Antibiofilm and Anti-Candidal Activities of the Extract of the Marine Sponge *Agelas dispar*

**Antonio Carlos Vital Júnior** · **Marcela de Castro Nogueira Diniz Pontes** · **Janaina Priscila Barbosa** · **José Francisco Höfling** · **Renata Mendonça Araújo** · **Douglas Boniek** · **Maria Aparecida de Resende Stoianoff** · **Vânia Sousa Andrade**

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

**Keywords** Biofilms · *Candida* species · Marine sponge · Antimicrobials

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A. C. V. Júnior · V. S. Andrade
Department of Microbiology and Parasitology, Center of Biosciences, Federal University of Rio Grande do Norte, UFRN, Natal, Rio Grande do Norte, Brazil
e-mail: ac.vitaljunior@outlook.com

V. S. Andrade
e-mail: vaniasandrade@gmail.com

M. de Castro Nogueira Diniz Pontes · R. M. Araújo
Center of Exact and Earth Sciences, Chemistry Institute, Federal University of Rio Grande do Norte, UFRN, Natal, Rio Grande do Norte, Brazil
e-mail: macanodi@gmail.com

R. M. Araújo
e-mail: renat.onca@gmail.com

J. P. Barbosa · J. F. Höfling
Department of Oral Diagnosis, School of Dentistry of Piracicaba, State University of Campinas, UNICAMP, Campinas, São Paulo, Brazil
e-mail: janaina.priscila@hotmail.com

J. F. Höfling
e-mail: hofling@fop.unicamp.br

D. Boniek · M. A. de Resende Stoianoff
Department of Microbiology, Institute of Biological Sciences, Federal University of Minas Gerais, UFMG, Belo Horizonte, Minas Gerais, Brazil
e-mail: maresend@icb.ufmg.br

D. Boniek
e-mail: douglasboniek@yahoo.com.br
Introduction

Candida species, as well as other fungi, have developed virulence mechanisms that increase their abilities of survival, pathogenesis, and the maintenance of infectious conditions in human hosts. Resistance to most commercial antifungal agents is often observed in conventional therapy. Among the virulence factors, the ability of yeast fungi to produce biofilms contributes to a favorable environment for the development of microorganisms[1].

Fungal biofilms are predominantly related to biomaterial-associated chronic infections, 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 favor the development of antifungal resistance and suppress the host immune responses [2].

Considering the antifungal resistance profile, the greatest challenge for 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], alternatives to conventional new compounds that are potentially antifungal drugs are urgently needed.

Due to their 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]. In the search for new antifungal drugs, many plants have been tested for their antifungal activities, mainly compounds extracted from essential oils, listing in plants, such as cinnamon, anise, clove, citronella, peppermint, pepper, and camphor [6]. β-Caryophyllene, β-citronellol, α-cymene, thymol, carvacrol, and γ-terpinene against Candida species [7–10]. Previous studies have shown 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 [11, 12].

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 [13, 14]. In Brazil, the diversity of marine sponges constitutes the main source of natural product research in this ecosystem, when compared to marine microorganisms, corals, and algae. Their ecological importance has not yet been fully elucidated; however, it is known that marine sponges are responsible for reinforcing marine substrates, filtering water, recycling nutrients, and being symbionts in the relationship with micro-and macroorganisms [15].

Studies on the biological activity of Agelas species are still scarce, but these sponges are of great interest to the scientific community. Antimicrobial, antiprotozoal, anticancer, and anti-inflammatory activities have been reported [16–18].

The Agelas species with proven antimicrobial activities consist of the following compounds in their chemical structure: bromopyrrole alkaloids (such as oroidin), carboxylic acids, such as bromopyrrole-2-carboxamide, 4,5-dibromopyrrole-2-carboxylic acid, its methyl ester 4,5-dibromopyrrole-2-carbonitrile, and 4,5-dibromopyrrole-2-carboxamide [19, 20], halogenated derivatives (especially in brominated forms), and other structures [21–23].

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

Materials and Methods

Marine Sponge

A. dispar (Ag) sponge samples were 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”W. A voucher of A. dispar was deposited under the 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 3 d, after which the remaining solution was filtered through a quantitative paper filter with a grammage of 80 g/m² and an air permeability of 26 l/s.m², and the majority of pores were 25 μm (JProlab®), concentrated under reduced pressure at temperatures of up to 40 °C to obtain the crude extract. The Ag extract was suspended in 6:1 (v/v) methanol (MeOH): water (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 a roto evaporator (FISATOM®) and stored for partition and chromatographic fractionation to obtain fraction 2(Ag2). The chemical characterization of the fractions was performed by high-pressure liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) using a Dionex Ultimate 3000 liquid chromatography system. Thus, the constituents were separated into a Phenomenex Hydro C-18 column (2.0 × 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) and a type ion source (AB Sciex TurboIonSpray®). The PeakView® v.2.2 software was used for chromatograms and spectra acquisition. The spectral data of the Ag2 fraction were similar to those found in the literature, and thus, used as the object of study in this research [14, 24].

Fungal Strains and Inoculum Preparation

Thirteen strains of Candida species were tested. The strains tested included the species Candida albicans (ATCC 90028), C. tropicalis (ATCC 13803), C. krusei (ATCC 6258), C. glabrata (ATCC 2001), and C. parapsilosis (ATCC 22019). The other samples were also sequenced and are available from GenBank (Table 1). For inoculum preparation, the specimens were grown on Sabouraud dextrose agar (SDA) with chloramphenicol (KASVI, PR, Brazil) at 35 °C for 24–48 h; colonies were suspended in sterile 0.85% sodium chloride (NaCl) solution and adjusted according to the McFarland standard 0.5, which is equivalent to 10⁶ colony-forming units (CFU)/mL [25–27].

Antifungal Activity Assays

The susceptibility of planktonic cells to the Ag2 sponge methanolic extract fraction of A. dispar was determined by the minimal inhibitory concentration (MIC) [27] and minimum fungicidal concentration (MFC) methodologies. The antifungal activity was quantitatively evaluated by the broth microdilution method using the Roswell Park Memorial Institute (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) at a ratio of up to 10% (v/v) and diluted with sterile distilled water. Serial dilutions of the Ag2 fraction were performed, and concentrations of 5 mg/mL (maximum), 2.5, 1.25, 0.625, 0.3125, 0.1562, 0.078, and 0.039 mg/mL (minimum) were used. DMSO was used as the growth control [25, 28]. MIC is defined as the lowest extract concentration capable of producing visible fungal growth inhibition in the wells. MIC results were compared to the standard amphotericin B antifungal drug (16 μg/mL), which was used as a positive control.

MFC is defined as the lowest concentration of the compound that is capable of killing the fungal strains [29, 30]. Following MIC assays, 10 μL of the supernatant from the wells with complete visual inhibition of fungal growth was transferred to Petri dishes containing SDA with chloramphenicol (KASVI, Brazil) [31]. Subsequently, the plates were incubated at 35 ± 2 °C for 48 h. Results were expressed as the arithmetic means of the MIC and MFC values obtained in three independent trials and triplicate.

Biofilm Formation in Candida Species

Biofilms were evaluated according to the method proposed by Jin et al. [32] and modified by Melo et al. [33]. For the adhesion phase, 100 μL of a standard cell suspension of 10⁷ cells/mL in sterile 0.85% NaCl solution was transferred to flat-bottomed microtiter plates and incubated for 1.5 h at 37 °C on a shaker at
Biofilm Quantification

After 66 h of yeast biofilm development, biomass quantification was performed according to the technique described by Djordjevic et al. [34], with minor modifications. Briefly, biofilm-coated microdilution wells were washed twice with 150 µL of sterile phosphate-buffered saline (PBS). Then, 100 µL of the yeast nitrogen base medium (YNB Himedia Laboratories, Mumbai, India) with 50 mM glucose was added to each of the assays, washed, and incubated at 37 °C for 66 h under the agitation of 0.62 g.

Antibiofilm Activity

Antibiofilm activity was evaluated according to a methodology adapted from Barbosa et al. [36]. The Ag2 fraction was tested at concentrations of 2.5 and 1.25 mg/mL for their antibiofilm activity against the formation and maturation of biofilm from C. 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 (10⁷ cells/mL) in sterile 0.85% NaCl solution was transferred to each well. No cells were added to the final column for use as negative control. The plate was incubated at 37 °C for 1.5 h with shaking at 0.62 g (Tecnal, Brazil) to facilitate the attachment of the cells to the surfaces of the wells.

Following the attachment phase, unattached cells were removed and the wells were washed twice with 150 µL PBS. After the adhesion phase [32, 33] for biofilm formation, 100 µL of the YNB medium (Himedia, India) was added. The Ag2 fraction was added at each concentration tested (2.5 and 1.25 mg/mL) and incubated for 24 h with shaking at 0.62 g (Tecnal, Brazil) at 37 °C. Concomitantly, for the mature biofilm, 100 µL of YNB medium (Himedia, India) was added and incubated for 24 h, shaking at 0.62 g 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 was added to 100 µL of YNB medium and incubated for 24 h, with stirring at 0.62 g at 37 °C. The post-treated biofilms were quantified using a 0.4% aqueous violet crystal solution.

For the analysis of biofilm cell viability inhibition (post-treatment), the formula from Jadhav et al. [37] 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 significant if 50% (IC50%) or higher inhibition of the formation and maturation of biofilms was observed. All assays were performed in triplicate.

Table 1

| Species_Samples | GenBank access number | Main results from BLAST search |
|-----------------|-----------------------|-------------------------------|
| Candida orthopsilosis_15 | MN450868 | LC389313.1 |
| C. parapsilosis_21 | MN450870 | LC389138.1 |
| C. parapsilosis_28 | MN450872 | KU200443.1 |
| C. parapsilosis_31 | MN450873 | KY178309.1 |
| C. parapsilosis_33 | MN450875 | LC389750.1 |
| C. tropicalis_40 | MN450880 | KX664582.1 |
| C. parapsilosis_50 | MN450881 | LC390144.1 |
| C. parapsilosis_58 | MN450883 | KX652405.1 |
Scanning Electron Microscopy (SEM)

Three strains were selected for SEM: C. krusei (ATCC 6258), C. glabrata (ATCC 2001), and C. parapsilosis 50. The 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 24 h 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 24 h at 37°C with 100 µL of YNB medium. Then, the plate was treated with 1.25 and 2.5 mg/mL of Ag2 fraction and incubated for 24 h at 37°C. The biofilms were fixed with 2% (v/v) glutaraldehyde (Sigma Aldrich, St. Louis, MO, USA) and dehydrated with an ethanol solution series (50, 70, 90, and 100%). Samples were taken, dried, glued on microscopy slides, metallized with 24 k gold, and then subjected to visualization on the SEM apparatus (JEOL, JSM 5600LV, Japan). Biofilm for the SEM assay was performed on 13 mm circular glass coverslips, which were inserted into the holes (wells) of 24-well polystyrene plates. After the biofilms were fixed and dehydrated, the coverslips were removed from the 24-well plate with the help of tweezers, dried, and glued to the microscope slides. Newly formed and mature biofilms not exposed to the Ag2 fraction were used as controls. Treated and untreated biofilm samples were prepared following the protocol described by Barbosa et al. [36].

Statistical Analysis

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

Results

Identification of the Ag2 Fraction Chemical Compounds

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

In vitro Susceptibility Testing

The MICs of the Ag2 fraction for all Candida isolates were determined. Five (38%) of the 13 Candida spp. were susceptible at a concentration of 0.625 mg/mL. The MIC values for the other species were 2.5 (7.7%), 1.25 (23.1%), 0.3125 (15.4%), and 0.15625 mg/mL (15.4%), as shown in Table 2. All 13 strains of the yeast were sensitive to 2.5 and 5.0 mg/mL of Ag2 fraction of the A. dispar extract, which was able to completely inhibit the growth of the microorganisms (Table 2).

The MFC assay showed that the strain sensitivity to the Ag2 fraction ranged from 5.0 to 0.3125 mg/mL. The concentration that eliminated 90% of the population was 5.0 mg/mL (Table 2). In addition, the extract exhibited fungicidal activity against all Candida species evaluated.

Evaluation of Biofilm Formation in Candida spp.

Thirteen strains (100%) were biofilm producers. Ten (77%) were weak biofilm producers, and three (23%) showed moderate production. By detailing the type of production on OD570nm, C. albicans (ATCC 90028) presented 0.092 ± 0.002, C. tropicalis (ATCC 13803) 0.07 ± 0, C. parapsilosis (ATCC 22019) 0.064 ± 0.002. Samples 15, 21, 28, 31, 33, 40, and 58 presented OD values ranging from 0.062-0.078; therefore, 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 C. parapsilosis 50 stood out when compared to the other
strains, and presented higher and relevant production values of OD (0.186 ± 0.013). All results were compared to the negative control, which was treated under the same conditions without the inoculum. All the data are shown in Fig. 3.

Antibiofilm Activity of the Ag2 Fraction of the A. dispar Methanolic Extract

Only C. krusei (ATCC 6258), C. glabrata (ATCC 2001), and C. parapsilosis 50 strains that showed moderate biofilm formation were treated with the Ag2 fraction. 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 ± 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 76.9 ± 1.31% (Fig. 4a).

For *C. glabrata*, 1.25 mg/mL of the Ag2 fraction inhibited the fungal community formation by 57.6 ± 1.20%, while 2.5 mg/mL inhibited it by 73.2 ± 1.68%. The same inhibitory profile was seen in biofilm formation for *C. parapsilosis* 50, indicating values of 57.1 ± 2.16% and 70.5 ± 1.42% for concentrations of 1.25 and 2.5 mg/mL, respectively. The Ag2 fraction antibiofilm activity results of concentrations of 1.25 and 2.5 mg/mL evaluated against

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**Table 2** Results of the evaluation of the minimum inhibitory concentration (MIC) (mg/mL) and minimum fungicidal concentration (MFC) (mg/mL) of the Ag2 fraction on *Candida* species

| Fungal strains                  | Ag2_MIC | Ag2_MFC | Amphotericin B (16 µg/mL) | Yeast control |
|---------------------------------|---------|---------|---------------------------|---------------|
| *Candida albicans* ATCC90028    | 1.25    | 5.0     | –                         | +             |
| *C. tropicalis* ATCC13803       | 0.3125  | 0.3125  | –                         | +             |
| *C. krusei* ATCC6258            | 0.3125  | 1.25    | –                         | +             |
| *C. glabrata* ATCC2001          | 0.15625 | 0.625   | –                         | +             |
| *C. parapsilosis* ATCC22019     | 2.5     | 2.5     | –                         | +             |
| *C. orthopsilosis* _15_         | 0.625   | 2.5     | –                         | +             |
| *C. parapsilosis* _21_          | 0.15625 | 0.3125  | –                         | +             |
| *C. parapsilosis* _28_          | 0.625   | 2.5     | –                         | +             |
| *C. parapsilosis* _31_          | 1.25    | 5.0     | –                         | +             |
| *C. parapsilosis* _33_          | 1.25    | 5.0     | –                         | +             |
| *C. tropicalis* _40_            | 0.625   | 1.25    | –                         | +             |
| *C. parapsilosis* _50_          | 0.625   | 1.25    | –                         | +             |
| *C. parapsilosis* _58_          | 0.625   | 2.5     | –                         | +             |

MIC, Minimum Inhibitory Concentration. MFC, Minimum Fungicidal Concentration. (+) presence of yeast growth. (-) absence of yeast growth.
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% ± 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% ± 0.88) and C. glabrata ATCC 2001 (65.4% ± 0.92). All strains showed susceptibility to 2.5 mg/ml of Ag2 fraction and the cell viability inhibition was 78.8% ± 0.84%, 76.2% ± 1.26%, and 63.3% ± 2.77% for C. krusei, C. glabrata, and C. parapsilosis, respectively. All tested concentrations showed statistical differences compared to the control ($p < 0.001$) (Fig. 4b).

**Forming Biofilm Treated with Ag2**

SEM analysis showed that the control of C. krusei forming biofilm (ATCC 6258) showed blastoconidia clusters in its basal layer, a thin extra polymeric coating, and few pseudohyphae formations. For C. glabrata (ATCC 2001), cells with intact morphologies were observed, and for C. parapsilosis 50, a smaller number of blastospores were observed. In contrast, this specimen produced pseudohyphae grouped with substantial amounts of biofilm formation.

When C. krusei forming biofilms were treated with 1.25 mg/mL of Ag2, a significant decrease in yeast cells with cell deformities and extravasation was observed. The original morphology was lost when 2.5 mg/mL was used. At this concentration, the 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 the 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 the destruction of 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, as shown in Fig. 6. *C. glabrata* (ATCC 2001) control showed only buds and blastoconidia, which 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, and 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. By applying 2.5 mg/mL of Ag2, free yeast cells were identified in the basal surface layer and 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 of Ag2, the amount of pseudohyphae decreased when compared to the control. In addition, extravasations and cellular deformities were observed 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 activities and can be safely administered for infection control has motivated various areas of scientific research, especially the attempt to elucidate antifungal compounds. Microorganisms are rapidly becoming resistant to a variety of antimicrobials employed in clinical practice, which makes the search for novel antifungals even more urgent. Natural products of aquatic origin have been reported to provide a variety of chemically bioactive compounds, especially antifungal substances [38, 39].

Due to the few studies reported on the antimicrobial activity of marine sponges, it is presented in the current literature related to manzacidine A and bromohimenalside as chemical compounds that are mostly found in marine sponges, mainly in the species of *Agelas* [40, 41]. Bromopyrol alkaloids, such as manzacidine A, have already been explored for their antiparasitic activities, as highlighted in the study...
by Scala et al. [17], who evaluated the compound against three enzymes involved in the fatty acid biosynthesis of a *Plasmodium falciparum* strain (PfFAS-II).

The biological activity of the Ag2 fraction found in the methanolic extract of *A. dispar* showed significant antifungal potential for the inhibition and elimination of different *Candida* species. The fact that the MIC values vary between 0.15625 and 2.5 mg/mL is probably due to the large variations related to the phenotypic and genotypic characteristics of each *Candida* species, as shown in Table 2. Similar to our findings, Nazemi et al. [42] found variations in MIC values when evaluating the antifungal activity of the methanolic extract of *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 90,028) and *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, Galeano and Martinez [43] verified the antifungal potential of 24 marine sponge extracts, among which 14 compounds showed inhibitory activity against *C. albicans*. In the same study, it was emphasized that species of marine sponges *Leucetta aff. floridana* and *Cinachyrella* presented higher anti- *C. albicans* activity.

Recently, Dogan and colleagues [44] 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 \(\mu\)g/mL, and found MICs of 16 and 32 \(\mu\)g/mL, respectively. It is possible that the extracts from marine sponges are more effective against *Candida* yeasts, especially *C. glabrata*. Similarly, this species 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 of this fraction, which revealed excellent MIC 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 they already have an innate resistance profile to the azole class [45].

The bioactive capacity of oroidin isolated from other *Agelas* species was evaluated against yeasts, in which Zidar et al. [46] verified the alkaloid derivative activity against *C. albicans* (ATCC 90028) and demonstrated inhibitory activity at 50 \(\mu\)g/mL. Additionally, the antifungal potential of agelasidine has already been reported by Stout et al. [47]. Agelasidine
was isolated from *A. citrina* and tested against *Cryptococcus grubii*, *Cryptococcus gattii*, and *Candida* strains, whose growth was inhibited by this metabolite. Consistent with these findings, Hammami et al. [48] 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. These data suggest that the antifungal activity of *Agelas* sponges may be associated with their secondary metabolites, which are also found in other genera of marine sponges [49]. 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 has been reported 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, not widely reported [50]. Contrary to the literature, this study showed that the *C. albicans* strain (ATCC 90028) showed weak biofilm production. Notably, the ATCC strains of *C. krusei* (6258) and *C. glabrata* (2001) showed moderate biofilm production, and *C. parapsilosis* 50 proved to be a potential biofilm-forming strain, also showing moderate biofilm production, as well as its ability to adhere to catheters and other medical devices and form biofilms [51]. However, *C. parapsilosis* 50 stooded out compared to other species.

Modrzewska and Kurnatowski [52] reported that the ability to adhere to material surfaces and human host cells varies among *Candida* species. Chaves and colleagues [53] showed that *C. parapsilosis* is strongly adherent to different surfaces when compared to *C. albicans*. From the previous evidence, it is possible to suggest that the *C. parapsilosis* strain (sample 50) in this 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 the 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 expression when compared to the other species reported in previous studies [54, 55].

Fifty strains of *C. krusei* (ATCC 6258), *C. glabrata* (ATCC 2001), and *C. parapsilosis* were treated with the Ag2 fraction of the marine sponge and showed a reduction in biomass formation and mature biofilm capacity at both concentrations tested. When subjected to both treatments, a quantitative decrease in the cell viability of all strains was observed. The effectiveness of the highest concentration (2.5 mg/mL) was also demonstrated in the mature biofilm, indicating that the Ag2 fraction remained active even after 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 biofilm formation and after formation, which can be plausibly explained by the lower amount of bioactive secondary metabolites present at this concentration. Corroborating these data, Cepas et al. [56] 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 (biofilms) 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 in biofilms from all *Candida* strains analyzed. Both concentrations used in this study induced pseudohyphae and blastoconidia deformities, roughness, cell extravasation, disintegration, and reduction in biomass formation and mature biofilm capacity. These include 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 is described for its ability to produce farnesol (*quorum-sensing* signaling molecule), which in turn is responsible for the inhibition of hyphal forms (regulatory response) and prevents biofilm formation [58, 59].

Another possible explanation for the biofilm effect is the affinity of terpenes, such as agelasidine A, to the fungal plasma membrane. This affinity is generated by the agelasidine A structure, which presents an elongated aliphatic chain and some unsaturated and polar functional groups, indicating a higher fat solubility profile and interaction with the fungal structure. Besides, the structure of agelasidine A resembles the chemical chains of antifungals, such as amphotericin B and nystatin [60, 61]. Additionally, this activity against the 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 the methanolic extract of *A. dispar* and present nitrogenous heterocyclics linked to pyrrolic rings, which makes them similar to the chemical structure of azoles, especially triazoles [62, 63].

**Conclusions**

The Ag2 fraction of the *A. dispar* methanolic extract showed antifungal and fungicidal activities, in addition to being efficient in decreasing the microbial load and modifying 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 the plasma membrane and/or cell wall, suggesting that the Ag2 fraction may be a promising antifungal and antibiofilm therapeutic strategy against different Candida species. Moreover, the Ag2 fraction may be useful in the treatment and/or prevention of fungal infections, especially those caused by the biofilm-forming *Candida* species, which are known to be the most resistant species to conventional antifungal therapy.

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**Author Contributions** MCNDP and RMA executed, analyzed, and interpreted the data for the sponge *Agelas dispar* extraction and fractionation, liquid chromatography, and mass spectrometry. ACVJ and VSA executed and analyzed the data for the antifungal and antibiofilm activities in the formation and maturation of biofilm among 13 strains of Candida species, designed the work, prepared the article, and translated it into English. JPB and JFH executed and analyzed the data for treated and untreated biofilm samples for visualization using scanning electron microscopy (SEM). DB and MARS identified the *Candida* species and participated in the preparation of the article.

**Declarations**

**Conflict of interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

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