Journal of Medicinal Plants Research Vol.6 (27), pp. 4409-4415, 18 July, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.344
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Cytotoxic saikosaponins from *Bupleurum yinchowense*

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Accepted 15 March, 2012

Activity-guided fraction of the ethanolic extract of the root from *Bupleurum yinchowense* resulted in the isolation of 13 saikosaponins. Their structures were identified to be saikosaponin a (1), saikosaponin c (2), saikosaponin d (3), saikosaponin b_2 (4), saikosaponin f (5), saikosaponin b4 (6), 6"-*O*-acetylsaikosaponin a (7), 3β,16α,23,28-tetrahydroxy-olean-11,13(18)-dien-29-oic acid 3-*O*-*β*-D-glucopyranosyl-(1→3)-*β*-D-fucopyranoside (8), chikusaikosidel(9), saikogenin F (10), saikosaponin e (11), 6"-*O*-acetylsaikosaponin d (12), saikosaponin 14 (13) on the basis of their spectral data and chemical evidences. Compound 8 is a new natural product and the other 12 compounds were separated from this plant for the first time. Compounds 1 to 10 were evaluated *in vitro* for their inhibitory ability against the growth of human esophageal cancer cell lines (Eca-109), human colon cancer cell lines (W-48), human cervical cancer cell lines (Hela), human ovarian cancer (SKOV3). Compounds 1, 3 and 7 exhibited significant inhibitory activities against the tested cell lines, with the IC_{50} values not more than 15 µg/ml.

Key words: *Bupleurum yinchowense*, umbelliferae, saikosaponins, cytotoxic, 3β,16α,23,28-tetrahydroxy-olean-11,13(18)-dien-29-oic acid 3-*O*-*β*-D-gluco-pyranosyl-(1→3)-*β*-D-fucopyranoside.

INTRODUCTION

The genus *Bupleurum* is a well-known and very important crude drug in traditional oriental medicine, especially in Chinese and Japanese traditional medicine. Preparations containing the roots of *Bupleurum* species have been prescribed for more than 2000 years in China where the first record about their use appeared in *Shen-Nong's* Herbal (Xie et al., 2009). Many *Bupleurum*-containing herbal drugs have been traditionally used in the treatment of tumours and cancers. Much research has shown that the genus *Bupleurum* mainly accumulates saikosaponins (Ding et al., 1986; Luo et al., 1993; Ebata et al., 1996;Zhao et al., 1996; Tan et al., 1999; Sánchez-Contreras et al., 2000). Moreover, several saikosaponins of *Bupleurum* have been evaluated for the anti-tumour and anti-proliferative effects. The extracts from *Bupleurum scorzonerifolium and Bupleurum kaoi* were assessed against A549 human lung cancer cells (Cheng et al., 2003). The saikosaponins from *Bupleurum rotundifolium* and *Bupleurum wenchuanense* demonstrated cytotoxicity against human gastric adenocarcinoma (MK-1), human cervical carcinoma (HeLa), murine melanoma (B16F10) cell lines, leukaemia P-388 cells and nasopharynx carcinoma KB cells (Fujioka et al., 2003, 2006). In our search for antitumour agents from Chinese herbs, we found out that the crude saikosaponins from the root of *Bupleurum yinchowense* specifically inhibited human esophageal cancer cell lines (Eca-109), human colon cancer cell lines (W48), human

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Abbreviations: ELISA, Enzyme-linked immuno sorbent assay; **MTT,** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PBS,** phosphate-buffered saline; **EDTA,** ethylenediaminetetraacetic acid; **DMSO,** dimethyl sulfoxide; **TBS,** tris buffered saline.

ovarian cancer cell lines (SKOV3) and human cervical cancer cell lines (Hela) *in vitro*. *B. yinchowense* is abundantly distributed in the Northwest of China and widely used in Chinese folk medicine. No evidence is available in the literatures concerning its constituents and pharmacological activities. To systematically evaluate its potential anticancer activity, the constituents were studied by activity-guided fraction and result in the isolation of 13 saikosaponins. Their structures were identified on the basis of spectral data. The isolation, structure elucidation and evaluation for cytotoxic activities were described in this study.

MATERIALS AND METHODS

General experimental procedure

Nuclear magnetic resonance (NMR) spectra were recorded on a Brucker DRX-500 spectrometer (Brucker Biosciences Corporation, Billerica, MA) with tetramethylsilane (TMS) as internal standard operating at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. Fast atom bombardment-mass spectra (FAB-MS) and high resolution-fast atom bombardment-mass spectra (HR-FABMS) were recorded on a Micromass Autospec-Q instrument (Micromass Ltd., Manchester, UK). Infrared (IR) spectra were recorded in KBr discs using a Perkin-Elmer 983G spectrophotometer (Perkin-Elmer Ltd., USA). Gas chromatography (GC) analysis was carried out on an Agilent 6890N gas chromatography (Agilent Co., USA) using an HP-5 capillary column. Column chromatography was performed with silica gel (100 to 200 mesh, Qingdao Marine Chemical Co., Qingdao, P. R. China), Sephadex LH-20 (25 to 100 μm, GE Healthcare Biosciences AB, Uppsaki, Sweden), octadecyl silica (25 to 40 μm, Merck, USA), D101 macroporous resins (Tianjin Gujiao Factory, Tianjin, P. R. China), MCI Gel CHP20P (75 to 150 μm, Mitsubishi Chemical, Japan). Thin layer chromatography (TLC) was performed on precoated silica gel GF_{254} (0.2 mm thick, Qingdao Marine Chemical Co., Qingdao, P. R. China) and spots were detected by spraying with 10% ethanolic H_2SO_4 reagent. 3-(4, 5-dimethylthiiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant collection

The roots of *B. yinchowense* were collected from Dingxi County, Gansu province, China, in August, 2009 and identified by the author, Professor Ruile Pan of the Institute of Medicinal Plant, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, where a voucher specimen (No. 20090815) was deposited.

Extraction

The dried and powdered roots (800 g) of *B. yinchowense* were extracted with 60% ethanol containing 0.5% ammoniae aqua (three times, 1 L each) at room temperature for 12 h. The ethanol extracts were combined and evaporated *in vacuo*, to yield a dark brown residue (120 g), which was dissolved in H_2O-MeOH (5:95) solution (200 ml), and then portioned with n-hexane of 200 ml) to get the n-hexane-soluble fraction. The H_2O-MeOH (5:95) layer was evaporated to remove residual MeOH, and then distilled water (200 ml) was added. This aqueous solution was subjected to a column contained 1 kg D101 macroporous resin and was eluted successively with water (2 L), 90% ethanol (2 L), respectively. Evaporation of the respective solvents gave n-hexane (12 g), water (42 g) and 90% ethanol (32 g) fractions. The 90% ethanol fraction is saponin-enriched part. Each fraction was evaluated for the cytotoxic activity on the tumor cell lines (Table 1). It was shown that the activity resided in the saponin-enriched part.

Isolation

Saponin-enriched part (32 g) was subjected to MCI column, eluting with a gradient of water-methanol (from 100:0 to 5:95), to yield 5 fractions. Fraction 2 (8 g) was chromatographed repeatedly on silica gel using chloroform-methanol (8:2) and octadecylsilane (ODS)-C_{18} with the elution of methanol-water (7:3) to afford 1 (80 mg), 2 (55 mg), 3 (75 mg), 11 (5 mg) and 9 (17 mg). Fraction 3 (10 g) was separated into three sub-fractions by ODS column using methanol-water (7:3) as elution and the second sub-fraction was subjected to repeated column chromatography, first on silica gel, chloroform:methanol (8:2) and purified on pharmadex LH-20 (methanol) to obtain 4 (51 mg), 5 (38 mg), 6 (20 mg) and 12 (8 mg). Compound 7 (35 mg), 8 (37 mg), 13 (6 mg) and compound 10 (22 mg) were purified from Fraction 4 (5 g) by repeated semipreparative high performance liquid chromatography (HPLC) using methanol-water (60:40) as fluent.

Characterization of isolation

Saikosaponin a (1): White amorphous powder, mp 229 to 230°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.91, 0.92, 0.99, 1.12, 1.31, 1.37 (each 3H, s, tert-Me×6), 1.47 (3H, d, *J*=6.6 Hz, Fuc-CH_3), 4.94 (1H, d, *J*=7.8 Hz, Fuc-1′-H), 5.35 (1H, d, *J*=8.4 Hz, Glc-1″-H), 5.66 (1H, dd, *J*=10.2, 2.4 Hz, 11-H), 6.00 (1H, d, *J*=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin c (2): White amorphous powder, mp 207 to 209°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.86, 0.91, 0.96, 0.97, 1.14, 1.28, 1.35 (each 3H, s, tert-Me × 7), 1.66 (3H, d, *J*=6.0 Hz, Rha-CH_3), 4.94 (1H, d, *J*=7.8 Hz, Glc-1′-H), 4.52 (1H, d, *J*=9.0 Hz, Rha-1″-H), 4.79 (1H, d, *J* =7.8 Hz, Glc-1‴-H), 5.64 (1H, dd, *J*=10.2, 2.4 Hz, 11-H), 5.90 (1H, d, *J*=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin d (3): White amorphous powder, mp 227 to 228°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.91, 0.92, 0.99, 1.12, 1.31, 1.37 (each 3H, s, tert-Me×6), 1.47 (3H, d, *J*=6.6 Hz, Fuc-CH_3), 4.94 (1H, d, *J*=7.8 Hz, Fuc-1′-H), 5.35 (1H, d, *J*=8.4 Hz, Glc-1″-H), 5.66 (1H, dd, *J*=10.2, 3.0 Hz, 11-H), 6.02 (1H, d, *J*=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin b_2 (4): White amorphous powder, mp 201 to 203°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.89, 0.92, 0.99, 1.01, 1.04, 1.68 (each 3H, s, tert-Me×6), 1.45 (3H, d, *J*=6.0 Hz, Fuc-CH_3), 4.99 (1H, d, *J*=7.8 Hz, Fuc-1′-H), 5.40 (1H, d, *J*=7.8 Hz, Glc-1″-H), 6.70 (1H, dd, *J*=10.2, 1.8 Hz, 11-H), 5.72 (1H, d, *J*=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin f (5): White amorphous powder, mp198 to 200°C ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.82, 0.95, 0.99, 1.00, 1.01, 1.29, 1.35 (each 3H, s, tert-Me×7), 1.65 (3H, d, *J*=6.0 Hz, Rha-CH_3), 4.94 (1H, d, *J*=7.8 Hz, Glc-1′-H), 4.52 (1H, d, *J*=9.0 Hz, Rha-1″-H), 4.79 (1H, d, *J*=7.8 Hz, Glc-1‴-H), 5.86 (1H, br, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin b4 (6): White amorphous powder, mp 206 to 208°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.97, 1.01, 1.01, 1.12, 1.14, 1.88 (each 3H, s, tert-Me×6), 3.26 (3H, s, OCH_3), 1.45 (3H, d, J=6.0 Hz, Fuc-CH_3), 4.96 (1H, d, J=7.2 Hz, Fuc-1′-H), 5.33 (1H, d, J=7.8 Hz, Glc-1″-H), 5.60 (1H, d, J=3.0 Hz, 12-H).^{13}C-NMR data (Table 1).

6″-*O*-acetylsaikosaponin a (7): White amorphous powder, mp 204 to 205°C, ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.89, 0.92, 0.92, 0.98,

Table 1. ^{13}C-NMR data for compounds 1 to 12 (C_5D_5N, 125 MHz).

	1	2	3	4	5	6	7	8	9	10	11	12
1	38.6	38.5	38.6	38.4	38.8	40.1	38.6	38.4	38.6	38.4	39.8	38.6
2	26.6	26.5	26.2	26.1	26.6	26.4	25.8	26.1	26.6	26.1	26.3	25.7
3	88.9	89.0	81.6	81.7	89.1	81.9	81.6	81.7	88.6	81.7	88.8	81.6
4	39.8	39.7	43.7	43.7	39.5	43.8	43.7	43.7	39.8	43.6	39.8	43.7
5	55.4	55.3	47.3	47.4	55.7	48.4	47.3	47.3	55.4	47.4	55.7	47.4
6	18.2	18.1	17.5	18.9	18.5	18.3	17.3	18.2	18.2	17.3	18.3	17.6
7	31.9	31.9	31.5	32.3	33.4	33.4	31.6	32.3	31.9	31.9	33.3	31.6
8	42.2	42.2	41.9	41.1	40.2	40.7	42.2	41.1	42.2	41.9	41.0	42.2
9	53.0	52.8	53.0	54.0	47.1	51.7	53.1	54.1	53.0	53.0	52.0	53.2
10	36.4	36.3	36.8	35.5	36.8	38.2	36.3	36.5	36.4	36.3	38.1	36.3
11	132.0	132.1	132.0	126.2	24.0	76.0	132.2	126.1	132.0	132.0	76.0	132.2
12	131.2	131.2	131.9	126.3	122.7	122.5	131.2	126.8	131.2	131.9	122.4	131.2
13	84.0	84.0	84.8	136.1	144.0	149.8	83.9	137.4	84.0	84.9	148.1	84.0
14	45.7	45.7	45.3	41.9	43.8	41.9	45.6	42.1	45.7	43.6	43.9	45.7
15	36.2	36.2	36.3	32.6	36.8	37.2	36.2	31.9	36.2	35.4	36.8	36.1
16	64.0	64.0	77.2	67.7	66.6	74.2	64.0	67.7	64.0	77.1	66.2	64.1
17	47.0	47.0	54.4	45.3	41.1	43.4	47.0	45.5	47.0	45.3	43.6	46.9
18	52.2	52.2	51.4	133.0	44.5	41.9	52.1	131.4	52.2	51.4	43.9	52.1
19	37.8	38.5	37.8	39.0	47.1	47.8	37.7	34.0	37.8	38.4	47.0	37.7
20	31.6	31.6	31.9	31.9	31.1	31.3	31.6	44.0	31.6	31.9	31.1	31.6
21	34.7	34.7	36.8	36.5	34.3	35.0	34.7	31.1	34.7	36.8	33.4	34.7
22	25.7	25.7	31.3	24.4	26.6	30.9	25.7	24.2	25.7	31.3	26.3	25.8
23	64.1	28.0	64.1	64.7	28.2	64.3	64.0	64.1	27.8	64.1	28.2	64.2
24	13.3	16.3	13.1	13.1	17.0	13.6	13.0	13.1	16.3	13.1	17.0	13.0
25	18.0	18.1	18.8	18.3	15.7	17.9	18.7	18.9	18.0	18.9	17.3	18.7
26	20.0	19.9	19.5	17.2	17.0	18.4	20.0	17.3	20.0	19.6	18.5	20.1
27	20.9	20.9	20.6	21.9	27.1	26.4	20.8	21.9	20.9	18.1	26.3	20.8
28	73.1	72.7	77.6	64.1	69.1	70.0	73.0	65.1	73.0	78.1	69.1	73.0
29	33.7	33.7	33.7	25.1	33.4	33.4	33.6	181.2	33.7	33.8	33.3	33.6
30	23.8	23.8	24.4	32.6	24.1	24.6	23.8	21.5	23.8	24.4	24.0	23.8
OCH_3	-	-	-	-	-	53.8	-	-	-	-	54.2	-
1′	106.8	106.7	106.0	106.0	106.7	106.0	106.1	106.0	106.8	106.1	106.7	105.1
2′	71.6	75.2	71.0	71.8	75.2	71.5	71.4	71.6	71.6	71.5	75.1	71.3
3′	85.2	76.8	85.3	85.3	76.8	85.5	85.4	85.3	85.2	84.9	76.8	85.9
4′	71.8	80.0	72.2	72.2	80.0	72.2	71.4	72.2	71.8	72.1	79.9	71.7
5′	71.0	75.2	71.5	71.0	75.6	71.0	71.0	71.1	71.0	71.0	75.5	70.9
6′	17.3	69.2	17.3	17.2	69.1	17.2	17.3	17.3	17.3	17.3	68.5	17.2
1″	106.8	102.9	106.8	106.7	102.9	106.7	106.4	106.7	106.8	106.5	105.3	104.5
2″	75.9	72.6	75.9	75.8	72.7	75.8	75.3	75.8	75.9	75.3	74.8	86.8
3″	78.5	72.5	78.8	78.8	72.6	78.4	78.1	78.3	78.5	77.8	78.3	77.6
4″	71.8	73.8	71.8	71.6	73.8	72.2	71.6	71.8	71.8	72.1	71.4	70.5
5″	78.4	70.5	78.9	78.5	70.5	78.4	75.3	78.7	78.4	75.6	78.3	78.1
6″	62.7	18.1	62.7	62.7	18.4	62.7	64.8	62.7	62.7	64.8	62.5	62.2
1‴	-	105.2	-	-	105.1	-	-	-	-	-	103.0	107.8
2‴	-	74.8	-	-	74.8	-	-	-	-	-	72.5	76.1
3‴	-	78.4	-	-	78.5	-	-	-	-	-	72.7	77.9
4‴	-	71.4	-	-	71.4	-	-	-	-	-	73.8	71.0
5‴	-	78.4	-	-	78.4	-	-	-	-	-	70.5	67.6
6‴	-	62.6	-	-	62.6	-	-	-	-	-	18.3	-
$\underline{C}OCH_3$	-	-	-	-	-	-	170.8	-	-	170.8	-	-
$CO\underline{C}H_3$	-	-	-	-	-	-	20.8	-	-	20.8	-	-

1.10, 1.39 (each 3H, s, tert-Me×6), 1.94 (3H, s, $COCH_3$), 1.48 (3H, d, J=6.0 Hz, Fuc-CH_3), 4.98 (1H, d, J=7.2 Hz, Fuc-1′-H), 5.25 (1H, d, J=7.8 Hz, Glc-1″-H), 6.00 (1H, d, J=10.2 Hz, 12-H), 5.66 (1H, dd, J=10.2, 2.4 Hz, 11-H). ^{13}C-NMR data (Table 1).

- 3β,16α,23,28-tetrahydroxy-olean-11,13 (18)-dien-29-oic acid 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside(8): White powder, mp 243 to 245°C (MeOH). The HR-ESI-MS *m/z* 811.4832 $[M+H]^+$ (calcd. for $C_{42}H_{66}O_{15}$, 811.0039), IR (KBr)vmax 1699 cm^{-1}. ^{1}H-NMR (C_5D_5N, 500 MHz): δ 0.88, 0.91, 0.99, 1.60, 1.70 (each 3H, s, tert-Me×5), 6.74 (1H, d, *J*=10.5 Hz, H-11), 5.72 (1H, d, *J*=10.5 Hz, H-12), 4.99 (1H, d, *J*=7.5 Hz, Fuc-1′-H), 5.34 (1H, d, *J*=7.5 Hz, Glc-1″-H). ^{13}C-NMR data (Table 1).

Chikusaikoside (9): White amorphous powder, mp 207 to 209C. ^{1}H-NMR (500 MHz, C_5D_5N) δ 0.89, 0.92, 0.93, 0.99, 1.10, 1.39 (each, 3H, s, tert-Me×6), 1.42 (3H, d, J=6.6 Hz, Fuc-CH_3), 4.94 (1H, d, J=7.8 Hz, Glu-CH_3), 5.15 (1H, d, J=7.8 Hz, Xyl-CH_3), 6.70 (1H, d, J=10.2 Hz, 11-H), 5.99 (1H, d, J=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikogenin F (10): White amorphous powder, mp 215 to 217°C, ^{1}H-NMR (500 MHz, CD_3OD): δ 0.70, 0.92, 0.94, 0.97, 1.03, 1.09 (3H, s, tert-Me×6), 1.90 (1H, br s, H-9), 3.04 (1H, d, J=7.2 Hz, H-28), 3.90 (1H, d, J=6.6 Hz, H-28), 4.17 (1H, dd, J=10.5, 6.0 Hz, H-16), 5.38 (1H, dd, J=10.5, 3.0 Hz, H-12), 5.95 (1H, d, J=10.8 Hz, H-11).^{13}C-NMR (125 MHz, CD_3OD):δ 12.1 (C-24), 18.4 (C-6), 18.8 (C-25), 20.2 (C-26), 21.2 (C-27), 24.1 (C-30), 26.1 (C-22), 27.3 (C-2), 32.1 (C-7), 32.3 (C-20), 33.9 (C-29), 35.2 (C-21), 36.0 (C-15), 37.2(C-10), 38.5(C-19), 39.2(C-1), 42.9(C-8), 43.5(C-4), 46.5(C-14), 47.6(C-14), 48.2(C-5), 53.1(C-30), 53.9(C-9), 65.3(C-16), 66.8(C-23), 73.4(C-28), 73.5(C-3), 85.7(C-13), 130.6(C-12) and 134.1(C-11).

Saikosaponin e (11): White amorphous powder, mp 227 to 228°C. ^{1}H-NMR (600 MHz, C_5D_5N): δ 0.91, 0.91, 0.96, 0.96, 1.12, 1.31, 1.37 (each, 3H, s, tert-Me×7), 1.47 (3H, d, J=6.6 Hz, Fuc-CH_3), 4.74 (1H, d, J=7.8 Hz, Fuc-1′-H), 5.41(1H, d, J=8.4 Hz, Fuc-1′-H), 5.66 (1H, d, J=10.2 Hz, 11-H), 5.97 (1H, d, J=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

6"-*O*-acetyl saikosaponin d (12): White amorphous powder, mp 197 to 198°C. ^{1}H-NMR (600 MHz, C_5D_5N): δ 0.90, 0.93, 0.99, 0.99, 1.38, 1.61 (each, 3H, s, tert-Me×6), 1.94 (3H, s, $COCH_3$), 1.49 (3H, d, J=6.0 Hz, Fuc-CH_3), 4.98 (1H, d, J=7.8 Hz, Fuc-1′-H), 5.25 (1H, d, J=7.8 Hz, Fuc-1′-H), 6.02 (1H, d, J=10.2 Hz, 11-H), 5.70 (1H, dd, J=10.2, 2.4 Hz, 12-H). ^{13}C-NMR data (Table 1).

Saikosaponin 14 (13): White amorphous powder, mp 199 to 200°C. ^{1}H-NMR (500 MHz, C_5D_5N): δ 0.91, 0.99, 1.00, 1.02, 1.04, 131, 1.45 (each, 3H, s, tert-Me×7), 3.24 (3H, s, OCH_3), 1.65 (3H, d, J=6.0 Hz, Rha-CH_3), 4.56 (1H, d, J=9.0 Hz, Glc-1′-H), 4.80 (1H, d, J=7.8 Hz, Rha-1″-H), 4.92(1H, d, J=7.8 Hz, Glc-1‴-H), 5.64 (1H, dd, J=10.2, 2.4 Hz, 11-H), 5.90 (1H, d, J=10.2 Hz, 12-H). ^{13}C-NMR data (Table 1).

Sugar identification of compound 8

Compound 8 (10 mg) was refluxed with 5% HCl in MeOH-water (1:1, 5 ml) for 6 h. The MeOH was then removed and the solution was extracted with EtOAc (2 ml × 3). The aqueous fractions were evaporated and the residues were prepared to their derivatives for GC analysis according to the methods described in the literature (Tang et al., 2005). The D-fucose and D-glucose were confirmed by comparison of their retention time (t_R, 4.6 and 11.5 min, respectively) with those of authentic standards. The authentic samples were purchased from the Pfanstienl Chemical Corporation, Waukengan, IL (Lot no, 1279).

Cytotoxicity assay

The cytotoxicity was measured by MTT assay (Alley et al., 1988; Zhou et al., 1993). Briefly, cells were plated in 96-well plates (5 × 10^4 cells/well) and incubated in a humidified atmosphere, 95% air, 5% CO_2 at 37°C. After 24 h, additional medium (100 µl) containing the test compounds (100, 80, 40, 20, 10 and 5 µg/ml) and vehicle (DMSO, final concentration of 0.1%) was added to each well. After 68 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/ml). 4 h later, the MTT formazan product was dissolved in 150 µl DMSO, and the optical density (OD) was read on a microplate ELISA reader (BioRad 680, Molecular Devices, USA) at 570 nm. The assays were repeated three times. Media and DMSO control wells, in which sample was absent, were included in all the experiments, in order to eliminate the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

Growth inhibition (%) = (ODcontrol – ODtreated / ODcontrol) × 100%

The cytotoxicity of sample on tumor cell lines was expressed as IC_{50} values (the drug concentration reducing by 50% the absorbance in treated cells, with respected to untreated cells), which were calculated by LOGIT method.

RESULTS AND DISCUSSION

Phytochemical investigation

The saponin-enriched part of *B. yinchowense* was subjected to a succession of chromatographic procedures, including silica gel chromatography, pharmadex LH-20, ODS-C_{18} and semipreparative HPLC to afford 13 compounds. On the basis of spectroscopic data analysis (IR, UV, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, DEPT, HMBC, HMQC, FAB-MS and HR-FABMS) and comparison with reports in the literatures, compounds 1 to 13 were identified to be saikosaponin a (1) (Liang et al., 1998), saikosaponin c (2) (Tori et al., 1976), saikosaponin d (3) (Liang et al., 1998), saikosaponin b_2 (4) (Ishii et al., 1980), saikosaponin f (5) (Tori et al., 1976), saikosaponin b_4 (6) (Ishii et al., 1980), 6"-O-acetylsaikosaponin a (7) (Ding et al., 1986), 3β, 16α, 23, 28-tetrahydroxy-olean-11, 13 (18)-dien-29-oic acid 3-O-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside (8) (Yoshikawa et al., 1997), chikusaikoside I (9) (Ebata N et al., 1996), saikogenin F (10) (Wang et al., 1998), saikosaponin e (11) (Hiroshi et al., 1997), 6"-*O*-acetyl saikosaponin d (12) (Ding et al., 1986) and saikosaponin 14 (13) (Ding et al., 1986). Compound 8 is a new natural compound which had been previously synthesized and now was isolated from natural source for the first time. The other 12 compounds were isolated from this plant for the first time. All the 13 compounds were oleanane-type saikosaponins (Figure 1).

3β,16α,23,28-tetrahydroxy-olean-11,13 (18)-dien-29-oic acid 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside (8) was obtained as white powder, mp 243 to 245°C (MeOH). The high resolution-electrospray ionization-mass spectrometry (HR-ESI-MS) displayed a molecule ion peak at m/z 811.4832 $[M+H]^+$ (calculate 811.0039), consistent with the molecular formula $C_{42}H_{66}O_{15}$, which also was confirmed by ^{13}C-NMR and ^{1}H-NMR spectral data.

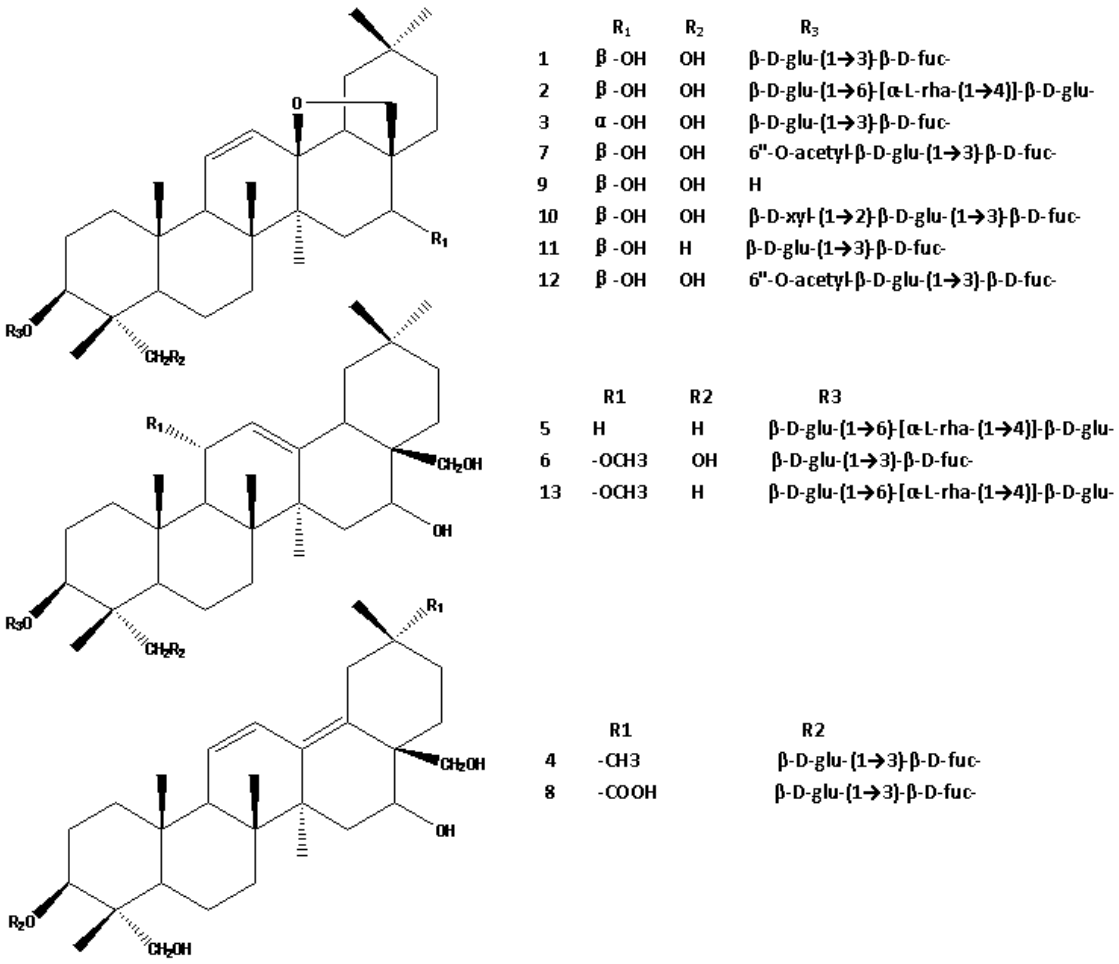

Figure 1. Chemical structures of compounds 1 to 13.

The ^{1}H-NMR spectra of compound 8 exhibited five typical angular methyl proton signals (δ 0.88, 0.91, 0.99, 1.60 and 1.70), which indicated that compound 8 was a triterpenoid saponin. Its UV spectrum also showed absorbances at 242, 251 and 261 nm, suggesting a heteroannular diene system at C-11, C-12, C-13 and C-18. This was further confirmed by its ^{1}H-NMR signals at δ 6.74 (1H, d, J=10.5 Hz, H-11), 5.72 (1H, d, J=10.5 Hz, H-12) and ^{13}C-NMR signals at δ 137.4, 131.4, 126.8 and 126.1, corresponding to C-13, C-18, C-12 and C-11, respectively. The ^{1}H-NMR and ^{13}C-NMR spectra also showed two anomeric proton signals at 4.99 (1H, d, J=7.5 Hz) and 5.34 (1H, d, J=7.5 Hz) and two sugar amoneric carbons at δ 106.0 and 106.7. All aforementioned evidences suggested that compound 8 was a diglycoside with a heteroannular diene system at C-11, C-12, C-13 and C-18 of the aglycone.

The distortionless enhancement by polarization transfer (DEPT) spectrum of compound 8 also showed that the aglycone moiety possessed four hydroxyl groups at δ 81.7, 67.7 (CHOH) and at δ 64.1, 65.1 (CH_2OH) and carbonyl group at δ 181.2. The ^{13}C-NMR signals of the aglycone moiety were in good agreement with those of saikosaponin v (Tan et al., 1999) except for the signal at C-30. A comparison of the ^{13}C-NMR data for their aglycone moieties showed that the signals for C-30 of compound 8 underwent a downfield shift (+2.6) on going from saikosaponin v to compound 8.

The IR spectrum of compound 8 showed a carbonyl band at 1699 cm^{-1}, and ^{13}C-NMR spectrum exhibited a carbonyl signal at δ 181.2. According to the formula of compound 8, it should contain a substitute of carbonyl. In heteronuclear multiple bond correlation (HMBC) experiments, significant correlation of the carbonyl signal (δ 181.2) with the methyl protons [δ1.6 (29-CH_3)], indicating that the carbonyl group should be at C-30. Therefore, the aglycone was ultimately determined as 3β, 16α, 23, 28-tetrahydroxy-olean-11,13 (18)-dien-30-oic acid.

On acid hydrolysis of compound 8, D-fucose and D-glucose were detected from the aqueous fraction by GC analysis comparing with the authentic samples. Through analysis of the ^{1}H-^{1}H COSY, HMQC, HMBC spectral data,

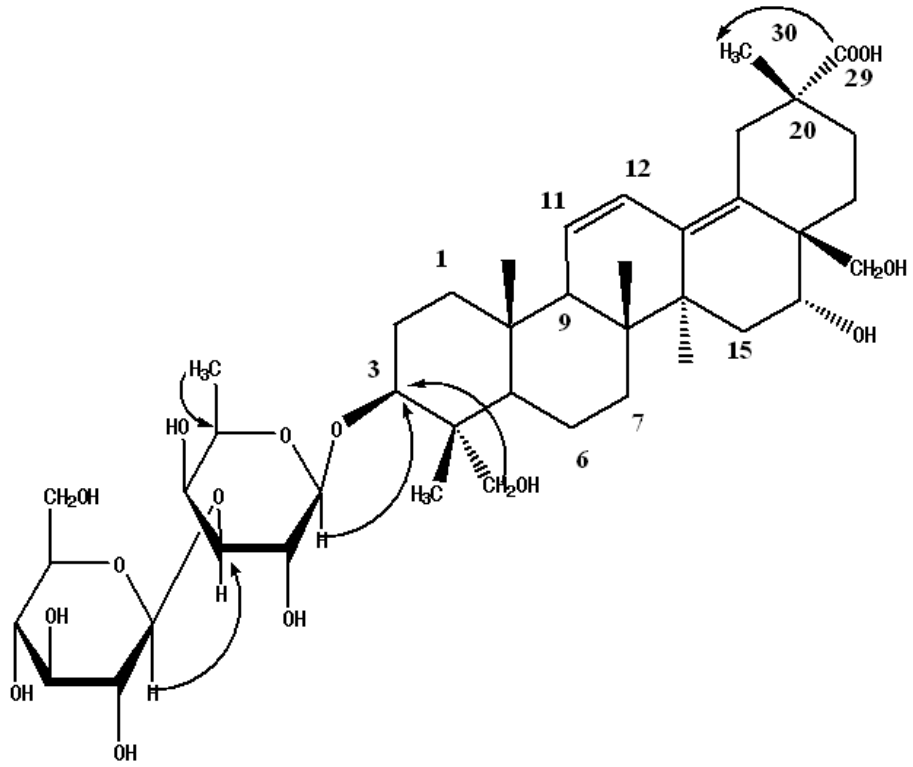

Figure 2. Structure of 8 and key correlations observed from HMBC.

Table 2. Cytotoxicity of different fractions and isolates from *B. yinchowense*.

Sample	IC_{50} value (μg/ml)			
	SKOV3	SW48	Eca-109	Hela
n-Hexane fraction	>100	>100	>100	>100
Water fraction	>100	>100	>100	>100
95% Ethanol fraction	14.46	12.12	13.73	22.51
1	5.14	5.81	4.92	7.82
2	-	-	-	-
3	5.32	6.12	5.85	9.21
4	37.2	35.8	42.3	42.7
5	-	-	-	-
6	-	-	-	-
7	4.53	5.32	4.61	8.67
8	-	-	-	-
9	35.2	36.9	30.2	45.4
10	-	87.5	-	-
5-Fu	11.32	9.31	11.23	14.30

^{1}H-NMR and ^{13}C-NMR signals for compound 8 were fully assigned. ^{13}C-NMR signals are as shown (Table 1). The HMBC spectrum showed obvious correlations between δ 4.99 (H-1') and 81.7 (C-3) and δ 5.34 (H-1") and 85.3 (C-3'), suggesting the sugar moiety was located at C-3 position, the glucosyl was connected with C-3' of the D-fucose.

According to the coupling constants of sugar anomeric proton signals δ 4.99 (1H, d, J=7.5 Hz) and 5.34 (1H, d, J=7.5 Hz), the fucose and glucose should be assigned as *β*-anomeric configurations. Thus, compound 8 was elucidated as 3β, 16α, 23, 28-tetrahydroxy-olean-11,13 (18)-dien-29-oic acid 3-O-β-D-glucopyranosyl- (1→3)-β-D-fucopyranoside (Figure 2). The spectra data of compound 8 were reported for the first time.

Cytotoxic activity

Compounds 1 to 10 were evaluated *in vitro* for their inhibitory ability against Eca-109, SW48, Hela, and SKOV3 by MTT assay, using cisplatin as a positive control. The 50% growth inhibition (IC_{50}) values are summarized in Table 2. Saikosaponin a (1), saikosaponin d (3) and 6''-O-acety-saikosaponin a (7) displayed strong cytotoxic activities against the tested cell lines in a dose-dependent manner, with IC_{50} values of 4.53 to 14.3 μg/ml. The other compounds showed no or weak antiproliferative activity.

Previous studies have shown many oleanane-type *Bupleurum* saponins, especially those that contained the epoxy bridge at C-13 and C-28 in their skeleton were very potent against many cancer cells (Chiang et al., 2003; Fujioka et al., 2006). Saikosaponin d exerted very

potent activity against the HepG2 cell line with an IC_{50} value of 12.5 µg/ml (Chiang et al., 2003). 3′-*O*-acetyl derivatives of saikosaponins a and d exhibited a potent activities against leukaemia P-388 cells and nasopharynx carcinoma KB cells with IC_{50} values of 0.5 and 6.3 µg/ml for the former and 1.2 and 6.3 µg/ml for the latter (Luo et al., 1993). In the present study, 10 oleanane-type *Bupleurum* saponins from *B. yinchowense* were tested against Eca-109, W-48, Hela and SKOV3. The result revealed that saikosaponin a, saikosaponin d and 6''-*O*-acetylsaikosaponin a exhibited significant inhibitory activities against the tested cell lines. The present study further demonstrated that *Bupleurum* saponins with an epoxy bridge and their acetyl derivatives were very potent against many cancer cells. To the best of our knowledge, this is the first report for the cytotoxic activity of compound 7.

ACKNOWLEDGEMENTS

This work was financially supported by National Science and Technology major projects (2011ZX09307-002-01 and 2012ZX09301002-001) and by the International Science and Technology Collaboration Project (0901).

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