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Original Article

Antifungal effect of (*R*) and (*S*)-citronellal enantiomers and their predictive mechanism of action on *Candida albicans* from voriconazole-resistant onychomycoses

Efeito antifúngico dos enantiômeros (*R*) e (*S*)-citronelal e seu mecanismo de ação preditivo sobre *Candida albicans* de onicomicoses resistentes ao voriconazol

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Abstract

Onychomycosis is the most common disease affecting the nail unit and accounts for at least 50% of all nail diseases. In addition, *Candida albicans* is responsible for approximately 70% of onychomycoses caused by yeasts. This study investigated the antifungal effect of (*R*) and (*S*)-citronellal enantiomers, as well as its predictive mechanism of action on *C. albicans* from voriconazole-resistant onychomycoses. For this purpose, *in vitro* broth microdilution and molecular docking techniques were applied in a predictive and complementary manner to the mechanisms of action. The main results of this study indicate that *C. albicans* was resistant to voriconazole and sensitive to the enantiomers (*R*) and (*S*)-citronellal at a dose of 256 and 32 µg/mL respectively. In addition, there was an increase in the minimum inhibitory concentration (MIC) of the enantiomers in the presence of sorbitol and ergosterol, indicating that these molecules possibly affect the integrity of the cell wall and cell membrane of *C. albicans*. Molecular docking with key biosynthesis proteins and maintenance of the fungal cell wall and plasma membrane demonstrated the possibility of (*R*) and (*S*)-citronellal interacting with two important enzymes: 1,3-β-glucan synthase and lanosterol 14α-demethylase. Therefore, the findings of this study indicate that the (*R*) and (*S*)-citronellal enantiomers are fungicidal on *C. albicans* from onychomycoses and probably these substances cause damage to the cell wall and cell membrane of these micro-organisms possibly by interacting with enzymes in the biosynthesis of these fungal structures.

Keywords: (*R*) and (*S*)-citronellal, voriconazole, onychomycosis, mechanism of action.

Resumo

A onicomicose é a doença mais comum que afeta a unidade ungueal e representa pelo menos 50% de todas as doenças ungueais. Além disso, a *Candida albicans* é responsável por aproximadamente 70% das onicomicoses causadas por leveduras. Nesse estudo, foi investigado o efeito antifúngico dos enantiômeros (*R*) e (*S*)-citronelal, bem como seu mecanismo de ação preditivo sobre *C. albicans* de onicomicoses resistentes ao voriconazol. Para este propósito, foram aplicadas técnicas *in vitro* de microdiluição em caldo e docking molecular de forma preditiva e complementar para os mecanismos de ação. Os principais resultados deste estudo indicam que *C. albicans* foi resistente ao voriconazol e sensível aos enantiômeros (*R*) e (*S*)-citronelal na dose de 256 e 32 µg/mL respectivamente. Além disso, houve aumento da concentração inibitória mínima (CIM) dos enantiômeros na presença do sorbitol e do ergosterol, indicando que estas moléculas possivelmente afetem a integridade da parede e da membrana celular de *C. albicans*. O docking molecular com proteínas chave da biossíntese e manutenção da parede celular e da membrana plasmática fúngica, demonstraram a possibilidade do (*R*) e (*S*)-citronelal interagir com duas importantes enzimas: 1,3-β-glucan sintase e lanosterol 14α-demetilase. Portanto, os achados desse estudo indicam que os enantiômeros (*R*) e (*S*)-citronelal são fungicidas sobre *C. albicans* de onicomicoses e provavelmente essas substâncias causem danos a parede e a membrana celular desses microrganismos possivelmente por interagir com as enzimas da biossíntese destas estruturas fúngicas.

Palavras-chave: (*R*) e (*S*)-citronelal, voriconazol, onicomicose, mecanismo de ação.

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1. Introduction

Onychomycosis is an infection of the nail unit caused by fungi (dermatophytes, non-dermatophyte moulds and yeasts) characterized by discolouration of the nail, onycholysis and thickening of the nail plate (Köhler et al., 2017; Almeida et al., 2019). Onychomycosis is the most common disorder affecting the nail unit and accounts for at least 50% of all nail diseases (Piraccini and Alessandrini, 2015; Almeida et al., 2019; Paškevičius et al., 2020). In addition, *C. albicans* is responsible for approximately 70% of onychomycoses caused by yeasts (Köhler et al., 2017). The worldwide prevalence of onychomycosis in the general population is approximately 5.5%, based on recently published epidemiological studies (Piraccini and Alessandrini, 2015; Fernandes et al., 2017; Lipner and Scher, 2019).

This clinical condition can bring several discomforts to its bearers, such as: difficulty in wearing shoes, aesthetic embarrassment, low self-esteem, clinical conditions of chronic pain and functional limitations. Occasionally, these infections can also disseminate and cause systemic infections, especially in diabetic and immunosuppressed individuals. These situations result from the morphological changes that set in during active infection and permanent nail dystrophy may persist after the cure as sequelae (Christenson et al., 2018).

The treatments for Onychomycosis have low cure rates, related to the difficulty of drug penetration in the keratinised structure that makes up the nail plate, with constant need for mechanical removal of the affected portion (Perusinha et al., 2019). Additionally, the long duration of the treatment favours its discontinuation, which corroborates with the establishment of resistant strains, which limits its effectiveness even further (Lipner and Scher, 2019; Vieira et al., 2019; Aggarwal et al., 2020). Thus, it is necessary to expand the possibilities of current treatments, in the search for molecules with antifungal potential that may play a viable alternative in the therapy of these infections.

Natural products, such as essential oils, are an important source of drugs, which present therapeutically important bioactive compounds, such as monoterpenes (Dehsheikh et al., 2020; Belhadj-Salah et al., 2022). Among these compounds, citronellal presents a range of biological activities described in the literature, such as antimicrobial, antioxidant, antinociceptive and anti-inflammatory (Santos et al., 2019). The enantiomers of citronellal: (*R*)-(+)-citronellal and (*S*)-(-)-citronellal [(*R*)-CIT and (*S*)-CIT], already have antifungal activity described in academic literature (Fernandes et al., 2017; Medeiros et al., 2017), however, their antifungal activity against *C. albicans* strains from nail infections, as well as the elucidation of their mechanism of action has not yet been described.

Therefore, this study aimed to investigate the antifungal activity of the monoterpenes (*R*)-CIT and (*S*)-CIT against *C. albicans* strains isolated from nail infections, as well as the predictive elucidation of their possible mechanisms of action.

2. Material and Methods

2.1. Substances

The substances used in the trials: (*R*)-(+)-citronellal [(*R*)-CIT] and (*S*)-(-)-citronellal [(*S*)-CIT], fluconazole (FLU), voriconazole (VOR) and amphotericin B (AMB), were purchased from Sigma-Aldrich® (São Paulo-SP). To carry out the pharmacological tests, the substances were solubilised in DMSO and diluted in sterile distilled water.

2.2. Strains

The *C. albicans* strains used in this study, belong to the fungal library of the Research Laboratory of Antibacterial and Antifungal Activity of Natural and/or Synthetic Bioactive Products of the Federal University of Paraiba (UFPB) and include: LM 441, LM 123, LM 37, LM 157, LM 600, LM 615, LM 74, LM 95, LM 35, LM 20, LM 72, LM 73, LM 65, LM 522 and LM 122. The American Type Culture Collection strain (*C. albicans* ATCC 76645) was used as a control. For the in vitro assays, the fungal suspensions were prepared in 0.85% saline solution of fresh cultures and turbidity was equivalent to 0.5 on McFarland's standard scale, which corresponds to an inoculum of approximately 1-5 × 10^6 colony forming units per millilitre (CFU/mL) (Hadacek and Greger, 2000; CLSI, 2017).

2.3. Minimum Inhibitory Concentration (MIC)

The MIC values of the substances were determined against *C. albicans* strains by broth microdilution technique. Initially, 100 µL of RPMI-1640 broth (Sigma-Aldrich®) were dispensed in the orifices of the plates. Then, 100 µL of the test substance emulsions were deposited in the first horizontal row of the plate and serial dilutions by a factor of two were performed, where a 100 µL aliquot was taken from the most concentrated well to the next well, resulting in concentrations of 1,024-0.5 µg/mL. Finally, 10 µL of the fungal inoculum suspensions were added to each well of the plate, where each column represented a fungal strain. Sterility controls were also performed with Amphotericin B, cell viability assay and interference of solubilising agents (5% DMSO and 2% Tween 80) (Diniz-Neto et al., 2022). The plates were then sealed and incubated at 35 ± 2 °C for 48 hours. After the incubation time, the presence (or absence) of microbial growth was observed visually (Hadacek and Greger, 2000; Ncube et al., 2008; CLSI, 2017). The MIC was defined as the lowest concentration at which the substance produced visible inhibition of yeast growth. The antifungal activity of the substances was interpreted as active or non-active according to the criteria proposed by Morales et al. (2008): strong/good activity (MIC <100 µg/mL); moderate activity (MIC >100 to 500 µg/mL); weak activity (MIC >500 to 1,000 µg/mL).

2.4. Minimum Fungicide Concentration (MFC)

The MFC was determined after reading the MIC, in which 10 µL aliquots of the supernatant from the wells where complete inhibition of fungal growth was observed (MIC, MIC × 2 and MIC × 4) in the microdilution plates, were transferred to the wells of a new microplate containing

100 µL of RPMI-1640 broth, which were incubated for 24 h at 35 ± 2 °C.

All controls were then performed after 24-48 h of incubation, and a reading was carried out to assess MFC based on the controls. The MFC is defined as the lowest concentration capable of causing complete inhibition of fungal growth after 24-48 h (Ncube et al., 2008; Siddiqui et al., 2013). The MFC/MIC ratio was performed to specify the nature of the antimicrobial effect. The product will be considered as fungicide when the MFC/MIC ratio is between 1:1 to 2:1, on the other hand, if the ratio is greater than 2:1, the mode of action is more likely to be fungistatic (Ncube et al., 2008).

2.5. Effect on fungal cell wall (assay with sorbitol)

Based on the results of the MIC and MFC, the clinical strain of *C. albicans* LM 600 and the standard strain of *C. albicans* ATCC 76645 were considered representative for the next essays. Therefore, the determination of the MIC of the substances in the presence of sorbitol (Sigma-Aldrich®) (osmotic protector of fungal protoplasts) was performed by microdilution in 96-well plates, where the microorganisms were exposed to different concentrations of the test substances in a medium containing sorbitol (0.8M). All controls were then performed as already described in the previous sections.

2.6. Action on the cell membrane (ergosterol assay)

The determination of the MIC of (*R*)-CIT and (*S*)-CIT against *C. albicans* strains (LM 600 and ATCC 76645) in the presence of exogenous ergosterol was performed by microdilution in 96-well plates. If the antifungal activity of the substance tested is caused by its binding to ergosterol, the exogenous ergosterol will prevent the compound from binding to ergosterol in the fungal cell membrane. Therefore, RPMI-1640 broth was used with the addition of 400 µg/mL of ergosterol (Sigma-Aldrich®). The same procedure was carried out with AMB, whose mechanism of action is known and involves interaction with ergosterol from the fungal cell membrane to serve as a positive control of the results. Growth control of the microorganisms was performed with 100 µL of culture medium and ergosterol at equal concentrations and 10 µL of each standard fungal inoculum. The plates were then sealed and incubated at 35 ± 2 °C for 24-48 h for further reading (Escalante et al., 2008).

2.7. Molecular docking

Hard molecular docking simulations were performed with the respective proteins: 1,3-β-glucan synthase (1,3-β-GS) (PBD ID: 1EQC) 1.85 Å, and the lanosterol 14α-demethylase (CYP51) (PBD ID: 5TZ1) 2.00 Å of *C. albicans*, whose structures were taken from the Protein Data Bank (PDB) and loaded into PyMol 2.5.3, for the removal of water molecules and the co-crystallised ligands (castanospermine and oteseconazole respectively). The structures of the (*R*)-CIT and (*S*)-CIT ligands were obtained from *PubChem*, and using the software Avogadro 1.2.0 at pH 7.4 and Mopac2012 at the PM6 level, energy minimisation and molecular optimisation were performed with the MMFF94 force field (Halgren, 2002; Hanwell et al., 2012).

Subsequently, the proteins were loaded in the AutoDock Tools software (Morris et al., 2009), for the addition of hydrogens and Kollman charges, in addition to the mixing of non-polar hydrogens. Next, docking simulations were performed with the identification of the active sites of the targets with the grid centres: 1,3-β-GS (34.282; 35.261; 56.222 Å), dimensions (40 x 40 x 40 Å) and spacing of 0.375 Å; CYP51 (71.381; 65.319; 4.808 Å), dimensions (40 x 40 x 40 Å) and spacing of 0.375 Å.

After locating the active sites, the docking was developed in AutoDock 4.2, with 100 Lamarckian genetic algorithm operations and the standard parameters of the AutoDock Tools. With this, values of Free Energy of Binding (ΔG) and Inhibitory Constant (Ki) were generated, and the conformations with the lowest ΔG value were selected. Finally, with the aid of PyMol 2.5.3 and Discovery Studio 2021 software, the results were analysed, determining the binding regions of the target with the ligand molecule, the types of interactions and the amino acids in the active site that participate in the bonds. Validation of the methodology was performed by molecular redocking, which consists of reflecting the position and orientation of the ligand found in the crystallographic structure and selecting the conformation of the ligand with the lowest mean square deviation (RMSD) the distances between the atoms should be ≤ 2,0 Å (Bell and Zhang, 2019).

3. Results

3.1. Fungicidal effect of (R) and (S)-citronellal against C. albicans strains

(*R*)-CIT showed a MIC of 256 µg/mL for 12 (75%) strains, while (*S*)-CIT showed a MIC of 32 µg/mL for 11 (≈ 69%) of the strains. Fluconazole and voriconazole, used as the positive control, showed MIC values between 1-64 µg/mL. All *C. albicans* strains were found to be resistant to voriconazole, while 4 strains (25%) were resistant and 12 (75%) were dose-dependent sensitive (S-DD) to fluconazole. The data presented indicated that the fungal strains analysed are sensitive to (*R*)-CIT and (*S*)-CIT enantiomers and that these presented MFC/MIC fungicidal activity ≤ 4 (Table 1).

3.2. Effect of (R) and (S)-citronellal on the cell wall of C. albicans

The effect of (*R*)-CIT and (*S*)-CIT on the fungal cell wall was investigated based on the results of the previous experiments. *C. albicans* strains LM 600 and *C. albicans* ATCC 76645 were chosen as representative in the analysis of the subsequent results. The fungal growth was evaluated in the presence and the absence of 0.8 M sorbitol (osmotic protoplast protector of fungal protoplasts). The MIC of the monoterpenes for both strains increased in the presence of sorbitol indicating that these enantiomers interfere with the viability of fungal strains through molecular mechanisms that probably involve cell wall disruption (Table 2).

Table 1. MIC values and MFC (µg/mL) of (*R*) and (*S*)-citronellal, fluconazole and voriconazole against *C. albicans* strains by broth microdilution.

Strains	[1](*R*)-CIT				[1](*S*)-CIT				[2]FLU		3VOR		GC
	MIC	MFC	MFC/MIC	Effect	MIC	MFC	MFC/MIC	Effect	MIC	MFC	MIC	MFC	
LM 20	512	1,024	2	Fungicidal	256	128	2	Fungicidal	4	2	1	2	+
LM 35	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+
LM 37	256	1,024	4	Fungicidal	128	128	1	Fungicidal	4	2	1	2	+
LM 65	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+
LM 72	512	1,024	2	Fungicidal	256	64	0.250	Fungicidal	4	4	1	2	+
LM 73	256	512	2	Fungicidal	128	128	1	Fungicidal	4	2	1	2	+
LM 74	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+
LM 95	256	512	2	Fungicidal	32	64	2	Fungicidal	8	4	1	2	+
LM 122	256	512	2	Fungicidal	32	64	2	Fungicidal	4	4	1	2	+
LM 123	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+
LM 157	256	512	2	Fungicidal	32	128	4	Fungicidal	8	2	1	2	+
LM 441	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+
LM 522	512	512	1	Fungicidal	32	64	2	Fungicidal	4	4	1	2	+
LM 600	256	512	2	Fungicidal	32	64	2	Fungicidal	64	4	1	2	+
LM 615	512	1,024	2	Fungicidal	128	128	1	Fungicidal	8	2	1	2	+
ATCC 76645	256	512	2	Fungicidal	32	64	2	Fungicidal	4	2	1	2	+

GC: growth control of the microorganism in RPMI-1640, DMSO (10%), and Tween 80 (2%), without monoterpenes or antifungals. S: susceptible; S-DD: susceptible dose-dependent; R: resistant. [1]Cutoff points: fungistatic (MFC/MIC > 4) and fungicidal (MFC/MIC ≤ 4) (Siddiqui et al., 2013). [2]Cutoff points: MIC of fluconazole ≤ 2 µg/mL (S); 4 µg/mL (S-DD); ≥ 8 µg/mL (R), document M60 (CLSI, 2017). [3]Cutoff points: MIC of voriconazole ≤ 0.12 µg/mL (S); ≥ 1 µg/mL (R) (CLSI, 2017).

Table 2. Effect of (*R*) and (*S*)-citronellal against *C. albicans* LM 600 and *C. albicans* ATCC 76645 in the absence and presence of 0.8M sorbitol.

Drug	MIC (µg/mL)			
	C. albicans LM 600		*C. albicans* ATCC 76645	
	Absence of sorbitol	Presence of sorbitol	Absence of sorbitol	Presence of sorbitol
(*R*)-CIT	256	>1,024	256	>1,024
(*S*)-CIT	32	>1,024	32	>1,024

3.3. Effect of (R) and (S)-citronellal on C. albicans cell membrane

As a possible mechanism of action, the interference of (R)-CIT and (S)-CIT enantiomers with fungal cell membrane ergosterol was investigated. The enantiomers in the presence of exogenous ergosterol showed an increased MIC, indicating that the compounds may interfere with fungal cell viability through mechanisms involving the fungal cell membrane (Table 3).

3.4. Iterations of (R) and (S)-citronellal with 1,3-β-glucan synthase and lanosterol 14α-demethylase in silico

In this study, when redocking was performed, which consists in removing the co-crystallized ligands and performing docking simulations, it was possible to obtain RMSD values lower than 2.0 Å (Table 4), allowing the validation of the applied methodology. Moreover, with redocking, structures with spatial arrangements (3D) were obtained similar to the crystallographic structures and the interactions of cocrystals with the active sites of enzymes (2D) (Figures 1 and 2).

Following the validation of the methodology, docking was performed with the enantiomers (R)-CIT and (S)-CIT and the 1,3-β-GS and CYP51 enzymes, in which they showed moderate affinities in relation to the co-crystallized ligands when comparing the binding energies (ΔG) of the molecules. Although cocrystals showed lower ΔG values and consequently, higher affinity compared to monoterpenes, the latter are also capable of binding to the enzymes studied (Table 4).

The ΔG between the (*R*)-CIT and (*S*)-CIT enantiomers for the enzymes under study, as well as the inhibitory constants (Ki) also showed discrete differences, and probably the ΔG of (*R*)-CIT is slightly lower compared to (*S*)-CIT because of the total amount of bonds formed, 14 and 12 respectively with the 1,3-β-GS enzyme. However, for CYP51 the ΔG and Ki of (*R*)-CIT were slightly lower than that of (*S*)-CIT probably because the former binds to an extra amino acid

Table 3 . Effect of (*R*), (*S*)-citronellal and amphotericin B against *C. albicans* LM 600 and *C. albicans* ATCC 76645 in the absence and presence of ergosterol at 400 µg/mL.

Drug	MIC (µg/mL)			
	***C. albicans* LM 600**		***C. albicans* ATCC 76645**	
	Absence of ergosterol	**Presence of ergosterol**	**Absence of ergosterol**	**Presence of ergosterol**
(*R*)-CIT	256	>1,024	256	>1,024
(*S*)-CIT	32	>1,024	32	>1,024
AMB	32	>256	8	>256

Table 4. Binding energies (Δ*G*) and inhibition constants (Ki) of the cocrystal ligands (castanospermine and oteseconazole) and the experimental enantiomeric ligands (*R*) and (*S*)-citronellal against the enzymes 1,3-β-GS (PBD ID: 1EQC) and CYP51 (PBD ID: 5TZ1).

Enzymes	Classification	Δ*G* (kcal/mol)	RMSD (Å)	K_i	Δ*G* (kcal/mol)	K_i	Δ*G* (kcal/mol)	K_i
					(*R*)-CIT		(*S*)-CIT	
1,3-β-GS	Hydrolase	- 5.05	0.273	197.15µM	- 4.45	548.91µM	- 4.41	580.98µM
CYP51	Oxidoreductase	- 8.47	0.632	613.44nM	- 5.23	147.70µM	- 5.13	173.30µM

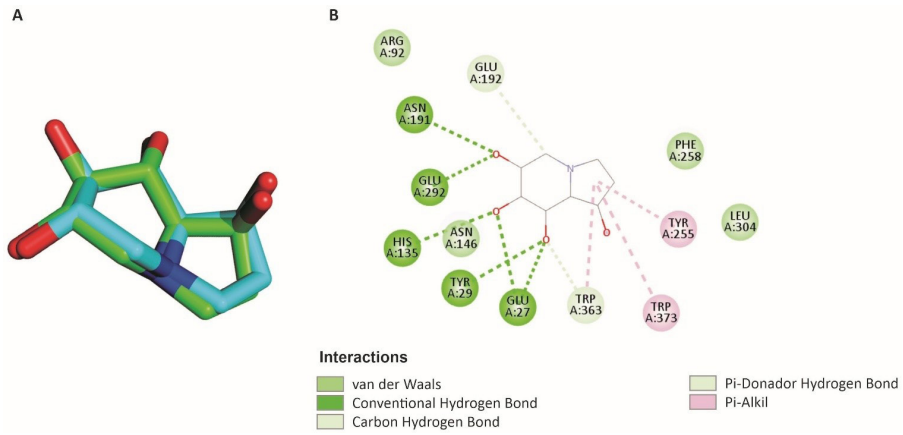

Figure 1. Redocking of crystallised castanospermine with 1,3-β-GS (PBD ID: 1EQC). **A**. The 3D diagram of the cocrystal ligand in green and the redocking ligand in cyan. **B**. The 2D diagram of the cocrystal showing the types of interactions with the active site of the enzyme.

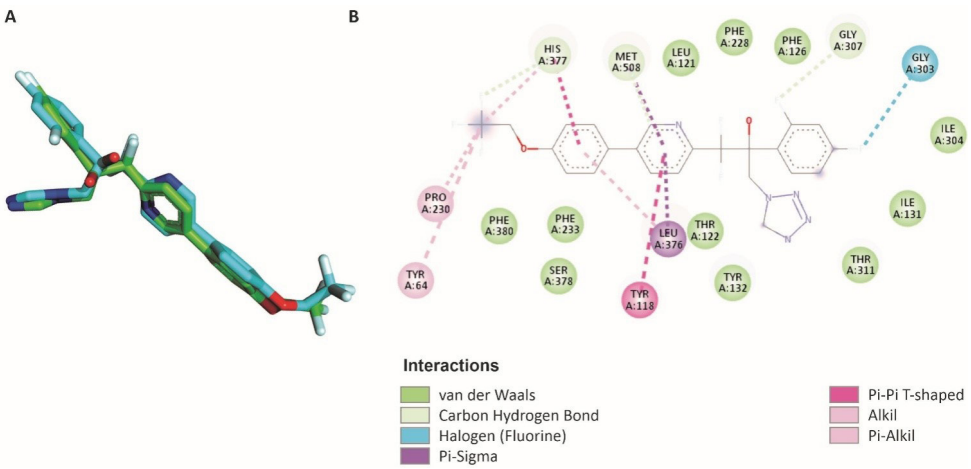

Figure 2. Redocking of crystallised oteseconazole with CYP51 (PBD ID: 5TZ1). **A**. The 3D diagram of the cocrystal ligand in green and the redocking ligand in cyan. **B**. The 2D diagram of the cocrystal showing the types of interactions with the active site of the enzyme.

(Phe126) and the respective enantiomer is in a more open conformation. In addition, the monoterpene (*R*)-CIT was able to interact with the active site of the enzyme 1,3-β-GS forming various types of bonds. Among them, van der Waals, Pi-sigma, alkyl and Pi-alkyl bonds stand out, in addition to the hydrogen bond between the oxygen of the enantiomer and the amino acid Asp145 with a binding distance of 2.05 Å (Figure 3A, B).

It is still possible to observe the hydrophobicity surfaces and the hydrogen donor and acceptor regions of (*R*)-CIT within the active site of the enzyme, given the high liposolubility of the monoterpene (Figure 3C, D). Furthermore, (*S*)-CIT also showed the potential to bind to the active site of the enzyme 1,3-β-GS, with practically the same types of interactions (Figures 4A, B). However, it is worth noting the hydrogen bonds between the oxygen of the monoterpene (S)-CIT and the amino acid Asn191 (1.92 Å) and the carbon-hydrogen bond with the amino acid Glu292 (3.01 Å). For this enantiomer, also observed on hydrophobicity and hydrogen donor-acceptor surfaces the ratios of the chemical groups of the monoterpene and the relationships of the chemical groups of the monoterpene with the active site of the enzyme, highlighting the coherence of these interactions (Figures 4C, D).

The enantiomers (*R*)-CIT and (*S*)-CIT also interacted with the active site of the CYP51 enzyme through various types of bonds such as van der Waals, hydrogen bonding, Pi-sigma, alkyl and Pi-alkyl. It is important to highlight that in general the binding distances of (*S*)-CIT to the amino acids of the active site of the enzyme are shorter than for (*R*)-CIT. In prominence is the interaction of the oxygen of (R)-CIT to the amino acid Tyr132, which has a bond distance of 2.16 Å. In contrast to (*S*)-CIT, this same interaction takes place with a binding distance of 1.93 Å (Part A and B of Figures 5 and 6). Moreover, it is observed that the active site of the CYP51 enzyme is rather hydrophobic and contains a porphyrin ring with an iron atom in its centre, but due to the lipophilic character of monoterpenes, these do not perform more stable interactions with this structure (Part C and D of Figures 5 and 6).

4. Discussion

Onychomycosis is notoriously difficult to treat due to the deep-seated nature of the fungus in the nail plate, the prolonged treatment necessary for resolution, the low patient compliance and the frequent recurrences (Christenson et al., 2018). Thus, research into new therapeutic alternatives, as well as bioactive molecules against *C. albicans* needs to be studied, especially substances with characteristics that have the potential to penetrate the structures of the nail unit. In this sense, monoterpenes stand out as being molecules of low molecular weight and hydrophobic character. Thus, citronellal is a naturally occurring monoterpene with two main enantiomeric forms, (*R*)-CIT and (*S*)-CIT derived mainly from plants of

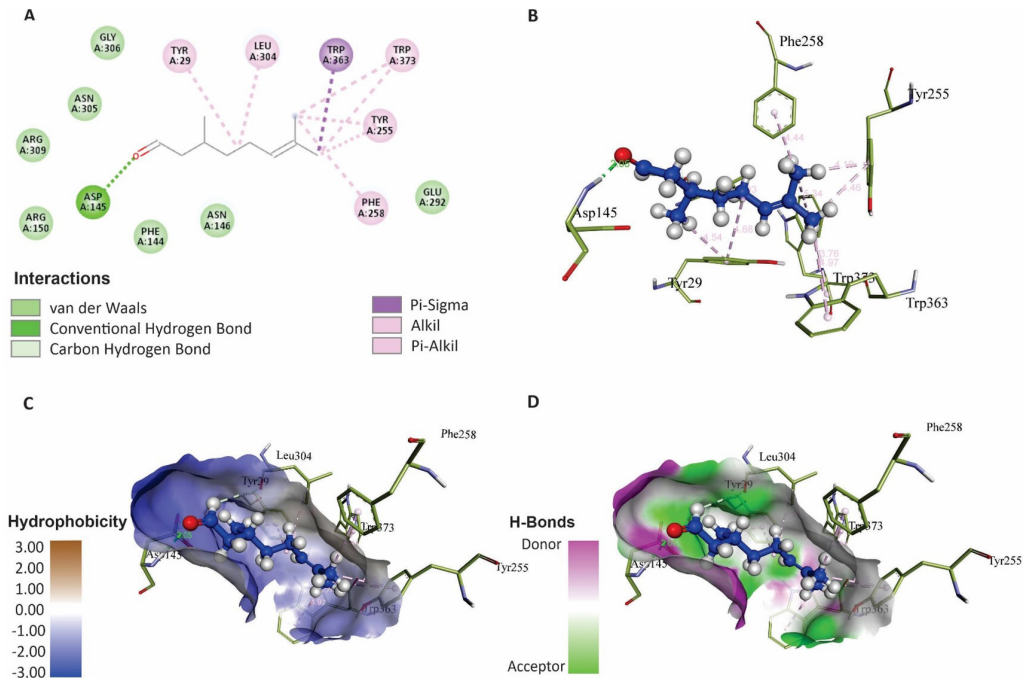

Figure 3. Molecular docking of (*R*)-citronellal with the active site of the enzyme 1,3-β-GS (PBD ID: 1EQC). **A**. The main types of ligand interactions with the active site of the enzyme in 2D. **B**. The 3D distribution and chemical bond distances of the monoterpene (blue) with the amino acids (green) of the enzyme's active site. **C**. The 3D surface model showing the regions of the ligand with a higher or lower degree and hydrophobicity. **D**. The 3D surface model of the region of the active site of the enzyme occupied with the ligand (blue) and the hydrogen donor and acceptor sites.

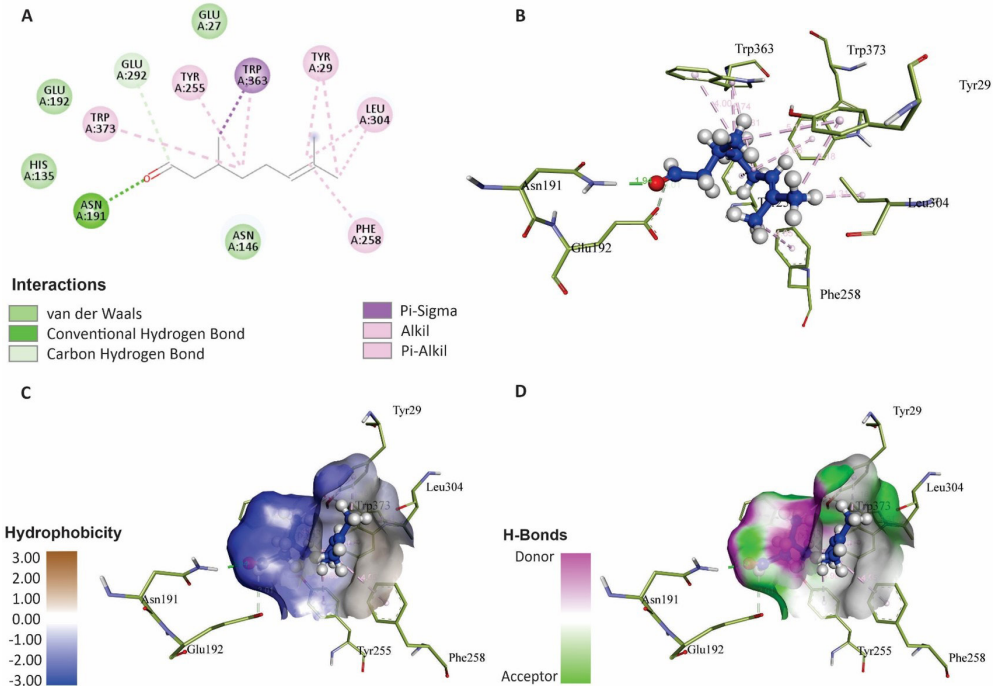

Figure 4. Molecular docking of (*S*)-citronellal with the active site of the enzyme 1,3-β-GS (PBD ID: 1EQC). **A**. The main types of ligand interactions with the active site of the enzyme in 2D. **B**. The 3D distribution and chemical bond distances of the monoterpene (blue) with the amino acids (green) of the enzyme's active site. **C**. The 3D surface model showing the regions of the ligand with a higher or lower degree and hydrophobicity. **D**. The 3D surface model of the region of the active site of the enzyme occupied with the ligand (blue) and the hydrogen donor and acceptor sites.

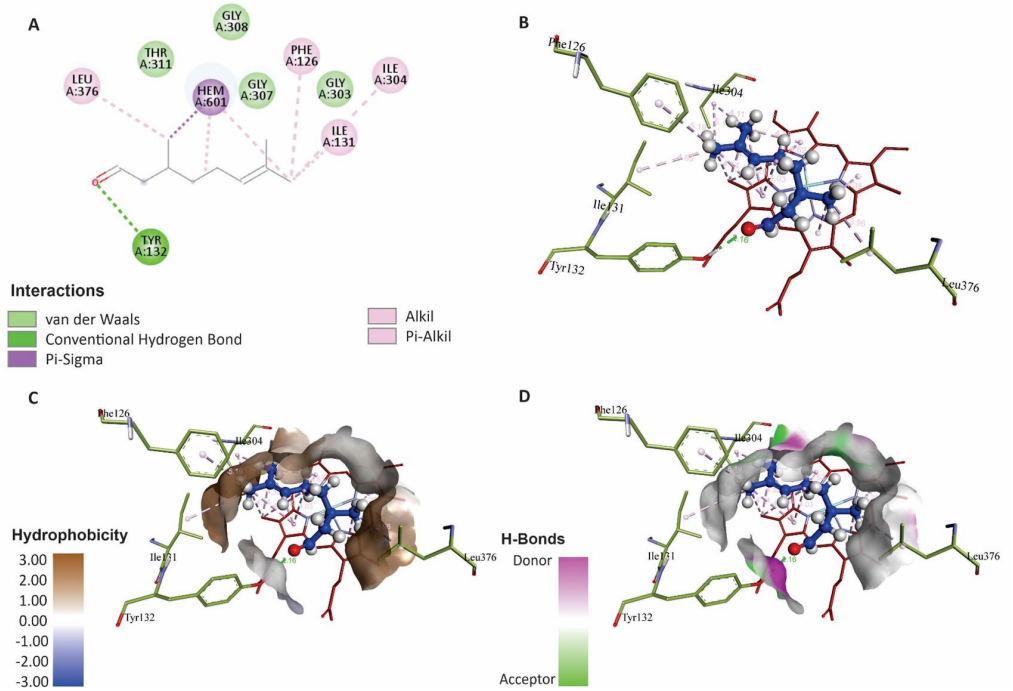

Figure 5. Molecular docking of (*R*)-citronellal with the active site of the CYP51 enzyme (PBD ID: 5TZ1). **A**. The main types of ligand interactions with the active site of the enzyme in 2D. **B.** The 3D distribution and chemical bond distances of the monoterpene (blue) with the amino acids (green) of the enzyme active site containing the porphyrin group and the iron atom in its centre. **C**. The 3D surface model of ligand hydrophobicity. **D**. The 3D surface model of the region of the active site of the enzyme occupied with the ligand (blue) and the hydrogen donor and acceptor sites.

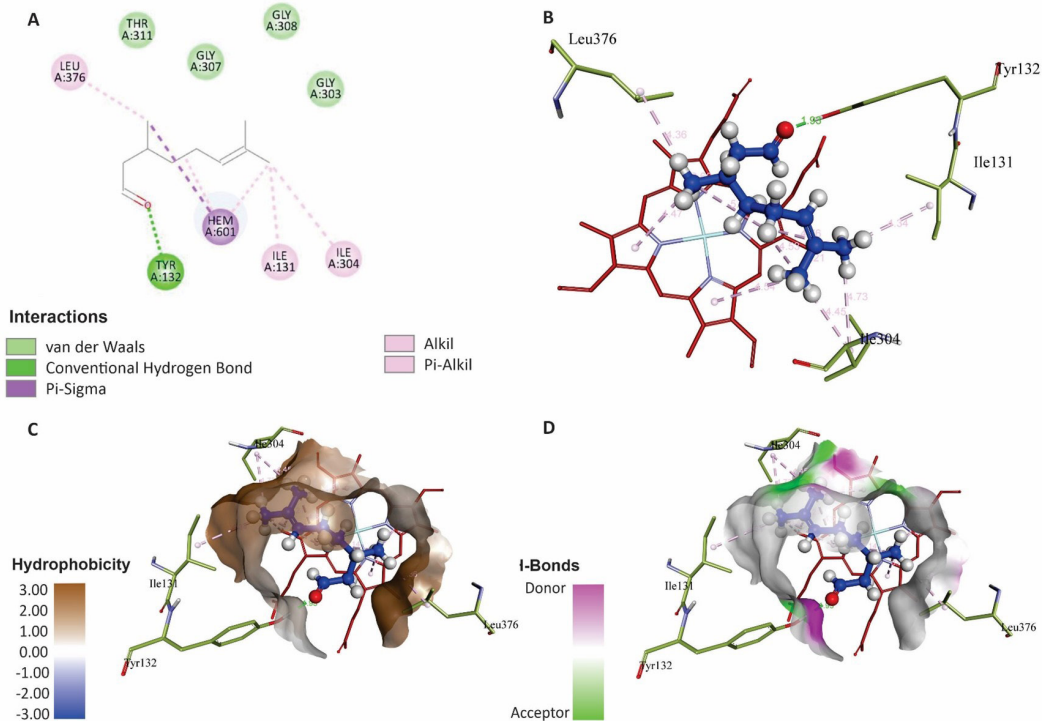

Figure 6. Molecular docking of (*S*)-citronellal with the active site of the CYP51 enzyme (PBD ID: 5TZ1). **A.** The main types of ligand interactions with the active site of the enzyme in 2D. **B**. The 3D distribution and chemical bond distances of the monoterpene (blue) with the amino acids (green) of the enzyme active site containing the porphyrin group and the iron atom in its centre. **C**, the 3D surface model of ligand hydrophobicity. **D.** The 3D surface model of the region of the active site of the enzyme occupied with the ligand (blue) and the hydrogen donor and acceptor sites.

the genus *Cymbopogon* which have various bioactivities such as anti-inflammatory, antifungal, antinociceptive, antioxidant, among others (Sharma et al., 2019).

Therefore, taking together the *in vitro* results of this study that point out the enantiomers (*R*)-CIT and (*S*)-CIT with fungicidal activity against *C. albicans* (CFM/CIM ≤ 4) (Table 1), as well as the interference of these monoterpenes with the fungal cell wall and plasma membrane; as sorbitol is an osmotic protector of the fungal protoplasts when added exogenously, the MICs of the enantiomers were higher than the MICs in the absence of sorbitol, indicating activity of (*R*)-CIT and (*S*)-CIT against this microbial structure (Table 2) and predictive molecular docking with the enzyme 1,3-β-GS directly involved in chitin and glucan synthesis, important components of the fungal cell wall, indicate that these molecules are active to interact with and possibly inhibit the synthesis of this microbial structure, and may eventually cause cell death (Zhou et al., 2022). It is important to note that the redocking ligand (castanospermine) binds to the active site of 1,3-β-GS with a lower Δ*G* than the enantiomers and with higher affinity, as well as a lower K_i, due to the greater number of bonds, mainly hydrogen (Figure 1). However, the (*R*)-CIT and (*S*)-CIT ligands, showed no significant difference in Δ*G*, also presenting practically the same affinity (Table 4).

Interestingly, (*R*)-CIT and (*S*)-CIT also appear to be avid against the plasma membrane of *C. albicans*, given that the MICs of the monoterpenes increased when exogenous ergosterol was added (main lipid of the fungal cell membrane) in relation to the MICs in the absence of this lipid, suggesting that possibly these citronellal enantiomers bind to ergosterol or interfere with the biosynthesis of this molecule (Table 3). Therefore, the CYP51 enzyme, oteseconazole (redocking ligand) establishes several interactions with the active site of the respective enzyme, generating a Δ*G* = -8.47 kcal/mol probably due to the size of the ligand, thus enabling better molecular complementarity (Figure 2).

In this study, (*R*)-CIT and (*S*)-CIT also showed no significant difference in ΔG and consequently, the affinities are quite close to each other (Table 4). The types of bonds of the enantiomers to the enzymes studied were also the same, however, the binding distances for (*S*)-CIT were shorter compared to (*R*)-CIT (Figures 3 and 4). Furthermore, the high hydrophobicity of the CYP51 active site may account for the lower Δ*G* when comparing the activities of the enantiomers of the two enzymes (Figures 5 and 6). Therefore, predictively, these differences may justify the better results of the *in vitro* tests of (*S*)-CIT against *C. albicans* strains.

Therefore, this monoterpene seems promising as an antifungal with possible action on the fungal cell wall, because this microbial structure has been widely exploited as a target for selective antifungal

therapy, in addition, there is a significant amount of evidence that several monoterpenes exert a fungicidal effect on *C. albicans*, interfering with the cell wall and plasma membrane of these cells (Zore et al., 2011; Pereira et al., 2018). Furthermore, the last class of drugs approved for clinical use that interfere with 1,3-β-GS were the echinocandins (caspofungin, anidulafungin and micafungin), which block glucan biosynthesis. However, these drugs can be costly and require patient hospitalisation due to their low bioavailability in oral administration (Chang et al., 2017).

Azole antifungals are the most commonly used drugs in the clinic for the treatment of mycoses, due to their broad-spectrum activity. Their mechanism of action is to inhibit the cytochrome P-450-dependent enzyme (CYP51) encoded by the *ERG11* gene which converts lanosterol to ergosterol in the cell membrane inhibiting fungal growth and replication (McManus and Shah, 2019). However, the use of these drugs can have disadvantages; such as the emergence of strains resistant to azoles due to frequent use, and interaction with the cytochrome P-450 isoenzymes in the mammalian liver produces an increase in the transaminases which are characteristic of this class of drugs. In addition, first-generation imidazoles and triazoles (Clotrimazole, Miconazole, Ketoconazole, Fluconazole and Itraconazole) are fungistatic and not fungicidal against *Candida* (Revie et al., 2018).

In turn, the enantiomers (*R*)-CIT and (*S*)-CIT were fungicidal against *C. albicans* and active against CYP51, which predictively can interfere with the ergosterol content of fungal cells or possibly in a similar way to polygenic antifungals such as amphotericin B, by incorporating into membrane lipids and promoting the formation of permeability pores, as well as cell membrane rupture, oxidative damage and fungal cell death (Pereira et al., 2018; McManus and Shah, 2019).

5. Conclusion

In summary, this research indicated that the (*R*)-CIT and (*S*)-CIT enantiomers are fungicidal against clinical strains of *C. albicans* from onychomycosis resistant to voriconazole. And based on the results of the sorbitol and ergosterol assays, these monoterpenes seem to affect the integrity of the cell wall and membrane of *C. albicans* and consequently its viability. Furthermore, molecular docking analyses predictively suggest the possibility of (*R*)-CIT and (*S*)-CIT interacting with key enzymes in the biosynthesis and maintenance of these microbial structures. However, further *in vitro* and *in vivo* investigations, as well as molecular dynamics analyses are required to better elucidate the mechanisms of action of these molecules.

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References

AGGARWAL, R., TARGHOTRA, M., KUMAR, B., SAHOO, P.K. and CHAUHAN, M.K., 2020. Treatment and management strategies of onychomycosis. *Journal de Mycologie Médicale*, vol. 30, no. 2, pp. 100949. http://dx.doi.org/10.1016/j.mycmed.2020.100949. PMid:32234349.

ALMEIDA, F., RODRIGUES, M.L. and COELHO, C., 2019. The still underestimated problem of fungal diseases worldwide. *Frontiers in Microbiology*, vol. 10, pp. 214. http://dx.doi.org/10.3389/fmicb.2019.00214. PMid:30809213.

BELHADJ-SALAH, K., SHEIKH, H.M., AL-HASAWI, Z.M., SELIM, E.M. and TOULIABAH, H.E., 2022. In vitro antifungal and antibacterial potentials of organic extracts of Avicennia marina collected from Rabigh Lagoon, Red Sea Coasts in Saudi Arabia. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 82, pp. e265038. http://dx.doi.org/10.1590/1519-6984.265038. PMid:36259892.

BELL, E.W. and ZHANG, Y., 2019. DockRMSD: an open-source tool for atom mapping and RMSD calculation of symmetric molecules through graph isomorphism. *Journal of Cheminformatics*, vol. 11, no. 1, pp. 40. http://dx.doi.org/10.1186/s13321-019-0362-7. PMid:31175455.

CHANG, C.C., SLAVIN, M.A. and CHEN, S.C., 2017. New developments and directions in the clinical application of the echinocandins. *Archives of Toxicology*, vol. 91 no. 4, pp. 1613-1621. https://doi.org/10.1007/s00204-016-1916-3. PMid: 28180946.

CHRISTENSON, J.K., PETERSON, G.M., NAUNTON, M., BUSHELL, M., KOSARI, S., BABY, K.E. and THOMAS, J., 2018. Challenges and opportunities in the management of onychomycosis. *Journal of Fungi*, vol. 4, no. 3, pp. 87. http://dx.doi.org/10.3390/jof4030087. PMid:30042327.

CLINICAL AND LABORATORY STANDARDS INSTITUTE – CLSI, 2017. *Performance standards for antifungal susceptibility testing of yeasts*. Wayne: CLSI, no. M60.

DEHSHEIKH, A.B., SOURESTANI, M.M., DEHSHEIKH, P.B., MOTTAGHIPISHEH, J., VITALINI, S. and IRITI, M., 2020. Monoterpenes: essential oil components with valuable features. *Mini-Reviews in Medicinal Chemistry*, vol. 20, no. 11, pp. 958-974. http://dx.doi.org/10.2174/1389557520666200122144703. PMid:31969098.

DINIZ-NETO, H., SILVA, S.L., CORDEIRO, L.V., SILVA, D.F., OLIVEIRA, R.F., ATHAYDE-FILHO, P.F., OLIVEIRA-FILHO, A.A., GUERRA, F.Q.S. and LIMA, E.O., 2022. Antifungal activity of 2-chloro-N-phenylacetamide: a new molecule with fungicidal and antibiofilm activity against fluconazole-resistant Candida spp. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 84, pp. e255080. http://dx.doi.org/10.1590/1519-6984.255080. PMid:35262564.

ESCALANTE, A., GATTUSO, M., PÉREZ, P. and ZACCHINO, S., 2008. Evidence for the mechanism of action of the antifungal phytolaccoside B isolated from Phytolacca tetramera Hauman. *Journal of Natural Products*, vol. 71, no. 10, pp. 1720-1725. http://dx.doi.org/10.1021/np070660i. PMid:18816139.

FERNANDES, H.M.B., OLVEIRA-FILHO, A.A., LIMA, E.O. and SIQUEIRA-JÚNIOR, J.P., 2017. Antifungal effect of synthetic isomer (*r*)-(+)-citronellal against *Candida* Strains. *Latin American Journal of Pharmacy*, vol. 36, pp. 408-411.

HADACEK, F. and GREGER, H., 2000. Testing of antifungal natural products: methodologies, comparability of results and assay choice. *Phytochemical Analysis*, vol. 11, no. 3, pp. 137-147. http://dx.doi.org/10.1002/(SICI)1099-1565(200005/06)11:3<137::AID-PCA514>3.0.CO;2-I.

HALGREN, T.A., 2002. Force Fields: MMFF94. In: P.R. SCHLEYER, ed. *Encyclopedia of Computational Chemistry*. New York: Wiley. https://dx.doi.org/10.1002/0470845015.cma012m.

HANWELL, M.D., CURTIS, D.E., LONIE, D.C., VANDERMEERSCH, T., ZUREK, E. and HUTCHISON, G.R., 2012. Avogadro: an advanced semantic chemical editor, visualization, and analysis platform. *Journal of Cheminformatics*, vol. 4, no. 1, pp. 17. http://dx.doi.org/10.1186/1758-2946-4-17. PMid:22889332.

KÖHLER, J.R., HUBE, B., PUCCIA, R., CASADEVALL, A. and PERFECT, J.R., 2017. Fungi that infect humans. *Microbiology Spectrum*, vol. 5, no. 3, pp. 5.3.08. http://dx.doi.org/10.1128/microbiolspec.FUNK-0014-2016. PMid:28597822.

LIPNER, S.R. and SCHER, R.K., 2019. Onychomycosis: treatment and prevention of recurrence. *Journal of the American Academy of Dermatology*, vol. 80, no. 4, pp. 853-867. http://dx.doi.org/10.1016/j.jaad.2018.05.1260. PMid:29959962.

MCMANUS, D.S. and SHAH, S., 2019. Antifungal drugs. In: S.D. RAY, ed. *Side effects of drugs annual*. 1st ed. Amsterdam: Elsevier, pp. 285-292. http://dx.doi.org/10.1016/bs.seda.2019.09.002.

MEDEIROS, C.I.S., SILVA, D.F., COSTA, A.O.C., ALMEIDA-FILHO, G.G., OLIVEIRA FILHO, A.A. and LIMA, E.O., 2017. Sensitivity of *C. albicans* to the (*S*)-(-)-Citronellal alone and in combination with four antifungal drugs in vulvovaginal candidiasis. *Latin American Journal of Pharmacy*, vol. 36, no. 5, pp. 993-999.

MORALES, G., PAREDES, A., SIERRA, P. and LOYOLA, L., 2008. Antimicrobial activity of three baccharis species used in the traditional medicine of Northern Chile. *Molecules (Basel, Switzerland)*, vol. 13, no. 4, pp. 790-794. http://dx.doi.org/10.3390/molecules13040790. PMid:18463580.

MORRIS, G.M., HUEY, R., LINDSTROM, W., SANNER, M.F., BELEW, R.K., GOODSELL, D.S. and OLSON, A.J., 2009. AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. *Journal of Computational Chemistry*, vol. 30, no. 16, pp. 2785-2791. http://dx.doi.org/10.1002/jcc.21256. PMid:19399780.

NCUBE, N.S., AFOLAYAN, A.J. and OKOH, A., 2008. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. *African Journal of Biotechnology*, vol. 7, no. 12, pp. 1797-1806. http://dx.doi.org/10.5897/AJB07.613.

PAŠKEVIČIUS, A., ŠVEDIENĖ, J., KIVERYTĖ, S., BRIDŽIUVIENĖ, D., VAITONIS, G. and JABLONSKIENĖ, V., 2020. Candida distribution in onychomycosis and in vitro susceptibility to antifungal agents. *Acta Dermatovenerologica Croatica*, vol. 28, no. 7, pp. 204-209. PMid:33834991.

PEREIRA, I., SEVERINO, P., SANTOS, A.C., SILVA, A.M. and SOUTO, E.B., 2018. Linalool bioactive properties and potential applicability in drug delivery systems. *Colloids and Surfaces. B, Biointerfaces*, vol. 171, pp. 566-578. http://dx.doi.org/10.1016/j.colsurfb.2018.08.001. PMid:30098535.

PERUSINHA, C.G., ROSADO, C. and LENCASTRE, A., 2019. Novos avanços no tratamento da onicomicose. *Journal of the Portuguese Society of Dermatology and Venereology*, vol. 77, no. 3, pp. 239-243. http://dx.doi.org/10.29021/spdv.77.3.1104.

PIRACCINI, B.M. and ALESSANDRINI, A., 2015. Onychomycosis: a review. *Journal of Fungi*, vol. 1, no. 1, pp. 30-43. http://dx.doi.org/10.3390/jof1010030. PMid:29376897.

REVIE, N.M., IYER, K.R., ROBBINS, N. and COWEN, L.E., 2018. Antifungal drug resistance: evolution, mechanisms and impact. *Current Opinion in Microbiology*, vol. 45, pp. 70-76. http://dx.doi.org/10.1016/j.mib.2018.02.005. PMid:29547801.

SANTOS, P.L., MATOS, J.P.S.C.F., PICOT, L., ALMEIDA, J.R.G.S., QUINTANS, J.S.S. and QUINTANS-JÚNIOR, L.J., 2019. Citronellol, a monoterpene alcohol with promising pharmacological activities - a systematic review. *Food and Chemical Toxicology*, vol. 123, pp. 459-469. http://dx.doi.org/10.1016/j.fct.2018.11.030. PMid:30453001.

SHARMA, R., RAO, R., KUMAR, S., MAHANT, S. and KHATKAR, S., 2019. Therapeutic potential of citronella essential oil: a review. *Current Drug Discovery Technologies*, vol. 16, no. 4, pp. 330-339. http://dx.doi.org/10.2174/1570163815666180718095041. PMid:30019646.

SIDDIQUI, Z.N., FAROOQ, F., MUSTHAFA, T.N.M., AHMAD, A. and KHAN, A.U., 2013. Synthesis, characterization and antimicrobial evaluation of novel halopyrazole derivatives. *Journal of Saudi Chemical Society*, vol. 17, no. 2, pp. 237-243. http://dx.doi.org/10.1016/j.jscs.2011.03.016.

VIEIRA, J.N., GONÇALVES, C.L., VILLARREAL, J.P.V., GONÇALVES, V.M., LUND, R.G., FREITAG, R.A., SILVA, A.F. and NASCENTE, P.S., 2019. Chemical composition of essential oils from the Apiaceae family, cytotoxicity, and their antifungal activity in vitro against candida species from oral cavity. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 79, no. 3, pp. 432-437. http://dx.doi.org/10.1590/1519-6984.182206. PMid:30328892.

ZHOU, A.-A., LI, R.-Y., MO, F.-X., DING, Y., LI, R.-T., GUO, X., HU, K. and LI, M., 2022. Natural Product Citronellal Can Significantly Disturb Chitin Synthesis and Cell Wall Integrity in *Magnaporthe oryzae*. *Journal of Fungi*, vol. 8, no. 12, pp. 1310. http://dx.doi.org/10.3390/jof8121310. PMid:36547643.

ZORE, G.B., THAKRE, A.D., JADHAV, S. and KARUPPAYIL, S.M., 2011. Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. *Phytomedicine*, vol. 18, no. 13, pp. 1181-1190. http://dx.doi.org/10.1016/j.phymed.2011.03.008. PMid:21596542.