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Antibacterial and Antifungal Activity of Organic and Peptidic Extracts of Ecuadorian Endophytic Fungi

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Abstract

Antibacterial and antifungal activities of 32 organic and 14 peptidic extracts obtained from twelve endophytic fungi of the Collection of Endophytes Quito-Catolica were tested against the pathogenic bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli* and *Salmonella enteritidis*, and against the fungal oomycete *Pythium ultimum*. It was observed that the extracts of *Marasmiellus candidus* (CEQCA-O1113), *Xylaria laevis* (CEQCA-O1399), *Fusarium solani* (CEQCA-O1393), *Diaporthe helianthi* (CEQCA-O1394) and *Xylaria* sp. (CEQCA-O1400) partially or totally inhibited the microorganisms tested. The extracts CEQCA-O1399.P1 and CEQCA-O1113.D1 showed fungistatic and fungicidal effects against *P. ultimum*, respectively. Six extracts strongly inhibited *S. aureus*, and *E. coli* was strongly inhibited by the extract CEQCA-O1113.E2. Based on a bioassay-guided approach, the extracts with strong bioactivity in the antibacterial assays by an agar diffusion method were also tested by a disk diffusion method, and the minimal inhibitory concentrations were determined by a microdilution assay. The most bioactive extract, CEQCA-O1113.D1, was also purified by high performance liquid chromatography. The extract recovered its initial bioactivity against *S. aureus* in two fractions after the purification. Overall, the results of this study highlight the potential of the fungal endophytes as producers of bioactive compounds.

Keywords

Bioactive Compounds, Fungal Endophytes, Growth Inhibition Assays, Pathogenic Bacteria

1. Introduction

The ability of the microorganisms to synthesize a variety of compounds has attracted the attention of researchers to bioprospecting for natural products in bacteria, fungi and actinomycetes [1]. Current challenges such as emerging antibiotic-resistant bacteria, environmental degradation, severe fungal infections and the lack of treatment for diseases such as neglected tropical diseases and cancer could be overcome by compounds produced by microorganisms [2] [3] [4].

Endophytic fungi live within plant tissues asymptomatically and are synthesizers of chemical compounds that provide protection to the plant host against herbivores and pathogens [1] [2] [3] [5] [6]. The diversity of the endophytic fungi and the variety of compounds they can produce (alkaloids, terpenoids, aldehydes, esters, alcohols, etc.), are in close association with their ability to inhibit other organisms [7] [8]. Thus, fungal endophytes comprise an interesting source of compounds useful for diverse applications in medicine, agriculture and industry.

Ecuador's geography, which ranges from seacoast to arid mountains and to Amazonian jungle, provides a diversity of ecosystems that contain a uniquely high and underexplored biological diversity [9]. The dynamics of the ecological interactions among different taxonomic groups present at each ecosystem may enhance the production of natural products with high potential for bioactivity. Moreover, exploration of unique habitats has been shown to be an effective strategy [2]. The Amazonian forest in particular is touted as potentially the largest drug dispensary in the world [7] [10]. This potential of the Ecuadorian plants to host interesting fungal endophytes with diverse biotechnological applications inspired the creation of the Collection of Endophytes Quito-Catolica, CEQCA by its acronym. Currently, the Collection counts with around 4000 endophytic fungi isolated from plants collected in tropical rainforests, lowlands, and Andean ecosystems.

The aim of our study is to evaluate the antibacterial and antifungal activities of extracts obtained from a selection of twelve endophytic fungi stored at the CEQCA. This study constitutes an important basis for the knowledge of the potential of Ecuadorian endophytes for bioprospection of natural products, and also gives an added value to Ecuadorian plant biodiversity, promoting the conservation of both ecosystems and plants as hosts of novel endophytes with promising biotechnological applications.

2. Materials and Methods

2.1. Fungal Cultures

The fungi stored at the CEQCA were previously identified by comparing the consensus sequences of the internal transcribed spacer (ITS) regions 1 and 4 with the sequences available at the NCBI GenBank using the Basic Local Alignment Search Tool (BLAST). Twelve endophytic fungi of the CEQCA were selected for this study based mainly on two criteria: 1) potential as new species (identity percent < 95% with other sequences in BLAST or identification not resolved at least until genus after comparing the sequences in BLAST) and 2) bio-

activity of the fungal isolates reported in previous assays (Table 1). Two isolates (CEQCA-M1179 and CEQCA-M1214) did not meet the criteria but were included since they were not tested for bioactivity before. The fungi were cultivated on Potato Dextrose Agar (PDA) (Difco, USA) plates at 21˚C for seven days, and then upscaled to 1 L of Potato Dextrose Broth (PDB) (Difco, USA) until abundant mycelia was observed in culture.

2.2. Organic Extraction of Secondary Metabolites

The extraction of secondary metabolites with organic solvents was performed as suggested by Bascom-Slack *et al.* [11] with modifications. The mycelia were filtered through cheesecloth and then through Whatman N˚1 filter paper, and discarded. The broth was extracted twice with 300 mL of dichloromethane (DCM). The filtrate was collected and reserved. After the DCM extraction, the broth was extracted with ethyl acetate (EA) following the same procedure. Each fraction (DCM and EA) was concentrated separately either with an evaporator Büchi Rotavapor RII (Fischer Scientific, USA) or a GeneVac concentrator (Fisher Scientific, USA) in 2 mL glass vials. The extracts were resuspended in methanol and labelled accordingly with the letters D for DCM or E for EA and stored at +4˚C.

Table 1. Endophytic fungi selected for this study. Information of their plant hosts is included.

Fungal species	Fungus ID	Province of collection/ecosystem	Reference GenBank accession number	Identity percent in BLASTn	Bioactive in previous assays	Plant host	
						Family	Species
Periconia sp.	CEQCA-M1179	Manabi/ Coastal dry forest	KC771468	100%	No tested	Violaceae	Unknown
Virgaria boninensis	CEQCA-M1193	Manabi/ Coastal dry forest	KC771473	91%	Yes	Arecaceae	Unknown
Entonaema pallida	CEQCA-M1214	Manabi/ Coastal dry forest	KC771479	100%	No tested	Rubiaceae	*Simira* sp.
Xylaria sp.	CEQCA-M1240	Manabi/ Coastal dry forest	KC771483	100%	Yes	Mimosaceae	Unknown
Pleosporales sp. 1	CEQCA-O1055	Orellana/ Amazon rainforest	KC771506	100%	No	Piperaceae	*Piper* sp.
Pleosporales sp. 2	CEQCA-O1090	Orellana/ Amazon rainforest	KC771509	100%	No	Meliaceae	*Guarea kunthiana*
Ascomycota sp.	CEQCA-O1111	Orellana/ Amazon rainforest	KC771513	100%	Yes	Violaceae	*Corynostylis arborea*
Marasmiellus candidus	CEQCA-O1113	Orellana/ Amazon rainforest	MN173348	100%	Yes	Combretaceae	Unknown
Fusarium solani	CEQCA-O1393	Orellana/ Amazon rainforest	JX476948	100%	Yes	Malvaceae	*Herrania mariae*
Diaporthe helianthi	CEQCA-O1394	Orellana/ Amazon rainforest	AY746005	100%	Yes	Malvaceae	*Herrania mariae*
Xylaria laevis	CEQCA-O1399	Orellana/ Amazon rainforest	JX476949	100%	Yes	Malvaceae	*Theobroma cacao*
Xylariaceae sp.	CEQCA-O1400	Orellana/ Amazon rainforest	JX476952	100%	Yes	Malvaceae	*Theobroma cacao*

2.3. Extraction of Peptides

The protocol followed by Panizel *et al.* [12] was modified for the peptide extraction. The fungal liquid cultures were filtered, and the mycelia discarded. The broth was placed together with 50 g of Amberlite XAD-7HP beads (Acros Organics, USA) and was agitated overnight, after which the aqueous phase was discarded, and the beads were placed into a clean tray and washed three times with MilliQ water. The wet beads were placed over filter paper until they dried completely and then covered with methanol. After five minutes, the beads were mixed for ten minutes with a magnetic stirrer and the methanol was collected. At this point, the methanol presented a yellowish color. The beads washed until the methanol collected was completely transparent. All the methanol was concentrated with a Büchi Rotavapor RII evaporator (Fischer Scientific, USA). The extracts were resuspended in methanol and distilled water. A total of 14 peptidic extracts were obtained and labelled accordingly with the letter P to differentiate from EA and DCM extracts. The extracts were stored at +4˚C.

2.4. Thin Layer Chromatography (TLC) of Peptidic Extracts

Before testing the peptidic extracts against the selected microorganisms, a TLC was run to confirm the presence of peptides in the samples and to validate the extraction protocol previously performed. The mobile phase consisted in ethyl acetate, methanol, and distilled water in proportion 120:60:20. Baker-flex Silica Gel IB2-F plates (20 × 20 cm) (Fischer Scientific, USA) were used as stationary phase. The plate was heated for three minutes and the samples were loaded 1 cm above a reference line. The samples were let dry for approximately one minute before the run was performed. The samples ran until they reached two thirds of the plate. The plate was let dry and then sprayed with ninhydrin monohydrate (Sigma, USA) previously prepared in methanol (33.3 g in 1 L). After 15 minutes, purple spots were observed on the plate, confirming the presence of peptides in the samples [13].

2.5. Microorganisms Assayed

Assays were performed with human pathogenic bacteria *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Serratia marcescens* (ATCC 13880), *Escherichia coli* (ATCC 25922) and *Salmonella enteritidis* (ATCC 13076) as well as the fungal oomycete *Pythium ultimum* stored at the CEQCA.

2.6. Bioassays against *P. ultimum*

The bioassays against *P. ultimum* were performed as indicated by Bascom-Slack *et al.* [11] with modifications. *P. ultimum* was grown on PDA plates for three days at room temperature. Each plate was inoculated with two extracts, a negative control (methanol only) and a positive control (fungus only). A total of 10 µL of each extract were placed on the plates, applying 5 µL first, letting the extract dry and then applying the last 5 µL. Agar plugs (3 × 3 mm) of the three-

day-old *P. ultimum* cultures were cut and placed with the side of the mycelium directly touching the spot where the extracts were previously applied, and over the positive control spot. The plates were kept at room temperature for three days. The diameter of growth of *P. ultimum* in each plate was recorded daily. On the fourth day, those plugs that did not show any fungal growth were transferred to fresh PDA plates to verify if the extracts had a fungistatic or fungicidal effect over the fungus. The average of the growth diameter was expressed as growth percentage compared to the methanol control.

2.7. Antibacterial Assays

2.7.1. Agar Diffusion Method

The antibacterial activity of organic and peptidic extracts where first tested with an agar diffusion method modified from Bascom-Slack *et al.* [11]. Plates containing Luria-Bertani (LB) agar (Difco, USA) were marked and divided into four quadrants. Each plate was inoculated with two extracts, one methanol control and one bacterial growth control (no methanol or extract applied). The selected bacteria where grown on LB agar for 24 hours at 37˚C. A bacterial suspension was prepared in a 0.8% (w/v) NaCl solution, and the turbidity was adjusted to 0.5 MacFarland scale prior to the inoculation of the plates [14]. After that, 10 µL of the extracts were pipetted on the plates. The extracts were let dry and the adjusted bacterial suspensions were inoculated with cotton swabs. The plates were incubated at 37˚C for 18 hours. The antibacterial activity was detected by the formation of an inhibition zone on the spots were the extracts were applied. The antibacterial activity of the extracts was catalogued as total inhibition, partial inhibition or no inhibition, according to the growth of the bacteria observed on the plates.

2.7.2. Disk Diffusion Method

Only the extracts that showed total inhibition using the agar diffusion method were tested by the disk diffusion method. The disk diffusion method followed the recommendations given by the Clinical and Laboratory Standards Institute [15]. The bacteria were grown on Mueller-Hinton agar (MHA) (Difco, USA) for 18 hours at 37˚C. A bacterial suspension was prepared as described in section 2.7.1. [14]. Whatman diffusion disks of 6 mm of diameter (Sigma Aldrich, USA) were loaded with the extracts of interest. Each MHA plate contained two disks loaded with the extracts of interest, one disk loaded with methanol only and one empty disk (no methanol or extract loaded). The adjusted bacterial suspensions were inoculated evenly with cotton swabs and the disks onto the agar. The plates kept at 37˚C for 18 hours. The diameter of the inhibition zones was measured and recorded as the average of three replicates with their standard deviation.

2.8. Determination of the Minimal Inhibitory Concentration (MIC)

The MIC was only determined for those extracts that presented a total inhibition of the microorganisms tested in both agar diffusion and disk diffusion methods.

Briefly, the bacteria were grown on Mueller-Hinton agar (MHA) (Difco, USA) for 18 hours at 37˚C. A bacterial suspension was prepared as described in section 2.7.1 [14]. Eight two-fold dilutions of the extracts were prepared and added to polystyrene 96-well plates (Corning, USA) with 90 µL of Mueller-Hinton broth (MHB) (Difco, USA), 10 µL of the inoculum and 10 µL of each dilution [14] [16]. Additionally, three controls were prepared: 90 µL of MHB plus 10 µL methanol, 90 µL of MHB plus 10 µL of inoculum, and 90 µL of MHB plus 10 of µL inoculum and 10 µL of the antibiotic Gibco Penicillin Streptomycin (10,000 U/mL) (Thermo Fischer Scientific, USA). The plates were incubated at 37˚C for 18 hours and read with an iMark microplate reader (BioRad, USA) at 630 nm.

2.9. Purification of Bioactive Extracts

The extracts that showed partial and total inhibition in both agar diffusion and disk diffusion methods were purified by high performance liquid chromatography (HPLC), following the method suggested by Forcina *et al.* [17] with modifications. The analyses were performed in an HPLC system (Gilson, USA) located in the West Campus at Yale University, New Haven (USA). The extracts were filtered with 2 mL Millipore Ultrafree column filters (Merck Millipore, USA) by centrifugation at 3000 rpm, and at room temperature (21˚C) for one minute. A second centrifugation was performed by adding methanol to recover as much of extract as possible from the column filters. The filtered extract was transferred to a sterile glass vial and injected to the HPLC equipment. The HPLC run was performed using a Waters SunFire C18 column (5 µM; 20 × 250 mm) (Waters, USA). The elution was carried out using a gradient of 0% - 100% acetonitrile at a rate of 10 mL/min for 40 minutes, at 254 nm and 215 nm. The results were analysed with the software Trilution LC (Gilson, USA). The fractions that showed sharp peaks were collected and lyophilized overnight in a GeneVac concentrator (Fisher Scientific, USA). The bioactivity of the fractions was verified by a microdilution assay performed in polystyrene 96-well plates (Corning, USA). Briefly, 90 µL of media was added to each well, followed by 10 µL of bacterial inoculum previously adjusted to 0.5 MacFarland scale and 10 µL of the corresponding HPLC fraction. Each fraction was tested in triplicate. The plate was read by a microplate reader integrated with the Gen5 Microplate Reader and Imager software (BioTek, USA). The reading was performed every ten minutes for 12 hours at 600 nm, under continuous agitation at medium speed and under constant temperature (35˚C). The results were observed as bacterial growth curves generated by the software GraphPadPrism (GraphPad Software, USA).

2.10. Mass Spectrometry (MS) Analysis

The HPLC fractions that preserved their bioactivity after fractionation were sent to MS analysis to the Molecular Innovations Center in the West Campus at Yale University. The results were analyzed with the software MassHunter Workstation (Agilent Technologies, USA).

2.11. Statistical Analyses

The data obtained from the antifungal and antibacterial assays were analyzed by a one-way analysis of variance (ANOVA) and a Tukey test to determine statistical significance. A p-value of <0.05 was considered as statistically significant. The experiments were carried out with a completely randomized design with three replicates. The analyses were performed with the IBM SPSS Statistics software (IBM, USA).

3. Results

A total of 46 extracts were tested and 42 of them showed bioactivity against at least one of the microorganisms tested. All the ninhydrin monohydrate tests were positive, which confirmed the presence of peptides within the samples.

3.1. Antifungal Assays against *P. ultimum*

The extract of *Marasmiellus candidus* CEQCA-O1113.D1, and the peptidic extract of *Xylaria laevis* CEQCA-O1399.P1, significantly inhibited the growth of *P. ultimum* after the three days of the experiment (0% of growth, $p < 0.05$). The fungal agar plugs that were exposed to the extract CEQCA-O1399.P1 showed mycelial growth after being transferred to a fresh PDA plate (fungistatic effect), whereas the fungal agar plugs that were exposed to the extract CEQCA-O1113.D1 showed no mycelial growth on a fresh PDA plate (fungicidal effect).

3.2. Antibacterial Assays

By using the agar diffusion method, we observed that *S. aureus* was totally inhibited by six extracts, while only one extract totally inhibited *E. coli*. None of the extracts showed total inhibition against the other bacteria but 29 extracts partially inhibited at least one of the bacteria tested, as shown in Table 2.

Regarding the peptidic extracts, a partial inhibition of *S. aureus* was mostly observed in those extracts obtained from *Xylaria* sp. (CEQCA-M1240.P1), Pleosporales sp. 1 (CEQCA-O1055.P1), *Diaporthe helianthi* (CEQCA-O1394.P1 and P2), and Ascomycota sp. (CEQCA-O1111.D1). The latter extract partially inhibited *S. enteritidis* as well. The peptidic extract of Xylariaceae sp. (CEQCA-O1400.P2) was the only one that totally inhibited *S. aureus*.

The extracts that totally inhibited *S. aureus* and *E. coli* were tested by the disk diffusion method. The extract CEQCA-O1113.D1 presented the biggest inhibition zone (17.67 mm ± 1.2) against *S. aureus* (Figure 1, Table 3).

3.3. MIC Determination

Table 3 shows the MIC values obtained after performing a microdilution test of the crude extracts that showed total inhibition against *E. coli* and *S. aureus*. The MIC values ranged from 4.4 to 237.42 mg/mL. The lowest MIC observed was of the extract CEQCA-O1113.D1 against *S. aureus*. The MIC of the extract CEQCA-O1113.E1 was not detected, suggesting that the MIC is found within a

non-assayed concentration between 15.83 mg/mL (concentration of crude extract) and 7.93 mg/mL (first dilution).

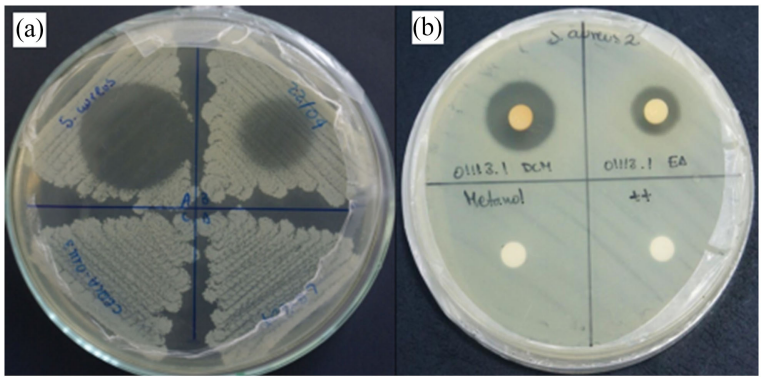

Figure 1. Comparison of the bioassays performed using the agar diffusion and the disk diffusion methods against *S. aureus*. The upper quadrants of both plates correspond to the extracts CEQCA-O1113. D1 (left) and CEQCA-O1113.E1 (right), whereas the quadrants below correspond to the methanol control (down left) and bacterial control (down right). (a) Agar diffusion method; (b) Disk diffusion method.

Table 2. Bioactivity of 46 fungal extracts evaluated by the agar diffusion method.

Fungal species	Extract ID	Bacteria tested*				
		S. aureus ATCC 25923	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922	*S. marcescens* ATCC 13880	*S. enteritidis* ATCC 13076
E. pallida	CEQCA-M1214.D1	–	–	–	–	–
	CEQCA-M1214.E1	–	–	–	–	–
	CEQCA-M1214.P1	–	–	–	–	–
Xylaria sp.	CEQCA-M1240.D1	–	–	–	–	–
	CEQCA-M1240.E1	–	–	–	–	–
	CEQCA-M1240.P1	+	–	–	–	–
Pleosporales sp. 1	CEQCA-O1055.D1	+	–	–	–	–
	CEQCA-O1055.E1	–	–	–	–	–
	CEQCA-O1055.P1	+	–	–	–	–
Periconia sp.	CEQCA-M1179.D1	–	–	–	–	–
	CEQCA-M1179.E1	–	–	–	–	–
	CEQCA-M1179.P1	–	–	–	–	–
Pleosporales sp. 2	CEQCA-O1090.D1	–	–	–	–	–
	CEQCA-O1090.E1	–	–	–	–	–
	CEQCA-O1090.P1	–	–	–	–	–
Ascomycota sp.	CEQCA-O1111.D1	+	–	–	–	–
	CEQCA-O1111.E1	–	–	–	–	–
	CEQCA-O1111.P1	+	–	–	–	+
V. baninensis	CEQCA-M1193.D1	–	–	–	–	–
	CEQCA-M1193.E1	–	–	–	–	–
	CEQCA-M1193.P1	–	–	–	–	–

Continued

M. candidus	CEQCA-O1113.D1	++	−	−	−	−
	CEQCA-O1113.D2	+	−	+	−	−
	CEQCA-O1113.E1	++	−	−	−	−
	CEQCA-O1113.E2	+	−	++	+	−
	CEQCA-O1113.P1	−	−	−	−	−
F. solani	CEQCA-O1393.D1	−	−	−	−	−
	CEQCA-O1393.D2	−	−	−	−	−
	CEQCA-O1393.E1	−	+	−	+	+
	CEQCA-O1393.E2	++	+	+	+	+
	CEQCA-O1393.P1	−	−	−	−	−
Xylariaceae sp.	CEQCA-O1400.D1	−	−	−	−	−
	CEQCA-O1400.E1	−	−	−	−	−
	CEQCA-O1400.P1	+	−	−	−	−
	CEQCA-O1400.P2	++	−	−	−	−
D. helianthi	CEQCA-O1394.D1	−	−	−	−	−
	CEQCA-O1394.D2	−	−	−	−	−
	CEQCA-O1394.E1	+	−	−	−	−
	CEQCA-O1394.E2	++	+	+	+	−
	CEQCA-O1394.P1	+	−	−	−	−
	CEQCA-O1394.P2	+	−	−	−	−
X. laevis	CEQCA-O1399.D1	+	−	−	−	−
	CEQCA-O1399.D2	+	−	−	+	+
	CEQCA-O1399.E1	+	+	−	+	+
	CEQCA-O1399.E2	++	+	+	+	+
	CEQCA-O1399.P1	−	−	−	−	−

*Symbols indicate −, no inhibition; +, partial inhibition; ++, full inhibition.

Table 3. Minimal inhibitory concentrations (MICs) of the extracts that showed strong bioactivity by the disk diffusion method.

Extract ID	Bacterium tested	Initial concentration of the extract (mg/mL)	Average diameter of the inhibition zone (mm)*	MIC (mg/mL)
CEQCA-O1113.D1	*S. aureus* ATCC 25923	35.13	17.67 ± 1.2^a	4.4
CEQCA-O1113.E1	*S. aureus* ATCC 25923	15.85	12.33 ± 0.6^a	Between 7.93 and 15.85
CEQCA-O1399.E2	*S. aureus* ATCC 25923	102.6	8 ± 1^a	25.65
CEQCA-O1393.E2	*S. aureus* ATCC 25923	383.2	8.67 ± 0.6	47.9
CEQCA-O1394.E2	*S. aureus* ATCC 25923	105.8	8.33 ± 0.6	26.45
CEQCA-O1400.P2	*S. aureus* ATCC 25923	949.67	9 ± 0	237.42
CEQCA-O1113.E2	*E. coli* ATCC 25922	91.2	10.33 ± 1.6	45.6

*The same superscript letter "a" indicates a significant difference ($p < 0.05$) between the diameters of the inhibition zones.

3.4. Purification of Extracts by HPLC

The organic extract CEQCA-O1113.D1, that presented the strongest bioactivity in the antimicrobial assays, was fractioned by HPLC. Only two fractions of the extract (fraction 45 and 46) preserved the bioactivity of the crude extract against *S. aureus* after the purification. A bacterial growth curve (absorbance vs time) was built based on the results of the microdilution test (**Figure 2**). The fraction 45 fully inhibited *S. aureus* growth, as observed in **Figure 2(a)**. The fraction 46 showed inhibition as well (**Figure 2(b)**), but not as strong as the bioactivity observed with fraction 45. The MS results showed that fraction 45 required additional purification, since there were too many possible compounds present in the sample and the chromatogram was not fully resolved.

4. Discussion

Fungal endophytes are well-known producers of a variety of secondary metabolites with a wide range of bioactivities against bacteria, fungi, and other microbes [2]. It has been hypothesized that the endophytes produce those compounds in nature to inhibit the growth of their competitors [5]. Remarkably, when produced *in vitro*, these compounds maintain bioactivity and can be potentially used in medicine, industry, and agriculture.

In the present study, a screening of 46 extracts obtained from 12 endophytic fungi selected from the CEQCA was performed to test their inhibitory capacities against the ATCC bacteria *S. aureus*, *E. coli*, *S. marcescens*, *P. aeruginosa* and *S. enteritidis*, and against the plant pathogenic oomycete *P. ultimum*. We found

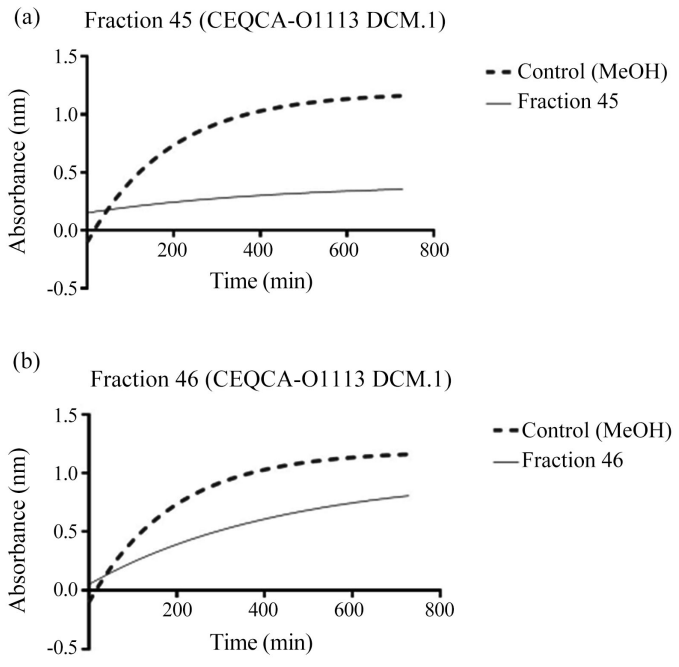

Figure 2. Curves of bacterial growth of *S. aureus* against the fractions 45 and 46 of the extract CEQCA-O1113.D1. Dotted line: fraction tested. Solid line: methanol control. (a) Fraction 45. (b) Fraction 46.

that 42 out of the 46 extracts tested showed partial or total inhibition against at least one of the microorganisms tested, therefore demonstrating the potential utility of plant fungal endophytes for mining biomolecules that may be developed into new therapeutic agents.

The culture conditions were especially important to harvest the secondary metabolites when culturing the fungus *Marasmiellus candidus* (CEQCA-O1113). The bioactivity of this fungus against *S. aureus* and *P. ultimum* was apparently conditioned by the guttation of dark brown droplets produced by the mycelia. These exudates were not abundant in the second round of organic extraction, and this might be why the extracts CEQCA-O1113.D2 and E2 did not showed the strong bioactivity observed in the extracts from the first round of extraction (D1 and E1). Guttation is the active secretion of water and dissolved materials, and is a common phenomenon observed in plants and fungi [18]. The composition of the droplets has not been studied in detail. However, it has been reported that fungal guttation droplets are important reservoirs of enzymes, mycotoxins and secondary metabolites such as penicillin, as reported in the droplets of *Penicillium* sp. [18] [19] [20]. Therefore, it has been hypothesized that guttation droplets take part in important physiological processes such as the maintenance of the mycelial growth rate under poor water availability and defense against other microorganisms [21]. Nevertheless, it is important to note that the formation of fungal guttation droplets occur at random conditions *in vitro*, which makes it difficult to study their composition and determine the conditions to enhance their production *in vitro*. According to Schmidt and Spiteller [20], there is no biological trigger for their production identified so far, which may explain why we did not find the droplets in all the plates of *M. candidus*.

The production of antioxidants, sesquiterpenoids and a mellein derivative with potential to inhibit acethylcholinesterase, which can potentially treat Alzheimer disease, have been reported in *Marasmiellus* species [22] [23] [24]. The production of terpenes such as 1,8-cineole has been also studied in other genera within the Basidiomycota and in the ascomycetous family Xylariaceae, suggesting the potential of endophytic fungi to produce insect-repellent and insecticidal compounds, as well as adjuvants for the attenuation of the inflammatory responses in viral infections such as the H1N1 influenza in mice [25], among other uses (flavoring and fragrance production) [26] [27]. In our study, we observed that the spectrum of activity of the extracts of *M. candidus* was broad, with strong bioactivity against *S. aureus*, *E. coli* and *P. ultimum*. It would be interesting to find out if the extracts of *M. candidus* can inhibit other microorganisms and have other bioactivities, such as anticancer activity, since in a study performed by Strobel *et al.* [28], one fungal extract that previously showed bioactivity against *P. ultimum*, showed cytotoxic activity against breast cancer cell lines BT-20 as well.

The extracts of *Xylaria laevis* (CEQCA-O1399), *Fusarium solani* (CEQCA-O1393), *D. helianthi* (CEQCA-O1394) and *Xylaria* sp. (CEQCA-O1400) either partially or totally inhibiting the bacteria tested and *P. ultimum*. A wide range of

bioactivities has been reported in fungi of these genera. A wide variety of bioactivities have been found in *Xylaria* species, including antibacterial activity of peptides, terpenes, diterpenes, among others against methicillin-resistant *S. aureus*, *E. coli*, *Shigella* sp., *Mycobacterium tuberculosis*, *Listeria monocytogenes* and other human pathogenic bacteria [29]. Other biological activities of *Xylaria* include anticancer activity against the cell lines KB, MCF-7 and NCI-H187, antimalarial activity against *Plasmodium falciparum* K1, antifungal activity against *Candida albicans* [30] [31], as well as anti-inflammatory activity [32]. Xyolide, a compound isolated from *Xylaria feejeensis*, showed a strong inhibitory capacity against the fungus *P. ultimum* [33]. Thus, we confirmed that the genus *Xylaria* is a good source for mining of secondary metabolites with potential in drug discovery.

Fungi of the genus *Diaporthe* are also good producers of bioactive secondary metabolites. Their genomes encode for enzymes such as sesquiterpenes synthases with a wide variety of bioactivities [27]. In the present study, we found strong bioactivity against *S. aureus* and partial activity against the other bacteria tested with the extracts of *D. helianthi*. Antibacterial activities have been previously reported in fungi of the genus *Diaporthe*. Diaporone A, a compound isolated from an EA extract of *Diaporthe* sp. showed moderate antibacterial activity against the soil bacterium *Bacillus subtilis* and also moderate anticancer activity [34]. A potent triterpenoid active against *S. aureus*, *P. aeruginosa*, *Streptococcus pyogenes*, *E. coli*, and *B. subtilis* has been also isolated from *Diaporthe* species, as well as variety of polyketides, peptides, and terpenoids [35] [36].

The EA extracts we obtained from *Fusarium solani* (CEQCA-O1393) were bioactive against all the bacteria tested. The wide array of metabolites secreted by fungi of the genus *Fusarium* is due to a great genetic variability within the genus, which affects its biology and ecology [37] [38]. A diversity of quinones with antimicrobial activities against *S. aureus*, *E. coli*, *P. aeruginosa*, *Aspergillus flavus* and *A. niger* have been previously observed [39]. Focusing on *F. solani*, well-known drugs have been isolated from this species, including quinine, camptothecin and taxol [37]. Altogether, these findings show the potential of *Fusarium* species as synthesizers of chemically diverse compounds that can be exploited in drug discovery.

The peptidic extracts obtained in this study showed moderate inhibitory activities against the bacteria tested, and it was notable the fungistatic activity of the extract CEQCA-O1400.P2 from *Xylaria* sp. against *P. ultimum*. Antimicrobial peptides are small molecules highly appreciated in drug discovery due to their low toxicity, easy diffusion through membranes and low chance of the microorganisms to develop resistance against the peptide [40]. The antimicrobial peptides also possess different mechanisms of action against the microorganisms, including perforation of cell membranes, inhibition of protein synthesis, inhibition of cell wall synthesis and direct DNA damage [41] [42]. These characteristics supported our idea of extracting and testing the bioactivity of the peptidic extracts from our fungal isolates. Excluding the bioactivity of the extract

CEQCA-O1400.P2 against *P. ultimum*, only partial inhibitions were observed against the other pathogens tested. However, the protocol for peptide extraction worked well, allowing future extractions from other fungal isolates of the CEQCA.

Other studies support the potential of the peptides produced by endophytic fungi as therapeutic agents and for industrial applications. Ibrahim *et al.* (2020) found new cyclic pentapeptides from the fungus *Xylaria ellisii* with strong bioactivities against Gram negative bacteria [43]. Besides, cyclic peptides produced by fungi of the genus *Epichloë* might help the plant to be resistant against herbivores [44], feature that could be useful in the development of pesticides. Other cyclic peptides isolated from *Fusarium* species and *Aspergillus tamarii* showed inhibitory activities against plant pathogens such as *P. ultimum* and *Rhizoctonia solani*, and also cytotoxic activity, respectively [45].

It is interesting to notice that the fungi *F. solani*, *Xylaria laevis* and Xylariaceae sp. that showed a wide range of bioactivities, were isolated from the plants *Herrania mariae* and *Theobroma cacao*. According to the Encyclopedia of Useful Plants of Ecuador (2008), these plant species have ethnobotanical uses in the Ecuadorian Amazon. The leaves are used to treat snake bites, as well as tumours and ulcers [46]. The endophytic fungi might have a role in the medicinal properties observed in *H. mariae* and *T. cacao*, since the endophytes influences the production of metabolites in the host and vice versa [47] [48]. Future studies should be performed to see the interaction of fungi and host plants in the production of bioactive secondary metabolites.

5. Conclusions

Our results indicate that the endophytic fungi of the CEQCA isolated from Ecuadorian plants have potential to produce antibacterial and antifungal compounds. Ecuadorian endophytic diversity is higher compared to temperate sites and other tropical locations, as observed in Panama by Arnold and Lutzoni [49]. Therefore, we may expect that the diversity of chemically interesting compounds is also high, and may show a wide range of bioactivities as observed in Ecuadorian endophytic fungi of the family Xylariaceae [7] [26] [33].

Further studies are needed to purify and identify the molecules responsible of the bioactivities observed. Additionally, efforts should be put in the identification of those fungi that were bioactive but lack taxonomic identification. The knowledge of their identity would shed light on the metabolites that they can potentially produce.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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