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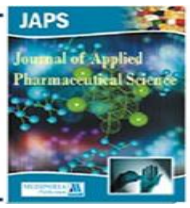

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Antimicrobial Activity of Crude Extracts of Endophytic Fungi Isolated from Medicinal Plant *Trichilia elegans* A. Juss

Rhoden S.A., Garcia A., Bongiorno V. A., Azevedo J.L. and Pamphile, J.A.

ABSTRACT

Endophytes are microorganisms which inhabit inside plants. These microorganisms are identified as being fungi or bacteria and can be useful for prospectionof bioactive compounds that may have medical and pharmaceutical applications.*Trichilia elegans* (Meliaceae) is a native tree in Brazil. Preparations using leaves, seeds, bark and roots and some members of the *Trichilia* genus are used in Brazilian popular medicine.The aim of the present work was to investigate biotechnological potential of fungal endophytes (*Cordyceps memorabilis, Phomopsis longicolla, Dothideomycete* sp. and one non-indentified) isolated from *T. elegans*, that have been assayed against five pathogenic bacteria. The fungi were incubated in Potato Dextrose and the secondary metabolites was extracted from fermentation medium with ethyl acetate, also was used directly extraction with methanolfrom mycelium. The extraction by ethyl acetate from *C. memorabilis* inhibited growing of *Enterococus hirae*, *Micrococcus luteus* and *Escherichia coli*. Fungal *Phomopsis* sp. inhibited *M. luteus, E. hirae* and *Salmonella typhi*, *Dothideomycetes* sp. and G8-25 inhibited *M. luteus* and *E. hirae.* Noextract by ethyl acetate inhibited *Staphylococcus aureus* and also no extract obtained by methanol inhibited the growing of tested bacteria. The present study helped justified the traditional use of *T. elegans* against human pathogenic bacteria.

Keywords: *Trichilia elegans*, fungal endophytes, bioative compounds, pathogenic bacteria.

Rhoden S.A., Garcia A., Bongiorno V. A., Azevedo J.L. and Pamphile, J.A.
Departamento de Biotecnologia, Genética e Biologia Celular Universidade Estadual de Maringá CEP 87020-900 – Maringá – Paraná – Brasil.

For Correspondence
Sandro Augusto Rhoden
Departamento de Biotecnologia, Genética e Biologia Celular Universidade Estadual de Maringá CEP 87020-900 – Maringá – Paraná - Brasil
Tel: (055) 44 3011-4683 (office)
(055) 45 99847053 (celular)

INTRODUCTION

All microorganisms that inhabit, at least for one period of their life cycle, the interior of higher plants, may be considered as an endophyte (Azevedo *et al.,* 2000). These microorganisms can have a biotechnological potential mainly on antimicrobial activity (Molina *et al.,* 2012). Actually, it is constant the search by newer antimicrobial agents because the drugs resistance in human pathogens have increased in last years. Thus, the endophytics studies have been important to discovery substances that can be used in a wide variety of harmful disease-causing agents (Strobel and Daisy, 2003).

The production of metabolites by microorganisms is known and explored, because the major of antibiotics are produced by fungi and bacteria (Strobel, 2003), in this way,antimicrobial activities have been demonstrated in a variety of metabolites biosynthesized by the plant endophytes (Strobel *et al.,* 2001). There is increasing effort to characterize and identify endophytic fungi isolated from medicinal plants. Many studies have shown that some medicinal properties of plants may be related to endophytic fungi hosted by these plants (Azevedo *et al.,* 2000).

Trichilia elegans A. Juss. belongs to Meliaceae family. About 70 species of this genus occur along the American tropical region. Preparations using the leaves, seeds, bark and roots of many plants from the Meliaceae family have been largely used in traditional medicine and some plants of *Trichilia* genus have been used to treat rheumatism and malaria, for inducing vomit and also for having a purgative characteristic (Garcez *et al*., 1996). However, it is not known well about the endophytes bioprospecting in *T. elegans.*

According Kuo *et al.* (2005), fungi belonging to the *Cordyceps* species have long been used as food and herbal medicines in Asia and are especially popular as powdered supplements. The genus *Cordyceps* is famous for entomopathogenic property and it have received significant interest by their potential as health foods and in medical and pharmaceutical applications (Xiao *et al.,* 2010). The genus *Phomopsis* is a rich source of biologically active secondary metabolites with antimicrobial activity (Rukachaisirikul *et al*., 2008). The *Phomopsis longicolla* usually found in plants and soil was first report of human infection (Garcia-Reyne *et al*., 2011).

The aim of the present work was to investigate biotechnological potential of fungal endophytes (*Cordyceps memorabilis, Phomopsis longicolla, Dothideomycete* sp. and one non-indentified) isolated from *T. elegans*, that have been assayed against five pathogenic bacteria.

MATERIAL AND METHODS

Biological Material

Four crude extracts of fungi endophytes isolates from *T. elegans* were tested, three were identified molecularly according Rhoden *et al*.(2012). The sequences weredeposited in NCBI: isolates *Cordyceps memorabilis* (GQ461583), *Phomopsis* sp. (GQ461584) and *Dothideomycetes* sp. (GQ461591) and another fungi not identified G8-25 (non-identified – NI.).

Obtainment of secondary metabolites

In order to obtain the secondary metabolites, a slightly modified version of the methodology described by Li *et al.* (2005) was used. The fungi were incubated in PD (Potato Dextrose) medium at 28º C for 15 days. The fermentation medium 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 difference. The process was repeated twice. The obtained ethyl acetate extract was 98% concentrated in a R-3000 Büchi rotary evaporator at 40º C and the material obtained from the evaporation was suspended with 1 ml of absolute methanol and stored at 4º C.
The metabolic extracts were also used directly from the produced mycelium. The mycelium was filtered and kept for 48 hours in methanol. After this period it was centrifuged and the collected supernatant was 98% concentrated in rotary evaporator. The material obtained from evaporation was suspended with 1 ml of absolute methanol and stored at 4º C.

Assessment of antimicrobial activity

The antimicrobial activities were tested using qualitative biological analysis in triplicate. The pathogenic bacteria used in this study were: *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (ATCC 19430), *Micrococcus luteus* (ATCC 9341) and *Enterococus hirae* (ATCC 1227).

The antimicrobial activity of metabolite extracts was assessed by cup plate diffusion technique. The test bacteria were grown on liquid LB (Luria Bertani) medium (Sambrook and Russel, 2001) for 24 hours, adjusted at a concentration of 1×10^6 cells/mL. The bacteria (100 µL) were inoculated on the Petri dishes containing solid LB medium and spread with a Drigalsky spatula. Afterwards, four sterile Whatman No. 4 filter paper disks were placed (Ø 6 mm) equidistant and inoculated with 10 µl of the metabolite extract. The plates remained incubated at 37ºC for 24 hours. The antimicrobial activity was detected by the formation of an inhibition halo. The diameter of the halo was measured in triplicate and compared with control. Control was Tetracycline (Sigma) (50 $\mu g.ml^{-1}$ in absolute ethanol) employed as a positive control.

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 antimicrobial activity from endophytes isolated of *T. elegans* G4-2 *(Cordyceps memorabilis),* G9-10 *(Phomopsis longicolla*G8-25 (molecularly non-identified) and G5-32 *(Dothideomycete* sp.) obtained through fermentation and extraction with ethyl acetate and through incubation of the mycelium with methanol was tested against five pathogenic bacteria (Table-1).When the metabolic extracts of the endophytic fungi, obtained through the mycelium incubation with methanol, were assessed, it was verified that no extract had differed from the control regarding the antimicrobial activity. Considering the metabolic extracts obtained by fermentation of the medium through the fungus and the extraction with ethyl acetate, positive outcomes were observed.
The antimicrobial activity statistically significant of metabolite extracts showed that the isolate G4-2 *(Cordyceps memorabilis)* is the most successful in control of pathogens because it produced an inhibition halo against *E. coli*, *M. luteus* and*E. hirae* followed by the isolate G9-10 *(Phomopsis longicolla)* which inhibited *S. typhi* and *E.hirae* growth. The isolates G5-32 (*Dothideomycete* sp.)and G8-25 (non-identified) showed inhibition halos only against *E. hirae.* Bernardi-Wenzel (2008), with isolates from *Luhea divaricata* and metabolic extracts extracted with ethyl acetate, also did not have positive data towards *S. aureus* inhibition. However, satisfactory results towards *E. coli* were obtained. Similarly, the isolate G4-2 (*Cordyceps memorabilis*) from *T. elegans* was also effective against *E. coli.* Using similar methodology Gomes-

Figueiredo *et al.*, (2007), performed bioprospections of highly diverse endophytic *Pestalotiopsis* spp. with antibacterial properties from *Maytenus ilicifolia*, a medicinal plant from Brazil, and detected that two isolates were successful in inhibiting the growth of the tested microorganisms *Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus* and S*taphylococcus aureus*. Phongpaichit *et al.* (2006)with isolates from *Garcinia* sp., a medicinal plant, verified that 70 isolates showed antimicrobial activity against for at least one pathogen microorganism tested, such as *Staphylococcus aureus, Candida albicans, Cryptococcus neoformans* and *Microsporum gypseu*, using extracted with ethyl acetate. Chareprasert *et al.* (2006), isolated endophytes fungi from *Tectona grandis* L. and *Samanea saman* Merr., from 37 isolated fungi, 18 shown antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and three isolates inhibited the growing of *Candida albicans.* Weber *et al.* (2007), demonstrated antifungic activity from metabolities of endophyte fungi group ascomicete against *Candida albicans.* Souza *et al.* (2004), tested antimicrobial activity from endophytes isolated of toxic plants from Amazon: *Palicourea longiflora* (Rubiacea) and *Strychnos* cogens (Loganiaceae). Some extracts from these fungi showed positive results against: *Bacillus sp., Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Candida albicans, Trichoderma* sp. and *Aspergillus flavus.*

The results showed that extracts metabolites from these fungi are important for help future studies of this plant in applications medicinal and pharmaceutical, others studies are still needed for identification of bioactive compounds and another implications of these extracts in humans.

Table. 1: Fungal endophyte isolates from *T. elegans* and pathogenic bacteria tested.

Metabolites	Pathogenic bacteria				
	E. coli	*M. luteus*	*S.typhi*	*E. hirae*	*S. aureus*
G4-2 (*Cordyceps memorabilis)*	+	+	–	+	–
G9-10 *(Phomopsis longicolla)*	–	-	+	+	–
G5-32 *(Dothideomycete sp.)*	–	+	–	+	–
G8-25 (non-identified - NI)	–	+	–	+	–

+ = Produced inhibition halo greater and statistically different from the negative control (BUGS - Bayesian Inference using Gibbs Sampling).
– = Did not produce inhibition halo statistically different from the negative control (BUGS - Bayesian Inference using Gibbs Sampling).

CONCLUSION

Endophyte from *T. elegans* has been 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. The present study helped justified the traditional use of *T. elegans* against human pathogenic bacteria.

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