Abietane Diterpenoids and a Lignan from *Pinus yunnanensis*

Tao Feng[a,b], Xiang-Hai Cai[a], Qin-Gang Tan[a], and Xiao-Dong Luo[a]

[a] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, 132 Lanhei Road, Kunming 650204, P. R. China
[b] Graduate School of Chinese Academy of Sciences, Beijing 100039, P. R. China

Reprint requests to Dr. Xiao-Dong Luo. Fax: +86-871-5150227. E-mail: xdluo@mail.kib.ac.cn

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Two new abietane diterpene acids, pinyunins A (**1**) and B (**2**), a new lignan, 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(2,3-dihydroxyphenyl)propane-1,3-diol (**3**), and eight known diterpenoids (**4** – **11**) were isolated from the bark of *Pinus yunnanensis* Franch. Their structures were elucidated by spectroscopic methods. Compounds **1** – **3** exhibited high inhibitions on Cox-2 (> 80 %) and low inhibitions on Cox-1 (< 50 %), showing the trend of selective inhibition of Cox-2, while the positive controls NS-398 (Cox-2) and SC-560 (Cox-1) gave inhibitions of 97.09 % and 61.30 %, respectively. Compounds **1** – **11** were evaluated for their cytotoxicity against five human cancer cell lines with Cisplatin as a positive control.

Key words: Pinus yunnanensis, Pinaceae, Diterpenoids, Lignan, Anti-inflammatory Activity, Cytotoxicity

Introduction

The genus *Pinus*, comprised of more than 80 species [1], is known to be rich in abietane diterpenoids [2 – 5]. Some abietane diterpenoids have been reported to possess antitumor activity [6 – 8], inhibit nitric oxide (NO) production [9], and show anti-inflammatory activity [4, 9]. *P. yunnanensis* Franch., a tree 10 to 30 m high distributed in the southwest of China, has been cultivated as economic and ecological forest on a huge scale (more than 3 million hectare) in Yunnan province, P. R. China [10], which could be a good source to search for new and bioactive abietane diterpenoids. Furthermore, the pine nodular branch of *P. yunnanensis* is used for the treatment of inflammations by people in the southwest of China [10]. In the course of our search for novel and active natural products, we carried out a phytochemical investigation on the bark of *P. yunnanensis*, which led to the isolation of two new abietane diterpenoids, pinyunins A (**1**) and B (**2**), and a new lignan, 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(2,3-dihydroxyphenyl)propane-1,3-diol (**3**), together with eight known diterpenoids, abietic acid (**4**) [5], 12α-methoxyabietic acid (**5**) [11], 12α-hydroxyabietic acid (**6**) [5], 15-hydroxydehydroabietic acid (**7**) [5], 7-oxo-12α,13β-dihydroxyabiet-8(14)-en-18-oic acid (**8**) [12], 8(14)-podocarpen-13-on-18-oic acid (**9**) [5], 13-hydroxy-8,11,13-podocarpatrien-18-oic acid (**10**) [5], and pimaric acid (**11**) [5] (Fig. 1). The new structures were established by means of spectroscopic methods, and the known compounds were identified by the comparison with data in the literature. Compounds **1** – **3** were evaluated for their anti-inflammatory activity against cyclooxygenases (Cox-1 and Cox-2) and 5-lipoxygenase (5-Lox). Compounds **1**–**11** were evaluated for their cytotoxicity against five human cancer cell lines. Reported herein are the isolation, structure elucidation, and bioactivities of these compounds.

Results and Discussion

Pinyunin A (**1**) was obtained as colorless crystals. The IR spectrum displayed absorption bands at ν = 3431, 1707, and 1665 cm^{-1} for -OH, -CO-, and -COO- groups, respectively. The HRESIMS displayed an $[M–H]^+$ peak at *m/z* = 331.1916, consistent with a molecular formula $C_{20}H_{28}O_4$ with seven degrees of unsaturation. The ^{13}C NMR and DEPT spectra displayed twenty signals (Table 1). Of them, two carbonyl carbons were observed at δ_C = 196.3 and 183.8, and two olefinic carbons were detected at δ_C = 164.1 and 125.5, indicating three degrees of unsaturation, which suggested a tetracyclic ring system for compound **1**. According to the characteristic signals for normal abietane diterpenoid acids at δ_C = 47.1 (s, C-4), 48.7 (d,

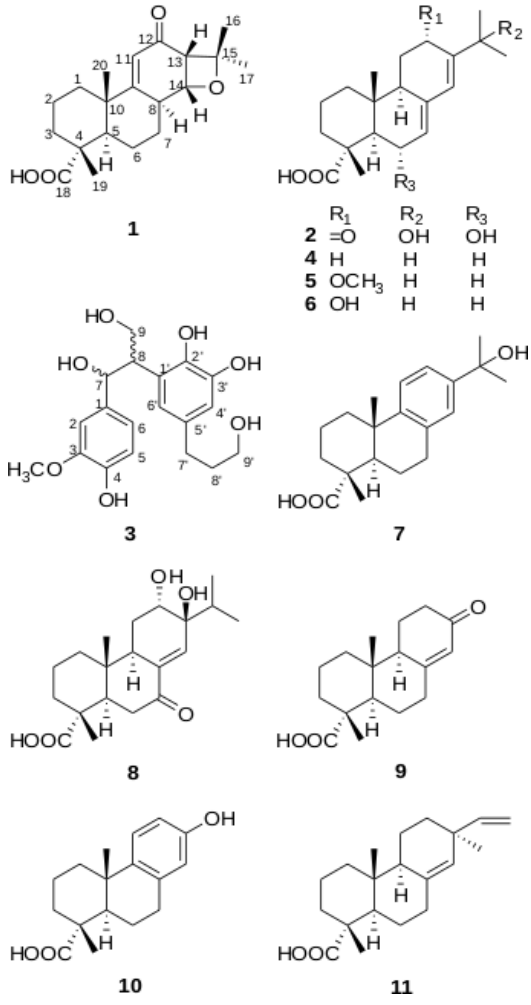

Fig. 1. Chemical structures of compounds **1** – **11**.

C-5), 40.2 (s, C-10), 183.8 (s, C-18), 16.1 (q, Me-19), and 16.1 (q, Me-20), compound **1** should derive from an abietane diterpene acid [5, 11, 12]. Analysis of the ^{1}H-^{1}H COSY spectrum gave two C-linkage fragments (**a**: C-1/C-2/C-3, **b**: C-5/C-6/C-7/C-8) (Fig. 2). The UV absorption band at λ_{max} = 246 nm suggested an α,β-unsaturated ketone moiety in the structure of **1**, corresponding to three carbons at δ_C = 196.3, 164.1 and 125.5, respectively, in the ^{13}C NMR spectrum. This moiety was identified as a system of a 12-ketone conjugated with a 9,11-double bond according to the HMBC correlations (Fig. 2). In the ^{13}C NMR spectrum, signals at δ_C = 70.5 and 85.4 were assigned to two oxygenated carbons (C-14 and C-15, respectively), according to the HMBC correlations of C-14 with H-13 and H-8, and of C-15 with Me-16, Me-17, and H-13, respectively (Fig. 2). To fulfill the MS analysis and the degrees of unsaturation, C-14 and C-15 should to be connected through an oxygen atom to form a four-membered ring. The planar structure of **1** was therefore elucidated as 14,15-epoxyabiet-9(11)-en-12-oxo-18-oic acid. The relative configuration of **1** was determined by a ROESY experiment (Fig. 2). The ROESY correlation of H-20 with H-19 indicated β-orientations of C-19 and C-20. The key ROESY correlations of H-20 with H-13 and H-14 indicated β-orientations of H-13 and H-14. Because of the above ROESY correlations, H-8 could only be α-oriented, which was supported by the molecular model as a 3D structure. In addition, the ROESY correlation of H-13 with H-16 indicated the β-orientation of Me-16 and the α-orientation of Me-17. Finally, compound **1** was elucidated as 14α,15-epoxyabiet-9(11)-en-12-oxo-18-oic acid.

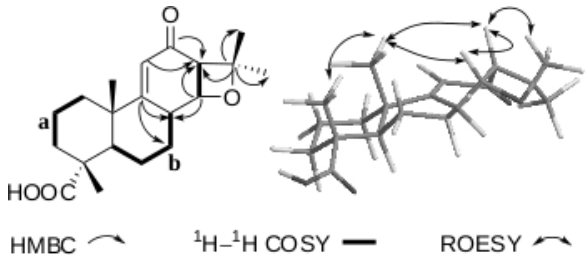

Fig. 2. Key HMBC, ^{1}H-^{1}H COSY, and ROESY correlations of **1**.

Pinyunin B (**2**) was isolated as a colorless powder. Its negative HRESIMS gave a molecular ion peak at *m/z* = 347.1850 $[M–H]^-$ and established the molecular formula $C_{20}H_{28}O_5$. The IR spectrum displayed absorption bands at ν = 3439 (-OH), 1700 (-CO-), and 1651 (-COOH) cm^{-1}. The UV absorption band at λ_{max} = 289 nm indicated the existence of conjugated groups in the structure. The ^{1}H and ^{13}C NMR data of **2** were similar to those of 15-hydroxy-7,13-abietadien-12-on-18-oic acid [5] (Table 1) except for the presence of one additional oxygenated methine group instead of one methylene function. This led to the assumption that **2** was an oxygenated derivative of 15-hydroxy-7,13-abietadien-12-on-18-oic acid. The ^{1}H-^{1}H COSY correlations of δ_H = 4.26 (1H, d, J = 11.0 Hz, H-6) with δ_H = 2.27 (1H, d, J = 11.0 Hz, H-5) and δ_H = 6.09 (1H, br s, H-7), as well as HMBC correlations of H-6 with C-5, C-7 and C-8, indicated that the additional hydroxyl group was located at C-6. The ROESY correlation of H-6 with H-20 suggested the β-orientation of H-6. A detailed analysis of the 2D NMR data (HMBC, HSQC, ^{1}H-^{1}H COSY, and ROESY) supported the structure

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

	— **1** —		— **2** —	
No.	δ_H	δ_C	δ_H	δ_C
1	1.31 (1H, m)	37.1 (t)	1.21 (1H, m)	37.8 (t)
	1.97 (1H, m)		1.70 (1H, m)	
2	1.64 (1H, m)	17.9 (t)	1.56 (1H, m)	17.4 (t)
	1.69 (1H, m)		1.70 (1H, m)	
3	1.71 (1H, m)	36.9 (t)	1.55 (1H, m)	37.9 (t)
	1.79 (1H, m)		1.72 (1H, m)	
4		47.1 (s)		43.4 (s)
5	2.07 (1H, m)	48.7 (d)	2.27 (1H, d, 11.0)	49.5 (d)
6	1.53 (1H, m)	26.9 (t)	4.26 (1H, d, 11.0)	67.7 (d)
	1.61 (1H, m)			
7	2.33 (1H, m)	37.4 (t)	6.09 (1H, br s)	137.2 (d)
	2.47 (1H, m)			
8	2.11 (1H, br s)	55.9 (d)		133.7 (s)
9		164.1 (s)	2.62 (1H, m)	48.1 (d)
10		40.2 (s)		36.3 (s)
11	6.16 (1H, m)	125.5 (d)	2.30 (1H, m)	37.8 (t)
			2.42 (1H, m)	
12		196.3 (s)		201.8 (s)
13	2.98 (1H, d, 6.7)	51.3 (d)		141.7 (s)
14	5.23 (1H, d, 6.7)	70.5 (d)	7.00 (1H, s)	141.3 (d)
15		85.4 (s)		71.6 (s)
16	1.58 (3H, s)	25.5 (q)	1.41 (3H, s)	28.7 (q)
17	1.15 (3H, s)	29.5 (q)	1.43 (3H, s)	28.9 (q)
18		183.8 (s)		183.2 (s)
19	1.10 (3H, s)	16.1 (q)	1.36 (3H, s)	16.5 (q)
20	0.64 (3H, s)	16.1 (q)	0.93 (3H, s)	15.3 (q)

of **2** as 6α,15-dihydroxy-7,13-abietadien-12-oxo-18-oic acid.

Compound **3** was obtained as a colorless oil. Its molecular formula $C_{19}H_{24}O_7$ was determined by the negative HRESIMS at *m/z* = 363.1447 $[M–H]^-$. A UV absorption band at λ_{max} = 282 nm, as well as the IR absorption band at ν = 1609 cm^{-1} suggested the presence of an aromatic system. The ^{13}C NMR spectrum displayed nineteen carbon resonances. Besides one methoxyl group, the ^{13}C NMR spectrum showed twelve sp^2 carbons and nine sp^3 carbons for two C_6–C_3 units, characteristic for a lignan skeleton. In the ^{1}H NMR spectrum, three aromatic signals at δ_H = 6.79 (1H, d, J = 8.0 Hz), 6.86 (1H, dd, J = 8.0, 1.9 Hz), and 7.03 (1H, d, J = 1.9 Hz), ascribed to H-5, H-6 and H-2, respectively, suggested an ABX system. Two singlet peaks at δ_H = 6.60 (1H) and 6.61 (1H) were ascribed to the protons of C-4′ and C-6′ with *meta* orientations. In the HMBC spectrum, three methylene signals at δ_H = 1.74 (2H, m), 2.52 (2H, t, J = 7.8 Hz) and 3.54 (2H, m) for H-8′, H-7′ and H-9′, respectively, established a side chain -CH_2-CH_2-CH_2OH, and three signals at δ_H = 3.48 (1H, m), 3.77 (2H, m), and 5.49 (1H, d, J = 6.6 Hz) for H-8, H-9, and H-7, respectively, suggested another side chain. All of the above information indicated that **3** had a similar structural pattern to that of 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(4-hydroxy-3-methoxyphenyl)propane-1,3-diol [13], except for a OH group instead of a OCH_3 group at C-3′, as supported by the loss of the signals of the methyl group in the ^{1}H and ^{13}C NMR spectra and MS analysis. The above information, combined with HMBC correlations, established the planar structure of **3** as 2-[2-hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(2,3-dihydroxyphenyl)propane-1,3-diol. Unfortunately, the configuration at C-7 and C-8 could not be identified due to the limited quantity of compound **3**.

Table 2. *In vitro* evaluation of anti-inflammatory activity of **1**–**3**.[a]

	Cox-1	Cox-2	5-Lox
1	47.74	84.83	49.42
2	40.57	83.71	57.40
3	45.69	88.25	59.37
SC-560[b]	61.30	–	–
NS-398[b]	–	97.09	–
Zileuton[b]	–	–	83.05
DMSO[c]	0	0	0

[a] Percent inhibition (compound concentration 100 μM); [b] used for positive control; [c] used for negative control.

Compounds **1**–**3** were evaluated for their anti-inflammatory activity (Table 2), which exhibited high inhibition on Cox-2 with percent inhibitions of 84.83, 83.71 and 88.25, respectively. Simultaneously, they all displayed low inhibition on Cox-1 (< 50 %), showing the trend of selective inhibition of Cox-2. In addition, compounds **1**–**11** were tested for their cytotoxicity against five human cancer cell lines, but no significant activity was found ($IC_{50} > 40$ μM).

Experimental Section

General

Melting points were obtained on an X-4 micro melting point apparatus. Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for IR spectroscopy using KBr pellets. 1D and 2D spectra were run on Bruker DRX-500 and AM-400 spectrometers with TMS as internal standard. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. HRMS ((–)-ESI) was performed on an API-Qstar-Pulsar-1 spectrometer. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., P.R. China), RP-18 gel (20–45 μm, Fuji Silysia Chemical Ltd., Japan) and Sephadex

LH-20 (Pharmacia Fine Chemical Co., Ltd., Sweden). Fractions were monitored by TLC (GF 254, Qingdao Haiyang Chemical Co., Ltd., P. R. China), and spots were visualized by heating silica gel plates sprayed with 10 % H_2SO_4 in EtOH.

Plant material

The bark of *P. yunnanensis* Franch. was collected in Kunming, Yunnan Province, P. R. China, and identified by Dr. Chun-Xia Zeng. A voucher specimen (FT20080810) has been deposited at Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

Extraction and isolation

The bark (10 kg) of *P. yunnanensis* was extracted three times with methanol at r. t. After removal of MeOH under reduced pressure, the viscous concentrate was partitioned with EtOAc (4 × 3 L) to afford EtOAc and H_2O extracts. The EtOAc extract (73 g) was chromatographed on a prepacked silica gel column (1.0 kg, 120 × 10 cm^2; $CHCl_3$: Me_2CO = 1:0 → 1:1) to give 6 fractions (1 – 6). Fraction 1 (13 g) was chromatographed over silica gel (300 g, 100 × 5 cm^2; petroleum ether : Me_2CO = 8:1 → 2:1) to give **4** (18 mg) and **11** (130 mg). Fraction 2 (7.0 g) was subjected to a silica gel column (300 g, 100 × 5 cm^2; $CHCl_3$: Me_2CO = 6:1 → 2:1) to afford three fractions (2a – 2c). Fraction 2a (1.7 g) was further purified on a silica gel column (80 g, 40 × 2.5 cm^2; petroleum ether : Me_2CO = 2 : 1) to afford **9** (11 mg) and **7** (17 mg). Fraction 2b (1.0 g) was purified on an RP-18 column (30 g, 30 × 1.5 cm^2; MeOH : H_2O = 6 : 4) to afford **5** (14 mg). Compound **1** (11 mg), colorless crystals, precipitated from fraction 2c (2.1 g). Fraction 3 (11 g) was subjected to a silica gel column (500 g, 100 × 6 cm^2; $CHCl_3$: MeOH = 9 : 1) to afford **6** (18 mg). Fraction 4 (7.2 g) was subjected to a silica gel column (350 g, 100 × 5 cm^2; $CHCl_3$: MeOH = 8 : 1 → 4 : 1) to afford four fractions (4a – 4d). Fraction 4a (1.2 g) was purified on an RP-18 column (40 g, 30 × 1.5 cm^2; MeOH : H_2O = 1 : 1) to afford **10** (5 mg). Fraction 4c (200 mg) was purified on a Sephadex LH-20 column (45 g, 180 × 1.0 cm^2; MeOH) to afford **2** (3 mg). Fraction 4d (1.1 g) was subjected to an RP-18 column (35 g, 30 × 1.5 cm^2; MeOH : H_2O = 1 : 9 → 3 : 7) to **8** (6 mg). Fraction 5 (3 g) was subjected to an RP-18 column (100 g, 45 × 2.5 cm^2; MeOH : H_2O = 1 : 9), then purified on a Sephadex LH-20 column (45 g, 180 × 1.0 cm^2; MeOH) to afford **3** (3 mg).

*Pinyunin A = 14α,15-epoxyabiet-9(11)-en-12-oxo-18-oic acid (**1**)*

Colorless crystals (MeOH). – M. p. 230 – 233 °C. – UV (MeOH): λ_{max} (log ε) = 246 (3.83), 221 (3.59), 201 (3.80) nm. – $[\alpha]_D^{16}$ = −21.9 (c = 0.14, MeOH). – IR (KBr) ν = 3431 (OH), 2940, 2877, 1707 (C=O), 1665 (C=O), 1230, 955, 866 cm^{-1}. – ^{1}H NMR (400 MHz, $CDCl_3$, 20 °C, TMS) and ^{13}C NMR (100 MHz, $CDCl_3$, 20 °C, TMS) spectral data: see Table 1. – HRMS ((–)-ESI): m/z = 331.1916 (calcd. 331.1909 for $C_{20}H_{27}O_4$, $[M–H]^-$).

*Pinyunin B = 6α,15-dihydroxy-7,13-abietadien-12-oxo-18-oic acid (**2**)*

Colorless powder. – M. p. 258 – 260 °C. – UV (MeOH): λ_{max} (log ε) = 289 (3.76), 202 (3.91) nm. – $[\alpha]_D^{16}$ = −23.2 (c = 0.04, MeOH). – IR (KBr): ν = 3439 (OH), 2931, 2844, 1700 (C=O), 1651 (C=O), 1349, 1178, 882 cm^{-1}. – ^{1}H NMR (500 MHz, $CDCl_3$/[D_4]methanol, 20 °C, TMS) and ^{13}C NMR (100 MHz, $CDCl_3$/[D_4]methanol, 20 °C, TMS) spectral data: see Table 1. – HRMS ((–)-ESI): m/z = 347.1850 (calcd. 347.1858 for $C_{20}H_{27}O_5$, $[M–H]^-$).

*2-[2-Hydroxy-5-(3-hydroxypropyl)-3-methoxyphenyl]-1-(2,3-dihydroxyphenyl)propane-1,3-diol (**3**)*

Colorless oil. – UV (MeOH): λ_{max} (log ε) = 282 (3.50), 211 (4.32) nm. – $[\alpha]_D^{16}$ = +6.4 (c = 0.14, MeOH). – IR (KBr): ν = 3416 (OH), 2938, 1609, 1516, 1275, 1032, 807 cm^{-1}. – ^{1}H NMR (500 MHz, [D_6]Me_2CO, 20 °C, TMS): δ = 1.74 (2H, m, H-8′), 2.52 (2H, t, J = 7.8 Hz, H-7′), 3.48 (1H, m, H-8), 3.54 (2H, m, H-9′), 3.77 (2H, m, H-9), 3.78 (3H, s, OMe), 5.49 (1H, d, J = 6.6 Hz, H-7), 6.60 (1H, s, H-4′), 6.61 (1H, s, H-6′), 6.79 (1H, d, J = 8.0 Hz, H-5), 6.86 (1H, dd,J = 8.0, 1.9 Hz, H-6), 7.03 (1H, d, J = 1.9 Hz, H-2). – ^{13}C NMR (100 MHz, [D_6]Me_2CO, 20 °C, TMS): δ = 32.3 (t, C-7′), 35.6 (t, C-8′), 55.1 (d, C-8), 56.1 (q, OMe), 61.8 (t, C-9′), 64.6 (t, C-9), 87.9 (d, C-7), 110.3 (d, C-2), 115.5 (d, C-5), 116.2 (d, C-6′), 116.7 (d, C-4′), 119.4 (d, C-6), 129.6 (s, C-1′), 134.5 (s, C-1), 136.1 (s, C-5′), 141.4 (s, C-3′), 145.8 (s, C-2′), 146.9 (s, C-4), 148.2 (s, C-3). – HRMS ((–)ESI): m/z = 363.1447 (calcd. 363.1443 for $C_{19}H_{23}O_7$, $[M–H]^-$).

In vitro anti-inflammatory assay

The *in vitro* anti-inflammatory activity tests were performed according to the literature with minor modifications [14]. Briefly, the reaction system was incubated for 5 min at 25 °C by sequential addition of the buffer, heme, compounds **1** – **3** (purity: > 90 %), and Cox-1 or Cox-2 (human recombinant, Cayman, USA) to the system followed by mixing with *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) (Cayman, USA) and arachidonic acid (Cayman, USA), and soft agitation for several seconds. The absorbance value was recorded at a wavelength of 590 nm after another 15 min of incubation at 25 °C. The performance of the assay was checked using SC-560 (purity: 95 %, Cayman, USA) and NS-398 (purity: 95 %, Cayman, USA) as positive controls

for Cox-1 and Cox-2, respectively, which gave the inhibition of Cox-1 (61.3 %) and Cox-2 (97.1 %), respectively. Different from the method mentioned above, the reaction system was added to the assay buffer with 5-Lox (human recombinant, Cayman, USA) in the presence of the colorimetric substrate and compounds **1** – **3** and then incubated for a period of 5 min at 25 °C. After the completion of the reaction, the chromogen was added, and the plate was shaken softly for a few seconds. Then another incubation period of 5 min was performed at 25 °C. The inhibitory effect against 5-Lox was determined by measuring the absorbance at a wavelength of 500 nm. The performance of the assay was checked using zileuton (purity: 95 %, Cayman, USA) as a positive control, which led to an inhibition of 83.05 %. The percent inhibition values were calculated: inhibitory ratio (%) = [(negative control absorbance – compound absorbance)/(negative control absorbance – positive control absorbance)] × 100.

Cytotoxicity assay

Five human cancer cell lines, breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1, and lung cancer A-549 cells, were used in the cytotoxic assay. All the cells were cultured in RPMI-1640 or DMEM medium (Hyclone, USA), supplemented with 10 % fetal bovine serum (Hyclone, USA) in 5 % CO_2 at 37 °C. The cytotoxicity assay was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method in 96-well microplates [15]. 100 μL adherent cells were seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before drug addition, while suspended cells were seeded just before drug addition with initial density of 1×10^5 cells mL^{-1}. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, 8, and 40 μM in triplicates for 48 h, with Cisplatin (sigma, USA) as positive control. After treatment, the cell viability was detected, and the cell growth curve was graphed. The IC_{50} value was calculated by the Reed and Muench's method [16].

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