



Universidade de São Paulo

Biblioteca Digital da Produção Intelectual - BDPI

Departamento de Física e Ciência Interdisciplinar - IFSC/FCI

Artigos e Materiais de Revistas Científicas - IFSC/FCI

2008-01

Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*

FEMS Immunology and Medical Microbiology, Oxford, v. 52, n. 1, p. 134-144, 2008

<http://www.producao.usp.br/handle/BDPI/49106>

Downloaded from: Biblioteca Digital da Produção Intelectual - BDPI, Universidade de São Paulo

Biological activities from extracts of endophytic fungi isolated from *Viguiera arenaria* and *Tithonia diversifolia*

Denise O. Guimarães¹, Warley S. Borges¹, Cristina Y. Kawano¹, Patrícia H. Ribeiro¹, Gustavo H. Goldman¹, Auro Nomizo¹, Otávio H. Thiemann², Glaucius Oliva², Norberto P. Lopes¹ & Mônica T. Pupo¹

¹Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil; and ²Instituto de Física de São Carlos, USP, São Carlos, SP, Brazil

Correspondence:

Mônica T. Pupo,
Faculdade de Ciências Farmacêuticas de
Ribeirão Preto, USP, Avenida do Café, s/n,
14040-903, Ribeirão Preto, SP, Brazil. Tel.:
+55 16 36024710; fax: +55 16 36024879;
e-mail: mtpupo@fcrfp.usp.br

Received 9 July 2007; revised 11 October 2007;
accepted 12 October 2007.

First published online 13 December 2007.

DOI:10.1111/j.1574-695X.2007.00354.x

Editor: Alex van Belkum

Keywords

endophytic fungi; *Viguiera arenaria*;
Tithonia diversifolia; *Glomerella cingulata*;
cytotoxic activity; nectriapyrone.

Abstract

A total of 39 endophytic fungi have been isolated from *Viguiera arenaria* and *Tithonia diversifolia*, both collected in São Paulo State, Brazil. The isolates were identified based on their ribosomal DNA sequences. The ethyl acetate (EtOAc) extracts of all endophytic fungi were evaluated for their antimicrobial, antiparasitic and antitumoral activity. Antimicrobial screening was conducted using an agar diffusion assay against three pathogenic microorganisms: *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. Antiparasitic activity was determined by enzymatic inhibition of gGAPDH of *Trypanosoma cruzi* and adenine phosphoribosyltransferase (APRT) of *Leishmania tarentolae*. Antitumoral activity was tested against human T leukemia cells by the Mosmann colorimetric method. All extracts showed activity in at least one assay: 79.5% of the extracts were cytotoxic against leukemia cells, 5.1% of the extracts were active against *S. aureus*, 25.6% against *E. coli* and 64.1% against *Candida albicans*. Only one extract showed promising results in the inhibition of parasitic enzymes gGAPDH (95.0%) and three were found to inhibit APRT activity. The cytotoxic extract produced by the strain VA1 (*Glomerella cingulata*) was fractionated and yielded nectriapyrone and tyrosol. Nectriapyrone showed relevant cytotoxic activity against both human T leukemia and melanoma tumor cell lines.

Introduction

The significance of natural products in the drug discovery and development processes has been reported extensively (Koehn & Carter, 2005). The importance of natural products as sources of innovative therapeutic agents can be illustrated by the drugs used in the control of infectious diseases, cancer, lipid disorders, immunomodulation and hypertensive diseases (Clardy & Walsh, 2004). However, one crucial aspect to be considered for a successful natural product-based drug discovery program is the selection of the natural source to be studied. It is important to take into account that unexplored and/or underexplored sources of biological diversity are often related to novel chemical diversity. Endophytic fungi have been considered to be untapped sources of natural products and should be included in the search for new and innovative biologically active compounds (Clardy & Walsh, 2004).

The term endophytic fungus has been used to describe those fungi that can be detected at a particular moment

within the tissues of an apparently healthy plant host. The colonization can be inter- or intracellular, localized or systemic (Schulz & Boyle, 2005), and their metabolic interactions with the environment increase the possibilities of the production of bioactive compounds. In fact, endophytic fungi have been shown to be a promising source of new natural bioactive agents (Pupo *et al.*, 2006). Moreover, several crude extracts from different culture broths have shown antimicrobial activity against pathogenic fungi, bacteria and yeasts, cytotoxic activity on human cell line, anti-Herpes simplex virus type 1 activity (anti-HSV) and antimalarial activity against the protozoan *Plasmodium falciparum* (Rodrigues *et al.*, 2000; Huang *et al.*, 2001; Liu *et al.*, 2001; Corrado & Rodrigues, 2004; Wiyakrutta *et al.*, 2004). Therefore, the investigation of endophytic fungi can lead to useful and new natural products.

The aim of this study was to isolate, identify and cultivate endophytic fungi from two *Asteraceae* species: *Viguiera arenaria* and *Tithonia diversifolia*. *Tithonia diversifolia*, also known as Mexican sunflower, was selected because its

extracts have been used traditionally in the treatment of malaria, diarrhea, fever, hepatitis and wounds (Cos *et al.*, 2002; Goffin *et al.*, 2002; Gu *et al.*, 2002). Anti-inflammatory, amebicidal, antispasmodic, antifungal, antibacterial and antiviral activities have also been described for *Tithonia diversifolia* extracts (Cos *et al.*, 2002; Goffin *et al.*, 2002). Bioactive diterpenes have been isolated from *V. arenaria* (Ambrosio *et al.*, 2006). Moreover, there are no previous reports on the isolation and cultivation of endophytes from *Tithonia diversifolia* and *V. arenaria*.

The organic extracts were evaluated in antimicrobial assays against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*, in cytotoxic assays using leukemia cells and inhibition of parasitic enzymes glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) from *Trypanosoma cruzi* and adenine phosphoribosyltransferase (APRT) from *Leishmania tarentolae*. The assays were selected on the basis of the need for new antibiotics and anticancer agents, due to the development of resistance by infectious microorganisms and the high worldwide mortality caused by cancer (Pisani *et al.*, 1999). In addition, there is an urgent need for efficient drugs for the treatment of protozoan and nematodal infectious such as malaria, leishmaniasis, trypanomiasis and filariasis (Strobel & Daisy, 2003). *Glomerella cingulata* was found to be the most common endophytic fungus associated with *V. arenaria*. For this reason, the cytotoxic extract VA1 was selected for chemical studies, leading to the isolation and identification of the bioactive compound nectriapyrone (1) and also of tyrosol (2).

Materials and methods

Isolation

The leaves and roots of *V. arenaria* were collected in São Carlos city, SP, and the leaves of *Tithonia diversifolia* were collected in Ribeirão Preto city, SP.

The general procedures adopted for isolation of the microorganisms followed the methodology described by Weber *et al.* (2004). After collection, the plant material was washed with water and surface sterilized by immersion in 70% aqueous ethanol (3 min), followed by 5% aqueous sodium hypochlorite (60'', 90'' and 5') and finally with 70% aqueous ethanol (1 min). After these procedures, the leaves and roots were rinsed with sterilized water. This latter water was incubated in Petri dishes to ensure the elimination of all epiphytic microorganisms. Small pieces of the leaves and roots were excised and placed on agar in Petri dishes containing potato dextrose agar (PDA) medium at 30 °C. Individual hyphal tips of the emerging fungi were removed and placed on PDA slants. Endophytic fungi isolated from the leaves *Tithonia diversifolia* have been codified as

TD1–TD5. Endophytic fungi isolated from the leaves *V. arenaria* have been codified as VA1–VA19, VA25, VA26 and VA29–VA37, and those isolated from the roots of *V. arenaria* have been codified as VA20, VA21, VA27 and VA28.

All the isolated endophytic fungi strains have been deposited at 'Laboratório de Enzimologia Industrial', FCFRP, USP. The strains are maintained by periodic transfers onto PDA (Nam *et al.*, 2000) at 4 °C, and also in silica gel (six to 12 mesh, grade 40, desiccant activated) at 10 °C.

Identification of the endophytic fungi

The isolates were identified based on their ribosomal DNA (rRNA gene) sequences. The fungi were grown on YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] for 72 h at 30 °C on a shaking platform at 120 r.p.m., harvested by vacuum filtration, frozen in liquid nitrogen and stored at –70 °C. Genomic DNA was isolated using the phenol–chloroform extraction technique, and the pellets were allowed to dry. DNAs were then dissolved in 100 µL sterile water and 1 µL of DNA solution was used for the amplification procedure. The DNAs were quantified with a fluorometer. Amplification of products of the correct size was verified on 1% agarose gels. The ITS1–ITS2 and ITS3–ITS4 primer pairs were used to amplify a large portion of the ITS1 region, 5.8S rRNA gene and the adjacent ITS2 region. PCR amplification was performed with a volume of 50 µL: 1 µL of genomic DNA (500 ng), 2.5 µL of 4 mM dNTP, 0.5 µL of 5 U of Amplitaq platinum Taq polymerase µL⁻¹, 5 µL of 10X buffer [100 mM Tris-HCl (pH 8.3), 2 µL of 50 mM MgCl₂], 1 µL of universal primers ITS1, ITS2, ITS3 and ITS4 (21 pM) and 37 µL distilled water. Amplification was completed in a thermal cycle under cycling conditions: 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min, and a final step of 72 °C for 10 min. PCR products were purified in Qiagen columns. Full DNA sequence analysis of the PCR products obtained with universal fungal primers ITS3–ITS4 specific for rRNA gene was used to confirm the species identification. Automated DNA sequencing was performed in both directions using the initial amplifications primers, and the resulting DNA sequences were aligned and analyzed with SEQUENCHER software. Comparison of sequences from isolates and GenBank sequences was performed using an advanced BLAST search (Henry *et al.*, 2000) and CLUSTAL W (Thompson *et al.*, 1994). Identities higher than 98% were taken into account for the classification.

Fermentation and extraction

The fermentation procedure was carried out in a two-step process in which the suspensions of spores or fungal mycelia were inoculated into 1-L flasks containing 200 mL of seed medium (Jackson *et al.*, 1993). After inoculation, the flasks

were incubated with agitation (120 r.p.m.) at 30 °C for 2 days (preculture). The resulting mycelia were harvested, rinsed and transferred into 2-L flasks containing 400 mL of Czapek medium (Alviano *et al.*, 1992) for an additional fermentation period of 6 days at 30 °C, with shaking at 120 r.p.m.

After the fermentation processes, the culture broths were separated from the mycelia by filtration under vacuum, and submitted to liquid-liquid partition with EtOAc (3 × 150 mL). After this procedure, the organic solvent was evaporated under reduced pressure to dryness to yield an EtOAc extract.

Isolation of compounds

The EtOAc crude extract (88.0 mg) obtained from *G. cingulata* (VA1) was fractionated in a silica gel column (0.063–0.200 nm) with hexane:EtOAc (9:1); hexane:EtOAc (1:1); EtOAc; and MeOH. The subfraction 19 (7.6 mg), obtained with Hexane:EtOAc (1:1), was submitted to preparative TLC in silica gel PF₂₅₄ eluted with hexane:EtOAc (7:3), yielding nectriapyrone (1) (rf: 0.41, 4.5 mg) (Fig. 1). The sub fraction 25 (6.4 mg), obtained with hexane:EtOAc (1:1), was submitted to preparative thin layer chromatography (TLC) in silica gel PF₂₅₄ eluted with CH₂Cl₂:MeOH (9:1), yielding tyrosol (2) (rf: 0.40, 5.0 mg) (Fig. 1). Both compounds were extracted with acetone:MeOH (4:1).

Structure elucidation

Nuclear magnetic resonance (NMR) spectra were acquired in Bruker spectrometers (DRX-400 and DRX-500), working at 400 and 500 MHz for ¹H and at 100 and 125 MHz for ¹³C. The spectra were recorded in CDCl₃, and the solvent signals at δ 7.26 for proton, and δ 77.0 for carbon, were used as references. Mass spectra were carried out in a mass spectrometer ESI-MS (Micromass Quattro LC – electro-spray ionization).

Tumor cell line culture and cytotoxicity assay

The human acute T-cell leukemia tumor cell line (JURKAT) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The tumor cell line was maintained

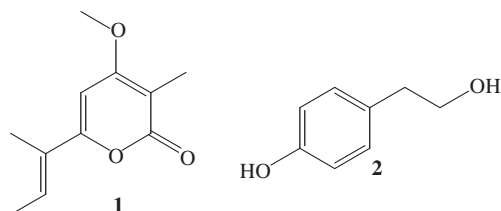


Fig. 1. Chemical structures of nectriapyrone (1) and tyrosol (2) produced by *Glomerella cingulata* (VA1).

in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mM L-glutamine, 1.5 g L⁻¹ sodium bicarbonate, 4.5 g L⁻¹ glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 100 U mL⁻¹ of penicillin and 100 µg mL⁻¹ of streptomycin. The tumor cells cultures were maintained at 37 °C in an atmosphere of 5% CO₂ and 95% air with more than 95% humidity.

The cytotoxic activity was determined against Jurkat cells and assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983). Briefly, the tumor cells line was cultured in appropriate flasks and maintained in continuous exponential growth. Tumor cells were removed from the flasks, washed and were placed in 96-well plates at a density of 1 × 10⁵ cells well⁻¹. EtOAc extracts and isolated compounds were dissolved in dimethyl sulfoxide (1000 × concentrated solution). Tumor cells were cultured with or without different concentrations of relevant compound methotrexate (Whitehall Co., São Paulo) (1000–0.1 µg mL⁻¹), and incubated for 24 h. At the end of the treatment period, 20 µL of MTT (5 mg mL⁻¹) was added to the wells, and cells were incubated for a further 4 h. Finally, 50 µL of 20% sodium dodecyl sulfate solution was supplied to each well. Formazan crystals were dissolved at 37 °C, overnight. The absorbance of each well was read on a micro plate reader (µQUANT, Biotek Instruments Inc.) at a wavelength of 570 nm. Cytotoxicity rate was calculated as follow: % of cytotoxicity of compounds = 1 – absorbance drug treated / absorbance control × 100.

Antimicrobial assays

The agar plate diffusion assay was used to evaluate the antimicrobial activity (Rios *et al.*, 1988; Freitas *et al.*, 2002). The EtOAc extracts of all endophytic fungi and isolated compounds were screened for antimicrobial activity against *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *Candida albicans* (ATCC 1023). All bacteria were maintained on tryptic soy agar at 37 °C and *Candida albicans* was maintained on Sabouraud at 37 °C. The microorganisms were grown onto antibiotic medium no. 1 for 24 h at 30 °C, and aliquots were placed in 0.9% NaCl until they reached a turbidity value of 0.5 on the McFarland scale (about 10⁷ CFU mL⁻¹). Each microorganism suspension (0.5% in antibiotic medium no. 1) was used for the agar-well diffusion method. In this procedure, 1 mg of each extract was dissolved in 1 mL of dimethylsulfoxide (50%) and the penicillin G (1 µg mL⁻¹ for *S. aureus*), miconazole (4 mg mL⁻¹ for *Candida albicans*) and streptomycin sulfate (0.2 mg mL⁻¹ for *E. coli*) were used as positive controls. All samples (organic extracts, isolated compounds and positive controls) were placed (50 µL) in templates on plates containing antibiotic medium no. 1 with the test

microorganisms. Dimethylsulfoxide (50%, 50 μ L) was used as a negative control. After 3 h of preincubation at room temperature to allow diffusion of the substances into the agar, the plates were incubated for 24 h at 37 °C and overlaid with 5 mL of 1% agar solution containing 2,3,5-triphenyl tetrazolium chloride (2 mg mL⁻¹). After 15 min, the plates were examined for the detection of clear zones of inhibition around the fungal extract. This experiment was performed in triplicate. For data analysis, the clear zones of inhibition around the fungal extracts were measured in millimeter with a ruler and compared with the positive controls. Extracts were considered to be active when the clear zones were equal to or superior to 12 mm.

gGAPDH activity assay

The gGAPDH used in the assays is a recombinant enzyme obtained in an *E. coli* expression system. The preparation and purification of the gGAPDH followed the procedure described previously (Souza *et al.*, 1998). The assays were performed as described (Vieira *et al.*, 2001). Reduced NADH was measured spectrophotometrically at 340 nm at a 30-s interval. The reaction medium contained 50 mmol L⁻¹ Tris-HCL, pH 8.6, buffer with 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ β -mercapto-ethanol, 30 mmol L⁻¹ Na₂HAsO₄ 2.5 mmol L⁻¹ NAD⁺, 0.3 mmol L⁻¹ glyceraldehyde-3-phosphate and 1.5 μ g protein, in a total volume of 1000 μ L. The reaction was initiated by addition of enzyme. All extracts and isolated compounds were evaluated at a concentration of 100 μ g mL⁻¹. All the measurements were run in triplicate. A negative control with 10% dimethylsulfoxide was used.

The APRT activity assay

The APRT used in the assays is a recombinant enzyme obtained in an *E. coli* expression system. APRT activity was determined by spectrophotometric measurements of formation of AMP at 259 nm after 60 s (Tuttle & Krenitsky, 1980). The reaction medium contained 100 mmol L⁻¹ Tris-HCL, pH 7.4, 5 mmol L⁻¹ MgCl₂, 100 mmol L⁻¹ adenine, 560 mmol L⁻¹ PRPP and 7.5 μ g APRT, in a total volume of 1000 μ L. All extracts and isolated compounds were evaluated at a concentration of 50 μ g mL. All the measurements were run in triplicate. A negative control with 10% dimethylsulfoxide was used.

Results and discussion

A total of 39 endophytic strains were isolated from *V. arenaria*, 30 from the leaves and four from the roots, while five endophytes were isolated from the leaves of *Tithonia diversifolia*. The molecular identification of the strains showed that 13 isolates obtained from *V. arenaria* were classified as *Glomerella cingulata* and two were classi-

Table 1. Endophytic fungi strains isolated from *Tithonia diversifolia* (TD) and *Viguiera arenaria* (VA)

Strain	Identification (homology %)
TD1	<i>Fusarium</i> sp. (98%)
TD2	<i>Diaporthe helianthi</i> (93%) or <i>Phomopsis</i> sp. (93%)
TD4	<i>Cercospora kikuchii</i> (99%)
TD5	<i>Phoma sorghina</i> (96%)
VA1	<i>Glomerella cingulata</i> (100%)
VA2	<i>Colletotrichum gloeosporioides</i> (100%)
VA3	<i>Glomerella cingulata</i> (99%)
VA4	<i>Glomerella cingulata</i> (97%)
VA5	<i>Glomerella cingulata</i> (100%)
VA6	<i>Colletotrichum gloeosporioides</i> (97%)
VA7	<i>Glomerella cingulata</i> (98%)
VA8	<i>Glomerella cingulata</i> (99%)
VA9	<i>Diaporthe helianthi</i> (99%)
VA10	<i>Glomerella cingulata</i> (99%)
VA12	<i>Diaporthe helianthi</i> (99%)
VA13	<i>Diaporthe phaseolorum</i> (96%)
VA14	<i>Diaporthe phaseolorum</i> (97%)
VA15	<i>Guignardia mangiferae</i> (100%)
VA16	<i>Phyllosticta</i> sp. (98%) or <i>Guignardia mangiferae</i> (98%)
VA17	<i>Fusarium</i> sp. (98%)
VA18	<i>Glomerella cingulata</i> (99%)
VA19	<i>Phomopsis longicolla</i> (97%)
VA20	<i>Penicillium ochrochloron</i> (96%)
VA25	<i>Glomerella cingulata</i> (100%)
VA26	<i>Glomerella cingulata</i> (99%)
VA27	<i>Cryptosporiopsis rhizophila</i> (99%)
VA28	<i>Glomerella</i> sp. (99%)
VA29	<i>Diaporthe phaseolorum</i> (97%)
VA31	<i>Exserohilum mcginnisii</i> (<i>Alternaria</i> sp.) (95%)
VA32	<i>Phomopsis</i> sp. (99%)
VA33	<i>Glomerella cingulata</i> (99%)
VA34	<i>Glomerella cingulata</i> (100%)
VA35	<i>Phomopsis</i> sp. (97%)
VA36	<i>Glomerella cingulata</i> (100%)
VA37	<i>Phoma sorghina</i> (<i>Ampelomyces</i> sp., <i>Phomopsis</i> sp.) (97%)

fied as *Colletotrichum gloeosporioides* (Table 1). In fact, *G. cingulata* is the teleomorph (sexual) stage of *Colletotrichum gloeosporioides*. These strains were kept separately because they showed morphological differences, and they also produced extracts with different chemical profiles analyzed by HPLC and ¹H NMR (data not shown). The alignment of the *G. cingulata* isolates ITS1-2 and ITS3-4 regions showed very high identity, above 95% (supplementary Fig. S1a, b). The alignment of ITS3-4 regions from *G. cingulata* with *Colletotrichum gloeosporioides* displayed about 93% identity (supplementary Fig. S1c). Additional studies are being currently performed to understand the chemistry and biological properties of these two groups of isolates.

Colletotrichum species are implicated in plant diseases, usually referred to as anthracnose, and include some of the most destructive postharvest pathogens of cereals, legumes, fruits and vegetables (García-Pajón & Collado, 2003). Five

Diaporthe species were also identified from *V. arenaria* (three strains of *Diaporthe phaseolorum* and two of *Diaporthe helianthi*). Another strain of *D. helianthi* was also isolated from *Tithonia diversifolia* (Table 1). One strain from *Tithonia diversifolia* and five from *V. arenaria* have not been identified.

The EtOAc extracts from the endophytic fungi were evaluated against three pathogenic microorganisms: *S. aureus*, *E. coli* and *Candida albicans*. Antiparasitic activity was determined by enzymatic inhibition of gGAPDH of *Trypanosoma cruzi* and APRT of *L. tarentolae*. Antitumoral activity was determined against human T leukemia cells. The extract obtained from the culture medium (blank) was tested in all the assays and did not present significant activity, showing that positive results were due to the secondary metabolites produced by the endophytic fungi.

Antimicrobial activity

The antimicrobial activities of the fungal extracts are presented in Table 2. Two extracts (5.1%) presented significant antimicrobial activity against *S. aureus*, while ten extracts (25.6%) were active against *E. coli* and 25 (64.1%) showed activity against *Candida albicans*. Some extracts were found to be ineffective against microorganisms at the concentration used (1 mg mL^{-1}). The relative high proportion of active extracts against *Candida albicans* indicates that these endophytic fungi might produce potent antifungal compounds.

Endophytes have already been reported as being prolific producers of antimicrobial compounds. Wiyakrutta *et al.* (2004) screened extracts from 360 endophytic fungi in six different assays, resulting in *c.* 25% active extracts against *Mycobacterium tuberculosis*.

A total of 187 strains of endophytic fungi belonging to 136 species have also been tested in antimicrobial assays (Pelaez *et al.*, 1998). The production of antimicrobial substances was detected in 45 strains. In this study, differences were observed among isolates from the same species, with respect to their ability to produce antimicrobial metabolites (Pelaez *et al.*, 1998). Species of *Phomopsis* isolated from *Aspidosperma tomentosum* and *Spondias mombin* were screened for their antibacterial activity using the bioautographic TLC agar-overlay technique. Three of the 13 extracts effectively inhibited the growth of all test organisms (Corrado & Rodrigues, 2004).

Compounds isolated previously from endophytic fungi also displayed antimicrobial activities. The altersetin, a natural product isolated from two endophytic *Alternaria* species, showed potent minimum inhibitory concentration against several pathogenic Gram-positive bacteria (Hellwig *et al.*, 2002). A series of 15 structurally diverse families of diterpene natural products, the guanacastepenes A–O, pro-

duced by an unidentified endophyte isolated from a Costa Rican plant also presented antimicrobial activity (Brady *et al.*, 2001). Guanacastepene A showed antibacterial activity against methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* (Singh *et al.*, 2000), while guanacastepene I showed pronounced activity against *S. aureus* (Brady *et al.*, 2001). Phomoxanthone A and phomoxanthone B were isolated from the endophytic fungus *Phomopsis* sp. from Thailand and exhibited significant activity against *M. tuberculosis* (Isaka *et al.*, 2001). Antibacterial dicerandrols A–C were isolated from *Phomopsis longicolla*, an endophyte of *Dicerandra frutescens* (Wagenaar & Clardy, 2001).

The continued development of new antimicrobial agents is important to overcome the difficulties related to the treatment of infections caused by resistant pathogens (Petersen *et al.*, 2004), and endophytic fungi have emerged as an alternative source for the production of new antimicrobial compounds.

Enzymatic activity for gGAPDH from *Trypanosoma cruzi* and APRT from *L. tarentolae*

gGAPDH is a homo-tetramer protein that catalyzes the oxidation of glyceraldehyde-3-phosphate with a concomitant reduction of β -nicotinamide adenine dinucleotide (NAD⁺). It represents a target to find trypanocidal compounds, because the bloodstream form of *Trypanosoma cruzi* is highly dependent on the glycolysis for ATP production (Souza *et al.*, 1998). The screening of the 39 extracts showed that only one extract (VA14A) exhibited a good level of gGAPDH activity inhibition (95%), and another one (VA17A) was found to have a moderate inhibitory activity (40%). In a previous screening using 32 plant extracts, four active extracts (12.5%) were found (Vieira *et al.*, 2001).

The phosphoribosyltransferases family is involved in the mechanism of purine formation (Thiemann *et al.*, 1998). The adenine phosphoribosyltransferase (APRT) enzyme from *L. tarentolae* has been proposed as a target for the rational search of new leishmanicidal drugs. It has been found that three extracts were able to decrease the APRT catalytic activity (inhibitory activity $\geq 50\%$): VA14A (60.7%), VA16A (57.4%) and VA35A (61.4%). These extracts might be considered for further fractionating in order to yield inhibitors of the purine biosynthetic pathway of *L. tarentolae*.

Crude extracts from *Meliaceae* and *Rutaceae* plants have already shown promising APRT activity (Ambrozini *et al.*, 2005), but endophytic fungi extracts had not been exploited in this assay.

The present results suggest that endophytic fungi can also produce antiparasitic compounds. The smaller number of active extracts in the enzymatic inhibition might be due to the higher selectivity of these assays, compared with the whole cell-based antimicrobial and antitumoral assays.

Table 2. Biological activities of the endophytic fungi extracts

EtOAc extracts	Antimicrobial activity						Enzymatic inhibition (%)		Cytotoxic activity IC ₅₀ (mg mL ⁻¹)
	<i>S. aureus</i>		<i>E. coli</i>		<i>C. albicans</i>		Tc gGAPDH (100 µg mL ⁻¹)	Lt APRT (50 µg mL ⁻¹)	
	R	I.Z. (mm)	R	I.Z. (mm)	R	I.Z. (mm)			
VA1A	-	10.5 ± 0.71	+	12.7 ± 2.31	-	11.4 ± 2.31	18.0	0.0	0.24 ± 0.04
VA2A	-	8.4 ± 0.58	+	12.0 ± 1.41	-	1.07 ± 1.15	17.3	27.9	0.22 ± 0.07
VA3A	-	8.7 ± 0.58	-	11.5 ± 2.12	-	11.4 ± 2.31	12.9	32.2	0.30 ± 0.01
VA4A	-	8.4 ± 0.58	+	12.0 ± 2.83	-	11.4 ± 2.31	19.7	0.0	0.18 ± 0.08
VA5A	-	8.7 ± 0.58	+	12.4 ± 2.08	-	11.7 ± 2.87	7.7	1.5	0.32 ± 0.06
VA6A	-	8.0 ± 0.0	+	12.4 ± 2.08	-	10.7 ± 1.15	21.1	0.0	0.27 ± 0.07
VA7A	-	8.4 ± 0.58	-	11.4 ± 2.08	-	11.7 ± 2.08	5.5	11.3	0.29 ± 0.02
VA8A	-	8.0 ± 0.0	-	11.0 ± 1.73	-	12.5 ± 2.12	10.9	7.5	0.35 ± 0.05
VA9A	-	8.4 ± 0.58	-	11.0 ± 1.73	+	13.0 ± 2.83	26.0	30.5	0.23 ± 0.08
VA10A	-	8.0 ± 0.0	-	11.4 ± 2.08	-	11.5 ± 0.71	20.4	4.0	0.35 ± 0.06
VA11A	+	12.5 ± 0.71	-	11.7 ± 2.31	+	13.0 ± 1.41	10.0	0.0	122.4 ± 2.14
VA12A	-	9.5 ± 0.71	+	12.0 ± 2.64	+	13.5 ± 2.12	10.0	18.2	202.7 ± 3.89
VA13A	-	8.4 ± 0.58	-	9.5 ± 0.71	+	14.4 ± 0.58	7.9	35.4	0.25 ± 0.08
VA14A	-	8.7 ± 0.58	-	10.5 ± 0.71	+	15.0 ± 1.00	95.0	60.7	0.38 ± 0.1
VA15A	-	9.7 ± 0.71	-	10.5 ± 0.71	+	14.5 ± 0.71	12.0	9.1	151.8 ± 2.8
VA16A	-	8.7 ± 1.15	-	10.0 ± 0.0	+	14.5 ± 0.71	6.3	57.4	0.30 ± 0.1
VA17A	-	9.5 ± 0.71	-	10.5 ± 0.71	-	11.5 ± 0.71	40.0	27.3	53.3 ± 1.4
VA18A	-	11.4 ± 0.58	-	10.0 ± 0.0	-	10.5 ± 0.71	0.0	4.2	0.18 ± 0.08
VA19A	-	11.7 ± 0.58	-	9.7 ± 0.58	+	14.7 ± 0.58	0.0	4.5	0.24 ± 0.07
VA20A	-	9.5 ± 0.71	-	9.4 ± 0.58	+	13.4 ± 1.15	0.0	9.1	155.8 ± 3.34
VA21A	-	10.0 ± 1.73	-	9.7 ± 0.58	+	14.5 ± 0.71	4.4	7.2	0.55 ± 0.01
VA25A	-	10.7 ± 0.58	-	9.0 ± 0.0	+	14.0 ± 1.41	9.2	30.7	0.18 ± 0.08
VA26A	-	11.4 ± 1.15	-	9.0 ± 0.0	+	14.0 ± 1.41	0.0	20.1	0.16 ± 0.08
VA27A	+	12.5 ± 0.71	-	9.4 ± 0.58	+	13.7 ± 1.53	0.0	36.4	112.9 ± 3.0
VA28A	-	9.5 ± 0.71	-	10.0 ± 2.00	+	12.7 ± 1.53	0.0	18.2	129.9 ± 2.9
VA29A	-	11.0 ± 1.00	-	10.0 ± 1.73	+	13.0 ± 0.0	0.0	23.5	0.50 ± 0.01
VA30A	-	11.0 ± 0.0	-	10.4 ± 1.53	+	13.0 ± 0.0	0.0	26.3	0.82 ± 0.01
VA31A	-	9.0 ± 1.73	-	10.4 ± 1.53	+	13.0 ± 1.41	6.0	2.1	0.41 ± 0.08
VA32A	-	8.7 ± 1.15	-	10.0 ± 1.73	+	14.5 ± 0.71	5.5	10.1	0.36 ± 0.07
VA33A	-	9.0 ± 1.73	-	10.4 ± 1.43	+	13.0 ± 1.73	4.0	22.2	0.35 ± 0.06
VA34A	-	9.4 ± 1.53	-	9.7 ± 1.58	+	13.0 ± 1.41	5.7	5.3	0.41 ± 0.01
VA35A	-	8.7 ± 1.15	-	9.0 ± 1.00	+	14.0 ± 0.0	32.2	61.4	0.64 ± 0.02
VA36A	-	9.4 ± 1.15	-	8.7 ± 1.53	+	14.4 ± 1.15	0.5	0.0	0.89 ± 0.02
VA37A	-	10.0 ± 1.73	-	9.7 ± 0.58	+	14.4 ± 1.15	1.2	0.0	0.49 ± 0.01
TD1	-	10.4 ± 1.15	+	12.7 ± 2.31	-	11.5 ± 0.71	0.0	30.0	0.78 ± 0.09
TD2	-	10.4 ± 1.15	+	12.4 ± 2.08	+	13.5 ± 0.71	6.8	10.0	0.53 ± 0.01
TD3	-	10.7 ± 0.58	-	11.7 ± 1.53	-	11.5 ± 0.71	7.5	0.0	0.50 ± 0.01
TD4	-	11.0 ± 1.73	+	12.0 ± 1.73	+	13.5 ± 0.71	2.7	40.0	0.71 ± 0.01
TD5	-	10.7 ± 0.58	+	12.7 ± 2.31	-	9.0 ± 0.0	9.4	20.0	0.22 ± 0.01
Culture control	-	9.4 ± 0.58	-	9.5 ± 0.71	-	10.0 ± 0.0	6.0	11.8	5.95 ± 0.85
Diluent	-	9.7 ± 1.15	-	9.4 ± 0.58	-	10.0 ± 1.41	*	*	0.00 ± 0.0
Penicillin G	+	23.7 ± 1.53	*	*	*	*	*	*	*
Streptomycin	*	*	+	20.7 ± 0.58	*	*	*	*	*
Miconazole	*	*	*	*	+	19.5 ± 0.71	*	*	*
Methotrexate	*	*	*	*	*	*	*	*	3.99 ± 3.40

*Not assayed.

R, results; +, active; -, inactive; I.Z., inhibition zone.

However, if an inhibitor is identified in these enzymatic screenings it could be used as a hit in the structure-based drug design of new trypanocidal or leishmanicidal compounds.

The results obtained in this screening showed that endophytic fungi are a promising source of bioactive natural products. The most active extracts have been selected for fractioning in order to isolate the active compounds.

Antitumoral activity

Table 2 shows the results of the antitumoral assay. These tests were carried out using human T leukemia cells and methotrexate as a positive control. A high number of EtOAc extracts were found to be active (79.5%). Several cytotoxic compounds have been isolated from endophytic fungi, showing the potential of these fungi in the search of antitumoral agents. Some anticancer drugs have been identified in endophytic fungi cultures. Taxol has been isolated from *Taxomyces andreanae*, an endophytic fungus from *Taxus brevifolia* (Stierle et al., 1993). Camptothecin has been produced by an endophytic fungus associated with *Nothapodytes foetida* (Puri et al., 2005). In addition, vincristine has been isolated from *Mycelia sterilia*, an endophyte from *Catharanthus roseus* (Yang et al., 2004), and podophyllotoxin was found to be produced by a novel endophyte, *Trametes hirsute*, associated with *Podophyllum hexandrum* (Puri et al., 2006). Therefore, endophytic fungi play an important role in the search for antitumoral compounds and might also represent an alternative source for the production of therapeutic agents that are not easily obtained by chemical synthesis.

Glomerella cingulata was found to be the most common endophytic fungus associated with *V. arenaria*. In addition, all the *G. cingulata* strains produced cytotoxic extracts (Table 2). The EtOAc extract from the VA1 showed a remarkable cytotoxic activity against the JURKAT tumor cell line ($IC_{50} = 0.24 \text{ mg mL}^{-1}$), and this activity was found to be higher than the control drug methotrexate ($IC_{50} = 3.99 \text{ mg mL}^{-1}$) (Table 2). Importantly, the control EtOAc extract obtained from culture medium alone did not show a significant cytotoxicity, indicating that the VA1 cytotoxic activity was mediated by fungal-derived compounds. The EtOAc extract from *G. cingulata* (VA1) culture supernatant was submitted to chromatographic procedures, yielding two major compounds, and also a mixture of nonidentified fatty acids. The structures of the major compounds were established on the basis of their spectroscopic data, mainly 1D and 2D NMR and ESI-MS, and are in agreement with the structures reported previously for nectriapyrone (Avent et al., 1992) and tyrosol (Chen et al., 2004). Both compounds have been tested against tumor cell lines. Tyrosol was inactive, but nectriapyrone was found to have relevant cytotoxic activity against both JURKAT T leukemia cells and B16F10 melanoma cells. Also, the results showed that nectriapyrone is more active on JURKAT cells ($IC_{50} = 476.4 \text{ } \mu\text{g mL}^{-1}$) than B16F10 cells ($IC_{50} = 1463 \text{ } \mu\text{g mL}^{-1}$) (Fig. 2). It is noteworthy that nectriapyrone showed higher activity compared with methotrexate on both tumor cell lines. Nectriapyrone also showed certain selectivity to the tumor cells, because it did not show remarkable cytotoxic effect towards normal

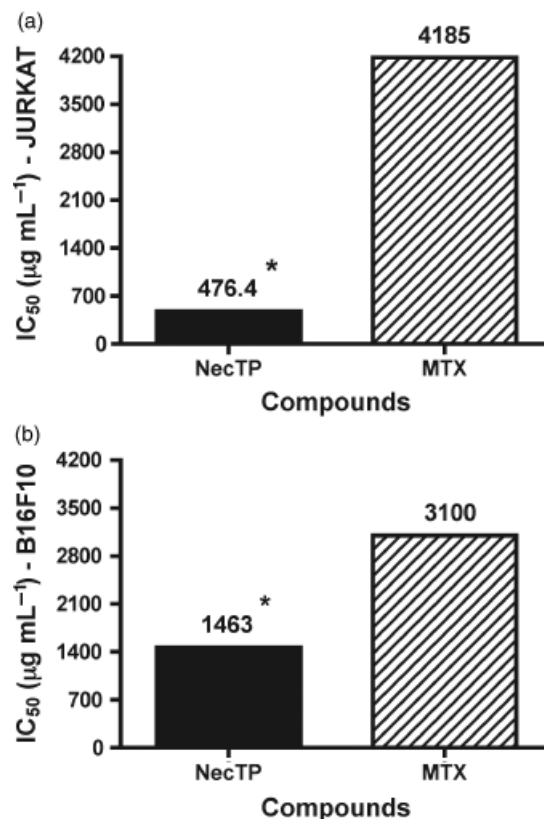


Fig. 2. Nectriapyrone (NecTP) cytotoxic activity against JURKAT (a) and B16F10 (b) tumor cell lines. Nectriapyrone was isolated as described in 'Material and methods'. Tumor cells were treated with different concentrations of nectriapyrone or methotrexate ($0.001\text{--}10 \text{ mg mL}^{-1}$), cultured for 24 h and cytotoxicity measured as described before. The data represent the IC_{50} mean value \pm SD, from three independent experiments ($n=5$). $P < 0.001$ was considered to be significant. *Methotrexate vs. nectriapyrone.

cells as peripheral blood mononuclear cells (PBMC) ($IC_{50} = 2667 \text{ } \mu\text{g mL}^{-1}$) (Fig. 3). Finally, nectriapyrone presented an IC_{50} value against PBMC normal cells between the positive controls methotrexate ($IC_{50} = 3709 \text{ } \mu\text{g mL}^{-1}$) and gemcitabine ($IC_{50} = 1381 \text{ } \mu\text{g mL}^{-1}$). The higher activity of the crude extract VA1A ($IC_{50} = 249 \text{ } \mu\text{g mL}^{-1}$) compared with that observed for nectriapyrone ($476.4 \text{ } \mu\text{g mL}^{-1}$) towards JURKAT cells could be related to a synergistic activity between fatty acid derivatives present in the extract and nectriapyrone. Cytotoxic synergism has already been reported between fatty acids and anticancer drugs such as taxanes (Menendez et al., 2005), vinorelbine (Menendez et al., 2002), gemcitabine and 5-fluorouracil (Whitehouse et al., 2003). In addition, it has been demonstrated that unsaturated fatty acids can be used as modulators of tumor cell chemosensitivity (Menendez et al., 2002).

Some α -pyrones have been reported to be produced by fungi belonging to several genera, including *Alternaria*,

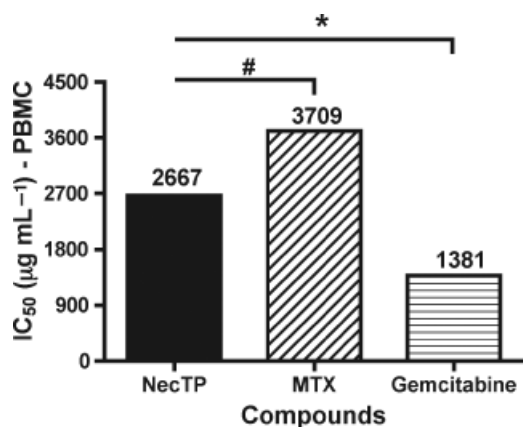


Fig. 3. Nectriapyrone (NecTP) cytotoxic activity towards normal human PBMC. Nectriapyrone was isolated as described in 'Materials and methods'. PBMC was collected, counted, placed in the wells on tissue culture plates as described and treated with different concentrations of nectriapyrone or methotrexate or gemcitabine (0.001–10 mg mL⁻¹), cultured for 24 h and cytotoxicity was measured as described before. The data represent the IC₅₀ mean value ± SD, from four independent experiments ($n=6$). $P < 0.001$ was considered to be significant. *Nectriapyrone vs. gemcitabine, #nectriapyrone vs. methotrexate.

Aspergillus, *Fusarium*, *Penicillium* and *Trichoderma*, and exhibit a wide range of biological activities, such as anti-biotic, antifungal, cytotoxic, neurotoxic and phytotoxic (Dickinson, 1993).

Nectriapyrone has already been isolated as an antibiotic against *Staphylococcus aureus* from the fungus *Gyrostroma missouriense* (Seeler) (Nair & Carey, 1975). The fungi *Phomopsis oblonga* (Claydon *et al.*, 1985) and *Gliocladium vermoesonii* (Avent *et al.*, 1991) also biosynthesized nectriapyrone. Recently, nectriapyrone has also been identified in the crude extracts of four endophytic fungi from the medicinal plant *Erythrina crista-galli* (Weber *et al.*, 2005). In addition, nectriapyrone was reported as a monoamine oxidase (MAO) inhibitor (Lee *et al.*, 1999). MAO inhibitors are therapeutically useful as antidepressants.

Interestingly, nectriapyrone was found to induce melanin production in B16-F1 melanoma cell lines (Thines *et al.*, 1998). Melanin accumulation in melanoma cells induce them to undergo apoptosis and/or increase the response to chemotherapy (Siracky *et al.*, 1984), possibly by the binding of divalent Zn or Cu to melanin that induces a pro-oxidant response under oxygen, generating superoxide and hydroxyl radicals (Farmer *et al.*, 2003). The mechanism by which nectriapyrone induces cytotoxicity requires further appropriate investigation. However, the previous results on the induction of melanin production by nectriapyrone might be related to the cytotoxic activity that has been determined for this compound. Furthermore, nectriapyrone cytotoxicity was associated with apoptosis of target cells, as JURKAT cells (data not shown).

The 2-pyrone subunit of nectriapyrone is found in a number of natural products possessing broad-spectrum biological activity and has inspired organic chemists to synthesize novel derivatives (Fairlamb *et al.*, 2004). Several 2-pyrone natural and synthetic compounds have shown cytotoxic activity (Fairlamb *et al.*, 2004; McGlacken & Fairlamb, 2005) against tumor cells, such as human ovarian carcinoma (A27080), human colorectal adenocarcinoma (SW480) (Suzuki *et al.*, 1997), human cervix carcinoma cell line (HeLa), human ovarian carcinoma cell line (A27080), Kirsten murine sarcoma virus-transformed rat kidney cell line (K-NRK), mouse macrophage tumor cells *in vivo* (P-388D1) (Kondoh *et al.*, 1998) and human chronic myelogenous leukemia cells (K562) (Marrison *et al.*, 2002), suggesting the potential use of these class of compounds as hits for the development of new anticancer drugs.

The present results have been shown that nectriapyrone was produced by the endophytic fungus *G. cingulata* (VA1) and also provide evidence that this compound presented a relevant cytotoxic activity towards tumor cells. The 2-pyrone sub-unit could be used as a good model for studies on the mechanism(s) of action and structure–activity relationships of this class of compounds. In addition, the data reinforce that endophytes are an outstanding source of bioactive small molecules.

Nectriapyrone and tyrosol did not present significant activity in the other bioassays.

The high incidence of antimicrobial and cytotoxic activity detected in the assays suggests that the bioactive secondary metabolites might be produced as an adaptation for specific functions in nature. Endophytic fungi live in a complex web of interactions with the plant host, other endophytes and also phytopathogens, and hence they are expected to produce bioactive compounds that can improve their ability to survive in such complex environment.

Acknowledgements

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP, for the financial support by the grants 03/07535-5 and 04/07935-6 (Bioprosecta/Biota). W.S.B. thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and D.O.G. thanks FAPESP for their scholarships during their doctoral works. The authors also thank Molecular Biology Laboratory (Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto/USP) and graduate student Andréa Carla Quiapim for support in DNA sequencing.

References

- Alviano CS, Farbiarz SR, Travassos LR, Angluster J & Souza W (1992) Effect of environmental-factors on *Fonsecaea-pedrosii*

- morphogenesis with emphasis on sclerotic cells induced by propanolol. *Mycopathologia* **119**: 17–23.
- Ambrosio SR, Tirapelli CR, da Costa FB & de Oliveira AM (2006) Kaurane and pimarane-type diterpenes from the *Viguiera* species inhibit vascular smooth muscle contractility. *Life Sci* **79**: 925–933.
- Ambrozin ARP, Leite AC, Silva M, Vieira PC, Fernandes JB, Thiemann OH, Da Silva MFGF & Oliva G (2005) Screening of *Leishmania* APRT enzyme inhibitors. *Pharmazie* **60**: 781–784.
- Avent AG, Hanson JR & Truneh A (1991) Two pyrones from *Gliocladium vermoeseni*. *Phytochemistry* **31**: 1065–1066.
- Avent AG, Hanson JR & Truneh A (1992) The biosynthesis of nectriapyrone and vermopyrone. *Phytochemistry* **31**: 3447–3449.
- Brady SF, Bondi SM & Clardy J (2001) The guanacastepenes: a highly diverse family of secondary metabolites produced by an endophytic fungus. *J Am Chem Soc* **123**: 9900–9901.
- Chen H, Fujita M, Feng QH, Clardy J & Fink GR (2004) Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc Natl Acad Sci USA* **101**: 5048–5052.
- Clardy J & Walsh C (2004) Lessons from natural molecules. *Nature* **432**: 829–837.
- Claydon N, Grove JF & Pople M (1985) Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*. *Phytochemistry* **24**: 937–943.
- Corrado M & Rodrigues KF (2004) Antimicrobial evaluation of fungal extracts produced by endophytic strains of *Phomopsis* sp. *J Basic Microb* **44**: 157–160.
- Cos P, Hermans N, De Bruyne T, Apers S, Sindambiwe JB, Vanden Berghe D, Pieters L & Vlietinck AJ (2002) Further evaluation of Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. *J Ethnopharmacol* **79**: 155–163.
- Dickinson JM (1993) Microbial pyran-2-ones and dihydropyran-2-ones. *Nat Prod Rep* **10**: 71–98.
- Fairlamb IJS, Marrison LR, Dickinson JM, Lu F-J & Schmidt JP (2004) 2-Pyrones possessing antimicrobial and cytotoxic activities. *Bioorg Med Chem* **12**: 4285–4299.
- Farmer PJ, Gidanian S, Shahandeh B, Di Bilio AJ, Tohidian N & Meyskens FL (2003) Melanin as a target for melanoma chemotherapy: pro-oxidant effect of oxygen and metals on melanoma viability. *Pigm Cell Res* **16**: 273–279.
- Freitas TPS, Furtado NAJC, Bastos JK & Said S (2002) Active substances against trypomastigote forms of *Trypanosoma cruzi* and microorganisms are produced in sequence by *Talaromyces flavus*. *Microbiol Res* **157**: 201–206.
- García-Pajón CM & Collado IG (2003) Secondary metabolites isolated from *Colletotrichum* species. *Nat Prod Rep* **20**: 426–431.
- Goffin E, Ziemons E, De Mol P, de Madureira MDC, Martins AP, da Cunha AP, Philippe G, Tits M, Angenot L & Frederich M (2002) *In vitro* antiplasmodial activity of *Tithonia diversifolia* and identification of its main active constituent: tagitinin C. *Planta Med* **68**: 543–545.
- Gu JQ, Gills JJ, Park EJ *et al.* (2002) Sesquiterpenoids from *Tithonia diversifolia* with potential cancer chemopreventive activity. *J Nat Prod* **65**: 532–536.
- Hellwig V, Grothe T, Mayer-Bartschmid A, Endermann R, Geschke FU, Henkel T & Stadler M (2002) Altersetin, a new antibiotic from cultures of endophytic *Alternaria* spp. taxonomy, fermentation, isolation, structure elucidation and biological activities. *J Antibiot* **55**: 881–892.
- Henry T, Iwen PC & Hinrichs SH (2000) Identification of *Aspergillus* species using internal transcribed spacer regions 1 and 2. *J Clin Microbiol* **38**: 1510–1515.
- Huang YJ, Wang JF, Li GL, Zheng ZH & Su WJ (2001) Antitumor and antifungal activities in endophytic fungi isolated from pharmaceutical plants *Taxus mairei*, *Cephalataxus fortunei* and *Torreya grandis*. *FEMS Immunol Med Microbiol* **31**: 163–167.
- Isaka M, Jaturapat A, Rukseree K, Danwisetkanjana K, Tanticharoen M & Thebtaranonth Y (2001) Phomoxanthones A and B, novel xanthone dimers from the endophytic fungus *Phomopsis* species. *J Nat Prod* **64**: 1015–1018.
- Jackson M, Karwowski JP, Humphrey PE, Kohl WL, Barlow GJ & Tanaka SK (1993) Calbistrins, novel antifungal agents produced by *Penicillium-restrictum*. 1. Production, taxonomy of the producing organism and biological-activity. *J Antibiot* **46**: 34–38.
- Koehn FE & Carter GT (2005) The evolving role of natural products in drug discovery. *Nature Rev Drug Discov* **4**: 206–220.
- Kondoh M, Usui T, Kobayashi S, Tsuchiya K, Nishikawa K, Nishikiori T, Mayumi T & Osada H (1998) Cell cycle arrest and antitumor activity of pironetin and its derivatives. *Cancer Lett* **126**: 29–32.
- Lee IK, Yun BS, Oh S, Kim YH, Lee MK & Yoo ID (1999) 5-Methylmellein and nectriapyrone, two new monoamine oxidase inhibitors. *Med Sci Res* **27**: 463–465.
- Liu CH, Zou WX, Lu H & Tan RX (2001) Antifungal activity of *Artemisia annua* endophyte cultures against phytopathogenic fungi. *J Biotechnol* **88**: 277–282.
- Marrison LR, Dickinson JM & Fairlamb IJS (2002) Bioactive 4-substituted-6-methyl-2-pyrones with promising cytotoxicity against A2780 and K562 cell lines. *Bioorg Med Chem Lett* **12**: 3509–3513.
- McGlacken GP & Fairlamb IJS (2005) 2-Pyrone natural products and mimetics: isolation, characterisation and biological activity. *Nat Prod Rep* **22**: 369–385.
- Menendez JA, Ropero S, del Mar Barbacid M, Montero S, Solanas M, Escrich E, Cortes-Furnes H & Colomer R (2002) Synergistic interaction between vinorelbine and gamma-linolenic acid in breast cells. *Breast Cancer Res Treat* **72**: 203–219.
- Menendez JA, Lupu R & Colomer R (2005) Exogenous supplementation with omega-3-polyunsaturated fatty acid docosahexaenoic acid (DHA; 22: 6n-3) synergistically enhances taxane cytotoxicity and downregulates Her-2/neu (c-erbB-2) oncogene expression in human breast cancer cells. *Eur J Cancer Prev* **14**: 263–270.

- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival – application to proliferation and cyto-toxicity assays. *J Immunol Methods* **65**: 55–63.
- Nair MSR & Carey ST (1975) Metabolites of pyrenomycetes II: nectriapyrone, an antibiotic monoterpenoid. *Tetrahedron Lett* **19**: 1655–1658.
- Nam JY, Kim HK, Kwon JY, Han MY, Son KH, Lee UC, Choi JD & Kwon BM (2000) 8-O-methylsclerotiorinamine, antagonist of the Grb2-SH2 domain, isolated from *Penicillium multicolor*. *J Nat Prod* **63**: 1303–1305.
- Pelaez F, Collado J, Arenal F *et al.* (1998) Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycol Res* **102**: 755–761.
- Petersen PJ, Wang TZ, Dushin RG & Bradford PA (2004) Comparative *in vitro* activities of AC98-6446, a novel semisynthetic glycopeptide derivative of the natural product mannopeptimycin alpha, and other antimicrobial agents against gram-positive clinical isolates. *Antimicrob Agents Chemoth* **48**: 739–746.
- Pisani P, Parkin DM, Bray F & Ferlay J (1999) Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* **83**: 870–873.
- Pupo MT, Guimaraes DO, Furtado NAJC & Borges WS (2006) Microbial natural products: a promising source of bioactive compounds. *Modern Biotechnology in Medicinal Chemistry and Industry* (Taft CA, ed), pp. 51–78. Research Signpost, Kerala, India.
- Puri SC, Verma V, Amna T, Qazi GN & Spitteller M (2005) An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *J Nat Prod* **68**: 1717–1719.
- Puri SC, Nazir A, Chawla R *et al.* (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *J Biotechnol* **122**: 494–510.
- Rios JL, Recio MC & Villar A (1988) Screening methods for natural products with antimicrobial activity: a review of the literature. *J Ethnopharmacol* **23**: 127–149.
- Rodrigues KF, Hesse M & Werner C (2000) Antimicrobial activities of secondary metabolites produced by endophytic fungi from *Spondias mombin*. *J Basic Microb* **40**: 261–267.
- Schulz B & Boyle C (2005) The endophytic continuum. *Mycol Res* **109**: 661–686.
- Singh MP, Janso JE, Luckman SW, Brady SF, Clardy J, Greenstein M & Maiese WM (2000) Biological activity of guanacastepene, a novel diterpenoid antibiotic produced by an unidentified fungus CR115. *J Antibiot* **53**: 256–261.
- Siracky J, Sparrow S & Jones M (1984) Relationship between melanogenesis, proliferative activity and response to chemotherapy of human-melanoma xenografts. *Neoplasma* **31**: 545–549.
- Souza DHF, Garratt RC, Araújo APU, Guimaraes BG, Jesus WDP, Michels PAM, Hannaert V & Oliva G (1998) *Trypanosoma cruzi* glycosomal glyceraldehyde-3-phosphate dehydrogenase: structure, catalytic mechanism and targeted inhibitor design. *FEBS Lett* **424**: 131–135.
- Stierle A, Strobel G & Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* **260**: 214–216.
- Strobel G & Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. *Microbiol Mol Biol R* **67**: 491–502.
- Suzuki K, Kuwahara A, Yoshida H, Fujita SI, Nishikiori T & Nakagawa T (1997) NF00659A(1), A(2), A(3), B-1 and B-2, novel antitumor antibiotics produced by *Aspergillus* sp. NF 00659 .1. Taxonomy, fermentation, isolation and biological activities. *J Antibiot* **50**: 314–317.
- Thiemann OH, Alfonzo JD & Simpson L (1998) Cloning and characterization of *Leishmania tarentolae* adenine phosphoribosyltransferase. *Mol Biochem Parasit* **95**: 141–146.
- Thines E, Anke H & Sterner O (1998) Scytalols A, B, C and D and other modulators of melanin biosynthesis from *Scytalidium* sp. 36–93. *J Antibiot* **51**: 387–393.
- Thompson JD, Higgins DG & Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.
- Tuttle JV & Krenitsky TA (1980) Purine Phosphoribosyltransferases from *Leishmania donovani*. *J Biol Chem* **255**: 909–916.
- Vieira PC, Mafezoli J, Pupo MT, Fernandes JB, Da Silva MFGF, de Albuquerque S, Oliva G & Pavão F (2001) Strategies for the isolation and identification of trypanocidal compounds from the Rutales. *Pure Appl Chem* **73**: 617–622.
- Wagenaar MM & Clardy J (2001) Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an endangered mint. *J Nat Prod* **64**: 1006–1009.
- Weber D, Gorzalczany S, Martino V, Acevedo C, Sterner O & Anke T (2005) Metabolites from endophytes of the medicinal plant *Erythrina crista-galli*. *Z Naturforsch* **60c**: 467–477.
- Weber RWS, Stenger E, Meffert A & Hahn M (2004) Brefeldin A production by *Phoma medicaginis* in dead pre-colonized plant tissue: a strategy for habitat conquest? *Mycol Res* **108**: 662–671.
- Whitehouse PA, Cooper AJ & Johnson CD (2003) Synergistic activity of gamma-linolenic acid and cytotoxic drugs against pancreatic adenocarcinoma cell lines. *Pancreatol* **3**: 367–373.
- Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwisetkanjana K, Ruangrunsi N & Meevootisom V (2004) Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. *World J Microb Biot* **20**: 265–272.

Yang X, Zhang L, Guo B & Guo S (2004) Preliminary study of vincristine-producing endophytic fungus isolated from leaves of *Catharanthus roseus*. *Zhongcaoyao* 35: 79–81.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1a. Alignment of the *Glomerella cingulata* ITS-1 and -2 regions.

Fig. S1b. Alignment of the *G. cingulata* ITS-3 and -4 regions.

Fig. S1c. Alignment of the ITS-3 and -4 regions *Colletotrichum gloeosporioides* with *G. cingulata*.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574.695X.2007.00354.x> (This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.