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ORIGINAL ARTICLE

Evaluation of interactions of silibinin with the proteins ALS3 and SAP5 against *Candida albicans*

Avaliação das interações da silibinina com as proteínas ALS3 e SAP5 contra *Candida albicans*

Cecília Rocha da Silva[1,4], Francisca Lariza Damacieira Moura[3], André Luis Almeida Alves Filho[3], Rosana de Sousa Campos[3], Vitória Pessoa de Farias Cabral[1,4], Lívia Gurgel do Amaral Valente Sá[1,3,4], Anderson Ramos da Silva[5], Jacilene Silva[2], Akenaton Onassis Cardoso Viana Gomes[3], Letícia Bernardo Barbosa[3], Hélcio Silva dos Santos[2], Emmanuel Silva Marinho[2], Bruno Coelho Cavalcanti[4], Hélio Vitoriano Nobre Júnior[1,4], João Batista de Andrade Neto[1,3,4]

1. School of Pharmacy, Laboratory for Bioprospection of Antimicrobial Molecules (LABIMAN), Federal University of Ceará, Fortaleza, CE, Brazil. 2. Department of Chemistry, Group for Theoretical Chemistry and Electrochemistry (GQTE), State University of Ceará, Ceará, Brazil. 3. Christus University Center (UNICHRISTUS), Fortaleza, Ceará, Brazil. 4. Drug Research and Development Center, Federal University of Ceará, Fortaleza, CE, Brazil. 5. Department of Surfactants and Nanobiotechnology, IQAC-CSIC, Barcelona, Spain.

Abstract

Objective: to evaluate the molecular interaction of silibinin with the targets ALS3 and SAP5. **Methodology:** Molecular docking protocols were conducted to analyze the binding interaction of silibinin with ALS3 and SAP5. **Results**: Eleven interactions of ALS3 with silibinin and four with fluconazole were found, while six interactions were observed of SAP5 with silibinin and four with fluconazole. **Conclusion**: Molecular docking between silibinin and ALS3 identified important interactions, but no significant interactions were observed with SAP5, even though silibinin can exhibit affinity and interactions with other SAP5 sites.

Keywords: Silibinin; Fungal Infection; *Candida* spp; Molecular Docking; Adhesion Proteins.

Resumo

Objetivo: Avaliar a interação molecular da silibinina com os alvos ALS3 e SAP5. **Metodologia**: Protocolos de docking molecular foram conduzidos para analisar a interação de ligação da silibinina com ALS3 e SAP5. **Resultados**: Foram encontradas onze interações de ALS3 com silibinina e quatro com fluconazol, enquanto seis interações foram observadas de SAP5 com silibinina e quatro com fluconazol. **Conclusão**: Docking molecular entre silibinina e ALS3 identificou interações importantes, mas não foram observadas interações significativas com SAP5, embora a silibinina possa apresentar afinidade e interações com outros sítios SAP5.

Palavras-chave: Silibinina; Infecção fúngica; *Candida* spp; Ancoragem Molecular; Proteínas de Adesão.

INTRODUCTION

Species of the genus *Candida* are important threats to public health since among systemic fungal infections they are responsible for most hospital infections and have a high financial cost for Brazil's National Health System[1]. These infections are a global concern. Species of *Candida* spp. belong to the normal human microbiota, mainly colonizing the gastrointestinal tract, vulvovaginal region, and oral cavity.[2–4] However, the overuse of broad-spectrum antibiotics and immunosuppressive therapies can result in immune imbalance, triggering changes in the normal human microbiota and leading to the development of pathogenic strains that are responsible for causing serious infections[5].

The main clinical species are *Candida albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*. Of all the species isolated from humans, *C. albicans* is the most prevalent and virulent and is the main cause of candidiasis[6,7]. These clinical isolates are responsible for causing severe superficial or systemic opportunistic mycoses, especially in some risk groups. Among these are patients in intensive care units (ICUs), immunocompromised and transplanted patients, elderly people, and patients who use catheters[8].

Candida spp. cause disease due to the presence of certain virulence factors, such as enzyme production and biofilm

Correspondence: João Batista de Andrade Neto. Universidade Federal do Ceara. Telephone number: # 55 85 3366 8274. E-mail: label_ufc@yahoo.com.br
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formation. The formation of biofilms is one of the main virulence mechanisms of *Candida* spp., facilitating the development of hyphae, making treatment difficult[4], since this mechanism prevents the penetration of antifungal substances in the extracellular matrix, and in addition prevents cell phagocytosis[7]. Other factors are the production of hydrolytic enzymes, which facilitate cell invasion besides helping the morphological transition and colonization of the affected tissues. Among these enzymes are lipases, phospholipases, and proteases[6].

Secreted aspartic proteases (SAPs) produced by *Candida* spp. are classified into 10 types, ranging from SAP 1 to SAP10. The expression of these genes induces greater pathogenicity of the infection since these enzymes can degenerate proteins, facilitating invasion in the host's epithelial tissue. These enzymes have high specificity against the substrates present in human proteins that belong to the immune system9, mainly immunoglobulins, complement system, and cytosine. In this way, they can circumvent the innate immune system.10 The literature reports that SAP genes are more strongly expressed in *C. albicans* biofilms and that different SAP genes can be expressed in blood infections associated with *C. albicans* biofilms, with SAP5 being the most important[11].

In addition to genes expressing SAPs, another group of genes also important in the virulence and pathogenicity of *Candida* species is the agglutinins (ALS), which encode cell wall proteins responsible for the pathogen's adhesion to host cells. They consist of a group of genes ranging from ALS1 to ALS8, of which ALS3 has the greatest effect on adhesion to the species of C. albicans[12]. In addition, ALS3 facilitates the formation of biofilms, as well as being able to invade epithelial and endothelial tissues, causing endocytosis of host cells and binding to ferritin to obtain iron. These are important mechanisms for the development and resistance of infection[13].

In this sense, natural sources have been extensively explored to find new pharmacological compounds.14 Phytotherapeutic studies are viable alternatives for the exploration of new antimicrobial drugs, since plants are rich in chemical compounds with antimicrobial action, with wide biodiversity and easy access.7 Flavonoid compounds derived from plants have been widely investigated as new antifungal and antibacterial drugs since they have a variety of therapeutic actions[15,16].

For the production of new antifungals, evaluating factors associated with macromolecules, such as existing molecular interactions with enzymatic sites, facilitates the recognition of possible therapeutic effects at a predetermined enzymatic site. Molecular docking performs a structural comparison of the macromolecule of interest, taking into account the polarity and number of hydrogen bridges that can be formed, deduced through computer programs to ascertain the energy required for the bond and the interaction force between the molecule and its target[17].

Silybum marianum, popularly known as milk thistle, is a plant widely used in traditional medicine, belonging to the Asteracea family, native to Southern Europe and North Africa. It adapts very well to tropical climates with a dry atmosphere and warm soil.18 The extract of its fruits is rich in chemical compounds, with silymarin being its main active ingredient. Among the flavonolignans present in silymarin is silibinin,19 a flavonoid that has anti-inflammatory, anti-fibrotic, hepatoprotective, and antioxidant activities as well as synergism with ampicillin and gentamicin against pathogenic bacteria in the oral cavity[18,20]. Silibinin is thus a promising natural compound for the development of new antifungal therapies, especially for *Candida* spp. This study aims to evaluate the molecular interaction of silibinin with the molecular targets ALS3 and SAP5 in C. albicans.

MATERIALS AND METHODS

Preparation of *Candida albicans* targets

The crystalline structures of ALS3 complexed with hepta-threonine (hepta-Thr) and SAP5 complexed with pepstatin A (pepA), a classic aspartic proteinase inhibitor, were selected using PDB codes 4LEB and 2QZX in the Protein Data Bank (https://www.rcsb.org/), where they are deposited with a resolution of 1.4 Å and 2.5 Å respectively, determined from X-ray diffraction[21,22]. In the preparation process of the enzymes *C. albicans* ALS3 and SAP5, the residues present in the protein structures were removed and polar hydrogen was added[23].

Binder preparation

The chemical structure of silibinin was obtained in the PubChem repository (https://pubchem.ncbi.nlm.nih.gov/) employing CID 31553 and optimized using the Avogadro code24, configured for cycles of 50 interactions of the descending steepest algorithm, applying the MMFF94 force field[25].

Molecular docking

The molecular docking routines between the binder and the *C. albicans* strains were performed using AutoDockVina code (version 1.1.2) configured to use three-way multithreading, Lamarkian Genetic algorithm[26] and the center_x search parameters = -5.806, center_y = 2,952, center_z = -13.754, size_x = 102, size_y = 126, size_z = 92, spacing = 0.592 and exhaustiveness = 8 for ALS3 and center_x = 20.664, center_y = 21.527, center_z = 45.515, size_x = 80, size_y = 82, size_z = 124, spacing = 0.919 and exhaustiveness = 8 for SAP5. The figures were created from Chimera[27] and Discovery Studio Visualizer[28].

RESULTS

Table 1 reports the values of root mean square deviation (RMSD) and free binding energy in the molecular docking simulations between silibinin and the targets *C. albicans* ALS3 and SAP5. The best simulations were selected from values less than or equal to -6.0 kcal/mol for free binding energy[29] and up to 2.0 Å RMSD[30].

Table 1. RMSD and affinity energy values calculated in molecular docking simulations

Candida albicans	Ligand	Affinity (kcal/mol)	RMSD (Å)
ALS3	Silibinin	-9.0	1.518
ALS3	Fluconazole	-6.0	1.704
SAP5	Silibinin	-9.9	1.829
SAP5	Fluconazole	-7.2	1.676

Docking of ALS3

Molecular docking of silibinin with the adhesion enzyme ALS3 of *C. albicans* occurred close to the hepta-Thr binding site complexed in the target enzyme (Figure 1). Comparing silibinin with hepta-Thr indicated that silibinin had a shorter distance with four residues from the active site (Table 2), highlighting the residues of the catalytic triad Lys59 (adhesion role) and Ala116, in which the analysis of distances showed the proximity of silibinin compared to the hepta-Thr and fluconazole controls. Analyzing the interactions of ALS3 with silibinin (Figure 2A) revealed the existence of 11 interactions: four of the conventional hydrogen bond type; one with Gly27 (2.05 Å), Lys59 (2.08 Å), Thr168 (1.93 Å) and Tyr226 (2.45 Å); three Pi-Alkyl interactions; one with Pro29, Val161, and Arg171; a carbon-hydrogen bond interaction with Arg171; a Pi-Pi T-shaped interaction with Trp295; an unfavorable acceptor-acceptor interaction with Tyr166; and an unfavorable donor-donor interaction with Val172. Four interactions were found for fluconazole (Figure 2B), two of the conventional hydrogen bond type, one with Asn22 (2.03 Å) and the other with Tyr23 (2.17 Å). A Pi-Sigma interaction with Val161 and a carbon-hydrogen bond interaction with Asp169 were also observed. Furthermore, silibinin showed an affinity of -9 kcal/mol and 11 molecular interactions with the target ALS3, among which the main one was a conventional hydrogen bond type, with the Lys 59 (2.08 Å) binding site standing out with the best interaction when assessing the distance between silybin and the target protein.

Figure 1. Silibinin binding site compared to hepta-threonine and fluconazole.

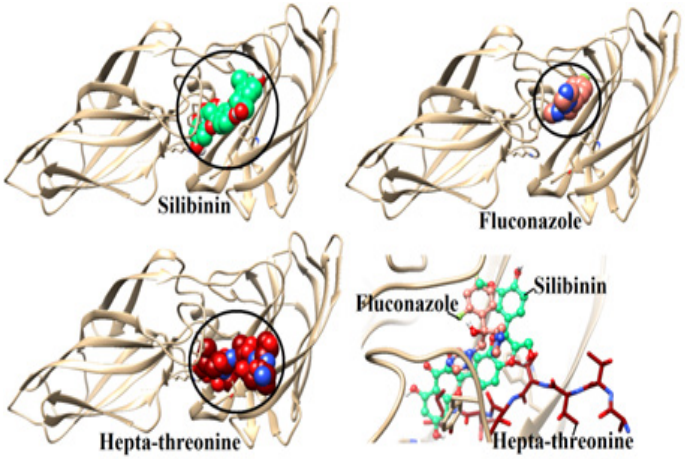
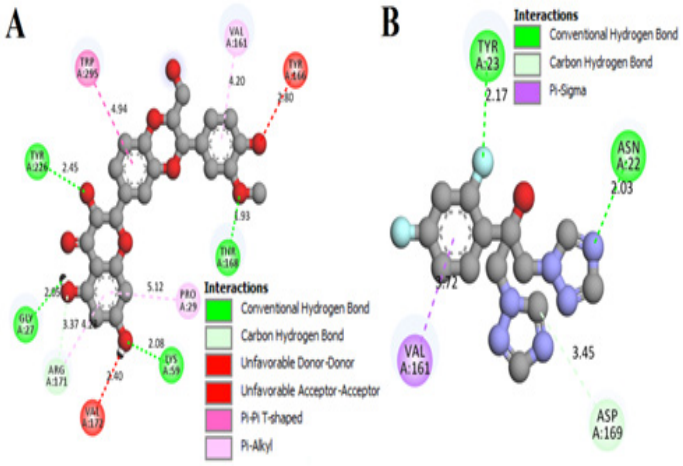


Table 2. Distances between the ALS3 *Candida albicans* residues and the ligand

ALS3 Candida albicans residue	Silibinin	Fluconazole	Hepta-threonine
Ala19	7.8 Å	8.2 Å	3.9 Å
Pro29	4.3 Å	6.3 Å	4.2 Å
Phe58	5.8 Å	11.0 Å	6.0 Å
Lys59	2.1 Å	8.4 Å	2.8 Å
Thr61	6.4 Å	11.4 Å	6.2 Å
Ala116	4.2 Å	9.4 Å	5.3 Å
Ser170	3.5 Å	4.4 Å	3.5 Å
Leu293	5.5 Å	7.1 Å	4.8 Å
Trp295	3.7 Å	4.7 Å	4.0 Å
Gly297	6.9 Å	10.1 Å	3.6 Å

Figure 2. Molecular interactions of Silibinin (A) and fluconazole (B) with ALS3.



Docking of SAP5

Although silibinin showed excellent binding free energy in the molecular docking with SAP5 of C. albicans, the fit did not occur close to the pepstatin A binding site co-crystallized in SAP5, as shown in Figure 3. The SAP5 enzyme of *C. albicans* has a region called the N-ent loop composed of bulky side chains with Lys50, Trp51, and Arg52, which extend to the substrate-binding gap, and therefore can form an additional barrier against the entry of substrates in the crack in the SAP5 active site. Analysis of the distances between silibinin and the residues of the active enzyme site indicated that the ligand was further from the catalytic site than pepstatin A (Table 3), which may be related to the SAP5 N-ent loop. Because of this, no significant distances were found for the silibinin-SAP5 complex. The complex formed with silibinin (Figure 4A) had six interactions: two of the conventional hydrogen bond type; one with Ser180 (2.22 Å) and Val330 (2.51 Å); two of the carbon-hydrogen bond; one with Thr261 and Ser273; interaction of the Pi-Alkyl type with Pro329; and interaction of the Pi-donor hydrogen bond type

with Ser273. There were four interactions with fluconazole (control) (Figure 4B): two of the Pi-sulfur type (one with Cys256 and the other with Cys294); one of the conventional hydrogen bond type with Gln282 (2.25 Å); and a Pi-Alkyl type interaction with Ala162.

Figure 3. Silibinin binding site compared to pepstatin A and fluconazole.

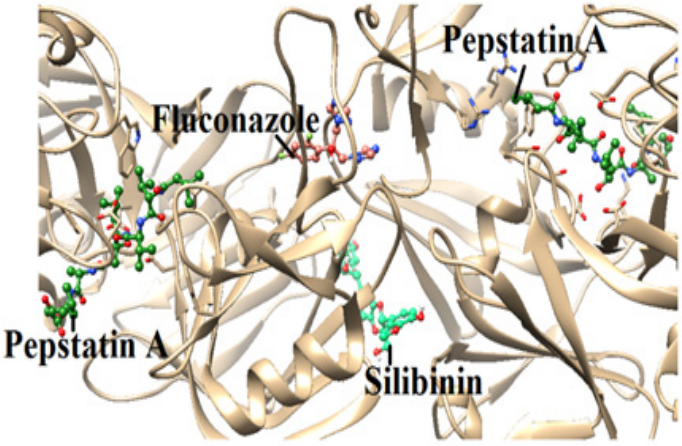
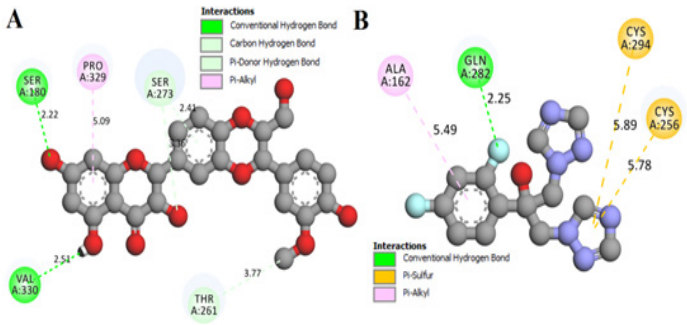


Table 3. Distances between the SAP5 *Candida albicans* residues and the ligand

SAP5 *Candida albicans* residue	Silibinin		Fluconazole		Pepstatin A	
	Chain A	Chain B	Chain A	Chain B	Chain A	Chain B
Ile12	20.4 Å	18.8 Å	8.6 Å	15.4 Å	3.8 Å	3.5 Å
Asp32	23.1 Å	21.0 Å	18.5 Å	25.0 Å	3.3 Å	3.3 Å
Gly34	22.5 Å	20.5 Å	19.2 Å	25.7 Å	2.9 Å	2.8 Å
Ser35	25.2 Å	22.9 Å	21.9 Å	28.5 Å	4.0 Å	4.5 Å
Lys50	30.7 Å	29.5 Å	19.4 Å	25.2 Å	5.6 Å	6.7 Å
Trp51	27.8 Å	24.7 Å	15.1 Å	18.6 Å	3.5 Å	3.5 Å
Arg52	33.1 Å	32.2 Å	17.7 Å	21.2 Å	9.8 Å	9.6 Å
Lys83	31.6 Å	29.8 Å	24.9 Å	31.0 Å	3.3 Å	2.7 Å
Tyr84	29.2 Å	27.1 Å	21.6 Å	27.8 Å	3.2 Å	3.3 Å
Gly85	27.9Å	26.8 Å	20.5 Å	25.7 Å	1.7 Å	3.1 Å
Asp86	27.7 Å	25.8 Å	17.7 Å	23.7 Å	2.2 Å	2.5 Å
Ile123	27.5 Å	26.0 Å	20.8 Å	27.5 Å	3.7 Å	3.7 Å
Gly220	20.0 Å	17.9 Å	14.7 Å	21.6 Å	3.1 Å	3.9 Å
Thr221	17.9 Å	16.0 Å	11.3 Å	17.5 Å	3.5 Å	3.4 Å
Thr222	16.6 Å	15.0 Å	8.2 Å	14.3 Å	2.0 Å	2.2 Å
Ile223	17.3 Å	15.6 Å	8.6 Å	13.4 Å	4.0 Å	4.0 Å
Tyr225	18.1 Å	17.4 Å	14.2 Å	18.7 Å	3.4 Å	2.9 Å
Ile305	17.3 Å	16.4 Å	15.1 Å	19.3 Å	4.2 Å	4.0 Å

Figure 4. Molecular interactions between Silibinin (A) and fluconazole (B) with SAP5.



DISCUSSION

The data on biological macromolecules deposited in the Protein Data Bank (PDB) has opened the possibility to investigate the correlation with biological information, constituting a favorable scenario for the application of computer systems for the prediction of binding affinity with target proteins, in addition to studies in the area of drug development and design[31]. In this context, the expansion of models for in silico analysis has significantly contributed to the assessment of hypotheses in the field of pharmacology. Among the tools used in this context, molecular docking stands out in microbiology for the ability to prospect for protein targets in microorganisms, in addition to molecules having high affinity with them[32].

The analysis of the interaction with SAP5 and ALS3 targets, important for the pathogenesis of C. albicans, is described in the literature concerning the assessment of potential antifungal activity33,34, so these were investigated in this study about the molecular interaction with silibinin. The observed interactions of silybinin with ASL3 and SAP5 were varied, among them the hydrogen bond type, which is recognized as playing a significant role in several chemical and biological processes.35 The conventional type stood out in the evaluation against ALS3. The hydrogen bond between a protein and its ligands provides interaction directionality and specificity, essential for molecular recognition 36, thus indicating its importance.

Furthermore, interactions were also observed, namely Pi-Alkyl, Pi-Pi T-Shaped, Pi-donor hydrogen bond, unfavorable acceptor-acceptor, and unfavorable donor-donor interactions. Regarding Pi-type interactions, the literature report they play an important role in the field of biochemistry so the occurrence of aliphatic (CH-Pi) and aromatic (Pi-Pi) hydrogen bonds in biological macromolecules suggests their functional role in the stability of 3D structures in molecular recognition events, as well as in the folding of macromolecules.37 Associated with the observed interactions, silibinin showed promising results regarding the free energy of binding with ALS3 and SAP5, and regarding the distances of residues of ALS3 and ligands. However, considerable distances to the pepstatin A binding site of SAP5 were found. Thus, future research can be directed toward optimizing the interaction with SAP5.

A previous study elucidated possible mechanisms of action by which silibinin acts against C. albicans, and confirmed that the treatment with 100 µM of this substance led to the generation of reactive oxygen species (ROS), which could cause apoptosis in yeast through oxidative stress, in addition to disturbances in ionic homeostasis, with the release of intracellular K+ and accumulation of cytoplasmic and mitochondrial Ca2+. Marks of early apoptosis were also verified, such as mitochondrial membrane depolarization, cytochrome c release, caspase activation, phosphatidylserine (PS) exposure, and DNA damage in response to treatment with silibinin, suggesting a possible induction of apoptosis of yeast mediated by mitochondrial Ca2+ signaling.38 Another study showed that silibinin induces fungal membrane rupture and effectively inhibits the growth of hyphae at a concentration of 100 µg/ml and exerts an antibiofilm effect in the early stage of formation at concentrations greater than 100 µg/ml.4

Regarding the clinical application area of silibinin, there are some limitations, such as low solubility, poor penetration into intestinal cells, high metabolism, and rapid systemic elimination. However, drug delivery systems based on nanotechnology have excellent potential for developing phytochemical compounds to improve solubility, bioavailability, stability, and even modify pharmacological activity. Successful examples of the use of silibinin in nanoformulations are its use of liposomes, lipid nanoparticles, polymeric nanoparticles, and nanoemulsions, along with complexation with cyclodextrins, among other possibilities.39, thus having many alternatives for future formulations.

CONCLUSIONS

The results of molecular docking between silibinin and ALS3 identified significant interactions with residues from the active enzyme site, one with the residue that has an adhesion role (Lys59) and belongs to the residues of the catalytic triad of ALS3 in C. albicans. As for the results of molecular docking with SAP5 of C. albicans, there were no significant interactions with silibinin. However, silibinin can exhibit affinity and interactions with other SAP5 sites.

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