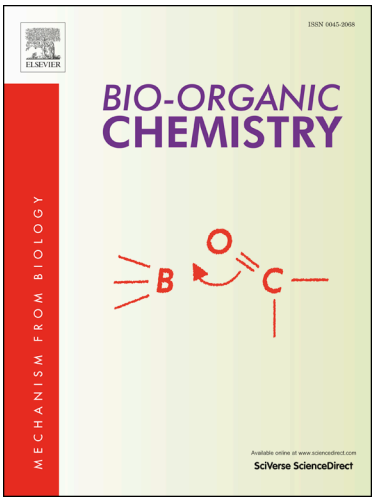

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Cytotoxic *ent*-Abietane-type Diterpenoids from the Roots of *Euphorbia ebracteolata*

Chunhui Han,[†a] Yulin Peng,[†b] Yifei Wang,[†b] Xiaokui Huo,[b] Baojing Zhang,[b] Dawei Li,*[a] Aijing Leng,[a] Houli Zhang,[b] Xiaochi Ma,*[b] and Chao Wang*[b]

[a] The first affiliated hospital of Dalian Medical University, No.222 Zhongshan Road, Dalian 116011, People's Republic of China

[b] College of Pharmacy, Dalian Medical University, Dalian 116044, People's Republic of China

ABSTRACT

Euphoroids A–C (**1**–**3**), three new *ent*-abietane-type diterpenoids, together with ten known analogues (**4**–**13**) were obtained from the roots of *Euphorbia ebracteolata*. The structures of these compounds were determined by extensive spectroscopic data analysis, including UV, HRESIMS, 1D-, and 2D-NMR data. The inhibitory effects of compounds **1**–**13** on human cancer cells were determined using the MTT assay. The results revealed that new compounds **2** and **3** showed moderate cytotoxic activities against human cancer cell lines. Especially, compound **3** displayed selective cytotoxic effect agains cancer cell lines.

Keywords: Euphorbia ebracteolata; diterpenoids; abietane; cytotoxic activities

1. Introduction

*Corresponding authors.
E-mail addresses: lidw87@163.com (D.W. Li), maxc1978@163.com (X. Ma), wach_edu@sina.com (C. Wang)
[†]These authors contributed equally to this work.

Euphorbia ebracteolata Hayata (Euphorbiaceae) is a biennial herbaceous plant that is widely distributed and cultivated in China. Its dried roots, named "Lang Du" in Chinese, are used in traditional Chinese medicine for the treatments of edema, cough, indigestion, asthma, and chronic bronchitis [1]. Chemical studies of the roots of *E. ebracteolata* revealed that these roots contain diterpenoids, triterpenoids, sesquiterpenoids, flavonols, and acetophenones, etc. of which diterpenoids are the main active constituents [2-6]. As part of a program to assess the chemical and biological diversity of traditional Chinese medicines, the ethanol extract of the roots of *E. ebracteolata* has been investigated. Our previous studies on the ethanol extracts led to the isolation of 27 new compounds including monoterpenoids, rosane, *ent*-kaurane, *ent*-atisane, tigliane, ingenane, heterodimeric diterpenoids, and phenolic glycosides. Some of them exhibited the DPPH scavenging effects, anti-tuberculosis effects, and inhibitory effects against α-glucosidase and human carboxylesterase 2 [7-10]. In this study, three new *ent*-abietane-type diterpenoids Euphoroids A–C (**1**–**3**), together with ten known analogues (**4**–**13**) were isolated from the ethanol extracts of the roots of *E. ebracteolata* by several chromatographic technologies (Fig. 1). Their structures were determined by spectroscopic analyses. The cytotoxicities against six human cancer cell lines of **1**–**13** were evaluated. The structure-activity relationship of *ent*-abietane-type diterpenoids and their cytotoxic activities were firstly also reported. We present herein the isolation and structural characterization of Euphoroids A–C (**1**–**3**), as well as their cytotoxic activities.

2. Materials and methods

2.1. General methods

UV spectra were recorded on a JASCO P2000 automatic digital polarimeter. 1D- and 2D-NMR spectra were obtained at 600 for ^{1}H and 150 MHz for ^{13}C, respectively, on a Burker 600 MHz spectrometer with solvent peaks as references. HRESIMS data were obtained with an Agilent 1290 Infinity liquid chromatography system and an Agilent 6540 UHD Accurate-Mass Q-TOF mass spectrometer. Analytical HPLC data

were collected on an UltiMate 3000 instrument (Thermo Scientific Dionex) equipped with a diode array detector (DAD). Preparative HPLC separation was performed on an Agel instrument with a UV detector and a YMC C_{18} column (250 × 20mm, 5 mm). Column chromatography (CC) was performed with silica gel (200−300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China). TLC was conducted on precoated silica gel GF254 plates (5 × 10 cm, 2.5 × 7.5 cm, Qingdao Marine Chemical Inc.). Chromatographic grade methanol were purchased from Sigma-Aldrich. All other solvents were of chemical grade (Kermel Chemical Co. Ltd, Tianjin, China). Spots were visualized under UV light (254 or 365 nm) or by spraying with 10% sulfuric acid in EtOH, followed by heating at 105 °C.

2.2. Plant material

The roots of *E. ebracteolata* were purchased from Bozhou city of Anhui province in China. The plant material was identified by Prof. Qing-Shan Yang (Anhui University of Chinese Medicine). A voucher specimen (No. P-231) was deposited in the College of Pharmacy, Dalian Medical University.

2.3. Extraction and isolation

The pulverized plant material (15 kg) was decocted with 80% EtOH (150 L; 3 × 1.5 h). The extracts were combined and evaporated under reduced pressure to yield the aqueous residue. The aqueous residue was diluted with H_2O and sequentially partitioned with petroleum ether, EtOAc, and n-BuOH. EtOAc extract (504 g) was chromatographed over silica gel, eluting with petroleum ether/acetone (50:1−2:1) mixtures, to yield 55 fractions. Fractions 3 (35 g) and 5 (77 g) were further separated into 14 fractions (A1–A14) and 20 fractions (B1–B20) by MPLC (ODS) using MeOH:H_2O (50:60–100:0) mixtures as the eluent, respectively. Subfraction A4 (350 mg) was purified by preparative HPLC (RP C_{18} column, 8 ml/min, detected at 210 and 250 nm) with a 60:40 MeOH:H_2O mixture as the eluent to yield compounds **4** (17.9 mg; t_R 33.1 min) and **9** (6.6 mg; t_R 39.0 min). Compounds **8** (7.1 mg; t_R 42.1 min) and **10** (4.0 mg; t_R 53.0 min) were isolated from subfraction A5 (200 mg) by preparative HPLC with a 65:35 MeOH:H_2O mixture as the eluent. Subfraction B6 (450 mg)

was separated by preparative HPLC (MeOH/H_2O, 66:34) and afforded the production of compounds **1** (4.5 mg; t_R 19.5 min), **2** (3.0 mg; t_R 23.0 min), **3** (4.5 mg; t_R 75.0 min), **11** (2.2 mg; t_R 21.3 min), **12** (3.6 mg; t_R 15.2 min), and **13** (5.4 mg; t_R 17.0 min). Compounds **5** (45 mg; t_R 31.0 min), **6** (2.2 mg; t_R 36.0 min), and **7** (18.3 mg; t_R 38.0 min) were isolated from subfraction B7 (400 mg) by preparative HPLC (MeOH/H_2O, 68:32).

2.3.1. Euphoroid A (**1**)

White amorphous powder; $[\alpha]_D^{20}$ + 11.0 (*c* 0.05 CH_3OH); UV (CH_3OH) λ_{max} 232.1 nm; ^1H NMR ($CDCl_3$, 600 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table 1; (+)-HR-ESIMS *m/z* 333.2069 $[M + H]^+$ (calcd for $C_{20}H_{29}O_4$, 333.2060).

2.3.2. Euphoroid B (**2**)

White amorphous powder; $[\alpha]_D^{20}$ – 8.2 (*c* 0.05 CH_3OH); UV (CH_3OH) λ_{max} 290.9 nm; ^1H NMR ($CDCl_3$, 600 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table 1; (+)-HR-ESIMS *m/z* 315.1995 $[M + H]^+$ (calcd for $C_{20}H_{27}O_3$, 315.1955).

2.3.3. Euphoroid C (**3**)

White amorphous powder; $[\alpha]_D^{20}$ – 13.0 (*c* 0.05 CH_3OH); UV (CH_3OH) λ_{max} 202.8, 220.5 nm; ^1H NMR ($CDCl_3$, 600 MHz) data, see Table 1; ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table 1; (+)-HR-ESIMS *m/z* 635.4278 $[M + Na]^+$ (calcd for $C_{38}H_{60}O_6Na$, 635.4278).

*2.4. Hydrolysis of **3***

Compound **3** (2 mg) was dissolved in 95% MeOH (1.0 mL), and K_2CO_3 (10 mg) was added. The solution was stirred at 40 °C for 12 h, acidified with HCl (2 N), and then evaporated under reduced pressure. The residue was dissolved in MeOH and subjected to HPLC analysis.

2.5. Cells and culture conditions

A549 (human lung cancer cell line), MCF-7 (human breast cancer cell line), Mewo (human melanoma cell line), SH-SY5Y (human neuroblastoma cell line), Lovo (human colon adenocarcinoma cell line), and HepG2 (human liver cancer cell line) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in RRMI1640 supplemented with 10% fetal

bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were incubated at 37 ℃ in a humidified 5% CO_2 atmosphere.

2.6. Cells proliferation assay

A549, MCF-7, Mewo, SH-SY5Y, Lovo, and HepG2 were seeded in 96-well microtiter plates at 1200 cells/well. After 24 h, the compounds **1**–**13** were added to the cells. After 96 h of drug treatment, cell viability was determined by measuring the metabolic conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) into purple formazan crystals by active cells. MTT assay results were read using a MK 3 Wellscan (Labsystem Drogon) plate reader at 570 nm. Compounds **1**–**13** were tested at five concentrations and were dissolved in 100% DMSO to give a final DMSO concentration of 0.1% in each well. Each concentration of the compounds was tested in three parallel wells. IC_{50} values were calculated using Microsoft Excel software [11].

3. Results and discussion

Compound **1** was obtained as a white amorphous powder. The molecular formula was determined as $C_{20}H_{28}O_4$ by HRESIMS at *m/z* 333.2069 $[M + H]^+$ (calcd for $C_{20}H_{29}O_4$, 333.2060), combined with the NMR data (Table 1). The ^{1}H NMR spectrum of **1** exhibited resonances attributable to an olefinic group at δ_H 6.11 (m, H-7); three oxymethine groups at δ_H 4.92 (s, H-14), 5.03 (m, H-12), and 3.32 (dd, J = 10.8, 8.4 Hz, H-11); a vinylic methyl group at δ_H 1.88 (d, J = 1.8 Hz, H-17); and three tertiary methyl groups at δ_H 0.92 (s, H-18), 0.90 (s, H-19), and 0.92 (s, H-20). The ^{13}C NMR and HSQC spectra displayed 20 carbon signals (Table 1) corresponding to four methyls, four methylenes, six methines (including one olefinic carbon at δ_C 133.4 and three oxygenated carbons at δ_C 84.3, 77.9, and 70.3), and six quaternary carbons (one carbonyl at δ_C 174.5 and three olefinic carbons at δ_C 135.9, 156.1, and 122.3). The ^{1}H–^{1}H COSY cross peaks between H-1/H-2/H-3, H-5/H-6/H-7, and H-9/H-11/H-12 and HMBC correlations from H-17 to C-13, C-15, and C-16; from H-12 to C-11, C-13, and C-15; from H-11 to C-8, C-9, C-10, C-12, and C-13; from H-14 to C-7, C-9, C-12, and C-15 (Fig. 2); together with chemical shifts of

these proton and carbon resonances, revealed **1** has the same planar structure as 11*β*,14*β*-dihydroxy-ent-abieta-7(8),13(15)-dien-16,12*β*-olide (fischeriolide A)[12]. However, comparison of the NMR data of **1** with fischeriolide A indicated that the C-13 and C-15 resonance in **1** were shielded by $\Delta\delta_C$ −3.4 and −4.6 ppm, respectively, whereas the C-7, C-8, C-9, C-11, and C-12 resonances were deshielded by $\Delta\delta_C$ +2.5 ~ + 12.2 ppm. Meanwhile, compound **1** has the reverse rotation as the reported fischeriolide A. These suggested that the configuration of **1** should be different from that of fischeriolide A, which was further supported by a NOESY experiment. The NOE correlations of H-9/H-12, H-9/H-14, and H-11/H-14 and CH_3-20 indicated *α*-orientations for H-9, H-11, H-12 and H-14 (Fig. 2). Therefore, the structure of **1** was determined to be Euphoroid A.

Compound **2**, a white amorphous powder, has a molecular formula of $C_{20}H_{26}O_3$ as determined by HRESIMS and NMR data (Table 1), which showed 18 less mass units than **1**. The 1D NMR spectra (Table 1) displayed similar pattern to **1**, while the major difference was the hydroxy group at C-11 in **1** was replaced by one double bond which was placed at C-11-C-12 in **2**, consistent with the HMBC correlations (Fig. 3) of H-11 with C-8, C-9, and C-12. The NOESY correlations (Fig. 3) of H-5/H-9, and H-9/H-14 indicated that these protons were co-facial and assigned a *β*-orientation. Thus, compound **2** was elucidated to be Euphoroid B.

Compound **3** was obtained as a white powder. Its molecular formula was determined to be $C_{38}H_{60}O_6$ based on the HRESIMS ion at *m/z* 635.4278 $[M + Na]^+$ (calcd for $C_{38}H_{60}O_6Na$, 635.4278) and NMR data (Table 1). The ^1H NMR spectrum suggested the presence of partially overlapped aliphatic methylenes and/or methines. The NMR data for the tetracyclic substructure of **3** were almost indistinguishable from those of yuxiandajisu E [13], and ester moiety linked to C-14 of **3** were similar to those of linoleic acid [14], with the only difference being the position of carbonyl group (δ 172.3), which were also proved by the 2D NMR data (Fig. 4). The two parts were linked with an ester bond between C-14 and C-2′ by the key HMBC cross peak of H-14 to C-1′. The standard linoleic acid and hydrolysate of **3** were also

subjected to HPLC analysis under the same condition. The same retention time confirmed the existence of linoleic acid in the structure of **3**. The relative configuration was determined by the NOESY spectrum (Fig, 4) in combination with yuexiandajisu E. The correlations of H11/ CH_3-20, H-12/ CH_3-20, H-5/ H-9, and H-12/ H-14 indicated an α-orientation for H-11, H-12, and H-14 and a β-orientation for H-5 and H-9. Thus, the structure of compound **3** was named Euphoroid C.

The ten known compounds were identified as jolkinolide E (**4**) [15], *ent*-11α-hydroxyabieta-8(14),13(15)-dien-16,12-olide (**5**) [16], jolkinolide F (**6**) [17], helioscopinolide E (**7**) [18], jolkinolide B (**8**) [19], jolkinolide A (**9**) [20], euphopilolide (**10**) [21], fischeriolide A (**11**) [12], yuexiandajisu D (**12**) [22], yuexiandajisu E (**13**) [22] from their spectroscopic data upon comparisons with values reported in the literatures.

In the bioassay experiment using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, the cytotoxic effects of isolated diterpenoids against six human cancer cell lines A549, MCF-7, Mewo, SH-SY5Y, Lovo, and HepG2, have been evaluated (Table 2). Compounds **2**, **3**, **8**, and **11** displayed moderate cytotoxicities on cancer cell lines. Especially, the bioactive compounds **2** and **3** were determined to be new diterpenoids. Additionally, new diterpenoid **3** inhibit the proliferation of four cancer cell lines A549, MCF-7, Lovo, and HepG2 with IC_{50} values < 30 μM. But, no cytotoxicity was observed for **3** against Mewo and SH-SY5Y (IC_{50} > 100 μM). Thus, diterpenoid **3** displayed selective cytotoxicity against human cancer cell lines.

4. Conclusions

In summary, the three new and ten known *ent*-abietane-type diterpenoids were isolated from the roots of *E. ebracteolata*. Additionally, **2**, **3**, **8**, **9**, and **11** showed cytotoxic effects against human cancer cell lines, especially for compound **3** showed selective cytotoxic activities against six human cell lines. The result, combined with our previous studies [7-10], shows that diverse compounds contribute towards pharmacological efficacy that supports and enriches the traditional uses of *E. ebracteolata* roots.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

^{1}H NMR, ^{13}C NMR, 2D NMR, and HRESIMS of new compounds associated with this article can be found online. Supplementary data associated with this article can be found in the online version.

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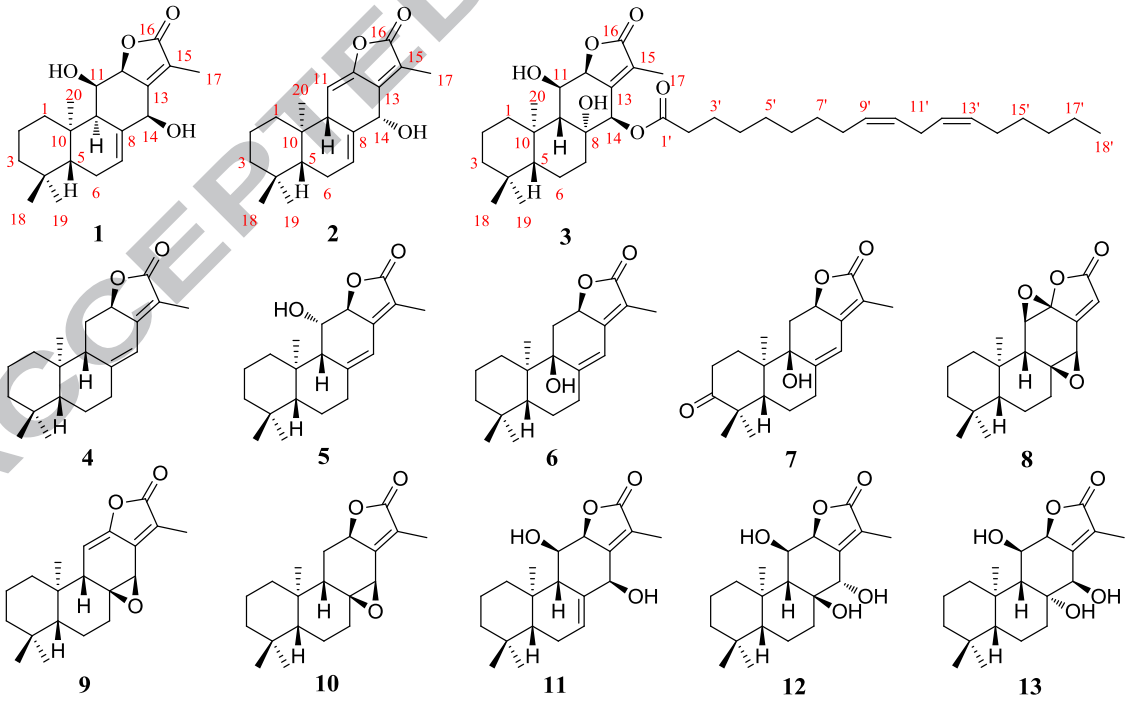


Fig. 1. Structures of *ent*-abietane diterpenoids **1**–**13** from the roots of *E. ebracteolata*.

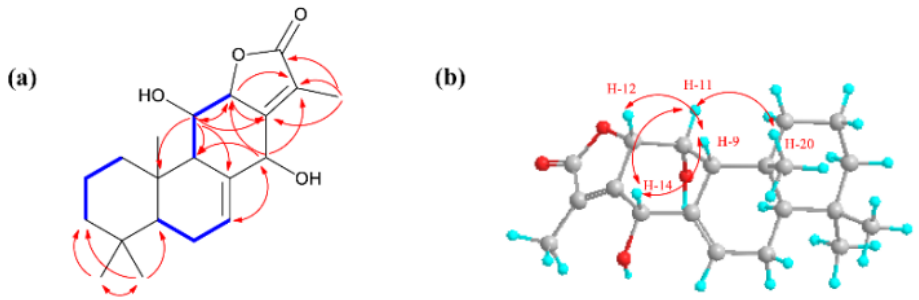


Fig. 2. (a) Main ^{1}H-^{1}H COSY (blue thick lines) and HMBC correlations (red arrows, from ^{1}H to ^{13}C) of **1**; (b) The key NOESY correlations of **1**.

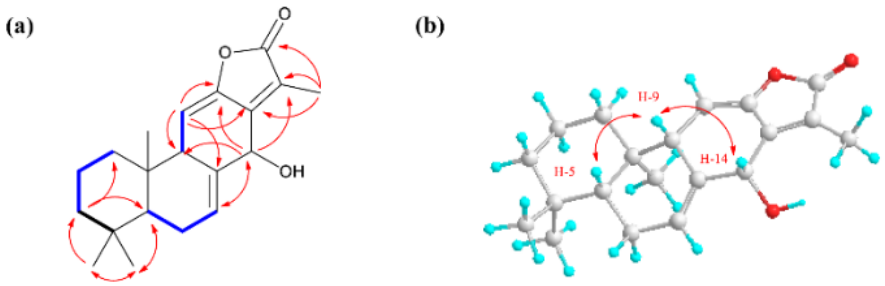


Fig. 3. (a) Main ^{1}H-^{1}H COSY (blue thick lines) and HMBC correlations (red arrows, from ^{1}H.

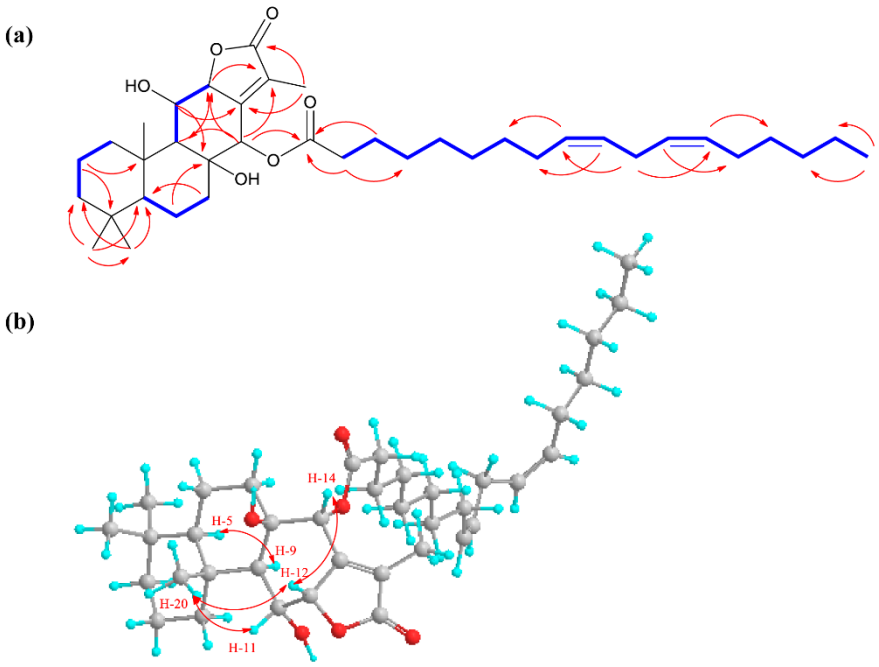


Figure 4. (a) Main ^{1}H-^{1}H COSY (blue thick lines) and HMBC correlations (red arrows, from ^{1}H to ^{13}C) of **3**; (b) The key NOESY correlations of **3**.

Table 1

The ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data of compounds **1**, **2**, and **3** (δ in ppm, J in Hz).

	1		**2**		**3**[a]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.57 m 1.20 m	42.0	1.96 m 1.18 m	38.8	1.95 m 1.64 m	41.6
2	1.51 m 1.42 m	19.0	1.59 m 1.55 m	18.7	1.62 m 1.54 m	18.9
3	1.42 m 1.20 m	42.0	1.48 m 1.23 m	42.1	1.45 m 1.22 m	41.7
4		33.4		33.1		33.4
5	1.41 m	50.3	1.37 dd (12.0,4.8)	49.1	1.07 m	55.1
6	2.07 m 1.92 m	23.5	2.20 m 1.92 m	24.0	1.62 m 1.42 m	20.4
7	6.11 m	133.4	6.02 t (2.4)	133.1	2.04 m 1.72 m	40.2
8		135.9		133.8		74.2
9	2.45 m	50.3	3.05 brs	47.8	2.09 s	62.2
10		36.9		36.3		37.9
11	3.32 dd (10.8,8.4)	77.9	5.77 d (2.4)	108.2	4.58 brs	68.6
12	5.03 m	84.3		148.4	5.11 m	78.9
13		156.1		146.6		151.8
14	4.92 s	70.3	5.02 s	67.7	5.50 s	73.6
15		122.3		122.9		129.9
16		174.5		171.3		174.2
17	1.88 d (1.8)	8.8	2.02 s	8.7	2.01 d (1.8)	8.94
18	0.92 s	22.8	0.90 s	21.8	0.92 s	34.2
19	0.90 s	33.9	0.90 s	33.1	0.87 s	22.1
20	0.92 s	14.4	0.70 s	14.6	1.71 s	17.9

[a]Data for the linoleoyl unit in **3**: δ_H 5.30−5.37 (4H, m), 2.76 (2H, t, J = 6.6 Hz), 2.34 (2H, m), 2.03 (4H, m), 1.62 (2H, m), 1.27−1.30 (14H, m), 0.88 (3H, t, J = 7.2 Hz); δ_C 172.3 (C-1'), 34.5 (C-2'), 24.9 (C-3'), 29.2 (C-4'), 29.2 (C-5'), 29.3 (C-6'), 29.7 (C-7'), 27.3 (C-8'),

130.1 (C-9'), 128.3 (C-10'), 25.8 (C-11'), 128.0 (C-12'), 130.4 (C-13'), 27.3 (C-14'), 29.5 (C-15'), 31.7 (C-16'), 22.7 (C-17'), 14.2 (C-18').

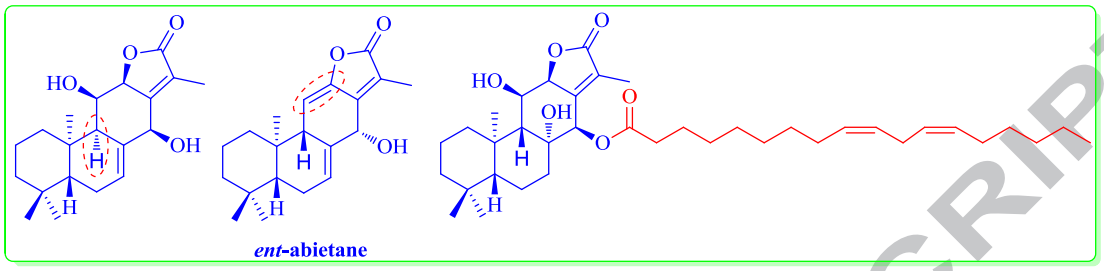
Table 2

Cytotoxicities of compounds **1**–**13**.

	Cytotoxicity IC_{50} (μM)					
No.	A549	MCF-7	Mewo	SH-SY5Y	Lovo	HepG2
1	>100	>100	>100	>100	>100	>100
2	22.87	85.7	80.50	58.63	95.68	95.63
3	28.7	28.57	>100	>100	27.0	28
4	>100	>100	>100	>100	>100	>100
5	>100	>100	>100	>100	>100	>100
6	>100	>100	>100	>100	>100	>100
7	>100	>100	>100	>100	>100	>100
8	94.7	73.6	62.7	85.8	63.2	95
9	18.56	>100	>100	>100	>100	>100
10	>100	>100	>100	>100	>100	>100
11	69.87	>100	88.45	57.56	85.0	>100
12	>100	>100	>100	>100	>100	>100
13	>100	>100	>100	>100	>100	>100
cisplatin[a]	2.5	3.0	2.5	4.4	2.8	5.1

[a]Cisplatin was used as the positive control. The data points represented the mean value of triplicate experiments.

Graphical abstract



Highlights

1. Three **NEW** and ten known *ent*-abietane diterpenoids were obtained from *Euphorbia ebracteolata*.
2. New diterpenoids **2** and **3** showed moderate cytotoxic activities against cancer cell lines.
3. New diterpenoid **3** displayed selective cytotoxic effects against cancer cell lines.