

**Chemical composition and *in vitro* cytotoxicity of *Corynespora olivacea* (V18) associated with *Vochysia divergens***

**Composição química e citotoxicidade *in vitro* de *Corynespora olivacea* (V18) associada a *Vochysia divergens***

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**ABSTRACT**

Chemical investigation into the ethyl acetate extract of the endophytic fungus *Corynespora olivacea* (V18) associated with *Vochysia divergens* identified compounds **1** and **2**. The compounds were characterized by spectroscopic methods including NMR (1D and 2D) and HRMS. The cytotoxicity of the crude extract and the isolated compounds against normal human lung fibroblast cells, lineage GM07492A, was evaluated by the

XTT assay. To the best of our knowledge, this is the first chemical and biological screening of the cytotoxicity of *C. olivacea* derived from *V. divergens*.

**Keywords:** Cambará, Cerrado, Endophytic fungus, Vochysiaceae

## RESUMO

A investigação química do extrato em acetato de etila do fungo endofítico *Corynespora olivacea* (V18) associado a *Vochysia divergens* identificou os compostos **1** e **2**. Os compostos foram caracterizados por métodos espectroscópicos incluindo RMN (1D e 2D) e EMAR. A citotoxicidade do extrato bruto e dos compostos isolados contra células de fibroblastos de pulmão humano normal, linhagem GM07492A, foi avaliada pelo ensaio do XTT. Até onde sabemos, esta é a primeira triagem química e biológica da citotoxicidade de *C. olivacea* associada à *V. divergens*.

**Palavras-chave:** Cambará, Cerrado, Fungo Endofítico, Vochysiaceae

## 1 INTRODUCTION

Endophytic fungi are microbes that live symbiotically inside healthy plant tissues at least in some period of their life cycle. They engage into various ecological relationships without causing visible symptoms of infection or morphological alterations in the host (Soares *et al.*, 2016). Endophytic fungi confer the host plant with competitiveness, fitness, and resistance to biotic and abiotic stress (Naik 2019; Naik, Ravikanth, Dayanandan, 2019, Maia *et al.*, 2020).

*Vochysia divergens* Pohl (Vochysiaceae), commonly known as Cambará, is an invasive species in the wetlands of the Brazilian Pantanal. *Vochysia divergens* has high tolerance to seasonal variations in hydrology, which allows it to spread rapidly and extensively under the extreme water stress of Pantanal during prolonged flooding and dryness. This results in extensive monospecific forests known as Cambarazal (Dalmolin *et al.*, 2018). This plant hosts numerous endophytic fungi (Biz *et al.*, 2017), which in turn help the host plant to mitigate environmental stress like salinity (Farias *et al.*, 2019).

Because the association of *V. divergens* with endophytic microorganisms is poorly understood, and since the chemical profile and biological potential of its microbiota remains partially unknown, our research group has been dedicated to the chemical study of this plant species (Pimenta *et al.*, 2015) and its chemical interaction with its endophytic microbiota (Parpinelli *et al.*, 2017; Pimenta *et al.*, 2017; de Oliveira *et al.*, 2018; Farias *et al.*, 2019).

According to the literature, *Microbispora* sp. LGMB259, an endophytic Actinomycetes isolated from *V. divergens*, produces antibacterial  $\beta$ -carboline and

indoles (Savi *et al.*, 2015; Gos *et al.*, 2017). Additionally, Savi *et al.*, (2018) isolated the new endophyte *Phaeophleospora vochysiae* from *V. divergens* and found that this endophyte produces (+)-cercosporin, (+)-isocercosporin, and the new compound 3-(sec-butyl)-6-ethyl-4,5-dihydroxy-2-methoxy-6-methylcyclohex-2-enone. Noriler *et al.*, (2018) conducted a bioprospection of the *V. divergens* endophytic community. Another study by the same research group mentioned that *Diaporthe vochysiae* isolated as endophyte from *V. divergens* produces the antibacterial Vochysiamides A and B (Noriler *et al.*, 2019). However, studies on the chemical composition and biological potential of *V. divergens* microbiota are scarce. For this reason, here we have investigated the chemical composition and the cytotoxic activity of the ethyl acetate extract of the endophytic fungus lineage *Corynespora olivacea* (V18) and isolated compounds against normal human lung fibroblast cells, lineage GM07492A. To the best of our knowledge, this is the first chemical evaluation of the fungus *C. olivacea* (V18) associated with *V. divergens*.

## 2 MATERIALS AND METHODS

### GENERAL EXPERIMENTAL PROCEDURES

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of compounds **1** and **2** were recorded in methanol- $d_4$  on a Bruker® DRX-400 spectrometer operating at 400 MHz for  $^1\text{H}$  NMR and 100 MHz for  $^{13}\text{C}$  NMR; TMS was used as internal standard. The preparative HPLC analyses were carried out on a Shimadzu LC-6AD binary system equipped with a DGU-20A5 degasser, an SPD-20A series UV-VIS detector, a CBM-20A communication bus module, and a Reodyne manual injector. The Gemini ODS column (250 × 10 mm, 5  $\mu\text{m}$ ; Phenomenex) was equipped with a precolumn, and the mobile phase consisted of  $\text{CH}_3\text{OH}/\text{H}_2\text{O}$  (75:25 v/v) employed in an isocratic mode for 60 min. The flow rate was 5.0 mL  $\text{min}^{-1}$ ; the injection volume was 500  $\mu\text{L}$ ; and UV detection was set at 254 nm and 25 °C. The data were acquired with a computer. HPLC-grade MeOH from J.T. Baker was used in the experiments. Ultrapure water was obtained by passing redistilled water through a Direct-Q UV3 system from Millipore. The ESI mass spectrometry analyses were performed on a micrOTOF Q II - ESI-TOF Mass Spectrometer (Bruker Daltonics) by direct infusion. The experimental conditions were capillary voltage of 3.5 kV and nitrogen as drying (Temperature = 180 °C; flow = 4 L  $\text{min}^{-1}$ ) and nebulizer gas (pressure = 0.4 Bar). Internal calibration was conducted with 10 mg/mL sodium trifluoroacetate (Na-TFA) solution, and the capillary voltage was set to 3500 V.

## ENDOPHYTIC FUNGUS EXTRACTION AND ISOLATION

The endophytic fungus *Corynespora olivacea* (V18) was obtained from the roots of *Vochysia divergens* Pohl (Vochysiaceae), which occurs in the wetland known as Pantanal (Biz *et al.*, 2017). This strain was obtained from the microorganism bank of the Laboratory of Biotechnology and Microbial Ecology of the Federal University of Mato Grosso-UFMT, Cuiabá, MT, Brazil. The KJ 439183 number represents the sequences of the ITS rDNA deposited in GenBank for *C. olivacea* (V18). The ethyl acetate (EtOAc) extract was obtained according to da Silva *et al.*, (2017). The endophytic strain was cultured at  $25 \pm 2$  °C in Petri dishes containing 20 mL of PDA (potato dextrose and agar) for 15 days. The mycelium was fragmented, macerated in flasks containing EtOAc (1:1 ratio) under stirring at 100 rpm for 24 h, and further submitted to ultrasonic bath (1 h) and vacuum filtration. To remove the solvent, the extract was concentrated in a rotary evaporator, which yielded 0.48 g of V18 crude extract. The obtained extract was separately submitted to solid phase extraction with a silica gel 90 (230–400 mesh, Sigma–Aldrich®) reverse phase ODS chromatographic column; MeOH/H<sub>2</sub>O (30:70 v/v), MeOH/H<sub>2</sub>O (1:1 v/v), and 100% MeOH were employed as eluents. After solid phase extraction in the ODS column, the V18 extract afforded the fraction V18-C (MeOH 100%, 315.2 mg). This fraction was sequentially purified by semi-preparative reverse phase HPLC with MeOH/H<sub>2</sub>O (75:25, v/v) as eluent, which yielded compounds **1** (5.1 mg; Rt = 19.25 min,  $\lambda_{\max}$  = 257, 298, and 341 nm) and **2** (24.5 mg, Rt = 20.77 min,  $\lambda_{\max}$  = 252, 297, and 343 nm). spectral data: Compound **1** ((*E*)-5,7-di-hydroxy-2-(pent-1-en-1-yl)-4*H*-benzopyran-4-one) spectral data: <sup>1</sup>H NMR (400 MHz,  $\delta$ , CD<sub>3</sub>OD): 6.52 (dd, J= 15.5, 7.5, H-1'), 6.35 (s, H-3), 6.31 (d, J= 2.2, H-8), 6.28 (d, J= 2.2, H-6), 6.13 (dt, J=15.5, 1.4, 1.4, H-2'), 4.60 (s, OH), 2.23 (dq, 7.4, 7.4, 7.4, 1.20, H-3'), 1.53 (sext, 7.4, H-4'), 0.98 (t, J=7.4, H-5'). <sup>13</sup>C NMR (100 MHz,  $\delta$ , CD<sub>3</sub>OD): 152.8 (C-2), 137.1 (C-2'), 123.2 (C-1'), 104.8 (C-3), 103.2 (C-8), 98.0 (C-6), 35.8 (C-3'), 23.2 (C-4'), 14.0 (C-5'), C-4a (n.d.), C-4 (n.d.), C-5 (n.d.), C-7 (n.d.).

Compound **2** (*E*)-7-hydroxy-5-methoxy-2-(pent-1-en-1-yl)-4*H*-1-benzopyran-4-one. spectral data: <sup>1</sup>H NMR (400 MHz,  $\delta$ , CD<sub>3</sub>OD): 6.55 (d, J=15.5, H-1'), 6.51 (d, J= 2.3, H-8), 6.46 (d, J= 2.3, H-6), 6.43 (s, H-3), 6.14 (dt, J= 15.5, 1.45, 1.45, H-2'), 4.61 (s, OH), 3.87 (s, OCH<sub>3</sub>), 2.24 (dq, J=7.6, 7.6, 7.6, 1.45, H-3'), 1.53 (sex, J= 7.6, H-4'), 0.98 (t, J= 7.6, H-5'). <sup>13</sup>C NMR (100 MHz,  $\delta$ , CD<sub>3</sub>OD): 168.6 (C-5), 167.1 (C-7), 164.8 (C-8a), 153.6 (C-2), 141.3 (C-4a), 137.6 (C-2'), 123.2 (C-1'), 105.8 (C-3), 102.8 (C-8), 101.7 (C-

6), 56.3 (OCH<sub>3</sub>), 35.8 (C-3'), 23.2 (C-4'), 14.0 (C-5'). HR EIMS  $m/z$  283.0974 [M+Na]<sup>+</sup>(calcd for C<sub>15</sub>H<sub>16</sub>O<sub>4</sub>Na, 283.0946).

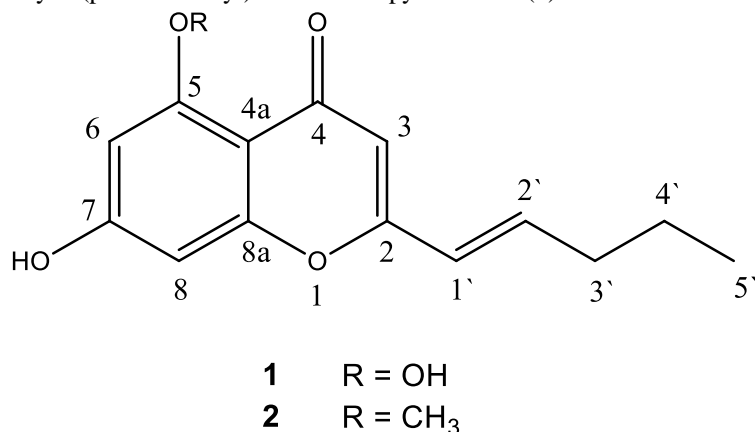
### IN VITRO CYTOTOXICITY ASSAY

The cytotoxic activity of the extract and compounds isolated from the fungal strain *C. olivacea* (V18) against GM07492A cells (normal human lung fibroblasts) was evaluated by *in vitro* colorimetric toxicological assay – XTT Kit (Roche Diagnostics). The manufacturer's guidelines were followed. For the experiment, 1 x 10<sup>4</sup> cells were seeded in microplates containing 100 µL of culture medium (HAM F10 + DMEM, Sigma-Aldrich, 1:1) supplemented with 10% fetal bovine serum (Nutricell) containing a concentration of the natural products under study, which ranged from 1.25 to 2500 µg/mL. Negative (untreated), solvent (dimethylsulfoxide, DMSO, Sigma-Aldrich, 0.02%), and positive (25% DMSO) controls were included. After incubation at 36.5 °C for 24 h, the culture medium was removed, and the cells were washed with 100 µL of phosphate buffered saline and exposed to 100 µL of HAM-F10 culture medium without phenol red (Sigma-Aldrich) plus 25 µL of XTT. The cells were then incubated at 36.5 °C for 17 h. The absorbance of the samples was determined at 450 nm and reference length of 620 nm by using a microplate reader (ELISA –Asys - UVM 340/Microwin 2000). The cytotoxicity was assessed by means of the IC<sub>50</sub> response parameter (50% inhibition of cell viability), which was calculated by the GraphPad Prism program. The experiments were performed in triplicate.

### 3 RESULTS AND DISCUSSION

The crude ethyl acetate extract of *C. olivacea* (V18) was purified by reverse phase chromatography, and a final purification by preparative reverse phase HPLC afforded the chromones (*E*)-5,7-di-hydroxy-2-(pent-1-en-1-yl)-4*H*-benzopyran-4-one (**1**) and (*E*)-7-hydroxy-5-methoxy-2-(pent-1-en-1-yl)-4*H*-1-benzopyran-4-one (**2**) (Figure 1). The spectral data allowed us to propose the structures of compounds **1** and **2**. 5,7-dihydroxychromones of analogous structures obtained from *Horsfieldia irya* (Myristicaceae) have been described (Gonzalez *et al.*, 2002) and were compared to the data obtained herein.

FIGURE 1 - Chemical structure of (*E*)-5,7-di-hydroxy-2-(pent-1-en-1-yl)-4*H*-benzopyran-4-one (1) and (*E*)-7-hydroxy-5-methoxy-2-(pent-1-en-1-yl)-4*H*-1-benzopyran-4-one (2).



The cytotoxicity of the ethyl acetate extract of V18 and the isolated compounds **1** and **2** was evaluated against normal human lung fibroblasts, cell lineage GM07492A (Table 1). The crude extract of V18 was the most cytotoxic (IC<sub>50</sub> = 165.47 µg/mL). In contrast, compounds **1** and **2** presented weak cytotoxicity activity or were inactive toward the GM07492A cells. Comparison of the structural difference between compounds **1** and **2** suggested that the presence of hydroxyl in compound **1** contributed to the higher toxicity of this chromone.

TABLE 1 - *In vitro* effects of the ethyl acetate extract of *Corynespora olivacea* (V18) and compounds 1 and 2 on the viability of GM07492A cells after incubation for 24 h.

Sample	IC <sub>50</sub> ±SD cell viability (µg/mL)	IC <sub>50</sub> ±SD cell viability (µM)
<b>V18</b>	165.47±5.3	-
<b>1</b>	544.4±20.3	2212.34±82.6
<b>2</b>	1250±0.0	≥4805.75±0.0

GM07492A, normal human lung fibroblasts. Positive control (25% DMSO) 20.0±0.0 µg/mL. Values are mean ± SD, n = 3.

Chromones **1** and **2** isolated from *C. olivacea* have not been previously reported.

Zhao *et al.*, (2015) described 12 chromone derivatives, designated corynechromones A–L, isolated from a sponge-derived strain of the fungus *Corynespora cassiicola*. The cytotoxic activity, antibacterial activity, acetylcholinesterase and topoisomerase I inhibitory activities, and antifouling action of the isolated compounds were tested, but they were not active in any of the assays (Zhao *et al.*, 2015).

Other classes of compounds isolated from *Corynespora* include decalactones, xestodecalactones, coryoctalactones, and corynesidones. Corynesidone B is worthy of



highlight: it inhibited protein kinase PIM1 with an IC<sub>50</sub> value of  $3.5 \times 10^{-7}$  M (Ebrahim *et al.*, 2012; Ebrahim *et al.*, 2013).

To date, the chemical constituents of the fungus *Corynespora olivaceae* are unknown.

This work has demonstrated the *in vitro* cytotoxic activity of the ethyl acetate extract of an endophytic fungus obtained from the roots of *V. divergens* and isolated compounds. The low toxicity observed for compounds **1** and **2** suggested that these compounds can be safely used in other trials at the evaluated doses. These data might be useful to guide the application of the endophytic extract and/or isolated compounds in future biological studies. This is the first time that the chromones from an endophytic fungus associated with *V. divergens* have been identified.

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