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DIVERSITY AND ANTIMALARIAL ACTIVITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH *Myrciaria dubia* FROM THE CANTÃO STATE PARK, TOCANTINS - BRAZIL

DIVERSIDADE E ATIVIDADE ANTIMALÁRICA DE FUNGOS ENDOFÍTICOS ASSOCIADOS À *Myrciaria dubia*, DO PARQUE ESTADUAL DO CANTÃO, TOCANTINS - BRASIL

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ABSTRACT

Camu-camu (*Myrciaria dubia*) is an Amazonian plant with great economic potential due to the high concentration of vitamin C in its fruits; the pulp is used in the pharmacological, cosmetic and food industries. Endophytic fungi are microorganisms that colonise the internal tissues of plants and, associated with vegetation, represent an unexplored source of new bioactive natural products. In this context, the objective of this study was to identify the diversity of endophytic fungi isolated from leaf and stem samples of *M. dubia* and to analyse the antimalarial potential of fungal extracts against the protozoan *Plasmodium falciparum* in an anti-HRPII (a histidine-rich protein) immunoenzymatic assay. The cytotoxicity assays of the extracts were performed against a human hepatoma cell line (HepG2) and determined by the MTT colorimetric method (3-(4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazoline bromide). A total of 308 fungal isolates were obtained and identified as belonging to 22 taxa using molecular methods. Extracts from the endophytic fungi *Diaporthe* sp. 2 (IC_{50} = 2.05), *Diaporthe miriciae* (IC_{50} = 2.31), *Diaporthe* sp. 4 (IC_{50} = 9.28), and from the family Xylariaceae (IC_{50} = 39.00), were active against *P. falciparum*. The identification of the fungal microbiota associated with plants contributes to taxonomic knowledge, collaborating for the bioprospection of endophytes that can be used mainly in agriculture and the pharmaceutical industry.

Keywords: Camu – camu. Fungal microbiota. *Plasmodium falciparum*. HepG2.

RESUMO

O camu-camu (*Myrciaria dubia*) é uma planta Amazônica de grande potencial econômico, devido à alta concentração de vitamina C em seus frutos, sendo a polpa utilizada na indústria farmacológica, de cosméticos e de alimentos. Os fungos endofíticos são micro-organismos que colonizam os tecidos internos das plantas e, associados aos vegetais, representam uma fonte inexplorada de novos produtos naturais bioativos. Neste contexto, este trabalho teve como objetivo identificar a diversidade de fungos endofíticos isolados de amostras de folhas e caules de *M. dubia* e analisar o potencial antimalárico dos extratos fúngicos contra o protozoário *Plasmodium falciparum* em ensaio imunoenzimático anti-HRPII (proteína rica em histidina II). Os ensaios de citotoxicidade dos extratos foram realizados contra linhagem celular de hepatoma humano (HepG2), sendo determinado pelo método colorimétrico MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina). Um total de 308 isolados fúngicos foram obtidos e identificados como pertencentes a 22 táxons por meio de métodos moleculares. Os extratos dos fungos endofíticos *Diaporthe* sp. 2 (IC_{50}= 2,05), *Diaporthe miriciae* (IC_{50}= 2,31), *Diaporthe* sp. 4 (IC_{50}= 9,28), e da família Xylariaceae (IC_{50}= 39,00), foram ativos contra o *P. falciparum*. A identificação da microbiota fúngica associada a vegetais contribui com o conhecimento taxonômico, colaborando para a bioprospecção dos endofíticos que podem ser utilizados principalmente na agricultura e indústria farmacêutica.

Palavras – chave: Camu – camu. Microbiota fúngica. *Plasmodium falciparum*. HepG2.

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1. INTRODUCTION

Among the most important microorganisms used as a source of natural products, fungi stand out, accounting for 38% of all biologically active compounds of microbial origin [1]. They present great potential for the search for new drugs and produce a wide variety of secondary metabolites, such as phenolic acids, benzopyranones, flavonoids, quinones, terpenoids, tetralones and xanthones, among others. Many of these compounds have properties that make them useful as agrochemicals, antibiotics, anticancer agents, antiparasitics, antioxidants, immunosuppressants and in the activation of the insulin receptor [2-5]. It is estimated that every plant species has endophytic microorganisms that have yet to be identified and have little known properties, but are potentially suitable for some biotechnological application [6].

Endophytic fungi inhabit the internal tissues of plants during at least one phase of their biological cycle [7]. This mutualistic interaction between endophyte and host plant may influence the composition of the plant community, maintaining ecological balance. In association with vegetation, endophytic fungi are ubiquitous, since all the plants examined to date shelter from one to hundreds of species of them [8, 9].

Studies on endophytic fungi are extremely relevant because they provide fundamental information for the evaluation of global fungal diversity and distribution, as well as revealing new taxa and new occurrences for existing species [10]. Endophytic fungi can stimulate plant growth, protect the host against disease, produce phytohormones, toxins, enzymes and confer to the plant resistance/tolerance to biotic and abiotic factors [11-16]. On the other hand, they can receive protection and nutrients from their respective hosts [17].

Camu-camu (*Myrciaria dubia* (Kunth) McVaugh, 1963) is a plant found on the banks of Amazonian rivers and lakes, used for medicinal and commercial purposes, because its fruits present a high content of ascorbic acid, proteins, fibres, calcium, phosphorus, potassium, iron, and vitamins such as thiamine, riboflavin, and niacin. In addition, it presents other bioactive compounds, such as carotenoids [18-21]. It is also characterised by a high content of phenolic compounds, relevant substances due to their antioxidant activity [22]. In this context, the objectives of the present study were to identify the biodiversity of endophytic filamentous fungi associated with *M. dubia* and to evaluate the inhibitory potential of fungal dichloromethanolic extracts against the protozoan *Plasmodium falciparum* Welch, 1897.

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2. MATERIALS AND METHODS

2.1 STUDY AREA

Thirty-one samples of leaves and stems from the plant *Myrciaria dubia* (Kunth) McVaugh were collected in August 2012 on the banks of the Javaés River (09°58' S and 50°03' W), in the Cantão State Park, located in the municipality of Pium-Tocantins, Brazil. The samples were georeferenced using GPS (Global Positioning System), preserved, exsicates were made to confirm the identity of the species and they were then registered (registration number 10.592) in the Herbarium at the Federal University of Tocantins, Porto Nacional campus.

2.2 FUNGAL ISOLATION

The plant material was submitted to the surface disinfection process for the elimination of epiphytic microorganisms, by means of serial washes in 70% (v/v) ethanol (1 min), 2% sodium hypochlorite (3 min) and sterilised milli-Q H_2O (2 min). Next, nine fragments (1.0 x 1.0 cm) were aseptically removed from each leaf and stem sample and transferred to six Petri dishes containing Potato Dextrose Agar (PDA), pH 6.8, supplemented with 100 μg ml^{-1} of chloramphenicol to inhibit the growth of contaminating bacteria [23]. An aliquot of the water used after disinfection was plated in culture medium to confirm the external disinfection of the plant fragments used. The isolated endophytic fungi were deposited in the Carlos Augusto Rosa Culture Collection of the Federal University of Tocantins, Brazil.

2.3 DATA ANALYSIS

Colonisation frequency (%CF) was calculated by means of the equation: ($CF = Ni/Nf$ x 100), where (Ni) = number of isolates obtained; (Nf) by the total number of fragments, according to Araújo [24]. The chi-square test was used to compare the absolute frequency of endophytic fungi that colonise stem and leaf tissues. To quantify species diversity, richness and dominance, we used Fisher's α, Margalef's and Simpson's indices respectively. The index calculations were performed using the program Paleontological statistics (PAST), version 2.16 [25]. The species accumulation curve was computed using the Jaccknife 1 wealth estimator obtained using the program EstimateS, version 9.1.0 [26].

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2.4 FUNGAL IDENTIFICATION

The protocol for DNA extraction was described by Rosa et al [27]. The internal transcribed spacer (ITS) region was amplified with the universal primers ITS1 and ITS4 [28]. β-tubulin amplification was performed with the primers Bt2a and Bt2b, respectively [29, 30]. The sequences obtained were analysed and a consensus sequence was obtained, both using electropherogram quality analysis (http://asparagin.cenargen.embrapa.br/phph/). To achieve species-rank identification based on ITS and β-tubulin data, the consensus sequence was aligned with all sequences from related species retrieved from the NCBI GenBank database using BLAST [31]. Taxa that displayed query coverage and identities ≤ 97% or an inconclusive taxonomic position were subjected to phylogenetic ITS and β-tubulin analysis in comparison with sequences of type species deposited in the GenBank database, with estimations conducted using MEGA version 6.0 [32]. The maximum composite likelihood method was employed to estimate evolutionary distances with bootstrap values calculated from 1000 replicate runs. The information about fungal classification generally follows [33], MycoBank (http:// www.mycobank.org) and Index Fungorum (http:// www.indexfungorum.org) databases.

2.5 PREPARATION AND DILUTION OF THE FUNGAL EXTRACT

The fungal extract was prepared using dichloromethane P.A. (Vetec-Sigma-Aldrich, St. Louis, Missouri, USA) as the solvent, following the protocol proposed by Rosa et al. [34]. The crude extract was diluted in Phosphate-Buffered Saline (PBS 1X, pH 7.4) in the bioassays and tested at the following concentrations: (100, 50.0, 25.0, 12.5, 6.2, 3.1, 1.5, 0.75, 0.37, 0.18) μg mL^{-1}.

2.6 CONTINUOUS CULTURE AND SYNCHRONISATION OF THE ERYTHROCYTIC PHASE OF THE PARASITE *P. falciparum*

In the antimalarial activity assays, the blood forms of a resistant *P. falciparum* W2 chloroquine (CQ) clone were used. The parasites were cultured in human O Rh^+ type erythrocytes, with 2% haematocrit and 50 mg mL^{-1} albumax (Gibco) under conditions established by Trager and Jensen [35]. The cultures with a predominance of rings (young trophozoites) used in the chemotherapy trials were submitted to the sorbitol synchronisation process, as described by Lambros and Vanderberg [36].

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2.7 PLATE PREPARATION FOR CHEMOTHERAPY AND ANTI-HRPII IMMUNOENZYMATIC ASSAYS

Cultures of synchronized parasites were distributed in 96-well microplates, with 180 µL of RPMI culture medium (created by the Roswell Park Memorial Institute) containing 0.05% parasitaemia and 1.5% haematocrit in each well. Initially, 20 µL of the extract was added to the culture plate, in triplicate, and at serial concentrations from 100 to 0.18 µg mL^{-1}. Control wells (six per test) contained infected erythrocytes with no addition of test compounds (negative control). The antimalarial standard, Artemisinin (ART), was tested in parallel in all experiments performed (positive control) at serial dilutions from 50 to 0.7812 ng mL^{-1}.

The plates were incubated for 72 h at 37 °C in a CO_2 incubator for the development of *Plasmodium*. After this period, the plates were frozen at -20 °C and thawed twice for erythrocyte lysis [37]. The anti-HRPII immunoenzymatic assay (histidine II-rich protein) followed the protocol of Noedl et al. [38]. Each assay was performed in triplicate in three independent experiments. Growth inhibition of 50% of the parasites (IC_{50}) was determined by means of dose-response curves as a function of non-linear regression. The program Origin (OriginLab Corporation, Northampton, MA, USA) was used to determine the IC_{50} value. According to Reynertson et al. [39], samples with IC_{50} of less than 50 µg mL^{-1} are considered highly active, between 50 and 100 µg mL^{-1}, moderately active, between 100 and 200 µg mL^{-1}, lowly active and values above 200 µg mL^{-1} were considered inactive.

2.8 CYTOTOXICITY TEST

The HepG2 cell lines (derived from a human hepatoma) were cultured according to Calvo-Calle et al. [40]. Cytotoxicity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) colorimetric method, according to Madureira et al. [41]. The cell concentration was adjusted to $2x10^4$/well and after 24 h of treatment with the fungal extract (500, 250, 125, 62.5, 31.2, 15.6, 7.8 µg mL^{-1}), 20 µL of MTT was added to each well at a concentration of 5 mg mL^{-1} in phosphate buffered saline-PBS (w/v). The plate was incubated for 4 h in an incubator at 37 °C. At the end of that period, the culture medium, together with the excess MTT, was discarded, then 100 µL of Dimethylsulphoxide (DMSO - Sigma-Aldrich) was added to each well. The optical density reading was performed in a

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microplate spectrophotometer at a λ of 570 nm. Cells with no addition of the fungal extract were used as the negative control.

The cytotoxic concentration for 50% of the cells (CC_{50}) was determined from non-linear regression of the serial dilutions of the extracts tested using the program Origin. The selectivity index (SI) of the samples was evaluated by calculating the ratio between the CC_{50} value and the IC_{50} value. Values higher than 10 were considered indicative of an absence in toxicity, while values below 10 were considered toxic [42].

3. RESULTS

Sample collection of leaves and stems of *M. dubia* resulted in the isolation of 308 colonies, consisting of 199 obtained from stem fragments and 109 colonies isolated from leaf fragments. These values indicate that the difference in colonisation between stems and leaves was significant, which can be verified through the statistical test ($\chi^2 = 26.30$, $p<0.01$). In relation to the frequency of global colonisation for *M. dubia*, a percentage of 59.7% of colony growth was obtained from the total number of plant fragments sampled. The colonisation frequency in the stem was 72.9%, and in the leaves 44.8%.

The fungal isolates obtained were identified by molecular methods and grouped into 22 different taxa (Table 1). It was observed that nine groups colonised the stem, eight both plant tissues, and five restricted their habitat to the leaves (Figure 1).

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Table 1: Molecular identification of the endophytic fungi associated with the plant *Myrciaria dubia*.

Codes[a]	Top BLAST search results (GenBank accession number)[b]	Query cover (%)	Identity (%)	Nº of bp analysed	Proposed taxa
5482 [b,c]	*Diaporthe miriciae* (KJ197262)	100	99	480	*Diaporthe miriciae*
5342 [b,c]	*Diaporthe masirevicii* (KJ197257)	99	94	450	*Diaporthe* sp. 1
5398 [b,c]	*Diaporthe acaciarum* (KP004509)	98	94	385	*Diaporthe* sp. 2
5480 [b,c]	*Diaporthe sclerotioides* (KC343193)	100	96	539	*Diaporthe* sp. 3
5438 [b,c]	*Diaporthe anacardii* (NR111841)	100	98	423	*Diaporthe* sp. 4
5502 [b]	*Diaporthe endophytica* (NR111847)	100	97	470	*Diaporthe* sp. 5
5591 [b]	*Diaporthe miriciae* (KJ197282)	100	96	470	*Diaporthe* sp. 6
5598 [b]	*Diaporthe inconspicua* (NR111849)	100	99	479	*Diaporthe* sp. 7
5491 [b]	*Melanconio hedericola* (KP004461)	100	96	516	Diaportheales
5322 [b]	*Muscodor equiseti* (JX089322)	100	100	475	*Muscodor equiseti*
5546 [b]	*Folliocryphia eucalypti* (NR135975)	97	84	425	Sordariomycetes
5349 [b,c]	*Neofusiococum umdonicola* (EU821904)	100	99	490	*Neofusiococum umdonicola*
5355 [b]	*Lasiodiplodia brasilliense* (JX464063)	100	100	479	*Lasiodiplodia* sp. 1

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Continuation

Codes[a]	Top BLAST search results (GenBank accession number)[b]	Query cover (%)	Identity (%)	Nº of bp analysed	Proposed taxa
5396 [b]	*Preussia persica* (GQ292750)	98	98	466	*Preussia* sp. 1
5400 [b,c]	*Neopestalotiopsis natalenses* (KM199377)	100	86	545	Xylariales
5442 [b,c]	*Fusarium sublunatum* (NR111606)	92	90	391	Nectriaceae
5450 [b,c]	*Penicillium citrinum* (NR121224)	98	99	465	*Penicillium citrinum*
5461 [b]	*Colletotrichum nupharicola* (NR120134)	100	99	423	*Colletotrichum* sp. 1
5526 [b]	*Guignardia musicola* (FJ538334)	100	100	558	*Guignardia* sp. 1
5569 [b]	*Curvularia pseudorobusta* (NR130653)	100	97	567	*Curvularia* sp.1
5592 [b]	*Colletotrichum aeschynomenes* (NR120133)	100	99	464	*Colletotrichum* sp. 2
5602 [b,c]	*Nemania abortiva* (NR121350)	100	92	375	Xylariaceae

[a]Codes of Culture Collection of Microorganisms from the Federal Universidade of Tocantins. [b]Reference sequence obtained through BLASTn and used for comparison. [c]Identification performed with BLASTn using fragments from the internal transcribed region ITS-5.8S in the rRNA gene region. [d]Identification performed with BLASTn using β-tubulin gene fragments.

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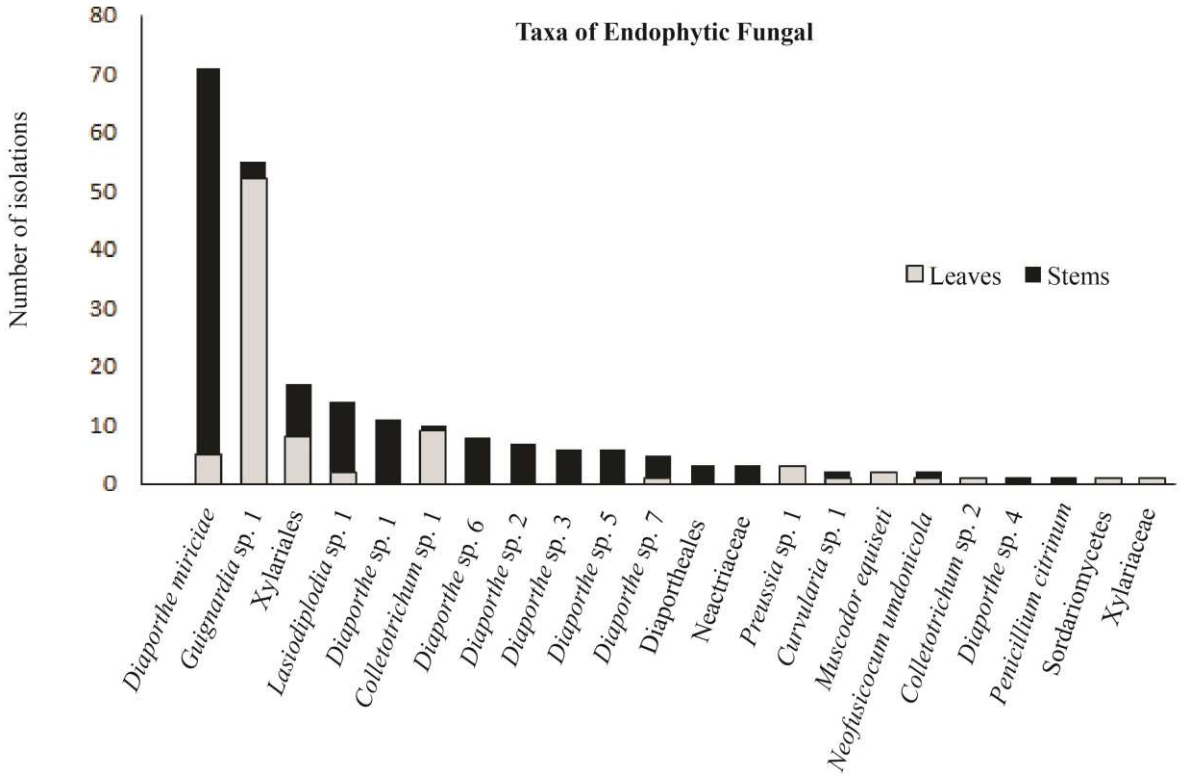

Figure 1. Isolation frequency of endophytic fungi taxa associated with *Myrciaria dubia*

The fungal community of *M. dubia* showed moderate richness (Margalef index = 3.86), moderate diversity (Fisher's Alpha = 5.99) and presented dominant species (Simpson = 0.83). The species accumulation curve (Figure 2) shows that saturation was not encountered, so it is possible to observe a slow accumulation of species throughout the sampling.

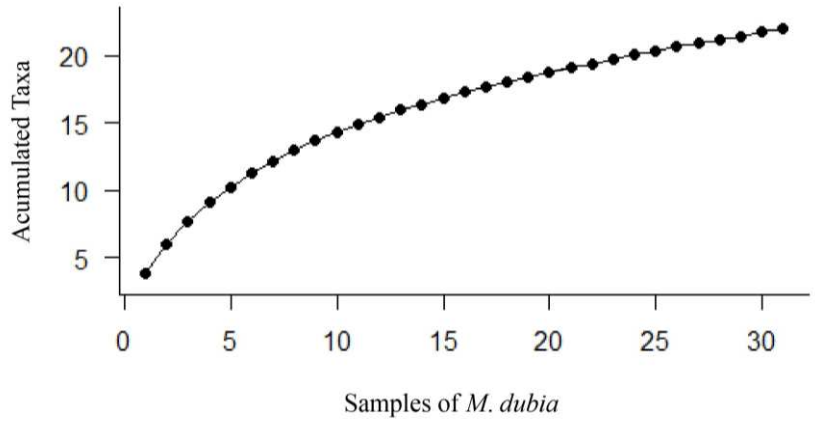

Figure 2. Species accumulation curve of *Myrciaria dubia* endophytic fungi (Jackknife Estimator 1).

Among the 308 fungal extracts tested, four showed activity against *P. falciparum* (W2 - resistant chloroquine strain) in an enzyme-linked immunosorbent assay (ELISA) using anti-HRPII monoclonal antibodies, a parasite-specific protein (Table 2)

The positive control used in the antiplasmodial bioassay was Artemisinin (IC_{50} = 6.77 ng mL^{-1}). Phosphate buffered saline (PBS 1X) used as a solvent of the extract did not present an antiplasmodial or cytotoxic effect, excluding the possibility of the action of the solvent as an inhibiting agent.

Table 2: Evaluation of fungal extracts that showed activity against *P. falciparum* in relation to their IC_{50} (inhibition of 50% growth of the parasite), CC_{50} (cytotoxic concentration for 50% of HepG2 cells) and SI (selectivity index).

Codes	Fungal Extracts	IC_{50} (µg mL^{-1})	CC_{50} (µg mL^{-1})	SI
5313	*Diaporthe* sp. 2	2,05	> 500	243,90
5430	*Diaporthe miriciae*	2,31	> 500	216,45
5438	*Diaporthe* sp. 4	9,28	> 500	219,30
5602	Xylariaceae	39,00	> 500	12,82
ART	Artemisinina	0,006	≥ 1000	149253,73

As for the cytotoxic activity of the extracts, in comparison to the human hepatoma-derived cell line (HepG2), it was not possible to calculate the CC_{50} as a dose-response function, because values above 500 µg mL^{-1} were observed in all replicates, indicating absence of toxicity of the compounds present in the extracts. The selectivity indices in all the extracts were higher than 10, confirming that the extracts are not toxic to the HepG2 cell.

4. DISCUSSION

Leaf and stem fragments have been the most used plant parts to obtain endophytic fungi in biodiversity and bioprospection studies [43-47]. This may be related to the way the endophyte enters the plant and also to the favorable conditions of certain tissues for fungal protection [48].

Similar to the present study, some authors also recorded high colonisation rates in the stem [49-51]. Although the cause of these variations is unclear, colonisation and distribution

of endophytic fungi in plants may very well be influenced by the type and amount of nutrients available in the different tissues of the host plant [52].

The overall colonisation frequency for *M. dubia* was 59.7%. The composition of endophytic fungi species may vary according to host, geographic distribution, plant age, ecological and seasonal conditions, such as altitude and rainy periods [53]. Pinto [54] isolated endophytic fungi from the leaves and stems of *Myrcia sellowiana*, observing a lower colonisation frequency (12.7%). Banhos et al. [55] obtained a higher colonisation frequency of endophytic fungi in *Myrcia guianensis* (53.3%), using the same methods of surface disinfection, and the same medium for isolation (PDA). Taylor et al. [56] obtained colonisation frequencies ranging from 23.4 to 57.3% in *Trachycarpus fortunei*, due to the location and climatic conditions where the plant was collected. Murali et al. [57] observed a low diversity of endophytic fungi in dry forests compared to tropical rain forests, as a result of the action of abiotic (annual precipitation, fires) and biotic factors (herbivore diversity, canopy opening).

Another relevant aspect is the dominance of one or a few species of endophytic fungi in the host [53, 58], which was also observed in this study (Figure 1), where the species *Diaporthe miriciae* and *Guignardia* sp. 1 were dominant, with 72 and 55 isolates, respectively, while the other species were less frequent. Vieira et al. [59] propose a possible antagonistic effect among endophytic fungi, where the high frequency of certain genera could be causing a decrease in others. Carvalho et al. [60] suggested that among less frequently occurring species, rare, endemic cryptic species or new species may be included and could play an important ecological role for their host plants.

The species accumulation curve (Figure 2) shows that saturation was not found, so a greater number of specimens or use of other methodologies could result in greater success in the isolation of new taxa.

The 308 fungal isolates were grouped into 22 taxa, of which 17 taxa were not identified at the species level; this fact may be related to the presence of new species that can be described in subsequent studies and contribute to a better knowledge of endophytic fungal biodiversity.

In the present study (*Diaporthe miriciae*, *Guignardia* sp.1, *Xylariales* sp.1, *Lasiodiplodia* sp.1, *Colletotrichum* sp.1, *Diaporthe* sp.7, *Curvularia* sp.1, *Neofusicocum umdonicola*) were generalists in relation to the tissues studied. Magalhães et al. [50] analysed

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the presence of endophytic fungi in candeia (*Eremanthus erythropappus*, DC.), and found that the genera *Xylaria* and *Phomopsis* were generalists, since they were found in all the analysed tissues (stems, leaves and seeds). The predominance of fungi in the plant organs is explained by the adaptation of these fungi, not showing specificity to the chemical compounds of the plant tissues, due to their function in the symbiotic relationship with the plant; thus, their presence in several tissues is observed, and some taxa, since they are dominant, keep their spaces in competition with the other endophytes.

Endophytic species of the genera *Phomopsis*, *Colletotrichum* and *Phyllosticta/Guignardia* present a variety of hosts and colonise several species of taxonomically distant plants [61-64], presenting adaptations for survival in different host types [65]. The high prevalence of species belonging to the *Phomopsis/Diaporthe* complex has been verified in several medicinal plants [66] and in temperate and tropical regions [67].

In relation to the bioassays against *P. falciparum*, the extracts of three endophytic fungi of the genus *Diaporthe* and one from the Xylariaceae family presented antiplasmodial action equivalent to the study by Correia et al. [68] with a dichloromethane extract from *M. dubia* leaves (IC_{50} 2.35 µg mL^{-1}). The ability of endophytes to produce the same substance as the host plant can be explained by coevolution between them over thousands of years [69]. Another example that confirms this hypothesis is quinine, one of the alkaloids of the Cinchona group, widely known and produced as a secondary metabolite of the bark of *Cinchona* sp. plants. Radiastuti et al. [70] detected the quinine alkaloid present in 16 cell lines of 39 *Diaporthe* spp. endophytic fungi, isolated from *Cinchona calisaya*, so that the quinine concentrations produced by these cell lines ranged from 1.1 mg L^{-1} to 155.2 mg L^{-1}.

The genus *Diaporthe* presents species with the potential for biological control [71], antibacterial properties [72, 73] and is a producer of secondary enzymes and metabolites [74-76], with anti-cancer activity [77], fungicide [78, 79] and antiplasmodial activity [80-82].

The genus *Phomopsis*, an anamorph of *Diaporthe* is mentioned by several authors, due to its metabolites with antiplasmodial potential. According to Isaka et al. [74], the secondary metabolite Phomopxanthone A, B produced by *Phomopsis* sp. BCC 1323, isolated from *Tectona grandis*, showed *in vitro* anti - *P. falciparum* K1 activity (IC_{50} 0.11 µg mL^{-1} and IC_{50} 0.33 µg mL^{-1}, respectively). Kornsakulkarn et al. [83] isolated from *Phomopsis* sp. BCC 45011, cytosporone E, cytosporone P, exhibiting antimalarial action and cytotoxic activity. *Phomopsis archeri* is an endophytic fungus isolated from *Vanilla albindia* (Blume), and

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produces aromatic sesquiterpenes - phomoarcherins A-C with activity against *P. falciparum* [84].

Martínez-Luis et al. [85] observed that the fungal extract from the Xylariaceae family (strain F0307) inhibited the protozoan *P. falciparum* in approximately 70.6% (samples with GI≥65% were considered active). The metabolites benzoquinone 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinone A, obtained from *Xylaria* sp., were active *in vitro* against *P. falciparum* K1 strains with IC_{50} values of 1.84 and 6.68 μM, respectively [86].

In regards to the antiparasitic properties of the endophytic fungi, it is relevant to cite the study by Correia et al. [87] which demonstrated the *in vitro* action of fungi isolated from *M. dubia* against the phytopathogens *Monilinia fructicola*, *Colletotrichum gloeosporioides* and *Aspergillus parasiticus*, producing diffusible and/or volatile substances in the antagonistic process. Among the antagonists tested by the authors, the fungal individuals 5322 (*Muscodor equiseti*) and 5450 (*Penicillium citrinum*) were considered promising because they had percentages above 80% inhibition.

According to Strobel [88], species from the genus *Muscodor* produce a mixture of toxic volatile organic compounds. Consequently, species from the genus *Muscodor* are considered promising for biocontrol, being used to control diseases of the root system, through applications of biological fumigation, as demonstrated by Suwannarach et al. [89]. Volatile compounds isolated from *Muscodor* present a potential for biological control of bacteria, yeasts and filamentous fungi [90].

Penicillium is a promising genus among the endophytic fungi isolated from *M. dubia*, widely investigated for the production of bioactive secondary metabolites with potential application in the pharmaceutical and chemical industries. Fill et al. [91] verified the production of substances with antibiotic activity in *Penicillium brasilianum* isolated from *Melia azedarach* (Meliaceae). Wang et al. [92] described the activity of secondary metabolites of *Penicillium* sp. isolated from the leaves of *Hopea hainanensis*, reporting their action against *Candida albicans*, *C. krusei* and *Aspergillus niger*.

Endophytes inhabit an ecological niche similar to that occupied by phytopathogens, enabling them to be controlled through competition for nutrients, production of antagonistic substances, parasitising the pathogen, or even inducing the development of resistance by the plant [6]. The ability of endophytes in the *in vitro* production of substances that inhibit the

growth of other species of microorganisms has stimulated research on bioprospection of endophytic fungi and biological control [93].

The ability of endophytic fungi to produce bioactive compounds is a consequence of their chemical defense, adaptive response to survive and colonise host plants in the wild [94]. Many medicinal properties of plants are related to metabolites produced by endophytes [95]. It is questioned whether bioactive phytochemicals from plants are produced exclusively by them or as a result of the mutualistic relationship with the microorganism. Thus, it is suggested that the therapeutic properties of a medicinal plant are related to the interaction between both [96].

Plants and endophytic fungi are closely related to the production of secondary metabolites in a phenomenon known as "Horizontal Genetic Transfer", defined by the ability some endophytic fungi have acquired to produce the same substances produced by plants. This phenomenon is explained by the exchange of genetic material between cells or unrelated genomes between endophytic fungi and the host plant [97].

The variability of biotic (such as species involved in the interaction) and abiotic factors (such as soil types, cultivation periods and water content) also contribute to the increase in diversity of the metabolites produced during the plant-endophytic fungus interaction [16]. Therefore, endophytic fungi are an important source of natural bioactive compounds with application in several areas, mainly in the pharmaceutical industry.

5. CONCLUSIONS

The colonisation frequency and the diversity index indicated that *M. dubia* presents a moderate diversity of associated endophytic fungi; however, four fungal extracts demonstrated antiparasitic and nontoxic activity, indicating a new path to be explored in a sustainable manner in the area of bioprospecting new drugs. The identification of fungal microbiota associated with plants contributes to taxonomic knowledge and consequently to the protection of biodiversity, since microbial diversity represents an important source of genetic resources for the advancement of biology and biotechnology. However, new studies should be carried out to characterise the substances present in the extracts and subsidise their use in the pharmaceutical industry.

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