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Isolation and Biocidal Activity of Endophytic Fungi de *Acrocomia aculeata* (Jacq.) Lodd. ex. Mart.

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Abstract

In order to evaluate potential biocidal activities, endophytic fungi present in the leaves of *Acrocomia aculeata* were isolated and identified in the expectation of finding new strains and new fungal products with pharmacological potential. Fifty fungi were isolated, 25 of which were identified in the genus *Fusarium*. Of these, eight strains with divergent morphological characteristics had their genre confirmed by sequencing the internal transcribed spacer region (ITS) of the fungal ribosomal DNA (rDNA). The nucleotide sequences obtained were compared with similar sequences on GenBank using the local BLAST NCBI, with a 100% similarity homology. The eight fungi were identified as *Fusarium sp.* Ethyl acetate extract from the fungi was subjected to biological tests demonstrating inhibition of bacterial growthand and an important anti-*Candida albicans* activity.

Keywords: *Antimicrobial agents, Endophytes fungi, Fusarium,*

1. Introduction

Acrocomia aculeata, a palm widely distributed in the Brazilian biome of the Cerrado (Ramos *et al.*, 2008), are widely appreciated and used in local cuisine in an artisanal manner due to their nutritional properties. However, research on endophytic fungi present in this plant remains scarce.

Endophytic fungi are responsible for the synthesis of several important compounds in the pharmaceutical and agricultural industry. Its biological active metabolites have potential for several applications, such as antibiotics, antimycotics, antitumor, antimicrobial and immunosuppressants (Kaul *et al.*, 2012).

The recent report by the Food and Drug Administration (FDA) shows that of the 38% of drugs discovered in natural products, microbes contributed to about 25% (Calixto, 2019). These discoveries really highlighted the critical role of microorganisms as a sustainable channel for the discovery of new drugs (Toghueo, 2020).

The aim of this study was to isolate and identify the endophytic fungi of *A. aculeata* leaves and to evaluate the potential antimicrobial activities of their secondary metabolites.

2. Materials and Methods

2.1 *Acromomia aculeata* leaves collection

Leaves were collected from eight individuals of the *A. aculeata* in the Cerrado, near Campo Grande,

Mato Grosso do Sul, Brazil (20°25'24"S, 54°40'07"W) in August 2015. The collected material was packed and sealed in sterile plastic bags to avoid environment contamination.

2.2 Isolation of endophytic fungi

In a sterile laminar flow chamber, *A. aculeata l*eaves was sanitized in commercial detergent 10% (15 minutes), hypochlorite solution 10% (15 minutes), sterile distilled water (briefly), alcohol 70% (10 minutes) and washed in distilled water five times. From the last water wash, aliquots (2 mL) were recovered and applied in PDA (potato dextrose agar) medium plates and treated with antibiotics as aseptic control. The dried leaves were cut aseptically, and fragments (1 cm) were distributed in PDA malt plates containing gentamicin (250 µg/mL) and chloramphenicol (100 µg/mL), and incubated at 35 °C, for 7 days. Then, the isolated fungi colonies were stored at 4 °C for further analysis (modified from Araújo *et al*., 2010).

2.3 Morphological identification

Morphological identification of fungi at genus level was performed by observing macroscopic aspects (color, appearance, consistency and edges of the colony) from central colony cultivation; and microscopic aspects (vegetative and reproductive structures) by slide culture (Dalmau, 1930; Riddell, 1950) and analysis under a microscope according to specialized literature (Leslie and Summerell, 2008).

2.4 Molecular identification

2.4.1 Genomic DNA extraction

Genomic DNA from fungal isolates grown on PDA at 25 °C, for 5 days, was extracted using the cetyltrimethylammonium bromide (CTAB) protocol (Warner, 1996). For this, a plug of the fungal mycelium was transferred to 1.5 mL tubes containing 500 µL of CTAB 2X extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCL pH 8.0 and EDTA 20) preheated at 60 °C. The tubes were homogenized and left in a water bath at 60 °C for 16 h. After this period, 500 µL of chloroform-isoamyl alcohol (24:1; v/v) was added followed by tube homogenization by inversion. The tubes were centrifuged at 9700 *g* for 30 min, and the upper aqueous phase was recovered and transferred to new tubes. To equivalent of 2/3 of the volume collected in the previous step was added 100% isopopanol and centrifuged at 9700 *g* for 10 min and the supernatant discarded. The precipitate was washed with 400 µL of ice cold 70% ethanol and centrifuged at 9700 *g* for 10 min, and the supernatant discarded. After the complete evaporation of ethanol, the obtained DNA was eluted in 50 µL Tris-HCl (10 mM, pH 8.0) with 20 µg/µL RNAse (Sigma-Aldrich, Saint-Louis, MI, USA). Concentrations and qualities of DNA extractions were evaluated by absorbance measurements on a Nanodrop® ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA), checked by 0.8% agarose gel electrophoresis and revealed with SYBR® Safe DNA (Invitrogen, USA).

2.4.2 Amplification and sequencing of ITS – rDNA regions

Molecular identification of the fungi was performed by sequencing of the internal transcribed spacer (ITS) regions 1 and 2, including the 5.8S using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990). The polymerase chain reaction (PCR) was performed in a final volume of 50 µL, containing 20 ng of genomic DNA, 20 mM Tris-HCl (pH 8.4); 3.0 mM $MgCl_2$; 0.2 mM of each dNTP; 0.5 µM of each primer; and 1.0 unit of Taq DNA polymerase (GoTaq, Promega, USA). PCRs were performed on the thermal cycler (Eppendorf Mastercycler® Hamburg, Germany) programmed for an initial denaturation step (3 min at 94 °C), followed by 35 cycles of 1 min at 94 °C, 1 min at 52.5 °C and 1.5 min at 72 °C. The last cycle was followed by a final extension of 10 min at 72 °C. The amplicons were analyzed by 1% (w/v) agarose gel electrophoresis revealed with SYBR® Safe DNA (Invitrogen, USA). Products were purified using the Wizard® SV Gel and PCR Cleaning System (Promega Corporation, Madison, WI, USA). The concentration of the purified products was checked by measuring the absorbance at 260 nm on the Nanodrop® ND-1000 spectrophotometer. DNA sequencing was performed by Macrogen Inc., Seoul, Korea using ITS1 and ITS4 starters and the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA). The partial sequences provided by Macrogen Inc. were high quality (Phred> 20) and used to generate consensus sequences using the Codon Code Aligner version 6.0.2 program (Codon Code Corp, USA). The nucleotide sequences were deposited at NCBI-GenBank.

2.4.3 Sequence analysis

The nucleotide sequences were compared to GenBank database of the National Center for National Center for Biotechnology Information (NCBI), through BLAST algorithm. The best score was used to ground the specie identification.

2.5 Fungal organic extraction

Those fungi molecularly identified as belonging to genus *Fusarium* were grown in PDA medium for 10 days. A litter of PD broth were inoculated with 12 fragments (7 mm^2) from each fungal isolate and incubated at room temperature for 28 days. After, the

fermented media were vacuum filtered to separate the mycelium. The filtrate was submitted to liquid-liquid extraction with ethyl acetate (EtOAc) 1:1 (v/v). The aqueous phase was separated and discarded. The organic phase was concentrated by complete solvent evaporation at 45 °C (Phongpaichit *et al*., 2007). The final product was diluted in DMSO to a concentration of 100 mg/mL of EtOAc extracts and up to a maximum of 7% DMSO (Marcellano *et al*., 2017) and reserved to following assays.

2.6 Antimicrobial Susceptibility Tests

The strains challenged were *Acinetobacter baumannii* (ATCC19606), *Enterobacter cloaceae* (ATCC81304), *Escherichia coli* (ATCC 35218), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa (*ATCC9027), *Salmonella enterica* (ATCC 51741), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus haemoliyticus* (ATCC 29970), *Candida albicans* (ATCC MYA-2876) and *C. tropicalis* (ATCC 750). The broth microdilution assay was performed in 96-well microplates according the M07-A10 (CLSI, 2017) and M27-A2 (CLSI, 2008) protocol standard. Briefly, the microorganisms stock was incubated in BHI broth for 24 h at 37 °C. Subsequently, plated on MHA (bacterial) or SDA (yeast), incubated for 24 h at 37 °C. Sterile 0.9% saline were inoculated with grown colonies until reaches the 0.5 McFarland turbidity scale, then diluted (1:20; v/v) in same solution. After microplate inoculation, the bacterial count was of approximately 10^5 CFU/mL to bacteria and 10^3 CFU/mL to yeast. Chloramphenicol or Amphotericin B was used as control; 7% DMSO and saline as growth control. Initially, all samples had their antimicrobial potential evaluated at 10 to bacterial and 40 mg/mL concentration to yeast. Those that reached 90% inhibition of bacterial growth, had the minimum inhibitory concentration (MIC) determined through serial dilution from 40 mg/mL to 0.31 µg/mL.

2.7 Anti-biofilm activity of extract sp. 34 and sp. 51 against *Candida albicans*

Biofilms were formed in 96-well plates. A 200 µL aliquot of a suspension of 1x 10^8 cells/mL of *Candida albicans* in 0.9% saline were added in wells for the initial pre-adhesion for 2h at 37 ºC. After that time, the saline was removed along with the non-adherent cells. The compounds were diluted in Yeast Nitrogen Base (YNB) with 1% glucose in concentrations 1X and 3X at MIC and left for 24h in an oven at 37 ºC. After this period, the wells were washed twice with saline to remove the culture medium, extracts and also dead and non-adherent cells. For the formation of mature biofilms were left for 24 h for formation and then treated for another 24 h. Then, the biofilms were plated on BHI plates. The percentage of viable cells was determined based on the survival of comparatively untreated biofilms (Bombarda *et al*, 2019). Amphotericin B (gold standard) was used as a control and the experiments were carried out in triplicate at three different times.

2.8 Systemic toxicity in the invertebrate model *G. mellonella* larvae.

The test was carried out to assess acute toxicity of the extracts (sp. 31, sp. 34, sp. 51) described by Bombarda *et al*. (2019). In experiments, 10 larvae were used for each group, weighing between 0.2 to 0.25 g without signs of melanization. 10 µL of the extracts in different concentrations (100 mg/mL, 60 mg/mL and 10 mg/mL) were injected into the hemocele of each larva through the last left proleg using a Hamilton syringe (Hamilton Inc, NV, USA). The larvae were incubated and analyzed at intervals for up to 72 h. Larvae without movement or showing high melanization were counted as dead.

3. Results and discussion

A total of 50 fungi were isolated, being 25 identified through morphological identified as *Fusarium* sp. Of these, eight strains with morphological characteristics divergent from those commonly described for *Fusarium* genus were select and had their peculiar characteristics analyzed. These *Fusarium* strains were named Sp.5, Sp. 24, Sp.31, Sp.32, Sp.34, Sp.50, Sp.51 and Sp.53. Morphologically, the fungi colonies grown in PDA had pale white, cream, lilac, pink and salmon coloration, the cottony hyphae and sometimes partially immersed in the middle (Figure 1). Those characteristics are determinants for the identification of *Fusarium* species, which takes into account the colony color, medium pigments, growth velocity, and mycelium appearance (if immersed in the middle, aerial or spars) (Leslie and Summerell, 2008).

In the microscopical analysis, it was observed: shape and size of the conidia (macro or microconidia); aseptate or septate conidia, and its quantities (if applied); presence or absence of chlamydospores; presence or absence of the phialides; the phialides shape and size, if simple, compounded or spiraled (Booth, 1971; Leslie and Sumerell, 2008). The main findings in the fungi here analyzed was show in Figure 2, being salient the large presence of trap hyphae (coileds), as indicated by the letter G, which are hygrophilous hyphae, specialized in killing nematodes or phytopathogenic fungi. In Table 1, the measurements of the reproduction structures were presented, according to the standard procedure for *Fusarium* identification (Leslie and Sumerell, 2008; Sumerell and Leslie, 2011; Sumerell, 2019)

At molecular level, the isolated fungi were identified by comparing their nucleotide sequences to those deposited in GenBank database of the National Center for National Center for Biotechnology Information (NCBI), through BLAST algorithm. All isolated fungi matched with 100% similarity, 100% cover and 0.0 e-value to *Fusarium* sp. However, due to the high conservation of ITS1 and ITS4 sequences into *Fusarium* species, it was not possible to identify the specie of which isolated with precision, only at the genus level was ratified. The eight sequences were deposited in the NCBI-GenBank under accession number indicated in table 2.

Although few studies about endophytic fungi of *A. aculeate* had been published, the genus *Fusarium* is always present in Arecaceae family (Lodge *et al*., 1996). Song *et al*, (2015), investigated the presence of endophytic fungi in 10 species of healthy Arecaceae and found 10 different fungal genera (*Cladosporium, Phialophora, Pestalotiopsis, Phoma, Phomopsis, Nigrospora, Xylaria, Fusarium, Colletotrichum* and *Rhizoctonia*), and other 20 fungi isolated belonging to mycelia sterilia fungus. Morales-Lizcano *et al*. (2017) evaluated microbial diversity in coconut palms (*Cocos nucifera* L.) with symptoms of fungal diseases and without any symptoms and the *Fusarium* species was present in both populations. Like demonstrated by Tibpromma *et al* (2018) that reported the presence of 22 endophytic fungi isolated in Mycelia sterilia, one unidentified and 13 mitosporic fungi including *Cladosporium* sp., *Colletotrichum* gloeosporioides, *Corynespora-like*, *Fusarium* sp., *Guignardia cocaicola*, *Paecilomyces* sp. *Pestalotiopsis* sp., *Phialophora* sp., *Phoma* sp., *Phoma-like* sp.

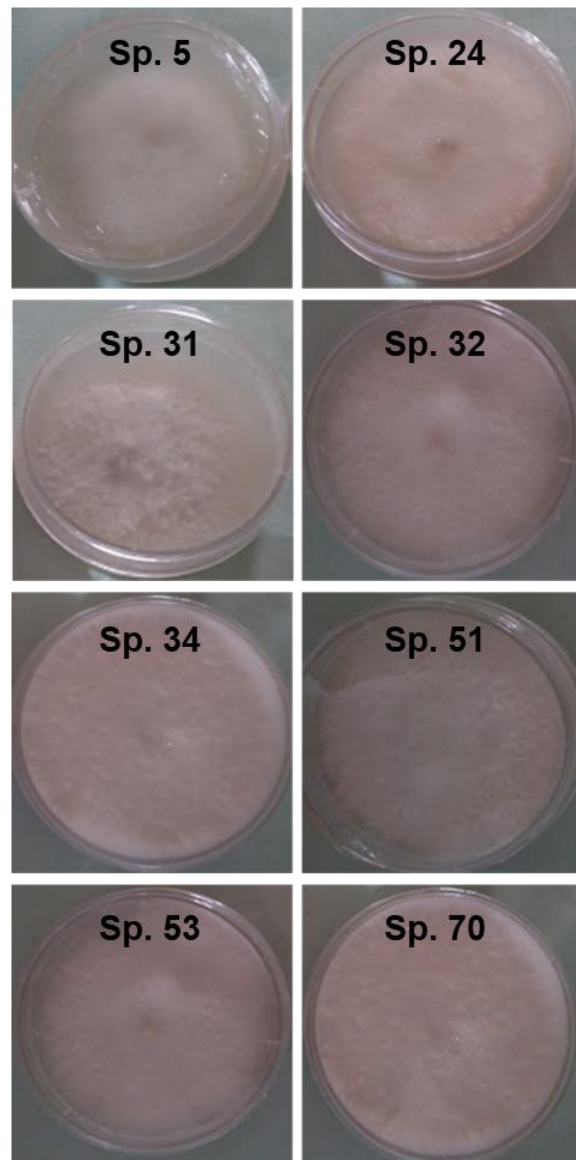

Figure 1. Morphology of isolated fungi colonies grown in Potato dextrose agar plates for 10 days at room temperature.

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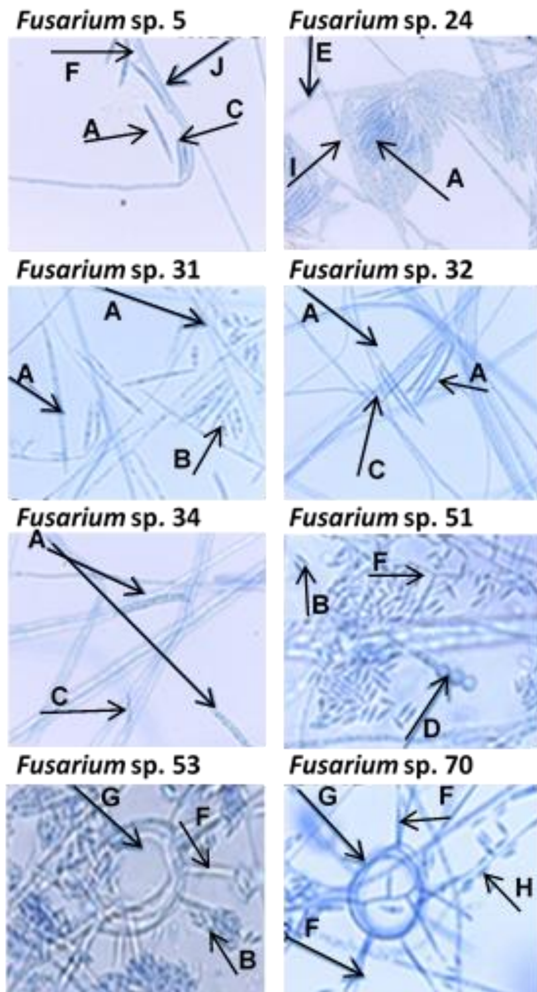

Figure. 2. Microscopic features of *Fusarium* on the Potato dextrose agar media. A: Macroconidia; B: Microconidia; C: Mesoconidia; D: Chlamydospores; E: Sporodoquia set F: Phialides; G: Coiled hypha; H: Microconidia chain; I: Sporodoquia; J: Hifas.

The evolution between plants and fungi has produced modified and stored chemical structures that form an important and unexplored reservoir that is believed involved in the protection and communication between host plants (Farrar *et al*., 2014; Yadav, 2019).

With this thought, this study evaluated the antimicrobial, and anticancer potential of compounds found into EtOAc extracts of isolated endophyte fungi of *A. aculeata* of the genus *Fusarium*. In a preliminary assay, the isolates sp. 31, sp. 34 e sp. 51 showed the best antimicrobial results, being able to control the bacterial growth of almost all challenged bacterial strains. Then, these isoletes had their MIC determined (Tab. 3).

Table 1. Characteristics and measurements of fungal reproductive structures.

	Macroconidia	
Species	Size (µm/µm)	Shape
Sp. 5	81/162	Straight, Curved
Sp. 24	32.4/43.2	Curved
Sp. 31	27/86.4	Straight
Sp. 32	27/81	Curved
Sp. 34	78/98	Straight
Sp. 51	27/75	Curved
Sp. 53	40.5/32.4	Straight
Sp. 70	30/5.3	Straight
	Microconidia	
Species	Size (µm/µm)	Shape
Sp. 5	8.1/13.5	Straight
Sp. 24	Absent	Absent
Sp. 31	7/10.5	Straight
Sp. 32	15/9	Straight
Sp. 34	11.5/8.3	Straight
Sp. 51	18.9/19.8	Straight
Sp. 53	8.1/13.5	Ovals
Sp. 70	8.0/11	Straight
	Sporodochium	Phialides
Sp. 5	Absent	Monophialides
Sp. 24	Present	Monophialides
Sp. 31	Absent	Monophialides
Sp. 32	Absent	Polyphialides
Sp. 34	Absent	Monophialides
Sp. 51	Absent	Polyphialides, verticiled
Sp. 53	Absent	Monophialides
Sp. 70	Absent	Polyphialides

Table 2. Accession numbers of *Fusarium* sequences deposited in the NCBI-GenBank.

Isolated	GenBank accession
Fusarium sp. 5	MN428023
Fusarium sp. 24	MN428024
Fusarium sp. 31	MN428025
Fusarium sp. 32	MN428026
Fusarium sp. 34	MN428027
Fusarium sp. 51	MN428028
Fusarium sp. 53	MN428029
Fusarium sp. 70	MN428230

The EtOAc extract of *Fusarium* sp.31 inhibited the growth of all bacterial strains, with the exception of *A. baumannii* and *P.* mirabillis, in which caused an extension of the lag phase of bacterium growth. With the EtOAc extract of *Fusarium* sp. 34, it was possible to achieve MIC to all bacterial and yeast strains in the tested concentrations. Indeed, sp. 34 extract showed the lowest MICS among tested extracts for *A. baumannii* (5 mg/mL), *E. cloacae* (5 mg/mL) and *E. coli* (2.5 mg/mL). In its turn, the EtOAc extract of *Fusarium* sp. 51 inhibited the growth of six of the bacterial and two yeast strains

used in this assay, but in *A. baumannii* and *E. cloacae* it only extended the lag phase.

The mechanism inherent to the extension of the lag phase was evaluated in the kinetic of growth curve of bacteria in presence of β-lactam antibiotics (Li *et al*., 2016), being observed the delay time extended with the increase in the concentrations of antibiotics as a response to the caused stress. In the current study, to the majority of bacterial strains in which MIC cannot be reached, the lag phase always was extended between 10 to 12 hours suggesting dose-dependency. However, this data can only be propriely determinated in further studies, after the purification of active molecule(s).

Table 3. Minimum inhibitory concentrations de EtOAc extracts of isolated *Fusarium* species. G-: Gram-negative; G+: Gram-positive; EtOAc: ethyl acetate.

Microorganisms	ATCC	Gram Type	MIC (mg/mL) sp. 31	sp. 34	sp. 51
A. baumannii	19606	G-	>10	5	>10
E. coli	35218	G-	10	2.5	5
E. cloacae	81304	G-	10	5	>10
P. aeruginosa	9027	G-	9	9	8
P. mirabillis	2453	G-	>10	10	>10
S. enterica	51741	G-	10	10	10
S. aureus	80958	G+	10	10	10
S. haemoliyticus	29970	G+	10	10	>10
C. albicans	2876	-	>40	20	40
C. tropicalis	750	-	>40	20	40

The current work evaluated of biocidal potencial of fungi metabolic extracts, and the results found are similar to those recurrently observed in the literature. Here, the most susceptive bacterial strain to *Fusarium* extracts was *E. coli*, with MIC values between 2.5 and 5 mg/mL. Marcellano *et al.* (2017) observed similar susceptibility of *E. coli* strain to EtOAc extract of endophytic fungi *Cinnamomum mercadoi*, with a MIC of 4.4 mg/mL. This extract also showed moderate antibacterial activity against the other bacterial strains with 10 mg/mL.

Kyekyeku *et al* (2017) showed antibacterial activity of EtOAc extract of *F. solani* against *S. aureus*, *Acinetobacter* sp., *E. coli* and *B. subtillis*, and found that the crude extract had the same yield as the compounds obtained with MICS of equally low values. Zhang *et al*. (2016) reported that a methanolic extract of the endophyte *Fusarium* sp. JY2, isolated from Honeysuckle (*Lonicera caprifolium*), was efficient in to control the growth the *P. aeruginosa* with MIC and MBC values of 0.2625 mg/mL and 1.05 mg/mL, respectively. This extract exhibited a broad spectrum of action toward both Gram-negative and Gram-positive bacteria. Obtained saponins from extracts of *F. oxysporum* PN8 and *A. niger* PN17 showed moderate to strong inhibitory activity against Gram-positive and Gram-negative bacteria, being observed MICs from 1.6 to 3.2 mg/mL (Jin *et al*., 2017).

As described in the literature, *Fusarium* species are known to producers secondary metabolics with a broad spectrum of antimicrobial action, like beta-lactam antibiotics, such as penecillins and cephalosporins (Kuck *et al*., 2014). As well, Ibrahim *et al*. (2016) demonstrated the antimicrobial and cytotoxic activity of fusarithioamide A, produced by the endophytic fungus *F. chlamydosporium* isolated from the leaves of *Anvillea garcini* showed a potent activity against *B. cereus*, *S. aureus, E. coli* and *Candida albicans* with MICs values of 3.1, 4.4, 6.9 and 2.6 µg/mL respectively.

Another endophytic fungus *Fusarium* sp., isolated from the invasive cactus *Opuntia dilleniid*, produced the antimicrobial secondary metabolite equisetin (Ratnaweera *et al.,* 2015). In addition, sanguinarine is an antimicrobial benzylisoquinoline alkaloid isolated from the endophytic fungal strain *F. proliferatum* found in the poppy *Macleaya cordata* (Wang *et al*., 2014). Likewise, Ibrahim *et al.* (2018) found the Fusaripeptide A, a new antifungal and anti-malarial cyclodepsipeptide from the endophytic fungus *Fusarium* sp. of *Mentha longifolia* L. (Labiatae) plant. In addition, a biofilm study was performed with *Candida albicans* strain with extracts sp. 34 and sp. 51. The results showed that these extracts were able to inhibit biofilm formation as well as having an action on mature biofilms, as seen in Figure 3. Our results demonstrated a reduction in the viability of the biofilm and are in line with those obtained by Chatterjee *et al.* (2020).

In addition, acute toxicity tests on *Galleria mellonella* alternative model larvae were performed. As seen in Figure 4, the larvae treated with different concentrations of sp. 31, sp. 34 and sp. 51 showed low toxicity, maintaining viability around 70% for the highest concentration. Thus, demonstrating the low toxicity of these extracts.

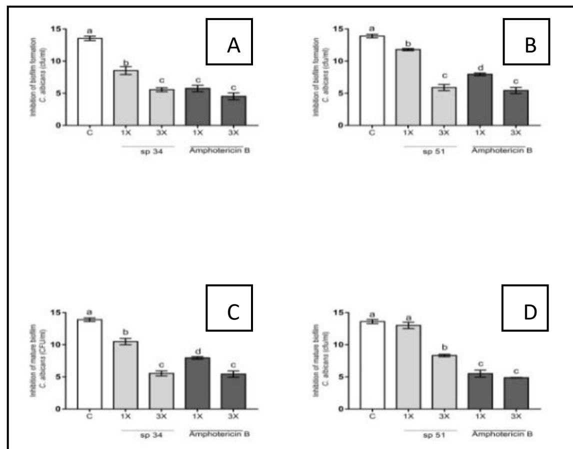

Figure 3. Quantitative analysis (CFU determination/mL) of inhibitory effects (mean ± SD) of sp. 34 and sp. 51, amphotericin B in (A,B) *Candida albicans* biofilm and (C,D) mature biofilm *Candida albicans* at concentrations 1 X MIC and 3 X MIC ($p<0.05$) ANOVA with Tukey's post-test.

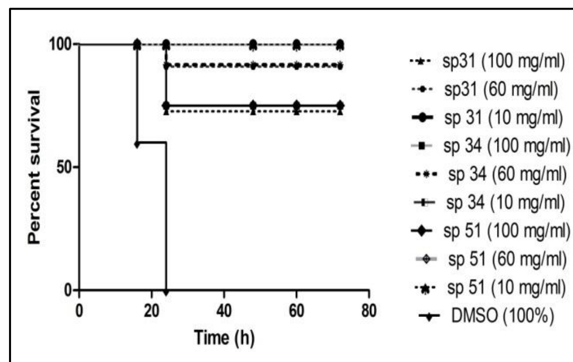

Figure 4. Percentage survival over time of *G. mellonella* larvae injected with sp. 31, sp. 34 and sp. 51 at concentrations 100 mg/mL, 60 mg/mL and 10 mg/mL.

4. Conclusion

At the end of this study we concluded that the endophytic fungi present in the *A. aculeata* species (sp. 31, sp. 34 and sp. 51), have potential as producers of secondary metabolites with antimicrobial action. These fungal endophytes of *A. aculeata* could be an appropriate source to produce antibacterial and antifungal agents. It will be interesting to investigate further the fungi found in this plant. As well as their, antioxidant and anticancer capacities among others as we can see in several species of the genus *Fusarium*.

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