

Antimicrobial Activity of Endophytes from Brazilian Medicinal Plants

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1. Introduction

The increased use of antibiotics has become the bacteria resistant. Currently, there are increasing problems worldwide with multiresistant bacteria. Examples of the resistance problems on a global scale are the methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci and *Enterobacteriaceae* producing beta-lactamases. A study of the *World Health Organization (WHO)* revealed that 90% of the bacteria strains are resistant to drugs of first choice. Bioprospecting studies of endophytic microorganisms for pharmaceutical and biotechnological purposes are fundamental for the discovery of new substances for human therapeutics including antibiotics, antimalarials, and anticarcinogenics (Strobel & Long 1998; Strobel 2002; Strobel & Daisy 2003). Endophytic fungi of medicinal plants are currently being widely studied in the search for new potentially useful secondary metabolites. The production of bioactive secondary metabolites by medicinal plants and by the endophytes provided countless drugs selected as important therapeutic options for innumerable disease. The endophytes still have wide potential to be explored what could expand even more the phenomenal contribution to health and well being. Aware of the reality of multi-resistant pathogenic microorganisms and the producing capacity of antimicrobial compounds by endophytes it is indispensable the search of antibiotic substances with new mechanisms of action, less toxic effect and/or medication enhance through this apparently inexhaustible bioactive metabolites source (Demain &

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Sanchez, 2009). Some studies show a relation between the endophyte secondary metabolites producing and the plant where it is found increasing the interest in endophytic microbiota of medicinal plants. Gene transference from the plant to the endophyte and the other way around is said to happen allowing common secondary metabolites production (Peixoto Neto et al., 2004). The classical example was the isolation of the fungi *Taxomyces andreanae* from the plant *Taxus brevifolia*, both taxol producers, making possible the use of this antitumor and the preservation of the medicinal plant (Stierle et al., 1993).

Recent and promising reports of the some Brazilian medicinal plant and endophytes fungi as a source of important bioactive compounds and novel structures (Table 1). Endophytic fungi from *Lippia sidoides* demonstrate pharmaceutical potential and can be seen as an attractive source of biologically active compounds (Souza-Motta et al., 2011). One isolate of *Penicillium janthinellum*, endophytic from fruits of *Melia azedarach* (Meliaceae) producers of polyketides citrinin, emodin, 1,6,8-trihydroxy-3-hydroxymethylanthraquinone, and a new modified anthraquinone, named janthinone. The authors reported citrinin inhibited 100% of *Leishmania* growth after 48h at a concentration of 40 mg mL⁻¹ (Marinho et al., 2005). Oliveira et al. (2010), in study with metabolites produced by the fungus *Pestalotiopsis guepinii* isolated from *Virola michelii* reported a new anthraquinone derivative, named guepinone, along with the known substances isosulochrin and chloroisosulochrin. *In vitro* quantitative and qualitative information obtained in study with endophytic fungi isolated from comfrey (*Symphytum officinale* L.) leaves indicates potential against the phytopathogenic fungus *S. sclerotiorum* (Rocha et al., 2009). Therefore, the use of bioactive products from the endophytic strains and/or the biological control with *S. sclerotiorum* needs investigation. Two novel benzopyrans have been isolated from *Curvularia* sp., an endophytic fungus from *Ocotea corymbosa* showed weak *in vitro* antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides* (Teles et al., 2006). Endophytic fungi recovered from leaves of the bioactive Brazilian plant species *Ageratum myriadenia*, *Palicourea tetraphylla*, *Piptadenia adiantoides* and *Trixis vauthieri* could be a promising source of antitumoral, leishmanicidal and trypanocidal secondary metabolites, which could be used for the development of new drugs (Rosa et al., 2010). Dendryol E and dendryol F, two novel anthraquinone derivatives, have also been isolated from *Phoma sorghina*, endophyte found in association with the medicinal plant *Tithonia diversifolia* (Borges & Pupo, 2006). *Chaetomium globosum* was isolated as an endophytic fungus from the healthy leaves of *Viguiera robusta* (Momesso et al., 2008) and were identified genera *Alternaria*, *Cochliobolus*, *Diaporthe*, *Epicoccum*, *Guignardia*, *Phoma*, and *Phomopsis* from *Luehea divaricata*, known popularly in Brazil as açoita-cavalo (Bernardi-Wenzel et al., 2010). Crude extracts of endophytic fungi isolated from *Smallanthus sonchifolius* also showed antimicrobial effectiveness (Ramos et al., 2010) The antibacterial activity of the azaphylones, citrinin and citrinin H-1, were identified in *Penicillium* species isolated as endophytic fungi from *Melia azedarach* and *Murraya paniculata* (Pastre et al., 2007). *Xylaria* sp., an endophytic fungus from *Piper aduncum* were evaluated against the fungi *C. cladosporioides* and *C. sphaerospermum* and cytotoxicity *in vitro* against HeLa and CHO cells lines were investigated, the cytochalasins showed a strong activity against HeLa (Silva et al., 2010). Oliveira et al. (2009) isolated as endophytes two strains of *Penicillium* sp. from *Alibertia macrophylla* (Rubiaceae), producers of orcinol and 4-

hydroxymellein, which exhibited detection limits of 5.00 and 10.0 μg against *Cladosporium cladosporioides* and *C. sphaerospermum*. Silva et al. (2005 and 2006) reported a isolate of *Phomopsis cassiae* endophytic from *Cassia spectabilis* (Fabaceae) producer of ethyl 2,4-dihydroxy-5,6-dimethylbenzoate and phomopsilactone. Both displayed strong antifungal activity against the phytopatogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum*, as well as cytotoxicity against human cervical tumor cell line (HeLa), in *in vitro* assays. *Bacillus pumilus* was isolated from cassava (*Manihot esculenta*) cultivated by Brazilian Amazon Indian tribes, which produces other metabolite with antifungal activity, the pumilacidin (Melo et al., 2009).

Medicinal plant	Endophytes fungi	References
<i>Ageratum myriadenia</i>	<i>Alternaria arborescens</i> , <i>Bipolaris</i> sp., <i>Penicillium citrinum</i> and <i>Penicillium griseofulvum</i>	Rosa et al., 2010
<i>Cassia spectabilis</i>	<i>Phomopsis cassiae</i>	Silva et al., 2005 and 2006
<i>Lippia sidoides</i>	<i>Colletotrichum gloeosporioides</i> , <i>Alternaria alternata</i> , <i>Guignardia bidwellii</i> and <i>Phomopsis archeri</i>	Souza-Motta et al., 2011
<i>Luehea divaricata</i>	<i>Alternaria</i> , <i>Cochliobolus</i> , <i>Diaporthe</i> , <i>Epicoccum</i> , <i>Guignardia</i> , <i>Phoma</i> and <i>Phomopsis</i>	Bernardi-Wenzel et al., 2010
<i>Maytenus ilicifolia</i>	<i>Pestalotiopsis</i> sp., <i>Pestalotiopsis vismae</i> and <i>Pestalotiopsis microspora</i>	Figueiredo et al., 2007
<i>Melia azedarach</i> L.	<i>Penicillium</i> sp. and <i>P. janthinellum</i>	Marinho et al., 2005
<i>Murraya paniculata</i>	<i>Penicillium</i> sp.	Pastre et al., 2007
<i>Ocotea corymbosa</i>	<i>Curvalaria</i> sp	Teles et al., 2006
<i>Palicourea tetraphylla</i>	<i>Arthrium</i> sp., <i>Fusarium oxysporum</i> , <i>Penicillium griseofulvum</i> and <i>Penicillium citrinum</i>	Rosa et al., 2010
<i>Piper aduncum</i>	<i>Xylaria</i> sp.	Silva et al., 2010
<i>Piptadenia adiantoides</i>	<i>Arthrium</i> sp. <i>Gibberella</i> sp.	Rosa et al., 2010
<i>Smallanthus sonchifolius</i>	<i>Papulaspora immersa</i> and <i>Arthrinium arundinis</i>	Ramos et al., 2010
<i>Spondias mombin</i>	<i>Guignardia</i> sp.	Rodrigues-Heerklotz et al., 2001
<i>Symphytum officinale</i> L.	<i>Trichophyton</i> sp., <i>Chrysosporium</i> sp., <i>Candida pseudotropicalis</i> and <i>Candida tropicalis</i>	Rocha et al., 2009
<i>Tithonia diversifolia</i>	<i>Phoma sorghina</i>	Borges & Pupo, 2006
<i>Trixis vauthieri</i>	<i>Arthrium</i> sp. and <i>Xylaria</i> sp.	Rosa et al., 2010
<i>Viguiera robusta</i> ,	<i>Chaetomium globosum</i>	Momesso et al., 2008
<i>Virola michelii</i>	<i>Pestalotiopsis guepinii</i>	Oliveira et al., 2011

Table 1. Some Brazilian medicinal plants and endophytic fungi associated

An important medicinal plant in this context is *Maytenus ilicifolia*, commonly known as espinheira santa. *M. ilicifolia* is native to South America, being most commonly found in southern Brazil, and is widely used in the treatment of stomach ulcers and other gastric problems. The heavy exploitation of this plant because of its medicinal properties led it to be included in the current list of endangered species (SEMA, 1995; Bittencourt, 2000). *Schinus terebinthifolius* Raddi (peppertree) is other important medicinal plant in Argentina, Brazil and Paraguay (Mytinger & Williamson, 1987). In Brazil the bark, leaves and fruits have been used in popular medicine due to their medicinal properties (Guerra et al., 2000; Lorenzi, 2002; Dgáspari et al., 2005; Ribas et al., 2006). Actions anti-inflammatory and antiseptic for treatment of wounds, urinary and respiratory infections are listed as medicinal properties popularly known (Lima et al., 2006). Studies showed antimicrobial (Degáspari et al., 2005; Schmourlo et al., 2005; Fenner et al., 2006; Ribas et al., 2006; Johann et al., 2007; Soares et al., 2007) and antitumor activities (Queires et al., 2006). *Vochysia divergens*, popularly known as camarará, is a tree commonly found in wet soils of "Pantanal Matogrossense" in Brazil. This tree has great economic importance for the local population, especially in the production of wood. Despite the economic interest and broad popular medicinal usage of the *V. divergens*, there are very few reports on the chemical composition and biological activity of this plant. In respect to the biological activities related to this species, it was verified that the etanolic extract of *V. divergens* barks presented bactericide activity against *Staphylococcus aureus* and antinociceptive activity. Leaves and barks are used in popular medicine against respiratory and gastrointestinal problems (Hess et al., 1995).

Looking forward to find a solution for the advance of multi-resistant bacteria the present study made a comparison between the composts with antimicrobial activity produced by the leaves of the medicinal plants *S. terebinthifolius* and from its endophytes and from the plants *M. ilicifolia* and *V. divergens*. The antimicrobial activity and the chemical composition of the crude extract and fractions of the *S. terebinthifolius* leaves, were analyzed. Parallel with it, endophytes from the same tree were isolated and selected in order to extract its active secondary metabolites. Those extracts with positive result were also chemist evaluated and compared with the extract and fractions from the leaves. Endophytes were isolated from these 3 medicinal plants and selected in order to extract their active compounds. Similarities and differences between active compounds produced by *S. terebinthifolius* leaves and some of their endophytes were analysed.

2. Methods

2.1 Plant material

The peppertree (*S. terebinthifolius*) leaves were collected from a tree found at the latitude -25°26.827S, longitude - 49°13.997O. The botanical identification has been made at the herbarium of the Botanical Department of Federal University of Parana (UFPR - UPCB), where a specimen of the plant can be found under the registration: UPCB-30848. *M. ilicifolia* leaves were collected from Centro Nacional de Pesquisa de Floresta (CNPQ) of EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária), Paraná, Brazil (latitude -25.369227, longitude -49.189301). The *V. divergens* leaves were collected from 10 tree from Pantanal, Brazil (latitude -19.254108, longitude -57.030029).

2.2 Isolation of endophytes from plant

To the endophytic isolation, preference was given to leaves with no marks, scratches or wounds, according to methodology described by Petrini (1991). The leaves were washed in running water. The petioles were paraffin-embedded and went through this battery of solutions: sterile distilled water for 1 minute, ethanol 70% for 1 minute, sodium hypochlorite 3% for 4 minutes, ethanol 70% for 30 seconds and sterile distilled water for 6 minutes. The leaves were cut in fragments that were later cultivated for 20 days at 28°C in a potato-dextrose-agar medium or selective agar for actinomycete (AC) (Küster & Williams, 1964). To eliminate the epiphytic microorganisms of *V. divergens* leaves we used the purification protocol of six steps (Bettiol, 2008), in medium AC added of Tetracycline (100 µg/mL) and Cycloheximide (50 µg/mL). The living cultures were deposited in the LabGeM collection, Federal University of Paraná, Curitiba, Paraná, Brazil (<http://www.labgem.ufpr.br/>).

2.3 Endophytes identification

An analysis based on a polyphasic approach integrating taxonomic information, morphological traits and the sequencing of the ITS1-5.8S-ITS2 of the rDNA or 16S was used, as described by Gomes-Figueiredo et al. (2007). Isolates were initially identified based on their microscopic and macroscopic characteristics including their morphology and characteristics when grown on the following culture media: PDA, oatmeal agar (OA) (20 g l⁻¹ oat, 20 g l⁻¹ glucose, 15 g l⁻¹ agar), malt extract agar (MEA), and complete medium (CM) (Pontecorvo et al., 1953). Isolates were incubated for 7 days at 22 or 28°C and a 12 h light: 12 h dark photoperiod. The experimental design was completely randomized with 3 replicates. Colonies were analyzed with respect to their average diameter (cm), the aspect of their borders, the aspect and coloration of the mycelium, sporulation, mycelium characteristics, the production of acervuli, the coloration of the reverse of the Petri dish, the viscosity and coloration of the medium, and the size and coloration of the conidia. A total of 20 conidia from each culture medium were observed under light microscopy (x 1000 magnification) after being grown for 7, 14, and 21 days. Conidia were assessed with respect to their width and length and the length of the apical appendages. The coloration of the median cells was also recorded. For actinomycetes identification, characteristics of colonies were used, after growth in AC medium. The isolates Gram-stained were observed under light microscopy (x 1000 magnification).

The fungi isolates were randomly selected as morphotypes according to Arnold et al. (2000), and the endophytes that presented at least one of the extracts with antimicrobial activity were submitted to identification using ITS sequences of the rDNA. DNA extraction followed method described by Raeder & Broda (1985), modified by Glienke-Blanco et al. (2002). For the fungi, the primers V9G (De Hoog et al., 2003) and ITS4 (White et al., 1990) were used to amplify the ITS1-5.8S-ITS2 of the nuclear ribosomal RNA, in the following reaction mixture (50 µl): 0,2 mM of each dNTP, 1X Tris/HCl, 1,5 mM MgCl₂, 1,5 U Taq polymerase, 0,06 µM each primer and 50ng of DNA; the PCR was processed in a Mastercycler Gradient (Eppendorf®) with the following program: 94 °C for 2 min at the start followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 3 min. For the actinomycete the primers Sm6F

(5'GGTGGCGAAGGCGGA 3') and Sm5R (5' GAACTGAGACCGGCTTTTGA 3') were used to amplify the 16S rDNA. Amplification conditions followed Arzanlou et al. (2008) for the fungi and Monciardini et al. (2002) for the actinomycete. Amplicons were sequenced using both PCR primers and DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Biosciences). Sequences were manually aligned using Mega v. 5 software (Kumar et al., 2004) by inserting gaps. The obtained sequences were aligned according to existing sequences at the data base NCBI through the BLASTn program. Phylogenetic analyses of the aligned sequence data were performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 1998).

2.4 Endophytes extracts

Endophytes were selected for the extraction of active metabolites by fermentation. After the growth in potato-dextrose-agar medium in Petri dishes for 7-14 days at 28°C, fragments of the endophytes with a diameter of 10mm were removed and sowed in Erlenmeyers with 50mL and 100mL of the liquid medium Czapeck (Silva et al., 2004), MPE (Hamada et al., 1974) and malt extract broth (20 g l⁻¹ malt extract, 1 g l⁻¹ peptone, 20 g l⁻¹ glucose), and were incubated at 28°C at 120rpm. The 50mL cultures were incubated for 24 hours, while the ones with 100mL of medium were cultivated for 7 days. After the predetermined period the mycelium was separated of the metabolic medium by paper Whatman n°4 vacuum filtration and then stored. Either compounds from the culture and the ones retained on the cell structures were extracted with ethyl acetate p. a. (EtOAc; Merck). Solvent evaporation was carried out using a rotaevaporator at 45°C. The final extract was weighed and diluted in methanol, methanolic extracts (ME) at a concentration of 10 mg/mL (Corrado & Rodrigues, 2004). The fermentative liquid was lyophilized, weighed and also diluted in ultrapure sterilized water, aqueous extracts (AE) at concentration of 10 mg/mL.

2.5 Antimicrobial activity of endophytes extracts

For the evaluation of the antimicrobial activity of the secondary metabolites obtained from the culture of the endophytes was used bioautographic TLC agar overlay assay (Corrado & Rodrigues, 2004). To evaluate the activity of the extracts obtained through the maceration of the endophytic cell mass, an adaptation of a manual patterned by Clinical and Laboratory Standards Institute (2003a) was used. The results were collected through the measurement of the growth inhibition halo formed around the well. The microorganisms used on the tests were: *Staphylococcus aureus* (ATCC 27213 and ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Enterococcus faecalis* (ATCC 29212), *Klebsiella pneumonia* (ATCC 700603), *Micrococcus luteus* (ATCC 9314), methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* (ATCC 10231). Test organisms were grown overnight in a Müeller-Hinton broth (MH, Merck) at 37 °C and were diluted until reaching the concentration of 10⁶ cells/mL. As positive control chloranphenicol 1mg/mL for bacterial strains and nystatin 100000UI/mL for *C. albicans* were used. Methanol was applied as solvent control and saline solution was used as negative control.

2.6 Peppertree crude methanolic extract and fractions

The dried leaves were put in contact with petroleum ether for six days, having the solvent freshened when saturated. After removing the filtrate with petroleum ether the leaves were exposed to methanol for nine days having the solvent freshened when necessary (Harbone, 1998). The concentrated crude methanolic extract was partitioned using the following gradient elution: petroleum ether, petroleum ether: dichloromethane 1:1, dichloromethane, dichloromethane: ethyl acetate 1:1, ethyl acetate, ethyl acetate: methanol 1:1 and methanol.

2.7 Antimicrobial activity of peppertree crude methanolic extract and fractions

For this evaluation an adaptation from the macrodilution method (Clinical and Laboratory Standards Institute 2003b) has been used. In a test tube with Müller-Hinton broth already with the extract/fraction in a known concentration, the microorganism to be combated (*Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Candida albicans*) was inoculated. To isolate the solvent influence (dimethyl sulfoxide) at the activity of the extract/fraction used, controls having different solvent concentration were prepared. The test tubes were incubated and after that the turbidity standard was analyzed. For an exact analysis of the results an aliquot of 100 µL of each test tube was sowed in a Petri dish with Müller-Hinton agar and incubated at 35°C for 24 hours for posterior growth analysis by colonies counting. For the yeast the incubation period was 48 hours at 35°C. The test was carried out in duplicate.

2.8 Chemical comparison

The bioactive extracts obtained from peppertree endophytes were compared with the compounds present in the crude methanolic extract of the leaves and fractions active by thin-layer chromatography. The revealing substances used were: Dragendorff reactive, potassium hydroxide, sulfuric vanillin, ferric chloride - 1.5%, anisaldehyde and ninhydrin (Ordóñez et al., 2006; Rodrigues et al., 2009).

3. Results

3.1 Isolation and identification of endophytes

One hundred thirty-one endophytes were isolated from peppertree leaves. Nine endophytes active metabolites producers were identified. These, 2 were identified as *Alternaria* sp., 3 as *Phomopsis* sp., 1 as *Penicillium roseopurpureum*, 1 as a basidiomycete, and 1 as *Streptomyces* sp. One hundred ninety-one endophytes were isolated from leaf fragments of *M. ilicifolia*, belonging to 6 genera of fungi: *Alternaria*, *Phyllosticta*, *Xylaria*, *Phomopsis*, *Pestalotiopsis* and *Colletotrichum*. Eighteen actinomycetes were isolated from 4000 samples *Vochysia divergens*, with isolation rates of 0.47%, of these 61.1% (11) were isolated from petiole and 38.9% (07) leaves. Three taxa were identified: *Microbispora* sp. (10 isolates), *Micromonospora* sp. (2 isolates) and *Streptomyces sampsonii* (2 isolates).

3.2 Antimicrobial activity

Antibacterial activity of the methanolic extracts from endophytes of *S. terebinthifolius* was evaluated (Table 2). From the twenty isolates selected to fermentation, three released

bioactive compounds in the medium culture: *Phomopsis* sp. (LGMF655) and *Alternaria* sp. (LGMF692) released active metabolites against *S. aureus*; and *Streptomyces* sp. (LGMF696) released active metabolites against *C. albicans*. Eight isolates had secondary metabolites with antimicrobial activity on their cell structures (Table 3). Thirteen endophytic *Pestalotiopsis* spp. Isolates obtained from *M. ilicifolia* were used for evaluation of the antimicrobial activity. *Pestalotiopsis* sp. (14JES) was effective in inhibiting MRSA, *K. pneumoniae*, *M. luteus*, *S. aureus*, and *E. coli*; and the isolate *Pestalotiopsis microspora* showed similar results, except that it was unable to inhibit *K. pneumoniae*. *P. vismae* showed traces of inhibition against *S. aureus* and *E. coli*; and 2 isolates of *Pestalotiopsis* sp. (10JAES and 11JAES) showed inhibition against *S. aureus* and *M. luteus*, respectively (Table 2).

The extract of endophytic actinomycetes isolated from *V. divergens* showed activity against pathogenic bacteria (Table 2). The *Microbispora* genus isolates showed activity against several clinical strains. The isolate (N4P61) inhibited *P. aeruginosa* while the isolate N34C1 had activity against *S. aureus* and *E. coli*. The isolate N5P3 was effective in inhibiting *S. aureus* MRSA, *E. coli*, *P. aeruginosa* including *C. albicans*. Besides that two isolates identified as *Microbispora* sp. (N43B2 e N4P61) and two isolates of *Streptomyces sampsonii* (A3F5 e A1P10) showed activity against *C. albicans*.

The crude extract from all the plants studied presented antimicrobial activity. So far, we have got detailed data of the *Schinus* plant's extract fractionation, and the minimum inhibitory concentration (MIC) of the crude extract from leaves and the fractions were evaluated (Table 4).

	<i>E. faecalis</i>		MRSA		<i>K. pneumoniae</i>		<i>P. aeruginosa</i>		<i>M. luteus</i>		<i>S. aureus</i>		<i>E. coli</i>		<i>C. albicans</i>	
	ME	ME	AE	ME	ME	AE	ME	ME	AE	ME	AE	ME	AE	ME	AE	
	<i>Pestalotiopsis</i> sp. (10JAES)	0	0	nt	0	0	nt	0	Tr	nt	0	nt	0	nt	0	nt
<i>Pestalotiopsis</i> sp. (11JAES)	0	0	nt	0	0	nt	Tr	0	nt	0	nt	0	nt	0	nt	
<i>Pestalotiopsis</i> sp. (14JAES)	0	+	nt	+	0	nt	+	+	nt	+	nt	0	nt	0	nt	
<i>P. microspora</i>	0	+	nt	0	0	nt	+	+	nt	+	nt	0	nt	0	nt	
<i>P. vismae</i>	0	Tr	nt	0	0	nt	0	0	nt	Tr	nt	0	nt	0	nt	

		<i>E. faecalis</i>		MRSA		<i>K. pneumoniae</i>		<i>P. aeruginosa</i>		<i>M. luteus</i>		<i>S. aureus</i>		<i>E. coli</i>		<i>C. albicans</i>	
		ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE
<i>S. terebinthifolius</i>	<i>Phomopsis</i> sp.	0	0	nt	0	0	nt	0	+	nt	0	nt	0	nt	0	nt	
	<i>Alternaria</i> sp.	0	0	nt	0	0	nt	0	+	nt	0	nt	0	nt	0	nt	
	<i>Streptomyces</i> sp.	0	0	nt	0	0	nt	0	0	nt	0	nt	0	nt	+	nt	
<i>V. divergens</i>	N43B2	nt	0	0	nt	0	0	nt	0	0	0	0	0	0	+	0	
	N2P10	nt	0	0	nt	0	0	nt	0	0	0	0	0	0	0	+	
	N4P61	nt	0	0	nt	+	0	nt	0	0	0	0	0	0	+	0	
	A3F5	nt	0	0	nt	0	0	nt	0	0	0	0	0	0	+	+	
	A1F10	nt	0	0	nt	0	0	nt	0	0	0	0	0	0	+	0	
	N34C1	nt	0	0	nt	0	0	nt	0	+	0	0	+	0	0	0	
	N5P3	nt	0	+	nt	0	+	nt	0	+	0	0	+	0	0	+	

NOTE: O: no inhibition; Tr: traces of inhibition; + inhibition zone between 4 – 5 mm in diameter. Nt: not tested

Table 2. Antibacterial activity of the methanolic (ME) and aqueous (AE) extracts

	<i>S. aureus</i>	<i>C. albicans</i>	<i>P. aeruginosa</i>
<i>Alternaria</i> sp. (LGMF626)	X		
<i>Alternaria</i> sp. (LGMF692)	X		
Basidiomycete (LGMF713)	X		
<i>Penicillium roseopurpureum</i> (LGMF698)	X	X	
<i>Phomopsis</i> sp. (LGMF627)		X	
<i>Phomopsis</i> sp. (LGMF694)	X		X
<i>Streptomyces</i> sp. (LGMF696)	X	X	
Not identify (LGMF673)	X	X	

X = represent the pathogen that was inhibited by the extract

Table 3. Endophytes with active metabolites on their cell structures

3.3 Chemical comparison of active compounds

Results of the TLC suggest the presence of phenolic and anthraquinone compounds at the crude extract of the leaves of peppertree and active fractions. Yet, according to the present results, the crude extract and the ethyl acetate fraction had alkaloids and terpenoids.

The chemical analysis of the compounds with antimicrobial activity extracted from the endophytes of the peppertree leaves suggest the presence of alkaloids in all the tested extracts. Two microorganisms (*Alternaria* sp. - LGMF692 and *Streptomyces* sp. - LGMF696) also produced anthraquinones while one of them (*Phomopsis* sp. - LGMF655) produced terpenoids.

4. Discussion

Resistance to antimicrobial drugs today, remains a major problem in modern health care, about the impact on treatment options, mortality, infection control and economic issues. All identified taxon in this study, *Alternaria* (Kjer et al., 2009), *Phomopsis* (Du et al., 2008), *Penicillium* (Bertinetti et al., 2009), *Pestalotiopsis* (Liu, 2011), basidiomycete (Suay et al., 2000) and *Streptomyces* (Maruna et al., 2010) had already been described as producers of metabolite with antimicrobial activity. Although the TLC method is only qualitative, it is a simple and relatively cheap technique to recognize the inhibitory activity of a large quantity of organic extracts. However, our study has shown that fungal endophytes isolated from Brazilian native plant species have chemical and biochemical properties potentially useful. In the Table 1 there is a list of other Brazilian medicinal plant and endophytes fungi with antimicrobial activity. Further investigation may yield novel compounds with practical applications in a variety of biotechnological areas, with countless useful drugs as important therapeutics options for innumerable disease. The mechanical removal of cell metabolites from the inside part of their structure amplified the action spectrum of the extracted compounds besides revealing a higher number of endophytes producing active substances in relation to the attempt on releasing bioactive compounds on the culture medium. Apparently the microorganisms store these compounds for a future competition situation. The pursuit of cell release of these substances by different fermentation means would provide better biotechnological conditions for production of these compounds in higher amount for a more detailed study to be carried out.

The results of the bioautographic TLC agar overlay assay are at present only qualitative; however, it indicates that the endophytic extracts have antimicrobial potential, suggesting the need for further investigations to elucidate the chemical structure of the secondary metabolites that provide the antimicrobial properties to these endophytic isolates. Owing to the high genetic variability among *Pestalotiops* species found in the present study, new isolation efforts of "espinheira santa" endophytes should be carried out with the goal of bioprospecting, given the importance of the genus *Pestalotiopsis* in the biotechnological study of secondary metabolites, in particular, the inhibition of the activity of cells in gastric tumors (Lee et al. 1996; Strobel et al. 1998).

In the present study, isolates 6JAES and 29JES, which showed antimicrobial activity and were suggested as belonging to the species *P. microspora*, are of special importance, given that this species has become important in the past few years in the production of taxol and other secondary metabolites of antifungal, anticarcinogenic, and antioxidant properties (Strobel 2002; Strobel & Daisy 2003).

Despite the economic interest and broad popular medicinal usage of the *Vochysia divergens* plant there are very few reports on the chemical composition and biological activity of this plant. In respect to the biological activities related to this species, it was verified that the ethanolic extract of *V. divergens* barks presented bactericide activity against *Staphylococcus aureus* and antinociceptive activity (Hess et al., 1995). The endophytic actinomycetes of the *V. divergens* plant showed activity against *C. albicans*, *S. aureus*, *E. coli*, *P. aeruginosa* and MRSA, suggesting a higher potential to the antimicrobial activity than the one found on the plant by Hess (1995). Bioprospecting studies of endophytic actinomycetes for pharmaceutical and biotechnological purposes are fundamental for the discovery of new substances for human therapeutics including antibiotics, antimicotic, and anticarcinogenics (Bi et al. 2011).

The peppertree crude methanolic extract present higher activity against *C. albicans* followed by *S. aureus*, and less active against *P. aeruginosa*. The fractions dichloromethane: ethyl acetate and ethyl acetate were more active against the Gram positive microorganism followed by the Gram negative and with less action against the yeast tested. There is a difference between the antimicrobial activities found to the crude extract of the plant in relation to their fractions, probably due to the existence of an interaction of compounds on the crude extract, what would enhance the activity against *C. albicans*. Therefore, when the extract is fractionated these compounds are put apart, reducing their potential to act. According to another study about peppertree antimicrobial activity it was verified that the aqueous extract when fractionated would lose activity against *C. albicans* (Schmourlo et al., 2005), confirming the importance of synergism in this case. Apparently compound interactions that help crude extract activity in relation to fractions against yeast do not show the same effect to the bacteria tested. It indicates a higher concentration of active compounds against these microorganisms or an elimination or decrease of compounds interfering, mainly the in the dichloromethane: ethyl acetate fraction.

It is suggested that most the active extracts of endophytes studied are compound by alkaloids. Other compound classes were also revealed in these extracts, however less frequent, two endophytes, an isolate from *Alternaria* sp. and another from *Streptomyces* sp. had produced anthraquinones and an isolate from *Phomopsis* sp. had produced terpenoids. Results of TLC reveal there are strong evidences that phenolic compounds present on peppertree, found either on the crude extracts as well as the two active fractions, were responsible for the antimicrobial activity of the plant. Other authors also address the activity of the plant to a group of phenolic compounds, the polifenoles (Ceruks et al., 2008; Degáspari et al., 2005; Queires & Rodrigues, 1998).

Yet, the crude extract and active fractions of the plant also presented anthraquinones (Table 4), creating a new hypothesis that the antimicrobial substances linked to the peppertree could be connected to this group of compounds. Another study had identified anthraquinones, fenoles and triterpenes in the extract with antimicrobial activity of *S. terebinthifolius* bark, however not in the leaves extract (Lima et al., 2006).

With data obtained it was not found direct connection between the secondary metabolites with antimicrobial activity produced by the plant with the ones produced by the studied endophytes, once none endophytic chemical profiles studied showed the presence of phenolic compounds. This fact shows the enormous diversity of secondary metabolites present on nature and the importance of looking for active substances in medicinal plants and their endophytes.

	<i>S. aureus</i>	<i>C. albicans</i>	<i>P. aeruginosa</i>
Crude Extract (methanolic)	2300µg/mL	2000µg/mL	>3600µg/mL*
Dichloromethane:Ethyl Acetate Fraction	500µg/mL	>2300µg/mL*	1700µg/mL
Ethyl Acetate Fraction	1000µg/mL	>2300µg/mL*	2200µg/mL

* In these cases it was observed a reduction on the colonies number indicating activity. To prevent the interference of the solvent used on the results the extract/fraction volumes should not be over 250µL.

Table 4. Minimum inhibitory concentration (MIC) of the crude extract from leaves and fractions

5. References

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