BMC Microbiology

RESEARCH Open Access

Comprehensive screening of potential inhibitors from ZINC15 database for Metallo-L1 B -Lactamase from *Stenotrophomonas maltophilia* via in Silico and in vitro approaches

K H Sreenithya[1], Salim Manoharadas[2] and Shobana Sugumar[1*]

Abstract

Background Antibiotic resistance caused by pathogenic microbes have become a serious issue in health field as most of the antibiotics discovered are rendered ineffective for the treatment of numerous microbial infections. *Stenotrophomonas maltophilia* is such a type of pathogen and the treatment of this bacterial infection is extremely difficult due to its intrinsic multi-drug resistance property. Production of β-lactamases (L1 and L2) by the organism is one of the main causes of resistance to a broad spectrum of antibiotics. β -lactamase inhibitor and β-lactam drug combination can be a promising alternative.

Result In the current study, approximately 500,000 compounds from ZINC15 database were subjected to virtual High Throughput screening (vHTS). Compounds with binding energies in the range of − 8.1 kcal/mol to − 7.2 kcal/mol were shortlisted for further analysis After molecular docking and ADMET analysis, ZINC393032 (-7.3 kcal/mol) and ZINC616394 (-7.6 kcal/mol) were selected for 300 ns Molecular Dynamics (MD) simulation. Analysis of RMSD, RMSF and Hydrogen bond concluded ZINC393032 as the best compound. In vitro validation assays with the screened inhibitor on recombinant Metallo-L1 β-lactamase like enzyme inhibition (IC_{50} obtained at 22.96 µM), MIC (Minimum inhibitory concentration), checkerboard synergy assay and time kill assay showed good inhibitory property. Five different concentration combinations of the inhibitor with imipenem were tested against the bacteria and found to have bactericidal effects.

Conclusion The study validates a promising compound for overcoming resistance caused by L1 β-lactamase in *Stenotrophomonas maltiphilia*. These results highlight the potential of combining computational and experimental approaches to develop novel therapies. The findings provide a foundation for future strategies targeting β-lactamase-mediated resistance in *Stenotrophomonas maltophilia*.

*Correspondence:
Shobana Sugumar
shobanasrmist@gmail.com

Full list of author information is available at the end of the article

Keywords Antibiotic resistance, Metallo L1 β-lactamase, ZINC15 database, Docking, MD simulation, GROMACS, Checkerboard synergy assay

Background

Bacterial cells have evolved mechanisms to contravene the bacteriostatic and bactericidal effects of antibiotics, a phenomenon referred to as bacterial resistance [1]. This adaptation is largely attributed to the widespread and excessive use of antibiotics. Antibiotic resistance has caught the eye as a copious global challenge, posing a serious hindrance to effective treatment and escalating into a critical public health concern. This problem is particularly worrying in India. Over 56,000 newborn deaths occur each year in India due to sepsis caused by organisms that are resistant to first-line antibiotics [2]. Among the various drug resistance, carbapenem resistance in gram-negative bacteria is a severe healthcare menace. High level of resistance to carbapenem is detected in multidrug-resistant (MDR) and extensive drug-resistant (XDR) gram-negative bacteria, especially due to the intrinsic production of extended-spectrum β-lactamase enzyme [3]. The enzyme targets the β-lactam ring antibiotics and renders them ineffective by hydrolysis of the drugs catalysed by SBL (serine β-lactamases) and MBL (Metallo- β-lactamases) [4].

Stenotrophomonas maltophilia is one among the MDR gram-negative bacteria that can produce β-lactamases intrinsically. The bacterium is responsible for various infectious diseases and death in hospitalized patients especially among the immunocompromised, immunosuppressed and patients with medical implants. In SENTRY surveillance program conducted between 1997 and 2016, it was found that *Stenotrophomonas maltophilia* infection was the highest with a range between 51.7% and 62.6% among nosocomial pneumonia patients in Asia-Pacific, Europe, and North America (Gales, A.C et al., 2019). It rapidly colonizes abiotic surfaces like catheters, nebulizers etc. It has been reported as etiological agents in bacteraemia, ocular infection, endocarditis and RTIs (associated with cystic fibrosis), wound infection and urinary tract infections (UTI). It is also an etiologic agent of meningitis, sepsis, skin and soft tissue infections (SSTI) and it has been diagnosed with rare cases of pyomyositis. In recent years, *Stenotrophomonas maltophilia* has emerged as a paradigm of intrinsically resistant, opportunistic bacterial pathogens with an environmental origin. Studies on *Stenotrophomonas maltophilia* demonstrate that this bacterium contains many antibiotic resistance determinants. The best-characterized among these are carbapenem-resistant determinants, which involve a zinc-dependent Metallo-β-lactamase L1, intrinsically embedded in the genome of *Stenotrophomonas maltophilia.* The L1 β lactamases or Zn β lactamases and L2 β lactamase with serine in the active site are the two β lactamases produced by *Stenotrophomonas maltophilia* [5–7]. Treatment options for *Stenotrophomonas maltophilia* infections are minimal due to the production of these extended-spectrum β-lactamases (ESBL).

The growing resistance of *Stenotrophomonas maltophilia* to β-lactam antibiotics, especially carbapenems, is primarily attributed to the production of the Metallo-β-lactamase L1. This enzyme, which functions as a tetramer consisting of αβ/βα structural motifs, contains an active site situated between two β-sheets that coordinate two Zn^{2+} ions. These zinc ions, stabilized by conserved amino acid residues at the base of the active site, are essential for the enzyme's catalytic activity, enhancing its ability to hydrolyse a wide range of β-lactam antibiotics.

Due to the lack of clinically approved inhibitors against Metallo-β-lactamases, the development of effective molecules that can be used alongside β-lactam drugs is of urgent importance. Historically, β-lactamase inhibitors such as clavulanic acid, discovered in 1976, and the synthetic agents sulbactam and tazobactam have been used in combination therapies to counter serine-β-lactamases. However, these are ineffective against the Metallo class, necessitating the search for novel alternatives.

Advancements in computational methods have significantly transformed drug discovery. Computer-aided drug design (CADD) enables rapid and cost-effective identification of promising drug candidates. Among these, virtual high-throughput screening (vHTS) has emerged as a widely adopted technique that allows researchers to screen large chemical libraries in silico against specific biological targets. This approach not only accelerates the early stages of drug development but also improves the efficiency of lead identification by filtering out compounds with unfavourable properties prior to in vitro testing.

[8, 9].

In the current study, a computational approach was adopted for data-driven drug discovery. Approximately 500,000 compounds were downloaded from the ZINC15 database, using molecular weight, pH, and log P value as initial filtering parameters. These compounds were screened against L1 β-lactamase based on their structural information available in the database. After conducting ADMET analysis, the top hit compounds were subjected to molecular dynamics (MD) simulations to evaluate their binding stability. The shortlisted compounds were then tested in vitro for experimental validation.

Methods

In silico screening

Virtual High Throughput screening (vHTS) by molecular docking was performed to screen the best ligand for the target protein (L1 β-lactamase) from a large set of compounds downloaded from ZINC15 database [10]. The computational screening of compounds through vHTS was carried out completely in silico based on binding energy values obtained after docking the compounds with target protein. vHTS was chosen as a reliable method to identify potential lead compounds in a cost-effective and time-efficient manner.

Protein Preparation

The protein used for the study was Metallo L1 β -lactamase present in *Stenotrophomonas maltophilia*. The 3D X-ray crystallography structure of the protein (PDB ID-6UAF) was downloaded in PDB format from RCSB-PDB [11]. The protein contained a hydrolysed ligand, imipenem, at the active site region. It is a 293 amino acid long structure. There are 266 amino acids in the crystal protein structure (residue number 23 to 289) with two Zn^{2+} ions. Since the N-terminal and C-terminal residues were away from the active site and did not affect the binding of ligands, the missing residues in these regions were kept unresolved. Prior to docking, the protein was prepared using UCSF Chimera [12] where the ligand and water molecules not involved in ligand interactions were removed, hydrogen atoms were added, and the protonation states of ionizable residues were adjusted appropriately at physiological pH. The active site residues, including His105, His107, His110, and Asp109, which coordinate the catalytic Zn^{2+} ions, were preserved in their original conformation. The bound water molecule mediating interaction between Zn^{2+} ions and the bound ligand (imipenem) in the original structure was retained where necessary to maintain physiological relevance.

Ligand library Preparation

The ligand library for virtual screening of suitable inhibitor molecules for L1 β-lactamase was prepared from the ZINC15 database [10]. A primary filtering of the ligands was done based on logP value, molecular weight, pH and charge. 3D structures of the ligands in PDBQT format were downloaded as CURL files. The ligand files were obtained as a single file with many compounds. It was split using the vina_split program of Auto Dock Vina suite [13].

Molecular Docking

Protein-ligand docking is an important step in computer aided drug designing. Using this technique, the orientation and conformation of the ligand molecules in the active site region of the target can be predicted and thus serves as a helpful tool for finding the molecules that can act as a potential drug candidate. In the current study structure-based screening was performed using Auto Dock Vina [13] in order to identify the inhibitor molecule for the target protein, L1 β-lactamase (6UAF). The Metallo protein containing two Zn^{2+} ions was docked with ligands downloaded from ZINC15 database. The quantity of grid point x, y and z was 29.194Å, 28.467Å and 0.771Å respectively and grid box focus point was allotted as 40 on the protein. Other docking computation parameters were kept at a default esteem. The target protein, the ligands and the configuration text files were maintained in the same folder along with the shell script file for docking.

ADMET studies

The absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the compounds were studied to reduce the risk of potential toxicity. The pharmacokinetic properties of the compounds obtained after screening via molecular docking were assessed. Lipinski's rule, the Ghose filter, Verber's rule, Muegge's rule, Egan's rule, water solubility, lipophilicity, hepatotoxicity, and other drug-likeness criteria were considered. The presence of PAINS (pan-assay interference compounds) could lead to false-positive results during the prediction of binding sites. Therefore, compounds containing PAINS were removed from the study, as they are more likely to generate false positives. Freely available online tools, such as SWISS-ADME [14] and ADMETsar [15], were used to analyze and list the ADMET properties.

Molecular dynamics

Molecular Dynamics (MD) simulation was performed to analyse how the complex would behave if it was subjected to some perturbations. The motion of all the individual particles was calculated as a function of time using GROMACS 4.6.5 [16]. Gromacs-53a6 was the selected force-field. After developing the topology file, the ligand information was included in it. After placing the complex in a triclinic box, it was solvated using spc216 water molecules. For charge neutralization, sodium ions or chloride ions were added to the system. The entire system (protein, ligand, water molecules and ions) was then subjected to energy minimization by utilizing the Particle Mesh Ewald (PME) algorithms. In the current study, the entire system was scaled at 310 K with the PME order set to 4 and Fourier spacing of 0.16 nm. After producing the index file and topology file with position restrain information of the ligand, the data of position restrain was added to the topology file. Thermal equilibration of the system was carried out by 100 ps NVT step using the modified Berendsen thermostat followed by pressure equilibration by maintaining an NPT ensemble at 300 K

for 100 ps using V-rescale and appropriate P-coupling and T-coupling parameters. The reference point for pressure equilibration was 1 bar. Once the temperature and pressure get equilibrated, MD production run was carried out for 300ns. The long-range electrostatic interactions were calculated using the PME and PBC algorithm of gromacs. The MD run results were analysed by plotting the RMSD, RMSF, and H-bond.

Binding free energy calculation using MMPBSA method

Binding free energy calculations were performed post-MD simulation to evaluate the affinity and overall stability of the protein–ligand complexes. In the context of drug design and discovery, understanding the molecular interactions and associated thermodynamic properties is essential. To this end, we applied the widely used MM/PBSA (Molecular Mechanics/Poisson–Boltzmann Surface Area) method, implemented via the *g_mmpbsa* tool [17]. This approach integrates MD simulation data with energy decomposition analyses, providing estimates of van der Waals, electrostatic, polar solvation, solvent-accessible surface area (SASA), and total binding energies. Such detailed insights strengthen the prediction of binding strength and complex stability under dynamic conditions.

In vitro **validation**.

Expression and purification of Recombinant Metallo-L1 β-lactamase

The *blaL1* gene was cloned into pET30b and the cloned plasmid was transformed into BL-21 (DE3) and plated on an LB agar plate with kanamycin (50 µg/ml) for screening the transformed colonies. Colony PCR was conducted using T7-F and T7-R primers for confirmation. The protein was overexpressed in BL-21 (DE3) cells with Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations varying from 0.2 mM to 0.8 mM. Harvested cells were disrupted by ultrasonication followed by centrifugation to remove cell debris. Supernatant was subjected to Ni-NTA chromatography and the elution was dialysed using 14KDa membrane which was quantified to get the total protein concentration.

Enzyme Inhibition assay

The inhibitor and nitrocefin (substrate) were purchased from Sigma-Aldrich and dissolved using 5% dimethyl sulfoxide (DMSO). All the working stocks were prepared using 50 mM HEPES buffer in neutral pH. 1 nM of enzyme was supplemented with 10 µM zinc sulphate in order to incorporate zinc ions to the active site. Ethylenediaminetetraacetic acid (EDTA) was used as the positive control. The inhibitor, substrate and control were dissolved in 5% DMSO and the same solvent was used as a negative control. 1 nM of enzyme was added to the inhibitor (10 µM to 50 µM) at 30 C and pH 7 was maintained using 50 mM HEPES buffer, for 10 min. The substrate, nitrocefin (60 µM) was then added to the mixture and the absorbance was measured at 490 nm [18] The experiment was performed in triplicate. IC_{50} values were calculated using Graph Pad Prism.

Determination of minimum inhibitory concentration (MIC)

MIC of a compound is defined as the lowest concentration at which the growth of an organism is inhibited. Broth dilution method as per CLSI guidelines [19, 20] was followed and the experiment was performed in a microtiter plate with a holding capacity of 200 µl per well. Fresh overnight colony suspension of *Stenotrophomonas maltophilia* at 0.5 McFarland standard was used for the test. A serial two-fold dilution of the compounds was made using MH broth in the microtiter plate. 10 µl of bacterial culture was added and incubated overnight at 37˚C. A well containing broth without inhibitor served as a growth control. Absorbance was measured at 600 nm using a 96-well plate reader.

Checkerboard Synergy Assay

The synergy of the inhibitor and β-lactam antibiotic (imipenem) combination test was performed using Checkerboard synergy method as described previously [21]. Different combination of the inhibitor and imipenem at increasing concentrations were tested against the bacterial suspension at 0.5 McFarland standard by incubating the set up overnight at 37˚C. Fractional inhibitory concentration (FIC) of each compound was calculated and the FIC index (FICI) was determined. Synergy ($FIC \leq 0.5$), Additive ($0.5 > FIC \leq 1$) and Indifferent ($1 < FIC \leq 4$) are the FICI ranges for drug combinations.

Time kill assay

Time-kill studies for the determination of synergy were performed in accordance with CLSI guideline [22]. The antibiotic and inhibitor synergistic combination concentrations were used in time-kill assay. Bacterial cultures were sampled at 1-hour intervals over a 24-hour period, and colony-forming unit (CFU) counts were determined at each time point.

Results

Virtual screening

Structure based virtual screening was performed by molecular docking for approximately 500,000 compounds downloaded from ZINC15 database against the target protein (PDB ID-6UAF). After successfully performing molecular docking, top 37 ligands with least dock scores were selected as their binding affinity towards the target is high. The dock scores ranged from – 8.1 kcal/mol to -7.2 kcal/mol. The 37 compounds along with their

dock score and 2D structure is given in Fig. 1. The highest binding affinity of -8.1 kcal/mol was observed for ZINC000000487753 and ZINC000003894728. Figure 2 represents the 3D visualization of the compounds at the binding pocket of the protein.

ADMET analysis

Top 37 compounds were studied for ADMET. All the compounds had molecular weight less than 250 daltons and logP < 5. ZINC000000487753, ZINC000301829834 and ZINC000192605700 with dock scores – 8.1 kcal/mol, -7.4, -7.3 respectively showed PAINS alert. Except for ZINC000000393032, ZINC000742221528,

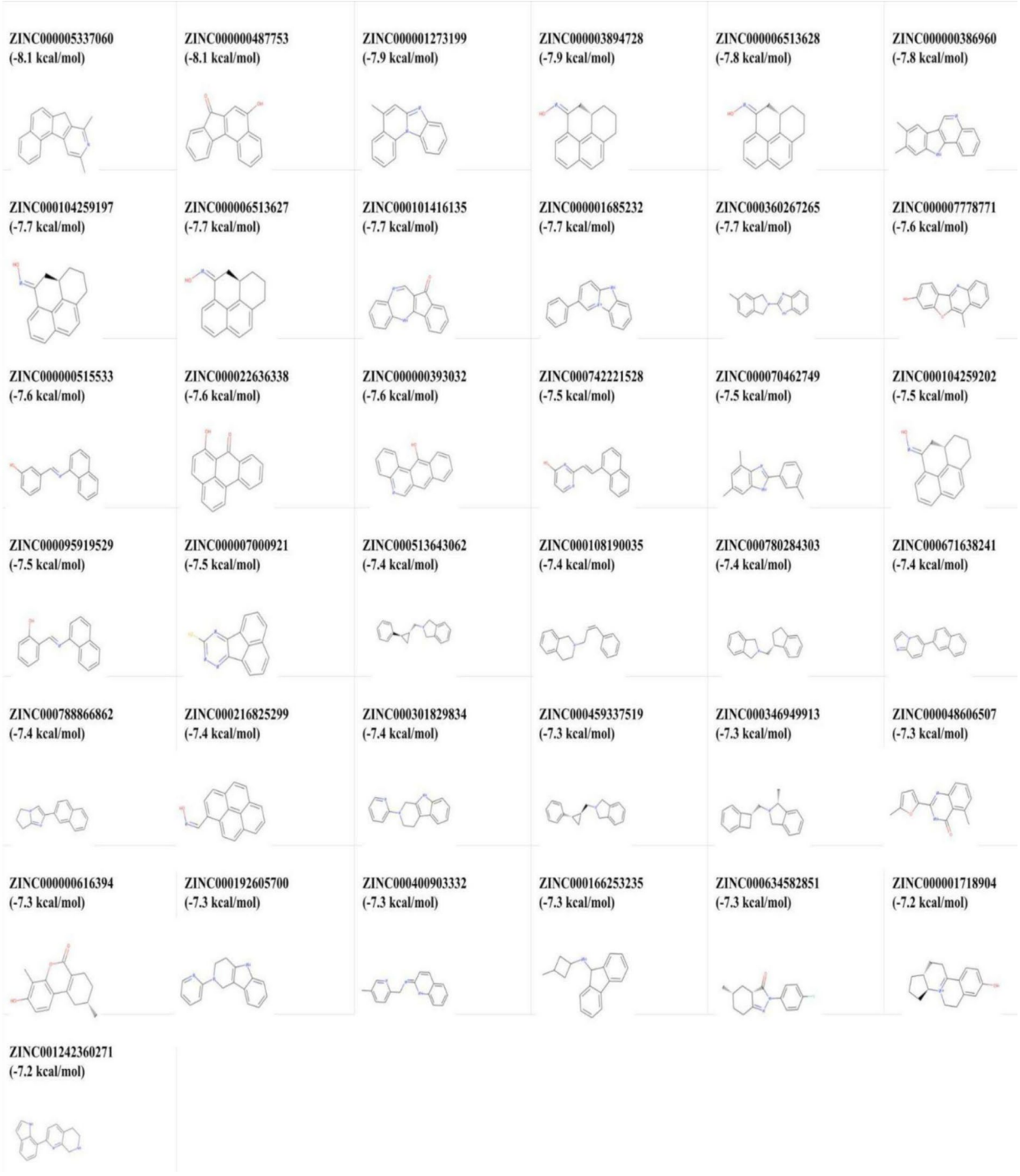


Fig. 1 2D chemical structures of the top 37 screened compounds from the ZINC15 database, along with their docking scores and ZINC IDs. The compounds are ranked based on their binding affinity (in kcal/mol), as determined by molecular docking studies

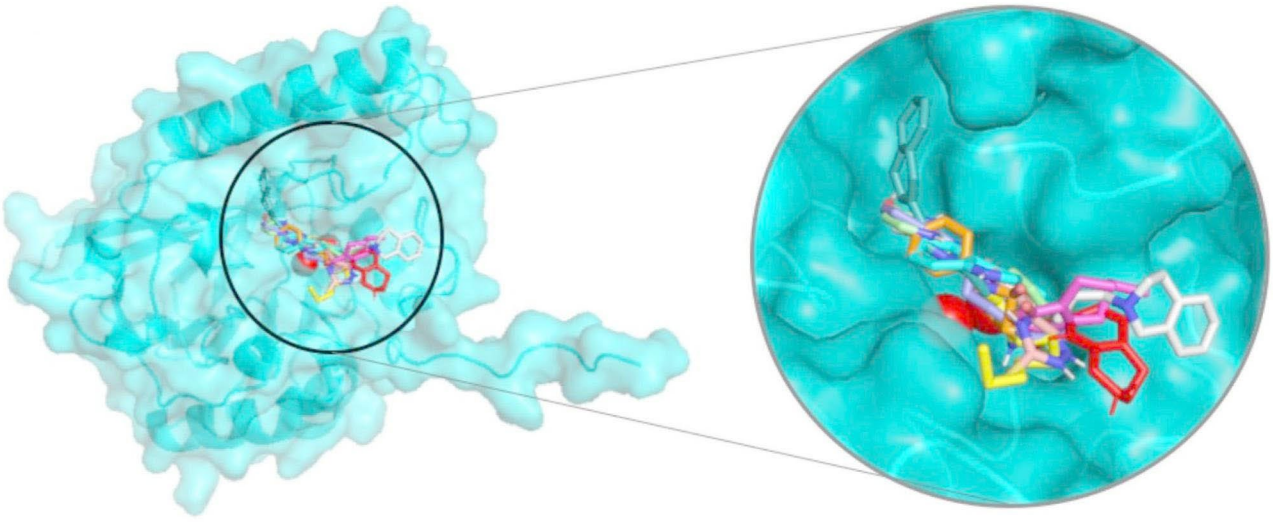


Fig. 2 3D representation of the top hit compounds bound at the active site of L1 β-lactamase. The binding pocket is shown with surface or cartoon representation, highlighting the positioning of the top-ranked compounds within the catalytic site. Key interacting residues and metal ions (Zn^{2+}) are visualized to demonstrate the molecular interactions responsible for binding affinity

Table 1 ADMET profiling of the top 10 non-toxic screened compounds, highlighting key Pharmacokinetic and toxicity parameters relevant for drug-likeness assessment

ZINC ID	Mol wt. (g/mol)	Log *p*	Lipinski	Ghose	Verber	Egan	Muegge	PAINS	GI solubility	AMES toxicity	CYP inhibition
ZINC000000393032	245.28	4.25	yes	yes	yes	yes	yes	0 alert	High	Non toxic	low
ZINC000742221528	248.28	3.51	yes	yes	yes	yes	yes	0 alert	High	Non toxic	low
ZINC000007000921	239.3	2.96	yes	yes	yes	yes	yes	0 alert	High	non toxic	high
ZINC000108190035	249.35	3.76	yes	yes	yes	yes	no	0 alert	High	non toxic	high
ZINC000301829834	249.31	3.13	yes	yes	yes	yes	no	1 alert	High	non toxic	high
ZINC000000616394	244.29	2.93	yes	yes	yes	yes	yes	0 alert	High	non toxic	low
ZINC000192605700	249.31	3.13	yes	yes	yes	yes	yes	1 alert	High	non toxic	high
ZINC000634582851	246.28	2.97	yes	yes	yes	yes	yes	0 alert	High	non toxic	high
ZINC000001718904	242.43	2.71	yes	yes	yes	yes	yes	0 alert	High	non toxic	low
ZINC001242360271	249.31	2.88	yes	yes	yes	yes	yes	0 alert	High	non toxic	high

ZINC000007000921, ZINC000108190035, ZINC000788866862, ZINC000301829834, ZINC000000616394, ZINC000634582851, ZINC000001718904 and ZINC001242360271, all the other compounds were AMES toxic. ZINC000108190035 and ZINC000301829834 violated Muegee rule. ZINC000007000921, ZINC00788866862, ZINC000634582851 and ZINC001242360271 had a high cyt-p450 inhibition. The ADMET results of the 10 non-toxic compounds are given in Table 1.

Molecular Docking analysis

The ten non-toxic compounds after ADMET studies were further analysed to check whether the compounds are occupying the active site. It gave the details on the number of hydrogen bonds, hydrophobic bonds along with its length as well as the residues responsible for the bond formation. The Zn^{2+} ions at the active side were coordinated by residues at the base of the active site. The Zn^{2+} ions at position 302 was bonded to Asp109, His110 and His246 at a distance of 2.1 Å, 2,2 Å and 2.1 Å respectively and the Zn^{2+} ions at position 303 was coordinated by His181, His105 and His107 at a distance of 2.2 Å, 2.0 Å and 2.2 Å respectively. The binding affinity along with the residues undergoing hydrogen and hydrophobic bond formation as well as Zn^{2+} ions interaction is given in Table 2.

Among the 10 compounds, ZINC000000393032 (Fig. 3A) possessed the highest binding affinity (-7.6 kcal/mol) and showed interaction with one Zn^{2+} ion (ZN-302). It formed hydrogen bonds with active site moieties like Ser206 and Ser208, whereas the hydrophobic bonds were established with Phe145, Ile149 and Pro210 ZINC000000616394 (Fig. 3B) on the other hand interacted with both the Zn^{2+} ions (ZN-302 and ZN-303) and had a binding affinity of (-7.3 kcal/mol) by forming

Table 2 Binding affinity, hydrogen bonding, hydrophobic bonding and zinc atom interaction of the top 10 compounds

Compound	Binding Affinity kcal/mol	Hydrogen bond Residue	Hydrophobic bond Residue	ZINC Atom	
				ZN-302	ZN-303
ZINC000000616394	-7.3	His181, Ser208	Phe145, Ile149	Ligand, Asp109, His,110, His246	Ligand, His105, His107, His181
ZINC000000393032	-7.6	Ser206, Ser208	Phe145, Ile149, Pro210	Ligand, Asp109, His110, His246	His105. His107, His181
ZINC000634582851	-7.3	Asp109, His246	Trp38, Phe145, Ile149, Pro210	Ligand, Asp109, His110, His246	His105. His107, His181
ZINC000108190035	-7.4	Ser208, His246	Phe145, Ile149, Ala249	Asp109, His110, His246	His105. His107, His181
ZINC000192605700	-7.3	Ser206, Ser208, His246	Phe145, Ile149, Pro210, Ala249	Asp109, His110, His246	His105. His107, His181
ZINC000301829834	-7.4	Asp109, Ser208	Phe145, Ile149, Pro210, His246, Ala249	Asp109, His110, His246	His105. His107, His181
ZINC000007000921	-7.5	Ser208	Tyr32, Trp38, Phe145, Ile149	Asp109, His110, His246	His105. His107, His181
ZINC001242360271	-7.2	Ser208	Trp38, Phe145, Pro210, His246, Ala249	Asp109, His110, His246	His105. His107, His181
ZINC000001718904	-7.2	-	Trp38, Phe145	Asp109, His110, His246	His105. His107, His181
ZINC00742221528	-7.5	Ser206, Ser208	Phe145, Ile149, Pro210, His246, Ala249	Asp109, His110, His246	His105. His107, His181
EDTA (control)	-4.6	Ser206, Ser208, Tyr32	-	Asp109, His110, His246	His105. His107, His181

hydrogen bonds with His181 and Ser208, and hydrophobic bonds with Phe145 and Ile149 at the active site. ZINC000634582851 (Fig. 3C) also showed interaction with one Zn^{2+} ions (ZN-302) and hydrogen and hydrophobic bonds were established with Asp109, His246 and Trp38, Phe145, Ile149, Pro210 respectively. EDTA, a known Metallo-β-lactamase inhibitor was docked with the target protein and compared with the docking results of the compounds. Binding energy of EDTA was found to be -4.9 kcal/mol and it showed hydrogen bond interactions with both zinc atoms as well as active site residues like Ser 206, Ser 205 and Tyr32 (Fig. 3D) The rest of the compounds did not interact with zinc atoms, but formed hydrogen and hydrophobic bonds with the active site residues.

Molecular dynamics

Based upon ADME results and docking analysis, ZINC000000616394 ((9R)-3-hydroxy-4,9-dimethyl-7,8,9,10-tetrahydrobenzo[c]chromen-6-one) and ZINC000000393032 (Benzo[j]phenanthridin-12-ol) were finalized for MD simulation studies as both the compounds satisfied all the drug likeness properties, were AMES non-toxic, showed zero PAINS alert, low CyP inhibition and interacted with zinc atoms at the active site. Simulation studies of the protein ligand complexes were carried out after the successful screening of compounds from ZINC15 database. Using the GROMACS tool, the protein and the ligand complexes were subjected to 300ns simulation. The results were obtained in the form of RMSD, RSMF, and Hydrogen bonds (H-bond). The graphs were plotted using Xmgrace (5). The change in the secondary structure of the protein was checked when the compounds are bound to the active site of the protein.

The RMSD (Root Mean Square Deviation) analysis serves valuable insights into the conformational stability of the α-carbon of the protein ligand complex throughout the Molecular Dynamics (MD) Simulation (Fig. 4A). An RMSD of less than 0.3 nm is considered as a stable binding [23]. In the initial stage, both the complexes exhibited a sudden RMSD elevation. This phenomenon is due to the ligand binding at the active site. The RMSD of the protein-ZINC393032 complex (blue) showed an average deviation (difference between the upper limit and lower limit in the plot) of approximately 0.2 nm from 0 to 60 ns. From 60 ns to approximately 100ns, the complex exhibited a stable RMSD with a very minute deviation of 0.05 nm. The RMSD gradually decreased till approximately 126 ns, after which it increased gradually with very less deviation of 0.05 nm till the end of 247 ns. After that a slight elevation was observed in the RMSD plot which remained stable till the end of 300 ns. The RMSD of the protein-ZINC616394 complex showed an initial elevation to 0.42 nm. Graph showed a sudden depression at 12 ns and the deviation observed was approximately 0.27 nm. RMSD gradually increased after 12 ns till 60 ns with a deviation of 0.08 nm. From 60 ns to 124 ns, the graph deviated up to 0.09 nm. After 124 ns, there was a sudden elevation and the graph showed high frequency fluctuations till 184 ns. For another 100 ns, RMSD a deviation of 0.13 nm was observed. After 284 ns, till the end of 300ns, RMSD deviated by 0.13 ns and remained stable. These observations indicate that each protein-ligand complex exhibits unique dynamic behaviours and stability profiles throughout the MD simulation.

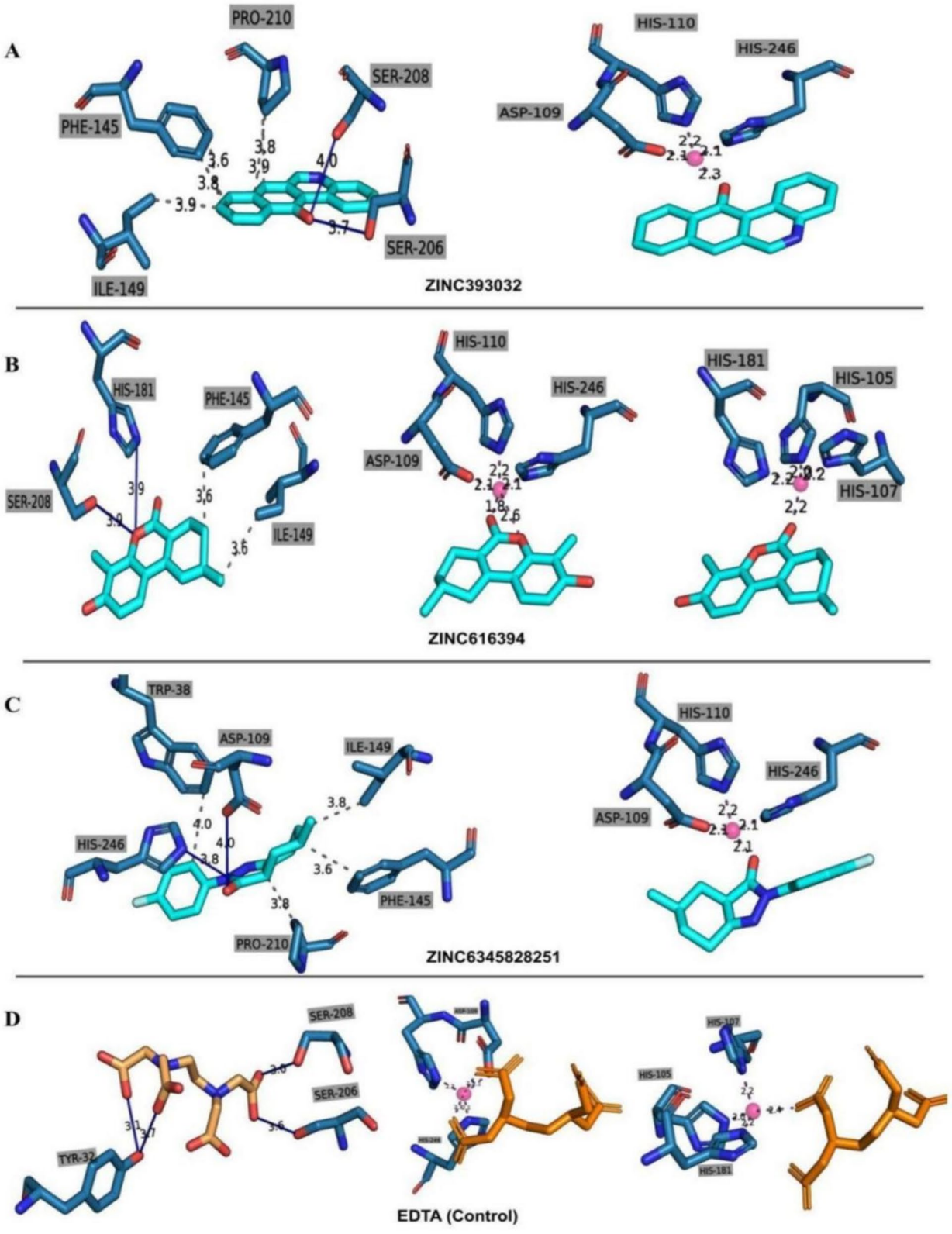


Fig. 3 3D structural representation of the top screened compounds (blue) having interaction with Zn^{2+} and control compound (orange) within the active site of L1 β-lactamase. Key interacting amino acid residues are shown in deep blue, and the catalytic Zn^{2+} ions are represented in pink. The visualization highlights the binding orientation and molecular interactions of the ligands with the active site residues and metal ions

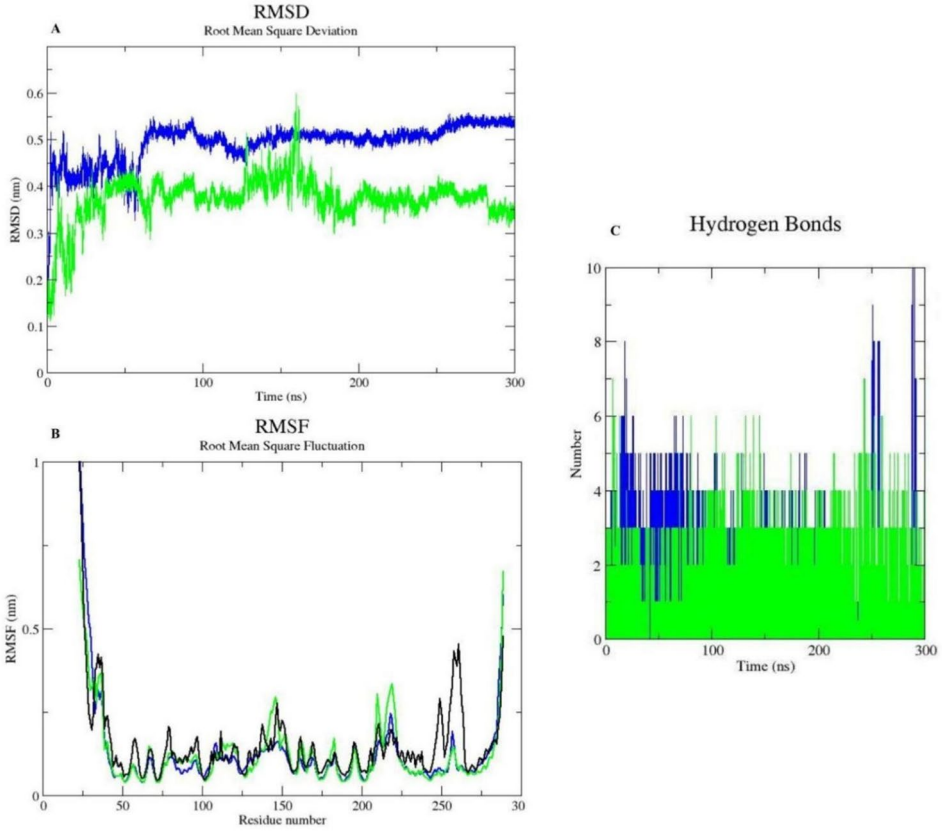


Fig. 4 Molecular Dynamics (MD) simulation analysis of ZINC393032 (blue) and ZINC616394 (green). *(A)* Root Mean Square Deviation (RMSD) of the protein-ligand complexes over 300 ns. *(B)* Root Mean Square Fluctuation (RMSF) of protein residues in complex with the ligands, along with the apoprotein (black) for comparison. *(C)* Number of hydrogen bonds formed between the ligands and the protein during the simulation. The results provide insights into the stability, flexibility, and interaction profiles of the ligand-bound systems

Root Mean Square Fluctuation or RMSF (Fig. 4B) was analysed for quantifying the flexibility and stability of the amino acid residues. An RMSF threshold of less than 0.2 nm [24] indicated stable binding. RMSF for the starting and ending residues of both the complexes showed higher values which can be attributed to the linearity and hence less stability in the region at C-terminal and N-terminal of the polypeptide chain. Amino acids at the non-reactive site of the protein were more stable and hence RMSF is low. A protein alone simulation was performed to compare the RMSF with the complex. From 50 to 100, the apo-protein showed higher fluctuation than the complexes. Same trend was repeated in the region between 249 and 262. The ZINC616394 complex fluctuated more than the apo-protein in the region between 140 and 146 and 210 to 221. ZINC393032 remained stable throughout, with minute fluctuations only at the active site regions (81, 95, 109, 145, 149, 181, 210, 258 etc.) due to the ligand binding to the residues. For the complex with ZINC393032, the RMSF was more stable (Fig. 4B) when compared to ZINC616394. ZINC616394 formed peaks higher than the apo protein indicating the instability of the protein-ligand binding. These high fluctuations indicated the chance of ligand breaking away from the protein. For both the complexes, the active site residues mainly showed small peaks, indicating the ligand binding. As a result, the active site region would be rendered unavailable for executing the inhibitory property of the protein. Hydrogen bonds formed throughout the simulation (Fig. 4C) was plotted to analyse how strong the ligand binds to the target. ZINC393032 formed a maximum of 9 hydrogen bonds and ZINC616394 formed 7 hydrogen bonds with the protein, indicating ZINC393032 was comparatively more stable than ZINC616394.

Binding free energy

Binding free energy calculations were performed using the MM/PBSA method. The estimated binding free energy for the ZINC616394 complex was -52.23 ± 6.02 kJ/mol, while ZINC393032 exhibited a more favourable value of -68.02 ± 8.006 kJ/mol. The presence of positive polar solvation energy alongside negative SASA energy for both compounds indicated a significant contribution of hydrophobic interactions to binding stability. Additionally, both hit compounds demonstrated strong van der Waals interactions, further supporting

their favourable binding profiles. A detailed summary of the energy components is provided in Table 3.

Expression and purification of Recombinant MetalloL1 β-lactamase

Recombinant protein was observed at approximately 31 KDa. The cells induced with 0.6 mM IPTG at 30˚C were found to have better expression (Fig. 5). A total protein concentration of 4.903 mg/ml was obtained after purification.

Enzyme Inhibition

Initially yellow coloured substrate nitrocefin changed to dark pink colour due to hydrolysis by the enzyme. Upon incubation with the inhibitor, the pink colour intensity gradually reduced and regained the yellow colour. OD readings were measured every 5 min for 35 min. Percentage inhibition was calculated from the OD vs. time plot.

% Inhibition = [1 - (OD of inhibitor ÷ OD maximum)] * 100.

IC_{50} value of EDTA was found to be 2.24 µM and for benzo[j]phenanthridin-12-ol (ZINC393032) it was 22.96 µM (Fig. 6).

MIC and checkerboard synergy test

CLSI (Clinical and Laboratory Standards Institute) standard breakpoint for Imipenem against gram negative bacteria was 16 *µg/ml* for resistance. MIC obtained in our case is 250 *µg/ml*, indicated that *Stenotrophomonas maltophilia* was highly resistant to imipenem. MIC for Benzo[j]phenanthridin-12-ol was found to be 15.6 *µg/ml* (Fig. 7) The combination of inhibitor (Benzo[j]phenanthridin-12-ol) and antibiotic (imipenem) resulted in a significant decrease in the MICs for both the compounds. Five different synergistic combinations with FIC indices of 0.37, 0.31, 0.28, 0.25 and 0.19 were found from the study (Table 4).

Specifically, the RMSD, RMSF, and hydrogen bond analyses revealed that the top hit compound, Benzo[j] phenanthridine-12-ol (ZINC393032), maintained stable interactions with key active site residues and Zn^{2+} ions throughout the simulation. These consistent and strong interactions likely contribute to the compound's ability to inhibit the enzyme efficiently, resulting in a measurable reduction in MIC. Thus, the MD simulation provides structural and dynamic insight into how the observed decrease in bacterial resistance may be occurring at the molecular level.

Table 3 MM/PBSA-based binding free energy and energy component analysis of the top two screened compounds (ZINC616394 and ZINC393032) in complex with L1 β-lactamase

	ZINC616394 (kJ/mol)	ZINC393032 (kJ/mol)
Binding free energy	-52.23 +/- 6.02	-68.02 +/- 8.006
Electrostatic energy	-0.816 +/- 32.12	-12.54 ± 2.47
Polar solvation energy	45.241 +/- 16.965	33.701 ± 3.21
SASA energy	-6.235 +/- 4.226	-7.65 ± 1.22
Van der Waals energy	-25.071 +/- 52.021	-69.94 ± 9.02

Time kill test

Benzo[j]phenanthridin-12-ol/Imipenem (BP/IMP) combination concentrations, 3.9/31.25 *µg*/ml, 1.95/31.25/ *µg*/ml, 3.9/15.3 *µg*/ml, 1.95/15.3 *µg*/ml and 3.9/7.8 *µg*/ml were tested against 6.22 ± 0.08 $\log_{10}$ CFU/ml of *Stenotrophomonas maltophilia* in MHB (Fig. 8). There was an abrupt increase in bacterial densities of up to 8.91 ± 0.08 $\log_{10}$ CFU/ml and remained steady till 24 h for control bacteria. BP/IMP-treated cultures reached log phase slowly, and the maximum density did not exceed 6.8 ± 0.10 $\log_{10}$ CFU/ml. The lowest density (2.1 ± 0.03 $\log_{10}$ CFU/ml) was observed for BP/IMP concentrations of 3.9/31.25 *µg*/ml and 3.9/15.3 *µg*/ml. No regrowth of bacteria was observed after 24 h in any of the cases.

Discussion

Resistance to antibiotics by bacteria has been a menace in the clinical field. Not just bacteria, almost all the pathogenic microbes have genetically evolved to shield themselves from a variety of antibiotics that are currently on the market. In such a scenario, combination drug therapy has become an approachable alternative as it is a promising method to enhance treatment success, prolong the activity of the otherwise resistant drug, and is also economically efficient. The current study targets *Stenotrophomonas maltophilia* due to its versatile multidrug-resistant mechanisms and nosocomial nature, making it one of the most serious threats in hospitals. Several combination therapies, once used for *Stenotrophomonas maltophilia* infection treatment, like TMP-SMX (Trimethoprim-Sulfamethoxazole) with β-lactams, fluoroquinolones with TMP-SMX, and colistins with β-lactam combinations have now become ineffective, due to the development of intrinsic resistance by the bacteria [25–27]. A recent study has reported withanolides A, Q, R, and 2,4-deoxy withaferin to be potential β-lactamase inhibitors using a computer-based drug screening technique [27].

A novel combination of an inhibitor and β-lactam antibiotic (BP/IMP) was discovered after rigorous virtual screening followed by in vitro validation. From as high as 500,000 compounds downloaded from the ZINC15 database docked with Metallo-L1 β-lactamase, 37 compounds with the best dock score were selected. The least binding energy (more negative value) indicated a strong affinity of the molecule to the protein domain. ADMET analysis was carried out for the 37 compounds as it was crucial to check the pharmacokinetic and toxicological

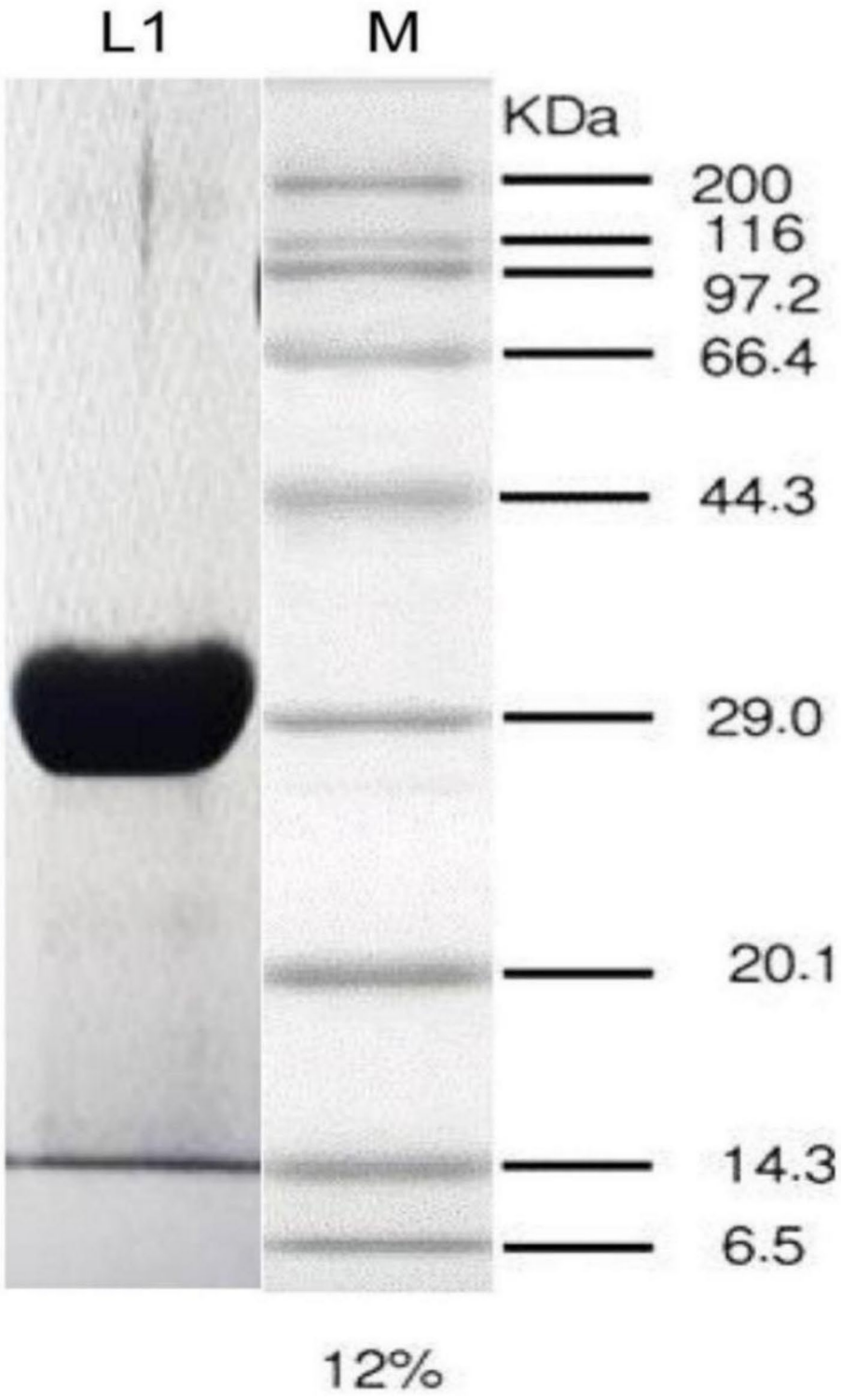


Fig. 5 SDS-PAGE analysis of purified recombinant Metallo L1 β-lactamase after dialysis. Lane M: Protein marker (Premixed Protein Marker (Broad), Takara Bio, Code: 3597); Lane L1: Dialyzed L1 β-lactamase protein. The gel confirms the presence of the target protein at the expected molecular weight

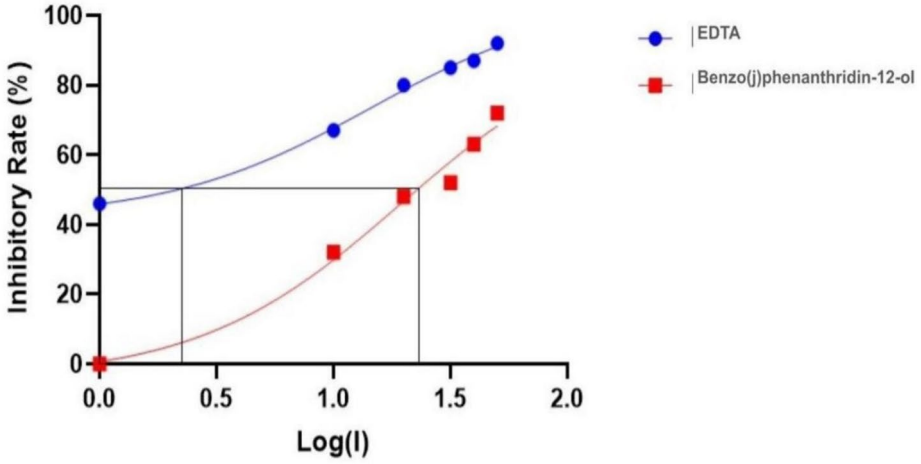
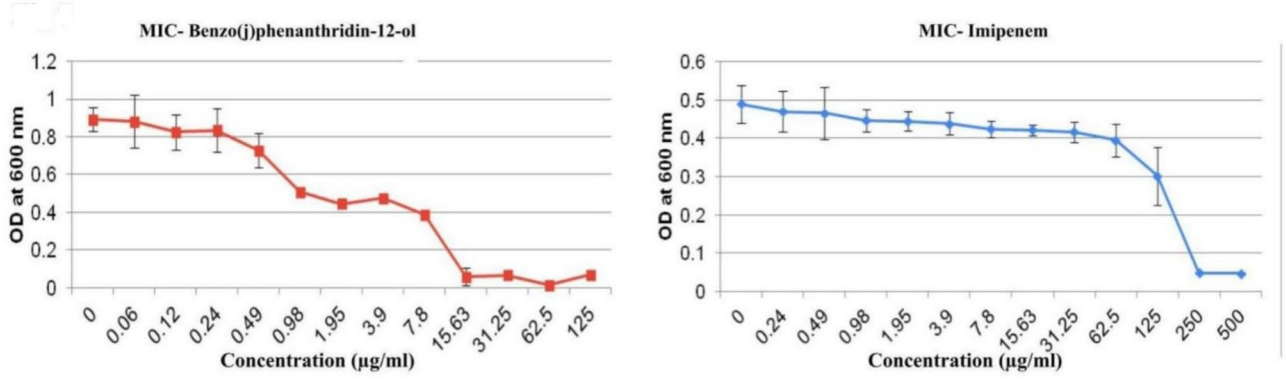


Fig. 6 Enzyme inhibition assay of recombinant L1 β-lactamase using Benzo[j]phenanthridin-12-ol. The percentage of enzyme inhibition is plotted against the logarithm of inhibitor concentrations



	Range Tested	MIC90
Imipenem	(0.24-500) µg/ml	250 µg/ml
Benzo[j]phenanthridine-120ol	(0.06-125) µg/ml	15.63 µg/ml

Fig. 7 Minimum Inhibitory Concentration (MIC) of Benzo[j]phenanthridin-12-ol and imipenem against *Stenotrophomonas maltophilia*

properties of compounds that can be potential drug candidates and to predict their behaviour in the human body. Based on the molecular docking and ADMET results, two compounds, ZINC393032 (isoquinoline alkaloid) and ZINC616394 (flavonoid-like compound), were finalized for molecular dynamics (MD) simulation. ZINC393032 and ZINC616394 formed hydrogen bond interactions with zinc atoms at the active site, rendering it unavailable for β-lactam ring hydrolysis. MBLs require the participation of active site metals (one or two $Zn2+$ ions in vivo) to activate an active site water molecule for ring-opening hydrolysis of the β-lactam ring. We attained a deeper understanding of drug and target interaction by simulating the two complexes for 300 ns using the GROMACS tool. RMSD, RMSF, and H-bond graphs were studied to interpret the efficacy of binding. The RMSD analysis gave the idea that the system did not expand and contract too much, and it remained in a steady state for ZINC393032, but showed greater fluctuations in the case of ZINC616394. The residue level fluctuation was obtained through the RMSF graph. The active site residues in the ZINC393032 complex were more stable when compared to those of the apoprotein, indicating that the compound remains attached to the active site and the otherwise reactive residues at the active site have attained a stable state. ZINC616394 showed fluctuations greater than the apo-protein at certain regions. RMSD and RMSF analysis gave the idea that ZINC393032 formed a more stable complex with the target protein. The hydrogen bond data also supports the information.

Table 4 Checkerboard synergy assay and FICI values for the combination of Benzo[j]phenanthridin-12-ol (B) and Imipenem (I) against *Stenotrophomonas maltophilia*, indicating potential synergistic antibacterial effects

S. No.	Antibiotic and Compound	MIC (µg/ml)	MIC in combination (µg/ml)	Reduction fold	FIC < 0.5 Synergy
1	I	250	31.25	8	0.37
	B	15.63	3.9	4	
2	I	250	31.25	8	0.25
	B	15.63	1.95	8	
3	I	250	15.63	16	0.31
	B	15.63	3.9	4	
4	I	250	15.63	16	0.19
	B	15.63	1.95	8	
5	I	250	7.8	32	0.28
	B	15.63	3.9	4	

Thus, MD simulation analysis and interpretation helped in concluding ZINC393032 as a better inhibitor.

To validate the in silico findings, ZINC393032 (Benzo[j]phenanthridin-12-ol) was selected for in vitro assays. This compound belongs to the class of polyaromatic nitrogen-containing alkaloids. Notably, to the best of our knowledge, there are no prior scientific reports detailing the biological activity of this compound. However, derivatives of this compound exhibit antibacterial and antiviral activities [28]. Initially, the compound was tested to check its inhibitory activity on recombinant Metallo-L1 β-lactamase using nitrocefin as the substrate. This chromogenic cephalosporin formed a spectroscopically detectable anionic intermediate, giving a dark pink colour, due to β-lactamase activity. The colour changed back to yellow upon incubation with the inhibitor. The colour change indicated the inhibition of β-lactamase due to the binding of the inhibitor at the active site, making it unavailable for nitrocefin to form the anionic intermediate. From the enzyme inhibition assay, the IC_{50} was found to be 22.96 µM for the inhibitor (Benzo[j]phenanthridin012-ol while the known inhibitor EDTA attained the IC_{50} value at 2.24 µM. Both compounds inhibit the enzyme by chelating the Zn^{2+} ions at the active site. This outcome can be compared with the docking result, where we found both Benzo[j]phenathridin-12-ol and EDTA binding to Zn^{2+} ions. After confirming that the compound can act as a potential inhibitor, the next quest was to find whether the compound, in combination with a β-lactam antibiotic (imipenem), can kill the bacteria. Imipenem is a substrate specific to L1 β-lactamase but not to L2 β-lactamase [29]. Checkerboard synergy testing gave five different combinations of BP/IMP with FICI of 0.37, 0.31, 0.28, 0.25, and 0.19. All five combinations were used in the time kill assay. The BP/IMP concentrations of 3.9/31.25 *µg/ml* and 3.9/15.3 *µg/ml* showed the deepest descent in cell density. This might be because the concentration of the inhibitor was the highest in these two combinations. However, all the combination concentrations killed the bacteria, and none of the combinations observed any bacterial regrowth after 24 h. The findings illuminated the compound as a good β-lactamase inhibitor and can be combined with imipenem to attain bacterial death. Through this study, we were able to propose Benzo[j] phenanthridin-12-ol (ZINC393032) as a potential inhibitor using in silico methods. The in vitro

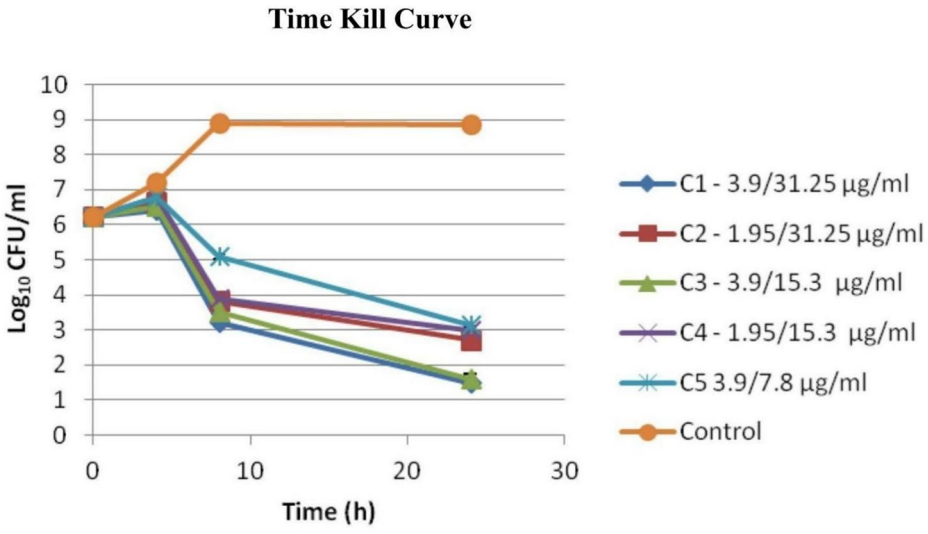


Fig. 8 Time-kill curves for five different concentration combinations (C1 to C5) of Benzo[j]phenanthridin-12-ol and imipenem against *Stenotrophomonas maltophilia*. Bacterial viability was monitored over time to assess the bactericidal activity of the combinations. The results demonstrate the synergistic effect of the compounds, with varying degrees of bacterial reduction across the combinations

study on Benzo[j]phenanthridin-12-ol validated the computational results as it was able to efficiently inhibit the recombinant L1 β-lactamase enzyme. ZINC616394 could also be tested in vitro, as it showed good docking results by binding to both the zinc atoms. Detailed research can be done on these two compounds to find the exact mechanism behind the inhibition process.

Conclusion

Antimicrobial resistance mediated by β-lactamase activity significantly limits therapeutic options, as most β-lactam antibiotics become ineffective due to enzymatic hydrolysis of their β-lactam ring. This study focused on targeting the Metallo-L1 β-lactamase enzyme produced by *Stenotrophomonas maltophilia*. Utilizing a virtual high-throughput screening approach, compounds from a large dataset were screened based on their binding affinity to the enzyme's active site. Among them, ZINC393032 and ZINC616394 exhibited favourable docking scores, fulfilled key drug-likeness criteria, and demonstrated structural stability during 300 ns molecular dynamics simulations. Subsequent in vitro validation of ZINC393032 (Benzo[j]phenanthridin-12-ol) confirmed its inhibitory potential against Metallo-L1 β-lactamase. Moreover, the combination of Benzo[j]phenanthridin-12-ol with imipenem (BP/IMP) exhibited bactericidal effects, highlighting its potential as an effective therapeutic strategy. This study, therefore, proposes two promising Metallo-L1 β-lactamase inhibitors and introduces a novel BP/IMP combination that may offer an improved alternative for treating *Stenotrophomonas maltophilia* infections.

Abbreviations

MD	Molecular Dynamics
vHTS	Virtual High Throughput Screening
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
H bond	Hydrogen Bond
MIC	Minimum inhibitory concentration
MDR	Multi-Drug Resistance
XDR	Extensive Drug Resistance
MBL	Metallo beta lactamase
UTI	Urinary tract Infection
SSTI	Skin and soft tissue infection
ESBL	Extended spectrum beta lactamase
CADD	Computer-aided drug discovery
ADMET	Absorption, Distribution, Metabolism, Excretion, and Toxicity
PAINS	Pan Assay Interference Compounds
PME	Particle mesh algorithm
NVT	Constant Number of particles, Volume, Temperature
NPT	Constant Number of particles, Pressure, and Temperature
PBC	Periodic Boundary Conditions
MM/PBSA	Molecular Mechanics/Poisson-Boltzmann Surface Area
SASA	Solvation Solvent Accessible Surface Area
LB	Luria-Bertani agar
IPTG	Isopropyl β-D-1-thiogalactopyranoside
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony Forming Unit
BP/IMP	Benzo[j]phenanthridine-12-ol/Imipenem

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03994-6.

Supplementary Material 1

Acknowledgements

The authors thanks HPCC of SRM Institute of Science and Technology, Kattankulathur for high performance computer facilityThe authors acknowledge the Researchers Supporting Project number (RSPD2025R708), King Saud University, Riyadh, Saudi Arabia, for funding open access.

Author contributions

S. K. H. performed the experiments, analysis and manuscript preparation.S. M. contributed in funding acquisition reviewing.S. S. designed the project and methodology and manuscript reviewing.

Funding

This research received no fund from any funding agency. Open access funding provided by King Saud University.

Data availability

The datasets generated and/or analysed during the current study are available in the ZENODO repository, https://doi.org/10.5281/zenodo.14615214

Declarations

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

Clinical trial number

Not Applicable.

Author details

[1]Department of Genetic Engineering, School of Bioengineering, College of Engineering and Technology, SRM Institute of Science and Technology, Kattankulathur, India
[2]Department of Botany and Microbiology, College of Science 5, King Saud University, Riyadh 11451, Saudi Arabia

Received: 25 November 2024 / Accepted: 23 April 2025
Published online: 06 May 2025

References

1. Munita JM, Arias CA. Mechanisms of antibiotic resistance. Virulence mechanisms of bacterial pathogens. Jun. 2016;22:481–511.
2. Wattal C, Kler N, Oberoi JK, Fursule A, Kumar A, Thakur A. Neonatal sepsis: mortality and morbidity in neonatal sepsis due to multidrug-resistant (MDR) organisms: part 1. Indian J Pediatr. 2020;87:117–21.
3. Zango UU, Ibrahim M, Shawai SA, Shamsuddin IM. A review on β-lactam antibiotic drug resistance. MOJ Drug Des Develop Ther. 2019;3(2):52–8.
4. Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother. 2010;54(3):969–76.
5. Huang HH, Chen PY, Hu RM, Lin YT, Li LH, Yang TC. Impacts of L1 promoter variation and L2 clavulanate susceptibility on ticarcillin-clavulanate susceptibility of Stenotrophomonas maltophilia. Antimicrob Agents Chemother. 2018;62(11):10–128.

6. Özdemir ZC, Turhan AB, Bör Ö. Stenotrophomonas Maltophilia/s. Maltophilia sepsis presenting with perianal cellulitis and pneumonia in a leukemic child.
7. Chatwin CL, Hamrick JC, Trout RE, Myers CL, Cusick SM, Weiss WJ, Pulse ME, Xerri L, Burns CJ, Moeck G, Daigle DM. Microbiological characterization of VNRX-5236, a broad-spectrum β-lactamase inhibitor for rescue of the orally bioavailable cephalosporin Ceftibuten as a carbapenem-sparing agent against strains of enterobacterales expressing extended-spectrum β-lactamases and Serine carbapenemases. Antimicrob Agents Chemother. 2021;65(8):10–128.
8. Rizzuti B, Grande F. Virtual screening in drug discovery: A precious tool for a still-demanding challenge. InProtein Homeostasis Diseases. 2020 Jan 1 (pp. 309–327). Academic Press.
9. Ligand-based SB. Computer-Aided drug design. Curr Drug Synthesis. 2023:339.
10. ZINC15 database. https://zinc15.docking.org/
11. Burley SK, Bhikadiya C, Bi C, Bittrich S, Chao H, Chen L, Craig PA, Crichlow GV, Dalenberg K, Duarte JM, Dutta S. RCSB protein data bank (RCSB.org): delivery of experimentally-determined PDB structures alongside one million computed structure models of proteins from artificial intelligence/machine learning. Nucleic Acids Res. 2023;51(D1):D488–508.
12. Meng EC, Goddard TD, Pettersen EF, Couch GS, Pearson ZJ, Morris JH, Ferrin TE. UCSF chimerax: tools for structure Building and analysis. Protein Sci. 2023;32(11):e4792.
13. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of Docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem NA-NA
14. SWISS-ADME. http://www.swissadme.ch/
15. ADMEtsar. http://lmmd.ecust.edu.cn/admetsar1
16. Bekker H, Berendsen HJ, Dijkstra EJ, Achterop S, Vondrumen RV, Vanderspoel D, Sijbers A, Keegstra H, Renardus MK. Gromacs-a parallel computer for molecular-dynamics simulations. In 4th International Conference on Computational Physics (PC 92), 1993 (pp. 252–256). World Scientific Publishing.
17. Kumari R, Kumar R, Lynn A. g_mmpbsa—A GROMACS tool for High-Throughput MM-PBSA calculations. J Chem Inf Model. 2014;54(7):1951–62.
18. Wiegand I, Hilpert K, Hancock RE. Agar and broth Dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.
19. CLSI. Methods for Dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. 7th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2006. (CLSI document M7-A7).
20. Vasudevan A, Kesavan DK, Wu L, Su Z, Wang S, Ramasamy MK, Hopper W, Xu H. Silico and in vitro screening of natural compounds as Broad-Spectrum β-Lactamase inhibitors against Acinetobacter baumannii new Delhi Metallo-β-lactamase-1 (NDM-1). Biomed Res Int. 2022;2022(1):4230788.
21. CLSI. Performance standards for antimicrobial susceptibility testing: approved standard. 17th informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute; 2008. (CLSI document M100-S18).
22. Landrum ML, Conger NG, Forgione MA. Trimethoprim-sulfamethoxazole in the treatment of Stenotrophomonas maltophilia osteomyelitis. Clin Infect Dis. 2005;40(10):1551–2.
23. Hollingsworth SA, Dror RO. Molecular dynamics simulation for all. Neuron. 2018;99(6):1129–43.
24. Genheden S, Ryde U. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. Expert Opin Drug Discov. 2015;10(5):449–61.
25. Junco SJ, Bowman MC, Turner RB. Clinical outcomes of Stenotrophomonas maltophilia infection treated with Trimethoprim/sulfamethoxazole, Minocycline, or fluoroquinolone monotherapy. Int J Antimicrob Agents. 2021;58(2):106367.
26. Jade D, Harrison MA, Sugumar S. Identification of a natural inhibitor against L1 β-lactamase present in Stenotrophomonas maltophilia. J Mol Model. 2022;28(11):342.
27. Anza M, Endale M, Cardona L, Cortes D, Eswaramoorthy R, Cabedo N, Abarca B, Zueco J, Rico H, Domingo-Ortí I, Palomino-Schätzlein M. Cytotoxicity, antimicrobial activity, molecular docking, drug likeness and DFT analysis of benzo [c] phenanthridine alkaloids from roots of Zanthoxylum [c]halybeum. Biointerface Res Appl Chem. 2022;12:1569–86.
28. Rasia RM, Vila AJ. Mechanistic study of the hydrolysis of Nitrocefin mediated by B. cereus Metallo-β-lactamase. Arkivoc. 2003;3:507–16.
29. Akova M, Bonfiglio G, Livermore DM. Susceptibility to β-lactam antibiotics of mutant strains of Xanthomonas maltophilia with high- and low-level constitutive expression of L1 and L2 β-lactamases. J Med Microbiol. 1991;35(4):208–13.

Publisher's note