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New pregnane saponins from *Ecdysanthera rosea* and their cytotoxicity

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ABSTRACT

Two new pregnane saponins elucidated as ecdysantheroside A (**1**) and ecdysantheroside B (**2**) and six known compounds (**3–8**) based on spectral data (MS, IR, 1D and 2D NMR) were isolated from the stem bark of *Ecdysanthera rosea*. The cytotoxicity against six cell lines of these compounds was tested by MTT assay. The results revealed that compounds **5** and **7** showed cytotoxicity against all the cell lines. Compound **2** showed cytotoxicity against cells A549, MDA435, HepG2, and HUVEC, while compound **4** showed cytotoxicity against cells A549, CEM, and HUVEC. Compound **6** had cytotoxicity against the others except cell HepG2.

1. Introduction

Ecdysanthera rosea Hook. et Arn., mainly distributed in tropical Asia, is an Apocynaceous liana, whose roots and stems have been used as analgesic, antiphlogistic, and spasmolytic agents in Chinese folk medicine [1]. Researchers have isolated some compounds such as ecdysantherin [2], 20-Epi-Kibataline, 3β, 14β, 20-trihydroxy-18oic (18→20) lactone pregnen-5 [3], 5-O-caffeoylquinic derivers, scopoletin [4], D-friedours-14-en-11α, 12α-epoxy-3β-yl palmitate [5] and some triterpenoids [6] from this plant. In previous studies, we have isolated six compounds such as hydroquinone diglycoside acyl esters, ecdysanrosin A, sesquiterpene, 5β-hydroperoxycostic acid and apocarotenoid, 2, 4, 7-trimethyl-2, 4, 6, 8-tetraene-dialdehyde [7]. However, the potential medicinal importance of the plant and our interest in new active compounds prompted us to investigate *E. rosea* further and two new steroidal glucosides were obtained further from *E. rosea*. The cytotoxicity of the two new steroidal glucosides, together with five other compounds isolated from *E. rosea* was tested against human acute lymphoblastic T-cell leukemia cells CEM, human umbilical vein endothelial cells HUVEC, human erythroleukemia cells K562, hepatocellular carcinoma cells HepG2, human carcinomic alveolar basal epithelial cells A549, and human mammary carcinoma cells MDA435 by MTT cytotoxicity assay [8].

2. Experimental

2.1. General

Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV Spectra were obtained with a Shimadzu 210A double-beam spectrophotometer. IR spectra were recorded with a Bio-Rad FTS-135 infrared spectrophotometer using the potassium bromide pellet technique. 1D and 2D-NMR Spectra

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were recorded with Bruker AM-400 and DRX-500 instruments with TMS as internal standard. EI–MS was measured with VG AutoSpec 3000 spectrometers. ESI–MS and HR–ESI–MS were measured with API QSTAR Pulsarimass spectrometers. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used as stationary phase for column chromatography. Silica gel (GF_{254}, Qingdao Marine Chemical Inc., China) was used for stationary phase for TLC.

2.2. Plant material

Aerial parts of *E. rosea* were collected in May, 2004 in Xishuangbanna, Yunnan province, People's Republic of China. The plant was identified by Dr. Li Rong, Kunming Institute of Botany, Chinese Academy of Sciences (CAS). A sample (Kun No.20040501) has been deposited in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, PR China.

2.3. Extraction and isolation

The dried powdered stems and leaves (11 kg) of *E. rosea* were extracted three times for 2 h under reflux with 60, 50, and 40 L EtOH, successively. Then the combined extracts were concentrated under reduced pressure to yield a dark residue which was suspended in water and partitioned with petroleum ether, EtOAc and *n*-BuOH, respectively. The EtOAc part (79 g) was subjected to silica gel column chromatography (CC, Ø × L, 15 × 150 cm) eluting with a gradient mixture of $CHCl_3$–MeOH (100:0, 95:1, 90:10, 80:20, 70:30v/v) to give seven fractions (Fr.1–Fr.7). And Fr. 2 (10 g) was further isolated by silica gel column chromatography (CC) (Ø × L, 20 × 60 cm) eluting with a gradient mixture of $CHCl_3$–MeOH (95:1–90:10v/v) to obtain twelve subfractions named as Fr2.1–Fr2.12. Furthermore Fr2.3 (1.6 g) was chromatographed on silica gel eluted with petroleum ether–Me_2CO (9:1 → 7:3, v/v) followed by Sephadex LH-20 column chromatography (CC) (Ø × L, 1 × 150 cm) (MeOH) to afford compound **4** (21 mg), **5** (11 mg), and **6** (16 mg). And Fr2.6 (1.2 g) was chromatographed on silica gel eluted with $CHCl_3$–MeOH (10:1, 8:1, 7:3) and further purified on sephadex-LH-20 eluted with $CHCl_3$–MeOH (1:1, v/v) and then on reverse phase (RP-18) column chromatography (CC) (Ø × L, 1 × 60 cm) eluted with CH_3OH: H_2O 1: 1 to afford **1** (87 mg), **7** (12 mg) and **8** (6 mg). Moreover, F6 (22 g) was further isolated by silica gel column chromatography (CC) (Ø × L, 20 × 100 cm) eluting with a gradient mixture of $CHCl_3$–MeOH (95:5–80:20v/v) to give ten subfractions named as F6.1–Fr6.10 respectively. And Fr6.6 (1.2 g) was further chromatographed on silica gel eluted with $CHCl_3$–MeOH (10:1, 8:1, and 7:3) followed by purifying on Sephadex LH-20 column chromatography (CC) (Ø × L, 1 × 150 cm) eluted with $CHCl_3$–MeOH 1:1 and then on reverse phase (RP-18) column chromatography (CC) (Ø × L, 1 × 60 cm) eluted with CH_3OH: H_2O 1:1 to afford **2** (127 mg).

2.4. Cytotoxicity assay

The cytotoxicity assay was performed against human acute lymphoblastic T-cell leukemia (CEM) cells, human umbilical vein endothelial cells (HUVEC), human erythroleukemia type cell line K562, hepatocellular carcinoma HepG2 cell line, carcinomic human alveolar basal epithelial cells A549, and human mammary carcinoma MDA435 cell line by MTT assay. Cell lines were maintained in RPMI-1640 medium (Hyclone) containing 10% heat inactivated fetal bovine serum (Hyclone), 100 μg/ml penicillin sodium salt and 100 μg/ml streptomycin sulphate, and kept in humidified incubator at 5% CO_2 at 37 °C. After 80% confluence was reached cells were harvested and seeded in a 96-well plate with 6000 cells per well to which tested compounds of concentration at 100 μM were added and incubated for 48 h followed by MTT assay at the wave length of 490 nm.

2.5. Acidic hydrolysis

Compounds **1** and **2** (each 5 mg) were dissolved in a mixture of CH_3OH (1.0 ml) and 2 M HCl (1.0 ml) and hydrolyzed for 2 h by refluxing in a boiling water bath. The hydrolysate was partitioned between water and EtOAc after it was cooled to room temperature and diluted two-fold with distilled water and then the aqueous layer was neutralized and concentrated *in vacuo* to obtain a residue which was identified by TLC compared with standard samples with solvent A: petrol ether/CH_3COCH_3 (3:2), solvent B: CH_2Cl_2/C_2H_5OH (9:1). Glucose was identified by TLC compared with authentic sample with solvent $CHCl_3$/CH_3OH/H_2O (4:3:1).

Ecdysantheroside A (**1** = 14β, 20-dihydroxy-18-oic (18 → 20) lactone-5-pregnene 3-*O*-β-D-cymaropyranoside).

White solid, molecular formula, $[\alpha]_D^{25} = +22.4$ (*c* 0.57, MeOH). Negative FAB-MS *m/z*: 489 $[M-H]^-$, negative HRFABMS *m/z*: 489.2863 $[M-H]^-$ calc. for $C_{28}H_{41}O_7$ 489.2852. UV: 217, 241 nm; IR bands (KBr) 3441 (OH), 2930, 2856, 1744 (C=O), 1640 (C=C), 1453, 1088 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 1.

Ecdysantheroside B (**2** = 14β, 20-dihydroxy-18-oic (18 → 20) lactone-5-pregnene 3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-1 → 4)-α-L-cymaropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside).

White solid, molecular formula, $[\alpha]_D^{25} = -21.7$ (*c* 0.58, CH_3OH). Negative FAB-MS *m/z*: 1101 $[M-H]^-$, negative HRFABMS *m/z*: 1101.5474 $[M-H]^-$ calc. for $C_{54}H_{85}O_{23}$ 1101.5481. UV: 224, 249 nm; IR bands (KBr) 3441 (OH), 2933, 2968, 1746 (C=O), 1633 (C=C), 1452, 1368, 1088 cm^{-1}; ^1H and ^{13}C NMR spectral data see Table 2.

3. Results and discussion

Ecdysantheroside A **(1)** was found to possess a molecular formula of $C_{28}H_{41}O_7$ based on the negative FAB-MS (m/z 489 $[M-H]^-$), negative HR-FAB-MS (*m/z* = 489.2863 $[M-H]^-$, calcd.: 489.2852) and the NMR spectroscopic data (Table 1). IR spectrum showed the absorption bands due to hydroxyl (3441 cm^{-1}), carbonyl (1744 cm^{-1}) and olefinic-bond (1640 cm^{-1}) groups. The ^{13}C NMR spectrum of compound **1** displayed 28 carbon signals including one methoxy, 21 carbons of which were assigned to the aglycon part and 6 to the glycoside. This can be further identified by acid hydrolysis of **1** that led to the isolation of the aglycone unit, identified as 3β, 14β, 20-trihydroxy-18oic (18 → 20) lactone 5-pregnene (**3**) [3] and a monosaccharide, cymarose, identified by comparison with a standard sample (Fig. 1). The ^1H NMR

Table 1
The ^{1}H and ^{13}C NMR spectral data, and HMBC correlations for **1** in $CDCl_3$.

Position	^{1}H (α, β)	^{13}C	HMBC (H-C)
1	1.90 m, 1.17 m	37.3 t	C-2, 3, 5, 19
2	1.74 m, 1.44 m	29.4 t	C-1, 3, 4
3	3.53 m	77.0 d	C-1, 4, 5, 1′
4	2.28 m, 2.15 m	39.0 t	C-2, 3, 5, 10
5		140.0 s	
6	5.37 brs	120.6 d	C-4, 5, 7, 8, 10
7	2.24 m, 1.92 m	26.6 t	C-6, 8, 9, 14
8	1.94 m	39.1 d	C-6, 7, 9, 10, 14
9	1.13 m	45.7 d	C-8, 10, 19, 12, 14
10		37.3 s	
11	2.05 m, 1.84 m	25.9 t	C-8, 9, 10, 12, 13
12	1.56 m, 1.24 m	33.4 t	C-9, 13, 14, 17, 18
13		59.9 s	
14		85.5 s	
15	2.03 m, 1.48 m	20.7 t	C-8, 13, 14, 16, 17
16	1.82 m	35.4 t	C-13, 14, 15, 17, 20
17	2.20	56.1 d	C-12, 13, 14, 16, 18, 20, 21
18		178.5 s	
19	0.97 s	19.3 q	C-1, 5, 9, 10
20	4.37 m	83.2 d	C-13, 16, 17, 18, 21
21	1.24 (d, 6.2)	21.4 q	C-17, 20
1′	4.80 (dd, 9.6, 2.0)	99.3 d	C-3, 2′, 3′, 5′
2′	1.97 m, 1.51 m	33.7 t	C-1′ , 3′ , 4′
3′	3.60 m	77.3 d	C-1′ , 2′ , 4′, 5′
4′	3.51 m	72.3 d	C-2′, 3′ , 5′
5′	3.83 m	68.4 d	C-3′, 4′ , 6′
6′	1.19 (d, 6.4)	18.3 q	C-4′, 5′
OMe	3.41 s	57.2 q	C-3′

and ^{13}C NMR spectra of compound **1** revealed the presence of three methyl groups [δ_H 0.97 (3H, s), 1.19 (3H, d, $J=6.4$ Hz) and 1.24 (3H, d, $J=6.2$ Hz)], one olefinic proton [δ_H 5.37, brd] and one anomeric proton [δ_H 4.80 (dd, $J=9.6$, 2.0 Hz)]. The ^{1}H and ^{13}C NMR data for **1** pointed to the presence in the molecule of an O-methylated 2,6-dideoxy sugars which were identified from the ^{1}H NMR data and HMQC–TOSCY analyses. An analysis of the ^{13}C chemical shift values for the anomeric carbon atom of the 2-deoxy sugars of a large number of steroid glycosides reveals that C-2 of the β-D-sugars resonates at δ_C 33–34 and that of α-L-sugars at δ_C 30–32 [9,10]. The chemical shift values for C-2 of the sugar moieties in **1** show that the cymarose moiety (δ_C 33.7) has the L-configuration and the β-configuration by the coupling constant (9.6, 2.0 Hz) of the proton peak at δ_C 4.80 (H-1′). In addition, comparing with **3**, the glycosidation shifts were observed at C-3(+5.8), C-2(−4.3), C-4(−4.3) as well as C-1(−0.4) and C-5(−0.9) in the aglycone moiety. Moreover, the correlations observed between signals at δ_H 3.53 (H-3) and δ_C 99.3 (C-1′), δ_H 4.83 (H-1′) and δ_C 77.0 (C-3) in the HMBC experiment indicated that β-D-cymaropyranosyl was located at C-3. Therefore, ecdysantheroside A (**1**) was identified as 14β, 20-dihydroxy-18oic (18→20) lactone-5-pregnene 3-*O*-β-D-cymaropyranoside.

Ecdysantheroside B **(2)** was obtained as amorphous powder. Its molecular formula of $C_{54}H_{86}O_{23}$ was assigned by high-resolution negative ion (HRFABMS) at *m/z* 1101.5474 ($[M–H]^-$ calc. 1101.5481) and its NMR spectroscopic data (Table 2). The IR spectrum showed absorption due to hydroxyl (3441 cm^{-1}), carbonyl (1746 cm^{-1}) and olefinic-bond (1633 cm^{-1}) groups. Acid hydrolysis of **2** led to the isolation of the aglycone, identified as 3β, 14β, 20-trihydroxy-18oic (18→20) lactone 5-pregnene (**3**) [3] and two single sugar, glucose and cymarose, identified by comparison with a standard sample. The ^{1}H NMR spectrum of **2** displayed two methyl signals at δ_H 1.01 (3H, s, H-19), 1.31 (3H, d, $J=6.8$ Hz, H-21), one olefinic proton signal at δ_H 5.41 (m, 1H, H-6) and two protons adjacent to oxygen at δ_H 3.48 (1H, m, H-3) and 4.38 (1H, m, H-20) of the aglycone moiety. Moreover, the ^{1}H and ^{13}C NMR data also indicated there are five hexose units in their pyranose form in the molecule based on their coupling constant, which were identified as two glucose and three cymarose moieties as shown in the ^{1}H NMR data. The magnitude of the (^{1}H, ^{1}H) coupling constants indicated that one anomeric proton (δ_H 4.56) formed a part of α-cymaropyranose whose configuration was determined to be L-form and other anomeric protons (δ_H 4.82, 4.78) formed a part of β-cymaropyranose which had a D-configuration based on the chemical shift of C-2. An analysis of the ^{13}C chemical shift values for the anomeric carbon atom of the 2-deoxy sugars of a large number of steroid glycosides reveals that C-2 of the β-D-sugars resonates at δ_C 35–38 and that of α-L-sugars at δ_C 30–32[9–11]. The chemical shift values for C-2 of the sugar moieties in **2** showed that an α-linked

Table 2
The ^{1}H and ^{13}C NMR spectral data for **2** in CD_3OD.

Position	^{1}H (α, β)	^{13}C	Position	^{1}H (α, β)	^{13}C
1	1.78 m, 1.07 m	38.3 t	1′	4.82 (dd, 9.6, 1.8)	97.1 d
2	1.78 m, 1.52 m	30.5 t	2′	1.58 m, 1.56 m	36.5 t
3	3.48 m	78.9 d	3′	3.83 m	78.4 d
4	2.34 m, 2.16 m	39.7 t	4′	3.25 m	83.7 d
5		140.6 s	5′	3.83 m	69.7 d
6	5.41 brs	122.4 d	6′	1.22 (d, 6.1)	18.4 q
7	2.27 m, 1.78 m	27.7 t	1″	4.78 (dd, 9.4, 1.6)	101.0 d
8	2.01 m	39.5 d	2″	2.22 m, 2.16 m	35.9 t
9	1.18 m	47.0 d	3″	3.77 m	78.1 d
10		38.4 s	4″	3.27 m	83.6 d
11	2.08 m, 1.75 m	26.2 t	5″	3.78 m	69.8 d
12	1.80 m, 1.41 m	34.3 t	6″	1.18 (d, 6.6)	18.6 q
13		60.7 s	1‴	4.56 (dd, 4.7, 1.5)	103.2 d
14		86.0 s	2‴	1.98 m, 1.80 m	33.0 t
15	2.11 m, 1.50 m	21.9 t	3‴	3.46 m	80.3 d
16	1.75 m	35.6 t	4‴	3.98 m	74.7 d
17		57.7 d	5‴	3.27 m	71.6 d
18		180.7 s	6‴	1.29 (d, 6.6)	17.9 q
19	1.01 s	19.9 q	1‴′	4.53 (d, 7.5)	104.5 d
20	4.38 m	84.8 d	2‴′	3.27 m	75.7 d
21	1.31 (d, 6.1)	21.4q	3‴′	3.19 m	77.8 d
OMe	3.42 s	58.0 q	4‴′	3.30 m	71.6 d
OMe	3.44 s	57.7 q	5‴′	3.43 m	77.2 d
OMe	3.40 s	58.4 q	6‴′	4.11 (dd, 1.6, 11.7), 3.77 (dd, 6.3, 11.7)	70.1 t
			1‴′′	4.39 (d, 7.8)	104.9 d
			2‴′′	3.21 m	75.0 d
			3‴′′	3.27 m	77.8 d
			4‴′′	3.52 m	71.5 d
			5‴′′	3.43 m	77.9 d
			6‴′′	3.65 (dd, 6.0, 12.0), 3.84 (brd, 12.0)	62.6 t

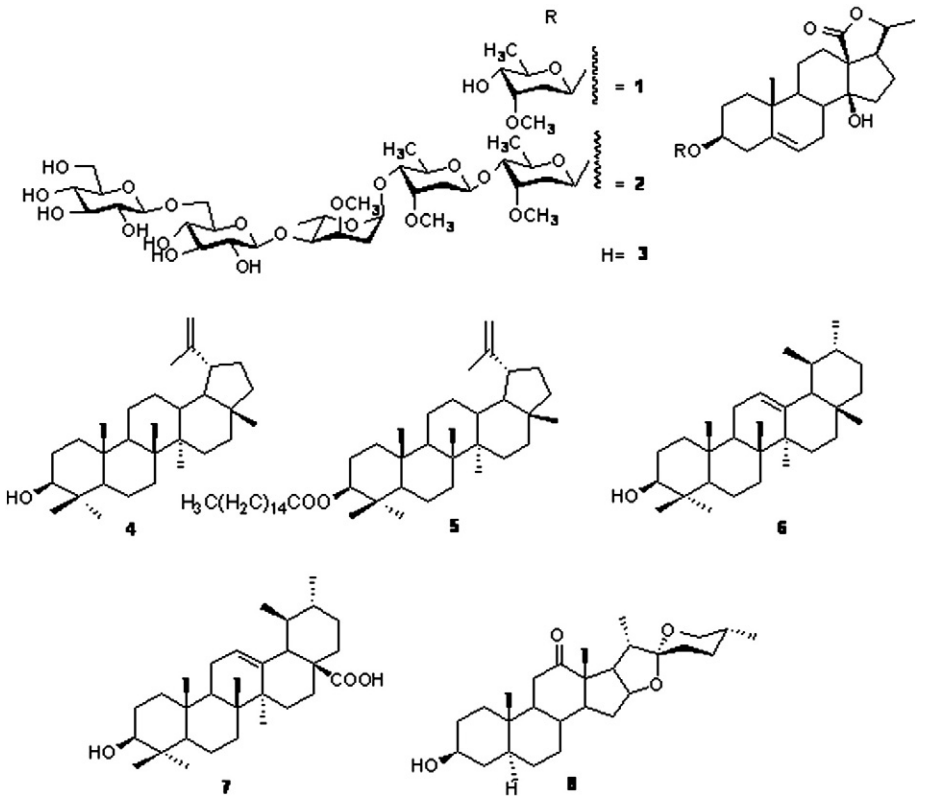

Fig. 1. The structure of compounds **1**–**8**.

cymarose (δ_C, 33) had the L-configuration and the two β-linked cymaroses (δ_C, 35.9 and 36.5), the D-configuration. And the configuration of two glucopyranoses was determined to be L-form according to the magnitude of the (^{1}H, ^{1}H) coupling constants at δ_H 4.53 (7.5Hz) and δ_H 4.39 (7.8Hz). The glycosylation shifts effects of C-3 (+7.7), C-2 (−3.7), C-4 (−3.6) and C-5 (−0.3) showed the linkage position of the sugar moiety was at the C-3 hydroxyl group of the aglycone. The three anomeric protons and three methoxy protons were used as emanating points to assign the other protons and carbon signals, and the sugar linkage pattern, and the sugar sequence of **2** by examining the long-range connectivities given in the HMBC diagram and the heteronuclear correlation from the cross-sections in the HSQC and HSQC–TOSCY diagram. The heteronuclear correlation (HSQC) and the ^{1}H-^{13}C long-range correlation (HMBC) of the anomeric protons (H1′ at δ_H 4.82, H1″ at δ_H 4.78, H1‴ at δ_H 4.56) readily enabled to recognize three fragments composed of three carbons each, one belonged to aglycone and/or other sugar whereas the glycoside bonds were located. The correlations of the anomeric protons (H-1⁗ at δ_H 4.53, H-1⁗′ at δ_H 4.39) of two D-glucopyranoses for carbons (C-4‴ at δ_C 80.3, C-6⁗ at δ_C 70.1) are also shown in the HMBC diagram (see Fig. 2). Thus, the structure of ecdysantheroside B (**2**) was determined to be 14β, 20-dihydroxy-18oic(18→20) lactone-5-pregnene3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-α-L-cymaropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

Besides the two new pregnane saponins, two known steroids and four known triterpenoids were isolated from the plant. Comparison of the physico-chemical and NMR properties with the reported data allowed for their structures to be identified as 3β, 14β, 20-trihydroxy-18oic(18→20) lactone pregnen-5 (**3**)[3], lupenol (**4**)[12], docosanoyl (**5**), α-amyrin (**6**) [13], oleanolic acid (**7**) [14] and (25R)-3β-hydroxyspirostan −12-one (**8**) [15].

The cytotoxicity of compounds **1**–**7** was assayed by MTT method. Paclitaxel, a widely used anti-cancer medicine, was used as a positive control. The results revealed that there was

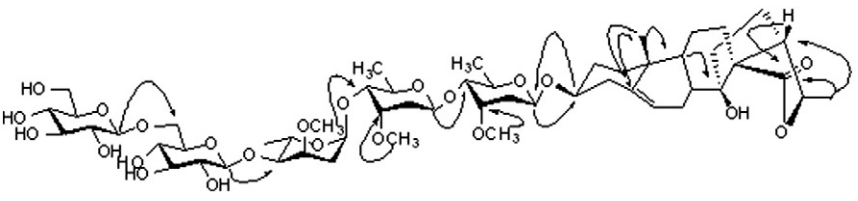

Fig. 2. Key HMBC correlations of compound **2**.

Table 3
The cytotoxicity of compounds against cells tested (the unit for IC_{50} was μM).

	A549	MDA435	K562	CEM	HepG2	HUVEC
2	18.3% [b]	20.4%	- [a]	-	11.6%	26.7%
4	26.4%	-	-	10.6%	-	22.4%
5	42.3%	51.2 (IC_{50}) [c]	29.3 (IC_{50})	36.2 (IC_{50})	83.7 (IC_{50})	73.8 (IC_{50})
6	26.2%	80.9 (IC_{50})	70.5 (IC_{50})	83.7 (IC_{50})	-	29.1%
7	44.6%	38.0 (IC_{50})	48.8 (IC_{50})	72.6 (IC_{50})	81.0 (IC_{50})	81.4 (IC_{50})

[a] "-" indicates no cytotoxicity against cell A549, MDA435, K562, CEM, HepG2 and HUVEC at concentration of 100 μM for compounds tested. Compounds **1** and **3** have no cytotoxicity against above-mentioned cells.
[b] The percentage data indicate the inhibition rate against cells with the concentration of compounds at 100 μM.
[c] The IC_{50} values for cytotoxicty of compounds against cells were given when IC_{50} values were less than 100 μM.

no cytotoxic effect against all the cells tested for compounds **1** and **3** at a concentration of 100 μM level (Table 3), while compounds **5** and **7** showed cytotoxicity against all the cells tested. Compound **2** showed cytotoxicity against cells A549, MDA435, HepG2, and HUVEC, while compound **4** showed cytotoxicity against cells A549, CEM, and HUVEC. Coumpound **6** had cytotoxicity against the others except cell HepG2. However, the IC_{50} value for cytotoxicty of compounds against cells tested was less than 100 μM. Compound **8** was not tested because of a few samples. Since the *E. rosea* has been used as analgesic, antiphlogistic, and spasmolytic agents in Chinese traditional medicine [1], further investigation of their bioactivity and its targets are necessary.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2011.01.022.

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