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Abstract: Almost fifty years after the introduction of antibiotics as an antimicrobial agent, we are facing an increasing number of multi-resistant microbials. The search for new antimicrobial compounds emerged as a promising alternative to treatments for diseases caused by microorganisms such as fungi and bacteria. Thus, endophytic fungi are gaining recognition for their varied metabolites that may possess antimicrobial properties. In our research, we isolated, from *Calea pinnatifida,* six endophytic fungi identified as *Colletotrichum siamense, Colletotrichum karstii, Hypomontagnella barbarensis, Neopestalotiopsis clavispora, Nigrospora sacchari-officinarum* and *Annulohypoxylon moriforme*. Analyses of microbial secondary metabolites by utilizing UHPLC-HR-ESI-MS/MS in conjunction with molecular networking, allowed the annotation of 9 compounds, 5 of them from GNPS spectral library match, and 4 from prediction-based on the molecular network, and allowed the isolation of two pairs of Acyl-tetronic acid derivatives distereoisomeric identified by NMR spectrum. In this sense, the present study underlines the value of endophytic fungi as a reservoir of potentially new antimicrobial agents.

Keywords: *Calea pinnatifida*; endophytic fungi; *Hypomontagnella barbarensis*; antimicrobial activity; dereplication

1. Introduction

The search for bioactive compounds as an alternative treatment for diseases caused by microorganisms such as fungi and bacteria has proved to be of great interest to researchers, since some treatments has already demonstrated a certain specific resistance to some common strains, thus triggering a threat to a global scale [1]. Modern medicine, which relies heavily on effective antimicrobial drugs, faces significant challenges in the face of these new demands [2,3]. Some actions can be linked to resistance to existing antimicrobials, such as their excessive and inappropriate use, in human treatment, food production, and the environment [4,5]. In response to this threat, over the last almost one hundred years, research has been dedicated to discovering and applying bioactive compounds from various organisms as a strategy for alternative solutions to the problem [6]. The use of natural products from fungi, including terpenes, peptides, and mainly polyketides has demonstrated properties that can be effective against bacteria and fungi and has emerged as an alternative to treatments [7–13]. Thus, from *Ampelomyces* sp., isolated from *Urospermum picroides* (Asteraceae), are described the compounds 6-O-Methylalaternin and Altersolanol A, which displayed antimicrobial activity against the gram-positive pathogens, *Staphylococcus aureus*, *S.*

epidermidis and *Enterococcus faecalis* [14]. Antimicrobial metabolites are also described from extract of the endophyte *Nodulisporium* sp., isolated from the plant *Erica arborea* (Ericaceae), including Nodulisporins D–F with antibacterial and fungicidal properties [15]. Asteraceae, a family noticeable for its medicinal uses, comprises approximately 33,000 species grouped into 1,911 genera [16], some of which harbor endophytic fungi [9]. Although there is a great diversity and abundance of Asteraceae, the associated species of fungi are small, and a low percent had their endophytic fungi studied. According to Caruso et al. [9], studies carried out on the endophytes of some Asteraceae species have shown interesting compounds, such as Isofusidiene I, A, B, C and D from endophytes of the genus *Chalara* associated with *Artemisia vulgaris*, showing antibacterial and antifungal activity [17] and Phomosin K with antimicrobial activity, from *Phomopsis* fungi inhabiting the internal tissues of *Notobasis syriaca* [18]. In this context, *Calea pinnatifida*, has emerged as a plant with remarkable pharmacological potential, due to its traditional medicinal properties, including use in folk medicine as an antimalarial, analgesic, anti-inflammatory, and for the treatment of gastrointestinal diseases [19]. However, despite their relevance in traditional medicine, the endophytic fungi associated with *C. pinnatifida* remain largely unknown and little explored. So, this research gap offers a unique opportunity to investigate and identify bioactive compounds with pharmaceutical potential [9,17,20,21]. Therefore, this study investigates endophytic fungi associated with *C. pinnatifida* to identify new antimicrobial compounds. To achieve that, the analysis of antimicrobial extracts was carried out by UHPLC-HRMSMS combined with molecular networking. This strategy led to selecting one endophytic fungi whose extract allowed the isolation of two pairs of diastereoisomeric Tetronic acid derivatives that includes the novel compound Noduslifuranol.

2. Materials and Methods

2.1. General Experimental Procedures

The ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, on Ultrashield 300 Avance III spectrometer (Bruker–BioSpin, Ettlingen, Germany). It was equipped with a 5 mm trinuclear inverse detection probe with z-gradient (TXI). The temperature was controlled by a BCU I accessory at 25°C. Deuterated methanol (MeOD-d_4) (Sigma-Aldrich, St. Louis, USA) was used as the solvent and internal standard. High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) analysis was carried out on the Ultimate 3000 liquid chromatograph with UV-DAD detector (Dionex, Sunnyvale, USA), using a Kinetex C18 analytical column (Core-Shell – 4.6 μm – 150.0 mm × 4.6 mm) for method standardization and chemical profile analysis and a Kinetex column C18 semi-preparative column (Core-Shell – 4.6 μm – 150.0 mm × 10.0 mm) for compound isolation. The analyses by UHPLC-HR-MS/MS were performed in a mass spectrometer Q-ToF maxis 3G (Bruker–Daltonics, Billerica, USA), equipped with an electrospray ionization source (ESI) operating with a resolution of 60,000 FWHM in positive mode with detection in a range of masses from m/z 50 to 1500 Da. The capillary voltage was fixed at 4500 V and the nozzle at 500 V. The conditions applied for the drying gas (N$_2$) were 8.0 mL/min, pressure 4.0 Bar, and temperature 200°C for the flow of 0.3 mL/min. UV-Vis detection (PDA) was performed at a rate of 4.6 Hz in a spectral window from 200 to 700 nm [22].   Calibration was set at less than 2 ppm using sodium formiate.

2.2. Plant Material, Isolation, and Cultivation of Endophytic Fungi

Branches and leaves of *C. pinnatifida*, were collected at the Botanical Garden of São Paulo – SP (coordinates 23 38'29.47" S, 46 37'15.83" W). The specimen (Ferreira, M. J. P. 27) has been deposited in the SPF Herbarium of the Botany Department from the Biosciences Institute of the University of São Paulo.

The methodology used for the isolation of endophytic fungi was based on the procedure described by Santos [23]. Briefly, from the fungi isolated on agar plates, fragments of cultivated mycelium were inoculated in 150 mL Erlenmeyer flask containing 70 mL of potato dextrose broth (PDB) (Becton Dickinson, São Paulo, Brazil), kept under stirring at 28°C for 7 days in an incubator (28°C) in order to obtain a seed culture for the solid medium inoculation. An aliquot of 1 mL of seed

culture was inoculated in rice medium, in a 250 mL Erlenmeyer flask containing 50 g of autoclaved rice grains and 90 mL of water. This culture remained in a static medium for 28 days in an incubator at 28°C [24]. After cultivation, the fungi was inactivated and extracted with ethyl acetate (50 mL) under stirring for 24 h, then vacuum filtered (total volume of 150 mL). The filtrate was dried in a rotatory evaporator under low pressure at 40°C and the crude extracts were obtained [25].

The isolated fungi were preserved using the Castellani's method [26], which consists of storing fungi in sterilized water or saline solution [27] under refrigeration (4°C). This technique is preferably applied to young cultures to reduce the metabolism and consequently the latency of the cells in the face of restricted nutritional sources [28], guaranteeing the preservation of the original characteristics of the culture for long periods and promoting the absence of contamination [29,30].

2.3. Identification of Endophytic Fungi

Total DNA was obtained from a 2-day-old at 37°C monosporic colonies on yeast extract sucrose agar (YES) (10 g yeast extract, 75 g sucrose, 10 g agar, and 500 mL distilled water) by PrepMan Ultra kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's recommendations. For accurate species identification was sequenced: i) fragments of internal transcribed spacer (ITS) of ribosomal DNA region using the primer pairs ITS1 and ITS4 and fragments of tubulin gene (*TUB2*) using the primer primers Bt-2a and Bt-2b. The amplification reactions were performed in a final volume of 25 μL containing 1 μL DNA (40 ng), 12.5 μL 2 PCR Master Mix (Promega Corporation, Madison, WI, USA) containing 3 mM $MgCl_2$, 400 mM each dNTP, and 50 U/mL of Taq Polymerase, 9.5 μL Milli-Q water, and 1 μL of each primer. The amplification protocol included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s (at 52°C and 55°C for ITS and *TUB2*, respectively), extension final for 10 min at 72°C. Amplicons were purified using Gel and PCR Clean-Up Kit (Promega Corporation, Madison, WI, USA) as manufacturer's recommendations. All cycling reactions were performed in Eppendorf Mastercycler X50 (Eppendorf Norge AS, Oslo, Norway). The amplified fragments were sequenced in both directions using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The samples were run on a SeqStudio Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Laboratory of Emerging Fungal Pathogens (Federal University of São Paulo, São Paulo, Brazil) under previously described conditions [31].

The obtained sequences were assembled and edited using the DNA sequence assembly software Sequencher 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Successful assembly of the contigs required a minimum match percentage of ≥85 and a minimum overlap of 20. The consensus sequences were compared with the sequences deposited in public genomic databases GenBank (http://www.ncbi.nlm.nih.gov/genbank/)   and   Mycobank   (http://https://www.mycobank.org/Pairwise_alignment) using BLASTn tool. We considered the parameters of E-value $<10^{-5}$ and identity ≥99% for accurate species identification.

To determine the phylogenetic distance of strains in the same family, phylogenetic trees were elaborated. Thus, ITS sequences from the fungi were aligned with MAFFT [32], and visualized with AliViewer. Poorly aligned regions were removed using trimal v1.2 (parameter - automated1) [33]. A maximum likelihood tree was built with the ITS sequences from the samples (CPFF12, CPFF14, CPFF16, CPFF41, CPFF42, and CPFF52), and ITS sequences from similar related genera. *Amanita muscaria* ITS sequence was used as an outlier (AB015700.1). The tree was built with IQ-TREE v.2 [34] with model finder (parameters –bb 1000 – bnni -nt AUTO). The resulting trees were visualized using iTOL [35].

For morphological characterization, the isolates were incubated on PDA at 25°C for 7 to 15 days to evaluate their viability and purity. For genus identification, each isolate was cultured on PDA, Oatmeal agar (OA), and Synthetic nutrient agar (SNA) [36] at 25°C for 14 days. The macromorphology features such as colony color, diffused pigment, and texture were observed. The vegetative and reproductive mycelium characteristics, including hyphae, conidia, and conidiogenous cells, were examined. Micromorphological observations were carried out using a light microscope (Leica® DM500) on mounts prepared in lactophenol [37,38].

After cultivation, the fungi were inactivated and extracted with ethyl acetate (50 mL) under stirring for 24 h, then vacuum filtered (total volume of 150 mL). The filtrate was dried in a rotatory evaporator under low pressure at 40°C and the crude extracts were obtained [25].

2.4. Data Acquisition by UHPLC-HRMS/MS and Molecular Networking

Each extract was solubilized in methanol (1 mg/mL), filtered in a 0.45 μm syringe filter (Milipore PTFE, 13 mm, 22 μm – Allcrom, São Paulo, Brazil) and analyzed by UHPLC-HRMS/MS. Analysis conditions are in mobile phase gradient mode (A) H_2O + 0.01% formic acid and (B) methanol, flow rate 300 μL/min: 0-3 min: 2% B; 3-18 min from 2% to 100%; 18-21 min: 100% B constant; 21-25 min returns to the initial condition of 2% B; 25-28 min. with 2% B for column reconditioning, using a reverse phase column (Kinetex EVO C18, 100 mm x 2.1 mm, 2.6 μm, 100 Å) at a constant temperature of 50°C.

The files generated in the UHPLC-HRMS/MS analyses were converted from the original ".d" format to the open ".mzXML" extension for the construction of molecular networking. The newly converted files were checked by SeeMS software (Proteowizard®). The ".mzXML" files were uploaded to the Global Natural Products Social Molecular Networking (GNPS) platform server using WinSCP software [39]. A molecular network was created using the online workflow on the GNPS platform (http://gnps.ucsd.edu) based on MSCluster algorithm [40]. Data were filtered by removing all ions from MS/MS fragments within +/- 17 Da of the *m/z* precursor. MS/MS spectra acquired on a quadrupole hyphenated time-of-flight (QTof) type mass analyzer were filtered by picking only the top 6 fragment ions in the +/- 50Da window across the spectrum. The precursor ion mass tolerance was set to 0.02 Da and the MS/MS fragment ion tolerance to 0.02 Da. A network was created where edges were filtered to have a cosine score above 0.7 and more than 6 corresponding peaks. In addition, edges between two nodes were kept in the network if and only if each of the nodes appeared in the top 5 most similar nodes to each other. Finally, the maximum size of a molecular family was set to 100, and the lowest-scoring edges were removed from molecular families until the molecular family size was below this threshold. The network spectra were then searched against the GNPS spectral libraries and for dereplication, the library spectra were filtered in the same way as the input data. All matches maintained between the network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks [39]. Clusters detected in blank were removed from the networking, which includes spectral data from solvents used in the extraction and chromatographic procedures (dried hexane, chloroform, ethyl acetate, methanol). The molecular networking view and edition were performed in Cytoscape version 3.10.2 [41].

2.5. Chromatographic Fractionation and ISOLATION of compounds

The extract obtained from *Hypomontagnella barbarensis* - CPFF41 was selected from profile in molecular networking for further purification. So, part of the extract (150 mg) was subjected to HPLC-DAD in semi-preparative RP-18 in H_2O with 0.01% formic acid (A) and methanol (MeOH) (B) in gradient mode: 0 min.: 40% B; 7 min.: 15% B; 9 min.: 85% B and gradually returns to the initial condition up to 10 min and oven temperature at 45°C (flow rates 1.8 mL/min, UV 255 nm) to afford compound **1** (25 mg) and compound **2** (8 mg). These compounds were then analyzed by NMR and HRMS-MS for structural identification.

2.6. Antimicrobial Activity Assay - Strains and Growth of Microorganisms in Solid Culture

The antimicrobial activity assays of the extracts were evaluated against both yeast strains (Table 1) and bacterial strains (Table 2).

Table 1. Yeast strains used in yeast inhibitory activity tests.

Species	Cell line
Cryptococcus neoformans	KN99α (serotype A)
Cryptococcus neoformans	JEC21 (serotype D)

Cryptococcus gattii	NIH312 (serotype C)
Cryptococcus gattii	R265 (serotype B)
Saccharomyces cerevisiae	BY4647
Candida krusei	Clinical isolate 9602
Candida parapsilosis	Clinical isolate 68
Candida albicans	CBmay 560
Candida tropicalis	ATCC 1303
Candida dubliniensis	ATCC 7876

Table 2. Bacterial strain used for disc diffusion and minimum inhibitory concentration assays.

Species	Cell line
Enterococcus faecium	ATCC CCB076
Pseudomonas aeruginosa	ATCC 27853
Klebsiella pneumoniae	ATCC 700603
Escherichia coli	ATCC 25922
Shigella flexneri	ATCC 12022
Salmonella enterica	ATCC 14028
Staphylococcus aureus	ATCC25923
Acinetobacter baumanii	ATCC 19606

The cultures were performed from the inoculum of the colonies isolated from the strains of interest in standard medium for YPD yeasts (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) and Luria–Bertani medium (Bio basic inc. S516), containing 25 mL of culture medium in each Petri dish. Temperature and growth time ranged from 24 to 48 h and from 30 to 37 ° C, depending on experimental planning. Eugenol (Sigma Aldrich, ST Louis, USA) was used as positive control for yeasts and kanamycin 50 μg/mL and the same concentration for ampicillin (AppliChem) as positive control for bacteria.

2.7. Disc Diffusion Method

The disc diffusion method to assess the antimicrobial susceptibility of the microorganisms was performed following the CLSI protocol (M2-A8) [42]. The microorganisms were cultured in LB (bacteria) or YPD (yeast) 24 to 48 h before. The inoculum was made by picking 3 colonies and transferred to a test tube containing 5 mL of 0.9% saline solution. The suspension was adjusted cell density similar to the Mcfarland scale of 0.5 (corresponds to 1.5×10^8 cells per mL). Subsequently, with a sterile swab, the solution was spread evenly under the plate containing Mueller Hinton for bacteria or Mueller Hinton (BD 212322) supplemented with 2% D-glucose + 0.5 μL methylene blue for yeasts. Then, sterile filter paper discs of 5 mm diameter that received 5 to 10 μL of the compounds of interest with a final concentration ranging from 200 μg to 400 μg, a positive control was also placed (disc containing 200 μg/disc of eugenol for yeasts or kanamycin 50 μg/disc for bacteria) and a negative control (disc containing water). The plates were incubated in a bacteriological oven at 30°C for 24 to 48 h depending on the microorganism cultivated, after which the halos were measured and the data duly recorded. The results are presented as mean of three biological replicates and statical analysis (mean comparation) were performed using the PrismGraph version 8.0.

2.8. Minimum Inhibitory Concentration (MIC)

Broth microdilution assays were performed in a 96-well plate for bacteria using the EUCAST (European Committee on Antimicrobial Susceptibility Testing) method [43]. Briefly, the microorganisms were grown on a solid LB medium and incubated for 24 h at 37°C. Three fresh colonies were used and transferred to 5 mL of saline solution (0.9% NaCl), this cell suspension was adjusted to a cell density of approximately $1-2 \times 10^8$ x CFU/mL (0.5 McFarland scale). The extracts were diluted in water and adjusted to an initial concentration of 0.0007 μg/μL, then a serial dilution

was made, and the extracts were tested, the growth control contained only 100 μL of control contained only 100μL of the liquid culture, and the negative control was made with 100 μL of the sterile RPMI medium the 96–well plates were incubated at 30°C for 24 h, after this period the optical density (0.0 μL) was measured. During this period, the optical density (0.23-0.27) was determined in a microplate reader (Epoche 2 from Biotek). After reading the OD530, 50 μL of a 0.005% resazurin solution was added to check the microorganisms' viability, and the plate was again incubated on a shaker at a temperature of 30°C for 2 h. Experiments were conducted with two technical replicates and in biological triplicates.

3. Results

3.1. Endophytic Fungi Obtained from Leaves

Seven endophytic fungi (designated as CPFF12, CPFF14, CPFF16, CPFF41, CPFF42, CPFF52 and CPFC2) were isolated from *Calea pinnatifida* leaves (Figure S1- Supplementary Material). Five different genera were identified through macro and microscopic visualization of conidiophores (reproductive structures), hyphae, and growth aspects (Figure S2 A-E - Supplementary Material). Furthermore, molecular analyses using sequences obtained from ITS region and fragment of the *TUB2* gene allowed the identification of the genus *Colletotrichum* for three isolates, CPFF16 being *Colletotrichum siamense* (Figure S2C -- Supplementary Material) and CPFF12 / CPFF14 as *Colletotrichum karstii* (Figure S2A and S2B - Supplementary Material), having been confirmed as identical strains only after molecular analysis. Another species found for CPFF41 was *Hypomontagnella barbarensis* (Figure S2D - Supplementary Material), segregated from the *Hypoxylon* group by a polyphasic taxonomic approach [44], CPFF42 was identified as *Neopestalotiopsis clavispora* (Figure S2E - Supplementary Material), a species inserted in the recently known genus within the *Pestalotiopsis* group. CPFF52 was found to be *Nigrospora sacchari-officinarum* (Figure S2F - Supplementary Material), and finally, CPFC2 was identified as *Annulohypoxylon moriforme* (Figure S2G - Supplementary Material). The database used to identify endophytic fungi was the NCBI platform, using the BLAST tool, and the sequences obtained were deposited in GenBank (Table 3). The blast of the nucleotide sequence of endophytic fungi are in Table S1 - Supplementary Material.

Table 3. Species identification of isolates based on sequences obtained from ITS region and fragment of the *TUB2* gene.

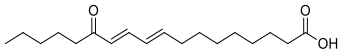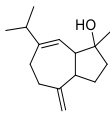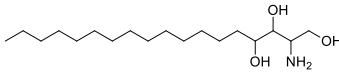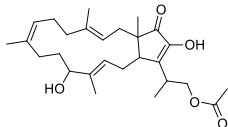| Strain Number	Species	% Identity*			
		ITS	Cod.	*TUB2*	Cod.
CPFF12	*Colletotrichum karstii*	100	PP831945	100	PP840075
CPFF14	*Colletotrichum karstii*	99.39	PP829193	99.72	PP840076
CPFF16	*Colletotrichum siamense*	100	PP829196	99.60	PP840077
CPFF41	*Hypomontagnella barbarensis*	99.23	PP829195	93.24	PP840078
CPFF42	*Neopestalotiopsis clavispora*	100	PP829194	99.09	PP942538
CPFF52	*Nigrospora sacchari-officinarum*	99.08	PP832015	100.00	PP840079
CPFC2	*Annulohypoxylon moriforme*	98.50	PP834406	98.09	PP840080
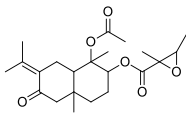
* Blast NCBI.

3.2. Dereplication Based on Molecular Networking Organization from HRMS-MS Data

The crude EtOAc extracts obtained from the seven endophytic fungi isolated from *C. pinnatifida* were analyzed by UHPLC-HRMS/MS and, subsequently, dereplicated using the GNPS platform, to generate molecular networking and to make annotations of secondary metabolites (Figure S3A and B - Supplementary Material). The dereplication process showed, for the analyzed extracts, a total of 5 library matches in positive ionization mode and annotations made by GNPS suggest 3 compounds from terpenoid class, Fusaproliferin (cembrane diterpene), Alismol (guaiane sesquiterpene) annotated for the *C. karstii* and *N. sacchari-officinarum* and 1-Acetoxy-7-isopropylidene-1,4a-dimethyl-

6-oxodecahydro-2-naphthalenyl-2,3-dimethyl-2-oxiranecarboxylate (eudesmane sesquiterpene), annotated for the *Colletotrichum* genus (Table 4).

**Table 4.** Annotation of compounds on GNPS spectral library match.

	Compound name	Molecular formula	Calculated mass	Precursor	Error (ppm)	Sample	Structural formula
1	1-Acetoxy-7-isopropylidene-1,4a-dimethyl-6-oxodecahydro-2-naphthalenyl 2,3-dimethyl-2-oxiranecarboxylate	$C_{22}H_{32}O_6$	415.2097 [M+Na]$^+$	415.2097	0.1	CPFF12 CPFF16	
2	13-keto-9Z,11E-octadecadienoic acid	$C_{18}H_{30}O_3$	295.2273 [M+H]$^+$	295.2275	0.7	CPFF41 CPFF42	
3	Alismol	$C_{15}H_{24}O$	203.1799 [M+H]$^+$	203.1797	1.0	CPFF14 CPFF52	
4	Phytosphingosine	$C_{18}H_{39}NO_3$	318.3008 [M+H]$^+$	318.3000	2.6	CPFF12 CPFF16 CPFF52	
5	Fusaproliferin	$C_{27}H_{40}O_5$	445.2954 [M+H]$^+$	445.2949	1,1	CPFF12 CPFF14	

Furthermore, considering that no known compounds were annotated by GNPS from the extract from *Hypomontagnella barbarensis* (CPFF41), besides fatty acid derivative (Table 4), the molecular networking of this extract was analyzed in detail. Thus, molecular networking for this extract showed a cluster in which the major compound at *m/z* 309.134 was observed, in agreement with the node size in the molecular network, indicating a more significant number of detected spectra (Figure 1). This compound (**1**) was then isolated in a more substantial proportion from the *H. barbarensis* extract and identified as *E/Z* Nodulisporacid A (see item 3.3) [45]. In addition to that, other nodes referring to analogous compounds were observed in the cluster, which includes the methyl ester of Nodulisporacid A at *m/z* 323.147 ([M+H]$^+$) (compound **3**), already described in the literature [45]; a derivative of Nodulisporacid A with one less methyl at *m/z* 295.116 ([M+H]$^+$), reported here for the first time, for which the name *nor*-Nodulisporacid A is proposed (compound **4**); and the compound at *m/z* 291.123 ([M+H]$^+$) whose proposed structure includes an additional furane ring, Nodulisfuranol. The additional furan ring is proposed based on a biogenesis hypothesis involving

the nucleophilic attachment of a hydroxyl group at C-3 (formed from the enolic tautomer of Tetronic acid A) to the C-6 position of the carboxylic acid, followed by dehydration. This last derivative, Nodulisfuranol - compound **2**, was isolated from the extract, and its structure was determined by NMR (see section 3.3) and HR-ESI-MS-MS² (Figures 1 and 3).

The structures **1** to **4** share similar MS² spectra, as observed in the molecular networking (Figure 1). Based on this observation, fragmentation proposals were put forth to corroborate and complement their structural similarity and annotation, considering characteristic and complementary fragmentation mechanisms. In the fragmentation spectra of structures **1**, **3**, and **4**, dehydration of the carboxylic acid (**1** and **4**) or elimination of MeOH from the methyl ester (**3**) was observed, resulting in the loss of 18 Da (H_2O) or 32 Da (MeOH), respectively, corresponding to mechanism **(a)** in the Figure 2. Another pathway, which combines mechanisms **(a)** and **(b)**, leads to concurrent loss of H_2O or MeOH along with the McLafferty rearrangement (loss of 70 Da, C_5H_{10}) in structures of **1**, **3** and **4**. This rearrangement occurs due to the deprotonation of the methyne carbon C-6′ in the side chain by the nucleophilic attach of π electrons of the dihydrofuran ring, resulting in an expected loss of 70 Da in structures **1**, **3**, and **4**. Additionally, based on the proposed alkene loss mechanism (70 Da), it was suggested that structure **4** lacks a methyl group at position C-10′ to maintain similarity with structures **1** and **3**. Consequently, structure **4** has no a stereogenic center at position C-4′. Furthermore, via pathway **(a)**, can occur a loss of C_2H_2O (42 Da), leading to fragments with *m/z* ratios of 249 (compounds **1** and **3**) and *m/z* 235 (compound **4**). Subsequently, the intermediate ion can lose 28 Da in the form of CO, supporting an alternative pathway to form ions with ratios of *m/z* 221 (compounds **1** and **3**) and *m/z* 207 (compound **4**) (Figure 2). Finally, the compound **2** exhibits a base peak with an *m/z* ratio of 221, corresponding to the previously proposed McLafferty rearrangement mechanism for structures **1**, **3**, and **4**. The additional furane ring also corroborate to the less fragmentation pattern in comparison to the structures **1**, **3** and **4** (Figure 1 and 3).
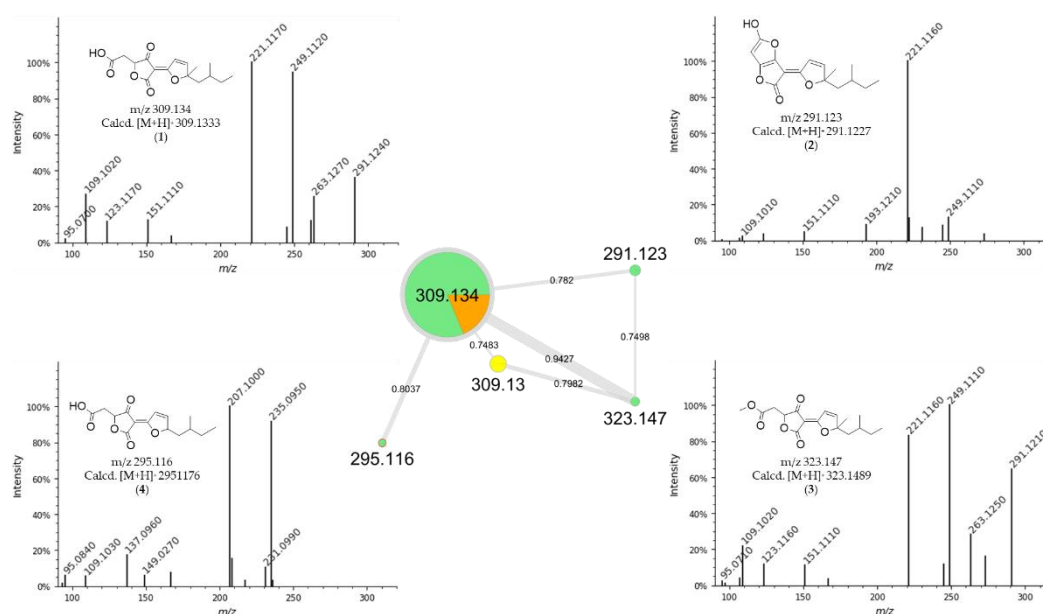
Figure 1. Consensus MS² mass spectra and Molecular network created by GNPS. Colors of the nodes: Green: CPFF41; Orange: CPFF42; Yellow: CPFF52. Edge labels between nodes represent *m/z* differences. Node labels represent parent masses (*m/z*). Edge labels between nodes represent cosine scores. The size of the nodes is proportional to the spectral detection rate.
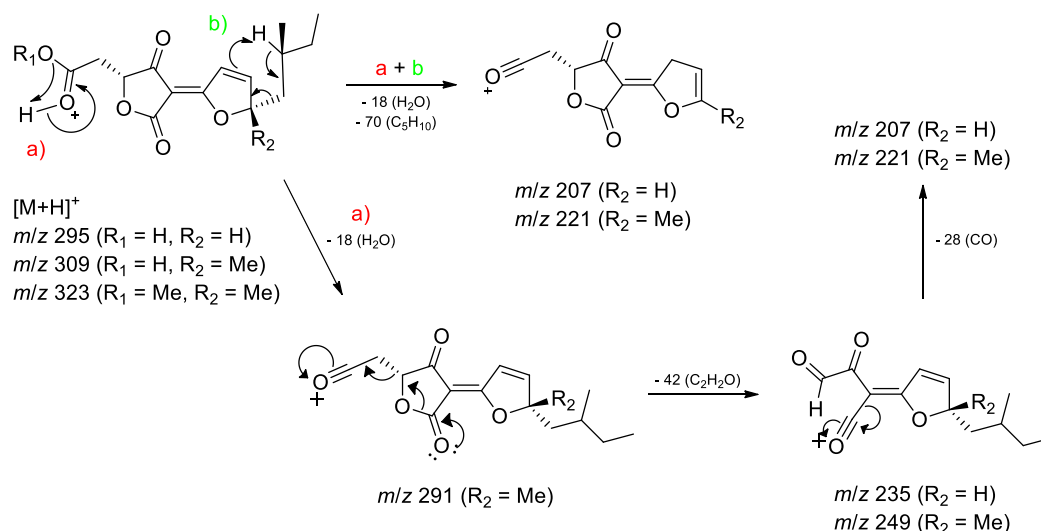
Figure 2. Fragmentation proposes for compounds **1** (m/z 309), **3** (m/z 323) and **4** (m/z 295) in ESI-(+)-QTof.
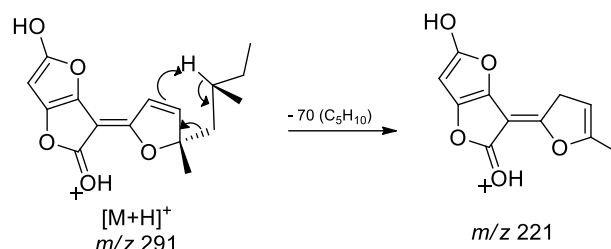
**Figure 3.** Fragmentation proposes for compound **2** (m/z 291) in ESI-(+)-QTof.

3.3. Identification of Compounds from Fungi Hypomontagnella barbarensis

Considering a molecular family of Tetronic acid derivatives and the annotations corresponding to Nodulisporacid A and three other derivatives (Figure 1), associated with antimicrobial activity evaluation with lower values of MIC against *S. aureus*, the extract from *H. barbarensis* was then selected for chromatographic fractionation. Thus, purification by HPLC allowed the isolation of compound **1** as a pair of isomers (*E* and *Z*). The compound was subjected to ^{1}H and ^{13}C NMR and UHPLC-HR-MS/MS analyses for structural elucidation. The ^{13}C NMR data indicated the presence of 32 carbon signals, including six methyl groups, six methylenes, four methines, and twelve fully substituted carbons. The carbon resonance at δ 170.7/172.6 was attributed to the carboxylic function. Signals were also assigned to an ester (δ 163.9/164.0) and an α,β-unsaturated ketone (δ 196.9/198.3). Other signals included a 1,2-disubstituted olefin unit (δ 123.0/123.1 and 164.0/164.1) and fully substituted carbons (δ 180.9/181.7, 103.5/104.1, and 94.5/94.9). Thus, the framework of this compound contains a furandione and a furylidene fragments connected by a double bond characteristic of Tetronic acid derivatives. Additionally, both *E* and *Z* isomers were observed for the double bond, and the *E*- and *Z*-isomers were interconverted spontaneously and reached an equilibrium state at a *ca*. ratio of 1:1. Therefore, the data were consistent with the structures diastereoisomers *Z* and *E* of Nodulisporacid A (Figure 4) and confirmed by comparison of NMR data with those reported in the literature [46]. For compound **2**, the ^{13}C NMR showed the same skeleton containing sixteen carbon signals duplicated, also revealing spontaneous inter-conversion between *E*- and *Z*-isomers, with the ratio of 1:1. The difference to compound **1** is the absence of a ketone carbonyl signal, indicating that the tetronic ring could be in the enolic form, which may have favored the attack of the enol hydroxyl on the carboxyl leading to the formation of a new furan ring after dehydration in this second ring, evidenced by signals at δ 5.67/5.68 (*s*, H-5) referring to the two E/Z stereoisomers in ^{1}H-NMR. These signals, in the HSQC spectrum presents a cross peak with shifts at 98.3/98.4 (C-5). Furthermore, it is

possible to observe the cross peaks in the HMBC spectrum between H-5 with signals at δ 153.2/153.6 (C-3), in addition to the signal at δ 182.3/182.5 (C-4). The structure with the additional furan ring was also suggested based on the analysis of the mass spectrum that showed [M+H]$^+$ at *m/z* 291.1232 corresponding to the molecular formula of $C_{16}H_{18}O_5$ indicating the presence of 8 unsaturations. Thus, the proposed structure is a derivative of compound **1** and, being the first time reported in the literature, the name Nodulisfuranol is proposed (Figure 4).

Compound **1**: Nodulisporacid A (*E:Z* mixture); yellow amorphous solid; *Z*-isomer: ^{1}H NMR (MeOH-d4, 300 MHz) δ 4.84 (*m*, H-4), 2.87 (*m*, H-5a), 2.73 (*m*, H-5b), 7.32 (*d*, J = 5.7 Hz, H-2'), 7.81 (*d*, *J* = 5.7 Hz, H-3'), 2.06 (*m*, H-5' a), 1.65 (*m*, H-5' b), 1.25 (*m*, H-6'), 1.25 (*m*, H-7'a), 1.14 (*m*, H-7'b), 0.78 (*t*, J = 7.0 Hz, H-8'); 0.85 (*d*, J = 6.1 Hz, H-9'); 1.54 (*s*, H-10'). ^{13}C NMR (MeOH-d4, 75 MHz) δ 172.5 (C, C-1), 94.9 (C, C-2), 198.3 (C, C-3), 80.7 (CH, C-4), 36.8 (CH$_2$, C-5), 173.1 (C, C-6), 181.6 (C, C-1'), 123.1 (CH, C-2'), 164.1 (CH, C-3'), 103.4 (C, C-4'), 45.2 (CH$_2$, C-5'), 31.3 (CH$_3$, C-6'), 31.5 (CH$_2$, C-7'), 11.4 (CH$_3$, C-8'); 21.3 (CH$_3$, C-9'), 23.8 CH$_3$, C-10'). *E*-isomer: ^{1}H NMR (MeOH-d4, 300 MHz) δ 4.87 (*m*, H-4), 2.93 (*m*, H-5a), 2.79 (*m*, H-5b), 7.47 (*d*, J = 5.8 Hz, H-2'), 7.83 (*d*, J = 5.7 Hz, H-3'), 2.12 (*m*, H-5'a), 1.72 (*m*, H-5'b), 1.25 (*m*, H-6'), 1.25 (*m*, H-7'a), 1.14 (*m*, H-7'b), 0.77 (*t*, J = 7.0 Hz, H-8'); 0.80 (*d*, J = 6.1 Hz, H-9'); 1.54 (*s*, H-10'). ^{13}C NMR (MeOH-d4, 75 MHz) δ 170.7 (C, C-1), 94.5 (C, C-2), 196.8 (C, C-3), 80.3 (CH, C-4), 36.7 (CH$_2$, C-5), 172.9 (C, C-6), 180.9 (C, C-1'), 123.0 (CH, C-2'), 163.9 (CH, C-3'), 103.4 (C, C-4'), 45.1 (CH$_2$, C-5'), 31.3 (CH, C-6'), 31.4 (CH$_2$, C-7'), 11.4 (CH$_3$, C8'), 21.2 (CH$_3$, C-9'), 23.7 (CH$_3$, C-10').

Compound **2**: Nodulisfuranol (*E:Z* mixture); yellow amorphous solid; *Z*-isomer: ^{1}H NMR (MeOH-d4, 300 MHz) δ 5.67 (*s*, H-5), 7.25 (*d*, J = 5.8 Hz, H-2'), 7.86 (*d*, J = 5.8 Hz, H-3'), 2.01 (*m*, H-5'a), 1.60 (*m*, H-5'b), 1.15-1.19 (*m*, H-6'), 1.15-1.19 (*m*, H-7'a), 1.04 (*m*, H-7'b), 0.74 (*t*, J = 7.0 Hz, H-8'), 0.78 (*d*, J = 6.0 Hz, H-9'), 1.47 (*s*, H-10'), 7.87 (*s*, OH). ^{13}C NMR (MeOH-d4, 75 MHz) δ 166.1 (C, C-1), 92.5 (C, C-2), 153.2 (C, C-3), 182.3 (C, C-4), 98.3 (C, C-5), 165.6 (C, C-6), 182.4 (C, C-1'), 123.1 (CH, C-2'), 163.6 (CH, C-3'), 104.7 (C, C-4'), 44.9 (CH$_2$, C-5'), 31.3 (CH, C-6'), 31.5 (CH$_2$, C-7'), 11.4 (CH$_3$, C-8'), 21.1 (CH$_3$, C-9'), 23.4 (CH$_3$, C-10'). *E*-isomer: ^{1}H NMR (MeOH-d4, 300 MHz) δ 5.68 (*s*, H-5), 7.40 (*d*, J = 5.7 Hz, H-2'), 7.89 (*d*, J = 5.7 Hz, H-3'), 2.03 (*m*, H-5'a), 1.68 (*m*, H-5'b), 1.15-1.19 (*m*, H-6'), 1.15-1.19 (*m*, H-7'a), 1.04 (*m*, H-7'b), 0.74 (*t*, J = 7.0 Hz, H-8'), 0.79 (*d*, J = 6.0 Hz, H-9'), 1.50 (*s*, H-10'), 7.90 (*s*, OH). ^{13}C NMR (MeOH-d4, 75 MHz) δ 167.9 (C, C-1), 93.0 (C, C-2), 153.6 (C, C-3), 182.5 (CH, C-4), 98.4 (C, C-5), 165.9 (C, C-6), 182.5 (C, C-1'), 123.2 (CH, C-2'), 163.9 (CH, C-3'), 105.4 (C, C-4'), 45.0 (CH$_2$, C-5'), 31.4 (CH$_3$, C-6'), 31.5 (CH$_2$, C-7'), 11.4 (CH$_3$, C-8'), 21.2 (CH$_3$, C-9'), 23.5 (CH$_3$, C-10').
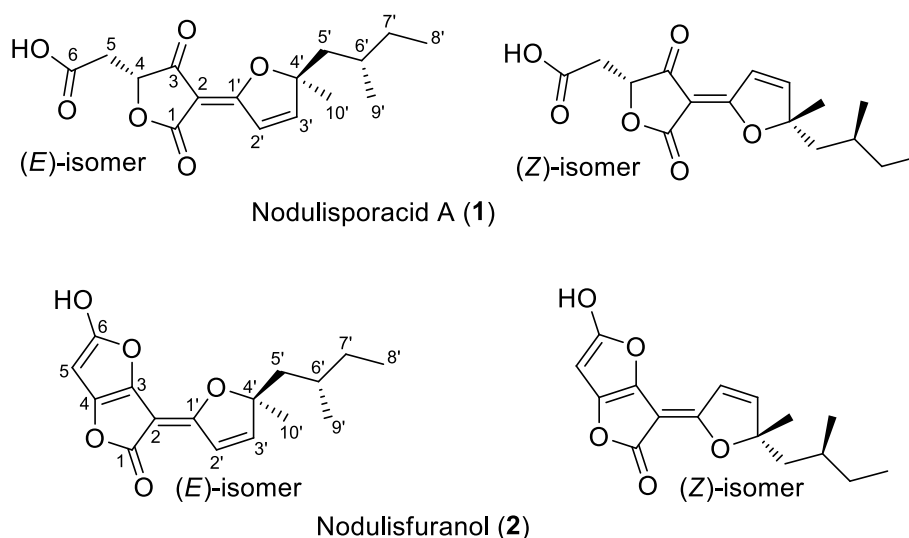
Figure 4. Structures of the *Z* and *E* isomers of Nodulisporacid A (**1**) and Nodulisfuranol (**2**).

3.4. Antimicrobial Activity

Initially, disk diffusion assays were conducted with the yeasts and bacterial strains listed in Table 1 and 2 in order to screen fungal extracts for antimicrobial activity. The CPFF14 and CPFF41 extracts inhibited the *S. aureus* strain and CPFF12 extract proved promising against two yeasts strains, *S. cerevisiae* and *C. albicans* in disk diffusion assay. The other extracts evaluated did not show significant inhibition values (Figure 5 and Table 5).
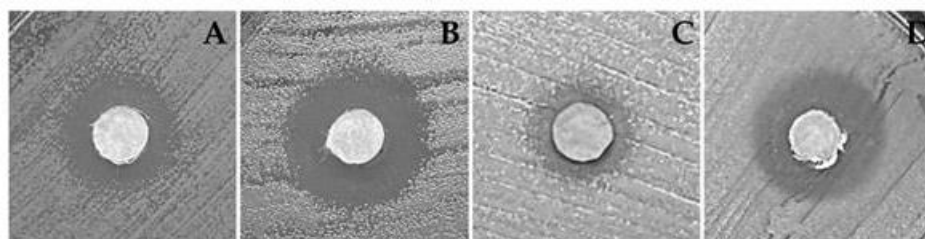
Figure 5. Evaluation of antimicrobial activity of crude extracts (CPFF12 and CPFF14) by disc diffusion test. Inhibition halo formed by the crude extract CPPF12 against A) *C. albicans*; B) *S. cerevisiae*, crude extract CPPF12 against C) *S. aureus* and crude extract CPPF41 against D) *S. aureus*.

Table 5. Disk diffusion method (DDM) – Zone of inhibition (mm).

Extract (400 μg)	Species (lineage)		
	S. cerevisiae (BY4647)	*C. albicans* (CBmay 560)	*S. aureus* (ATCC 25923)
CPFF12	1.4 ± 0.26	1.23 ± 0.25	n/a
CPFF14	n/a	n/a	0.73 ± 0.23
CPFF41	n/a	n/a	1.37 ± 0.32
Control*	2.07 ± 0.12[a]	2.07 ± 0.12[b]	2.5 ± 0.0[b]

* [a]EEugenol (200 μg); [b]Kanamicin (50 μg).   n/a – not active.

In sequence, the species listed in Table 1 and 2 were tested to determine the minimum inhibitory concentration (MIC). The results showed that yeasts were more sensitive to the extract CPFF12 (*C. karstii*), while the bacteria *S. aureus* was more sensitive to the extracts CPFF14 and CPFF41.   The *H. barbarensis* (CPFF41) extract showed 90% growth inhibition at 0.05 mg/mL (Table 6). Other extracts also had no significant activity against microbial strains.

Table 6. Minimum inhibitory concentration *(MIC)*.

Specie	Extract - MIC90 (mg/mL)			Control*
	CPFF12	CPFF14	CPFF41	
S. aureus (ATCC25923)	n/a	0.20 (91.6±1.5)	0.05 (92.0±1.0)	0.004[a]
C. albicans (CBmay 560)	0.01 (92.0±1.9)	n/a	n/a	0.025[b]
S. cerevisiae (BY4647)	0.02 (90.3±1.5)	n/a	n/a	0.013[c]

*[a] Vancomycin; [b] Chloramphenicol; [c] Fluconazole. n/a – not active.

4. Discussion

Asteraceae family, to which *C. pinnatifida* belongs, has common or cosmopolitan endophytic species [47]. The genera found, *Colletotrichum*, *Nigrospora,* and *Pestalotiopsis*, according to Caruso [9] and Nicoletti [48], were isolated from most plant species in this family, while the genus *Xylaria*, was observed in a lower percentage. Also, these fungi were described as phytopathogens, a diverse group of endophytic fungi that can affect a wide range of economically important crops, including fruit, vegetables, ornamental plants, and grasses, and some species in this group have also become the object of study for biotechnological applications, seeking new bioactive compounds that can be used in new environments [49].

From C. pinnatifida were isolated 3 species of endophytic fungi from the Colletotrichum genus including C. siamense and C. karstii, besides species of Hypomontagnella barbarensis, Neopestalotiopsis clavispora, Nigrospora sacchari-officinarum and Annulohypoxylon moriforme. These genera of endophytes have already been described as endophytes of other species and produce metabolites of interest, such as Colletotrichum gloesporioides, which has stood out in studies in terms of the production of toxic metabolites and promising biological activity [50]. Wang [51] reported that Pestalachlorides E and F, isolated from fungi of the genus Pestalotiopsis, showed potent agronomic activity, which was also reported by Yang [52], regarding the isolation of the anticancer Taxol present in the species Pestalotiopsis microspora. Other compounds with interesting activities come from the Nigrospora genus, ranging from herbicides to insecticides and antimicrobials [53–56]. These secondary metabolites have demonstrated a wide range of biological activities, such as antifungal, antibacterial, antiviral, antitumor, and cytotoxic properties [57–59]. Hypoxylaceae family was recently elevated from Xylariaceae, representing one of the predominant groups of fungal endophytes. Furthermore, from this family, it was identified the species Hypomontagnella barbarensis, a new genus of the Hypoxylaceae family, segregated from Hypoxylon by a polyphasic taxonomic approach [44]. Regarding to chemical composition of extracts, some metabolites, already described in the literature, isolated from other organisms were annotated by the platform, such as Fusaproliferin, a compound with anti-inflammatory action, isolated from Fusarium proliferatum [60] (Figure S3A Supplementary Material and Table 4).   Most of these bioactive compounds are derived from the secondary metabolism of these organisms, through which they generate compounds belonging to the most diverse chemical classes, such as terpenes, alkaloids, polyketides, and peptides, among others [61]. The molecular networking analysis also allowed the dereplication of a molecular family of Tetronic acid derivatives. It made possible the annotation of derivatives from H. barbariensis, which drives chromatographic fractionation. Furthermore, compound **1**, isolated from H. barbariensis, identified as two isomers (E and Z) of Nodulisporacid A, has been characterized by Kasettrathat et al [62], and it has already been reported by Sumiya et al. [63] being isolated from the marine fungi Nodulisporium sp. and by Huang et al., [45] isolated from an endophytic fungus Hypomontagnella monticulosa, belonging to the same genus as the fungus found. This compound has been tested for its bioactivity and cytotoxicity, showing moderate activity against cancer cell lines developed by Kasettrathat et al. [63] and moderate antimalarial activity by Huang et al [45]. However, compound **2,** which was isolated from the same extract, was identified as a derivative of Nodulisporacid A named Nodulisfuranol, and has not yet been described in the literature, suggesting that it is a new substance, as well as compound **4** (nor-Nodulisporacid A) of m/z 295 annotated in the Nodulisporacid A cluster.

The antimicrobial activity of the endophytic fungal extracts was evaluated by disk diffusion and MIC assays against 8 bacterial strains including *S. aureus*, and 10 yeast strains. However, only the extracts of *C. Karstii* and *H. barbarensis* were active against, at least, one of the strains, with the extract of *H. barbarensis* being active against *S. aureus*. It was also demonstrated that this extract is composed of Noduliporacid A and the triple-bonded derivative called Nodulisfuranol. Considering that Nodulisporacid A has already been evaluated against *S. aureus* in the work of Huang et al [45], and that it showed no activity, it can be suggested that the activity observed for the CPFF41 extract may be due to the presence of the derivative, or even due to the synergistic action between the two compounds.

Furthermore, combining the data from the GNPS annotations and the microbiological assays, we can suggest that the presence of the annotated compounds, such as Fusaproliferin and alismol, is related to the antimicrobial activity of the extracts. For the CPFF12 extract, was observed the annotation of these two compounds, already described as active and with cytotoxic/antimicrobial action [64,65]. They may justify the inhibition, especially of *C. albicans*, with a low extract concentration. This makes it clear that synergy between compounds can occur, and thus, the activity can be associated with more than one compound.

Due to these promising activities, knowing the metabolic profile of these individuals was of great interest. The presence of compounds already described as active, and the possibility of isolating

new ones leaves open the opportunity for new discoveries. Additionally, considering the large number of secondary metabolites that fungi might contain, dereplication tools such as LC-MSMS and molecular networking, have become important in prospecting metabolites, saving time and work on isolation and characterization of natural products. However, whereas genome analyses have revealed a great diversity of biosynthetic gene clusters (BGCs) that far exceeds the number of known substances, most of its secondary metabolites remain still unknown [66]. Thus, dereplication of the extracts from fungi, with the aid of GNPS to annotate metabolites, can be complemented with genomic analysis integrated with metabolomics, to identify gene clusters related to the biosynthesis of the annotated molecules, and thus can allow access full biosynthetic potential of the fungi.

5. Conclusions

Investigating endophytic fungi offers a promising avenue for discovering new bioactive compounds with potential pharmaceutical uses. Through the methods outlined in this study, we isolated 6 endophytic fungi from *C. pinnatifida* leaves, belonging to 4 distinct genera: *Colletotrichum*, *Neopestalotiopsis*, *Nigrospora*, *Hypomontagnella* and *Annulohypoxylon*. Although these genera are recognized for their phytopathogenic roles, they have also demonstrated biotechnological promise in producing bioactive metabolites. Additionally, our findings regarding chemical composition of extracts from fungi indicate that the molecular network approach based on GNPS showed to be a rapid and efficient tool to organize into clusters and annotate bioactive metabolites from a crude extract, not only based on the annotation of known molecules, but also for new ones, as Acyl tetronic acid derivatives suggested for the first time in the present study. Moreover, it was isolated two Tetronic acid derivatives identified by NMR, from the most active extract against *S. aureus*. Thus, this approach can be used for drug discovery. Remarkably, the array of bioactive compounds synthesized by these endophytic fungi underscores their potential as valuable reservoirs for new pharmaceuticals, agrochemicals, and industrial compounds. Overall, this study underscores the significance of investigating endophytic fungi associated with medicinal plants like *C. pinnatifida*. It provides a fertile ground for discovering new bioactive compounds with a wide range of pharmacological properties.

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