BIOPROSPECTING OF ENDOPHYTIC FUNGI FROM SANTA CATARINA ISLAND MANGROVES FOR ANTIFUNGAL ACTIVITY

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ABSTRACT

New resistant pathogenic and opportunistic fungi represent an issue with the potential to cause significant harm worldwide. This problem is exacerbated by the indiscriminate use of agrochemical agents in plantations, which promotes the selection of resistant fungi strains. Mangroves, on the other hand, are rich environments in biodiversity, especially concerning microbiome. In this context, endophytic fungi stand out for their production of secondary metabolites with inhibitory potential, which offers a promising perspective for discovery new molecules. In this study, we aimed to evaluate the anti-fungal activity of extracts obtained from endophytic fungi from Florianópolis - SC mangroves. The tests were conducted with 17 extracts, selected based on their activity against *Candida* spp. in previous studies. It was evaluated the biological activity of the extracts against 5 filamentous fungi: *Alternaria alternata, Aspergillus niger, Cladophialophora exuberans, Fusarium oxysporum*, and *Rhizopus stolonifera* in 96-well plates with RPMI. Extracts that exhibited biological activity were further evaluated to determine fungistatic or fungicidal activity. In the screening step, five compounds were selected and two showed activities against *A. niger* and three *against R. stolonifer* and *C. exuberans*. An extract obtained from the endophytic fungus *Arthrinium sp*. demonstrated MIC assay of 300 µg/ml and 150 µg/ml against *C. exuberans* and *R. stolonifer* respectively. This work presented results that shows the potential of fungal secondary metabolites from Florianopolis mangroves, which may lead future biotechnological applications.

Keywords: Fungi. Secondary metabolites. Endophytic. Anti-fungal molecules

1 INTRODUCTION

Fungi play a crucial role in nutrient recycling, but their presence challenge agriculture, with direct impact in the food sector[1]. The species diversity, along with their pathogenic potential and ability to adapt to different conditions, represents a considerable obstacle to control these organisms. Contaminations by mycotoxins largely depend on the production, storage, and transportation conditions of food, and is more prevalent in grain and cereal crops[2]. Additionally, the presence of these toxins in animal products such as beef and pork has already been documented[2]. Furthermore, the emergence of resistant species has been observed, mainly due to the use of agrochemicals in plantations which, promotes the selection of resistance strains [2,3]. For example, *Aspergillus niger* and *Aspergillus fumigatus* strains are known for their reduced sensitivity to azole agents and amphotericin B[3], which may lead for the outbreaks development[4,5].

Mangroves are unique ecosystems characterized by abundant biodiversity and remarkable resilience in biotic and abiotic stressful environments. This resilience is attributed to their rich endophytic microbiota, which plays a crucial role in plant protection against pathogenic microorganisms [6,7]. Among these beneficial agents, endophytic fungi stand out through the production of biomolecules with inhibitory capacity[8]. Therefore, the bioprospecting of mangrove endophytic fungi is a potential source for obtaining new molecules with biological activities[9]. In this way, the lack new molecules against fungi highlight the importance of screening new potential producer strains to develop new products and process with biotechnological application.

3 MATERIAL & METHODS

Oliveira [10] cultivated endophytic fungi obtained from the Mário Steindel collection, which included material collected from the mangroves of the Itacorubi and Ratones regions of Florianopolis, Santa Catarina. These fungi were previously isolated and identified based on their morphology using micro-cultivation techniques and ITS1 region sequencing. These isolates were then cultivated on rice for 21 days at 28°C. Following this cultivation stage, secondary metabolites were extracted through solid-liquid extraction using solvents of different polarities (dimethyl sulfoxide and methanol). After 7 days, the extracts were concentrated by rotary evaporation, lyophilized, and stored at -80°C.

Five fungal isolates were chosen for the study: *Fusarium oxysporum* MUCL909, *Cladophialophora exuberans* CMRP1227, *Aspergillus niger* DR02, *Alternaria alternata* CCT2816, and *Rhizopus stolonifer* CCT0276. These species were chosen due to the negative impact in Agriculture, Carcinoculture, and opportunist human infections. The isolates were cultured on potato dextrose agar (PDA) pH 5.0 at 28°C until abundant sporulation. After this period, spores were harvested by addition of 5 ml of sterile Tween 20 (0.1% w/w). Subsequently, fungal colonies were carefully scraped off using a Drigalski loop and transferred to a sterile tube. The suspension was homogenized for 15 seconds and filtered using a sterile cotton gauze until absent of hyphae were detected. All spore suspensions were adjusted to 2-5 x 10 spores/ml by counting in a Neubauer chamber. The spores were stored in a solution of 30% glycerol and 0.1% peptone at -80°C until use in the experiments.

In the initial screening step was performed with a standard concentration of 100 µg/ml of fungal extracts (Table 1.) based on their ability to cause total, partial inhibition, or hyphae morphology modification. Tests were conducted according to the guidelines of the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST). Fungal inoculums were standardized to 1-2 x 10^5 spores/ml and inoculated in RPMI 1640 with 1% DMSO. Amphotericin B was used as a positive control, and RPMI 1640 with 1% DMSO as a negative control. The plates were incubated at 28°C and analyzed at 24, 48, and 72 h. Spores germination and hyphae morphology was visualized (400 x) at an inverted microscope (Olympus) and documented with a digital camera (17-megapixel Olympus DP73).

The Minimum Inhibitory Concentration (MIC) tests were conducted according to the Brazilian Committee on Antimicrobial Susceptibility Testing guidelines (BrCAST). Fungal inoculums were standardized to a concentration of 1-2 x 10^5, and the extracts were tested at a maximum concentration from 25 to 400 µg/ml. The assay was performed as described in the screening section. Amphotericin B was used as a positive control, and RPMI 1640 with 1% DMSO served as a negative control. Wells with no growth, were inoculated (50 µl) onto PDA medium to confirm fungistatic or fungicidal properties of the extract.

4 RESULTS & DISCUSSION

The extracts were subjected to separation processes using solvents with different polarities. This process was repeated several times, resulting in the extracts used in this study (Table 1). In this study it was used the same extracts produced by Oliveira[10] , which were tested against *Candida* spp. and observed significant inhibitory activity.

Table 1: Crude Extracts Obtained from Endophytic Fungi of Florianópolis mangroves

Idetification	Molecular identification	Solvents	Extract
ILRR1A20047	*Curvularia* sp.	DM	EDCM/MET0047
IASF1A10001	*Neofusicoccum parvum*	D-M	EDCM001 and EMET001
RASC1B20231	*Arthrinium* sp.	D-M	EDCM231 and EMET231
IASF2A10006	*Stemphylium* sp.	D-M	EDCM006 and EMET006
ILRF2A10022	*Trichoderma* Section Longibrachiatum	D-M	EDCM022 and EMET022
IASCM10015	*Bjerkandera* sp.	D-M	EDCM015 and EMET015
RASCM10119	*Bjerkandera* sp.	DM	EDCM/MET119
ILRF1A10021	*Penicillium* Glabra series	DM	EDCM/MET021
RASFM10100	*Phlebia* sp.	D-M	EDCM100 and EMET100
ILRF2A10023	*Buergerula spartinae*	D-M	EDCM023
IASFM30068	*Dothideomicetos*/*Muyocopron* sp.	D-M	EMET068

M – Methanol / D- Dimethyl sulfoxide / DM – Methanol and dimethyl sulfoxide

In the initial screening step, it was used a standard concentration (100 µg/ml) for the 17 extracts (Table 1.) due to extract solubility in aqueous solution. The selection criteria in this step were total/partial growth inhibition, or hyphae morphology modification. The screening was conducted in 96 well plates, using strains of *F. oxysporum, A. alternata, R. stolonifer, A. niger*, and *C. exuberans*. None of the extracts showed visible activity against *F. oxysporum* and *A. alternata*. However, extracts showed activity against *R. stolonifer, A. niger*, and *C. exuberans* (Table 2). These extracts were derived from *Arthrinium* sp., *Stemphylium* sp., *Phlebia* sp., and *Trichoderma* section *Longibrachiatum*. The screening results are consistent with Oliveira[10] study, which also observed activity from the same extracts, except for *Phlebia* sp. extracts (EDCM100 and EMET100), which showed activity only in this study.

Table 2 Fungi extracts that presented antifungual activity in screening assays (100 µg/ml)

Identification	Extract	Solvent
Aspergillus niger DR02	EDCM231, EDCM006	D - D
Cladophialophora exuberans CMRP1227	EDCM231, EDCM100, EMET100	D-D-M
Rhizopus stolonifer CCT0276	EDCM231	D

*D – Dimethyl sulfoxide / *M – Methanol

Further, extracts that presented activity was subjected to MIC (Minimum Inhibitory Concentration) determination (400, 300, 200, 150, 125, 100, and 25 µg/ml). It was measured the pH of the medium before and after incubating to confirm that growth inhibition was not due to pH variations. There were no pH variations even at the highest extract concentration (400 µg/ml), this is due to the presence of sodium bicarbonate buffer in the culture medium (RPMI 1640).

Two extracts (EDCM231 and EDCM006) were used to determine MIC for *A. niger*. For this species it was selected extracts based on a visible reduction of growth rate. Even though, higher extracts concentrations were chosen, spores germination was observed for all concentrations, but lower growth rate was observed from 100 to 400 µg/ml. For *C. exuberans*, compounds EDCM100, and EMET100 did not exhibit spores germination inhibition at the highest concentration (400 µg/ml). However, the extract EDCM231 demonstrated complete growth inhibition at a concentration of 300 µg/ml. As expected, with a decrease in the extract concentration hyphal development was observed. The evaluation of the extract profile in PDA medium revealed a fungicidal profile. No information has been found in literature regarding to fungal compounds with biological activity against *C. exuberans.*

In the screening step, none extract exhibited fungicidal activity against *R. stolonifera*. Although a reduced growth was detected for EDCM231, MIC assay showed complete inhibitory growth at 150 µg/ml. Besides no fungal growth was observed on PDA, which suggested a fungicidal activity against *R. stolonifer.* This result corroborated with Oliveira[10], which observed anti-fungal activity for the EDCM231 extract with MIC of 150 µg/ml for *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, and *Candida parapsilosis* and 100 µg/ml for *Candida albicans.* The biological activity of EDCM231 extract against the *R. stolonifer* is promising result, as this fungus is responsible for cause post-harvest rot in fruits and vegetables. Even though in previous assays, high cytotoxicity for human cells was observed for EDCM231[10], there is potential for applications on films and surfaces to extend the preservation of fresh fruits and vegetables.

5 CONCLUSION

The extract EDCM231 obtained from the endophytic fungus *Arthrinium* sp. presented activity against strains of *C. exuberans* and *Rhizopus stolonifer* with MIC of 300 and 150 µg/ml respectively, which may lead future biotechnological applications. One of the main bottleneck in bio active molecules development is isolating and purification steps, given the wide variety of organic compounds produced. In this way further studies are necessary to isolated and elucidate the molecules with biological activity.

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