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Original Article

Antifungal activity of *Annona crassiflora* Mart. dichloromethane fraction against strains of *C. albicans*

Atividade antifúngica da fração diclorometano *de Annona crassiflora Mart* (FDAcM) contra cepas de *C. albicans*

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Abstract

Products derived from medicinal plants with antimicrobial activity are considered a promising alternative in the treatment of fungal infections. In this perspective, this study proposed to evaluate the antifungal activity of the dichloromethane fraction of *Annona crassiflora* Mart. against *C. albicans* strains. Tests were carried out to determine Minimum Inhibitory Concentration (MIC), Minimum Fungicide Concentration (MFC), microbial growth kinetics, fungal cell wall and membrane mechanisms of action, antifungal biofilm activity, and cytotoxic effects on human erythrocytes. The extract presented MIC and MFC values that ranged from 256 µg/mL to 1,024 µg/mL, with fungicidal activity in the microbial growth kinetics assay. The mechanism of action did not occur through damage to the cell wall or via binding to ergosterol in the membrane, though the fraction presents activity against biofilm and is not cytotoxic in human erythrocytes. The dichloromethane fraction of *Annona crassiflora* Mart. presented antifungal activity and reduced biofilm growth, without toxicity against human erythrocytes; however, further studies are needed to define its mechanism of action.

Keywords: *Annona crassiflora* Mart., Annonaceae, candida, candidiasis, medicinal, plant.

Resumo

Produtos derivados de plantas medicinais com atividade antimicrobiana são considerados uma alternativa promissora no tratamento de infecções fúngicas. Nesta perspectiva, o estudo propôs avaliar a atividade antifúngica da fração diclorometano da *Annona crassiflora* Mart. frente às cepas de *C. albicans*. Realizou-se ensaios para determinação de Concentração Inibitória Mínima (CIM) e Concentração Fungicida Mínima (CFM), interferência sobre a cinética de crescimento microbiano, mecanismos de ação sobre a parede e a membrana celular fúngica, atividade antifúngica sobre o biofilme e os efeitos citotóxicos sobre eritrócitos humanos. Esse extrato apresentou valores da CIM e CFM que variaram de 256 µg/mL a 1,024µg/mL e no ensaio de cinética de crescimento microbiana, demonstrou atividade fungicida. O mecanismo de ação não ocorreu por danos na parece celular, tampouco por via ligação ao ergosterol da membrana. Demonstrou-se, ainda, que a fração tem ação frente ao biofilme e não é citotóxico em eritrócitos humanos. Assim, a fração diclorometano de *Annona crassiflora* Mart possui atividade antifúngica com capacidade de promover redução do crescimento do biofilme, não demosntrou toxicidade frente eritrócitos humanos, no entanto, necessita-se de mais estudos para definição do seu provável mecanismo de ação.

Palavras-chave: *Annona crassiflora* Mart., Annonaceae, *candida*, candidiases, plantas medicinais.

1. Introduction

Oral cavity candidiasis is principally observed in users of dental prostheses (Alves et al., 2021). Despite being considered a superficial infection, as patients need more complex medical procedures, such as transplants, oncological therapy, hemodialysis, HIV treatment, and the use of intravenous catheters or immunosuppressive drugs, the condition easily progresses to systemic candidiasis with high mortality (45-75%) (Hamdy et al., 2020;

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Robbins et al., 2017; Ribeiro et al., 2006; Rodrigues et al., 2004; Höfling et al., 2001).

In recent years, especially after the coronavirus pandemic (COVID-19), fungal infections have increased both in incidence and in resistance to existing treatments (Arastehfar et al., 2020; Diniz-Neto et al., 2024). The World Health Organization (WHO, 2022) recently published a list of the 19 main fungi that pose a threat to public health. Among these priority pathogens was *Candida albicans*, the main cause of candidemia which especially affects immunocompromised patients.

Depending on the clinical picture, conventional treatment of candidiasis involves azole derivatives (miconazole, clotrimazole, ketoconazole, itraconazole and fluconazole), polyenic derivatives (nystatin and amphotericin B), or echinocandins (caspofungin and micafungin) (Santos and Vieira, 2017). For systemic infections, echinocandins and amphotericin B are the principal antifungal agents used. In the case of oral candidiasis, nystatin, in the form of oral suspension and miconazole, available in gel, are routinely used, however, systemic therapy is also indicated with fluconazole or ketoconazole to treat cases of non-regression (Hamdy et al., 2020; Marquez and Quave, 2020).

In recent years, fungal resistance in *Candida* spp. (especially to azoles), and the observed antifungal toxicity of amphotericin B have encouraged the search for therapeutic alternatives (Alves et al. 2021; Marquez and Quave, 2020; Diniz-Neto et al., 2024; Santos et al., 2024; Debiasi et al., 2023).

Annona crassiflora Mart. (AcM), popularly known as *araticum*, *marolo*, or *pinha-do-cerrado*, is a plant found in the Cerrado biome of Brazil, with great food and economic potential (Brasil, 2018).

Studies have shown that extracts from different parts of its structure contain bioactive phenolic compounds with biological benefits that include antibacterial, nematicidal, antimalarial, antimutagenic, chemo-preventive, and anti-inflammatory activity, this without yet investigating its antifungal activity (Vilar et al., 2008; Dragano et al., 2010; Pimenta et al., 2014; Machado et al., 2015; Formagio et al., 2015; Carvalho et al., 2022).

In a previous unpublished screening study, the antifungal potential of the dichloromethane fraction of *A. crassiflora* was verified. Our study aimed to investigate this antifungal activity against strains of *C. albicans* isolated from the oral cavity.

2. Materials and Methods

2.1. Chemicals and microorganisms

Reference Candida spp. strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): ATCC 90028, ATCC 60193 ATCC 76485 e ATCC 76645, e and six strains of *C. albicans* clinical isolates from leukemic patients (PL), PL A1, PL A5, PL A8, PL A10, PL A11 and PL A15. All these clinical strains were previously collected (CAAE protocol: 43911715.8.0000.5188), these clinical strains were provided by the Cell Cultivation and Analysis Laboratory (LACEC)/UFPB for this research.

Nistatin, caspofungin, Tween 80%, DMSO, RPMI 1,640 with glutamine and ergosterol were obtained from Sigma-Aldrich® Chemical Co. (St. Louis, MO, EUA), and sorbitol (D-sorbitol anidro) from INLAB® (São Paulo, Brasil). The culture medium Sabouraud Dextrose Broth (CDS) e Sabouraud Dextrose Agar were obtained from KASVI® (Curitiba, Brasil).

2.2. Dichloromethane fraction from Annona crassiflora Mart. (FDAcM)

Dichloromethane fraction of the bark of the plant *Annona crassiflora* Mart. was kindly provided by Prof. Foued Salmen Espíndola from the Institute of Biotechnology, Federal University of Uberlândia, Uberlândia/MG. *A. crassiflora* Mart was collected in natural resources of the Cerrado Biome, in the northern region of Minas Gerais, in March 2015, and was identified by André Vito Scatigna, from the Institute of Biology of the Federal University of Uberlândia (UFU), in Uberlândia – MG, Brazil (Santos and Vieira, 2017).

The voucher specimen (HUFU68467) was deposited in the UFU herbarium (Herbarium Uberlandense) and the plant barks were carefully transported to the Biochemistry and Molecular Biology Laboratory of the Federal University of Uberlândia and stored at -20 °C until analysis (Santos and Vieira, 2017). The National Council for Scientific and Technological Development (CNPq), under Resolution 246/2009, of the Genetic Heritage Management Council, authorized the access and shipment of components of the genetic heritage, n. 010743/2015-4 (Santos and Vieira, 2017).

2.3. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The MIC was determined through the microdilution technique described by the Clinical and Laboratory Standards Institute (CLSI, 2018). Yeast suspensions were prepared in RPMI broth (Roswell Park Memorial Institute) media and adjusted by turbidity equivalence to $2.5x10^3$ UFC / mL, at 530 nm, abs 0.08 - 0.1.

Serial dilutions of the test substances were made in 96-well U-bottom microtiter plates containing sterile RPMI, in triplicate. The plates were incubated for 24 h at 35 ºC, and the results were read by visual observation of cell aggregates at the bottom of the wells. FDAcM was tested at concentrations ranging from 8 µg/mL to 1.024 µg/mL. Nystatin (Sigma-Aldrich, São Paulo, SP, Brazil) was used as a positive control and tested at concentrations ranging from 0.93 µg/mL a 120 µg/mL. Strain viability and media sterility controls were included simultaneously in the assay; DMSO (dimethyl sulfoxide) (Sigma-Aldrich, São Paulo, Brazil) and tween 80 (Sigma-Aldrich) was used for the preparation of nystatin and FDAcM solutions.

The MFC was defined as the lowest concentration of the drug able to inhibit visible growth on solid media. Aliquots from the wells corresponding to the MIC and higher concentrations were subcultured onto Sabouraud Dextrose agar (KASVI1, Kasv Imp and Dist. Prod/Laboratórios LTDA, Curitiba, Brazil). The plates were incubated for 24 h at 35 °C, and reading was performed by visual observation of fungal growth on the solid media based on the counting

of Colony-Forming Units (CFU). The MFC/MIC ratio was calculated to determine whether the substance had fungistatic (MFC/MIC ≥ 4) or fungicidal (MFC/MIC < 4) activity (CLSI, 2012).

2.4. Microbial growth curve

For the following tests, *C. albicans* ATCC 90028 strain and *C. albicans* clínicals (PL A11) ($2.5x10^3$ UFC / mL) strain of each species which showed MIC results were randomly selected and used. The MIC assay results were confirmed by verifying the interference of different product concentrations MIC (512 µg/mL), MICx2 (1,024 µg/mL) and MIC/2 (256 µg/mL), on the microbial growth curve in a 24h period. Following the same procedure in the MIC assay, the plates were incubated for 24 hours at 35 ± 2 °C in a microplate reader (BIOTEK™ EON™) and absorbance values were read at 530 nm every four hours. Nystatin was used as a positive control and tested at concentrations ranging from 1.5 at 12 µg/mL. The experiment was carried out in triplicate (Klepser et al., 1998).

2.5. Effects of FDAcM on biofilm reduction

The effects of different concentrations of FDAcM against mixed-species biofilm reduction (*C. albicans* ATCC 90028, *C. albicans* PL A10, *C. albicans* PL A11, *C. albicans* PL A15) were determined according to a microdilution protocol adapted from (Rodrigues et al., 2015). Briefly, 100 µL of Sabouraud Dextrose Broth (SDB, KASVI, Curitiba, Brazil) were added to 96-well U-bottom microdilution plates, then 100 µL of FDAcM solution (30, 20, 10 e 5 mg/mL) were added to the wells. Lastly, 100 µL of yeast inoculum (25 µL of each strain at 2.5×10^6 CFU/mL) prepared with Sabouraud Broth plus sucrose (2%) were added to the wells.

Nystatin (Sigma-Aldrich, São Paulo, Brazil) was used as a control. Media sterility and untreated growth controls were also included in all assays. The plates were incubated at 35 ºC for 48 h. Biofilm was quantitated using 0.4% crystal violet (w/v), followed by dissolution in 95% ethanol. The optical density of 95% ethanol was measured at 595 nm (Multiskan GO; Thermo Fisher Scientific). Inhibition of adherence was measured indirectly considering the yeast growth group as 100% of fungal adherence (Rodrigues et al., 2015).

2.6. Effects of FDAcM on the fungal cell wall and membrane permeability

2.6.1. Sorbitol Test (Effect on Cell Wall)

For this assay, the MIC value was defined as the lowest concentration of the substance inhibiting visible microbial growth in the presence of sorbitol (D-sorbitol anhydrous) (INLAB, São Paulo, Brazil) (Leite et al., 2014; Freires et al., 2014). The microdilution technique was used to compare the MIC values of FDAcM against *C. albicans* ATCC 90028 and *C. albicans* clínics (PL A11) in the absence and presence of sorbitol at 0.8 µM. The technique was performed following the same procedures described in Section MIC. The plates were incubated at 35º C, and readings were performed 24 h after incubation (Leite et al., 2014; Freires et al., 2014).

The positive control for this assay was caspofungin at an initial concentration of 4 µg/mL (caspofungin diacetate—Sigma-Aldrich, St. Louis, MO, USA), which is known to disrupt the yeast cell wall (Kartsonis et al., 2003; Pierce et al., 2013).

2.6.2. Ergosterol test (effect on cell membrane)

For this assay, the MIC was defined as the lowest concentration of the substance inhibiting visible microbial growth in the presence of exogenous ergosterol. The assay was performed using the microdilution technique, as previously described, in the presence of exogenous ergosterol (Sigma-Aldrich, São Paulo, Brazil) at a concentration of 400µg/mL. The strains used in this test were the same as those described in Section MIC.

The plates were incubated at 35°C, and the readings were performed after 24 h. Nystatin was used as a positive control at the concentration of 0.93 µg/mL a 120 µg/mL for its known activity on yeast cell membranes, binding to membrane sterols and thereby disrupting membrane permeability. A control with 96% ethanol and tween 80% (used to prepare ergosterol solutions) was also included (Djordjevic et al., 2002).

2.6.3. Cytotoxic effects of FDAcM on human erythrocytes

The hemolytic activity of FDAcM was determined using human red blood cells. Briefly, 80 µL of a 5% erythrocyte/PBS suspension was mixed with of 20 µL FDAcM at different concentrations (MIC at MICx8) and incubated at 37°C for 1 h. Then, 200 µL of phosphate-buffered saline (PBS; 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 136.9 mM NaCl, and 2.6 mM KCl, pH 7.2) was added to stop the hemolysis process, and the samples were centrifuged for 10 min at 1000g.

The supernatant was collected, and hemolysis was measured spectrophotometrically (550 nm). The hemolysis percentage was determined as [(Abssam− Abscon)/(Abstot − Abscon) × 100], where Abssam was the absorbance of the samples, Abscon corresponded to the absorbance of the blank control (without drugs), and Abstot was the absorbance of total hemolysis (replacing the sample solution by an equal volume of Milli-Q water). Study volunteers authorized their participation by signing an informed consent form. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Research Ethics Committee at the Federal University of Paraiba under protocol n° 16861519.8.0000.5188 (Rodrigues et al., 2015).

2.7. Statistical analysis

The data were analyzed by the Tukey Analysis of Variance (ANOVA) with the Bonferroni *post hoc* test, using the GraphPadPrism software (version 7.0 for Windows, San Diego, CA, USA). The data were considered significant when $P < 0.05$.

3. Results and Discussion

3.1. Annona crassiflora Mart (FDAcM) dichloromethane fraction analysis

Table 1 presents the phytochemical prospection results for total phenolics, and proanthocyanidins contents of

fractions purified from the peels of *Annona crassiflora* Mart. fruit (FDAcM), potentially related to antifungal activity (Justino et al., 2016; Justino et al., 2020).

The ethanolic extraction of peels from *A. crassiflora* M. (EtOAcM) presented a total yield of about 5%. Of the organic fractions in the ethanolic extract, the FDAcM fraction presented a yield of approximately 5.5%. Proanthocyanidins were purified from the EtOAcM fraction using column chromatography, and resulted in the 12 fractions (F1-F12) grouped according to the Rf values. F6 and F7 presented respectively higher yields of 24.6% and 14.0%, followed respectively by F4, F5, F8, and F3 (5.2, 3.7, 1.6, and 1.4%). The other fractions presented yields below 1% (Justino et al., 2020).

In general, all of the fractions presented total phenolics and proanthocyanidins respectively greater than 150 mg of GAE g^{-1} and 250 mg of EC g^{-1}. As expected, the FDAcM fraction also contained a substantial amount of phenolic compounds. Of the analyzed fractions, F7 presented the highest values for total phenolics (660.6 mg GAE g^{-1}) and proanthocyanidins (1,295.7 mg EC g^{-1}) (Justino et al., 2020).

From ^{1}H and ^{13}C NMR analyses of the FDAcM fraction, it was possible to relate the profile to characteristic signs of aporphine alkaloids, which are considered markers for the Annonaceae family. The chemical shifts of the signals at higher intensities corroborate the presence of the alkaloids crassiflorin and stephalagin, both reported for *Annona crassiflora* (Peña-Hidalgo et al. 2021). It was also possible to detect signals of a fatty acid derivative $CH_3(CH_2)nCOOH$.

Table 1. Total content of phenols and proanthocyanidins in fruit peel fractions of A. crassiflora Mart.

Fractions	Total phenolic content (mg GAE g^{-1})	Proanthocyanidin content (mg EC g^{-1})
F1	205.5±15.1	385.3±19.5
F2	483.9±52.7	720.4±66.8
F3	446.9±33.4	650.2±54.2
F4	468.9±24.2	840.2±95.3
F5	502.1±16.4	902.7±126.5
F6	545.1±65.8	1102.2±135.7
F7	660.6±71.9	1295.7±91.4
F8	455.8±32.5	878.3±165.8
F9	448.8±22.3	778.4±116.0
F10	384.1±53.6	628.8±49.0
F11	232.8±32.4	443.0±31.8
F12	151.3±14.0	278.3±31.8
FDAcM	497.9±38.7	757.5±12.0

Note: Values expressed as mean ± standard deviation. Note: FDAcM = dichloromethane fraction .

3.2. Determination of minimum inhibitory (MIC) and minimum fungicide (MFC) concentration values

FDAcM was evidenced in the 10 fungal species tested. MIC and MFC values for FDAcM and nystatin (standard drug) are shown in Table 2. FDAcM was able to inhibit the growth of 8 (80%) of 10 strains of *C. albicans* up to a concentration of 512 µg/ml. One of these strains (*C. albicans* ATCC 60193) suffered inhibition at a concentration of 256 µg/ml. Only two strains (*C. albicans* PL A10 and *C. albicans* PL A15) were inhibited at the concentration of 1,024 µg/ml. For nystatin, MIC and MFC values ranging from 7.5 µg/mL to 60 µg/mL were observed.

The MFC of FDAcM ranged between 256 and 1,024 µg/mL. In comparative MIC and MFC analyses (Table 2) it was observed that 4 of 10 strains tested presented MFC values two times higher than their MIC values, all other tested strains (6/10) presented MFC values equal to their MIC values. This characterized the observed fungicidal activity of

Table 2. Results of the evaluation of the minimum inhibitory and fungicidal concentration (MIC/MFC) of the ethanolic extract of A. crassiflora Mart and nystatin on C. albicans. MIC and MFC expressed their values in µg/mL.

STRAINS	***A. crassiflora* Mart**				**NYSTATIN**		
	MIC	**MFC**	**MFC/MIC**	**ANTIFUNGAL ACTIVITY**	**MIC**	**MFC**	**MFC/MIC**
C. albicans ATCC 90028	512 µg/mL	512 µg/mL	1	Fungicidal	7.5µg/mL	7.5µg/mL	1
C. albicans ATCC 60193	256 µg/mL	256 µg/mL	1	Fungicidal	7.5µg/mL	7.5µg/mL	1
C. albicans ATCC 76485	512 µg/mL	1,024 µg/mL	2	Fungicidal	7.5µg/mL	7.5µg/mL	1
C. albicans ATCC 76645	512 µg/mL	1,024 µg/mL	2	Fungicidal	7.5µg/mL	7.5µg/mL	1
C. albicans PL A1	512 µg/mL	1,024 µg/mL	2	Fungicidal	7.5µg/mL	7.5µg/mL	1
C. albicans PL A5	512 µg/mL	512 µg/mL	1	Fungicidal	60 µg/mL	60 µg/mL	1
C. albicans PL A8	512 µg/mL	512 µg/mL	2	Fungicidal	15 µg/mL	15 µg/mL	1
C. albicans PL A10	1,024 µg/mL	1,024 µg/mL	1	Fungicidal	15 µg/mL	15 µg/mL	1
C. albicans PL A11	512 µg/mL	512 µg/mL	1	Fungicidal	7.5 µg/mL	7.5 µg/mL	1
C. albicans PL A15	1,024 µg/mL	1,024 µg/mL	1	Fungicidal	7.5 µg/mL	7.5 µg/mL	1

MFC/MIC > 4 signify it is fungistatic, <4 means it is fungicidal

FDAcM against the tested strains, which are in accordance with established criteria (Balouiri et al., 2016). The MFC/MIC ratio indicated fungicidal effect against all tested strains

In the literature, the MIC values of natural products of plant origin are interpreted and considered active or not, according to the following criteria: 50-500 µg/mL = strong/optimal activity; 600-1,500 µg/mL = moderate activity; above 1,500 µg/mL = weak activity or inactive product (Sartoratto et al., 2004; Houghton et al., 2007. Webster et al. (2008) proposed that satisfactory MIC values occur at 1,000µg/mL or less. It can be concluded that FDAcM presents strong/great antifungal activity.

Plant molecules are enormously diverse in terms of structure, physicochemical, and biological properties, and thus the growing interest of the pharmaceutical industry in the discovery of drugs from these natural sources (Bresolin and Cechinel-Filho, 2009). Extracts, fractions, and isolated compounds extracted from natural sources have been revealed by researchers to present various significant properties, including antimicrobial activity (Höfling et al., 2010; Albernaz et al., 2010).

Costa et al. (2013) tested the antimicrobial activity of three aporphinoids (liriodenine 1, anonaine 2, and asymilobin 3), a benzyltetrahydroisoquinoline (reticuline 4), and an azaanthracene (cleistofoline 5). These alkaloids were extracted from *Annona salzmanni* bark (a plant of the same genus as *A.* classifora), and tested on bacteria such as *K. rhizophila, and S. epidermidis,* and against yeasts such as *C. dubliniensis, C. albicans,* and *C. parapsilosis.* The authors found that against *Candida* spp., reticulin presented an MIC of more than 100 mg/mL.

Through previous studies it was observed that the crude extract of the leaves of *A. crassiflora* Mart. (enriched alkaloid phase) present bactericidal effect (Peña-Hidalgo et al., 2021. Additionally, extracts from different parts of the *A. crassiflora* fruit present antibacterial, antimalarial, antimutagenic, chemo-preventive, and anti-inflammatory activities. In studies corroborating the observations of this work, these activities have also been associated with the presence of bioactive compounds, such as phenolic molecules (Justino et al., 2016; Egydio et al., 2013; Lage et al., 2014).

However, none of the studies verified the antifungal activity of the FDAcM dichloromethane fraction, making this study pioneering.

Considering the observed MIC values and the fungicidal character demonstrated for all tested strains, these promising results point to new antifungal agents.

3.3. Microbial growth curve

Based on the results of the observed MIC values, growth time curves were constructed for two randomly selected strains of *C. albicans* (*C. albicans* ATCC 90028 and clinical strain *C. albicans* PL A11) to evaluate the effect of the differing concentrations of FDAcM over time.

The results of the microbial kinetics assays are presented as absorbance curve graphs for *C. albicans* ATCC 90028 (Figure 1A) and *C. albicans* PL A11 (Figure 2B) in function of time (0, 4, 8, 12, 16, 20, and 24 hours), in the absence of the fraction (control), and in the presence of FDAcM at MICx2 (1,024 µg/mL), MIC (512 µg/mL) and MIC/2 (256 µg/mL).

Figure 1A demonstrates that after 12 hours of fungal growth, the number of *C. albicans* ATCC 90028 viable cells was significantly reduced compared to the untreated control. Reductions of 57.5% and 68.36%, were respectively observed for the fungi exposed to concentrations of MIC and MICx2. These reductions were even greater after 24-hours when fungal growth reductions of 77%, 85%, and 88% were respectively observed for the concentrations of MIC/2, MIC, and MICx2, as compared to the control (Figure 1).

Figure 1B reveals that at 12h of treatment, the number of viable *C. albicans* PL A11 cells (clinical isolate) was significantly reduced compared to the control when treated with the three studied FDAcM concentrations ($P \leq 0.001$). At 24 hours there were respective reductions in fungal growth of 88%, 92%, and 94% for the concentrations of MIC/2, MIC, and MICx2 as compared to the control. Thus, it was concluded that the antifungal activity of FDAcM it is not time dependent for the tested strains.

Differing growth results have been observed with other natural extracts and *Candida* spp., though similar studies

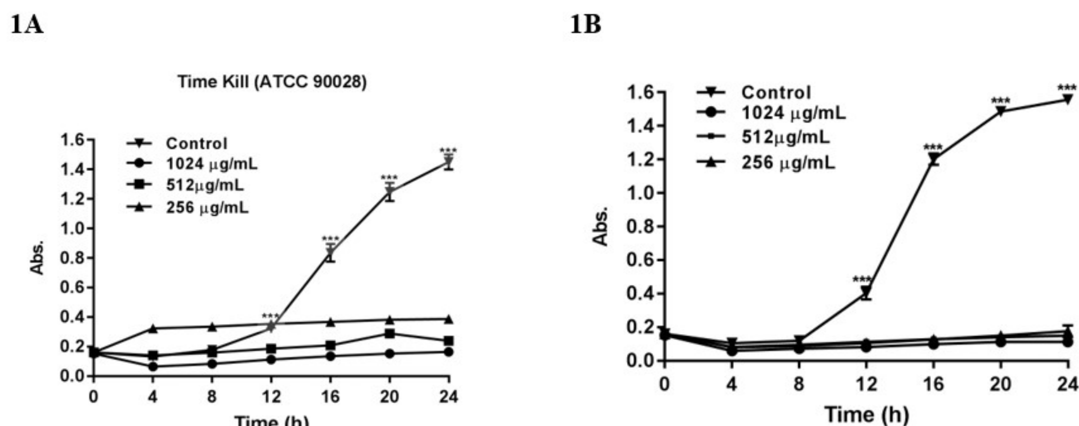

Figure 1. Microbial growth curve of the strain C.albicans 90028 **(1a)** and PL A11 **(1b),** under the action of different concentrations of ethanolic extract of A. crassiflora Mart. The graph represents the mean ± standard deviation of the mean. ***p ≤ 0.001 compared to control.

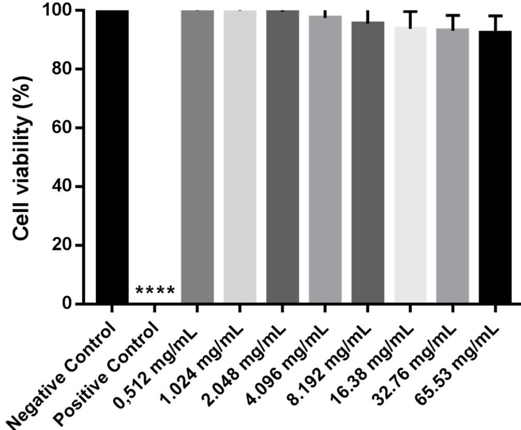

Figure 2. Effect of dichloromethane extract from A. crassiflora Mart. on cell viability in human erythrocytes within 1 h of exposure.

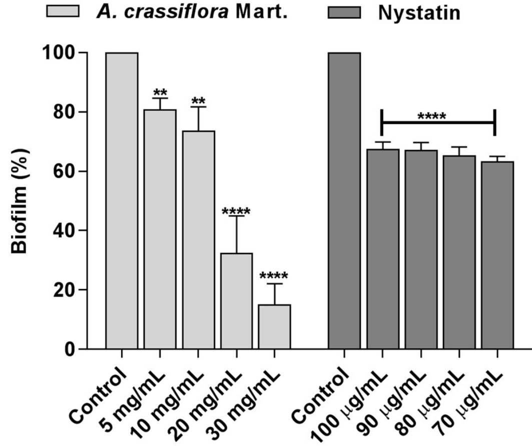

Figure 3. Percentage of C. albicans biofilm treated with different concentrations of diclomethane extract from A. crassiflora Mart. and Nystatin.

with FDAcM were not found in the literature, and our results are thus unprecedented. Yet in contrast, propolis extract (EP) presents growth inhibition effect for up to 12 hours (450 µg /ml) for strains of the genus *Candida* (Tobaldini-Valerio et al., 2016).

3.4. Assessment of FDAcM activity against fungal biofilm

Biofilm growth confers advantages to microorganisms, including resistance to antimicrobial agents, increased virulence, and protection against host defenses. Biofilm is the principal form of growth of many opportunistic fungi, such as *Candida yeasts.* (Mello et al., 2016).

To analyze the anti-biofilm activity of FDAcM, mature biofilm (48 hours after formation) was treated with FDAcM at concentrations of MIC x 10 (5mg/mL), MIC x 20 (10mg/mL), MIC x 40 (20mg/mL), and MIC x 60 (30mg/mL). During the analysis, it was observed that FDAcM reduced the biofilm significantly in all analyzed concentrations ($p<0.05$), with respective reductions of 19.29%, 26.42%, 67.75%, and 85.1% as compared to the growth control (Figure 3). Further, nystatin reduced mature biofilm from the lowest concentration analyzed (70 µg/mL), this reduction being 36.9% as compared to the control (Figure 3). To date, there have been no studies of FDAcM with regard to anti-biofilm activity.

Prolonged antibiotic therapy, parenteral nutrition, chemotherapy, and breakdown of mucosal skin barriers are important determinants for the development of biofilms. Thus, this test was of great importance, as infections related to *Candida* spp. biofilms, when not treated correctly, can lead to invasive fungal infections and bloodstream infections with a high risk of mortality (Diniz-Neto et al., 2024).

3.5. FDAcM activity on the cell wall and interaction with ergosterol

3.5.1. Sorbitol assay

One way to assess whether a new drug acts on the cell wall is through the sorbitol assay, based on the ability of sorbitol to act as an osmotic protector in the fungal cell wall, higher MIC values in the presence of sorbitol (standard medium) suggest that the cell wall is a likely cellular target of the compound under analysis. (Kartsonis et al., 2003; Pierce et al., 2013; Hao et al., 2013).

In the presence of sorbitol there was no increase in the MIC, both for the *C. albicans strains* ATCC 90028 or *C. albicans* PL A11, with the values remaining the same (512 µg/mL) (Table 3). For caspofungin (positive control), the respective mean MIC values were 0.25 µg/mL, in the absence of sorbitol, and 2 µg/mL in the presence of sorbitol - (0.8 M) in *C. albicans* ATCC and *C. albicans* clinical isolates, as already reported in the literature (Santos and Vieira, 2017).

Although the basic structure of the fungal cell wall is the same in all species (chitin and β - glycans), each fungal cell wall presents a different chemical composition. The genes that encode for chitin synthesis and the signals by which they are activated depend on specific environmental conditions and cell cycle stage, and thus vary greatly between species (Santos and Vieira, 2017). The sorbitol assays carried out in this research demonstrated that the FDAcM does not exert its antifungal effect through the cell wall.

3.5.2. Interaction with Ergosterol

Ergosterol is an important pharmacological target found in fungi yet absent in human cells. Ergosterol is important to fungal plasma membrane maintenance and against drugs that interact directly with the plasma membrane by forming pores, such as polyenes (Minnebruggen et al., 2010). The next pharmacological target studied regarding the mechanism of action of FDAcM was therefore the plasma membrane.

The exogenous ergosterol assays demonstrated that FDAcM does not exert its antifungal effect through interaction with the ergosterol present in the fungal cell membrane, since the MIC values in the presence and absence of exogenous ergosterol were the same (512 µg/mL),

Table 3. MIC values (μg / mL) of drugs and extract in the absence and presence of sorbitol (0.8M) and ergosterol (400 μg / mL) against C. albicans ATCC 90028 and C. albicans PL A11.

Microorganisms	**MIC values µg/mL**					
	C. albicans	***C. albicans***	***C. albicans***	***C. albicans***	***C. albicans***	***C. albicans***
	ATCC 90028	**PL A11**	**ATCC 90028**	**PL A11**	**ATCC 90028**	**PL A11**
Drug	Control		+Sorbitol		+Ergosterol	
FDAcM	512	512	512	512	512	512
Nystatin	7.5	7.5	-	-	120	120
Caspofungin	7.5	7.5	120	120	-	-

both for the *C. albicans* strains ATCC 90028 and *C. albicans* PL A11 (Table 3). For nystatin, average MIC values of 7.5 µg/mL in the absence of ergosterol, and 30 µg/mL in the presence of ergosterol were observed for tested strains. Nystatin is recognized for interfering in the fungal cell membrane via ergosterol (Mathew and Nath, 2009).

In view of these results, it was verified that the observed antifungal activity of FDAcM did not occur from activity involving two classic pharmacological targets of current antifungals, and thus requires further study.

3.5.3. Evaluation of hemolytic potential in human erythrocytes

Medicinal plants have in their composition active principles responsible for the therapeutic properties attributed to them. Despite this, adverse reactions may appear as a result of misuse or direct contact (Veiga-Junior et al., 2005). Hemolytic activity was used to evaluate the potential of FDAcM to cause lesions in the blood cell plasma membrane.

Hemolysis is characterized as rupture of the erythrocyte with the release of hemoglobin and free hemoglobin into the plasma. This is harmful to health, causing serious damage to vital organs, such as the liver, kidneys, and heart. These results are significant for the use of this plant, which revealed a significant antioxidant potential, as well as promising antifungal activity (Silva et al., 2011; Trentin et al., 2015). In this assay, FDAcM using MIC concentrations of even MICx8 did not cause damage to the erythrocyte plasma membrane, demonstrating compatibility with human red blood cells (Figure 2). Thus, there are perspectives for its use with complete safety and for continuation of tests for future application in humans.

References

ALBERNAZ, L.C., DE PAULA, J.E., ROMERO, G.A., SILVA, M.R., GRELLIER, P., MAMBU, L. and ESPINDOLA, L.S., 2010. Investigation of plant extracts in traditional medicine of the Brazilian Cerrado against protozoans and yeasts. *Journal of Ethnopharmacology*, vol. 131, no. 1, pp. 116-121. http://doi.org/10.1016/j.jep.2010.06.011. PMid:20600775.

ALVES, D.N., MARTINS, R.X., FERREIRA, E.S., ALVES, A.F., DE ANDRADE, J.C., BATISTA, T.M., LAZARINI, J.G., AMORIM, L.S., ROSALEN, P.L., FARIAS, D.F. and DE CASTRO, R.D., 2021. Toxicological parameters of a formulation containing cinnamaldehyde for use in treatment of oral fungal infections: an in vivo study. *BioMed Research International*, vol. 2021, pp. 23056995. http://doi.org/10.1155/2021/2305695. PMid:34722758.

ARASTEHFAR, A., CARVALHO, A., NGUYEN, M.H., HEDAYATI, M.T., NETEA, M.G., PERLIN, D.S. and HOENIGL, M., 2020. COVID 19-associated candidiasis (CAC): an underestimated complication in the absence of immunological predispositions? *Journal of Fungi (Basel, Switzerland)*, vol. 6, no. 4, pp. 211. http://doi.org/10.3390/jof6040211. PMid:33050019.

BALOUIRI, M., SADIKI, M. and IBNSOUDA, S.K., 2016. Methods for in vitro evaluating antimicrobial activity: a review. *Journal of Pharmaceutical Analysis*, vol. 6, no. 2, pp. 71-79. http://doi.org/10.1016/j.jpha.2015.11.005. PMid:29403965.

BRASIL. Ministério do Meio Ambiente, 2018 [viewed 15 October 2018]. *Biodiversidade Brasileira* [online]. Available from: http://www.mma.gov.br/biodiversidade/biodiversidade-brasileira

BRESOLIN, T.M.B. and CECHINEL-FILHO, V., 2009. *Fármacos e medicamentos: uma abordagem multidisciplinar*. São Paulo: Santos, 416 p.

CARVALHO, N.C.C., MONTEIRO, O.S., DA ROCHA, C.Q., LONGATO, G.B., SMITH, R.E., DA SILVA, J.K.R. and MAIA, J.G.S., 2022. Phytochemical analysis of the fruit pulp extracts from Annona crassiflora Mart. and evaluation of their antioxidant and antiproliferative activities. *Foods*, vol. 11, no. 14, pp. 2079. http://doi.org/10.3390/foods11142079. PMid:35885322.

CLINICAL AND LABORATORY STANDARDS INSTITUTE - CLSI, 2018. *Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard; CLSI document M27-A3*. Wayne: Clinical and Laboratory Standards Institute.

CLINICAL AND LABORATORY STANDARDS INSTITUTE - CLSI, 2012. *Clinical and Laboratory Standards Institute. Reference Method for Broth Dilution Antifungal Susceptibility Testing of yeasts; Fourth Informational Supplement - M27-s4*. Wayne: Clinical and Laboratory Standards Institute.

COSTA, E.V., DA CRUZ, P.E., DE LOURENÇO, C.C., MORAES, V.R.S., NOGUEIRA, P.C.L. and SALVADOR, M.J., 2013. Antioxidant and antimicrobial activities of aporphinoids and other alkaloids from the bark of Annona salzmannii A. DC. (Annonaceae). *Natural Product Research*, vol. 27, no. 11, pp. 1002-1006. http://doi.org/10.1080/14786419.2012.688044. PMid:22582985.

DEBIASI, B.W., RAISER, A.L., DOURADO, S.H.A., TORRES, M.P.R., ANDRIGHETTI, C.R., BONACORSI, C., BATTIROLAD, L.D., RIBEIROB, E.B. and VALLADÃO, D.M.S., 2023. Phytochemical screening of Cordia glabrata (MART.) A.DC. extracts and its potential antioxidant, photoprotective, antimicrobial and antiviral activities. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 83, pp. e248083. http://doi.org/10.1590/1519-6984.248083. PMid:34190769.

DINIZ-NETO, H., SILVA, S.L., CORDEIRO, L.V., SILVA, D.F., OLIVEIRA, R.F., ATHAYDE-FILHO, P.F., OLIVEIRA-FILHO, A.A., GUERRA, F.Q.S. and LIMA, E.O., 2024. Antifungal activity of 2-chloro-N-phenylacetamide: a new molecule with fungicidal and antibiofilm activity against fluconazole-resistant Candida spp. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 84, pp. e255080. http://doi.org/10.1590/1519-6984.255080.

DJORDJEVIC, D., WIEDMANN, M. and MCLANDSBOROUGH, L.A., 2002. Microtiter Plate Assay for Assessment of Listeria monocytogenes Biofilm Formation. *Applied and Environmental Microbiology*, vol. 68, no. 6, pp. 2950-2958. http://doi.org/10.1128/AEM.68.6.2950-2958.2002. PMid:12039754.

DRAGANO, N.R., DE VENANCIO, V.P., PAULA, F.B.A., DELLA, F.L., FONSECA, M.J. and AZEVEDO, L., 2010. Influence of Marolo (Annona crassiflora Mart.) pulp intake on the modulation of mutagenic/antimutagenic processes and its action on oxidative stress in vivo. *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, vol. 65, no. 4, pp. 319-325. http://doi.org/10.1007/s11130-010-0191-3. PMid:20878359.

EGYDIO, A.P.M., VALVASSOURA, T.A. and SANTOS, D.Y.A.C., 2013. Geographical variation of isoquinoline alkaloids of *Annona crassiflora Mart.* from Cerrado, Brazil. *Biochemical Systematics and Ecology*, vol. 46, pp. 145-151. http://doi.org/10.1016/j.bse.2012.08.006.

FORMAGIO, A.S.N., VIEIRA, M.C., VOLOBUFF, C.R.F., SILVA, M.S., MATOS, A.I., CARDOSO, C.A.L., FOLIO, M.A. and CARVALHO, J.E., 2015. In vitro biological screening of the anticholinesterase and antiproliferative activities of medicinal plants belonging to Annonaceae. *Brazilian Journal of Medical and Biological Research*, vol. 48, no. 4, pp. 308-315. http://doi.org/10.1590/1414-431x20144127. PMid:25714885.

FREIRES, I.A., MURATA, R.M., FURLETTI, V.F., SARTORATTO, A., ALENCAR, S.M., FIGUEIRA, G.M., RODRIGUES, J.A.O., DUARTE, M.C. and ROSALEN, P.L., 2014. Coriandrum sativum L. (Coriander) essential oil: antifungal activity and mode of action on Candida spp., and molecular targets affected in human whole-genome expression. *PLoS One*, vol. 9, no. 6, pp. e99086. http://doi.org/10.1371/journal.pone.0099086. PMid:24901768.

HAMDY, R., FAYED, B., HAMODA, A.M., RAWAS-QALAJI, M., HAIDER, M. and SOLIMAN, S.S.M., 2020. Essential oil-based design and development of novel anti-Candida azoles formulation. *Molecules (Basel, Switzerland)*, vol. 25, no. 6, pp. 1463. http://doi.org/10.3390/molecules25061463. PMid:32213931.

HAO, B., CHENG, S., CLANCY, C.J. and NGUYEN, M.H., 2013. Caspofungin kills Candida albicans by causing both cellular apoptosis and necrosis. *Antimicrobial Agents and Chemotherapy*, vol. 57, no. 1, pp. 326-332. http://doi.org/10.1128/AAC.01366-12. PMid:23114781.

HÖFLING, J.F., ANIBAL, P.C., OBANDO-PEREDA, G.A., PEIXOTO, I.A.T., FURLETTI, V.F., FOGLIO, M.A. and GONÇALVES, R.B., 2010. Antimicrobial potential of some plant extracts against Candida species. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 70, no. 4, pp. 1065-1068. http://doi.org/10.1590/S1519-69842010000500022. PMid:21180915.

HÖFLING, J.F., ROSA, E.A.R., PEREIRA, C.V., BORIOLLO, F.G. and RODRIGUES, J.A.O., 2001. Differentiation and numerical analysis of oral yeasts based on SDS-Page profiles. Influence of the culture media on the whole-cell protein extracts. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 61, no. 3, pp. 507-516. http://doi.org/10.1590/S1519-69842001000300021. PMid:11706579.

HOUGHTON, P.J., HOWES, M.J., LEE, C.C. and STEVENTON, G., 2007. Uses and abuses of in vitro tests in ethnopharmacology: visualizing an elephant. *Journal of Ethnopharmacology*, vol. 110, no. 3, pp. 391-400. http://doi.org/10.1016/j.jep.2007.01.032. PMid:17317057.

JUSTINO, A.B., COSTA, M.S., SARAIVA, A.L., SILVA, P.H., VIEIRA, T.N., DIAS, P., LINHARES, C.R.B., DECHICHI, P., AVILA, V.M.R., ESPINDOLA, F.S. and SILVA, C.R., 2020. Protective effects of a polyphenol-enriched fraction of the fruit peel of Annona crassiflora Mart. on acute and persistent inflammatory pain. *Inflammopharmacology*, vol. 28, no. 3, pp. 759-771. http://doi.org/10.1007/s10787-019-00673-7. PMid:31845053.

JUSTINO, A.B., PEREIRA, M.N., VILELA, D.D., PEIXOTO, L.G., MARTINS, M.M., TEIXEIRA, R.R., MIRANDA, N.C., DA SILVA, N.M., DE SOUSA, R.M., DE OLIVEIRA, A. and ESPINDOLA, F.S., 2016. Peel of araticum fruit (Annona crassiflora Mart.) as a source of antioxidant compounds with α-amylase, α-glucosidase and glycation inhibitory activities. *Bioorganic Chemistry*, vol. 69, pp. 167-182. http://doi.org/10.1016/j.bioorg.2016.11.001. PMid:27842248.

KARTSONIS, N.A., NIELSEN, J. and DOUGLAS, C.M., 2003. Caspofungin: the first in a new class of antifungal agents. *Drug Resistance Updates*, vol. 6, no. 4, pp. 197-218. http://doi.org/10.1016/S1368-7646(03)00064-5. PMid:12962685.

KLEPSER, M.E., ERNST, E.J., LEWIS, R.E., ERNST, M.E. and PFALLER, M.A., 1998. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrobial Agents and Chemotherapy*, vol. 42, no. 5, pp. 1207-1212. http://doi.org/10.1128/AAC.42.5.1207. PMid:9593151.

LAGE, G.A., MEDEIROS, F.S., FURTADO, W.L., TAKAHASHI, J.A., DE SOUZA FILHO, J.D. and PIMENTA, L.P., 2014. The first report on flavonoid isolation from Annona crassiflora Mart. *Natural Product Research*, vol. 28, no. 11, pp. 808-811. http://doi.org/10.1080/14786419.2014.885518. PMid:24571732.

LEITE, M.C.A., BEZERRA, A.P.B., SOUSA, J.P., GUERRA, F.Q.S. and LIMA, E.O., 2014. Evaluation of antifungal activity and mechanism of action of citral against Candida albicans. *Evidence-Based Complementary and Alternative Medicine*, vol. 2014, pp. 378280. http://doi.org/10.1155/2014/378280. PMid:25250053.

MACHADO, A.R., FERREIRA, S.R., DA SILVA, F.M., FUJIWARA, R.T., DE SOUZA FILHO, J.D. and PIMENTA, L.P., 2015. Nematicidal activity of Annona crassiflora leaf extract on Caenorhabditis elegans. *Parasites & Vectors*, vol. 8, no. 1, pp. 113. http://doi.org/10.1186/s13071-015-0708-6. PMid:25885032.

MARQUEZ, L. and QUAVE, C.L., 2020. Prevalence and therapeutic challenges of fungal drug resistance: role for plants in drug discovery. *Antibiotics (Basel, Switzerland)*, vol. 9, no. 4, pp. 150. http://doi.org/10.3390/antibiotics9040150. PMid:32244276.

MATHEW, B.P. and NATH, M., 2009. Recent approaches to antifungal therapy for invasive mycoses. *ChemMedChem*, vol. 4, no. 3, pp. 310-323. http://doi.org/10.1002/cmdc.200800353. PMid:19170067.

MELLO, T.P., AOR, A.C., GONÇALVES, D.S., SEABRA, S.H., BRANQUINHA, M.H. and SANTOS, A.L., 2016. Assessment of biofilm formation by Scedosporium apiospermum, S. aurantiacum, S. minutisporum and Lomentospora prolificans. *Biofouling*, vol. 32, no. 7, pp. 737-749. http://doi.org/10.1080/08927014.2016.1192610. PMid:27309801.

MINNEBRUGGEN, G.V., FRANCOIS, I.E.J.A., CAMMUE, B.P.A., THEVISSEN, K., VROOME, V., BORGERS, M. and SHROOT, B., 2010. A general overview on past, present and future antimycotics. *The Open Mycology Journal*, vol. 4, no. 1, pp. 22-32. http://doi.org/10.2174/1874437001004010022.

PEÑA-HIDALGO, M., FURTADO, L.C., COSTA-LOTUFO, L.V., FERREIRA, M.J.P. and SANTOS, D.Y.A.C., 2021. Alkaloids from the Leaves of Annona crassiflora and Their Cytotoxic Activity. *Revista*

Brasileira de Farmacognosia, vol. 31, pp. 244-248. http://doi.org/10.1007/s43450-021-00147-4.

PIERCE, C.G., SRINIVASAN, A., UPPULURI, P., RAMASUBRAMANIAN, A.K. and LÓPEZ-RIBOT, J.L., 2013. Antifungal therapy with an emphasis on biofilms. *Current Opinion in Pharmacology*, vol. 13, no. 5, pp. 726-730. http://doi.org/10.1016/j.coph.2013.08.008. PMid:24011516.

PIMENTA, L.P., GARCIA, G.M., GONÇALVES, S.G., DIONÍSIO, B.L., BRAGA, E.M. and MOSQUEIRA, V.C., 2014. In vivo antimalarial efficacy of acetogenins, alkaloids and flavonoids enriched fractions from Annona crassiflora Mart. *Natural Product Research*, vol. 28, no. 16, pp. 1254-1259. http://doi.org/10.1080/14786419.2014.900496. PMid:24678811.

RIBEIRO, E.L., SCROFERNEKER, M.L., CAVALHAES, M.S., CAMPOS, C.C., NAGATO, G.M., SOUZA, N.A., FERREIRA, W.M., CARDOSO, C.G., DIAS, S.M.S., PIMENTA, F.C. and TOLEDO, A.O., 2006. Phenotypic aspects of oral strains of *Candida albicans* in children with Dow's syndrome. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 66, no. 3, pp. 939-944. http://doi.org/10.1590/S1519-69842006000500020. PMid:17119842.

ROBBINS, N., CAPLAN, T. and COWEN, L.E., 2017. Molecular Evolution of Antifungal Drug Resistance. *Annual Review of Microbiology*, vol. 71, no. 1, pp. 753-775. http://doi.org/10.1146/annurev-micro-030117-020345 PMid:28886681.

RODRIGUES, J.A.O., HÖFLING, J.F., TAVARES, F.C.A., DUARTE, K.M.R., GONÇALVES, R.B. and AZEVEDO, R.A., 2004. Evaluation of biochemical and serological methods to identify and clustering yeast cells of oral *Candida* species by CHROMagar test, SDS-Page and ELISA. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 64, no. 2, pp. 317-326. http://doi.org/10.1590/S1519-69842004000200018. PMid:15462306.

RODRIGUES, K.A., DIAS, C.N., NÉRIS, P.L., ROCHA, J.C., SCOTTI, M.T., SCOTTI, L., MASCARENHAS, S.R., VERAS, R.C., DE MEDEIROS, I.A., KEESEN, T.S., DE OLIVEIRA, T.B., DE LIMA, M.C., BALLIANO, T.L., DE AQUINO, T.M., DE MOURA, R.O., MENDONÇA JUNIOR, F.J. and DE OLIVEIRA, M.R., 2015. 2-Amino-thiophene derivatives present antileishmanial activity mediated by apoptosis and immunomodulation in vitro. *European Journal of Medicinal Chemistry*, vol. 6, pp. 1-14. http://doi.org/10.1016/j.ejmech.2015.10.011. PMid:26513640.

SANTOS, J.I. and VIEIRA, A.J.H., 2017. Mechanisms of resistance of Candida albicans to the antifungals fluconazole, amphotericin B and caspofungin. *Revista Brasileira de Análises Clínicas*, vol. 3, pp. 235-239.

SANTOS, L.S., FERNANDES, C.C., SANTOS, L.S., DIAS, A.L.B., SOUCHIE, E.L. and MIRANDA, M.L.D., 2024. Phenolic compounds and antifungal activity of ethyl acetate extract and methanolic extract from Capsicum chinense Jacq. ripe fruit. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 84, pp. e258084. http://doi.org/10.1590/1519-6984.258084. PMid:35195174.

SARTORATTO, A., MACHADO, A.L.M., DELARMELINA, C., FIGUEIRA, G.M., DUARTE, M.C.T. and REHDER, V.L.G., 2004. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Brazilian Journal of Microbiology*, vol. 35, pp. 275-280. http://doi.org/10.1590/S1517-83822004000300001.

SILVA, L.C., DA SILVA JUNIOR, C.A., DE SOUZA, R.M., MACEDO, A.J., DA SILVA, M.V. and CORREIA, M.T.S., 2011. Comparative analysis of the antioxidant and DNA protection capacities of Anadenanthera colubrina, Libidibia ferrea and Pityrocarpa moniliformis fruits. *Food and Chemical Toxicology*, vol. 49, no. 9, pp. 2222-2228. http://doi.org/10.1016/j.fct.2011.06.019. PMid:21712063.

TOBALDINI-VALERIO, F.K., BONFIM-MENDONÇA, P.S., ROSSETO, H.C., BRUSCHI, M.L., HENRIQUES, M., NEGRI, M., SILVA, S. and SVIDZINSKI, T.I.E., 2016. Propolis: a potential natural product to fight Candida species infections. *Future Microbiology*, vol. 11, no. 8, pp. 1035-1046. http://doi.org/10.2217/fmb-2015-0016. PMid:27501739.

TRENTIN, D.S., SILVA, D.B., FRASSON, A.P., RZHEPISHEVSKA, O., DA SILVA, M.V., PULCINI, E.L., JAMES, G., SOARES, G.V., TASCA, T., RAMSTEDT, M., GIORDANI, R.B., LOPES, N.P. and MACEDO, A.J., 2015. Natural Green coating inhibits adhesion of clinically important bacteria. *Scientific Reports*, vol. 5, pp. 8287. http://doi.org/10.1038/srep08287. PMid:25655943.

VEIGA JUNIOR, V.F., PINTO, A.C. and MACIEL, M.A.M., 2005. Plantas medicinais: cura segura? *Quimica Nova*, vol. 28, no. 3, pp. 519-528. http://doi.org/10.1590/S0100-40422005000300026.

VILAR, J.B., FERREIRA, F.L., FERRI, P.H., GUILLO, L.A. and CHEN, L., 2008. Assessment of the mutagenic, antimutagenic and cytotoxic activities of ethanolic extract of araticum (Annona crassiflora Mart. 1841) by micronucleus test in mice. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 68, no. 1, pp. 141-147. http://doi.org/10.1590/S1519-69842008000100020. PMid:18470389.

WEBSTER, D., TASCHEREAU, P., BELLAND, R.J., SAND, C. and RENNIE, R.P., 2008. Antifungal activity of medicinal plant extracts; preliminary screening studies. *Journal of Ethnopharmacology*, vol. 4, no. 115, pp. 140-146. http://doi.org/10.1016/j.jep.2007.09.014. PMid:17996411.

WORLD HEALTH ORGANIZATION - WHO, 2022, *WHO fungal priority pathogens list to guide research, development and public health action*. Geneva: WHO.