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Three new diterpenes with cytotoxic activity from the roots of *Euphorbia ebracteolata* Hayata

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ABSTRACT

From the roots of *Euphorbia ebracteolata* Hayata, three new diterpenes, Ebracteolatas A–C, based on the rosane (**1**–**2**) and lathyrane (**3**) skeleton, were isolated together with four known ones (**4**–**7**). Their structures and relative configurations were elucidated on the basis of spectroscopic methods, especially 2D NMR techniques. Compounds **1**, **6**, and **7** exhibited moderate cytotoxic effects against five cancer cell lines.

1. Introduction

The genus *Euphorbia*, belonging to the Euphorbiaceae family, is the source of a large number of biologically active compounds, which have attracted a lot of attention of chemists and pharmacologist (Vasas and Hohmann, 2014). *E. ebracteolata* is distributed throughout the China, and its roots, named Lang Du (Chinese Pharmacopoeia, 2010), is used in traditional Chinese medicine to treat cancer, swelling, and warts (Zhong Hua Ben Cao, 2010). A number of diterpenes with a wide spectrum of bioactivities, such as antihepatotoxic and cytotoxic activities, have been isolated from this species (Liu et al., 2016; Liang et al., 2014; Shi et al., 2005; Xu et al., 2005). In our continuing research for novel bioactive compounds from the genus of *Euphorbia* (Li et al., 2016; Mu et al., 2013; Deng et al., 2010), three new diterpernes and four known analogues were isolated from roots of the title plant. Herein, we presented the isolation, structural elucidation, and bioactivity evaluation of these compounds.

2. Results and discussion

The 95% EtOH extracts of the roots of *Euphorbia ebracteolata* were fractionated by solvent partition. Repeated silica gel, MCI gel and Sephadex LH-20 column chromatography led to the isolation of three new diterpernes, Ebracteolatas A–C (**1**–**3**), as well as four known ones, jolkinol A (**4**) (Valente et al., 2004), jolkinol A' (**5**) (Valente et al., 2004), Yuexiandajisu F (**6**) (Shi et al., 2005), and jolkinol B (7) (Wang and Ding, 1998). (Fig. 1).

Ebracteolata A (**1**), a white, amorphous power, has the molecular formula $C_{20}H_{30}O_2$, as deduced by HRESIMS at *m/z* 325.2128 $[M+Na]^+$ (calcd. 325.2138). Its IR spectrum showed absorption bands for OH (3425 cm^{-1}), C=O (1712 cm^{-1}), and C=C (1637 cm^{-1}) groups. The ^1H and ^{13}C NMR data of **1** implied the typical signal for one ketocarbonyl (δc 213.0), one monosubstituted olefinic bond (δ_H 5.79, 4.91 and 4.84, δc 150.9 and 109.0), one 1,1,2-trisubsituted double bond (δ_H 5.54, δc 148.3 and 115.5), and one hydroxymethyl (δ_H 4.05 and 3.58, δc 65.7) (Table 1). These functionalities accounted for three out of six indices of hydrogen deficiency, and thus, three rings must exist in **1**.

Extensive analysis of NMR data of **1** indicated that **1** is a rosane-type diterpene, and had the same skeleton as **6** but with different substitution patterns in ring A (Shi et al., 2005). 2D NMR experiments, especially HMBC (Fig. 2A), further confirmed this

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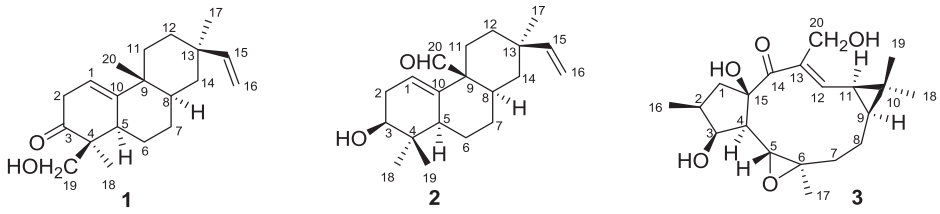

Fig. 1. Structures of compounds **1**–**3**.

Table 1
^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **1**–**2** (δ in ppm, J in Hz).

Position	**1**[a]		**2**[b]	
	δ_C	δ_H	δ_C	δ_H
1	115.5	5.54, dd (3.4,6.6)	122.1	5.57, m
2a	39.0	3.14, dt (2.6,16.5)	32.1	2.39, m,
2b		2.81, m		1.97, m
3	213.0		74.5	3.54, dd (5.9,10.1)
4	52.3		37.1	
5	44.7	2.54, m	43.9	2.10, d (14.0)
6a	19.5	1.72, m	19.4	1.73[c], m
6b		1.48, m		1.10, m
7a	25.4	1.61, m	26.2	1.93, m
7b		1.27, m		1.73[c], m
8	31.3	1.65, m	30.3	1.58, m,1.90, m
9	37.4		54.0	
10	148.3		138.7	
11a	35.0	1.70, m	29.4	2.17, m
11b		1.47, m		1.30, m
12a	32.7	1.50, m	33.2	1.46, m
12b		1.27, m		1.32, m
13	36.3		36.1	
14a	39.7	1.19, m	40.5	1.58, m
14b		1.13, m		1.28, m
15	150.9	5.79, dd (10.8,17.6)	150.2	5.75, dd (10.8,17.5)
16a	109.0	4.91, dd (1.3,17.5)	109.3	4.90, d (17.5)
16b		4.84, dd (1.3,12.1)		4.85, d (10.7)
17	22.2	0.94, S	21.6	0.94, S
18	16.7	1.17, s	23.9	1.03, S
19a	65.7	4.05, d (10.7)	12.8	0.62, S
19b		3.58, d (10.8)		
20	20.6	0.99, s	202.5	9.29, S

[a] Recorded in $CDCl_3$ at 400 and 125 MHz.
[b] Recorded in $CDCl_3$ at 600 and 150 MHz.
[c] Overlapped.

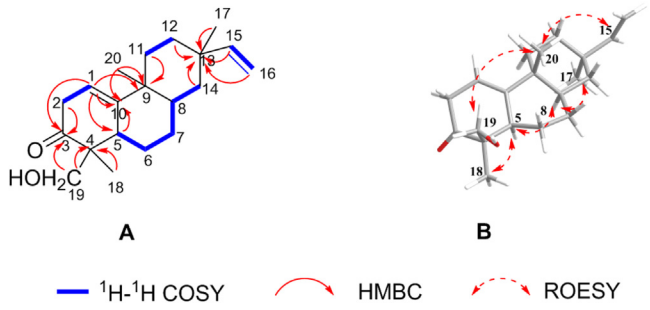

Fig. 2. (A) ^{1}H–^{1}H COSY (Bold) and Key HMBC Correlations (Arrow, H → C) of 1. (B) Key ROESY Correlations of 1.

suggestion. The HMBC correlations of H-1 with C-5, C-9, and C-10 located the trisubstituted double bond between C-1 and C-10. The ketone group was placed at C-3 by the HMBC correlations of H-1 and H-2/C-3. The cross peaks of H_2-12, H_2-14, H-15, H_2-16, and Me-17/C-13 in HMBC spectrum indicated the presence of a $\Delta^{15(16)}$ double bond. The hydroxymethyl was located at C-19 by the HMBCs from H_2-19 and Me-18 to C-4. Therefore, the planar structure of compound **1** was established as shown, and the relative configuration of **1** was determined by the ROESY spectrum and 3D computer modeling (Fig. 2B). The ROE correlations of H_2-19/Me-20, Me-20/H-15 indicated that both groups are cofacial, and were assigned arbitrarily as β-orientation. In turn, the ROE correlations of Me-18/H-5, H-5/H-8, and H-8/Me-17 suggested the α-orientation of these groups. Thus, the structure of compound **1** was unambiguously assigned as shown.

The molecular formula of Ebracteolata B (**2**) was assigned as $C_{20}H_{30}O_2$ by the (+)-HRESIMS ion at m/z 325.2138 $[M+Na]^+$ (calcd 325.2138). Analysis of the NMR data of **2** showed that it possessed the same skeleton as **6**. The main difference between them was that the methylene and aldehyde group (δ_C 202.5) replaced the hydroxymethine at C-2 and methyl at C-20 in the latter, respectively. The ^{1}H–^{1}H COSY from H_2-2 to H-1 and H-3 located the methylene group at C-2. The aldehyde unit was located at C-20 by the HMBC correlation (Fig. 3A) from CHO-20 (δ_H 9.29) to C-8 and C-9. The remaining planar and relative configurations of **2** were identical to those of **6**, as determined by the HMBC, and ROESY data.

Ebracteolata C (**3**) gave the molecular formula $C_{20}H_{30}O_5$ from (+)-HRESIMS ion at m/z 373.1989 $[M+Na]^+$ (calcd 373.1985). The ^{13}C NMR and DEPT spectra of **3** showed signals corresponding to four CH_3, four CH_2 (one oxygenated at δ_C 58.4), seven CH (one sp^2 at δ_C 152.6, and two oxymethines at δ_C 79.8 and 58.5), and five quaternary carbons (one carbonyl group at δ_C 199.2, one olefinic carbon at δ_C 136.9, and two C—OH at δ_C 89.2 and 63.7). Based on the six degrees of unsaturation deduced by the molecular formula, four additional rings were required.

By detailed comparison of NMR data (Table 2) between **3** and jolkinol A (**4**), **3** was implied to resemble closely **4**, except for the absence of the benzoyl group in the latter, thus indicating that **3** was 15-debenzoyl jolkinol A. This conclusion was further confirmed by 2D NMR data (Fig. 4).

All compounds were evaluated for their *in vitro* growth inhibitory effects against five human cancer cell lines, namely, HL-60 (human promyelocytic leukemia cell line), SMMC-7721 (human hepatocellular carcinoma cell line), A-549 (human lung cancer cell line), MCF-7 (human breast cancer cell line), and SW480 (colorectal cancer cell line), using a previously described protocol 3.4. Among them, compounds **1**, **6**, and **7** showed the moderate

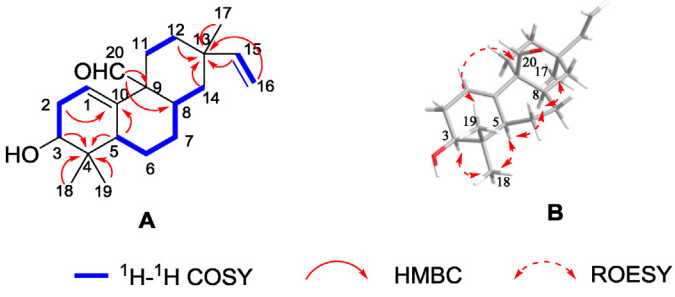

Fig. 3. (A) ^{1}H–^{1}H COSY (Bold) and Key HMBC Correlations (Arrow, H → C) of 2. (B) Key ROESY Correlations of 2.

Table 2
^{1}H and ^{13}C NMR Spectroscopic Data for Compounds **3** Recorded in $CDCl_3$ at 600 and 150 MHz. (δ in ppm, J in Hz).

position	δ_c	δ_H
1a	46.8	3.32, dd (8.5,13.7)
1b		1.56, m
2	38.5	1.92, m
3	79.8	4.17, m
4	53.2	1.44, dd (3.2,9.6)
5	58.5	3.44, dd (9.5)
6	63.7	
7a	38.8	2.02, m
7b		1.58, m
8a	23.2	2.04, m
8b		1.62, m
9	35.9	1.27, m
10	27.9	
11	29.9	1.72, dd (7.9,11.8)
12	152.6	7.84, d (11.9)
13	136.9	
14	199.2	
15	89.2	
16	13.7	1.11, S
17	20.3	1.22, S
18	29.0	1.21, S
19	16.2	1.12, S
20a	58.4	4.46, d (12.0)
20b		4.35, m

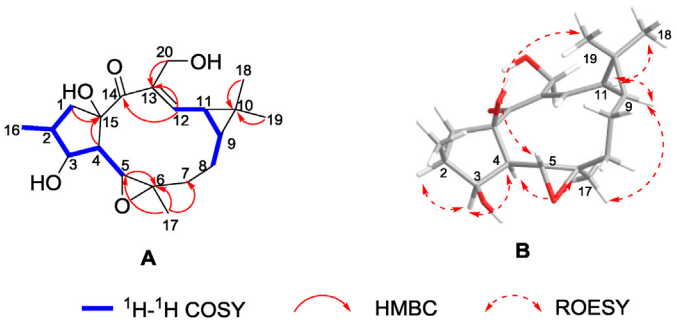

Fig. 4. (A) ^{1}H–^{1}H COSY (Bold) and Key HMBC Correlations (Arrow, H → C) of 3. (B) Key ROESY Correlations of 3.

cytotoxic activity with IC_{50} values in the range of 3.5–19.7 μM (Table 3).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a Jasco P-1020 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for IR spectra as KBr pellets. 1D and 2D NMR spectra were recorded on Bruker spectrometer with TMS as internal standard. HRESIMS was performed on a triple quadrupole mass spectrometer. Semi-preparative HPLC was performed on an Agilent 1100 liquid chromatograph with a Waters X-Bridge Prep Shield RP18 (10 × 150 mm) column. Column chromatography (CC) was performed using silica gel (100–200 mesh and 300–400 mesh, Qingdao Marine Chemical, Inc., Qingdao, P. R. China) and Sephadex LH-20 (40–70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Table 3
IC_{50} Values (μM) of Diterpenoids from *Euphorbia ebracteolata* Hayata for Human Tumor Cell Lines.

Compound[a,b]	HL-60	A549	SMMC-7721	MCF-7	SW480
1	17.5	11.0	16.8	17.5	18.0
6	16.8	19.7	18.4	15.3	15.3
7	5.0	11.5	3.5	15.8	9.5
DDP	19.1	12.5	17.7	19.7	11.9
Taxol	<0.008	<0.008	<0.008	<0.008	<0.008

[a]Other compounds than selected ones were inactive(IC_{50} > 40 μM)
[b]DDP(cisplatin) and Taxol were used as positive controls.

3.2. Plant material

The roots of *Euphorbia ebracteolata* were collected from Anhui Province, People's Republic of China, in November 2014. The plant samples were identified by Prof. Ji-Ming Xv of Kunming Institute of Botany, Chinese Academy of Science (CAS). A voucher specimen (HXJ20141108) was deposited at the State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

3.3. Extraction and isolation

The air-dried, powdered plant materials (20 kg) were extracted with 95% EtOH (3 × 50 L) under reflux three times (4, 3, and 3 h, respectively). The combined EtOH extracts were concentrated under vacuum to give a crude residue (2.1 kg), which was suspended in water and then partitioned with petroleum ether. The petroleum ether portion (788 g) was subjected to passage over a silica gel column, eluted with a gradient of petroleum ether-acetone (from 1:0 to 0:1), to yield five major fractions (1–5). Fr.3 (60 g) was then separated over a C18 silica gel column (MeOH—H_2O from 4:6 to 10:0) to obtain seven further fractions (3A–3G). Fr.3C (9 g) was chromatographed on a silica gel column eluted with petroleum ether-acetone (50:1 to 10:1), to afford five subfractions (3C1-3C5). Fr3C2 (2.2 g) was purified by Sephadex LH-20 (Acetone) and then chromatographed on a silica gel column eluted with petroleum ether-acetone (25:1) to obtain **7** (200 mg) and **2** (3 mg). Fr.3E (5 g) was separated by Sephadex LH-20 (MeOH—$CHCl_3$, 1:1) to obtain **1** (100 mg) and a major fraction (Fr.3E3). Fr.3E3 (500 mg) was separated by semipreparative HPLC (CH_3CN—H_2O, 6:4) to give **3** (5 mg), **4** (200 mg), and **5** (100 mg).

3.3.1. Ebracteolata A (*1*)

White, amorphous powder; $[\alpha]_D^{21}$ +37.7 (*c* 0.2 MeOH); UV (MeOH) λ_{max} (log ε) 204 (3.74) nm, 314 (2.71) nm, 394 (1.98); IR (KBr) ν_{max} 3425, 3081, 2969, 2927, 2882, 2863, 1712, 1637, 1468, 1453, 1414, 1379, 1349, 1326, 1288, 1214, 1095, 1033, 1001, 909 cm^{-1}; positive ESIMS *m/z* 325 $[M+Na]^+$; HRESIMS *m/z* 325.2128 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_2Na$, 325.2138); ^{1}H and ^{13}C NMR data, see Table 1.

3.3.2. Ebracteolata B (*2*)

White, amorphous powder; $[\alpha]_D^{21}$ −77.5 (*c* 0.2 MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.00) nm; IR (KBr) ν_{max} 3418, 2925, 2867, 1750, 1693, 1630, 1448, 1387, 1370, 1332, 1269, 1247, 1169, 1102, 1062, 1033, 974, 632, 586, 558 cm^{-1}; positive ESIMS *m/z* 325 $[M+Na]^+$; HRESIMS *m/z* 325.2138 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_2Na$, 325.2138); ^{1}H and ^{13}C NMR data, see Table 1.

3.3.3. Ebracteolata C (*3*)

White, amorphous powder; $[\alpha]_D^{21}$ −6.3 (*c* 0.1 MeOH); UV (MeOH) λ_{max} (log ε) 267 (4.09) nm, 201 (3.77) nm; IR (KBr) ν_{max} 3442, 3428, 2952, 2928, 2872, 1622, 1455, 1412, 1383, 1265, 1206, 1178, 1152, 1121, 1060, 1042, 1009, 994, 861, 561; positive ESIMS *m/z* 373 $[M+Na]^+$; HRESIMS *m/z* 373.1989 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_5Na$, 373.1985); ^{1}H and ^{13}C NMR data, see Table 2.

3.4. Cytotoxicity assays

The following human tumor cell lines were used: HL-60, SMMC-7721, A-549, MCF-7, and SW-480. All cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium (MTS) (Sigma, St. Louis, MO, USA). Briefly, 100 μL of adherent cells was seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h before test compound addition, while suspended cells were seeded just before test compound addition, both with an initial density of 1×10^5 cells/mL in 100 μL of medium. Each cell line was exposed to the test compound at various concentrations in triplicate for 48 h, with cisplatin and paclitaxel (Sigma) used as positive controls. After the incubation, MTS (100 μg) was added to each well, and the incubation continued for 4 h at 37 °C. The cells were lysed with 100 μL of 20% SDS-50% DMF after removal of 100 μL of medium. The optical density of the lysate was measured at 595 nm in a 96-well microtiter plate reader (Bio-Rad 680). The IC50 values of each compound were calculated by Reed and Muench's method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2016.10.008.

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