



# Natural trypanocidal product produced by endophytic fungi through co-culturing

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## Abstract

Endophytic fungi live inside vegetal tissues without causing damage to the host plant and may provide lead compounds for drug discovery. The co-culture of two or more endophytic fungi can trigger silent gene clusters, which could lead to the isolation of bioactive compounds. In this study, two endophytic strains isolated from *Handroanthus impetiginosus* leaves, identified as *Talaromyces purpurogenus* H4 and *Phanerochaete* sp. H2, were grown in mixed and axenic cultures. The meroterpenoid austin was detected only in the extracts from the mixed culture. Once isolated, austin displayed very interesting trypanocidal activity, with an IC<sub>50</sub> value of 36.6 ± 1.2 µg/mL against *Trypanosoma cruzi* in the epimastigote form. The results obtained highlight the importance of the co-culturing of endophytic fungi to obtain natural bioactive products. The findings also enhance our understanding of the ecological relationships between endophytic fungi.

## Introduction

The group designated as neglected tropical diseases (NTDs) includes, among other parasitic illnesses, Chagas disease, which is caused by the protozoan parasite *Trypanosoma cruzi*. The World Health Organization has reported that 8 million people are infected by *T. cruzi* worldwide, mostly in Latin America, and Chagas disease causes around 10,000 deaths per year. The main drugs currently used in the treatment of Chagas disease during the acute phase are nifurtimox and benznidazole, which have been used for more than three

decades. However, these drugs have low efficacy and cause serious side effects in patients (WHO 2018).

Secondary metabolites currently play a crucial role in the search for new lead compounds (Cragg and Newman 2013). Due to the ubiquitous occurrence of microorganisms, along with the wide chemodiversity that can occur within a given species (Bertrand et al. 2014), they are interesting sources of natural bioactive scaffolds (Demain 2014). In this context, the endophytic fungi merit attention (de Carvalho et al. 2016; Silva et al. 2017). Endophytic microorganisms naturally occur in vegetal tissue, without causing notable damage to the host plant (Arnold and Lutzoni 2007). Several studies have provided evidence that the interaction between endophytic community members can play a major role in the onset of metabolite production, such as the biosynthesis of defense metabolites and quorum-sensing signals (Akone et al. 2016).

The genes encoding these biosynthetic pathways are usually clustered (Yamanaka et al. 2014). In many cases, they are not expressed under standard laboratory culture conditions because their activation relies on environmental cues and, therefore, only a minority of the potential secondary metabolites can be investigated in vitro (Scherlach and Hertweck 2009).

Silenced biosynthesis pathways, mainly those related to defense compounds, may be activated when the microbes are grown together in co-cultures or in confrontation

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experiments (Bertrand et al. 2014). Interestingly, these experiments may result either in an enhanced production of known metabolites or in the discovery of new compounds. Recently, the co-culturing of the endophytic fungus *Fusarium tricinctum* with the bacterium *Bacillus subtilis* 168 trpC2 resulted in a 78-fold increase in the accumulation of secondary metabolites and also in the isolation of some compounds which were present in neither of the pure cultures (fungal and bacterial) (Ola et al. 2013). In addition, the endophytic fungus *Alternaria tenuissima* significantly increased the production of some polyketides in response to the presence of the endophytic fungus *Nigrospora sphaerica* (Chagas et al. 2013).

The study reported herein was focused on the search for bioactive metabolites produced by the co-culture of two endophytic fungi isolated from *Handroanthus impetiginosus* leaves.

## Material and methods

### General experimental procedures

One- and two-dimensional NMR spectra were recorded at 500 MHz with a Bruker Avance III spectrometer. Chemical shifts ( $\delta$ ) were referenced to the residual deuterated chloroform ( $\text{CDCl}_3$ ) peak at  $\delta_{\text{H}}$  7.27 for  $^1\text{H}$  and  $\delta_{\text{C}}$  77.0 for  $^{13}\text{C}$ . High-resolution electrospray ionization mass spectroscopy (HRESIMS) spectra were recorded with a micrOTOF mass spectrometer operating in the negative ion mode and using the following conditions: capillary voltage of 4500 V, dry gas flow of 4 L/min, and nitrogen as the nebulizer gas. HPLC analysis was performed using a Shimadzu chromatographic system with a Thermo Scientific Acclaim® 120 C<sub>18</sub> column (100 × 2.1 mm, 5  $\mu\text{m}$ , 120 Å) and MeOH-H<sub>2</sub>O gradient. The analysis was carried out at 0.5 mL/min and all of the chromatograms were recorded at  $\lambda$  254 nm.

### Isolation and preservation of endophytic fungi

The botanical material of *Handroanthus impetiginosus* (Mart. ex DC.) Mattos was collected in Alfenas, Minas Gerais (S 21° 18' 49.15", W 45° 57' 28.53"), Brazil. It was identified by Dr. Lúcia G. Lohmann from the Botanical Department of the Bioscience Institute of the University of São Paulo. A voucher specimen was deposited in the herbarium of the Federal University of Alfenas, under voucher code 2535. Seventy-two endophytic fungal isolates were recovered as described in the literature (Gallo et al. 2009). Briefly, the collected leaves were washed with running water and surface-sterilized by immersion in 70% (v/v) ethanol for 1 min, 2.5% (v/v) sodium hypochlorite solution for 3 min, and 70% (v/v) ethanol again for 1 min. Finally, the leaves were rinsed with sterilized water

and cut aseptically into fragments (0.5 × 0.5 cm). The fragments were inoculated in Petri dishes containing potato dextrose agar (PDA) supplemented with 0.5 g/L chloramphenicol to inhibit the bacterial growth. In order to evaluate the efficiency of the epiphytic microorganism elimination process, 50  $\mu\text{L}$  of the final washing water was also inoculated on PDA. The plates were incubated at 28 °C in a BOD (biochemical oxygen demand) chamber with daily monitoring of fungal growth. Each fungus grown from the plant tissue was transferred to new plates containing PDA medium until pure cultures were obtained. The pure cultures were stored in sterilized water at room temperature (Rodrigues et al. 1992).

### Identification of endophytic fungi

The two selected endophytic strains were identified by classical phenotypic and phylogenetic analysis. Extractions of fungal genomic DNA were carried out by standard procedures and the ITS1–5.8S–ITS2 region was amplified using the primer pair ITS1–ITS4 (White et al. 1990). Amplification of the D1/D2 domain of the LSU rRNA gene was performed using the primers ITS1-F (TCCGTAGGTGAACCTGCGG) and NL-4 (5'-TCCTCCGCTTATTGATATGC-3'). The resulting PCR products were sequenced with an Applied Biosystems automatic sequencer ABI 3730XL (Macrogen Corp.). Sequences were compared with those already present in the GenBank database using the BLAST program (National Center for Biotechnology Information) (Altschul et al. 1990). The sequences were then deposited in GenBank database under the accession numbers MK737061 and MK749843 for *Phanerochaete* sp. H2 and *Talaromyces purpurogenus* H4, respectively. Phylogenetic relationships were calculated using the version 4.0 of the software MEGA (Tamura et al. 2007).

### Endophytic fungi cultures

The endophytic fungi were cultivated in mixed (co-culture) and single (axenic) cultures, in solid and liquid media. Single cultures were established by adding plugs (6 mm diameter) from the same fungus, while for the mixed cultures, plugs from two different fungi were placed simultaneously in the culture medium.

The cultivation in solid media was carried out on Petri dishes containing 20 mL of PDA using a plug from each fungus as the inoculum. All cultures were carried out in triplicate and incubated in a BOD chamber for 7 days, at 28 °C. After this period, the mycelia were removed from the whole plate. **The secondary metabolites were extracted using 40 mL of ethyl acetate (EtOAc), followed by sonication for 20 min.** Additionally, liquid cultivation was carried out in 125-mL Erlenmeyer flasks containing 50 mL of PDB using the same inoculation method. The cultures were incubated in a BOD

chamber for 7 days, at 28 °C. The mycelia were then removed by filtration and the culture broth was extracted with EtOAc.

All extracts were dried over anhydrous sodium sulfate and concentrated under vacuum to yield the liquid and solid crude extracts. Each extract was then dissolved in MeOH (1 mg/mL) and 20  $\mu$ L of this solution was analyzed by HPLC-DAD.

### Isolation of natural product

To isolate the secondary metabolites, 40 plates of solid medium cultures were extracted with EtOAc to afford 104.0 mg of crude extract. This was then subjected to column chromatography (CC) using silica gel 60H (Sigma-Aldrich®, particle size 0.040–0.063 mm) and mixtures of *n*-hexane-EtOAc followed by EtOAc-MeOH as the eluting solvent. Ten fractions were collected and the seventh fraction contained austin (1).

**Austin (1):** 17.0 mg (16.3% yield) white powder;  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{Cl}$ )  $\delta$  6.63 (1H, d,  $J$  = 10.0 Hz, H-1), 6.10 (1H, d,  $J$  = 10.0 Hz, H-2), 5.72 (1H, d,  $J$  = 1.0 Hz, H-1'), 5.49 (1H, d,  $J$  = 1.0 Hz, H-1'), 4.42 (1H, q,  $J$  = 6.2 Hz, H-5'), 3.55 (1H, m, H-7), 2.03 (3H, s, H-12'), 1.87 (3H, s, H-13), 1.67 (1H, m, H-7), 1.62 (3H, s, H-9'), 1.60 (1H, m, H-6), 1.54 (3H, s, H-12), 1.53 (1H, m, H-6), 1.38 (3H, s, H-15), 1.29 (3H, d,  $J$  = 6.2 Hz, H-10'), 1.19 (3H, s, H-14);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{Cl}$ )  $\delta$  171.1 (C-4'), 170.5 (C-8'), 168.7 (C-11'), 163.9 (C-3), 146.7 (C-1), 144.1 (C-10), 137.8 (C-2'), 132.8 (C-9), 120.5 (C-2), 118.3 (C-1'), 85.8 (C-4), 84.5 (C-3'), 80.9 (C-6'), 79.0 (C-5'), 74.9 (C-11), 63.1 (C-7'), 46.9 (C-5), 42.4 (C-8), 27.3 (C-6), 26.8 (C-7), 26.2 (C-15), 23.8 (C-12), 22.7 (C-14), 20.9 (C-12'), 20.5 (C-9'), 15.7 (C-13), 11.6 (C-10'); HRESIMS  $m/z$  499.1972 [ $\text{M}^-$ ] (calcd for [ $\text{M}^-$ ] 499.1968).

### Fungal antagonism assays with austin and extract from co-culture

One plug from the 7-day-old fungal colony grown in PDA was transferred to another PDA plate. Austin (1) and the crude extract from the solid co-culture were dissolved in 1:1 methanol:water (0.05 mg/ $\mu$ L), and 20  $\mu$ L of each solution was separately placed in wells placed 4 cm apart of the fungal colony. A mixture of 1:1 methanol:water was used as the negative control. Plates were incubated at 28 °C for 7 days. All assays were performed in duplicate.

### In vitro trypanocidal and cytotoxicity assays

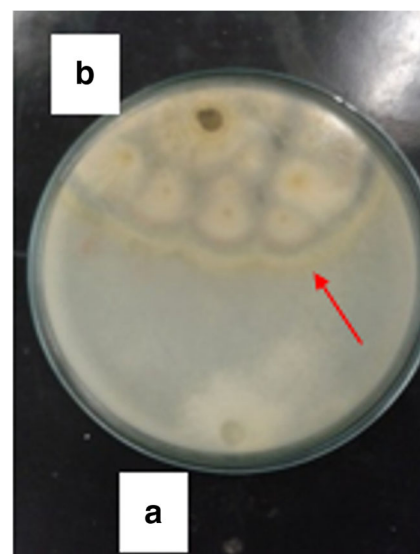
The trypanocidal assay was carried out using the *Trypanosoma cruzi* Y strain (DTU II). The epimastigotes were maintained in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum (FBS) at 28 °C. The cytotoxicity assay was performed with uninfected H9c2 ATCC CRL 1446, which was obtained from neonatal rat

cardiomyoblasts and cultured in Dulbecco's modified Eagle's medium (DMEM) in an incubator with 5%  $\text{CO}_2$ . Austin (1) and benznidazole were tested at the same concentrations, in triplicate, for both the trypanocidal and cytotoxicity assays, as described in the literature (Brancaglioni et al. 2018). The concentrations required to inhibit 50% of the epimastigotes ( $\text{IC}_{50}$ ) and 50% of H9c2 uninfected cells ( $\text{CC}_{50}$ ), along with the selectivity index (SI,  $\text{CC}_{50}/\text{IC}_{50}$ ) values, were calculated with the GraphPad Prism (version 5.0) software.

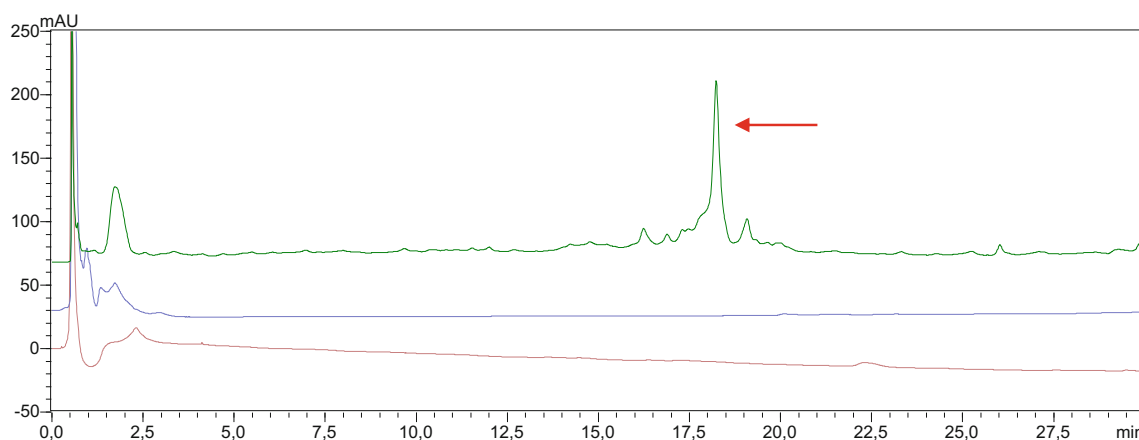
## Results

The 72 isolates obtained from the leaves of *H. impetiginosus* were named H1–H72, and those that presented distinct morphological characteristics were selected. These were used to conduct tests of dual confrontation on PDA plates and determine the growth inhibition areas (data not shown). From these experiments, the strains H2 and H4 were selected because they showed a notable inhibition area in the solid medium assay (Fig. 1). Both strains were identified by molecular methods up to genus and species levels as *Phanerochaete* sp. H2 and *Talaromyces purpurogenus* H4, by analyzing their ITS sequences. The corresponding phylogenetic trees are shown in the Supplementary data (Figs. 1 and 2).

*Phanerochaete* sp. H2 and *T. purpurogenus* H4 were used to perform axenic and dual cultures in both solid (PDA) and liquid (PDB) media. From these cultures, crude extracts were obtained with ethyl acetate and their chemical profiles were analyzed by HPLC-DAD (Fig. 2). Notably, in the organic extracts obtained from the mixed cultures, a metabolite (1)



**Fig. 1** Antagonist activity of 7-day-old cultures of *Phanerochaete* sp. H2 (A) against *Talaromyces purpurogenus* H4 (B) cultured in PDA medium. There is a visible inhibition zone (red arrow)



**Fig. 2** Metabolic profiles of the solid crude extracts obtained from the 7-day-old mixed and single cultures of two selected endophytic fungi from *Handroanthus impetiginosus*. The chromatograms corresponding to the mixed culture extract (green) and the *Phanerochaete* sp. H2 and the

*Talaromyces purpurogenus* H4 pure cultures (brown and blue chromatograms, respectively). The production of austin (red arrow) was observed only in the mixed culture

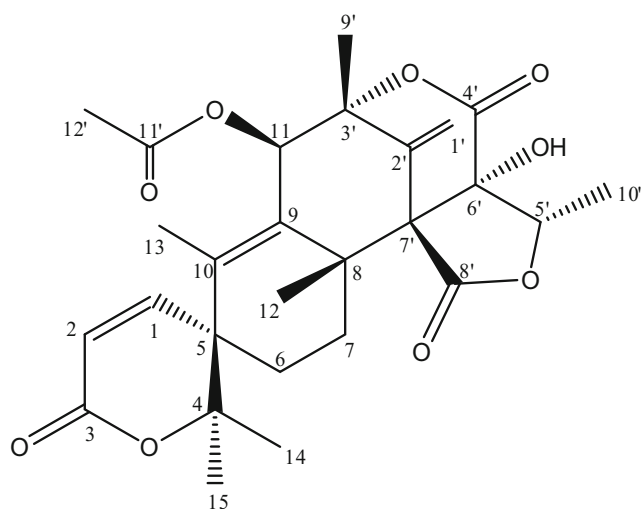
appeared at a retention time of 18.0 min, and this was not present in any of the axenic cultures.

Due to the accumulation of compound **1**, measured as a percentage of the chromatogram total area, the amount in the dual cultures grown in solid media was almost twice that of the cultures grown in liquid media, with values of 20.46% and 12.49%, respectively (see Figs. 3 and 4 in the Supplementary data). Thus, the solid medium cultures were chosen to resize the assay aimed at the isolation of **1**. A pooled sample of the organic solid medium extract samples was subjected to CC and product **1** was isolated in 16.3% yield. Its molecular formula was determined as  $C_{27}H_{32}O_9$  by HRESIMS ( $m/z$  499.1972 [M - H]<sup>-</sup>, Fig. 5 in the Supplementary data). The <sup>1</sup>H NMR spectrum analysis showed typical terpenoid hydrogen signals and the <sup>13</sup>C NMR spectrum evidenced 27 carbons, consistent with a meroterpenoid. The chemical structure of **1** was then identified with the aid of 2D-NMR analysis (see

spectra in Figs. 6–9 in the Supplementary data) as an unusual meroterpenoid named austin (**1**; Fig. 3). All of the spectral data are in agreement with those reported in the literature (Hayashi et al. 1994).

Subsequently, growth inhibition tests were carried out with both axenic fungal strains, exposing them alternatively to the solid medium extract and dilution of pure austin (**1**). It was evident that the solid medium extract inhibited the development of *T. purpurogenus* H4, while the austin (**1**) solution had no effect on the growth of the two strains (see Fig. 10 in the Supplementary data).

On the other hand, when the bioactivity of austin (**1**) toward *T. cruzi* epimastigotes was assessed, inhibition rates of 100% and 96.39% were observed at concentrations of 200  $\mu$ g/mL and 100  $\mu$ g/mL, respectively. The  $IC_{50}$  value for austin (**1**) against the epimastigote form was  $36.60 \pm 1.20$   $\mu$ g/mL, while the reference drug benznidazole showed an  $IC_{50}$  value of  $8.01 \pm 1.31$   $\mu$ g/mL. In the cytotoxicity assay against uninfected H9c2 cells, austin (**1**) showed a  $CC_{50}$  value of  $175.65 \pm 1.20$   $\mu$ g/mL, while in comparison the  $CC_{50}$  value for benznidazole was  $187.85 \pm 2.15$   $\mu$ g/mL. The selectivity indexes for austin (**1**) and for benznidazole were calculated as 4.79 and 23.45, respectively.



**Fig. 3** Chemical structure of austin (**1**), a meroterpenoid isolated from the *Phanerochaete* sp. H2 and *Talaromyces purpurogenus* H4 co-culture

## Discussion

In order to identify the potential of the endophytic fungi isolated from *H. impetiginosus* leaves for the biosynthesis of chemical compounds with antibiotic activity, dual cultures were performed in order to assess the effects of the microbial interaction on the metabolomic profiles. The stress caused by the presence of the two species in the same Petri dish seems to be efficient in triggering biosynthetic pathways. Although fungal cultures in agar plates are not ideal substrates in



ecological terms, this culture method allows the sampling of different regions of the mycelia as well as the observation of inhibition zones (Hiscox et al. 2010). Therefore, in the study reported herein, interspecies interactions between *Phanerochaete* sp. H2 and *T. purpurogenus* H4 were evaluated on PDA plates.

Moreover, since the endophytic strains belong originally to the same habitat, the co-culture assay could mimic the natural interaction between these microorganisms and visual analysis allowed us to select the strains with the potential to produce bioactive compounds.

To the best of our knowledge, this is the first time the isolation of the meroterpenoid austin (**1**) from fungi in a co-culture has been reported. This approach efficiently enhanced the yield of this important meroterpenoid.

The meroterpenoids are natural products that are widespread in fungi. Austin (**1**) was isolated for the first time in 1976 from *Aspergillus ustus* cultures (Chexal et al. 1976). Later, a mixed polyketide-terpenoid biosynthetic pathway in *A. ustus* was elucidated by the incorporation of <sup>13</sup>C-labeled acetate and methionine groups into the chemical structure of **1** (Ahmed et al. 1989). Other authors have reported the isolation of **1** from fungal cultures, for instance, a *Penicillium* sp. MG-11 soil culture (6.0% yield) (Hayashi et al. 1994), the endophytic fungus *Penicillium* sp. T2-8 (0.065% yield) (Duan et al. 2016), and the plant pathogen *Verticillium albo-atrum* (0.027% yield) (Wu et al. 2018).

We postulate that the biosynthesis of the meroterpenoid **1** occurred as a fungal defense mechanism or as a signaling molecule triggered by stress caused by the co-culture. It has been reported that microorganisms interact with each other via metabolic exchange mechanisms (Phelan et al. 2012). The biosynthesis of some secondary metabolites is important for the endophyte survival when it is in competition with other microorganisms (Ola et al. 2013).

Austin (**1**) has been previously identified as a potential natural insecticide (Wu et al. 2018; Kataoka et al. 2011) and also as mycotoxin (Chexal et al. 1976). Our results corroborate the hypothesis of the important role of the endophytic microbiota in the plant defense strategy against pathogens. In the antagonism assays, we tested the action of austin (**1**) and the extracts of the co-culture against the endophytic fungi investigated in this study. The results obtained show that **1** may act in synergy with other compounds as an antifungal agent.

The inhibition zone observed on the plate containing the two endophytic strains was probably due to the secretion of the natural products exclusively detected in the mixed cultures. Since these secondary metabolites demonstrated antifungal activity in the assays, it is presumed that these chemical compounds may display other types of interesting antibiotic activities as well.

The antiparasitic activity of the natural meroterpenoids is well documented (Gray et al. 2006; Agostinho et al. 2013).

Prompted by the interesting biological potential displayed by austin (**1**), its trypanocidal activity was investigated. Although the selectivity index of austin (SI = 4.79) was less favorable than that observed for benznidazole (SI = 23.45), the meroterpenoid was about fivefold less toxic to the host cell than to the epimastigote. The results demonstrate that **1** shows good potential for use in further studies on the development of trypanocidal drugs.

Considering that endophytic fungi can influence the metabolism of other microorganisms or the host plant in a sophisticated crosstalk (Kusari et al. 2013), we hypothesize that both endophytic fungi contributed to the biosynthesis of austin (**1**). Two different organisms in the same environment may contribute to the biosynthesis of the same secondary metabolite when each organism contains part of the enzymes necessary to complete the biosynthetic pathway. In this case, the biosynthetic pathways of the two organisms are combined and each is induced by the other (Ludwig-Müller 2015).

In summary, in this study, the interactions between endophytic fungi from the same host plant were found to stimulate the biosynthesis of the meroterpenoid austin (**1**) in good yield.

Additionally, austin (**1**) can act as a chemical signal included in the network established between the endophytes. The potential antiparasitic activity of **1** reinforces the pharmacological importance of natural products for which their biosynthesis is activated by microbial interactions. It is clear that future studies on mixed cultures of endophytic microorganisms will open up possibilities for the identification of new lead compounds and will provide a better understanding of the mechanisms involved in the interactions between endophytic fungi.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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