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Analysis through molecular docking of alliodorin on the enzyme laccase and its inhibitory activity on *Trametes versicolor*

Análisis mediante docking molecular del alliodorin sobre la enzima lacasa y su actividad inhibitoria sobre *Trametes versicolor*

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ABSTRACT

This study examined the use of molecular docking to predict the biological activity of a molecule by assessing its binding or interaction with a biological system (enzyme). The case in point is the compound isolated from the *Cordia elaeagnoides* tree, named alliodorin, whose potential use as a preservative or antifungal agent was determined. An *in-silico* analysis was performed on the enzyme laccase, which is the primary enzyme secreted by the wood-decaying fungus *Trametes versicolor*. Laccase is responsible for degrading key components of wood (lignin, cellulose, and hemicellulose), a mechanism through which the fungus obtains nutrients for its development. Therefore, it is hypothesized that inhibiting or inactivating laccase would inhibit the growth of *T. versicolor*. This hypothesis was later confirmed through inhibition tests using the disk diffusion method, demonstrating the inhibitory effect of alliodorin on *T. versicolor*.

KEY WORDS: Docking, in-silico, natural products, enzyme, laccase, fungal

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RESUMEN

Este estudio examinó el empleo de docking molecular, para predecir la actividad biológica de una molécula, mediante el acoplamiento/interacción de esta sobre un sistema biológico (enzima). Como es el caso del compuesto aislado del árbol de *Cordia elaeagnoides* denominado alliodorin, con el cual se determinó su posible uso como conservante o antifúngico. Esto al realizarse un análisis *in silico* sobre la enzima lacasa, la cual es la principal enzima segregada por el hongo xilófago *Trametes versicolor* y la que lleva a cabo los procesos de degradación de los principales componentes de la madera (lignina, celulosa y hemicelulosa), siendo este mecanismo por el cual el hongo obtiene nutrientes para su desarrollo. Por lo tanto, se considera que al inhibir/inactivar a la enzima lacasa se inhibe al hongo *T. versicolor*, lo cual fue demostrado posteriormente al realizarse las pruebas de inhibición mediante el método de difusión en placa con el alliodorin sobre *T. versicolor*.

PALABRAS CLAVE: Docking, *in-silico*, productos naturales, enzima, lacasa, hongos.

Introduction

Any biological action originates from the formation of a complex between a molecule and its receptor site on a biological macromolecule, such as an enzyme. Thus, the specificity of the response to a molecule is largely determined by the ability of various cellular receptors to recognize it as an agonist or antagonist, enabling the elicitation or inhibition of a response (García *et al*., 2004; Warshel, 2014). Most biological processes depend on interactions between molecular receptors and their ligands, highlighting the importance of molecular recognition in biomolecular events, such as enzyme-substrate interactions, protein-inhibitor interactions, and nucleic acid-inhibitor interactions (Mohan *et al*., 2005; Heldin *et al*., 2016).

In recent years, significant advances have been made in theoretical calculations, promoting the use and popularity of *in-silico* (computational) studies for the analysis of biologically relevant molecules. These studies have become an integral part of both industrial and academic research, focusing on the design and discovery of molecules with biological activity (Velásquez *et al*., 2013; Macalino *et al*., 2018). A notable example is molecular docking, a valuable tool for understanding biological systems and the intra- or intermolecular interactions that small molecules can have within these systems (receptor-ligand). This approach allows for the identification of specific sites and/or interactions that can lead to significant biological effects (Honarparvar *et al*., 2014; Ferreira *et al*., 2015).

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Through such theoretical analyses, valuable insights have been gained into the mechanism and action sites of the enzyme laccase, which is the primary enzyme produced by the wood-decaying fungus *Trametes versicolor*. This fungus, known for its ability to cause white rot, develops fruiting bodies or mushrooms (basidiomata) that grow throughout the tree and exhibit concentric rings of various colors, ranging from brown to white and from brown to ochre, sometimes displaying iridescence depending on the ambient humidity (Bari *et al*., 2015).

T. versicolor is one of the most important wood degraders and is considered particularly aggressive, as it can break down the main components of wood—lignin, cellulose, and hemicellulose—to obtain nutrients necessary for its growth. For these reasons, the Annual Book of Standards (ASTM, 1994) recommends it for durability testing (Archibald *et al*., 1997; Tišma *et al*., 2021).

The mechanism of wood component degradation by laccase is a redox process mediated by the four Cu atoms that compose this enzyme. These are classified into three types (T1, T2, and T3), each with distinct properties. The type 1 Cu (T1) acts as the primary electron acceptor from substrates, while the T2 and T3 Cu atoms form a trinuclear cluster (TNC) where oxygen reduction occurs (Gochev & Krastanov, 2007; Strong & Claus, 2011; Singh & Gupta, 2020).

The mechanism of this process begins with the T1 site, which receives electrons that are sequentially transferred to the T2-T3 center. Upon receiving four electrons, this center reduces a molecule of oxygen to water (Augustine *et al*., 2008; Götze & Bühl, 2016) (Figure 1). Consequently, this enzyme exhibits broad substrate specificity, as it catalyzes the oxidation of diphenols, aminophenols, methoxyphenolic acids, organic ions, diamines, arylamines, aromatic amines, and both phenolic and non-phenolic lignin dimers (Madhavi & Lele, 2009; Chiranjeevi *et al*., 2014; Janusz *et al*., 2020).

Once this redox mechanism was identified, various authors have mentioned three important sites or cavities in laccase: (1) the T1 Cu cavity, where compounds are oxidized; (2) the oxygen entry cavity, through which oxygen is transported to the TNC for reduction; and (3) the water exit cavity, which allows the release of water at the end of the mechanism. Based on this, some studies suggest that the inhibition or deactivation of laccase can be induced by molecules that dock at the T1 site and the oxygen entry site, as they can disrupt the redox mechanism (Martínez-Sotres *et al*., 2015; Götze & Bühl, 2016). In this work, a molecular docking analysis was performed with the compound alliodorin, aiming to determine the inhibitory activity of this compound on the laccase enzyme and, consequently, on the fungus *T. versicolor*.

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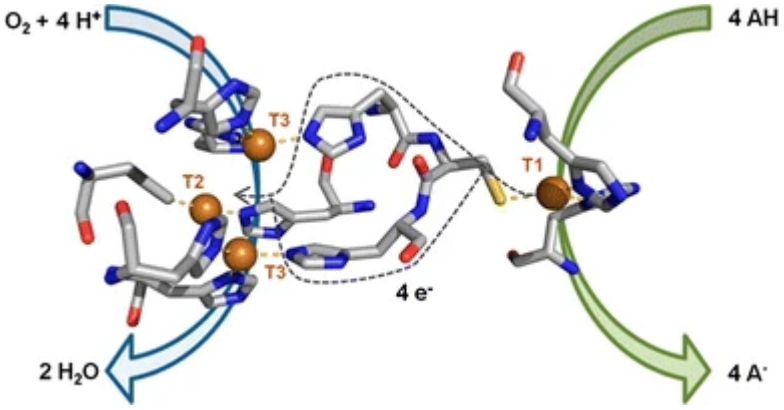

Figure 1. Mechanism of laccase, initiated by the oxidation and obtaining of electrons from substrates (lignin, cellulose, and hemicellulose) in type 1 Cu and continuing with the reduction of the electrons and oxygen in the TNC to generate wáter (Pardo & Camarero, 2015).

Source: Pardo & Camarero, 2015.

Materials and Methods

Molecular Modeling and Docking

Initially, the virtual model of the compound alliodorin was constructed using Gaussian 16 software. A conformational analysis was performed using density functional theory (DFT), employing the hybrid functional B3LYP and the 6-311G** basis set (Frisch *et al*., 2016). The three-dimensional structure of the laccase enzyme from the fungus *T. versicolor* was obtained from the Protein Data Bank (PDB), with the code 1GYC (Piontek *et al*., 2002; Roy, 2022). Molecular docking was carried out using the open-source software AutoDock 4.2 (Morris *et al*., 2009; Reynaldi *et al*., 2024).

First, a blind docking was performed using AutodockTools. Polar hydrogen atoms were added, Kollman partial charges were assigned, and the Lamarckian Genetic Algorithm (LGA) was used for the conformational search of the protein-ligand complex. This process included an initial random population of substrate conformations with up to 250 arbitrary orientations, a mutation rate of 0.02, and a crossover rate of 0.8. The simulations considered 2.5 million energy evaluations with a maximum of 27,000 generations. Each simulation was run 1,000 times, generating 1,000 conformations to calculate the binding energy of the complex and determine whether alliodorin interacts with the sites of interest identified in a free search (Morris *et al*., 1998; El-Hachem *et al*., 2017).

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Additionally, the process automatically assigns the number of allowed torsions for alliodorin, favoring more flexible poses in molecular docking. Interaction specifications in the 1GYC protein were considered, focusing on a grid box composed of 126 x 126 x 126 grid points, with a spacing of 0.375 Å between each point. It is important to note that the force field implemented by AutoDock does not include parameters for Cu atoms or their ionic forms, so the AD4_parameters.dat file was modified to include Cu parameters, following the instructions in the AutoDock 4.2 user guide. Finally, interactions with amino acids at the binding sites were visualized using BIOVIA Discovery Studio Visualizer.

Antifungal Assays

The antifungal assays were conducted using the agar diffusion method, employing potato dextrose agar (3.9%) and incorporating alliodorin at concentrations of 50, 75, 100, and 150 mg/L. After the medium solidified, it was inoculated with the mycelium of the fungus *T. versicolor* (verification number ATCC-32745) and incubated for seven days at 28 ± 2 °C. A control test was also performed, consisting of untreated agar, which was considered as 0% inhibition for comparison with the treated samples. Each treatment was repeated three times, and the percentage of inhibition was calculated using the formula according to (Rutiaga-Quiñones, 2001; Martínez-Sotres *et al*., 2015):

$$\%\ inhibicion = \left(\frac{crecimiento\ control - crecimiento\ tratamiento}{crecimiento\ control}\right) * 100$$

This formula compares the average diameter of fungal growth for each treatment with the control and thus expresses the percentage of inhibition.

Results and Discussion

Isolation of Alliodorin

The isolation of alliodorin was carried out by our research group and has been previously reported (Guevara-Martínez *et al*., 2024). This compound was obtained through extractions from the heartwood flour of the *Cordia elaeagnoides* tree, using a Soxhlet apparatus with tetrahydrofuran (THF) as the solvent. Subsequently, the solvent was evaporated to obtain the crude extract. This extract was subjected to purification in a chromatographic column with a polarity of 9:1 (Hex:AcOEt), successfully identifying a compound through thin-layer chromatography. Finally, ^{1}H and ^{13}C NMR spectroscopy were performed to characterize the compound (Figures 2 and 3).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.74 (s, 3H, H-10´), 1.75 (s, 3H, H-9´), 2.26 (t, J = 7.2 Hz, 2H, H-5´), 2.51 (q, J = 7.3 Hz, 2H, H-4´), 3.30 (d, J = 7.2 Hz, 2H, H-8´), 4.87 (s, 1H, OH), 5.14 (s, 1H, OH), 5.32 (tq, J = 8.5, 1.2 Hz, 1H, H-7´), 6.49 (tq, J = 7.3, 1.3 Hz, 1H, H-3´), 6.54 (d, J = 3.0, Hz, 1H, H-6), 6.57 (dd, J = 8.4, 3.0 Hz, 1H, H-4), 6.65 (d, J = 8.4 Hz, 1H, H-3), 9.36 (s, 1H, H-1´). ^{13}C NMR (101 MHz, $CDCl_3$) δ 9.28 (C-10´), 15.98 (C-9´), 27.09 (C-4´), 28.95 (C-8´), 37.91 (C-5´),

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113.72 (C-6), 116.22 (C-3), 116.35 (C-4), 123.10 (C-7´), 128.05 (C-6´), 135.82 (C-1), 139.58 (C-2´), 147.56 (C-5), 149.47 (C-2), 154.70 (C-3´), 196.09 (C-1´), Rf: 0.22 (Hex: AcOEt, 8:2).

With the NMR results obtained, a literature search was conducted, revealing a compound with similar ^{13}C NMR chemical shifts reported by Manners (1983), identified as 8-(2,5-dihydroxyphenyl)-2,6-dimethylocta-2,6-dienal or alliodorin (Figure 4).

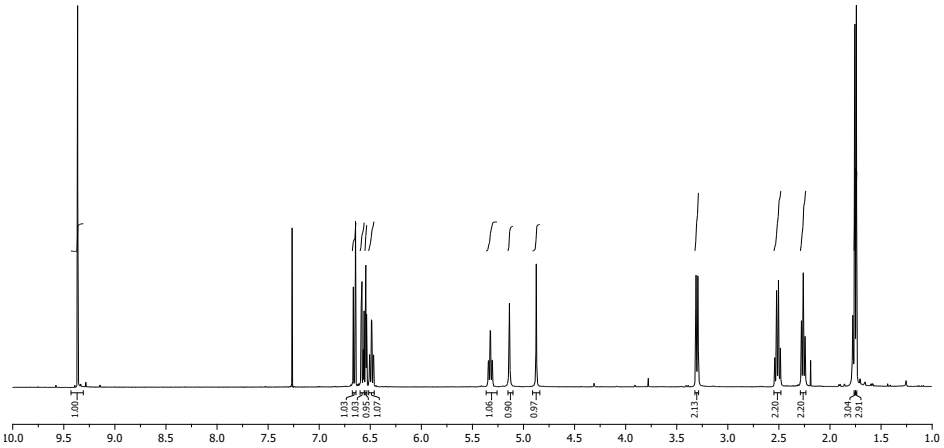

Figure 2. ^{1}H NMR spectrum of alliodorin, obtained with $CDCl_3$.

Source: Own elaboration, visualized using MestReNova.

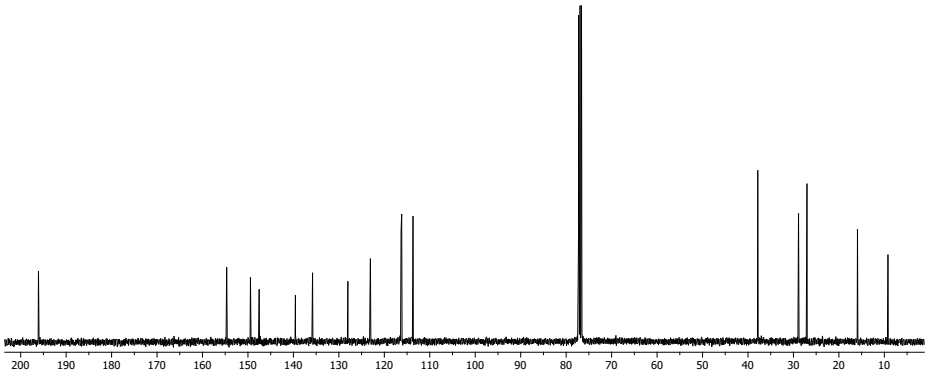

Figure 3. ^{13}C NMR spectrum of alliodorin, obtained with $CDCl_3$.

Source: Own elaboration, visualized using MestReNova.

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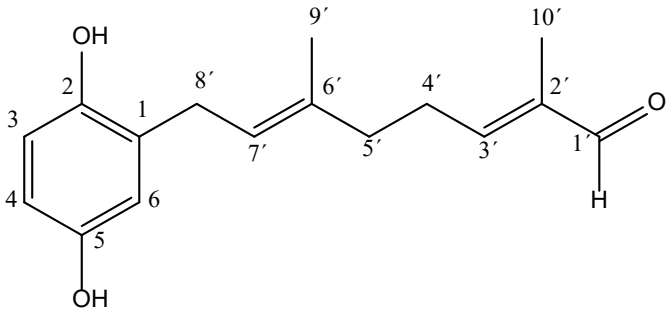

Figure 4. Compound isolated from the THF extract of *C. elaeagnoides* called *alliodorin.*

Source: Own elaboration, visualized on ChemDraw Professional.

Docking Analysis of Alliodorin-Laccase Complex

The results obtained in this work are based on the description of the laccase inhibition mechanism, as outlined in the introduction. Additionally, studies investigating laccase inhibition suggest that inhibitory compounds are small molecules capable of binding to the Cu ions of the trinuclear cluster (TNC), thereby disrupting the internal electron transfer process. Among the inhibitors that have demonstrated this activity are sodium azide (Johannes & Majcherczyk, 2000), small halides, heavy metals, and EDTA (Xu, 1996; Sun *et al*., 2023).

However, bulky natural compounds, such as medicarpin (isolated from *Dalbergia congestiflora* Pittier), have also shown the ability to inhibit laccase activity (Martínez-Sotres *et al*., 2015). In that study, it is suggested that the inhibitory effect is due to the blockage of oxygen entry to the TNC and the obstruction of the T1 cavity by medicarpin. Furthermore, the importance of binding and/or interacting with the amino acids surrounding the Cu atoms of the TNC and the T1 Cu is highlighted. These amino acids include ALA80, GLN102, SER110, HIS111, SER113, TYR116, PHE162, PRO163, ASP206, ASN208, ASP224, ASN264, PHE265, ALA393, LEU399, ASP424, HIS454, ILE455, and HIS458 (Götze & Bühl, 2016; Martínez-Sotres *et al*., 2015).

Considering the above, a blind docking was performed, allowing the ligand (alliodorin) to interact freely and randomly with the entire protein (laccase: 1GYC), without restrictions. The goal was to determine whether the ligand could interact with the previously reported sites and form interactions with amino acids of interest. The simulation results revealed three main anchoring sites for alliodorin on laccase: the T1 site, the oxygen entry channel, and a site near the TNC (Figure 5).

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Figure 5. Main anchoring sites of alliodorin in the laccase protein (1GYC): in yellow, anchoring at the T1 site (-6.19 Kcal/mol); in red, site near the TNC (-7.35 Kcal/mol); and in blue, anchoring at the entrance of the oxygen channel (-7.31 Kcal/mol).

Source: Own elaboration, visualized with BIOVIA Visualizer.

It is important to highlight that the results of this work were compared with those previously reported for medicarpin, a natural compound known for its inhibitory activity on the laccase enzyme and the fungus *T. versicolor* (Martínez-Sotres *et al*., 2012). The docking energy at the T1 site with alliodorin was -6.19 kcal/mol, lower than that reported for medicarpin, which was -7.35 kcal/mol. However, in the oxygen entry cavity, alliodorin showed a more favorable docking energy than medicarpin, with values of -7.31 kcal/mol and -7.24 kcal/mol, respectively. Additionally, another anchoring site not reported for medicarpin was identified, which presented the best docking energy for alliodorin at -7.35 kcal/mol (Figure 5).

Upon confirming the anchoring of alliodorin at the laccase sites of interest, a description of the molecular interactions was carried out. The molecular docking results showed anchoring in the T1 cavity, with notable proximity to the Cu, forming a hydrogen bond with His458 and establishing interactions through Van der Waals forces with Ile455, indicating that these amino acids surround the T1 Cu (Figure 6). Of particular interest was the anchoring of alliodorin at a site near the TNC, which resulted in Van der Waals interactions with two of the Cu atoms, as well as with the covering amino acids, such as His111, His109, and His452. Additionally, strong interactions (hydrogen bonds) were observed with Ser113, Arg157, and Glu460 (Figure 7).

Based on these findings and the reported information, it is evident that alliodorin interacts with the sites of interest for the inactivation of the laccase enzyme, offering better interactions than those observed for medicarpin. This suggests that the inhibition mechanism of alliodorin involves the obstruction of the T1 site and the inactivation of the TNC through interactions with the amino acids near the Cu atoms, similar to medicarpin but with superior results. This conclusion

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is supported by the strong interactions and hydrogen bonds observed in the complexes formed between alliodorin and the laccase enzyme.

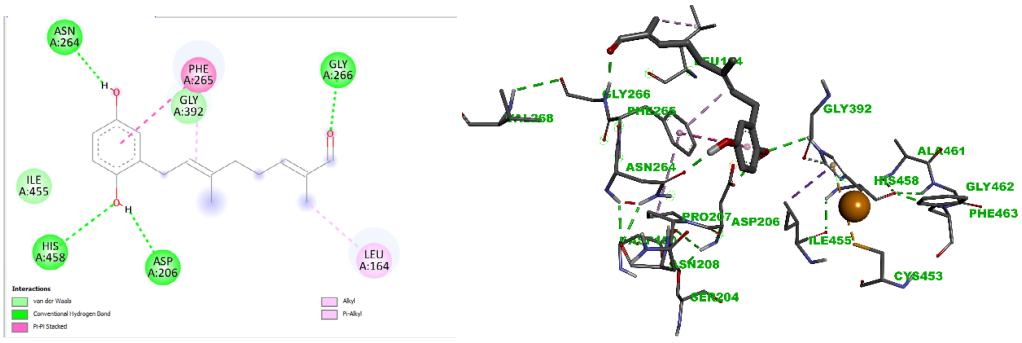

Figure 6. Interaction of alliodorin at the anchoring site near T1 with the amino acids His458 (hydrogen bond) and Ile455 (Van der Waals forces), which surround Cu T1.

Source: Own elaboration, visualized with BIOVIA Visualizer.

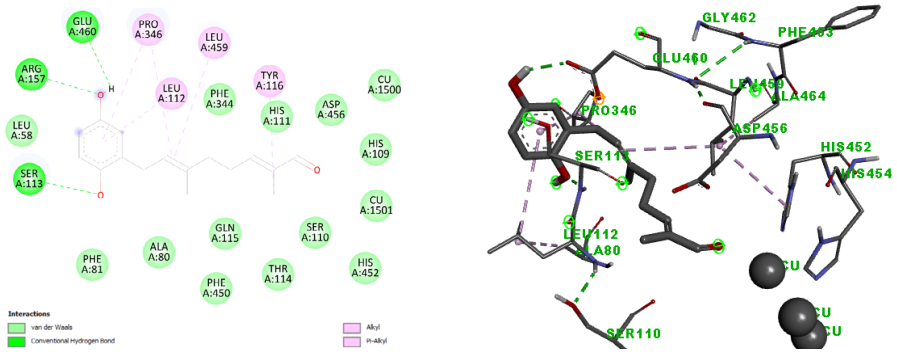

Figure 7. Van der Waals interactions of alliodorin with two copper atoms of the TNC and the amino acids His452, Ser110, and Ile455, which surround the Cu in a site very close to the TNC.

Source: Own elaboration, visualized with BIOVIA Visualizer.

Antifungal Activity

After obtaining promising results in the molecular docking of alliodorin, biological tests were conducted. This sequence was followed because natural compounds are isolated in small quantities, and a certain degree of purity must be achieved. Once the compound alliodorin

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was identified, docking was performed to verify its potential biological activity on laccase and, consequently, on the wood-decaying fungus *T. versicolor*.

The inhibition assays were carried out using the agar diffusion method, approved by ASTM standards for laboratory durability testing with wood-decaying fungi. Although the method is designed to use wood blocks inoculated with fungal mycelium on agar and evaluate their resistance through weight loss (Mielnichuk & Lopez, 2007; de Castro *et al*., 2019), this methodology has been modified to directly test secondary metabolites (extractives) and evaluate fungal mycelium growth/inhibition (Velásquez *et al*., 2006; Vega-Ceja *et al*., 2022; Vovchuk *et al*., 2024) (Figure 8).

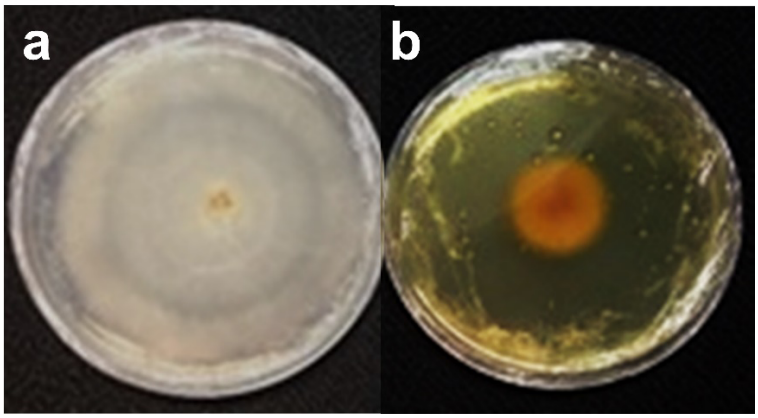

Figure 8. Inhibition tests using the plate diffusion method, (a) control growth of the fungus *T. versicolor*, (b) inhibition test at a concentration of 75 mg/L (80% inhibition).

Source: Own elaboration.

The concentrations used were based on previous studies with medicarpin (Martínez-Sotres *et al*., 2012), but lower levels were chosen because the docking results indicated greater effectiveness of alliodorin. Thus, the assays were conducted at concentrations of 50, 75, 100, and 150 mg/L. The results showed complete inhibition of the fungus at concentrations of 100 and 150 mg/L, while at 75 mg/L, the inhibitory activity was reduced to 80% on the mycelium of *T. versicolor* (Graph 1).

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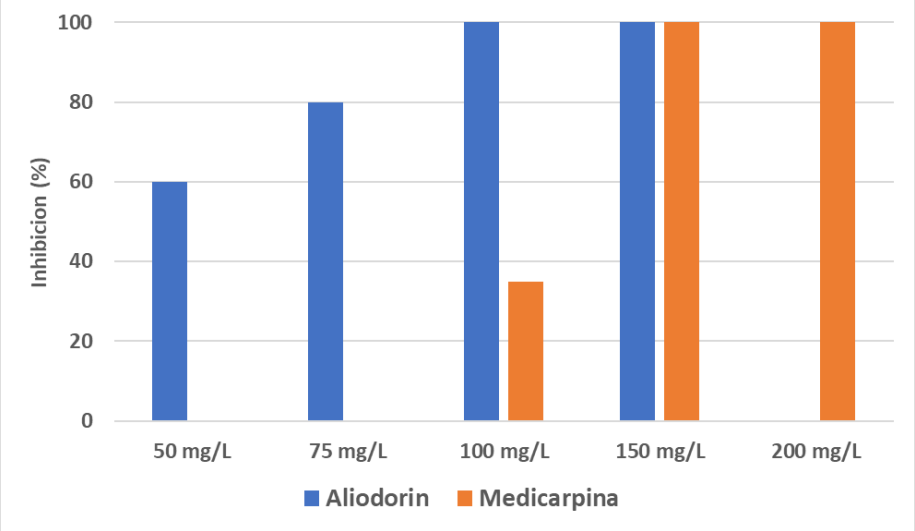

Figure 9. Inhibitory activity (%) of alliodorin and that reported for medicarpin (Martínez-Sotres *et al*., 2012).

Source: Own elaboration, visualized with Excel.

Conclusions

This research analyzed the inhibitory effect of alliodorin, a compound isolated from *Cordia elaeagnoides*, on the laccase enzyme of the fungus *Trametes versicolor*. The primary methodology of the study focused on the use of molecular docking to predict and understand this inhibitory activity.

Molecular docking was successfully employed to predict the interaction between alliodorin and laccase. This *in-silico* approach identified three main binding sites for alliodorin on the laccase enzyme: the T1 site, the oxygen channel, and a site near the trinuclear copper cluster (TNC).

The docking results suggest an inhibition mechanism where alliodorin interacts with key amino acid residues surrounding the Cu ions at these binding sites, thereby interfering with the enzyme's redox reactions and substrate binding. This aligns with existing literature on laccase inhibition mechanisms, where blocking the T1 site or the oxygen channel can inactivate the enzyme. Importantly, the *in-silico* results of this study suggest that alliodorin may be a more effective inhibitor than medicarpin, a previously studied laccase inhibitor.

The in vitrio antifungal assays using the agar diffusion method confirmed the inhibitory activity of alliodorin against *T. versicolor*. The experimental results support the *in-silico* predictions, demonstrating that alliodorin effectively inhibits the growth of *T. versicolor* at certain concentrations.

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The identification of the precise locations where alliodorin interacts with laccase was a significant achievement, as it contributes to a better understanding of the mechanism of action. The docking data allowed for the proposal of the inhibition mechanism, suggesting that alliodorin's interaction with specific sites disrupts laccase function. Additionally, the *in-silico* results guided the selection of concentrations used in the *in vitro* antifungal assays.

In summary, this study successfully integrated *in-silico* molecular docking with in vitro experiments to elucidate the inhibitory mechanism of alliodorin against *T. versicolor* laccase. The findings highlight the value of molecular docking as a valuable tool in drug discovery and the potential of alliodorin as a natural antifungal agent. Future research could focus on refining docking parameters, exploring other strains of *T. versicolor*, and investigating the potential of alliodorin in real-world applications.

Author Contributions

Conceptualization, F.G.M-P., S.J.G-M. and R.H-B.; Writing—original draft preparation, F.V-M., F.G.M-P., S.J.G-M. and R.H-B.; Writing—review and editing, F.V-M., F.G.M-P., S.J.G-M., A.Z-O. and R.H-B; Visualization, F.V-M., F.G.M-P. and S.J.G-M.; Project administration, F.G.M-P. and R.H-B.; Supervision, A.Z-O., F.G.M-P. and R.H-B.

All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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