

## Aromatic compounds produced by *Periconia atropurpurea*, an endophytic fungus associated with *Xylopia aromatica*

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### Abstract

6,8-Dimethoxy-3-(2'-oxo-propyl)-coumarin (**1**) and 2,4-dihydroxy-6-[(1'E,3'E)-penta-1', 3'-dienyl]-benzaldehyde (**2**), in addition to the known compound periconicin B (**3**), were isolated from the ethyl acetate extract of *Periconia atropurpurea*, an endophytic fungus obtained from the leaves of *Xylopia aromatica*, a native plant of the Brazilian Cerrado. Their chemical structures were assigned based on analyses of MS, 1D and 2D-NMR spectroscopic experiments. Biological analyses were performed using two mammalian cell lines, human cervix carcinoma (HeLa) and Chinese hamster ovary (CHO). The results showed that compound **1** had no effect when compared to the control group, which was treated with the vehicle (DMSO). Compound **2** was able to induce a slight increase in cell proliferation of HeLa (37% of increase) and CHO (38% of increase) cell lines. Analysis of compound **3** showed that it has potent cytotoxic activity against both cell lines, with an IC<sub>50</sub> of 8.0 µM. Biological analyses using the phytopathogenic fungi *Cladosporium sphaerospermum* and *C. cladosporioides* revealed that also **2** showed potent antifungal activity compared to nystatin.

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**Keywords:** *Periconia atropurpurea*; Endophytic fungi; Coumarin; Benzaldehyde; Antifungal; Cell proliferation

### 1. Introduction

This study was conducted as part of our ongoing bioprospecting program Biota-FAPESP, that aims to discover potential antitumor, antifungal and antioxidant agents produced by endophytic fungi associated with species of native plants from the Brazilian Cerrado. Among sixty screened fungi, *Periconia atropurpurea*, isolated from the leaves of *Xylopia aromatica*, was chosen for detailed chemical inves-

tigation due to its antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides*, i.e. as demonstrated by the ethyl acetate extract which in turn led to isolation of two new compounds: 6,8-dimethoxy-3-(2'-oxo-propyl)-coumarin (**1**) and 2,4-dihydroxy-6-[(1'E,3'E)-penta-1',3'-dienyl]-benzaldehyde (**2**), as well as the known compound periconicin B (**3**) (Kin et al., 2004). In this paper, we report the isolation, structure elucidation and biological evaluation of compounds **1–3** using two mammalian cell lines, human cervix carcinoma (HeLa) and Chinese hamster ovary (CHO), as well as two phytopathogenic fungi, *Cladosporium sphaerospermum* and *C. cladosporioides*.

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Table 1

<sup>1</sup>H (500 MHz) and <sup>13</sup>C (125 MHz) NMR spectroscopic data for compounds **1** (<sup>1</sup>H, DMSO-*d*<sub>6</sub>; <sup>13</sup>C, CDCl<sub>3</sub> + CD<sub>3</sub>OD)<sup>a</sup> and **2** (DMSO-*d*<sub>6</sub>)<sup>a</sup>

Position	<b>1</b>				<b>2</b>			
	<sup>1</sup> H (δ)	<sup>13</sup> C (δ)	gHMBC	NOESY	<sup>1</sup> H (δ)	<sup>13</sup> C (δ)	gHMBC	NOESY
1						111.2		
2		161.0 <sup>b</sup>				165.3		
3		125.0 <sup>b</sup>				101.4	C-1, C-2, C-4	–
4	7.80 <i>s</i>	142.1	C-9	H-5, H-1'	6.18 <i>d</i> (2.5)	165.1		
5	6.76 <i>d</i> (2.5)	100.0	–	H-4, 6-OCH <sub>3</sub>	6.49 <i>d</i> (2.5)	105.8	C-3, C-4, C-6, C-1'	H-2'
6		156.0 <sup>b</sup>				143.7		
7	6.87 <i>d</i> (2.5)	102.9	C-9	8-OCH <sub>3</sub>				
8		147.0 <sup>b</sup>						
9		139.0 <sup>b</sup>						
10		119.6						
1'	3.68 <i>s</i>	44.2	C-2', C-2, C-4	H-4, H-3'	7.16 <i>d</i> (15.5)	124.8	C-5, C-6, C-3'	1-COH, H-3'
2'		205.0 <sup>b</sup>			6.71 <i>dd</i> (10.5, 15.5)	134.0	C-6, C-4'	H-5, H-4'
3'	2.19 <i>s</i>	29.6	C-2'	H-1'	6.27 <i>ddd</i> (0.5, 10.5, 15.0)	131.7	–	–
4'					5.95 <i>dq</i> (6.5, 15.0)	132.3	C-2', C-5'	H-2', H-5'
5'					1.80 <i>dd</i> (0.5, 6.5)	18.1	C-3'	H-4'
1-COH					10.10 <i>s</i>	192.3	C-1, C-2, C-6	H-1'
6-OCH <sub>3</sub>	3.80 <i>s</i>	55.7	C-6	H-5				
8-OCH <sub>3</sub>	3.89 <i>s</i>	56.5	C-8	H-7				

<sup>a</sup> Chemical shifts (relative to TMS) are in (δ) ppm, coupling constants in Hz in parentheses. Assignments were aided by <sup>1</sup>H-<sup>1</sup>H gCOSY and gHMQC.<sup>b</sup> Chemical shifts obtained from gHMBC.

## 2. Results and discussion

The known compound periconicin B (**3**) was identified by comparing its physical and spectroscopic data with literature values (Kin et al., 2004). It was described for the first time from the fungus *Periconia* sp., and has not been reported from any another fungal source.

The new compound 6,8-dimethoxy-3-(2'-oxo-propyl)-coumarin (**1**), was obtained as a brown amorphous powder and had a molecular formula of C<sub>14</sub>H<sub>14</sub>O<sub>5</sub>, as revealed by analysis of the positive ion HRESI-MS data. The UV spectra showed four bands at 215, 231 (sh), 249 (sh) and 287 nm, with the latter being typical of an aromatic chromophore. The IR spectrum had bands at 1740 and 1630 cm<sup>-1</sup>, suggesting conjugated carboxyl and carbonyl groups, respectively. In the <sup>1</sup>H NMR spectrum (Table 1) of **1**, two methoxy moieties were identified by observation of signals at δ 3.80 (3H, *s*, 6-OCH<sub>3</sub>) and δ 3.89 (3H, *s*, 8-OCH<sub>3</sub>). A spin-system corresponding to a 1,2,3,5-tetrasubstituted benzene ring was also assigned on the basis of the signals at δ 6.76 (1H, *d*, *J* = 2.5 Hz, H-5) and δ 6.86 (1H, *d*, *J* = 2.5 Hz, H-7). In addition, the <sup>1</sup>H NMR spectrum displayed an isolated olefinic hydrogen signal at δ 7.80 (1H, *s*, H-4) and resonances at δ 3.68 (2H, *s*, H-1') and δ 2.19 (3H, *s*, H-3') attributable to a 2'-oxo-propyl group. These data were consistent with the corresponding <sup>13</sup>C NMR spectroscopic data (Table 1) and with similar information from analogues published in the literature (Dittmer et al., 2005), which suggested that **1** could possess a coumarin skeleton. This was inferred by signals at δ 142.1 (C-4), δ 100.0 (C-5), δ 102.9 (C-7) and δ 119.6 (C-10) observed in the <sup>13</sup>C NMR spectrum, and additional resonances at δ 161.0 (C-2), δ 125.0 (C-3), δ 156.0 (C-6), δ 147.0 (C-8) and δ 139.0 (C-9) observed in the gHMBC spectrum. In

addition, two signals at δ 55.7 (6-OCH<sub>3</sub>) and δ 56.5 (8-OCH<sub>3</sub>), and three at δ 44.2 (C-1'), δ 205.0 (C-2') and δ 29.6 (C-3') were observed and attributable to methoxy and oxo-propyl groups, respectively. Data from the gHMBC and NOESY experiments (Table 1) supported these conclusions and enabled completion of the structure of **1**. Correlations of the H-1' protons with olefin (C-4) and carboxyl (C-2) carbons supported the location of a 2'-oxo-propyl group at C-3. NOESY experiments were conducted on all hydrogens of **1** and showed a NOE correlation between 6-OCH<sub>3</sub>/H-5, H-5/H-4, H-4/H-1', H-1'/H-3' and H-7/8-OCH<sub>3</sub> thereby confirming unambiguously

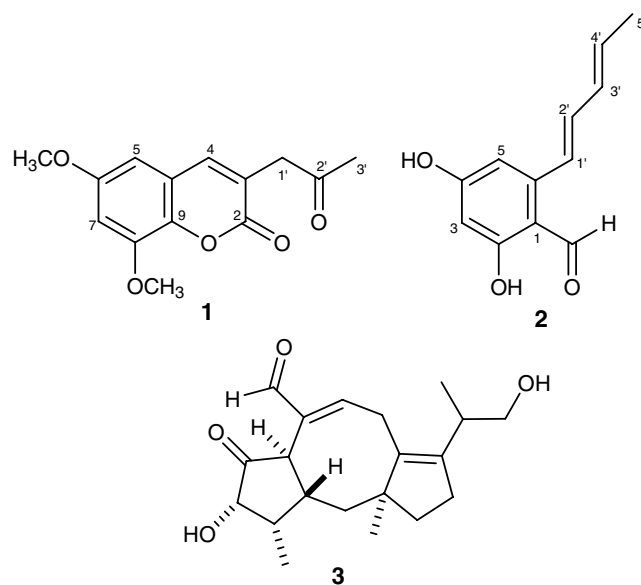


Fig. 1. Compounds isolated from the ethyl acetate extract of *Periconia atropurpurea*.

the positions of the methoxy and oxo-propyl groups (Fig. 1).

2,4-Dihydroxy-6-[(1'*E*,3'*E*)-penta-1',3'-dienyl]-benzaldehyde (**2**) was obtained as a colorless amorphous powder and was assigned the molecular formula of  $C_{12}H_{12}O_3$ , via analysis of the negative ion HRESI-MS. The UV spectra showed bands at 261, 294 and 339 nm, assuring the presence of an aromatic ring. The IR spectrum had bands at 3470 and 1629  $cm^{-1}$  accounting for the aromatic hydroxyl and carbonyl groups, respectively. The  $^1H$  NMR spectrum (Table 1) of **2** displayed a signal at  $\delta$  10.10 (1H, s, 1-COH) attributable to an aldehyde group. The spin-system corresponding to a tetra-substituted benzene ring was identical to that obtained for **1** and was observed at  $\delta$  6.18 (1H, d,  $J = 2.5$  Hz, H-3) and  $\delta$  6.49 (1H, d,  $J = 2.5$  Hz, H-5). The spectrum also displayed four resonances at  $\delta$  7.16 (1H, d,  $J = 15.5$  Hz, H-1'),  $\delta$  6.71 (1H, dd,  $J = 10.5, 15.5$  Hz, H-2'),  $\delta$  6.27 (1H, ddd,  $J = 0.5, 10.5, 15.0$  Hz, H-3') and  $\delta$  5.95 (1H, dq,  $J = 6.5, 15.0$  Hz, H-4') attributable to olefinic hydrogens in an *S-trans* arrangement, suggesting the presence of a conjugated diene with a *trans/trans* configuration (*E/E*). In addition, a signal at  $\delta$  1.80 (3H, dd,  $J = 0.5, 6.5$  Hz, H-5') was attributable to a vinyl methyl moiety in a *vicinal* coupling with H-4' and allylic coupling with H-3'. The  $^{13}C$  NMR spectrum showed 12 signals (Table 1), including one for the aldehyde group at  $\delta$  192.3 (1-COH). The penta-1',3'-dienyl moiety was confirmed by assignment of the carbon resonances at  $\delta$  124.8 (C-1'),  $\delta$  134.0 (C-2'),  $\delta$  131.7 (C-3'),  $\delta$  132.3 (C-4') and  $\delta$  18.1 (C-5'), these being substantiated by the gHMQC correlations observed. Data from the gHMBC experiment (Table 1) also supported these conclusions. Correlation of the 1-COH hydrogen with C-2 and C-6, and between H-2'/C-6 and H-5'/C-1' confirmed the linkage of the aldehyde at C-1 and of the penta-dienyl unit at C-6, respectively. 1D-TOCSY experiments, showed a complete set of sequence correlations for the penta-dienyl unit. 1D-NOE experiments were also conducted showing NOE between 1-COH/H-1', H-1'/H-3', H-5/H-2', H-2'/H-4' and H-4'/H-5' corroborating the *E/E* suggested arrangement (Fig. 1). To the best of our knowledge, this is the first report of an endophytic fungus from *Xylopiia aromatica* (Annonaceae) and the first isolation of this class of compounds from *P. atropurpurea*.

Compounds **1–3** were evaluated against *Cladosporium sphaerospermum* and *C. cladosporioides* using direct bioautography (Rahalison et al., 1991). Only compound **2** exhibited strong antifungal activity against both fungi, showing a detection limit of 1.0  $\mu g$ , comparable to nystatin (used as a positive control). Compound **1** did not show any antifungal activity, and **3** showed a relatively weak detection limit of 25.0  $\mu g$ .

Compounds **1–3** were also evaluated for cytotoxicity using HeLa and CHO mammalian cell lines. Compound **1** showed no activity to either cell lines, when compared to the vehicle-treated cells. Compound **2** was able to induce proliferation of HeLa cells (maximum of 37% of increased proliferation at 2.0  $\mu M$ ) and CHO cells (maximum of 38%

of increased proliferation at 20.0  $\mu M$ ). Results obtained with compound **3** showed that it is a potent cytotoxic agent against the two mammalian cell lines analyzed. Compound **3** decreased cell viability of HeLa and CHO cells, with an  $IC_{50}$  of 8.0  $\mu M$ , showing potency similar to that of cisplatin, a well known antineoplastic agent ( $IC_{50}$  5.0  $\mu M$ ), which was used as a cytotoxic positive control.

### 3. Experimental

#### 3.1. General experimental procedure

UV spectra were recorded using a Perkin Elmer UV-Vis Lambda-14P spectrophotometer. IR spectra were recorded using a Nicolet Impact-400 spectrophotometer.  $^1H$  (500 MHz) and  $^{13}C$  (125 MHz) NMR spectra were recorded using a VARIAN DRX-500 spectrometer, with TMS as an internal standard. HRESI mass spectra were obtained using a Bruker Daltonics (UltraTOF-Q) mass spectrometer. Analytical HPLC was performed with a Varian Pro Star 230 using a Phenomenex C-18 column (250 mm  $\times$  4.6 mm). Column chromatography (CC) was performed over reversed-phase silica gel 230–400 mesh and normal-phase silica gel G60 (Merck). TLC was performed using Merck silica gel 60 (>230 mesh) and pre-coated silica gel 60 PF<sub>254</sub> plates. Spots on TLC plates were visualized under UV light and by spraying with anisaldehyde- $H_2SO_4$  reagent followed by heating at 120  $^{\circ}C$ . Preparative HPLC was performed on a Varian Prep-Star 400 system using a Phenomenex C-18 (250 mm  $\times$  21.2 mm) preparative column.

#### 3.2. Plant material

Authenticated *Xylopiia aromatica* (Lamarck) Martius plant material was collected in “Estação Experimental de Araraquara”, Araraquara, São Paulo, Brazil, in 2001. The botanical identification was made by Professor Maria Cláudia Marx Young and a voucher specimen was deposited at the Herbarium of the Botanic Garden of São Paulo, Brazil (Voucher No SP 142.360).

#### 3.3. Isolation of the endophytic fungus

For isolation of the endophytic fungus, adult and healthy leaves were selected and subjected to surface sterilization. They were first washed with water and soap, and then immersed in a 1% aqueous sodium hypochlorite solution for 5 min and aqueous ethanol (3:7, %) for 1 min. A second washing with water and soap was performed and finally the leaves were immersed in sterile water for 10 min. The sterilized leaves were cut into 2  $cm^2$  pieces and deposited on a Petri dish containing PDA (potato-dextrose-agar) and gentamicin sulfate (0.5 mg/ml) with approximately 3–4 pieces on each dish. The material was incubated at 25  $^{\circ}C$  for 10 days and the endophyte *P. atro-*

*purpurea* was isolated by replication and preserved in sterile water (Maier et al., 1997). The fungus was identified by Dr. Ludwig H. Pfenning using rRNA internal transcribed spacer (ITS) region and deposited in the (Coleção Micologica de Lavras, at the Universidade Federal de Lavras fungal herbarium with the accession number CML 631).

### 3.4. Growth and production of the crude extract

The fungus was cultivated using three different culture media: PDB (4.0 g of potato extract, 20.0 g dextrose/1.0 l H<sub>2</sub>O), Czapek (30.0 g saccharose; 3.0 g NaNO<sub>3</sub>; 1.0 g K<sub>2</sub>PO<sub>4</sub>; 0.5 g MgSO<sub>4</sub>; 0.5 g KCl; 0.01 g Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>/1.0 l H<sub>2</sub>O) and Czapek + polystyrene resin XAD-2. For the first medium, were used 2 Erlenmeyer flasks (500 ml), each containing 0.8 g of potato extract, 4.0 g dextrose and 200 ml distilled water which were autoclaved at 125 °C for 15 min. Approximately 10 small pieces (1 cm<sup>2</sup>) of PDA (potato extract, dextrose and agar) medium from the Petri dish containing biomass of the *P. atropurpurea* isolated were inoculated into each flask and the flasks were sealed with cotton to permit aerobic growth. After incubation for 28 days at 25 °C on rotary shakers at 150 rpm, the mycelia biomass accumulated in the flasks was separated from the aqueous medium by filtration, and the filtrate was partitioned with EtOAc (3 × 200 ml). Collection and evaporation of the organic phase *in vacuo* yielded a brown solid residue (129 mg). The above process was scaled up (4.0 l of PDB in 20 Erlenmeyer flasks) after the antifungal bioactivity against *Cladosporium cladosporioides* and *C. sphaerospermum* was detected, affording 1.44 g of EtOAc extract (A). For the other two culture media the process described was directly scaled up, using 5.0 l of Czapek medium [affording 200 mg of EtOAc extract (B)] and 1.0 l of Czapek + 50.0 g of XAD-2. After incubation, the resin XAD-2 was separated from medium by flotation and extracted with MeOH affording 700 mg of MeOH extract (C).

### 3.5. Antifungal assay

*C. cladosporioides* (Fresen) de Vries SPC 140 and *C. sphaerospermum* (Perzig) SPC 491 were used in the antifungal assay, and were maintained at the Instituto de Botânica, São Paulo, SP, Brazil. Compounds **1–3** were applied on pre-coated Si-gel TLC plates using solution (10 µl) containing 100.0, 50.0, 25.0, 10.0, 5.0 and 1.0 µg. After eluting with CHCl<sub>3</sub>–CH<sub>3</sub>OH (9:1), they were sprayed with spore suspensions of the fungi (Homans and Fuchs, 1970). Nystatin was employed as positive control.

### 3.6. Biological assays using mammalian cell lines

Biological assays aiming to determine cell viability after treatment with the compounds were performed as described previously (Teles et al., 2005). Briefly, HeLa and CHO cells were cultured using Dulbecco's modified

Eagle's medium (DMEM, Life Technologies Inc., Gaithersburg, MD) supplemented with 10% Fetal Calf Serum (FCS, Life Technologies Inc., Gaithersburg, MD). A stock solution (20.0 mM) was prepared by dissolving compounds **1–3** in DMSO (vehicle). The final concentrations (200.0, 20.0 and 2.0 µM) were achieved by direct dilution into the cell medium. The compound **3** was also diluted in the following concentrations: 5.0, 10.0, 15.0 and 50.0 µM. After addition of the compound or the vehicle, cells (10<sup>4</sup> cells/well plated 24 h before) were analyzed after a period of 48 h using the MTT assay (Mosmann, 1983; Rubinstein et al., 1990).

### 3.7. Extraction and isolation

EtOAc extract A (1.44 g) was fractionated by CC using silica gel and eluted with a CHCl<sub>3</sub>–MeOH gradient (95:5 → 100% MeOH) affording 15 fractions. Fraction 01 (290 mg) was fractionated by CC using Sephadex LH-20 and eluted with hexane–CH<sub>2</sub>Cl<sub>2</sub> (1:4, 200 ml); CH<sub>2</sub>Cl<sub>2</sub>–acetone (3:2, 200 ml), CH<sub>2</sub>Cl<sub>2</sub>–acetone (1:4, 200 ml), acetone (150 ml) and MeOH (150 ml), affording six sub-fractions. Sub-fraction 01 (11 mg) was further purified using reversed-phase prep. HPLC [ $\lambda$  = 235 nm, 10.0 ml/min, CH<sub>3</sub>CN–H<sub>2</sub>O (9:1)] affording **1** (2.8 mg, *R*<sub>t</sub> = 17 min). EtOAc extract B (200 mg) was fractionated by CC using reversed-phase silica gel and eluted with a MeOH–H<sub>2</sub>O gradient (25:75 → 100% MeOH) affording four fractions. Fraction 4 (80.5 mg) was purified by HPLC using phenyl–hexyl phase in preparative column [ $\lambda$  = 220 nm, 15.0 ml/min, MeOH–H<sub>2</sub>O (6:4)] affording **3** (16 mg, *R*<sub>t</sub> = 20 min). The MeOH extract C (700 mg) was fractionated by CC using reversed-phase silica gel and eluted with a MeOH–H<sub>2</sub>O gradient (20:80 → 100% MeOH) affording 05 fractions. Fractions 4 (160 mg) and 3 (41 mg) were purified by HPLC using a phenyl–hexyl phase preparative column and using the following chromatographic conditions: [ $\lambda$  = 225 nm, 15.0 ml/min, MeOH–H<sub>2</sub>O (63:37)] and [ $\lambda$  = 233 nm, 15.0 ml/min, MeOH–H<sub>2</sub>O (50:50)], affording **2** (20.0 mg, *R*<sub>t</sub> = 22 min) and an additional sample of compound **1** (6.4 mg, *R*<sub>t</sub> = 20 min), respectively.

#### 3.7.1. 6,8-Dimethoxy-3-(2'-oxo-propyl)-coumarin (**1**)

Brown amorphous powder. *R*<sub>f</sub> 0.78 on SiO<sub>2</sub>-TLC [CHCl<sub>3</sub>–MeOH (9:1)]; UV (MeOH)  $\lambda_{\max}(\log \epsilon)$  215 (3.65), 231 (sh) (3.59), 249 (sh) (3.51) and 287 (3.31) nm; IR (KBr) $\nu_{\max}$  2930, 1740, 1630, 1380 and 1070 cm<sup>-1</sup>; For <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) and <sup>13</sup>C NMR (CDCl<sub>3</sub> + CD<sub>3</sub>OD) spectra, see Table 1; HRESI-MS *m/z* 285.0753 [M + Na]<sup>+</sup> Calc. for C<sub>14</sub>H<sub>14</sub>O<sub>5</sub>Na, 285.0739.

#### 3.7.2. 2,4-Dihydroxy-6-[1'E,3'E]-penta-1',3'-dienyl]-benzaldehyde (**2**)

Colorless amorphous powder. *R*<sub>f</sub> 0.50 on SiO<sub>2</sub>-TLC [CHCl<sub>3</sub>–MeOH (9:1)]; UV (MeOH)  $\lambda_{\max}(\log \epsilon)$  261 (3.75), 294 (3.67) and 339 (3.43) nm; IR (KBr) $\nu_{\max}$  3470 and 1629 cm<sup>-1</sup>; For <sup>1</sup>H NMR and <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)

spectra, see Table 1; HRESI-MS  $m/z$  203.0722  $[M - H]^-$   
Calc. for  $C_{12}H_{11}O_3$ , 203.0708.

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