Regular Article

Four New Compounds Isolated from the Aerial Part of Belamcanda chinensis (L.) and Their Effect on Vascular Smooth Muscle Cell (VSMC) Proliferation

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Bio-guided fractionation of the 70% ethanol extract of Belamcanda chinensis (L.) DC. revealed four new compounds, including 6′-O-acetylenolin (5), 3′-O-acetylenolin (6), irigenin 3′-O-β-glucopyranoside (8), and 2′-acetyl-1,3-O-diferuloylsucrose (9), along with five known compounds (1–4, 7). Their chemical structures were determined using extensive NMR data, mass spectroscopy, and comparison with published literature. Among the isolates, compounds 1 and 4–7 achieved good regulation of the growth and proliferation of vascular smooth muscle cells.

Key words Belamcanda chinensis; Iridaceae; flavonoid glycoside; 2′-O-acetyl-1,3-O-diferuloylsucrose; vascular smooth muscle cell (VSMC) proliferation

Introduction

Belamcanda chinensis (L.) DC. (BC) is a popular ornamental and medicinal plant in the Iridaceae family. The dried rhizomes of BC are used in Vietnamese traditional medicine for treatment of inflammation and respiratory disorders, such as asthma, coughing, tonsillitis, and pharyngitis.13 Previous phytochemical studies have identified isoflavones and isoflavone aglycones as the main components of this plant. Other chemical groups found in this plant include benzoquinones, phenolics, iridal-type triterpenoids, and steroids.21 The pharmacological effects of the BC rhizomes have been demonstrated by modern biological assays. BC rhizome properties include anti-inflammatory,3 anti-tumor,4,5 antioxidant, hepatoprotection,6,7 estrogen receptor modulation,8,9 and aldose reductase inhibition.10 Recent studies have reported the hypoglycemic effect of BC leaf extract in normal and streptozotocin (STZ)-induced diabetic rats,11,12 due to the presence of isoflavones.13 In this study, we focused on the phytochemical extraction, isolation, and identification of new compounds from the aerial part of BC and evaluated effects on vascular smooth muscle cell (VSMC) proliferation.

Results and Discussion

The 70% ethanol extract from the aerial parts of BC was suspended in distilled water and then successively partitioned with n-hexane and EtOAc. The total extract and all the fractions, including n-hexane and EtOAc and the remaining water layer, were screened for their effects on the proliferation of VSMCs. Since the EtOAc-soluble fraction showed the most potent inhibitory activity on VSMC proliferation at the concentration of 20 µg/mL, this fraction was used to isolate compounds using repeated silica gel, reversed phase column chromatography and preparative (p)-HPLC purifications. The isolation process revealed four new compounds (5, 6, 8, and 9) and five known compounds (1–4, 7).

Compound 5 was obtained as a yellow amorphous solid. The maximum UV absorptions at 216, 273, and 329 nm suggested that this compound was a flavone.14 The IR spectrum showed absorption bands for hydroxyl (3360 cm−1), conjugated ketone (1649 cm−1), conjugated olefin (1606 cm−1), and aromatic moiety (1489 cm−1). The molecular formula was determined by high-resolution electrospray ionization (HR-ESI)-MS at m/z 647.1995 [M−H]+ (Calcd for C31H29O12, 647.1976). The 1H-NMR data (Table 1) of 5 showed the signals of two AA′BB′ systems of 4′-substituted B-rings at δH 8.10 (or 8.09) (2H, d, J = 9.0 Hz, H-2′ and H-6′) and 7.13 (2H, d, J = 9.0 Hz, H-3′ and H-5′); a pair of singlet signals for H-3 at δH 6.97 and 6.95; a pair of singlet signals for H-8 at δH 6.88 and 6.89; a singlet signal for chelated 5-OH at δH 13.43; a pair of singlet signals for 7-OCH3 at δH 3.91 and 3.90; and a singlet signal for 4′-OCH3 at δH 3.87. The signals of anomeric protons at δH 4.69 (or 4.70) (1H, d, J = 10.0 Hz, H-1′) and 5.07 (or 4.98) (1H, d, J = 10.0 Hz, H-1′) together with heteronuclear multiple bond correlations (HMBCs) of the proton at δH 5.07 (4.98) with the carbon signal at δC 74.3 (75.8) indicated the existence of the disaccharide moiety in 5. The chemical shifts of the anomeric carbons at δC 71.1 (or 70.8) and 100.3 (or 100.8) indicated C- and O-glycosides, respectively. The doublet methyl signal at δH 0.47 (or 0.59) (3H, d, J = 6.0 Hz, H-6″) in the 1H-NMR revealed a rhamnopyranosyl unit. The α-rhamnopyranosyl unit was assigned by the small coupling constant (1.0 Hz) of H-1″. The other sugar was determined to be a β-glucopyranosyl unit. The doublet methyl signal at δH 0.55 (1H, d, J = 6.6 Hz) and a multiplet at δH 0.48 (1H, d, J = 6.6 Hz) in the 13C-NMR revealed a glucose unit. The α-glucopyranosyl unit was assigned by the high coupling constant (2.0 Hz) of H-1‴. The other sugar was determined to be a β-glucopyranosyl unit.
scopic data of 5 were similar to those of embinin (4), except for an additional proton chemical shift of an acetoxy group at δH 1.99 (3H, s) and 13C chemical shifts at δC 20.7 and 170.4. The obvious downfield shift of H-6" (δH 4.39 and 3.86) in 5 compared with that of 4 (δH 3.73 and 3.36), as well as the HMBC correlation of H-6" with the carboxy carbon of an acetoxy group, confirmed the position of the additional acetoxy group at C-6". Based on the above analysis, the structure of 5 was determined to be 5-hydroxyl-7,4′-dimethoxy-6-[2-O-(α- \text{rhamnopyranosyl})]-6-O-acetyl-glucopyranosyl]flavone and was named 6"-O-acetylembinin (Fig. 1).

The 1H- and 13C-NMR signals of 6 (Table 1) shared similarities with those of 4, except for the presence of an additional acetoxy group (δH 2.07 [or 2.08], δC 21.2 and 170.3 [or 170.2]). The position of this acetoxy group at C-3" was determined based on the HMBC correlation between the signals of δH 4.97 (H-3") and δC 170.3/170.2 and the correlation spectroscopy (COSY) correlations of H-3" (δH 7.97) with both H-2" (δH 4.50/4.33) and H-4" (δH 3.31). Moreover, the chemical shift of H-3" (δH 4.97) in 6 differed from that of 4 (δH 3.36), indicating that acetoxy group was connected to C-3" position. This result was supported by HMBC analysis (Fig. 2). Thus, the chemical structure of 6 was shown to be 3"-O-acetylembinin (Fig. 1).

Duplicate signals shown in the NMR spectra of 5 and 6 were caused by rotational hindrance at the C(6)–C(1") linkage, which is very common for 6-C-glycosylflavones.

Compound 8 was purified as a yellow amorphous solid. The UV spectrum was characteristic of isoflavones showing absorption maxima at 244 and 270 nm. The molecular formula of 8 was determined to be C23H29O13 using HR-ESI-MS at m/z 521.3238 [M+H] (Caled for C23H28O13, 521.1295). The 1H-NMR spectrum of 8 displayed one C-8 proton at δH 6.45 (1H, s) in an A-ring, one C-2' proton at δH 8.20 (1H, s), three methoxy groups at δH 3.89 (3H, s, 5'-OCH3), 3.87 (3H, s, 4'-OCH3), and 3.88 (3H, s, 6-OCH3), and an anomic proton at δH 4.98 (1H, d, J = 7.5 Hz, H-1'). Two protons in ring B appeared as two doublets at δH 7.03 (1H, d, J = 1.5 Hz, H-2') and 6.99 (1H, d, J = 1.5 Hz, H-6'). The position of methoxy groups at C-6, C-4', and C-5' was identified by the HMBC correlations between the proton signals of methoxy groups at δH 3.89, 3.87, 3.86, and 3.85, respectively.

### Table 1. NMR Data of Compounds 5 and 6 (DMSO-d6)

| No. | Major δCb | Minor δCb (J in Hz) | Major δHb | Minor δHb (J in Hz) |
|-----|------------|--------------------|------------|--------------------|
| 2   | 163.3      | 163.3              | 163.6      | 163.6              |
| 3   | 103.6      | 9.67 s             | 103.8      | 6.95 s             |
| 4   | 182.3      | 181.9              | 182.5      | 182.0              |
| 5   | 160.3      | 159.2              | 160.6      | 159.6              |
| 6   | 109.6      | 109.4              | 108.9      | 108.8              |
| 7   | 164.8      | 163.3              | 165.1      | 163.6              |
| 8   | 90.5       | 6.88 s             | 91.5       | 6.89 s             |
| 9   | 156.9      | 157.1              | 157.2      | 157.4              |
| 10  | 104.9      | 104.2              | 105.0      | 104.3              |
| 5-OH| 13.43      | s                  | 13.50      | s                  |
| 7-OMe| 56.6      | 3.91 s             | 56.8       | 3.91 s             |
| 1'  | 122.5      | 122.6              | 122.6      | 122.6              |
| 2', 6' | 128.4     | 8.10 d (9.0)       | 128.5      | 8.06 d (9.0)       |
| 3', 5' | 114.7     | 7.13 d (9.0)       | 114.8      | 7.10 d (9.0)       |
| 4'  | 162.5      | 162.5              | 162.7      | 162.6              |
| 4'-OMe| 55.6      | 3.87 s             | 55.7       | 3.85 s             |
| 6-C-Glc' | 71.1     | 4.69 d (10.0)      | 71.3       | 4.79 d (10.0)      |
| 2'  | 74.3       | 4.32 t (10.0)      | 74.1       | 4.50 t (9.5)       |
| 3'  | 77.8       | 3.37 m             | 80.3       | 4.97 t (9.0)       |
| 4'  | 70.5       | 3.59 m             | 68.4       | 3.31 m             |
| 5'  | 79.5       | 3.37 m             | 81.5       | 3.31 m             |
| 6'  | 64.4       | 4.39 m             | 61.4       | 3.37 m             |
| 3'-OAc| 3.86 m | s                  | 3.43 m     | s                  |
| 6'-OAc| 20.7      | 1.99 s             | 20.7       | 1.99 s             |
| 1'w | 100.3      | 5.07 d (1.0)       | 100.8      | 4.98 d (1.0)       |
| 2'w | 70.4       | 3.06 ddd (9.0, 6.0, 3.0) | 70.4       | 3.12 ddd (9.0, 6.0, 3.0) |
| 3'w | 70.3       | 3.17 m             | 70.3       | 3.06 dd (9.5, 3.0) |
| 4'w | 71.5       | 2.90 td (9.0, 4.5) | 71.6       | 2.90 td (9.0, 4.5) |
| 5'w | 68.2       | 2.12 dp (12.0, 6.0) | 68.2       | 2.24 dp (12.0, 6.0) |
| 6'w | 17.5       | 0.47 d (6.0)       | 18.0       | 0.59 d (6.0)       |

a) Recorded at 125MHz. b) Recorded at 500MHz.
3.88 (3H each, s) and C-5′ (δC 154.0), C-4′ (δC 140.0), and C-6 (δC 133.2), respectively. The existence of a β-glucopyranosyl moiety was suggested by the coupling constant of the anomeric proton and 13C-NMR data. The 1H- and 13C-NMR spectra of 8 resembled those of irigenin,17 except for the presence of a β-glucopyranosyl moiety. The HMBC correlations of the anomeric proton at δH 4.98 and H-2′ at δH 7.03 with a carbon signal at δC 152.1 suggested that the β-glucopyranosyl moiety was placed on C-3′ (Fig. 2). From the results described above, the structure of 8 was determined to be irigenin 3′-O-β-glucopyranoside (Fig. 1).

Compound 9 was isolated as a pale amorphous solid. The UV spectrum exhibited absorption maxima at 264 and 328 nm. The IR spectrum showed absorption bands at 3331 cm⁻¹ (OH), 1651 cm⁻¹ (conjugated CO), and 1604 cm⁻¹ (aromatic C=C). The molecular formula was determined to be C34H40O18 using HR-ESI-MS at m/z 735.2176 [M−H]⁻ (Calcd for C34H39O18, 735.2136). The 1H-NMR spectrum of 9 showed two sets of signals for the trans olefinic protons at δH 7.65 (1H, d, J = 16.0 Hz, H-7″), 6.37 (1H, d, J = 16.0 Hz, H-8″), 7.71 (1H, d, J = 16.0 Hz, H-7‴) and 6.49 (1H, d, J = 16.0 Hz, H-8‴); two sets of 1,3,4-trisubstituted aromatic ring protons at δH 7.18 (1H, d, J = 2.0 Hz, H-2″), 6.77 (1H, d, J = 8.0 Hz, H-5″), 7.06 (1H, dd, J = 8.0, 2.0 Hz, H-6″), 7.23 (1H, d, J = 2.0 Hz, H-2‴), 6.82 (1H, d, J = 8.0 Hz, H-5‴), and 7.12 (1H, dd, J = 8.0, 2.0 Hz, H-6‴); and two methoxy groups at δH 3.90 (3H, s, 3″-OCH3) and 3.84 (3H, s, 3‴-OCH3), which suggested the existence of two feruloyl units in 9. A doublet signal with a small coupling constant at δH 5.65 (1H, d, J = 3.5 Hz, H-1′), as well as signals at δC 91.2, 74.5 (2C), 72.3, 71.3 and 62.2
indicated the presence of an \( \alpha \)-glucopyranosyl moiety in 9.\(^{(18)} \)

Furthermore, signals at \( \delta_c \) 66.4, 103.4, 79.8, 73.4, 84.0, and 63.1 suggested that 9 contained a fructofuranosyl moiety. The existence of a sucrose moiety in 9 was revealed due to the HMBC correlation of H-1 (\( \delta_H \) 4.29 and 4.20) with C-9 (\( \delta_C \) 168.4) (Fig. 2). The spectroscopic data of 9 was similar to that of 1,3-O-diferuloylsucrose,\(^{(10)} \) except for the presence of an acetoxy group at \( \delta_H \) 2.11 (3H, s) and \( \delta_C \) 172.8 and 21.1, which linked to C-2 (\( \delta_C \) 74.5) based on the HMBC cross-peak from H-2 (\( \delta_H \) 4.64) to the carboxy carbon of the acetoxy group (\( \delta_C \) 172.8), in conjunction with the COSY correlations of H-2 (\( \delta_H \) 4.64) with both H-1 (\( \delta_H \) 5.65) and H-3 (\( \delta_H \) 3.86). Based on the above spectroscopic data, 9 was determined to be 2′-acetyl-1,3-O-diferuloylsucrose (Fig. 1).

Based on spectroscopic analysis and comparison with the literature, the structures of known compounds were determined to be isoswertisin (1),\(^{(20)} \) 2′-O-\( \alpha \)-l-rhamnosyl-4′-O-methylisovitexin (2),\(^{(21)} \) 2′-O-rhamnosyliswertisin (3),\(^{(22)} \) em-binin (4),\(^{(25)} \) and iridin (7)\(^{(23)} \) (Fig. 1).

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall.\(^{(24)} \) VSMC proliferation plays an important role in vessel-wall remodeling and is a crucial event in the formation of atherosclerosis. Thus, inhibition of VSMC proliferation can potentially decrease the incidence of vascular occlusive diseases.\(^{(25,26)} \)

In a preliminary cell proliferation assay, 70% ethanol extract (BCL 70EtOH), \( n \)-hexane (BCL HF), ethyl acetate (BCL EF), and water fractions (BCL WF) were screened for their effects on VSMC proliferation in human VSMC cells. The results showed that the BCL EF significantly inhibited VSMC proliferation at a concentration of 20 \( \mu \)g/mL (Fig. 3A). Eight compounds (1–7 and 9) were obtained from the most active BCL EF fraction and were screened for their effect on VSMC proliferation. Compounds 1 and 4–7 showed considerable inhibitory effects, whereas compounds 2, 3, and 9 demonstrated weak inhibitory effects on VSMC proliferation at a concentration of 30 \( \mu \)M (Fig. 3B). These results provide initial evidence and incentive to further study the aerial part of BC and associated compounds on VSMC proliferation as a treatment for atherosclerosis.

### Experimental

**General Experimental Procedures** UV spectra were recorded in MeOH on a JASCO V-550 UV/Vis spectrometer with a 0.5 nm resolution, and IR spectra were measured in MeOH on Shimadzu FT-IRAffinity-1S and presented in cm\(^{-1}\). NMR spectra were recorded on a Bruker Avance Digital 500 MHz NMR spectrometer (Karlsruhe, Germany) with tetramethylsilane (TMS) as the internal standard, \( J \) in Hz at 294 K. HR-ESI-MS data were analyzed via an Agilent
After 24 h incubation, they were treated with 25 ng/mL PDGF-BB (Upstate Biotechnology, NY, U.S.A.) and further incubated for another 24 h. After the incubation period, add 10 μL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) labeling reagent and follow protocol from cell proliferation kit (11465007001, Sigma).

**Statistical Analysis** Quantitative data are presented as mean ± standard error of the mean (S.E.M.). Data were analyzed using GraphPad Prism Software 5. Statistical analysis was performed by Student’s t-test or one-way ANOVA for multiple comparisons. Differences were considered significant when p < 0.05.

**Plant Material** The aerial parts of *Belamcanda chinensis* were collected in Thanh Hoa province (Vietnam), in July 2013 and botanically identified by Tran The Bach, Ph.D., Department of Herbarium, Institute of Ecology and Biological Resources, Vietnam. A voucher specimen (BUTHIBINH-I) has been deposited at the Herbarium of NIMM, Hanoi, Vietnam.

**Extraction and Isolation** The dried leaves of BC (2.0 kg) were extracted and refluxed with 70% ethanol (10 L × 3× 3 times) at 70°C. The combined extracts were filtered and evaporated under reduced pressure to yield a residue (500.0 g, BCL 70EtOH), which was suspended in H2O and successively partitioned with organic solvents, then concentrated to yield 3 fractions of n-hexane (78.0 g, BCL-HF), EtOAc (54.0 g, BCL EF), and a water-soluble layer (335.0 g, BCL WF). The EtOAc extract (54.0 g) was fractionated via silica gel column chromatography and eluted with MC–MeOH (from 100 : 0 to 0:100, v/v) to yield 12 fractions (1A–1M). Fraction 1F (226.2 mg), 2E (226.2 mg), and 2G (1.12 g) were recrystallized to yield compounds 1 (BCLE-2D1, 161.6 mg), 7 (BCLE-2E1, 45.2 mg), and 5 (BCLE-2G1, 115.3 mg), respectively. Fraction 2F (1.50 g) was chromatographed on MCI gel CC with an elution mixture of MeOH–H2O (50–100%, v/v) to yield 7 fractions (2A–2G).

**Cell Culture** Human vascular smooth muscle cells were obtained from ATTCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F12 medium (Biochrom, FG 4815) supplemented with 10% fetal bovine serum (FBS, Biochrom, S 0115) and 50 μg/mL penicillin/streptomycin (Biochrom, A 2212) at 37°C and 5% CO2.

**Cell Proliferation** Initially, the cells were seeded into 96-well culture plates at 5 × 103 cells/well and cultured in DMEM containing 10% FBS at 37°C for 24 h. When cells reached up to 70% confluence, the medium was replaced with serum-free DMEM containing 20 μg/mL of the aerial extract and fractions of BC or 30 μM of the isolated compounds.

### Table 3. NMR Data of Compound 9 (CD3OD)

| No. | δH (J in Hz) | δC |
|-----|--------------|----|
| 1   | 66.4         | 4.29 H, d, 11.5 | 114.9 H, d, 16.0 |
| 2   | 79.8         | 5.51 H, d, 8.0  |
| 3   | 73.4         | 4.40 H, t, 8.0  |
| 4   | 84.0         | 3.95 H, m       |
| 5   | 84.0         | 3.86 H, m       |
| 6   | 91.2         | 5.65 H, d, 3.5  |
| 7   | 74.5         | 4.64 H, d, 10.0 |
| 8   | 72.3         | 3.86 H, m       |
| 9   | 71.3         | 3.49 H, m       |
| 10  | 74.5         | 3.95 H, m       |
| 11  | 62.2         | 3.89 H, m       |
| 12  | 7.77 H, d, 12.0 | 2E1 R = 21.0 min |
| 13  | 21.1         | 2.11 H, s       |
| 14  | 127.6        |                |
| 15  | 111.6        | 7.18 H, d, 2.0  |
| 16  | 149.4        |                |
| 17  | 150.9        |                |
| 18  | 116.5        | 6.77 H, d, 8.0  |
| 19  | 124.4        | 7.06 H, d, 8.0  |
| 20  | 147.5        | 7.65 H, d, 16.0 |
| 21  | 114.9        | 6.37 H, d, 16.0 |
| 22  | 168.4        |                |
| 23  | 127.6        |                |
| 24  | 112.0        | 7.23 H, d, 2.0  |
| 25  | 149.4        |                |
| 26  | 150.8        |                |
| 27  | 116.5        | 6.82 H, d, 8.0  |
| 28  | 124.4        | 7.12 H, d, 8.0  |
| 29  | 148.1        | 7.71 H, d, 16.0 |
| 30  | 114.7        | 6.49 H, d, 16.0 |
| 31  | 168.3        |                |
| 32  | 56.5         | 3.90 H, s       |
| 33  | 56.4         | 3.84 H, s       |

**6-O-Acetylambinin (5)** Yellow amorphous solid. UV max (MeOH) 329, 273, 216 nm. IR (νmax): 3360 (OH), 1649 (conjugated CO), 1606 (aromatic C–C), 1489 (aromatic moiety) cm−1. 1H- and 13C-NMR (DMSO-d6) data, see Table 1. HR-ESI-MS m/z 647.1995 [M+H]− (Calcd for C31H35O15, 647.1976).

**3′-O-Acetylambinin (6)** Yellow amorphous solid. UV max (MeOH) 331, 273, 215 nm. IR (νmax): 3381 (OH), 1649 (conjugated CO), 1602 (aromatic C–C), 1489, 1442 (aromatic moiety) cm−1. 1H- and 13C-NMR (DMSO-d6) data, see Table 1. HR-ESI-MS m/z 647.1999 [M+H]− (Calcd for C31H35O15, 647.1976).
Irigenin 3′-O-β-D-Glucopyranoside (8)

Yellow amorphous solid. UV \( \lambda_{\text{max}} \) (MeOH) 270, 244 nm. \(^1\text{H}\) and \(^{13}\text{C}\)-NMR (CD3OD) data, see Table 2. HR-ESI-MS m/z 521.1328 [M−H]− (Calcd for C24H25O13, 521.1295).

Pale amorphous solid. UV \( \lambda_{\text{max}} \) (MeOH) 328, 264 nm. IR (\( \nu_{\text{max}} \)) 3331 (OH), 1651 (conjugated CO), 1604 (aromatic C=O) cm\(^{-1}\). \(^1\text{H}\) and \(^{13}\text{C}\)-NMR (CD3OD) data, see Table 3. HR-ESI-MS m/z 735.2176 [M−H]− (Calcd for C34H39O18, 735.2136).

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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