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Microbial transformation of methyl protodioscin by *Cunninghamella elegans*

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Abstract

Biotransformation of methyl protodioscin (**1**) by *Cunninghamella elegans* (AS 3.1207) was investigated. Nine bioconversion products were isolated and identified. Eight of the bioconversion products were pregnane glycoside or steroidal saponins. It was found that steroidal saponin skeleton could be converted to pregnenolone skeleton only using microbial methods, which must have chemical procedures in the reported literatures. The found enriched the types of bioconversion reaction and provided a new way for the production of androstenedione. Most bioconversion products showed considerable cytotoxic activities against HepG2, NCI-H460, MCF-7 and HeLa cell lines compared to methyl protodioscin.

Keywords: Methyl protodioscin; Microbial transformation; *Cunninghamella elegans*; Anticancer compound; Cytotoxicity

1. Introduction

Methyl protodioscin (**1**) is a furostanol biglycoside with the chemical name of 3-*O*-[α-L-rhamnopyranosyl-(1 → 2)-{α-L-rhamnopyranosyl-(1 → 4)}-β-D-glucopyranosyl]-26-*O*-[β-D-glucopyranosyl]-22-methoxy-(25*R*)-furost-5-ene-3β,26-diol. In continuing efforts to seek bioactive components from the traditional Chinese herbal medicine, 14 steroidal saponins with anticancer activities have been isolated in our groups from the rhizome of *Dioscorea collettii* var. *hypoglauca* (Dioscoreaceae), a Chinese herbal remedy for the treatment of cervical carcinoma, carcinoma of urinary bladder and renal tumor for centuries [1–3]. It was included in the 1985, 1990, 1995 and 2000 versions of the pharmacopoeia of the People's Republic of China. Among the tested compounds, methyl protodioscin showed the most potent activity against most cell lines from leukemia and solid tumors in the National Cancer Institute's (NCI) human cancer panel [4]. Except widely existed in *Dioscorea* plants, methyl protodioscin was also isolated from the rhizomes of *Smilax* [5,6], the seeds of *Asparagus* [7] and *Costus* species [8]. Methyl protodioscin has been synthesized for the first time from diosgenin through nine steps in one of our cooperative group [9], which guaranteed the material for the further research and applications in medicine.

The importance of microbial biotechnology in the production of steroid drugs and hormones was realized for the first time in 1952 when Murray and Peterson patented the process of 11α-hydroxylation of progesterone by a *Rhizopus* species. Since then, microbial reactions for the transformation of steroids have proliferated, and specific microbial transformation steps have been incorporated into numerous

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partial syntheses of new steroids for evaluation as drugs and hormones. These biotransformations have provided adequate tools for the large-scale productions of natural or modified steroid analogues. The latter are currently favored when compared to their natural counterparts due to some therapeutic advantages, such as an increased potency, longer half-lives in the blood stream, simpler delivery methods and reduced side effects. The preferential use of whole cells over enzymes as biocatalysts for the production of these pharmaceutical derivatives mostly results from the costs of the latter enzyme isolation, purification and stabilization. Furthermore, the use of microbial models to mimic mammalian metabolism is well known [10,11].

The chemical conversion of methyl protodioscin into steroidal alkaloid glycoside was investigated [12]. The preclinical investigations of methyl protodioscin have been carrying out in our group, and the present work was an attempt to get the microbial transformation products of methyl protodioscin (**1**) by *Cunninghamella elegans* AS 3.1207 and provide some information about the metabolism of methyl protodioscin in mammalian. The effects of these bioconversion products on some human tumor cells, such as HepG2, NCI-H460, MCF-7 and HeLa were studied.

2. Results and discussion

After 48 h incubation, nine products (Fig. 1) were isolated from the broth of *C. elegans* in potato medium. Some of them have potent anti-tumor effects in vitro compared to parent drug – methyl protodioscin (**1**).

Compound **2** was obtained as white amorphous powder, which was positive in the Liebermann–Burchard reaction and Molish test, indicating its glycosidic nature. The molecular formula of $C_{51}H_{82}O_{23}$ was thus drawn based on all ^{1}H NMR, ^{13}C NMR and IR data. The positive high-resolution second ionization mass spectrum (HR-SI-MS) showed the quasi-molecular ion $[M+Na]^+$ at *m/z* 1085.5183 corresponding to $C_{51}H_{82}O_{23}Na$, which confirmed the molecular formula further. The positive ESI–MS^n spectrum showed *m/z*

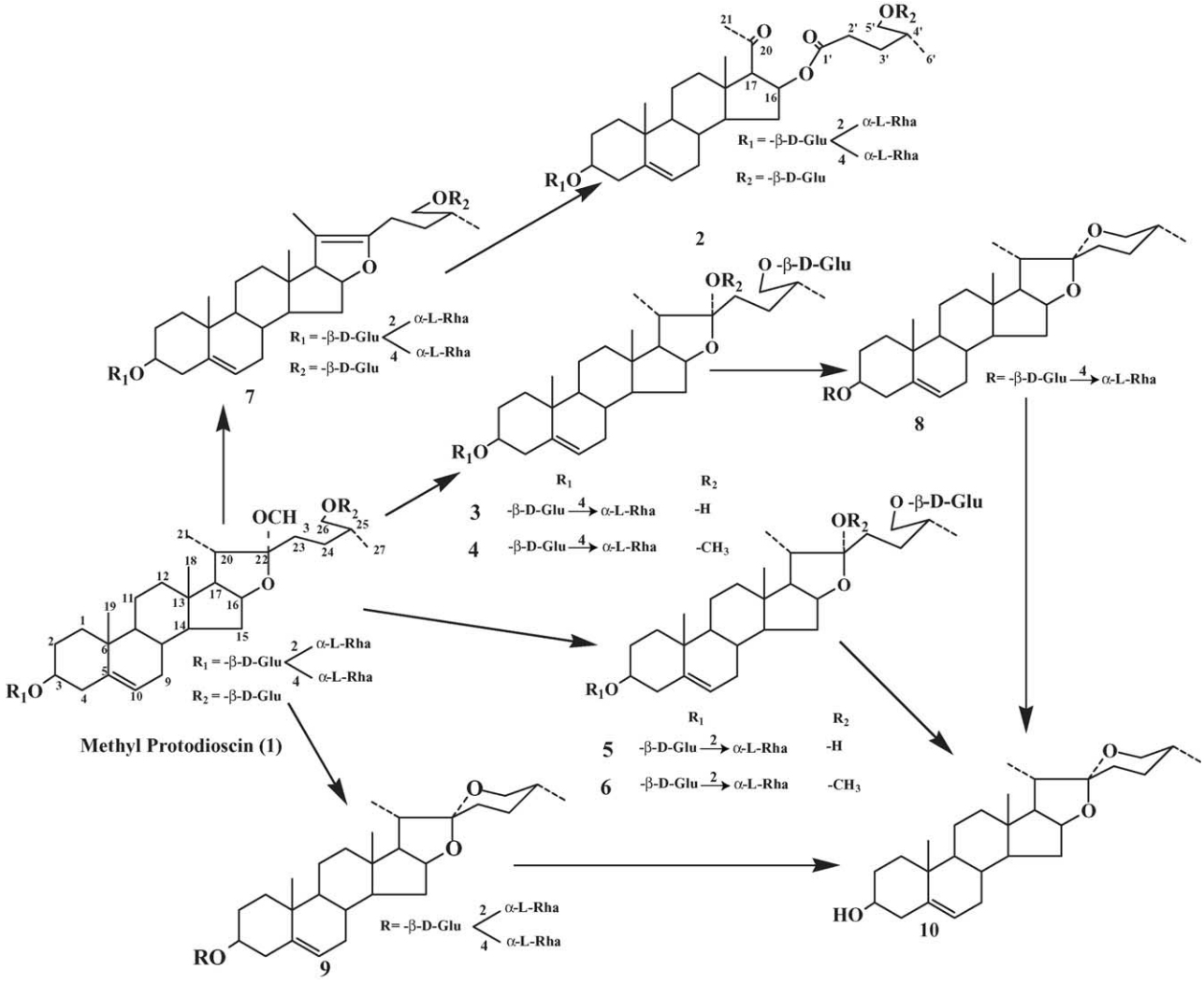

Fig. 1. Biotransformation pathways of methyl protodioscin by *Cunninghamella elegans*.

1085 $[M + Na]^+$, 939 $[M + Na\text{-}Rha]^+$, 793 $[M + Na\text{-}2Rha]^+$, 791 $[M + Na\text{-}294]^+$ and 645 $[M + Na\text{-}Rha\text{-}294]^+$. The (+) ESI–$MS^n$ spectra were characterized by the loss of *m/z* 294 fragments, which indicated that the 20(22) carbon–carbon bond of methyl protodioscin (**1**) was disconnected and a lactone moiety chain was formed at C-16. On acid hydrolysis with 1 M hydrochloric acid in dioxane–H_2O, glucose and rhamnose were identified with the authentic sugars. The precipitate of the acid hydrolysis products was identified as pregna-5,16-dien-3β-ol-20-one by comparison with the authentic compounds.

The ^1H NMR spectrum of compound **2** showed the presence of six methyl groups at δ 0.90 (3H, d, $J = 6.6$ Hz, Me-6′), 1.04 (3H, s, Me-19), 1.21 (3H, s, Me-18), 1.61 (3H, d, $J = 6.2$ Hz, Rha Me-6′), 1.74 (3H, d, $J = 6.2$ Hz, Rha Me-6), and 2.12 (3H, s, Me-21); four anomeric protons at δ 6.38 (1H, d, $J = 1.0$ Hz, Rha H-1), 5.84 (1H, d, $J = 1.0$ Hz, Rha H-1′), 4.93 (1H, d, $J = 7.6$ Hz, Glc H-1), and 4.78 (1H, d, $J = 7.5$ Hz, Glc H-1′); and an olefinic proton at δ 5.30 (1H, brs, H-6). In the carbon and DEPT NMR spectra, there were 51 carbon signals, which consisted of 5 quarternary carbons, 28 methines, 12 methylenes, and 6 methyls. There were two carbonyl carbons at δ 205.4 (C-20) and 173.2 (C-1′), two olefinic carbons at δ 140.9 (C-5) and 121.5 (C-6). The β-configuration of the anomeric carbons of two glucopyranosyl units was determined by J_{H1-H2} values ($J > 7.0$ Hz) and the NMR and MS spectra. The α-configuration of the anomeric carbons of two rhamnopyranosyl units was confirmed by comparison of the chemical shift values of carbons 3 and 5 with those of the corresponding carbons of methyl α- and β-rhamnopyranoside [13,14]. The β-configuration of the side chain attached to C-16 was determined by the *J* value between H-16 and H-17 ($J = 7.9$ Hz).

Combined with ^1H ^1H COSY, HMQC, HMBC and TOCSY spectra, the pregnane skeleton, the C-16 side chain of the aglycone, and the sugar moieties could be determined and assigned. The linkage of sugar side chains were determined by HMBC spectra.

Based on all the above data, the structure of compound **2** was established as 16β-(4′- methyl-5′-*O*-β-D-glucopyranosyl-pentanoxyl)-pregn-5-en-3β-ol-20-one-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside (hypoglaucin G). It was also isolated from the rhizome of *D. collettii* [3].

Compound **3**, obtained as white amorphous powder, and was positive to Liebermann–Burchard, Molish, anisaldehyde and Ehrlich reactions, which suggested it is a furostanol saponin, just like the parent compound – methyl protodioscin. The positive ESI–MS spectrum showed the quasi-molecular weight *m/z* 925 $[M + Na]^+$. The molecular formula of compound **3** was deduced as $C_{45}H_{74}O_{18}$ combined with ESI–MS, ^1H NMR, ^{13}C NMR spectra. On acid hydrolysis, glucose and rhamnose were identified with the authentic sugars. Three anomeric signals of sugars appearing at δ 104.9, 102.6 and 102.4 in the ^{13}C NMR indicated that there were three sugars in the molecular. The negative ESI–MS^n spectrum showed *m/z* 901 $[M - H]^-$, 755 $[M - H\text{-}Rha]^-$ and 575 $[M - H\text{-}Rha\text{-}Glc\text{-}H_2O]^-$. The sugars were consisted of two glucoses and a rhamnose judged from its mass spectra and the results of acid hydrolysis. Compared with the NMR spectra of methyl protodioscin (**1**), and analyzed its 2D NMR spectra, compound **3** was identified as 26-*O*-β-D-glucopyrannosyl-(25*R*)-furan-5-ene-3β,22α,26-trihydroxy-3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside [15].

Compound **4**, white amorphous powder, was positive to Liebermann–Burchard, Molish, anisaldehyde and Ehrlich reactions, which suggested a furostanol saponnins. The positive ESI–MS spectrum showed the quasi-molecular weight *m/z* 939 $[M + Na]^+$. The molecular formula of compound **4** was deduced as $C_{46}H_{76}O_{18}$ combined with ESI–MS, ^1H NMR, ^{13}C NMR spectra. Compound **4** could be converted to compound **3** fully when it was refluxed in 50% acetone–H_2O for 24 h. On the other hand, compound **3** could be converted to compound **4** fully when it was refluxed in methanol for 36 h. Therefore, compound **4** was the C-22 methyl ether corresponding to compound **3**. From above analysis, compound **4** was identified as 26-*O*-β-D-glucopyrannosyl-(25*R*)-furan-5-ene-22α-methoxy-3β,26-dihydroxy-3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside [15].

Compounds **5** and **6** were obtained as white amorphous powder, both exhibited a purple coloration with the anisaldehyde and Ehrlich reactions, suggesting compounds **5** and **6** were furostanol saponins. Compound **6** could be converted to compound **5** fully when it was refluxed in 50% acetone–H_2O for 24 h. Similarly, compound **5** could be converted to compound **6** fully when it was refluxed in methanol for 36 h. Compounds **5** and **6** were a pair of C-22 hydroxyl-methoxyl furostanol saponins. The positive ESI–MS spectrum of compound **5** showed the quasi-molecular weight *m/z* 925 $[M + Na]^+$. The molecular formula of compound **5** was deduced as $C_{45}H_{74}O_{18}$ combined with ESI–MS, ^1H NMR, ^{13}C NMR spectra. On acid hydrolysis, glucose and rhamnose were identified with the authentic sugars. Three anomeric signals of sugars appearing at δ 104.8, 101.9 and 100.2 ppm in the ^{13}C NMR indicated that there were three sugars in the molecular. Except for the ^{13}C NMR data of sugar side chain at C-3, the ^{13}C NMR data of compound **5** was almost the same to compound **3**. Combined with the reported data [13,16], compound **5** was identified as 26-*O*-β-D-glucopyrannosyl-(25*R*)-furan-5-ene-3β,22α,26-trihydroxy-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside. Accordingly, compound **6** was identified as 26-*O*-β-D-glucopyrannosyl-(25*R*)-furan-5-ene-22α-methoxy-3β,26-dihydroxy-3-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

Using the same methods and comparing with the literatures, compounds **7**, **8**, **9** and **10** were identified as 26-*O*-β-D-glucopyrannosyl (25*R*)-furan-5,20(22)-diene-3β,26-dihydroxy-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, diosgenin 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-β-D-glucopyranoside, diosgenin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-[α-L-rhamn-

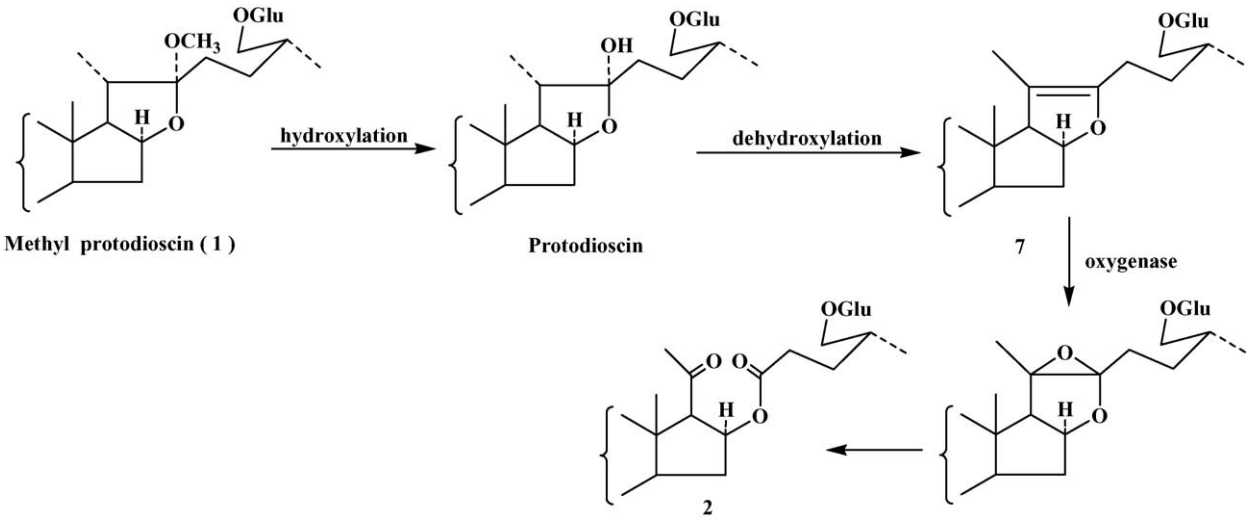

Fig. 2. The proposed biosynthetic pathways of the microbial conversion products of methyl protodioscin (**1**).

opyranosyl-(1 → 4)]-β-D-glucopyranoside, and diosgenin, respectively [13,16].

Compound **2** are the bioconversion products which methyl protodioscin have undergone the opening of E-ring, and formed pregnenolone. Bioconversion steroidal saponins to pregnenolone were chemical transformation procedures in the reported literatures, as we have ever known [11]. It was found that steroidal saponin skeleton could be converted to pregnenolone skeleton only using microbial methods in this research, which must have chemical procedures in the reported literatures. The proposed biosynthetic pathways of the microbial conversion products of methyl protodioscin were shown in Fig. 2. This find enriched the types of bioconversion reaction and provided a new thought way for the androstenedione production. Most of bioconversion products were furostanol saponins, which kept good solubility in water. Generally, furostanol saponin had no or very slight hemolysis effects compared to steroidal saponin, which had more clinical advantages. The steroidal skeleton had no change of most products, even hydroxylation. These conversion characters were benefit to keeping the bioactivities of parent compound.

2.1. Cytotoxic activity

The cytotoxic activities of the bioconversion products of methyl protodioscin were evaluated. Most bioconversion products showed considerable cytotoxic activities against HepG2, NCI-H460, MCF-7 and HeLa cell lines compared to parent drug – methyl protodioscin (Table 1). Among these compounds, compound **7** has the strongest activity. The IC_{50} of compound **7** against HepG2 and NCI-H460 cell lines were 19.6 and 12.1 μM, respectively. The IC_{50} of compound **9** against human HepG2 and NCI-H460 cell lines were 6.2 and 16.6 μM, respectively. Generally, the cytotoxic activities were decreased when the sugars were hydrolyzed from methyl protodioscin. These results provided useful clues in the process of methyl protodioscin development.

3. Experimental

3.1. General experimental procedures

IR spectra were determined on a Bruker IFS 55 spectrometer in KBr pellets. UV spectra were measured on a Shimadzu UV-2201 spectrometer. ESI–MS spectra were recorded on a Bruker ESQUIRE 2000 mass spectrometer. High-resolution SI-MS spectra were recorded on a Bruker second ionization mass spectrometer. NMR spectra were measured on Bruker AV-400 spectrometers. Analysis and prepared HPLC were carried on Waters 600 instruments equipped with RI and PDA detectors.

Table 1
Cytotoxic activity of compounds **1**–**10** against human cancer cell lines

Cell lines	IC50 (μM)									
	1	2	3	4	5	6	7	8	9	10
HepG2	8.2	23.5	34.5	30.6	40.6	50.2	19.6	89.7	6.2	–
NCI-H460	7.6	38.5	42.0	38.9	60.5	45.0	12.1	40.6	16.6	230.1
MCF-7	10.5	18.6	56.7	49.7	100.2	88.5	34.6	71.3	78.1	–
Hela	12.6	50.6	45.6	45.3	20.4	31.2	40.5	78.4	34.3	100.5

No effect (–).

Methyl protodioscin (**1**) was isolated from the rhizome of *D. collettii* var. *hypoglauca* (Dioscoreaceae). The purity was above 98% determined by HPLC method.

3.2. Microorganisms and culture

C. elegans AS 3.1207 was purchased from China General Microbiological Culture Collection Center. All culture and biotransformation experiments were performed in potato medium. Potato medium was prepared by the following procedure: 200 g of mincing husked potato, added 800 mL water, was boiled in water for half-an-hour. Then the solution was filtered and the filtrate was added with water and 20 g glucose to 1 L.

3.3. Culture and biotransformation procedures

Screening scale biotransformation of Methyl Protodioscin by *C. elegans* was carried out in 250 mL Erlenmeyer flasks containing 60 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers, operating at 180 rpm at 28 °C. The substrate was dissolved in 80% methanol with a concentration of 10 mg/mL. After 48 h of culture, 0.5 mL of the solution was added into the fermentation flasks and these flasks were maintained under the same conditions for an additional 2 days. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but with the same amount of 80% methanol alone. When the fermentation finished, the broths were filtered and the filtrates were extracted with the same volume of *n*-butanol for three times. The cells were refluxed with methanol. The extracts were evaporated to dryness under reduced pressure and the residues were dissolved in methanol. The solutions were spotted on silica gel plates, which were developed by chloroform–methanol–H_2O (65:35:7), and visualized by spraying with 10% H_2SO_4 solution, followed by heating at 110 °C for 10 min. TLC analyses revealed that *C. elegans* could bioconversion the substrate.

Preparative scale biotransformation of methyl protodioscin by *C. elegans* were carried out in 500 mL Erlenmeyer flasks containing 200 mL of potato medium. A total of 800 mg of compound **1** was transformed. Other procedures were the same as screening scale biotransformations.

3.4. Extraction and isolation

A 9500 mg of brown residue was obtained from the fermented broth of *C. elegans*. The residues were subjected to Diaion HP-20 (30 mm × 500 mm) and eluted with H_2O, 30% MeOH, 70% MeOH and MeOH stepwisely. 70% MeOH elution (2500 mg) was further subjected to ODS, Sephadex LH-20, and prepared Rp-HPLC (10 mm × 250 mm, Purospher STAR, Rp-18e, Merck Company). Compound **2** (15.0 mg, 1.9% yield) was obtained by prepared Rp-HPLC (70% MeOH/H_2O). Compound **3** (102.0 mg, 12.8% yield) and 4 (75.0 mg, 9.4% yield) were obtained from 78% MeOH/H_2O prepared Rp-HPLC. Compounds **5** (82.0 mg, 10.2% yield) and **6** (75.3 mg, 9.4% yield) were obtained from open ODS column (15 mm × 300 mm) eluted with 70% MeOH/H_2O. Compound **7** (192.1 mg, 24.0% yield) were obtained from 80% MeOH/H_2O prepared Rp-HPLC. Compounds **8** (12.5 mg, 1.6% yield) and **9** (8.0 mg, 1.0% yield) were obtained from 85% MeOH/H_2O elution of ODS column. Compound **10** (85.0 mg, 10.6% yield) was obtained from the chloroform–methanol (100:2) fraction.

Compound **2**, colorless amorphous powder, mp 160–162 °C, $C_{51}H_{82}O_{23}$, Liebermann–Burchard reaction and Molish test: positive. UV (MeOH) λ_{max}: 195.8 nm. IR (KBr) ν_{max} 3398 (OH), 2951, 1702 (C=O), 1040 (C–O). HR–ESI–MS *m/z* $[M + Na]^+$ 1085.5183 (calcd. 1085.5145); (+) ESI–MS^n *m/z*: 1085, 939, 793, 791, 645, 495, 317. ^{1}H NMR (C_5D_5N, 400 MHz): 6.38 (1H, d, *J* = 1.0 Hz, Rha H-1), 5.84 (1H, d, *J* = 1.0 Hz, Rha H-1′), 5.30 (1H, brs, H-6), 4.93 (1H, d, *J* = 7.6 Hz, 3-*O*-Glc H-1), 4.78 (1H, d, *J* = 7.5 Hz, 26-*O*-Glc H-1′), 2.12 (3H, s, Me-21), 1.74 (3H, d, *J* = 6.2 Hz, Rha Me-6), 1.61 (3H, d, *J* = 6.2 Hz, Rha Me-6′), 1.21 (3H, s, Me-18), 1.04 (3H, s, Me-19), 0.90 (3H, d, *J* = 6.6 Hz, Me-6′). ^{13}C NMR (C_5D_5N, 100 MHz): 205.4 (s, C-20), 173.2 (s, C-1′), 140.9 (s, C-5), 121.6 (d, C-6), 104.9 (d, 26-*O*-Glc-1), 102.8 (d, Rha-1′), 101.9 (d, Rha-1), 100.2 (d, 3-*O*-Glc-1), 78.6 (d, 26-*O*-Glc-5), 78.5 (d, 26-*O*-Glc-3), 78.5 (d, 3-*O*-Glc-4), 78.0 (d, C-3), 78.0 (d, 3-*O*-Glc-3), 77.7 (d, 3-*O*-Glc-2), 76.9 (d, 3-*O*-Glc-5), 75.1 (d, 26-*O*-Glc-2), 74.6 (d, C-5′), 74.6 (d, C-16), 74.0 (d, Rha-4), 73.8 (d, Rha-4′), 72.8 (d, Rha-3), 72.7 (Rha-3′), 72.5 (d, Rha-2), 72.5 (d, Rha-2′), 71.6 (d, 26-*O*-Glc-4), 70.3 (d, Rha-5′), 69.4 (d, Rha-5), 66.6 (d, C-17), 62.8 (t, 26-*O*-Glc-6), 61.2 (t, 3-*O*-Glc-6), 54.0 (d, C-14), 50.4 (d, C-9), 42.2 (s, C-13), 38.9 (t, C-4), 38.1 (t, C-12), 37.4 (t, C-1), 37.0 (s, C-10), 35.4 (t, C-15), 33.4 (d, C-4′), 32.2 (t, C-2′), 31.8 (t, C-7), 30.9 (d, C-8), 30.4 (q, 21-CH_3), 30.1 (t, C-2), 29.0 (t, C-3′), 20.6 (t, C-11), 19.3 (q, 19-CH_3), 18.6 (q, Rha-6), 18.2 (q, Rha-6′), 16.8 (q, 6′-CH_3), 13.7 (q, 18-CH_3).

Compound **3**, white amorphous powder, $C_{45}H_{74}O_{18}$, Liebermann–Burchard reaction and Molish test: positive. UV (MeOH) λ_{max}: 202.0 nm. IR (KBr) ν_{max} 3412 (OH), 2938, 1040 (C–O). (+) ESI–MS^n *m/z*: 925, 907, 903, 779, 763. ^{1}H NMR (C_5D_5N, 400 MHz): 6.30 (1H, d, *J* = 1.2 Hz, Rha H-1), 5.28 (1H, brs, H-6), 4.95 (1H, d, *J* = 7.5 Hz, Glc H-1), 4.80 (1H, d, *J* = 7.6 Hz, Glc H-1″), 1.65 (3H, d, *J* = 6.5 Hz, Rha Me-6), 1.24 (3H, s, Me-18), 1.20 (3H, s, Me-21), 1.06 (3H, s, Me-19), 0.95 (3H, d, *J* = 6.8 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 140.9 (s, C-5), 121.8 (d, C-6), 110.7 (s, C-22), 104.9 (d, 26-*O*-Glc-1), 102.6 (d, Rha-1), 102.4 (d, 3-*O*-Glc-1), 81.2 (d, C-16), 78.7 (d, 3-*O*-Glc-4), 78.6 (d, 26-*O*-Glc-5), 78.4 (d, 26-*O*-Glc-3), 78.2 (d, C-3), 77.1 (d, 3-*O*-Glc-5), 76.8 (d, 3-*O*-Glc-3), 75.2 (d, 26-*O*-Glc-2), 75.2 (d, C-26), 74.1 (d, Rha-4), 72.6 (d, 3-*O*-Glc-2), 72.6 (d, Rha-3), 72.4 (d, Rha-2), 71.8 (d, 26-*O*-Glc-4), 70.4 (d, Rha-5), 63.9 (d, C-17), 62.9 (t, 26-*O*-Glc-6), 61.7 (t, 3-*O*-Glc-6), 56.7 (d, C-14), 50.5 (d, C-9), 40.9 (d, C-20), 40.7 (s, C-13), 40.0 (t, C-12), 39.1 (t, C-4), 37.6 (t, C-1), 37.2 (t, C-23), 37.1 (s, C-10), 34.4 (d, C-25),

32.4 (t, C-15), 32.4 (t, C-7), 31.6 (d, C-8), 30.3 (t, C-2), 28.4 (t, C-24), 21.2 (t, C-11), 19.5 (q, 19-CH_3), 18.6 (q, Rha-6), 17.5 (q, 27-CH_3), 16.5 (q, 21-CH_3), 16.4 (q, 18-CH_3).

Compound **4**, white powder, $C_{46}H_{76}O_{18}$, Liebermann–Burchard reaction and Molish test: positive. UV (MeOH) λ_{max}: 200.0 nm. IR (KBr) ν_{max} 3410 (OH), 2942, 1042 (C–O). (+) ESI–MS^n *m/z*: 939, 921, 793, 631, 613. ^{1}H NMR (C_5D_5N, 400 MHz): 6.28 (1H, d, *J* = 1.0 Hz, Rha H-1), 5.32 (1H, brs, H-6), 4.98 (1H, d, *J* = 7.6 Hz, Glc H-1), 4.84 (1H, d, *J* = 7.4 Hz, Glc H-1″), 3.50 (3H, s, 22-*O*-CH_3), 1.67 (3H, d, *J* = 6.5 Hz, Rha Me-6), 1.25 (3H, s, Me-18), 1.23 (3H, s, Me-21), 1.16 (3H, s, Me-19), 0.99 (3H, d, *J* = 6.8 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 141.3 (s, C-5), 122.2 (d, C-6), 113.2 (s, C-22), 105.1 (d, 26-*O*-Glc-1), 102.6 (d, Rha-1), 102.5 (d, 3-*O*-Glc-1), 81.7 (d, C-16), 78.7 (d, 26-*O*-Glc-5), 78.7 (d, 26-*O*-Glc-3), 78.6 (d, 3-*O*-Glc-4), 78.4 (d, C-3), 77.6 (d, 3-*O*-Glc-5), 76.7 (d, 3-*O*-Glc-3), 75.4 (d, 26-*O*-Glc-2), 75.3 (d, C-26), 74.2 (d, Rha-4), 72.8 (d, Rha-3), 72.7 (d, Rha-2), 72.4 (d, 3-*O*-Glc-2), 72.1 (d, 26-*O*-Glc-4), 70.1 (d, Rha-5), 64.5 (d, C-17), 63.1 (t, 26-*O*-Glc-6), 61.9 (t, 3-*O*-Glc-6), 56.9 (d, C-14), 50.6 (d, C-9), 47.5 (q, 22-*O*-CH_3), 41.2 (s, C-13), 40.8 (d, C-20), 40.1 (t, C-12), 39.3 (t, C-4), 37.8 (t, C-1), 37.5 (s, C-10), 34.7 (d, C-25), 32.7 (t, C-15), 32.5 (t, C-7), 32.0 (d, C-8), 31.2 (t, C-23), 30.5 (t, C-2), 28.5 (t, C-24), 21.4 (t, C-11), 19.8 (q, 19-CH_3), 18.6 (q, Rha-6), 17.5 (q, 27-CH_3), 16.7 (q, 21-CH_3), 16.5 (q, 18-CH_3).

Compound **5**, white amorphous powder, $C_{45}H_{74}O_{18}$, Liebermann–Burchard reaction and Molish test: positive. Anisaldehyde and Ehrlich reactions: positive. UV (MeOH) λ_{max}: 196.5 nm. IR (KBr) ν_{max} 3414 (OH), 2931, 1041 (C–O). (+) ESI–MS^n *m/z*: 925, 779, 599. ^{1}H NMR (C_5D_5N, 400 MHz): 6.35 (1H, d, *J* = 1.0 Hz, Rha H-1), 5.25 (1H, brs, H-6), 4.98 (1H, d, *J* = 7.6 Hz, Glc H-1), 4.76 (1H, d, *J* = 7.4 Hz, Glc H-1″), 1.68 (3H, d, *J* = 6.5 Hz, Rha Me-6), 1.30 (3H, s, Me-21), 1.25 (3H, s, Me-18), 1.04 (3H, s, Me-19), 0.97 (3H, d, *J* = 6.8 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 140.6 (s, C-5), 121.4 (d, C-6), 110.5 (s, C-22), 104.8 (d, 26-*O*-Glc-1), 101.9 (d, Rha-1), 100.2 (d, 3-*O*-Glc-1), 81.0 (d, C-16), 78.6 (d, 26-*O*-Glc-5), 78.5 (d, 26-*O*-Glc-3), 78.4 (d, 3-*O*-Glc-5), 78.0 (d, C-3), 78.0 (d, 3-*O*-Glc-2), 76.8 (d, 3-*O*-Glc-3), 75.2 (d, 26-*O*-Glc-2), 75.0 (d, C-26), 74.5 (d, Rha-4), 72.6 (d, Rha-3), 72.4 (d, Rha-2), 71.9 (d, 3-*O*-Glc-4), 71.6 (d, 26-*O*-Glc-4), 70.4 (d, Rha-5), 63.7 (d, C-17), 62.6 (t, 26-*O*-Glc-6), 62.8 (t, 3-*O*-Glc-6), 56.7 (d, C-14), 50.5 (d, C-9), 40.6 (d, C-20), 40.5 (s, C-13), 40.3 (t, C-12), 39.3 (t, C-4), 37.5 (t, C-1), 37.3 (t, C-23), 37.1 (s, C-10), 34.6 (d, C-25), 32.8 (t, C-15), 32.5 (t, C-7), 31.8 (d, C-8), 30.5 (t, C-2), 28.3 (t, C-24), 21.3 (t, C-11), 19.6 (q, 19-CH_3), 18.6 (q, Rha-6), 17.8 (q, 27-CH_3), 16.8 (q, 21-CH_3), 16.5 (q, 18-CH_3).

Compound **6**, white powder, $C_{46}H_{76}O_{18}$, Liebermann–Burchard reaction and Molish test: positive. Anisaldehyde and Ehrlich reactions: positive. UV (MeOH) λ_{max}: 195.3 nm. IR (KBr) ν_{max} 3410 (OH), 2933, 1039 (C–O). (+) ESI–MS^n *m/z*: 939, 793, 761, 631. ^{1}H NMR (C_5D_5N, 400 MHz): 6.36 (1H, d, *J* = 1.0 Hz, Rha H-1), 5.23 (1H, brs, H-6), 4.96 (1H, d, *J* = 7.6 Hz, Glc H-1), 4.82 (1H, d, *J* = 7.8 Hz, Glc H-1″), 1.65 (3H, d, *J* = 6.5 Hz, Rha Me-6), 1.26 (3H, s, Me-21), 1.22 (3H, s, Me-18), 1.10 (3H, s, Me-19), 0.95 (3H, d, *J* = 6.8 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 140.8 (s, C-5), 121.3 (d, C-6), 113.4 (s, C-22), 105.1 (d, 26-*O*-Glc-1), 102.4 (d, Rha-1), 100.5 (d, 3-*O*-Glc-1), 81.6 (d, C-16), 78.6 (d, 26-*O*-Glc-5), 78.5 (d, 26-*O*-Glc-3), 78.4 (d, 3-*O*-Glc-5), 78.3 (d, C-3), 78.1 (d, 3-*O*-Glc-2), 76.8 (d, 3-*O*-Glc-3), 75.6 (d, 26-*O*-Glc-2), 75.5 (d, C-26), 74.2 (d, Rha-4), 72.7 (d, Rha-3), 72.5 (d, Rha-2), 72.2 (d, 3-*O*-Glc-4), 72.0 (d, 26-*O*-Glc-4), 70.2 (d, Rha-5), 64.5 (d, C-17), 63.1 (t, 26-*O*-Glc-6), 62.9 (t, 3-*O*-Glc-6), 56.9 (d, C-14), 50.8 (d, C-9), 47.9 (q, 22-*O*-CH_3), 40.8 (d, C-20), 40.3 (s, C-13), 40.0 (t, C-12), 39.3 (t, C-4), 37.5 (t, C-1), 37.3 (s, C-10), 34.6 (d, C-25), 32.7 (t, C-15), 32.5 (t, C-7), 32.0 (d, C-8), 31.3 (t, C-23), 30.8 (t, C-2), 28.6 (t, C-24), 21.5 (t, C-11), 19.8 (q, 19-CH_3), 18.6 (q, Rha-6), 17.5 (q, 27-CH_3), 16.9 (q, 21-CH_3), 16.6 (q, 18-CH_3).

Compound **7**, white amorphous powder, $C_{51}H_{82}O_{21}$, Liebermann–Burchard reaction and Molish test: positive. Anisaldehyde and Ehrlich reactions: positive. UV (MeOH) λ_{max}: 193.5 nm. IR (KBr) ν_{max} 3412 (OH), 2930, 1042 (C–O). (+) ESI–MS^n *m/z*: 1053, 907, 761, 581. ^{1}H NMR (C_5D_5N, 400 MHz): 6.46 (1H, d, *J* = 1.2 Hz, Rha H-1), 5.86 (1H, d, *J* = 1.0 Hz, Rha H-1′), 5.30 (1H, brs, H-6), 4.96 (1H, d, *J* = 7.6 Hz, 3-*O*-Glc H-1), 4.84 (1H, d, *J* = 7.5 Hz, 26-*O*-Glc H-1), 2.03 (3H, s, Me-21), 1.76 (3H, d, *J* = 6.2 Hz, Rha Me-6), 1.60 (3H, d, *J* = 6.2 Hz, Rha Me-6′), 1.22 (3H, s, Me-18), 1.06 (3H, s, Me-19), 0.91 (3H, d, *J* = 6.8 Hz, 27-Me). ^{13}C NMR (C_5D_5N, 100 MHz): 152.2 (s, C-22), 140.7 (s, C-5), 121.6 (d, C-6), 104.8 (d, 26-*O*-Glc-1), 103.4 (s, C-20), 102.8 (d, Rha-1′), 101.9 (d, Rha-1), 100.2 (d, 3-*O*-Glc-1), 84.4 (d, C-16), 78.6 (d, 26-*O*-Glc-5), 78.5 (d, 26-*O*-Glc-3), 78.5 (d, 3-*O*-Glc-4), 78.4 (d, C-3), 78.3 (d, 3-*O*-Glc-3), 78.0 (d, 3-*O*-Glc-2), 76.9 (d, 3-*O*-Glc-5), 75.1 (d, 26-*O*-Glc-2), 74.9 (d, C-26), 74.1 (d, Rha-4), 73.9 (d, Rha-4′), 72.7 (d, Rha-3), 72.7 (Rha-3′), 72.6 (d, Rha-2), 72.5 (d, Rha-2′), 71.6 (d, 26-*O*-Glc-4), 70.3 (d, Rha-5′), 69.4 (d, Rha-5), 64.4 (d, C-17), 62.8 (t, 26-*O*-Glc-6), 61.2 (t, 3-*O*-Glc-6), 54.9 (d, C-14), 50.2 (d, C-9), 43.4 (s, C-13), 39.6 (t, C-12), 38.9 (t, C-4), 37.5 (t, C-10), 37.0 (s, C-1), 34.4 (t, C-23), 33.4 (d, C-25), 32.3 (t, C-7), 31.4 (t, C-15), 31.3 (d, C-8), 30.1 (t, C-2), 23.6 (t, C-24), 21.2 (t, C-11), 19.3 (q, 19-CH_3), 18.6 (q, Rha-6), 18.2 (q, Rha-6′), 17.3 (q, 27-CH_3), 14.0 (q, 18-CH_3), 11.7 (q, 21-CH_3).

Compound **8**, colorless needle crystal (methanol), $C_{39}H_{62}O_{12}$, Liebermann–Burchard reaction and Molish test: positive. Anisaldehyde: positive. UV (MeOH) λ_{max}: 200.2 nm. IR (KBr) ν_{max} 3410 (OH), 2933, 1042 (C–O), 970, 914 < 896, 850. (+) ESI–MS^n *m/z*: 745, 599, 413. ^{1}H NMR (C_5D_5N, 400 MHz): 6.32 (1H, d, *J* = 1.2 Hz, Rha H-1), 5.26 (1H, brs, H-6), 4.96 (1H, d, *J* = 7.5 Hz, Glc H-1), 1.65 (3H, d, *J* = 6.5 Hz, Rha Me-6), 1.14 (3H, s, Me-21), 1.08 (3H, s, Me-19), 0.83 (3H, s, Me-18), 0.68 (3H, d, *J* = 6.6 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 140.8 (s, C-5), 121.7 (d, C-6), 109.2 (s, C-22), 102.7 (d, Rha-1), 102.45 (d, 3-*O*-Glc-1), 81.1 (d, C-16), 78.7 (d, 3-*O*-Glc-4), 78.3 (d, C-3), 77.1 (d, 3-*O*-Glc-5), 76.8 (d, 3-*O*-Glc-3), 74.1 (d, Rha-4), 72.8 (d,

Rha-3), 72.6 (d, 3-*O*-Glc-2), 72.5 (d, Rha-2), 70.4 (d, Rha-5), 66.8 (d, C-26), 62.9 (d, C-17), 61.7 (t, 3-*O*-Glc-6), 56.6 (d, C-14), 50.2 (d, C-9), 42.0 (d, C-20), 40.4 (s, C-13), 39.8 (t, C-12), 39.6 (t, C-4), 37.5 (t, C-1), 37.1 (s, C-10), 32.3 (t, C-15), 32.2 (t, C-7), 31.8 (t, C-23), 31.7 (d, C-8), 30.6 (d, C-25), 30.2 (t, C-2), 29.2 (t, C-24), 21.1 (t, C-11), 19.4 (q, 19-CH_3), 18.6 (q, Rha-6), 17.3 (q, 27-CH_3), 16.3 (q, 18-CH_3), 15.0 (q, 21-CH_3).

Compound **9**, white needle crystal, $C_{45}H_{72}O_{16}$, Liebermann–Burchard reaction and Molish test: positive. Anisaldehyde: positive. UV (MeOH) λ_{max}: 202.2 nm. IR (KBr) ν_{max} 3413 (OH), 2930, 1040 (C–O), 978, 918 < 895, 866. (+) ESI–MS^n *m/z*: 891, 745, 599. ^{1}H NMR (C_5D_5N, 400 MHz): 6.38 (1H, d, *J* = 1.0 Hz, Rha H-1), 5.84 (1H, d, *J* = 1.0 Hz, Rha H-1′), 5.30 (1H, brs, H-6), 4.93 (1H, d, *J* = 7.6 Hz, 3-*O*-Glc H-1), 1.76 (3H, d, *J* = 6.2 Hz, Rha Me-6), 1.61 (3H, d, *J* = 6.2 Hz, Rha Me-6′), 1.14 (3H, s, Me-21), 1.04 (3H, s, Me-19), 0.83 (3H, s, Me-18), 0.69 (3H, d, *J* = 6.6 Hz, Me-27). ^{13}C NMR (C_5D_5N, 100 MHz): 140.6 (s, C-5), 121.6 (d, C-6), 109.2 (s, C-22), 102.9 (d, Rha-1′), 101.9 (d, Rha-1), 100.2 (d, 3-*O*-Glc-1), 81.1 (d, C-16), 78.5 (d, 3-*O*-Glc-4), 78.1 (d, C-3), 78.0 (d, 3-*O*-Glc-3), 77.7 (d, 3-*O*-Glc-2), 76.9 (d, 3-*O*-Glc-5), 74.0 (d, Rha-4), 73.8 (d, Rha-4′), 72.8 (d, Rha-3), 72.7 (Rha-3′), 72.5 (d, Rha-2), 72.5 (d, Rha-2′), 70.3 (d, Rha-5′), 69.4 (d, Rha-5), 66.8 (d, C-26), 62.8 (d, C-17), 61.2 (t, 3-*O*-Glc-6), 56.6 (d, C-14), 50.2 (d, C-9), 41.9 (d, C-20), 40.4 (s, C-13), 39.8 (t, C-12), 38.9 (t, C-4), 37.4 (t, C-1), 37.2 (s, C-10), 32.3 (t, C-15), 32.2 (t, C-7), 31.8 (t, C-23), 31.6 (d, C-8), 30.6 (d, C-25), 30.1 (t, C-2), 29.2 (t, C-24), 21.1 (t, C-11), 19.4 (q, 19-CH_3), 18.6 (q, Rha-6), 18.5 (q, Rha-6′), 17.3 (q, 27-CH_3), 16.3 (q, 18-CH_3), 15.0 (q, 21-CH_3).

Compound **10**, white cubic crystal (in chloroform), $C_{27}H_{42}O_3$, Liebermann–Burchard reaction: positive. (+) ESI–MS^n *m/z*: 437. ^{1}H NMR ($CDCl_3$, 400 MHz): 5.42 (1H, brs, H-6), 1.16 (3H, s, Me-21), 1.06 (3H, s, Me-19), 0.90 (3H, s, Me-18), 0.60 (3H, d, *J* = 6.6 Hz, Me-27). ^{13}C NMR ($CDCl_3$, 100 MHz): 141.2 (s, C-5), 121.6 (d, C-6), 109.5 (s, C-22), 81.3 (d, C-16), 77.6 (d, C-3), 66.8 (d, C-26), 62.8 (d, C-17), 56.2 (d, C-14), 50.4 (d, C-9), 42.2 (d, C-20), 40.3 (s, C-13), 40.0 (t, C-12), 37.2 (t, C-4), 37.1 (s, C-10), 37.0 (t, C-1), 32.3 (t, C-15), 32.3 (t, C-7), 31.8 (d, C-8), 31.6 (t, C-23), 30.6 (d, C-25), 29.6 (t, C-2), 29.2 (t, C-24), 21.2 (t, C-11), 19.5 (q, 19-CH_3), 17.2 (q, 27-CH_3), 16.5 (q, 18-CH_3), 15.0 (q, 21-CH_3).

*3.5. Acid hydrolysis of compound **2** to **9***

Each compound (a few milligrams) was dissolved in 1 M HCl (dioxane–H_2O, 1:1, 10 mL) was heated at 100 °C for 2.5 h in a sealed glass tube. After dioxane was removed, the solution was extracted with EtOAc (3 mL × 5 mL). The extraction was washed with H_2O and evaporated to dryness in a vacuum. The residue was chromatographed on silica gel eluting with $CHCl_3$–MeOH (20:1, 1:1). The monosaccharide portion was neutralised by passing through an exchange resin (Amberlite MB-3, Sigma) column, concentrated. The monosaccharides were identified by HPTLC analysis with authentic sugars.

3.6. Cell culture and assay for cytotoxic activity

All the cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. After they were digested with 0.25% trypsin solution, the cells were washed, resuspended in the above medium to 1×10^5 cells/mL, then 90 μL of this cell suspension were placed in each well of a 96-well flat-bottom plate. The cells were incubated for 24 h at 37 °C in 4% CO_2. After incubation, 10 μL of MeOH–medium (1:1) solution containing the sample was added to give final concentrations of 0.1–50 μg/mL; 10 μL of MeOH–medium (1:1) was added into control wells. The cells were incubated for a further 48 h in the presence of each agent, and then cell growth was evaluated with an MTT assay procedure. After termination of cell culture, 20 μL of 5 mg/mL MTT in phosphate buffered saline was added to every well and the plate was reincubated at 37 °C in 4% CO_2 for a further 4 h, then the supernatant was removed from every well, and 150 μL DMSO was added to dissolve the formazan crystals. The plate was shaken on a microshaker for 10 min, and then read on a microplate reader at 570 nm. A dose response curve was plotted for these samples that showed more than 90% of cell growth inhibition at the sample concentration of 50 μg/mL, and a concentration giving 50% inhibition (IC50) was calculated.

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