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Antimicrobial Activity from Endophytic Fungi *Arthrinium* state of *Apiospora montagnei* Sacc. and *Papulaspora immersa*

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ABSTRACT

Papulaspora immersa *and* Arthrinium *state of* Apiospora montagnei *Sacc. were isolated from the roots of* Smallanthus sonchifolius *(yacón). The crude extracts from their cultures inhibited the growth of* Staphylococcus aureus, Kocuria rhizophila, Pseudomonas aeruginosa *and* Escherichia coli. *The more relevant results were observed in the ethyl acetate extract from* P immersa *against* P aeruginosa *(90 µg/mL) and ethyl acetate extract from* Arthrinium *state of* A montagnei *Sacc. against* P aeruginosa *(160 µg/mL). The two endophytic fungi isolated from yacón roots as well as their antimicrobial activity detected in the crude extracts cultures were being reported for the first time.*

Key words: *Arthrinium* state of *Apiospora montagnei*, *Papulaspora immersa*, *Smallanthus sonchifolius*, yacón

INTRODUCTION

Resistance to drugs used in the treatment of many infectious diseases, not only bacterial, but also of fungal or parasitic origin is increasing and new resistance problems are emerging, further complicating and rendering treatment of critical infectious illness more difficult. Increasing prevalence of multi-resistant bacteria has turned the search for new antimicrobial agents an important strategy for alternative therapies useful in the handling of difficult infections (Levy, 2005). Natural products continue to be an important source of new pharmaceutical products (Newman and Cragg, 2007). Since some of them are produced by the organisms as a result of selection in favor of improved defense against competing deleterious microorganisms. The production of defensive secondary metabolites is of importance due to their original natural function in response to environmental challenges (Lu and Shen, 2004).

Endophytes may be isolated from different parts of plants. Araujo et al. (2000) obtained 53 isolates of actinomycetes from the leaves and roots of maize and 297 endophytic fungi were isolated from the leaves and steams from soybean by Pimentel et al., (2006). Metabolites isolated from the fungal endophytes are good sources of novel secondary metabolic products having diverse structural groups and showing antibacterial, antifungal, anticancer, antiviral, antioxidant, insecticide, antidiabetic and immunosuppressive activities (Demain, 1999; Tan and Zou, 2005). The endophytes are also potential enzyme producers. Endophytic bacteria isolated from the leaves and steams of *Jacaranda decurrens* presented proteolytic, amilolytic, lipolytic and esterasic activities (Carrim et al., 2006). There have not

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been reports of studies on endophytic fungi isolated from *Smallanthus sonchifolius* (Asteraceae), known as yacón, an ethnobotanical historical plant originated from the Andes (Zardini, 1991). Its leaves and roots extracts have shown fungicidal (Inoué, 1995), antibacterial (Lin et al., 2003), hypoglycemic (Grau and Rea, 1997) and antioxidant activity (Yan et al., 1999; Valentová et al., 2003). Due to such an ethnobotanical history, yacón was selected in the present study to a search for bioactive substances showing antimicrobial effectiveness in the crude extracts of endophytic fungi isolated from *S. sonchifolius*.

MATERIALS AND METHODS

Sample collection and fungal isolation

Healthy *Smallanthus sonchifolius* were collected at an altitude of 800 m in Ribeirão Preto city, S. P. State, Brazil. Samples were transferred to the laboratory and thin roots were gently rinsed in fresh water. Sterilization was performed by sequential immersion for 2.5 min in 70% (v/v) ethanol, 1.5 min in 5% sodium hypochlorite, 1 min in 70% ethanol and sterile distilled water. Then 1 x 1 cm segments were plated on potato dextrose agar (PDA) supplemented with penicillin G (30 mg/L) and streptomycin sulfate (30mg/L) and incubated at room temperature. Emergent fungi were isolated and inoculated into fresh PDA antibiotic-free medium and incubated at room temperature for seven days. Water washings from the surface-sterilized samples showed no microbial growth on PDA after a 10-day incubation under the same conditions. *Papulaspora immersa* and *Arthrinium* state of *Apiospora montagnei* Sacc. (=*Arthrinium arundinis*) were identified by Cristina Maria de Souza Motta from "Coleção de Culturas - Micoteca URM - Departamento de Micologia/CCB/ UFPE", Av. Prof. Nelson Chaves. s/n Cidade Universitária, CEP 50670-420, Recife/PE, Brazil. Strains were maintained by periodic transfers onto PDA at 4 ºC.

Fermentation and treatment of the fermentation broth

The strains were separately cultured in 200 mL of pre-fermentative liquid medium, pH 4.5 (Jackson et al., 1993) for 2 days at 30 °C, 120 rpm. The resulting mycelia were harvested, rinsed with sterile distilled water and transferred to 400mL of Czapek liquid medium, pH 5 (Alviano et al., 1992), and incubated for 20 days at 30 °C, 120 rpm. The mycelial mass was separated from the broth by filtration, and the culture broth was partitioned with ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) (3 x 200 mL). The separated mycelium biomass was macerated with ethanol (EtOH) for seven days. The organic solvents were evaporated under vacuum and water extracted fractions were lyophilized, which resulted EtOAc, *n*-BuOH, water and EtOH extracts.

Antimicrobial activity

The antibacterial activity was performed using microbroth dilution assay on 96-wells microplates according to the National Committee for Clinical Laboratory Standards approved document M7-A6 (NCCLS, 2003). Crude extracts were dissolved in DMSO at 0.1% (v/v) and each microdilution well containing 50µL of the twofold crude extracts serially diluted in Mueller Hinton Broth was inoculated with 50µL of the diluted inoculum suspension of *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853 or *Escherichia coli* ATCC 25922 at final concentration of $5x10^5$ CFU mL^{-1}. After incubation at 37 °C for 24h, 40 µL of 0.7%, 2,3,5-triphenyl tetrazolium chloride were added to each well and the microplates were reincubated for an additional 3 h. Bioactivity was recorded as the absence of red coloration in wells.

Morphological alteration

The effect of endophytic fungi crude extracts on the morphological alterations were tested against the strains from our private collection, *Aspergillus fumigatus* and *Neurospora crassa*. Vegetative hyphae were grown into solid Vogel's minimal medium (Vogel, 1956) for *N. crassa* and PDA for *A. fumigatus* and incubated at 30 °C. The spore suspensions obtained after seven days were diluted in sterile distilled water and aliquots of the diluted suspensions inoculated ($1.3x10^2$ CFU mL^{-1}) onto 96 well plates. The aliquots of liquid Vogel's minimal medium to *N. crassa* and PDB to *A. fumigatus* were poured into the plates. Different concentrations of crude extracts dissolved in DMSO were applied to the plates and incubated at 30 °C for 6, 12, 18 or 24 h. The cultures were then observed and recorded under a Zeiss stereomicroscope.

RESULTS AND DISCUSSION

Eleven crude extracts from endophytic fungi were screened for their antibacterial activities against the standard ATCC bacterial strains. In this study, EtOAc and *n*-BuOH extracts showed higher antimicrobial activities against Gram-positive and Gram-negative bacteria than water and ethanol extracts (Tables 1 and 2). The best result (Table 1) was obtained from *Papulaspora immersa* EtOAc extract against *P. aeruginosa* (90µg/mL). The MIC of crude extracts of *Arthrinium* state of *A. montagnei* Sacc. against ATCC bacteria (Table 2) showed the best result again in EtOAc extract against *P. aeruginosa* (160µg/mL). Extracts showed no antifungal activity or caused morphological alterations even when tested at up to 1mg/mL (not shown). According to Cabello et al. (2001), *Arthrinium* state of *A. montagnei* Sacc. produced arundifungin, a glucan synthesis inhibitor of a class of antifungal agents showing activity against a broad panel of human pathogenic filamentous fungi and yeasts and against *S. aureus.* This class of antifungals are known to alter the morphology of filamentous fungi. Results of morphological alteration assays showed that this compound was not produced by *Arthrinium* state of *A. montagnei* Sacc. strain. Thus, the antibacterial activity reported here might be due another bioactive substance. Till now, no data on the antimicrobial activity of *P. immersa* had been reported in the literature.

Table 1 - MIC of crude extracts obtained from liquid culture (Czapek) of endophytic fungus *Papulaspora immersa* from *S. Sonchifolius* against ATCC bacteria test.

Tested extract	*E. coli*	*P. aeruginosa*	*S. aureus*	*K. rhizophila*
EtOAc	-	90	240	220
n-BuOH	190	190	-	-
EtOH	-	-	-	-
Water	280	190	-	-
negative control	-	-	-	-
positive control	0.184	0.184	0.023	0.023

MIC values are expressed in µg/mL, penicillin G was used for positive control to *S. aureus* and *K. rhizophila* and streptomycin sulfate was used to *E. coli* and *P. aeruginosa.* DMSO was used to negative control of solvent activity. (-) absence of activity.

Table 2 - MIC of crude extracts obtained from liquid culture (Czapek) of endophytic fungus *Arthrinium arundinis* from *S. Sonchifolius* against ATCC bacteria test.

Tested extract	*E. coli*	*P. aeruginosa*	*S. aureus*	*K. rhizophila*
EtOAc	260	160	-	-
n-BuOH	190	200	-	-
EtOH	-	-	-	-
Water	-	-	-	-
negative control	-	-	-	-
positive control	0.184	0.184	0.023	0.023

MIC values are expressed in µg/mL, penicillin G was used for positive control to *S. aureus* and *K. rhizophila* and streptomycin sulfate was used to *E. coli* and *P. aeruginosa.* DMSO was used to negative control of solvent activity. (-) absence of activity.

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RESUMO

Mesmo havendo grandes investimentos, a resistência a fármacos continua sendo um grave problema de saúde pública. Produtos naturais têm prevalecido como a maior fonte de novas drogas e muitos deles são isolados de sistemas simbióticos:

microorganismos-plantas. Os fungos endofíticos *Papulaspora immersa* e *Arthrinium* state of *Apiospora montagnei* Sacc. foram isolados de raízes de *Smallanthus sonchifolius* (yacón). Extratos de culturas destes fungos apresentaram atividade antibacteriana frente *Staphylococcus aureus*, *Kocuria rhizophila*, *Pseudomonas aeruginosa* e *Escherichia coli*, sendo as mais relevantes observadas nas frações acetato de etila provenientes dos fungos *P. immersa* e *Arthrinium* state of *Apiospora montagnei* Sacc. frente a *P. aeruginosa*, apresentando CIM de 90µg/mL e 160µg/mL, respectivamente.

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