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Gerberdriasins A–F, six undescribed coumarin derivatives from *Gerbera anandria* (Linn) Sch-Bip and their protective effects on scopolamine-induced injury in PC12 cells†

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A chemical investigation on the herb *Gerbera anandria* (Linn) Sch-Bip led to the isolation and identification of six previously undescribed coumarin derivatives, named Gerberdriasins A–F (**1–6**). Structurally, their chemical structures and absolute configurations were determined by nuclear magnetic resonance (1D and 2D NMR), high resolution electrospray ionization mass spectroscopy (HR-ESI-MS), experimental and quantum mechanical nuclear magnetic resonance (QM-NMR) methods, Mosher's method and calculated electronic circular dichroism (ECD) experiments. The biological activity of the obtained compounds showed that they displayed significant neuroprotective effects against scopolamine-induced injury in PC12 cells at the concentrations 12.5, 25.0 and 50.0 nM. Further study demonstrated that **1** could inhibit cell apoptosis, decrease malondialdehyde (MDA) levels and increase superoxide dismutase (SOD) activity in scopolamine-treated PC12 cells.

Introduction

The large genus *Gerbera* belonging to the family Asteraceae comprises about 80 species. Many of them exist in Africa and Asia, and twenty of them are widely distributed in most South-western Asian countries, especially Yunnan Province in China.[1–4] In addition, they had been widely used as Traditional Chinese Medicine (TCM) to treat enteritis, coughs, lung heat, dysentery, *etc.*[5–7] Previous phytochemical investigations of this genus *Gerbera* yielded coumarins, terpenoids, sterols, flavonoids and phenylacetones.[8–13] Some of them exhibited a wide array of bioactivities, including anti-bacterial, anti-inflammatory, anti-coagulant and antitumor activities.[14–16] To date, research on the phytochemistry and pharmacological activity of *Gerbera anandria* (Linn) Sch-Bip has rarely been reported. In this study, six undescribed coumarin derivatives, Gerberdriasins A–F (**1–6**), were isolated from the herb *Gerbera anandria* (Linn) Sch-Bip. In bioactivity assays, all compounds exhibited neuroprotective effects on scopolamine-induced injury in PC12 cells. The isolation, structural elucidation and neuroprotective activities of these compounds (Fig. 1) are described in this paper.

Experimental

General experimental procedure

Column chromatography (CC): silica gel H (10–40 μm) and silica gel (200–300 mesh) (Marine Chemical Factory, Qingdao, P. R. China); MCI gel CHP–20P: (Daiso, Co., Japan) and RP-C_{18} gel (40–63 μm; Daiso, Co., Japan) were used for column chromatography; Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, USA). TLC: silica gel plates, visualization by

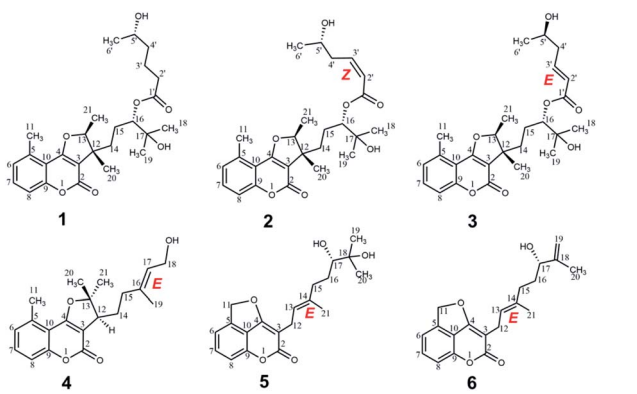

Fig. 1 Chemical structures of **1–6**.

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spraying with 10% H_2SO_4 in EtOH and Dragendorff's reagent. Semi-preparative HPLC: Agilent 1260 series (Agilent Technologies, US) with a Zorbax SB-C_{18} (5 μM, 9.4 mm × 25 cm) column. NMR Spectra: Bruker Avance III-500 spectrometer (Bruker, Switzerland). MS: Agilent MSD-Trap-XCT (for ESI) and Agilent-6520 Q-TOF mass spectrometer (for HR-ESI). IR: Thermo Scientific Nicolet 6700 (Thermo Scientific, US). UV spectra: Agilent 1260 series DAD detector (Agilent Technologies, US). CD spectrum: Brighttime Chirascan (Applied Photophysics Ltd, UK). Optical rotation: Rudolph Autopo V (Rudolph Research Analytical, Hackettstown, NJ).

Plant material

The herb of *Gerbera anandria* (Linn) Sch-Bip was collected from Yunnan province of China in October 2019, and identified by Prof. Bao-kang Huang (Department of pharmacognosy, Second Military Medical University, Shanghai, China). A voucher specimen (no. 201910-VS) is deposited in the department of pharmacognosy, Second Military Medical University.

Extraction and isolation

The dried herb of *Gerbera anandria* (Linn) Sch-Bip (50.0 kg) was extracted by maceration with 95% methanol overnight at room temperature (3 × 60 L). After remove of solvent, the methanol extract (8.60 kg) was partitioned between water and petroleum ether (PE)/ethyl acetate (EtOAc) (3 times with 10 L each). PE extract (2.15 Kg) was segmented by Silica gel H (10–40 μm) column chromatography (PE/EtOAc, 100 : 1 to 0 : 1 v/v) to give 21 fractions (Fr. 1–21). Fraction 18 (5.5 g) was purified through MCI column chromatography (MeOH/H_2O, 20 : 80 to 100 : 0 v/v) to give 9 subfractions (Fr. 18.1–18.9). Then, subfraction 18.5 was isolated by ODS (CH_3CN/H_2O, 20 : 80 to 50 : 50 v/v) to afford 7 subfractions (Fr. 18.5.1–18.5.7). Subfractions 18.5.3 and 18.5.5 were further separated using semi-preparative RP-C_{18} HPLC (CH_3CN/H_2O, 55 : 45 v/v 1.0 mL min^{-1}) to produce compounds **1** (9.3 mg), **2** (8.5 mg) and **3** (7.6 mg). Subfraction 18.6 was also subjected to ODS column chromatography (CH_3CN/H_2O, 20 : 80 to 50 : 50 v/v) to afford 6 subfractions (Fr. 18.6.1–18.6.5). Subfraction 18.6.2 was purified by semi-preparative RP-C_{18} HPLC (CH_3CN/H_2O, 50 : 50 v/v 1.0 mL min^{-1}) to afforded compounds **5** (10.3 mg) and **6** (7.9 mg). Subfraction 18.7 was separated by a Sephadex LH-20 column eluted with MeOH/H_2O (70 : 30, v/v) to yield 5 subfractions (Fr. 18.7.1–18.7.5), and subfraction 18.7.4 was separated through semi-preparative RP-C_{18} HPLC (CH_3CN/H_2O, 65 : 35 v/v 1.0 mL min^{-1}) to get compound **4** (6.5 mg). Above all, compounds **1–6** were obtained (Fig. 1).

Compound characterizations of 1–11

Gerberdriasin A (1). Colorless oil; $[\alpha]_D^{25}$ −15.83 (*c* 0.06, CH_3OH); IR (KBr) ν_{max} 3430, 2964, 2927, 2869, 1720, 1700, 1623, 1479, 1380, 1238, 1166, 1083, 1039, 989, 788, cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 483.2351 ($[M + Na]^+$, calcd for $C_{26}H_{36}O_7Na$, 483.2353).

Gerberdriasin B (2). Colorless oil; $[\alpha]_D^{25}$ −14.45 (*c* 0.075, CH_3OH); IR (KBr) ν_{max} 3432, 2973, 2933, 2875, 1716, 1704, 1623, 1602, 1563, 1463, 1413, 1382, 1170, 1010, 948, 788 cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 481.2201 ($[M + Na]^+$, calcd for $C_{26}H_{34}O_7Na$, 481.2197).

Gerberdriasin C (3). Colorless oil; $[\alpha]_D^{25}$ −5.28 (*c* 0.06, CH_3OH); IR (KBr) ν_{max} 3432, 2967, 2929, 2871, 1716, 1702, 1623, 1602, 1479, 1463, 1382, 1168, 1101, 1081, 985, 788 cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 481.2200 ($[M + Na]^+$, calcd for $C_{26}H_{34}O_7Na$, 481.2197).

Gerberdriasin D (4). Colorless oil; $[\alpha]_D^{25}$ +13.33 (*c* 0.06, CH_3OH); IR (KBr) ν_{max} 3332, 2969, 2929, 2869, 1720, 1623, 1602, 1562, 1479, 1386, 1049, 1018, 788, 742 cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 351.1569 ($[M + Na]^+$, calcd for $C_{20}H_{24}O_4Na$, 351.1567).

Gerberdriasins E (5). Colorless oil; $[\alpha]_D^{25}$ −16.0 (*c* 0.025, CH_3OH); IR (KBr) ν_{max} 3434, 2973, 2931, 2875, 1731, 1670, 1648, 1602, 1484, 1402, 1278, 1164, 1076, 1043, 779, 755, cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 367.1514 ($[M + Na]^+$, calcd for $C_{20}H_{24}O_5Na$, 367.1516).

Gerberdriasins F (6). Colorless oil; $[\alpha]_D^{25}$ −9.17 (*c* 0.02, CH_3OH); IR (KBr) ν_{max} 3430, 2969, 2931, 2871, 1716, 1698, 1623, 1602, 1562, 1479, 1461, 1382, 1238, 1166, 1081, 1039, 788, 742 cm^{-1}; ^1H- and ^{13}C-NMR data (500 MHz/125 MHz), see Tables 1 and 2; positive HRESIMS *m/z* 349.1407 ($[M + Na]^+$, calcd for $C_{20}H_{22}O_4Na$, 349.1410).

Table 1 ^{13}C NMR spectroscopic data for compounds **1–6**[a] (*δ*: ppm)

No.	1	2	3	4	5	6
2	160.8	160.7	160.8	161.5	166.4	166.3
3	108.5	108.4	108.5	105.4	99.0	98.9
4	167.5	167.5	167.5	166.9	167.8	167.8
5	136.5	136.4	136.5	136.5	138.5	138.5
6	126.3	126.3	126.3	126.3	116.5	116.5
7	131.8	131.7	131.8	131.7	133.4	133.4
8	114.3	114.2	114.3	114.3	112.6	112.6
9	155.8	155.8	155.8	155.7	149.1	149.1
10	111.7	111.7	111.7	111.8	118.9	118.9
11	20.0	20.0	20.0	20.0	80.9	80.9
12	45.9	46.0	46.0	47.0	22.3	22.2
13	89.5	89.4	89.4	94.7	120.4	120.4
14	34.7	34.7	34.6	26.7	136.3	135.9
15	24.4	24.5	24.5	37.3	36.4	35.3
16	79.5	79.1	79.6	137.8	29.1	32.8
17	71.3	71.3	71.4	124.1	77.4	74.6
18	24.1	24.1	24.0	57.9	72.3	147.4
19	24.9	25.0	25.0	14.7	24.2	110.0
20	18.0	18.1	18.1	21.3	23.5	16.1
21	14.5	14.4	14.4	28.4	14.9	14.8
1′	173.8	166.3	166.6			
2′	33.8	120.5	122.7			
3′	21.1	146.8	146.6			
4′	38.1	38.0	41.3			
5′	66.7	66.7	66.0			
6′	22.1	21.9	21.9			

[a] NMR Data were measured at 125 MHz in CD_3OD for **1–6**.

Table 2 ^{1}H NMR spectroscopic data for compounds **1–6**[a] (δ: ppm, J: Hz)

No.	1	2	3	4	5	6
6	7.13, d, (7.5)	7.10, d, (7.5)	7.12, d, (7.5)	7.10, d, (7.5)	7.27, d, (7.4)	7.28, d, (7.4)
7	7.48, tlike, (8.1)	7.45, tlike, (8.3)	7.47, tlike, (8.2)	7.46, tlike, (7.8)	7.60, tlike, (7.6)	7.60, tlike, (7.6)
8	7.20, d, (8.4)	7.18, d, (8.3)	7.20, d, (8.4)	7.20, d, (8.3)	7.15, d, (8.2)	7.15, d, (8.2)
11	2.67, s	2.66, s	2.67, s	2.65, s	5.85, s	5.84, s
12				3.07, dd, (8.3, 3.8)	3.17, d, (7.3)	3.16, d, (7.2)
13	4.91, dd, (13.3, 6.7)	4.89, dd, (13.0, 6.5)	4.91, dd, (13.3, 6.7)		5.33, m	5.31, m
14	1.82, 1.69, m	1.85, 1.67, m	1.85, 1.67, m	2.06, 1.77, m		
15	1.64, m	1.67, m	1.67, m	2.18, 2.06, m	2.25, 2.04, m	2.00, m
16	4.79, m	4.82, m	4.83, m		1.72, 1.34, m	1.60, m
17				5.41, m	3.22	3.95, t, (6.8)
18	1.12, s	1.12, s	1.13, s	4.08, d, (6.7)		
19	1.13, s	1.13, s	1.13, s	1.71, s	1.13, s	4.86, 4.76, s
20	1.27, s	1.26, s	1.26, s	1.60, s	1.10, s	1.67, s
21	1.48, d, (6.6)	1.46, d, (6.6)	1.46, d, (6.7)	1.56, s	1.77, s	1.76, s
2′	2.40, m	5.92, d, (11.4)	5.94, d, (15.7)			
3′	1.76, 1.68, m	6.40, m	7.04, m			
4′	1.46, m	2.79, m	2.36, m			
5′	3.73, m	3.86, m	3.89, m			
6′	1.15, d, (6.2)	1.17, d, (6.3)	1.19, d, (6.3)			

[a] NMR data were measured at 500 MHz in CD_3OD for **1–6**.

ECD calculations

In general, the conformational analyses were generated on the basis of the OPLS3 force field. Then, the generated conformers of compounds **1–4** whose energy threshold less than 3.0 kcal mol^{-1} were subjected to optimization with DFT calculation at the B3LYP/6-31G (d, p) level in the gas phase through the Gaussian 09 software. ECD calculations were performed using the TD-DFT methodology at the b3lyp/6–311 + g (d, p) level in methanol. Finally, the ECD spectrum was obtained by the Boltzmann-calculated and the SpecDis software. The ECD curves were drawn using the Origin Pro 8 program (Origin Lab Corporation, Northampton, MA, USA).

Preparation of the (*S*)-MTPA and (*R*)-MTPA esters of 1–3 and 5

To each compounds (each 1.5 mg) in pyridine-d_5 (130 μl) were separately added (*R*)-(−)-MTPA (5 μL) and (*S*)-(+)-MTPA (5 μL) at room temperature, followed by stirring at 40 °C for 8 h. Then the reaction was monitored through HPLC following the four time points: 0, 2, 4, and 8 h, and the reaction was found to be completed at 8 h. Finally, we transferred them into a 1.7 mm NMR tube, respectively.

(*S*)-MTPA ester of **1** (**1a**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.30 (3H, H_3-6′), 1.51 (1H, H-4′a), 2.13 (1H, H-3′a), 1.41 (3H, H_3-18), 1.42 (3H, H_3-19).

(*R*)-MTPA ester of **1** (**1b**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.32 (3H, H_3-6′), 1.45 (1H, H-4′a), 2.07 (1H, H-3′a), 1.39 (3H, H_3-18), 1.40 (3H, H_3-19).

(*S*)-MTPA ester of **2** (**2a**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.28 (3H, H_3-6′), 2.06 (1H, H-4′a), 6.11 (1H, H-3′), 5.42 (1H, H-2′), 1.42 (3H, H_3-18), 1.42 (3H, H_3-19).

(*R*)-MTPA ester of **2** (**2b**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.31 (3H, H_3-6′), 2.01 (1H, H-4′a), 6.01 (1H, H-3′), 5.36 (1H, H-2′), 1.39 (3H, H_3-18), 1.39 (3H, H_3-19).

(*S*)-MTPA ester of **3** (**3a**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.31 (3H, H_3-6′), 2.03 (1H, H-4′a), 6.04 (1H, H-3′), 5.35 (1H, H-2′), 1.23 (3H, H_3-18), 1.24 (3H, H_3-19).

(*R*)-MTPA ester of **3** (**3b**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.28 (3H, H_3-6′), 2.06 (1H, H-4′a), 6.18 (1H, H-3′), 5.43 (1H, H-2′), 1.27 (3H, H_3-18), 1.27 (3H, H_3-19).

(*S*)-MTPA ester of **5** (**5a**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.31 (3H, H_3-19), 1.36 (3H, H_3-20), 2.11 (1H, H-16a).

(*R*)-MTPA ester of **5** (**5b**). ^{1}H NMR (pyridine-d_5, 500 MHz): δ_H: 1.43 (3H, H_3-19), 1.45 (3H, H_3-20), 2.06 (1H, H-16a).

Cell culture and cell viability assay

The PC12 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere under 5% CO_2.

Cell viability was determined by the CCK-8 assay. In brief, PC12 cells were seed in 96-well plates at a density of 5×10^3 cells per well for 24 h. Then the cells were incubated with scopolamine for an additional 24 h and the compounds with various concentrations were pretreated for 12 h before treated with scopolamine. After treatment, each well with 10 μL CCK-8 reagent and incubated at 37 °C for 30 min in the dark. Afterward, the optical OD-value was measured at 450 nm through a microplate reader, respectively (BioTek Instruments, Inc).

Cell apoptosis assay

For apoptosis assay, the treated cells were washed with PBS and stained with PI followed by Annexin V–FITC at room

temperature for 20 min, then, the samples were examined by flow cytometry (BD Biosciences, San Jose, CA, USA).

MDA and SOD assay

Malondialdehyde (MDA) and superoxide dismutase (SOD) assay were determined by the commercial assay kits (Beyotime Institute of Biotechnology, Nantong, China) based on manufacture instructions. At the same time, protein concentrations were measured on the basis of the BCA protein assay kit (Beyotime Institute of Biotechnology, Nantong, China). Finally, all the samples were detected according to the manufacturer's protocol.

Statistical analysis

The statistical analysis of the values was analyzed according to Prism 8 (GraphPad, San Diego, CA, USA) software. $P < 0.05$ was considered to indicate statistical significance. One-way analysis of variance (ANOVA) and Tukey post hoc test were used for the dates of three separate studies. And, all values were expressed as mean ± standard deviation (SD).

Results and discussion

Compound **1** was isolated as colorless oil. Its molecular formula was established as $C_{26}H_{36}O_7$ based on the positive HRESIMS ion peak at *m*/*z* 483.2351 ([M + Na]$^+$, calcd for $C_{26}H_{36}O_7Na$, 483.2353), suggesting 9 indices of hydrogen deficiency. The IR spectrum showed the absorption bands 3430 cm^{-1} and 1720 cm^{-1}, which confirmed to the presence of the hydroxyl group and conjugated ester-carbonyl group.

The ^{1}H NMR spectra data (Table 1) showed three aromatic olefinic protons: δ_H 7.13 (1H, d, J = 7.5 Hz, H-6); 7.48 (1H, tlike, J = 8.1 Hz, H-7); 7.20 (1H, d, J = 8.4 Hz, H-8), three oxygenated methines: δ_H 4.91 (1H, dd, J = 13.3, 6.7 Hz, H-13); 4.79 (1H, H-16); 3.73 (1H, H-5′), six methyl peaks: δ_H 2.67 (3H, s, H_3-11); 1.12 (3H, s, H_3-18); 1.13 (3H, s, H_3-19); 1.27 (3H, s, H_3-20); 1.48 (3H, d, J = 6.6 Hz, H_3-21) and 1.15 (3H, d, J = 6.2 Hz, H_3-6′). The ^{13}C NMR and DEPT spectra data (Table 2) indicated 26 carbons, including six methyls (δ_C 14.5, 18.0, 20.0, 22.1, 24.1, 24.9), five methylenes (δ_C 21.1, 24.4, 33.8, 34.7, 38.1), six methines (including 3 sp^2 carbons at δ_C 114.3, 126.3, 131.8, 3 sp^3 carbons at δ_C 66.7, 79.5, 89.5), nine quaternary carbons (2 ester carbonyls at δ_C 160.8, 173.8, 5 sp^2 carbons at δ_C 108.5, 111.7, 136.5, 155.8, 167.5, 2 sp^3 carbons at δ_C 45.9, 71.3). Meanwhile, the presence of a 1,2,3-trisubstituted methylbenzene ring was determined by the NMR features as follows: δ_H 7.13 (H-6) to δ_C 126.3 (C-6), δ_H 7.48 (H-7) to δ_C 131.8 (C-7), and δ_H 7.20 (H-8) to δ_C 114.3 (C-8), which together with an aromatic methyl (δ_H 2.67, H_3-11, δ_C 20.0, CH_3-11) and a conjugated system (a conjugated ester at δ_C 160.8, two olefinic carbons at δ_C 167.5 and δ_C 108.5) determined that unit A should be a 5-methylcoumarin moiety.[17,18] Furthermore, two spin-coupling systems: H_2-14/H_2-15H-16 and H_2-2′/H_2-3′/H_2-4′/H-5′ as revealed by the ^{1}H–^{1}H COSY spectra and the HMBC cross-peaks from H_3-18 to C-16, C-17 and C-19, from H_3-20 to C-12 and C-14, from H_3-21 to C-12 and C-13, as well as from H_2-2′ to C-1′ (an ester carbonyl at δ_C 173.8), from H_3-6′ to C-5′ established the structure of unit B (the remaining sixteen carbons) could be a monoterpene moiety and six carbon short-chain fatty acid ester (Fig. 2). Meanwhile, they should be linked directly *via* C-16–O–C-1′ bond based on the HMBC correlation from H-16 (δ_H 4.79) to C-1′ (δ_C 173.8). Finally, the connectivity of unit A and unit B, *via* a furan ring, was deduced by the HMBC correlations from H-13 (δ_H 4.91) to C-3 (δ_C 108.5), C-4 (δ_C 167.5), C-12 (δ_C 45.9) and from H_3-20 (δ_H 1.27) to C-3 (δ_C 108.5) and C-14 (δ_C 34.7). Thus, the planar structure of **1** was determined.

The relative configuration was deduced from its NOESY spectrum. The cross-peaks from H_3-21 to H_3-20 indicated these protons were oriented on the same direction and assigned as β-oriented (Fig. 2). In order to further confirm its absolution configuration, the method of ECD calculation was applied. As seen from the Fig. 3, the calculated ECD curve of (12*R*, 13*S*, 16*S*, 5′*S*)-**1** was in good agreement with the experimental ECD spectrum of **1**. In addition, the observed $\Delta\delta_{H(S-R)}$ values of the (*S*)- and (*R*)-MTPA esters established the absolute configuration of C-5′ in **1** as *S* (Fig. 4), which was consistent with the ECD results. Thus, the structure of **1** was defined and named gerberdriasin A.

Compound **2**, isolated as colorless oil, possessed a molecular formula of $C_{26}H_{34}O_7$ as established by the ion peak at *m*/*z* 481.2201 ([M + Na]$^+$, calcd for $C_{26}H_{34}O_7Na$, 481.2197) in the positive HRESIMS spectrum, corresponding to 10 indices of hydrogen deficiency. Overall comparison of 1D NMR data of **2** (Tables 1 and 2) with those of **1** revealed that they share the similar skeleton with the exception of the presence of a double bond (δ_H 5.92, 6.40, H-2′, H-3′; δ_C 120.5, 146.8, C-2′, C-3′) at the C-2′ and C-3′ positions in the six carbon short-chain fatty acid ester in **2**. This deduction was supported by the 2 Da less than **1**, the one more unsaturation and the ^{1}H–^{1}H COSY correlations from H-2′ (δ_H 5.92) to H-3′ (δ_H 6.40) H_2-4′ (δ_H 2.79) and H-5′ (δ_H 3.86), as well as the HMBC correlations from H-2′ and H-3′ both to C-1′ (δ_C 166.3), C-4′ (δ_C 38.0). Additionally, the C-2′/C-3′ double bond should be *Z*-configured due to the coupling constants of $J_{2'/3'}$ = 11.4 Hz and the absence of the NOESY correlation from H-2′ to H_2-4′. Meanwhile, compound **2** shared the same relative configurations with **1** through the analysis of

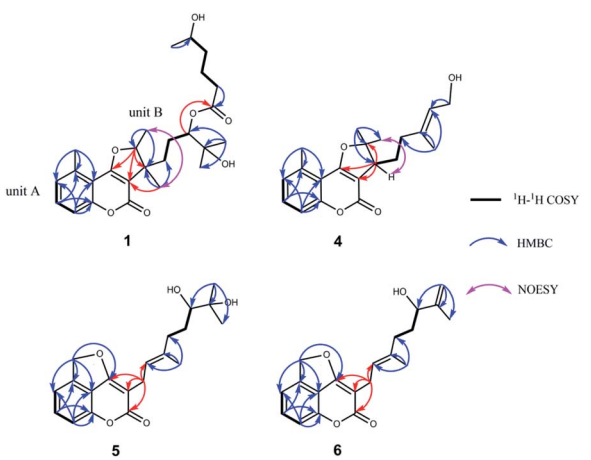

Fig. 2 Selected 2D NMR correlations of **1** and **4**–**6**.

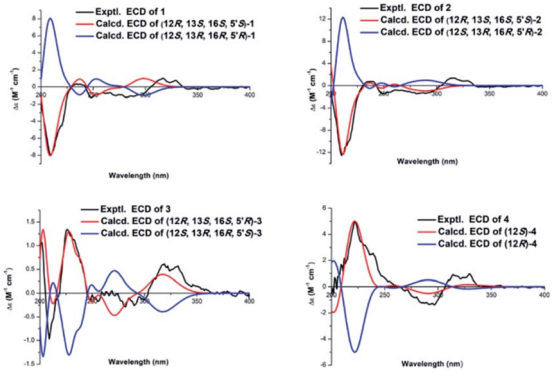

Fig. 3 Experimental and calculated ECD spectra of **1**–**4**.

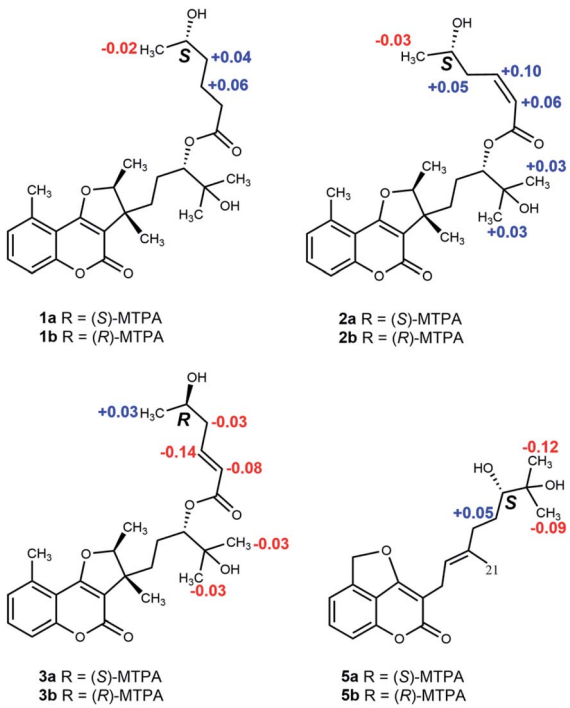

Fig. 4 Chemical shift differences ($\Delta\delta_{H\ (S-R)}$) between (*S*)-MTPA and (*R*)-MTPA.

its NOESY data. The absolute configuration of **2** was assigned as 12*R*, 13*S*, 16*S*, 5′*S* based on the good qualitative agreement between the experimental and calculated ECD spectra (Fig. 3). Additionally, the absolute configuration of C-5′ in **2** was also *S* determined by the $\Delta\delta_{H(S-R)}$ results (Fig. 4), which also was fitted well with the ECD results. Finally, the structure of **2** was determined and named Gerberdriasin B.

Compound **3** was isolated as colorless oil and assigned the same molecular formula as that of **2** according to the HRESIMS data: *m*/*z* 481.2200 ([M + Na]$^+$, calcd for $C_{26}H_{34}O_7Na$, 481.2197). Detail comparison of the 1D and 2D NMR spectroscopic data (^{1}H, ^{13}C, DEPT, ^{1}H–^{1}H COSY and HMBC) of **2** and **3** showed that they possessed the same 2D structure. The differences between them on NMR data were the C-4′ and H_2-4′, the chemical shift of C-4′ was down field shifted from δ_C 38.0 in **2** to δ_C 41.3 in **3**, at the same time, an obvious up field shifted of H_2-4′ from δ_H 2.79 in **2** to δ_H 2.36 in **3**. The de-shielding of C-4′ ($\Delta\delta$ +3.3) and the shielding of H_2-4′ ($\Delta\delta$ −0.43) in **3** were clearly implied the configurational change of C-5′. This deduction was further evidenced by the $\Delta\delta_{H(S-R)}$ results (Fig. 4). In addition, correlations between H-2′ and H_2-4′ and the large coupling constants of $J_{2'/3'}$ = 15.7 Hz indicated that the C-2′/C-3′ double bond was *E*-configured. By means of ECD calculation, the experimental ECD spectrum of **3** fitted well with the calculated spectrum of (12*R*, 13*S*, 16*S*, 5′*R*)-**3** (Fig. 3). Therefore, its structure was established and named Gerberdriasin C.

Compound **4**, isolated as colorless oil, displayed a molecular formula of $C_{20}H_{24}O_4$ as defined by the positive HRESIMS ion peak at *m*/*z* 351.1569 ([M + Na]$^+$, calcd for $C_{20}H_{24}O_4Na$, 351.1567), with 9 degrees of unsaturation. A detailed analysis of the 1D NMR spectra of **4** (Tables 1 and 2) indicated that **4** also had a 5-methylcoumarin moiety. The major difference was the side chain monoterpene moiety, as supported by the ^{1}H–^{1}H COSY correlations from H-12 to H_2-14 and H_2-15 and the HMBC correlations from H_3-19 (δ_H 1.71) to C-15 (δ_C 37.3), C-16 (δ_C 137.8) and C-17 (δ_C 124.1); from H_2-18 (δ_H 4.08) to C-17 (δ_C 124.1) and from H_3-20 (δ_H 1.60) to CH_3-21 (δ_C 28.4), C-12 (δ_C 47.0) and C-13 (δ_C 94.7) (Fig. 2). Meanwhile, the two moieties were also linked at C-3 and C-4 through a furan ring by the HMBC correlations from H-12 (δ_H 3.07) to C-3 (δ_C 105.4), C-4 (δ_C 166.9) and C-13 (δ_C 94.7) and the remaining one unsaturation (Fig. 2). The relative configuration of **4** was established by analyzing its NOESY data. Correlations from H_2-15 to H-17 determined that the C-16/C-17 double bond might be *E* conformation. And, the NOESY correlations from H_3-21 to H-12 suggested a same orientation of these protons. To further confirm the relative configuration of C-12, geometrical optimizations at TDDFT level for two isomers (12*S**)-**4a** and (12*R**)-**4b** were undertaken. Then, the NMR calculations performed through the QM-NMR method at the mPW1PW91/6-31G(d) level by the GIAO approach.[19,20] Further DP4+ analyses based on the ^{13}C NMR data indicated (12*S**)-**4a** (R^2 = 0.9980) was the correct structure with 100% probability (Fig. 5) Furthermore, the absolute configuration of **4** (12*S*) was determined by comparative analysis of calculated and experimental ECD spectra (Fig. 3). Finally, the structure of **4** was defined and named Gerberdriasin D.

Compound **5** was isolated as colorless oil. Its molecular formula was confirmed to be $C_{20}H_{24}O_5$ based on the positive HRESIMS data: 367.1514 ([M + Na]$^+$, calcd for $C_{20}H_{24}O_5Na$, 367.1516). Analyzing the ^{1}H and ^{13}C NMR spectroscopic data (Tables 1 and 2) in detail established that compound **5** consisted of a coumarin moiety and a monoterpene moiety. Explicit structural information was derived from 2D NMR spectroscopic data (^{1}H–^{1}H COSY and HMBC) (Fig. 2). The ^{1}H–^{1}H COSY correlations of H-6/H-7/H-8 and the HMBC correlations from H_2-11 (δ_H 5.85) to C-5, C-6 and C-10, from H-8 to C-6, C-9 and C-10 together with a conjugated system (a conjugated ester at δ_C 166.4, two olefinic carbons at δ_C 167.8 and δ_C 99.0), a characteristic of the 5-methylol-4-hydroxycoumarin moiety was composed. Furthermore, a furan ring newly generated fused with the coumarin moiety at C-4, C-10 and C-5 through C4–O–

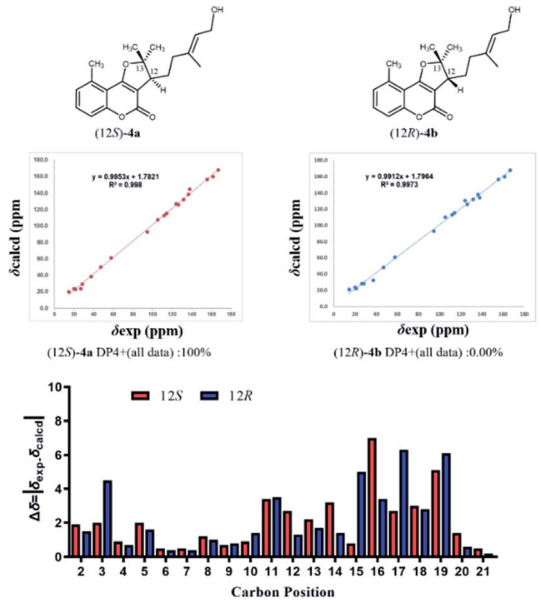

Fig. 5 NMR calculations with DP4+ analyses for (12*S*)-**4a** and (12*R*)-**4b**.

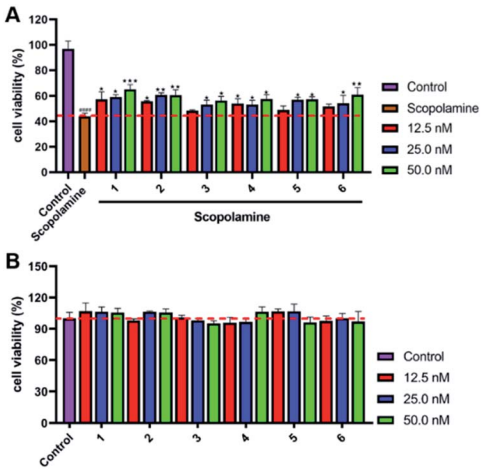

Fig. 6 Neuroprotective activity study *in vitro*, (A) the neuroprotective effects of compounds **1–6** on scopolamine-injured PC12 cells by CCK8 assay at 12.5, 25 and 50 nM dose concentrations. (B) The cytotoxic effect of these compounds on PC12 cells by CCK8 assay. All results were expressed as mean ± SD (n = 3). $^{\#\#\#\#}p < 0.0001$ *vs.* control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the scopolamine-treated group.

C11 bond. This conclusion was revealed by the HMBC cross-peaks from H_2-11 (δ_H 5.85) to C-4 (δ_C 167.8), C-5 (δ_C 138.5) and C-10 (δ_C 118.9). Another the spin-coupling system, H_2-15/H_2-16/H-17, in the ^1H–^1H COSY spectrum, as well as the HMBC correlations from H_3-19 to C-17, C-18 and C-20, from H_3-21 to C-13, C-14 and C-15, and from H_2-12 to C-13 revealed the presence of a monoterpene moiety. Meanwhile, the HMBC correlations from H_2-12 (δ_H 3.17) to C-3 (δ_C 99.0), C-2 (δ_C 166.4), C-4 (δ_C 167.8) and C-13 (δ_C 120.4) defined that this monoterpene moiety was attached to the 5-methylol-4-hydroxycoumarin moiety *via* a C3–C12 single bond. Meanwhile, the C-13/C-14 double bond was confirmed to be *E* conformation based on the NOESY correlations between H-13 and H_2-15. Additionally, based on the Mosher's method, the 17*S* configuration in **5** was established (Fig. 4). Therefore, its structure was depicted and named Gerberdriasin E.

Compound **6** was assigned a molecular formula of $C_{20}H_{22}O_4$ as established by the $[M + Na]^+$ ion peak m/z 349.1407 (calcd for $C_{20}H_{22}O_4Na$, 349.1410) in the positive HRESIMS data. Its ^1H and ^{13}C NMR data (Tables 1 and 2) were similar as those of **5** except that the appearance of an exocyclic $\Delta^{18(19)}$ double bond in **6** instead of an oxygenated quaternary carbon (C-18, δ_C 72.3) and a methyl (CH_3-19, δ_C 24.2) in **5**. This characteristic was verified by the less than 18 Da between their molecular weight, one more unsaturation as well as the HMBC correlations from H_2-19 (δ_H 4.86, 4.76) to C-17 (δ_C 74.6), C-18 (δ_C 147.4), C-20 (δ_C 16.1) (Fig. 2). Similar as **5**, it also tended to have an *E* double bond at C-13 and C-14 positions according to the NOESY data. Hence, the structure of **6** was deduced and named Gerberdriasin F.

All the isolated compounds (**1–6**) were tested for their protective effects on scopolamine-induced PC12 cells injury by the CCK8 assay. As shown in Fig. 6A and B, compounds **1–6** showed varying degrees of protection against scopolamine-damaged PC12 cells with no significant cytotoxicity at dose concentrations of 12.5, 25.0 and 50.0 nM. Furthermore, compound **1** showed stronger activities than others. Thus, we chose **1** as the material to further study neuroprotective effects.

Flow cytometry analysis was performed to investigate whether pre-treatment with **1** could reduce apoptosis in scopolamine-induced PC12 cells. As shown in Fig. 7, after treated with scopolamine, the apoptosis rate of PC12 cells was significantly increased (4.15% → 19.74%). However, the percentage of apoptotic cells was reduced to 13.99%, 8.70%, and 6.33% after pre-treatment with **1** at 12.5, 25.0 and 50.0 nM, respectively. Meanwhile, to examine whether **1** could enhance anti-oxidant activity, the MDA and SOD assay of PC12 cells were carried out. As shown in Fig. 8A, treatment with **1** prior to

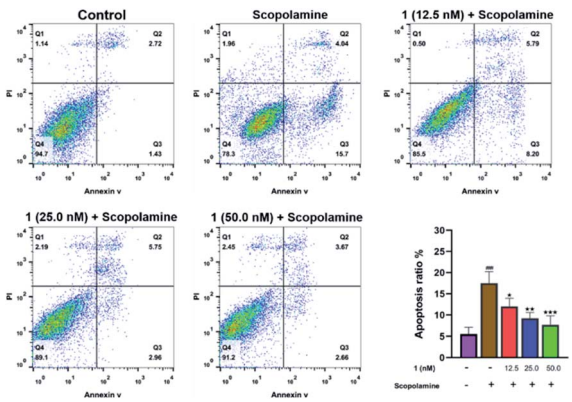

Fig. 7 Cell apoptosis was measured by flow cytometry analysis after Annexin V-FITC/PI staining, and the percentage of apoptotic cells was calculated in the bar chart. All results were expressed as mean ± SD (n = 3). $^{\#\#\#\#}p < 0.0001$ *vs.* control group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the scopolamine-treated group.

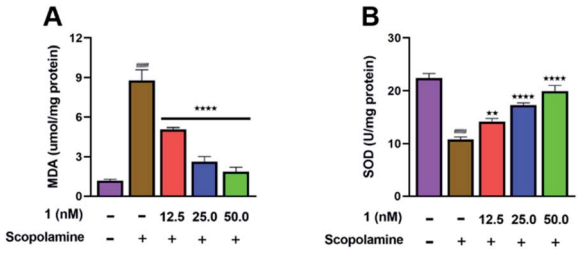

Fig. 8 The effect of **1** on the levels of MDA (A) and SOD (B). All results were expressed as mean ± SD ($n = 3$). $^{\#\#\#\#}p < 0.0001$ *vs.* control group. $^{**}p < 0.01$, $^{****}p < 0.0001$ *vs.* the scopolamine-treated group.

scopolamine evidently reduced the level of MDA, compare with the scopolamine group. Additionally, in comparison with the scopolamine group, pre-treatment with **1** significantly increased the SOD levels as shown in Fig. 8B. These above results exhibited that **1** could prevent apoptosis and improve anti-oxidant activity in scopolamine-induced PC12 cells.

Conclusions

In conclusion, Gerberdriasin A–F (**1–6**), six new coumarin derivatives were isolated and elucidated from the herb of *Gerbera anandria* (Linn) Sch-Bip. Their chemical structures including absolute configurations were identified by the analyses of their 1D and 2D NMR, HRESIMS spectra, Mosher's method, QM-NMR method and ECD calculation. In addition, all the isolated compounds revealed the neuroprotective effects on scopolamine-induced injury in PC12 cells. Meanwhile, pre-treatment with **1** significantly ameliorated apoptosis and oxidative stress in scopolamine-injured PC12 cells. Taken together, this research provided a series of rare coumarin derivatives as a candidate molecule for the treatment of neurodegenerative disease.

Conflicts of interest

The authors declare no conflicts of interest in this paper.

Acknowledgements

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