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In vitro evaluation of 2-bromo-*N*-phenylacetamide for antifungal activity against *Candida glabrata* oral cavity isolates

Gregório Márcio de Figueirêdo Rodrigues[1], Edeltrudes de Oliveira Lima[2], Daniele de Figueirêdo Silva[1], Laísa Vilar Cordeiro[1], Francisco Patricio de Andrade Júnior[3]*, Helivaldo Diógenes da Silva Souza[1], Rafael Farias de Oliveira[1] & Petrônio Filgueiras de Athayde-Filho[1]

[1] Federal University of Paraíba (UFPB), João Pessoa, Paraíba, Brazil
[2] Program in Bioactive Natural and Synthetic Products, UFPB, João Pessoa, Paraíba, Brazil
[3] State University of Piauí, 2335 Olavo Bilac Street, Centro (South), Teresina, Piauí, 64001-280, Brazil

*Correspondent author E-mail: juniorfarmacia.ufcg@outlook.com

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SUMMARY

Objective: To evaluate the antifungal potential of 2-bromo-*N*-phenylacetamide (A1Br) against *Candida glabrata* strains isolated from the oral cavity. **Methods**: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A1Br were determined by the broth microdilution technique and via the checkerboard method for pharmacological interaction assessment. Micromorphological changes induced by the compound and the effect of its combination with standard antifungals (nystatin and miconazole) were also evaluated using the checkerboard method, with determination of the fractional inhibitory concentration index (FICI). **Results**: A1Br showed an MIC of 16 μg/mL and an MFC of 32–64 μg/mL against all tested *C. glabrata* strains, indicating predominantly fungicidal activity. The A1Br–nystatin combination exhibited antagonism (FICI = 4.5), whereas the A1Br–miconazole combination showed indifference (FICI = 1.25). Micromorphological analysis revealed reduction of all fungal structures, achieving 100% inhibition at 4× MIC. **Conclusion**: 2-Bromo-*N*-phenylacetamide demonstrated excellent antifungal activity against *C. glabrata* and emerges as a potential drug candidate.

Keywords: Oral candidiasis; *Candida glabrata*; acetanilide; mycobioma.

RESUMO

Avaliação *in vitro* da atividade antifúngica da 2-bromo-*N*-fenilacetamida contra isolados de *Candida glabrata* da cavidade oral

Objetivo: Avaliar o potencial antifúngico da 2-bromo-*N*-fenilacetamida (A1Br) contra cepas de *Candida glabrata* isoladas da cavidade oral. **Métodos:** A concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) da A1Br foram determinadas pela técnica de microdiluição em caldo e pelo método checkerboard para avaliação da interação farmacológica. As alterações micromorfológicas induzidas pelo composto e o efeito de sua combinação com antifúngicos padrão (nistatina e miconazol) também foram avaliados pelo método checkerboard, com determinação do índice de concentração inibitória fracionada (ICF). **Resultados:** A1Br apresentou uma CIM de 16 μg/mL e uma CFM de 32–64 μg/mL contra todas as cepas de *C. glabrata* testadas, indicando atividade predominantemente fungicida. A combinação A1Br-nistatina apresentou antagonismo (FICI = 4,5), enquanto a combinação A1Br-miconazol demonstrou indiferença (FICI = 1,25). A análise micromorfológica revelou redução de todas as

estruturas fúngicas, atingindo 100% de inibição a 4 × CIM. **Conclusão:** A 2-Bromo-*N*-fenilacetamida demonstrou excelente atividade antifúngica contra *C. glabrata* e surge como um potencial candidato a fármaco.

Palavras-chave: Candidíase oral; *Candida glabrata*; acetanilida; micobioma.

RESUMEN

Evaluación *in vitro* de 2-bromo-*N*-fenilacetamida para actividad antifúngica contra aislados de *Candida glabrata* de la cavidad oral

Objetivo: Evaluar el potencial antifúngico de la 2-bromo-*N*-fenilacetamida (A1Br) frente a cepas de *Candida glabrata* aisladas de la cavidad oral. **Métodos**: Se determinaron la concentración inhibitoria mínima (CIM) y la concentración fungicida mínima (CFM) de A1Br mediante la técnica de microdilución en caldo y mediante el método de checkerboard para la evaluación de interacciones farmacológicas. También se evaluaron los cambios micromorfológicos inducidos por el compuesto y el efecto de su asociación con antifúngicos estándar (nistatina y miconazol) mediante el método de checkerboard, con la determinación del índice de concentración inhibitoria fraccionada (FICI). **Resultados**: A1Br presentó una CIM de 16 μg/mL y una CFM de 32–64 μg/mL en todas las cepas de *C. glabrata* evaluadas, evidenciando una acción predominantemente fungicida. La combinación A1Br–nistatina mostró antagonismo (FICI = 4,5), mientras que la combinación A1Br–miconazol demostró indiferencia (FICI = 1,25). El análisis micromorfológico reveló reducción de todas las estructuras fúngicas, alcanzando un 100 % de inhibición a 4× CIM. **Conclusión**: La 2-bromo-N-fenilacetamida demostró excelente actividad antifúngica contra *C. glabrata* y se perfila como un posible candidato a fármaco.

Palabras clave: Candidiasis oral; *Candida glabrata*; acetanilida; micobioma.

1. INTRODUCTION

The infectious process called candidiasis is caused by fungi of the Candida genus; opportunistically manifesting in the host in different anatomical regions such as the vaginal or oral mucosa, the respiratory tract, orin various organs [1]. Species such as *Candida albicans* are most often associated with candidiasis lesions, but other species such as *C. tropicalis*, *C. glabrata* and *C. krusei* are also frequently identified [2].

Prosthetic stomatitis is a pathological condition characterized as an inflammatory process that affects the oral mucosa. Colonization of the fungi in the oral cavity is favored by the presence of acrylic resin that due to its porosity favors biofilm accumulation and consequent fungal infection. In the presence of a total prosthesis, tissue changes are observed, including palate injuries and soft tissue damage with the presence of petechiae or reddish areas in the region covered by the prosthesis, often associated with a lack of hygiene. Some patients are asymptomatic for the infection; however, they usually report a variety of symptoms including pain, swelling, xerostomia, halitosis, and bleeding, which often make the prosthesis impossible to use [3].

Epidemiological data reveal that in Brazil, the absence of teeth (edentulism) is indeed present in the young, resulting in the need to use total dentures in 13.7% in the 15-19 year old age group. Other age groups reveal a significant increase evolving to 68.8% in 35-44 year olds, and reaching 92.7% in 65-74 year olds. In total, 17.9% use at least one full denture in one arch and 15.4% in both arches. In a large number of dental prosthesis users, the need for early care is strongly associated with pathological lesions and candidiasis [4].

Candida albicans and *C. glabrata* are responsible for most systemic candidiasis infections, followed by *C. parapsilosis* and *C. tropicalis*. Distinctions between *C. albicans*, *C. parapsilosis,* and *C. tropicalis* relate to CUG (a single leucine-serine exchange).*C.glabrata* maintains several distinctions: in genome duplication, pathogenicity, division, and cell structure [5].

Interactions between the pathogenic fungi *C. albicans* and *C.glabrata* increase with inflammation, suggesting synergistic interactions between the two species and expression of virulence-related genes which may depend on the co-culture composition [6]. In associations of *C. glabrata* with *C. albicans,* the cells do not adhere to each other, but *C. glabrata* presents adhesion along the length of the *C. albicans* hyphae, demonstrating an interdependent relationship. *Candida glabrata* can utilize the destruction of epithelial tissue caused by *C. albicans* to harness nutrients, and even to access the bloodstream [7]. This can bring serious clinical consequences since as *C. glabrata* reaches the internal organs, its high resistance to commonly used antifungals makes treatment more difficult [5-7].

Patients with oral candidiasis are often treated with nystatin or miconazole, antifungals used topically to treat superficial infections. Other agents such as fluconazole, itraconazole, or voriconazole are mainly indicated for deep infections yet are also used in cases of recalcitrant oral candidiasis when topical treatment has failed [3, 8]. Reduced susceptibility of *C. glabrata* to azoles can become a problem in combating infections, especially when infections are associated with patients with HIV [9].

Synthesis of new acetamide substances, is of great interest to researchers since they present several biological activities, among them antibacterial and antifungal [10]. Within the amide class we find the 2-halo-N-arylacetamides, which are an important class of amides used as intermediates in organic synthesis of different products, following principles established by Peixoto [11] and Kristensen [12]. The high incidence of fungal resistance motivates current research to synthesize and discover new substances. The antimicrobial character of bis-pyramidine acetamide has been observed as comparable to the common drugs, cefadroxil (antibacterial) and fluconazole (antifungal) [13].

The high prevalence of candidiasis in users of complete dentures, coupled with the rise of resistant strains, highlights the urgent need for new therapeutic alternatives. Especially *Candida glabrata*, one of the main agents responsible for resistant oral infections, requires the development of effective substances for its control. In this context, acetamides emerge as a promising class, with antifungal properties already observed in previous studies and used in drug formulations [14-16]. This study is justified by the search for new molecules that can expand the therapeutic arsenal available for the treatment of oral candidiasis, particularly in patients with dental prostheses.

The aim of this study was to evaluate the antifungal potential of 2-bromo-*N*-phenylacetamide (A1Br) and its association with other antifungals against *Candida glabrata* strains isolated from the oral cavity.

2. METHODOLOGY

2.1. Chemistry

All reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Brazil) and used without further purification. The progress of the reaction was monitored by thin layer chromatography (TLC) on silica gel plates. ^{1}H-NMR and ^{13}C-NMR spectra were obtained

using a Bruker Avance Ultrashield™ instrument (400 MHz for ^{1}H and 101 MHz for ^{13}C). Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane (TMS) was used for the internal standard. Chemical shifts (δ) were measured in parts per million (ppm), and the coupling constants (J), in hertz (Hz). The compound was purified by recrystallization and confirmed by determining the melting point range on an MQAPF-3 brand hotplate.

2.2. Preparation of 2-bromo-*N*-phenylacetamide (A1Br)

The preparation of 2-bromo-*N*-phenylacetamide (A1Br), as show in Fig. 1, was obtained according to the procedure of Kaushik *et al.* [17]. In a 50 mL flask containing aniline (1.86 g, 0.020 moles) and K_2CO_3 (3.31 g, 0.024 moles) solubilized in 20 mLofCH_2Cl_2 (dichloromethane) at a temperature of 0 °C, 2-bromoacetyl bromide (4.84 g, 0,024 moles) is slowly added. The ice bath was then removed, and there action stayed under agitation for 20 h at room temperature. The reaction mixture was monitored by TLC (hexane/methyl acetate 1:1). At the end of the reaction, there action mixture was filtered and the solvent was evaporated under reduced pressure, yielding a precipitate. The solid was recrystallized from an ethanol/water mixture (8:2). Yield: 80% M.P. 130-132 °C (Lit. 129-131°). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.30 (t, J = 8.0 Hz, 2H), 7.09 (t, J = 7.4 Hz, 1H), 3.97 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.53, 137.76, 128.25, 123.73, 119.36, 29.29 [17].

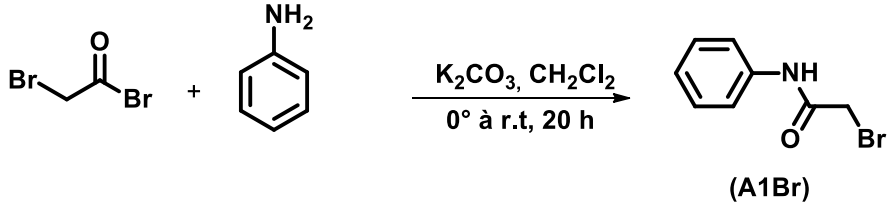

Figure 1. Synthetic route to obtain the target molecule.

The products were weighed and properly solubilized in 5% dimethyl sulfoxide (DMSO) and 2% Tween 80. The final volume was completed with sterile distilled water to yield an emulsion of the products for initial concentrations of from 0.5 μg/mL to 1024 μg/mL [18-20]. Nystatin and miconazole used in the assays as controls were purchased from Sigma-Aldrich® with batches SLCC2040 (nystatin) and BCBD5966D (miconazole), Cotia/SP, CNPJ: 68.337.658/0001-27.

2.3. Culture mediums

For maintenance of strains for assays of antifungal activity, Sabouraud Dextrose Agar – ASD, and RPMI 1640 (INLAB, São Paulo, Brazil) were used, which were prepared according to the manufacturers' descriptions.

2.4. Microorganisms

For the antifungal activity assays, 13 strains of *C. glabrata*, isolated from the oral mucosa were used: LM-16, LM-17, LM-28, LM-35, LM-46, LM-106, LM-108, LM -116, LM-186, LM-188, LM-302, LM-418, LM-533, and a standard ATCC-90030 strain. All were kept on Sabouraud Dextrose-ASD Agar (DIFCO Laboratories LTD/USA) at 4 °C (refrigerator). All belong to the collection of the Antibacterial and Antifungal Activity Research Laboratory, of the Bioactive Natural and Synthetic Products/Health Sciences Center (CCS), of the Department of Pharmaceutical Sciences (DCF), at Paraíba Federal University (UFPB).

2.5. Ethical aspects

As an observational, qualitative-quantitative, analytical/descriptive study, an informed consent waiver was performed since it used *Candida glabrata* strains from the oral cavity; from biological materials collected and stored in the Antibacterial and Antifungal Activity Research Laboratory, of the Bioactive Natural and Synthetic Products/Health Sciences Center (CCS), of the Department of Pharmaceutical Sciences (DCF), at the Federal University of Paraíba (UFPB). The study phases were performed without risk to the research participants. The project linked to the study was approved by the research ethics committee of the Health Sciences Center of the Federal University of Paraíba (CAEE: 12364419.3.0000.5188).

2.6. Inoculum

The inoculum was prepared from colonies obtained from fresh cultures (24-48 hour 35-37°C) of *C. glabrata* in ASD medium. The colonies were suspended in sterile 0.9% physiological solution (Fresenius Kabi Brazil LTDA Barueri-SP), and adjusted according to the 0.5 McFarland scale standard to obtain an inoculum of 10^6 CFU/mL [18, 20-23].

2.7. Determination of Minimum Inhibitory Concentration - MIC

Initially, 100 μL of double concentrated RPMI broth was distributed to the microdilution plate wells. Then 100 μL of each substance was dispensed to the wells of the first line of the plate. By serial dilution at a ratio of two, concentrations of from 0.5 μg/mL to 1024 μg/mL were obtained. Finally, 10 μL of the yeast suspensions were added to the wells, where each column of the plate specifically referred to one yeast. At the same time, the microorganism (RPMI + yeast), and culture medium (RPMI) controls were performed to verify the respective viability of the strains and the sterility of the medium. Controls were also performed with standard antifungals: nystatin and miconazole. The prepared plates were aseptically sealed and incubated at 35-37°C for 24 - 48 hours. MIC determination was performed by the liquid microdilution technique (RPMI broth) with 96 bottom U-shaped wells, (ALAMAR ®). After the assay incubation time, reading was performed and the MIC for each product was defined as the lowest concentration capable of visually inhibiting microbial growth. The antifungal activity of the products was interpreted and considered as being either active or inactive according to the following criteria: 50-500 μg/mL = strong activity; 600-1500 μg/mL = moderate activity; > 1500 μg/mL = weak activity or inactive [24-27]. The assays were performed in duplicate and the results expressed as the arithmetic means of the MFCs obtained in the two assays [27].

2.8. Determination of Minimum Fungicidal Concentration –MFC

After MIC reading, 10 μL aliquots were removed from the well supernatants with no visible fungal growth with the two subsequent dilutions (MIC, MIC × 2 and MIC × 4), and transferred to new microdilution plates containing 100μL of RPMI-1640 in each well. The plates were incubated at 35-37 °C for 24-48 hours. The MFC was considered as the lowest concentration at which there was no growth of yeast in the culture medium. The assays were performed in duplicate and the results expressed as the arithmetic MFC mean [27]. According to Saddiq and Khayyat [28], a substance is fungistatic when the MFC/MIC ratio ≥ 4 and fungicidal when the MFC/MIC ratio<4. For Hafidh and colleagues [29], the respective ratios of 1:1 and 2:1 are considered; where a product is considered both fungicidal and fungistatic at a ratio greater than 2:1.

2.9. Effect of 2-bromo-*N*-phenylacetamide, nystatin, and miconazole on the viability and morphology of *C. glabrata*

This study was performed to analyze *C. glabrata* ATCC-90030 and LM-302 cell viability and morphological characteristics against the products as used in the antifungal activity assays; based on MIC, MIC × 2, and MIC × 4. The fungi were sub-cultured against differing concentrations of 2-bromo-*N*-phenylacetamide, nystatin, and miconazole. First, 100 μL of RPMI 1640 broth was distributed to the wells of the microdilution plates. Then, 100 μL of product was dispensed into the wells of the first line of the plate, this at a concentration corresponding to MIC × 4. By two-fold serial dilutions, the corresponding concentrations of MIC × 2 to MIC were obtained. Then 10 μL of microorganism inoculum was added to the wells (each column of the plate corresponded to a specific fungal lineage). The plates were then incubated at 35 ± 2 °C for 24-48 hours. After the incubation period, a 10 μL aliquot was taken and placed between a slide and coverslip for optical microscopy analysis at 400X magnification. Cell viability assessment of yeast characteristics was thus performed [30].

The experiment was performed in duplicate and the arithmetic mean was calculated from the structures present in five fields (100 μL of RPMI 1640 + 10 μL of inoculum). Evaluation of the results was performed in SPSS® version 13.0statistical software, available as a trial version, and the graphs were elaborated in Excel 2016.

2.10. 2-Bromo-*N*-phenylacetamide (A1Br) molecule association assay (checkerboard method)

The effect of association with standard antifungals was evaluated using microdilution - checkerboard technique for derivation of the FICI (fractional inhibitory concentration index). The tests used solutions of the products in concentrations determined from their respective MICs. Initially, 100 μL RPMI was added to the 96-well plate wells. Then 50 μL of miconazole and nystatin (standard antifungals) at: (MIC × 8, MIC × 4, MIC × 2, MIC, MIC ÷ 2, MIC ÷ 4 and MIC ÷ 8) were added vertically, and then 50 μL of the test product (A1Br) was added horizontally. Finally, 20 μL of fungal suspension was added. The assay was performed in triplicate and the assay incubated at 35-37 °C for 24-48 hours [31].

The FICI (Fractional Inhibitory Concentration Index) was calculated by summing the FIC^A + FIC^B, where A represents the test product and B the standard antifungal. The FIC^A, is calculated as FIC^A= (MIC^A combined)/(MIC^A alone), whereas FIC^B is calculated as FIC^B = (MIC^B combined)/(MIC^B alone). The index is interpreted as follows: synergism (FICI ≤ 0.5), antagonism (FICI> 4.0) and indifference (0.5 <FICI ≤ 4) [32-36].

2.11. Statistical analysis

Analysis of the results was performed according to statistical guidelines for each methodology used; selecting the most appropriate tests in relation to each variable. The results for the micromorphology study were evaluated using SPSS® software version 13.0, available in a trial version. Graphs were elaborated in Excel 2016 for Windows. The non-parametric samples were submitted to statistical analysis using the Mann-Whitney U test, considering significance at $p < 0.05$.

3. RESULTS

Test results for the antifungal activity of the A1Br molecule and the standard antifungals nystatin and miconazole are shown in Table 1. For all studies the media sterility control in RPMI presented no contamination, and all wells in the fungal growth control (no product or antifungal addition) were positive.

The A1Br molecule at a concentration of 16 μg/m inhibited the growth of 13 of the 14 *C. glabrata* strains (92.8%) used in the microbiological assays. The MIC was set at 16 μg/mL and the MFC between 32-64 μg/ml. The antifungal evaluation of results for nystatin were also recorded. The antifungal nystatin inhibited the growth of 11 of the 14 *C. glabrata* strains (78.54%). Its MIC was established at 8 μg/mL. The MFC of nystatin was determined to be between 8-32 μg/mL. For the antifungal activity results of miconazole, the antifungal inhibited yeast growth of11 of the 14 *C. glabrata* strains (78.5%), with an MIC determined at a concentration of 64 μg/mL and the respective MFC at between 512-1204 μg/mL.

Table 1. MIC and MFC results for A1Br, nystatin and miconazole with respective fungicidal or fungistatic activity against *C.glabrata* isolates - microdilution technique.

Strains	Acetamide A1Br (μg/mL)				Nystatin (μg/mL)				Miconazole (μg/mL)			
	MIC[A]	MFC[A]	MFC[a]/MIC[a]	Antifungal Activity	MIC[B]	MFC[b]	MFC[b]/MIC[b]	Antifungal Activity	MIC[c]	MFC[c]	MFC[c]/MIC[c]	Antifungal Activity
ATCC 90030	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 16	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 17	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 28	16	32	2	Fungicide	16	32	2	Fungicide	64	1024	16	Fungistatic
LM 35	32	32	2	Fungicide	16	32	2	Fungicide	64	512	8	Fungistatic

LM 533	LM 418	LM 302	LM 188	LM 186	LM 108	LM 106	LM 78	LM 46
16	16	16	16	16	16	16	16	16
32	32	32	64	64	64	64	32	32
2	2	2	4	4	4	4	2	2
Fungicide	Fungicide	Fungicide	Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungicide	Fungicide
8	8	8	8	8	8	8	8	8
8	8	8	8	8	16	16	8	8
1	1	1	1	1	1	1	1	1
Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide
64	64	64	128	64	512	128	64	64
512	512	512	1024	1024	1024	1024	512	512
8	8	8	8	16	2	8	8	8
Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungicide	Fungistatic	Fungistatic	Fungistatic

Micromorphological analysis was performed to evaluate the presence or absence of hyphae (virulence structures) as well as the fungalshape. For this experiment, a clinical sample - LM-302 was randomly selected from those with the most common MICs of the strains tested (16 μg/mL) and the ATCC 90030 standard. The data are presented in Figure 1, 2 and 3 (A-B), the effects at MIC, MIC × 2 and MIC × 4 revealed no significant differences.

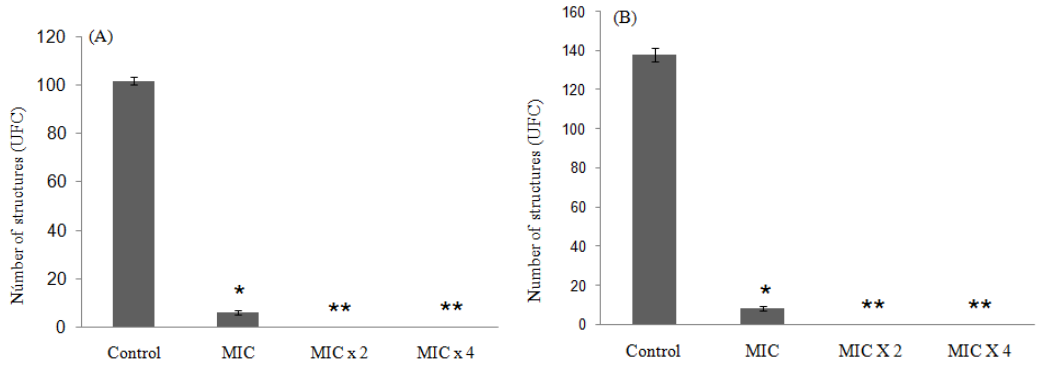

Figure 1. (A-B) - Effect of A1Br at MIC (MIC, MIC × 2, MIC × 4) on standard and clinical lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures.

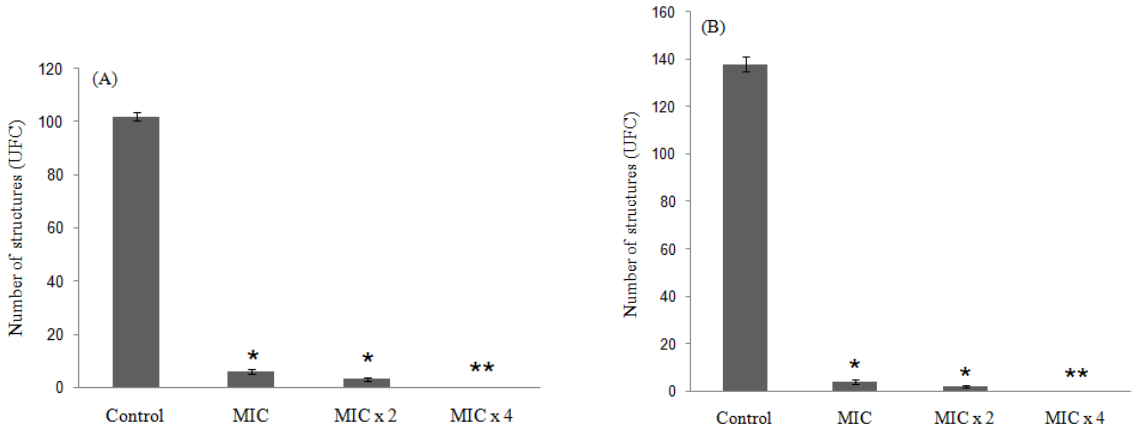

Figure 2. (A-B) - Effect of nystatin at MIC (MIC, MIC × 2, MIC × 4) on standard and clinical lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures.

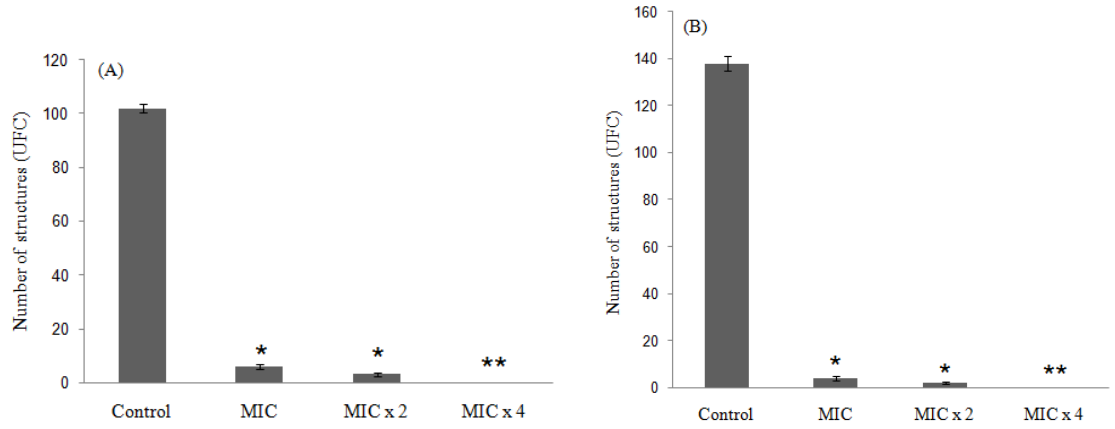

Figure 3. (A-B) - Effect of miconazole at MIC (MIC, MIC × 2, MIC × 4) on the standard and clinical /lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures. Source: Elaborated by the author.

For both the *C. glabrata* strain LM 302 and the ATCC 90030 standard, micromorphology was observed at 400x magnification. Agglomeration of rounded-shaped fungal cells without structural hyphae, consistent with the species was noted.

The association study was performed using the checkerboard technique, which allows construction of a two-dimensional matrix by combining the differing concentrations of the evaluated substances; interactions with nystatin (antagonism)and miconazole (indifference)were respectively obtained (Table 2 and 3).

Table 2. Association study (checkboard) of A1Br molecule with nystatin against *C. glabrata*

Isolated fungi	**MIC (μg/mL)**			**FICI Index**	**Type of Interaction**
	A1Br	Nystatin	A1Br/Nystatin		
C. glabrata ATCC 90030	4	4	2/16	4.5	Antagonism
C. glabrata LM-302	16	2	16/2	4.5	Antagonism

Table 3. Association study (checkboard) of A1Br molecule with miconazole against *C. glabrata*

Isolated fungi	**MIC (μg/mL)**			**FICI Index**	**Type of Interaction**
	A1Br	Miconazole	A1Br/Miconazole		
C. glabrata ATCC 90030	4	128	1/128	1.25	Indifference
C. glabrata LM- 302	16	32	4/32	1.25	Indifference

4. DISCUSSION

The development of antimicrobial research involves analysis of substances with proven effects as well as substances with potential for further development into new compounds. This makes it possible to treat infectious diseases using safer and more effective drugs.

In this context, acetamides and their derivatives appear in several studies revealing antimicrobial and antifungal power [8, 10, 36-39]. Studies such as those by Oliveira and collaborators [37], attaching a methoxide to the amide aromatic ring in the "para" position contributed to antifungal activity, and reinforce the importance of research involving this type of compound; where changes in the molecule's structure may alter its antifungal activity.

The present study confirmed antifungal activity of the A1Br molecule as well as the standard antifungals nystatin and miconazole against clinical samples of *C. glabrata.* When performing the MIC test, the A1Br product was observed inhibiting the growth of 92.8% of the tested strains; this after several dilutions from 1024 μg/mL to 16 μg/mL. The tested substance thus presented excellent antifungal activity values according to the Sartoratto *et al.* classification [25].

When performing the MIC test for antifungals it was observed that nystatin presents an MIC of 8 μg/mL with reductions for 78.54% of the *C. glabrata* strains tested, and presenting low concentration activity against most of these fungi. For miconazole, the most commonly used antifungal for oral cavity fungal infections, an MIC of 64 μg/mL against the tested *C. glabrata* strains was observed. This in relation to the A1Br product, demonstrates inhibition against

fewer fungi. It also corroborates most studies which report reduced azole activity (miconazole fluconazole, itraconazole, voriconazole, and metronidazole). Resistance to these antifungals can lead to systemic infection and death [7, 8].

The results for antifungal activity as obtained by the A1Br molecule in this study corroborate those already obtained by Oliveira and colleagues [37] who tested ten amides from a vanillic group against *Candida albicans* and all showed antifungal effect on the yeast when MIC testing was performed.

Pejchal *et al.* [38] evaluated the MICs of both triazolic fluorobenzene amide and 13 structurally modified derivatives for antimicrobial and antifungal effect, this, in 5 distinct dilutions ranging from 200 μg/mL to 6.25 μg/mL. One of the fungi studied was *C. glabrata* for which 8 of the amides presented no antifungal action at the highest concentration (200 μg/mL); only 5 substances presented a combination effect at 100 μg/mL, 50 μg/mL, and 25 μg/mL. The authors demonstrated the efficacy of amides, but modification of their structure bonds, either by position (ortho and para) or by binding to other elements, can lead to both greater and/or lesser activity against fungi, whether fungicidal or fungistatic.

In yet another case, Çavuşoğlu *et al.* [39] showed that 10 molecular derivatives of an acetamide (obtained from triazole oxadiazoles) against *C. glabrata* presented MICs of from 65.5 μg/mL to 500 μg/mL for 90% of the tested population, with antifungal and apoptotic effect, and presenting good antifungal potential; yet less than A1Br which at 16 μg/mL itself inhibited 90% of the yeast tested.

The MFC was determined. This assay allows understanding at which concentration a given substance is fungicidal, which may result in values higher than the MIC [40].

With respect to the MFC, Bardiot and colleagues [41] found that fungicidal activity is generally preferred over fungistatic activity because it identifies the inhibition of targets essential for fungal growth and/or induces activation of pathways leading to cell death. It also contributes to reducing the emergence of potential resistance phenomena [40]. The MFC obtained for A1Br was 32–64 μg/mL, characterizing the compound as predominantly fungicidal, promoting cell death at low concentrations against *C. glabrata*.

The micromorphological study, in the presence and absence of A1Br, was visualized at 400 X; indicating the presence of rounded yeast, consistent with the structure of *C. glabrata*. To ensure the validity of the study, the standard ATCC 90030 and LM 302 were randomly selected from those with the most prevalent MIC (16μg/mL) for the tested strains. Yeast growth was analyzed by counting their structures when facing A1Br, nystatin, and miconazole at their MIC, MIC × 2, and MIC × 4 concentrations. Fungicidal or fungistatic action was evaluated in function of concentration in relationship to microbial activity [9, 10]

There was a significant decrease in the number of microorganism structures; reaching 100% for the substance A1Br at MIC × 2 for the two strains studied, an excellent result in relation to the antifungals that at a MIC × 2 concentration obtained only partial reductions of the structures. For all of the products tested, and against both strains, A1Br at MIC × 4 presented a 100% reduction in the number of microorganism structures [30]. In this context, it is important to highlight that the morphological changes induced by acetamide are significant findings, as by preventing the formation of invasion and reproduction structures, the infection is halted and controlled.

After confirming the antifungal activity of A1Br, a combination assay was performed using acetamide and drugs commonly used in clinical practice, such as miconazole and nystatin. According to Andrade Júnior *et al*. [42], the combination of antifungal agents can reduce the

selection of resistant strains, in addition to allowing the use of lower concentrations of each individual drug, thereby minimizing adverse effects and enhancing treatment effectiveness.

In accordance with the results obtained in the combination assay, the FICI was 4.5 (four and a half) for the standard strain ATCC 90030 and LM 302; an antagonism ratio of FICI> 4.0for nystatin with A1Br, and demonstrating activity for only one of the substances against the analyzed strains; imply in inhibition. The value was obtained using the Inhibitory Concentration Index (FICI), which considers the isolated and combined MICs of the products and yields the index value. The association of miconazole with A1Br obtained a value of 1.25 for both ATCC 90030 and LM 302, and an indifferent relationship between them (0.5 <FICI<4). Thus, the union to these two compounds neither potentiates nor antagonizes the activity against the studied yeasts [32-36].

Pharmacological indifference occurs when one drug does not interfere with the mechanism of action of the other, neither reducing nor increasing the response, while antagonism happens when there is interference between the mechanisms of action of the drugs. In the latter case, the combined administration of the substances is unfeasible, although their clinical application individually is perfectly possible [42]. In pharmacological indifference, the drugs can be administered together, even without enhancing each other's therapeutic effect.

Furthermore, it is important to highlight that the variability in the responses of substances against fungi of the same species is related to the genetic and phenotypic characteristics of each strain, emphasizing the need for more in-depth research on this topic [43].

Thus, although no synergy was observed in the combinations tested between A1Br and standard antifungals, combination therapy remains a promising strategy, especially against resistant pathogens [44].

There are no studies in the literature reporting on association effects against *Candida glabrata* for A1Br with either nystatin or miconazole, leaving room for further research evaluating both mechanisms of action and pharmacological receptors, and to further evaluate potential effects of this new substance whether by modification of the ligand site, or through its activity in combination with other drugs.

The A1Br molecule demonstrated excellent antifungal activity against *C. glabrata*, with a predominantly fungicidal character. A significant reduction in fungal structures was observed, being more effective in eliminating fungal cells compared to the other antifungals tested. Regarding the combination, antagonism of A1Br with nystatin and indifference with miconazole was observed. Therefore, despite its promising potential against fungi, further studies are needed to better understand the pharmacological and toxicological characteristics of 2-bromo-*N*-phenylacetamide.

CONFLICT OF INTEREST

All authors report that they do not have any conflicts of interest.

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