JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IDENTIFICATION OF ANTIBACTERIAL AGENTS AGAINST KLEBSIELLA PNEUMONIAE TARGETING THE CTX-M-15 PROTEIN USING INTEGRATED STRUCTURE MODEL-BASED VIRTUAL SCREENING METHODS

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https://doi.org/10.55251/jmbfs.11876

ARTICLE INFO

Received 11. 6. 2024
Revised 25. 9. 2024
Accepted 30. 9. 2024
Published 1. 10. 2024

Regular article

OPEN ACCESS

ABSTRACT

Background: The spread of carbapenem-resistant Gram-negative bacteria, particularly those having the *bla* $_{CTX-M-15}$ gene, has stimulated a global challenge for β-lactam antimicrobial effectiveness. This study aims to identify commercially available compounds that demonstrate strong antibacterial properties with high efficacy and low toxicity to combat the increasing problem of antibiotic resistance. Methods: Virtual screening and molecular docking techniques were employed to generate a pharmacophore model based on the molecular structure. Eleven potential compounds were selected based on their IC_{50} values. Subsequently, a molecular docking approach was employed to select the best three small molecules for further comprehensive investigation. Moreover, absorption, distribution, metabolism, and excretion (ADME) and toxicity analysis ensured that it can be used as a drug without the effect of health. Finally, molecular dynamic (MD) simulations and generalized Born model and solvent accessibility (MMGB/SA) were carried out to evaluate the stability of these compounds against the receptor. Results: Three selected compounds, specifically ZINCCID (ZINC94211493, ZINC20528448, and ZINC04331046), have exhibited strong binding affinities of -8.4, -8.1, and -7.7 kcal/mol, respectively. This has been predicted as a potential competitive inhibitor targeting CTX-M-15. However, further assessment through experimental lab studies is required to evaluate the efficacy of these compounds. Conclusion: Initially, a structure-based model was constructed, and subsequently, molecular docking, MD simulation, ADMET analysis, and MMGB/SA were performed. Through the A-to-Z virtual screening process, the top three natural compounds were identified as potential lead molecules in the development of novel drugs targeting CTX-M-15 for combating Klebsiella infections.

Keywords: *Klebsiella*; Structure-based; Molecular docking; Pharmacophore modeling; CTX-M-15 protein; ADMET; MMGB/SA, MD simulation

INTRODUCTION

The global rise in resistance to multiple antimicrobial agents among Gram-negative bacteria, particularly in healthcare-associated infections, is a growing public health concern (**Breijyeh *et al.*, 2020**). In 1983, Germany made the groundbreaking discovery of the initial plasmid-encoded β-lactamase with the ability to counteract extended-spectrum β-lactam antibiotics. This enzyme, associated with a variant of the SHV-1 enzyme found in Klebsiella pneumoniae, marked the beginning of a series of similar hydrolytic enzymes such as TEM-1 and TEM-2. In 1989, CTX-M enzymes were detected in Germany and Argentina, and their prevalence has since increased worldwide, making them the most prevalent extended-spectrum β-lactamases (ESBLs). CTX-M β-lactamases, classified as ESBLs, are highly prevalent in various bacterial strains. These types of enzymes have been classified into four groups, namely CTX-M-1, CTX-M-8, CTX-M-2, and CTX-M-9, based on comparisons of amino acid sequence. Among these enzymes CTX-M variants, the CTX-M-15 belongs to a specific group known for its heightened ceftazidime-hydrolyzing activity (**Ali *et al.*, 2018**). Notably, these enzymes have been found not only in healthcare settings but also in community settings (**Altinkum *et al.*, 2013**).

The blaCTX-M-15 gene encodes the β-lactamase enzyme CTX-M-15, which possesses the ability to hydrolyze cefotaxime. This gene is commonly found on plasmids in different bacteria, including Klebsiella pneumoniae **(Prakoso *et al.*, 2024)**. The extensive spread of bacteria carrying the CTX-M-15 gene poses a global threat in both hospital and community settings, as these bacteria exhibit resistance to multiple clinically important antibiotics (**Hyeon *et al.*, 2023; Rehman *et al.*, 2015**). The prevalence of CTX-M-15 in *K. pneumoniae* is a significant concern due to its strong ability to hydrolyze and deactivate extended-spectrum cephalosporins, including cefotaxime and ceftazidime (**Lee *et al.*, 2011**). As a result, the emergence of resistance in the bacterium against these crucial antibiotics reduces the available treatment options and raises the probability of treatment failure. Consequently, there is a need to explore alternative therapeutic approaches and options to address this issue (**Al-Masaudi *et al.*, 2020; Farhadi *et al.*, 2018; Malik *et al.*, 2023; Qaeed *et al.*, 2023a, b**).

Several modern strategies for identifying new therapeutic leads rely on the tertiary structure of receptors (**Farhadi *et al.*, 2015; Farhadi *et al.*, 2016**). In order to identify biologically active compounds, theoretical and computational drug design methods are utilized to define new hits or leads. Computer-aided drug design (CADD) tools, including virtual screening, pharmacophore modeling, molecular docking, and dynamic simulation, are extensively employed in drug discovery, analysis, and development to identify the most promising compounds from a vast pool of possibilities (**Aljahdali and Molla, 2021; Molla, and Aljahdali, 2023; Aljahdali *et al.*, 2023**). The utilization of a structure-based pharmacophore model

aims to identify analogous active molecules that target a specific protein. In-silico molecular docking techniques are utilized to evaluate the binding affinity of numerous compounds on a large scale **(Aini *et al.* 2023 a, b; Turista *et al.* 2023; Widyananda *et al.* 2023; Herdiansyah *et al.* 2024).**

Docking analysis provides insights into molecular docking-based scoring functions and interactions (**Farhadi *et al.*, 2016; Farhadi and RS, 2017; Panda *et al.*, 2019; Vijayalakshmi *et al.*, 2014**). Although this process is relatively straightforward, it can be time-consuming, yet it remains a reasonable approach for predicting compounds against specific proteins. To assess the toxicity and efficacy of the compounds, absorption, distribution, metabolism, and excretion (ADME) screening is conducted. Molecular dynamic modeling is employed to verify the stability of the drug in relation to the target protein. Significant advancements have been made in computational approaches to predict the efficacy and toxicity of compounds (**Islamiati *et al.*, 2022; Mawaddani *et al.*, 2022; Jakhmola *et al.*, 2022**). Molecular dynamics simulations have played a crucial role in confirming the stability of therapeutic candidates when interacting with specific target proteins (**Aljahdali *et al.*, 2021; Abdulrazik *et al.*, 2022**).

Despite the challenges in developing new drugs, there is a pressing need to explore novel protein classes and conduct extensive research to discover bioactive compounds. Consequently, this study aimed to use structure-based drug discovery strategies, such as virtual screening, pharmacophore modeling, molecular docking, ADMET, and dynamic simulation, have been employed to identify a natural antagonist against the CTX-M-15 protein for the treatment of Klebsiella infections. The introduction of novel drug-like compounds through these strategies holds promise for the discovery of effective drugs against bacterial infections **(El Omari *et al.* 2021; Ansari *et al.* 2022; Muthukrishnan *et al.* 2022).**

MATERIALS AND METHODS

Methods

Structure-based

Structure-based Pharmacophore Modelling (SBPM).

The methods employed in this study involved the development of pharmacophore-based structural features targeting a specific protein. The process included the estimation of potential active sites and the identification of co-crystallized ligands. The active antagonists of the CTX-M-15 protein were determined through target annotations using the ChEMBL database and related literature sources **(Makhouri *et al.*, 2018; Molla *et al.*, 2023**). Subsequently, a meticulous selection process identified a set of eleven active antagonists for generate a SBPM (Table 1). These compounds were obtained by searching the comprehensive ChEMBL database (**https://www.ebi.ac.uk/chembl/ 2024**) and conducting an extensive literature review to identify similar molecules. Docking studies were carried out using PyRx AutoDock Vina software, and compounds were selected based on their scoring function (**Molla *et al.*, 2023; Obaid *et al.*, 2023**). The compounds with the highest scores, indicating maximum binding affinity (kcal/mol), were chosen to create the model. To observe the interaction between the top scoring compounds and the CTX-M-15 protein, natural compound retrieval hits were considered(**Molla *et al.*, 2023)**. The molecular design software LigandScout 4.4 Essential was employed to generate SBPM. Additionally, the LigandScout 4.4 software was utilized to examine the interactions between inhibitors and crucial amino acids located at the protein's active sites. The pharmacophore model revealed various features such as hydrophilic and hydrophobic regions, hydrogen bond donors, charge transfer, and hydrogen bonds, which exhibited dissimilarities compared to the ligand-receptor complex. Additionally, sequential algorithms were applied to determine the hybridization state, binding pattern, the number of aromatic rings, and distance between the binding site and the receptor molecule. Consequently, the hydrophilic characteristics were not only eliminated from the CTX-M-15 protein but were also selectively incorporated or excluded as attributes at the active site utilizing the LigandScout 4.4 Essential software **(Bernal and Coy-Barrera, 2015).**

Pharmacophore model validation

During the process of validating the pharmacophore, potential features associated with the interaction between specific proteins and ligands were identified. To validate the protein-ligand complexes, the widely used web-based database DUD-E decoys was employed. This database helps distinguish active compounds and assesses their performance by screening a set of 11 known actives and their corresponding 500 decoy compounds (**Haider *et al.*, 2020**). From the ChEMBL database (**https://www.ebi.ac.uk/chembl/2024**), 19 antagonists that were identified as active against the CTX-M protein were selected. These compounds were further validated experimentally before being chosen for subsequent experiments. In order to screen the DUD-E database, LigandScout 4.4 was utilized to convert it to the .idb format **(Molla *et al.*, 2023).**

Dataset generation for pharmacophore-base screening

A virtual screening approach was employed to identify active and novel compounds, relying extensively on the pharmacophore model. The ZINC chemical database (**https://zinc.docking.org/2024**) played a crucial role in our search for potential lead compounds. Its extensive resources and data were instrumental in guiding our exploration and identifying promising compounds for further investigation **(Pal *et al.*, 2019**). The compound of interest was retrieved from the database by inputting its name, structure, or chemical ID. Various physical and chemical properties, such as 3D and 2D structures, boiling and melting points, were examined and recorded. The identification of the desired compound was based on factors such as biological information, molecular weight, and crystal structure. Priority was given to compounds with the most similar characteristics that could readily interact with the specified protein. The selection of compounds with the highest matches was determined by counting the potential hits. Subsequently, the ZINC natural product library, specifically the ZINC-Pharmer server (**http://zincpharmer.csb.pitt.edu/pharmer.html/2024**), was employed for the initial screening of the CTX-M target.

Pharmacophore-based virtual screening

A structure-based pharmacophore characteristic that had undergone validation was utilized to generate a database using ZINCPharmer. The validation of the interaction between the ligand and protein in a three-dimensional model was confirmed utilizing Ligand Scout 4.4 Essential. Moreover, the file format was transformed into a designated (idb) format. All chosen compounds were extracted from the database to produce a pharmacophore feature via virtual screening. (**Sangande *et al.*, 2020**). The selected relevant pharmacophore characteristic was used to identify a subset of compounds by excluding certain features that appeared more than twice. The pharmacophore feature score was then applied to fit the hit compounds, which underwent further validation.

Molecular docking

Ligand and protein preparation

The in-silico drug design procedure was utilized to modify the molecular structure for enhanced compatibility with protein preparation. Before the docking process, improvements were made to the protein crystal structure by introducing additional and refined hydrogen bonds, addressing atomic collisions, and applying other methods not encompassed in the X-ray crystal structure refinement. The 3D structure of the CTX-M-15 protein was experimentally determined through X-ray diffraction, yielding a resolution of 1.50 and R-values of 0.147 (free score), 0.132 (Work), and 0.133 (Observed). The protein structure was obtained from the Protein Data Bank (PDB) with the identification code (PDB ID: 4XUZ (**Makhouri and Ghasemi, 2018**). To facilitate the construction of the desired protein structure for X-ray crystallography, certain components such as metal ions, water molecules, and cofactors were eliminated. Subsequently, both polar and non-polar hydrogen bonds were incorporated, and the Gasteiger charge calculation was executed using AutoDockTools (ADT). The selected hit compounds from Ligand Scout 4.4 Essential underwent subsequent energy minimization and optimization procedures, which included necessary adjustments to bond angles utilizing the default settings of the Universal Force Field (UFF) for each ligand (**Valasani *et al.*, 2014)**.

Grid generation and active site identification

Identifying active sites within a target protein is a crucial strategy for treating certain diseases. However, it is important to ensure proper binding or interaction between the protein and ligand to avoid potential side effects or increased toxicity. The binding affinities of a chemical compound can be influenced by factors such as hydrogen bonding, hydrophilic or hydrophobic interactions, chelation, and ionization with zinc compounds (**Xie *et al.*, 2009**). The BIOVIA Discovery Studio Visualizer Tool 16.1.0 was utilized to precisely locate the binding sites or active pockets within the target protein. Additionally, the PrankWeb server (**https://prankweb.cz/2024**) was employed to validate the predicted protein binding sites. Utilizing a machine learning-based methodology, this server predicts the locations of ligand binding sites by analyzing the provided protein structure. After selecting the protein of interest, a receptor grid was created using the PyRx program.

Molecular docking

To conduct molecular docking on the selected compounds acquired through virtual screening, we employed the PyRx virtual screening software. PyRx, a well-regarded program, was chosen for its ability to evaluate various drug designs targeting a range of diseases. In the molecular docking process, we utilized the Lamarckian Genetic Algorithm (LGA) as a scoring function, in conjunction with AutoDock and AutoDock Vina **(Molla and Aljahdali, 2022**). In this study, the PyRx tool AutoDock Vina was utilized to facilitate the analysis of interactions between ligands and proteins. Following that, the assessment of complex binding

poses was conducted using the visualization tools provided by the BIOVIA Discovery Studio (**Forli *et al.*, 2016**).

ADME analysis

Evaluating the properties of absorption, distribution, metabolism, and excretion (ADME) is essential in crafting potent and successful drug candidates. In the early phases of drug design, conducting ADME analysis is indispensable to anticipate potential adverse effects of compounds, ultimately helping to minimize or predict the failure rate during clinical trials. This proactive approach contributes to significant time and resource savings (**Krüger *et al.*, 2019**). The elimination of a drug through feces and urine directly impacts its ADME profile in humans (**Hasan *et al.*, 2022**). The excretion process directly impacts factors like lipophilicity, hydrophobicity, gastrointestinal (GI) tract absorption, and permeability through the blood-brain barrier. To evaluate the ADME parameters of chosen drugs, including solubility profile, gastrointestinal absorption, and bioavailability, the Swiss-ADME server (**http://www.swissadme.ch/ 2024**) was utilized.

Toxicity test

To ensure the safety of substances intended for human consumption or their potential as therapeutic candidates, toxicity tests are conducted. These tests evaluate the harmful effects that the substances may have on human health or their suitability for use as therapeutic molecules. In silico toxicity tests provide a means to quantitatively and qualitatively analyze various parameters such as mutagenicity, carcinogenicity, LD50 value, and immunotoxicity. To evaluate the potential hazardous effects of the chosen chemicals, we utilized the ProTox-II free server (**http://tox.charite.de/protoxII/ 2024**) (**Islam *et al.*, 2022**). Moreover, the Toxicity Estimation Software Tool (TEST) was employed to assess the toxicity of the substances of interest, eliminating the requirement for additional software**(Fan *et al.*, 2018).**

MD simulation

To examine the stability interactions between ligands and receptor binding pockets, the Desmond v3.6 program from the Schrödinger software suite was utilized. Molecular dynamics (MD) simulations lasting 100 ns were carried out to evaluate the stability of chosen compounds with their respective target proteins. These simulations incorporated free energy perturbation (FEP) calculations to estimate the equation of state (EOS) across different temperatures (**Aljahdali *et al.*, 2021**). The setup utilized a pre-established TIP3P water model, and an orthorhombic periodic boundary box with a volume of 10 was employed to encompass both sides of the system. After creating the solvated system, which included the protein-ligand complex, the OPLS-2005 force field was applied to refine and optimize the entire system (**Ivanova *et al.*, 2018**). The results of the molecular dynamics simulations were validated and scrutinized using the simulation interaction diagram (SID) provided in the Schrödinger software. The stability of the protein-ligand complex was assessed by scrutinizing parameters obtained from the trajectory, including root-mean-squared deviation (RMSD), root-mean-squared fluctuation (RMSF), protein-ligand (P-L) interactions, and hydrogen bond interactions.

MM-GBSA analysis

In this study, the determination of bound free energies in a complex system involving molecules and proteins was carried out through the utilization of MM-GBSA analysis. This approach involved the calculation of the free energy of binding by assessing the trajectory obtained from MD simulation. Specifically, MM-GBSA was employed to evaluate the free energy of binding (Gbind) for specific chemical compounds with the CTX-M-15 protein. The computational software package Schrodinger Maestro was utilized to perform the MM-GBSA calculations, as described by **Forouzesh and Onufriev (2020**).

Results

Structure-based pharmacophore modelling

A pharmacophore presents a thorough depiction of both structural and electronic characteristics, offering insights into how a compound interacts with the active site of a particular biological macromolecule.Pharmacophore characteristics have proven valuable in studying ligand-protein interactions and screening large chemical databases to identify potential lead small molecules. In this study, we utilized the PDB entry 4XUZ, along with associated chemicals, to identify a lead drug that targets the CTX-M-15 protein. Subsequently, a pharmacophore model was constructed. Instead of synthesizing a small-molecule drug candidate based on the identified antagonist, we opted to purchase a compound library. We selectively screened the library for active antagonists against CTX-M-15 through well-established databases like ChEMBL and extensive literature reviews. Molecular docking was performed using PyRx, and the antagonist CHEMBL3622898 displayed the highest binding score (PubChem CID: 56949451).

The flow chart, depicted in (**Figure 1**), serves as a comprehensive schematic diagram outlining the research methodology employed in our study. As an integral step in drug design, determining the 3D structure of the target protein is crucial, necessitating the validation of protein structures obtained from numerous protein data banks. The crystal X-ray structure of the CTX-M-15 protein (PDB: 4XUZ) was specifically selected and documented in conjunction with the antagonist compound. A structure-based pharmacophore model was then generated for the enzymatic cavity of the protein. The interactions between the ligands and the CTX-M-15 protein were experimentally validated and determined using the X-ray diffraction method. The binding of the inhibitor to the active site of the CTX-M-15 protein was found to regulate the overall protein expression. The effectiveness of the protein-inhibitor interaction was assessed by evaluating the binding effectiveness. Consequently, the active sites of the inhibitor were investigated to ascertain sufficient interaction, aiming to enhance the biological activity compared to existing compounds. The essential chemical features derived from the pharmacophore model were generated using the LigandScout 4.4 software, as illustrated in (**Figure 2**).

Eleven distinct chemical properties were identified through our analysis. To illustrate a protein-ligand complex interaction, we presented a set of 28 characteristics, comprising 1 hydrophobic property, 1 negative ionizable bonds, 3 hydrogen bond donors, 7 hydrogen bond acceptors, and 16 with an exclusion volume (**Figure 2**). In order to preserve the optimal features of the pharmacophore, certain aspects were intentionally omitted during the development process. The observed pharmacophore characteristics were derived from the interaction between the protein and the ligand. Notably, the hydrophobic interactions with specific amino acid residues in the selected protein were found to be significant. Additionally, multiple bonds interacted with the protein such as ASN79A, ASN107A, HOH403A, THR210A, SER212A, SER105 A, ARG42A, THR146A, ASN145A, SER212A and HOH607A (**Figure 3**).

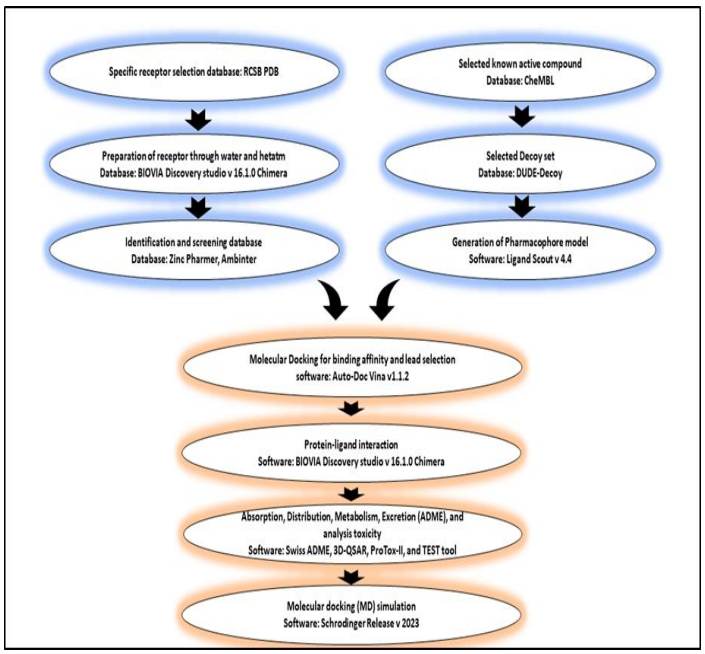

Figure 1 The flow chart presents the comprehensive visual representation of the research workflow in our study.

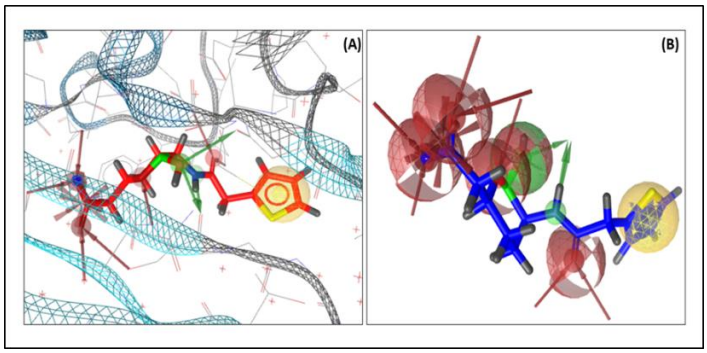

Figure 1 (A)The study utilized the X-ray crystal structure of the CTX-M-15 protein (PDB ID: 4XUZ) to create a 3D pharmacophore model. This model incorporated ligands from CHEMBL3622898. (B)The generated pharmacophore features included hydrophobic interactions, positive interactions, hydrogen bond donors, and hydrogen bond acceptors.

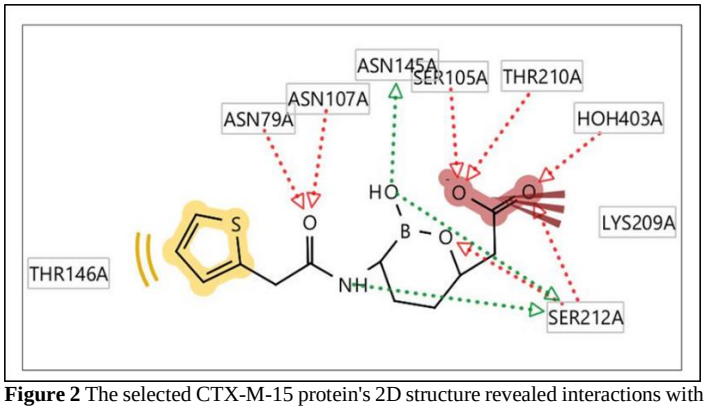

Figure 2 The selected CTX-M-15 protein's 2D structure revealed interactions with specific hydrophobic contacts, and amino acid residues, visually represented in yellow. The prevailing interactions between the protein and the ligand, specifically the hydrogen bond donors (HBDs), were visually represented in red. Furthermore, the interactions between the ligand's oxygen and nitrogen atoms and the benzene ring, along with its diverse side chains, were distinctly highlighted in green.

Pharmacophore model validation

Validation plays a significant role in verifying the accuracy of pharmacophore analysis and the reliability of molecular models. The generation of structure-based pharmacophore models precedes the screening of databases due to the models' ability to differentiate active compounds from decoy sets. In this study, a set of nineteen known CTX-M-15 antagonists, along with corresponding 500 decoy compounds, were validated using the enhanced Database of Useful Decoys (DUDe). The IC50 values were combined with the decoy compounds, and the initially screened compounds were evaluated against the validated model. The receiver operating curve (ROC) was employed to estimate the area under the curve (AUC) value and the enrichment factor (EF) value. The AUC value represents the performance of the classification model, providing an indication of its predictive capability, while ranging between 0 and 1. Remarkably, the model achieved a perfect prediction accuracy of 100% based on the AUC value. Furthermore, the model exhibited an early enrichment factor (EF1%) of 100, indicating excellent discrimination between true actives and decoy compounds (**Figure 4**).

The Dataset generation for pharmacophore-base screening

The crucial task of generating a dataset for screening involved identifying the most suitable lead molecules. In this study, we utilized the ZINC dataset, which consists of commercially available chemical compounds. The dataset provided comprehensive information, including chemical structure, molecular weight, physical and chemical properties, as well as the biological activity of macromolecules. The ZINC database consists of more than 206 million chemical substances in a 3D format, accessible to the public through a website and suitable for docking. Additional compound information, including natural compounds, was obtained from the Ambinter database, which serves as a library for natural compounds. The database for virtual screening, utilizing pharmacophores, was compiled, and individual pharmacophore models were created for each active chemical. These models were subsequently uploaded to ZINCPharmer. In the initial stage, hits were computed utilizing the ZINC database, specifically the sections "ZINC natural products" and "ZINC natural derivatives," which encompassed millions of drug-like small molecules, FDA-approved medications, and natural products. The RMSD sphere center values of 0.5Å were used as the expected criteria in ZINCPharmer, resulting in the retrieval of approximately 11,000 compounds for further screening. Lastly, the hits were recorded, saved, and downloaded for subsequent screening processes.

Pharmacophore-based virtual screening

The structure of the protein-ligand complex has been recorded using a library of purchasable compounds in order to create a pharmacophore feature. The relative pharmacophore, which is used in the screening process to assess the compounds, excludes up to four features from all of the query features. To enhance the accuracy of the pharmacophore fit score, certain features are deliberately excluded during screening, as evaluating all features can be challenging. A higher score signifies superior activity against the target macromolecules and a greater compatibility with the desired environment. The ROC curve visually represents the pharmacophore fit value for the geometric feature fit to the 3D structure-based pharmacophore model (**Figure 4**). By comparing the protein match score with the validated pharmacophore model, we identified the protein that exhibited the highest match score. This protein demonstrated activity against our target, the CTX-M-15 protein. As a result, a potential hit compound was discovered, retrieved, and stored for further analysis in future studies.

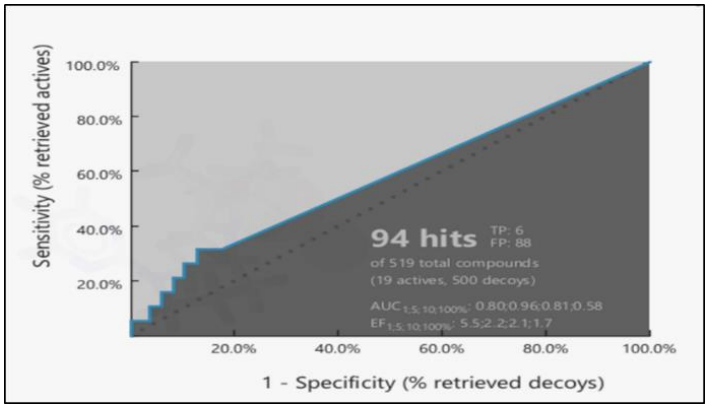

Figure 3 The SBPM demonstrated its capability to identify both active and decoy compounds, enabling the construction of a receiver operating characteristic (ROC) curve. In this evaluation, the pharmacophore model was tested using nineteen active compounds and five hundred and nineteen inactive compounds against CTX-M-15.

Molecular Docking Based Virtual Screening.

The CTX-M-15 protein, which was of interest, exhibited two ligands bound to it (PDB ID: 4XUZ). The protein possesses multiple binding pockets with diverse shapes for ligand attachment. The active site of the protein, responsible for catalyzing the reaction, consists of specific amino acid residues that form a region capable of transiently interacting with the substrate, recognized as the binding site. The binding site of the protein enables the identification of ligands and facilitates strong binding interactions to stabilize intermediate reactions. To identify the active sites of the CTX-M-15 protein, the CASTpi server was employed, which provided the combined positions of the active sites. Verification of the active sites was conducted utilizing the PrankWeb server (**https://prankweb.cz/ 2024**), confirming the presence of twenty active sites in total. The active site pocket was investigated, and the binding site positions were identified at MET43, THR46, SER47, VAL49, ALA51, VAL123, PHE126, ALA127, LEU130, ASP132, PHE135, SER157, THR156, PRO158, ALA160, THR164, LEU165, ILE221, VAL235, and TRY237, represented by different colors such as red, green, orange, blue, and white (**Figure 5**). The receptor grid for the molecular docking simulation was established using the binding sites identified by the server. The dimensions of the grid were set as follows: X = 35.9740 Å, Y = 40.2899 Å, and Z = 34.6741 Å.

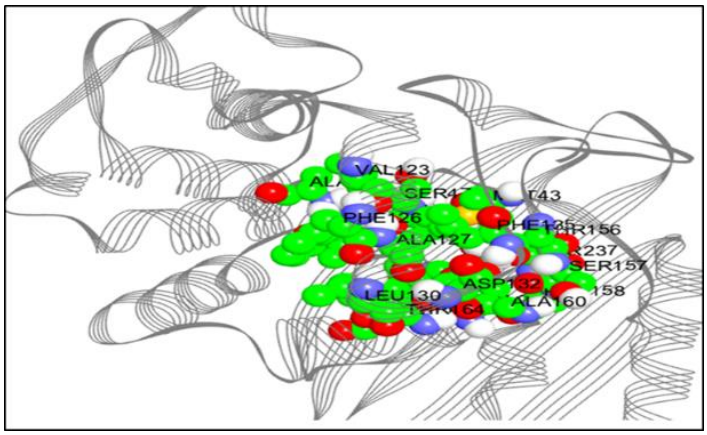

Figure 4 The active site and its associated binding site of the CTX-M-15 protein are visually presented. The active site is depicted using ball-shaped atoms, with each atom represented by colors such as green, red, orange, white, and blue, corresponding to specific amino acids within the site.

Molecular docking

In this research, molecular docking plays a pivotal role in the drug discovery process, assessing the binding capacities of identified compounds to the target protein CTX-M-15. Employing the pharmacophore model assumptions as a guide, the Autodock Vina tool within PyRx was applied to facilitate the docking of drugs onto CTX-M-15, enabling the determination of their binding affinities. Through this analysis, three compounds (ZINC ID 94211493, ZINC ID 20528448, and ZINC ID 04331046) were identified as having higher binding affinities compared to the CTX-M-15 antagonist ZINC ID 94211493 (−8.4 kcal/mol), which was used to create the initial pharmacophore model (**Table 1**). Additional chemicals listed in table 1 also displayed slightly varying binding affinities. Notably, it was hypothesized that drugs with superior docking affinities would exhibit stronger interactions with the CTX-M-15 protein, suggesting their potential as promising candidates for further investigation.

Table 1 List of 11 currently identified active antagonists of the CTX-M-15 protein, along with their respective binding affinities to the protein as determined through the molecular docking technique.

SI No.	PubChem CID	Zinc ID	Molecular Formula (MF)	Molecular weight (MW)	Chemical structure	Binding Affinity (Kcal/mol)
1	CID 19579838	ZINC94211493	$C_{14}H_7F_3N_4O_5$	368.22 g/mol		- 8.4
2	CID 29020424	ZINC20528448	$C_9H_6ClN_6O_5^-$	313.63 g/mol		- 8.1
3	CID 19660912	ZINC04331046	$C_{16}H_{14}N_4O_5$	342.31 g/mol		-7.7
4	CID 34909230	ZINC27546493	$C_{16}H_{16}ClN_6O_2^-$	359.79 g/mol		-6.8
5	CID 3739219	ZINC02571768	$C_{16}H_{14}N_4O_4$	326.31 g/mol		-7.6
6	CID 4944183	ZINC14033838	$C_{23}H_{21}N_3O_5$	419.4 g/mol		-7.5
7	CID 5356180	ZINC06045827	$C_{16}H_{12}N_4O_3$	308.29 g/mol		-7.7
8	CID 60586673	ZINC72033223	$C_{18}H_{18}F_3N_3O_5$	413.3 g/mol		-7.5
9	CID 9315627	ZINC08182183	$C_{18}H_{20}N_2O_4$	328.4 g/mol		-6.9
10	CID 9316188	ZINC08182775	$C_{18}H_{20}N_2O_4$	328.4 g/mol		-6.9
11	CID 9658192	ZINC02543901	$C_{17}H_{16}N_4O_4$	340.33 g/mol		-7.6

Interpretation of protein–ligands interactions

The analysis of protein-ligand interactions revealed specific details for the ZINC ID: 94211493 compound. It formed five conventional hydrogen bond interactions with amino acid residues ASN79, SER105, ASN107, UNK1, and UNK1:H - A. Additionally, it formed two hydrogen bonds with GLY211 and GLY214, and a halogen (fluorine) interaction with PRO142. Furthermore, there was one Pi-Donor Hydrogen Bond with SER45, and a Pi-Pi T-shaped interaction with UNK (**Figure 6**). In the case of ZINC20528448, the compound exhibited seven conventional hydrogen bonds with SER45, LYS48, ASN79, SER105, ASN107, SER212, and ASN145. Moreover, it formed a Pi-Pi Stacked interaction with TYR80 (**Figure 7**). Regarding the compound ZINC04331046, it formed six conventional hydrogen bonds with SER45, LYS48, ASN79, ASN107, and UNK1. Additionally, it was observed to form four Pi-Pi Stacked interactions in three positions involving TYR80 and UNK1 (**Figure 8**).

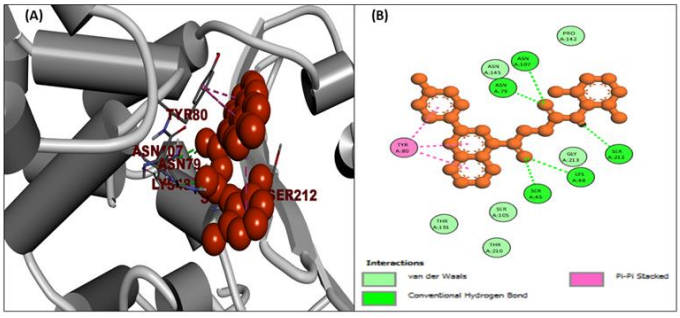

Figure 5 The ligand-protein complexes 3D interaction(A) and (B) 2D. ZINC ID: 94211493 depicts the interaction between the ligand and the protein CTX-M-15 after molecular docking, different types of bonds were indicated by employing different colors.

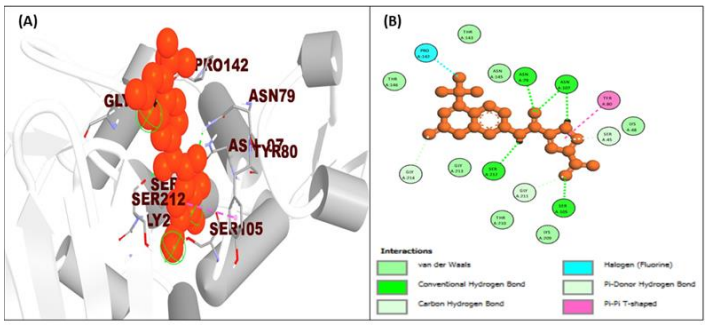

Figure 6 The ligand-protein complexes (3D) shows in figure (A) and 2D shows in figure (B). ZINC ID: 20528448 depicts the interaction between the ligand and the protein CTX-M-15 after docking, different types of bonds were indicated by employing different colors.

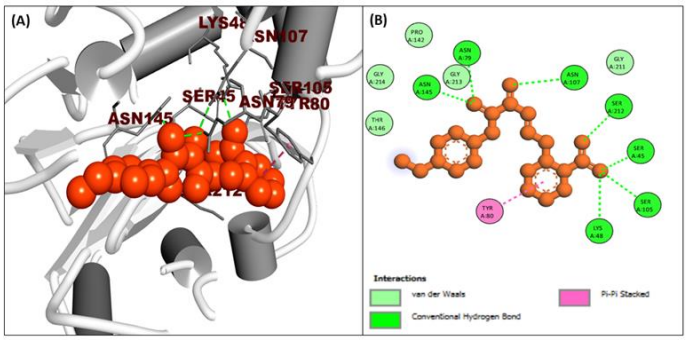

Figure 7 The ligand-protein complexes (3D) show in figure (A) and 2D shows in figure (B). ZINC ID: 04331046 depicts the interaction between the ligand and the protein CTX-M-15 after molecular docking, different types of bonds were indicated by employing different colours**.**

ADMET

The drug undergoes processes of ADME following administration to either an animal model or a human, leading to its transport to the target site through active or passive means. The interaction between a biological macromolecule and a pharmacological agent can result in either a beneficial or detrimental impact. Designing a drug involves a systematic approach, and failure to do so can result in its rejection, which is a costly outcome for the company.

A drug's bioavailability is contingent upon its effectiveness, safety, and the absence of these factors is a primary contributor to drug failures. These attributes are frequently assessed through the drug's ADME properties. Swiss-ADME was employed to assess the ADME characteristics of the three compounds, including parameters such as lipophilicity, drug-likeness, water solubility, and medicinal chemistry. Lipophilicity indicates the potential for a substance to diffuse across cell membranes, making it unsuitable for oral administration. Considering limited gastrointestinal absorption, an injectable dose form may be more effective in achieving a rapid onset of action (**Table 2**).

Table 2 The pharmacokinetics inventory consists of the ADME characteristics of the three selected medications. Additionally, the inventories encompass the diverse physicochemical attributes of the three compounds.

Properties	Parameters	ZINC 94211493	ZINC 04331046	ZINC 20528448
	MW (g/mol)	368.22 g/mol	342.31 g/mol	313.63 g/mol
	Heavy atoms	26	25	21
	Rotatable bonds	5	8	5
	Arom. Heavy atoms	15	12	10
	H-bond donor	2	2	2
	H-bond acceptors	9	6	7
	Molar Refractivity	82.67	92.05	68.70
Lipophilicity	Log Po/w	1.03	1.89	0.27
Water solubility	Log S (ESOL)	-3.46	-3.20	-2.37
Pharmacokinetics	GI absorption	Low	High	Low
Medi. Chemistry	Synth. accessibility	2.98	2.84	2.63
Drug likeness	Lipinski, Violation	No	No	Yes 1

Pharmacophore features analysis

A pharmacophore is a crucial concept that encompasses a set of specific steric or electronic properties. These properties play a vital role in facilitating optimal supramolecular interactions during the virtual screening process of large molecule databases. By identifying the ideal pharmacophore, researchers can effectively pinpoint molecules that have the potential to induce or inhibit specific macromolecular activities. To achieve this, molecular docking emerges as an efficient and powerful method. By utilizing molecular docking, scientists can comprehensively analyse and evaluate the interactions between molecules and target macromolecules. This technique aids in the identification of potential drug candidates that possess the ability to modulate specific biological activities, thus opening up new avenues for drug discovery and development. Compounds exhibiting similar or relevant properties are expected to display comparable or improved activity compared to the reference compound. In this study, the docking scores and pharmacophore features of ZINC94211493, ZINC04331046, and ZINC20528448 were analysed, as depicted in Figure 9.

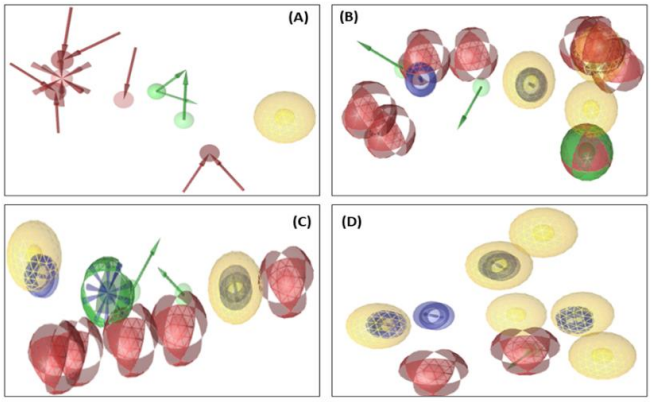

Figure 8 The pharmacophore properties generated from the three chosen compounds attach to the desired CTX-M-15 Protein. A Ligand, B ZINC94211493, C ZINC04331046, and D ZINC20528448.

Table 3 The drug-induced toxicity profile of selected chemicals.

Endpoint	Target	ZINC 94211493	ZINC 04331046	ZINC 20528448
	Hepatotoxicity	Light active	Light active	Light active
	Cytotoxicity	Inactive	Inactive	Inactive
The organ toxicity	Immunotoxicity	Inactive	Inactive	Inactive
	Carcinogenicity	Inactive	Inactive	Inactive
Toxicity endpoints	Mutagenicity	Active	Active	Active
	LD50 (mg/kg) Toxicity class	4	4	4
Tox21-Nuclear receptor	Aryl hydrocarbon Receptor (AhR)	Inactive	Inactive	Inactive
signaling pathways	Androgen Receptor (AR)	Inactive	Inactive	Inactive
Tox21-Stress response	ATPase family AAA domain-containing protein 5 (ATAD5)	Inactive	Inactive	Inactive
pathway	Heat shock factor response element (HSE)	Inactive	Inactive	Inactive
Fathead minnow LC50 (96 h)	-Log10(mol/L)	5.52	6.54	5.15
48-h Daphnia magna LC 50	-Log10(mol/kg)	4.00	4.18	4.15
Developmental toxicity	Value	1.00	0.96	0.73
Oral rat LD50	-Log10(mol/kg)	2.32	2.54	N/A
Bioconcentration factor	Log10	0.60	0.11	1.00

Before embarking on clinical trials, assessing in-silico toxicity is a vital step to identify more effective lead compounds. Computer-based toxicity assessments have gained popularity due to their precision, speed, and accessibility. These tools can provide toxicity information for both natural and synthetic molecules. In our

investigation, we employed the TEST tool and ProTox II server to assess potential hazards associated with the selected four substances. Using these software packages, various toxicological parameters were assessed, such as acute toxicity, hepatotoxicity, carcinogenicity, mutagenicity, cytotoxicity, and immunotoxicity. Through result analysis, we determined the median lethal dosage (LD50) in milligrams per kilogram (mg/kg). As per the ProTox-II service, compounds ZINC94211493, ZINC04331046, and ZINC20528448 were classified as class 4, and the respective LD50 ranges were collected and presented in Table 3.

Molecular dynamics (MD) simulation

Molecular dynamics simulations are employed in the investigation of the stability of protein-ligand docking interactions. In addition, MD simulations offer the advantage of unveiling specific details pertaining to intermolecular interactions within a specified timeframe. In this study, the binding interactions of three natural compounds and a reference antagonist with the CTX-M-15 Protein were thoroughly examined using MD simulation techniques. The primary objective was to evaluate the stability of the protein-molecule complex and the strength of the intermolecular connections within a remarkably short time span of less than 100 nanoseconds. By employing MD simulation, a dynamic approach that captures the intricate movements and interactions of molecules, the study aimed to provide a comprehensive understanding of how these compounds interact with the CTX-M-15 Protein at a molecular level. This approach allowed for the exploration of the complex's behavior and structural changes over time, shedding light on the stability and durability of the protein-molecule complex. The Maestro Desmond interface, in conjunction with SID, was utilized for the extraction of the MD trajectory. The simulation results were presented through various statistical analyses, including root-mean-square fluctuation (RMSF), root-mean-square deviation (RMSD), and Protein-Ligand (P-L) interaction mapping.

RMSD analysis

In molecular dynamics simulations, the root mean square deviation (RMSD) is employed to assess the average distance caused by atom displacement within a defined time interval, relative to a reference time frame. RMSD values are computed for distinct components of the protein structure, including Cα atoms, sidechain, backbone, and heavy atoms. Specifically, the RMSD of the protein-bound ligand is examined throughout all time frames within the reference period, typically set at 100 nanoseconds in this case. The calculation of the RMSD value is performed using the X frame, which allows for evaluating whether the simulation has achieved equilibrium. By analysing fluctuations in the range of 1-3 Å relative to the reference protein structure, one can determine the acceptability of these variations. Larger RMSD values indicate instability within the system, suggesting deviations from the equilibrium state. In our four protein-ligand docking complexes, except for the combination involving CID 19579838, the Cα atoms of the CTX-M-15 protein exhibited acceptable fluctuations. However, during the 100 nanoseconds simulation experiment, CID 19579838 displayed significant variation of 2.5 Å and a maximum fluctuation of 3.5 Å (between 26 and 28 nanoseconds) (Figure 10). These findings indicate that the binding of CID 19579838 induces substantial conformational changes in the CTX-M-15 Protein. Additionally, as the 100-nanosecond simulation neared completion, the measurement of RMSD using data extracted from protein-bound ligands exhibited remarkably minimal fluctuations, with a value as low as 1.24 Å. This observation underscores the stability and consistency of the protein-ligand complex throughout the simulation duration.

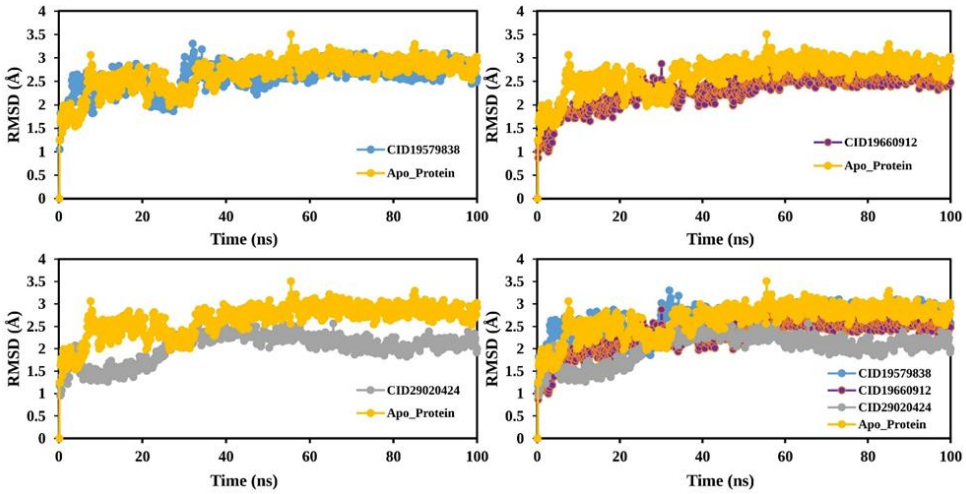

Figure 9 The RMSD values of the CTX-M-15 Protein in complex with the chosen four compounds ZINC94211493 (CID 19579838), ZINC04331046 (CID 19660912), and ZINC20528448 (CID 29020424), by extracting data from the Cα atoms of the complex system.

RMSF analysis

The RMSF is utilized to detect and quantify local conformational changes in both ligand molecules and the protein chain. In the case of the CTX-M-15 Protein interacting with natural compounds, fluctuations in the residue index C were used to assess local structural variations. Notably, with the exception of the N-terminal region ranging from 0.7 to 5.89, all protein residues exhibited low RMSF values. Validating potential drug compounds against the CTX-M-15 protein involved examining the RMSD and RMSF values for all ligand-protein complexes. The most significant alteration in the Apo protein occurred between amino acid positions 40 and 70aa, with a variation of 3.81. The compound with CID: 19579838 exhibited the narrowest average root mean square fluctuation (RMSF) range, ranging from 0.8 to 1.1. Additionally, it displayed the lowest variation in fluctuation, spanning from amino acid positions 81 to 110, compared to the Apo protein structure as shown in Figure 11. The RMSF graph demonstrated that the Apo protein, along with CID: 19660912 (0.5 to 2.6) and CID: 29020424 (0.5–2.8), exhibited notably lower and statistically significant average values compared to the reference Apo structure, as seen in Figure 11. As mentioned earlier, a lower root mean square fluctuation (RMSF) value indicates greater protein stability. In this investigation, it was observed that the RMSF values for each protein-ligand system were comparatively lower than those for the Apo protein. This finding suggests that the presence of ligands contributes to the stabilization of the protein structure, leading to reduced fluctuations in atomic positions.

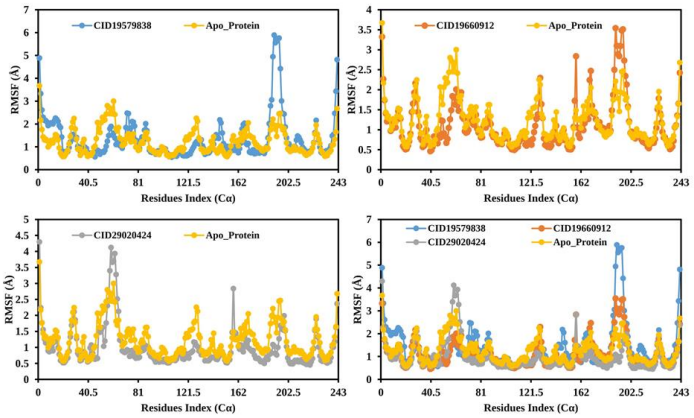

Figure 10 The RMSF values of the CTX-M-15 Protein in complex with the chosen four compounds ZINC94211493 (CID 19579838), ZINC04331046 (CID 19660912), and ZINC20528448 (CID 29020424), by extracting data from the Cα atoms of the complex system.

Ligand– Protein interaction analysis

Ionic bonding, water bridges, Hydrogen bonding, and hydrophobic bonding collectively contribute to the transformation of a molecule into an efficacious drug. In the case of the CTX-M-15 protein, interactions between the protein and ligand were observed, and three natural chemicals, namely CID 19579838, CID 29020424, and CID 19660912, were selected from the MD trajectories for further investigation using the default parameters of the Desmond module. Notably, all three natural chemicals demonstrated substantial interactions with a majority of the protein residues (**Figure 12**). Moreover, the four compounds subjected to analysis using diverse filtering techniques displayed notable intermolecular interactions.

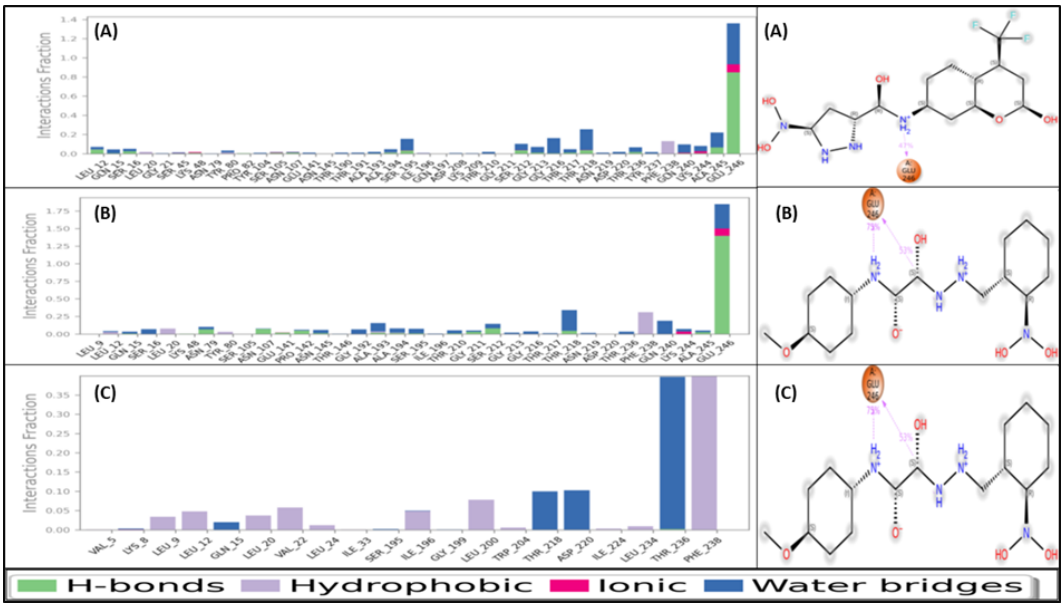

Figure 11 The interactions between ligands and proteins are display for 100 ns. The interaction of chosen three compounds A ZINC94211493 (CID 19579838), B ZINC04331046(CID 19660912), and C ZINC20528448 (CID 29020424) in complex with the CTX-M-15 Protein.

Solvent accessible surface area (SASA)

The solvent-accessible surface area (SASA) of biological macromolecules significantly influences both their function and structure. Active sites on the protein surface, composed of amino acid residues, play a vital role in ligand binding and provide valuable insights into the hydrophilic or hydrophobic characteristics of molecules and protein-ligand complexes. In Figure 13, the calculated SASA values of the protein are presented in relation to the chemicals (A) CID 19579838, (B) CID 29020424, and (C) CID 19660912. In complex systems, an average SASA value ranging from 0 to 40 Å signifies a substantial exposure of amino acid residues to the molecule of interest.

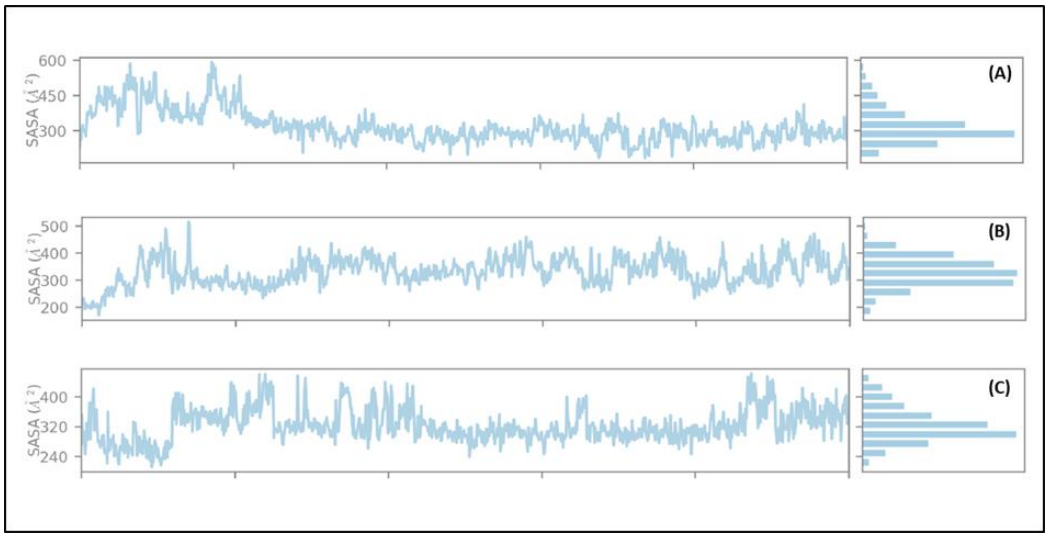

Figure 12 The solvent accessible surface area of the protein-ligand complex was computed from the compounds Zinc ID: A ZINC94211493, B ZINC04331046, and C ZINC20528448 until 100 ns simulation.

MM-GBSA analysis

The assessment of the binding free energy of small molecules to the CTX-M-15 protein involved a combination of molecular energies with the Born and surface area continuum solvation methods. This approach lies between empirical scoring and rigorous alchemical perturbation methods, as depicted in Figure 14. The calculated negative binding free energy values for three compounds, namely CID 19579838, CID 29020424, and CID 19660912, with the CTX-M-15 protein were -22.793±6.7, -28.542±7.8, and -40.249±4.07 kcal/mol, respectively (**Table S4**).

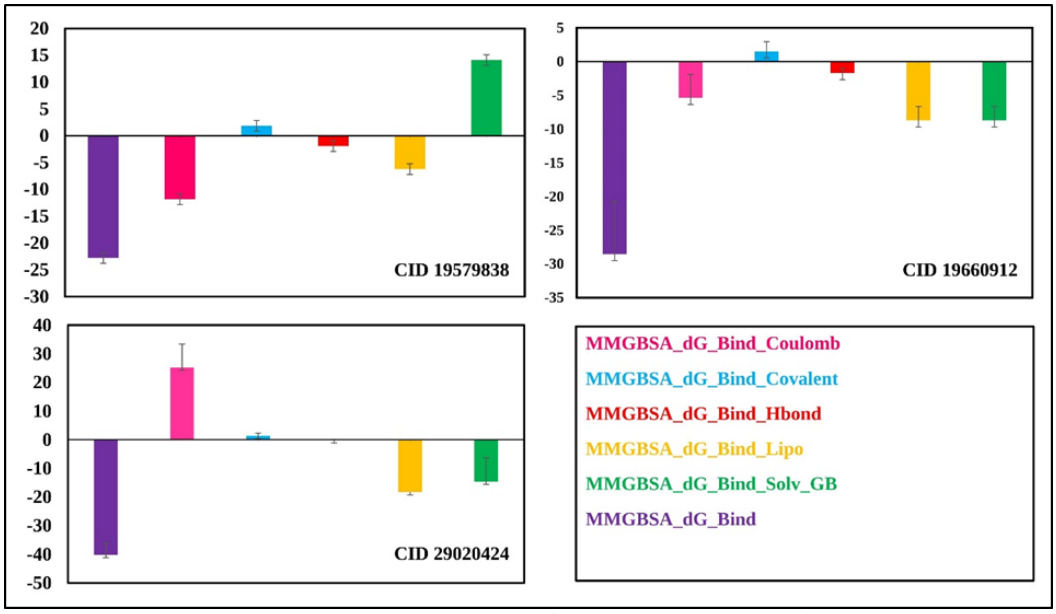

Figure 13 Molecular mechanics (MM) with the generalized Born surface area (GBSA)calculation from the selected three compounds

DISCUSSION

The spread of CTX-M around the globe has grown to be a serious health risk. The strongest hydrolytic activity against cefotaxime is exhibited by CTX-M type ESBLs in comparison to ceftazidime **(Ali *et al.*, 2018; González *et al.*, 2015)**. Given the escalating resistance of *K. pneumoniae*, the development of new drugs is increasingly challenging. Therefore, there is an urgent requirement to investigate novel protein classes and undertake comprehensive research to uncover bioactive compounds. In contemporary drug design, computer-aided drug design (CADD) has revolutionized the field of medicine by enabling cost-effective processes, time savings, and reduced labor costs, thus enhancing the feasibility of drug discovery (**Kapetanovic, 2008; Talevi, 2023**). CADD serves as an essential component and tool in medication development, providing scientists and researchers with a framework for biological and synthetic investigations. Techniques such as molecular docking, ADME, and molecular dynamics (MD) simulations are employed to evaluate the biological efficacy of potential drug candidates. By studying the mechanism of a disease causing by bacteria, identifying relevant proteins, and designing ligand-binding strategies for these proteins, CADD can contribute to mitigating the severity of the disease(**De Vivo *et al.*, 2016**). Moreover, CADD facilitates the identification of specific target molecules by utilizing information on their behavior and ligand-binding characteristics. Molecular docking elucidates the prevalent binding modes between ligands and proteins, while MD simulations provide insights into the intricate interactions between the protein and ligand. Consequently, CADD enables the discovery of small molecule candidates that hold promise for the treatment of specific diseases (**Pinzi and Rastelli, 2019**).

To identify potential candidates for targeting the CTX-M-15 protein against Klebsiella infections, a compressive drug design approach was utilized to screen a collection of natural compounds. Through molecular docking analysis, the three most favorable compounds with the highest binding affinity were selected from the compound library. These selected compounds hold promise for their potential in combating Klebsiella infections by specifically targeting the CTX-M-15 protein. Specifically, compounds ZINC94211493, ZINC04331046, and ZINC20528448 exhibited stronger bonds with scores of −8.4, −8.1, and −7.7 kcal/mol, respectively. ADME analyses were carried out to explore the kinetics of metabolites in the small molecule candidates. ADME primarily shapes the pharmacokinetic properties of drugs, which can be challenging to assess quickly. Traditional methods, including animal testing, are frequently necessary to evaluate promising drug candidates before advancing to standard clinical trials. Therefore, optimizing the PK parameters is crucial in the early stages of the drug design process. These parameters influence the ability of small molecules to navigate biological systems, taking into account factors such as the topology of their polar surfaces (TPSA) and molecular weightTop of Form **(Tibbitts *et al.*, 2016**). Increased molecular weight can potentially result in decreased permeability, whereas the introduction of a larger topological polar surface area (TPSA) can enhance the permeability of smaller molecules. LogP is a valuable parameter used to assess the solubility of a particular chemical compound in polar and nonpolar solvents. The logarithm and coefficients of relevant molecules play a crucial role in determining their partitioning behavior in the aqueous phase. Consequently, LogP affects the absorption of medication molecules in the body, with higher values associated with slower absorption rates. LogS takes a conservative approach and contributes to assessing the solubility of candidate compounds, particularly those with lower values. The ability of a drug molecule to traverse a bilayer membrane is contingent on the number of hydrocarbon bonds established between the hydrogen bond donors and acceptors within the (**Bennion *et al.*, 2017)**. Rotatable bonds play a significant role in oral bioavailability due to their substantial impact on rational barriers. All three compounds underwent evaluations of their PK properties, and satisfactory values were obtained for each compound.

Toxicity tests are essential for evaluating potential adverse effects that could harm or damage an organism. Approximately 20% of drug development failures are linked to toxicity issues. However, conducting toxicity tests using animal models is both costly and time-consuming, making it a critical and resource-intensive step in drug experimentation. To overcome these challenges and avoid unnecessary animal testing, in-silico alternatives are favored in the early stages of drug development. Through this approach, the study identified three compounds that demonstrated low toxicity and optimal pharmacokinetic (PK) properties (**Forouzesh and Onufriev, 2020**).

Molecular dynamics (MD) simulation has emerged as a valuable tool in computer-aided drug design (CADD). It allows for the exploration of how a compound interacts within a macromolecular environment and offers insights into the stability of a drug candidate when bound to the target macromolecule (**González *et al.*, 2015**). Through MD simulation, analyses of root mean square deviation (RMSD), root mean square fluctuation (RMSF), and ligand-protein interactions were conducted on the complex system. The findings revealed favorable RMSD and RMSF values for all three compounds, along with significant protein-ligand contacts (**Kapetanovi, 2008**). Consequently, these three selected compounds can serve as a foundation for designing novel drug candidates targeting CTX-M-15, offering potential therapeutic options against Klebsiella infections.

CONCLUSION

In conclusion, this study identifies three highly promising compounds, ZINC94211493, ZINC04331046, and ZINC20528448, which demonstrate robust binding affinities with the CTX-M-15 protein, offering potential for treating antibiotic-resistant *K. pneumoniae* infections. Computational toxicity tests indicate lower toxicity, while ADME analysis highlights their high solubility and efficient tissue absorption. Employing a structured approach, including a structure-based model (SBM), ADME analysis, molecular docking, and MD simulation, these compounds emerged as lead molecules through virtual screening. Despite the reliance on computational analysis, further experimental validation is essential to confirm their effectiveness. The insights gained here contribute valuable knowledge for designing new drugs to combat Klebsiella infections.

Funding: The Deanship of Scientific Research at Northern Border University, Arar, KSA, funded this work through project number NBU-FFR-2024-420-02.

Acknowledgements: The authors extend their appreciation to the Deanship of Scientific Research at Northern Border University, Arar, KSA for funding this research work through the project number NBU-FFR-2024-420-03.

Data Availability: The data used to support the findings of this study are included within the article.

Conflicts of interest: There are no conflicts of interest.

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