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In vitro and *silico* Analysis of Amentoflavone against *Candida albicans* with a Competitive Action about Amphotericin B

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Antifungal resistance is a global health problem, and alternatives for control and treatment must be sought. Thus, substances isolated from plants with antifungal capacity have received considerable attention in the pharmaceutical sector. *Ouratea fieldingiana*, popularly known

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as batiputá, is a tree species found in northeastern Brazil, with its leaves rich in biflavonoids such as amentoflavone. Biflavonoids are well-studied due to their high antifungal capacity.
Objectives: Carry out the isolation, identification, and characterization of the compound amentoflavone (AMT), as well as evaluate its antifungal activity and modulatory effect against strains of *Candida albicans*, also including the computational study of the mechanism of action of the compound AMT against the Als3 and Sap5 enzymes of *C. albicans*.
Methodology: Column chromatography was used to isolate the AMT compound from the species *O. fieldingiana*, and its characterization was performed by high-performance liquid chromatography (HPLC). The antifungal activity was analyzed by the broth microdilution method. Modulatory activity assays were performed by the checkerboard technique using AMT and amphotericin B (AmB) as a standard. For the *in silico* study, computational simulations of the interaction between the enzymes Als3 and Sap5, and AMT were performed using the Vina AutoDock Code.
Results: The AMT compound showed an inhibitory effect against all strains of *C. albicans*. Regarding modulatory activity, an indifferent and antagonistic effect was observed for all *C. albicans* strains tested. Molecular docking with AMT showed higher affinity energy for the Als3 and Sap5 enzymes than AmB. The results obtained in this study suggest that AMT has antifungal effects, as well as a high affinity for the Als3 and Sap5 enzymes of *C. albicans*.
Conclusion: These results prove that the AMT compound could be a potential source of a new biotechnological product, acting as a natural antifungal agent.

Keywords: Amentoflavone; antifungal; modulating effect and natural products.

1. INTRODUCTION

"Candida albicans is a common commensal fungus that colonizes the oropharyngeal cavity, gastrointestinal and vaginal tracts, and the skin of healthy individuals. In 50% of the population, *C. albicans* is part of the natural flora of animals. However, when the homeostasis of the host and/or endogenous microbiota is disturbed, this phenomenon will result in a transition from normal flora to pathogenic and opportunistic infections. The various clinical manifestations of *Candida* species range from superficial and localized mucocutaneous disorders to invasive diseases involving multiple organ systems and are fatal" [1-3]. **"**The co-evolution of *C. albicans* with humans has established a complex balance between commensalism and pathogenicity for this yeast fungus. In recent decades, advances in modern medicine have enabled this pathobiont to become one of the most common human pathogens causing life-threatening healthcare-associated infections, the prevalence of which continues to be significant. Becoming a serious health threat worldwide" [4-5].

"The pathogenicity of *C. albicans* is attributed to many virulence determinants, among which are host tissue adhesion, response to environmental stresses, production of hydrolytic enzymes, transition from yeast to hyphae, and production of biofilms. Among the proteins that directly contribute to the pathogenicity of *C. albicans* are the cell surface glycoproteins of the Als family. Among these ALS3 adhesin is commonly associated with fungal adhesion to host cells, as well as more complex interactions, such as invasion and biofilm formation" [6-7].

"The secreted aspartic proteinases (Saps) are considered putative virulence factors important for the pathogenicity of *C. albicans* [8-9]. *C. albicans* has a large gene family encoding Saps, and the individual Sap isoenzymes likely evolved for optimal adaptation to specific functions or host niches" [10,11]. "Expression of SAP5 during infection might be activated by signals that also induce hyphal growth, possibly involving the same signal transduction pathways, but might be independent of the hyphal morphology itself" [11].

"The continuous increase in fungal infections, combined with the phenomenon of resistance, has led to the search for new bioactive substances of biological and pharmacological interest, so attempts to enhance the effect of conventional antifungals through interactions between natural products and commercial drugs have been tested against isolates from clinical trials and multiresistant *Candida* species. Currently, to study new compounds and their possible biological activities, it is essential to know the structure of the molecule in question, define the reaction sites, and characterize atomic properties and bonds to then enable the synthesis of the molecule. In this context, molecular modeling, a set of tools for

manipulating and analyzing complex molecular systems, using *in silico* methods and theoretical calculations, allows the characterization of molecular structures and their properties, as well as predicting the mechanism of interaction between molecules and possible biological targets, for the development of potential candidate molecules for new drugs" [12-14].

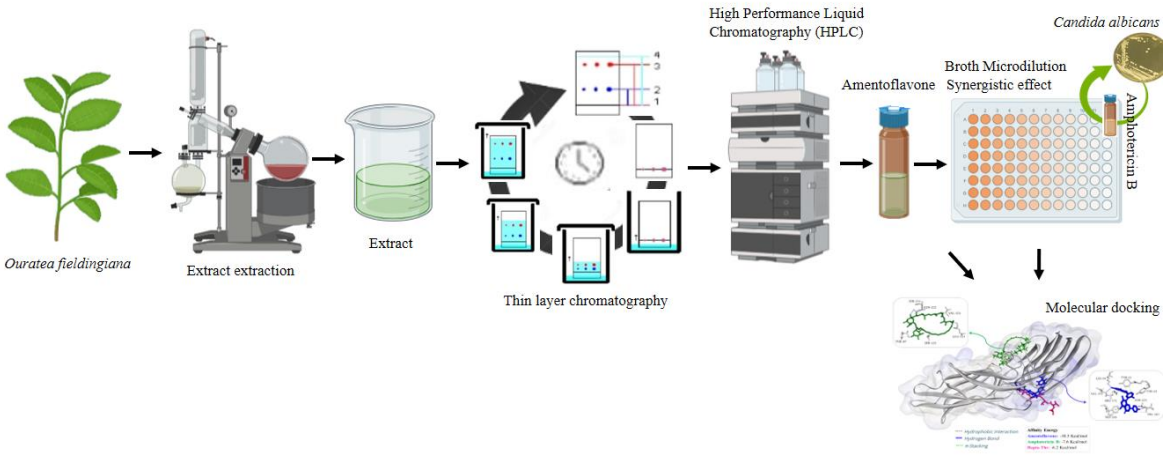
"Naturally occurring structures have been used as targets in the development of new drugs. In this context, we can highlight the plants of the Ochnaceae family, such as *Ouratea fieldingiana,* popularly known as batiputá, which is rich in biflavonoids, such as amentoflavone (AMT). This compound presents several promising pharmacological properties, such as antifungal, antioxidant, antitrypanosomal, and antiviral, among others. Due to these properties, amentoflavone is a compound that can be exploited for the treatment of several diseases, including those of fungal origin" [12-15].

"AMT exhibited potent antifungal activity against several pathogenic fungal strains but had a very low hemolytic effect on human erythrocytes. In particular, amentoflavone induced the accumulation of intracellular trehalose in *C. albicans* as a stress response to the drug and disrupted the dimorphic transition that forms pseudo-hyphae during pathogenesis" [16]. "Furthermore, the antibacterial effects and combined effects of AMT and conventional antibiotics such as ampicillin, cefotaxime, and chloramphenicol were investigated. These results showed that amentoflavone had a considerable antibacterial effect and synergistic interaction with antibiotics against various bacterial strains" [17].

However, the modulating potential of AMT isolated from *O. fieldingiana* with the antifungal Amphotericin B (AmB), as well as its mechanism of action against the enzymes ALs3 and Sap5 of *C. albicans* have not yet been studied. Therefore, evaluating the in vitro antifungal activity of the compound AMT on *C. albicans* and evaluating its modulating potential with the antifungal AmB, as well as molecular docking studies, are ways to promote both knowledge about this biflavonoid and also enhance the discovery of new phytopharmaceuticals and new therapeutic strategies.

2. MATERIALS AND METHODS

Pic. 1 shows the scheme illustrating the methodology used in the isolation, identification, and evaluation of the antifungal activity of amethoflavone, obtained from *O. fieldingiana*. The plant was subjected to extraction and fractionation, followed by the isolation of AMT by thin-layer chromatography. The structure of the isolated compound was confirmed by high-performance liquid chromatography. The antifungal activity of AMT was evaluated against *C. albicans* by broth microdilution tests, including synergism studies with AmB. The molecular interaction of AMT with the fungal target was investigated by *in silico* studies.



Pic. 1. General scheme of the methodology used in the isolation, identification, and evaluation of the antifungal activity of amethoflavone, obtained from *O. fieldingiana*

2.1 Plant Material

The leaves of *O. fieldingiana* were collected in the municipality of Trairi, Ceará, Brazil, in March 2019, at the geographic location (latitude 3° 13'01, 90', longitude 39° 23'20, 10'' W). A voucher sample (62392) was deposited at the Herbarium Prisco Bezerra of the Federal University of Ceará (UFC) and authenticated by the botanist Luiz Wilson Lima-Verde.

2.1.1 Amentoflavone (AMT) extraction

To prepare the extract, 1 kg of *O. fieldingiana* leaves were dried, ground, and macerated in a closed glass bottle at room temperature for seven days with 70% ethanol. The extract was then filtered into a round-bottomed flask and concentrated on a rotary vacuum evaporator at 50°C. After this process, a green material was obtained together with an aqueous solution. The green waxy material was separated from the aqueous solution by filtration in a Buchner funnel coupled to a kitazate and a vacuum pump, and the brownish solution, after lyophilization, resulted in the defatted ethanolic extract of the leaves (EEF) of *O. fieldingiana*, with 16.5% yield [18].

For the isolation of AMT, a G-60 silica column was used, which was eluted with ethyl acetate and chloroform solvents in mixtures of increasing polarities. Purification of the AMT compound was carried out on a Sephadex column (LH-20), using the solvent methanol for elution. AMT was identified using Thin Layer Chromatography (TLC), comparing it with an authentic sample previously isolated in our laboratory [19].

High-performance liquid chromatography (HPLC): To confirm the purification of the AMT compound, the fraction containing amt was subjected to high-performance liquid chromatography (HPLC-DAD). to identify the phenolic compounds, a methanolic solution of the fraction with a concentration of 20 µl ml^{-1} was injected into the equipment. Standards were purchased from sigma chemical co. (St. Louis, MO, USA). the solvents used for the extraction were of analytical grade (Vetec®), in the analyses the solvents used were of HPLC grade (J.T. BAKER®). Chromatographic analyses were performed on a shim-pack reversed phase column (CLC) ODS GOLD (4.6x250mm, 5µm). Mobile phases C and D were acetonitrile and Milli-Q water acidified to ph 2.8 with phosphoric acid, correspondingly, solvent gradient was used as follows: 0-15 min, an isocratic elution with C:D (20:80 v/v); 17-25 min, linear variation up to C:D (40:60 v/v); 25-40 min, an isocratic elution with C:D (20:80 V/V).The flow rate was 1.0 $mL.min^{-1}$, with an injection volume of 20 μL and a wavelength of 350 nm. The peaks relating to the constituents present in the HPLC chromatogram were confirmed by comparing their retention time with that of the reference standard and by DAD spectra (200 to 400 nm).

2.2 *In vitro* Antifungal Assay (Inoculum Preparation for Antifungal Susceptibility Testing)

Antifungal activity was determined by the broth microdilution method, according to the M27-A3 guidelines for yeast, according to the Clinical and Laboratory Standards Institute protocol CLSI M27 –A3, 2008 [20] with modifications for natural products. *C. albicans* strains were obtained from the fungal collection of the Vale do Acaraú State University. In this study, a total of 5 strains of *C. albicans* were used, 5 strains of clinical isolates of *C. albicans* were used: LABMIC 0101 (blood culture), LABMIC 0102 (blood culture), LABMIC 0104 (tracheal aspirate), LABMIC 0105 (blood culture) and *C. albicans* ATCC 90028 used as the standard strain for the analysis. Microorganisms were grown overnight on potato dextrose agar (Difco, Detroit, MI, USA) and incubated at 37 °C. From this culture, saline-free suspensions were prepared according to the McFarland 0.5 scale. Suspensions were diluted 1:2000 in RPMI 1640 medium (with l-glutamine without sodium bicarbonate buffered to pH 7.0 with 0.165 M MOPS), to obtain an inoculum of $2.5^{-5}x10^{3}$ CFU mL^{-1}.

2.2.1 Broth microdilution method

The minimum inhibitory concentration (MIC) was determined according to the CLSI guideline document [20-21]. AMT (10 mg L^{-1}) was diluted in Dimethylsuffoxide (DMSO) 5%. AmB was prepared in DMSO and placed in concentrations ranging from 16 µg/mL to 0.125 µg mL. For antimicrobial activity, AMT was tested at concentrations ranging from 0.002 to 2.5 mg L^{-1}. The microdilution test was performed in 96-well microdilution plates and incubated at 37 °C, and the antifungal effect was visually analyzed after 24 h.

2.2.2 Synergism by Checkboard

The synergistic activity between AmB and AMT was determined by the checkboard technique. Initially, 50 µL of RPMI-1640 was added to all

wells of the 96-well microdilution plate. Then, 50 µL of each dilution of AMT was added in vertical orientation, with concentrations ranging from 5 mg L^{-1} to 0.03 mg L^{-1}. In horizontal orientation, 50 µL of AmB (standard antifungal) were placed at concentrations ranging from 16 µg/mL to 0.125 µg ml, then 100 µL of the *C. albicans* suspension (2.5^{-5} x 10^3 CFU mL -1) were added to all wells and incubated at 37 ºC for 24 h. Assays were performed in triplicate. To interpret the results, the fractional inhibitory concentration index (FICI) was calculated, in which FICI ≤ 0.5 will have a synergistic effect; FICI = 0.5 to 1.0 will be an additive effect; FICI > 1.0 < 4.0 indifferent effects; and FICI > 4.0 antagonistic effects [22-23].

2.3 Molecular Docking (Preparation and Optimization of the Amentoflavone (AMT) Compound

The two-dimensional AMT and AmB coordinates were obtained from the PubChem repository (https://pubchem.ncbi.nlm.nih.gov/) [24]. The structures were rendered using MarvinSketch software (https://chemaxon.com/products/Marvin) and then subjected to structural optimization using the classical force field formalism MMFF94 (Merck Molecular Force Field 94) [25]. The optimization calculations were performed using the Avogadro® software, [26] configured to perform force field simulations MMFF94, using the Steepest Descent algorithm, [27] 500 numerical steps, and a convergence parameter 10e -7 [28].

2.3.1 Obtaining the 3D structures of the target proteins

To investigate the antifungal effect of AMT present in the ethanol extract of *O. fieldingiana* against *C. albicans*, the structures of the enzymes Als3 and Sap5 from *C. albicans* were obtained from the Protein Data Bank database (https://www.rcsb.org/), PDB ID: 4LEB and 2QZX respectively [29-30]. In the receptors preparation, the polar hydrogens and the calculated Gasteiger charges using the software Autodocktools ™ [31-32].

2.3.2 Molecular docking procedures

After the AMT, AmB, and enzyme preparation phase, the compounds were then subjected to molecular docking. The AutoDock Vina software (version 1.1.2) was used to perform the docking simulations, using 3-way multithreading, exhaustiveness 64, and Lamarkian Genetic Algorithm [33]. Centered on the entire protein, the grid box was defined with parameters of 102Åx126Åx92Å and dimensions (x, y, z) = (-5.806, 2.952, -13.754) for the Als3 receptor and parameters of 80Åx82Åx124Å and dimensions (x, y, z) = (20.664, 21.527, 45.515) for the Sap5 receptor. As a standard procedure, 50 independent simulations were performed, obtaining 20 poses each [34]. Redocking procedures were performed for methodological and statistical validation of the simulations. Results analysis was performed using Discovery Studio Visualizer ™ viewer, PyMOL, and UCSF Chimera software [35-36].

3. RESULTS AND DISCUSSION

3.1 Antifungal Activity

The antifungal activity of the AMT (Fig. 1) compound against several strains of *C. albicans* is represented in (Table 1). In the broth microdilution assay against yeast, the compound AMT inhibited the growth of all *C. albicans* strains tested, with values of MIC that ranged from 0,15 to1,25 mg/mL.

Table 1. Minimum inhibitory concentration of amentoflavone and Amphotericin B

Strains	MIC Amentoflavone (mg/mL)	Amphotericin B (µg/mL)
C. albicans ATCC 90028	0.15	1.0
C. albicans LABMIC 0101	0.62	1.0
C. albicans LABMIC 0102	1.25	1.0
C. albicans LABMIC 0104	1.25	1.0
C. albicans LABMIC 0105	1.25	1.0

**LABMIC: Microbiology Laboratory.*
** MIC Minimum Inhibitory Concentration*

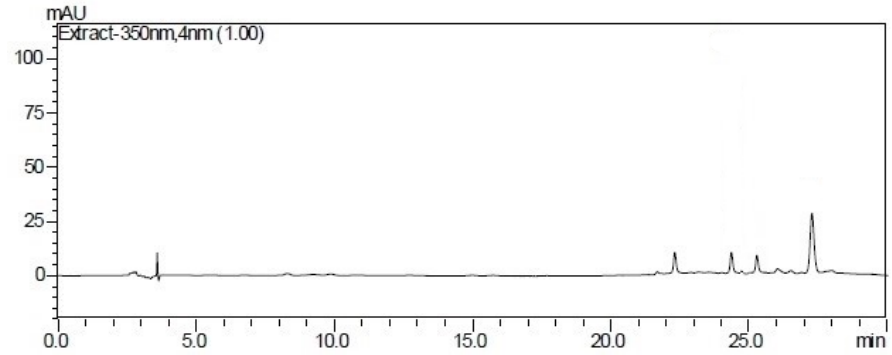


Fig. 1A.

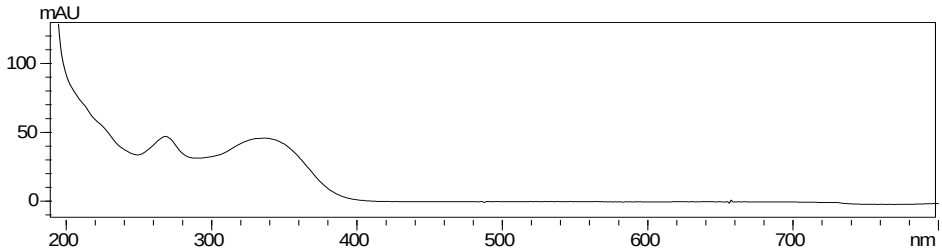


Fig. 1B.

Fig. 1. Confirmation of amentoflavone at the retention time of 27.33 obtained on the High-Performance Liquid Chromatograph (Fig. 1A) and its ultraviolet (UV) spectrum (Fig. 1B)

"Plant-derived natural products are powerful sources in traditional medicine due to their low cost, high availability, and fewer side effects. Several plant-derived bioactive compounds exhibit a wide variety of pharmacological effects, such as AMT. AMT is a biflavonoid found in several plant species, and although there are studies in the literature on its biological and pharmacological activities, there are few reports on its antifungal effect against human pathogens. Antifungal studies conducted with this biflavonoid demonstrated that AMT exhibited antifungal activity against *C. albicans*, *C. parapsilosis*, and *C. krusei"* [16,37-38]. "Research on the antifungal mechanism of AMT in *C. albicans* suggested that this active phytochemical arrested cell cycles during the S phase and inhibited cell proliferation and division. The anti-candida activity was shown to be related to apoptotic cell death, which may be associated with mitochondrial dysfunction. Furthermore, AMT-induced hydroxyl radicals may play a significant role in apoptosis" [39].

3.1.1 Modulatory activity

To determine the modulatory activity, 5 strains of *C. albicans* were used. The results are shown in (Table 2). The combination of the AMT compound with AmB did not show a significant reduction in the MIC values of the AMT against *C. albicans* strains. The MIC values of the compound AMT with AmB were the same as found for the AMT tested alone against the strains (LABIMIC 0102, LABIMIC 0104, and LABIMIC 0105). While the compound AMT alone showed a better MIC against *C. albicans* strains (LABIMIC 0101 and ATCC 90028) when tested in combination with AmB. From these values, the fractional inhibitory concentration index (FICI) was calculated, which showed an indifferent and antagonistic effect on the modulatory activity for all tested *C. albicans* strains. The MIC values for AmB associated with the compound AMT, despite demonstrating a reduction in the MIC value for AmB, showed antagonistic and indifferent effects against the strains of *C. albicans*. Therefore, suggesting that there was an influence of AMT on the action of AmB, demonstrating that the antifungal effect of both is dependent. Thus, they compete for the same protein virulence targets in the *C. albicans* structure. It is worth mentioning that no studies were found in the literature on the synergistic potential of the compound AMT isolated from *O.*

fieldingiana against clinical and ATCC strains of *C. albicans*, this being the first report of a synergistic study for the aforementioned compound.

"Previous studies have shown that the MIC of AmB was reduced when used in combination with quercetin or rutin for the ATCC strain of *Cryptococcus neoformans* and reduced when combined with rutin for a clinical isolate of *C. neoformans.* AmB is an antifungal from the polyene class that selectively binds to ergosterol, which is an analogue of cholesterol present in mammalian cell membranes, directly interrupting the integrity of the fungal membrane. Thus, its use has been limited in many patients because it almost always results in some degree of renal failure, which varies in severity depending on the total dose" [40-41].

"Thus, a more promising treatment would be the association of AmB with compounds that enhance its action, so that smaller doses of the drug can be used, or even with compounds that protect target cells or tissues from toxicity mediated by this antifungal. This effect was also observed when AmB was combined with the natural compounds berberine, allicin, epigallocatechin gallate and with essential oils of *Melaleuca alternifolia*, *Origanum vulgare*, *Pelargonium graveolens*, *Coriandrum sativum*, *Thymus maroccanus*, *Thymus broussonetii* and *Lippia alba* against *Candida species"* [42-48]. "The mechanism of action of the synergistic effects with AmB and most natural compounds is not well elucidated. However, several studies suggest that subinhibitory concentrations of AmB facilitate the absorption of natural compounds, resulting in increased death of fungal cells" [41].

3.2 Molecular Docking

Molecular docking positions the ligand in different orientations in the active site of the target receptor, to obtain the best interaction, allowing a classification to be established between compounds with greater and lesser affinity for a given receptor [14]. In the *in silico* test, after the molecular docking simulations between the AMT compound and the Als3 (Fig. 2) and Sap5 (Fig. 3) enzymes from *C. albicans*, it was possible to notice that the AMT presented a RMSD (Root Mean Square Deviation) values within the ideal parameter, less than 2 Å (Table 3) [49]. The affinity energy to evaluate the formation of the complex between AMT, Als3, and Sap5 should present more negative values than -6.0 kcal/mol, [50] which was observed since the AMT compound showed high affinity for the Als3 and Sap5 receptors, -10.5 kcal/mol and -11.3 kcal/mol respectively, evidencing more favorable affinity energy than the AmB (Table 3). AmB is an antifungal from the polyene class that selectively binds to ergosterol, which is a cholesterol analog present in mammalian cell membranes, directly interrupting the integrity of the fungal membrane. Thus, its use has been limited in many patients because it almost always results in some degree of renal failure, which varies in severity depending on the total dose [40-41]. The formed complexes presented RMSD in the order of 1.026 Å to 1.707 Å, affinity energy in the order of -11.3 to -7.6 kcal/mol (Table 3).). Previous *In silico* studies revealed that the AMT compound isolated from *O. fieldingiana* coupled to the CYP450 protein of *C. albicans* due to low energy stabilization (-9.39 kcal/mol), indicating a possible mechanism of action by inhibition of ergosterol biosynthesis of *Candida* fungi [38].

Table 2. MIC in the presence and absence of the amentoflavone compound

Strains	Amenthoflavone (mg/mL)		Amphotericin B (µg/mL)		
	MIC isolated	MICcombined	MIC isolated	MIC combined	FICI
C. albicans ATCC 90028	0.15	1.25	1.0	0.5	8.8
C. albicans LABMIC 0101	0.62	1.25	1.0	0.5	2.5
C. albicans LABMIC 0102	1.25	1.25	1.0	0.5	1.5
C. albicans LABMIC 0104	1.25	1.25	1.0	0.5	1.5
C. albicans LABMIC 0105	1.25	1.25	1.0	0.5	1.5

**FICI: Fractional Inhibitory Concentration Index; *LABIMIC: Microbiology Laboratory;*
** MIC Minimum Inhibitory Concentration*

Table 3. Affinity energy, RMSD values, and interaction types of the amentoflavone complex e amphotericin B (control) formed after docking simulations against Als3 and Sap5 *C. albicans*

Als3/Ligand	Affinity energy (kcal/mol)	RMSD (Å)	Residue	Interaction	Distance (Å)
Amentoflavone	-10.5	1.707	Tyr 21A	Hydrophobic	3.19
			Tyr 21A	Hydrophobic	3.60
			Tyr 23A	Hydrophobic	3.67
			Val 161A	Hydrophobic	3.55
			Val 161A	Hydrophobic	3.81
			Arg 171A	Hydrophobic	3.29
			Lys 59A	Hydrogen Bond	2.39
			Val 172A	Hydrogen Bond	2.66
			Asn 225A	Hydrogen Bond	2.10
			Trp 295A	π-Stacking	4.45
Amphotericin B	-7.6	1.429	Phe 87A	Hydrophobic	3.83
			Ser 125A	Hydrogen Bond	3.22
			Ser 211A	Hydrogen Bond	1.85
			Asn 212A	Hydrogen Bond	2.10
			Val 215A	Hydrogen Bond	2.41
			Glu 231A	Hydrogen Bond	3.18
Sap5/Ligand	**Affinity energy (kcal/mol)**	**RMSD (Å)**	**Residue**	**Interaction**	**Distance (Å)**
Amentoflavone	-11.3	1.026	Ala 162A	Hydrophobic	3.61
			Lys 257B	Hydrophobic	3.61
			Lys 257B	Hydrophobic	3.93
			Pro 290B	Hydrophobic	3.35
			Glu 278A	Hydrogen Bond	3.06
			Gln 282A	Hydrogen Bond	2.00
			Tyr 284A	Hydrogen Bond	3.26
			Tyr 284A	Hydrogen Bond	3.57
			Arg 312A	Hydrogen Bond	2.59
Amphotericin B	-8.4	1.172	Glu 10B	Hydrophobic	3.94
			Asn 160B	Hydrogen Bond	2.76
			Ser 161B	Hydrogen Bond	3.30
			Glu 163B	Hydrogen Bond	2.26
			Glu 163B	Hydrogen Bond	2.98
			Ser 165B	Hydrogen Bond	2.55
			Ser 165B	Hydrogen Bond	3.08
			Thr 166B	Hydrogen Bond	2.06
			Thr 166B	Hydrogen Bond	2.22
			Lys 293A	Hydrogen Bond	2.43

Analyzing the interaction patterns of the AMT compound and AmB against *C. albicans,* it was possible to identify interactions hydrophobic, π-Stacking, and hydrogen bonds with distances between 1.85 and 4.45 Å (Table 3).

The ALS family constitutes a large group of proteins that includes eight genes that encode large cell surface glycoproteins, of these eight proteins, Als3 is especially important for adhesion and is associated with the pathogenesis of *C. albicans* [51]. Thus, Als3 adhesin is considered a promising target involved in the design of antifungals through molecular docking and can be used to prospect molecules capable of inhibiting the function of this protein and the adhesion capacity of *C. albicans*, as well as its pathogenicity, thus providing new anti-*Candida* drugs [52].

“Among the most studied isoenzymes related to *Candida* virulence, they are Secreted aspartic proteinases (Saps) which are a family of 10 related proteases, among them SAP5. These

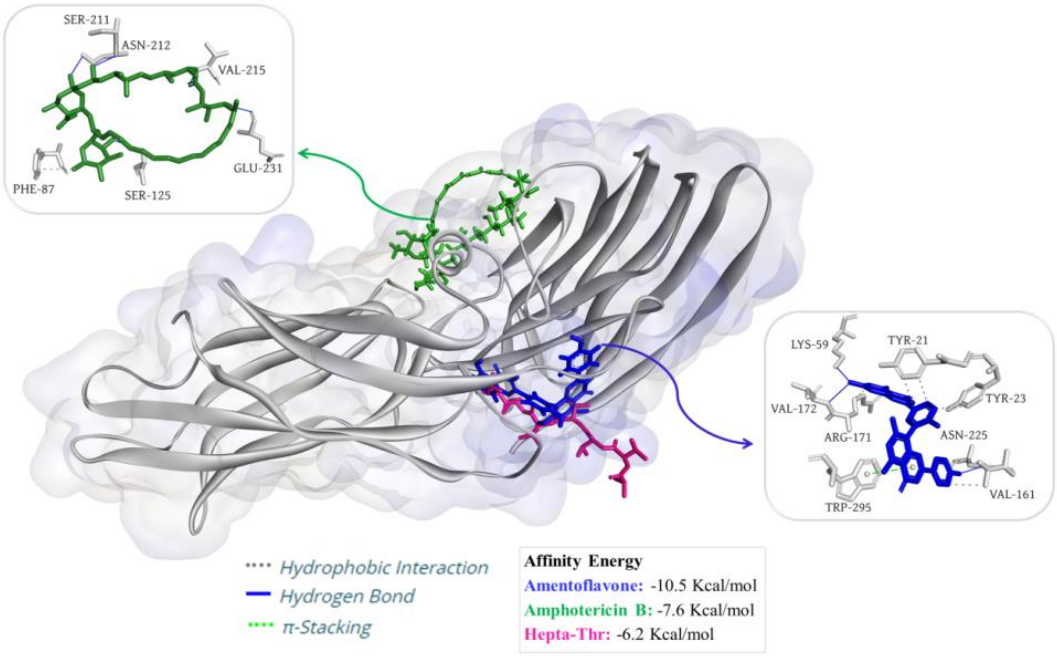


Fig. 2. Interaction complex of amentoflavone, antifungal Amphotericin B, and Hepta-Thr (co-crystallized inhibitor) with Als3 receptor

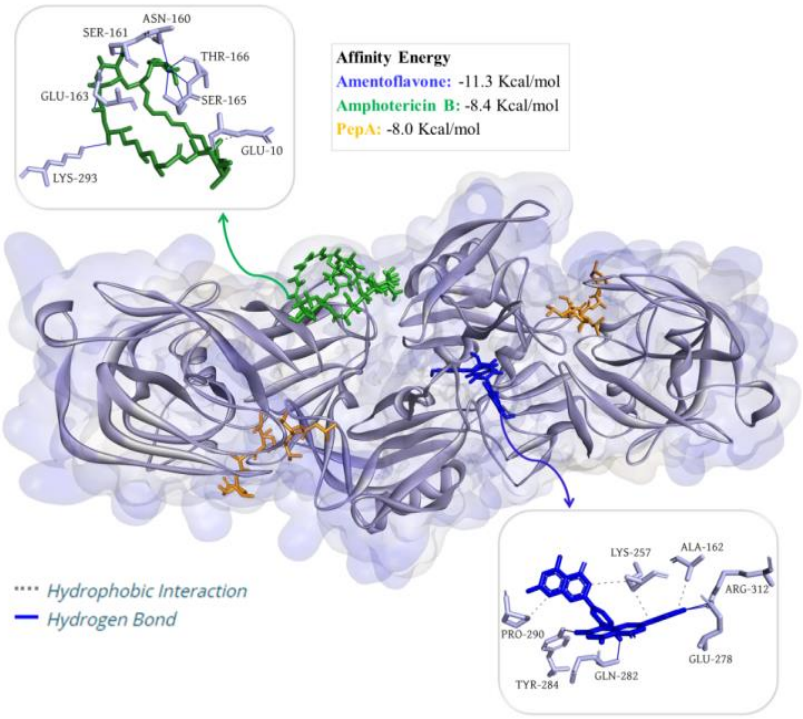


Fig. 3. Interaction complex of the amentoflavone, antifungal Amphotericin B, and PepA (co-crystallized inhibitor) with Sap5 receptor

proteases are virulence factors due to their proteolytic activity, as well as their roles in adherence and colonization of host tissues that participate in the infection process by, degrading several host cell proteins, such immunoglobulins, proteins of the complement system and

extracellular matrix, contributing to tissue damage and the resulting invasion by the microorganism" [53-54].

The redocking of the co-crystallized hepta-Thr inhibitor against Als3 showed an RMSD value of 1.707 Å and an affinity energy of -10.5 kcal/mol. The sNT-Als3 (shorter version of NT-Als3 that excludes the C-terminal AFR) and hepta-Thr binding site is formed by residues from the A1-B1 loop (Ala 19, Asn 22, Gly 27 and Thr 28), E1 β-strand (Ala 116), A2 β-strand (Tyr 166, Thr 168, Ser170 and Val 172), G2 β-strand (Arg 294, Trp 295, Thr 296 and Tyr 298) e β-strand C1* (Thr 61 and invariant Lys 59). Thus, we observed that AMT binds in the same region as the binding site of the co-crystallized inhibitor, having common interactions with residues Lys 59, Val 172, and Trp 295, indicating that has a similar action to hepta-Thr. The results of *in vitro* and *silico* studies coincide with the assumption that the AMT can act as an effective antifungal compound, including inhibition of the Als3 enzyme responsible for virulence and adhesion in *C. albicans.* Comparing the AmB (control), we observed that AMT does not compete for the control binding site, thus, confirming the results of *in vitro* assays.

The redocking of the inhibitor Pepstatin A (PepA) co-crystallized in the A and B chains of Sap5 showed an RMSD value of 1.026 Å and an affinity energy of -11,3 kcal/mol. The PepA binding site is formed by residues Ile 12, Asp 32, Gly 34, Ser 35, Lys 83, Tyr 84, Gly 85, Asp 86, Ile 123, Gly 220, Thr 221, Thr 222, Ile 223 and Ile 305 [46]. The analysis of interactions showed that AMT does not compete for the PepA and AmB (control) binding site, thus, confirming the results of in vitro assays.

4. CONCLUSION

Given the current limitations in antifungal therapy, natural products are attractive prototypes for this purpose, due to their abundance in nature, low cost, and broad spectrum of biological activity. AMT showed antifungal activity against all strains of *C. albicans*. The experimental data were corroborated by the molecular docking study, where AMT showed better affinity energy than AmB against the als3 and sap5 enzymes of *C. albicans*. Modulatory activity assays with the combination of AMT and AmB against *C. albicans* showed that they have indifferent and antagonistic actions. However, future research will be needed to elucidate the *in vivo* antifungal mechanisms, as well as the bioavailability and metabolic pathways involved.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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