Fitoterapia 116 (2017) 99–105

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Isolation of novel stilbenoids from the roots of *Cyrtopodium paniculatum* (Orchidaceae)

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ARTICLE INFO

Article history:
Received 4 November 2016
Received in revised form 20 November 2016
Accepted 22 November 2016
Available online 28 November 2016

Keywords:
Cyrtopodium paniculatum
Orchidaceae
Stilbenoids
Phenanthrenes
Phenanthrene dimers
Human glioblastoma

ABSTRACT

Four new stilbenoids, named cyrtopodinone (**1**), cyrtopodinol (**2**), and coeludols A and B (**3**–**4**) were isolated from the roots of *Cyrtopodium paniculatum*, together with 21 known stilbenoids derivatives (**5**–**25**). Their structures were elucidated by comprehensive spectroscopic analysis including extensive 1D and 2D-NMR techniques. The cytotoxic activities of the isolated compounds were tested on human glioblastoma U-87MG cell line with fluorescence-based image cytometry. Only four compounds (**18**, **19**, **22** and **25**) displayed moderate cytotoxicity with IC_{50} values of 45.2, 39.9, 58.2 and 48.0 μM, respectively.

1. Introduction

The family Orchidaceae is considered as one of the largest family of flowering plant and is widely distributed in tropical and subtropical regions. The family consists of approximately 736 genera with over 30,000 species [1]. Several species in the family Orchidaceae are known to accumulate stilbenoids, known as phytoalexins as a means of protection against predation [2–4]. Stilbenoids are characterized by a C-6—C-2—C-6 skeleton [5] and they exhibit a fairly large structural diversity such as stilbenes [6], bibenzyls [7], 9,10-dihydrophenanthrenes [8,9], phenanthrenes [10], (dihydro)-phenanthrenequinones [11,12], *p*-hydroxybenzylphenanthrenes [13,14], 9,10-dihydrophenanthrofurans [15,16] and phenanthrene dimers [17,18]. Many of these compounds exhibited antimicrobial, anti-inflammatory, spasmolytic, antifibrotic and cytotoxic activities [10,19–21].

The genus *Cyrtopodium* comprises of 47 identified endemic species from Southern and Central America [22–24]. The terrestrial growing orchid *Cyrtopodium paniculatum* (Ruiz & Pav.) Garay is mostly distributed over Venezuela [24], Colombia [25], Peru [26] and Bolivia [27]. Previous phytochemical investigation done in our laboratory on the aerial parts of *C. paniculatum*, revealed the presence of a large stilbenoids composition [28]. These results compelled us to further investigate the stilbenoid content in the underground parts in order to discover potentially new compounds. Thus, the phytochemical investigation of the CH_2Cl_2 extract from *C. paniculatum* roots led us to the isolation of four new phenanthrene derivatives; a 9,10-dihydrophenanthrenequinone named cyrtopodinone (**1**), a 9,10-dihydrophenanthrene named cyrtopodinol (**2**) and two phenanthrene dimer named coeludols A and B (**3**–**4**), together with 21 known compounds. We herein report the isolation, structural elucidation and cytotoxic activities of the isolated compounds.

2. Experimental

2.1. General experimental procedure

Optical rotations were recorded on a Jasco P-2000 polarimeter (Jasco, Lisses, France). UV spectra were measured on a Shimadzu UV-2401 PC spectrometer (Shimadzu, Kyoto, Japan). IR spectra were obtained on a 380 FT-IR spectrophotometer (Thermo Electron Corporation, Saint Herblain, France). The NMR experiments were recorded with a Bruker 500 MHz Avance III spectrometer (Bruker Biospin, Rheinstetten Germany) equipped with a DCH $^{13}C/^{1}H$ Cryoprobe (Bruker Biospin, Rheinstetten Germany). Acetone-d_6 (Euriso-Top, Saint-Aubin, France) was used as deuterated solvent and its protonated residual signals were used as internal standard at 2.05 ppm relative to TMS. The HR-ESI-MS analyses were performed on a HPLC-DAD/UV-MS Agilent 1200 Series 6520 Q-ToF instrument (Agilent Technologies, Santa Clara, CA, USA) and the acquisition of mass spectra was conducted in ESI positive

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http://dx.doi.org/10.1016/j.fitote.2016.11.015
0367-326X/

ion mode. Vacuum Liquid Chromatography was carried out using a 40–60 μm silica gel (Sigma Aldrich, Saint-Louis, MO, USA). Fractions were monitored by TLC and the spots were visualized under UV light (254 nm) and with 2% sulfuric vanillin reagent. Sephadex LH-20 (Sigma Aldrich, St-Louis, MO, USA) was used for gel chromatography. Semi-preparative purification by RP-HPLC was carried out on a Gilson LC system (Gilson Inc., Limburg an der Lahn, Germany) equipped with a semi preparative Kinetex Axia C-18 column (100 mm × 21.2 mm, 5 μm; Phenomenex, Torrance, CA, USA). Preparative TLC were carried out on a glass supported silica gel $60F_{254}$ (0.5 mm thickness; Merck, Darmstadt, Germany). Analytical grade solvents of HPLC quality were purchased from Sigma Aldrich (Saint-Louis, MO, USA).

2.2. Plant material

Fresh specimens of *C. paniculatum* (Ruiz & Pav.) Garay were purchased in October 2013 from the orchid farm *Orquidea del Valle*, located in Ginebra (Colombia). They were imported into France in compliance with the CITES convention on biodiversity. Voucher specimens (No. 58054-58056) were deposited at the Herbarium CUVC Universidad del Valle in Cali, Colombia.

2.3. Extraction and isolation

The roots of *C. paniculatum* (171 g) were lyophilized and grinded into powder and then sequentially extracted at room temperature with cyclohexane, CH_2Cl_2 and CH_3OH (1 g/15 mL of solvent). The extracts were evaporated under reduced pressure to yield cyclohexane (0.88 g, 0.51% yield), CH_2Cl_2 (1.88 g, 1.10% yield) and CH_3OH (4.49 g, 2.63% yield) extracts. The CH_2Cl_2 extract was subjected to vacuum liquid chromatography (VLC) on silica gel eluted with cyclohexane, EtOAc and CH_3OH to afford 6 major fractions (A-F). Fraction B (471.1 mg) was subjected to Sephadex LH-20 (CH_3OH) to afford 15 sub-fractions (B1–B15). Sub-fraction B3 (25.8 mg) was subjected to a semi-preparative RP-HPLC (12% B for 3 min, 12% to 22.5% in 1 min, 22.5% B for 26 min, flow rate 26 mL/min, monitoring at λ = 260 nm, with A = H_2O + 0.05% FA; B = CH_3OH + 0.05% FA) to afford **1** (1.1 mg) and **2** (1.2 mg). Sub-fraction B4 (46.2 mg) was purified on semi-preparative RP-HPLC (using the same method as sub-fraction B3) to afford **5** (2.0 mg), **6** (1.4 mg), **7** (1.2 mg), **8** (3.4 mg) and **9** (2.8 mg). Sub-fraction B5 (61.7 mg) led to the isolation of **10** (6.4 mg), **11** (11.9 mg), **12** (1.5 mg) and **13** (5.2 mg) using semi-preparative RP-HPLC (12% B for 3 min, 12% to 22.5% in 1 min, 22.5% B for 26 min, flow rate 26 mL/min, monitoring at λ = 260 nm, with A = H_2O + 0.05% FA; B = CH_3OH + 0.05% FA). An aliquot (76 mg) of the sub-fraction B6 (127 mg) afforded compounds **14** (12.5 mg), **15** (14.5 mg) and **16** (5 mg) using semi-preparative RP-HPLC (12% B for 3 min, 12% to 22.5% in 1 min, 22.5% B for 26 min, flow rate 26 mL/min, monitoring at λ = 260 nm, with A = H_2O + 0.05% FA; B = CH_3OH + 0.05% FA). Sub-fraction B9 (10.6 mg) permitted the isolation of compounds **17** (1.5 mg), **3** (0.7 mg) and **4** (0.6 mg) through preparative TLC ($CHCl_3$/CH_3OH; 94:6). Sub-fraction B10 (12.7 mg) was subjected to preparative TLC ($CHCl_3$/CH_3OH 94:6) to afford the purification of **18** (1.0 mg), **19** (0.9 mg), **20** (0.7 mg) and **21** (0.6 mg). Sub-fraction B11 (5.7 mg) was subjected to preparative TLC ($CHCl_3$/CH_3OH 94:6) to afford the isolation of **22** (1.0 mg) and **23** (0.8 mg). Fraction B13 (3.8 mg) was subjected to preparative TLC ($CHCl_3$/CH_3OH 94:6) to afford **24** (0.6 mg). Sub-fraction B14 (5.2 mg) was subjected to preparative TLC ($CHCl_3$/CH_3OH 94:6) to purify compound **25** (1.3 mg).

2.3.1. Cyrtopodinone (*1*)

Orange amorphous powder (1.1 mg); UV (CH_3OH) λ_{max}(log ε): 203 (3.86), 219 (4.03), 256 (3.50), 269 (3.65), 338 (3.28), 468 (2.98), 488 (2.98) nm ; IR (FT-IR) ν_{max}: 3387, 2931, 2849, 2777, 1656, 1602, 1562, 1355, 1215, 1083, 1044, 842, 789, 769, 511 cm^{-1}, ^1H NMR and ^{13}C NMR see Table 1; HR-ESI-MS: *m/z* 317.1025 $[M + H]^+$ (calcd. $C_{17}H_{17}O_6$ for 317.1020).

Table 1
NMR data of compounds **1–2** (acetone-d_6, 500 MHz for ^1H, 125 MHz for ^{13}C).

Position	**1** δ_H (*J* in Hz)	δ_C	**2** δ_H (*J* in Hz)	δ_C
1		180.8	6.58 (s)	111.9
2		159.2		150.5
3	6.03 (s)	108.1		140.8
4		185.7		152.6
4a		142.2		120.2
4b		116.3		129.7
5		153.2	7.67 (s)	111.7
6		140.3		149.4
7		154.0		140.0
8	6.61 (s)	111.3		150.7
8a		137.8		123.2
9	2.47 (m)	29.0	2.65 (m)	22.3
10	2.58 (m)	20.8	2.58 (m)	30.7
10a		138.4		136.1
2-OCH_3	3.85 (s)	56.6		
3-OCH_3			3.86 (s)	60.9
4-OCH_3			3.73 (s)	60.5
5-OCH_3	3.85 (s)	60.5		
6-OCH_3	3.76 (s)	61.0		
7-OCH_3			3.84 (s)	61.1
8-OCH_3			3.80 (s)	60.9

2.3.2. Cyrtopodinol (*2*)

Yellow amorphous powder (1.2 mg); UV (CH_3OH) λ_{max}(log ε): 218 (4.37), 281 (4.04), 311 (3.80); IR (FT-IR) ν_{max}: 3365, 2930, 2843, 1584, 1454, 1409, 1321, 1134, 1208, 1165, 1114, 1089, 1062, 1010, 936, 843, 760, 510 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 1; HR-ESI-MS: *m/z* 333.1340 $[M + H]^+$ (calcd. $C_{18}H_{21}O_6$ for 333.1333).

2.3.3. Coeludol A (*3*)

Pale yellow amorphous powder (0.7 mg); UV (CH_3OH) λ_{max}(log ε): 213 (4.70), 263 (4.79), 297 (4.39), 309 (4.27), 349 (3.34), 367 (3.39), IR (FT-IR) ν_{max}: 3271, 2931, 2835, 1608, 1585, 1451, 1393, 1351, 1210, 1162, 1118, 1076, 999, 948, 865, 830, 527 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2; HR-ESI-MS: *m/z* 511.1751 $[M + H]^+$ (calcd. $C_{31}H_{27}O_7$ for 511.1751).

2.3.4. Coeludol B (*4*)

Pale yellow amorphous powder (0.6 mg); UV (CH_3OH) λ_{max}(log ε): 213 (4.80), 264 (4.89), 297 (4.49), 310 (4.36), 349 (3.49), 367 (3.43) cm^{-1}, IR (FT-IR) ν_{max}: 3268, 2930, 2835, 1609, 1582, 1450, 1393, 1352, 1211, 1164, 1112, 1076, 1000, 945, 865, 831, 525 cm^{-1}; ^1H NMR and ^{13}C NMR see Table 2; HR-ESI-MS: *m/z* 511.1777 $[M + H]^+$ (calcd. $C_{31}H_{27}O_7$ for 511.1751).

2.4. Biological assay

2.4.1. Chemical and reagents

DMSO-Hybrimax and Dulbecco's modified eagle's medium (DMEM-Glutamax) were purchased from Sigma Aldrich (Saint-Louis, MO, USA). Calcein-AM was purchased from ATT Bioquest® (Interchim, Montluçon, France), propidium iodide was purchased from Miltenyi Biotec (Paris, France) and Hoechst 33342 was purchased from Life Technologies (Courtaboeuf, France). The U-87 MG human cells line from the American Type Culture Collection (ATCC®, Manassas, VA, USA) was kindly provided by Professor M. Lehmann, of the Department of Biophotonic and Pharmacology, Strasbourg University.

2.4.2. Cell viability assessment

The human glioblastoma cell line U-87 MG cells were maintained in EMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 200 IU/mL penicillin/streptomycin and 0.6 mg/mL glutamine, in a 37 °C humidified incubator with 5% CO_2. Cells (50,000 cells/