Antifungal activity, antibiofilm and synergic effect of diallyl disulfide and diallyl trisulfide against *Candida albicans*

Atividade antifúngica, antibiofilme e efeito sinérgico do dissulfeto de dialila e trissulfeto de dialila frente a espécie *Candida albicans*

Actividad antifúngica, antibiopelícula y efecto sinérgico de disulfuro de dialilo y trisulfuro de dialilo frente a *Candida albicans*

Abstract
The objective of the present study was to determine the antifungal activity, antibiofilm and synergic effect of the compounds diallyl disulfide and diallyl trisulfide in combination with antifungal agents against clinical isolates of *Candida albicans*, including also a computational study. Antimicrobial sensitivity tests were performed using the broth microdilution method to determine the minimum inhibitory concentration against *C. albicans* strains and modulatory activity using the checkerboard technique. The biofilm formation was evaluated by biomass quantification using the violet crystal staining method. For the study of molecular docking computer simulations. The constituents showed relevant antifungal activity against strains of *C. albicans*. In the modulatory activity assay, demonstrated a synergistic interaction with fluconazole and amphotericin B, with an increase in its antifungal action. The diallyl disulfide, diallyl trisulfide and fluconazole ligands formed complexes with ALS3 enzyme. Then, both compounds were considered promising products for the development of new drugs to prevent candidiasis.

Keywords: Candidiasis; Antifungal; Biofilm; *Allium sativum*; *In silico*.

Resumo
O objetivo do presente estudo foi determinar a atividade antifúngica, antibiofilme e efeito sinérgico dos compostos dissulfeto de dialila e trissulfeto de dialila em combinação com agentes antifúngicos contra isolados clínicos de *Candida albicans*, incluindo também um estudo computacional. Testes de sensibilidade antimicrobiana foram realizados pelo
método de microdiluição em caldo para determinar a concentração inibitória mínima contra cepas de *C. albicans* e a atividade modulatória usando a técnica checkerboard. A formação de biofilme foi avaliada pela quantificação da biomassa pelo método de coloração por cristal violeta. Para o estudo de simulações computacionais de encaixe molecular. Os constituintes apresentaram atividade antifúngica relevante contra cepas de *C. albicans*. No ensaio de atividade modulatória, demonstrou interação sinérgica com fluconazol e anfotericina B, com aumento de sua ação antifúngica. Os ligantes disulfeto de dialila, trissulfeto de dialila e fluconazol formaram complexos com a enzima ALS3. Então, ambos os compostos foram considerados produtos promissores para o desenvolvimento de novos medicamentos para prevenir a candidíase.

**Palavras-chave:** Candidíase; Antifúngico; Biofilme; *Allium sativum*; In silico.

1. Introduction

The increasing in microbial infections together with the resistance developed to conventional antimicrobials has led to a constant search for effective therapeutic alternatives that can offer better treatment options to patients (Pierce *et al.* 2013).

Infectious diseases are the second leading cause of mortality worldwide, and this problem linked to the high resistance rates of microorganisms, especially in hospital environments, justifies the urgency in the development of new antimicrobial agents (Guimaraes *et al.* 2010). Infectious diseases are considered a serious collective health problem, due to the impact they have on society. They are caused by pathogenic microorganisms that invade the host's organism, avoid their defenses and cause tissue damage (Guido *et al.* 2010).

As opportunistic fungal pathogens, *Candida* species are usually harmless commensals in the gastrointestinal tract, genitourinary or oropharyngeal tract of most healthy individuals (Lim *et al.* 2012). *Candida albicans* species are the fourth most common cause of systemic infections acquired in hospitals, being responsible for a gross mortality rate of up to 50% (Pfaller and Diekema 2010).

Many fungal factors contribute to active penetration, including the progressive stretching of the hyphae, physical force exerted by the extending hyphae (Villar *et al.* 2007; Wachtler *et al.* 2012), secretion of hydrolytic enzymes, ALS3 and other factors still unknown (Wachtler *et al.* 2011). ALS3 refer to *Candida* surface invasive proteins that bind to host receptors (Phan *et al.* 2007; Fu *et al.* 2013). Medicinal plants have been a good source of new pharmacologically active molecules. For example, natural products can be a potential alternative to control the disease-associated pathogen (Beshbishy *et al.* 2019, Batiha *et al.* 2019, Batiha *et al.* 2020). Plants representative of the *Allium* genus are commonly used by humans, due to their medicinal properties determined by the incidence of various sulfur compounds (Gunther 2013).

Garlic (*Allium sativum* L.; Family: Amaryllidaceae) is an annual aromatic herb spice and one of the oldest authenticated and most important herbs that has been used since ancient times as traditional medicine. *Allium* species and their active components reduce the risk of diabetes and cardiovascular disease, protect against infections by activating the immune system and have antimicrobial, antifungal, anti-aging and anti-cancer properties, confirmed by epidemiological data from clinical human studies (Ayaz and Alposy 2007; Badal *et al.* 2019).
Thus, the aim of the present study was to determine the antifungal and antibiofilm activity of diallyl disulfide and diallyl trisulfide and their synergistic effect with the antifungal amphotericin B and fluconazole against clinical isolates of *C. albicans*, including also the computational study of the mechanism of action against adhesion enzyme ALS3.

2. Methodology

2.1 Chemical constituents

The main constituents of the essential oil of *A. sativum* diallyl disulfide and diallyl trisulfide were acquired by Sigma-Aldrich.

2.2 Antifungal activity

In this study, strains from *C. albicans* clinical isolates were used LABMIC 0101 (blood culture), LABMIC 0102 (blood culture), LABMIC 0103 (urine), LABMIC 0104 (tracheal aspirate) and LABMIC 0105 (blood culture), isolated from Santa Casa de Misericordia de Sobral and *C. albicans* ATCC 90028 used as a standard strain for the analyzes. The entire research was approved by the Ethics Committee of the Universidade Estadual Vale do Acaraú, under the number 644.365.

The isolates were obtained from primary cultures and the yeast strains were presumptively identified according to the morphological characteristics and color of the colonies grown in CHROMagar-*Candida* medium (Paris, France), as well as in the automated system VITEK 2 (BioMérieux Vitek, Hazelwood, France) and PCR-AGE analysis.

The antifungal activity test of constituents was carried out according to the standards of the Clinical and Laboratory Standards Institute (CLSI 2008a; CLSI 2008b), with some modifications proposed by Fontenelle et al. (2007) and Fontenelle et al. (2008). The fungal strains came from the potato agar stock at -20 °C. They were seeded into tubes containing potato dextrose agar (Difco, Detroit, MI, USA), subsequently incubated at 37 °C for 24 hours. The yeast suspension was obtained by dilution 1:100 followed by a 1:20 dilution of the standard suspension with liquid medium RPMI 1640, with L-glutamine, without sodium bicarbonate, buffered to pH 7.0 with MOPS (2-[N-morpholino]-propanesulfonic; MPOS 0.165M), in order to result in concentrations of 5.0 x 10^2 to 2.5 x 10^3 UFC.mL^-1.

2.3 Minimum Inhibitory concentration (MIC) and Minimum fungicidal concentration (CFM)

The constituents were prepared with dimethyl sulfoxide (DMSO) - (CH₃)₂SO and its concentrations were analyzed between 0.03 to 2.5 mg/mL, whereas amphotericin B (Sigma, Chemical Co., USA) was prepared in distilled water and fluconazole with DMSO 5%. 100 μL of sterile RPMI 1640 medium was inoculated into each well of the microdilution plate, followed by 100 μL of the test solution added to the first line, from which serial dilutions were made up to line G. Also 100 μL of the suspension was added fungal in all wells. The fungal growth control wells presented 100 μL of sterile medium, free of drugs and constituents, added with 100 μL of the inoculum suspensions. The plates were incubated at 37 °C for 24 hours, after which the plates were visually read, observing the macroscopic reduction of fungal growth. MIC was defined by the smallest test fraction capable of inhibiting visually detected fungal growth. CFM corresponded to the lowest concentration that resulted in fungal death after 24h with the sowing of 100 μL of solution from wells without turbidity on potato dextrose agar.

2.4 Antibiofilm activity

The test was carried out based on serial microdilution tests and violet crystal staining in 96-well polyethylene plates according to Stepanovic et al. (2000), with modifications. The preparation of the plates for the tests was similar to the procedure used in the MIC test. Strains of *C. albicans* LABMIC 0101, LABMIC 0102, LABMIC 0104, LABMIC 010 and ATCC 90028 were used. To evaluate the action the constituents on preformed biofilms, each well of the polystyrene plate was filled with 100
µL of the yeasts plus 100 µL of sterile RPMI 1640 in suspension at a concentration of 2x10^6 UFC.mL^-1 and incubated at 37 °C for 24h. After the incubation time, the supernatant was removed and replaced with 200 µL of medium with of diallyl disulfide and diallyl trisulfide in different concentrations for another 24h at 37 °C.

2.5 Quantification of biomass

The quantification of biofilm biomass was determined using the violet crystal (CV) staining method. After 24 hours of incubation, the plates were washed with sterile distilled water three times to remove non-adhered planktonic cells. Subsequently, the wells were filled with 200 µL of methanol (CH₃OH) for 5 minutes to fix the biofilms. Then, 200 µL of 1% violet crystal was added for another 5 minutes. Then, the excess dye was removed and the plates washed with distilled water. The remaining dye was removed with 33% acetic acid and then the biomass was quantified by measuring the optical density at 590nm (OD₅₉₀) with the aid of a microplate reader.

2.6 Synergism with antifungals

The effect of the major constituents diallyl disulfide and diallyl trisulfide of *A. sativum* combined with the antifungal amphotericin B (C₄₇H₇₃NO₁₇) and fluconazole (C₁₃H₁₂F₂N₆O) was determined by the checkerboard technique, this method being used to determine the interaction of drugs by calculating the Fractional Inhibitory Concentration Index (ICIF). The ICIF is calculated by adding the Fractional Inhibitory Concentration (FIC) for each compound tested, being defined as the addition of the MIC values of each drug in the combination and MIC of the same product alone (White *et al.* 1996). Strains of *C. albicans* LABMIC 0104, LABMIC 0105 and ATCC 90028 were used.

The products tested were used in the concentrations of their respective MIC values. Initially 50µL of the RPMI medium was added to all 96 wells of the microdilution plate. Then, 50µL of the major constituents diluted in DMSO 5% was added to the first line and the serial dilution was performed vertically. In the first column, 50µL of each antifungal were placed in different concentrations according to the MIC.

Finally, 100µL of the inoculum was placed in all wells. The RPMI medium with the inoculum was used as a negative control. The plates were incubated at 37 °C for 24h. According to the results obtained, ICIF values ≤ 0.5 will be indicative of synergistic effect, ICIF values > 0.5 and ≤ 1.0 will be indicative of additive effects and ICIF values > 1.0 will be indicative of effect antagonistic (Lechartier, Hartkoorn and Cole 2012; Rosato *et al.* 2007).

2.7 Molecular docking with ALS3 enzyme

The structure of protein *Als3* was obtained from the Protein Data Bank database (https://www.rcsb.org/). For molecular docking simulations, proteins were prepared by removing all residues and adding polar hydrogens (Schimmel *et al.* 1998), producing favorable protonation states for the simulations (Milite *et al.* 2019).

The *Als3* protein was identified in the repository as “Structure of the Als3 adhesin from *Candida albicans*, residues 1-299 (mature sequence)” (PDB 4LE8). The structure of *Als3* is deposited in Protein Data Bank with a resolution of 1.75 Å, determined by X-ray diffraction (R-Value Free: 0.291, R-Value Work: 0.257), classified as cell adhesion, *C. albicans* organism and expression *Escherichia coli* BL21.

For the computational study of molecular docking, the two major compounds of the essential oil of *A. sativum* were selected: diallyl disulfide and diallyl trisulfide. As reference drugs was fluconazole (C₁₃H₁₂F₂N₆O), one of the main antifungals used for *Candida* spp (Sueth-Santiago *et al.*, 2015). The ligands must be in their best potential energy state for the simulations; therefore, they were optimized by the semiempirical parametric method 7 (PM7) (Stewart 2013; Almeida-Neto *et al.* 2020) using the MOPAC® software.
For the study of molecular docking, computer simulations of interaction between proteins and ligands were performed using the AutoDock Vina code (version 1.1.2), using 3-way multithreading, Lamarckian Genetic Algorithm (Trott and Olson, 2009), with docking parameters: center_x = -45.642, center_y = -14.402, center_z = 22.495, size_x = 126, size_y = 90, size_z = 102, spacing = 0.642. The grid box parameters were configured to fit the whole protein, seeking greater amplitude in the selection of poses. 100 independent simulations were carried out for all protein targets, obtaining 10 poses each, as a standard procedure.

For the selection of the simulations with the best poses, the simulations with RMSD (Root Mean Square Deviation) value less than 2 Å were used as criteria (Yusuf et al., 2008; Shityakov and Förster 2014). To analyze the results and generate two-dimensional maps of chemical interactions, the Discovery Studio Visualizer (Biovia et al. 2000) and UCSF Chimera codes were used (Pettersen et al. 2004).

2.8 Statistical analysis

All tests were performed in triplicates and with a significance level of \( p < 0.05 \). For the tests, the difference between the means of the triplicates was verified through the application of the One-way ANOVA test with Bonferroni post-test, performed with the aid of the GraphPad Prism version 8.0 for Windows (San Diego, California, USA).

3. Results and Discussion

*Allium sativum* bulbs are reported to contain hundreds of phytochemicals, including sulfur-containing compounds, such as ajoenes (E-ajoene, Z-ajoene), thiosulfinates (allicin), vinilditiins (2-vinyl- (4H) -1, 3 -ditiin, 3-vinyl- (4H) -1,2-dithine), sulfides (diallyl disulfide (DADS), diallyl disulfide (DATS)) and others that represented 82% of the total sulfur content of garlic (Al-Snafi, 2013). The chemical structures are shown in Fig. 1.

**Figure 1** - Structural representation of the main constituents of the essential oil of *A. sativum* Diallyl disulphide (1) and Diallyl trisulfide (2).

Regarding antifungal activity, the constituent diallyl trisulfide was able to inhibit the microbial growth of all strains of *C. albicans* at a concentration of 2.5 mg. mL\(^{-1}\) and the Minimum Fungicide Concentration was 5.0 mg.mL\(^{-1}\). Diallyl disulphide was only able to inhibit the growth of *C. albicans* ATCC 90028 at a concentration of 2.5 mg. mL\(^{-1}\) and the CFM was 5.0 mg.mL\(^{-1}\). In relation to amphotericin B and fluconazole, the concentration was 1μg. mL\(^{-1}\) (Table 1).
DADS and DATS separated from garlic essential oil showed antifungal activity against several fungi (C. albicans, C. tropicalis and Blastoschizomyces capitatus). In addition, saponins extracted from A. sativum exhibited antifungal activity against Botrytis cinerea and Trichoderma harzianum (Zhen et al. 2006).

Previous reports have demonstrated the antifungal activity of alllicin, a compound present in the essential oil of A. sativum, in vitro against Aspergillus, Trichophyton and Candida spp. (Yamada & Azuma 1977; Aala et al. 2010). Allicin exhibited antifungal activity with MIC between 0.195 and 6.25 µg/ml and, when used in synergy with antimicrobial agents, increases the effectiveness of therapeutic agents (Aala et al. 2010; Khodavandi et al. 2010). For example, the combination of allicin with amphotericin B and fluconazole has been shown to have a significant synergistic effect in a model of systemic candidiasis in mice (An et al. 2009; Guo et al. 2010).

Most of these abilities are related to the disulfide bond of alllicin affects thiol-containing compounds, such as adhesion proteins; however, the main targets of alllicin in Candida are not well known. It has been shown that the antifungal activity of alllicin in vivo can be related to other secondary metabolites, such as ajoene, diallyl trisulfide and diallyl disulfide, since the molecule of alllicin is very unstable and immediately converts to those products (Miron et al. 2004; Khodavandi et al. 2010).

Although the antimicrobial activities of diallyl trisulfide and diallyl disulfide have already been reported, little attention is paid to their antibiofilm potential. As shown in Figure 2 and Figure 3 on the inhibition of biofilm formation, diallyl disulfide and diallyl trisulfide significantly reduced the biofilm biomass of all yeasts tested in this study. Concentrations ranged from 2.5 mg.mL⁻¹ to 0.039 mg.mL⁻¹ in which secondary metabolites reduced the amount of biomass to values between 20 to 100%. The results of the biomass quantification of the preformed biofilms showed that there was a reduction of 20 to 90% of the biomass for the yeasts tested for disulphide and diallyl trisulfide (Figure 4 and 5).

Table 1 - Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC) of the constituents against C. albicans species.

| C. albicans | Origin            | Diallyl disulphide | Diallyl trisulfide | AMB | FLC |
|-------------|-------------------|--------------------|--------------------|-----|-----|
|             | MIC mg. mL⁻¹ | MFC mg. mL⁻¹ | MIC mg. mL⁻¹ | MFC mg. mL⁻¹ | MIC µg. mL⁻¹ | MFC µg. mL⁻¹ |
| LABMIC 0101 | Blood culture   | -                  | 2.5               | 5.0  | 1.0 | 1.0 |
| LABMIC 0102 | Blood culture   | -                  | 2.5               | 5.0  | 1.0 | 1.0 |
| LABMIC 0103 | Urine           | -                  | 2.5               | 5.0  | 1.0 | 1.0 |
| LABMIC 0104 | Tracheal aspirate| -                  | 2.5               | 5.0  | 1.0 | 1.0 |
| LABMIC 0105 | Blood culture   | -                  | 2.5               | 5.0  | 1.0 | 1.0 |
| ATCC 90028  | Culture Collection | 2.5             | 5.0               | 1.0 | 1.0 |

Source: Elaborated by the authors.
Figure 2 - Antibiofilm effect of diallyl disulphide at different concentrations on the inhibition of biomass of *C. albicans* biofilms.

Figure 3 - Antibiofilm effect of diallyl trisulfide at different concentrations on the inhibition of biomass of *C. albicans* biofilms.

Source: Elaborated by the authors.
Figure 4 - Antibiofilm effect of diallyl disulphide in different concentrations on the biomass inhibition of preformed *C. albicans* biofilms.

Source: Elaborated by the authors.

Figure 5 - Antibiofilm effect of diallyl trisulphide in different concentrations on the biomass inhibition of preformed *C. albicans* biofilms.

Source: Elaborated by the authors.
The combination of antimicrobial agents can be used to increase the spectrum of action, to prevent the emergence of resistant mutants and to promote synergism between two or more drugs. In relation to the synergistic activity, the results are shown in tables 2 and 3.

**Table 2** - Determination of FIC indices (FICI) for *Candida albicans* and amphotericin B.

| *C. albicans* | Diallyl trisulfide | Amphotericin B | FICI | EFFECT |
|--------------|------------------|----------------|------|--------|
|               | MIC µg/mL (Individual) | MIC µg/mL (Combined) | MIC µg/mL (Individual) | MIC µg/mL (Combined) |
| LABMIC 0104   | 2.500            | 625            | 1.0  | 0.25   | 0.50  | Synergism |
| LABMIC 0105   | 2.500            | 625            | 1.0  | 0.25   | 0.50  | Synergism |
| ATCC 90028    | 2.500            | 625            | 1.0  | 0.25   | 0.50  | Synergism |

Source: Elaborated by the authors.

**Table 3** - Determination of FIC indices (FICI) for *Candida albicans* and fluconazole.

| *C. albicans* | Diallyl trisulfide | Fluconazole | FICI | EFFECT |
|--------------|------------------|-------------|------|--------|
|               | MIC µg/mL (Individual) | MIC µg/mL (Combined) | MIC µg/mL (Individual) | MIC µg/mL (Combined) |
| LABMIC 0104   | 2.500            | 625          | 1.0  | 0.25   | 0.50  | Synergism |
| LABMIC 0105   | 2.500            | 1.250        | 1.0  | 0.25   | 0.75  | Additive  |
| ATCC 90028    | 2.500            | 625          | 1.0  | 0.25   | 0.50  | Synergism |

Source: Elaborated by the authors.

The combination trial of diallyl trisulfide with the standard antifungal amphotericin B and fluconazole showed that there was a reduction in MIC values in all tested strains and also MICs of antifungals. From these values, it was possible to calculate the fractional inhibitory concentration index (ICIF), which showed a synergistic effect for *C. albicans* LABMIC 0104, *C. albicans* LABMIC 0105 and *C. albicans* ATCC 90028 in combination with amphotericin B, combination with fluconazole, presented a synergistic effect for *C. albicans* LABMIC 0104 and *C. albicans* ATCC 90028 and additive effect for *C. albicans* LABMIC 0105. Fungal infections caused by *Candida* species pointed out as one of the main causes of mortality in immunocompromised patients, (Sueth-Santiago et al. 2015). One of the main classes of antifungals, triazoles, such as itraconazole and fluconazole, have a broad spectrum of action against *Candida* species but have resistance problems (Silva et al. 2012; Colombo et al. 2013). In this context, it is necessary to search for new antifungal drugs, in isolation or in combination therapy (Guo 2008).

Molecular docking is essential for understanding the interaction between receptor and ligand, so, after docking, all simulations of ligands showed an RMSD value within the ideal parameter, less than 2 Å (Yusuf et al. 2008). Diallyl disulfide had an RMSD value of 1.323 Å, diallyl trisulfide was 1.771 Å and Fluconazole 1.387 Å (Table 4). The diallyl disulfide, diallyl trisulfide and fluconazole ligands formed complexes with the protein target, with interaction distances ranging between 1.98 and 5.24 Å (Table 5) and binding energy -3.4 kcal/mol, -3.3 kcal/mol and -6.9 kcal/mol respectively.

**Table 4** - Interaction energy and RMSD in molecular docking assay with Als3.

| Inhibitor   | Als3     |
|-------------|----------|
|             | Energy   | RMSD   |
| Diallyldisulfide | -3.4     | 1.323  |
| Diallyltrisulfide | -3.3     | 1.771  |
| Fluconazole  | -6.9     | 1.387  |

Source: Elaborated by the authors.
Diallyl disulfide presented a hydrophobic interaction varying between 3.68 and 3.80 Å with TYR21A, TYR23A and TRP295A. Diallyl trisulfide showed hydrophobic interactions with ALS3, ranging from 3.68 to 3.78 Å, with residues VAL161A, TRP295A and ARG299A. Fluconazole showed interactions with residues VAL161A, VAL161A, ASN22A and TYR21A.

Table 5 - Interactions between Als3 and major compounds diallyldisulfide, diallyl trisulfide and fluconazole.

| Ligand          | Receptor    | Interaction | Distance (Å) |
|-----------------|-------------|-------------|--------------|
| Diallyldisulfide| TYR21A      | Hydrophobic | 3.73         |
|                 | TYR23A      | Hydrophobic | 3.68         |
|                 | TRP295A     | Hydrophobic | 3.80         |
| Diallyltrisulfide| VAL161A     | Hydrophobic | 3.63         |
|                 | TRP295A     | Hydrophobic | 3.68         |
|                 | ARG299A     | Hydrophobic | 3.78         |
| Fluconazole     | VAL161A     | Hydrophobic | 3.63         |
|                 | VAL161A     | H-Bond      | 3.63         |
|                 | ASN22A      | H-Bond      | 1.98         |
|                 | TYR21A      | π-stacking  | 5.24         |

Source: Elaborated by the authors.

Regarding the co-crystallized inhibitor, the two ligands under study coupled in the region of the catalytic site of fluconazole (Figure 6), with diallyl disulfide having a hydrophobic interaction with residues TYRA21 and TYRA23. Diallyl trisulfide showed hydrophobic interactions with the TRP295A and ARG299A residues from the enzyme's catalytic site. Fluconazole showed hydrophobic interaction with residue from the active site (VAL161A), coupling in a different region from the other ligands (Figure 7).

Figure 6 - Binding site of the compounds under study at ALS3.

Source: Elaborated by the authors.
Figure 7 - 2D maps of interactions between Als3 complexes- Diallyl disulfide (A), ALS3- Diallyl trisulfide (B) and ALS3- Flucnazole (C).

4. Conclusion

The major constituents of the essential oil of A. sativum diallyl disulfide and diallyl trisulfide have antifungal and antibiofilm activity on clinical isolates of C. albicans, responsible for the development of pathologies in humans. In addition, diallyl trisulfide showed a synergistic effect with the commercial drugs amphotericin B and fluconazole.

The compounds diallyl disulfide and diallyl trisulfide coupled in the region of the catalytic site in different regions occupied by fluconazole, making it possible to infer the potential use of these compounds synergistically with fluconazole as a pharmacological tool in the treatment of fungal infections caused by Candida spp.

References

Aala, F., Yusuf, U. M., Khodavandi, A., & Jamal, F. (2010) In vitro antifungal activity of allicin alone and in combination with two medications against six dermatophytic fungi. Afr. J Microbiol Res 4: 380-385.

Almeida-Neto, F. W. Q., Silva, L. P., Ferreira, M. K. A., Mendes, F. R. S., Castro, K. K. A., Bandeira, P. N., Menezes, J. E. S. A., Santos, H. S., Monteiro, N. K. V., Marinho, E. S., & Lima-Neto, P. (2020) Characterization of the structural, spectroscopic, nonlinear optical, electronic properties and antioxidant activity of the N-[4-{(E)-3-(Fluorophenyl)-1-(phenyl)-prop-2-en-1-one}]-acetamide. J. Mol. Struct 1220: 1-53.

Al-Snafi, A. (2013) Pharmacological effects of Allium species grown in Iraq. An overview. Int. J. Pharm. Health Care Res 1: 132-147.

An, M., Shen, H., Cao, Y., Zhang, J., Cai, Y., Wang, R., & Jiang, Y. (2009). Allicin enhances the oxidative damage effect of amphotericin B against Candida albicans. Int J Antimicrob Ag 33, 258-263.

Ayaz, E., & Alposy, H. C. (2007). Garlic (Allium sativum) and traditional medicine. Turkiye Parazitoloji Derg. 31, 145-149.

Badal, D. S., Dwivedi, A. K., Kumar, V., Singh, S., Prakash, A., Verma, S., & Kumar, J. (2019). Effect of organic manures and inorganic fertilizers on growth, yield and its attributing traits in garlic (Allium sativum L.). J. Pharmacogn. Phytochem. 8, 587-590.
Bathi G. E. S., Beshbisy A. A., Adeyemi O. S., Nadwa E., Rashwan E., Yokoyama N., & Igarashi I. (2020). Safety and efficacy of hydroxyurea and eflorenithine against most blood parasites Babesia and Theileria. PLoS ONE. 15, e0228996.

Bathi G. E. S., Beshbisy, A. A., Tayebwa, D. S., Shaheen M. H., Yokoyama N., & Igarashi I. (2019). Inhibitory effects of Szczygium aromaticum and Camellia sinensis methanolic extracts on the growth of Babesia and Theileria parasites. Ticks Tick Borne Dis. 10, 949-958.

Beshbisy A. M., Batiga G. E. S., Adeyemi O. S., Yokoyama N., & Igarashi I. (2019). Inhibitory effects of methanolic Olea europaea and aceticAcacia laeta on the growth of Babesia and Theileria. Asian Pac. J. Trop. Med. 12, 425-434.

Clinical and Laboratory Standards Institute. Reference Method for Broth Dilation Antifungal Susceptibility Testing of Yeasts (Approved Standard. Document M27. CLSI). Third ed. vol. M27-A3. Clinical and Laboratory Standards Institute 2008a: Wayne, PA.

Clinical and Laboratory Standards Institute. Reference Method for Broth Dilation Antifungal Susceptibility Testing of Filamentous Fungi (Approved Standard. Document M38. CLSI). Second ed. vol. M38-A2. Clinical and Laboratory Standards Institute 2008b: Wayne, PA.

Colombo A L., Guimarães T., Camargo L. F. A., Richtmann R., Queiroz-Telles F. Salles M. J. C., Cunha C. A., Yasuda M. A. S., Moretti, M. L., & Nuccio M., 2013. Brazilian guidelines for the management of candidiasis - a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical. Brazilian J. Infect. Dis. 17, 283-312.

D. S. Biocia et al., “Dassault Systèmes BIOVIA, Discovery Studio Visualizer, v.17.2, San Diego: Dassault Systèmes, 2016.

Fontenelle R. O. S., Morais S. M., Brito E. H. S., Brilhante R. S. N., Cordeiro R. A., Nascimento N. R. F., Sidrím J. J. C., & Rocha M. F. G. (2008). Antifungal activity of essential oils of Crotus species from the Brazilian Caatinga biome. J. Appl. Microbiol. 104, 1383-1390.

Fontenelle R.O.S., Morais S.M., Brito E.H.S., Kerntopf M. R., Brilhante R. S. N., Cordeiro R. A., Tomé A. R., Queiroz M. G. R., Nascimento N. R. F., Sidrím J. J. C., & Rocha M. F. G. (2007). Chemical composition, toxicological aspects and antifungal activity of essential oil from Lippia sidoides Cham. Journal of Antimicrobial Chemotherapy, 59, 934-940.

Fu Y., Phan Q. T., Luo G., Solis N. V., Liu Y., Cormack B. P., Edwards J. J., Ibrahim A.S., & Filler S. G. (2013). Investigation of the function of Candida albicans Als3 by heterologous expression in Candida glabrata. Infect Immun. 81, 2528-2535.

Guido, R. C., Andricopulo, A. D. & Oliva, G. (2010). Planejamento de fármacos, biotecnologia e química medicinal: aplicações em doenças infecciosas. Estudos Avançados. 24, 81-98.

Guimaraes, D. O., Momesso, L. S., & Pupo, M. T. (2010). Antibióticos: importância terapêutica e perspectivas para a descoberta e desenvolvimento de novos agentes. Química Nova. 33, 667-679.

Guo N., Wu X., Yu L., Liu J., Meng R., Jin J., Lu H., Wang X., Yan S., & Deng X. (2010). In vitro and in vivo interactions between fluconazole and allicin against clinical isolates of fluconazole-resistant Candida albicans determined by alternative methods. FEMS Immunol Med Microbiol. 58, 193-201.

Guo Q., Sun S., Yu J., Li Y., & Cao L. (2008). Synergistic activity of azoles with amiodarone against clinically resistant Candida albicans tested by checkerboard and time-kill methods. J. Med. Microbiol. 57, 457-462.

Khodavandi A., Alizadeh F., Aala F., Sekawi Z., & Chong P. P. (2010). In vitro investigation of antifungal activity of allicin alone and in combination with azoles against Candida species. Mycopathologia. 169, 287-295.

Lechartier B., Hartkoorn R. C., & Cole S. T. (2012). In vitro combination studies of benzothiazinone lead compound BTZ043 against Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy. 56, 5790-5793.

Lim C. S., Rosli R., Seow H. F., & Chong P. P. (2012). Candida and invasive candidiasis: back to basics. Eur J Clin Microbiol Infect Dis. 31, 21-31.

Milite C., Amendola G., Nocentini A., Bua S., Cipriano A., & Barresi E. (2019). Novel 2-substituted-benzimidazole-6-sulfonamides as carbonic anhydrase inhibitors: synthesis, biological evaluation against isozymes I, II, IX and XII and molecular docking studies. J. Enzyme Inhib. Med. Chem. 34, 1697-1710.

Miron T., Bercovici T., Rabinkov A., Wilchek M., & Mirelman D. (2004). [HI] Allicin: preparation and applications. Anal Biochem. 331, 364-369.

Petteresen E. F., Goddard T. D., Huang C. C., Couch G. S., Greenblatt D. M., Meng E. C., & Ferrin T. E. (2004). UCSF Chimera - A visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605-1612.

Pfaffler M. A., & Diekema D. J. (2010). Epidemiology of invasive mycoses in North America. Crit Rev Microbiol. 36, 1-53.

Phan Q. T., Myers C. T., Adeyemi O. S., Nadwa E., Rashwan E., Yokoyama N., & Igarashi I. (2020). Aspects of antifungal activity of allicin alone and in combination with azoles against Candida species. Mycopathologia. 189, 1697-1710.

Pierce, C. G., et al. (2013). Antifungal therapy with an emphasis on biofilms. Current Opinion in Pharmacology. 13, 1-5.

Rosato A., Vitali C., Gallo D., Balenzo L., & Mallamaci R. (2008). The inhibition of Candida species by selected essential oils and their synergism with Amphotericin B. Phytomedicine. 15, 635-638.

Schimmel P., Tao J., & Hill J. (1998). Aminoacyl (RNA synthetases as targets for new anti-infectives. FASEB J. 12, 1599-1609.

Shiriakov S., & Förster C. (2014). In silico predictive model to determine vector-mediated transport properties for the blood-brain barrier choline transporter. Adv. Appl. Bioinforma. Chem. 7, 23-36.

Silva S., Negri M., Henriques M., Oliveira R., Williams D. W., & Azeredo J. (2012). Candida glabrata, Candida parapsilosis and Candida tropicalis: Biology,
epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiology Reviews*. 36, 288-305.

Stepanovic S, Vukovic D, Dakic I et al., Savić B., & Švabić-Vlahović M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J. Microbiol. Methods*. 40 (2):175-179.

Stewart J. J. P. (2013). Optimization of parameters for semiempirical methods VI: More modifications to the NDDO approximations and re-optimization of parameters. *J. Mol. Model*. 19, 1-32.

Sueth-Santiago V., Franklim T. N., Lopes N. D., & Lima M. E. F. (2015). CYP51: Is it a good idea?. *Rev. Virtual Quim*. 7, 539-575.

Trott O., & Olson A. J. (2009). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem*. 31, 455-461.

Villar C. C., Kashleva H., Nobile C. J., Mitchell A. P., & Dongari-Bagtzoglou A. (2007). Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. *Infect Immun*. 75, 2126-2135.

Wachtler B., Citiulo F., Jablonowski N., Forster S., Dalle F., Schaller M., Wilson D., & Hube B. (2012). *Candida albicans*-epithelial interactions: dissecting the roles of active penetration, induced endocytosis and host factors on the infection process. *PLoS ONE*. 7, 1-9.

Wachtler B., Wilson D., Haedicke K., Dalle F., & Hube B. (2011). From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PLoS ONE*. 6, 1-14.

White R. L., Burgess D. S., Manduri M., & Bosso J. A. (1996). Comparison of three different in vitro methods of detecting synergy: Time-kill, checkerboard and E-test. *Antimicrobial agents and chemotherapy*. 40, 1914-1918.

Yamada Y., & Azuma K. (1977). Evaluation of the in vitro antifungal activity of allicin. *Antimicrob Agents Ch*. 11, 743-749.

Yusuf D., Davis A. M., Kleywegt G. J., & Schmitt S. (2008). An alternative method for the evaluation of docking performance: RSR vs RMSD. *J. Chem. Inf. Model*. 48, 1411–1422.

Zhen H., Fang F., Ye D. Y., Shu S. N., Zhou Y. F., Dong Y. S., Nie X. C., & Li G. (2006). Experimental study on the action of allitridin against human cytomegalovirus in vitro: Inhibitory effects on immediate-early genes. *Antiviral Res*. 72, 68-74.