.65 (each 1H, d, J = 10.4 Hz). The ^{13}C-NMR spectrum revealed that **4** have carbon bearing hydroxys (δ 76.7, 64.9, 64.0 and 63.9) and three methyl carbons (δ 28.3, 23.3 and 17.0), while suggesting **4** to be an *ent*-pimarane-type diterpene (Tables 1 and 2). Therefore, **4** were elucidated as kirenol by comparing its spectral data in the literature (Jiang *et al* , 1992).

The cytotoxicity of compounds **1-5** from *S pubescens* in Caki cells, renal carcinoma cells, was assayed by a cell counting kit and shown in Fig. 2. Only one group treated with kirenol (**4**), an *ent*-pimarane-type diterpenoid, showed the inhibition of the cell growth in Caki cells. But other compounds (**1**, **2**, **3** and **5**), *ent*-kaurane-type diterpenoids, have no inhibition of the cell growth in Caki cells. Therefore, a cytotoxic compound, kirenol (**4**) from *S pubescens*, can be suitable for the anti-tumor agent. Further studies will be needed to clarify the mechanism of inhibition of Caki cell growth by kirenol (**4**).

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