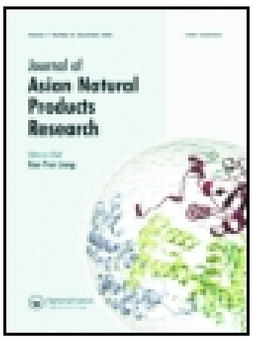

Journal of Asian Natural Products Research

ISSN: 1028-6020 (Print) 1477-2213 (Online) Journal homepage: https://www.tandfonline.com/loi/ganp20

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To cite this article: Yu-Bo Zhang, Kar-Ho Lam, Li-Feng Chen, Hei Wan, Guo-Cai Wang, Kai-Fai Lee, Chun-Wang Yip, Kwun-Hung Liu, Pak-Heng Leung, Hai-Yong Chan, Yi-Gang Shi, Jia Zhao, Li-Xing Lao, Yao-Lan Li, Yan-Bo Zhang & Wei Meng (2020): Chemical constituents from the thorns of *Gleditsia sinensis* and their cytotoxic activities, Journal of Asian Natural Products Research, DOI: 10.1080/10286020.2020.1731799

To link to this article: https://doi.org/10.1080/10286020.2020.1731799

JOURNAL OF ASIAN NATURAL PRODUCTS RESEARCH
https://doi.org/10.1080/10286020.2020.1731799

Chemical constituents from the thorns of *Gleditsia sinensis* and their cytotoxic activities

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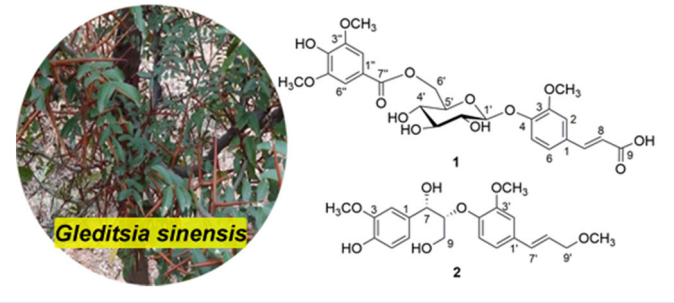
ABSTRACT

A new aromatic glycoside (**1**) and a new natural product, neolignan (**2**), along with twenty-three known compounds (**3-25**), were isolated from the thorns of *Gleditsia sinensis*. According to the spectroscopic analyses (IR, UV, HRESIMS, NMR and ECD), the structures of isolates were elucidated. Herein, compounds **4**, **6-8**, **10-13**, **15**, **16**, **18**, **20**, **23** were isolated from the plant of *G. sinensis* for the first time. Moreover, compounds **4, 6**, **15** and **24** showed cytotoxic effects on human ovarian cancer (SKOV-3) cells with IC_{50} values of 24.83 ± 4.90, 48.86 ± 9.11, 80.13 ± 5.62, 15.38 ± 2.21 μM, respectively.



ARTICLE HISTORY
Received 22 August 2019
Accepted 15 February 2020

KEYWORDS
Leguminosae; *Gleditsia sinensis*; chemical constituent; structural identification; cytotoxic activity

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Supplemental data for this article is available online at https://doi.org/10.1080/10286020.2020.1731799.

1. Introduction

The perennial plant *Gleditsia sinensis* Lam. (Leguminosae), is widely distributed in China, such as Jiangsu, Hubei, Henan, Shandong and Sichuan Provinces [1]. The thorns of *G. sinensis*, which is known as 'Zao-Jiao-Ci' in China, have been used for the treatment of several illnesses, including cancer, carbuncle and skin diseases [2]. Moreover, the thorns of *G. sinensis* and seven other medicinal materials are mixed as a Chinese medicine formula called "Kun Tai No. 1", which is clinically used to treat polycystic ovary syndrome [3]. Modern pharmacology researches have shown that the plant of *G. sinensis* possesses anti-tumor [4, 5], anti-inflammatory [6], anti-allergic [7], anti-angiogenic [8], and antibacterial [9] activities. In particular, the crude extract and chemical constituents of the thorns of *G. sinensis* possessed a certain inhibitory effect on various tumor cells, such as cervical, breast, leukemia, liver, oral, and gastric cancer cells [5]. Previous phytochemical investigations on this plant have led to the isolation of triterpenoid saponins [5], flavonoids [10], phenolic [11], and steroidal [12] derivatives.

In our efforts to discover new anti-cancer metabolites from traditional medicinal plants [13–15], we investigated the chemical constituents of *G. sinensis*. As a result, a new aromatic glycoside (**1**) and a new natural product, neolignan (**2**), along with twenty-three known compounds (**3–25**), were isolated from the thorns of *G. sinensis* (Figure 1). Their chemical structures were determined by analysis of spectroscopic and physical data. Then, all the isolates were evaluated for their cytotoxic activities on human ovarian cancer cells by using the 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay.

2. Results and discussion

Compound **1** was isolated as a white amorphous powder with $[\alpha]_D^{25}$ − 35.8 (*c* 1.0, CH_3OH), and has the molecular formula $C_{25}H_{28}O_{13}$ as indicated by the HRESIMS at *m/z* 559.1440 $[M+H]^+$. The IR spectrum of **1** showed absorption bands for aromatic ring (1609, 1513, 1455 cm^{-1}), carbonyl (1699 cm^{-1}), and hydroxyl group (3392 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1), interpreted with the help of an HSQC experiment, showed signals for a 1,2,4-trisubstituted aromatic group [δ_H 7.30 (1H, br s), 7.07 (1H, d, $J = 8.6$ Hz), and 6.89 (1H, br d, $J = 8.6$ Hz)], a symmetrically 1,3,4,5-tetrasubstituted aromatic group [δ_H 7.20 (2H, s)], a pair of mutually coupled aliphatic protons [δ_H 7.41 (1H, d, $J = 15.6$ Hz) and 6.40 (1H, d, $J = 15.6$ Hz)], and three methoxyls [δ_H 3.75 (6H, s) and 3.73 (3H, s)], and an anomeric proton [δ_H 5.08 (1H,

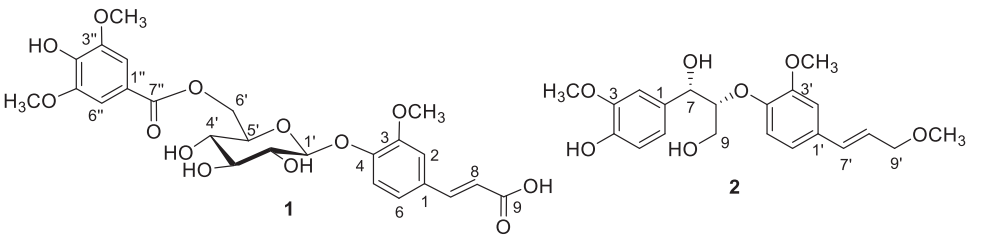

Figure 1. The chemical structures of **1-2**.

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **2** (J in Hz).[a]

	1			2	
No.	δ_H	δ_C	No.	δ_H	δ_C
1	–	128.4	1	–	134.1
2	7.30 (br s)	111.1	2	7.02 (d, 1.8)	111.9
3	–	149.1	3	–	148.7
4	–	148.0	4	–	147.0
5	7.07 (d, 8.6)	114.7	5	6.73 (d, 8.1)	115.7
6	6.89 (br d, 8.6)	121.7	6	6.84 (dd, 8.1, 1.8)	121.0
7	7.41 (d, 15.6)	143.3	7	4.83 (d, 5.8)	74.1
8	6.40 (15.6)	118.2	8	4.36-4.38 (m)	86.1
9	–	168.2	9	3.82[b]	62.2
1′	5.08 (d, 6.7)	99.3		3.77 (dd, 12.2, 3.7)	
2′	3.33[b]	73.1	1′	–	132.7
3′	3.33[b]	76.6	2′	7.00 (br s)	111.4
4′	3.26[b]	70.3	3′	–	151.9
5′	3.79[b]	74.0	4′	–	149.2
6′	4.62 (d, 11.2)	64.1	5′	6.88 (br s)	118.8
	4.18 (dd, 11.2, 7.6)		6′	6.88 (br s)	120.8
1″	–	119.3	7′	6.54 (dt, 15.3, 1.1)	133.8
2″,6″	7.20 (s)	107.3	8′	6.18 (dt, 15.3, 6.3)	125.2
3″,5″	–	147.7	9′	4.05 (dd, 6.3, 1.1)	74.2
4″	–	141.2	3-OMe	3.79 (s)	56.3
7″	–	165.5	3′-OMe	3.80 (s)	56.5
3-OMe	3.73 (s)	55.8	9′-OMe	3.36 (s)	58.0
3″,5″-OMe	3.75 (s)	56.2			

[a]Measured at 500 (^{1}H) and 125 (^{13}C) MHz in DMSO-d_6 for **1** and CD_3OD for **2**.
[b]Overlapped with other signals.

d, $J = 6.7$ Hz)]. The ^{13}C NMR spectrum of **1** (Table 1) in conjunction with the DEPT-135 spectrum showed the existence of 25 carbon signals, including fourteen aromatic or olefinic carbons (δ_C 149.1, 148.0, 147.7 × 2, 143.3, 141.2, 128.4, 121.7, 119.3, 118.2, 114.7, 111.1, and 107.3 × 2), two carbonyls (δ_C 168.2, 165.5), three methoxyls (δ_C 55.8, 56.2 × 2), five oxygenated methines (δ_C 99.3, 76.6, 73.1, 74.0 and 70.3) and an oxygenated methylene (δ_C 64.1).

Detailed analysis of the 1D NMR data of **1** showed that compound **1** possessed ferulic acid (δ_C 168.2, 149.1, 148.0, 143.3, 128.4, 121.7, 118.2, 114.7, 111.1, 55.8) [16], 4-hydroxy-3,5-dimethoxybenzoyl (δ_C 165.5, 147.7 × 2, 141.2, 119.3, 107.3 × 2, 56.2 × 2) [17], and glucose units (δ_C 99.3, 76.6, 74.0, 73.1, 70.3, 64.1) [17]. In the HMBC spectrum of **1**, the correlations between H-1′ (δ_H 5.08) and C-4 (δ_C 148.0), and between H_2-6′ (δ_H 4.62, 4.18) and C-7″ (δ_C 165.5) indicated that the ferulic acid and 4-hydroxy-3,5-dimethoxybenzoyl moieties were connected to C-1′ and C-6′ of glucose unit, respectively. Moreover, the type of sugar residue was confirmed by the acid hydrolysis of **1**, which was detected as D-glucose by HPLC analysis. The $^{3}J_{H1\text{-}H2}$ coupling constant demonstrated β-glycosidic linkages of D-glucose ($J = 6.7$ Hz). Thus, the structure of **1** was determined and named as 4-[6-*O*-(4-hydroxy-3,5-dimethoxybenzoyl)-β-D-glucopyranosyloxy]-ferulic acid.

Compound **2** was obtained as yellow oil, which was assigned the molecular formula of $C_{21}H_{26}O_7$ based upon an $[M+H]^+$ ion peak at m/z 413.1571 $[M+H]^+$ in HRESIMS. The IR spectrum of **2** showed absorption bands for hydroxyl (3441 cm^{-1}). The ^{1}H NMR spectrum of **2** revealed the presence of the signals for eight olefinic protons [δ_H 7.02 (1H, d, $J = 1.8$ Hz), 7.00 (1H, br s), 6.88 (2H, br s), 6.84 (1H, dd,

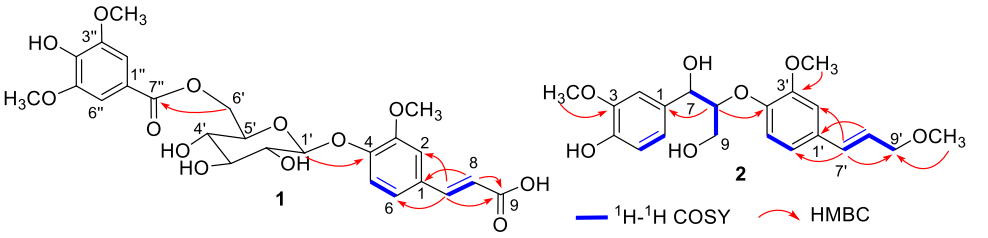

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of **1** and **2**.

$J = 8.1$, 1.8 Hz), 6.73 (1H, d, $J = 8.1$ Hz), 6.54 (1H, dt, $J = 15.3$, 1.1 Hz) and 6.18 (1H, dt, $J = 15.3$, 6.3 Hz)], six deshielded protons [δ_H 4.83 (1H, d, $J = 5.8$ Hz), 4.37 (1H, m), 4.05 (2H, dd, $J = 6.3$, 1.1 Hz), 3.82 (1H, overlapped) and 3.77 (1H, dd, $J = 12.2$, 3.7 Hz)], and three methoxyls [δ_H 3.80, 3.79 and 3.36 (each, 3H, s)]. The ^{13}C NMR and DEPT spectra exhibited twenty-one signals, including six quaternary carbons, ten methines, two methylenes, and three methyls. Detailed analysis of the ^{1}H and ^{13}C NMR data of **2** (Table 1) showed that **2** possessed two 1,3,4-trisubstituted benzene rings [δ_C 148.7, 147.0, 134.1, 121.0, 115.7,111.9; δ_C 151.9, 149.2, 132.7, 120.8, 118.8, 111.4], a double bond [δ_C 133.8 and 125.2], two oxygenated methines [δ_C 86.1 and 74.1], two oxygenated methylenes [δ_C 74.2 and 62.2], and three methoxyls [δ_C 58.0, 56.5 and 56.3]. The NMR spectroscopic features of **2** were close to those of viburfordoside G [18]. The main difference was that the NMR signals of the glucopyranosyl moiety in **2** disappeared. In the HMBC spectrum, the protons of three methoxyls showed cross-peaks with the carbons of C-3/C-3′/C-9′, indicating that three methoxyls were linked to C-3/C-3′/C-9′, respectively. The planar structure of **2** was further confirmed by ^{1}H-^{1}H COSY and HBMC spectra (Figure 2). The erythro form of H-7 and H-8 in **2** was established by a small coupling constant ($J_{7,8}$ = 5.8 Hz) [18]. Furthermore, the $\Delta\delta_{C8-C7}$ value of **2** (12.0) was identical to viburfordoside G [18], which further verified that **2** should possess the same relative stereochemistry as that of viburfordoside G (7,8-erythro). Finally, experimental ECD spectrum of **2** was in good agreement with calculated ECD spectrum of 7*S*,8*R*-**2** using a TDDFT [B3LYP/6-31G(d)] (Figure 3), indicating that the absolute configurations of **2** were assigned to be 7*S*, 8*R*. Thus, compound **2** was named as glensin A, which was previously obtained (without detailed spectroscopic data) by a hydrolysis of viburfordoside G. Herein it was reported for the first time as a molecule isolated from nature [18].

Twenty-three known compounds were identified as (7*S*,8*R*)−4,9′-dihydroxyl-3,3′-dimethoxyl-7,8-dihydrobenzofuran-1′-propylneolignan (**3**), (7*S*,8*R*)-threo-1′-[3′-hydroxy-7-(4-hydroxy-3-methoxyphenyl)−8-hydroxymethyl-7,8-dihydrobenzofuran]acrylaldehyde (**4**), (2*R-trans*)−2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)−3-(hydroxymethyl)−7-methoxy-5-benzofuranpropanol acetate (**5**), (7*R*,8*S*)-dehydrodiconiferyl alcohol (**6**), (-)-secoisolariciresinol (**7**), (+)-pinoresinol (**8**), (+)-medioresinol (**9**), syringaresinol (**10**), garbanzol (**11**), 7,4′-dihydroxy-5,3′-dimethoxyflavanonol (**12**), 4′-*O*-methyldihydroquercetin (**13**), (+)-aromadendrin (**14**), (-)-eriodictyol (**15**), 5-hydroxy-7-methoxyflavanone (**16**), quercetin (**17**), scopoletin (**18**), 3-formylindole (**19**), indazole (**20**), isovanillic acid (**21**), friedelin (**22**), lupenone (**23**), betulin (**24**), sitosterol (**25**) respectively, by comparing their spectroscopic and physical data with those of related

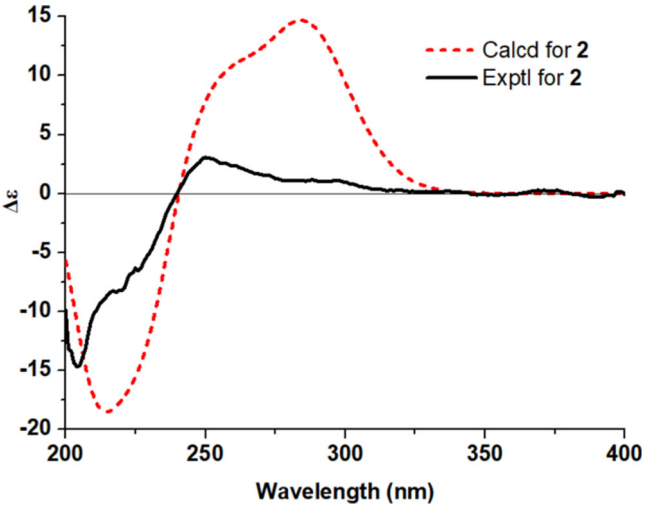

Figure 3. Experimental and calculated ECD spectra of **2**.

literatures. Herein, compounds **4**, **6–8**, **10–13**, **15**, **16**, **18**, **20**, **23** were isolated from the plant of *G. sinensis* for the first time. The references and chemical structures have been listed in supporting material.

Then, the isolated compounds (**1–25**) were subjected to test their cytotoxic activities on human ovarian cancer SKOV-3 cells and human normal ovarian IOSE80 cells using MTT assay. Cisplatin was adopted to be positive control in this experiment. As a result (Table 2), compounds **4** and **24** showed moderate cytotoxic activities against ovarian cancer cells SKOV3 with IC_{50} values of 24.83 ± 4.90 and 15.38 ± 2.21 μM, and SI values of 15.20, 1.09, respectively. Compounds **6** and **15** showed weak cytotoxic activities with IC_{50} values of 48.86 ± 9.11 and 80.13 ± 5.62 μM, respectively. However, compounds **1–3**, **5**, **7–14** and **16–24** had not obvious activities (IC_{50}>100 μM).

3. Experimental

3.1. General experimental procedures

IR and UV spectra were obtained on JASCO FI/IR-480 Plus Fourier transform spectrometer and JASCO V-550 UV/vis spectrophotometer (JASCO, Tokyo, Japan), respectively. Optical rotations were measured on JASCO P-1020 polarimeter (JASCO, Tokyo, Japan). NMR spectra were performed on Bruker AV-500 spectrometer (Bruker, Faellanden, Switzerland). CD spectra were recorded on JASCO J-810 spectropolarimeter (JASCO, Tokyo, Japan). HRESIMS data were tested on Agilent 6210 LC/Q TOF mass spectrometer (Agilent Technologies, CA, USA). Preparative HPLC were performed on Varian Prostar system equipped with UV detector (Varian, California, USA) and a preparative C_{18} column (Cosmosil, 5 µm, 20×250 mm, Nacalai Tesque, Japan). Analytical HPLC were performed on Dionex chromatograph (Dionex, California, USA) with an analytical column (Cosmosil, 5 µm, 4.6×250 mm, Nacalai Tesque, Japan), a P680 pump, and a PDA-100 photodiode array detector. Open column chromatography (CC) was performed using ODS (50 μm; YMC,

Table 2. Cytotoxic activities of the active compounds.[a]

Compounds	SKOV3 IC_{50} (μM)[b]	IOSE80 CC_{50} (μM)[c]	SI[c]
4	24.83 ± 4.90	73.42 ± 13.24	15.20
6	48.86 ± 9.11	>100[b]	>2.05
15	80.13 ± 5.62	85.34 ± 7.91	1.06
24	15.38 ± 2.21	16.75 ± 3.70	1.09
Cisplatin[d]	11.08 ± 0.18	13.25 ± 2.53	1.20

[a]Data are represented as the mean value ± SD.
[b]The IC_{50} value of the sample was higher than 100 μM.
[c]SI value equals to IC_{50} (SKOV3 cells)/IC_{50} (IOSE80 cells).
[d]Positive control.

Tokyo, Japan), silica gel (200–300, 300–400 mesh, Qingdao Haiyang Chemical Company, Ltd., Qingdao, China), and Sephadex LH-20 (25–100 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Thin layer chromatography (TLC) was carried out using precoated silica gel GF_{254} plates (Yantai Jiangyou Silica Gel Technology Development Co., Ltd., Yantai, China). All the reagents were purchased from Tianjin Damao Chemical Company (Damao, Tianjin, China).

3.2. Plant material

The dried thorns of *Gleditsia sinensis* were purchased in April 2018 from Luoyang City, Henan Province of China, and identified by Prof. Guang-Xiong Zhou, Jinan University, Guangzhou, China. A voucher specimen (No.20180318) was deposited at the Institute of Traditional Chinese Medicine and Natural Products, Jinan University.

3.3. Extraction and isolation

The thorns of *Gleditsia sinensis* (30 kg) were powdered and extracted three times with 75% EtOH (50 L × 3, 24 h, each) at ambient temperature. The combined extracts were evaporated *in vacuo* to afford a crude extract (1.8 kg), which was sequentially partitioned into petroleum ether (PE), EtOAc, and *n*-BuOH. The PE part (126.5 g) was chromatographed on silica gel column with a PE-EtOAc step gradient system (100:0 to 0:100) to give eight fractions (1-8). Fr. 3 (4.5 g) was purified with the aid of silica gel column (PE-acetone, 100:0 to 1:1) to yield **18** (5.8 mg) and **24** (19.2 mg). Fr. 4 (278.0 mg) was purified by Sephadex LH-20 ($CHCl_3$/CH_3OH, 1:1) to obtain **22** (30.5 mg), and Fr. 6 (18.3 g) was separated by silica gel column (PE/EtOAc, 95:5 to 1:1) to obtain **23** (15.7 mg) and **25** (20.6 mg).

The EtOAc part (510.8 g) was subjected to silica gel column and eluted with $CHCl_3$/CH_3OH (99:1 to 50:50) to afford five fractions (A-E). Fraction B (103.6 g) was further separated with the aid of ODS column (CH_3OH/H_2O, 30:70 to 100:0) to give six subfractions (B1-B6). Fr. B2 (8.7 g) was separated by silica gel column (PE/EtOAc, 90:10 to 0:100) and then purified by Sephadex LH-20 column ($CHCl_3$/CH_3OH, 1:1) to obtain **19** (7.4 mg). Fr. B3 (2.2 g) was applied to Sephadex LH-20 column ($CHCl_3$/CH_3OH, 1:1) to give **16** (10.0 mg) and **21** (15.9 mg). Fr. B5 (25.7 g) was separated by silica gel column ($CHCl_3$/CH_3OH, 95:5 to 70:30) and further purified by preparative HPLC (CH_3OH/H_2O, 85:15, 8 ml/min) to obtain **8** (9.3 mg), **9** (12.0 mg) and **10**

(18.1 mg). Fr. B6 (1.5 g) was applied to silica gel column ($CHCl_3/CH_3OH$, 95:5 to 80:20) to yield **5** (17.7 mg). Fr. D (92.8 g) was loaded onto a ODS column and eluted with CH_3OH/H_2O (50:50 to 100:0) to give nine subfractions (D1-D9). Fr. D2 (3.6 g) was chromatographed on a Sephadex LH-20 column ($CHCl_3/CH_3OH$, 1:1) and then purified by preparative HPLC (CH_3OH/H_2O, 85:15, 8 ml/min) to yield **3** (15.7 mg), **4** (10.8 mg) and **6** (20.5 mg). Fr. D3 (2.1 g) was subjected to Sephadex LH-20 column ($CHCl_3/CH_3OH$, 1:1) to yield **20** (14.6 mg), and Fr. D8 (4.7 g) was subjected to a silica gel column ($CHCl_3/CH_3OH$, 95:5 to 70:30) to yield **7** (22.9 mg). Fr. D5 (15.8 g) was separated by silica gel column ($CHCl_3/CH_3OH$, 100:0 to 80:20) and purified by Sephadex LH-20 column ($CHCl_3/CH_3OH$, 1:1) to yield **11** (21.7 mg), **12** (18.0 mg) and **13** (16.5 mg). Fr. D8 (1.5 g) was separated by ODS column (CH_3OH/H_2O, 20:80 to 90:10) to yield **1** (16.8 mg). Compounds **14** (20.5 mg) and **15** (13.2 mg) were isolated from Fr. D9 (2.1 g) by Sephadex LH-20 column eluting with $CHCl_3$-CH_3OH (1:1). Fraction E (39.4 g) was sequentially separated by ODS column (CH_3OH/H_2O, 30:70 to 90:10) and Sephadex LH-20 CC (MeOH) to afford **2** (11.8 mg) and **17** (23.5 mg).

3.3.1. 4-[6-O-(4-Hydroxy-3,5-dimethoxybenzoyl)-β-D-glucopyranosyloxy]-ferulic acid (1)

Brown oil; $[\alpha]_D^{25} -35.8$ (*c* 1.0, CH_3OH); UV (CH_3OH) λ_{max} 209 and 276 nm; IR (KBr) ν_{max} 3392, 2941, 1699, 1609, 1513, 1455, 1423, 1336, 1269, 1228, 1071 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS: *m/z* 559.1440 $[M+H]^+$ (calcd for $C_{25}H_{28}O_{13}Na$, 559.1422).

3.3.2. Glensin A (2)

Yellow oil; $[\alpha]_D^{25} +42.4$ (*c* 1.0, CH_3OH); UV (CH_3OH) λ_{max} 205, 277 nm; IR (KBr) ν_{max} 3441, 1630, 1510, 1382, 1267, 1115, 1027, 563 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS: *m/z* 413.1571 $[M+H]^+$ (calcd for $C_{21}H_{26}O_7Na$, 413.1571).

3.4. Acid hydrolysis of compound 1

Compound **1** (1 mg) was dissolved in 4.0 M HCl (10 ml) and refluxed at 90 °C for 6 h. The reaction mixture was concentrated under reduced pressure and then partitioned with equal volumes of chloroform and water. After the H_2O layer was concentrated, the sugar residue was obtained. Then, the residue was dissolved in anhydrous pyridine (1 ml), followed by addition of L-cysteine methyl ester hydrochloride (2 mg) at 60 °C for 1 h. The reaction solvent was dried under a stream of N_2. Subsequently, 0.2 ml of trimethylsilyl imidazole was added at 60 °C for another 1 h. The final product was analyzed by HPLC and compared with standard sugar [flow rate: 0.8 ml/min; injection volume: 10 μl; column: Cosmosil 5C18-MS-II column (4.6 × 250 mm); mobile phase: 25% CH_3CN-H_2O (0.05% acetic acid); wavelength: 210 nm; column temperature: 25 °C)]. The same treatment was applied for standard sugar. The retention time of standard sugar was detected at 16.42 min (D-glucose).

3.5. Cytotoxicity assay

Compounds **1-25** were evaluated for their cytotoxic effects against human ovarian cancer SKOV-3 cells and human normal ovarian IOSE80 cells by MTT assays. The

cells were cultured in DMEM (dulbecco's modified eagle medium) medium supplemented with 10% heat-inactivated fetal bovine serum. Then, cells were planted in 96-well plates with approximately 3000 cells per well and treated with 0-100 μM of isolated compounds for 48 h at 37 °C in 5% CO_2. Subsequently, experiments were terminated by adding 20 ml of MTT (10 mg/ml) into each well of the plates and then incubated for 4 h. The supernatant was replaced by 100 μl of DMSO to dissolve the formazan. MTT assay results were detected using a microplate reader spectrophotometer (Thermo Labsystems Multiskan MK3) at 570 nm.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by grants from Innovation and Technology Support Programme, Government of Hong Kong (Project code: UIM/321), National Key R&D Program of China (No. 2017YFC1703800), and China Postdoctoral Science Foundation (No. 2019T120795).

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