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In vitro Antibacterial Activity of Crude Extracts Produced by Endophytic Fungi Isolated from *Piper hispidum* Sw.

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ABSTRACT

Endophytic fungi inhabit the interior of plants without causing apparent harm to them and constitute an alternative for the control of human pathogens, since they can synthesize bioactive compounds. The plant *Piper hispidum* Sw. has several medicinal properties and harbors a diversity of endophytes. In this present study, four endophytic fungi from *P. hispidum* were used for obtaining crude ethyl acetate extracts that were tested against *Enterococcus hirae*, *Escherichia coli*, *Micrococcus luteus*, *Salmonella typhi* and *Staphylococcus aureus*, using cup plate technique. The obtainment resulted in crude ethyl acetate extracts with a final concentration between 19.9 and 61.4 mg/ml. the antibacterial tests presented satisfactory results, where the pathogenic bacteria were inhibited by the four extracts tested, except for *E. hirae* that was inhibited by two extracts. Means of inhibition halos ranged from 9.42±0.63 to 14.08±1.70 mm. Analysis of variance showed that the extract produced by endophyte *Lasiodiplodia theobromae* was the most effective against all bacteria except for *S. typhi*, which was more inhibited by the extract of an endophyte from Diaporthales order. Therefore, this study indicates that endophytes from medicinal plant *P. hispidum* could be potential sources of antibacterial substances, with emphasis on *L. theobromae*.

INTRODUCTION

The term endophyte, that means "in the plant" (from Greek *endon*, within; *phyton*, plant) (Schulz and Boyle, 2005), was first used to referred to any organism found within tissues of living plants (Arnold, 2008). Actually, endophytes are considered microorganisms that colonize internal plant tissues without causing any apparent harm or disease to their host (Arnold, 2008; Petrini, 1991; Stone *et al*., 2000; Wilson, 1995). Many studies have emphasized endophytes from medicinal plants, since its isolation until their application in different areas (Bernardi-Wenzel *et al*., 2010; Garcia *et al.*, 2012; Gazis and Chaverri, 2010; Huang *et al.*, 2008; Mitchell *et al*., 2008; Orlandelli *et al.*, 2012; Rhoden *et al.*, 2012; Specian, 2010; Targa *et al.*, 2011; Tejesvi *et al.*, 2007). Studies have shown that endophytic fungi can synthesize bioactive products identical or similar to those produced by plants, being a source of potentially new and useful medicinal compounds (Strobel, 2003). Therefore, considering that the self-medication habit and antibiotics overuse can cause the selection of resistant bacteria strains, endophytes can be researched for new medication. However, Ramasamy *et al.* (2010) emphasized that the endophytic therapeutic properties have not been widely explored although a single endophyte may be capable of producing a variety of biological compounds. Firáková *et al.* (2007) questioned whether bioactive compounds of medicinal plants are really produced by plant itself or as a consequence of their association with endophytes, due to the fact that some of them may have developed genetic systems allowing for the transfer of information between

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themselves and their hosts. The medicinal plant *Piper hispidum* Sw. (Piperaceae family) is distributed throughout the Antilles, Central and South America, including several Brazilian States (Guimarães and Giordano, 2004). This shrub is commonly known as "platanillo-de-Cuba" (Cuba), comdocillo" (Mexico), "higuillo" (Puerto Rico), "cordoncillo blanco" (Venezuela), "oijú-yú" (Trinidad), "jaborandi" and "falso-jaborandi" (Brazil) (Albiero *et al.*, 2006; Roíg y Mesa, 1945). It is used as astringent, diuretic, stimulant, healing wound; for stopping hemorrhages, treating malaria and cutaneous leishmaniasis, and has antifungal and antibacterial action (Alécio *et al.*, 1998; Estevez *et al.*, 2007; Milliken, 1997; Nair and Burke, 1990; Roíg y Mesa, 1945).

P. hispidum harbors a diversity of endophytic fungi, as shown recently by Orlandelli *et al.* (2012). In virtue of the shortage of information about antimicrobial activity of endophytes isolated from it, this present study evaluated the potential of four *P. hispidum* endophytic fungi for producing extracts with inhibitory activity against five human pathogenic bacteria.

MATERIAL AND METHODS

Biological Material and Culture media

The endophyte *Lasiodiplodia theobromae* (JF766989), an endophyte from Diaporthales order (JF767007) and two non-identified endophytes (isolates G33-73 and G53-83) were isolated from *Piper hispidum* leaves by Orlandelli *et al.* (2012) and the sequences of those molecularly identified are deposited in GenBank. These endophytic fungi belong to the fungal culture collection of Laboratório de Biotecnologia Microbiana from Universidade Estadual de Maringá, Paraná, Brazil.

The pathogenic bacteria *Enterococcus hirae* ATCC 1227, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 9341, *Salmonella typhi* ATCC 19430 and *Staphylococcus aureus* ATCC 25923 were provided by the Laboratório de Microbiologia, Departamento de Análises Clínicas from Universidade Estadual de Maringá, Brazil.

Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were prepared according to Smith and Onions (1983) modified by Pamphile *et al.* (2004); Luria Bertani Agar (LBA) and Luria Bertani Broth (LBB) were prepared according to Sambrook and Russel (2001).

Obtainment of crude ethyl acetate (EtOAc) extracts from endophytic fungi

Crude EtOAc extracts were obtained according a slightly modification of Phongpaichit *et al.* (2007). The pure cultures of endophytes were re-cultivated on Petri dishes with PDA at 28º C for seven days for obtaining young colonies. Three mycelia fragments (5 mm^2) of each endophyte were inoculated into 500 ml Erlenmeyer flasks containing 250 ml of PDB and incubated at 28º C for two weeks under stationary condition. The broth cultures were filtered and centrifuged at 3,600 rpm for 20 min to separate the broth culture and mycelia. All filtrates were extracted three times with equal volume of EtOAc P.A. (Fmaia) in a separatory funnel, where, after strong agitation, the separation of phases occurred by polarity difference. The EtOAc phase was collected and the solvent was removed by 98% concentration in R-3000 Büchi rotary evaporator at 40º C. The extract residues were dissolved in 10 ml of absolute methanol P.A. (Fmaia) and stored at 4º C.

In vitro antibacterial activity of the crude EtOAc extracts

The in vitro antibacterial activity was assessed by qualitative biological analysis in triplicate, using the cup plate technique. The five pathogenic bacteria were grown for 24 h sequentially in both LBA and LBB, adjusted at a concentration of $1x10^6$ cells/ml and spread (100 µl) on Petri dishes with LBA. In each dish, equidistantly, were placed four 6 mm disks of sterile Whatman No. 4 filter paper inoculated with 10 µl of crude EtOAc extracts of endophytes. Paper disks were also inoculated with autoclaved distilled water and absolute methanol (negative controls) and Tetracycline (Sigma) (50 $\mu g.ml^{-1}$ in absolute ethanol) in the same concentration of each crude extract (positive controls). Dishes were incubated at 37º C for 24 h. Inhibition halos were measured and expressed in mm. All experiments were carried out using a completely randomized design (CRD) and were analyzed by ANOVA (variance analysis). In order to verify the efficiency of crude EtOAc extracts, means were compared by Tukey test ($p<0.05$) using statistical program SAS (2001).

RESULTS AND DISCUSSION

EtOAc has been frequently employed as solvent to obtain compounds produced by fungal endophytes (Hormazabal and Piontelli, 2009; Jayanthi *et al.*, 2011; Khan *et al.*, 2012; Pongcharoen *et al.*, 2008; Phongpaichit *et al.*, 2006, 2007; Radji *et al.*, 2011; Rhoden *et al.*, 2012; Specian, 2010; Sutjaritvorakul *et al.*, 2011).

Herein, the obtainment of crude EtOAc extracts produced by endophytes isolated from *P. hispidum* resulted in extracts with a final concentration between 19.9 and 61.4 mg/ml (Table 1). All bacteria were inhibited by the four extracts tested, except for *E. hirae* that was inhibited by only two extracts. The extract produced by *L. theobromae* was statistically the most effective against all bacteria except for *S. typhi*, being the extract of the Diaporthales endophyte more effective against it (Table 1).

L. theobromae represents the asexual (= anamorphic) state of *Botryosphaeria rhodina* (Mohali *et al.*, 2005), an important plant pathogenic fungus for both tropical and subtropical regions, causing leaf spots, necrosis, gummosis and even the death of many plants (Encinas, 1996; Encinas and Ahmad, 1999). However, some studies have shown its endophytic association with the host plant (Mohali et *al.*, 2005; Orlandelli *et al.*, 2012; Slippers and Wingfield, 2007). This fungus has been reported as producer of biological compounds, as shown by Pandi *et al.* (2011), which reported the production of the anticancer drug taxol by *L. theobromae* endophyte from medicinal plant *Morinda citrifolia.*

Table. 1: Antibacterial activity (mean±standard deviation), represented by inhibition halos in mm, of crude ethyl acetate extracts of endophytic fungi isolated from *Piper hispidum*.

Treatments	Concentration (mg/ml)	Pathogenic Bacteria				
		Enterococcus hirae	*Escherichia coli*	*Micrococcus luteus*	*Salmonella typhi*	*Staphylococcus aureus*
Extract of *L. theobromae*	61.4	10.17±0.52[b]*	9.42±0.63[c]	14.08±1.70[b]	7.67±2.10[c]	12.42±1.01[d]
Extract of Diaporthales	50.1	3.67±0.14[c]	3.08±1.70[d]	3.83±1.77[c]	9.00±1.32[c]	3.17±1.42[e]
Extract of G33-73	19.9	0.00±0.00[d]	4.17±0.38[d]	5.25±1.09[c]	6.33±4.30[c]	3.75±0.25[e]
Extract of G53-83	24.0	0.00±0.00[d]	3.67±1.66[d]	5.08±0.76[c]	7.33±1.84[c]	2.67±1.59[e]
Distilled Water (c-)	-	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[d]	0.00±0.00[d]	0.00±0.00[f]
Absolute Methanol (c-)	-	0.00±0.00[d]	0.00±0.00[e]	0.00±0.00[d]	0.00±0.00[d]	0.00±0.00[f]
Tetracycline (c+)	61.4	19.50±0.00[a]	20.75±0.66[a]	39.50±0.25[a]	32.75±0.25[a]	34.00±0.00[a]
	50.1	19.42±0.14[a]	19.75±0.66[ab]	39.25±0.00[a]	32.25±0.25[a]	33.58±0.14[ab]
	19.9	19.00±0.00[a]	18.08±0.52[b]	39.00±0.00[a]	27.00±0.25[b]	29.75±0.66[c]
	24.0	19.25±0.00[a]	19.50±0.87[ab]	39.25±0.25[a]	28.92±1.13[b]	32.25±0.25[b]

*Means in the same column followed by the same letter are not significantly different at $p<0.05$ according to Tukey test.
(c-) = negative control; (c+) = positive control

Diaporthales is an order that comprises about 94 genera, including several plant pathogenic fungi, such as the large genera *Cytospora* and *Phomopsis* (with more than 100 species each one) (Kirk *et al.*, 2001; Rossman *et al.*, 2007). Especially *Phomopsis* genus and its teleomorphic phase (*Diaporthe* genus) are often predominant as endophytes in their host assemblages (Chareprasert *et al.*, 2006; Murali *et al.*, 2006). They are producers of a variety of compounds (Bunyapaiboonsri *et al.*, 2010), including those with antibacterial activity (Jayanthi *et al.*, 2011; Specian, 2010).

The inhibition halos produced by crude EtOAc extracts of endophytes isolated from *P. hispidum* ranged from 9.42±0.63 to 14.08±1.70 mm. These data are in accordance with other studies, such as the one conducted by Hormazabal and Piontelli (2009), which tested the antimicrobial activity of 36 extracts of endophytic fungi from Chilean native gymnosperms, observing that the extract of *Curvularia protubera* had the best effect on *Bacillus subtilis*, *M. luteus*, and *S. aureus*, with growth inhibition of 12, 9 and 16 mm, respectively. All extracts were inactive against the Gram-negative bacteria, including *E. coli*. Differently, the present study shows *E. coli* inhibition by the four crude EtOAc extracts produced by *P. hispidum* endophytic isolates, with halos between 3.08±1.70 and 9.42±0.63 mm.

Endophytic fungi from *Garcinia* plants in Thailand were investigated about the biological activities of their crude extracts (Phongpaichit *et al.*, 2006). The authors emphasized the need of researches on effective antimicrobial agents since it is increasing the world health problems caused by drug-resistant bacteria and fungi. Antimicrobial activity was presented by 18.6% of extracts against at least one pathogenic microorganism (*S. aureus*, *Candida albicans* and *Cryptococcus neoformans*). Best results were observed for *Phomopsis* sp., *Botryosphaeria* sp. and a non-identified fungal endophyte, with inhibition zones that ranged from 7 to 19 mm. Similarly, Ramasamy *et al.* (2010) evaluated the antimicrobial activity of the crude EtOAc extracts produced by endophytic fungi from Malaysian medicinal plants, verifying that 16% of the extracts were effective against *B. subtilis*, and about 1% to 2% inhibited *M. luteus*, *S. aureus*, *E. coli* and *C. albicans*, with inhibition zones ranging from 8 to 24 mm.

The crude EtOAc extracts of 24 endophytic fungi from *Garcinia mangostana* were evaluated for their antibacterial activity by Radji *et al.* (2011) against six pathogenic bacteria using the agar diffusion method. As result, 41.6% of extracts inhibited at least one pathogen. The isolate *Microdiplodia hawaiiensis* presented the strongest antibacterial activity against *S. aureus*, *B. subtilis*, *M. luteus*, *E. coli*, *S. typhi* and *Pseudomonas aeruginosa* (minimum inhibitory concentrations between 25 and 200 g/ml).

An antimicrobial metabolite produced by endophytic fungus *Fusarium solani*, isolated from bark of Himalayan yew, was evaluated by Tayung *et al.* (2011) against *B. subtilis*, *Staphylococcus epidermidis* and *S. aureus*, *Klebsiella pneumoniae*, *Shigella flexneri*, *E.coli*, *Candida tropicalis* and *C. albicans*. The fungal metabolite inhibited all pathogens tested, with zones of inhibition that ranged from 10.6±1.1 to 27.0±1.7 mm. The authors cited that the same endophytic species was also reported as producer of the anticancer metabolite taxol, as reported by Chakravarthi *et al.* (2008).

Recently, Rhoden *et al.* (2012) also used the extraction with EtOAc for obtaining extracts of four endophytes from *Trichilia elegans*. The bacteria *E. hirae*, *M. luteus*, *E. coli* and *S. typhi* were inhibited at least by one extract; however, none extract inhibited *S. aureus*. On the contrary, in the present study, this bacterium was inhibited by the four crude extracts of endophytic fungi from *P. hispidum*, with inhibition halos up to 12.42±1.01 mm. Summarizing, the obtainment of crude EtOAc extracts of endophytes from *P. hispidum* resulted in extracts with a satisfactory antibacterial activity, even if its action was statistically inferior to the antibiotic (positive control). The endophyte *L. theobromae* can be emphasized, since it demonstrated the best action against four of the five human pathogenic bacteria tested.

CONCLUSION

The present study demonstrates the potential of fungal endophytes from *P. hispidum* for the production of extracts with antibacterial action, with emphasis on *L. theobromae*. Moreover, future pharmacological studies on isolation and identification, safety and efficacy can be applied for these fungal extracts aiming their pharmaceutical application.

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