Patologia: Doenças Bacterianas e Fúngicas

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APRESENTAÇÃO

No volume III da coleção Patologia intitulado: Doenças Bacterianas e fúngicas, apresentamos em capítulos, diversos artigos de pesquisas realizadas em diferentes regiões. A temática contempla a pesquisa básica que inclui estudos sobre os agentes infecciosos, dados epidemiológicos, diagnósticos e tratamentos, bem como temáticas correlacionadas.

O crescimento destas infecções se caracteriza como um grave problema de saúde pública, em especial pelo aumento da resistência microbiológica aos tratamentos disponíveis. Neste sentido, é extremamente importante que os profissionais que atuam na área da saúde conheçam os agentes infecciosos, suas características, seus agravos, suas incidências regionais e sistemas de prevenção e tratamento.

A multidisciplinaridade dos trabalhos apresentados tem como objetivo explorar a produção de conhecimentos sobre as infecções relevantes no Brasil, tais como a sífilis, a tuberculose, hanseníase, infecções fúngicas, entre outras.

A obra é fruto do esforço e dedicação das pesquisas dos autores e colaboradores de cada capítulo e da Atena Editora em elaborar este projeto de disseminação de conhecimento e da pesquisa brasileira. Espero que este livro possa somar conhecimentos e permitir uma visão critica e contextualizada; além de inspirar os leitores a contribuírem com pesquisas para a promoção de saúde e bem estar social.

Yvanna Carla de Souza Salgado

SUMÁRIO

CAPÍTULO 34

IN VITRO AND IN SILICO ANALYSIS OF THE MORIN ACTION MECHANISM IN YEAST OF THE *Cryptococcus neoformans* COMPLEX

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ABSTRACT: Cryptococcosis is an important opportunistic fungal disease that has as the etiological agent, yeasts belonging to the complex Cryptococcus neoformans. In recent years, the resistance of these yeasts to antifungals has increased, causing the need to seek for different therapies for the infection. The molecular docking simulation assists on the search for these new therapies by using a three-dimensional structure of a computationally simulated protein-ligand complex. This work aimed to evaluate the mechanism of action of the morin flavonoid on yeasts of the C. neoformans complex, as well as to elucidate potential inhibitors of important enzymes on the fungal cell membrane. First, the action of the compound on ergosterol was evaluated and the action of the morin on the cytoplasmic membrane of C. gattii ATCC 24065 was verified using flow cytometry. Afterwards, we applied the Guided Differential Evolution (DGE) method incorporated the Mocker Virtual Docker program

in order to obtain the crystallographic position of the binder for the prediction of protein-binder interaction. The results showed that the morin was able to reduce ergosterol and cause damage on the fungal cell membrane in MIC of 32 µg / mL. Re-docking results for the combination of four search algorithms, four scoring functions and the presence of water molecules tested with the structure of C. neoformans Trehalose-6-phosphate phosphatase, generated a RMSD of less than 2 Å. These results showed that the compound has action on the cell membrane of this fungus and trehalose 6-phosphate phosphatase becomes a potential target for elucidation of the mechanism of action involving the cell membrane.
KEYWORDS: Cryptococcus neoformans, flavonoid, cell membrane, molecular docking.

RESUMO: A criptococose é uma importante doença fúngica oportunística que tem como agente etiológico, leveduras pertencentes ao complexo Cryptococcus neoformans. A resistência dessas leveduras aos antifúngicos tem aumentado nos últimos anos, fazendo com que se busque diferentes terapias para a infecção. A simulação de docking molecular auxilia na busca destas novas terapias, onde utiliza-se uma estrutura tridimensional de um complexo proteína-ligante simulada computacionalmente. Este trabalho objetivou-se em avaliar o mecanismo de ação do flavonoide morina sobre leveduras do complexo C. neoformans bem como elucidar potenciais inibidores de enzimas importantes na membrana celular fúngica. Primeiramente foi avaliada ação do composto sobre o ergosterol, e verificada a ação da morina sobre a membrana citoplasmática de C. gattii ATCC 24065 usando citometria de fluxo. Em seguida aplicamos o método de evolução diferencial guiada (GDE) implementado no programa Molegro Virtual Docker para obter a posição cristalográfica do ligante para a previsão da interação proteína-ligante. Os resultados mostraram que a morina foi capaz de reduzir o ergosterol e causar lesão de membrana nas células fúngicas na CIM de 32 *µ*g/mL. Análise dos resultados de re-docking para a combinação de quatro algoritmos de busca, quatro funções scores e a presença de moléculas de água realizadas com a estrutura da Trealose-6-fosfato fosfatase de C. neoformans, geraram um RMSD menor que 2 Å. Estes resultados mostraram que o composto tem ação sobre a membrana celular deste fungo, e a trealose 6-fosfato fosfatase torna-se um alvo em potencial para elucidação do mecanismo de ação que envolve a membrana celular.
PALAVRAS-CHAVE: Cryptococcus neoformans, flavonoide, membrana celular, docking molecular.

1 I INTRODUCTION

During the last century, cryptococcosis has evolved into a major invasive fungal disease and its epidemiology has been substantiated on the main focus of the disease which is a reflex of both the environmental change in exposure to the fungus and of the increase of host risk factors such as the increase of the infection by the Acquired Immunodeficiency Virus (HIV)(HARRIS et al., 2013; PERFECT; BICANIC, 2014). Its etiological agent is yeast species belonging to the complex Cryptococcus neoformans,

Cryptococcus neoformans and Cryptococcus gattii which are subdivided into different serotypes and genotypes (KWON-CHUNG; VARMA, 2006; LIN; HEITMAN, 2006). The infection is acquired after the inhalation of infective propagules, basidiospores or dehydrated yeasts present in the environment. Upon reaching the pulmonary alveoli, primary infection begins in the lungs and through hematogenous dissemination it can cause from cutaneous lesions to systemic infections (Kwon-Chung et al. 2014). Cryptococcal meningitis is the most frequent clinical form of the disease reported among patients with human immunodeficiency virus, affecting approximately 80% of these patients (HUNG et al., 2007; JARVIS et al., 2014).

The antifungal treatment for cryptococcosis varies according to the extent of the disease, the severity, as well as the immunological state of the host (PERFECT et al., 2010). Although there are some clinical differences between cryptococcosis caused by C. neoformans from that caused by C. gattii, the recommended treatment for both species are currently identical. The antifungals amphotericin B and fluconazole are the drugs of choice for the treatment of cryptococcal meningitis with the first one being used for induction, while the second is used for consolidation, maintenance and prophylaxis (CHEN et al., 2013; CHEN; MEYER; SORRELL, 2014; GROSSMAN; CASADEVALL, 2016). Resistance to antifungals was previously rare. However, recent reports have described the increase in the minimal inhibitory concentration (MIC) of C. neoformans isolates for fluconazole and, to a lesser extent, for amphotericin B in the last decade (CHEN; MEYER; SORRELL, 2014; SMITH et al., 2015). Despite the scarcity of new classes of antifungal drugs that have hit the market in recent years, innovative approaches to drug discovery have driven research into alternative therapeutic strategies (KHAN et al., 2006; LOMBARDI et al., 2015). On this context, plants and their bioactive compounds have been very promising as candidates for the development of new drugs.

Molecular Docking, a computer simulation methodology that can predict the conformation of a protein-drug complex with relatively high accuracy when compared with experimental structures was previously analyzed. Analyses of the interactions between a protein target and a drug can be simulated by these evolutionary algorithms (HEBERLÉ; DE AZEVEDO, 2011).

Morin (3,5,7,2',4'-pentahydroxyflavone) is a yellow polyphenol pigment originally isolated from members of the Moraceae family. Present in many plants, fruits and wine, it has several biological and biochemical effects, such as anti-inflammatory, antioxidant, antidiabetic, antitumor antihypertensive, antibacterial, hypouryemic and neuroprotective actions. (AL-NUMAIR et al., 2014; CASELLI et al., 2016; HUSSAIN et al., 2014; LIN et al., 2013; SELEEM; PARDI; MURATA, 2016; TIAN, 2006; XIE et al., 2006; ZHANG et al., 2010) We evaluated the action of this compound against yeasts of the C. neoformans complex, in order to elucidate their possible mechanisms of action.

2 I MATERIALS AND METHODS

2.1 Compound

Morin was purchased from Sigma-Aldrich Company. Solvent for the compound test: dimethylsulfoxide (DMSO).

2.2 Evaluation of the mechanism of action

2.2.1 Ergosterol dosing

The methodology used for extracting ergosterol from yeasts of C. neoformans was performed according to the technique developed by ARTHINGTON-SKAGGS et al., (1999). The presence of ergosterol and the late sterol intermediate 24 (28) DHE (Dehydroergosterol) in the extracted sample resulted in a characteristic curve of four peaks. The ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations: % ergosterol + % 24(28)DHE = [(A_{281}.5/ 290) x F]/pellet weight, % 24(28)DHE = [(A_{230}/518) x F]/pellet weight, and %ergosterol = [% ergosterol+ %24(28)DHE] - %24(28)DHE. F is the dilution factor in ethanol and 290 and 518 are the values of E (in percentages per centimeter) determined for crystalline ergosterol and 24 (28) DHE, respectively.

2.2.2 Effect of morin on the cell membrane of yeasts of Cryptococcus neoformans

The evaluation of Morin's mechanism of action was carried out according to Ahmad et al. (2011), with modifications. Cells of *C. gattii* ATCC 24065 were cultured in ASD for 72 hours, inoculated in 10 ml of culture medium RPMI and incubated overnight at 35 ° C in Shaker at 200 rpm. The inoculum was adjusted in a spectrophotometer at a length of 530nm and 85% transmittance, obtaining a concentration of approximately 1-5x10^6 CFU / ml. The inoculum was added to 5 ml of RPMI broth plus the compound in 4x MIC concentration, 2x MIC, MIC and ½ MIC and incubated at 35°C for 2 hours. Afterwards, the cells were centrifuged for 5 minutes at 5000 rpm, the pellet was resuspended in 5 ml of phosphate buffer (PBS: 8.77 g NaCl, 1.02 g Na2 HPO4, 0.34 g NaH2 PO4 / L, PH 7.2), centrifuged again for 5 minutes at 5000 rpm and the content after vortexed were transferred to eppendorfs. Cells were centrifuged again for 10 minutes at 5000 rpm, the supernatant discarded and the pellet resuspended in 200 ul PBS.

For cell labeling, 5 µg/mL propidium iodide (PI-Sigma), a membrane integrity marker, was added in all treated cells and on the negative controls. The obtained marker suspension was maintained at 35 ° C for 30 minutes in the absence of light and subsequently submitted to analysis by the Accuri C6 (Becton Dickinson Biosciense) cytometer, with 10,000 events being acquired in a given population (gate), and fluorescence red light used on detector FL2 for PI. (PI) is a marker of membrane integrity with the ability to cross damaged cell membrane, therefore, inside the cell it binds to nucleic acids, emitting red fluorescence, however, when associated with the

intact cell membrane this marker does not emit fluorescence (PINA-VAZ et al., 2001; VALE-SILVA et al., 2006).

2.2.3 Analysis in silico

In the pre-docking analysis we used the enzyme trehalose-6-phosphate phosphatase of *C. neoformans,* PDB access code: 5DX9, resolution 2.5 Å (MIAO et al., 2016)Tps1, and trehalose-6-phosphate phosphatase, Tps2. Here, we report the structures of the N-terminal domain of Tps2 (Tps2NTD. We performed the docking simulation against the active site of 5DX9 and compared the docked poses with the crystallographic structure. In docking simulations, it is expected that the best results generate RMSD values less than 2.0 Å when compared with crystallographic structures

The Guided Differential Evolution (GDE) method was implemented in the Molegro Virtual Docker program to obtain the crystallographic position of the binder in order to predict the protein-binder interaction.

In the present work, all simulations were performed in a MacBook Air (Intel Processor Core i5 Duo, 1.4 GHz, 4 GB SDRAM DDR3 1600 MHz).

3 I RESULTS

Inhibition of ergosterol biosynthesis was evaluated through cellular ergosterol quantification which demonstrated that in 32 µg / mL (MIC), morin reduced fungal cell ergosterol by 59.65%, a higher value when compared to fluconazole MIC. Table 1 shows the results obtained in the test. Fluconazole, used as a positive control showed a reduction of ergosterol in MIC (2 µg / mL) of 53.08%.

Compound / antifungal	MIC	½ MIC
Morin	59,65%	59,43%
Fluconazole	53,08%	40,14%

Table 1. Reduction of ergosterol biosynthesis by the morin compound and antifungal fluconazole in C. gattii ATCC 24065

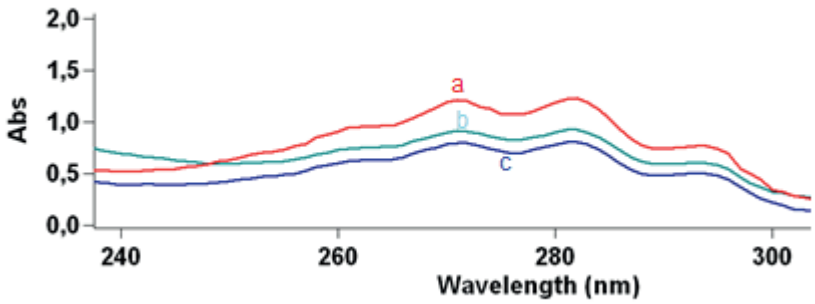

Figure 1. Spectrophotometric profile of C. gattii ATCC 24065 sterols, untreated in (a) and after

treatment with the morin compound at concentrations (µg/mL) 16 (b) and 32 (c).

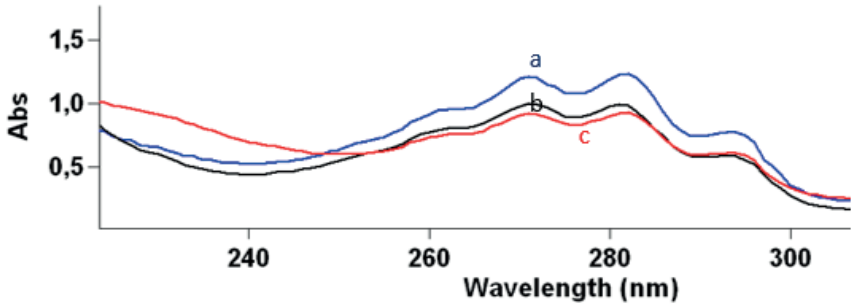

Figure 2. Spectrophotometric profile of C. gattii ATCC 24065 sterols, untreated in (a), treated with morin at 32 µg/mL (b), and treated with fluconazole at 2 µg/mL (c).

The effect of morin on the fungal plasma membrane was assessed by flow cytometry through the analysis of the cells labeled with the PI marker. As a positive control, 70% ethyl alcohol and 2 µg/mL amphotericin B were used on the cells of *C. gattii* ATCC 24065. Ethyl alcohol 70% caused membrane damage in 99.8% of the analyzed cells as shown in Figure 3 on histogram B and amphotericin presented 26.8% of fungal death as shown in Figure 3 on histogram C. The negative control was performed with untreated *C. gattii* labeled with PI, showing 0.0% of cells with membrane damage (Figure 3, histogram A). After morin incubation period of 2h at 37 ° C, it was observed that the compound promoted lesions in an average of 45.5% of the cells tested.

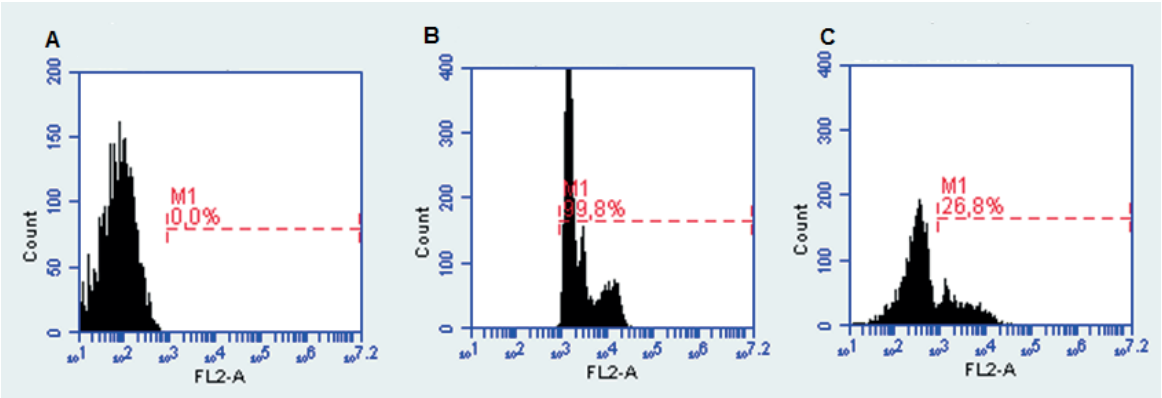

Figure 3. Histograms showing cells of C. gattii ATCC 24065. (A) Autofluorescence control, (B) Control with 70% alcohol, (C) Amphotericin B at concentration of 2 µg/ml. The X-axis shows, on a logarithmic scale, the fluorescence intensity of the PI-labeled cells.

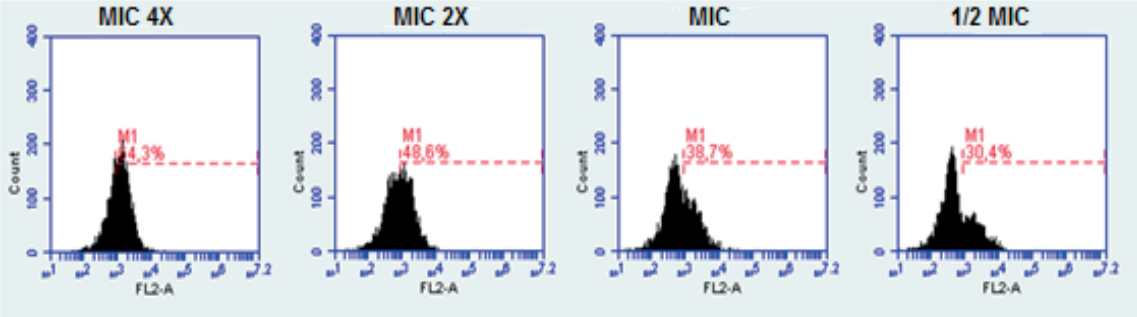

Figure 4. Histograms of C. gattii ATCC 24065 treated with different concentrations of Morin. The X-axis shows, on a logarithmic scale, the fluorescence intensity of the PI-labeled cells. Gate M1 shows the percentage of cells not non-viable labled with PI.

Re-docking simulations were performed with the structure of *C. neoformans* Trehalose-6-phosphate phosphatase. Re-docking results for the combination of four search algorithms, four scoring functions and the presence of water molecules (a total of 32 different docking protocols) generated an RMSD of less than 2 Å. The following parameters and their combinations were varied: radius of the docking sphere, number of series, maximum number of interactions and maximum population size demonstrating interactions of hydrogen bonds and van der walls with residues.

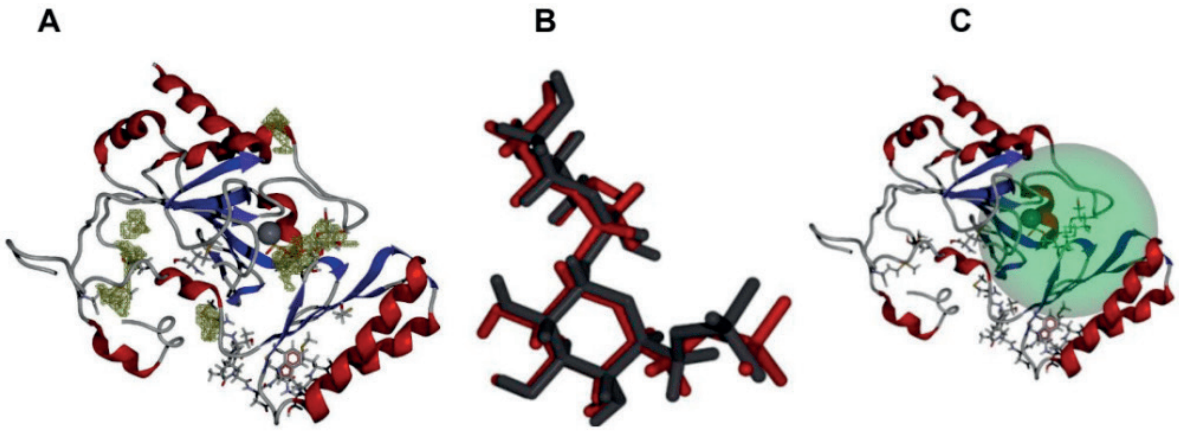

Figure 5. The crystalline structure of the enzyme trehalose-6-phosphate phosphatase of C. neoformans (X-ray diffraction (2.5 Å). A) In red are represented the α-helices and in blue the β-bands. The cavities with potential bonding capacity used in re-docking are in yellow. B) Overlap of the best docking structure generated in the MVD with the ligand Trehalose-6-phosphate (T6P). C) Doping sphere (green) used in molecular docking simulations. Parameters used: radius of the docking sphere (15 Å), number of series (20), maximum population size (50), atomic coordinates (X = 0.48, Y = 22.13, Z = 21.00).

4 I DISCUSSION

In docking simulations, it is expected that the best results generate RMSD values less than 2.0 Å when compared with crystallographic structures. The procedure of

obtaining the crystallographic position of the ligand is often called “re-docking” which is fundamentally a validation method that determines whether the molecular docking algorithm is able to recover the crystallographic position using computer simulation.

The ergosterol is the predominant sterol in the cytoplasmic membrane, as well as the main component of the fungi membrane. It is involved in several biological functions and it is the main target of the antifungals used to treat systemic fungal infections (AHMAD ET AL. 2011, ALCAZAR-FUOLI & MELLADO 2013). Therefore, in our studies we aimed to quantify this sterol in the presence of morin. We obtained promising results when using the morin, such as a reduction of 59.65% of the ergosterol from the standard strain of *C. gattii* ATCC 24065. Other flavonoids showed this same potential, as in the study carried out by Reis et al., (2016) which quantified the ergosterol of *C. neoformans* in the presence of fisetin, and observed a reduction of 25.4% in the concentration of 128 µg/mL. These results suggest that ergosterol is a possible target for flavonoids.

The flow cytometric analysis with PI marker used to evaluate the capacity of the compound in the plasma membrane of the cells, showed that the morin acted on 38.7% of the cells of species *C. neoformans* complex, causing lesion in its membrane. Similar results were found for other flavonoids related to morin, like in the study carried out by DA SILVA et al., (2014)vegetables, grains, flowers, tea, and wine. They differ in their chemical structures and characteristics. Such compounds show various biological functions and have antioxidant, antimicrobial, anti-inflammatory, and antiapoptotic properties. The aim of this study was to evaluate the in vitro interactions of flavonoids with fluconazole against Candida tropicalis strains resistant to fluconazole, investigating the mechanism of synergism. Three combinations formed by the flavonoids (+ which reported that the association of flavonoids such as Catechin and Quercetin altered the cell membrane structure of *Candida tropicalis* yeasts resistant to fluconazole, resulting in loss of plasma membrane integrity, causing higher permeability.

5 I CONCLUSION

Natural products offer many biological activities and have a great impact on the discovery of new drugs. The results obtained in our study showed the existence of antifungal activity by the Morin compound against yeasts of the *C. neoformans* complex, in addition to guiding to the possible places of action of this polyphenol.

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