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Six new triterpenes, 3β,6β-dihydroxyurs-12-en-27-oic acid (**1**), 3β,6β,24-trihydroxyurs-12-en-27-oic acid (**2**), 3β,6β,7α-trihydroxyurs-12-en-27-oic acid (**3**), 3β-acetoxy-6β-hydroxyurs-12-en-27-oic acid (**4**), 3β,6β,24-trihydroxyolean-12-en-27-oic acid (**5**), and 3β,6β,7α-trihydroxyolean-12-en-27-oic acid (**6**), were isolated from the rhizomes of *Astilbe chinensis*. Their structures were elucidated on the basis of 1D- and 2D-NMR analyses and HR-MS experiments. The isolated compounds exhibited significant cytotoxic activities against the SK-N-SH and HL-60 cell lines.

Keywords: *Astilbe chinensis*; triterpenes; cytotoxic activity

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1. Introduction

Astilbe chinensis (Maxim.) Franch. et Sav. (*A. chinensis*) is a perennial herbaceous plant found in China, Russia, Japan, and Korea. Its rhizomes are used to treat headache, arthralgia, chronic bronchitis, and stomachalgia in traditional Chinese medicine [1–5]. Previous chemical investigations of this plant have identified various compounds, including triterpenes and flavones [6–10].

In our search for bioactive compounds from medicinal herbs, we have studied the chemical constituents and reported two new triterpenes from *A. chinensis* [11]. This paper deals with the isolation and structure elucidation of six new triterpenes, 3β,6β-dihydroxyurs-12-en-27-oic acid (**1**), 3β,6β,24-trihydroxyurs-12-en-27-oic acid (**2**), 3β,6β,7α-trihydroxyurs-12-en-27-oic acid (**3**), 3β-acetoxy-6β-hydroxyurs-12-en-27-oic acid (**4**), 3β,6β,24-trihydroxyolean-12-en-27-oic acid (**5**), and 3β,6β,7α-trihydroxyolean-12-en-27-oic acid (**6**). These compounds were isolated from the rhizomes of *A. chinensis* by screening with antineoplastic tests *in vitro*, and their structures were established by spectroscopic methods, particularly 2D-NMR and HR-MS.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder. The HR-FAB-MS indicated a molecular formula of $C_{30}H_{49}O_4$ at *m/z* 473.3628 $[M + H]^+$, and the IR spectrum revealed the presence of OH (3443 cm^{-1}) and COOH (1698 cm^{-1}) groups. The ^1H NMR spectrum revealed the presence of five tertiary methyls at δ_H 1.34, 1.28, 1.14, 1.01, and 0.88 (each 3H, s), two secondary methyls at δ_H 0.83 (3H, d, $J = 6.5$ Hz) and 0.85 (3H, d, $J = 6.5$ Hz), two oxygenated methine at δ_H 4.42 (1H, brs) and 3.04 (1H, dd, $J = 4.0$, 11.4 Hz), a tri-substituted double bond at δ_H 5.56 (1H, brs). A direct comparison of the ^{13}C NMR spectral data of **1** (Table 2) with those

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of the reported compound [3β-hydroxyurs-12-en-27-oic acid] [8] indicated that they had the same skeleton. The difference between these two compounds lay in ring B, and **1** was deduced to be a 6-hydroxyl congener.

The $^{1}H-^{1}H$ COSY spectrum of **1** revealed two separated spin–spin systems (H-2/H-3 and H-5/H-6/H-7) in the A and B rings of the ursene-type skeleton. In the HMBC spectrum, H_3-23 and H_3-24 correlated with the C-3 at δ 80.0, C-4 at δ_C 40.6, and C-5 at δ 56.6; and the H-6 at δ_H 4.42 correlated with the C-5 at δ_C 56.6, C-7 at δ 45.1, C-8 at δ 40.2, and C-10 at δ_C 37.7. Thus, two hydroxyl groups could be located at C-3 and C-6, respectively.

The relative configuration of **1** was determined by extensive analysis of the ^{1}H NMR and NOESY spectra. The 3-OH group should be β-orientated considering the NOE correlations for H-3/H-23 and H-3/H-5, and the coupling constant J (2,3) of 11.4, 4.0 Hz for H-3. The NOE correlation for H-5/H-6 indicated that the 6-OH group was also β-oriented. Thus, the structure of **1** was assigned as 3β,6β-dihydroxyurs-12-en-27-oic acid (Figure 1).

The molecular formula $C_{30}H_{48}O_5$ for **2**, as determined by HR-FAB-MS at m/z 511.3394 $[M + Na]^+$, has one O-atom greater than that of **1**. Comparing the ^{1}H and ^{13}C NMR spectral data to those of **1** (Tables 1 and 2) indicated that **2** had an additional OH group located at C-23 or C-24. This structural deduction was confirmed by HMBC and NOESY experiments: the proton signals at δ_H 3.63 and 4.10 (H_2-24) showed long-range correlations with C-3, C-4, C-5, and C-23, while the signal at δ_H 4.10 (H-24) showed NOE correlation with H_3-25. Thus, the structure of **2** was elucidated as being 3β,6β,24-trihydroxyurs-12-en-27-oic acid (Figure 1).

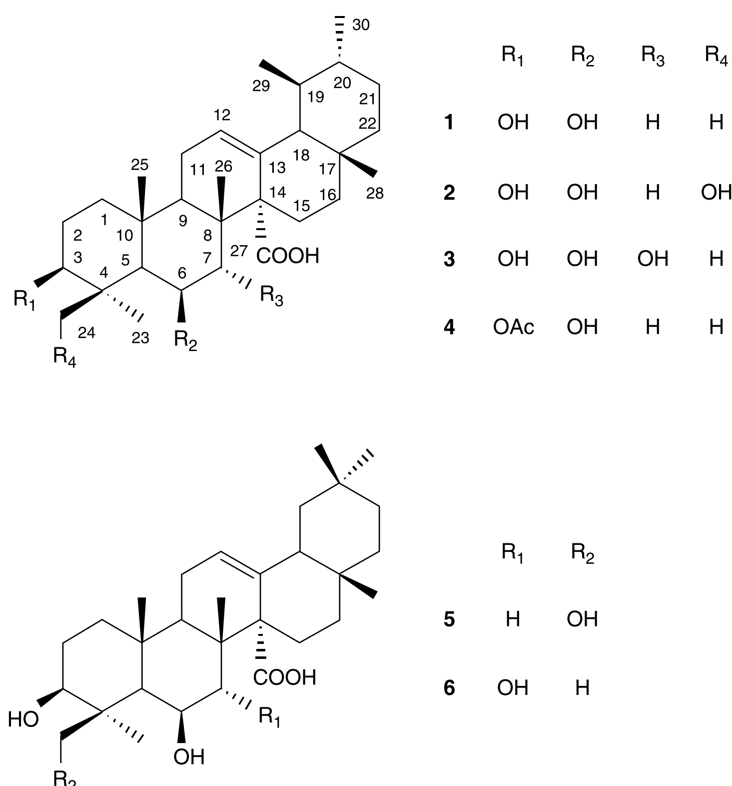

Figure 1. The structures of compounds **1**–**6**.

Table 1. ^{1}H NMR spectral data for compounds **1–4**.

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]
1	1.00–1.05 m	1.10–1.20 m	0.95–1.00 m	0.85–0.90 m
	1.60–1.65 m	2.00–2.05 m	1.65–1.70 m	1.70–1.75 m
2	1.60–1.65 m	1.60–1.80 m	1.60–1.80 m	1.20–1.30 m
	1.70–1.75 m			1.70–1.75 m
3	3.04 dd, 4.0, 11.4	3.20 dd, 4.7, 12.0	3.20 dd, 6.7, 8.7	4.43 m
4				
5	0.70 brs	0.85–0.90 m	1.05–1.10 m	0.85–0.90 m
6	4.42 brs	4.34 brs	4.56 brs	4.47 brs
7	1.30–1.40 m	1.30–1.40 m	4.26 d, 3.2	1.35–1.40 m
	1.80–1.90 m	1.90–2.00 m		1.90–1.95 m
8				
9	2.34 dd, 2.4, 9.4	2.41 t, 9.7	1.20–1.25 m	2.31 m
10				
11	2.00–2.10 m	2.00–2.20 m	1.95–2.05 m	2.05–2.15 m
12	5.56 brs	5.57 brs	5.56 brt, 3.2	5.59 brs
13				
14				
15	1.80–1.85 m	1.80–2.00 m	1.75–1.90 m	1.80–1.85 m
	1.95–2.00 m			1.95–2.05 m
16	1.30–1.40 m	1.25–1.35 m	1.25–1.30 m	1.30–1.40 m
			1.90–1.95 m	
17				
18	1.30–1.40 m	1.30–1.40 m	1.40–1.50 m	1.35–1.40 m
19	0.80–0.90 m	0.75–0.85 m	0.90–0.95 m	0.80–0.90 m
20	0.80–0.85 m	0.70–0.80 m	0.95–1.00 m	0.80–0.90 m
21	1.30–1.35 m	1.40–1.45 m	1.30–1.35 m	1.30–1.40 m
	2.10–2.15 m	2.00–2.05 m	1.95–2.00 m	2.00–2.05 m
22	1.20–1.25 m	1.25–1.30 m	1.20–1.25 m	1.20–1.25 m
	1.40–1.50 m	1.35–1.40 m	1.30–1.35 m	1.40–1.45 m
23	1.01 s	1.14 s	1.08 s	0.92 s
24	1.14 s	3.63 d, 11.4, 4.10 d, 11.4	1.18 s	1.23 s
25	1.34 s	1.39 s	0.92 s	1.37 s
26	1.28 s	1.29 s	1.21 s	1.29 s
27				
28	0.88 s	0.84 s	0.83 s	0.84 s
29	0.83 d, 6.5	0.84 d, 6.0	0.76 d, 6.4	0.83 d, 6.5
30	0.85 d, 6.5	0.89 d, 6.2	0.95 d, 6.1	0.85 d, 6.5

[a] CD_3OD.
[b] $CDCl_3$.

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Compound **3** had the same molecular formula ($C_{30}H_{48}O_5$) as **2**, and its ^{13}C NMR spectral data were similar to those of **2** (Table 2), except for an oxygenated methine (δ_C 82.9) in **3**. The $^1H-^1H$ COSY correlation of H-5/H-6/H-7 and the HMBC correlations of H-7 with C-5, C-6, C-8, and C-9 indicated that **3** was the 24-dehydroxy-6-hydroxy derivative of **2**. In the NOESY spectrum, the proton signal of H-7 correlated with H_3-26, and H_3-23 correlated with H-3, H-5, and H-6. Therefore, the OH groups possessed the 3β, 6β, and 7α configurations. Thus, the structure of **3** was assigned as 3β,6β,7α-trihydroxyurs-12-en-27-oic acid.

Compound **4** possessed the molecular formula $C_{32}H_{50}O_5$, as determined by HR-FAB-MS. Its ^{13}C NMR spectral data were similar to those of **1** (Table 2), except for an additional acetyl group. Compound **4** was assumed to be a 3-OAc congener of **1**. The HMBC correlations from H-3 (δ_H 4.43) to the ketone carbon of the acetyl group supported this conclusion. Accordingly, the structure of

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Table 2. ^{13}C NMR spectral data for compounds **1**–**4**.

Position	**1**[a]	**2**[a]	**3**[b]	**4**[b]
1	42.1	42.4	39.2	40.4
2	28.1	27.9	27.2	23.7
3	80.0	80.2	78.5	80.9
4	40.6	45.8	39.1	38.5
5	56.6	57.7	49.9	55.1
6	68.4	67.4	67.9	68.2
7	45.1	43.1	82.9	44.0
8	40.2	40.1	43.1	39.2
9	48.4	48.8	49.8	47.0
10	37.7	37.8	35.8	36.5
11	23.9	23.9	21.4	22.6
12	129.7	129.7	130.1	128.9
13	134.3	134.6	131.3	132.4
14	57.7	57.9	56.1	56.2
15	23.6	23.7	23.1	22.4
16	31.6	30.5	27.1	28.9
17	34.8	34.9	33.5	33.7
18	61.7	61.9	60.0	60.2
19	40.9	41.1	39.2	39.6
20	38.8	38.9	36.2	37.6
21	30.4	31.8	30.8	30.4
22	42.2	42.3	41.1	40.9
23	28.5	22.8	27.2	27.9
24	17.5	64.2	17.4	18.3
25	18.1	18.1	10.9	17.7
26	20.5	20.5	12.9	19.9
27	179.4	179.3	176.9	179.9
28	29.7	29.8	28.6	28.9
29	18.6	18.6	17.9	17.9
30	21.8	21.8	21.0	21.3

[a] Measured in CD_3OD.
[b] Measured in $CDCl_3$.

4 was determined to be 3β-acetoxy-6β-hydroxyurs-12-en-27-oic acid (Figure 1).

Compound **5** was isolated as an amorphous powder. Its HR-FAB-MS revealed a quasi-molecular ion at *m/z* 511.3349 $[M + Na]^+$, indicating a molecular formula of $C_{30}H_{48}O_5$. The IR spectrum revealed the presence of OH ($3385\,cm^{-1}$) and COOH ($1687\,cm^{-1}$) groups. The ^{1}H NMR spectral data of **5** (Table 3) showed signals including six methyls, two oxygenated methines, one oxygenated methylene, and a vinyl proton. The ^{13}C NMR spectral data of **5** (Table 3) were similar to those of the reported compound [3β,6β-dihydroxyolean-12-en-27-oic acid] [9], and was deduced to be a 24-hydroxy congener. In the HMBC spectrum, H_2-24 (δ_H 3.62 and 4.09) correlated with C-3, C-4, C-5, and C-23; and H_3-23 correlated with C-3, C-4, C-5, and C-24. In addition, H-24 (δ_H 4.09) showed NOE correlation with H_3-25, and H_3-23 correlated with H-3, H-5, and H-6. From these observations, the structure of **5** was determined as being 3β,6β,24-trihydroxy-olean-12-en-27-oic acid.

Compound **6** had the same molecular formula as **5**, and the ^{1}H and ^{13}C NMR spectral data of **6** were similar to those of **5**, except for the location of an OH group. The HMBC correlations from H-7 (δ_H 4.28) to C-5, C-6, C-8, and C-9 suggested that the OH group was located at C-7. The relative configurations of **6** were determined by the same method as that described above. Accordingly, the structure of **6** was determined to be 3β,6β,7α-trihydroxyolean-12-en-27-oic acid (Figure 1).

The antineoplastic activities of compounds **1**–**6** were determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay with two tumor cell lines: the human neuroblastoma cell line (SK-N-SH) and the human leukemia cell line (HL-60). Compounds **1**–**6** exhibited significant cytotoxic activity against these tumor cells *in vitro* (Table 4).

3. Experimental

3.1 General experimental procedures

The IR spectrum was detected by a Perkin-Elmer 577 spectrometer (in cm^{-1}) and optical rotations were obtained on a Perkin-Elmer 241-MC digital polarimeter. The NMR analysis of samples was performed with a Bruker Avance 300 instrument (^{1}H NMR, 300 MHz ^{13}C NMR and, 75 MHz), both with tetramethylsilane as an internal standard. HR-EI-MS data and EI-MS data were obtained on a JEOL JMS-SX102A instrument (in *m/z*). Column chromatography was performed on silica gel (Qingdao Haiyang Chemical Co. Ltd, Qingdao, China) and Toyopearl HW-40 (TOSOH). HPLC was a JASCO Gulliver Series with PU-2089 (pump), RI-2031, and

Table 3. ^{1}H and ^{13}C NMR spectral data of compounds **5** and **6**.

	5[a]		**6**[b]	
Position	^{1}H	^{13}C	^{1}H	^{13}C
1	1.25–1.30 m	42.4	1.25–1.30 m	39.3
	1.55–1.60 m		1.55–1.60 m	
2	1.60–1.80 m	27.9	1.60–1.80 m	27.1
3	3.18 m	80.2	3.17 dd, 6.9, 8.9	78.5
4		45.8		39.1
5	0.80–0.85 m	57.8	1.05–1.10 m	49.9
6	4.34 brs	67.3	4.56 brs	67.9
7	1.35–1.40 m	42.9	4.28 d, 3.0	82.8
	1.95–2.00 m			
8		40.1		43.0
9	1.35–1.45 m	49.0	1.20–1.30 m	49.6
10		37.8		35.9
11	1.95–2.05 m	24.1	1.90–2.00 m	21.4
12	5.61 brs	127.0	5.64 brt, 3.5	127.8
13		138.5		134.2
14		57.6		56.2
15	1.60–1.65 m	23.5	1.60–1.65 m	22.9
	1.85–1.90 m		1.90–1.95 m	
16	1.60–1.65 m	29.0	1.65–1.70 m	26.6
	2.00–2.05 m		2.05–2.11 m	
17		34.1		34.4
18	2.00–2.05 m	50.7	2.00–2.10 m	48.4
19	0.95–1.00 m	45.5	0.90–1.00 m	42.5
	2.27 dd, 1.8, 6.6		2.61 t, 13.8	
20		32.1		30.8
21	1.10–1.15 m	35.7	1.10–1.15 m	34.4
	1.35–1.40 m		1.35–1.40 m	
22	1.30–1.35 m	37.9	1.20–1.30 m	36.7
	1.45–1.50 m		1.50–1.55 m	
23	1.13 s	22.8	1.08 s	27.2
24	3.62 d, 11.4	64.1	1.18 s	17.4
	4.09 d, 11.4			
25	1.39 s	18.0	1.28 s	17.9
26	1.28 s	20.3	1.19 s	12.6
27		180.1		177.5
28	0.88 s	28.9	0.86 s	28.3
29	0.85 s	33.9	0.94 s	33.0
30	0.87 s	24.2	0.88 s	24.0

[a] Measured in CD_3OD.
[b] Measured in $CDCl_3$.

UV-2075 (detector). Preparative HPLC column was used as ODS (YMC-Pack ODS-A, SH-343-5).

3.2 Plant material

The rhizomes of *A. chinensis* (Maxim.) Franch. et Sav. were collected in November 2003 from Hefeng, Hubei Province, China. The plant material was identified by Prof. Ding-Rong Wan, School of Life Sciences, South-Central University for Nationalities. A voucher specimen (No. D20030802) has been deposited at the School of Pharmaceutical Sciences, Tianjin Medical University, China.

3.3 Extraction and isolation

The dried rhizomes (3.2 kg) of *A. chinensis* were crushed and extracted three times with

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Table 4. Cytotoxic activities of compounds **1**–**6**.

	IC_{50} (μg/ml)	
Compound	SK-N-SH	HL-60
1	26.42	10.19
2	31.82	18.41
3	44.28	19.04
4	17.45	19.44
5	17.94	37.00
6	34.98	38.19
5-FU[a]	0.72	
ATRA[b]		1.56

[a] Fluorouracil.
[b] All-*trans* retinoic acid.

95% EtOH (10 l) for 6 h under reflux (3×), and then concentrated *in vacuo* to afford a residue (1115 g), which was suspended in H_2O and then separately partitioned with petroleum ether (PE), EtOAc, and *n*-BuOH. The PE extract (20 g) was separated by column chromatography (1 kg silica gel; PE/EtOAc 10:1, 8:1, 6:1, 3:1, 2:1, 1:1, 1:2, and 1:3), EtOAc, EtOAc–MeOH (19:1 and 10:1), MeOH to yield 17 fractions (1–17). Fraction 8 (3.6 g) was chromatographed on silica gel [$CHCl_3$–MeOH (97:3 and 93:7)] to afford five fractions (8.1–8.5). Fraction 8.2 was chromatographed by column chromatography (Toyopearl HW-40; $CHCl_3$/MeOH, 2:1) to afford four fractions (8.2.1–8.2.4). Fraction 8.2.2 was purified by HPLC (ODS-A, eluted with MeOH/H_2O, 9:1, 3.0 ml/min) to afford **1** (78.8 mg) and **4** (27.3 mg). Fraction 8.3 was chromatographed by column chromatography (Toyopearl HW-40) to afford four fractions (8.3.1–8.3.4). Fraction 8.3.2 was purified by HPLC (ODS-A, eluted with MeOH/H_2O, 9:1, 3.0 ml/min) to afford **2** (40.1 mg), **3** (7.4 mg), **5** (25.4 mg), and **6** (15.8 mg).

*3.3.1 3β,6β-Dihydroxyurs-12-en-27-oic acid (**1**)*

Amorphous powder; $[\alpha]_D^{25}+87.1$ ($c = 1.2$, MeOH). IR max (KBr): 3443, 2994, 2864, 1698, 1456, 1375, 1224, 1096, 1015, and 921 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HR-FAB-MS *m/z*: 473.3628 $[M+H]^+$ (calcd for $C_{30}H_{49}O_4$, 473.3631).

*3.3.2 3β,6β,24-Trihydroxyurs-12-en-27-oic acid (**2**)*

Amorphous powder; $[\alpha]_D^{25}+93.2$ ($c = 1.15$, MeOH). IR max (KBr): 3385, 2925, 1687, 1455, 1374, 1232, 1059, 916, and 885 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HR-FAB-MS *m/z*: 511.3394 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

*3.3.3 3β,6β,7α-Trihydroxyurs-12-en-27-oic acid (**3**)*

Amorphous powder; $[\alpha]_D^{25} = 17.2$ (c=0.62, MeOH). IR max (KBr): 3494, 3352, 2927, 1750, 1459, 1369, 1173, 1135, 1073, 1031, and 882 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HR-FAB-MS *m/z*: 471.3477 $[M+H-H_2O]^+$ (calcd for $C_{30}H_{47}O_4$, 471.3474).

*3.3.4 3β-Acetoxy-6β-hydroxyurs-12-en-27-oic acid (**4**)*

Amorphous powder; $[\alpha]_D^{25}+66.9$ ($c = 1.09$, CH_3Cl). IR max (KBr): 3531, 2951, 1716, 1686, 1377, 1267, 1036, 985, 832, and 758 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Tables 1 and 2. HR-FAB-MS *m/z*: 537.3526 $[M+Na]^+$ (calcd for $C_{32}H_{50}O_5Na$, 537.3556).

*3.3.5 3β,6β,24-Trihydroxyolean-12-en-27-oic acid (**5**)*

Amorphous powder; $[\alpha]_D^{25}+91.5$ (c = 1.15, MeOH). IR max (KBr): 3379, 2924, 1676, 1466, 1363, 1263, 1065, 1018, and 1001 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Table 3. HR-FAB-MS: *m/z* 511.3349 $[M+Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

*3.3.6 3β,6β,7α-Trihydroxyolean-12-en-27-oic acid (**6**)*

Amorphous powder; $[\alpha]_D^{25}+27.3$ ($c = 0.67$, MeOH). IR max (KBr): 3451, 2928, 1756, 1467, 1387, 1259, 1132, 1083, 1009, and

936 cm^{-1}. ^{1}H and ^{13}C NMR spectral data, see Table 3. HR-FAB-MS: *m/z*: 471.3515 $[M+H-H_2O]^+$ (calcd for $C_{30}H_{47}O_4$, 471.3474).

3.4 Procedure of bioassay

The cytotoxicities of compounds **1**–**6** were assayed in two cell lines: the human neuroblastoma cell line (SK-N-SH) and human leukemic cell line (HL-60). One hundred and eighty microliters of the cell suspensions (5×10^4 cells/ml) were seeded in 96-well microplates, and incubated for 24 h in order to allow cell attachment. The tested compound solutions were then added to the cell cultures at final concentrations of 3.12, 6.25, 12.50, 25.00, 50.00, and 100.00 μg/ml, and the cells were incubated (37°C, 5% CO_2) for 72 h. The supernatants were then removed using a micropipette, and 100 μl of MTT solution (0.5 mg/ml) were added to each well of the 96-well plates, followed by incubation for 4 h. Subsequently, the cell culture medium was removed by pipetting and the purple formazan crystals were dissolved by adding 100 μl of DMSO to each well. The optical density was read at a wavelength of 490 nm using a microplate reader.

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