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Cytotoxic phenolics from *Bulbophyllum odoratissimum*

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Abstract

A phytochemical study of the whole plant of *Bulbophyllum odoratissimum* (J.E. Smith) Lindl (Orchidaceae) and cytotoxicity measurement of its isolated compounds have been carried out. The ethyl acetate extract yielded nine phenolics, identified as moscatin (**1**), 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene (**2**), coelonin (**3**), densiflorol B (**4**), gigantol (**5**), batatasin III (**6**), tristin (**7**), vanillic acid (**8**) and syringaldehyde (**9**). Compounds **4** and **8** were discovered in the *Bulbophyllum* genus for the first time and all compounds, except for **9,** have not been reported in this plant before. The isolated compounds were evaluated *in vitro* for their inhibitory ability against the growth of human leukaemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402 and human stomach cancer SGC-7901. Densiflorol B (**4**) the most active compound, exhibited IC_{50} values of 0.08–3.52 μg/ml against the five tested cell lines.

Keywords: *Bulbophyllum odoratissimum*; Orchidaceae; Phenolics; Densiflorol B; Cytotoxic

1. Introduction

Bulbophyllum odoratissimum (J.E. Smith) Lindl (Orchidaceae) is a plant widely distributed in China, Nepal, Sikkim, Bhutan, India, Burma, Thailand, Laos and Vietnam and used in folk medicine to treat tuberculosis, chronic inflammation and fracture (Qu, Qin, Yang, Li, & Peng, 2006; Wu, 1990). In China, plants of *Bulbophyllum* genus are also used as a substitute for "Shi-hu", an important Chinese herb prepared from the dried stems of *Dendrobium* species (Orchidaceae) (Bao, Shun, & Chen, 2001).

The genus *Bulbophyllum* contains mainly phenanthrenes and bibenzyls and some of these compounds possess antitumour and NO release inhibitory activities (Leong & Harrison, 2004; Leong, Harrison, & Powell, 1999; Leong, Kang, Harrison, & Powell, 1997; Majumder & Banerjee, 1989; Majumder, Kar, & Shoolery, 1985; Majumder, Pal, & Majumder, 1999; Majumder, Roychowdhury, & Chakraborty, 1997; Wu, He, & Pan, 2006; Yao, Wang, Bei, & Liu, 2005a; Yao, Wang, Bei, Liu, & Zhang, 2005b). Investigation of the constituents of *B. odoratissimum* have revealed the presence of phenanthrone, lignan, flavonoids, bibenzyls, bulbophyllispiradienone and its derivatives, phenolic glycosides, aldehydes and acids (Lin et al., 2006; Liu et al., 2006; Majumder & Sen, 1991; Yao et al., 2005a, 2005b). During the search for bioactive compounds from medicinal plants in Yunnan of China, we investigated the whole plant of *B. odoratissimum* and isolated a phenanthrene, moscatin (**1**), two dihydrophenanthrenes, 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene (**2**) and coelonin (**3**), a phenanthoquinone, densiflorol B (**4**), three bibenzyls, gigantol (**5**), batatasin III (**6**) and tristin (**7**), and two simple aromatic compounds, vanillic acid (**8**)

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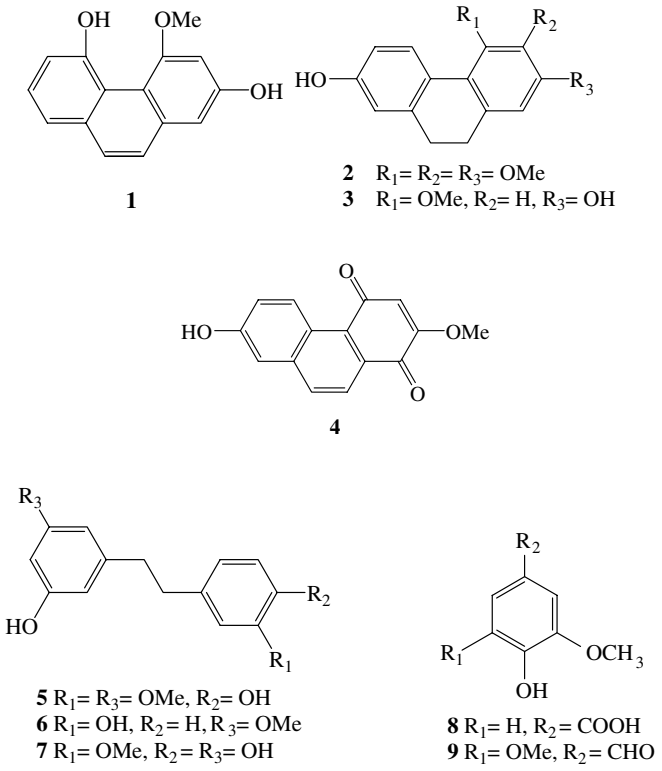

Fig. 1. Structures of constituents isolated from *B. odoratissimum*.

and syringaldehyde (**9**) (see Fig. 1). The isolation, structure elucidation and evaluation for cytotoxic activity of these compounds are described herein.

2. Materials and methods

2.1. General methods

MS were determined on an API Qstar Pulsar (Applied Biosystems, Foster City, CA) LC/TOF mass spectrometer. NMR spectra were measured on a Bruker DRX-500 spectrometer (Bruker Biosciences Corporation, Billerica, MA) with TMS as internal standard. Silica gel (200–300 mesh, Qingdao Marine Chemical Co., Qingdao, China) and Sephadex LH-20 (25–100 μm, GE Healthcare BioSciences AB, Uppsaki, Sweden) was used for column chromatography and silica gel GF_{254} for TLC (Qingdao Marine Chemical Co). Solvents were of industrial purity and distilled prior to use. MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Plant material

The whole plant of *B. odoratissimum* was collected from Simao County, Yunnan Province, China, in February, 2004, and identified by one of the authors, Dr. Hong Yu, School of Life Science, Yunnan University, where a voucher specimen (No. 0402017) is deposited.

2.3. Extraction and isolation

The dried powdered whole plant of *B. odoratissimum* (20 kg) was extracted with 95% ethanol (four volumes, each 20 l) at room temperature. The ethanol extract was evaporated *in vacuo*, to yield a dark brown residue (1 kg). Water (2.5 l) was added to the residue, and the resulting solution was extracted with petroleum ether, ethyl acetate and l-butanol successively (four volumes, each 1.5 l). Evaporation of the respective solvents gave petroleum ether (116 g, 0.58%), ethyl acetate (350 g, 1.75%) and l-butanol (500 g, 2.50%) extracts.

The ethyl acetate extract (350 g) was applied to a silica gel column, eluting with petroleum ether, containing increasing amounts of acetone to obtain 6 fractions. Fr. 2 (45 g) was purified on column chromatography (silica gel, petroleum ether: ethyl acetate 4:1) to afford **2** (32 mg, 0.00016%). Fr. 3 (77 g) was separated into two subfractions by silica gel column chromatography (petroleum ether/acetone 4:1, 7:3). The second subfraction (47 g) was subjected to repeated column chromatography, first on silica gel (chloroform:acetone 80:1) and then on Sephadex LH-20 (methanol:water 9:1) to obtain **1** (35 mg, 0.000175%), **3** (30 mg, 0.00015%), **4** (0.1 g, 0.0005%), **5** (0.3 g, 0.0015%), **6** (0.1 g, 0.0005%) and **9** (15 mg, 0.000075%). Fr. 4 (67 g) was subjected to repeated column chromatography (silica gel, chloroform:methanol 10:1), and then purified by chromatography over Sephadex LH-20 (methanol:water 9:1) to yield **7** (36 mg, 0.00018%) and **8** (9 mg, 0.000045%).

Moscatin (**1**) was obtained as a colourless powder. EI-MS: *m/z* (%) 240 (M^+, 100), 225 (49), 197 (45), 139 (18); ^{1}H NMR [$(CD_3)_2CO$, 500 MHz]: δ 9.57 (1H, s, 5-OH), 9.09 (1H, s, 2-OH), 7.62 (1H, d, $J = 8.8$, H-9), 7.50 (1H, d, $J = 8.8$, H-10), 7.43 (1H, dd, $J = 7.6$, 7.6, H-7), 7.40 (1H, dd, $J = 7.6$, 1.8, H-6), 7.13 (1H, dd, $J = 7.6$, 1.8, H-8), 7.09 (1H, d, $J = 2.5$, H-1), 7.01 (1H, d, $J = 2.5$, H-3), 4.15 (3H, s, 4-OMe); ^{13}C NMR [$(CD_3)_2CO$, 125 MHz]: δ 157.4 (C-4), 156.4 (C-2), 155.2 (C-5), 137.2 (C-10a), 135.0 (C-8a), 129.8 (C-7), 127.5 (C-9), 127.0 (C-10), 121.1 (C-8), 119.9 (C-4b), 117.1 (C-6), 114.0 (C-4a), 107.9 (C-1), 102.7 (C-3), 58.7 (4-OMe).

7-Hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene (**2**) was obtained as a colourless powder. EI-MS: *m/z* (%) 286 (M^+, 100), 271 (17), 228 (22), 157 (22); ^{1}H NMR [$(CD_3)_2CO$, 500 MHz]: δ 8.43 (1H, s, 7-OH), 8.09 (1H, d, $J = 8.4$, H-5), 6.74 (1H, d, $J = 2.6$, H-8), δ 6.72 (1H, dd, $J = 8.4$, 2.6, H-6), 6.69 (1H, s, H-1), 3.83 (3H, s, 3-OMe), 3.81 (3H, s, 2-OMe), 3.70 (3H, s, 4-OMe), 2.66 (4H, t, $J = 5.5$, $2CH_2$); ^{13}C NMR [$(CD_3)_2CO$, 125 MHz]: δ 156.8 (s, C-7), 152.6 (s, C-3), 152.3 (s, C-4), 142.6 (s, C-2), 140.3 (s, C-8a), 134.7 (s, C-10a), 129.3 (d, C-5), 125.1 (s, C-4a), 121.7 (s, C-4b), 115.3 (d, C-6), 114.1 (d, C-8), 108.7 (d, C-1), 60.9 (q, 2-OMe), 60.6 (q, 4-OMe), 56.3 (q, 3-OMe), 30.9 (t, C-9), 30.8 (t, C-10).

Coelonin (**3**) was obtained as a colourless powder. EI-MS: *m/z* (%) 242 (M^+, 100), 227 (17), 199 (37), 181 (24), 121 (26); ^{1}H NMR [CD_3OD, 500 MHz]: δ 7.99 (1H, d,

$J = 7.4$, H-5), 6.62 (1H, dd, $J = 7.4$, 2.5, H-6), 6.62 (1H, d, $J = 2.5$, H-8), 6.39 (1H, d, $J = 2.3$, H-3), 6.30 (1H, d, $J = 2.3$, H-1), 3.80 (3H, s, 4-OCH_3), 2.66 (4H, s, 2CH_2); ^{13}C NMR [CD_3OD, 125 MHz]: δ 158.6 (s, C-4), 156.9 (s, C-7), 155.5 (s, C-2), 141.4 (s, C-10a), 140.0 (s, C-8a), 129.5 (d, C-5), 125.7 (s, C-4b), 116.3 (s, C-4a), 114.6 (d, C-6), 113.1 (d, C-8), 107.9 (d, C-1), 98.9 (d, C-3), 55.4 (q, 4-OMe), 31.3 (t, C-9), 30.7(t, C-10).

Densiflorol B (**4**) was obtained as a reddish powder. EI-MS: *m*/*z* (%) 254 (M^+, 100), 225 (60), 197 (43), 155 (86); ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.13 (1H, d, $J = 9.5$, H-5), 7.86 (1H, d, $J = 8.5$, H-9), 7.75 (1H, d, $J = 8.5$, H-10), 7.19 (1H, dd, $J = 9.5$, 2.3, H-6), 7.09 (1H, d, $J = 2.3$, H-8), 6.00 (1H, s, H-3), 3.74 (3H, s, 2-OMe); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 188.6 (C-4), 180.5 (C-1), 158.4 (C-2), 157.7 (C-7), 139.1 (C-8a), 132.5 (C-9), 129.9 (C-10), 128.3 (C-10a), 126.9 (C-4a), 123.5 (C-4b), 122.6 (C-6), 122.0 (C-5), 111.0 (C-3), 110.0 (C-8), 56.5 (2-OMe).

Gigantol (**5**) was obtained as a reddish gum. EI-MS: *m*/*z* (%) 274 (M^+, 54), 137 (100), 122 (22), 107 (9), 94 (16), 77 (11); ^{1}H NMR [$(CD_3)_2CO$, 500 MHz]: δ 6.80 (1H, d, $J = 2.0$, H-2″), 6.74 (1H, d, $J = 8.0$, H-5″), 6.66 (1H, dd, $J = 2.0$, 8.0, H-6″), 6.33 (1H, dd, $J = 2.0$, 2.0, H-6′), 6.30 (1H, dd, $J = 2.0$, 2.0, H-4′), 6.26 (1H, dd, $J = 2.0$, 2.0, H-2′), 3.78 (3H, s, 5′-OMe), 3.69 (3H, s, 3″-OMe), 2.79 (2H, s, 2-CH_2), 2.78 (2H, s, 1-CH_2); ^{13}C NMR [$(CD_3)_2CO$, 125 MHz] δ 160.8 (C-5′), 158.2 (C-3″), 147.0 (C-3″), 144.5 (C-1′), 144.2 (C-4″), 133.1 (C-1″), 120.6 (C-6″), 114.6 (C-2″), 111.9 (C-5″), 107.9 (C-2′), 105.3 (C-6′), 98.7 (C-4′), 55.2 (OCH_3), 54.3 (OCH_3), 37.9 (C-2), 36.9(C-1).

Batatasin III (**6**) was obtained as a reddish gum. EI-MS: *m*/*z* (%) 244 (M^+, 30), 137 (100), 107 (43), 77 (8); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.12 (1H, dd, $J = 8.0$, 8.0, H-5″), 6.74 (1H, d, $J = 8.0$, H-6″), 6.67 (1H, dd, $J = 8.0$, 2.4, H-4″), 6.64 (1H, dd, $J = 2.4$, 2.4, H-2″), 6.34 (1H, dd, $J = 1.4$, 1.4, H-6′), 6.29 (1H, dd, $J = 1.4$, 1.4, H-2′), 6.27 (1H, dd, $J = 1.4$, 1.4, H-4′), 3.73 (3H, s, 5′-OMe), 2.80 (2H, m, 1-CH_2), 2.81 (2H, m, 2-CH_2); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 160.7 (C-5′), 156.4 (C-3′), 155.4 (C-3″), 144.4 (C-1′), 143.4 (C-1″), 129.3 (C-5″), 120.8 (C-6″), 115.4 (C-2″), 112.9 (C-4″), 108.2 (C-2′), 106.9 (C-6′), 99.3 (C-4′), 55.2 (5'-OCH_3), 37.3 (C-1), 36.9 (C-2).

Tristin (**7**) was obtained as a reddish gum. EI-MS: *m*/*z* (%) 260 (M^+, 22), 137 (100), 123 (5), 107 (2), 94 (50), 77 (3); ^{1}H NMR [$(CD_3)_2CO$, 500 MHz]: δ 6.80 (1H, d, $J = 1.9$, H-2″), 6.76 (1H, d, $J = 8.0$, H-5″) 6.67 (1H, dd, $J = 8.0$, 1.9, H-6″), 6.28 (2H, d, $J = 2.1$, H-2′, 6′), 6.26 (1H, t, $J = 2.1$, H-4′), 3.79 (3H, s, 3″-OMe), 2.88 (2H, m, 1-CH_2), 2.81 (2H, m, 2-CH_2); ^{13}C NMR [$(CD_3)_2CO$, 125 MHz]: δ 159.6 (s, C-3′), 159.6 (s, C-5′), 148.5 (s, C-3″), 145.7 (s, C-1′), 145.8 (s, C-4″), 134.8 (s, C-1″), 116.1 (d, C-5″), 122.1 (d, C-6″), 113.4 (d, C-2″), 108.5 (d, C-6′), 108.5 (d, C-2′), 101.7 (d, C-3′), 56.7 (q, 3″-OMe), 39.3 (t, 2-CH_2), 38.3 (t, 1-CH_2).

Vanillic acid (**8**) was obtained as colourless needles. ^{1}H NMR [$CDCl_3$, 500 MHz]: δ 7.59 (1H, dd, $J = 8.2$, 1.2, H-6), 7.55 (1H, d, $J = 1.2$, H-2), 6.90 (1H, d, $J = 8.2$, H-5), 3.90 (3H, s, 3-OMe); ^{13}C NMR [$CDCl_3$, 125 MHz]: δ 166.7 (s, –COOH), 151.2(s, C-3), 147.2 (s, C-4), 122.1 (d, C-2), 124.0 (d, C-5), 114.7 (d, C-6), 112.7(d, C-2), 55.5 (q, 3-OMe).

Syringaldehyde (**9**) was obtained as white needles. EI-MS: *m*/*z* (%) 182 (M^+, 51), 181 (46), 151 (26), 137 (100); ^{1}H NMR (CD_3OD, 500 MHz): δ 9.74 (1H, s, –CHO), 7.21 (2H, s, H-2, 6), 3.92 (6H, s, 3,5-OCH_3); ^{13}C NMR (CD_3OD, 125 MHz): δ 190.6 (s, –CHO), 147.2 (s, C-3,5), 141.2 (s, C-4), 126.8 (s, C-1), 105.8 (d, C-2,6), 54.4 (q, 3,5-OMe).

2.4. Cell growth inhibition assay

Growth inhibition by the sample of tumour cells was measured by microculture tetrazolium (MTT) assay, with minor modification (Alley et al., 1988; Mosmann, 1983; Zhou, Yue, Han, & Yang, 1993). Briefly, adherent cells were seeded into 96-well microculture plates and allowed to adhere for 24 h before drug addition, while suspended cells were seeded just before drug addition. The cell densities were selected based on the results of preliminary tests, in order to maintain the control cells in an exponential phase of growth during the period of the experiment and to obtain a linear relationship between the optical density and the number of viable cells. Each tumour cell line was exposed to sample at 0.01, 0.1, 1.0, 10 and 100 μM concentrations for different periods (adherent cells 72 h, suspended cells 48 h) and each concentration was tested in triplicate. At the end of exposure, 20 μl of 5 g per l MTT was added to each well and the plates were incubated for 4 h at 37 °C. Then triplex solution (10% SDS–5% isobutanol–0.012 M HCl) was added and the plates were incubated for 12–20 h at 37 °C. The optical density (OD) was read on a plate reader at 570 nm. Media and DMSO control wells, in which sample was absent, were included in all the experiments, in order to eliminate the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition } (\%) = (\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}/\text{OD}_{\text{control}}) \times 100\%.$$

The cytotoxicity of sample on tumour cells was expressed as IC_{50} values (the drug concentration reducing by 50% the absorbance in treated cells, with respect to untreated cells), which were calculated by LOGIT method.

3. Results and discussion

3.1. Phytochemical investigation

The 95% ethanol extract of *B. odoratissimum* was partitioned successively with petroleum ether, ethyl acetate and l-butanol. The ethyl acetate fraction was subjected to a succession of chromatographic procedures, including silica gel chromatography and gel permeation chromatography using Sephadex LH-20 to afford 9 isolates.

Table 1
Cytotoxicity of compounds **1**–**9** isolated from *B. odoratissimum*

Compound	IC_{50} value (μg/ml)				
	K562	HL-60	A549	BEL-7402	SGC-7901
Moscatin	29.6	4.60	8.74	7.16	10.9
7-Hydroxy-2,3,4-trimethoxy-9, 10-dihydrophenanthrene	>100	9.76	74.2	115	>100
Coelonin	>100	>100	33.1	17.1	48.4
Densiflorol B	3.52	0.08	2.9	1.38	2.34
Gigantol	19.2	8.19	13.4	3.96	5.29
Batatasin III	33.7	15.1	27.1	7.48	19.0
Tristin	28.8	5.02	8.76	4.06	2.08
Vanillic acid	>100	>100	>100	>100	66.8
Syringaldehyde	21.9	6.01	17.6	1.54	18.5
Cisplatin	0.08	0.70	0.44	0.18	0.21

On the basis of spectroscopic data analysis (NMR and MS) and comparison with reports in the literature, compounds **1**–**9** were identified to be moscatin (**1**) (Majumder & Sen, 1987), 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene (**2**) (Honda & Yamaki, 2001), coelonin (**3**) (Majumder, Laha, & Datta, 1982), densiflorol B (**4**) (Fan, Wang, Wang, Qin, & Zhao, 2001), gigantol (**5**) (Juneja, Sharma, & Tandon, 1987; Leong et al., 1997), batatasin III (**6**) (Juneja et al., 1987; Leong et al., 1997; Lin, Chang, Chen, Wang, & Tsao, 2001), tristin (**7**) (Majumder & Pal, 1993), vanillic acid (**8**) and syringaldehyde (**9**). Compounds **4** and **8** were discovered in the *Bulbophyllum* genus for the first time and the other compounds except for **9** had not been reported in *B. odoratissimum* previously.

3.2. Cytotoxic activity

As several phenanthrenes and bibenzyls from plants of Orchidaceae were found to possess anti-tumour activity (Gong et al., 2004; Lee, Park, Baek, Kim, & Ahn, 1995; Lin et al., 2001), the isolated compounds were evaluated *in vitro* for their inhibitory ability against the growth of human leukaemia cell lines K562 and HL-60, human lung adenocarcinoma A549, human hepatoma BEL-7402 and human stomach cancer SGC-7901, using cisplatin as a positive control. Densiflorol B (**4**), the most active compound, exhibited IC_{50} values of 0.08–3.52 μg/ml against five cell lines (Table 1). Tristin (**7**) displayed selective cytotoxicity against SGC-7901, with an IC_{50} value of 2.08 μg/ml whereas syringaldehyde (**9**) exerted activity against BEL-7402 with an IC_{50} value of 1.54 μg/ml. The other compounds evaluated were weak or inactive. Moscatin (**1**) has been previously tested on HL-60 cells *in vitro*, with an IC_{50} value of 48.24 μg/ml. Although the results presented here suggest moscatin is more active than reported in the literature, all published results showed that moscatin has weak cytotoxicity against HL-60 cells (Tan et al., 2006).

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