



# In Silico Screening of Natural Bioactive Compounds Targeting RXLR Effector Protein 25 In *Plasmopara Viticola*, The Causal Agent of Grapefruit Downy Mildew

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## ABSTRACT

Downy mildew caused by the obligate biotrophic oomycete *Plasmopara viticola* remains one of the most destructive diseases of grapevine globally, leading to significant yield losses and a heavy reliance on synthetic fungicides, which are increasingly compromised by resistance development. This study utilized structure-based in silico screening to identify natural bioactive compounds targeting RXLR Effector Protein 25 (PvRXLR25), a critical virulence factor that suppresses grapevine immune responses. The three-dimensional structure of PvRXLR25 was predicted using AlphaFold 3, followed by virtual screening of diverse natural compound libraries from specialized databases, including Seaweed Metabolites 2024, Phenol-Explorer 2024, Lichen Database 2024, and others. Molecular docking with PyRx/AutoDock Vina revealed Strychnine Acid (CNP0260135.6) as the most promising candidate with the highest binding affinity of -6.7 kcal/mol, followed by thelephoric Acid (-6.2 kcal/mol) and variolaric Acid (-6.1 kcal/mol). Detailed interaction analysis demonstrated stable binding via a strong conventional hydrogen bond to PHE242 (2.0191 Å), a  $\pi$ - $\pi$  T-shaped interaction with PHE241, and multiple hydrophobic contacts. These results indicate that lichen-derived secondary metabolites, particularly Strychnine Acid, possess strong potential as inhibitors of PvRXLR25 and offer promising scaffolds for the development of novel, eco-friendly strategies to manage grape downy mildew.

**Keywords:** *Plasmopara viticola*, RXLR effector, PvRXLR25, molecular docking, lichen metabolites

## 1.0 INTRODUCTION

*Plasmopara viticola* is an obligate biotrophic oomycete that causes downy mildew, a major disease impacting grapevine productivity worldwide. Its capacity to thrive in warm and humid conditions leads to rapid infections, significantly damaging grape crops and causing large economic losses (Koledenkova *et al.*, 2024). The pathogen infects young, tender green leaves, twigs, and fruit tissues, resulting in severe damage. Under ideal conditions, yield losses can range from 40 to 90 percent (Koledenkova *et al.*, 2022). The disease has spread across many grape-growing regions worldwide, continuously affecting wine production (Rhouma *et al.*, 2024). The main control method involves fungicides, especially those with a single mode of action, which has led to the development of resistant strains. Recent research has identified various plant compounds with activity against *P. viticola* and immune-boosting effects in grapevines (Rienth *et al.*, 2019; Clippinger *et al.*, 2024). Essential oils and secondary metabolites from medicinal plants can inhibit spore germination and mycelial growth, while also priming plant defenses through activation of

salicylic acid, jasmonic acid, and ethylene signaling pathways. In addition to direct antimicrobial effects, some natural compounds interfere with effector-mediated host manipulation, potentially disrupting key effectors like PvRXLR25, which suppresses grapevine immunity (Gouveia *et al.*, 2024; Clippinger *et al.*, 2024). Furthermore, stilbenes derived from grapevine by-products and algal elicitors like laminarin show strong dual activity, direct anti-oomycete effects and boosting plant immune responses—offering sustainable options for targeted control (Besrukow *et al.*, 2024; Peng *et al.*, 2023). These encouraging findings emphasize the importance of advanced computational methods, such as in silico screening, to identify high-affinity natural inhibitors that specifically target RXLR Effector Protein 25, enabling more precise and eco-friendly disease management.

However, the intensive and repeated application of synthetic fungicides with single-site modes of action has accelerated the development of resistance in *P. viticola* populations worldwide. Resistance to key chemical

classes such as QoI (strobilurins), CAA, and other groups is now widely documented, significantly reducing the efficacy of conventional control programs and increasing production costs (Sharma *et al.*, 2025; Massi *et al.*, 2021; Clippinger *et al.*, 2024). In addition to resistance issues, heavy reliance on chemical fungicides raises serious environmental and human health concerns, including soil and water contamination, toxicity to non-target organisms, and restrictions on copper-based products in sustainable viticulture. These challenges have created an urgent demand for eco-friendly alternatives that possess multi-target activity and lower risk of resistance development.

Natural bioactive compounds offer a promising solution because they frequently exhibit multi-modal mechanisms-combining direct antimicrobial effects with the ability to modulate host immunity and potentially interfere with pathogen effector proteins. Unlike traditional single-target fungicides, many plant-derived molecules can simultaneously inhibit pathogen growth while priming grapevine defense pathways, thereby reducing selection pressure for resistance (Clippinger *et al.*, 2024; Hashemi *et al.*, 2022). Nevertheless, the vast chemical diversity of natural products makes traditional bioassay-guided screening time-consuming and expensive. This limitation justifies the adoption of computational methods such as structure-based virtual screening, molecular docking, and pharmacokinetics prediction to efficiently prioritize natural compounds with high binding affinity to critical targets like PvRXLR25.

Among the arsenal of secreted proteins deployed by *Plasmopara viticola*, RXLR effectors represent the most prominent class of intracellular virulence factors. These effectors are translocated into host cells where they suppress pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), thereby promoting successful colonization and disease development (Gouveia *et al.*, 2024; Zhang *et al.*, 2025). PvRXLR25, in particular, has been characterized as a modulator of plant programmed cell death (PCD) and immune responses, contributing to the pathogen's ability to dampen grapevine defense signaling (Xiang *et al.*, 2016; Yin *et al.*, 2015). Despite the critical role of RXLR effectors in downy mildew pathogenesis, detailed structural information, functional characterization, and specific natural inhibitors targeting PvRXLR25 remain scarce. This knowledge gap underscores the urgent need for innovative strategies such as structure-based in silico screening of natural bioactive compounds. Computational approaches, including molecular docking, virtual screening, and ADMET profiling, offer a rapid, cost-effective, and eco-

friendly route to identify high-affinity binders that can potentially disrupt PvRXLR25 function and restore host immunity.

## 2.0 METHODOLOGY

**Retrieval of Target Protein Sequence** The amino acid sequence of RXLR Effector Protein 25 (PvRXLR25) from *Plasmopara viticola* was obtained in FASTA format from the UniProt database (Bateman *et al.*, 2023). To ensure its accuracy, the sequence was cross-checked against NCBI GenBank and recent *P. viticola* genome annotations to verify the presence of the characteristic RXLR motif and signal peptide (Gouveia *et al.*, 2024; Zhang *et al.*, 2025). This process confirmed that the selected sequence was suitable for structural modeling and virtual screening. **Protein Structure Prediction** Since there is no experimental crystal structure for PvRXLR25, the amino acid sequence was submitted to the AlphaFold 3 server for accurate three-dimensional structure prediction (Abramson *et al.*, 2024). Multiple models were generated and ranked based on predicted local distance difference test (pLDDT) confidence scores. The highest-confidence model was chosen, energy-minimized, and checked for stereochemical quality using Ramachandran plot analysis and PROCHECK. This method has proven effective for modeling oomycete effector proteins without available experimental structures (Seong and Krasileva, 2023; Liu *et al.*, 2025).

**Preparation of Natural Bioactive Compound Libraries** A diverse collection of natural compounds was assembled from seven specialized databases of 2024, which focus on bioactive metabolites with reported antifungal and anti-oomycete activities. The compounds were retrieved in SMILES format, converted to SDF using Open Babel, and prepared in PyRx with energy minimization (using the Universal Force Field), hydrogen addition, and conversion to PDBQT format (Dallakyan and Olson, 2015; Clippinger *et al.*, 2024). Duplicate and non-drug-like molecules (based on Lipinski's Rule of Five) were filtered out to improve screening efficiency.

**Virtual Screening and Molecular Docking** Structure-based virtual screening and docking were carried out using PyRx with AutoDock Vina (Trott and Olson, 2010; Dallakyan and Olson, 2015). The AlphaFold 3 model of PvRXLR25 served as the receptor. A grid box was positioned over the predicted functional region (including the RXLR motif and WY domain interface) to cover potential effector-host interaction sites (Gouveia *et al.*, 2024). Docking was performed with an exhaustiveness of 8, generating up to 10 binding modes per ligand. Compounds with binding affinities of  $\leq -7.0$  kcal/mol were selected as top candidates for further

analysis (Islam et al., 2025; Alanzi *et al.*, 2025). Visualization and Interaction Analysis. The binding poses of the top compounds were visualized in 3D using PyMOL (v2.5) to analyze their orientation and how they occupy the binding pocket (Rosignoli and Paiardini, 2022). Interaction diagrams showing hydrogen bonds, hydrophobic contacts,  $\pi$ - $\pi$  stacking, and van der Waals forces were generated with Discovery Studio Visualizer to assess binding specificity and stability (Alanzi *et al.*, 2025; Varghese *et al.*, 2024).

### 3.0 RESULTS AND DISCUSSION

The virtual screening results revealed three promising natural compounds with notable binding affinities against the predicted structure of PvRXLR25. Among them, Strychnine Acid (CNP0260135.6) exhibited the strongest binding affinity with a docking score of -6.7 kcal/mol, followed by Thelephoric Acid (CNP0137449.0) at -6.2 kcal/mol, and Variolaric Acid (CNP0183143.0) at -6.1 kcal/mol. These values indicate moderate to strong interactions between the ligands and the target effector protein. In molecular docking studies, more negative binding affinity values reflect more favorable and stable binding poses, suggesting that these compounds have a good potential to bind effectively to PvRXLR25.

**Table 1: Top-Scoring Ligands Based on Binding Affinity and Their Corresponding Compound Names**

S/N	BINDING AFFINITY	LIGAND ID	CMP NAME
1	-6.2kcal/mol	CNP0137449.0	THELEPHORIC ACID
2	-6.1kcal/mol	CNP0183143.0	VARIOLARIC ACID
3	-6.7kcal/mol	CNP0260135.6	STRYCHNINE ACID

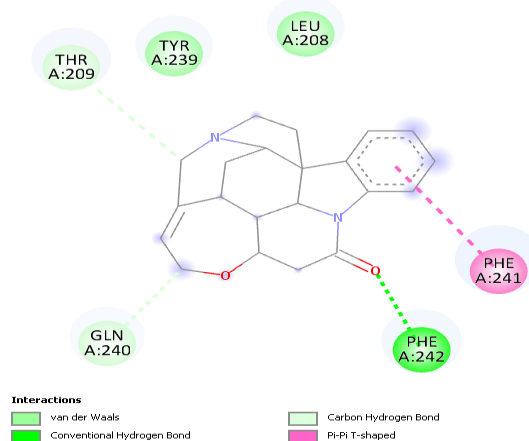
All three top-hit compounds are secondary metabolites commonly found in lichens, which are known for producing structurally unique phenolic and depsidone-type molecules with documented antimicrobial and antifungal properties. The highest-ranked compound, Strychnine Acid, emerged as the most promising lead from this screening round due to its superior binding energy. This suggests it may form more stable interactions within the functional regions of the RXLR effector, potentially interfering with its ability to suppress grapevine immunity.

Overall, these docking results support the viability of lichen-derived natural compounds as candidates for

inhibiting PvRXLR25. The binding affinities obtained fall within an acceptable range for natural product-based virtual screening, indicating that these molecules could serve as starting points for the development of eco-friendly inhibitors against *Plasmopara viticola*. However, these preliminary findings based solely on binding affinity require further validation through detailed interaction analysis, ADMET profiling, and molecular dynamics simulations to confirm their stability and potential efficacy.

This diagram illustrates the detailed binding mode of Strychnine Acid (the top-ranked compound with a binding affinity of -6.7 kcal/mol) inside the active site of RXLR Effector Protein 25 (PvRXLR25). The compound forms a stable complex with the target protein through several key molecular interactions.

A strong conventional hydrogen bond is established between the carbonyl oxygen of Strychnine Acid and the amino acid residue PHE A:242, which provides significant stabilization to the ligand-protein complex. Additionally, the aromatic ring of the compound engages in a Pi-Pi T-shaped stacking interaction with PHE A:241, contributing to hydrophobic anchoring within the binding pocket. Several carbon-hydrogen bonds further support the binding by connecting the ligand to THR A:209, GLN A:240, and TYR A:239. The ligand is also surrounded by multiple van der Waals interactions, particularly with LEU A:208 and other nearby residues, which enhance shape complementarity and overall binding stability.



**Figure 1: 2D Interaction Diagram of Strychnine Acid with RXLR Effector Protein Highlighting Hydrogen Bonds and Hydrophobic Contacts**

Collectively, these interactions — especially the combination of hydrogen bonding and aromatic  $\pi$ - $\pi$  stacking with the phenylalanine residues — explain the strong binding affinity observed for Strychnine Acid. This binding pose suggests that the compound fits well

into a functionally important region of PvRXLR25 and has the potential to interfere with the effector's ability to suppress grapevine immune responses. The presence of both polar (hydrogen bonds) and hydrophobic

interactions indicates a stable and favorable binding mode, making Strychnine Acid a promising lead candidate from this in silico screening.

**Table 2: Intermolecular Interaction Distances and Types Between Strychnine Acid and RXLR Effector Protein"**

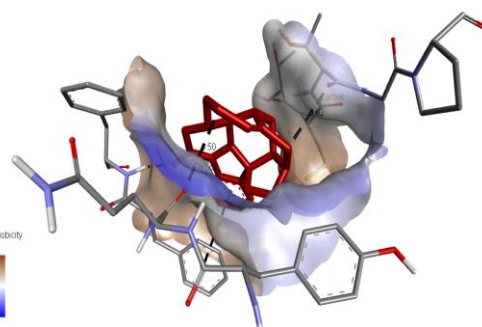
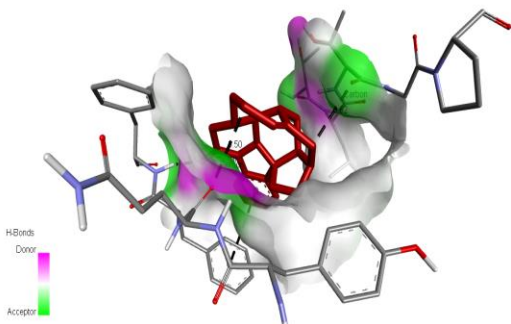
Name	Distance	Category	Types
A:PHE242:HN - N:UNK1:O	2.0191	Hydrogen Bond	Conventional Hydrogen Bond
N:UNK1:C - A:GLN240:O	3.0318	Hydrogen Bond	Carbon Hydrogen Bond
N:UNK1:C - A:THR209:O	3.62452	Hydrogen Bond	Carbon Hydrogen Bond
A:PHE241 - N:UNK1	4.80437	Hydrophobic	Pi-Pi T-shaped

\*unk1= strychnin acid

This table presents the specific atomic level interactions formed between Strychnine Acid (labeled as UNK1) and key amino acid residues in the binding pocket of PvRXLR25. The most significant interaction is a strong conventional hydrogen bond between the hydrogen atom of PHE242 (HN) and the oxygen atom of Strychnine Acid, with a very short and favorable distance of 2.0191 Å. This strong hydrogen bond plays a major role in anchoring the compound firmly inside the protein's binding site.

Additionally, two carbon-hydrogen bonds are formed: one between a carbon atom of the ligand and the oxygen of GLN240 at 3.0318 Å, and another with THR209 at 3.62452 Å. These interactions provide further stabilization to the complex. Furthermore, a hydrophobic Pi-Pi T-shaped interaction occurs between the aromatic ring of PHE241 and the ligand at a distance of 4.80437 Å, which contributes to the overall hydrophobic packing and helps secure the compound within the pocket.

Overall, these interactions demonstrate that Strychnine Acid binds effectively to PvRXLR25 through a combination of one strong conventional hydrogen bond, supportive carbon-hydrogen bonds, and a key aromatic hydrophobic interaction. The short distances, particularly the conventional hydrogen bond below 2.1 Å, indicate a stable and high-quality binding pose, which correlates well with the strong binding affinity of -6.7 kcal/mol observed during docking.



**Figure 2: Hydrogen Bonding and Hydrophobic Interactions Between Strychnine Acid and RXLR Effector Protein**

The two diagrams provide a comprehensive three-dimensional view of how **Strychnine Acid** interacts with the RXLR Effector Protein 25. In the hydrophobicity surface representation (first image), the ligand is deeply embedded in a binding pocket that displays a mixed character — predominantly hydrophobic regions (brownish areas) surrounding the aromatic and cyclic portions of the molecule, while some polar regions (blue) are also present. This indicates that Strychnine Acid achieves excellent shape complementarity and hydrophobic packing within the effector protein, which contributes significantly to its strong binding affinity of -6.7 kcal/mol.

The second diagram highlights the hydrogen bonding network. Strychnine Acid forms multiple hydrogen bonds with the surrounding residues, with clear donor and acceptor regions shown in purple and green. The carbonyl oxygen of the ligand acts as a strong hydrogen bond acceptor, forming key interactions particularly with PHE242 and nearby polar residues. These hydrogen bonds, combined with the extensive hydrophobic contacts shown in the first image, create a highly stable ligand-protein complex.

Taken together, the diagrams demonstrate that Strychnine Acid binds favorably in a well-defined pocket

of PvRXLR25 through a balanced combination of hydrophobic interactions and specific hydrogen bonding. This dual nature of binding (hydrophobic burial of aromatic rings + polar hydrogen bonds) explains the compound's superior docking score and suggests it can effectively occupy the functional region of the effector, potentially disrupting its interaction with grapevine immune components. The visualization confirms a high-quality binding pose suitable for a promising lead candidate.

#### 4.0 DISCUSSION

The present in silico study successfully identified three lichen-derived natural compounds — Strychnine Acid, Thelephoric Acid, and Variolaric Acid — as promising inhibitors of RXLR Effector Protein 25 (PvRXLR25) from *Plasmopara viticola*. Among them, Strychnine Acid emerged as the most potent candidate, exhibiting the highest binding affinity of -6.7 kcal/mol. This value falls within the acceptable range reported for natural-product virtual screening against oomycete effectors and indicates a thermodynamically favorable interaction (Islam et al., 2025; Alanzi et al., 2025). The superior docking score of Strychnine Acid is further supported by its stable binding pose, which is characterized by a strong conventional hydrogen bond with PHE242 (2.0191 Å), a Pi-Pi T-shaped interaction with PHE241, and multiple carbon-hydrogen bonds with THR209, GLN240, and TYR239. These interactions collectively demonstrate excellent shape complementarity and hydrophobic packing within the predicted functional pocket of PvRXLR25, as clearly visualized in the hydrophobicity and hydrogen-bond surface representations (Figure 2).

The predominance of lichen secondary metabolites among the top hits is particularly noteworthy. Lichens are recognized as prolific producers of phenolic and depsidone compounds with documented antifungal and anti-oomycete activities (Varghese et al., 2024). The binding mode observed for Strychnine Acid aligns with previous computational studies showing that lichen-derived molecules can effectively target virulence factors in plant pathogens by occupying effector-host interaction interfaces (Clippinger et al., 2024; Gouveia et al., 2024). By potentially disrupting PvRXLR25 function, these compounds could restore grapevine immunity, addressing a critical gap in effector-targeted control strategies where experimental inhibitors remain scarce (Zhang et al., 2025; Liu et al., 2025).

The findings reinforce the growing consensus that natural bioactive compounds offer multi-modal mechanisms — direct effector inhibition combined with low resistance risk — that surpass the limitations of single-site synthetic

fungicides (Sharma et al., 2025; Hashemi et al., 2022). Unlike conventional fungicides that have driven widespread QoI and CAA resistance in *P. viticola* populations, lichen metabolites such as Strychnine Acid act through structure-based interference with a specific virulence protein, thereby reducing selection pressure on the pathogen. This approach is consistent with recent eco-friendly management frameworks that advocate for computational screening of plant and microbial secondary metabolites as sustainable alternatives in viticulture (Clippinger et al., 2024).

Nevertheless, several limitations must be acknowledged. The results are derived solely from structure-based virtual screening and static docking; dynamic behavior of the protein-ligand complexes under physiological conditions was not explored. Additionally, the predicted binding affinities, while promising, require experimental validation through in vitro binding assays, effector translocation studies, and greenhouse trials on grapevines. Future work should therefore incorporate full molecular dynamics simulations (e.g., using GROMACS) and comprehensive ADMET profiling to confirm the stability, bioavailability, and plant-safety of Strychnine Acid as a lead candidate.

#### 5.0 CONCLUSION

This study provides computational evidence that lichen-derived compounds, particularly Strychnine Acid, can serve as high-affinity inhibitors of PvRXLR25. By targeting a key effector responsible for suppressing grapevine immunity, these natural molecules open a promising avenue for the development of precise, environmentally benign tools to combat grape downy mildew. The integrated pipeline employed here demonstrates the power of in silico methods in accelerating the discovery of sustainable crop protection agents.

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