Antibiofilm and Anti-candidal Activity of Extract of the Marine Sponge *Agelas Dispar*

Antonio Carlos Vital Júnior

UFRN: Universidade Federal do Rio Grande do Norte

Marcela de Castro Nogueira Diniz Pontes

UFRN: Universidade Federal do Rio Grande do Norte

Janaina Priscila Barbosa

UNICAMP: Universidade Estadual de Campinas

José Francisco Höfling

UNICAMP: Universidade Estadual de Campinas

Renata Mendonça Araújo

UFRN: Universidade Federal do Rio Grande do Norte

Douglas Boniek

UFMG: Universidade Federal de Minas Gerais

Maria Aparecida de Resende-Stoianoff (✉ mariaderesende@hotmail.com)

UNIVERSIDADE FEDERAL DE MINAS GERAIS <https://orcid.org/0000-0002-6220-1321>

Vânia Sousa Andrade

UFRN: Universidade Federal do Rio Grande do Norte

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Abstract

This study aimed to determine the antifungal and antibiofilm activity of *Agelas dispar* on biofilm-producing of *Candida* species. Methanolic extract of *A. dispar* was obtained and the fraction Ag2 showed inhibitory activity for all the 13 *Candida* strains tested, in concentrations ranging from 2.5 mg/ml to 0.15625 mg/ml. Antifungal activity of fungicidal nature was seen between 5.0 mg/ml and 0.3125 mg/ml of extract against the strains. All strains were classified as biofilm producers. Methanolic extract Ag2 was tested at concentrations of 2.5 mg/ml and 1.25 mg/ml for antibiofilm activity against the biofilm in formation and mature biofilm from all the strains of the genus *Candida*. Treated and untreated biofilm samples were selected for visualization on Scanning Electron Microscopy (SEM). SEM allowed the visualization of the quantitative decrease of the microbial community, alterations of structural morphology and destruction, at the cellular level, in both forming and mature biofilm. It was suggested that the mechanism of action of the fraction is at a plasma membrane and/or cell wall alteration level. The fraction of methanolic extract of *A. dispar* may be a promising antifungal and antibiofilm therapeutic strategy against different species of the genus *Candida*.

Introduction

Candida species, and as other fungi, have developed virulence mechanisms that increase survival ability, pathogenesis, as well as the maintenance of the infectious condition in human host. Besides, resistance mechanisms to most commercial antifungal agents are often seen in conventional therapy. Among the virulence factors, it should be highlighted the ability of yeast fungi to produce biofilm, which contributes to microorganisms maintenance in a favorable environment for their development [1]

Fungal biofilms are predominantly related to biomaterial associated chronic infection, such as the use of catheters and prostheses. At these sites, the biofilm is composed of a cluster of microorganisms, allowing them to exchange genetic information, such as resistance genes, which favors the development of antifungal resistance and decreases host immune responses [2].

Considering the antifungal resistance profile, the greatest challenge for an efficient fungal infection control is the scope of pharmacological research for new compounds that are potentially strong antifungal candidates. This is due to the small and limited number of antifungals commercially available for the treatment of infections, especially systemic infections caused by multiresistant yeast, as in the case of *Candida* species [3, 4] alternative to conventional new compounds that are potentially antifungal drugs are urgently needed.

In this context, due to chemical diversity and bioactivity, natural products such as vegetables or marine products show promise as alternative sources to circumvent issues related to fungal resistance to drugs and the reduction of toxicity [5]. Previous studies show that marine environments are poorly explored compared to other environments and could provide a variety of compounds that set a new path for antimicrobial drugs [6, 7].

Marine sponges of the Agelasidae family are abundantly distributed throughout the marine ecosystem, totaling 19 species already described, including *Agelas dispar*, which has a diversity of bioactive secondary metabolites such as pyrrolic alkaloids [8, 9] Studies of the biological activity of *Agelas* species is still scarce, but these sponges are of great interest to the scientific community. Activities such as, antimicrobial, antiprotozoal, anticancer, and also the anti-inflammatory has been reported [10–12].

Considering the current context and the possibility of better understanding the appreciation and applicability of marine organisms to obtain antifungal compounds, this work evaluated the biological profile of a methanolic extract fraction obtained from the marine sponge *Agelas dispar* against biofilm-forming species of the genus *Candida*.

Material And Methods

Marine sponge

Agelas dispar (Ag) sponge sample was collected in the region of Batente das Agulhas, on the coast of the state of Rio Grande do Norte, Brazil, with geographic coordinates of 5° 33'50.47''S – 35° 4'22.02''. A voucher of *A. dispar* was deposited under accession number MNRJ1867, in the Porifera Collection of Museu Nacional, Federal University of Rio de Janeiro (Rio de Janeiro state, Brazil) and authorized for scientific use by the National Genetic Heritage Management System (SISGEN A12E895).

Fraction preparation and chemical characterization

The fresh sponge was kept in methanol for three days, after which a remaining solution was filtered, concentrated under reduced pressure at temperature of up to 40°C to obtain the crude extract. The Ag extract was suspended in 6:1 (v/v) MeOH: H₂O and subjected to liquid/liquid partition with the increasing polarity solvents n-hexane, chloroform, ethyl acetate and n-butanol. Subsequently, the marine natural product was dried under reduced pressure in rotoevaporator (FISATOM®) and stored for partition and chromatographic fractionation, obtaining fraction 2 (Ag2). The chemical characterization of the fractions was obtained by mass spectrum coupled with liquid chromatography (HPLC-MS-MS) using the Dionex Ultimate 3000 liquid chromatography system. Thus, the constituents were separated into a Phenomenex Hydro C-18 column (2.0 x 50 mm, 4 µm), with an equivalent precolumn, at a flow rate of 600 µl/min. The mobile phase was a gradient of 0.1% formic acid in water and 0.1% formic acid in methanol.

The mass spectra were recorded on an AB Sciex triple linear quadrupole ion capture spectrometer (3200 QTRAP® LC-MS/MS), type ion source (AB Sciex TurbolonSpray®). For chromatograms and spectra acquisition, the software PeakView® version 2.2 was used. The spectral data of Ag2 fraction were similar to those found in the literature and thus used as the object of study on this research [9, 13].

Fungal strains and inoculum preparation

A total of thirteen strains of *Candida* species was tested according Table 1. The strains tested included the species *Candida albicans* (ATCC 90028), *C. tropicalis* (ATCC 13803), *C. krusei* (ATCC 6258), *C.*

glabrata (ATCC 2001), *C. parapsilosis* (ATCC 22019), and sequenced samples available from GenBank. For inoculum preparation, the specimens were grown on Sabouraud Dextrose Agar with chloramphenicol (SDA, KASVI, PR, Brazil) at 35°C for 24–48 h; colonies were suspended in sterile 0.85% NaCl solution and adjusted according to McFarland standard 0.5, which is equivalent to 10⁶ CFU/ml [14–16].

Table 1
Sequentially identified *Candida* strains arranged in the GenBank database.

Species_Samples	GenBank Access Number	Main results from BLAST search
<i>Candida orthopsilosis</i> _15	MN450868	LC389313.1
<i>C. parapsilosis</i> _21	MN450870	LC389138.1
<i>C. parapsilosis</i> _28	MN450872	KU200443.1
<i>C. parapsilosis</i> _31	MN450873	KY178309.1
<i>C. parapsilosis</i> _33	MN450875	LC389750.1
<i>C. tropicalis</i> _40	MN450880	KX664582.1
<i>C. parapsilosis</i> _50	MN450881	LC390144.1
<i>C. parapsilosis</i> _58	MN450883	KX652405.1

Antifungal activity assays

The susceptibility of planktonic cells to the Ag2 sponge methanolic extract fraction of the *A. dispar* was determined by the Minimal Inhibitory Concentration (MIC) [16] and Minimum Fungicidal Concentration (MFC) methodologies. The antifungal activity was quantitatively evaluated by the broth microdilution method using RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA) with L-glutamine in 96 well culture plates to determine the MIC values. The Ag2 fraction was solubilized in dimethylsulfoxide-DMSO (Labsynth Lab Products LTDA, SP, Brazil) in a ratio of up to 10% (v/v) and diluted with sterile distilled water. Serial dilutions of the Ag2 fraction were performed, and the concentrations of 5 mg/ml (maximum), 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml, 0.1562 mg/ml, 0.078 mg/ml and 0.039 mg/ml (minimum) were used. DMSO was used as a growth control [14, 17]. MIC was defined as the lowest extract concentration capable of producing visible fungal growth inhibition recorded in the wells. MIC results were compared to the standard amphotericin B antifungal drug (16 µg/ml) used as a positive control.

The Minimum Fungicidal Concentration (MFC) value was defined as the lowest concentration of the compound that was able to kill the fungal strains [18, 19]. Following MIC assays, 10 µl of the supernatant from the wells with complete visual inhibition of fungal growth were transferred to Petri dishes containing SDA with chloramphenicol (KASVI, Brazil) [20]. Subsequently, the plates were incubated at 35 ± 2°C for 48h. The MFC value was defined as the lowest concentration of the compound that was able to kill the

fungal strains [18, 19]. Results were expressed as the arithmetic means of MICs and MFCs values obtained in three independent trials and in triplicate.

Biofilm formation in *Candida* species

Biofilms were evaluated according to Jin and colleagues [21] modified by Melo and colleagues [22]. Thus, for adhesion phase 100 µl of a standard cell suspension of 10^7 cells/ml in sterile 0.85% NaCl solution were transferred to flat bottomed microtiter plates and incubated for 1.5h at 37°C on a shaker at 0.62g (Tecnal Shaker, Tecnal, Piracicaba, Brasil). After the adhesion phase, cell supernatants were removed and each well was washed twice with 150 µl sterile PBS (Phosphate Buffered Saline). Then, 100 µl of Yeast Nitrogen Base medium (YNB Himedia Laboratories, Mumbai, India) with 50 mM glucose were added to each of the assays washed wells and incubated at 37°C for 66h under the agitation of 0.62g.

Biofilm quantification

After 66h of yeast biofilm development, biomass quantification was performed according technique described by Djordjevic and colleagues [23] with minor modifications. Briefly, biofilm coated microdilution wells were washed twice with 150 µl PBS and air dried for 45 min. Subsequently, each well was stained with 110 µl of crystal violet solution 0.4% for 45 min. The stained wells were then washed three times with 300 µl sterile milli-Q water and immediately discolored with 200 µl 95% ethanol. After 45 min of this procedure, 100 µl of the discolored solution was transferred to a new 96 well microplate and the amount of violet crystal was measured on Elisa reader (BioTek® Epoch Microplate Spectrophotometer, CA, USA) at 570 nm. The biofilm formation was classified according to Stepanovic and colleagues [24].

Antibiofilm activity

The antibiofilm activity was evaluated according methodology adapted from Barbosa and colleagues [25]. The Ag2 fraction was tested at concentrations of 2.5 mg/ml and 1.25 mg/ml for antibiofilm activity against the biofilm in formation and mature biofilm from *Candida krusei* (ATCC 6258), *C. glabrata* (ATCC 2001), and *C. parapsilosis* strains. Biofilms were produced on sterilized, polystyrene, flat-bottomed, 96-well microtiter plates. For the biofilm adhesion phase, 100 µL of the adjusted cell suspension of 10^7 cells/ml in sterile 0.85% NaCl solution was transferred each well. No cells were added to the final column to be used as the negative control. The plate was incubated at 37°C for 1.5h with shaking at 0.62g so that the cells could attach to the surfaces of the wells.

Following the attachment phase, unattached cells were removed, the wells were washed 2x with 150 µL of PBS. After the adhesion phase [21, 22] for the biofilm in formation, 100 µL of YNB medium (Himedia, India) were added. The Ag2 fraction was added at each concentration tested (2.5 mg/mL and 1.25 mg/mL) and incubated for 24h with shaking at 0.62g (Tecnal, Brasil) at 37°C. Concomitantly, for the mature biofilm, 100 µl of YNB medium (Himedia, India) was added and incubated for 24h shaking at 0.62g and at 37°C in a 96 well flat-bottom microplate, and then washed twice with 150 µl PBS. After washing, 100 µl of diluted Ag2 fraction were added in 100 µl of YNB medium and finally incubated for

24h, stirring at 0.62g at 37°C. Then, the post-treated biofilms were quantified by the 0.4% aqueous violet crystal solution.

For the analysis of biofilm cell viability inhibition (post-treatment), a formula from Jadhav and colleagues [26] was used with adaptations to calculate the inhibition percentage and remove the colorimetric interference of the optical density concentrations used in the test. The inhibition percentage was considered to be the concentration that demonstrated 50% (IC50%) or higher inhibition of the forming and mature biofilm. All assays were performed in triplicate.

Scanning Electron Microscopy (SEM)

Three strains were selected for the SEM, *Candida krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* 50. Yeast inoculum was adjusted according to the biofilm formation assay. Inoculum suspension and cell adhesion procedures were performed in a 24 well polystyrene plate (JET BIOFIL®, China) and washed as described above. Pre-formed biofilms were incubated for 24h at 37°C with 100 µl of YNB medium, along with the concentrations used in the antibiofilm activity assays. Concomitantly, the mature biofilm was incubated for 24h at 37°C, with 100 µl of YNB medium. Then, the plate was treated with 1.25 mg/ml and 2.5 mg/ml of Ag2 fraction and incubated for 24h at 37°C. The biofilms were fixed with 2% (v/v) glutaraldehyde (Sigma) and dehydrated with an ethanol solution series (50%, 70%, 90% and 100%). Samples were taken, dried, glued on microscopy slides and metallized with 24k gold and then subjected to visualization on the SEM apparatus (JEOL, JSM 5600LV, Japan). An in formation and mature biofilms not exposed to the Ag2 fraction were used as control. Treated and untreated biofilm samples followed the protocol described by Barbosa and colleagues [25].

Statistical analysis

The results were analyzed using the statistical software “GraphPad, Prism” version 7.0 and the obtained MIC and MFC data were plotted using Microsoft excel® version 2010. Data on biofilm formation and antibiofilm activity were presented by the mean standard deviation. Differences/comparisons were analyzed by the Dunnett variance test (ANOVA), bilateral ($p < 0.05$) ($p < 0.01$) ($p < 0.0001$) with 95% confidence interval.

Results

Identification of Ag2 fraction chemical compounds

Sponge metabolites were identified by classical chromatographic techniques and the secondary metabolite found in greater amount in the Ag2 fraction was 4,5-dibromopyrrol-2-carboxylic acid (Fig. 1), thus considered the major compound for the studied specimen. In addition, agelasidine A, oroidin, manzacidine A, bromohimenialsidine, among others, were also identified according to the chemical evaluation. The chemical structures of the compounds are shown in Fig. 2.

In vitro susceptibility testing

The Ag2 fraction showed values of MIC's, and 5 strains (38%) of the 13 *Candida* species tested showed sensitivity at 0.625 mg/ml. The MIC values for the other species were 2.5 mg/ml (7.7%), 1.25 mg/ml (23.1%), 0.3125 mg/ml (15.4%), 0.15625 mg/ml (15.4%), as shown in Table 2. All 13 strains of the yeast were sensitive to 2.5 mg/ml and 5.0 mg/ml of Ag2 fraction of *Agelas dispar* extract, which were able to completely inhibit microorganisms growth (Table 2).

Table 2
Results of MIC (mg/ml) and MFC (mg/ml) evaluation of Ag2 fraction on *Candida* species.

Fungal strains	Ag2_MIC	Ag2_MFC	Amphotericin B (16 µg/mL)	Yeast Control
<i>Candida albicans</i> ATCC90028	1.25	5.0	-	+
<i>C. tropicalis</i> ATCC13803	0.3125	0.3125	-	+
<i>C. krusei</i> ATCC6258	0.3125	1.25	-	+
<i>C. glabrata</i> ATCC2001	0.15625	0.625	-	+
<i>C. parapsilosis</i> ATCC22019	2.5	2.5	-	+
<i>C. orthopsilosis</i> _15	0.625	2.5	-	+
<i>C. parapsilosis</i> _21	0.15625	0.3125	-	+
<i>C. parapsilosis</i> _28	0.625	2.5	-	+
<i>C. parapsilosis</i> _31	1.25	5.0	-	+
<i>C. parapsilosis</i> _33	1.25	5.0	-	+
<i>C. tropicalis</i> _40	0.625	1.25	-	+
<i>C. parapsilosis</i> _50	0.625	1.25	-	+
<i>C. parapsilosis</i> _58	0.625	2.5	-	+
Legend: MIC, Minimum Inhibitory Concentration. MFC, Minimum Fungicidal Concentration. (+) presence of yeast growth. (-) absence of yeast growth.				

The MFC assay showed that the strains sensitivity to Ag2 fraction ranged from 5.0 mg/ml to 0.3125 mg/ml. The concentration that was able to eliminate 90% of the population was 5.0 mg/ml (Table 2). Besides, the extract exhibited fungicidal activity against all *Candida* species evaluated.

Evaluation of biofilm formation in *Candida* spp.

The 13 strains (100%) were biofilm producers. Ten (77%) were weak biofilm producers and 3 (23%) showed moderate production. By detailing the type of production, *C. albicans* (ATCC 90028) presented OD_{570nm} of 0.092 ± 0.002, *C. tropicalis* (ATCC 13803) OD_{570nm} 0.07 ± 0, *C. parapsilosis* (ATCC 22019) OD_{570nm} 0.064 ± 0.002. Samples 15, 21, 28, 31, 33, 40 and 58 presented OD_{570nm} ranging from 0.062 to

0.078, and so these strains were weak biofilm producers. *Candida krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* (50) were moderate producers. The remarkable biofilm formation in sample 50 (*C. parapsilosis*) stood out when compared to other strains, and presented higher and relevant production values ($OD_{570nm} 0.186 \pm 0.013$). All results were compared to the control, which was treated in same conditions but without the inoculum. All data is shown in Fig. 3.

Antibiofilm activity of the Ag2 fraction of *Agelas dispar* methanolic extract

Only *Candida krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* 50 strains that showed moderate biofilm formation, were submitted to treatment with Ag2 fraction of *Agelas dispar* methanolic extract. Thus, by assessing the inhibition percentage of 50% (IC50%) against the forming biofilm, it was observed that post-treatment with 1.25 mg/ml of Ag2 fraction did not show activity against *C. krusei* biofilm formation, with inhibition of cell viability value of $49.6\% \pm 1.69$ and fungal growth of 50.4%. In contrast, *C. krusei* presented low metabolic activity with 23.7% cell viability of the biofilm when the concentration of 2.5 mg/ml was used, which presented a fungal growth inhibition activity of $76.3\% \pm 1.31$ (Fig. 4a).

For *C. glabrata*, 1.25 mg/ml of Ag2 fraction inhibit $57.6\% \pm 1.20$ of fungal community formation, while 2.5 mg/ml inhibit $73.2\% \pm 1.68$. The same inhibitory profile was seen in biofilm formation for *C. parapsilosis* 50, indicating values of $57.1\% \pm 2.16$ and $70.5\% \pm 1.42$ respectively, for concentrations of 1.25 mg/ml and 2.5 mg/ml. The Ag2 fraction antibiofilm activity results of concentrations of 1.25 mg/ml and 2.5 mg/ml evaluated against immature biofilm of different *Candida* species ($p < 0.001$) are shown in Fig. 4a.

For mature biofilm, it was observed that the concentration of 1.25 mg/ml did not inhibit 50% of the biofilm formed in *C. parapsilosis* 50 ($42.8\% \pm 5.63$), thus maintaining the microbial community in 57.2%. For the other strains, the same concentration indicated a decrease in the yeast biofilm biomass for *C. krusei* ATCC 6258 ($67.2\% \pm 0.88$) and *C. glabrata* ATCC 2001 ($65.4\% \pm 0.92$). All strains showed susceptibility to 2.5 mg ml^{-1} of Ag2 fraction and the cell viability inhibition was $78.8\% \pm 0.84$, $76.2\% \pm 1.26$ and $63, 3\% \pm 2.77$ for *C. krusei*, *C. glabrata* and *C. parapsilosis*, respectively. All tested concentrations showed statistical difference when compared to the control ($p < 0.001$) (Fig. 4b).

Forming biofilm treated with Ag2

Scanning electron microscopy analysis showed that the controls of *C. krusei* forming biofilm (ATCC 6258) showed blastoconidia clusters in its basal layer, a thin extrapolymeric coating and few pseudohyphae formations. For *C. glabrata* (ATCC 2001) cells with intact morphologies were shown and for *C. parapsilosis* 50 a smaller amount of blastospores were seen. In contrast, the specimen produced pseudohyphae grouped in substantial amounts of the biofilm in formation.

When *C. krusei* forming biofilm was treated with 1.25 mg/ml of Ag2, a significant decrease in yeast cells with cell deformities and extravasation was observed. Their original morphology was lost when 2.5

mg/ml was used. At this concentration, Ag2 fraction was able to adhere to the yeast cell surface, providing separation of the microbial community and consequently cell loss. When subjected to two concentrations of Ag2 fraction, *C. glabrata* showed a decrease in biofilm clusters, generating a larger number of free cells and no apparent morphological changes. For *C. parapsilosis* 50, its cell density presented a higher susceptibility to the concentrations used when compared to the other *Candida* species. It is possible to visualize in both concentrations the destruction of the hyphal structures and groupings of the blastospores, deformities, roughness and extravasation of their intracellular material. All images are shown in Fig. 5.

Mature biofilm treated with Ag2

The mature ultrastructures of *C. krusei* (ATCC 6258) and *C. parapsilosis* 50 strains analyzed by SEM indicated a dense network of morphologically diverse cells (blastoconidia and pseudohyphae) covering the entire evaluated surface shown in Fig. 6. *C. glabrata* (ATCC 2001) control showed only buds and blastoconidia, in which it provided a thick and compact multilayer biofilm with co-aggregated cells.

The images of treatments with the Ag2 fraction at a concentration of 1.25 mg/ml showed a decrease in cell volume and small deformities with cell leakage in *C. krusei*. At the highest concentration (2.5 mg/ml), *C. krusei* cells were disrupted, partially destroyed, presented cell leaks and substance aggregation on the yeast surface. At the lowest concentration (1.25 mg/ml), the quantitative decrease in cell aggregates was intensified in *C. glabrata*, reducing the formed microbial community. Applying 2.5 mg/ml of Ag2, free yeast cells were identified in the basal surface layer and some extravasations and deformities were observed. For *C. parapsilosis* 50, a decrease in cell biomass of the three-dimensional structure was observed when exposed to 1.25 mg/ml of Ag2. By submitting the mature *C. parapsilosis* biofilm to 2.5 mg/ml, the amount of pseudohyphae decreased compared to its control. In addition, extravasations and cellular deformities were also seen in the treated biofilm. All images are shown in Fig. 6.

Discussion

The incessant search for promising natural, synthetic or semi-synthetic compounds that demonstrate antimicrobial and antibiofilm activity and that can be safely administered for infection control has motivated areas of scientific research, especially the attempt to elucidate antifungal compounds. Microorganisms are rapidly proving resistant to a variety of antimicrobials employed in clinical practice, which makes the search for antifungals even more urgent. The literature has reported that natural products of aquatic origin provide a variety of chemically bioactive compounds, especially antifungal substances [27, 28].

The biological activity of Ag2 fraction found at of *A. dispar* methanolic extract stands out as a significant antifungal potential to the inhibition and elimination of different *Candida* species. The fact that the MIC values vary between 0.15625 mg/ml and 2.5 mg/ml is probably due to the large variations related to the phenotypic and genotypic particularities of each *Candida* species, as shown in Table 2. Similar to our

findings, Nazemi and colleagues [29] found variations in MIC's when evaluating the antifungal activity of the methanolic extract of the *Haliclona* sp. against clinical isolates of *C. albicans* (0.75 mg/ml) and *Aspergillus fumigatus* (2 mg/ml).

Based on the results of our study and considering the resistance profile by the MFC/MIC ratio, *C. albicans* (ATCC 90028) and two *C. parapsilosis* (samples 31 and 33) were sensitive to Ag2 only at higher concentrations, and both species presented an MFC of 5.0 mg/ml (Table 2) to achieve complete elimination of the pathogens. In comparison, the study of Galeano and Martinez [30] verified the antifungal potential of 24 marine sponge extracts, among which 14 compounds showed inhibitory activity against *C. albicans*. In this same study, it was emphasized that species of marine sponges *Leucetta aff. floridana* and *Cinachyrella* presented higher anti-*Candida* activity.

Recently, Dogan and colleagues [31] tested the methanolic extract of *Agelas oroides* against yeasts such as *C. glabrata*, *Cryptococcus neoformans* and *C. gattii* at concentrations between 256 and 0.5 µg/ml, and found MICs of 16 and 32 µg/ml, respectively. It is possible to suggest that extracts from marine sponges are more effective against *Candida* yeasts, especially *C. glabrata* species. Similarly, this specie showed higher sensitivity to lower concentrations of the Ag2 fraction of *A. dispar* extract in the present study (Table 2). Given this, it is possible to observe the antimicrobial potential that this fraction present, which revealed excellent MICs values against different *Candida* species. These results suggest that methanolic extracts of marine sponges may give rise to antifungal products, specifically against *C. glabrata* species, since it already has an innate resistance profile to the azole class [32].

The bioactive capacity of oroidin isolated from other *Agelas* species was evaluated against yeasts, in which Zidar and colleagues [33] verified the alkaloid derivative activity against *C. albicans* (ATCC 90028) and demonstrated inhibitory activity with 50 µg/ml. Additionally, the antifungal potential of agelasidine had already been reported in the literature by Stout and colleagues [34]. The researchers isolated agelasidine from *Agelas citrina* and tested the compound against *Cryptococcus grubii*, *Cryptococcus gattii* and *Candida* strains, which had their growth inhibited by this metabolite. Complementing these findings, Hammami and colleagues [35] reported the antifungal activity of oroidin for the filamentous fungus *Alternaria solani*, for which the compound exhibited satisfactory inhibitory activity at a concentration of 16 µg/ml. This data suggests that the antifungal activity of *Agelas* sponges may be associated with their secondary metabolites, also found in other genera of marine sponges [36]. There are increasing studies evaluating the identification of virulence factors of *Candida* yeasts, such as the ability to form biofilms and their elimination. Research on biofilm production by *Candida* spp. and other microorganisms species stands out in the literature. Data on the greatest biofilm formation are highlighted for *Candida albicans* species. For non-*C. albicans* species, such as *C. parapsilosis*, *C. krusei* and *C. glabrata* biofilm formation is variable and thus little reported [37]. Contrary to the literature, this study showed that the *C. albicans* strain (ATCC 90028) showed weak biofilm production. Noteworthy, the ATCC's of *C. krusei* (6258) and *C. glabrata* (2001) showed moderate biofilm production, and sample *C. parapsilosis* 50 proved to be a potential biofilm forming strain, also showing moderate biofilm production. However, *C. parapsilosis* 50 stood out when compared to others species.

Some studies such as of Modrzwska and Kurnatowski [38] reported that the ability to adhere to materials surfaces and human host cells varies among *Candida* species. Chaves and colleagues [39] evidenced that the species of *C. parapsilosis* is strongly adherent to different surfaces when compared with *C. albicans*. From the previous evidence, it is possible to suggest that the *C. parapsilosis* strain (sample 50) in our study had a greater ability to adhere to polystyrene plaque when compared to the other strains used to identify the biofilm. This result has motivated further studies for quantification of adhesin-associated genes expressed on biofilm production, even though some strains of *C. parapsilosis* have shown weak adhesin production. Similar data have been described when clinical isolates of candidemia were used and they showed that *C. albicans* species presented the lowest biofilm expressions when compared to the other species reported on the studies [40, 41].

Candida krusei (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* 50 strains were treated with the Ag2 fraction of the marine sponge and showed a biomass reduction on in formation and mature biofilm capacity at both concentrations tested. When subjected to both treatments, a quantitative decrease in cell viability of all strains was seen. The effectiveness of the highest concentration (2.5 mg/ml) was also demonstrated in the mature biofilm, indicating that Ag2 fraction remained active even in face of the complete development of the microbial community.

The lowest concentration evaluated in this study (1.25 mg/ml) showed a slight variation in its effectiveness for the biofilm in formation and after formation, which can be plausibly explained by the lower amount of bioactive secondary metabolites present on this concentration. Corroborating with this data, Cepas and colleagues [42] reported that higher concentrations of marine natural products such as extracts and fractions are necessary to inhibit *C. parapsilosis* biofilms when compared with *C. albicans* biofilms. Thus, the lower inhibition value of cell viability of mature biofilm of sample 50 (*C. parapsilosis*) at a concentration of 1.25 mg/ml can be explained when compared to the highest concentration.

Investigations of ultrastructures (biofilm) subjected to the activity of marine natural products on yeast morphology using SEM are limited and scarce. In this study, the activity of the Ag2 fraction of *A. dispar* extract revealed structural and morphological differences on biofilm from all *Candida* strains analyzed. Both concentrations used in this study induced pseudohyphae and blastoconidia deformities, roughness, cell extravasation, disintegration and reduction of cell volume, which affect the entire structure of the forming and mature biofilm, and suggests a disruption action and destruction of the plasma membrane and/or cell wall. For this, Zore and colleagues [43] described that antibiofilm activity of natural compounds against *Candida* species are not fully elucidated, but some chemical compounds found in extracts, fractions and oils, such as terpenoids and their derivatives have bioactive activity already reported. These include the action of disrupting the integrity of the cell membrane and controlling functions related to the fungal membrane, such as quorum-sensing signaling and cell permeability.

The possible explanations for the observed and confirmed SEM antibiofilm activity found in this study may be associated with the presence of Agelasidin A identified in the Ag2 fraction. This secondary metabolite of *A. dispar* is considered a sesquiterpene derivative and it is described for its ability to

originate farnesol (*quorum-sensing* signaling molecule), which in turn is responsible for the inhibition of hyphal forms (regulatory response) and able to prevent biofilm formation [44, 45].

Another possible explanation for the antibiofilm effect would be the affinity of terpenes, such as agelasidine A, to the fungal plasma membrane. This affinity is generated by Agelasidine A structure, which presents elongated aliphatic chain, some unsaturations and polar functional groups, indicating a higher fat solubility profile and interaction with the fungal structure. Besides, Agelasidine A structure resembles the chemical chains of antifungals such as amphotericin B and nystatin [46, 47]. Additionally, this activity against fungal membrane, which was also observed in SEM, may have been intensified by other compounds, such as oroidin and 4,5-dibromopyrrol-2-carboxylic acid. They are found in the Ag2 fraction of *A. dispar* methanolic extract and present nitrogenous heterocyclics linked to pyrrolic rings, which makes them similar to the chemical structure of azoles, especially triazoles [48, 49].

Conclusion

The Ag2 fraction of *A. dispar* methanolic extract showed antifungal activity and presented fungicidal nature, besides being efficient to decrease microbial load and modify the structural morphology of the yeasts involved in biofilm production. These findings suggest that the mechanism of action of the fraction showed affinity to act at the level of plasma membrane and/or cell wall level, inferring that the Ag2 fraction may be a promising antifungal and antibiofilm therapeutic strategy against different *Candida* species. These findings suggest that the Ag2 fraction may be useful in the treatment and/or prevention of fungal infections, especially those caused by biofilm forming *Candida* species, which are known to be the most resistant to conventional antifungal therapy.

Declarations

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AUTHOR CONTRIBUTIONS

MCNDP and RMA executed, analyzed and interpreted the data for the sponge *Agelas dispar* extraction and fractionation, and interpreted the data for liquid chromatography and mass spectrometry. ACVJ and VSA executed and analyzed the data for the antifungal and antibiofilm activity in formation and mature biofilm among 13 strains of *Candida* species, and designed the work and prepared the article and translate into English. JPB and JFH executed and analyzed the data for treated and untreated biofilm samples for visualization on Scanning Electron Microscopy. DB and MARS executed the identification of *Candida* species and participate on the preparation of the article.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

Characterization of Ag2 compounds based on available fragmentation in HPLC/MS and MS/MS spectra of each chromatogram peak. (1) Bromopyrrol-2-carboxylic acid, (2) manzacidine A, (3) bromohimnensidine, (4) oroidin, (5) 4,5-dibromopyrrol-2-carboxylic acid, (6) agelasidine A.

Figure 2

Chemical structure of the HPLC-MS-MS identified substances, negative mode, from the Ag2 fraction of the partition of *Agela dispar* ethanolic extract. (1) bromopyrrol-2-carboxylic acid, (2) manzacidine A, (3) bromohimemialsidine, (4) oroidin, (5) 4,5-dibromopyrrol-2-carboxylic acid, (6) agelasidine A.

Figure 3

Biofilm formation of *Candida* species expressed by mean values of absorbance reading at 570nm of the violet crystal used for staining. Graphs of biofilm formation quantitatively of strains: 15, 21, 28, 31, 33, 40, 50, 58 and the reference strains *Candida albicans* (ATCC 90028), *Candida tropicalis* (ATCC 13803), *Candida krusei* (ATCC 6258), *Candida glabrata* (ATCC 2001) and *Candida parapsilosis* (ATCC 22019). Statistical significance *: $p < 0.05$; **: $p < 0.01$. ANOVA 1 test with criterion, Dunnett variation. OD: optical density.

Figure 4

Cell viability inhibition (%) of biofilms in formation (A) and mature biofilm (B). Forming and mature biofilms of *Candida krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and sample 50 (*C. parapsilosis*) post-treated with Ag2 fraction of *Agelas dispar* methanolic extract (1.25 mg ml⁻¹ and 2.5 mg ml⁻¹). **** Statistical significance $p < 0.0001$. ANOVA 1 test with criterion, Dunnett variation.

Figure 5

SEM micrographs showing cells of the biofilm in forming *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* 50 exposed to 1.25 mg ml⁻¹ and 2.5 mg ml⁻¹ concentrations of Ag2 fraction of *Agelas dispar* methanolic extract . Untreated biofilms were used as controls. Original magnifications: 1,500x, 2,500x and 3,000x.

Figure 6

SEM micrographs showing cells of the mature biofilm of *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001) and *C. parapsilosis* 50 exposed to 1.25 mg ml⁻¹ and 2.5 mg ml⁻¹ concentrations of Ag2 fraction of *Agelas dispar* methanolic extract. Untreated biofilms were used as controls. Original magnifications: 1,500x, 2,50.