

Antimicrobial Activity of Crude Extracts of Endophytic Fungi Isolated from Medicinal Plant *Sapindus saponaria* L.

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ABSTRACT

Endophytic microorganisms are fungi or bacteria which live inside plant tissues or organs, without causing them any harmful symptoms. They may protect the plant from insect attacks and diseases, being also able to produce substances of biotechnological interest. *Sapindus saponaria* L. is a tree commonly known in Brazil as “sabão-de-soldado”. In folk medicine, its bark, root and fruit are used as producing anxiolytic, astringent, diuretic and expectorant substance, as well as tonic, blood depurative and cough medicine. Its leaves extracts present properties that neutralize bleeding. The fruit extract presents antifungal and larvicidal activities. The aim of the present work was to investigate biotechnological potential of crude extracts of fungal endophytes (G2-20 *Cochliobolus intermedius*, G1-74 non-identified - NI, G22-97 *Phomopsis* sp. and G23-100 NI) isolated from *S. saponaria*, that have been assayed against five pathogenic bacteria. The antibacterial activities with extracts obtained from the four endophytic lineages were promising, since all of them inhibited the growth of at least one of the tested bacteria. One metabolite, extracted from the lineage G2-20 (*Cochliobolus intermedius*) presented activity for all the bacteria tested. The results showed that *S. saponaria* isolates presented biotechnological potential for the control of pathogenic bacteria tested in vitro.

INTRODUCTION

Endophytic microorganisms are fungi and bacteria that colonize inter or intracellular spaces of plant tissues during at least one phase of their life cycle (Azevedo *et al.*, 2000; Kaneko *et al.*, 2010). Mostly, they maintain a stable relationship with the host plants, since they live asymptotically (Pamphile and Azevedo 2002), without causing apparent damage to them (Kaneko *et al.*, 2010).

Many substances found in plants were extracted from their endophytes (Azevedo *et al.*, 2000). Therefore, it is increasing the studies that focused on the isolation and/or application of endophytes from medicinal plants (Bernardi-Wenzel *et al.*, 2010; Garcia *et al.*, 2012; Li *et al.*, 2005; Orlandelli *et al.*, 2012; Pillegi *et al.*, 2002; Rhoden *et al.*, 2012a; Targa *et al.*, 2011; Visalakchi and Muthumary, 2009; Xue *et al.*, 2012). Through the interaction endophytes plants, these microorganisms can produce several

substances of biotechnological interest, including: secondary metabolites with pharmaceutical application (Schulz *et al.*, 1999; Schulz and Boyle, 2005; Strobel, 2003; Strobel, 2006), being used in the production of antimicrobials that inhibit the development of pathogens (Chareprasert *et al.*, 2006; Phongpaichit *et al.*, 2006; Strobel *et al.*, 1999; Weber *et al.*, 2007).

Also, they can protect the hosts against several biotic and abiotic factors, such as the attack of insects, pathogens and herbivores (Arnold *et al.*, 2003; Firáková *et al.*, 2007; Mejía *et al.*, 2008). The in vitro inhibition of pathogens by antagonists is considered an indication of antibiosis caused by antagonistic substances possibly produced in the culture media (Rocha *et al.*, 2009).

Sapindaceae family is known for its traditional medicinal uses (Tsuzuki *et al.*, 2007). *Sapindus saponaria* L. is a tree vulgarly known as “sabão-de-soldado”, “saboeiro”, “sabão-de-macaco”, “pau-de-sabão” and “saboneteiro” in Brazilian portuguese. It is distributed in Central and South America, from the luxuriant forest to the Cerrado. In Brazil, it is found from Pará to Rio Grande do Sul States (Albiero *et al.*, 2001; Lorenzi, 2004).

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The bark, root and fruits from *S. saponaria* are used in popular medicine as tranquilizer, astringent, diuretic, expectorant, tonic, blood cleanser, healing and to counter cough (Albiero *et al.*, 2001), being also a neutralizer of hemorrhage (Castro *et al.*, 1999). Its fruit extracts have activity as antifungal (Tsuzuki *et al.*, 2007), larvaecidal (Barreto *et al.*, 2006; Fernandes *et al.*, 2005; Silva *et al.*, 2004).

The genus *Phomopsis* is commonly found as endophyte in tropical medicinal plants, such as reported by Bernardi-Wenzel *et al.* (2010), Garcia *et al.* (2012), Orlandelli *et al.* (2012) and Rhoden *et al.* (2012a). This genus is a rich source of biologically active secondary metabolites with antimicrobial activity against several pathogens including *Mycobacterium tuberculosis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Micrococcus luteus* and *Candida albicans* (Jayanthi *et al.*, 2011; Rukachaisirikul *et al.*, 2008) and it is also potential for protecting plants from fungal diseases (Brayford, 1990).

Tilley and Walker (2002), isolated *Cochliobolus intermedius* from diseased crabgrass (*Digitaria* sp.) and evaluated its potential as a microbial herbicide for control of large crabgrass (*Digitaria sanguinalis*), observing that these fungus could be a potential microbial herbicide for control of large crabgrass in crops such as soybean, cotton, and peanut.

The aim of the present work was to obtain crude extracts of fungal endophytes (G2-20 *Cochliobolus intermedius*, G1-74 non-identified - NI, G22-97 *Phomopsis* sp. and G23-100 NI) isolated from *S. saponaria* and investigate their biotechnological potential against five pathogenic bacteria.

MATERIAL AND METHODS

Endophytic strains

The endophytic strains selected for the obtainment of crude extracts were isolated from leaves of a *S. saponaria* tree located at State University of Maringá, Paraná, Brazil and molecularly identified by Garcia *et al.* (2012). The sequences were deposited in NCBI: isolates G2-20 *Cochliobolus intermedius* (AF071327), G1-74 (non-identified - NI), G22-97 *Phomopsis* sp. (EF687936) and G23-100 (NI).

Obtainment of crude extracts

The crude extracts were obtained following the methodology described by Li *et al.* (2005) modified. The endophytic fungi were incubated in culture medium, at 25° C for 10 days in Potato Dextrose (PD), which prepared according to Smith and Onions (1983) modified by Pamphile *et al.* (2004). The fermented medium obtained was centrifuged at 3.600 rpm for 10 minutes. The supernatant was transferred to a separatory funnel to which was added the same volume of crude ethyl acetate. The funnel was strongly agitated and then the separation of the phases occurred by polarity differences. This process was repeated twice. Ethyl acetate solution containing the fungal metabolite was 98% concentrated in a Büchi R-3000 Rotavapor at 40° C and the material obtained from the evaporation was suspended with 1ml of

absolute methanol and stored at 4° C until its use.

The fungal mycelium obtained in the PD incubation was maintained for 48 hours in methanol. After this period, the methanol was centrifuged and the supernatant was collected and 98% concentrated a Büchi R-3000 Rotavapor at 40° C. The material obtained from the evaporation was suspended with 1 ml of absolute methanol and also stored at 4° C.

Assessment of antimicrobial activity

The antimicrobial activity was tested by qualitative biological analysis in triplicate, using the disk diffusion technique (cup plate). The microorganisms used in this test were the human pathogenic bacteria *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (ATCC 19430), *Micrococcus luteus* (ATCC 9341) and *Enterococcus hirae* (ATCC 1227).

To evaluate the antibacterial activity, bacteria were grown in liquid LB (Luria Bertani) medium (Sambrook and Russel, 2001) for 24 h, adjusted at a concentration of 10⁶ cells/ml and spread (100 µl) on Petri dishes containing solid LB medium. In each dish were placed, equidistantly, four disks of sterile filter paper Whatman n°. 4 (6 mm) inoculated with 10 µl of extracts. As negative controls, paper disks were inoculated with autoclaved distilled water and absolute methanol. As positive control, Tetracycline (50 µg.ml⁻¹ in absolute ethanol) was used. The dishes were incubated at 37° C in B.O.D for 24 h. The antibacterial activity was evaluated by the formation of inhibition halos, according to Souza *et al.* (2004).

All the experiments were carried out using a completely randomized design (CRD), with 3 repetitions. In order to test the efficiency of the metabolite extracts, statistical analyses through WinBUGS (Spiegelhalter *et al.*, 1994) software were employed, which is followed by the Bayesian analysis, admitting normal distribution due to the growth inhibition halo data.

RESULTS AND DISCUSSION

The increasing use of chemical products in order to implant and maintain healthy crops and high productivity has been caused negative effects for the biotic complex of nature, affecting animals, humans and plants (Mochi *et al.*, 2005).

Discovery of new and potential drugs molecules can be focused on the production of bioactive compounds by plants, microbial and marine organisms. Endophytic microorganisms isolated from plants constitute a source of search for novel secondary metabolites (Firáková *et al.*, 2007), since a single endophyte may be able to produce a variety of bioactive metabolites (Ramasamy *et al.*, 2010).

The antibacterial tests with the fungal isolates G2-20 (*Cochliobolus intermedius*), G22-97 (*Phomopsis* sp.), G1-74 and G23-100 (non-identified endophytes) showed that when the extracts were obtained by the incubation of mycelium with methanol, it was verified no difference in comparison to the negative controls. Considering the endophytic extracts obtained

from the fermented medium extracted with ethyl acetate, the results were statistically significant in comparison to the negative controls for at least one of the five human pathogenic bacterium tested (*E. coli*, *S. aureus*, *S. typhi*, *M. luteus* and *E. hirae*) (Figure-1 and Table-1). However, the only one extract that showed antibacterial activity against all pathogenic bacteria was the endophytic strain G2-20, molecularly characterized as *C. intermedius*.

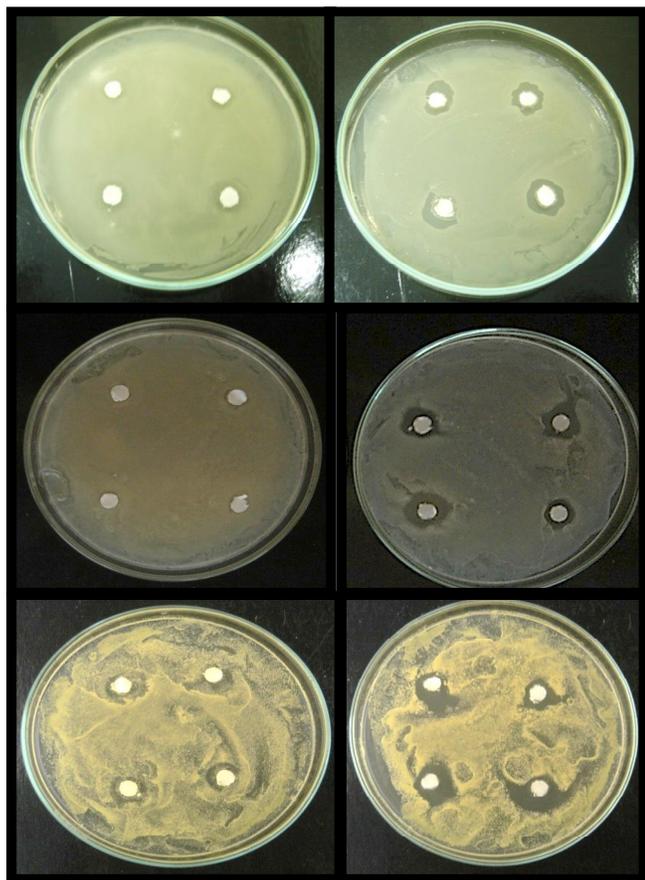


Fig. 1: Antimicrobial activity of crude extracts of endophytic fungi from *S. saponaria*.

(a) Control with methanol (*S. typhi*) (b) Inhibition halo produced by the extract of lineage G1-74 (NI) against *S. typhi*; (c) Control with methanol (*S. aureus*) (d) Inhibition halo produced by the extract of lineage G2-20 (*C. intermedius*) against *S. aureus*; (e) Control with methanol (*M. luteus*) (f) Inhibition halo produced by the extract of lineage G2-20 (*C. intermedius*) against *M. luteus*.

Tabel. 1: Antimicrobial activity of crude extracts of endophytic fungi from *S. saponaria*.

Crude Extracts	Pathogenic Bacteria				
	<i>E. coli</i>	<i>M.luteus</i>	<i>S.typhi</i>	<i>E. hirae</i>	<i>S. aureus</i>
G2-20 (<i>C. intermedius</i>)	+	+	+	+	+
G1-74 (NI)	-	+	+	+	+
G22-97 (<i>Phomopsis</i> sp.)	-	+	-	-	-
G23-100 (NI)	-	+	+	+	+

+ = Produced inhibition halo greater and statistically different from the negative control.

- = Did not produce inhibition halo statistically different from the negative control. (software WinBUGS, Spiegelhalter et al., 1994).

For *S. aureus*, extract from G2-20 (*C. intermedius*) showed the highest antimicrobial activity. Those produced by G1-74 (NI) and G23-100 (NI) also showed inhibitory activity, whereas the extract produced by G22-97 strain (*Phomopsis* sp.) was statistically no significant in relation to negative control. For *E. coli*, only one crude extract that showed antimicrobial activity was the produced by G2-20 (*C. intermedius*) strain. For *M. luteus*, all extracts tested showed positive results. Among them, the most efficient was also G2-20 (*C. intermedius*). Against *S. typhi*, highest indexes of antimicrobial activity were produced by the isolates G2-20 (*C. intermedius*) and G1-74 (NI), followed by G23-100 (NI). The same was observed for *E. hirae*.

Many studies are evaluating metabolic activity from endophytic fungi isolated, mostly, of plants with properties medicinal. Pileggi et al. (2002), studied antimicrobial action in endophyte fungi isolated from medicinal plant *Simphytum officinale*, popularly known as confrei. These isolated inhibit growing of pathogenic bacteria *S. aureus*.

According to Borges (2008), an important factor to be considered is the fungal growth condition, since the fungus requires excellent conditions for an optimal development, expressing thus its enzymatic potential in the production of metabolites. In his study, this author demonstrated that, for the same fungus, the metabolites production varied depending on the conditions of cultivation.

In our study, the most efficient method for the obtainment of fungal secondary metabolites was the extraction with ethyl acetate, where results statistically significant in relation to negative control were obtained for at least one bacterium tested. The obtainment by incubation of fungal mycelium in methanol did not present positive results for none of the isolates.

Rhoden et al. (2012b), using the same methodology and tested against same bacterial observed that endophytic fungi *Cordyceps memorabilis* inhibit growing against *E. coli*, *M. luteus* and *E. hirae*, *Phomopsis longicolla* inhibit *S. typhi* and *E. hirae*, and two another fungi not identified inhibit growing of *M. luteus* and *E. hirae*.

Similarly, Bernardi-Wenzel (2008) and Rhoden et al. (2012b) also observed minor efficiency of endophytic secondary metabolites extracted with methanol regarding antimicrobial tests. Bernardi-Wenzel (2008) researched the antimicrobial activity of fungal secondary metabolites produced by endophytes from *Luehea divaricata* against the human pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. None extracts showed antagonistic activity against *S.aureus*, while some extracts inhibited the *E. coli* growth.

Phongpaichit et al. (2006) isolated fungal endophytes from five medicinal *Garcinia* plants and verified that the metabolites produced by 70 isolates and extracted with ethyl acetate showed antimicrobial activity by agar diffusion test against at least a pathogen microorganism tested: *Staphylococcus aureus*, *C.albicans*, *Cryptococcus neoformans* and *Microsporium gypseu*. These authors identified the genera *Aspergillus*, *Botryosphaeria*, *Eutypella*, *Fusarium*, *Guignardia*, *Penicillium*, *Phomopsis* and

Xylaria as the ones which showed the greatest results for the inhibition of microbial growth. Among these endophytes, three strains showed most active for production of secondary metabolites were *Phomopsis* sp., *Botryo* sp., *haeria* sp. and a non-identified fungi.

In a subsequent study employing endophytes from the same plant genus, Phongpaichit *et al.* (2007) verified the biological activity of sixty-five fungal metabolites extracted with ethyl acetate. Their results were promising, which 80% of the extracts showed some bioactivity, such as: cytotoxic, antimycobacterial, antimalarial, antiviral, antioxidant and anticancer.

Souza *et al.* (2004), tested the antimicrobial activity of endophytes from Amazonian toxic plants *Palicourea longiflora* and *Strychnos cogens*. From total of 79 fungal isolates whose metabolites were tested, 19 inhibited at least one of the pathogenic microorganisms tested: *Bacillus* sp., *B.subtilis*, *S.aureus*, *E. coli*, *Candida albicans*, *Trichoderma* sp., and *Aspergillus flavus*.

Gomes-Figueiredo (2007) observed that the metabolic extracts produced by endophytes of *Pestalotiopsis* genus, isolated from medicinal plant *Maytenus ilicifolia*, have antimicrobial activity against a variety of human pathogens and some isolates showed be potential for the biological control of the phytopathogenic fungus *Guignardia citricarpa*. Similarly, some authors reported activity of metabolites from endophytic fungi in the inhibition of different pathogenic bacteria and fungi, showing that some plant properties can be present in their endophytes.

Using Chinese medicinal plants Li *et al.*(2005) isolated 130 endophytic fungi and tested the antitumour and antifungal activities from their extracts. As result, 9.2% of them presented antitumour activity and 30% had antifungal activity, what indicates that some fungal compounds can be associated with the host plants.

Similarly to the present study, Teles *et al.* (2006) also extracted secondary metabolites from *Periconia atropurpurea*, an endophyte from *Xylopi aromatica*, using ethyl acetate. Besides identifying these compounds, the authors tested their biological activity, proving their cytotoxic and antifungal potential.

Hoffman *et al.* (2008), working with a *Phoma* strain, endophytically isolated from *Saurauia caberrinae*, verified that it produced phomodione, a substance with inhibitory action against pathogenic bacterium *Staphylococcus aureus*.

Hormazabal and Piontelli (2009) showed that, among the endophytes isolated from Chilean native gymnosperms, the metabolite produced by *Curvularia protuberata* had the best effect on *Bacillus subtilis*, *M. luteus* and *S. aureus*, with growth inhibition zones of 12, 9 and 16 mm, respectively. None of the metabolites tested by these authors showed effect on *E. coli*, on the contrary to the result obtained by our research group, where one metabolite tested was effective against this bacterium.

Sutjaritvorakul *et al.* (2011) used the paper disk susceptibility test to evaluate the antimicrobial activity of metabolites produced by fungal endophytes against five reference human pathogenic microorganisms (*S. aureus*, *B. subtilis*,

Pseudomonas aeruginosa, *E. coli* and *C. albicans*). The fungal metabolites inhibited two or more pathogens and the growth of Gram positive bacteria were more inhibited than those Gram negative, with inhibition halos up to 20.1 mm, which was produced by extract of *Pestalotiopsis* sp. DO2 against *B. subtilis*.

The results reported in this present study demonstrate the potential of endophytic fungi from *S. saponaria* for the biological control of human pathogens. Tests with the crude extracts produced by the endophytic isolates showed promising results for growth inhibition of human pathogenic bacteria. Therefore, it indicates that these endophytes can be important sources of bioactive substances of biotechnological interest.

CONCLUSION

Crude extracts from endophytes of *S. saponaria* showed in this study a greater antimicrobial activity against some human pathogenic bacteria. So studies on safety and efficacy should be performed for these fungi for use as pharmaceutical drugs.

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