In silico screening of antifungal phytochemicals against *Glomerella cingulata* cutinase: Identification of potential inhibitors for *Colletotrichum* spp. Pathogenicity in chilli

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Abstract

Anthracnose, caused by Colletotrichum spp., is a significant disease affecting chilli, leading to considerable agricultural losses. This study aimed to identify natural inhibitors of Glomerella cingulata cutinase, a key virulence factor of Colletotrichum spp., through in silico methods. Thirty natural compounds known for their antifungal properties were selected and screened using SwissADME for drug-likeness and ProTox-II for toxicity, resulting in 20 non-toxic candidates. Molecular docking using AutoDock Vina revealed that Carvacrol and Eugenol exhibited the highest binding affinities (-5.4 kcal/mol and -5.1 kcal/mol, respectively), comparable to the standard inhibitor DCTC (-4.6 kcal/mol). Visualization and interaction analysis using Discovery Studio confirmed stable interactions of these compounds with key active site residues.

The study concludes that Carvacrol and Eugenol are promising eco-friendly antifungal agents, providing a basis for the development of sustainable treatments for anthracnose in chilli. Further experimental validation is warranted to confirm these findings and to explore their potential in agricultural applications.

Keywords: *Colletotrichum spp*., *Glomerella cingulata cutinase*, *In silico* analysis, Natural inhibitors, Antifungal properties.

Introduction

Anthracnose, caused by various species of the fungal genus *Colletotrichum*, is a significant plant disease affecting a wide range of crops including chilli[10]. This disease leads to substantial economic losses globally due to reduced yield and quality of the affected produce. Among the various *Colletotrichum* species, *Colletotrichum gloeosporioides* and *Colletotrichum capsici* are particularly notorious for causing anthracnose in chilli. The pathogenicity of these fungi is facilitated by the secretion of cutinases, allowing the fungus to penetrate and infect plant tissues. The cutinase enzyme from *Glomerella cingulata* (the sexual stage of *Colletotrichum gloeosporioides*), specifically the apo form of cutinase (PDB ID: 3DCN), has been identified as a critical virulence factor in the infection process. Inhibiting this enzyme could significantly reduce the pathogenicity of *Colletotrichum spp.* and provide an effective means of controlling anthracnose in chilli.

Traditional methods of controlling anthracnose include the use of chemical fungicides, which pose risks to human health and the environment[3]. Therefore, there is a growing interest in exploring natural compounds with antifungal properties as safer and more sustainable alternatives. Natural compounds have been extensively studied for their antimicrobial properties. These compounds, derived from plants, offer a diverse array of chemical structures and mechanisms of action, making them promising candidates for the development of novel antifungal agents[11].

In our study, we focus on identifying potential inhibitors of *Glomerella cingulata* cutinase through an *in silico* approach, leveraging the power of computational tools to screen and to evaluate the efficacy of natural compounds. This study involves the selection of 30 natural compounds known for their antifungal properties. These compounds were chosen based on their documented efficacy, diverse chemical structures and favourable drug-likeness properties. The initial screening involved ADME (Absorption, Distribution, Metabolism and Excretion) analysis using the SwissADME web server[4] followed by toxicity prediction using the ProTox-II web server[1].

Compounds exhibiting drug-likeness and non-toxic profiles were subjected to molecular docking studies using AutoDock Vina to evaluate their binding affinity with the target cutinase enzyme. The docking results were visualized and analyzed using Discovery Studio () to identify compounds with the highest binding energies and potential inhibitory effects.

Among the compounds tested, carvacrol and eugenol emerged as the most promising inhibitors, demonstrating higher binding affinities compared to the standard inhibitor[14] N-(3,4-dichlorophenyl)-1H-1,2,4-triazole-3-carboxamide (DCTC). DCTC is a known inhibitor of cutinase enzymes and serves as a reference compound in our study. The identification of carvacrol and eugenol as potent inhibitors provides valuable insights into the potential use of natural compounds in controlling anthracnose[7]. Therefore, this study not only contributes to the understanding of natural compounds as antifungal agents but also provides a basis for

further experimental validation and development of eco-friendly antifungal treatments.

Material and Methods

Selection of Natural Compounds: A total of 30 natural compounds known for their antifungal properties were selected for this study (Table 1). These compounds were chosen based on evidence from the literature on the basis of their antifungal activity, diversity in chemical structure, natural origin and favorable drug-likeness properties. The standard compound used for comparison was N-(3,4-dichlorophenyl)-1H-1,2,4-triazole-3-carboxamide (DCTC), a known inhibitor of cutinase enzymes.

Target Protein Selection: The target protein selected for this study is the apo form of cutinase from *Glomerella cingulata*, with the Protein Data Bank (PDB) ID: 3DCN (Table 2).

Table 1
List of selected natural compounds known for their antifungal properties.

S.N.	PubChem ID	Compound name
1.	2758	1,8-Cineole
2.	65036	Allicin
3.	6654	Alpha-pinene
4.	5280443	Apigenin
5.	2353	Berberine
6.	14896	Beta-pinene
7.	1549992	Bisabolol
8.	2537	Camphor
9.	10364	Carvacrol
10.	9064	Catechin
11.	637511	Cinnamaldehyde
12.	638011	Citral
13.	323	Coumarin
14.	969516	Curcumin
15.	5281855	Ellagic acid
16.	3314	Eugenol
17.	445070	Farnesol
18.	370	Gallic acid
19.	637566	Geraniol
20.	5317570	Germacrene D
21.	5280863	Kaempferol
22.	6549	Linalool
23.	5280445	Luteolin
24.	1254	Menthol
25.	439246	Naringenin
26.	6054	Phenethyl alcohol
27.	5280343	Quercetin
28.	445154	Resveratrol
29.	17100	Terpineol
30.	6989	Thymol

Table 2
Physiochemical parameters of target protein apo form of cutinase from *Glomerella cingulate*.

PARAMETERS	Crystal structure of apo form of cutinase from *Glomerella cingulata* (PDB ID: 3DCN)
Classification	Hydrolase
Mutation(s)	No
Expression System	*Escherichia coli*
Organism(s)	*Colletotrichum gloeosporioides*
Membrane Protein	*Yes*
Experimental method	X-Ray Diffraction
Resolution	1.90 Å

Cutinases are serine hydrolases that play a crucial role in the degradation of cutin, a major component of the plant cuticle, facilitating fungal penetration and infection[9]. *Glomerella cingulata*, the sexual stage of *Colletotrichum gloeosporioides*, is a significant pathogen responsible for anthracnose in a variety of crops including chilli[12]. The 3DCN protein was chosen due to its well-characterized structure, relevance to the pathogenicity of *Colletotrichum spp. and* its role as a virulence factor. By targeting this specific enzyme, we aim to identify natural inhibitors that can impede the cutin degradation process, thereby reducing the fungal infection and offering a potential strategy for controlling anthracnose[13].

ADME Analysis: The drug-likeness and ADME (Absorption, Distribution, Metabolism and Excretion) properties of the selected compounds were assessed using the SwissADME web server[4]. Parameters such as molecular weight, hydrogen bond donors and acceptors, lipophilicity (LogP) and the number of rotatable bonds were evaluated to ensure compliance with Lipinski's rule of five[8]. Compounds that exhibited favorable ADME profiles were shortlisted for further analysis.

Toxicity Prediction: The toxicity of the selected compounds was predicted using the ProTox-II web server[1]. The server provides a comprehensive toxicity profile based on five parameters: LD50 (median lethal dose), hepatotoxicity, cytotoxicity, carcinogenicity and immunotoxicity. Compounds that were predicted to be non-toxic across these parameters were selected for molecular docking studies.

Molecular Docking: The molecular docking studies were conducted using AutoDock Vina software[6]. The target protein, *Glomerella cingulata* apo cutinase (PDB ID: 3DCN), was obtained from the Protein data bank. The protein structure was prepared by removing water molecules and adding hydrogen atoms using AutoDocktools. The three-dimensional structures of the selected compounds were obtained from the PubChem database[15] and prepared for docking by energy minimization and conversion to PDBQT format. The docking grid was centered on the active site of the cutinase enzyme and the dimensions of the grid box were set to cover the entire binding pocket. Each compound was docked into the active site of the cutinase enzyme and the binding affinity was calculated in terms of binding energy (kcal/mol). The binding energies of the natural compounds were compared with the binding energy of the standard compound DCTC.

Compounds that exhibited higher or comparable binding energies were selected for detailed interaction analysis and comparison with DCTC. The docking results were visualized and analyzed using Discovery Studio Visualizer. The interactions between the docked compounds and the active site residues of the cutinase enzyme were examined, focusing on hydrogen bonds, hydrophobic interactions and other significant contacts. Compounds with the highest binding affinities and favorable interaction profiles were identified as potential inhibitors.

Statistical Analysis: The statistical significance of the docking results was evaluated using appropriate statistical methods. The mean binding energies of the top compounds were compared using one-way Anova followed by post-hoc analysis using Tukey's test to determine significant differences between the compounds[2]. A p-value of less than 0.05 was considered statistically significant[5].

Target Prediction: Swiss target prediction (http://www.swisstargetprediction.ch) is a web-based tool for predicting the macromolecular target of a tiny bioactive chemical. Identifying the phenotypical side effects and potential cross-reactivity of tiny biomolecules is essential. In order to forecast the target of our hit compounds, the canonical smile is entered and processed in the search field.

Results

ADME Analysis: The ADME (Absorption, Distribution, Metabolism and Excretion) properties of the 30 selected natural compounds were evaluated using the SwissADME web server. The analysis included parameters such as molecular weight, hydrogen bond donors and acceptors, lipophilicity (LogP) and the number of rotatable bonds, ensuring compliance with Lipinski's rule of five. Out of the 30 compounds, 29 met the criteria for drug-likeness and were shortlisted for further toxicity analysis. Based on the boiled egg diagram from the SwissADME web server, the molecules within the yellow and white regions are assumed to have drug-likeness properties (Figure 1). BBB (Blood-Brain Barrier) Permeant Compounds are the compounds within the yellow region. These compounds are assumed to have the ability to cross the blood-brain barrier, which is a desirable property for drugs targeting the central nervous system.

Similarly, HIA (Human Intestinal Absorption) compounds are the compounds within the white region. These compounds are assumed to have good absorption in the human intestine, which is a desirable property for oral drugs. Conversely, in the boiled egg diagram, molecule 20 is outside the colored regions and hence assumed not to have drug-likeness properties.

Toxicity Analysis: The shortlisted compounds were subjected to toxicity prediction using the ProTox-II web server which provided a comprehensive toxicity profile based on five parameters: LD50 (median lethal dose), hepatotoxicity, cytotoxicity, carcinogenicity and immunotoxicity.

Compounds predicted to be non-toxic across these parameters were selected for molecular docking studies. This screening resulted in 8 non-toxic compounds being further evaluated for their binding affinities (Table 3).

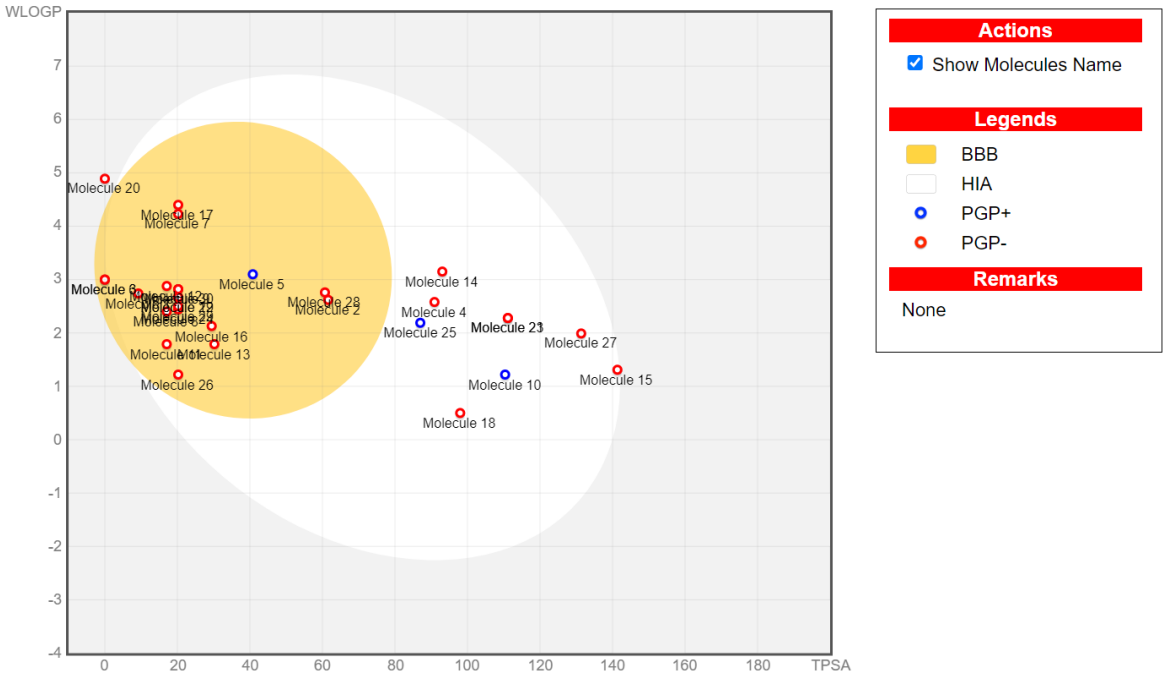

Figure 1: Boiled Egg Plot for Drug-likeness Assessment. The boiled egg plot depicts the predicted gastrointestinal absorption (HIA) and blood-brain barrier (BBB) permeation of various molecules. The X-axis represents the topological polar surface area (TPSA), while the Y-axis represents the lipophilicity (WLOGP) of the molecules.

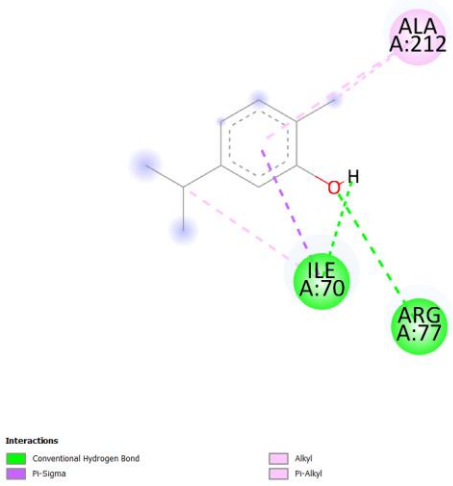

Figure 2: Visualization of molecular docking of *Glomerella cingulata* apo cutinase with Carvacrol.

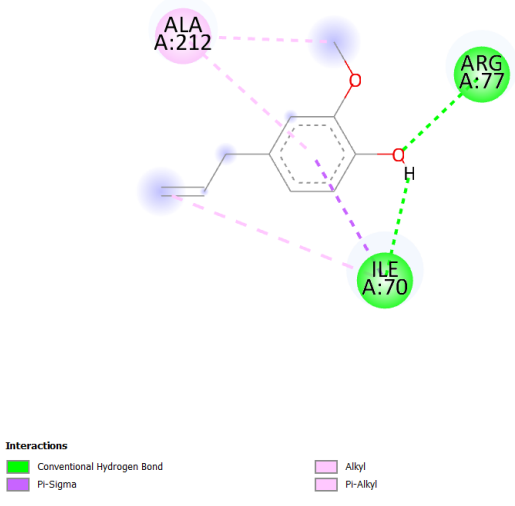

Figure 3: Visualization of molecular docking of *Glomerella cingulata* apo cutinase with Eugenol.

Table 3
Toxicity analysis of the screened natural compounds.

S.N.	Compounds	LD50 (median lethal dose)	Hepatotoxicity	Cytotoxicity	Carcinogenicity	Immunotoxicity
1.	1,8-Cineole	No	No	Yes	Yes	No
2.	Allicin	No	No	No	Yes	No
3.	Alpha-pinene	No	No	No	No	No
4.	Apigenin	No	No	Yes	Yes	No
5.	Berberine	No	No	Yes	No	No
6.	Beta-pinene	No	No	Yes	No	No
7.	Bisabolol	No	No	Yes	Yes	No
8.	Camphor	No	No	No	Yes	No
9.	Carvacrol	No	No	No	No	No
10.	Catechin	No	No	No	No	No
11.	Cinnamaldehyde	No	No	No	Yes	No
12.	Citral	No	No	Yes	No	No
13.	Coumarin	No	No	Yes	Yes	No
14.	Curcumin	No	No	No	No	No
15.	Ellagic acid	No	No	Yes	Yes	No
16.	Eugenol	No	No	No	No	No
17.	Farnesol	Yes	No	No	Yes	No
18.	Gallic acid	No	No	Yes	Yes	No
19.	Geraniol	No	No	No	Yes	No
20.	Kaempferol	No	No	No	No	No
21.	Linalool	Yes	No	Yes	Yes	No
22.	Luteolin	Yes	Yes	No	No	No
23.	Menthol	No	Yes	Yes	No	No
24.	Naringenin	Yes	No	No	No	No
25.	Phenethyl alcohol	No	No	No	Yes	No
26.	Quercetin	No	No	No	No	No
27.	Resveratrol	No	No	No	No	No
28.	Terpineol	No	Yes	No	Yes	No
29.	Thymol	No	No	No	Yes	No

Table 4
The binding energy of Carvacrol, Eugenol and DCTC with the target protein.

S.N.	Compound name	Target Protein	Binding affinity (kcal/mol)
Standard compounds			
1.	N-(3,4-dichlorophenyl)-1H-1,2,4-triazole-3-carboxamide (DCTC)	*Glomerellacingulata* apo cutinase (PDB ID: 3DCN)	-4.6
Screened natural compounds, known for their antifungal properties			
2.	Carvacrol	*Glomerellacingulata* apo cutinase (PDB ID: 3DCN)	-5.4
3.	Eugenol		-5.1

Molecular Docking: The molecular docking studies were conducted using AutoDock Vina to evaluate the binding affinities of the 8 non-toxic natural compounds with *Glomerella cingulata* apo cutinase (PDB ID: 3DCN). The binding affinities were calculated in terms of binding energy (kcal/mol) and the results are presented in table 4. Carvacrol

and eugenol demonstrated the highest binding affinities among the tested compounds, with binding energies of -5.4 kcal/mol and -5.1 kcal/mol respectively. These values were comparable to the binding energy of the standard compound, DCTC which had a binding energy of -4.6 kcal/mol.

The molecular docking results were further analyzed using Discovery Studio Visualizer to examine the interactions between the docked compounds and the active site residues of the cutinase enzyme. Figures 2, 3 and 4 illustrate the binding interactions of the target protein with carvacrol, eugenol and DCTC respectively. Carvacrol and eugenol formed stable hydrogen bonds and hydrophobic interactions with key active site residues, indicating their potential as effective inhibitors of *Glomerella cingulate* cutinase.

Statistical Analysis: The binding energies of the top compounds were statistically analyzed using one-way Anova, followed by post-hoc analysis using Tukey's test. The mean binding energies of carvacrol and eugenol were not significantly different from that of DCTC ($p > 0.05$), suggesting that these natural compounds could serve as effective alternatives to the standard inhibitor.

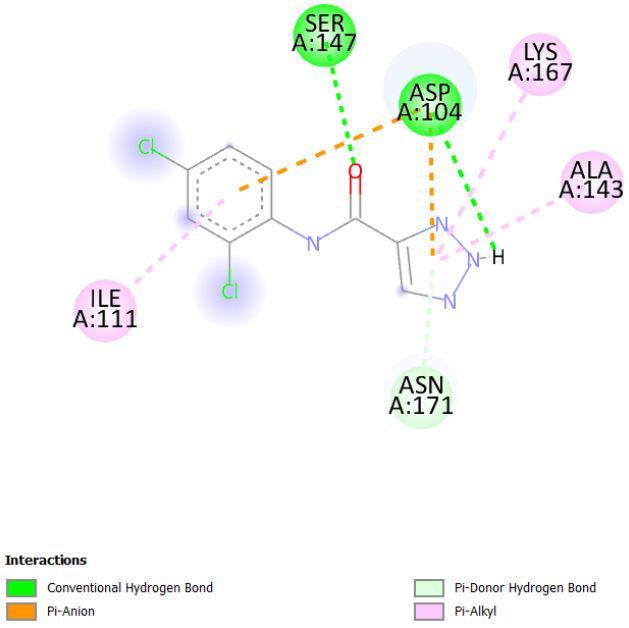

Figure 4: Visualization of molecular docking of *Glomerella cingulata* apo cutinase with the standard compound DCTC.

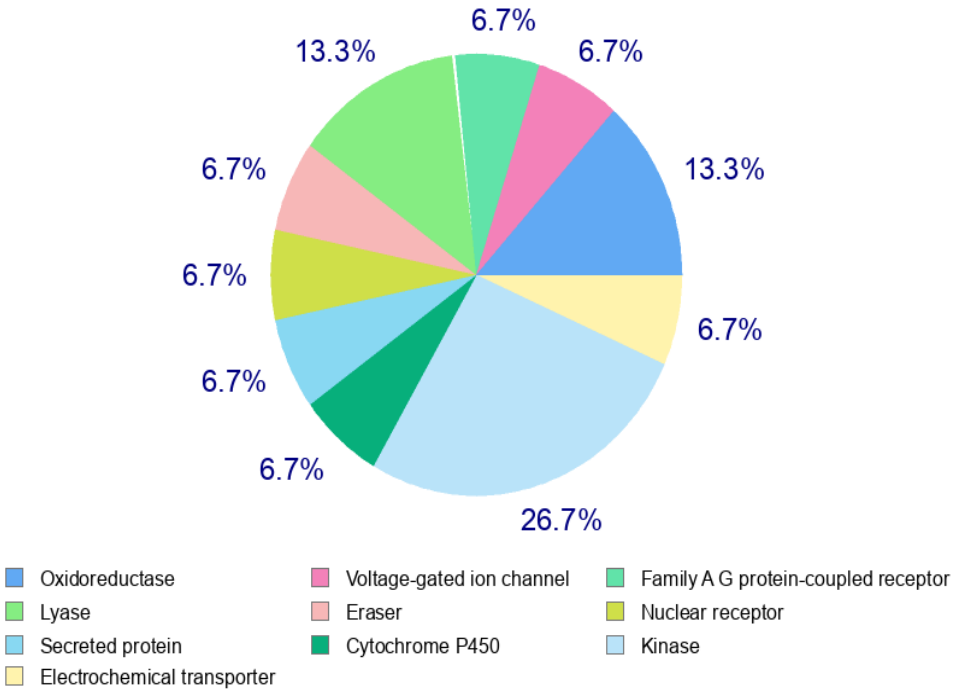

Figure 5: Molecular targets of Carvacrol.

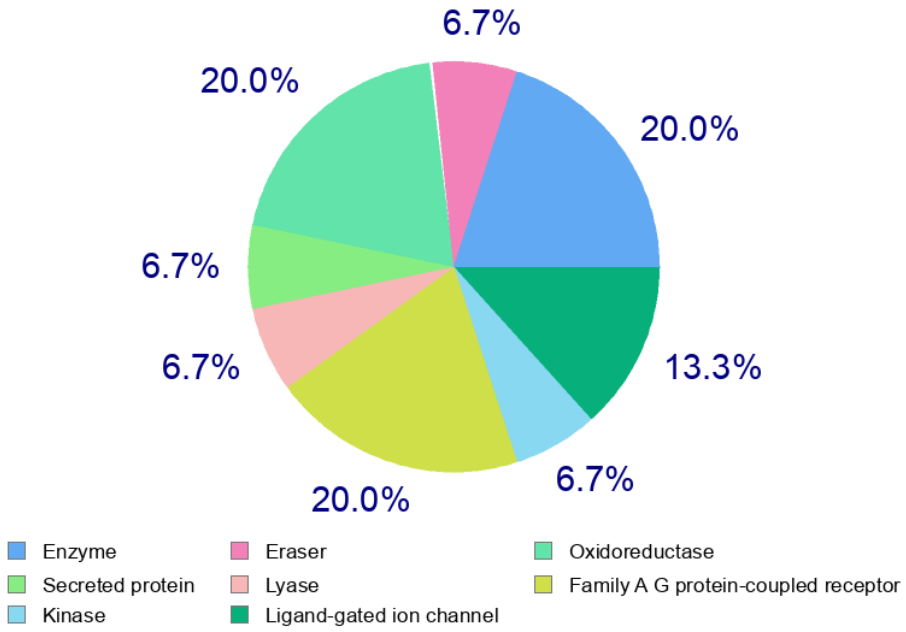

Figure 6: Molecular targets of Eugenol.

Molecular Target Analysis: After the screening and molecular docking study, the molecular targets of carvacrol and eugenol (Figure 5 and Figure 6) are investigated further. 6.7% and 20% of oxidoreductase, 26.7% and 6.7% of kinase, 13.3% and 6.7% of lyase,13.3 and 6.7% of secreted protein, 6.7% of eraser and 6.7% and 20% of family A G protein-coupled receptor were predicted for carvacrol and eugenol respectively.

Discussion

The ADME analysis ensured that the selected compounds complied with Lipinski's rule of five, indicating good drug-likeness properties. Out of the initial 30 compounds, 25 met the criteria, suggesting that these compounds have favorable pharmacokinetic properties including appropriate molecular weight, lipophilicity and hydrogen bonding characteristics. This preliminary screening is crucial as it predicts the compounds' ability to be absorbed, distributed, metabolized and excreted in the body which are essential factors for their efficacy as potential drugs.

The toxicity analysis using the ProTox-II web server further filtered the compounds based on their safety profiles. The exclusion of toxic compounds resulted in a final list of 20 non-toxic candidates, thereby reducing the risk of adverse effects and highlighting their potential as safe antifungal agents. This dual screening approach of ADME and toxicity ensures that the selected compounds exhibit antifungal activity and possess favorable pharmacokinetic and safety profiles, making them suitable for further development.

The molecular docking studies provided insights into the binding interactions between the selected natural compounds and the *Glomerella cingulate* cutinase. Carvacrol and eugenol showed the highest binding affinities, with binding energies of -5.4 kcal/mol and -5.1 kcal/mol respectively which are comparable to the binding energy of DCTC (-4.6 kcal/mol). The docking results indicated that these compounds interact effectively with key residues in the enzyme's active site, forming stable hydrogen bonds and hydrophobic interactions which are crucial for inhibiting enzyme activity.

The standard inhibitor, DCTC, is a known inhibitor of cutinase enzymes and served as a benchmark for this study. The binding energies of carvacrol and eugenol were comparable to that of DCTC, suggesting that these natural compounds have the potential to inhibit *Glomerella cingulata* cutinase with similar efficacy. The visualizations of molecular docking further supported this finding, as both carvacrol and eugenol formed stable interactions with the same active site residues as DCTC, indicating a similar mechanism of inhibition.

The identification of carvacrol and eugenol as potent inhibitors of *Glomerella cingulata* cutinase has significant implications for the development of antifungal treatments. These natural compounds offer a safer and more sustainable alternative to synthetic fungicides, aligning with the growing demand for eco-friendly agricultural practices. The ability of these compounds to effectively inhibit a key virulence factor of *Colletotrichum spp.* provides a targeted approach to controlling anthracnose, potentially reducing the disease's impact on chilli crops.

While the *in silico* findings are promising, further experimental validation is necessary to confirm the efficacy of carvacrol and eugenol as antifungal agents. *In vitro* and *in vivo* studies should be conducted to evaluate their inhibitory effects on *Glomerella cingulata* cutinase and their overall impact on fungal pathogenicity. Additionally, the potential synergistic effects of these compounds with other natural or

synthetic antifungal agents should be explored to enhance their effectiveness. The study also opens avenues for exploring other natural compounds with similar structures and properties, expanding the library of potential antifungal agents. Advances in computational biology and chemistry will continue to play a crucial role in the rapid screening and identification of such compounds, accelerating the development of novel and sustainable solutions for crop protection.

Conclusion

The present study successfully identified carvacrol and eugenol as potential natural inhibitors of *Glomerella cingulata* cutinase through comprehensive *in silico* analyses. By leveraging ADME and toxicity screening followed by molecular docking studies, these compounds demonstrated high binding affinities and favorable interaction profiles with the target enzyme, comparable to the standard inhibitor DCTC. The findings underscore the potential of Carvacrol and eugenol as eco-friendly antifungal agents, offering a promising approach in controlling anthracnose in chilli crops.

This research provides a solid foundation for further experimental validation and development of sustainable antifungal treatments, aligning with the global demand for safer agricultural practices. The study highlights the efficacy of *in silico* methods in rapidly screening and identifying natural compounds, paving the way for future explorations into natural product-based crop protection strategies.

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