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Cembrane-type diterpenoids from *Macaranga pustulata*

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ABSTRACT

Three new cembrane-type diterpenoids, deheiculatins M − O (**1** − **3**), together with five known analogues (**4** − **8**), were isolated from the twigs of *Macaranga pustulata* King ex Hook. The structures of new compounds **1** − **3** were elucidated by extensive spectroscopic analyses, modified Mosher's method, and the experimental and calculated electronic circular dichroism (ECD) experiments. All the isolates were evaluated for their cytotoxicity on three human cancer cell lines (CNE1, CNE2, and HCT 116), and all of them showed weak cytotoxicity (IC_{50} > 20 μM).

1. Introduction

Macaranga genus (Euphorbiaceae) comprises of more than 300 species worldwide [1, 2]. This genus plants have been traditionally used for wound cleaning, antitussive, and antipyretic treatments [3, 4]. The sap of *Macaranga* genus plants have been used to treat fungal infections, and the leaf decoction was used as a stomachache agent [5]. In China, this species is commercially manufactured as products, such as health drinks and toothpaste [6]. *Macaranga pustulata* King ex Hook, small trees or shrubs, are widely distributed in secondary forests, mountain slopes, and valleys in Tibet and Yunnan Provinces in China [7]. Several earlier phytochemical investigations of this genus plants lead to the isolation of diterpenoids [8–11], triterpenoids [10], flavonoids [2, 12–18], bergenins [19], and stilbenes [20–22]. As part of our ongoing search for novel or active chemical constituents from the Euphorbiaceae family plants, three new cembranoids (**1** − **3**), together with 5 known analogues (**4** − **8**), were isolated from the twigs of *M. pustulata* King ex Hook.

2. Results and discussion

Compound **1**, colorless oil, had the molecular formula $C_{20}H_{30}O_3$ as established by positive-ion HRESIMS, requiring for six degrees of unsaturation. Its IR spectrum displayed the strong absorption band at 1641 cm^{-1} and a broad absorption band at 3399 cm^{-1} (OH), which suggested an α,β-unsaturated carboxyl group in compound **1**. The IR absorption peak at 2713 cm^{-1} indicated the presence of an aldehyde group (CHO). The ^1H NMR spectrum suggested that **1** contained an isopropyl group (δ_H 0.88, 0.86), a vinylic methyl group (δ_H 1.52), three olefinic protons (δ_H 6.66, 5.86, and 4.95), and an aldehyde proton (δ_H 9.34). The ^{13}C NMR data indicated three double bonds (δ_C 155.5, 143.1, 125.1, 134.4, 146.6, and 129.8), an aldehyde carbon (δ_C 196.0), and a carbonyl group (δ_C 172.7). Thus compound **1** must consist of one ring except for three double bonds and two carbonyl groups. From the above mentioned, the structure of compound **1** was similar to that of poilaneic acid (**7**) [23], which has a cembrane-type diterpenoid skeleton. The key differences in the NMR spectra of these compounds were the absence of a Δ^2 olefin and the additional substitution of an aldehyde group at C-18 in compound **1** when compared to **7**. The HMBC correlations of H-3 with C-1, C-5, and C-18 suggested that the aldehyde group was connected to C-4. Detailed 2D NMR spectra analyses (Fig. 2) allowed for the complete structural determination of **1** as shown in Fig. 1. The NOESY cross peaks between H-3 (δ_H 6.66) and H-18 (δ_H 9.34) indicated the *E* configuration of Δ^3 olefin. The *E* configuration of Δ^7 olefin could be deduced based on the NOESY correlation between H-7 (δ_H 4.95) and H-9 (δ_H 2.05). This could be validated by the ^{13}C NMR chemical shift of C-19 at δ_C 15.6, which was similar to the chemical shift of the *trans*-methyl group reported in isoneocembrene A [24, 25]. The Δ^{11} olefin was established as the *E* configuration due to the chemical shift of H-11 (δ_H 5.86), which was consistent with an olefinic proton *trans* to a carboxylic group in a trisubstituted olefin (calcd for δ_H, *trans* = δ_H 6.19) [26]. This could be further elucidated by the absence of the NOESY correlations of H-11 with H-13. The experimental electronic circular dichroism (ECD) spectrum of **1** showed a positive Cotton effect at 242 (+3.39) nm and a negative one at 215 (−1.66) nm, which was consistent with the calculated ECD of (1*S*)-**1** (Fig. 3) using the Gaussian 09 program at the TD-DFT-B3LYP/6-31 + G (d,p) level in MeOH. Therefore, compound **1** was determined as (1*S*,3*E*,7*E*,11*E*)-4-aldehyde-3,7,11-trien-20-oic acid, and given a trivial name deheiculatin M.

Compound **2** was obtained as a colorless oil, having the molecular formula $C_{20}H_{30}O_3$ deduced from the HRESIMS ion at *m/z* 319.2255

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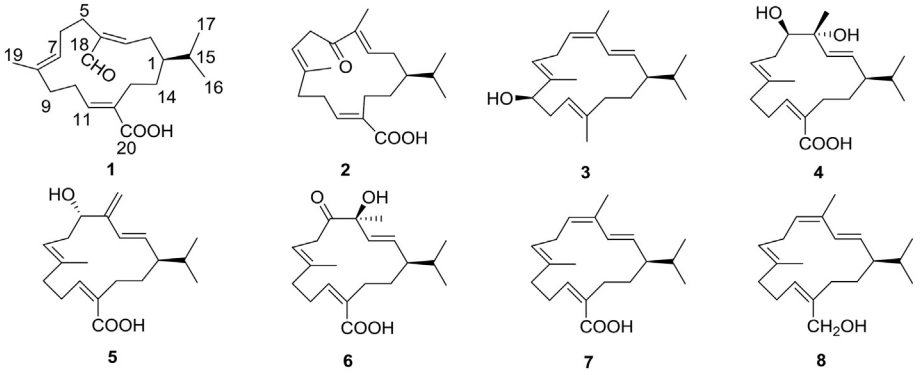

Fig. 1. Structures of compounds **1**–**8**.

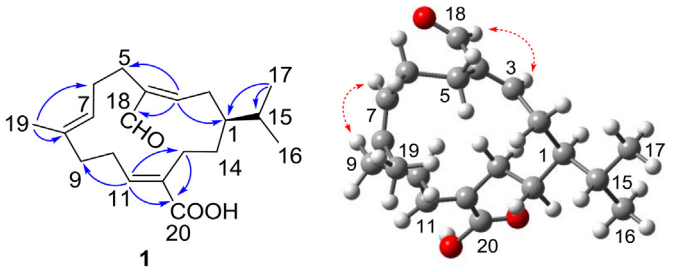

Fig. 2. Key $^{1}H - {}^{1}H$ COSY (—), HMBC (→), and NOESY (⋯) correlations of compound **1**.

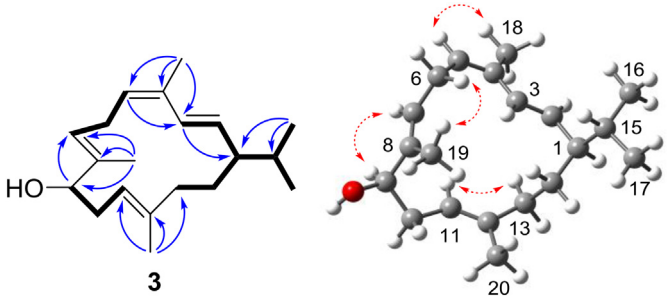

Fig. 4. Key $^{1}H - {}^{1}H$ COSY (—), HMBC (→), and NOESY (⋯) correlations of compound **3**.

$[M + H]^+$ (calcd for $C_{20}H_{30}O_3$, 319.2268), indicating six degrees of unsaturation. Its IR spectrum showed the presence of an *α,β*-unsaturated carboxylic carbonyl group (1643 cm^{-1} and 3392 cm^{-1}). The ^{1}H and ^{13}C NMR spectra was closely similar to those of **1**, except for the absence of an aldehyde group and a methylene and the presence of one ketone carbonyl group (δ_C 201.3) and one methyl group (δ_H 1.72, δ_C 11.7). The HMBC correlations from H_3-18 (δ_H 1.72, s) to C-3, C-4, and C-5 indicated the ketone group was attached to C-5 and the double bond was located at C-3/C-4. The configuration of C-3/C-4 was determined as *E* configuration based on the similar ^{13}C NMR chemical shift of C-18 with the reported compound deheiculatin E, isolated from *Macaranga deheiculata* [8]. The *E* configuration of Δ^7 olefin was established by the NOESY cross peaks between H-7 (δ_H 5.18) and H-9 (δ_H 2.26, 2.11). This could be further confirmed by comparison of the ^{13}C NMR chemical shift of C-19 at δ_C 15.7 with that of the *trans*-methyl group in the cembranoid skeleton. With the same method as used in compound **1**, the Δ^{11} olefin was determined as *E* configuration for the ^{1}H NMR chemical shift H-11 at δ_H 5.90. The absolute configuration of **2** was determined as 1*S*-configuration by comparison of the calculated and the experimental ECD spectra of **2** (Fig. 3). Thus, compound **2** was

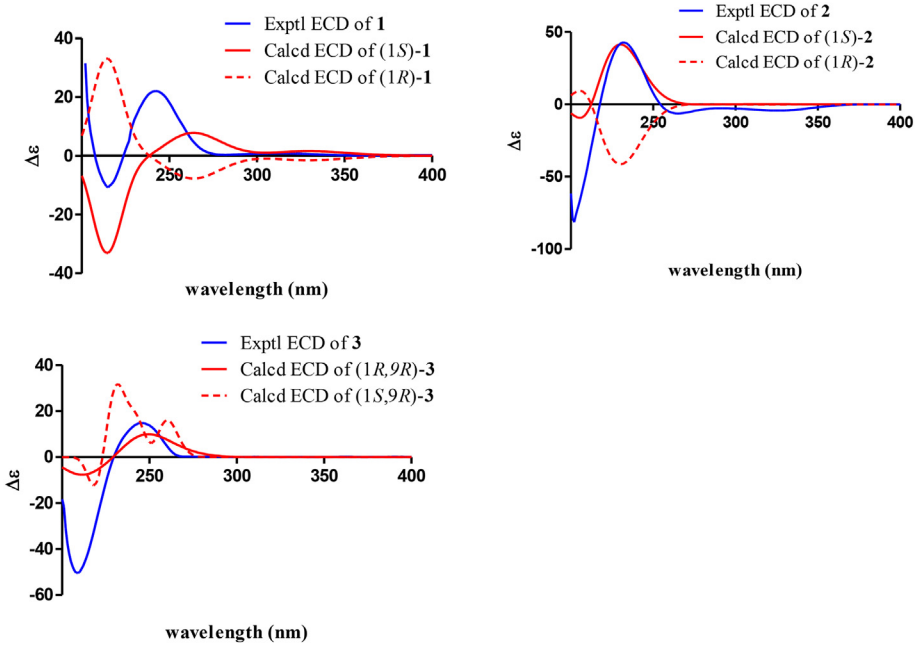

Fig. 3. Experimental ECD spectra (200–400 nm) and TD-DFT-calculated ECD spectra for compounds **1**–**3**.

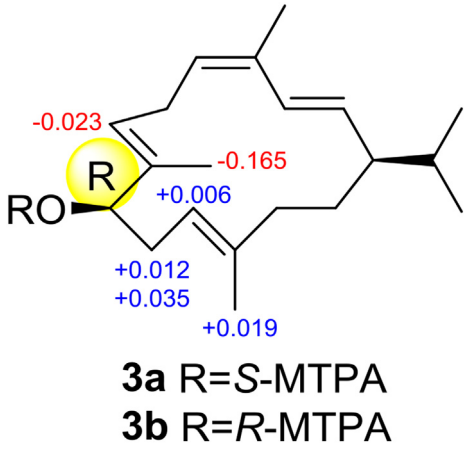

Fig. 5. $\Delta\delta_H$ ($S-R$) values (ppm) calculated from the 9-*O*-(*S*)- and 9-*O*-(*R*)-Mosher esters of compound **3**.

characterized as (1*S*,3*E*,7*E*,11*E*)-5-oxocembra-3,7,11-trien-20-oic acid, and named as deheiculatin N.

Compound **3**, colorless oil, was identified as (1*R*,2*E*,4*Z*,7*E*,9*R*,11*E*)-cembra-2,4,7,11-tetraen-9-ol. It had the molecular formula $C_{20}H_{32}O$ based on the HREIMS ion at *m/z* 288.2445 $[M]^+$ (calcd for $C_{20}H_{32}O$, 288.2448). The NMR data of **3** was similar to those of **7**, except for the absence of one methylene and carboxyl group and the presence of oxygenated methine and methyl group. The HMBC cross peaks from the hydrogen of oxygenated methine (H-9) to C-7 and from H_3-19 to C-9 suggested that the oxygenated methine was assigned to C-9. The additional singlets methyl located at C-20 was deduced through the HMBC correlations between H_3-20 and C-11/C-12/C-13. The $^1H-^1H$ COSY correlations indicated three spin couple fragments (Fig. 4), which further confirmed the planar structure of **3**. The configuration of the Δ^2-double bond was assigned as *E*, based on the coupling constant $J_{2,3}$ = 15.4 Hz [27], whereas the *Z* configuration of Δ^4 olefin was determined by the correlations of H-5/H-18 in the NOESY spectrum (Fig. 4). The key NOESY cross peaks of H_3-19/H-6, H-7/H-9, and H-11/H-13 accounted for the *E* configurations of Δ^7 and Δ^{11} olefins, respectively. To determine the absolute configuration of C-9, the OH group at C-9 was converted to *R*- and *S*-Mosher esters (Fig. 5). The absolute configuration of C-9 was assigned as *R* based on the $\Delta\delta_H$ (*S*-*R*) values of the ^1H NMR signals in $CDCl_3$. Based on the same biosynthetic pathway of the cembrane-type compounds, the absolute configuration of C-1 was assigned as *R*, which was further confirmed by comparison of the experimental and calculated ECD spectra of 1*R*,9*R*-**3**. Thus compound **3** was determined as (1*R*,2*E*,4*Z*,7*E*,9*R*,11*E*)-cembra-2,4,7,11-tetraen-9-ol and given a trivial name deheiculatin O.

The known compounds deheiculatin I (**4**) [8], deheiculatin C (**5**) [8], deheiculatin B (**6**) [8], poilaneic acid (**7**) [23], and 20-hydroxy *ent*-cembrene (**8**) [28–30] were identified based on the reported spectroscopic data.

All the isolates were assessed for cytotoxicity using the MTT method in CNE1, CNE2, and HCT 116 cell lines. None of them were cytotoxic toward these three human cancer cell lines ($IC_{50} < 20\,\mu M$).

3. Experimental section

3.1. General experimental procedures

Optical rotations were measured on a PerkinElmer 341 automatic polarimeter. UV spectra were recorded using a Shimadzu UV-2450 spectrophotometer. CD spectra were obtained on an Applied Photophysics Chirascan spectrometer. IR spectra were determined on a Bruker Tensor 37 infrared spectrophotometer. The ^1H (400 MHz), ^{13}C (100 MHz), and 2D NMR spectra were obtained on a Bruker AM-400 with TMS as an internal reference at 25 °C. Chemical shifts (δ) are expressed in ppm with reference to the solvent signals. HREIMS were measured on a Thermo MAT95XP high-resolution mass spectrometer, and EIMS on a Thermo DSQ EIMS spectrometer. HRESIMS were acquired on a Shimadzu LCMS-IT-TOF, and the ESIMS data were measured on an Agilent 1200 series LC-MS/MS system. RP-C_{18} silica gel (Fuji, 40–75 μm), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan), silica gel (200–300 Mesh Marine Chemical Ltd., Qingdao, China), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden) were used for column chromatography (CC). Analytical and semi-preparative HPLC separation were carried out on an LC-20AT Shimadzu liquid chromatography system with a ZORBA SB-C_{18} column (250 × 9.4 mm, 5 μm) or an Agilent SB-C_{18} column connected with an SPD-M20A diode array detector. TLC analysis was carried out on silica gel plates (Marine Chemical Ltd., Qingdao, China). Fractions were monitored by TLC and visualized by heating plates sprayed with 5% H_2SO_4 in EtOH. All solvents were of analytical grade (Guangzhou Chemical Reagents Company, Ltd.).

3.2. Plant material

The twigs of *M. pustulata* were collected in September 2014 from Lincang in Yunnan Province, People's Republic of China, and were authenticated by Dr. Chunyan Han of Kunming Institute of Botany, the Chinese Academy of Science. A voucher specimen (XG-2014006) has been deposited at the School of Pharmacy Sciences, Sun Yat-sen University.

3.3. Extraction and isolation

The air-dried and crushed twigs of *M. pustulata* (8 kg) were repeatedly extracted with 95% EtOH at room temperature. The EtOH extract was filtered and concentrated under reduced pressure and was suspended in H_2O (2 L) and then partitioned with EtOAc. The EtOAc extract (300 g) was subjected to silica gel column chromatography (CC), eluted with petroleum ether/EtOAc (20:1, 10:1, 3:1, 1:1), to obtain four corresponding fractions (A − D). Fraction A (120 g) was chromatographed on silica gel CC using petroleum ether/EtOAc (from 50:1 to 1:1) to get three fractions (A1 − A3). Fraction A3 was purified using a Sephadex LH-20 column, eluting with CH_2Cl_2/MeOH (1:1), to yield **7** (2 g). Fraction B was loaded on an RP-C_{18} column (MeOH/H_2O, 70% − 100%) to afford three major fractions (B1 − B3). Fraction B1 (4.6 g) was loaded on a Sephadex LH-20 column using CH_2Cl_2/MeOH (1:1) as solvent to yield three fractions (B1-a, B1-b, B1-c). Fraction B1-b (2.1 g) was separated on silica gel CC with petroleum ether/EtOAc (from 20:1 to 1:1) to yield **5** (3 mg), **6** (4 mg), and fraction B1-b-1. Fraction B1-b-1 was purified by semi-preparative HPLC (75% MeOH in H_2O, 1.5 mL/min) to yield **1** (5 mg) and **2** (8 mg). Fraction B2 (8 g) was fractionated over silica gel CC with petroleum ether/EtOAc (from 40:1 to 1:1) to get four fractions (B2-a, B2-b, B2-c, B2-d). Fraction B2-a was purified by semi-preparative HPLC (80% MeOH/H_2O, 1.5 mL/min) to yield **3** (8 mg) and **8** (30 mg). Compound **4** (15 mg) was isolated from fraction B2-c by semi-preparative HPLC (70% MeOH/H_2O, 1.5 mL/min).

3.3.1. Deheiculatin M (1)

Colorless oil; $[\alpha]$ $[25]_D$ + 69.8 (*c* 0.43, MeOH); UV (MeOH) λ_{max} (log ε) 228 (4.08) nm; ECD (MeOH) λ_{max} (log ε) 215 (−1.66), 242 (+3.39) nm; IR (KBr) ν_{max} 3396, 3188, 2961, 2924, 2853, 2715, 1687, 1645, 1445, 1416, 1384, 1261, 1095, 1020, 864, 799, and 703 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 319.2262 $[M + H]^+$ (calcd for $C_{20}H_{30}O_3$, 319.2268).

3.3.2. Deheiculatin N (2)

Colorless oil; $[\alpha]$ $[25]_D$ −129.4 (*c* 0.51, MeOH); UV (MeOH) λ_{max} (log ε) 226 (4.07) nm; ECD (MeOH) λ_{max} (log ε) 202 (−12.56), 232

Table 1
^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data of Compounds **1–3** in $CDCl_3$.

Position	1		2		3	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	41.7	1.43 (m)	41.6	1.29 (m)	48.6	1.57 (m)
2	30.5	2.23 (m)	29.2	2.12 (m)	131.6	5.16 (dd, 15.4, 9.8)
		2.23 (m)		2.21 (m)		
3	155.5	6.66 (t, 7.8)	146.1	6.42 (dd, 10.0, 4.2)	130.3	5.93 (d, 15.4)
4	143.1		136.7		135.9	
5	24.7	2.40 (m)	201.3		124.6	5.49 (t, 7.6)
		2.27 (m)				
6	25.4	2.48 (m)	40.4	3.65 (m)	26.0	3.07 (m)
		2.16 (m)		2.99 (dd, 14.0, 9.6)		2.41 (m)
7	125.1	4.95 (dd, 6.8, 6.0)	120.8	5.18 (dd, 8.8, 4.0)	129.2	5.28 (br d, 10.7)
8	134.4		136.2		133.4	
9	39.8	2.22 (m)	38.7	2.26 (m)	79.5	4.12 (dd, 11.2, 5.4)
		2.05 (m)		2.11 (m)		
10	26.4	2.62 (m)	26.5	2.99 (m)	32.1	2.35 (m)
		2.62 (m)		2.52 (m)		
11	146.6	5.86 (t, 7.8)	146.1	5.90 (t, 6.4)	121.7	4.64 (d, 8.0)
12	129.8		131.0		133.8	
13	32.2	2.36 (m)	28.6	2.47 (m)	36.7	1.99 (m)
		2.36 (m)		2.05 (m)		1.90 (td, 13.3, 3.6)
14	27.6	1.69 (m)	31.2	1.47 (m)	27.9	1.67 (m)
		1.39 (m)		1.28 (m)		1.21 (m)
15	29.8	1.82 (m)	28.8	1.88 (m)	33.0	1.47 (m)
16	19.4	0.88 (d, 6.8)	21.5	0.92 (d, 6.8)	21.0	0.84 (d, 6.8)
17	18.4	0.86 (d, 6.8)	16.8	0.79 (d, 6.8)	20.1	0.79 (d, 6.8)
18	196.0	9.34 (s)	11.7	1.72 (s)	20.1	1.76 (s)
19	15.6	1.52 (s)	15.7	1.65 (s)	9.5	1.62 (s)
20	172.7		173.5		14.7	1.50 (s)

(+6.61), 265 (−0.97) nm; IR (KBr) ν_{max} 3396, 3188, 2956, 2922, 2851, 1671, 1646, 1466, 1441, 1421, 1385, 1256, 1209, 1076, 1029, 947, 891, 830, 722, and 646 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 319.2255 $[M + H]^+$ (calcd for $C_{20}H_{30}O_3$, 319.2268).

3.3.3. Deheiculatin O (3)

Colorless oil; [α] $[25]_D$ −94.4 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 247 (4.29) nm; ECD (MeOH) λ_{max} (log ε) 209 (−10.83), 245 (+3.19) nm; IR (KBr) ν_{max} 3300, 2960, 2920, 1650, 1440, 1380, 1260, 1110, 1020, 966, 852, 796, and 698 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 288.2445 $[M]^+$ (calcd for $C_{20}H_{32}O$, 288.2448).

3.4. Cytotoxicity assay

The well-differentiated human nasopharyngeal carcinoma (CNE1), the poorly differentiated human nasopharyngeal carcinoma (CNE2), and human colorectal cancer (HCT 116) cell lines were obtained from Sun Yat-sen University Cancer Center and cultured in RPMI-1640 medium at 37 °C in a humidified atmosphere with 5% CO_2. The cells were seeded at 2000 − 4000 cells/well in 96-well plates. For the sample treatment experiments, the cells were treated in triplicate with graded concentration of compounds (predissolved in DMSO) for a period of three days. At the end of the compound treatment period, MTT solution (10 μL, 5 mg/mL) in PBS (PBS without MTT as the blank) was added to each well (containing 100 μL medium). After 4 h of incubation, the formazan crystal formed in the well was dissolved with 100 μL of DMSO for optical density reading at 492 nm [31]. All IC_{50} values were calculated by nonlinear regression analysis (GraphPad Prism).

4. Conclusions

Eight cembrane-type diterpenoids, including three new compounds, were isolated from the twigs of *M. pustulata*. All the isolates were evaluated for their cytotoxicity toward three human cancer cell lines (CNE1, CNE2, and HCT 116), and all of them showed weak cytotoxicity ($IC_{50} > 20\ \mu M$).

Conflict of interest

The authors declare no competing financial interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.06.020.

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