COMPUTATIONAL SCREENING OF ALKALOIDS TO ELIMINATE *PSEUDOMONAS AERUGINOSA* BASED ON THE INHIBITION OF PROTEINS INVOLVED IN PEPTIDOGLYCAN BIOSYNTHESIS

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative pathogen responsible for severe nosocomial infections, demonstrating high adaptability and multidrug resistance. The increasing prevalence of multidrug resistant *P. aeruginosa* necessitates novel antimicrobial strategies, primarily targeting essential bacterial enzymes. This study aimed to identify potential alkaloid inhibitors targeting D-alanine:D-alanine ligase and Alanine racemase, key enzymes involved in bacterial cell wall synthesis. A machine learning-based regression model was developed using a ChEMBL database dataset to predict alkaloids' inhibitory activity against *P. aeruginosa*. Molecular docking simulations assessed the binding interactions between selected alkaloids and D-alanine:D-alanine ligase or Alanine racemase. The study identified 10-Hydroxycamptothecin as a potent inhibitor, exhibiting the highest docking affinity against D-alanine:D-alanine ligase (-8.01 kcal/mol) and alanine racemase (-8.928 kcal/mol) and favorable interactions with key residues. Absorption, distribution, metabolism, excretion, and toxicity analysis revealed high gastrointestinal absorption for all selected compounds, with some exhibiting blood-brain barrier permeability. Toxicity predictions indicated a low risk of hepatotoxicity and cardiotoxicity but raised concerns regarding potential neurotoxicity and nephrotoxicity. These findings suggest that alkaloids, particularly camptothecin derivatives, hold promise as scaffolds for novel antibiotics targeting *P. aeruginosa*. Further wet experimental validation is required to confirm the efficacy and safety selected alkaloids.

SÀNG LỌC TÍNH TOÁN CÁC ALKALOID ĐỂ LOẠI BỎ *PSEUDOMONAS AERUGINOSA* DỰA TRÊN SỰ ỨC CHẾ CÁC PROTEIN THAM GIA VÀO QUÁ TRÌNH TỔNG HỢP PEPTIDOGLYCAN

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TỪ KHÓA

Alkaloid
Alanine racemase
Kháng sinh
D-alanine:D-alanine ligase
Pseudomonas aeruginosa

TÓM TẮT

Pseudomonas aeruginosa là một mầm bệnh Gram âm chịu trách nhiệm gây ra các nhiễm trùng bệnh viện nghiêm trọng, thể hiện khả năng thích nghi cao và tính đa kháng thuốc. Việc gia tăng sự xuất hiện của *P. aeruginosa* đa kháng thuốc đòi hỏi các chiến lược kháng khuẩn mới, chủ yếu nhắm vào các enzyme thiết yếu của vi khuẩn. Nghiên cứu này nhằm xác định các chất ức chế alkaloid tiềm năng nhắm vào D-alanine:D-alanine ligase và alanine racemase, hai enzyme quan trọng tham gia vào quá trình sinh tổng hợp màng tế bào vi khuẩn. Mô hình hồi quy dựa trên học máy đã được phát triển sử dụng dữ liệu từ cơ sở dữ liệu ChEMBL để dự đoán hoạt tính ức chế của các alkaloid đối với *P. aeruginosa*. Các mô phỏng liên kết phân tử đã đánh giá sự tương tác liên kết giữa các alkaloid chọn lọc và D-alanine:D-alanine ligase hoặc alanine racemase. Nghiên cứu này đã xác định 10-Hydroxycamptothecin là một chất ức chế mạnh, thể hiện ái lực liên kết cao nhất đối với D-alanine:D-alanine ligase (-8.01 kcal/mol) và alanine racemase (-8.928 kcal/mol) cùng với các tương tác thuận lợi tại các vị trí quan trọng. Phân tích hấp thu, phân bố, chuyển hóa, thải trừ, và độc tính cho thấy tất cả các hợp chất được chọn đều có khả năng hấp thu cao qua đường tiêu hóa, với một số hợp chất có khả năng thẩm thấu qua hàng rào máu-não. Dự đoán độc tính chỉ ra rủi ro thấp về độc tính gan và độc tính tim, nhưng có những lo ngại về khả năng độc tính thần kinh và độc tính thận. Các phát hiện này cho thấy alkaloid, đặc biệt là các dẫn xuất camptothecin, có tiềm năng trở thành nền tảng cho các kháng sinh mới nhắm vào *P. aeruginosa*. Cần tiến hành thêm thực nghiệm xác thực để khẳng định sự hiệu quả và mức độ an toàn an toàn của các alkaloid đã tuyển chọn.

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1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium responsible for numerous nosocomial infections and ranks among the top three pathogens causing healthcare-associated infections. It primarily affects burn wounds, urinary tracts in catheterized patients, immunocompromised hosts, and lungs in conditions like cystic fibrosis and pneumonia [1], [2]. Due to its adaptability, biofilm formation, pili, flagella, and antibiotic resistance mechanisms, this pathogen can thrive in harsh environments, including soil, water, surfaces, and hospital equipment. *P. aeruginosa* accounts for about 7.1%-7.3% of all nosocomial infections and is a leading cause of chronic infections in hospitalized individuals [3]. It is listed as a critical pathogen in the WHO's ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species) and exhibits high prevalence of multidrug resistance (MDR) in the Middle East and North Africa (35.2% in general clinics, 39.3% in ICUs). Carbapenem-resistant *P. aeruginosa* causes significant morbidity, with high mortality rates, particularly in the Middle East and South America [4]. This pathogen develops resistance to antibiotics like aminoglycosides, quinolones, and β-lactams through altered membrane permeability, efflux pumps, and antibiotic-modifying enzymes [5], [6]. The Center of Disease Control and Prevention (CDC) has identified *P. aeruginosa* as a serious threat, with an estimated 32,600 cases, 2,700 deaths, and an annual cost of $727 million in the U.S. As MDR *P. aeruginosa* infections rise, developing new antimicrobial agents is critical [7], [8].

However, antibiotic resistance is becoming a global medical challenge, highlighting the urgent need for new antibiotics. Many plants produce antimicrobial compounds, particularly secondary metabolites, to defend against microorganisms [9]. Alkaloids, found in various plants, are nitrogen-containing compounds with antimicrobial properties and have been used to develop drugs like metronidazole and quinolones. Alkaloids are promising for antibiotic development due to their broad antimicrobial spectrum, low adverse reactions, and reduced resistance potential. They also work through mechanisms such as inhibiting cell wall synthesis, altering membrane permeability, and affecting nucleic acids and protein synthesis [10].

A key strategy in developing new antibiotics is inhibiting peptidoglycan formation. D-alanyl-D-alanine, crucial for cell wall integrity, is formed by the enzyme D-alanine:D-alanine ligase (Ddl), making Ddl a potential antibiotic target [11]. Another important enzyme is alanine racemase (Alr), which converts L-alanine to D-alanine, essential for peptidoglycan synthesis. Alr is prevalent in bacteria but rare in humans, making it an ideal target for antibiotics. Inhibiting Alr disrupts D-alanine production, compromising bacterial cell walls and leading to bacterial death. As a result, Alr inhibitors are being explored as promising candidates for new antibiotics [12]. This study will screen potential alkaloid compounds from the open libraries for *P. aeruginosa* D-alanine:D-alanine ligase and Alanine racemase inhibition activities using computational methods.

2. Materials and Methods

2.1. Data preparation

The dataset utilized in this study for evaluating inhibitors of Pseudomonas aeruginosa was sourced from the ChEMBL database, a publicly available repository of bioactive molecules with drug-like properties. A data-cleaning process was implemented to ensure high-quality input data for computational modeling. Initially, molecular structures containing salts, metal ions, or non-organic atoms were filtered out. Subsequently, all remaining compounds were standardized and converted into canonical simplified molecular-input line-entry system (SMILES) representations to maintain consistency.

An independent test dataset was obtained from the Selleckchem database for external validation. This dataset underwent the same preprocessing steps to ensure uniformity with the training data. Compounds with molecular weights ranging from 250 to 500 Daltons were selected to focus on

drug-like candidates, aligning with commonly accepted guidelines for small-molecule drug discovery. Additionally, the protonation states of the molecules were adjusted to pH 7 using OpenBabel to reflect physiological conditions [13]. Further structural modifications, including generating possible stereoisomers and tautomeric forms, were performed using RDKit functions, enhancing the dataset's chemical diversity for robust model training and evaluation.

2.2. Regression model development

A machine learning-based regression approach was employed to develop a predictive model for Pseudomonas aeruginosa inhibition. The dataset consisted of molecular structures represented in SMILES format alongside their corresponding inhibition percentages. A deep learning model based on the Chemprop framework, which leverages message-passing neural networks (MPNNs), was trained to establish quantitative structure-activity relationships (QSAR) [14].

Before training, the dataset was randomly split into three subsets: training (80%), validation (10%), and test (10%). The splitting process was scaffold-aware, ensuring that structurally similar compounds were distributed across subsets to prevent data leakage and improve generalizability. Molecular graphs were then generated from SMILES representations, serving as input for the MPNN-based model. The network was trained for 100 epochs, optimizing parameters to minimize prediction error.

Upon completing the training phase, the model was used to predict the inhibition activity of compounds from the test dataset. Only molecules predicted to exhibit inhibitory activity (% inhibition > 0) were shortlisted for subsequent computational screening, including molecular docking simulations.

2.3. Molecular docking studies

Molecular docking simulations were conducted following our previous protocol to assess the binding interactions between candidate inhibitors and two target enzymes critical for bacterial cell wall synthesis: d-alanine-d-alanine ligase and alanine racemase [15]. The three-dimensional crystal structures of these proteins were retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB ID: 8EVX and 6A2F, respectively). All non-essential molecules, such as bound ligands and water molecules, were removed to prepare the proteins for docking. Polar hydrogen atoms were added, and Kollman charges were assigned using AutoDock Tools (version 1.5.6). The processed protein structures were then saved in pdbqt format for docking simulations. For ligand preparation, the three-dimensional structures of selected molecules were generated from their SMILES representations using Open Babel 3.1.1. Hydrogen atoms were added where necessary to ensure compatibility with the docking software. Energy minimization of protein structures was performed using the Swiss-PDBViewer to refine atomic coordinates and optimize docking accuracy.

Docking simulations were carried out using AutoDock Vina (version 1.1.2), employing a semi-flexible docking approach. The protein was maintained in a rigid conformation, while ligands were allowed to adopt flexible orientations within the active site. The docking grid was carefully defined to encompass the catalytic region, ensuring that ligand placement corresponded to the observed co-ligand binding position in the crystal structures. Following docking, molecular interactions, including hydrogen bonding, hydrophobic contacts, and π-π stacking interactions, were analyzed to identify key binding residues. The docking results were visualized using BIOVIA Discovery Studio Visualizer 2020, providing insights into the structural basis of ligand binding and aiding in selecting promising inhibitors for further evaluation.

2.4. Absorption, distribution, metabolism, excretion, and toxicity studies

The drug-likeness of the compounds was evaluated based on Lipinski's Rule of Five. In this study, we used the SwissADME server to analyze the absorption, distribution, metabolism,

excretion, and toxicity (ADMET) properties of these compounds [16]. Additionally, toxicity predictions on various organs were assessed using the ProTox 3.0 Prediction Server [17].

3. Results and Discussions

3.1. Deep learning regression model

The CheMBL dataset contains approximately 23,000 compounds that exhibit inhibitory activity against *P. aeruginosa*. The distribution of % inhibition is shown in Figure 1.a. Notably, two-thirds of the compounds in the dataset demonstrate antibacterial activity, while the remaining third do not inhibit *P. aeruginosa*. This data distribution helps prevent the model from overfitting when predicting the inhibitory potential of compounds. The compounds were represented as SMILES strings, then converted into molecular graphs and processed using a neural network integrated with the Chemprop model. The dataset was divided into three subsets: one for training, one for evaluating model performance after each epoch, and one for assessing the model's predictive capability. Finally, the model was trained for 100 epochs. The results indicated a general correlation between the model's predictions and the actual inhibitory activity of the compounds against *P. aeruginosa*, based on their chemical structures (Figure 1.b).

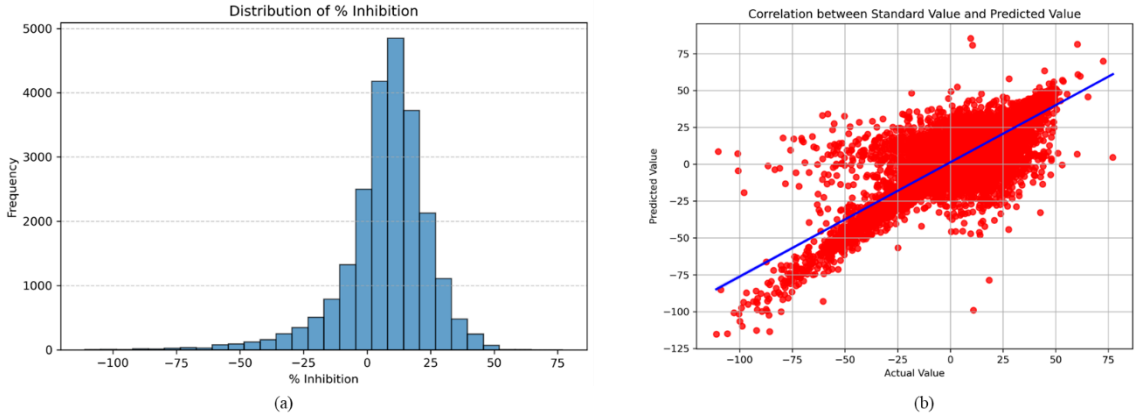

Figure 1. *Distribution of percentage inhibition in the training dataset from the ChEMBL library (a) and Correlation between the standard and predicted values of the trained model on the ChEMBL dataset (b)*

In this study, the regression model was developed to predict % inhibition values from SMILES representations of compounds. The dataset was divided into three subsets: 80% for training, 10% for validation, and 10% for final testing. The model's performance was evaluated on the independent test dataset. The model achieved a Mean Absolute Error (MAE) of 11.6% and a Root Mean Squared Error (RMSE) of 16.44% on the test set. Given that the inhibition values in the dataset ranged from -111% to 77.11%, these error metrics suggest a moderate predictive accuracy. Specifically, the MAE corresponds to approximately 6% of the total range of inhibition values, indicating that the model can capture general trends in the data. However, the higher RMSE compared to MAE indicates the presence of outliers or larger individual prediction errors, particularly in the prediction of negative inhibition values. Fortunately, the virtual screening process primarily focuses on identifying hit compounds that exhibit positive inhibition. Additionally, a subsequent structure-based virtual screening step will be applied to further enhance the accuracy and reliability of the screening workflow.

After completing the training process, the model was used to predict the antibacterial activity of alkaloids against *P. Aeruginosa* (Figure 2). The dataset of common alkaloids was obtained from the Selleckem library. This study focused on identifying potential hit compounds from small molecules. Therefore, we selected compounds with molecular weights ranging from 250 to 500 in the initial screening step. Next, we removed molecular components that could interfere with

predictions, such as metals, acids, and salts. The remaining compounds were then standardized to their protonation states at neutral pH to ensure consistency with experimental conditions. Finally, the alkaloid molecules were processed similarly to the training data and input into the regression model to predict their inhibitory activity against *P. aeruginosa*.

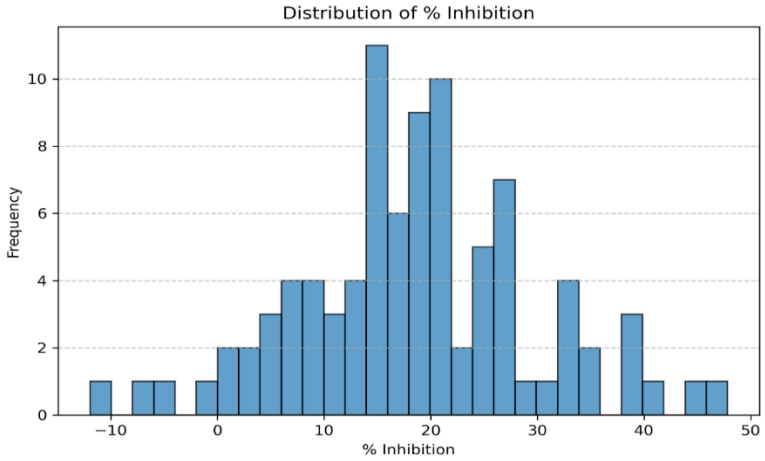

Figure 2. *Distribution of percentage inhibition alkaloids predicted by the regression model*

3.2. Molecular docking

Eighty-six alkaloids predicted by the regression model to inhibit the growth of *P. aeruginosa* were further screened for their potential to bind directly to two target proteins: D-alanine-D-alanine ligase and alanine racemase. Based on binding affinity, compounds that could inhibit enzyme activity through direct binding to the catalytic site were identified. Docking positions were selected based on the interaction sites of marketed cancer drugs that function through enzyme inhibition. The structures of the top highest-affinity compounds (docking score > -8.0 kcal/mol) are shown in Figure 3 and Figure 4. This study aims to design compounds that competitively bind to the catalytic site, thereby blocking enzyme activity and inhibiting bacterial growth.

We established a docking affinity cutoff of -8.0 kcal/mol to identify promising inhibitors. Following the screening process, one alkaloid met this criterion when docked to d-alanine-d-alanine ligase. The results, presented in Figure 3, highlight that 10-Hydroxycamptothecin formed two hydrogen bonds with Gly181 and Glu237, along with a donor-donor interaction and a Pi-Sigma interaction involving Val238 and Tyr241, respectively. The docking simulation yielded a binding energy of -8.01 kcal/mol, indicating favorable molecular interactions with the target enzyme.

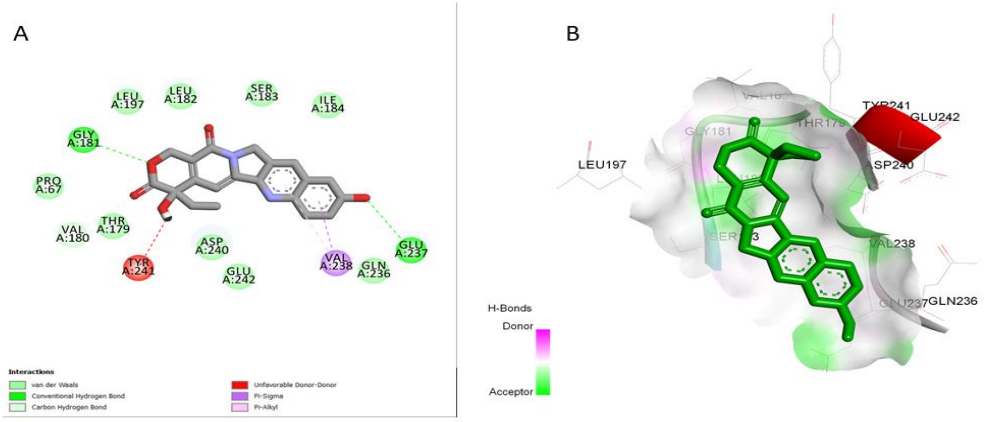

Figure 3. *2D interaction of 10-Hydroxycamptothecin and D-alanine:D-alanine ligase (pdb ID 8EVX)*

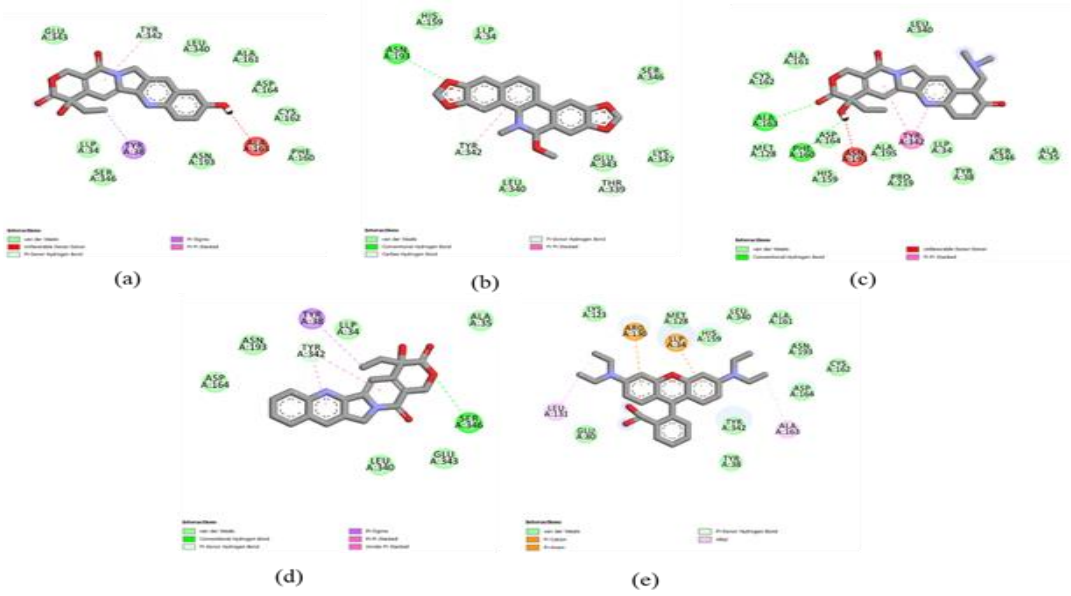

Figure 4. *2D interaction of compounds 10-Hydroxycamptothecin (a), 6-Methoxydihydrosanguinarine (b), Topotecan (c), Camptothecin (d), and Rhodamine B (e) with Alanine racemase (pdb ID 6A2F)*

A greater number of alkaloids exhibited binding affinities surpassing the established cutoff value when docked to alanine racemase, suggesting their potential as inhibitors of this enzyme. Notably, among the five highest potential compounds, including 10-Hydroxycamptothecin, 6-Methoxydihydrosanguinarine, Topotecan, Camptothecin, and Rhodamine B, 10-Hydroxycamptothecin demonstrated the strongest binding affinity among the tested compounds, with a docking score of -8.928 kcal/mol. The molecular interaction analysis revealed that this compound formed hydrogen bonds with Tyr38, Ala163, and Tyr342, indicating strong stabilization within the active site. These results suggest that 10-Hydroxycamptothecin could serve as promising alanine racemase inhibitors, potentially interfering with bacterial cell wall synthesis.

The structural features of alkaloids play a crucial role in their antibacterial potential, particularly in their ability to inhibit essential bacterial enzymes. The presence of fused ring systems, such as in camptothecin derivatives, contributes to their stability and strong interactions within enzyme active sites. The planar structure and hydrogen bond donor/acceptor groups in 10-Hydroxycamptothecin likely enhance its binding affinity by facilitating hydrogen bonding and hydrophobic interactions. Similarly, alkaloids with methoxy and hydroxyl substitutions, such as 6-methoxydihydrosanguinarine, may contribute to increased binding affinity through additional electronic and steric interactions.

The results showed that camptothecin and its derivatives exhibited notable binding affinity toward two key enzymes, D-alanine:D-alanine ligase (Ddl) and Alanine racemase (Alr), which are involved in bacterial peptidoglycan biosynthesis. Camptothecin is a well-known DNA topoisomerase inhibitor, widely studied for its role in cancer therapy. Previous studies have also highlighted the role of DNA topoisomerase as a potential antibacterial target [18]. In addition, several reports have suggested that camptothecin may affect the growth of both Gram-negative and Gram-positive bacteria [19]. However, to the best of our knowledge, this study is the first to reveal the potential binding of camptothecin derivatives, particularly 10-Hydroxycamptothecin, to two crucial bacterial enzymes, Ddl and Alr. These findings may provide a foundation for the development of a novel structural scaffold with the potential to be optimized as an antibacterial agent targeting dual enzymes involved in peptidoglycan biosynthesis.

3.3. ADMET analysis

In this study, we validate ADMET of candidates via Gastro-intestinal absorption; Per: Blood brain barrier permeability, and the interaction with major cytochrome P450 enzymes. These enzymes were selected because they are responsible for the metabolism of the majority of clinically used drugs. In particular, CYP3A4 contributes to approximately 55% of drug metabolism. CYP2D6 is involved in the metabolism of many central nervous system and cardiovascular drugs and is known for high genetic polymorphism. CYP2C9 and CYP2C19 play critical roles in metabolizing nonsteroidal anti-inflammatory drugs, proton pump inhibitors, and antiepileptic drugs. CYP1A2 is responsible for the metabolism of several xenobiotics, including caffeine and certain antidepressants. Inhibition of these enzymes can result in significant drug-drug interactions, altered drug exposure, and increased toxicity risk, making their evaluation crucial in early drug discovery.

Table 1. *ADMET Predictions of Compounds Computed by SwissADME*

Compound	Log Kp (cm/s)	GI Abs	BBB Per	Inhibitor interaction					
				P-gp Substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor
10-Hydroxycamptothecin	-7.54	High	No	Yes	Yes	No	No	No	No
6-Methoxydihydrosanguinarine	-5.61	High	Yes	Yes	Yes	Yes	Yes	No	Yes
Topotecan	-8.00	High	No	Yes	No	No	Yes	No	Yes
Camptothecin	-7.19	High	No	Yes	Yes	No	Yes	No	Yes
Rhodamine B	-7.84	High	Yes	Yes	No	No	No	No	No

GI Abs: Gastro-intestinal absorption; BBB Per: Blood brain barrier permeability; P-gp, P-glycoprotein; CYP, cytochrome-P

Table 1 shows that all five compounds are predicted to have high gastrointestinal absorption and act as P-glycoprotein (P-gp) substrates. Their log Kp values, which measure skin permeability, are all below -5.0, indicating poor skin absorption, meaning transdermal drug delivery may be ineffective. Among them, 6-Methoxydihydrosanguinarine has the least negative value (-5.61 cm/s), suggesting it has the highest skin permeability among the five compounds. In contrast, Topotecan has the most negative value (-8.00 cm/s), indicating it has the lowest skin permeability in the group. Of the five compounds, 6-Methoxydihydrosanguinarine and Rhodamine B are predicted to be capable of crossing the blood-brain barrier. Notably, Rhodamine B is the only compound predicted not to inhibit all CYP enzymes: CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4.

3.4. Toxicity prediction

Evaluating the effects of compounds on normal cells is crucial to ensure selectivity in disease treatment and to develop strategies that do not compromise patient safety. In this study, we used the ProTox 3.0 Prediction Server to evaluate their potential toxicity (Table 2). This software classifies compounds into five risk groups according to the globally harmonized system of classification of labeling of chemicals (GHS), including groups 1 to 5, respectively: fatal if swallowed ($LD50 \leq 5$), fatal if swallowed ($5 < LD50 \leq 50$), toxic if swallowed ($50 < LD50 \leq 300$), harmful if swallowed ($300 < LD50 \leq 2000$), may be harmful if swallowed ($2000 < LD50 \leq 5000$), non-toxic ($LD50 > 5000$). Notably, 10-Hydroxycamptothecin, Topotecan, and Camptothecin were all predicted to have an LD50 value of 50 mg/kg, placing them in Group 3 (toxic if swallowed). On the other hand, 6-Methoxydihydrosanguinarine had an LD50 value of 2000 mg/kg, classifying it in Group 5 (may be harmful if swallowed). Additionally, all five compounds appear safe for the liver and heart, indicating a low risk of hepatotoxicity and cardiotoxicity. However, there remains a potential risk of neurotoxicity, nephrotoxicity, and respiratory toxicity, which should be carefully considered. Although these compounds are predicted to inhibit essential bacterial enzymes, their potential cytotoxicity necessitates further biological evaluation. Future experimental validation should focus on neurotoxicity, nephrotoxicity, and respiratory toxicity to thoroughly assess the safety profile of these compounds. It can be explained that alkaloids often exhibit neurotoxicity and respiratory toxicity due to their nitrogen-containing heterocyclic structures, which allow them to interact with

neurotransmitter receptors and ion channels in the nervous system. This structural similarity can disrupt normal neuronal signaling, leading to CNS depression, seizures, or respiratory failure. Further studies focusing on structure–toxicity relationships and modification of toxic functional groups are needed to minimize adverse effects while retaining therapeutic potential.

Table 2. *Toxicity of selected compounds predicted by ProTox 3.0 Prediction Server*

Compound	Predicted LD50 mg/kg	Predicted Toxicity Class	Organ toxicity (Probability)				
			Hepatotoxicity	Neurotoxicity	Nephrotoxicity	Respiratory toxicity	Cardiotoxicity
10-Hydroxycamptothecin	50	3	Inactive (0.84)	Active (0.74)	Active (0.61)	Active (0.71)	Inactive (0.74)
6-Methoxydihydrosanguinarine	2000	4	Inactive (0.65)	Active (0.60)	Active (0.51)	Active (0.54)	Inactive (0.72)
Topotecan	50	3	Inactive (0.92)	Active (0.85)	Active (0.57)	Active (0.90)	Inactive (0.77)
Camptothecin	50	3	Inactive (0.83)	Active (0.74)	Active (0.61)	Active (0.70)	Inactive (0.73)
Rhodamine B	887	4	Inactive (0.67)	Active (0.57)	Active (0.60)	Active (0.83)	Inactive (0.66)

4. Conclusion

This study explored the potential of alkaloid compounds as inhibitors of D-alanine:D-alanine ligase and Alanine racemase in *P. aeruginosa* using a combination of machine learning and molecular docking approaches. The results identified 10-Hydroxycamptothecin as the most promising candidate, exhibiting strong binding affinity to Ddl and Alr through significant interactions with catalytic residues. ADMET and toxicity analyses highlighted favorable pharmacokinetic properties while indicating areas requiring further investigation. The structural characteristics of alkaloids suggest their potential as lead compounds for developing new antibiotics against MDR *P. aeruginosa*. Future research should focus on validation *in vitro* and *in vivo* to confirm their antimicrobial activity and safety. Given the rising threat of antibiotic resistance, these findings provide valuable insights into developing novel antimicrobial agents to combat persistent bacterial infections.

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