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neo-Clerodane diterpenoids from *Scutellaria barbata* with cytotoxic activities

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Abstract

Three *neo*-clerodane diterpenoids, named barbatins A–C (**1**–**3**), and the *neo*-clerodane diterpenoid nicotinyl ester, named scutebarbatine B (**4**), were isolated from the whole plant of *Scutellaria barbata* D. Don. Their structures were elucidated by spectroscopic analyses (UV, IR, HRFAB-MS, 1D NMR and 2D NMR). *In vitro*, compounds **1**–**4** showed significant cytotoxic activities against three human cancer lines, namely, HONE-1 nasopharyngeal, KB oral epidermoid carcinoma, and HT29 colorectal carcinoma cells, with IC_{50} values in the range 3.5–8.1 μM.

Keywords: *Scutellaria barbata* D. Don; Labiatae; *neo*-Clerodane diterpenoid; Barbatins A–C; Scutebarbatine B; Cytotoxic activity

1. Introduction

The traditional Chinese medicinal herb ''Banzhilian'', derived from the dry whole plant of *Scutellaria barbata* D. Don, is commonly used for the treatment of tumors, hepatitis, cirrhosis and other diseases (Wang et al., 1996). In recent years, more than thirty flavonoids, over ten *neo*-clerodane type diterpenoids, triterpene acids and sterol glucosides have been isolated, some of which exhibit interesting biological activities (Wang et al., 1996; Ducki, 1996; Kizu et al., 1997; Yu and Lei, 2004; Yin et al., 2004). In the course of our searches for anti-tumor compounds, we initiated a phytochemical study of *S. barbata*. The EtOH extract of *S. barbata* was successively partitioned with $CHCl_3$ and EtOAc, and the $CHCl_3$ fraction was sequentially subjected to column chromatography over silica gel, silica gel RP-18, and Sephadex LH-20 to give three new *neo*-clerodane diterpenoids, named barbatins A–C (**1**–**3**), and a new *neo*-clerodane diterpenoid nicotinyl ester, named scutebarbatine B (**4**). This paper deals with the isolation and structure elucidation of these four new compounds, as well as an evaluation of their cytotoxic effects.

2. Results and discussion

Compound **1** was obtained as white needles, and the molecular formula was established as $C_{34}H_{38}O_8$ by HRFAB MS, which displayed a quasi-molecular ion at m/z 575.2649 $[M+H]^+$. The IR spectrum showed absorption bands at 3430, 1776, 1668, 1630, 1600, 1581, 1460, 1382, 1021, 760, and 720 cm^{-1}, which were assignable to hydroxyl, conjugated carbonyl, benzene ring, and γ-lactone groups. The ^1H and ^{13}C NMR spectra indicated the presence of four tertiary methyl groups (δ_H 1.09 s, 1.11 s, 1.39 s, and 1.68 s, each 3H; δ_C 16.7 q, 19.8 q, 20.1 q, and 22.3 q), two benzoyloxy moieties (δ_H 7.80, 2H, m, H-3′ and H-7′; 7.39, 2H, m, H-4′ and H-6′; 7.43, br t, $J = 7.8$ Hz, H-5′; 7.79, 2H, m, H-3″ and H-7″; 7.46, 2H, m, H-4″ and H-6″; 7.40, br t, $J = 7.7$ Hz, H-5″; δ_C 166.7 s, C-1′; 128.9 s, C-2′; 130.0 d, C-3′ and C-7′; 129.2 d, C-4′ and C-6′; 133.4 d, C-5′; 168.3 s, C-1″; 129.4 s, C-2″; 129.8 d, C-3″ and C-7″; 128.6 d, C-4″ and C-6″; 133.7 d, C-5″), in addition to an 8,13-ether bridge (δ_H 1.11 s, H_3-17; δ_C 19.8 q, C-17; 83.7 s, C-8; 76.8

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s, C-13) and a 13-spiro-15,16-γ-lactone moiety (δ_H 2.54, 1H, d, $J = 17.6$ Hz, H_a-14; 3.19, 1H, d, $J = 17.6$ Hz, H_b-14; 4.05, 1H, d, $J = 8.5$ Hz, H_a-16; 4.20, 1H, d, $J = 8.5$ Hz, H_b-16; δ_C 43.9 t, C-14; 174.2 s, C-15; 76.3 t, C-16); several other *neo*-clerodane diterpenoids have this structural moiety as well (Malakov and Papanov, 1996, 1997; Bruno et al., 2002). Detailed examination of the ^{1}H-^{1}H COSY spectrum indicated the presence of another two spin systems. The first spin system included the signals of a methine (δ 2.86, 1H, dd, $J = 2.8$, 12.3 Hz, H-10), two methylenes (δ 1.67, 1H, m, H_a-1; 2.05, 1H, m, H_b-1; 2.17, 2H, m, H-2) and a tri-substituted double bond (δ 5.31, br s, H-3). Thus, H_2-1 coupled with the resonances of H-10 and H_2-2 which in turn was vicinally coupled with H-3. The latter, together with the crucial ^{1}H-^{13}C long-range correlations observed in the HMBC spectrum of **1** (Fig. 1) clearly indicated the presence of a double bond across C-3/C-4. The second spin system was comprised of two aliphatic protons on carbons bearing oxygen at δ 5.67 (1H, d, $J = 9.9$ Hz, H-6) and 3.74 (1H, d, $J = 9.9$ Hz, H-7). Observation of the cross-peaks in the HMBC spectrum from H-6 to C-1′ and from H-11 to C-1″ proved that two benzoyloxy moieties were connected to C-6 and C-11, respectively. Based on the above data and comprehensive 2D NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC), the structure of **1** was established as shown in Fig. 1. The relative stereochemistry of the chiral centers in **1** was resolved by a 2D ROESY analysis. In the ROESY spectrum (Fig. 2), the cross-peaks were observed from H_3-20 to H-7, H-11, H_b-16, H_3-17, and H_3-19, from H-6 to H-10, from H_3-17 to H-7, H-11, H_a-16, H_b-16, and H_3-20, as well as from H-11 to H_b-12, H_a-16, H_b-16, H_3-17, and H_3-20. Thus, H_3-17, H_3-19, H_3-20, H-7, H-11, and H_2-16 were in the same molecular plane (α-configuration) while H-6 and H-10 were on the opposite side (β-configuration).

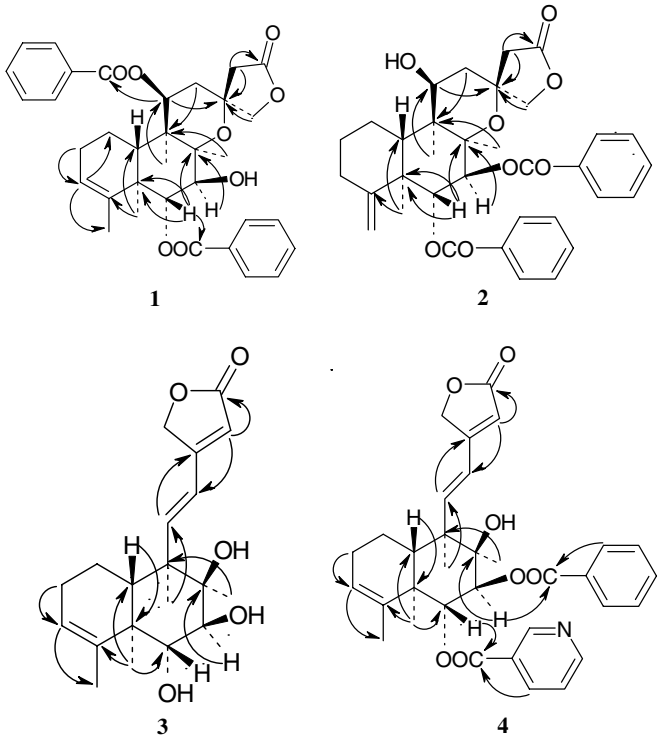
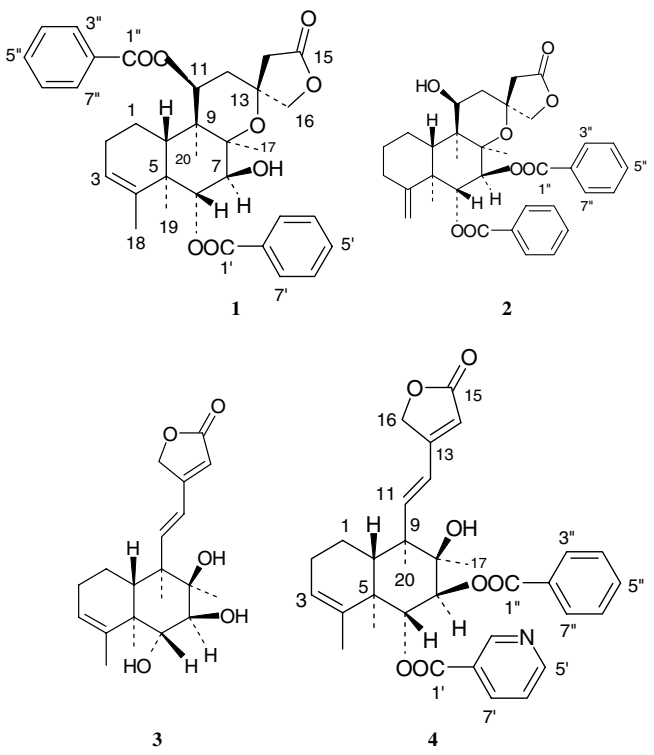


Fig. 1. Key HMBC correlations for compounds **1**–**4**.



Compounds isolated from *Scutellaria barbata*.

Compound **2** was isolated as white needles. The HRFAB mass spectrum showed a quasi-molecular ion peak at m/z 575.2653 $[M + H]^+$, consistent with a molecular formula $C_{34}H_{38}O_8$. The IR spectrum gave absorption bands due to hydroxyl, conjugated carbonyl, benzene ring, and γ-lactone moieties at 3455, 1770, 1661, 1629, 1608, 1577, 1458, 1380, 1013, 770, and 721 cm^{-1}. Comparison of its ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) with those of **1** showed that **2** had many spectroscopic features in common with **1**. The differences in their NMR spectra could be accounted for by the absence of the signals of both the C3═C4 double bond and the change of attachment of one benzoyloxy group. Instead, an exomethylene group (δ_H 4.60, 2H, br s, H-18; δ_C 104.9 t, C-18; 154.6 s, C-4) was present in **2** and a benzoyloxy function (δ_H 7.94, 2H, m, H-3″ and H-7″; 7.63, 2H, m, H-4″ and H-6″; 7.48, 1H, br t, $J = 7.7$ Hz, H-5″; δ_C 168.7 s, C-1″; 129.6 s, C-2″; 129.7 d, C-3″ and C-7″; 128.4 d, C-4″ and C-6″; 133.6 d, C-5″) was attached to C-7. The stereochemical assignments within **2** were accomplished in a similar manner as described for **1** (Fig. 2).

Compound **3** was obtained as white needles. The molecular formula was determined to be $C_{20}H_{28}O_5$ by HRFAB MS, which showed a quasi-molecular ion at m/z 349.2011 $[M + H]^+$. The IR spectrum showed absorption bands at 3438 (br), 1713, 1665, 1638, and 1012 cm^{-1}, which were

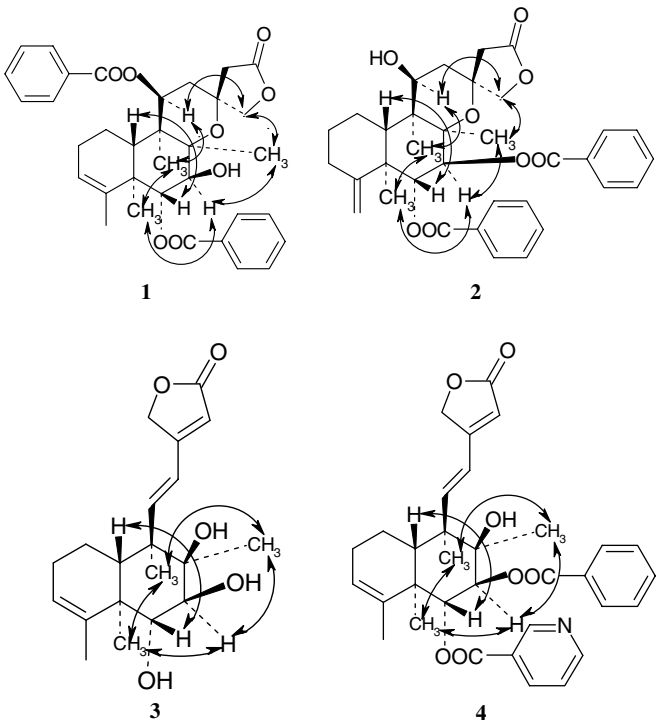


Fig. 2. Selected NOE correlations for compounds **1**–**4**.

in agreement with hydroxyl, conjugated carbonyl, and α,β-unsaturated γ-lactone moieties. The ^{1}H NMR spectrum of **3** revealed the presence of the following: Four tertiary methyl groups at δ 1.23 (3H, s, H-17), 1.42 (3H, s, H-20), 1.03 (3H, s, H-19), and 1.12 (3H, s, H-18); an α,β-unsaturated γ-lactone moiety at δ 5.91 (1H, br s, H-14),4.92 (1H, dd, $J = 1.2$, 16.4, H_a-16), and 5.06 (1H, dd, $J = 1.2$, 16.4, H_b-16); a double bond with an E configuration at δ 6.39 (1H, d, $J = 16.8$ Hz, H-11) and 6.30 (1H, d, $J = 16.8$ Hz, H-12). In addition, the ^{1}H-^{1}H COSY experiment revealed two spin systems. The first spin system included the signals of a methine (δ 2.09, 1H, dd, $J = 1.6$, 12.8 Hz, H-10), two methylenes (δ 1.28, 1H, m, H_a-1; 1.55, 1H, m, H_b-1; 2.01, 2H, m, H-2) and a tri-substituted double bond (δ 5.22, br s, H-3). Thus, H_2-1 was coupled with the signals of H-10 and H_2-2 which in turn was vicinally coupled with H-3. The latter, together with the crucial long-range correlations observed in the HMBC spectrum of **3** (Fig. 1), indicated the presence of the double bond across C-3/C-4. The second spin system was traced from two aliphatic protons on oxygenated carbons at δ 3.75 (1H, d, $J = 9.2$ Hz, H-6) and 3.62 (1H, d, $J = 9.2$ Hz, H-7). Based on all of the above data and from an HMBC analyses (Fig. 1), the structure of **3** was established as shown in Fig. 1. The relative stereochemistry of all the

Table 1
^{1}H NMR spectroscopic data for compounds **1**–**4** (400 MHz, in $CDCl_3$)[ab]

H	1	2	3	4
1	1.67 (m, H_a-1)	1.82 (m, H_a-1)	1.28 (m, H_a-1)	1.35 (m, H_a-1)
	2.05 (m, H_b-1)	2.46 (m, H_b-1)	1.55 (m, H_b-1)	1.66 (m, H_b-1)
2	2.17 (m, 2H)	1.47 (m, H_a-2)	2.01 (m, 2H)	2.06 (m, 2H)
		2.06 (m, H_b-2)		
3	5.31 (br s)	2.13 (m, H_a-3)	5.22 (br s)	5.25 (br s)
		2.29 (m, H_b-3)		
6	5.67 (d, 9.9)	5.70 (d, 10.6)	3.75 (d, 9.2)	5.93 (d, 10.8)
7	3.74 (d, 9.9)	5.61 (d, 10.6)	3.62 (d, 9.2)	5.72 (d, 10.8)
10	2.86 (dd, 2.8, 12.3)	2.38 (dd, 2.0, 12.4)	2.09 (dd, 1.6, 12.8)	2.36 (br d, 11.6)
11	5.80 (dd, 3.8, 11.7)	4.34 (dd, 3.8, 12.0)	6.39 (d, 16.8)	6.46 (d, 16.8)
12	1.76 (m, H_a-12)	1.57 (m, H_a-12)	6.30 (d, 16.8)	6.40 (d, 16.8)
	2.09 (m, H_b-12)	2.14 (m, H_b-12)		
14	2.54 (d, 17.6, H_a-14)	2.84 (d, 17.2, H_a-14)	5.91 (br s)	5.94 (br s)
	3.19(d, 17.6, H_b-14)	3.03 (d, 17.2, H_b-14)		
16	4.05 (d, 8.5, H_a-16)	4.23 (d, 9.2, H_a-16)	4.92 (dd, 1.2, 16.4, H_a-16)	5.00 (2H, br s)
	4.20 (d, 8.5, H_b-16)	4.39 (d, 9.2, H_b-16)	5.06 (dd, 1.2, 16.4, H_b-16)	
17	1.11 (s, 3H)	1.12 (s, 3H)	1.23 (s, 3H)	1.07 (3H, s)
18	1.68 (s, 3H)	4.60 (br s, 2H)	1.42 (s, 3H)	1.58 (3H, s)
19	1.39 (s, 3H)	1.43 (s, 3H)	1.03 (s, 3H)	1.45 (3H, s)
20	1.09 (s, 3H)	1.58 (s, 3H)	1.12 (s, 3H)	1.28 (3H, s)
3′	7.80 (m)	7.91 (m)		8.98 (br s)
4′	7.39 (m)	7.55 (m)		
5′	7.43 (br t, 7.8)	7.42 (br t, 7.7)		8.63 (br d, 4.6)
6′	7.39 (m)	7.55 (m)		7.25 (dd, 4.6, 7.8)
7′	7.80 (m)	7.91 (m)		8.05 (br d, 7.8)
3″, 7″	7.79 (m, 2H)	7.94 (m, 2H)		7.83 (m, 2H)
4″, 6″	7.46 (m, 2H)	7.63 (m, 2H)		7.33 (m, 2H)
5″	7.40 (br t, 7.7)	7.48 (br t, 7.7)		7.48 (br t, 7.6)

[a] Chemical shift values were in ppm and J values (in Hz) were presented in parentheses.
[b] The assignments were based on HMQC, HMBC, and ^{1}H-^{1}H COSY experiments.

Table 2
^{13}C NMR spectroscopic data for compounds **1**–**4** (100 MHz, in $CDCl_3$)[a]

Carbon	1	2	3	4
1	28.8 t	22.5 t	19.5 t	19.2 t
2	33.1 t	28.6 t	26.4 t	26.1 t
3	121.1 d	32.9 t	122.6 d	123.3 d
4	143.9 s	154.6 s	142.4 s	140.7 s
5	44.8 s	46.1 s	43.3 s	43.4 s
6	73.0 d	74.1 d	76.5 d	76.3 d
7	69.8 d	67.7 d	74.9 d	75.8 d
8	83.7 s	85.0 s	77.0 s	77.1 s
9	39.1 s	43.8 s	47.9 s	48.3 s
10	43.7 d	43.6 d	42.7 d	42.8 d
11	72.5 d	74.5 d	147.8 d	146.6 d
12	29.6 t	31.2 t	121.5 d	121.9 d
13	76.8 s	77.9 s	162.3 s	162.0 s
14	43.9 t	42.4 t	114.7 d	115.0 d
15	174.2 s	174.3 s	174.1 s	173.9 s
16	76.3 t	79.2 t	70.7 t	70.6 t
17	19.8 q	16.5 q	22.5 q	22.5 q
18	20.1 q	104.9 t	22.0 q	20.1 q
19	16.7 q	17.7 q	16.1 q	17.3 q
20	22.3 q	20.2 q	15.5 q	15.4 q
1′	166.7 s	166.5 s		164.5 s
2′	128.9 s	128.6 s		125.2 s
3′	130.0 d	129.9 d		150.3 d
4′	129.2 d	128.9 d		
5′	133.4 d	133.1 d		152.9 d
6′	129.2 d	128.9 d		123.2 d
7′	130.0 d	129.9 d		137.0 d
1″	168.3 s	167.8 s		165.5 s
2″	129.4 s	129.6 s		128.6 s
3″, 7″	129.8 d	129.7 d		129.6 d
4″, 6″	128.6 d	128.4 d		128.3 d
5″	133.7 d	133.6 d		133.4 d

[a] The assignments were based on HMQC, HMBC, and ^1H-^1H COSY experiments.

asymmetric centers of **3** was firmly established from the ROESY spectrum (Fig. 2). H-6 showed NOEs cross peaks with H-10, and H_3-20 showed NOEs with H-7, H_3-17, and H_3-19. Therefore, H_3-17, H_3-19, H_3-20 and H-7 were on the same molecular plane (α-configuration) while H-6 and H-10 were on the opposite side (β-configuration).

Compound **4** was isolated and purified as white needles, and showed a positive response to alkaloid reagents. In the HRFAB mass spectrum, **4** gave a quasi-molecular ion peak at *m/z* 558.2487 $[M+H]^+$, corresponding to the molecular formula $C_{33}H_{35}NO_7$. The IR spectrum exhibited absorption bands at 3342, 1780, 1743, 1727, 1643, 1591, 1501, 1451, 740, and 712 cm^{-1}, indicative of hydroxyl, conjugated carbonyl, benzene ring, and α,β-unsaturated γ-lactone groups. Comparison of the NMR spectra of **3** and **4** (Tables 1 and 2) showed similarities except for the substitution of two hydroxy groups at C-6 and C-7 in **3** with a benzoyloxy at C-7 and a nicotinic acid ester at C-6 in **4**. The NOE experiments proved that **4** possessed the same relative configurations as **3** (Fig. 2).

The four isolated compounds (**1**–**4**) were evaluated for their cytotoxic activities against HONE-1, KB, and HT29 cancer cell lines by using methylene blue dye assay and anti-cancer drugs, etoposide and cisplatin (Chang et al., 2002, 2004a), as positive controls. These compounds exhibited significant cytotoxicity as shown in Table 3.

Table 3
Cytotoxicity of compounds **1**–**4** against cultured HONE-1, KB and HT29 cancer cell lines

Compounds	Growth inhibition constant (IC_{50})[a] [μM]		
	HONE-1	KB	HT29
Etoposide[b]	0.6 ± 0.2	0.9 ± 0.3	2.3 ± 0.5
Cisplatin[b]	3.7 ± 0.4	4.2 ± 0.9	5.6 ± 1.8
1	4.7 ± 2.0	7.7 ± 2.2	5.9 ± 1.3
2	5.0 ± 2.1	8.1 ± 1.8	6.6 ± 1.5
3	4.1 ± 1.5	7.1 ± 2.6	4.3 ± 2.0
4	4.4 ± 1.9	6.1 ± 2.7	3.5 ± 2.1

[a] IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are means ± standard deviation of three independent replicates. The IC_{50} greater than 10 μM was considered to have no cytotoxicity.
[b] Positive control.

3. Experimental

3.1. General

Melting points were measured on an XT-4 micro-melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. UV spectra were obtained on a Shimadzu UV-160 spectrophotometer. IR spectra were recorded on a Perkin–Elmer 683 infrared spectrometer with KBr disks, whenever FABMS and HRFABMS employed an Autospec-Ultima ETOF MS spectrometer. NMR spectra were recorded on a Varian Unity BRUKER 400 at 400 MHz (^1H) and 100 MHz (^{13}C), with TMS as the internal standard. Silica gel (200–300 mesh) for column chromatography and silica gel GF254 for preparative TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, People's Republic of China.

3.2. Plant material

S. barbata D. Don was collected in Linyi district, Shandong Province, People's Republic of China, in September 2003, and identified by Professor Yan-yan Zhao, School of Pharmaceutical Science, Yantai University. The whole plant of *S. barbata* was harvested and air-dried at room temperature in the dark. A voucher specimen (YP03053) has been deposited at the Herbarium of School of Pharmaceutical Science, Yantai University.

3.3. Extraction and isolation

The air-dried whole plant (13.6 kg) of *S. barbata* was finely cut and extracted three times (1 h × 3) with refluxing EtOH (30 l × 3). Evaporation of the solvent under reduced

pressure provided the ethanolic extract (901.7 g). The extract was dissolved and suspended in H_2O (3.0 l), and partitioned with $CHCl_3$ (3 × 4 l) and EtOAc (3 × 4 l). The $CHCl_3$ fraction (94.6 g) was subjected to CC (10 × 120 cm) on silica gel (200–300 mesh), eluted with cyclohexane–acetone [95:5 (4.0 l), 90:10 (4.0 l), 85:15 (5.0 l), 80:20 (5.0 l), 75:25 (4.0 l), 70:30 (4.0 l), 60:40 (3.0 l), and 50:50 (2.5 l)] to give eight fractions. Fraction 5 (2.3 g) was separated by reversed-phase silica gel (60 g, 20–45 μ) CC [eluted by MeOH–H_2O, 55:45, v/v], to give a mixture (61 mg) and **4** (33 mg). The mixture was further separated by preparative TLC [$CHCl_3$–MeOH–CH_3COCH_3, 8:1:1, v/v] to afford **1** (23 mg) and **2** (21 mg), and subsequently purified on Sephadex LH-20 [50 g, eluting with $CHCl_3$–CH_3OH, 10:40, v/v] to give **1** (20 mg), and **2** (17 mg). Fraction 6 (2.0 g) was separated by reversed-phase silica gel (60 g, 20–45 μ) CC [eluted by MeOH–H_2O, 40:60, v/v] and purified by preparative TLC [$CHCl_3$–MeOH–CH_3COCH_3, 7:1:2, v/v] to give **3** (38 mg).

3.4. Barbatin A (*1*)

White needles, m.p. 150–151 °C, $[\alpha]_D^{29}$ − 63.6 (*c* 0.12, MeOH). UV ($CDCl_3$) λ_{max}: 219, 255 nm. IR (KBr) ν_{max}: 3430, 1776, 1668, 1630, 1600, 1581, 1460, 1382, 1021, 760, and 720 cm^{-1}. FABMS *m/z*: 575.2 $[M + H]^+$. HR-FABMS *m/z*: 575.2649 $[M + H]^+$ (calcd for $C_{34}H_{39}O_8$, 575.2645). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.5. Barbatin B (*2*)

White needles, m.p. 148–150 °C, $[\alpha]_D^{29}$ − 60.4 (*c* 0.13, MeOH). UV ($CDCl_3$) λ_{max}: 220, 256 nm. IR (KBr) ν_{max}: 3455, 1770, 1661, 1629, 1608, 1577, 1458, 1380, 1013, 770, and 721 cm^{-1}. FABMS *m/z*: 575.3 $[M + H]^+$. HR-FABMS *m/z*: 575.2653 $[M + H]^+$ (calcd for $C_{34}H_{39}O_8$, 575.2645). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.6. Barbartin C (*3*)

White needles, m.p. 156–158 °C, $[\alpha]_D^{29}$ − 103.8 (*c* 0.14, MeOH). UV ($CDCl_3$) λ_{max}: 220, 257 nm. IR (KBr) ν_{max}: 3438 (br), 1713, 1665, 1638, and 1012 cm^{-1}. FABMS *m/z*: 349.4 $[M + H]^+$. HR-FABMS *m/z*: 349.2011 $[M + H]^+$ (calcd for $C_{20}H_{29}O_5$, 349.2015). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.7. Scutebarbatine B (*4*)

White needles, m.p. 151–153 °C, $[\alpha]_D^{29}$ − 109.6 (*c* 0.13, MeOH). UV ($CDCl_3$) λ_{max}: 217, 222, 257 nm. IR (KBr) ν_{max}: 3342, 1780, 1743, 1727, 1643, 1591, 1501, 1451, 740, 712 cm^{-1}. FABMS *m/z*: 558.3 $[M + H]^+$. HR-FABMS *m/z*: 558.2487 $[M + H]^+$ (calcd for $C_{33}H_{36}NO_7$, 558.2492). For ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

3.8. Anti-tumoral cytotoxic bioassays

Human nasopharyngeal carcinoma HONE-1, oral epidermoid carcinoma KB, and colorectal carcinoma HT29 cells were maintained in RPMI-1640 medium supplied with 5% fetal bovine serum. Cells in logarithmic phase were cultured at a density of 5000 cells/ml/well in a 24-well plate. The cells were exposed to various concentrations of the tested drugs for 72 h. The methylene blue dye assay was used to evaluate the effects of the tested drugs on cell growth, as described previously (Chang et al., 2004b). The IC_{50} value resulting from 50% inhibition of cell growth was calculated graphically as a comparison with the control.

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