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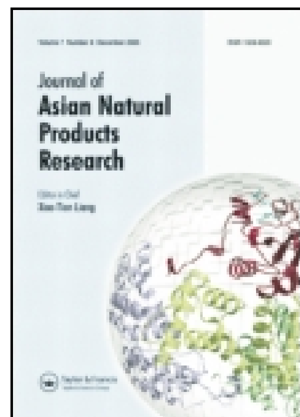

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Two new diterpenoids from Excoecaria acerifolia

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Two new diterpenoids from *Excoecaria acerifolia*

Xing-De Wu[a], Lan-Chun Zhang[b], Juan He[a], Gen-Tao Li[a], Lin-Fen Ding[b], Xiu Gao[a,c], Liao-Bin Dong[a,c], Liu-Dong Song[b,*], Yan Li[a] and Qin-Shi Zhao[a,*]

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A new tigliane diterpenoid, acerifolin A (**1**), and a new isopimarane diterpenoid, acerifolin B (**2**), together with two known compounds, were isolated from *Excoecaria acerifolia*. Their structures were elucidated on the basis of their spectroscopic methods, including 1D and 2D NMR techniques. All of the compounds were evaluated for cytotoxicity against five human cancer cell lines with cisplantin as a positive control.

Keywords: *Excoecaria acerifolia*; diterpenoids; cytotoxic activity; Euphorbiaceae

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1. Introduction

The genus *Excoecaria* (Euphorbiaceae), containing nearly 40 species, mainly grows in tropical Asia, Africa, and Oceania [1]. Previous phytochemical investigations on this genus revealed the presence of sesquiterpenoids, diterpenoids, triterpenoids, and flavonoids [2–6], some of which displayed potent cytotoxic and anti-HIV activities [7,8].

Excoecaria acerifolia, an evergreen shrub, mainly distributed in Yunnan and Guizhou Provinces of China. The whole plant has been used as traditional Chinese medicine to treat cough, malaria, and hepatitis in China [9]. In previous studies, a series of lignans, coumarins, diterpenoids, and flavonoids have been isolated from this plant [10–12]. In our current investigation, a new tigliane diterpenoid, acerifolin A (**1**), and a new isopimarane diterpenoid, acerifolin B (**2**), together with two known compounds (Figure 1), were isolated from the aerial parts of *E. acerifolia*. Herein, we report the isolation, structure elucidation, and cytotoxicity of these compounds.

2. Results and discussion

Acerifolin A (**1**) was isolated as colorless oil. Its molecular formula was determined as $C_{32}H_{42}O_{10}$ on the basis of HR-EI-MS at *m/z* 586.2791 $[M]^+$ (calcd 586.2778), indicating 12 degrees of unsaturation. The IR spectrum showed characteristic absorption bands at 3422, 1711, 1618, and 1454 cm^{-1} for hydroxy, carbonyl, and double bond groups. Analysis of its ^{13}C NMR and DEPT spectra (Table 1) established the presence of 32 carbon resonances, including 6 methyls, 3 methylenes (1 oxygenated), 14 methines (7 olefinic and 3 oxygenated), and 9 quaternary carbons (1 olefinic, 3 carbonyls, and 4 oxygenated). The ^1H and ^{13}C NMR (DMSO-d_6) spectroscopic data (Table 1) of compound **1** were similar to those of 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate [13], a known tigliane-type diterpene ester. The main difference was

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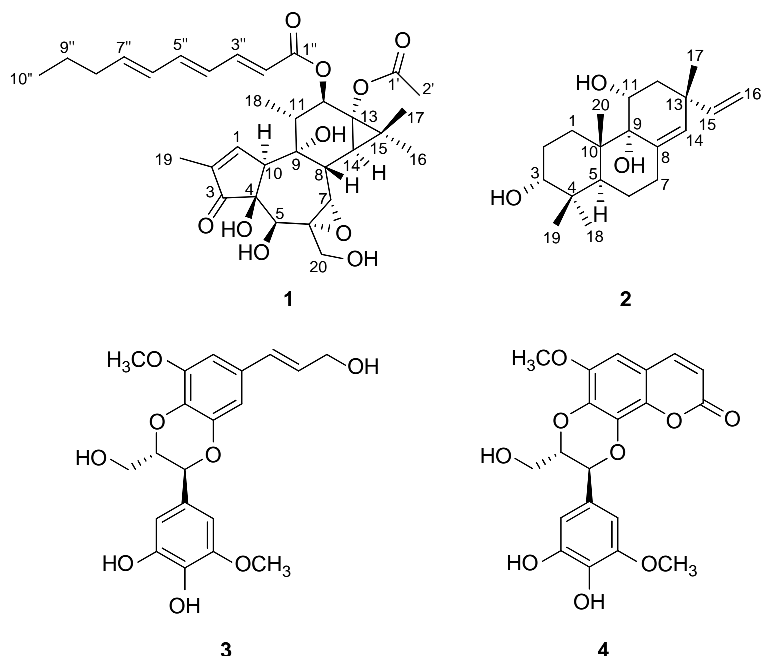

Figure 1. The structure of compounds **1**–**4**.

that an epoxy between C-6 and C-7 occurred in **1** instead of a double bond in 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate. The other difference was that a methylene at C-5 in 12-*O*-*n*-deca-2,4,6-trienoylphorbol-13-acetate was replaced by an oxygenated methine in **1**. The above deduction was inferred from the HMBC correlations (Figure 2) from H-7 (δ_H 3.14) to C-6 (δ_C 63.7), C-8 (δ_C 35.2), and C-20 (δ_C 62.5), from H-5 (δ_H 3.93) to C-3 (δ_C 207.6), C-4 (δ_C 73.3), C-6, and C-20, and from 5-OH (δ_H 5.20) to C-5, and confirmed by the molecular formula of **1**. Detailed 2D NMR analysis established the plane structure of **1** as shown in Figure 1.

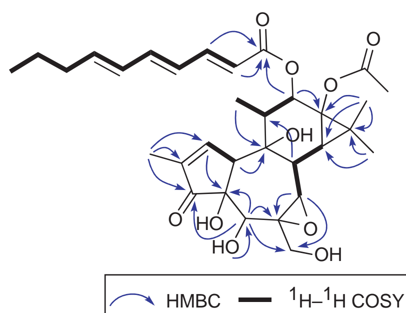

Figure 2. Key HMBC and ^{1}H–^{1}H COSY correlations of **1**.

The relative configuration of **1** was established on the basis of coupling constants and a ROESY experiment (Figure 3). Taking into consideration the typical tigliane skeleton with transfused A/B ring denoting H-10α and OH-4β, as well as the ROESY correlations of H-5/H-10, 5-OH/H_2-20, H-7/H_2-20, and 9-OH/H-10, established the α-orientation of H-5, 9-OH, and epoxide groups. Meanwhile, the cross peaks of 4-OH/H-8, H-8/H-11, and H-8/H_3-17 in ROESY spectrum indicated that 4-OH, H-8, and H-11 were β-oriented, and H-14 and acetoxy group were α-oriented. In addition, a α-orientation of H-12 was established in the light of the large coupling constant (10.1) between H-11 and H-12 and a 2″*E*, 4″*E*, 6″*E*-configuration for the conjugated double bonds was

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Table 1. ^{1}H and ^{13}C NMR spectroscopic data of compound **1**.

	1[a]		**1**[b]	
Position	δ_H (*J* in Hz)	δ_C mult.	δ_H (*J* in Hz)	δ_C mult.
1	7.60 br s	161.6	7.63 br s	163.2
2		132.9		135.1
3		207.6		209.9
4		73.3		74.5
5	3.93 d (6.3)	68.0	4.10 br s	70.3
6		63.7		64.5
7	3.14 br s	63.8	3.24 br s	65.7
8	2.94 d (6.5)	35.2	3.22 d (6.5)	36.9
9		76.7		79.0
10	4.05 br s	49.3	4.13 br s	50.5
11	1.83 m	45.7	2.03 m	46.9
12	5.33 d (10.1)	77.6	5.46 d (10.1)	78.4
13		65.6		67.4
14	1.29 d (6.5)	35.0	1.37 d (6.5)	36.8
15		26.9		28.1
16	1.13 s	23.5	1.23 s	23.8
17	1.18 s	17.2	1.28 s	17.5
18		14.7		15.3
19		9.9		9.9
20a	3.39 dd (12.4, 6.5)	62.5	3.60 d (12.4)	64.7
20b	3.83 dd (12.4, 6.5)		3.98 d (12.4)	
1′		172.2		174.9
2′	1.97 s	21.0	2.07 s	21.0
1″		166.5		168.8
2″	5.88 d (15.1)	120.1	5.86 d (15.2)	120.3
3″	7.18 dd (15.1, 11.4)	145.3	7.29 dd (15.2, 11.3)	147.2
4″	6.34 dd (14.9, 11.4)	128.0	6.31 dd (14.8, 11.3)	128.9
5″	6.69 dd (14.9, 10.8)	141.8	6.62 dd (14.8, 10.6)	143.3
6″	6.18 dd (15.1, 10.8)	130.3	6.19 dd (15.1, 10.6)	131.4
7″	5.97 m	140.6	5.99 m	141.8
8″a	2.09 m	34.6	2.13 m	36.1
8″b	2.09 m		2.13 m	
9″a	1.39 m	21.8	1.45 m	23.3
9″b	1.39 m		1.45 m	
10″	0.85 t (7.4)	13.8	0.92 t (7.4)	14.0
4-OH	5.54 br s			
5-OH	5.20 d (6.3)			
9-OH	5.22 br s			
20-OH	4.55 t (6.5)			

[a] Recorded in DMSO-d_6 at 600 MHz for ^{1}H and 150 MHz for ^{13}C.
[b] Recorded in CD_3OD at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

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determined by the large coupling constants of $J_{2'',3''}$ (15.1 Hz), $J_{4'',5''}$ (14.9 Hz), and $J_{6'',7''}$ (15.1 Hz) [14]. Therefore, compound **1** was assigned to be 6α,7α-epoxy-5β-hydroxy-12-*O*-*n*-deca-2*E*,4*E*,6*E*-trienoyl-phorbol-13-acetate.

Acerifolin B (**2**), a colorless oil, possessed a molecular formula $C_{20}H_{32}O_3$ as established by the HR-EI-MS at *m/z* 320.2342 $[M]^+$. The IR absorption bands of **2** revealed the presence of hydroxyl (3430 cm^{-1}) and olefinic (1634 cm^{-1}) functionalities. The ^{1}H and ^{13}C NMR (DMSO-d_6) spectra of **2** (Table 2) showed four tertiary methyls, five methylenes, one methine, three quaternary carbons, two

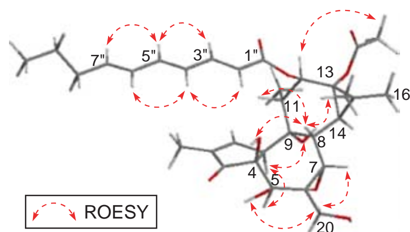

Figure 3. Key ROESY correlations of **1**.

oxygenated methines [δ_H 3.17 (1H, dd, $J = 4.9, 2.6$ Hz, H-3), 3.94 (1H, m, H-11); δ_C 73.5 (C-3), 63.8 (C-11)], one oxygenated quaternary carbon [δ_C 74.2 (C-9)], one trisubstituted double bond [δ_H 5.22 (1H, br s, H-14); δ_C 137.9 (C-8), 129.2 (C-14)], and one terminal double bond [δ_H 5.76 (1H, dd, $J = 17.5, 10.6$ Hz, H-15), 4.89 (1H, d, $J = 10.6$ Hz, H-16a), 4.93 (1H, d, $J = 17.5$ Hz, H-16b); δ_C 148.1 (C-15), 110.3 (C-16)]. These data suggested that **2** was a isopimarane diterpenoid with one tertiary hydroxy and two secondary hydroxy groups, which was similar to those of oryzalexin E [15], except for the presence of an additional hydroxyl group in **2**. The HMBC correlations (Figure 4) from H-5 (δ_H 2.22), H_2-12 (δ_H 1.43), H-14, and H_3-20 (δ_H 0.85) to C-9 indicated that the hydroxyl group was located at C-9. The ROESY correlations (Figure 4) of H-5/9-OH and H-5/H_3-18

Table 2. ^{1}H and ^{13}C NMR spectroscopic data of compound **2**.

	2[a]		**2**[b]	
Position	δ_H (J in Hz)	δ_C mult.	δ_H (J in Hz)	δ_C mult.
1a	1.17 m	26.0	1.36 overlapped	26.4
1b	2.03 overlapped		2.08 m	
2a	1.43 overlapped	25.3	1.66 m	25.0
2b	1.73 m		1.87 m	
3	3.17 dd (4.9, 2.6)	73.5	3.39 d (2.8)	75.6
4		37.1		37.4
5	2.22 dd (12.9, 2.7)	38.4	2.21 dd (12.8, 2.7)	39.2
6a	1.22 m	21.9	1.36 overlapped	22.0
6b	1.39 m		1.52 m	
7a	2.03 m	32.2	2.16 m	32.5
7b	2.33 m		2.44 m	
8		137.9		136.9
9		74.2		75.6
10		42.2		42.3
11	3.94 m	63.8	4.11 m	65.8
12a	1.43 overlapped	40.8	1.54 m	40.9
12b	1.43 overlapped		1.63 m	
13		37.9		38.1
14	5.22 br s	129.2	5.35 br s	131.3
15	5.76 dd (17.5, 10.6)	148.1	5.77 dd (17.5, 10.6)	147.6
16a	4.89 d (10.6)	110.3	4.92 d (10.6)	110.7
16b	4.93 d (17.5)		4.97 d (17.5)	
17	1.03 s	24.2	1.08 s	24.6
18	0.87 s	29.2	1.00 s	28.7
19	0.79 s	22.7	0.89 s	22.6
20	0.85 s	17.3	0.97 s	17.7
3-OH	4.15 d (4.9)			
9-OH	4.07 s			
11-OH	4.49 d (6.4)			

[a] Recorded in DMSO-d_6 at 600 MHz for ^{1}H and 150 MHz for ^{13}C.
[b] Recorded in $CDCl_3$ at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

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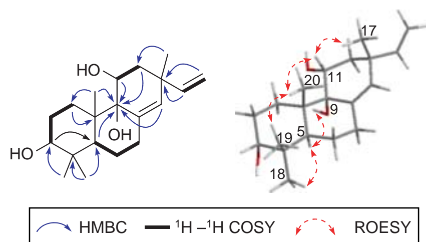

Figure 4. Key 2D NMR correlations of **2**.

indicated that they were cofacial and arbitrarily assigned as α-orientation. In addition, the ROESY cross peaks of H-11/H_3-20, H-11/H_3-17, H_3-19/H_3-20 revealed that these protons were β-oriented. The H-3 was also deduced to be β-oriented by the small coupling constants (2.6) of H-3 with H_2-2 [16]. Thus, the structure of **2** was determined as iso-pimara-8(14),15-diene-3α,9α,11α-triol.

Two known compounds, simplidin (**3**) [17] and malloapelin C (**4**) [18], were identified by the analysis of their NMR spectra and by comparison with the data reported in the literature.

Compounds **1–4** were tested for their cytotoxicities against human myeloid leukemia (HL-60), hepatocellular carcinoma (SMMC-7721), lung cancer (A-549), breast cancer (MCF-7), and colon cancer (SW-480) cell lines using the methyl thiazolyl tetrazolium (MTT) method as previously reported [19], with cisplatin (Sigma, St. Louis, MO, USA) as the positive control. Among them, compound **1** showed moderate cytotoxicity against the above five cancer cell lines, while compound **4** exhibited moderate cytotoxicity against HL-60 (Table 3).

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer (Shimadzu, Tokyo, Japan). IR spectra were obtained by a Bruker Tensor 27 spectrophotometer with KBr pellets (Bruker, Karlsruhe, Germany). 1D and 2D spectra were run on a Bruker AM-400 or an Avance III 600 spectrometer (Bruker, Karlsruhe, Germany) with tetramethylsilane as the internal standard. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. EI-MS and HR-EI-MS were recorded on a Waters Autospec Premier P776 spectrometer (Waters, Milford, MA, USA). Silica gel (100–200 and 200–300 mesh, Qingdao Marine Chemical Co. Ltd, Qingdao, China) and Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden) were used for column chromatography (CC). MPLC was performed on a Lisui EZ Purify III System including pump manager P03, detector modules P02, and fraction collector P01 (Shanghai Li Sui Chemical Engineering Co., Ltd, Shanghai, China) and columns packed with RP-18 silica gel (40–63 μm, Merck, Darmstadt, Germany). Semi-preparative HPLC was performed on an Agilent 1100 apparatus (Agilent, Santa Clara, CA, USA) equipped with a UV detector and a Zorbax SB-C-18 (Agilent, 9.4 mm × 25 cm) column. Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

Table 3. Cytotoxicity of compounds **1–4** against selected tumor cell lines (IC_{50}, μM).[a]

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW-480
1	17.60	20.27	13.01	18.38	17.59
4	15.79	>40	>40	>40	>40
Cisplatin[b]	1.29	7.18	5.16	16.07	14.70

[a] Compounds **2** and **3** were inactive for the selected cancer cell lines (IC_{50} > 40 μM).
[b] Positive control.

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3.2 Plant material

The aerial parts of *E. acerifolia* were collected from Xishangbanna Country, Yunnan Province, China, in September 2010, and identified by Prof. X. Cheng, Kunming Institute of Botany. A voucher specimen (KIB20100716e) is deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

3.3 Extraction and isolation

The air-dried and powdered aerial parts of *E. acerifolia* (9 kg) were extracted three times with 70% aqueous acetone at room temperature overnight and concentrated under vacuum to give a residue, which was partitioned between H_2O and EtOAc. The EtOAc extract (200 g) was chromatographed on MPLC eluting with MeOH–H_2O (from 10:95 to 100:0) to provide six fractions A–F. Fraction B (30 g) was subjected to silica gel CC using petroleum ether–Me_2CO (from 8:2 to 1:0) as eluent to obtain three subfractions B1–B3. Subfraction B2 was subjected to silica gel CC ($CHCl_3$–MeOH, 9:1), followed by Sephadex LH-20 (MeOH), and further purified by semi-preparative HPLC (MeOH–H_2O, 38:62; flow rate: 3 ml/min; wavelength: 254 nm) to afford **3** (61 mg, t_R 16.3 min) and **4** (40 mg, t_R 20.6 min). Fraction C (40 g) was separated into four subfractions C1–C4, by silica gel CC with petroleum ether–Me_2CO (from 8:2 to 1:0) as the eluent. Subfraction C1 was further subjected to CC over a silica gel column eluted with petroleum ether–EtOAc (9:1) to give **1** (8 mg). Subfraction C3 was subjected to silica gel CC (petroleum ether–EtOAc, 8:2), followed by Sephadex LH-20 (MeOH) to yield **2** (21 mg).

3.3.1 Acerifolin A (1)

Colorless oil; [α]25.1 D −42.70 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε): 303 (4.06), 255 (4.01), 202 (3.88) nm; IR (KBr) ν_{max} 3422, 2926, 1711, 1618, 1559, 1454, 1377, 1326, 1261, 1241, 1171, 1081, 1004, 939, and 616 cm^{-1}; for ^1H and ^{13}C NMR spectral data, see Table 1; EI-MS: *m/z* 586 $[M]^+$(10), 568 (9), 495 (8), 421 (20), 342 (18), 241 (17), 149 (100), 107 (66), and 83 (40); HR-EI-MS: *m/z* 586.2791 $[M]^+$ (calcd for $C_{32}H_{42}O_{10}$, 586.2778).

3.3.2 Acerifolin B (2)

Colorless oil; $[\alpha]_D^{25.1}$ −10.99 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε): 204 (3.97) nm; IR (KBr) ν_{max} 3430, 2937, 1634, 1458, 1410, 1386, 1367, 1236, 1175, 1067, 1044, 988, and 911 cm^{-1}; for ^1H and ^{13}C NMR spectral data, see Table 2; EI-MS: *m/z* 320 $[M]^+$(11), 302 (33), 284 (22), 252 (100), 234 (22), 180 (31), 136 (80), 121 (49), and 69 (42); HR-EI-MS: *m/z* 320.2342 $[M]^+$ (calcd for $C_{20}H_{32}O_3$, 320.2351).

3.4 Cytotoxicity assay

The cytotoxicity of compounds **1–4** against HL-60, SMMC-7721, A-549, MCF-7, and SW480 cell lines was assessed using the MTT method. Cells were plated in 96-well plates 12 h before treatment and continuously exposed to different concentrations of compounds. After 48 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well, which was incubated for another 4 h. Then 20% of SDS (100 μl) was added to each well. After 12 h at room temperature, the OD value of each well was recorded at 595 nm. The IC_{50} value of each compound was calculated by the Reed and Muench method [20].

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