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Diterpenes with potential treatment of vitiligo from the aerials parts of *Euphorbia antiquorum* L

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ABSTRACT

Six new diterpenes Euphonoids A-F including one ingenol (**1**), three lathyrane (**2**–**5**), one *ent*-abietane (**6**) and fifteen known derivatives (**7**–**21**) were isolated from the aerial parts of *Euphorbia antiquorum* L. Their structures were elucidated by physical data analysis. Compounds **1**, **12**, and **16** improve the melanogenesis in B16 cells in vitro.

1. Introduction

Euphorbia, is the largest genus of the family Euphorbiaceae. Chemical investigations on this genus have led to the isolation of a large array of diterpenoids with diverse structural classes, which showed important biological activities including cytotoxic, antimicrobial, anti-inflammatory, and anti-HIV activities [1,2]. *Euphorbia antiquorum* L. is a common thorny succulent species distributed in southwestern mainland China, its stems and leaves have been applied in Chinese folk medicine for treatment of toothache, dropsy, palsy, and amaurosis [3–5]. Previous investigation on chemical constituents of this plant has revealed an array of structurally diverse diterpenoids and triterpenoids [6–12]. As part of our continuous effort to search for bioactive natural products [13–18], six new dierpenoids, namely, Euphonoids A–F (**1**–**6**), including ingol esters, lathyane, *ent*-abietane, together with fifteen known ones, namely 3,8,12-*O*-triacetylingol-7-benzoate [19], ingol 3,8,12-O-triacetate-7-tiglate [20], 3,12-*O*-diacetylingol-7-benzoate-8-methoxyl [21], 3,12-diacetyl-7-angeloyl-8-methoxyingol [22], 3,12-diacetyl-7-tigloyl-8-methoxyingo l [23], Euphorantin I [7], 12-acetyl-7-angeloyl-8-methoxyingol [24], 3,12-diacetyl-ingol-7-tigliate [25], 3,12-diacetyl-7-angolyl-8-hydroxyingol [26], Euphorantin J [26], Tirucalicine [27], Eurifoloid A [28], 3-O-[(Z)-2-methyl-2-butenoyl]-20-*O*-acetylingenol [29], Eurifoloid L [28], and Antiquorine A [30], bearing various groups such as acetyl, benzoyl, nicotinyl, and angeloyl moieties, were isolated from the aerial parts of the title plant (Fig. 1). All isolated compounds were tested for treatment of vitiligo. Herein, the isolation, structural elucidation, and improve effects of **1**–**21** on B-16 are described.

2. Experimental

2.1. 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 chromatography with a Waters X-Bridge C18 (150 × 10 mm, 5 μm) 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).

2.2. Plant material

The aerial parts of *Euphorbia antiquorum* L.were collected from Xishuang Banna of Yunnan Province, P. R. China, in October 2014, and

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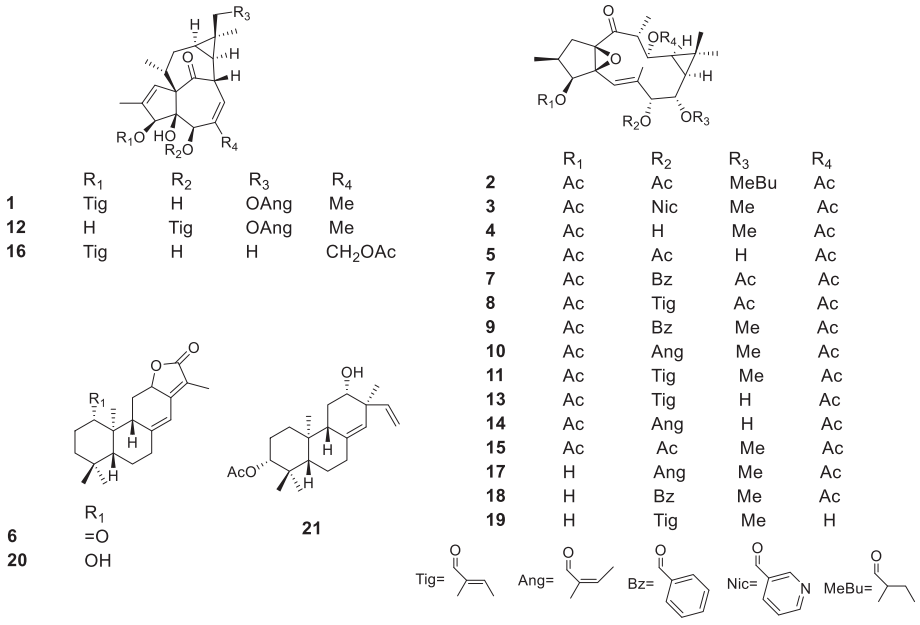

Fig. 1. Structures of compounds **1–21**.

authenticated by Prof. Xia Peng (Institute for Food and Drug Control of Xishuang Banna). The specimens were slipped and dried under 40 °C. A voucher specimens (HXJ20140126) were deposited at State Key Laboratory of Phytochemistry and Plant Resource in West China, Kunming Institute of Botany, Chinese Academy of Science (CAS).

2.3. Extraction and isolation

The air-dried powdered aerial parts (11.0 kg) were extracted 3 times (4, 3, and 3 h) with 95% ethanol (20 L × 3) at room temperature and concentrated *in vacum* at 60 °C to give a residue (1.0 kg). The residue was dispersed in water (4.0 L) and extracted with petroleum ether and ethyl acetate in turn. The petroleum ether and ethyl acetate portion (500 g) were combined and then subjected to pass over a silica gel column (100–200 mesh; petroleum ether-acetone, 1:0 → 0:1), to yield three major fractions (1–3). Then Fr.1 (30.0 g) was separated by RP-18 flash CC (40–60 μm, Merck), eluting with a step gradient of MeOH:H_2O (40:60–100:0) to give seven fractions (Fr.1a–1 g). Fr. 1c (5.0 g) was applied to a silica gel column (200–300 mesh; PE/EtOAc, 50:1–10:1) and further subjected to a Sephadex LH-20 column (i.d. 2.0 × 180 cm, 40–70 μm) eluted with MeOH to give 3,8,12-*O*-triacetylingol-7-benzoate (**7**, 500 mg) and ingol 3,8,12-O-triacetate-7-tiglate (**8**, 500 mg), and the Fr.1c3 (200 mg) was further separated by semi-preparative HPLC (Waters X-Bridge C18, i.d. 150 × 10 mm, 5 μm, 80% MeOH 3.0 mL/min, UV 210 nm) to give 3,12-*O*-diacetylingol-7-benzoate-8-methoxyl (**9**, 4.3 mg, t_R 8.5 min), 3,12-diacetyl-7-angeloyl-8-methoxyingol (**10**, 2.0 mg, t_R 11.7 min) and 3,12-diacetyl-7-tigloyl-8-methoxyingol (**11**, 14 mg, t_R 15.3 min). Fr. 1e (2.0 g) was subjected to a Sephadex LH-20 column (i.d. 2.0 × 180 cm, 40–70 μm) eluted with MeOH to give 3-O-[(Z)-2-methyl-2-butenoyl]-20-*O*-acetylingenol (**12**, 500 mg) and 3,12-diacetyl-ingol-7-tigliate (**13**, 500 mg). Subsequently, Fr.2 (220 g) was separated over an MCI column (MeOH–H_2O from 3:7 to 10:0) to obtain four subfractions (2a–2d). Fr.2b (50 g) was then chromatographed on a silica gel column eluted with Petroleum ether-ethyl acetate (50:1 to 5:1), to afford five subfractions (2b1–2b5). Fr.2b2 (2 g) was purified by Sephadex LH-20 (i.d. 2.0 × 180 cm, 40–70 μm) eluted with MeOH to obtain **4** (21 mg) and a major fraction (Fr.2b21). Fr.2b21 (200 mg) was separated by semi-preparative HPLC (Waters X-Bridge C18, i.d. 150 × 10 mm, 5 μm, 80% CH_3CN 3.0 mL/min, UV 210 nm) to give **1** (7 mg, t_R 9.5 min) and **3** (12 mg, t_R 17.5 min). Fr.2b3 (15 g) was separated on a silica gel column eluted with Petroleum ether-ethyl acetate (25:1) to obtain four subfractions (Fr.2b31–Fr.2b34). Fr.2b31 (150 mg) was purified by Sephadex LH-20 (i.d. 2.0 × 180 cm, 40–70 μm) eluted with acetone to obtain **2** (6 mg). Fr.2b32 (80 mg) was separated by semi-preparative HPLC (Waters X-Bridge C18, i.d. 150 × 10 mm, 5 μm, 80% CH_3OH 3.0 mL/min, UV 210 nm) to give **6** (11 mg, t_R 14.5 min). Fr.2b33 (110 mg) was chromatographed on a silica gel column eluted with Petroleum ether-ethyl acetate (8:1) to obtain **5** (20 mg). Fr. 3 (120 g) was separated by RP-18 flash CC (40–60 μm, Merck), eluting with a step gradient of MeOH:H_2O (50:50–100:0) to give five fractions (Fr.3a–3e). Fraction 3a (11 g) was chromatographed on a silica gel column eluted with Petroleum ether-ethyl acetate (25:1 to 1:1), to afford five subfractions (3a1–3a5). Fraction 3a3 (2.6 g) was subjected to a Sephadex LH-20 column (i.d. 2.0 × 180 cm, 40–70 μm) eluted with MeOH, and the fractions were further separated by semipreparative HPLC (Waters X-Bridge C18, i.d. 150 × 10 mm, 5 μm, 75% MeOH, 3.0 mL/min, UV 210 nm) to give 3,12-diacetyl-7-angolyl-8-hydroxyingol (**14**, 8.0 mg, t_R 11.5 min), Tirucalicine (**15**, 5.0 mg, t_R 15.5 min), Eurifoloid A (**16**, 8.1 mg, t_R 19.5 min), 12-acetyl-7-angeloyl-8-methoxyingol (**17**, 6.0 mg, t_R 20.8 min), and Euphorantin I (**18**, 4.5 mg, t_R 24.6 min). Fr. 3c (3.5 g) was subjected to a Sephadex LH-20 column (i.d. 2.0 × 180 cm, 40–70 μm) eluted with MeOH, and the fractions were further separated by semipreparative HPLC (Waters X-Bridge C18, i.d. 150 × 10 mm, 5 μm, 70% MeOH, 3.0 mL/min, UV 210 nm) to give Euphorantin J (**19**, 8.3 mg, t_R 10.5 min), Antiquorine A (**20**, 5.0 mg, t_R 12.5 min) and Eurifoloid L (**21**, 10.1 mg, t_R 19.5 min). The purities of compounds **1–21** were 95%, as determined by TLC and HPLC.

2.4. Euphonoid A

Amorphous oil (MeOH); $[\alpha]_D^{25}$: +49.6 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 215 (4.36) nm; IR (KBr) ν_{max}: 3437, 2963, 2926, 1711, 1650, 1456, 1383, 1357, 1306 cm^{-1}; ^1H and ^{13}C NMR: Table 1; HR-ESI-MS: *m/z* $[M + Na]^+$, calcd for: 535.2666, found: 535.2674.

2.5. Euphonoid B

Amorphous oil (MeOH); $[\alpha]_D^{25}$: −15.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.00) nm, 225 (3.52) nm; IR (KBr) ν_{max}: 3438, 2968,

Table 1
^{1}H and ^{13}C NMR spectroscopic Data for Euphonoid A (**1**) in $CDCl_3$ at 500 MHz.

Position	1		Position	1	
	δ_H (*J* in Hz)	δ_C		δ_H (*J* in Hz)	δ_C
1	6.02 (1H, d, 1.6)	132.1	17a 17b	4.31 (1H, d, 12.0) 4.17 (1H, d, 12.0)	65.6
2		136.0	18	0.98 (3H, d, 7.2)	16.8
3	5.50 (1H, brs)	82.8	19	1.77 (3H, d, 1.4)	15.6
4		85.1	20	1.74 (3H, s)	22.0
5	3.66 (1H, brs)	77.3		Ang	
6		137.9	1’		168.4
7	5.69 (1H, m)	123.1	2’		127.2
8	4.16 (1H, s)	43.0	3’	6.13 (1H, m)	139.8
9		206.4	4’	1.98 (3H, m)	20.8
10		72.0	5’	1.89 (3H, m)	15.9
11	2.45 (1H, m)	38.7		Tig	
12a 12b	2.33 (1H, m) 1.82 (1H, m)	30.9	1”		168.5
13	0.89 (1H, m)	24.1	2”		128.7
14	1.08 (1H, s)	23.7	3”	6.84 (1H, m)	137.3
15		27.7	4”	1.78 (3H, d, 1.2)	14.4
16	1.13 (3H, s)	24.5	5”	1.82 (3H, m)	12.1

2939, 1738, 1631, 1459, 1372 cm^{-1}; ^{1}H and ^{13}C NMR: Tables 2, 3; HR-ESI-MS: *m/z* [M + Na]$^{+}$, calcd for: 599.2827, found: 599.2832.

2.6. Euphonoid C

Amorphous oil (MeOH); $[\alpha]_D^{25}$: −11.5 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε): 205 (3.97) nm; IR (KBr) ν_{max}: 3431, 2938, 1735, 1633, 1592, 1531, 1451, 1424, 1374,1343 cm^{-1}; ^{1}H and ^{13}C NMR: Tables 2, 3; HR-ESI-MS: *m/z* [M + Na]$^{+}$, calcd for: 570.2698, found: 570.2701.

2.7. Euphonoid D

Amorphous oil (MeOH); $[\alpha]_D^{25}$: +11.7 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.07) nm; IR (KBr) ν_{max}: 3451, 2939, 1738, 1633, 1548, 1422, 1373 cm^{-1}; ^{1}H and ^{13}C NMR: Tables 2, 3; HR-ESI-MS: *m/z* [M + Na]$^{+}$, calcd for: 487.2302, found: 487.2290.

2.8. Euphonoid E

Amorphous oil (MeOH); $[\alpha]_D^{25}$: +12.3 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 205 (4.25) nm; IR (KBr) ν_{max}: 3470, 2936, 1737, 1709, 1641, 1448, 1372, 1304 cm^{-1}; ^{1}H and ^{13}C NMR: Tables 2, 3; HR-ESI-MS: *m/z* [M + Na]$^{+}$, calcd for: 515.2252, found: 515.2257.

2.9. Euphonoid F

A white, amorphous power (MeOH); $[\alpha]_D^{25}$: +118.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 275 (3.85) nm; IR (KBr) ν_{max}: 3458, 2951, 1739, 1703, 1669, 1609, 1441, 1394, 1328, 1302 cm^{-1}; ^{1}H and ^{13}C NMR: Table 4.; HR-ESI-MS: *m/z* [M + Na]$^{+}$, calcd for: 337.1774, found: 337.1783.

2.10. Cell culture

Murine B16 melanoma cell lines (B16F10) were obtained from CAS (Chinese Academy of Sciences, China). The B16F10 cells were grown in DMEM medium (GIBICO, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBICO, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO, USA) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.11. Melanin contents assay

Exponentially growing cells were seeded into 6-well plates at a concentration of 5×10^5 cells per well. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium containing the candidate compounds in different concentrations. The cells were incubated for another 48 h, washed with ice cold PBS,

Table 2
^{1}H NMR spectroscopic Data for Euphonoids B–E (**2–5**) in $CDCl_3$ at 500 MHz.

Position	2	3	4	5
	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1a 1b	2.77 (1H, dd, 9.0, 15.0)	2.78 (1H, dd, 9.1, 15.0)	2.81 (1H, dd, 9.3, 15.1)	2.81 (1H, dd, 9.2, 15.0)
	1.69 (1H, d, 15.0)	1.69 (1H, d, 15.0)	1.67 (1H, d, 15.0)	1.69 (1H, d, 15.0)
2	2.46 (1H, m)	2.46(1H, dd, 8.2, 16.1)	2.57 (1H, m)	2.56 (1H, m)
3	5.33 (1H, d, 8.6)	5.20 (1H, d, 8.6)	5.18 (1H, d, 8.5)	5.21 (1H, d, 8.5)
5	5.58 (1H, s)	5.68 (1H, s)	5.76 (1H, s)	5.79 (1H, s)
7	5.08 (1H, s)	5.54 (1H, s)	4.33 (1H, s)	4.21 (1H, s)
8	4.58 (1H, dd, 1.8, 10.9)	3.00 (1H, dd, 1.9, 10.1)	2.85 (1H, dd, 1.8, 10.2)	4.54 (1H, dd, 1.8, 10.2)
9	1.29 (1H, m)	1.26 (1H, m)	1.16 (1H, m)	1.38 (1H, m)
11	1.07 (1H, overlapping)	1.06 (1H, overlapping)	0.99 (1H, m)	1.09 (1H, m)
12	4.86 (1H, dd, 3.9, 11.1)	4.91 (1H, dd, 4.0, 11.0)	4.84 (1H, dd, 4.0, 11.1)	4.85 (1H, dd, 4.0, 11.1)
13	2.93 (1H, m)	2.94 (1H, m)	2.92 (1H, m)	2.89 (1H, m)
16	0.97 (3H, d, 7.5)	0.93 (3H, s)	0.89 (3H, d, 7.5)	0.90 (3H, d, 7.5)
17	2.10 (3H, overlapping)	2.12 (3H, d, 1.2)	1.97 (3H, s)	2.04 (3H, d, 7.5)
18	1.11 (3H, s)	1.13 (3H, s)	1.06 (3H, s)	1.07 (3H, s)
19	0.85 (3H, s)	0.99 (3H, s)	0.96 (3H, s)	0.85 (3H, s)
20	1.05 (3H, d, 7.3)	1.08 (3H, d, 7.3)	1.05 (3H, s)	1.06 (3H, d, 7.3)
	8-MeBu	7-nicotinate		
1’				
2’	2.33 (1H, m)			
3’		9.24 (1H, m)		
4’	0.88 (3H, d, 7.5)	8.79 (1H, dd, 1.7, 4.9)		
5’	1.10 (3H, d, 7.0)	7.41 (1H, dd, 4.9, 8,0)		
6’		8.28 (1H, dt, 8.0)		
3-OAc	2.07 (3H, s)	2.05 (3H, s)	2.10 (3H, s)	2.10 (3H, s)
7-OAc	2.13 (3H, s)			
8-OAc			2.08 (3H, s)	
12-OAc	2.10 (3H, overlapping)	2.13 (3H, s)	2.11 (3H, s)	2.09 (3H, s)
8-OCH_3		3.36 (3H, s)		3.35 (3H, s)

Table 3
^{13}C NMR spectroscopic Data for Euphonoids B–E (**2–5**) in $CDCl_3$ at 500 MHz.

Position	2	3	4	5
	δ_C	δ_C	δ_C	δ_C
1	31.4	31.5	31.7	31.7
2	29.4	29.5	29.5	29.7
3	76.3	76.6	77.8	77.6
4	73.4	73.4	73.8	73.7
5	116.9	117.6	116.7	116.8
6	140.0	139.2	140.6	141.3
7	76.9	75.1	73.2	76.5
8	71.1	78.8	80.8	74.0
9	24.9	27.2	26.0	23.4
10	19.3	19.5	19.1	19.1
11	30.8	30.6	30.5	30.7
12	70.8	71.0	71.1	70.7
13	24.1	43.0	43.1	43.2
14	207.6	207.5	207.8	207.6
15	71.1	71.3	71.5	71.5
16	17.0	17.0	17.0	17.0
17	17.4	17.8	17.8	17.5
18	29.2	29.4	29.2	29.0
19	16.3	16.6	16.6	16.3
20	13.4	13.4	13.4	13.4
	8-MeBu	7-nicotinate		
1’	175.9	164.3		
2’	40.9	126.0		
3’	26.7	151.0		
4’	11.5	153.7		
5’	16.4	11.5		
6’		16.4		
3-OAc	170.8	170.4	170.6	170.7
	20.6	20.6	20.7	20.7
7-OAc	169.7			
	21.0			
8-OAc				170.5
				21.2
12-OAc	170.5	170.8	170.8	170.5
	21.0	21.0	21.1	21.1
8-OCH_3		56.5	56.7	

Table 4
^{1}H and ^{13}C NMR spectroscopic Data for Euphonoid F (**6**) in $CDCl_3$ at 500 MHz.

Position	6		Position	6	
	δ_H (*J* in Hz)	δ_C		δ_H (*J* in Hz)	δ_C
1	–	215.7	11a 11b	2.59 (1H,dd, 6.6, 14.3)1.75 (1H,m)	30.0
2a 2b	2.90 (1H, m) 2.20 (1H,dt, 12.3)	36.1	12	4.57 (1H, dd, 6.5, 13.3)	76.2
3a 3b	1.88 (1H, m) 1.71 (1H, m)	43.7	13	–	155.4
4	–	33.8	14	6.34 (1H, s)	115.6
5	1.53 (1H, m)	57.5	15	–	116.9
6a 6b	1.88 (1H, m) 1.71 (1H, m)	24.1	16	–	175.1
7a 7b	2.50 (1H, dt, 12.3) 2.12 (1H, m)	36.7	17	1.82 (3H, s)	8.3
8	–	150.7	18	1.10 (3H, s)	22.3
9	2.80 (1H, dt, 8.7)	43.1	19	0.96 (3H, s)	32.5
10		56.0	20	1.29 (3H, s)	14.9

followed by lysis with RIPA buffer for 40 min on ice, and the lysates were centrifuged at 10,000 *g* for 20 min. Supernatants containing protein were subject to the protein assay and the pellets with intracellular melanin were solubilized in 200 μL of 1 M NaOH for 2 h at 60 °C. Melanin amount was determined spectrophotometrically at 405 nm by a multi-plate reader. The melanin amount was calculated by normalizing the total melanin values with protein content (abs melanin/lg protein).

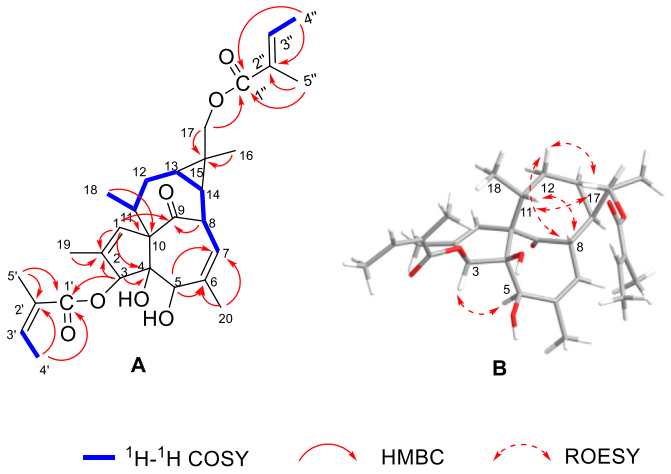

Fig. 2. Selected 2D NMR correlations for Euphonoid A (**1**).

3. Results and discussion

Euphonoid A (**1**), an amorphous oil, has the molecular formula $C_{30}H_{40}O_7$, as deduced by HRESIMS at *m/z* 535.2674 $[M + Na]^+$ (calcd for 535.2666), with 11 degrees of unsaturation. Its IR spectrum showed absorption bands for OH (3437 cm^{-1}), C=O (1712 cm^{-1}), and C=C (1455 cm^{-1}) groups. With the assistance of DEPT and HSQC spectra, its NMR data showed resonances for three carbonyl carbons, four trisubsituted double bonds, eight methyls, two methylenes (one oxygenated), six methines (two oxygenated), three quaternary carbon (one oxygenated). The carbonyls and the double bonds accounted for seven out of 11 degrees of unsaturation, which required the presence of four rings in compound **1**.

Further examination of the 2D NMR spectra constructed the 2D structure of **1**. First, three spin-coupling systems were identified via the $^{1}H-^{1}H$ COSY spectrum (Fig. 2). The connection of the three structural units with other functional groups was then made via the HMBC spectrum, in which the correlations of H_3–19/C-1, C-2, and C-3; H-1/C-2, C-4, C-9 and C-10; H-5/C-6 and C-7; H_3–20/C-6 and C-7; and H_3–18/C-10; H_3–16, H_2–17/C-15 that **1** was likely a diester of an ingenol diterpenoid. An angeloyl and a tigloyl were readily identified by ^{1}H and ^{13}C NMR data. The presence of the angeloyloxy and tigloyloxy groups was confirmed by the HMBC correlations within two motifs, which were located at C-5 and C-17 by the HMBC correlations from H-5 and H_2–17 to each of the corresponding carbonyls in the ester units, respectively (Fig. 2). The $^{1}H-^{1}H$ COSY correlations of H_3–4′/H-2′ and HMBC correlations of H_3–5′/C-1′, C-2′, and C-3′ confirmed the presence of angeloyl (δ_C 168.5), and the HMBC correlation from H-3 (δ_H 5.50) to C-1′ (δ_C 168.5) indicated that this unit was located at C-3. The other 17-tigloylate group as identified by the key HMBC correlation of H_3–5″/C-1″, C-2″, and C-3″ was allocated by the chemical shift of C-17 (δ_C 168.4). The presence of hydroxy groups of C-5 (δ_C 77.3) and C-4 (δ_C 85.1) were indicated by the chemical shifts of the relevant proton and/or carbons and confirmed by the HMBC correlations from H-1 (δ_H 6.02)/C-2, C-4, C-9 and C-10; H-5 (δ_H 3.66) to C-6 and C-7, respectively. The only ketocarbonyl was located at C-9 by the HMBC correlations from H-7, H-8, and H-11 to C-9. Thus, the planar structure of **1** was established as shown.

In the ROESY spectrum (Fig. 2), the cross-peaks of H_2–17/H-11, H_2–17/H-12β, H-8/H-11, and H-8/H-12β suggested that CH_2–17, H-11, and H-8 were cofacial and were assigned to be β-oriented randomly. The relative configurations of the C-3, C-4, C-5, and C-10 stereocenters were identical to those of known analogues based on their similar chemical shifts and coupling patterns of the protons and carbons in the A and B rings. This was supported by the key ROESY correlation (Fig. 2) between H-3 and H-5. Thus, the structure of **1** was assigned as depicted.

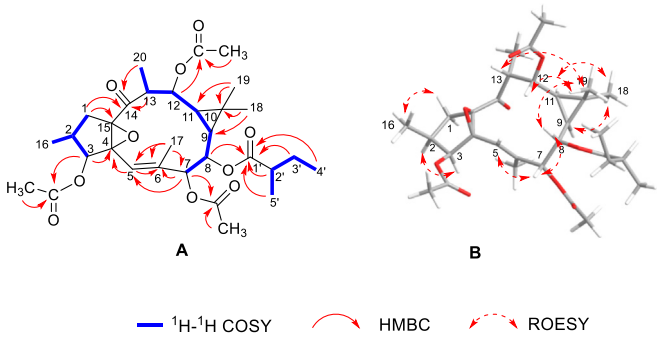

Fig. 3. Selected 2D NMR correlations for Euphonoid B (**2**).

Euphonoid B (**2**) gave a molecular formula of $C_{31}H_{44}O_{10}$ as deduced from the ^{13}C NMR and the sodium adduct (+)-HRESIMS ion at *m/z* 599.2832 $[M + Na]^+$ (calcd 599.2837), which indicated 10 indices of hydrogen deficiency (IHD). The 1D NMR spectra of **2** (Tables 1 and 2) revealed the presence of a carbonyl (δ_C 207.6), three acetyl units (δ_C 170.8, 170.5, 169.7), a 2-methylbutanoate moiety, a trisubstituted double bond, five methyls, a methylene, eight methines (four oxygenated) and three quaternary carbons (two oxygenated). These groups accounted for six out of ten IHDs, and the remaining indices required the presence of four additional rings. Scrutiny of its 1D and 2D NMR data showed that it is a structurally closely related analogue of euphorantin M [7], and the difference was the replacement of the hydroxyl in the latter by an acetyl at C-7 in the former. This conclusion was confirmed by HMBC correlations from H-7 to C-5, C-6, and C-1″. The relative configuration of **2** was determined by analyzing the ROESY spectrum (Fig. 3) and comparing the NMR data with euphorantin M.

Euphonoids C (**3**) displayed a molecular formula of $C_{31}H_{44}O_{10}$ as assigned by the ^{13}C NMR and the sodium adduct ion at *m/z* 570.2701 $[M + H]^+$ (calcd 570.2698) in the (+)-HRESIMS. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) suggested a strong resemblance of its structure to that of **2** with the differences resulting from the C-7 and C-8 substituent. A nicotinate group at C-7 for **3** was identified based on the HMBC correlation (S3.5, Supporting Information) from H-7 to C-1′ of the nicotinate unit, while methyloxy group at C-8 for **3** was identified based on the HMBC correlation from OMe to C-8. The relative configuration of **3** was assigned via the ROESY spectrum (S3.6, Supporting Information).

A molecular formula of $C_{25}H_{36}O_8$ was assigned for euphonoid D (**4**) by the ^{13}C NMR and the sodium adduct in at *m/z* 487.2290 (calcd 487.2302) in the (+)-HRESIMS. Scrutiny of the NMR data of **4** suggested the close similarity of its structure with that of **3**, and the only difference was the replacement of the nicotinate unit in **3** by a hydroxy unit at C-7 in **4** as judged by the shielded H-7 (δ_H 4.33). The above assignment was supported by analysis of the HMBC spectrum (S4.5, Supporting Information). The relative configuration of **4** was identical to **2** following analysis of the ROESY spectrum (S4.6, Supporting Information).

Euphonoids E (**5**) possessed a molecular formula of $C_{26}H_{36}O_9$ as determined by the (+)-HRESIMS and ^{13}C NMR data (Tables 1 and 2). Inspection of its 1D and 2D NMR spectra revealed that it is structurally closely similar to **2**. The only difference was the presence of hydroxyl (δ_H 2.10, δ_C 172.5 and 21.2) at C-8 in **5**, instead of the 2-methylbutanoate moiety in **2**, as judged by the shielded H-7 (δ_H 4.21). The relative configuration of **5** was established as identical with that of **2** by the ROESY data (S5.6, Supporting Information), as well as their similar NMR patterns.

Euphonoids F (**6**) gave a molecular formula of $C_{20}H_{26}O_9$ as deduced from the ^{13}C NMR data (Tables 3) and a sodium adduct ion in the (+)-HRESIMS at *m/z* 337.1783 (calcd 337.1734). Inspection of its 1D and 2D NMR spectra revealed that it is structurally similar to the co-existing known compound Antiquorine A [30]. The only difference was the presence of a ketone group located at C-1 in **6** replaced the hydroxymethyl group in **3** based on the chemical shifts of C-1 (δ_C 194.4), which was further confirmed by the HMBC correlations from H-2, H-9 and H_3–20 to C-1. The relative configuration of **6** was established by the ROESY data (Fig. 4). The structure of compound **6** was thereby assigned.

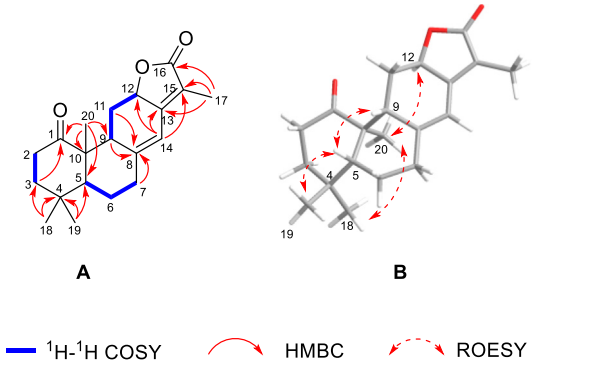

Fig. 4. Selected 2D NMR correlations for Euphonoid F (**6**).

3.1. *In vitro melanin synthesis evaluation*

All compounds were evaluated for their in vitro growth of B16 melanoma and 8-MOP-treated B16 melanoma cells with a known method [31]. As shown in Table 5, cell viability after treatment with **1**, **12**, **16** were more potent than the positive control (8-MOP), the commonly used drug for vitiligo in clinic. Noticeably, compounds **1** (159.9%), **12** (203.1%), **16** (177.4%) based on ingenol-type were stronger than 8-MOP (114.50%).

4. Concluding remarks

In summary, six new dierpenoids, along with 15 known ingenol esters, lathyane, *ent*-abietane, and isoprimarane were isolated from *E. antiquorum* L. Among them, compound **1**, **12**, and **16** exhibited better activities on melanin synthesis in vitro than positive control (8-MOP). It was noteworthy that the activity of ingenol diterpenoid **12** (203.1%) were nearly 2-fold stronger than 8-MOP (124.38%) on melanin synthesis in murine B16 cells. Compound **12** was specific and promising candidates against vitiligo, further studies on action mechanism of them and animal experiment on vitiligo transgenic mouse is under way.

Declaration of Competing Interest

Author's declares that they are no conflict of interest.

Table 5
The effects of Compounds (**1**, **12**, and **16**) on the cell proliferation of B16 melanoma cells and 8-MOP-treated B16 melanoma cells.

Compound	Amount(μM)	Cell viability(% of control)
Control	2 μL DMSO	100 ± 6.35
8-MOP	50 μM	124.38 ± 4.30
1	1 μM	117.64 ± 5.48
	10 μM	124.86 ± 8.64
	50 μM	159.89 ± 10.35
12	1 μM	103.81 ± 3.42
	10 μM	106.41 ± 2.34
	50 μM	203.11 ± 2.55
16	1 μM	110.40 ± 3.72
	10 μM	116.17 ± 8.39
	50 μM	177.43 ± 6.52

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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.2020.104583.

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