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Original Article

Molecular and morphological diversity, qualitative chemical profile and antioxidant activity of filamentous fungi of the digestive tract of *Phylloicus* sp. (Trichoptera: Calamoceratidae)

Diversidade molecular e morfológica, perfil químico qualitativo e atividade antioxidante de fungos filamentosos do trato digestivo de *Phylloicus* sp. (Trichoptera: Calamoceratidae)

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Abstract

This study aimed to identify by molecular analysis, morphology, chemistry and antioxidant extracts of filamentous fungi isolated from the digestive tract of *Phylloicus* sp, an aquatic insect that lives on leaf packages in tropical streams and participates together with fungi of the decomposition of plant substrates in aquatic habitats. Insect larvae of *Phylloicus* sp. were collected in streams in the state of Tocantins, Brazil. Fungi were isolated from the digestive tract of larvae after disinfection and dissection, then described and purified for identification purposes and testing for antioxidant activity. Molecular identity was performed of ITS1 and ITS4, TUB e TEF sequencing. Fungal extracts were produced in 70% ethanol solution and later lyophilized. For analysis of chemical groups of extracts, thin layer chromatography (TLC) was performed in two mobile phases and different developers. Morphology was performed by optical microscopy stained with Toluidine Blue and measurement performed using the ImageJ program. Antioxidant activity performed in TLC and by quantitative method for DPPH and hydrogen peroxide (H_2O_2) radicals. Four fungi were identified: *Endomelanconiopsis endophytica*, *Myxospora musae*, *Neopestalotiopsis cubana* and *Fusarium pseudocircinatum*. The TLC showed several spots with acetone/chloroform mobile phase and UV 254 nm developers and I^2 vapor. Fungal extracts demonstrate antioxidant action to reduce the DPPH free radical and especially for H_2O_2 above 50%, *E. endophytica* 91.6%, *M. musae* 87.8%, *N. cubana* 89.5% and 92.3% for *F. pseudocircinatum*. This study demonstrated that the molecular technique by PCR was satisfactory for identifying fungi, and extracts with numerous chemical groups and potent reducing agents. Thus future work, should be carried out evaluating these four species for industrial use.

Keywords: antioxidant activity, fungal metabolites, *E. endophytica*, *M. musae*, *N. cubana*, *F. pseudocircinatum*.

Resumo

Diversidade molecular, morfológica, perfil químico qualitativo e atividade antioxidante de fungos filamentosos do trato digestivo de *Phylloicus* sp. (Trichoptera: Calamoceratidae). Este estudo visou identificar por análises molecular, morfológica, química e por extrato antioxidante, fungos filamentosos isolados do trato digestivo de *Phylloicus* sp, um inseto aquático que vive entre folhagens em riachos tropicais, e participam junto com os fungos da decomposição de substratos vegetais em habitats aquáticos. Larvas de insetos de *Phylloicus* sp. foram coletadas em riachos no Estado do Tocantins, Brasil. Os fungos foram isolados a partir do trato digestivo das larvas após desinfecção e dissecção, depois descritos e purificados para fins de identificação e testes para atividade antioxidante. A identidade molecular foi realizada do sequenciamento ITS1 e ITS4, TUB e TEF. Os extratos fúngicos foram produzidos em solução etanólica 70% e posterior liofilização. Para análise de grupos químicos dos extratos, foi realizada cromatografia em camada delgada (CCD) em duas fases móveis e diferentes reveladores. A morfologia foi realizada por microscopia óptica corada com Azul de Toluidina e a mensuração realizada no programa ImageJ. A atividade antioxidante realizada em CCD e por método quantitativo para os radicais DPPH e peróxido de hidrogênio (H_2O_2). Foram identificados quatro fungos*: Endomelanconiopsis endophytica*, *Myxospora musae*, *Neopestalotiopsis cubana* e *Fusarium pseudocircinatum*. Na CCD foi evidenciado várias manchas com fase móvel acetona/clorofórmio e reveladores UV 254 nm e vapor de I^2. Os extratos fúngicos demonstram ação antioxidante para redução do radical livre DPPH e em especial para H_2O_2 acima de 50%, *E. endophytica* 91.6%, *M. musae* 87.8%, *N. cubana* 89.5% e 92.3% para *F. pseudocircinatum*. Este

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estudo demonstrou que a técnica molecular por PCR foi satisfatória para identificação dos fungos, e os extratos com inúmeros grupos químicos e potentes agentes redutores. Com isso, trabalhos futuros deverão ser realizados avaliando essas quatro espécies para uso industrial.

Palavras-chave: atividade antioxidante, metabólitos fúngicos, *E. endophytica*, *M. musae*, *N. cubana*, *F. pseudocircinatum*.

1. Introduction

The Trichoptera were distributed even in the Triassic period where they share a common ancestor with Lepitoptera (Pes et al., 2005). These organisms, still in the larval form, present superior morphology with a sclerotinized head, short antennae, developed mandibles, thorax with a sclerotinized pronotum, membranous abdomen, which may present gills, and the IX segment presents a pair of anal pseudolegs with claws. These organisms present fresh, lotic and lentic life mainly in streams of Central Amazonia, and are rarely found in maritime areas (Reyes-Torres and Ramírez, 2018; Pimentel et al., 2020).

Calamoceratidae is composed of eight genera and 197 species, with the genus *Phylloicus* Müller 1880 being the most representative with a neotropical distribution with 60 described species. In addition to being distributed throughout the New World, it is, however, particularly diversified with the largest number of species described in Brazil, Peru and Venezuela (Souza-Holanda et al., 2020). In Brasil, it is the only known genus of the family, with 25 species mainly in the North region with records of seven species *P. amazonas* Prather 2003, *P. auratus* Prather 2003, *P. brevior* Banks 1915, *P. dumsi* Santos & Nessimian 2010, *P elektoros* Prather 2003, *P. fenestratus* Flint 1974 and *P. flinti* Prather 2003 (Leite et al., 2016; Holzenthal and Calor, 2017; Prather, 2003; Santos and Nessimian, 2010).

The genus *Phylloicus* shares, among other groups of Trichoptera, the presence of a wide variety of fungal microorganisms (yeasts) in the digestive tract, although reports related to the different yeast strains associated with these aquatic insects are still little restricted in the literature (Santos et al., 2019). It is known that this beneficial interaction can occur throughout the insect's body, depending on the microorganism's needs to establish a full mutualistic colonization. In the interface of interaction between yeast and insects, yeast colonization are observed both on the external surface of the body, reproductive system, and in the digestive tract (DT) (Santos et al., 2019). Furthermore, insects are responsible for dispersing these microorganisms, acting as vectors in different natural environments (Christiaens et al., 2014).

In a study carried out by Santos et al. (2019) 20 yeast species of the genus *Candida*, *Papiliotrema*, *Rhodotorula* and *Issatchenkia* were described only in the digestive tract of *Phylloicus* larvae collected in an area of the Cerrado domain in Brasil. These yeasts play a role in increasing and maintaining health, favoring the antioxidant action and interacting with nutrition, thus providing supplements in the diet during the stages from larva to adult insect (Noda and Koizumi, 2003; Douglas, 2015; Stefani et al., 2016).

Several yeasts have important biological actions with high antioxidant activity, due to the contents of total phenolics and among other groups of compounds produced from their metabolism, such as in strains of *Chaetomium* sp., (Huang et al., 2007), in *Terfezia claveryi* and *Picoa juniperi* truffles, and raw mushrooms *Lepista nuda*, *Lentinus edodes*, *Agrocybe cylindracea*, *Cantharellus lutescens* and *Hydnum repandum* in the study by Murcia et al. (2002) and in *Ganoderma lucidum*, *Tremella fuciformis*, *Schizophyllum commune*, *Morchella esculenta*, *Flammulina velutipes*, *Agaricus bisporus*, *Dictyophora inausiata*, *Grifola frondosa*, *L. edodes*, *Coprinus comatus*, *Fermentum latissis*, *Hericium albumuceus*, *Hericium, andrinas*, *Boletus bicolor* and *Aspergillus cristatus* in the study by Yang et al. (2020) where they showed, in studies, potential reducing efficiency over assays with free radicals by lipid peroxidation ($LOO^{\cdot}$), deoxyribose ($OH^{\cdot}$), peroxidase (H_2O_2) and in the reduction of free radicals DPPH, FRAP and $ABTS^{+}$, these being some examples of yeasts involved in several processes that benefit both humans and animals.

Thus, it is known that many fungal species have important biological activities, favoring the well-being of various organisms, especially as an antioxidant agent, which is a feature in the reduction of different forms of free radicals capable of negatively interacting on biomolecules, producing failures in gene transcription on DNA and RNA strands, in addition to being involved in several types of cancers and premature aging. In addition, they may also be directly positively linked to the development of industrial bioprocesses capable of promoting increased productivity on an industrial scale in the food production chain, agricultural bioactives and energy production (biofuels) (Otero-Colina et al., 2010; Sha et al., 2017; Yang et al., 2020; Sulfahri et al., 2020).

Thus, it is necessary to know the genetic profile, morphological and biological activities, as a potential reducing action of oxidizing agents from mutualistic fungal species present in the digestive tract (DT) of *Phylloicus* larvae. The aim of this study was to describe the profile on molecular and morphological diversity, qualitative chemical, and antioxidant analysis of filamentous fungi associated with the digestive tract of aquatic crusher larvae of insects of the genus *Phylloicus* (Trichoptera: Calamoceratidae) in natural environments of the Brazilian Cerrado.

2. Material and Methods

2.1. Fungal selection

Four fungal strains were randomly selected and provide from the Carlos Rosa Collection (CRC) of the Universidade Federal do Tocantins, Palmas, TO, Brasil, which were isolated from *Phylloicus* sp. (Trichoptera: Calamoceratidae) collected near the riverbanks of the Lajeado State Park,

Tocantins, Brasil. Then, the strain were evaluated for potential antioxidante activity and identified.

2.2. Molecular identification, amplification and fungal sequencing

Each fungal sample was identified by the ribosomal DNA inner transcribed region (ITS) sequencing method. DNA extraction was performed as proposed by Doyle and Doyle (1987). The extracted DNA sample was subjected to polymerase chain reaction (PCR) for amplification of the ITS region of the rDNA of part of the β-tubulin and 1-α elongation factor genes. The ITS region oligonucleotide primers were: SR6R (5'-AAGWAAAAGTCGTAACAAGG-3') and LR1 (5'-GGTTGGTTTCTTTTCCT-3') as described by Vilgalys and Hester (1990).

For the β-tubulin gene, TUB2Fd (5'-GTBCACCTYCARACCGGYCARTG-3') and TUB4Rd (5'-CCRGAYTGRCCRAARACRAAGTTGTC-3') were used according to Aveskamp et al. (2009). For the elongation factor gene they were: EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') as described by Carbone and Kohn (1999) and EF2 (5'-GGARGTACCAGTSATCATGTT-3') by O'Donnell et al. (1998). The solution for the PCR assay consisted of an aliquot containing 1 µL of DNA, 1 µL for each conc primer. 10 µM, 10 µL 5X PCR buffer, 1 µL dNTPs conc. 10 mMol, 0.2 µL of 5 U µL GoTaq DNA polymerase (Promega) and 35.8 µL sterile ultra-distilled H_2O, for a total final volume of 50 µL.

The amplification program consisted of initial denaturation at 94 °C for 2 min., followed by 40 cycles of denaturation at 94 °C for 10 s, with annealing at 54 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 4 min. The verification of the amplified products was carried out using conc. agarose gel electrophoresis. 0.8% stained with ethidium bromide. The amplified products were purified by precipitation with polyethylene glycol (Schmitz and Riesner, 2006), followed by the sequencing reaction through the chain termination method, using Big Dye 3.1 reagent solution (Applied Biosystems). The analysis was performed in an automatic capillary sequencer (Applied Biosystems, Mod. 3500 xL).

2.3. Phylogenetic determination

After sequencing, the base pairs were analyzed using the GenBank database, allowing the identification of fungal isolates at the genus and species level, when it comes to the species already described, based on gene similarity. The phylogenetic tree was developed using the Neighbour-Joining method through the MEGA program (version 6.0, USA). The bootstrap was 2000 replications over the confidence level as described by Tamura et al. (2013), GenBank sequences were indicated by accession numbers.

2.4. Extracts from cultures of filamentous fungi

The filamentous fungal cultures were subjected to extraction process as described by Lim et al. (2021) modified. Briefly, the cultures were homogenized and extracted using (70%) hydroethanolic solution and ethyl acetate (5:2). The mixture was left to stir overnight at room temperature. The spatula full of sodium sulfate was added before filtering the using filter qualitative paper. The filtered was transferred into a Becker flask and dried to evaporation in 36 °C. The extract obtained was dissolved in DMSO and kept as stock solution conc. 100 mg mL^{-1} at -12 °C until further analysis.

2.5. Basic morphological characteristics

Microscopy slides containing samples of the four fungi were prepared for light microscopy using Toluidine blue conc. 1% (*w/v*). Then, the slides were observed under an optical microscope (Olympus, Mod. BX61), with a digital camera attached (Olympus, Mod. DP73). The micrographs obtained from the 10, 20, 40 and 100x magnifications and their diameter expressed in micrometers (µm) were evaluated using ImageJ software (free version, 2019), where the diameter, length and shape of 50 viable fungal cells were determined.

2.6. Thin-Layer Chromatographic (LTC)

In silica gel chromatoplates F_{254}, five (5) µL of the fungal extract was deposited in the baseline, after drying the solvent, the strips of chromatoplates (1 x 10 cm^2) were transferred to a chromatographic vat containing the following phases: acetone/chloroform (5:3), and acetone/petroleum ether (5:2). Following elution, separated compounds were visualised using UV 254 and 365 nm, iodine vapour, ferric chloride solution, sulfuric vanillin solution, green bromocresol solution and chromic acid solution. The retention factors (R*fs*) for each spot was calculated as the ratio of the distance (mm) that spot was eluted on a plate relative to the total distance (mm). Images of derivatised TLC were taken using a digital camera (Lim et al., 2021) modified.

2.7. Antioxidant activity by qualitative assay

On a silica gel chromatoplate, the evaluated fungal extracts were applied. After total evaporation of the sample solubilization vehicle, the chromatoplate was nebulized with conc. 0.2% (*w/v*). The antioxidant activity was evidenced by the presence of white or yellow spots resulting from the reduction of the DPPH free radical, against the background pink coloration, after 30 min at room temperature. As a positive standard, 5 µL of stock solution at 1 mg mL^{-1} of ascorbic acid and quercetin equivalent to conc. 1.64 mMol as described by Lim et al. (2021) modified. Antioxidants were visualized as bright zones against a purple background under white light.

2.8. Quantitative assay of antioxidant activity (DPPH) and inhibition by hydrogen peroxide (H_2O_2)

Antioxidant activity was obtained in 96-well flat-bottom plates using a Biotek Elisa reader (model ELX 800 with Gen5 software V2.04.11). To evaluate the antioxidant activity, the test against the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) was used by according Alves et al. (2020). In this test, the following solutions were used per well: 180 µL of methanolic solution of DPPH conc. 0.2% (*w/v*), 20 µL of the extract sample dissolved in methanol, at concentrations of 200-0.78 µg mL^{-1}. Results were

expressed as inhibition concentration IC_{50} µg mL^{-1}. As a positive control, a standard solution of ascorbic acid and quercetin was used.

The ability of the four fungal extracts to inhibit hydrogen peroxide (H_2O_2) was estimated following the method of Ruch et al. (1989) and cited by Yadav et al. (2014). The solution of H_2O_2 conc. 40 mMol L^{-1} was prepared in phosphate buffer conc. 50 mMol L^{-1} with pH 7.4. The concentration of H_2O_2 was determined by UV-*Vis* spectrophotometry in absorption at 230 nm. Fungal extracts conc. 1 mg mL^{-1} in distilled water was added to H_2O_2 and the absorbance at 230 nm was determined after 10 min against a blank solution containing a phosphate buffer solution without H_2O_2. The percentage of elimination of H_2O_2 was calculated according to Equation 1.

$$\text{Scavenged H2O2}(\%) = [(Ai - At)/Ai]*100 \quad (1)$$

Were: Ai is the absorbance of control and At is the absorbance of test samples.

2.9. Statistical analysis

All tests were performed in triplicate, followed by ± SD (standard deviation), when necessary. From the means, an analysis of variance (ANOVA) was adopted, followed by Duncan's test ($p < 5\%$) for significance.

3. Results

The four fungal strains were identified as *Endomelanconiopsis endophytica, Myxospora musae, Neopestalotiopsis cubana e Fusarium pseudocircinatum.* All isolated from DT of *Phylloicus* larvae

The phylogenetic relationship between fungal species is shown in Figures 1-3, in trate digestion of *Phylloicus* sp.

The genetic sequence of the four identified fungi is presented below: *Neopestalotiopsis cubana* CBS 600.96 (**1**), *Fusarium pseudocircinatum* CBS 449.97 (**2**), *Myxospora musae* CBS 265.71 (**3**) and *Endomelanconiopsis endophytica* CBS 120397 (**4**).

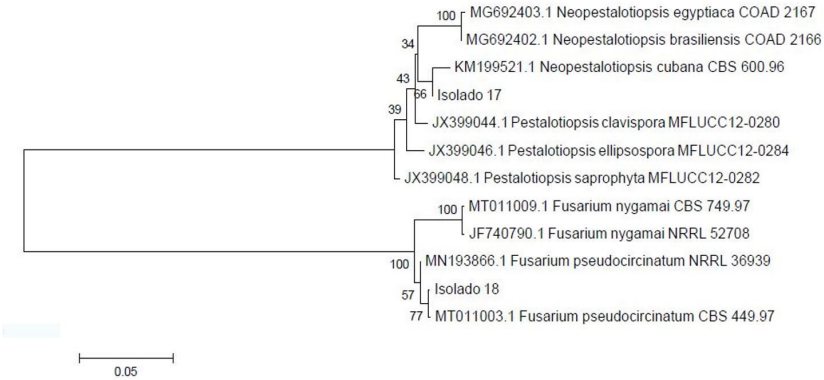

Figure 1. Phylogenetic relationship between fungal species for *Neopestalotiopsis cubana* and *Fusarium pseudocircinatum*. Source: Authors.

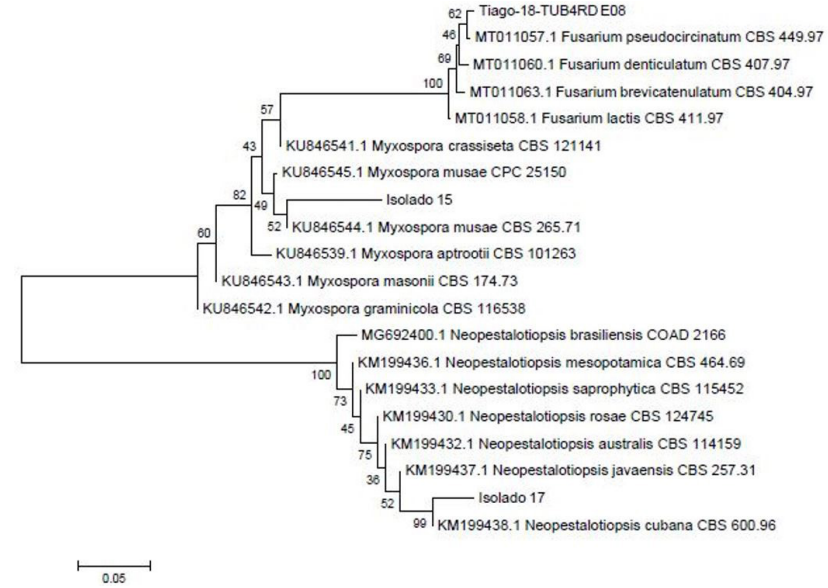

Figure 2. Phylogenetic relationship between fungal species for *Myxospora musae* and *Neopestalotiopsis cubana*. Source: Authors.

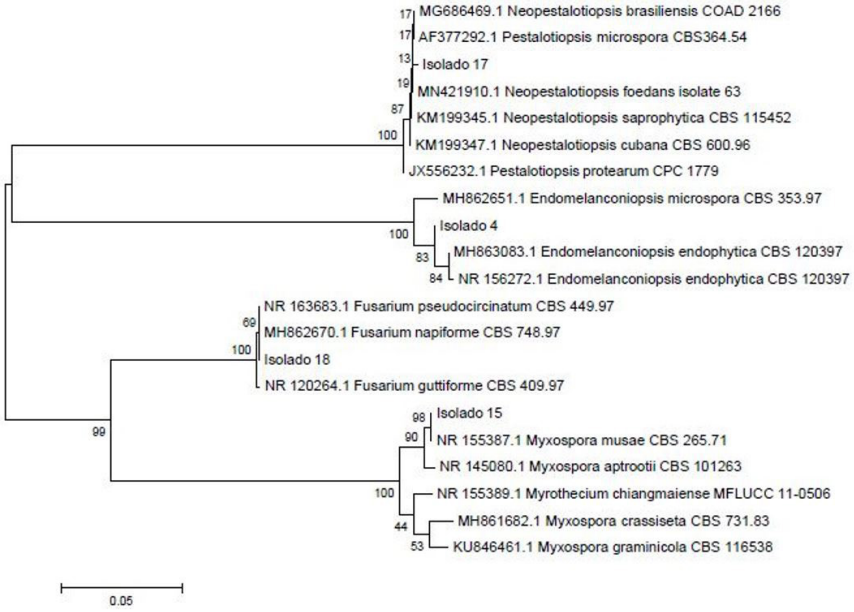

Figure 3. Phylogenetic relationship between fungal species for *Endomelanconiopsis endophytica* and *Myxospora musae*. Source: Authors.

(1): TEF

CATCGAGAAGTTCGAGAAGGTTAGT CATCTATTGATTCCCATCATCA TTCCCCTTCACT CCAGCGTCATTAT TTTCAACCTACGCGTTGAAA A TTATTTTCACTCCTTCCACACTTTTT CGCTGGTTACCCCGCCGCGAGG CACCCGCACGACCCCGCGG TGCAA ACGAAAAATTTCTTAT CACAGCCCCACCTTTCACAAGC AACCATGCATTG CTCATGAGACCCACTTTGAACAATTGCTAATGCCTTC ATACAGGAAGCCGC CGAGCTCGGTAAGGGTTCCTT CAAGTACGCCTG GGTTCTTGACAA GCTCAAGGCCGAGC GTGAGCGTG GTATCACCATCGATA TCGCTCTCTGGAAGTTCGAG ACCAACGAGTACAATGTCA CCGTCATTGGTTAGTACCCC TCCACCTATGCCATGTGCTG CTCCATAAGACACTTGACTA ACCTTGCTTCATAG ACGC TCCCGGTCACCGTGA TTTCATCAAGAACA TGATTACTGGTACT

(2): TEF

ATCGGCCACGTCGACTCT GGCAAGTCGACCACTGTGA GTACTACCCTCGACGATG AGCTTATCTGCCATCATAATC CCGACCAAAAACCTGGCGGGG TATTTCTCAAAAGCCAACA TGCTGACATTACTTCACAGACCGGTCA CTTGATCTACCAGTGCGGTGGTA TCGACAAGCGAAC CATCGAGAAGTTCGAGAAGG TTAGTCACTTTCCCTTCGATC GCGCGTCCTTTATCCATC GATTTCCCCTACGACTC GAAACGTGCCCGCTACCCCGCTC GAGTCCAAAATTTTTGCGAT ATGACCGT AATTTTTTTGGTGGGGCCTTTA CCCCG CCACTCGAGCGGCGCGTTTTTG CCCTCTCT CATTCCACAACCTCAC TGAGCGCATCGTC ACGTGTCAAGT AGTCACTAACCGTTCGACAATAGG AAGCCGCTGAGCTCGGTAAGGG TTCCTTCAAGTACGCCTGGGT TCTTGACAAGCTCAAGGC CGAGCGTGAGC GTGGTATCACCATCGATATTG CTCTCTGGAAGTTCGAGACTCCTC GCTACTATGTCA CCGTCATTGGTATG TTGCCGCTCATGC TTCATTCTACATC TCTTCTTACTAACATATC GCTCAGACGCCCCCGGT CACCGTGATTTCATC AAGAACATGATCA

(3): TUB

GTCAATGCGTAAGTGCTACCTAC CACGATTCTTGCTTCAA CCGCCGTCAGGCCACTCACG CGTTTTTCAGGGTAACCAAATTG GTGCTGCTTTCTGGCAGACCATCTCT GGCGAGCACGGCCTCGA CAGCAATG GTGTCTACAACGGAAC TTCCGAGCTCCAGCT CGAGCGCATGAACGTCTACTTCAA CGAGGTACGCCTTGCCATTGTTT ACTCGGCCTCGGGGCATCAAGC TAACCCACTGGTCAACAGGGTAG CGGTAACAAGTACGTTCC TCGCGCCGTCCTCGTCGAT CTCGAGCCCGGTACCATGGAC GCTGTCCGTGCCG

(4): ITS

GCGGCCGGCCCCCTAACCGGGGC TGGCCAGCGCCCGCCAGAGG ACTACCAAACTCCAGTCAGTAAACGT AGCTGTCTGATCAAAAGTTTAATAAACTAAA ACTTTCAACAACGGATCTCTTGGTTCTGGCAT CGATGAAGAACGCAGCGAA ATGCGATAAG TAATGTGAATTGCAGAATTC AGTGAATCATCGAATCTTTGAACGCACATT GCGCCCCTTGGTATTCCGAGGGGCATGCCT GTTCGAGCGTCATTTCACCACTCAAGCTCT GCTTGGTATTGGGCGCCGTCCTTCACCG GACGCGCCTCAAAGACCTCGGCGGTGGCGTC TTGCCTCAAGCGTAGTAG AAAACACCTCGCTTTGGAGGACGGGAC GTTCGCTCGCCGGACGAACCTT CTGAATTTTCTCAAGGTTGACCTCGGAT CAGGTAGGGATACCCGCTGAA

Figure 4 shows the microscopic image of the fungi isolates *E. endophytica*, *M. musae*, *Neopestalotiopsis cubana* and *F. pseudocircinatum*. Hyphae diameters were observed in *E. endophytica* hyphae with 11.23 ± 2.61 µm (a), *M. musae* hyphae with 3.41 ± 0.43 µm (c), *N. cubana* hyphae with 3.15 ± 0.95 µm (d) and in *F. pseudocircinatum* hyphae with 3.79 ± 0.73 µm (b) where they presented statistical difference between species according to Duncan's test (Figures 4A, 4B, 4C and 4D). In *E. endophytica* intense activity of lateral shoots was observed in the developed hyphae (Figure 4A). A large amount of conidia was observed in *M. musae* with polar diameter measurements of 4.59 ± 0.61 µm (a), and equatorial diameter of 2.71 ± 0.51 µm (b), and *F. pseudocircinatum* with polar diameter of 5.77 ± 0.92 µm (a) and equatorial diameter of 2.70 ± 0.23 µm (b), demonstrating inductive activity of conidiophores and dispersion (Figures 4B and 4D). Statistically, there was no statistical difference between polar and equatorial diameters between fungi species according to Duncan's test.

As for the TLC analysis, it was observed a large amount of spots with R*fs* in the mobile phase acetone/chloroform and acetone/petroleum ether with 9 and 7 R*fs* for *E. endophytica*, 6 and 7 R*fs* for *M. musae*, 6 and 4 R*fs* for *N. cubana* and 7 and 7 R*fs* for *F. pseudocircinatum*, respectively. The best developers were UV light 254 nm and I^2 vapor (Table 1).

In the qualitative assay by TLC, all plates exhibited spots with a color change to yellow against a purple background suggesting antioxidant activity in reducing DPPH. Similar results were observed by the quantitative test in the reduction of DPPH through a standard curve. The intensity of yellow color indicates the antioxidant capacity. Further analysis of fungi active extracts was shown in (Figure 5).

All samples of fungal extracts demonstrated antioxidant activity in reducing the DPPH radical above 60 µL mL^{-1}, although lower when compared to the standard antioxidant Ascorbic acid according to Duncan's test. The fungi *M. musae* and *N. cubana* did not show statistical difference according to Duncan's test (Figure 5).

In the reduction of hydrogen peroxide, the fungal extracts showed a reduction with values greater than 50%, *E. endophytica* 91.6%a, *M. musae* 87.8%b, *N. cubana* 89.5%a and 92.3%a for *F. pseudocircinatum*, respectively. It is observed that the fungi under study have high reduction activity in the peroxide test. Statistical difference was observed only for the fungal extract of *M. musae* according to Duncan's test.

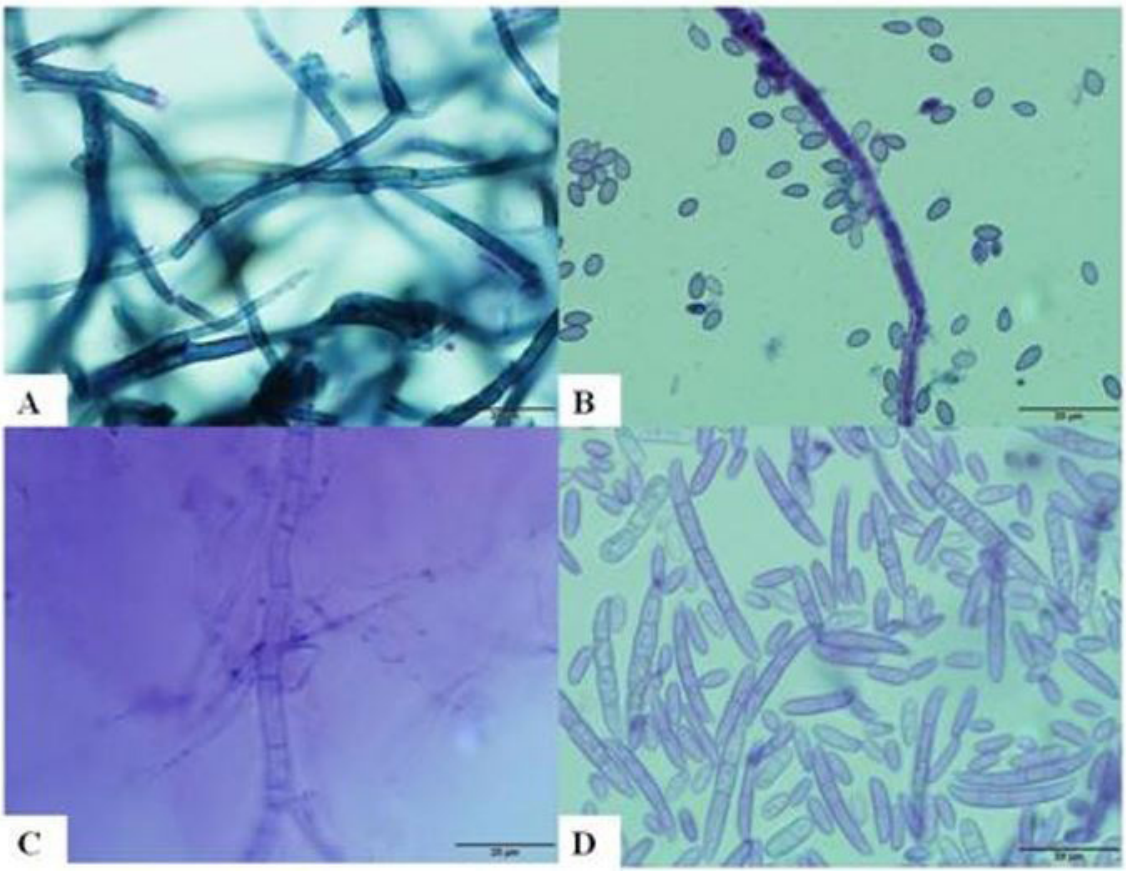

Figure 4. Morphology of hyphae and conidia of *Endomelanconiopsis endophytica* (A), *Myxospora musae* (B), *Neopestalotiopsis cubana* (C) and *Fusarium pseudocircinatum* (D). Source: Authors.

Table 1. Qualitative chromatographic profile by Thin-Layer Chromatographic, for the four fungi isolates *Endomelanconiopsis endophytica*, *Myxospora musae*, *Neopestaloiopsis cubana* and *Fusarium pseudocircinatum*.

Eluents	Revealing substances *E. endophytica*						
	1	2	3	4	5	6	7
Acetone/chloroform	0.15	-	0.16	0.44	-	-	0.71*
	0.40		0.22				
	0.78		0.31				
			0.40				
Acetone/petroleum ether	0.17	-	0.15	0.40	-	-	-
	0.37		0.22				
	0.44		0.31				
Eluents	**Revealing substances *M. musae***						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Acetone/chloroform	0.19	0.90	0.18	-	-	-	-
	0.33		0.45				
	0.69						
Acetone/petroleum ether	0.24	-	0.21	-	-	-	-
	0.37		0.30				
	0.77		0.38				
Eluents	**Revealing substances *N. cubana***						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Acetone/chloroform	-	-	0.25	0.91*	-	-	-
			0.37				
			0.40				
			0.44				
			0.53				
Acetone/petroleum ether	-	-	0.28	0.93*	-	-	-
			0.38				
			0.45				
Eluents	**Revealing substances *F. pseudocircinatum***						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Acetone/chloroform	0.48	-	0.20	0.40	-	-	0.88*
			0.27				
			0.47				
			0.56				
Acetone/petroleum ether	0.48	-	0.21	0.44	-	-	0.86*
			0.26				
			0.47				
			0.55				

Note: UV 254 mm (1) and 365 nm (2), iodine vapour (3), ferric chloride solution (4), sulfuric vanillin solution (5), green bromocresol solution (6) and chromic acid solution (7). (-) Absent. *Heating at 90 °C for 5 min. Results in millimeters (mm). Source: Authors.

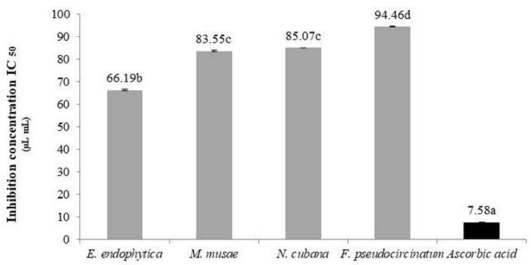

Figure 5. Antioxidant activity in reducing DPPH on fungi extracts (*Endomelanconiopsis endophytica*, *Myxospora musae*, *Neopestalotiopsis cubana* e *Fusarium pseudocircinatum*). Different letters show statistical difference by *Duncan's* test ($p < 5\%$). Source: Authors.

4. Discussion

In this study, great richness of species of morphospecies isolated from the digestive tract of *Phylloicus* sp. show great apparent antioxidant potential. According to Suh et al. (2005), Tegtmeier et al. (2016) and Santos et al. (2018) the digestive tract of insects, mainly in its larval form, is known to harbor a great diversity, as well as a promising source of new fungal species that can be used in industrial bioprocesses, in agriculture, nutrition and as bioindicator agents. This is the first record of the occurrence of *E. endophytica*, *M. musae*, *N. cubana* and *F. pseudocircinatum* in association with the digestive tract of an aquatic insect of the genus *Phylloicus*. The molecular method was highly

sensitive for TEF, TUB and ITS during the identification of fungi clades with 100% sensitivity. Similar results were also described by Lataeef et al. (2018) identifying by molecular techniques the diversity of *Pestalotiopsis*, *Neopestalotiopsis* and *Pseudopestalotiopsis* collected in Sarawak, on the island Borneo (Malaysia).

The preliminary identification based on molecular methods also allowed studies such as by Santos et al. (2018) the identification of four isolates, two identified as *Paraphaeosphaeria arecacearum*, one strain of *P. sclerotiorum* and one strain of *P. simplicissimum*, associated with the digestive tract of *Phylloicus* collected in Cerrado areas near the municipality of Santarém, Pará state, Brasil, where it was first isolated in soil under *Elaeis guianeensis* Jacq. in Suriname described by Verkley et al. (2014).

Several microorganisms isolated from the digestive tract of insect and insect larvae have distinct enzymes that are naturally produced. Furthermore, certain fungal groups are found in plant species being characterized and classified as endophytic (Sun et al., 2016a, b). The *E. endophytica* isolate was studied by Sun et al. (2016a) harmonically inhabiting *Ficus hirta* roots, being classified as a symbiotic organism, where, in addition, this fungus produces several compounds, two chemical structures of great industrial importance being 2,3-dimethylcyclopent-2-em-1-one and 2-hydroxymethyl -3-methylcyclopent-2-enone (Ferreira et al., 2015).

In addition to this harmonic characteristic between fungus-plant (symbiosis), these microorganisms produce an important enzyme mixture being used in several industrial segments (Sun et al., 2016b). This characteristic has been described in *M. musae* where it has cellulolytic activity (Vieto et al., 2022) and also beneficial effects on the rhizosphere in various plant groups in nitrogen fixation (Liang et al., 2019; Adeyemi et al., 2020) . Various organisms such as *Azospirillum* sp. and several mycorrhizal fungi, are used as inoculants in seeds and also directly in the leaf area, ensuring greater absorption of nutrients, increased productivity, leaf protection and less susceptibility to attacks by viruses, fungi and phytopathogenic bacteria (Fukami et al., 2018). However, a large number of fungi genera are considered phytopathogenic such as *N. cubana*, where in the study Pornsuriya et al. (2020) the researchers recorded an association with defoliation in rubber trees (*Hevea brasiliensis*) in Thailand for the genus *Neopestalotiopsis*, and Gerardo-Lugo et al. (2020) with mango gray leaf spot disease in Mexico. Not limited to this group, several species of the *Fusarium* genus are also responsible for serious agricultural losses annually, *F. pseudocircinatum* is also included in the class of genera that cause plant diseases such as malformation of mango fruits in Mexico (Freeman et al., 2014), in *Acacia koa* (Shiraishi et al., 2012) and in *Tabebuia rosea* (Montoya-Martínez et al., 2021).

Fungi isolated in the digestive tract of *Phylloicus* by different researchers emphasize that the species *P. sclerotiorum* has xylanolytic activity and in *P. simplicissimum* the cellulase enzyme (Zeng et al., 2006; Knob and Carmona, 2010; Santos et al., 2018). Fungi species with characteristics of plant material degradation have a remarkable industrial use. Xylanase for example, has a notable industrial use in the production of feed and food, and cellulases that can also be obtained from strains of *Penicillium* spp. has potential use in industrial processes (Dutta et al., 2008; Knob and Carmona, 2010; Bomtempo et al., 2017).

As for morphological characteristics, *N. endophytica* in this study showed septate hyphae. Similar results were also observed by Parambayil et al. (2018) evaluating *N. endophytica* isolates collected in different regions of Kerala, although in this study the diameter of the hyphae was greater when compared to the aforementioned study, which had an average diameter of 6.3 µm. Furthermore, *N. endophytica* was not observed in this study with conidiophores in culture medium. In younger cultures Rojas et al. (2008) analyzing species of the genus *Endomelanconiopsis* also did not observe the presence of conidia, only developing hyphae. Although in more established cultures, microconidia were observed with variable shapes, ellipsoidal to subcylindrical, hyaline and also with the appearance of phialidic conidiogenous cells.

In *M. musae* we noticed through microscopic observations the positive presence of both conidiophores and hyphae in a young established isolate. In the study by Liang et al. (2019) the group of researchers cites, through morphological characteristics, the standard mean value of conidia for *Myxospora* between 4-6 µm, with the value of this study being similar to the work cited.

N. cubana presented in this study only the developmental phase with hyphae. Similar results were also described in the study by Pornsuriya et al. (2020) where they evaluated the association of Neopestalotiopsis hyphae in *H. brasiliensis*. In comparison with another phytopathological species of the *Neopestalotiopsis* genus, *N. clavispora*, Daengsuwan et al. (2021) obtained in conidiophores cultures, where they determined the diameter of conidiomata between 150-250 mm, conidia length between 18-26 µm and conidia wide between 6.5-8.5 µm using an isolate of *Anthurium andraeanum* in Thailand. Superior results in conidia diameter were found *in Neopestalotiopsis zimbabwana* with values between 20.1-25.0 x 5.2-7.3 µm with fusiform to clavate, straight or erected, 5-cells morphology described by Hermawan et al. (2021) on *Xylaria* isolates.

In this work, *F. pseudocircinatum* presented aerial conidia with short clavate and zero-septate, fusiform, one- to three-septate aerial conidia without a foot-cell, and multiseptate sporodochial conidia with an acuminate apical beak-like cell and a distinct basal foot-like cell. Freeman et al. (2014) described characteristics for *F. pseudocircinatum* isolates in Mexico similar to those in this study. Isolates produced mostly 0-septate but occasionally 1-3 septate oval, obovoid, or elliptical aerial conidia (0 septate: 4 to 19 [avg. 8.7] × 1.5 to 4 [avg. 2.6] µm) in Freeman et al. (2014) described characteristics for *F. pseudocircinatum* isolates in Mexico similar to those in this study. The authors describe the morphology of *F. pseudocircinatum* in false heads in the dark and in short false chains under black light, unbranched or sympodially branched prostrate aerial conidiophores producing mono- and polyphialides, and sporodochia with straight or falcate conidia that were mostly 3-5 septate, but sometimes up to 7 septate (3 septate: 25 to 58 [avg. 41] × 2 to 3.3 [avg. 2.9] µm; 5 septate: 33.5 to 76.5 [avg.

56.7] × 2.5 to 6 [avg. 3.5] µm) and circinate sterile hyphae were rarely formed.

TLC analysis was used to determine the count and types of different constituents present in the crude extract of the evaluated strains. In TLC, the acetone/chloroform mobile phase showed greater aptitude for stain separation, which is discussed by Cai (2014) regarding mixtures of different solvents and polarities. As for the developer, 254 nm UV light and I^2 vapor revealed the greatest number of spots. It should also be emphasized that this is a qualitative analysis, which requires classical standards for comparison.

Although, it is a preliminary analysis to observe possible groups of molecules arising from special metabolism. It is possible to suggest that 254 nm UV light reveals phytochemicals that absorb ultraviolet light, where they quench the green fluorescence, producing dark purple or bluish patches on the plate. In addition, UV light usually reveals conjugated substances and aromatic systems; I^2 shows structures of organic compounds, this thesis is also defended by Menezes Filho et al. (2021).

Frisvad et al. (1989) evaluated fungal extracts by TLC where they described the presence of basic mycotoxins, alkaloids, roquefortine C & D, atramentins, fumigaclavines, penitrems, paxilline and aflatrem using as revealers a solution of $Ce(SO_4)_4$ in H_2SO_4 conc. 6N (15%), methanolic solution of acetic acid and conc. 0.5% anisaldehyde (17:2:1), and acidic mycotoxins, viridicatumtoxin, ochratoxin A, citrinin, rugulosin, luteoskyrin peptide like mycotoxins with I^2 vapor or chlorine, followed by spraying with *O*-tolidine solution (cyclochlorotine, simatoxin) blue fast B salt followed by NH_3 vapors (kojic acid, 3-nitropropionic acid), among other groups of molecules from the special metabolism of various fungal extracts. Complementary studies by high-performance liquid chromatography (HPLC) should be carried out, evaluating the groups of compounds isolated from the most suitable mobile phase, and thus, elucidating the groups of biomolecules present in the four strains studied.

Still on the TLC analysis, the extracts of the four fungi isolated in this study demonstrate DPPH free radical reducing activity. This is proven through a rapid qualitative result that shows a degree of confidence, where the violet color of the DPPH radical in the presence of antioxidant agents in the case of fungal extracts, turns yellow, showing the reduction of the radical compared to the control (Praptiwi et al., 2018). Although the qualitative assay has demonstrated activity, it is necessary to quantify this activity, and as noted above, fungal extracts exhibit antioxidant activity, although lower than the standard ascorbic acid as a first-line antioxidant and also in the peroxidation assay demonstrating an effect with high reducing activity of H_2O_2.

Quantitatively, the extracts exhibited activity in reducing the DPPH radical, although inferior to ascorbic acid, which is one of the main antioxidant agents found in fruits, vegetables, vegetables and among some restricted groups of algae (Njus et al., 2020). Studies have revealed that the use of extracts from fungal colonies, mainly of medicinal fungal species, has significant antioxidant activity. This corroborates our findings for the four species evaluated. Antioxidant activity values found by Caicedo et al. (2019) in *Fusarium oxysporum* culture showed DPPH radical scavenging activity of 51.5 and 26.5% evaluated in two types of culture media. Expressive free radical scavenging activities, were described by Cai et al. (2019) evaluating the content of total triterpenoids in *Sanghuangporus sanghuang* fungal mycelia were superior to our findings, with high activity in the reduction of free hydroxyl, superoxide anion, DPPH with a 6% reduction in conc. = 18.75 µg mL^{-1} and 96% with conc. = 350 µg mL^{-1}, and for 2,2'-Azino *Bis*-(3-ethylbenzthiazoline-6-sulfonate) the reduction between concentrations 9.37-300 µg mL^{-1} was between 20-96%. Superior antioxidant activity was also described by Zhou (2015) from *Ganoderma lucidum* extract in O_2 anion reduction between 30-90%, between 10-90% in hydroxyl free radical reduction and for DPPH between 10-70%.

The digestive tract of *Phylloicus* sp. larvae harboured *N. endophytica*, *M. musae*, *N. cubana* and *F. pseudocircinatum* and this is the first report on isolation of these fungi associated with *Phylloicus* sp.. The morphological structure also presented results similar to those in the cited literature, corroborating the molecular analysis, also suggesting that these fungi have a low evolution rate for different regions. All fungal extracts presented a high number of groups of separate compounds when acetone/chloroform were used as mobile phase and as main revealers UV light 254 nm and I^2 vapor, in addition to exhibiting potential reducing activity on DPPH free radical and hydrogen peroxide.

Future studies should proceed with investigations to characterize the endophytic potential and as an inoculant in biofactories and field, as well as to evaluate the cellulolytic and amylolytic enzymatic action already described in previous studies for the studied strains.

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