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Endophytic Actinomycetes from *Miconia albicans* (Sw.) Triana (*Melastomataceae*) and Evaluation of Its Antimicrobial Activity

Ana Candida Martins Toledo Piza[1,3], Carlos Osamu Hokka[3,4] and Cristina Paiva de Sousa[1,2,3*]

[1]Department of Morphology and Pathology/Via Washington Luis, Federal University of Sao Carlos (UFSCar), Monjolinho, P.O. Box: 676 - 13565-905 Sao Carlos, Brazil.
[2]Nursering Post Graduation Program, Via Washington Luis, Monjolinho, P.O. Box: 676 - 13565-905 Sao Carlos, São Paulo, Brazil.
[3]Biotechnology Post Graduation Program, Via Washington Luis, Monjolinho, P.O. Box: 676 - 13565-905 Sao Carlos, São Paulo, Brazil.
[4]Department of Chemical Engineering, Via Washington Luis, Federal University of Sao Carlos (UFSCar), Monjolinho, P.O. Box: 676 - 13565-905 Sao Carlos, São Paulo, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors COH and CPS designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author ACMTP managed the literature searches, analyses of the study managed the experimental process. All authors read and approved the final manuscript.

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ABSTRACT

The need for new and useful compounds to provide assistance and relief in all aspects of human condition is over-growing. Seeking to improve the quality of life, the natural products isolated from endophytic microorganisms are important to obtain new compounds that can be used in different

**Corresponding author: Email: prokarya@ufscar.br;*

industrial segments. The substances formation is coupled with the onset of development of the microorganisms and the use of parvome to find novel compounds from unique habitats is a new goal. In this scope, this study aimed to isolate potentially bioactive substances produced by endophytic microorganisms collected from endemic medicinal plants. *Miconia albicans* (Sw.) Triana (*Melastomataceae*) were collected in Brazilian Tropical Savannah and disinfected. Gram-positive, catalase-positive, not alcohol-acid-resistant and spore-forming microorganisms were isolated and characterized by phenotypic analyzes. From such characteristics the isolates were characterized as actinomycetes belonging to the genus *Streptomyces*. Two of the isolates were identified as *Nocardiopsis dassonvillei* and *Amycolatopsis orientalis* based on the analysis of 16S rRNA gene sequences reporting, for the first time, the association of endophyticactinomycetes with aerial parts of *Miconia albicans*. The bioactivity was determined by testing for antibiosis using *Staphylococcus aureus* (ATCC 29213), *Serratiamarcensis* (ITB 1475), *Enterococcus faecalis* (ATCC 29212), *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922) and *Shigella sonnei* (ATCC 1578) as indicator organisms. *Amycolatopsis orientalis* showed the best antimicrobial potential, verified by antagonist halos against *S. aureus*, *E. faecalis*, *C. albicans* and *S. sonnei* measuring 3.50 cm, 3.15 cm, 3.35 cm and 3.20 cm in diameter, respectively. These findings can contribute to the discovery and characterization of new and useful antimicrobial substances with possible medical and/or industrial applications.

Keywords: Antibiosis; endophytic microorganisms; bioactivity; antimicrobial potential; Amycolatopsis orientalis; Nocardiopsis dassonvillei.

1. INTRODUCTION

The continuous and sometimes uncontrolled use of antibiotics by people has led to the selection of microorganisms resistance to antimicrobials currently used, which emphasizes the need to exploit niches that harbor bacteria that produce new metabolites of low molecular weight. Thus, studies with new bioactive substances from natural sources yet unexplored are of great importance for the discovery of new compounds with potential applications to improve the quality of life.

Miconia albicans (Sw.) Triana, popularly known as "quaresmeira-branca" is a native abundant species that is found in Brazilian Tropical Savannah. These plants belong to the *Melastomataceae* family, which have 163 genera and 4,300 species [1]. *Miconia* is one of the largest genera of this family and just in Brazil there are about 250 species [2], and *Miconia stenostachya* as well as *Miconia albicans* are the most abundant [3]

According Damascos et al. [4], *M. albicans* grows in sunny areas as shrubs, and they are trees reaching approximately 3.0 meters. The insertionofthe leaves occurs in the opposite way and through inserts and features cross front leathery texture. Its foliage is permanent year round. Produces flowers, branches and leaves regularly.

Studies conducted by Vasconcelos et al. [5], suggest the analgesic property in crude plant extract. These authors found that *M. Albicans* showed satisfactory anti-inflammatory characteristics in their tests. The absence of herbivores as predators in that plant species may indicate an efficient anti-herbivore chemical defense mechanism [4]

Miconia albicans relations with endophytic microorganisms are not yet described in the literature. On the other hand, papers describing the activity of the plant extract can be found, such as Rodrigues et al. [6].

Brazilian Tropical Savannah is abiome that may contain potentially endophytic microorganisms producing bioactive metabolites. Endophytic microorganisms inhabit the interior of plants without causing, apparently, no damage to their hosts [7,8,9,10] or developing with them a symbiotic relationship [11].

One of the advantages in the use of endophytes is tied to its ability of producing a wide variety of bioactive molecules [12], potentially useful in the production of metabolites of biotechnological interest [13,14].

In soils of Brazilian Tropical Savannah the natural population of Actinomycetes may be greater than 80%, with a predominance of *Streptomyces* spp. [15] and this genus is responsible for the production of more than 80%

of current known antibiotics [16] and presenting an antibiosis [17] course. This plethora can be understood by the diversity of complex organisms and their different habitats are responsible for its prodigious microbial variety [18].

The study of endophytic microorganisms was awakened when the beneficial effects on plants, through symbiotic interactions with the host, were identified by their use of biotechnological techniques [19].

This work aimed to sort the isolation and characterization of endophyticactinomycetes from samples of *Miconia albicans*, chosen mainly by the absence on relationship with endophytic microorganisms beyond its crude extract presented antimicrobial activity. Another interesting characteristic of the chosen plant is its analgesic and anti-inflammatory features [6,5], emphasizing that on this biome the evaluation of substances can have antimicrobial and/or another applications.

2. MATERIALS AND METHODS

2.1 Geoecolocalization and Isolation of Samples

Leaf samples of *Miconia albicans* (Sw.), with healthy appearance, popularly known as "Quaresmeira-Branca" were collected in Brazilian Tropical Savannah from Federal University of Sao Carlos, Sao Carlos – SP/ Brazil (21° 58´S, 47 ° 52´ W) situated 850 m above sea level - and taken for further analysis. The leaves were washed in tap water for the removal of waste. To eliminate the epiphytic population samples were disinfected using 70% alcohol (3 min.), solution of sodium hypochlorite 3% (10 min.) and washed with sterile distilled water to remove the excess of alcohol according Araújo et al. modified [20].

After disinfection, 25 g of leaves were homogenized in 225 mL of saline (0.85%) and filtered. Decimal dilutions were performed (10^{-2} to 10^{-6}) and 200 mL aliquots of the sample were deposited in Petri dishes containing culture media Yeast Extract Agar (YE) and Yeast Extract and Malt Agar (ISP2A). The plates were incubated at 28ºC under the protection of visible light, for a period of 10 to 20 days. All experiments were performed in triplicate.

After growth, typical colonies of Actinomycetes selected by phenotypic and genotypic analysis were purified utilizing YE and ISP2A agar.

2.2 Phenotypic, Physiological and Biochemical Characterization

In phenotypic identification, morphological criteria such as size of colonies, aspect of their edges, as well as the consistency of their capacity to adhere to the agar were studied. To analyze the formation of pigments the microorganisms were observed in liquid medium YE [15].

According Good fellow & Williams [21], many strains of Actinomycetes, including *Streptomyces* are able to degrade complex polysaccharides such as starch, chitin and pectin for producing amylase. Therefore, for theassimilation of carbon test sources, it was used ISP2A medium, where the use of starch as the carbon source could be observed when there was that carbohydrate degradation, resulting in formation of halos. As a negative control we used ISP2 medium (without starch) whereas no formation of the halos shows that the starch was degraded in the ISP2A medium.

In biochemical analysis, the smell of wet earth produced was evaluated. This odor suggests the production of geosmin, the compound produced by certain groups of microorganisms including most *Streptomyces*, and some species of cyanobacteria, fungi and some myxobacteria [22].

Other biochemical analysis performed was the catalase production where from microbial cultures in TSB (broth Tryptone-Soya) at 15 days incubation (28℃) a small portion of the colony was transferred to the glass slide using a wooden spatula. Two drops of hydrogen peroxide (10%) were added and the reaction was observed. The release of gas bubbles showed the presence of catalase by the tested microorganism. A strain of *S. aureus* (ATCC 29213) was used as a positive control. Some members of the Actinomycetes as *Streptomyces coelicolor*, produces three distinct catalases [23] that protects themselves from osmotic and oxidative stress, and allow proper growth and differentiation. The mostimportantcatalase (Cat A) is induced by hydrogen peroxide and is required for efficient aerobic growth.

2.3 Staining Characteristics

For this analysis, spore microscopic examination, Gram staining and Ziehl-Neelsen (alcohol-acid resistant) staining was performed. Actinomycetesand*Streptomyces* are Gram +, non-alcohol-acid resistant and the most of them have spores.

2.4 Overlay Assay

Six typical colonies of Actinomycetes were selected and diluted in saline (0.85%). Drops of 10 µLwere deposited (with Pasteur pipette) in Petri dishes containing YE (Tryptone 5%, Yeast Extract 3%, Glucose 8%, Agar 15% and distilled water 1000 mL) for samples 1, 2 and 7 and ISP2A (Starch 10%, Yeast Extract 4%, Malt extract 10%, Dextrose 16%, Agar 15% and distilled water 1000 mL) for sample 9 and the plates were incubated at 28ºC for 10 days. The use of two different culture media was due to the easiest of growth of each microorganism in the above mentioned media.

After growth, colonies were inactivated with chloroform (20 min.) and the plates were opened (30 min.) for the evaporation of the substance [24]. *Staphylococcus aureus* ATCC 29213, *Serratiamarcensis* ITB 1475, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922 and *Shigella sonnei* ATCC 1578 were used as indicator microorganisms and tested for antibiosis. Therefore, the strains were reactivated in a tube containing Brain Heart Infusion broth (BHI) (24h/37ºC). After 24 h of incubation, 200 mL of previously reactivated cultures were transferred to a tube with 10 mL of semi-solid BHI. The tube was shaken in vortex and the solution deposited on the surface of the plate containing the microorganism previously inactivated. The plates were incubated at 37ºC for 24-48h to verify and measure the inhibition halos [17].

2.5 Genotypic Identification

The selected endophytes were identified by the method of amplification and sequencing 16S ribosomal RNA at Université de Montréal, Canada. The extraction of total genomic DNA of the isolates was performed using the QIAamp DNA mini kit (Qiagen). 5µL sample containing DNA template was added to 15µL of reaction mixture (Qiagen Fast Cycling PCR Kit). The primers used in the reaction were 27F 5 '- AGA GTT TGA TCM TGG CTC AG3' and 519R 5' - GWA TTA CCG CGG CKG CTG - 3 '.

The amplified PCR products were purified using the QIA quick PCR purification (Qiagen) commercial kit as indicated by the company. Both purified PCR products were sequenced using the same primers with standard sequencing methods (FMV Sequencing Laboratory Big Dye Terminator version 3.1 sequencer: AB1 310, Applied Biosystems, Foster City, California, USA).

The sequences were compared to NCBI GenBank using BLAST (National Center for Biotechnology Information - http://blast.ncbi.nlm.nih.gov/blast.cgi).

3. RESULTS AND DISCUSSION

Currently, the majority of work found with endophytic microorganisms shows the bioactivity of fungal isolates. Works with endophytic actinomycetes are more difficult to find besides the absence of reports about the association of these bacteria and the Brazilian Tropical Savannah and its bioactivity as a groundbreaking work.

Table 1 report the activity of six endophytes collected from healthy leaves of *M. albicans*, concerning the isolation and identification according to their phenotypic, macroscopic, physiological, biochemical and morpho-dying characteristics.

According Table 1, the phenotypic, macroscopic, physiological, biochemical and morpho-dying characteristics that conducted to the initial identification of the isolates were carried out. These explorations allowed us to categorize the isolated microorganisms according its similarities. Some authors [25] studied fungi highlighting its distribution and bioactivity similarly to the data we presented.

In 2014, Tanvir et al. [26] also isolated endophytic actinomycetes from plants of the *Asteraceae* family in Pakistan. The genus*Streptomyces*was the dominant isolated bacteria (52, 7%) and demonstrated strong bioactivity, potent cytotoxicity and additional oxidant action. Our work revealed some similarities with these authors, like the isolation of *Amycolatopsis*s pp. Considering another geographical location, These authors [26], obtained small percentual of this genus.

Ontheotherhand, our isolation procedure as well as the Brazilian biome characteristics allowed us to recovered Actinomycetes and particulary *Amycolatopsis* in larger numbers and presenting bioactivity.

In Brazilian state of Sao Paulo, Ratti et al. [17], isolated endophytic microorganism from *Prunus*spp. and *Cassia leptophylla* collected from the Brazilian Tropical Savannah. These authors observed that the endophytes isolated showed antimicrobial activity against important public health pathogen like *S. aureus*. In the present studythe bioactivity demonstrated by *Amycolatopsis orientalis* and *Nocardiopsis dassonvillei* Table 2 was effective against *Enterococcus faecalis* (2.60 cm and 1.90 cm, respectively). *A. oriental s*p resented antibiosis against *S. aureus*, *Candida albicans*, *E. coli* and *S. sonnei* (1.50 cm; 2.40 cm; 2.10 cm and 2.50 cm), respectively along with the *E. faecalis* (2.60 cm) bioactivity.

It can be observed Table 2 that samples 1 and 9 were effective against Gram-positive microorganisms, while sample 2 showed specific activity against *S. Marcensis* (3.60cm). The sample 7 presented antibiosis against a larger number of microorganisms, being effective against Gram-positive, Gram-negative bacteria and yeast.

In the bioactivity test, the sample 9 showed antimicrobial activity with inhibition halos of 1.80 cm in diameter against *E. faecalis* and 1.50 cm against *S. aureus* Figs. 1 and 2.There were no inhibitory activities against *S. marcensis, C. albicans, E. coli* and *S. sonnei.*

Table 1. Bioprospection and characterization of endophytic isolates from health leaves of *Miconia albicans*

Samples	Culturemedia	Colony characteristics	Physiological pattern	Biochemical profile
9	ISP2A	6-8 cm/diameters, white front, orange-brown reverse, cottony appearance, good agar adhesion	Geosmin smell, catalase +, clear orange pigment, starch degradation	Gram +, alcohol-acid resistance - , spores +
2	YE	4-5 cm/diameters, white front, brown reverse, cottony appearance, strong agar adhesion	Strong geosmin smell, catalase +, no pigments,no starch degradation	Gram +, alcohol-acid resistance -, spores +
1	YE	2-5 cm/diameters, white-grayish front, powder appearance, yellow reverse and strong agar adhesion	Strong geosminsmell, catalase +, no pigments,no starch degradation	Gram +, alcohol-acid resistance - and presence of spores
7	YE	1-2 cm/diameters, white front, small and elevated appearance. Orange reverse, strong agar adhesion	Smell of geosmin, catalase +, without production of pigments	Gram +,alcohol-acid resistance -, spores +
4 *	YE	2-3 cm/diameter cotton like front grayish-white appearance, dark green back, strong agar adhesion	Strong geosmin smell, catalase +, no pigments,no starch degradation	Gram +, alcohol-acid resistance -, spores +
12 **	ISP2A	2 to 3 mm diameter, concave, wrinkled, uneven edges, white front and cotton like back, yellow/orange, poor agar adhesion	Geosmin smell, catalase +, dark orange pigments +, starch degradation +	Gram +, alcohol-acid resistance -, spores +

4 Nocardiopsis dassonvillei, 12**Amycolatopsis orientalis*

Table 2. Antibiosis by endophytic microorganisms isolated from *Miconia albicans* against microorganisms and indicators measured by inhibition zones (cm)

Samples	*E. faecalis*	*S. aureus*	*S. marcensis*	*C. albicans*	*E. coli*	*S. sonnei*
9	1.80	1.50	_	_	_	_
2	_	_	3.60	_	_	_
1	1.30	_	_	_	_	_
7	3.15	3.50	_	3.35	_	3.20
4*	1.90	_	_	_	_	_
12**	2.60	1.50	_	2.40	2.10	2.50

*4 * Nocardiopsis dassonville, 12 **Amycolatopsis orientalis*

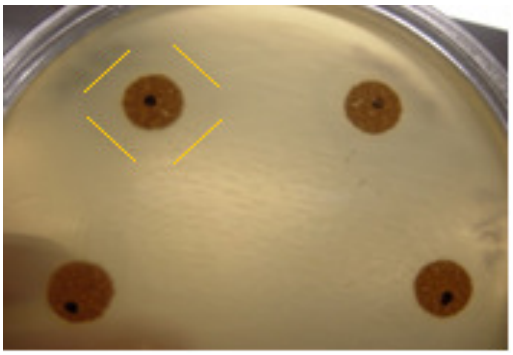

Fig. 1. Inhibition potential of *E. faecalis* by endophytic isolate of *M. albicans* (sample 9). The halos are represented by a yellow line

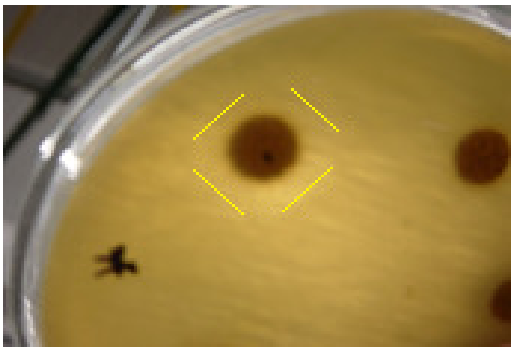

Fig. 2. Antagonistic activity of endophytic isolate of *M. albicans* (sample 9) against *S. aureus*. The halos are represented by a yellow line

On Fig. 3, the sample 2 showed inhibition zones of 3.60 cm against *S. marcensis*. This result is highlighted and shows the confluence of halos. This characteristic pattern may suggest that the substance produced by the endophytic actinomycetes, had strong potential to inhibit the indicator organism tested. However, this sample was not active against the other microorganisms tested.

Similarly to the work presented, Ibrahim et al. [27], also isolated endophytic microorganisms from leaves with different ages of *Swietenia macrophylla*. A total of 461 filamentous fungi were isolated and 68, 3% of them showed antimicrobial properties in a first screen as our work demonstrated with bacteria. Accordingly, as the leaf age increased, the percentage of antimicrobial activity also increased.

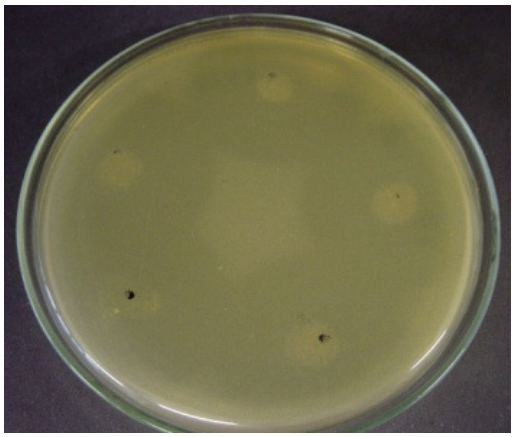

Fig. 3. Strong inhibition halos of endophytic isolate from *M. albicans* (sample 2) against S. *marcensis*

Fig. 4 showed inhibition halos (1.30 cm) corresponding to sample 1 against *E. faecalis*. In this same sample, there was no inhibitory activity against *S. aureus*, *S. marcensis*, *C. albicans*, *E. coli* and *S. sonnei*.

The endophytic isolate on sample 7 showed antimicrobial activity against 4 of 6 indicator microorganisms: *S. aureus*, *E. faecalis*, *C. albicans* and *Shigella* with 3.50 cm; 3.15 cm; 3.35 cm and 3.20 cm, respectively (Figs. 5 and 6). However, it wasn'tdetected bioactivity against *S. marcensis* and *E. coli.*

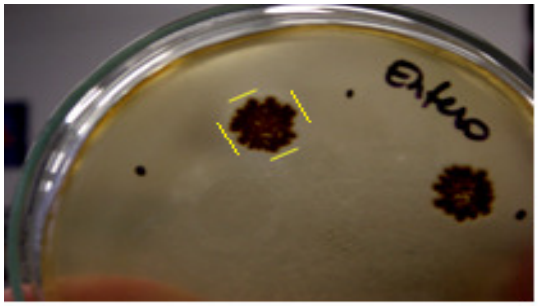

Fig. 4. Bioactivity measured by inhibition zone of endophytic isolate from *M. albicans* (sample 1) against *E. faecalis.* The halos are represented by a yellow line

Weber et al. [28] isolated phomol, an anti-inflammatory metabolite produced by endophytic fungus from the medicinal plant *Erythrinacrista-galli* L. This metabolite was also bioactive against various bacteria, yeast and fungi, including *Escherichia coli*. The presentworkshowed that only *A. orientalis* had the potential to inhibit *E. coli*.

Some authors [29] in China, isolated fungi of orchid species with antimicrobial action against *E. coli, B. subtilis, S. aureus, C. albicans, C. neoformans* and *A. fumigates,* showing some similarities with the present work such as the inhibition of *E. coli, S. aureus* and *C. albicans.*

As in some of our isolates (Fig. 6 A), Harzallah et al. [30], demonstrated antimicrobial activity against *C. albicans* and *E. coli* produced by an endophytic fungus isolated from wheat. Moreover, its extract showed antioxidant activity and growth promotion in wheat seeds.

In sample 4 is evidenced the activity against *E. faecalis*with 1.90 cm inhibitory halo (Fig. 7). The bacteria background in Fig. 7 is not as clear as in the other samples. The reason for this could be explained by the absence of this bacteria effect on the others tested microorganisms and/or this could indicate a minor potential of this sample. Castillo et al. [31], working with endophytic *Streptomyces* isolate from *Grevillea pteridifolia*, described and partially characterized some bioactive substances detected. Authors observed that kakadumycin A showed a broad spectrum of antimicrobial activity, especially against Gram-positive bacteria.

Of all the samples tested, 12 showed the highest activity against a number of of all the samples tested, *A. orientalis* showed the highest activity against the majority of themicroorganisms tested. From six of the indicator microorganisms tested, the bioactive substances were effective against five of them. Its inhibitory activity was through inhibition zones of 2.60 cm diameter against *E. faecalis*; 1.50 cm in diameter against *S. aureus*; 2.40 cm diameter against *C. albicans*; 2.10 cm in diameter against *E. coli*, and finally 2.50 cm in diameter against *S. sonnei*. There was no inhibitory halos against *S. marcensis* (Figs. 8 (A and B) and 9 (A and B)).

Recently, some authors [25] isolated fungi *Myrcia guianensis* (Myrtaceae) from the Brazilian Amazon through morphological analysis. The fermentation broth of these fungi have been studied and carried out tests in which bioactivity was demonstrated against *Staphylococcus aureus*, *Enterococcus faecalis*, *Candida albicans* and *Penicillium avellaneum*. In our studies, the bioactivity has also been presented, however, with actinomycetes. The data presented here with the bioactivity of endophytes bacteria isolated from medicinal plants can be interpreted as the pioneering spirit of our work.

Sardi et al. [32] observed antimicrobial activity of endophytic *Streptomyces* isolates from 28 plant species of northwestern Italy against *Escherichia coli* (ATCC 25922), *Micrococcus luteus* (ATCC 9341) and *Fusarium oxysporum*.

Castillo et al. [33] found that the compounds isolated from endophytic Streptomyces native plant *Kennedia nigriscans* showed broad spectrum of activity against important human pathogens.

Bomfim [34] obtained results against *Candida* spp. bioactive metabolites from *Streptomyces* spp. endophytic, similar to the data presented in this study.

Guimarães et al. [35] isolated from*Tithoniaarenaria* and *Viguieradiversifolia*, trees of Sao Paulo/Brazil, 39 endophytic fungi. Extracts of studied fungi were active against *S. aureus, E. coli* and *C. albicans*. Sample 12 showed similarity with the authors mentioned.

3.1 Genotypic Identification

Two of the isolated endophytic microorganisms were genotypically identified as *Nocardiopsis dassonvillei* (sample 4) and *Amycolatopsis orientalis* (sample 12), both belonging to the group of actinomycetes and *Streptomyces* genus, according to the characteristics previously studied in this work.

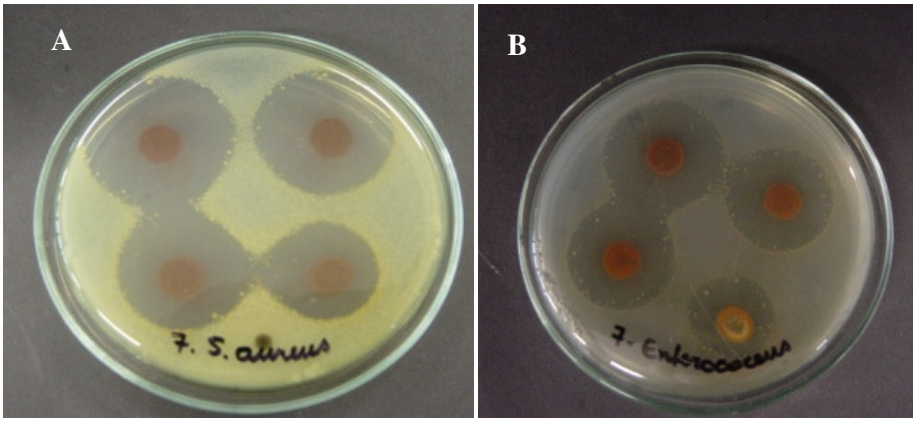

Fig. 5. Production of the inhibition zones of endophytic isolate from *M. albicans* (sample 7) against *S. aureus* (A) and *E. faecalis* (B)

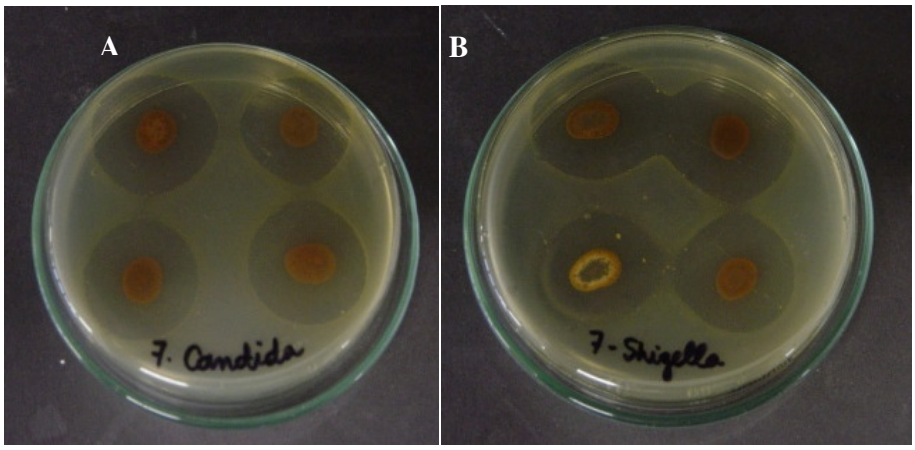

Fig. 6. Inhibition zones of endophyticisolate from *M. albicans* (sample 7) against *C. albicans* (A) and *S. sonnei* (B)

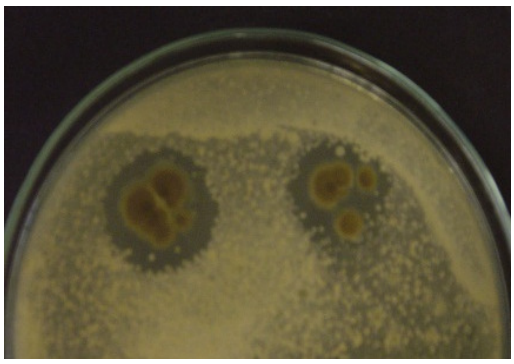

Fig. 7. Antagonistic activity from microorganism isolated against the indicator *E. faecalis* (sample 4)

*Nocardiopsis dassonvillei*is described in the literature as a microorganism isolated from soil. In this study, Aghamirian and Ghiasian [36] pursued the identification, prevalence and geographical distribution of aerobic actinomycetes in the soil of Iran. Selvin et al. [37] isolated from one marine sponge *Nocardiopsis dassonvillei*, testing its bioactivity against a number of pathogens. Tillnow, however, it was not found description with this respect to its endophytic characteristic.

Amycolatopsis orientalis in turn, was described as producing vancomycin [38] and have sought to optimize the production of this substance. Banskota et al. [39] found at least 10 secondary metabolites produced by *Amycolatopsis orientalis* in addition to vancomycin. The data obtained in this study suggests that these endophytic actinomycetes isolated can also be producers of antibiotics of interest to industry.

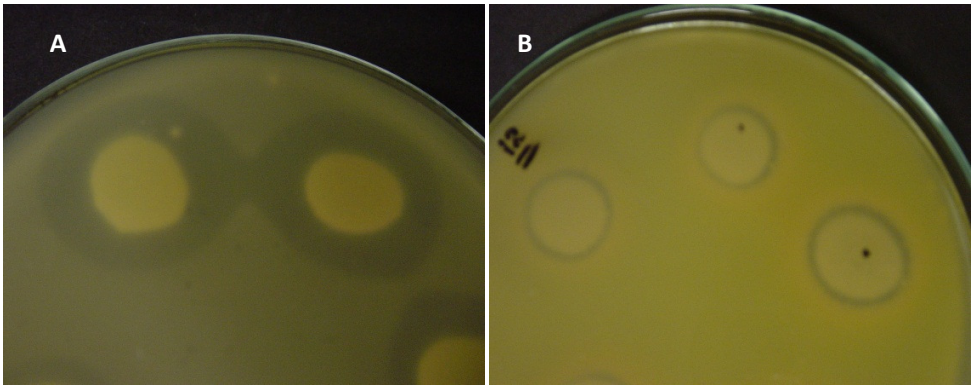

Fig. 8. Antagonistic activity from the endophytic isolated from *M. albicans* (sample 12) against *E. faecalis* (A) and *S. aureus* (B)

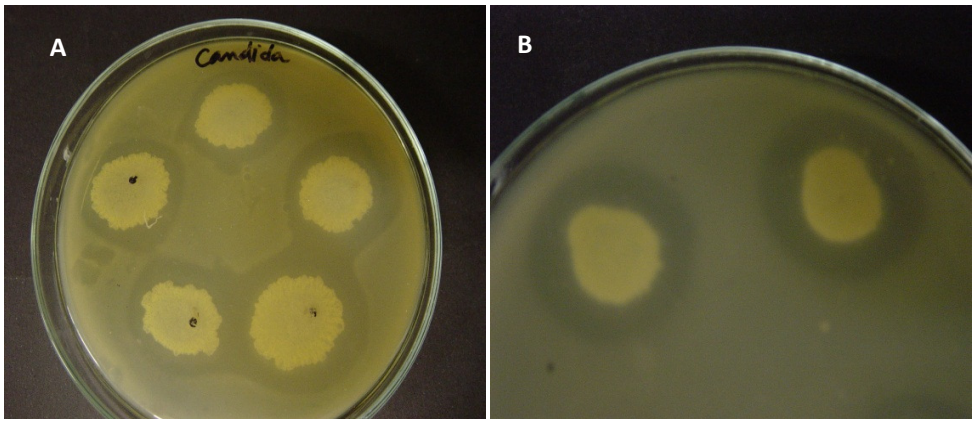

Fig. 9. Inhibition halos from sample 12 against *Candida albicans* (A) and *Shigella sonnei* (B)

The data presented in our work, in general, can lead us to consider the importance of this study involving the isolation of endophytic microorganisms harbored by plants from the Brazilian Tropical Savannah of Sao Carlos - SP. Thisecologicalassociation, as was demonstrated, induces the production of specific chemicals, bioactive secondary metabolites that can be applied to different uses. The phenotypic behavior seen in different isolates demonstrates the plethora of genetic information part in the make-up of actinomycetes and particularly in *Streptomyces*. The continuation of this work will be with the genotypic characterization of another isolates and cultivation in different conditions to verify the microbial ability to maintain and/or enhance the production of bioactive substances.

4. CONCLUSION

All samples were phenotypically identified by their characteristic growth of Actinomycetes. Two of the isolates were genotypically identified as *Nocardiopsis dassonvillei* (sample 4) and *Amycolatopsis orientalis* (sample 12), reporting for the first time, the association of these endophytic actinomycetes with aerial parts of *Miconia albicans*. All samples demonstrated some type of bioactivity against important clinical pathogens, and *Amycolatopsis orientalis* showed the best results, with inhibition zones of 1.50; 2.60; 2.40; 2.10 and 2.50 cm against *S. aureus*, *E. faecalis*, *C. albicans*, *E. coli* and *S. sonnei*, respectively. *A. orientalis* was not bioactive only against *S. marcensis*. This work shows the importance of the discovery of metabolites from the crude extracts against pathogens as well as its potential use in the pharmaceutical/medical industry.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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