UNIVERSIDADE FEDERAL DO PARÁ

INSTITUTO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

AVALIAÇÃO *IN SILICO* DA ATIVIDADE ANTIMICROBIANA DO TIMOL – COMPOSTO MAJORITÁRIO DO ÓLEO ESSENCIAL DE *Lippia thymoides* Mart. & Schauer (Verbenaceae)

JORDDY NEVES DA CRUZ

BELÉM – PA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas do instituto de Ciências da Saúde da Universidade Federal do Pará, como requisito para obtenção do título de Mestre em Ciências Farmacêuticas.

Orientador: Prof. Dr. Rafael Rodrigues Lima

Coorientadora: Prof. Dra. Eloisa Helena de Aguiar Andrade

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DEDICATÓRIA

À minha mãe Carminda Neves da Cruz (*in memorian*)

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Eu estabeleço uma norma para diferenciar bem as pessoas. Escolho os amigos pela beleza, os conhecidos pelas qualidades de carácter, e os inimigos pelas de inteligência. Todo o cuidado é pouco na escolha dos inimigos. Não tenho um único que seja estúpido. São todos homens de capacidade intelectual e, por conseguinte, todos me apreciam.

Oscar Wilde

RESUMO

Neste trabalho avaliamos as interações fármaco-receptor responsáveis pela atividade antimicrobiana do Timol, composto majoritário presente no óleo essencial (OE) de *Lippia thymoides (L. thymoides)* Mart. & Schauer (Verbenaceae). Foi relatado anteriormente que este OE possui atividade antimicrobiana contra *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*) e *Escherichia coli* (*E. coli*). Sendo assim, usamos docking molecular, simulações de dinâmica molecular e cálculos de energia de afinidade para investigar a interação do Timol com receptores farmacológicos de interesse terapêutico para combater esses patógenos. Descobrimos que o Timol interagiu favoravelmente com os locais de ligação dos alvos moleculares dos micro-organismos. Os resultados do MolDock Score para sistemas formados com CYP51 (*C. albicans*), Diidrofolato Redutase (*S. aureus*) e Diidropteroato Sintase (*E. coli*) foram -77,85, -67,53 e -60,88, respectivamente. Durante todas as simulações de dinâmica molecular, o Timol continuou interagindo com o local de ligação do alvo molecular de cada micro-organismo. O valor das interações de van der Waals (ΔE_{vdW}=−24,88, −26,44, −21,71 kcal/mol) energias de interações eletrostáticas (ΔE_{ele} = −3,94, −11,07, −12,43 kcal/mol) e as energias de solvatação não polar (ΔG_{NP} = −3,37, − 3,25, -2,93 kcal/mol) foram os principais responsáveis pela formação de complexos com CYP51 (*C. albicans*), Diidrofolato Redutase (*S. aureus*) e Diidropteroato Sintase (*E. coli*).

Palavras-chaves: Óleos essenciais, modelagem molecular, antimicrobiano, modo de interação

ABSTRACT

In this paper, we evaluated the drug-receptor interactions responsible for the antimicrobial activity of thymol, the major compound present in the essential oil (EO) of *Lippia thymoides (L. thymoides)* Mart. & Schauer (Verbenaceae). It was previously reported that this EO exhibits antimicrobial activity against *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*). Therefore, we used molecular docking, molecular dynamics simulations, and free energy calculations to investigate the interaction of thymol with pharmacological receptors of interest to combat these pathogens. We found that thymol interacted favorably with the active sites of the microorganisms' molecular targets. MolDock Score results for systems formed with CYP51 (*C. albicans*), Dihydrofolate reductase (*S. aureus*), and Dihydropteroate synthase (*E. coli*) were −77.85, −67.53, and −60.88, respectively. Throughout the duration of the MD simulations, thymol continued interacting with the binding pocket of the molecular target of each microorganism. The van der Waals (ΔE_{vdW} = −24.88, −26.44, −21.71 kcal/mol, respectively) and electrostatic interaction energies (ΔE_{ele} = −3.94, −11.07, −12.43 kcal/mol, respectively) and the nonpolar solvation energies (ΔG_{NP} = −3.37, −3.25, −2.93 kcal/mol, respectively) were mainly responsible for the formation of complexes with CYP51 (*C. albicans*), Dihydrofolate reductase (*S. aureus*), and Dihydropteroate synthase (*E. coli*).

Keywords: Essential oils, molecular modeling, antimicrobial, mode of interaction

SUMÁRIO

1. INTRODUÇÃO

Por muitos séculos os compostos de origem natural foram umas das poucas alternativas para o tratamento de doenças. Essas substâncias podem ser encontradas em plantas, bactérias, fungos, organismos marinhos e insetos. A utilização desses compostos para tratamento terapêutico é conhecida como medicina tradicional, inicialmente praticada por civilizações do oriente médio, pelos egípcios, chineses, povos da Grécia antiga e indígenas das Américas (PANG *et al.*, 2021).

Os registros mais antigos sobre o uso medicinal de plantas foram encontrados na Mesopotâmia (onde atualmente estão localizados o Iraque, Irã e Jordânia, Oriente Médio) e datam do ano de 2600 antes de Cristo (A.C.). Esses registros foram encontrados em tabuletas de argila com escritas cuneiformes que relatam o uso medicinal de óleos de espécies de *Cupressus sempervirens* (Cipreste) e *Commiphora myrrha* (Mirra) (CRAGG; NEWMAN, 2005). O Papiro de Ebers é um dos registros Farmacêuticos e Médicos mais importantes do mundo, sendo encontrado ocasionalmente à venda numa banca de papiros pelo monge Georg Ebers em 1873 na cidade de Luxor (antiga Tebas, capital do Egito). Neste papiro há descrição de mais de 700 medicamentos à base de plantas medicinais, além de uma rica descrição do sistema circulatório humano (HALLMANN-MIKOŁAJCZAK, 2004).

O médico grego, Dioscórides, (100 D.C.), registrou a coleta, armazenamento e os usos de ervas medicinais, enquanto o filósofo grego, Teofrasto (~300 A.C) tratou de ervas medicinais. Durante a Idade Média, os mosteiros da Inglaterra, Irlanda, França e Alemanha preservaram esse conhecimento ocidental, enquanto os árabes preservaram o conhecimento greco-romano e expandiram os usos de seus próprios recursos naturais, juntamente com ervas chinesas e indianas desconhecidas dos greco-romanos (CRAGG; NEWMAN, 2005). Foram os árabes os primeiros a possuir farmácias privadas (século VIII) com farmacêutico, médico e filósofo, contribuindo muito para as ciências farmacêuticas e médicas através de obras como o *Canon Medicinae* (HALLMANN-MIKOŁAJCZAK, 2004).

O uso de produtos naturais como alternativa terapêutica tem sido descrita ao longo da história como remédios, pomadas, porções e óleos com muitos desses compostos bioativos ainda não identificados. A fonte dominante de conhecimento sobre o uso de plantas medicinais é resultado do homem experimentando por

tentativa e erro, durante centenas de anos, os efeitos farmacológicos de diversas plantas (HOLZMEYER *et al.*, 2020). A utilização de plantas do gênero *Salvia* é um exemplo desse fato. Essas plantas crescem em toda a região sudoeste dos Estados Unidos e noroeste do México, e foram usadas por tribos indígenas do sul da Califórnia. Nessas tribos os bebês recém-nascidos do sexo masculino eram "cozidos" nas cinzas quentes de *Salvia*, pois acreditava-se que esses bebês cresciam mais fortes e saudáveis, sendo imunes a todas as doenças respiratórias (GHORBANI; ESMAEILIZADEH, 2017).

Atrelado ao uso terapêutico dos produtos naturais, cada cultura ao redor do mundo atribuiu crenças e costumes religiosos. Ao longo do tempo, todo esse conhecimento gerado permitiu, a partir de estudos etnobotânicos e etnofarmacológicos, que muitos produtos naturais pudessem ser avaliados cientificamente quanto as suas propriedades terapêuticas (HUSSEIN; EL-ANSSARY, 2018).

Em particular, os compostos de origem vegetal, possuem uma ampla riqueza de estruturas químicas privilegiadas de grande valor para a indústria farmacêutica. Essas estruturas foram descobertas a partir do conhecimento terapêutico empírico de populações locais que serviram de apoio para a investigação científica, realizada por pesquisadores de diversas áreas do conhecimento (VINET; ZHEDANOV, 2011).

Esses compostos são oriundos do metabolismo secundário das plantas e recebem, de forma geral, a nomenclatura de metabólitos secundários. Esses metabolitos não são essenciais, apesar de influenciarem no crescimento, desenvolvimento ou reprodução de um organismo e são produzidos como resultado da adaptação do organismo ao ambiente circundante ou são produzidos para atuar como um possível mecanismo de defesa contra predadores auxiliando a sobrevivência da planta (MAPLESTONE; STONE; WILLIAMS, 1992).

Os compostos vegetais podem ser categorizados como compostos fixos e voláteis. Os fixos são substâncias que possuem uma cadeia carbônica formada por vários átomos que devido seu peso molecular não são voláteis a temperatura de 100 ºC. Contudo, os compostos voláteis, são capazes de volatilizar nessa temperatura, devido serem estruturas químicas de baixo peso molecular. Sendo compostos principalmente, mas não exclusivamente, por cadeias com 11, 12 ou 13 átomos de carbonos (ALZOMAN *et al.*, 2021).

As substâncias voláteis podem ser encontradas em diferentes partes das plantas como folhas, ramos e frutos, sendo moléculas obtidas como óleo essencial a partir de métodos de extração como hidrodestilação, arraste a vapor, extração por micro-ondas e extração por fluido supercrítico (BEZERRA *et al.*, 2020).

O óleo essencial pode ser utilizado na indústria para a produção de cosméticos e perfumes, produção de embalagens inteligentes com propriedades antimicrobianas, mas também podem ser investigados quanto as suas propriedades fitotóxicas e terapêuticas utilizando métodos *in vitro*, *in vivo* e *in silico* (DA COSTA *et al.*, 2019; NETO *et al.*, 2020; PINTO *et al.*, 2019).

A planta que serviu de inspiração para esse trabalho faz parte da família das Verbenaceae, gênero *Lippia* tendo sua espécie denominada como *Lippia thymoides* (Figura 1). Essa planta possui reconhecida propriedade medicinal para o combate de doenças como bronquite, reumatismo e febre, sendo comumente encontrada nos cerrados e campos rupestres do Brasil. Suas propriedades farmacológicas estão associadas a presença de compostos fixos pertencentes a classe dos terpenos, flavonoides e alcaloides, mas seu uso medicinal, também é investigado devido a presença do Timol como composto majoritário do óleo essencial obtido dessa espécie (ESCOBAR *et al.*, 2020).

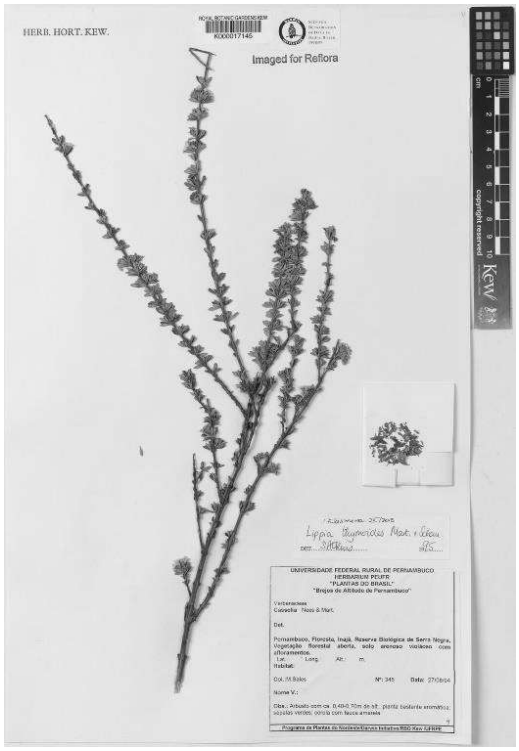

Figura 1. Exsicata de *L. thymoides* (Fonte: Royal Botanic Gardens).

O Timol (Figura 2) é um monoterpeno volátil oriundo do metabolismo secundária de várias plantas. Na espécie de *L. thymoides*, o Timol pode ser encontrado como composto majoritário do óleo essencial extraído a partir de suas folhas. Esse óleo essencial (rico em Timol) e o composto isolado, possuem diversificadas aplicações medicinais no combate a distúrbios que afetam os sistemas respiratório e digestivo (SALEHI *et al.*, 2018; WAN *et al.*, 2018), atividade contra doenças bucais como cárie (FLAMEE *et al.*, 2015), além de apresentar capacidade cicatrizante (MOLLARAFIE *et al.*, 2015), antioxidante (NIKOLIĆ *et al.*, 2014; SILVA, F. S. *et al.*, 2016), anti-inflamatória (OLIVEIRA *et al.*, 2017) e antimicrobiana (JAFRI; AHMAD, 2020; MEMAR *et al.*, 2017).

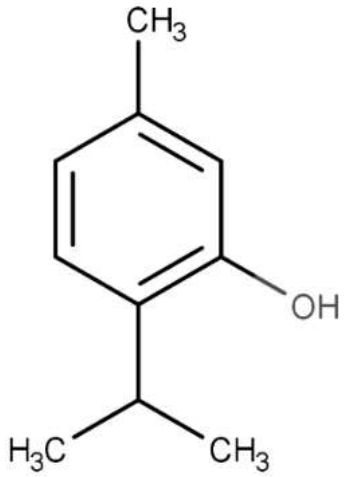

Figura 2. Estrutura molecular do Timol (Fonte: O autor).

A investigação *in silico* realizada nesta dissertação foi apoiada pelos resultados experimentais previamente publicados por Silva e colaboradores (2021) (SILVA, S. G. *et al.*, 2021), onde foi investigada a capacidade antimicrobiana do óleo essencial de *L. thymoides* rico em Timol. Esses autores obtiveram o óleo essencial por fluido supercrítico, nas condições de 50 ºC de temperatura e 200 bar de pressão, apresentando o Timol como composto majoritário que correspondendo a 88.56 ± 0.65 % da composição total do óleo essencial. Esses autores demonstraram que *Candida albicans*, *Escherichia coli* e *Staphylococcus aureus* foram sensíveis ao óleo essencial. O composto Timol isolado foi utilizado como controle positivo e os halos de inibição para os micro-organismos sensíveis ao óleo essencial foram semelhantes aos obtidos para os testes realizados com o Timol.

A partir disso, para compreender como o Timol é capaz de interagir com alvos moleculares essenciais para a sobrevida desses micro-organismos, utilizamos algumas abordagens de modelagem molecular como *docking*, simulações de dinâmica molecular e energia de afinidade. Buscamos na literatura proteínas previamente descritas como alvos moleculares de produtos naturais e sintéticos que combatem esses parasitas. Assim, foram selecionadas as proteínas CYP51 (*C. albicans*), Dihydrofolate reductase (*S. aureus*), and Dihydropteroate synthase (*E. coli*) que são essenciais para as vias metabólicas desses micro-organismos, dessa forma, se a inibição dessas proteínas por realizada pelo Timol, as interrupções de suas vias metabólicas podem comprometer a sobrevida desses micro-organismos, contribuindo assim para o combate da patogenia causada por esses parasitas.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o modo de interação do Timol com alvos moleculares importantes para a sobrevida de micro-organismos patogênicos como *Candida albicans*, *Staphylococcus aureus* e *Escherichia coli*.

2.2 Objetivos específicos

- Analisar o modo de interação do Timol como o local de ligação de proteínas importantes para a viabilidade de bactérias e fungos;
- Identificar as interações químicas estabelecidas entre o Timol e os resíduos do local de ligação de proteínas presentes em bactérias e fungos;
- Avaliar ao longo do tempo a formação e a estabilidade dos sistemas timol-proteínas;
- Quantificar a energia de afinidade dos complexos timol-proteínas.

3. ARTIGO PUBLICADO

molecules

MDPI

Article

In Silico Evaluation of the Antimicrobial Activity of Thymol—Major Compounds in the Essential Oil of *Lippia thymoides* Mart. & Schauer (Verbenaceae)

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Abstract: In this paper, we evaluated the drug-receptor interactions responsible for the antimicrobial activity of thymol, the major compound present in the essential oil (EO) of *Lippia thymoides (L. thymoides)* Mart. & Schauer (Verbenaceae). It was previously reported that this EO exhibits antimicrobial activity against *Candida albicans* (*C. albicans*), *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*). Therefore, we used molecular docking, molecular dynamics simulations, and free energy calculations to investigate the interaction of thymol with pharmacological receptors of interest to combat these pathogens. We found that thymol interacted favorably with the active sites of the microorganisms' molecular targets. MolDock Score results for systems formed with CYP51 (*C. albicans*), Dihydrofolate reductase (*S. aureus*), and Dihydropteroate synthase (*E. coli*) were −77.85, −67.53, and −60.88, respectively. Throughout the duration of the MD simulations, thymol continued interacting with the binding pocket of the molecular target of each microorganism. The van der Waals (ΔE_{vdW} = −24.88, −26.44, −21.71 kcal/mol, respectively) and electrostatic interaction energies (ΔE_{ele} = −3.94, −11.07, −12.43 kcal/mol, respectively) and the nonpolar solvation energies (ΔG_{NP} = −3.37, −3.25, −2.93 kcal/mol, respectively) were mainly responsible for the formation of complexes with CYP51 (*C. albicans*), Dihydrofolate reductase (*S. aureus*), and Dihydropteroate synthase (*E. coli*).

Keywords: molecular modeling; natural products; biological activity; interaction mechanism

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1. Introduction

Essential oils (EOs), formed as secondary metabolites of aromatic plants, are biosynthesized in different plant organs such as flowers, leaves, and stems, among others [1,2]. These EOs chemical compositions and yields can change due to natural factors such as physiological, environmental, geographic, genetic, and plant evolution [3,4]. These oils play essential roles in plant protection and communication [5,6].

In the industry, EOs have been widely studied, mainly because of their potential applications as antimicrobials [7]. Over the years, the volatile compounds present in them have been employed for several pharmacological activities, such as antioxidant, anticancer, antiprotozoal, antimicrobial, anti-inflammatory, phytotoxic, and neuroprotective activities [8–12]. In a recent study, Tanrıkulu et al. [13] demonstrated that species such as *Ocimum basilicum* and *Thymbra spicata* show good antioxidant and antimicrobial activities against *Staphylococcus aureus*, *Streptomyces murinus*, *Micrococcus luteus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Yersina enterocolitica*, *Proteus algilus vulgaris*, and *Candida algilis*, demonstrating the importance of EOs in the industry.

Although the antimicrobial action of EOs is not yet fully understood, it can be attributed to their ability to permeate the cell wall of microorganisms, which arises from their diverse and synergistic chemical compositions. The hydrophobic nature of EOs enables the partitioning of lipids from the cell membrane and mitochondria, making them more permeable; consequently, ions and critical cellular components (lipids, proteins, and nucleic acids) are extravasated from the cells, leading to eventual cell death. Generally, EOs have more action on gram-negative than on gram-positive bacterium, owing to the hydrophobic components of these oils interacting with the cell membranes of the former [14–16].

Different methods have been used to assess the antibacterial and antifungal properties of EOs. The most commonly used methods are the agar disk diffusion, minimum inhibition concentration (MIC), minimum bacterial concentration (MBC), and minimum fungicidal concentration (MFC) methods. Because the agar disk diffusion method is limited by the hydrophobic nature of EOs and plant extracts, preventing their uniform diffusion through the agar medium, most authors report their obtained results via the MIC, MBC, and MFC methods [17].

Silva et al. [18] evaluated the antimicrobial activity of EOs from *Lippia thymoides* Mart. & Schauer (Verbenaceae) against *C. albicans*, *S. aureus*, and *E. coli*. In this study, we used molecular modeling approaches to further analyze the investigations performed by Silva et al. We decided to conduct these investigations in silico to deepen our understanding of the interactions of volatile compounds with molecular targets that are vital for the viability of these microorganisms. Previous publications have shown that these approaches successfully reveal how drug-receptor interactions occur [19–22]. Therefore, we used molecular docking, molecular dynamics (MD) simulations, and affinity energy calculations to investigate how thymol (Figure 1)—the major compound of the EO from *L. thymoides*—interacts with the molecular targets from *C. albicans*, *S. aureus*, and *E. coli*.

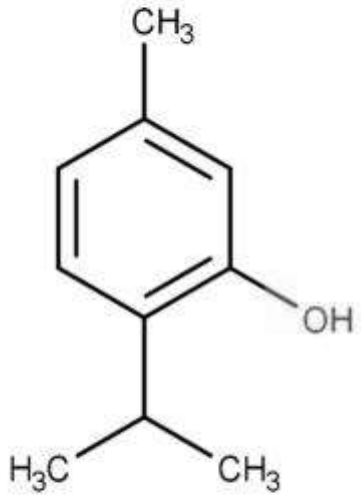

Figure 1. The molecular structure of thymol.

2. Results and Discussions

2.1. Molecular Binding Mode

According to the molecular docking results, the thymol interacted favorably with the binding sites of the proteins (Table 1).

Table 1. Scoring functions obtained with the MolDock score.

Drug Target	MolDock Score (Kcal/mol)
CYP51 (*C. albicans*)	−77.85
Dihydrofolate reductase (*S. aureus*)	−67.53
Dihydropteroate synthase (*E. coli*)	−60.88

We identified the residues with which the ligand interacted and the chemical nature of these interactions (Figure 2).

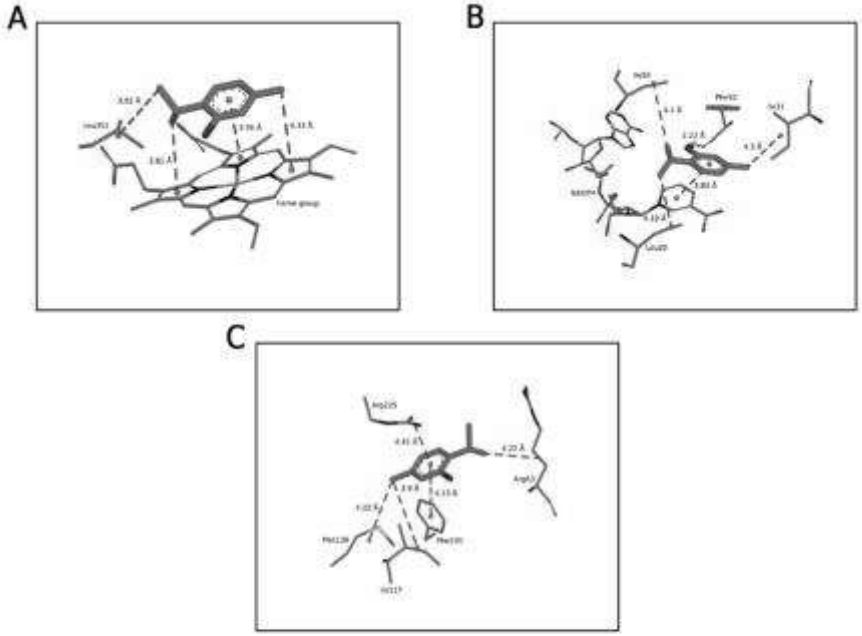

Figure 2. Intermolecular interactions of the drug-receptor systems. Molecular binding mode of thymol interacting with the active site residues of (**A**) CYP51 (*C. albicans*), (**B**) dihydrofolate reductase (*S. aureus*), and (**C**) dihydropteroate synthase (*E. coli*).

After molecular docking, we performed MD simulations of 100 ns to evaluate how thymol can interact with the active site of the molecular targets. We then performed per-residue free energy decomposition using the MM/GBSA approach to evaluate the energetic contribution of the residues to the formation of the receptor-ligand complex, as shown in Figure 3.

EOs target the membrane of microorganisms such as *C. albicans*, *S. aureus*, and *E. coli* [23]. The total or partial rupture of the membrane allows the extravasation of intracellular liquid, causing a hydroelectrolytic imbalance capable of causing the death of the microorganism [24]. In addition, after passage through the membrane, EO compounds are free to interact with molecular targets essential for bacterial and fungal viability [25]. Thymol, for example, in our results, was shown to be able to interact with CYP51, Dihydrofolate reductase, and dihydropteroate synthase proteins that are critical for parasite viability. But in addition to these molecular targets, there are reports that this same compound can interact with other proteins. Dutta et al. concluded that the thymol in the EO of Trachyspermum ammi interacted with the pocket binding of glucosamine-6-phosphate synthase. The observed molecular interactions were essentially hydrophobic [26]. Some authors have identified that thymol can interact with dihydro-folate reductase from *S. aureus* [27,28]. Barbosa et al. (2021) determined that thymol can also inhibit NorA efflux

pump inhibition in multidrug-resistant (MDR) *S. aureus* strains. In this paper, the authors evaluated that thymol establishes hydrophobic, hydrophilic, and electrostatic molecular interactions in the binding site [29]. Nagle et al. used thymol as a scaffold to perform a computer-aided drug design. Thymol and analogous compounds were investigated for their antimicrobial properties against *E. coli* and *S. aureus*. The molecular target chosen for the in silico studies was glucosamine-6-phosphate synthase GlcN-6-P synthase. The docking results showed that thymol and analogous showed a favorable interaction with the active site of the protein and their interactions were hydrophobic and electrostatic [30].

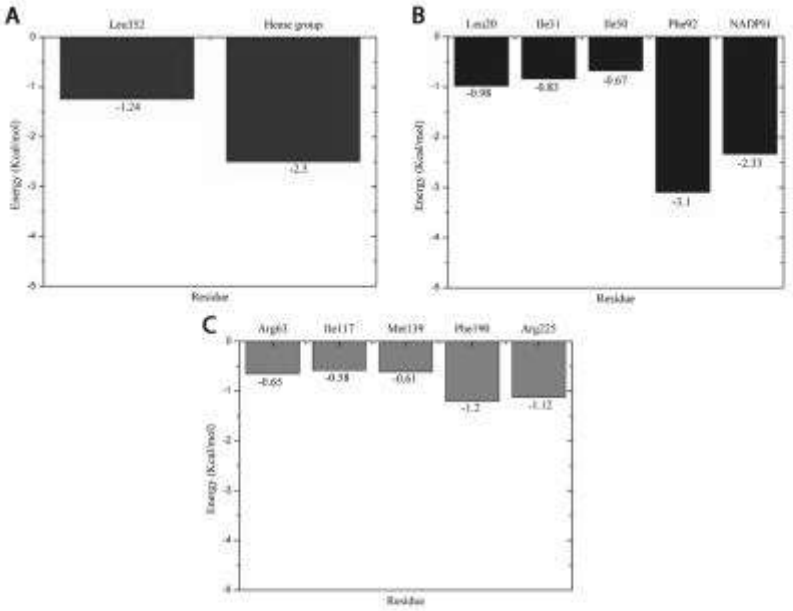

Figure 3. Per-residue free energy decomposition: **(A)** CYP51 (*C. albicans*), **(B)** dihydrofolate reductase (*S. aureus*), and **(C)** dihydropteroate synthase (*E. coli*).

At the active site of CYP51 (*C. albicans*), the thymol established interactions with the heme group and Leu352. The interactions with the heme group were hydrophobic; one was of the π-π type and the other two were of the π-alkyl type. In addition to these three interactions, the heme group participated in the binding of the compound with the binding pocket, contributing with an energy value of −2.5 Kcal/mol. With Leu352, the interaction was of the alkyl type, and throughout the simulations, this residue provided an energy value of −1.24 Kcal/mol.

In the binding pocket of dihydrofolate reductase (*S. aureus*), thymol established a hydrogen bond with Phe92, and the energy value of its interaction was −3.1 Kcal/mol. Alkyl hydrophobic interactions were formed with Ile20, Ile31, and Ile50 with energy contributions of −0.98, −0.83, −0.67 Kcal/mol, respectively. The docking simulations demonstrated that NAPH established π-π interaction with thymol reaching energy value of −2.33 Kcal/mol throughout the MD simulations.

At the active site of dihydropteroate synthase (*E. coli*), the ligand formed alkyl type hydrophobic interactions with Arg63, Ile117, and Met139 with energy contribution values for a compost binding of −0.65, −0.58 e 0.61 Kcal/mol, respectively. With Arg225, the interaction occurred with energy value of −1.12 and a π-π interaction was established between the benzene ring and the guanidino group of the amino acid. Another π–π interaction was formed between the thymol and Phe190 side chain, exhibiting an energy value of −1.2 Kcal/mol.

2.2. DM Trajectory Stability

We used root-mean-square deviation (RMSD) calculations to assess each protein's structural changes in the ligand and backbone. The RMSD of the ligand was calculated using its heavy atoms, while the Cα atoms were used for the RMSD plot of the protein backbone (Figure 4).

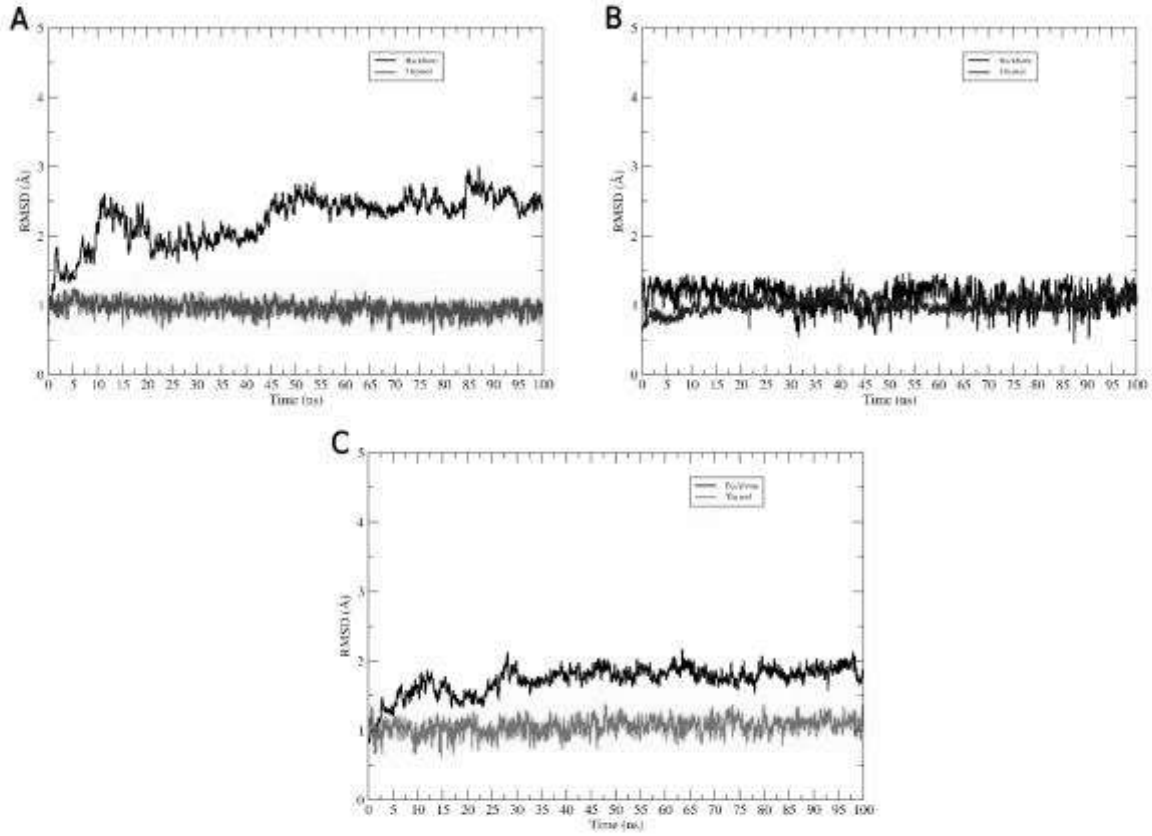

Figure 4. Intermolecular interactions of the drug-receptor systems. Molecular binding mode of thymol interacting with the active site residues of (**A**) CYP51 (*C. albicans*), (**B**) dihydrofolate reductase (*S. aureus*), and (**C**) dihydropteroate synthase (*E. coli*).

The RMSD values of the ligands in all systems showed minor variations along the trajectories. Thus, the ligand was accommodated in its binding site, preserving its interactions with the catalytic residues. The three-dimensional structures of the proteins did not undergo any change that would compromise the maintenance of the systems formed with thymol. Throughout the entire trajectory, the inhibitors remained in interaction with the proteins.

2.3. Free Energy Calculation of the Thymol-Receptor Complexes

In this study, the free energies of the thymol-receptor complexes were calculated to assess whether thymol interacts favorably with the target protein of each microorganism. For this, we used the MM/GBSA method, and the free energy and its energetic components were calculated using the last 500 frames of the MD trajectories. The results are presented in Table 2.

Table 2. Binding energy values (ΔG_{bind}) of the drug-receptor systems. ΔE_{vdW}, van der Waals contributions; $\Delta E_{electrostatic}$, electrostatic energy; ΔG_{GB}, polar solvation energy; ΔG_{nonpol}, nonpolar solvation energy. All values are in kcal/mol.

System	ΔE_{vdW}	ΔE_{ele}	ΔG_{GB}	ΔG_{NP}	ΔG_{bind}
CYP51(*C. albicans*)	−24.88	−3.94	12.15	−3.37	−20.04
dihydrofolate reductase (*S. aureus*)	−26.44	−11.07	16.03	−3.25	−24.73
dihydropteroate synthase (*E. coli*)	−21.71	−12.43	19.23	−2.93	−17.84

The main contributions to the formation of the complexes were the van der Waals (ΔE_{vdW}), electrostatic ($\Delta E_{electrostatic}$), and nonpolar solvation (ΔG_{nonpol}) energies. The negative values of ΔG_{bind} demonstrated that the interaction of the ligand with these molecular targets is favorable. Based on all our results, we can state that thymol is capable of interacting with and thereby inhibiting these molecular targets.

3. Materials and Methods

3.1. Molecular Docking

For the molecular docking study, thymol was selected since it was the major compound from the essential oil isolated from the leaves of *L. thymoides* Mart. & Schauer (Verbenaceae). Molecular docking was used to investigate the interaction between thymol and essential proteins of *C. albicans*, *S. aureus*, and *E. coli*. The proteins used as a molecular target are essential for the metabolic pathways of such microorganisms, in addition to being reported in the literature as targets for natural and synthetic products that combat these pathogens [31–34].

Thymol (2-Isopropyl-5-methylphenol) is a volatile substance present in the essential oil of several species. This substance is a monoterpenoid belongs being derived from a hydride of a p-cymene [35]. Thymol was drawn in GaussView 6 [36], and its structure was optimized via B3LYP/6-31G * [37] using the Gaussian quantum chemistry software 16 [38]. The three-dimensional structures of the proteins used as molecular targets were obtained from the Protein Data Bank (www.rcsb.org (1 January 2022). The corresponding PDB IDs are 5V5Z (*C. albicans*—SC5314) [39], 2W9H (*S. aureus*—ATCC) [33], and 1AJ2 (*E. coli*—ATCC) [34]. To study the interaction mode of this molecule with target proteins for drug action, the software Molegro Virtual Docker 5.5 [40]. The MolDock Score (GRID) function was used with a Grid resolution of 0.30 Å and radius of 7 Å, encompassing the entire crystallographic ligand-binding cavity found in the PDB of each protein. The MolDock SE algorithm was used with the number of runs equal to 10; 1500 max interactions, and max population size equal to 50. The full evaluation of 300 steps with neighbor distance factor equal to 1 and energy threshold equal to 100 was used during the molecular docking simulation. The RMSD limit for multiple cluster poses was set to <1.00 Å.

3.2. MD Simulations

The charges of the thymol atoms were calculated using the restrained electrostatic potential protocol using HF/6-31G * [41]. Parameter files were built using the Antechamber and General Amber Force Field [42]. The protonation states of the protein residues were studied from the results obtained using the PROPKA server [43].

The proteins were described by the ff14SB force field [44] in all simulations, with explicit water molecules described by the TIP3P model [45]. Each system was solvated in an octahedron periodic box with a 12 Å cutting radius in all directions from the solute. An adequate number of counterions were added to neutralize the partial charge of the systems.

The MD simulations were performed using the Amber 16 software [46,47]. Energy minimizations were performed with the sander module, while the heating, balance and production steps were performed with pmemd. CUDA.

The system's energy minimization took place in three steps. In the first stage, 2000 cycles were executed using the steepest descent method and conjugate gradient algorithms,

applying a harmonic force constant of 50 kcal·mol^{-1}·Å^{-2} about the solute. In the second stage, the harmonic force constant applied to the solute was 25 kcal·mol^{-1}·Å^{-2}, and 1000 more cycles were run using the steepest descent method and conjugate gradient algorithms. In the last step, the constraints were removed, and 1000 cycles were run using the steepest descent method and conjugate gradient algorithm.

900 ps simulations were run to increase the system temperature from 0 to 300 k. Warming up was carried out in three steps. In the first, the solute was constrained with a harmonic force constant of 25 kcal·mol^{-1}·Å^{-2}, in this way, only the solvent and counterions were free to move. In the next two steps, the harmonic force constant was removed. To balance the complexes, we run 2 ns simulations at constant temperature and without restrictions. Then, for each complex, 100 ns of MD simulation with NVT ensemble were generated.

The particle mesh Ewald method [48] was used for calculating the electrostatic interaction energies, and the bonds involving hydrogen atoms were restricted with the SHAKE algorithm [49]. The temperature was controlled using a Langevin thermostat [50] with a collision frequency of 2 ps^{-1}.

3.3. Free Energy Calculations

The free energy calculations were performed using the molecular mechanic's generalized boron surface area (MM/GBSA) method [51–53]. For these calculations, we used the last 5 ns of the MD simulation trajectories. The free energy was calculated as follows:

$$\Delta G_{bind} = \Delta H - T\Delta S \approx \Delta E_{MM} + \Delta G_{solv} - T\Delta S \quad (1)$$

where ΔG_{bind} is the free energy of the complex, resulting from the sum of the molecular mechanics energy (ΔE_{MM}), desolvation free energy (ΔG_{solv}), and entropy ($-T\Delta S$).

$$\Delta E_{MM} = \Delta E_{internal} + \Delta E_{electrostatic} + \Delta E_{vdW} \quad (2)$$

The energy of molecular gas phase mechanics (ΔEMM) can be described by the sum of the internal energy contributions ($\Delta E_{internal}$); sum of the connection, angle, and dihedral energies; electrostatic contributions ($\Delta E_{electrostatic}$); and van der Waals terms (ΔE_{vdW}).

$$\Delta G_{solv} = \Delta G_{GB} + \Delta G_{nonpol} \quad (3)$$

The desolvation free energy (ΔG_{solv}) is the sum of the polar (ΔG_{GB}) and nonpolar (ΔG_{nonpol}) contributions. The polar desolvation term was calculated using the implicit generalized born (GB) approach.

4. Conclusions

According to our results for the binding energies obtained using the MM/GBSA method, thymol interacts favorably with the molecular targets of microorganisms. The binding free energies (ΔG_{bind}) for thymol interacting with CYP51, dihydrofolate reductase, and dihydropteroate synthase proteins demonstrate that the formation of the complexes is favorable; the ΔG_{bind} values obtained were: −20.04, −24.73, −17.84 kcal/mol, respectively. During the 100 ns of MD simulations, thymol remained in interaction with the binding pockets of the enzymes. The RMSD values obtained over 100 ns of MD simulation showed that the thymol are stable in the binding pocket. The main interactions established by the ligand with the active site residues were found to be hydrophobic.

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Conflicts of Interest: The authors declare no conflict of interest.

Sample Availability: Samples of the *L. thymoides* Mart. & Schauer (Verbenaceae) are available from the Museu Emilio Goeldi.

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4. PERSPECTIVAS FUTURAS

Os resultados obtidos podem servir de base para o planejamento e desenvolvimento de futuros compostos com atividade antimicrobiana. Além de aumentar o conhecimento sobre as interações químicas estabelecidas pelo Timol durante as interações com receptores de interesse terapêutico para o combate de micro-organismos.

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