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ORIGINAL ARTICLE

Two new 4-arylcoumarins from the seeds of *Calophyllum polyanthum*

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Two new 4-arylcoumarins, 7,4'-dihydroxy-6,8-dimethoxy-4-phenylcoumarin (**1**) and 7-hydroxy-6,8,4'-trimethoxy-4-phenylcoumarin (**2**), together with four known compounds were isolated from the seeds of *Calophyllum polyanthum*. The structures of the new compounds were determined by extensive spectroscopic analyses, and the structure of compound **2** was confirmed by X-ray crystallography analysis. Both new compounds exhibited significant cell protective activities against H₂O₂-induced human umbilical vein endothelial cell damage.

Keywords: *Calophyllum polyanthum*; Guttiferae; 4-arylcoumarins; cell protecting activity

1. Introduction

The genus *Calophyllum*, which comprises more than 200 species, is widely distributed in the tropical rain forest. Four species of this genus, *Calophyllum inophyllum* L., *Calophyllum membranaceum* Gardn. et Champ, *Calophyllum blancoi* Planch et Triana, and *Calophyllum polyanthum* Wall. Et Choisy, are found in South and Southwest China [1]. A variety of coumarins [2–7] and xanthenes [8–12] have been isolated from this genus, and some of them exhibited remarkable biological activities. The plant *C. polyanthum* Wall. Et Choisy (Guttiferae) is an arbor, which has been used to treat traumatic bleeding and to relieve pain in Chinese folk medicine [13]. Chemical studies conducted previously on *C. polyanthum* reported the major presence of coumarins [14,15]. In the current study, two new 4-arylcoumarins, 7,4'-dihydroxy-6,8-dimethoxy-4-phenyl-

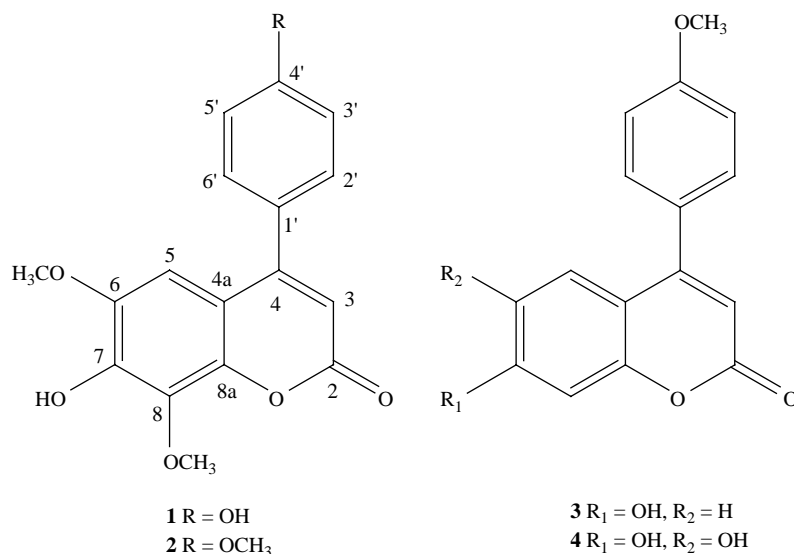
coumarin (**1**) and 7-hydroxy-6,8,4'-trimethoxy-4-phenylcoumarin (**2**), together with four known compounds, 7-hydroxy-4'-methoxy-4-phenylcoumarin (**3**), 6,7-dihydroxy-4'-methoxy-4-phenylcoumarin (**4**), 3,4-dihydroxybenzoic acid, 3-hydroxy-4-methoxybenzoic acid, were isolated from the seeds of *C. polyanthum* (Figure 1). The two new compounds **1** and **2** isolated from this plant material were tested for cell protecting activities against H₂O₂-induced human umbilical vein endothelial cell (HUVEC) damage, and both compounds showed significant activities.

We present herein the isolation and structural elucidation of these new compounds, and their cell protective activities.

2. Results and discussion

7,4'-Dihydroxy-6,8-dimethoxy-4-phenylcoumarin (**1**), a white amorphous powder,

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Figure 1. Structures of compounds **1**–**4**.

had a molecular formula of C₁₇H₁₄O₆ as determined by HR-ESI-MS at *m/z* 315.0896 [M + H]⁺ with 11 degrees of unsaturation. The IR absorption bands revealed the presence of hydroxyl (3215 cm⁻¹), carbonyl (1693 cm⁻¹), and

aromatic (1608 and 1502 cm⁻¹) functionalities. The ¹H NMR spectrum (Table 1) showed the presence of six aromatic proton signals at δ 7.42 (2H, d, *J* = 8.4 Hz), 6.94 (2H, d, *J* = 8.4 Hz), 6.75 and 6.12 (each 1H, s), two methoxyl

Table 1. ¹H NMR and ¹³C NMR spectral data of compounds **1** and **2**^a.

Position	1		2	
	δ _C	δ _H (<i>J</i> , Hz)	δ _C	δ _H (<i>J</i> , Hz)
2	160.5		159.9	
3	110.5	6.12 (s, 1H)	110.4	6.16 (s, 1H)
4	156.0		155.1	
4a	110.1		109.4	
5	103.4	6.75 (s, 1H)	102.8	6.72 (s, 1H)
6	145.7		145.1	
7	144.5		144.0	
8	135.6		135.1	
8a	143.8		143.3	
1'	126.2		127.3	
2'	130.6	7.42 (d, <i>J</i> = 8.4, 1H)	129.9	7.54 (d, <i>J</i> = 8.1, 1H)
3'	116.1	6.94 (d, <i>J</i> = 8.4, 1H)	114.2	7.12 (d, <i>J</i> = 8.1, 1H)
4'	159.4		160.2	
5'	116.1	6.94 (d, <i>J</i> = 8.4, 1H)	114.2	7.12 (d, <i>J</i> = 8.1, 1H)
6'	130.6	7.42 (d, <i>J</i> = 8.4, 1H)	129.9	7.54 (d, <i>J</i> = 8.1, 1H)
6-OCH ₃	56.4	3.71 (s, 3H)	55.8	3.70 (s, 3H)
8-OCH ₃	61.2	3.86 (s, 3H)	60.6	3.86 (s, 3H)
7-OH		10.00 (s, 1H)		10.10 (s, 1H)
4'-OH(OCH ₃)		9.99 (s, 1H)	55.2	3.85 (s, 3H)

Note: ^aData were measured in DMSO-*d*₆ at 600 MHz (¹H) and 150 MHz (¹³C).

signals at δ 3.86 and 3.71 (each 3H, s), and two exchangeable protons at δ 10.00 and 9.99 (each 1H, s). The ^{13}C NMR spectrum (Table 1) resolved 17 carbon resonances comprising two methyls (δ 61.2, 56.4), six sp^2 methines [δ 130.6 ($2 \times \text{C}$), 116.1 ($2 \times \text{C}$), 110.5, 103.4], and nine sp^2 quaternary carbons (including one ester carbonyl) as categorized by the DEPT experiment. The aforementioned data suggested that compound **1** likely possessed a scaffold of 4-arylcoumarin [16].

Extensive analysis of the 2D NMR (HSQC and HMBC) spectra further confirmed the 4-arylcoumarin feature for compound **1**, and finally allowed us to establish its structure. After the assignment of the protons to their direct bonding carbons by the HSQC spectrum, the structure of **1** was confirmed by the following HMBC correlations. In the HMBC spectrum (Figure 2), the correlations from the hydroxyl at δ_{H} 10.00 to C-6 (δ 145.7), C-7 (δ 144.5), and C-8 (δ 135.6) indicated that this hydroxyl was linked to C-7; the correlations from two methoxyl protons to C-6 and C-8 suggested that two methoxyls were located

at C-6 and C-8, respectively; combination of the correlations from H-5 to C-4a (δ 110.1), C-6, C-7, and C-8a (δ 143.8) revealed the presence of a pentasubstituted benzene ring (ring A) for **1**. The HMBC correlation of H-5/C-4 (δ 156.0) suggested that a sp^2 quaternary carbon C-4 was attached to C-4a. Furthermore, a *p*-hydroxyl substituted benzene ring (ring C) was attached to C-4 by the HMBC correlations from OH-4' to C-3' (δ 116.1), C-4' (δ 159.4), and C-5' (δ 116.1), from H-3' and H-5' to C-1' (δ 126.2), and from H-2' and H-6' to C-4' and C-4. The HMBC correlations from H-3 (δ 6.12) to C-1' (δ 126.2), C-4a and C-2 (δ 160.5) indicated that C-2, C-3, and C-4 were linked in order. The aforementioned functionalities accounted for 10 degrees of unsaturation, and the remaining one degree of unsaturation required the presence of an additional ring in **1**. According to the analysis of the IR and ^{13}C NMR (Table 1) spectral data, C-2 (δ 160.5) and C-8a (δ 143.8) were likely linked as an ester to furnish a characteristic feature of coumarin. The structure of **1** was thus determined as depicted in Figure 1.

7-Hydroxy-6,8,4'-trimethoxy-4-phenylcoumarin (**2**) was obtained as a colorless crystal (in MeOH) with mp 192–194°C, and had the molecular formula of $\text{C}_{18}\text{H}_{16}\text{O}_6$ as determined by the HR-ESI-MS ion at m/z 329.1046 $[\text{M} + \text{H}]^+$. A combined analysis of ^1H , ^{13}C NMR (Table 1), HSQC, and HMBC spectra of **2** indicated that it was a closely related analog of **1** sharing the same skeleton, and the only structural difference between the two compounds was the presence of a methoxyl (δ_{H} 3.85, 3H, s) attached at C-4' in **2** instead of a hydroxyl linked at C-4' in **1**. This conclusion was further confirmed by the HMBC correlations from the methoxyl proton signal at δ 3.85 to C-4' (δ 160.2), from H-3' and H-5' to C-1', and from H-2' and H-6' to C-4' and C-4. A single crystal X-ray diffraction analysis was successfully conducted to confirm the

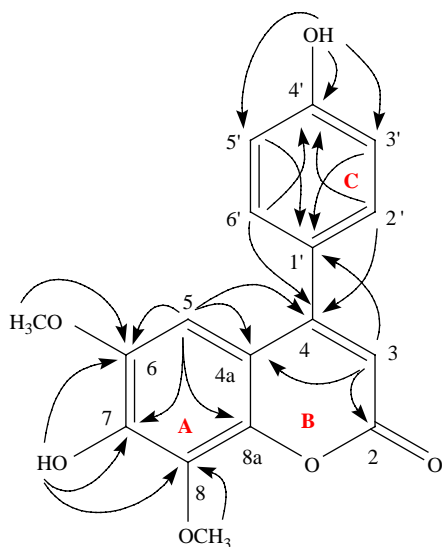


Figure 2. Key HMBC (H \rightarrow C) correlations of compound **1**.

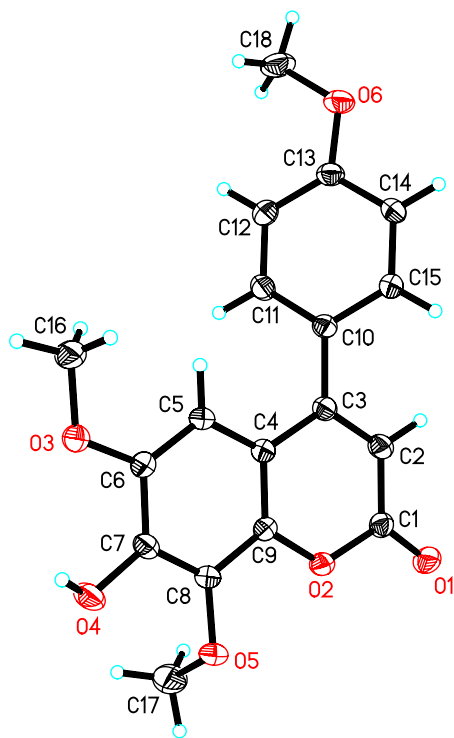


Figure 3. X-ray crystal structure of **2**.

structure of **2** (Figure 3), which also supported the structure assignments of compound **1**.

Four known compounds were identified as 7-hydroxy-4'-methoxy-4-phenylcoumarin (**3**) [17], 6,7-dihydroxy-4'-methoxy-4-phenylcoumarin (**4**) [18], 3,4-dihydroxy-

benzoic acid [19], and 3-hydroxy-4-methoxybenzoic acid [20], by comparison of spectroscopic data (^1H , ^{13}C NMR, and EIMS) with those reported in the literature.

In the current research, the cell protective activities against H_2O_2 -induced HUVEC damage of compounds **1** and **2** were evaluated according to the reported protocol [21] with minor modification. HUVECs were cultured *in vitro* and divided into three groups as a control group, H_2O_2 injury group, and compounds **1** and **2** with different concentrations plus an H_2O_2 group. Survival rate of HUVECs was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. The results are shown in Table 2, in which compound **1** can significantly attenuate the H_2O_2 -induced HUVEC damage at 1×10^{-5} and 4×10^{-7} mol/l, while at 1×10^{-5} , 2×10^{-6} , 4×10^{-7} and 8×10^{-8} mol/l, compound **2** showed remarkable cell protective activities. However, both compounds have not exhibited in a dose-dependent manner.

3. Experimental

3.1 General experimental procedures

IR spectra were recorded on a Perkin-Elmer 577 spectrometer with KBr discs. UV spectra were recorded on a Varian CARY 300 Bio spectrophotometer.

Table 2. Effects of compounds **1** and **2** on HUVEC survival rate^a.

Group	Concentration (mol/l)	Absorbance (OD value)	Cell survival rate (%)
Control group	—	0.99 ± 0.05	100
H_2O_2 injury group	—	0.83 ± 0.11	83
Compound 1	5×10^{-5}	0.88 ± 0.11	88
	1×10^{-5}	1.02 ± 0.15	102
	2×10^{-6}	0.87 ± 0.07	87
	4×10^{-7}	1.03 ± 0.13	103
	8×10^{-8}	0.85 ± 0.04	85
Compound 2	5×10^{-5}	0.82 ± 0.11	82
	1×10^{-5}	1.02 ± 0.05	102
	2×10^{-6}	1.05 ± 0.06	106
	4×10^{-7}	1.00 ± 0.15	100
	8×10^{-8}	1.01 ± 0.05	102

Note: ^a $n = 5$, $X \pm \text{SD}$.

Specific rotations were made on a Perkin-Elmer 341 polarimeter at room temperature. NMR spectra were measured on a Bruker AM-600 spectrometer. ESI-MS and HR-ESI-MS were made on an Agilent 6330 LC-MS and a Waters Q-ToF Ultima Global mass spectrometer, respectively. Semi-preparative HPLC was performed on a Shimadzu LC-6AD pump equipped with a Shimadzu SPD-20A UV detector (254 nm) and a YMC-Pack ODS-A column (250 × 10 mm, S-5 μm, 12 nm). Silica gel H (Qingdao Haiyang Chemical Co. Ltd, Qingdao, China), C₁₈ reversed-phase silica gel (150–200 mesh; Merck, Darmstadt, Germany), and Sephadex LH-20 gel (Amersham Biosciences, Little Chalfont, UK) were used for column chromatography (CC). Pre-coated silica gel GF₂₅₄ plates (Qingdao Haiyang Chemical Co. Ltd) were used for TLC.

3.2 Plant material

The seeds of *C. polyanthum*, collected from Xishuangbanna, Yunnan Province of China, were identified by Dr Wen-Zhao Tang, at the Institute of Materia Medica, Shandong Academy of Medical Sciences. A voucher specimen has been deposited in the Institute of Materia Medica, Shandong Academy of Medical Sciences (accession number: 20081202).

3.3 Extraction and isolation

The powder of the dried seeds of *C. polyanthum* (5.0 kg) was extracted with 95% EtOH three times at room temperature. Evaporation of the solvent under reduced pressure provided 620 g of ethanolic extract, which was then partitioned between water and EtOAc to give an EtOAc-soluble fraction (380 g). The EtOAc-soluble fraction was chromatographed over a silica gel column eluted with petroleum ether–acetone (100:1 to 1:1, v/v) in gradient to give five fractions A–E. Fraction B (32 g) was subjected to a

column of an RP-18 silica gel (MeOH–H₂O, 30:70–60:40, v/v) in gradient to obtain three fractions B1–B3. Fraction B1 (2.8 g) was chromatographed over a column of Sephadex LH-20 eluted with MeOH to give four sub-fractions B1a–B1d. Purification of fraction B1a (1.2 g) by CC eluted with CHCl₃–MeOH–HCOOH (100:10:0.1, v/v) gave 3-hydroxy-4-methoxybenzoic acid (85 mg). Fraction B1b (750 mg) was separated on a column of CC eluted with CHCl₃–MeOH–HCOOH (100:20:0.1, v/v) to give 3,4-dihydroxybenzoic acid (53 mg). Fraction D (4.5 g) was chromatographed over a column of an RP-18 silica gel (MeOH–H₂O, 40:60–80:20, v/v) in gradient to obtain four fractions D1–D4. D2 (900 mg) was subjected to CC eluted with petroleum ether–EtOAc (3:1, v/v) to give **1** (15 mg) and **2** (20 mg). Fraction D3 (1.5 g) was chromatographed by a column of Sephadex LH-20 eluted with MeOH to give three sub-fractions D3a–D3c. Purification of fraction D3c (530 mg) by semi-preparative HPLC (MeOH–H₂O: 80:20, 3 ml/min) gave **3** (25 mg) and **4** (18 mg).

3.3.1 7,4'-Dihydroxy-6,8-dimethoxy-4-phenylcoumarin (**1**)

Obtained as a white amorphous powder, UV (MeOH) λ_{max} (log ε): 315 (3.78) nm. IR (KBr) ν_{max}: 3215, 1693, 1608, 1502 cm⁻¹. ¹H and ¹³C NMR spectral data: see Table 1. Positive-ion ESI-MS: *m/z* 315.1 [M + H]⁺. HR-ESI-MS: *m/z* 315.0896 [M + H]⁺ (calcd for C₁₇H₁₅O₆, 315.0869).

3.3.2 7-Hydroxy-6,8,4'-trimethoxy-4-phenylcoumarin (**2**)

Obtained as a colorless crystal (in MeOH), mp 192–194°C. UV (MeOH) λ_{max} (log ε): 307 (3.85) nm. IR (KBr) ν_{max}: 3319, 1687, 1606, 1500 cm⁻¹. ¹H and ¹³C NMR spectral data: see Table 1. Positive-ion ESI-MS: *m/z* 329.1 [M + H]⁺. HR-ESI-MS: *m/z*

329.1046 [M + H]⁺ (calcd for C₁₈H₁₇O₆, 329.1025).

3.4 X-ray crystallography experiment

Empirical formula: C₁₈H₁₆O₆; formula weight: 328.31; temperature: 293(2) K; wavelength: 0.71073 Å; crystal system and space group: triclinic, *P*-1; unit cell dimensions: *a* = 9.3903(11) Å, *b* = 9.8323(11) Å, *c* = 9.8472(11) Å, *α* = 80.089(2)°, *β* = 67.881(2)°, *γ* = 67.357(2)°; volume: 776.95(15) Å³; *Z*, calculated density: 2, 1.403 mg/m³; absorption coefficient: 0.106 mm⁻¹; *F*(000): 344; crystal size: 0.411 × 0.369 × 0.237 mm; *θ* range for data collection: 2.23–26.00°; limiting indices: -11 ≤ *h* ≤ 7, -12 ≤ *k* ≤ 11, -12 ≤ *l* ≤ 8; reflections collected/unique: 4273/2995 [*R*(int) = 0.0219]; completeness to *θ* = 26.00: 98.3%; absorption correction: empirical; max. and min. transmission: 1.00000 and 0.49765; refinement method: full-matrix least squares on *F*²; data/restraints/parameters: 2995/0/225; goodness-of-fit on *F*²: 1.060; final *R* indices [*I* > 2σ(*I*): *R*1 = 0.0472, *wR*2 = 0.1309; *R* indices (all data): *R*1 = 0.0542, *wR*2 = 0.1374; extinction coefficient: 0.015(5); largest difference peak and hole: 0.200 and -0.255 e Å⁻³. Crystallographic data of compound **2** have been deposited in the Cambridge Crystallographic Data Centre (deposition number: CCDC 764242).

3.5 Cell protecting evaluation

HUVECs digested by 0.25% trypsin were made into a single-cell suspension in RPMI-1640 medium with 10% fetal calf serum, HUVECs were cultured for 24 h at a density of 1 × 10⁵ cells per well in 96-well plates with a humid atmosphere of 5% CO₂ and 95% air at 37°C. Then, cells were pretreated with various concentrations of the compounds for 24 h, followed by exposure to 1 × 10⁻⁴ mol/l of H₂O₂ in

the presence of the same concentrations of the compounds for another 4 h; in this step, the control group and H₂O₂ injury group were set up. To produce oxidative stress, H₂O₂ was freshly prepared from 30% stock solution prior to each experiment. Cell survival was evaluated by MTT reduction. Briefly, after 4 h exposure, 10 μl of MTT (5 mg/ml in PBS) was added to each well and the cells were incubated at 37°C for 4 h. The supernatants were aspirated carefully and 200 μl of dimethyl sulfoxide was added to each well to dissolve the precipitate and the absorbance (OD) at 535 nm was measured with a microplate reader (Victor 1420, Waltham, Massachusetts, USA). Cell survival rate was calculated according to the OD value. Cell survival rate (%) = (OD value for the test product group/OD value for the control group) × 100%.

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