INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

*Research Article*

# ANTITUMOR, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF SECONDARY METABOLITES EXTRACTED BY ENDOPHYTIC ACTINOMYCETES ISOLATED FROM *VOCHYSIA DIVERGENS*

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**ABSTRACT**

Endophytic actinomycetes encompass bacterial groups that are well known for the production of a diverse range of secondary metabolites, including various antibiotics, antitumor, immunosuppressive agents, plant growth hormones, and have capacity of survive inside of plants tissues. *Vochysia divergens* is a Brazilian medicinal plant common isolated in the Pantanal region, and was focus of many researches, but the community endophytic remains unknown. Therefore, the goals of the present work were to carry out an initial assessment of antimicrobial, antitumor and antioxidant activities of crude extract produced by endophytic actinomycetes isolated from *Vochysia divergens*. Using 16S sequences, 10 isolates were classified as *Microbispora* sp. and two isolates were classified as *Streptomyces sampsonii*. The other two isolates were identified as *Micromonospora* sp. and are apparently undescribed species. The isolates were able to produce secondary metabolites with antioxidant activity, antitumor activity against of Glioblastoma cell and antimicrobial activity against bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus aureus* and the yeast *Candida albicans*. Taking into consideration the lack of effective medicaments for the treatment of Glioblastoma multiforme, and the increasing number of bacterial strains expressing resistance, the basic research using microorganisms from unexplored environmental showed can be an alternative to discover new secondary metabolites to treat these diseases.

**Keywords**: Endophytic actinomycetes, Pantanal, Biological activity.

## INTRODUCTION

Endophytic actinomycetes are bacteria that reside in the internal tissue of plants via symbiotic, parasitic, or mutualistic without causing immediately negative effects[1]. Actinomycetes are well known for the decomposition of organic matter and for produce a diverse range of secondary metabolites, including antibiotics, antitumor, immunosuppressive agents and plant growth hormones[2,3]. The search for new natural products has been conducted extensively using soil actinomycetes, and this might have reduced the chance of finding new biologically active molecules from them. Thus, new microbial habitats need to be examined, in the search for new bioactive compounds[4].

We are particular interesting in microorganisms isolated from medicinal plants located in the Pantanal region (Brazil). The Pantanal is a periodic floodplain with area of approximately 138,183 km$^2$, belonging to the Paraguay River Basin [5]. Due to the dynamic character, few plants are able to tolerate long periods of flooding, that begins in November and in adjacent areas can last until mid-June. Among the plant species that have tolerance to high levels of flooding is Cambará - *Vochysia divergens*[6]. *V. divergens* is a medicinal plant, using to treat infection caused by *Staphylococcus aureus*, and respiratory problems[7].

Considering the associated limitations with the productivity and vulnerability of plant species as new metabolites sources, microorganisms serve as the ultimate, readily renewable, reproducible, and inexhaustible source of new structures bearing pharmaceutical potential[8,9]. Therefore, the goal of the present work was to carry out an initial assessment of antimicrobial, antitumor and antioxidant activities of endophytic actinomycetes obtained from *Vochysia divergens*.

## MATERIALS AND METHODS

### Isolation of endophytic actinomycetes

The *V. divergens* leaves were collected from 10 specimens located in two Pantanal regions, Nhecolândia (S18º10.07', W57º23.03') and Amolar (S20º10.10', W53º23.05') in Brazil. To the endophytic isolation, the preference was given to leaves with no marks, scratches or wounds. To eliminate epiphytic microorganisms, a purification protocol of six steps was used[10]. The leaves were fragmented and inoculated in Petri dishes with medium PDA (Potato Dextrose Agar). The plates were incubated at 28°C for 30 days, and the growth was daily verified. The living cultures were deposited in the LabGeM collection, Federal University of Paraná, Curitiba, Paraná, Brazil (http://www.labgem.ufpr.br/).

### Actinomycetes identification

Genomic DNA extraction was carried out using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA, USA) according to manufacturer's protocol. Amplification conditions followed Lee et al.[11] using the primers 9f (5' – GAGTTTGATCCTGGCTCAG) and 1541r (5'-AAGGAGGTGATCCAGCC) were used to amplify the 16S rDNA gene. Amplicons were sequenced using both PCR primers with BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, and sequences were analyzed on an ABI3100 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were compared with available sequences in the Genbank database of NCBI (http://www.ncbi.nlm.nih.gov/).

The Bayesian inference of phylogeny was made using MrBayes v3.1.2[12,13]. The putative stationary phase and burn-in were determined after multiple runs and post data analysis in Tracer v1.5[14] and AWTY[15]. The final trees were assembled in Sumtrees, from DendroPy v3.9.0 package[16]. Also, Maximum Likelihood analysis was performed, as implemented in GARLI version 2.0[17], using default parameters and 1000 bootstraps pseudoreplicates.

### Biological Activity

### Production of extracts

The isolates that already had biological activity in other studies[9] were selected here for complementary analysis. Crude extracts were obtained through fermentation process, in PD (Potato Dextrose) medium under agitation for 14 days (110rpm, 36°C and 12 h). After fermentation, the mycelium was separated of fermented liquid by Whatman® qualitative filter paper, Grade 4. The fermented liquid was lyophilized, weighed, and diluted in ultrapure sterilized water (10 mg/mL).

### Total phenolic compounds and antioxidant activity

### Phenolic compound quantification

The phenolic compound content in the extracts was estimated by a colorimetric assay according to Singleton and Rossi[18]. The Folin–Ciocalteau method was used with Gallic acid as a standard. The absorbance was then measured at 765 nm using an UV/Vis double beam spectrophotometer T-80 (PG Instruments Limited, Beijing, China). The results were expressed as Gallic acid equivalents (GAE) using a calibration curve over the range of 5–250 ppm.

**1,1-Diphenyl-2-picrylhydrazyl radical (DPPH•) assay**

The free radical scavenging activity was assessed with the DPPH• method as previously described by Mensor et al.[19]. Based on the total phenolic compound values, six deferent concentrations (50, 75, 125, 250 and 500 µg/mL in water) of the extract were used to perform the DPPH• assay.

**Coupled oxidation of *β*-carotene and linoleic acid**

The antioxidant activity was performed according to *β*-carotene-linoleic acid coupled oxidation assay was measured using the methodology of Emmons et al.[20] with modifications proposed by Prado[21]. Oxidation of the emulsion was monitored spectrophotometrically using an UV/Vis double beam spectrophotometer T-80 (PG Instruments Limited) by measuring absorbance at 470 nm over a period of 120 min. The degradation over time was nonlinear. Therefore, the antioxidant activity was expressed as percent inhibition relative to the control after incubation for 120 min using the following equation:

$$AOA = 100\ X\ \left(\frac{DR_c - DR_s}{DR_C}\right)$$

AOA stands for the antioxidant activity, $DR_C$ is the degradation rate of the control ($\ln(a/b)/120$), $DR_S$ is the degradation rate of the sample ($\ln(a/b)/120$), $a$ is the initial absorbance at time zero, and $b$ is the absorbance at 120 min.

**Antitumor Activity**

The U87MG human glioblastoma cell was seeded at a density of $1 \times 10^4$ cells into 96- well plates in 200 µL of DMEM high-glucose medium supplemented with 10% of fetal bovine serum (FBS), both obtained from Cultilab (Campinas, Brazil) and 50 µg/mL gentamycin. After 24 hours of incubation at 37°C and 5% $CO^2$ extracts was added to each well at the 50 µg/mL concentrations. After 24 h or 48 h of incubation 200µL of MTT in HBSS (final concentration of 0,5mg/mL) were added to each well and incubated at 37°C for a further 3 h. After that, the formazam crystals formed were dissolved in DMSO and the absorbance of the dissolved precipitate was measured using a Tecan-Infinite M200 microplate reader, in 550nm. The cell proliferation index was calculated as the ratio of the absorbance of extracts-treated cells to that of control cells. The assay was conducted five times for each cell line[22].

**Antibacterial Activity**

**Bioautographic TLC agar-overlay assay**

The antibacterial potential of the methanolic extracts of Actinomycetes endophytic was assessed against the following test organisms: *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), and methicillin-resistant *Staphylococcus aureus* (MRSA). The test organisms were grown overnight in a Mueller–Hilton broth (Merck) at 37°C and were diluted until reaching the concentration of 106 cells/mL. The bioautography followed the protocol described by Rahalison et al.[23].

**Statistical analysis**

To perform the statistical analyzes, we used the software R 3.0.0. The normality tests followed the methodology of Shapiro-Wilk test. Once the data was classified as normal was applied to parametric analysis of variance of a factor with post-hoc Tukey's HSD.

**RESULTS AND DISCUSSION**

**Isolation and molecular characterization**

Eighteen actinomycetes isolates were isolated from 4.000 analyzed leaves fragments. 55,5 % of isolates were collected in Nhecolândia and 45,5% in Amolar. 61.1% of them were obtained from stems and 38.9% from leaf tissues. The frequency of isolation was 0.47% using leaves and stem fragmentation. Actinomycetes are the largest and most dominant, comprising nearly 50% of the total population, of soil and root inhabiting, as probably saprophytes[24]. Moreover, Actinomycetes colonization in leaves and stems is less frequent. In the present study, the genera *Streptomyces* (two isolates) and *Micromonospora* (two isolates) were isolated from different regions in Pantanal: *Streptomyces* from Nhecolândia, and *Micromonospora* from Amolar. *Microbispora* isolates (14 isolates) were isolated of both regions, Nhecolândia and Amolar, probably due to their higher frequency of isolation in the leaves and stem tissues of *V. divergens* (77.78%).

The partial sequence of 16S of the rDNA gene revealed that the isolates Clade 2 belong to the genus *Microbispora* (Figure 1). However, the isolates formed a clade with two type strains, *Microbispora rosea* (D86936) and *Microbispora mesophila* (AF002266). The phylogenic analysis also shows that using only the 16S of rDNA gene, it was not possible to assign a single species to these isolates, due of conflicting topologies and no support to classification in species level. So we assumed that the 16S rRNA analysis is more appropriate for discrimination on the genus

level. The isolates LGMB260 and LGMB261 were identified as *Micromonospora* sp. however, the obtained sequences were not similar to any of the available sequences from the type species of the GenBank data base (Figure 2), and probably is new specie. The isolates LGMB262 and LGMB263 were identified as *Streptomyces sampsonii* with a high degree of similarity (Figure 3). This specie has been described producing an antibiotic of the polyenes group in the methanolic extract, with antifungal activity[25].

**Antioxidant activity**

In this study, the concentration of total phenolic ranged from 0.000337 mg/g (gallic acid equivalent) of extract of the endophytic LGMB262 (*Streptomyces sampsonii*), by 228.6364 mg/g of the extract from isolated LGMB259 (*Microbispora* sp.) (data not shown).

*In vitro* antioxidant activity of the isolates was determined by DPPH free radical scavenging ability. This technique had already been proven as a key method for detection and evaluation of antioxidant property of any molecules. The crude extracts from *Microbispora* sp. LGMB255, LGMB258 and LGMB259 had a noticeable DPPH free radical activity with EC50 of 163,90 µg/mL, 179,04 µg/mL and 153,24 µg/mL respectively (Figure 4). It was also observed that the DPPH scavenging activity was increased in a dose-dependent manner. For comparison, in a study performed by Mahapatra and Banerjee[26], of the crude extract from *Fusarium solani* showed a protective activity of 50% (EC50) in concentration of 578.541 µg/mL and which is comparable with standard antioxidant Vit-C 433.099 µg/mL. The extracts evaluated in our present study had better protective action even in lower concentration EC50 of 153.24 µg/mL (Figure 4).

The isolate LGMB255 (*Microbispora* sp.) showed strong inhibition of lipid peroxidation with EC50 of 181.68 µg/mL. The inhibition of β-carotene bleaching by the isolate LGMB255 (*Microbispora* sp.) was higher than produced by fungi and bacteria[27], and equivalent to the results reported by Chen et al.[28], who studied the antioxidant activity of secondary metabolites from a strain of endophytic *Aspergillus* sp. Therefore, the extracts of endophytic actinomycetes could become an alternative over synthetic antioxidants, as butylated hydroxytoluene (BHT) and butylated hydroxanisole (BHA). Compounds reported as carcinogenic and hepatotoxic[26].

**Antitumor activity**

The biological effects of the metabolites was also tested against tumor cell lines, to check if beyond a protective action by production of antioxidants compounds the extracts also have activity against tumors cells. Crude extracts from *Microbispora* sp. LGMB259, LGMB250, LGMB255 and LGMB256 showed antitumor activity against Glioblastoma multiforme cell higher than 98%. The extracts of the strains LGMB258 (*Microbispora* sp.) and LGMB262 (*Streptomyces sampsonii*) showed activity of 59% and 70% percent respectively (Figure 5). Glioblastoma multiforme (GBM) represents the most aggressive tumor among high grade gliomas (HGG), with a poor prognosis of about 14-15 months[29], and has been shown to be resistant to standard therapy, either because of distinct biophysical and genetic properties, or possibly due to migration outside of the treatment field[30]. Seznec et al.[31] in study with mithramycin compound isolated of Streptomyces strain, showed the activity in less concentration from glioblastoma cell line, by inactivation of enzyme Sp1. Thus, it is necessary to consider multimodal strategies that maximize the potency of available treatments through complementary and synergistic effects.

**Antibacterial activity**

Among the diseases that cause highs costs in public health, the bacterial infections have a great relevance, and this problem is increased by the developing of resistance[32]. For example, studies reporting that MRSA caused 250,000-300,000 hospital acquired infections[33]. The genus *Streptomyces* was showed many compounds for pharmaceutics industry, for example Vancomycin that was antibiotic chose for infections with bacteria multiresistance drugs[34]. Therefore, the search for new compounds can focus on the isolation of rare actinomycetes, among which, the genus *Microbiospora*[35]. Strains *Microbispora* sp. (LGMB250 and LGMB259) and *Streptomyces sampsonii* (LGMB262) showed activity against *Candida albicans* (Figure 6). Crude extract from LGMB255 (*Microbispora* sp.) also showed activity against to *S. aureus* and *E. coli* and metabolites from LGMB259 (*Microbispora* sp.) had antimicrobial activity against all tested microorganisms, including the bacteria Methicillin-resistant *S. aureus* (Table 1).

Our results show that *Microbispora* was the predominant genus of endophytic actinomycetes isolated from *V. divergens*. *Micromonospora* isolates probably belong to a new species, and a multigene analyzes is necessary to their identification. The

actinomycetes isolated in this study showed a promising biological activity, a notable antioxidant and antitumor activities, and the extract from *Microbispora* sp. LGMB259 had activity against Methicillin resistant *S. aureus* which is leading cause of nosocomial infections worldwide. These results evidence that isolation of microorganisms from unexplored environments can be one alternative to isolation of metabolites with wide biological activity.

**ACKNOWLEDGEMENTS**

We thank Hospital das Clínicas/ UFPR (Curitiba/Brazil) for providing some of the biological strains. The Brazilian agencies CAPES, CNPq and Fundação Araucária provided financial support for this study.

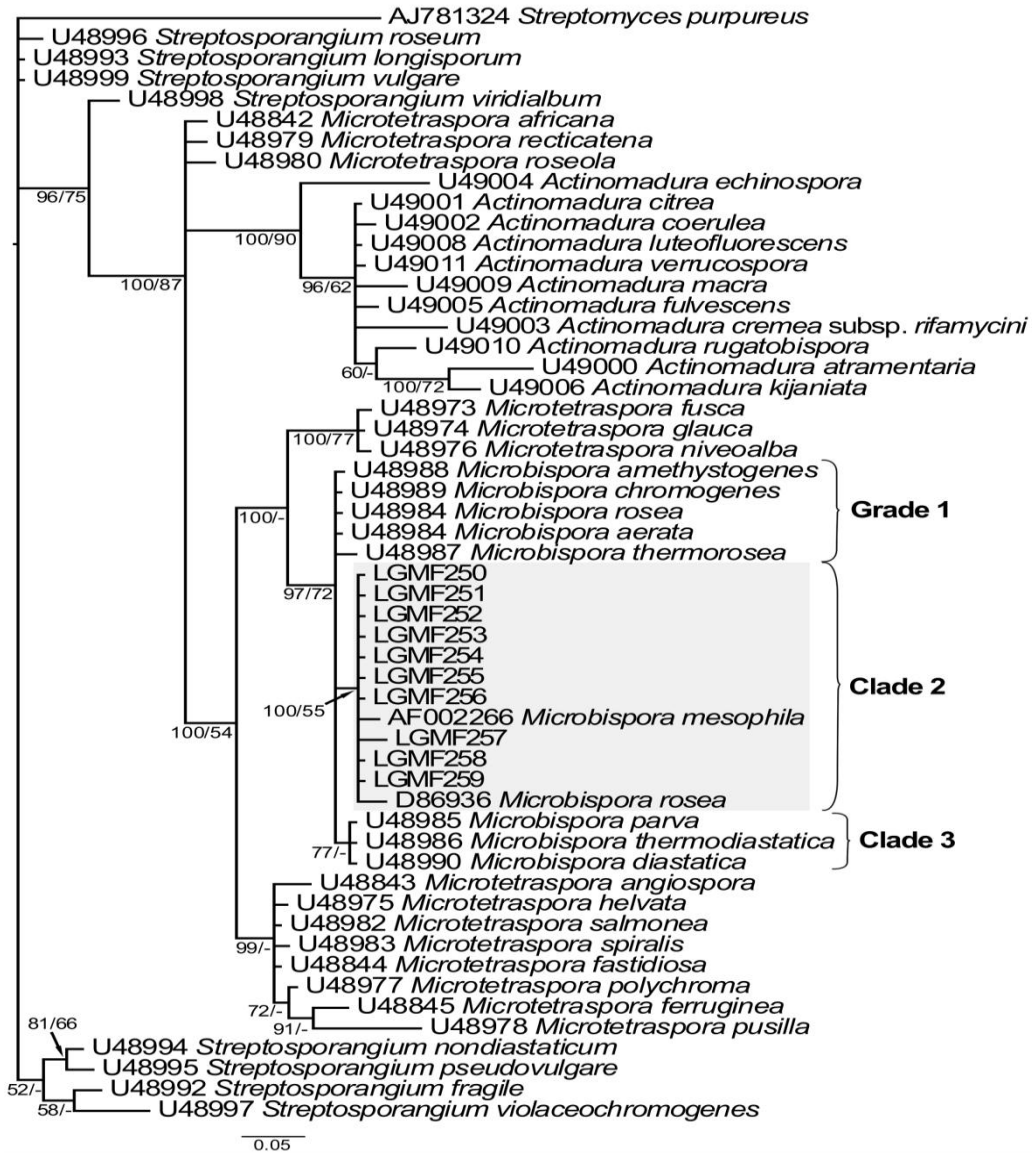


**Fig. 1: Bayesian Phylogenetic tree for *Microbispora* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Streptomyces purpureus* (AJ781324). The gray shade indicates the endophytic isolates analyzed in this study.**

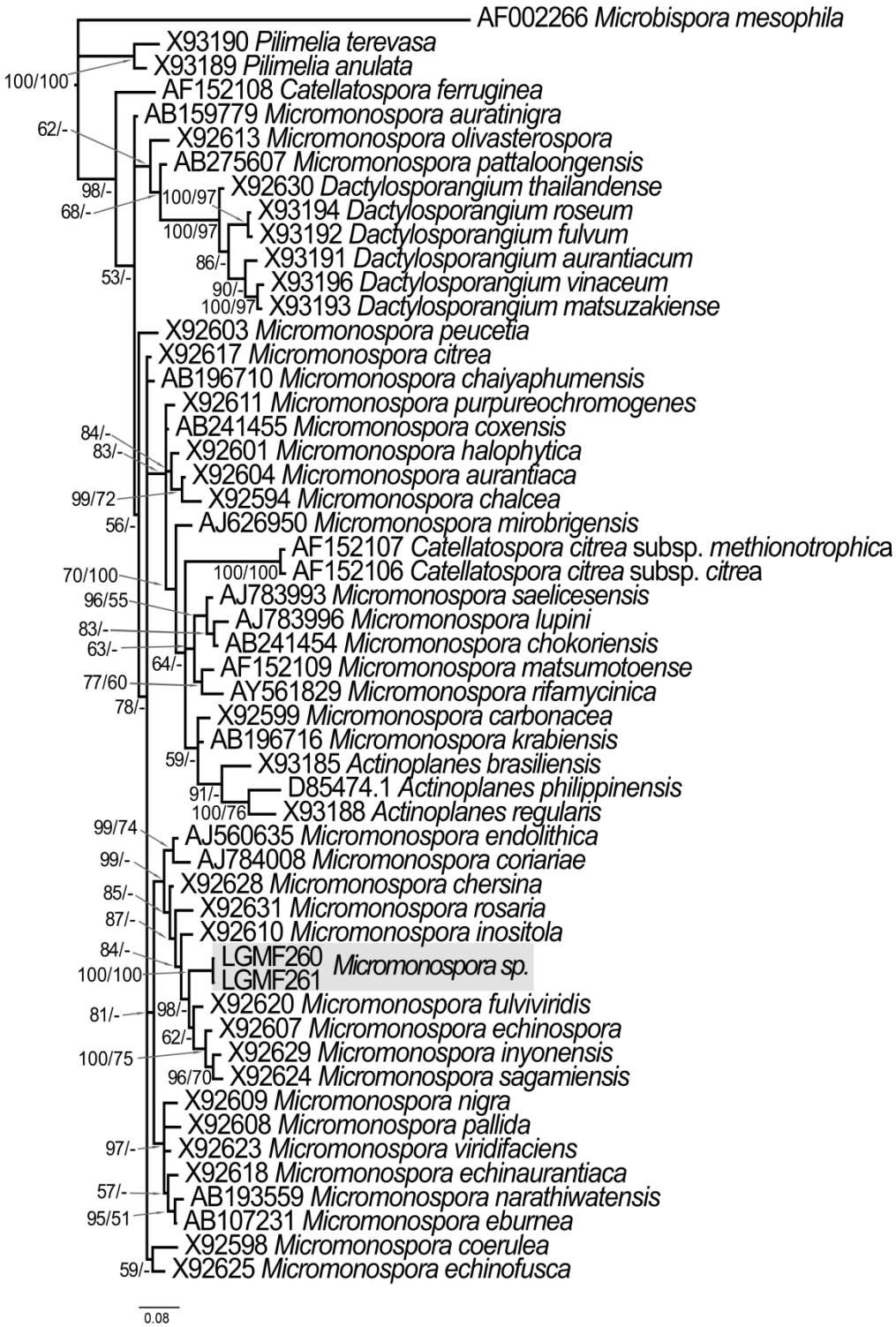


**Fig. 2: Bayesian Phylogenetic tree for *Micromonospora* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Microbispora mesophila* (AF002266). The gray shade indicates the endophytic isolates analyzed in this study.**

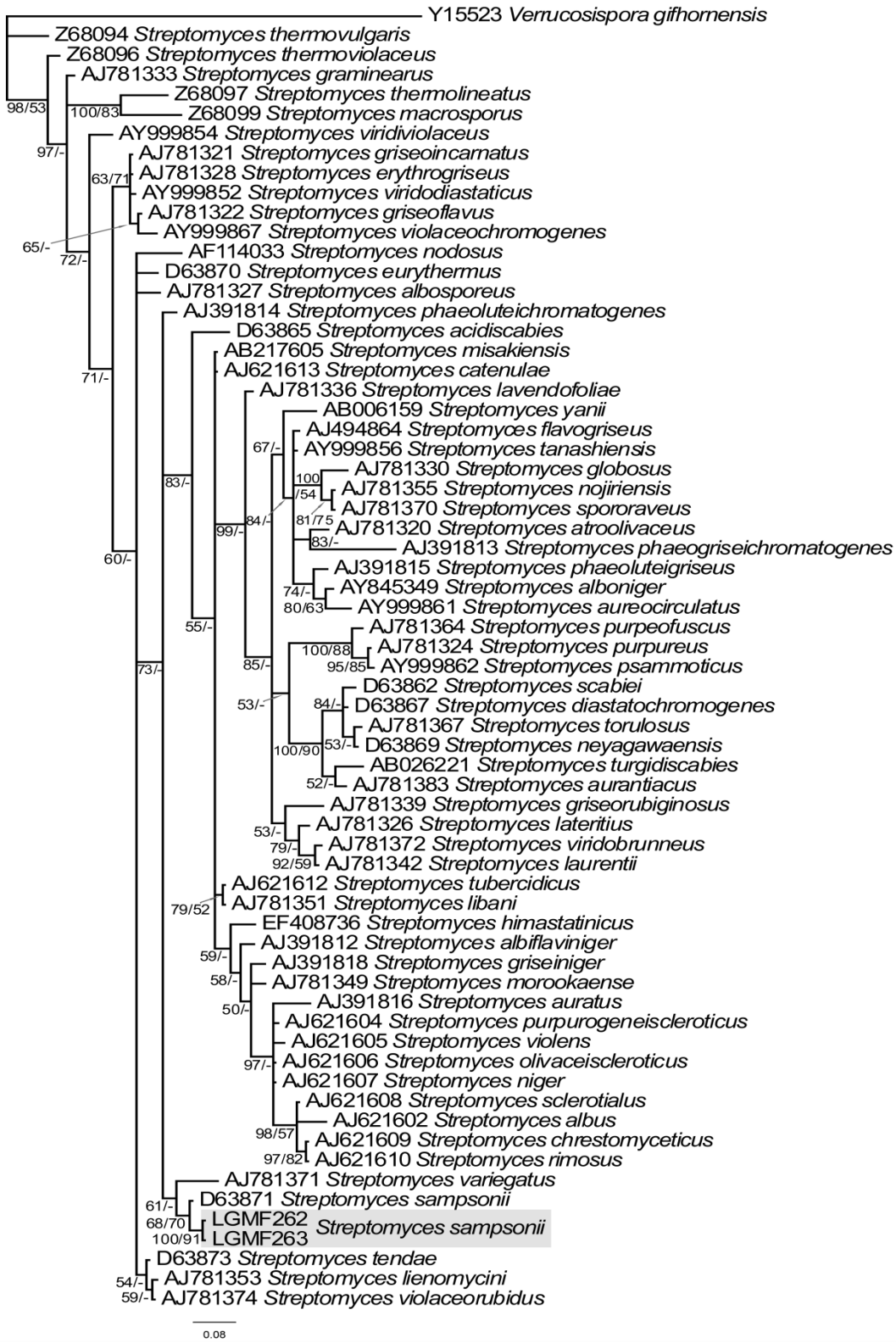


**Fig. 3: Bayesian Phylogenetic tree for *Streptomyces* genus using 16S rDNA gene. Posterior probabilities are shown on nodes, together with maximum likelihood bootstrap support, if it exists. Only clades with more than 50% of posterior probabilities are shown. The tree was rooted with *Verrucosspora gifhomesis* (Y1552302266). The gray shade indicates the endophytic isolates analyzed in this study.**

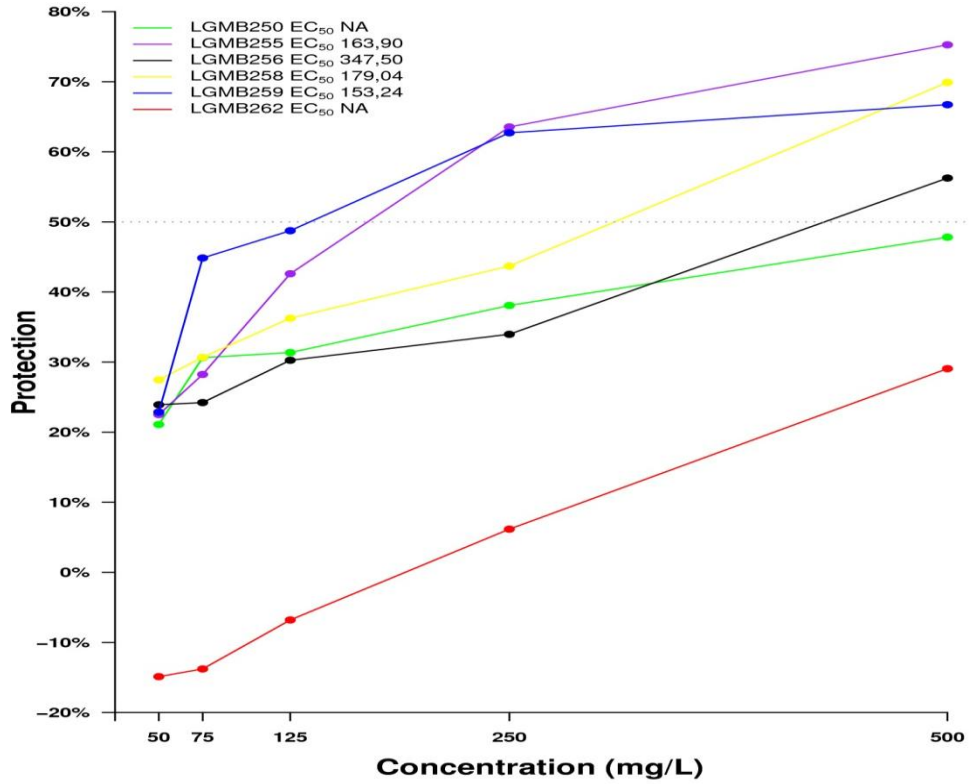


**Fig. 4: Antioxidant activity evaluation of crude extracts of the endophytic actinomycetes in different concentration (Y axis represents the percentage of protection, X axis represents the extract concentrations); NA: not achieved 50%.**

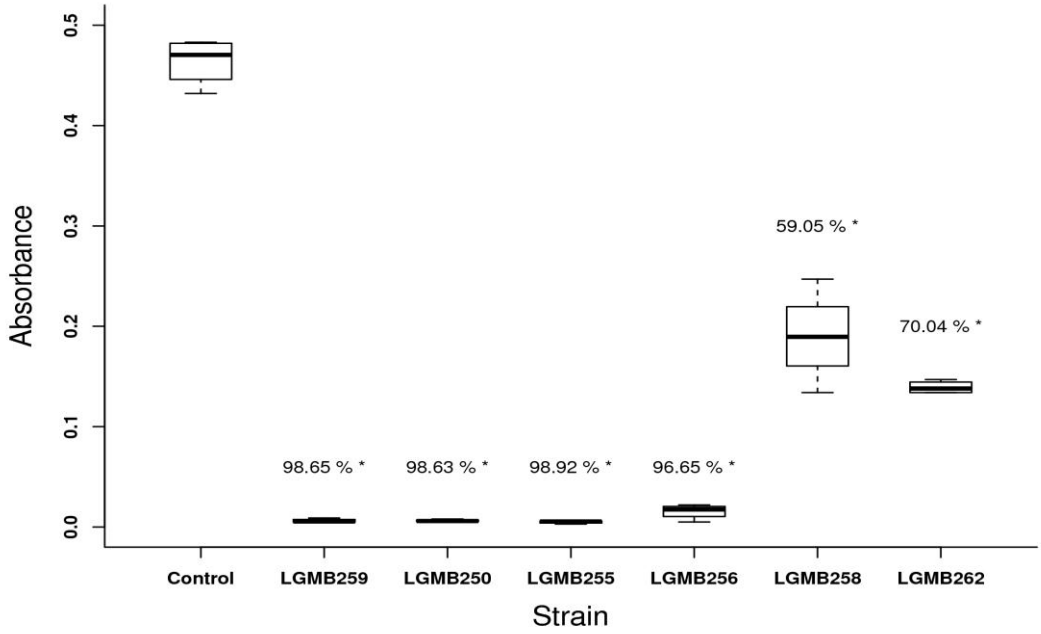


**Fig. 5: Evaluation of the antitumor activity of crude extracts of actinomycetes in the concentration of 50 µg/mL (Y axis represents the absorbance of the growth of tumor cells, de X axis represents the strain codes, * represents inhibition of growth).**

**Table 1: Evaluation of antibacterial activity of crude the extracts of endophytic actinomycetes by the Bioautographic TLC agar-overlay assay**

| | Antibacterial activity | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Staphylococcus aureus* | | *Escherichia coli* | | *Pseudomonas aeruginosa* | | Methicillin resistant *Staphylococcus aureus* | | *Candida albicans* | |
| Exctract | 5 ul | 10ul | 5 ul | 10ul | 5 ul | 10ul | 5 ul | 10ul | 5 ul | 10ul |
| LGMB258 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LGMB256 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | + |
| LGMB250 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LGMB262 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + | + |
| LGMB255 | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| LGMB259 | + | + | + | + | + | + | + | + | + | + |

Note: 0, No inhibition; +, inhibition zone between 4 and 5 mm in diameter

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