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Punica granatum leaf extract as a natural antibacterial agent explored by experimental and computational methods

Faria Tasnim[1,2], Md. Eram Hosen[2,3], Al Riyad Hasan[4], Md. Taohid Hasan[5], Md. Mizanur Rahaman[3], Md. Motaher Hossain[6], Md. Abdulla Al Jubayed[6], Md. Aktaruzzaman[3], Mansour Altayyar[7], Endalamaw Yihune[8]✉, Md. Tarikul Islam[9] & Magdi E. A. Zaki[10]✉

The rise of multidrug-resistant (MDR) microorganisms, particularly *Escherichia coli*, poses a significant threat to global public health, necessitating the development of alternative treatments. This study investigates the antibacterial properties of methanolic *Punica granatum* (pomegranate) leaf extract (MPGL) against MDR *E. coli* and explores its potential as a source of natural inhibitors for the CTX-M-9 beta-lactamase enzyme, a key contributor to antibiotic resistance. The disc diffusion assay revealed that MPGL exhibited significant antibacterial activity, with the highest inhibition zone of 16.67 ± 0.29 mm at 100 µg/disc. Additionally, an *in silico* approach was employed to identify potential inhibitors from *P. granatum* phytochemicals. Molecular docking studies demonstrated strong binding affinities of Epicatechin, Kaempferol, and Apigenin to the CTX-M-9 beta-lactamase protein (PDB ID: 1YLY), with binding energies of -6.25, -5.23, and – 5.21 kcal/mol, respectively. These compounds also showed favorable pharmacokinetic and toxicity profiles in ADMET analysis, indicating their potential as safe and effective therapeutic agents. Molecular dynamics simulations further confirmed the stability of these compounds in complex with the target protein over a 100 ns trajectory, with Epicatechin showing the most stable interactions. The study highlights the promising antibacterial activity of *P. granatum* leaf extract and identifies Epicatechin, Kaempferol, and Apigenin as potential lead compounds for developing novel therapies against MDR *E. coli*. These findings underscore the potential of plant-based compounds in combating antibiotic resistance and provide a foundation for further research into phytopharmaceuticals as alternative treatments for bacterial infections.

Keywords Antibacterial activity, *Punica granatum*, ADMET, Molecular Docking, Molecular dynamics simulation, CTX-M-9 beta-lactamase

Abbreviations

MPGL	Methanolic Punica granatum Leaf
PDB	Protein data bank
ADMET	Absorption, distribution, metabolism, excretion, and toxicity

[1]Department of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi 6205, Bangladesh. [2]Department of Microbiology, Shaheed Shamsuzzoha Institute of Biosciences, Affiliated with University of Rajshahi, Rajshahi6205, Bangladesh. [3]Biomedical Sciences and Molecular Biology, College of Medicine and Dentistry, James Cook University, Townsville, QLD 4811, Australia. [4]Department of Pharmacy, Faculty of Biological Science and Technology, Jashore University of Science and Technology, Jashore 7408, Bangladesh. [5]Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi 6205, Bangladesh. [6]Department of Microbiology, Rajshahi Institute of Biosciences, Affiliated with University of Rajshahi, Rajshahi, Bangladesh. [7]Department of Basic Medical Sciences, College of Medicine, University of Jeddah, Jeddah 23890, Saudi Arabia. [8]Department of Biology, College of Science, Bahir Dar University, P. O. Box 79, Bahir Dar, Ethiopia. [9]Department of Genetic Engineering and Biotechnology, Jashore University of Science and Technology, Jashore 7408, Bangladesh. [10]Department of Chemistry, College of Science, Imam Mohammad Ibn Saud Islamic University Riyadh, Riyadh, Saudi Arabia. ✉email: yihuneendalamaw3@gmail.com; Endalamaw.Yihune@bdu.edu.et; mezaki@imamu.edu.sa

MM-GBSA	Molecular mechanics with generalized Born and surface area solvation
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
rGyr	Radius of Gyration
SASA	Solvent accessible surface area

There is an alarming rise of multi-drug resistant (MDR) microorganisms and the emergence of new strains resistant to nearly all known antibiotics. The World Health Organization (WHO) has identified antimicrobial resistance (AMR) as one of the ten most critical global public health challenges facing humanity[1]. Global estimates indicate that in 2023, over 1.05 million deaths were directly attributed to AMR, and this number is projected to rise to approximately 10 million deaths annually by 2050 if adequate measures are not taken[1,2]. The rise of MDR bacteria causes antimicrobial treatments ineffective, prolongs the duration of illness and hospital stays, and elevates the mortality rate. The causes of AMR are diverse, but the excessive use of antibiotics has been a primary driver. Between 2000 and 2015, antibiotic consumption increased by 65% globally, with significant rises particularly in low- and middle-income countries. Bacteria, being a complex organism, can readily exchange DNA, often without significant detriment to themselves. Surprisingly, the presence of antibiotics has minimal effect on this DNA transfer but can favor the selection of resistant traits[1]. One solution of AMR involves combining antibiotics with other agents for synergistic effect. Several projects are developing new phyto-pharmaceuticals, offering alternative treatments alongside antibiotics[3]. This advancement could provide phytotherapy with renewed credibility and offer alternative treatments for diseases traditionally treated with synthetic drugs[4].

Punica granatum, commonly known as pomegranate, belongs to the *Punicaceae* family. It is a large deciduous shrub or small tree originating from Asia. This plant possess several health, nutritional, therapeutic benefits, and bioactive compounds[5]. The components of *P. granatum* have been associated with various biological and pharmacological effects. Specifically, phenolics found in pomegranate leaves are known for their health-promoting properties. These phenolics exhibit significant affinity for diverse molecular structures such as proteins or glycoproteins, which can antagonize bacterial resistance (Trabelsi et al., 2020). It is not unknown that a significant portion of pharmaceuticals worldwide originate from plants, only a small fraction is used as antimicrobial agents. Plant-derived natural compounds are increasingly capturing attention in modern times due to their apparent efficacy and minimal adverse effects. Moreover, natural plant-based compounds can be locally sourced, reducing production costs while promoting environmental and economic sustainability. Integrating traditional knowledge with modern research validates their therapeutic potential. Furthermore, Phytochemicals can synergize with antibiotics. This reduces required dosages, overcomes bacterial resistance, inhibits drug-inactivating enzymes like β-lactamases, and enhances drug bioavailability[7]. This combination can improve antimicrobial effectiveness while minimizing side effects, providing a promising approach to combat AMR[8].

Escherichia coli is a natural inhabitant of the gut and a frequent cause of various infections in humans, including urinary tract infections, enteric infections, and systemic or bloodstream infections. A subgroup of *E. coli*, known as extra-intestinal pathogenic *E. coli* is particularly responsible for infections outside the intestines and has been crucial in spreading antibiotic resistance[9]. The genetic adaptability of *E. coli* enables it to obtain numerous AMR mechanisms due to constantly changing environments. As versatile residents of the gut, commensal strains of *E. coli* face repeated challenges from antimicrobial agents throughout the host's life span. Consequently, these strains acquire resistance genes or develop resistant mutants to ensure survival and maintain microbial balance in the lower intestinal tract. Therefore, commensal *E. coli* strains serve as an indicator of the antimicrobial burden on their hosts[10]. One of the major resistance mechanisms in *E. coli* involves the production of beta-lactamases, specifically the CTX-M enzymes. CTX-M-9 beta-lactamase, in particular, has emerged as a predominant extended-spectrum beta-lactamase (ESBL) in MDR *E. coli*. CTX-M-9 hydrolyzes beta-lactam antibiotics via a serine active site, which results in resistance[11]. Despite its resistance to beta-lactam antibiotics, CTX-M-9 beta-lactamase exhibits high efficiency in hydrolyzing cefotaxime and ampicillin, indicating its ability to effectively degrade penicillins and cephalosporins[11]. While they have become the predominant ESBLs in some regions, effective inhibitors are inadequate and their precise mechanism remains poorly understood[12]. This complexity is compounded by the genetic variability and adaptability of these enzymes, which pose challenges for treatment and containment strategies[13]. Our research aims to identify promising natural lead compounds that could inhibit the pathogenesis of *E. coli* and serve as a potential therapy for antibiotic resistance in humans. Hence, the objective of this study was to determine the antibacterial activity of the methanolic leaf extract of *P. granatum* against the MDR *E. coli* bacteria. An in silico approach was also conducted as well to identify inhibitors against the CTX-M-9 beta-lactamase protein (PDB ID: 1YLY) of *E. coli* from the phytochemicals of *P. granatum* leaf. CTX-M-9 is highly relevant due to its major role in driving MDR in *E. coli*. Although alternative targets such as efflux pumps and porins exist, CTX-M-9 beta-lactamase remains a critical focus due to its clinical impact and lack of effective inhibitors. By identifying lead compounds capable of inhibiting CTX-M-9, this research seeks to develop novel therapeutic options to combat AMR in *E. coli*.

Materials and methods

Plant material

Fresh and mature leaves of *P. granatum* were collected from the Botanical Garden of University of Rajshahi, Bangladesh in August 2022, situated at latitudes of 24°36"82' N and 88°63"76' E. The plant was identified by Dr. A. H. M. Mahbubur Rahman, Professor in the Department of Botany at the University of Rajshahi, Bangladesh. Moreover, a voucher specimen with the accession number (BDRU144) has been preserved at the Herbarium for future reference.

Plant extract preparation

The collected leaves of *P. granatum* were thoroughly washed first to eliminate any impurities. Following this, the leaves were cut into small pieces and air-dried for a week at room temperature. Once completely dried, they underwent grinding using a grinder machine to produce a coarse powder. Powdered leaves (20 g) were immersed in 100 mL of methanol in a conical flask and kept in an orbital shaker at 25 °C and 150 rpm for 72 h. Thereafter the extract was filtered using Whatman No. 1 filter paper. The residue was again extracted with the same solvent twice. After filtration, the methanol was evaporated and concentrated at room temperature. The dried extract was stored at − 20 °C. For antibacterial assays, the methanolic *P. granatum* leaf (MPGL) extract was dissolved in the same adequate solvent.

Antibacterial activity

Antibacterial activity of MPGL extract was examined against the gram-negative bacteria *Escherichia coli* (ATCC25922). The methanolic extract was tested against bacterial strains obtained from the State Key Laboratory of Microbiology and Bioinformatics, Department of Microbiology, Shaheed Shamsuzzoha Institute of Biosciences, Affiliated with University of Rajshahi, Rajshahi, Bangladesh. The antibacterial assessment was conducted using the disc diffusion method, following a previously established protocol by *Hosen et al.*[14]. Sterile paper discs each with a diameter of 6 mm, were impregnated with varying concentrations of the extract and then air-dried. These concentrations included 25 µg/disc, 50 µg/disc, 75 µg/disc, and 100 µg/disc. Additionally, ciprofloxacin was used as a standard and solvent (methanol) was used as control. These prepared discs were then placed onto Muller-Hinton agar plates inoculated with the bacteria under investigation at a concentration of 10^8 CFU/mL. After 24 h of incubation at 37 °C, the diameter of the zone of inhibition was measured using a millimeter scale. The assay was conducted in triplicate.

Ligand preparation

The phytochemicals from *P. granatum* leaves, along with ceftazidime as a positive control, were identified using a combination of literature studies and database searches. A total of 29 phytochemicals were selected based on their reported bioactivity. The SDF files of each phytochemical were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and then optimized and converted to PDB format using Avogadro. Subsequently, for efficient analysis, the ligands were further optimized and converted to PDBQT format using the PyRx virtual screening tool. **Supplementary Table 1** provides the names of the phytocompounds, their corresponding PubChem CID.

Protein preparation

For our study, we utilized the CTX-M-9 beta-lactamase protein (PDB ID: 1YLY) due to its clinical relevance in *E. coli* antibiotic resistance for molecular docking. The X-ray crystallographic structure of CTX-M-9 beta-lactamase complexed with a ceftazidime-like boronic acid was obtained from the RCSB-PDB database (https://www.rcsb.org/)[12]. The protein was meticulously prepared by removing water molecules and heteroatoms with Discovery Studio 2021 to focus on the essential binding interactions of the active site. Subsequently, energy minimization was conducted using GROMOS96 43B1 parameters via SwissPDB viewer[15]. The energy minimization techniques in GROMOS96 help reduce steric clashes, leading to more stable protein structures. It uses thermodynamically calibrated force fields and integrates experimental data, like NMR and X-ray scattering, to refine protein models and achieve low-energy conformations[16,17].

Active site recognition

Active site amino acid residues in 1YLY were identified using the online server CastP[18]. CASTp assists in identifying a protein's topographical characteristics in a detailed, quantitative, and comprehensive manner. It allows for precise measurement and localization of three-dimensional structures.

ADMET analysis

ADMET (absorption, distribution, metabolism, excretion, and toxicity) analysis is crucial for determining drug-likeness, stability, safety profile and overall effectiveness of new drug candidates in the drug discovery process[19]. To evaluate the pharmacokinetic and drug-like characteristics of the selected 29 phytocompounds, we utilized the SwissADME (http://www.swissadme.ch/) server. Toxicity analysis is crucial in drug discovery for patient safety, regulatory approval, risk minimization, therapeutic optimization, and drug development guidance[20]. The toxicity analysis was conducted in protox-3 (https://tox.charite.de/protox3/) server.

Molecular Docking

Molecular docking was conducted to evaluate the binding affinities between the target protein and the phytocompounds as ligands that showed drug-likeness characteristics. Glide package from the Schrödinger Suite was employed to perform the process[21,22]. Docking was performed in standard precision (SP) mode to balance accuracy with computational efficiency. The OPLS3e force field was applied, ensuring reliable interaction modeling by accounting for van der Waals forces, hydrogen bonding, and electrostatic interactions[23]. The binding site coordinates derived from CastP analysis, with a grid box set to dimensions of X = 16.62 Å, Y = 53.48 Å, and Z = 30.9 Å, allowed the ligands to explore key regions of the active site. To visualize the interactions, the Maestro viewer was used to identify residues involved and the types of chemical bonds formed, providing insights into binding stability and potential inhibitory[24].

MM-GBSA analysis

Molecular Mechanics Generalized Born Surface Area (MM-GBSA) analysis was utilized to validate the molecular docking process in computer-aided drug design to calculate the binding-free energy of the finalized ligands. Prime MM-GBSA v-3.0 was applied to predict the MM-GBSA score[25]. To ensure precise energy calculations, this approach combines the OPLS3e force field with a novel energy solvation model known as VSGB (Variable Solvent Generalized Born)[26]. Accurate solvation energy predictions are essential to ascertain binding affinities, and the VSGB model improves these predictions. The other parameters were kept at their default settings to retain consistency and dependability in the outcomes.

Molecular dynamic simulation

In this experiment, a 100 nanosecond (ns) molecular dynamics (MD) simulation was conducted to assess the stability of the complex structures formed by our specified candidate compounds binding to the active site of the target protein. The MD simulation was performed using the Desmond program in Schrödinger 2020-3 on a Linux platform[27]. To maintain system neutrality, Na^+ and Cl^- ions were added to attain a 0.15 M salt concentration. After assembling the solvated system containing the protein-ligand complex, we applied system minimization and relaxation using the default Desmond protocol with OPLS3 force field parameters[28]. For maintaining the system at steady temperature at 310 K and pressure at 1.01325 bar, we utilized Nose–Hoover temperature coupling and isotropic scaling, with energy recorded at 100 picosecond intervals and a coupling constant of 1.2 [29]. The temperature remained constant approximately 310 K throughout the simulation. Snapshots of the MD simulation were captured using Maestro v-12.5. To assess the stability of the complex structure over the 100 ns trajectory, various metrics including Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), radius of gyration (rGyr), and Solvent Accessible Surface Area (SASA) were analyzed. These analyses were conducted using the Simulation Interaction Diagram (SID) tool within the Desmond module[30].

Statistical analysis

The statistical analysis for the antibacterial activity was conducted using Microsoft Excel-2016 to ensure rigorous evaluation of the experimental results. A one-way analysis of variance (ANOVA) was conducted, with statistical significance set at $p < 0.01$. All experiments were conducted in triplicates for reliability. Data were expressed as mean ± standard deviation (SD) for all replicates.

Results and discussion

Antibacterial assay

The *in vitro* antibacterial efficacy of MPGL extract against the gram-negative human pathogenic bacteria *E. coli* (ATCC25922) was evaluated using agar disc diffusion method. MPGL extract revealed varying patterns of antibacterial activity against *E. coli* at four different concentrations at 25, 50, 75 and 100 µg/disc (Fig. 1). At the lowest concentration of 25 µg/disc, no zone of inhibition was observed, indicating no significant antibacterial effect. However, at 50 µg/disc, the extract showed a moderate antibacterial effect with a zone of inhibition of 9.43 ± 0.40 mm. The activity increased further at 75 µg/disc, where the zone of inhibition reached 12.83 ± 0.76 mm. The highest antibacterial activity against *E. coli* was observed at the concentration of 100 µg/disc with zone of inhibition 16.67 ± 0.29 mm shown in Fig. 2. The statistical analysis showed a significant difference in antibacterial activity, with $p < 0.05$, indicating that the observed effects were statistically significant (**Supplementary Table 2**).

The observed antibacterial effects can be attributed to the presence of tannins, flavonoids and phenols in *P. granatum* leaf extract. These phytochemical groups are recognized for containing antimicrobial compounds[31,32]. Tannins exhibit broad-spectrum antimicrobial activity by inhibiting enzymatic functions and nucleic acid synthesis in pathogens, while flavonoids interfere with bacterial DNA and enzymes, leading to bacterial cell death[33,34]. Previous investigations have explored the antimicrobial properties of *P. granatum* leaves utilizing the disk diffusion method. These studies revealed that the methanolic extract displayed inhibition against several gram positive and gram negative bacterial strains including *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus mirabilis*, and *Salmonella typhi*[35,36]. Moreover, the methanolic leaf extract of *P. granatum* exhibited antibacterial activity against prevalent bacterial isolates found in septic wounds, including multidrug-resistant strains of *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and *E. coli*[37]. According to another study, the aqueous extracts demonstrated inhibition zones ranging from 15 mm to 22 mm against pathogens like *P. aeruginosa* and *Shigella dysenteriae*[38]. Our study similarly demonstrated comparable zones of inhibition, further corroborating the antibacterial efficacy of *P. granatum* leaf extracts against *E. coli* and highlighting its potential as a natural antimicrobial agent.

Active site determination

The CASTp server was utilized to examine the active site of the target protein and identify the amino acid residues within the active site. As per the prediction, the target protein's active residues (most significant active pocket with a surface area of 53.452) are SER70, LYS73, ASN104, TYR105, SER130, ASN132, LYS234, THR235, GLY236 and SER237 (Fig. 3).

ADMET analysis

The preclinical stage of drug research and development relies significantly on understanding the pharmacokinetic properties, which include absorption, distribution, metabolism, excretion as well as toxicity (ADMET) prediction (Table 1). While toxicity evaluations look at safety margins and side effects, pharmacokinetic assessments look at bioavailability, potential accumulation, and metabolic pathways[39]. These preliminary assessments aid in the identification of promising medication candidates with advantageous qualities and low safety risks[40].

The ADMET analysis of 29 phytochemicals derived from *P. granatum* leaves provided promising pharmacokinetic and safety profiles, supporting their potential as therapeutic agents (**Supplementary Tables**

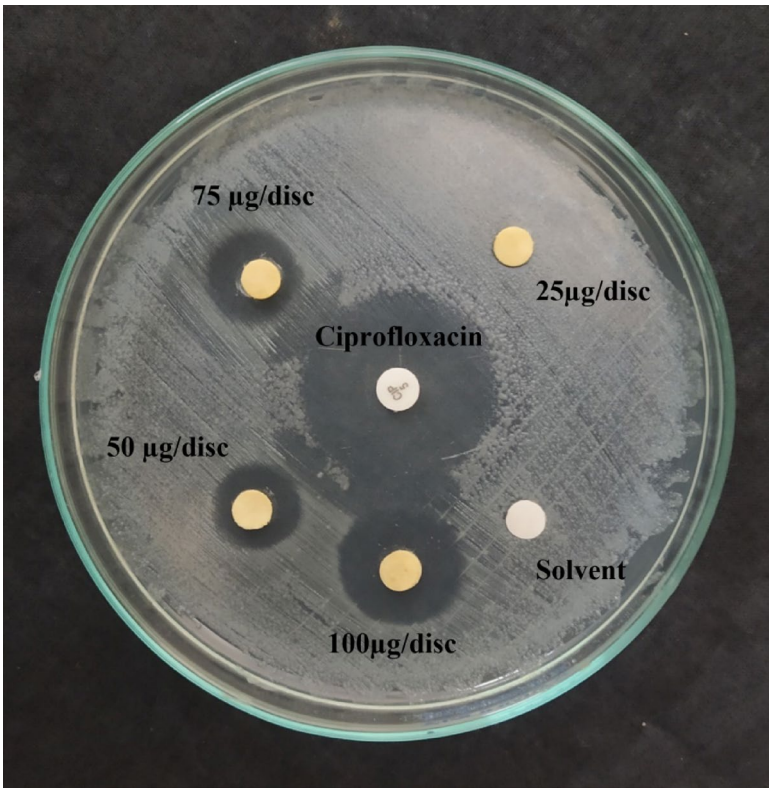

Fig. 1. Antibacterial activity of *P. granatum* leaf extract against *E. coli*.

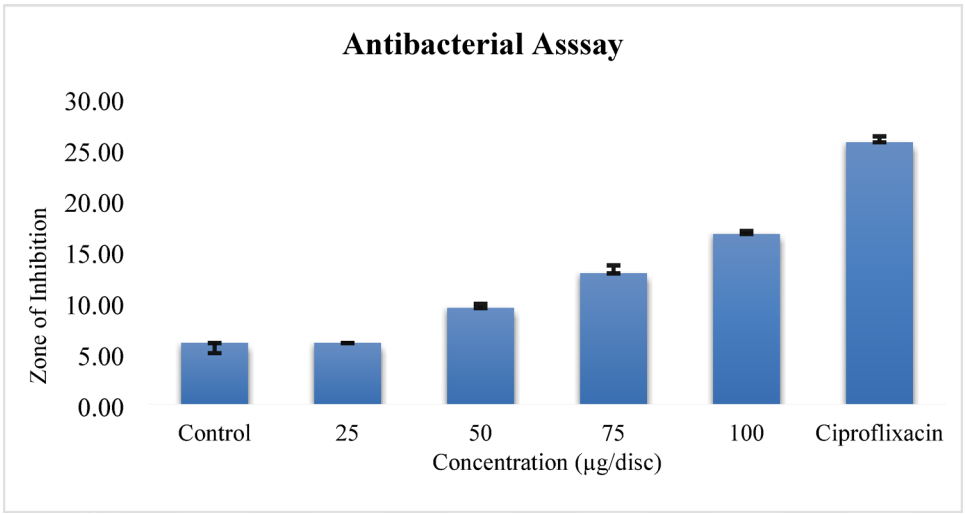

Fig. 2. Zone of inhibition of *P. granatum* leaf extract against tested bacteria. Where, Ciprofloxacin was used as a positive control. Data are presented as mean ± SD with three ($n = 3$) biological replications.

3 and 4). The drug-likeness of the compounds was assessed using Lipinski's Rule of Five, which states that an orally administered drug should have a molecular weight of ≤ 500, a $\log_p$ value of ≤ 5, no more than 5 hydrogen bond donors, and no more than 10 hydrogen bond acceptors. These criteria help predict a compound's potential as an effective oral drug. If a compound meets at least three of these four rules, it is considered to have drug-like properties[41]. Veber's Rule suggests that a compound should have ≤ 10 rotatable bonds and a topological polar surface area (TPSA) ≤ 140 Å² for optimal oral bioavailability[42]. Among all the phytochemicals, three lead compounds—Epicatechin (CID 72276), Kaempferol (CID 5280863), and Apigenin (CID 5280443)—demonstrated high drug-likeness based on their adherence to Lipinski's and Veber's rules.

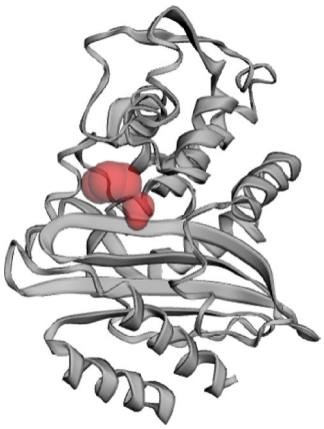

Fig. 3. The active site (red color) of the target protein PDB: 1YLY were determine by the online server CASTp[18].

Property	CID 72276 (Epicatechin)	CID 5280863 (Kaempferol)	CID 5280443 (Apigenin)
Lipinski rules			
MW (g/mol) < 500	290.27	286.24	270.24
HBA < 10	6	6	5
HBD < 5	5	4	3
Log Po/w ≤ 5	0.85	1.58	2.11
Lipinski violations	0	0	0
Veber's rules			
nRB ≤ 10	1	1	1
TPSA ≤ 140	110.38	111.13	90.9
Drug-likeness properties			
GI absorption	High	High	High
Skin permeability (cm/s)	−7.82	−6.70	−5.80
P-Glycoprotein substrate	No	No	No
CYP1A2 inhibitor	No	Yes	Yes
CYP2C19 inhibitor	No	No	No
CYP2C9 inhibitor	No	No	No
CYP2D6 inhibitor	No	Yes	Yes
CYP3A4 inhibitor	No	Yes	Yes
BBB permeability	No	No	No

Table 1. ADME profiling of selected phytoconstituents from *P. granatum* that have drug-likeliness properties.

High gastrointestinal (GI) absorption indicates that a compound can be readily absorbed, making it a promising candidate for oral drug formulations. All three lead compounds exhibited molecular properties within the optimal range for drug absorption. Their topological polar surface area (TPSA) values—110.38 Å² for epicatechin, 111.13 Å² for kaempferol, and 90.9 Å² for apigenin—fall within the acceptable threshold (< 140 Å²), indicating enhanced membrane permeability and efficient absorption through the intestinal wall[43]. Their compliance with Lipinski's Rule of 5 (no violations) further supports their potential as orally active drugs[44].

Regarding distribution, all three lead compounds do not cross the blood-brain barrier (BBB), suggesting limited central nervous system (CNS) penetration. Their low log $P_{o/w}$ values indicate moderate lipophilicity and a moderate balance between renal and hepatic elimination, meaning they are more likely to remain in circulation rather than accumulate in fatty tissues[45]. Additionally, their low number of rotatable bonds (nRB = 1) suggests a relatively rigid structure, which can affect distribution dynamics. The compounds show varied interactions with cytochrome P450 (CYP) enzymes. Kaempferol and apigenin inhibit multiple CYP enzymes (CYP1A2, CYP2D6, CYP3A4), whereas Epicatechin does not inhibit any. This suggests that Kaempferol and Apigenin may interfere with the metabolism of other drugs that rely on these enzymes, leading to potential drug-drug interactions.

The toxicity assessment revealed that all three compounds were inactive for cytotoxicity, immunogenicity, carcinogenicity, and mutagenicity, suggesting a strong safety profile. However, Kaempferol displayed a hepatotoxicity probability of 0.68, raising concerns about potential liver toxicity. This risk underscores the need for mitigation strategies, such as controlled dosing, formulation modifications like nanoparticle-based drug

delivery to minimize hepatic exposure, or co-administration with hepatoprotective agents to reduce adverse effects. Despite this consideration, Kaempferol is known for its potent antioxidant and anti-inflammatory properties, making it a valuable therapeutic candidate, particularly for short-term treatment regimens where its benefits outweigh potential risks. On the other hand, Epicatechin and Apigenin, with low toxicity profiles and high LD_{50} values, emerge as safer candidates for long-term therapeutic use. The predicted LD_{50} values for Epicatechin, Kaempferol, and Apigenin were found to be 10,000 mg/kg, 3919 mg/kg, and 2500 mg/kg, respectively (Table 2), recommending lower acute toxicity[46]. These findings propose that the selected phytoconstituents are lead candidates for further drug development processes, given their favorable ADMET properties and low toxicity risks.

Molecular docking score analysis

This current research is focused on finding CTX-M-9 beta-lactamase protein inhibitors by utilizing computer-based techniques. The most promising compounds that exhibited drug-likeness and toxicity-free characteristics were chosen for molecular docking study (**Supplementary Table 5**). On this basis, three lead compounds, CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443 (Apigenin) were selected and compared with a control compound CID 5481173(Ceftazidime). The compounds revealed higher binding affinities with the target protein than the control. Epicatechin demonstrated a binding energy of -6.25 kcal/mol, forming three hydrogen bonds at ASN132, GLY238, and ASP240 amino acid residues. Kaempferol exhibited a binding energy of -5.23 kcal/mol and established four hydrogen bonds with ASN104, ASN132 (2 hydrogen bonds), and ASP240 residues. Similarly, Apigenin showed a binding energy of -5.21 kcal/mol, also forming four hydrogen bonds at ASN104, ASN132, and ASP240 residues. Other interacting bonds were also observed at different residues including hydrophobic, polar, glycine, pi-pi stacked etc. On the contrary, Ceftazidime had a binding energy of -4.28 kcal/mol, with two hydrogen bonds at ASN170 and SER130 residues. These results indicate that the selected compounds not only have stronger binding affinities but also form more hydrogen bonds with the target protein, suggesting a potentially higher efficacy compared to the control compound. The interaction between particular phytocompounds and protein is shown in Fig. 4, and all docking scores are shown in Table 3.

The protein CTX-M-9 beta-lactamase (1YLY) is remains largely unexplored to be inhibited by phytocompounds through molecular docking studies. However, a study identified two compounds from drugbank, DB01753 and ZINC33264777 as a potent CTX-M-9 inhibitor through molecular modeling, with a docking energy of -16.62 kcal/mol and − 15.11 kcal/mol, respectively. Both compounds showed interactions with 1YLY via hydrogen bonds with residues SER237, ASN104, GLU166, SER274, and TYR105 [47]. Nonetheless, the lower binding energies and rigid bonding patterns in our study indicate that Epicatechin, Kaempferol and Apigenin efficiently bind to the active site of 1YLY protein and can cause inhibition[48].

MM-GBSA binding energy

The lead compounds were evaluated for their binding free energies against the target protein (PDB ID: 1YLY) using the MM-GBSA method. This method provides insights into the stability and strength of these interactions and is particularly valuable due to its capability to integrate molecular mechanics with implicit solvation models[49]. It results in more accurate predictions of binding energies compared to simpler scoring functions. The molecular mechanical energy, solvation free energy, and the entropy contribution are the key parameters in the MM-GBSA calculation, with each parameter representing a separate interaction contributing to the total binding energy[50]. Complexed with 1YLY protein, the selected three compounds CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443(Apigenin) had greater net negative binding free energy values, compared to the control CID 5481173 (Ceftazidime) according to the analysis of MM-GBSA showed in Table 4; Fig. 5.

The complex analysis of MM-GBSA revealed binding free energies of Epicatechin, Kaempferol, Apigenin and Ceftazidime at -37.953 kcal/mol, -28.107 kcal/mol, -27.587 kcal/mol and − 25.154 kcal/mol, respectively. The results suggest that the lead compounds may sustain a long-lasting interaction with the target protein, potentially comparable to the control compound.

Molecular dynamic simulation

RMSD of protein–ligand complex

The atomic Root Mean Square Deviation (RMSD) of the Cα backbone has been calculated in order to estimate the displacement of precise carbon atoms over a certain period of time in relation to a reference point. Protein-ligand

Types of toxicity	Chemical identifier		
	CID 72276 (Epicatechin)	CID 5280863 (Kaempferol)	CID 5280443 (Apigenin)
Cytotoxicity (probability)	0.84 (Inactive)	0.98 (Inactive)	0.87 (Inactive)
Hepatotoxicity (probability)	0.72 (Inactive)	0.68 (Inactive)	0.68 (Inactive)
Immunogenicity (probability)	0.96 (Inactive)	0.96 (Inactive)	0.99 (Inactive)
Carcinogenicity (probability)	0.51 (Inactive)	0.72 (Inactive)	0.62 (Inactive)
Mutagenicity (probability)	0.55 (Inactive)	0.52 (Inactive)	0.57 (Inactive)
Predicted LD_{50} mg/kg	10,000	3919	2500

Table 2. Toxicity profiling of selected phytoconstituents that have drug likeliness characteristic found in *P. Granatum*.

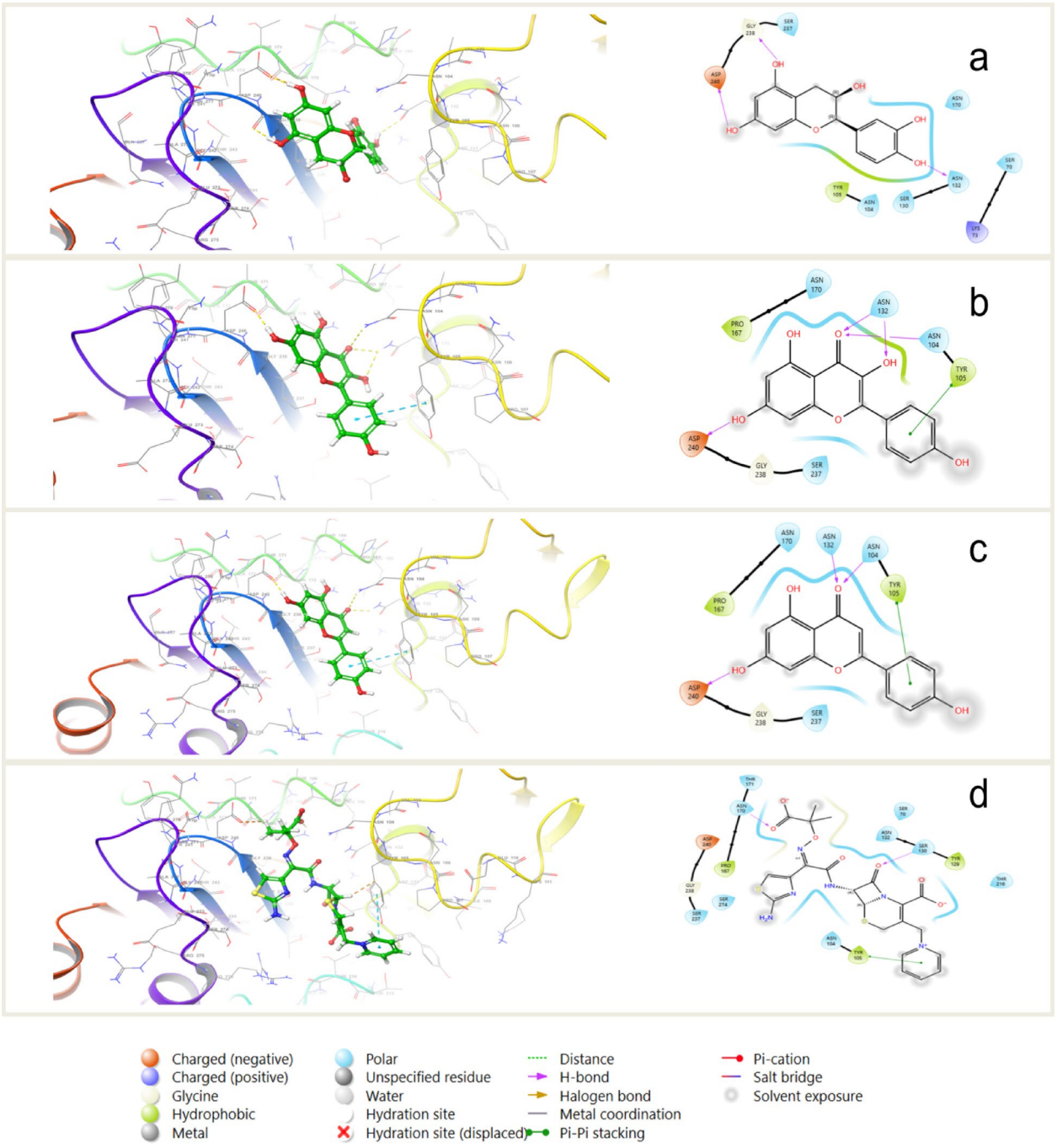

Fig. 4. Molecular docking interaction of the lead compounds. Left side three-dimensional interaction and right side is the two-dimensional interaction of the compounds with respective protein. Here, (**a**) CID 72276, (**b**) CID 5280863, (**c**) CID 5280443 and (**d**) Control CID 5481173.

complexes typically have RMSD changes between 1 to 3Å, but higher records indicate significant conformational changes in the protein structure[51]. For this, the structural stability of 1YLY with the lead compounds CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443(Apigenin) and control CID 5481173 (Ceftazidime) 100ns simulation was performed and RMSDs were calculated from the simulation trajectories and are plotted in Fig. 6a. RMSD values of Epicatechin, Kaempferol and Apigenin with 1YLY maintained stability without any major fluctuations throughout the 100 ns simulation. Compared to the control, Epicatechin showed slightly lower RMSD values with a maximum of 2.25 Å, minimum of 0.89 Å, and an average of 1.69 Å, indicating marginally better stability than the control. Epicatechin also showed closest resemblance with apo-protein. Kaempferol showed much variability among the three with a maximum, minimum and average RMSD of 2.72 Å, 0.96 Å, and 2.16 Å, respectively. Apigenin exhibited highest RMSD values at 3.05 Å and lowest and average at 0.77 Å, and 2.16 Å representing minor fluctuations in stability. Based on the comprehensive trajectory analysis,

Compound	Docking score (kcal/mol)	Hydrogen bonds	Other interacting bonds
CID 72276 (Epicatechin)	−6.25	ASN132, GLY238, ASP240	TYR105, SER237, ASN104, SER130, SER70, ASN170, ASP240, LYS73
CID 5280863 (Kaempferol)	−5.23	ASN104, ASN132, ASP240	PRO167, ASN170, TYR105, GLY238, SER237
CID 5280443 (Apigenin)	−5.21	ASN104, ASN132, ASP240	PRO167, ASN170, TYR105, GLY238, SER237
Control CID 5481173 (Ceftazidime)	−4.28	ASN170, SER130	TYR105, TYR129, PRO1676, ASN104, SER274, ASP240, GLY238, THR171, SER70, ASN132, THR216

Table 3. Docking scores and interacting residues with specific bonds. Here, other interacting bonds are denoted as hydrophobic, polar, glycine, pi-pi stacking and positive and negative charged bonds.

Compounds CID	MM-GBSA						
	ΔG Bind	ΔG Bind Coulomb	ΔG Bind Hbond	ΔG Bind Lipo	ΔG Bind Packing	ΔG Bind Solv GB	ΔG Bind vdW
CID 72276 (Epicatechin)	−37.953	−30.532	−3.630	−7.614	−2.145	24.540	−20.263
CID 5280863 (Kaempferol)	−28.107	−29.952	−2.684	−4.874	−2.359	31.461	−20.377
CID 5280443 (Apigenin)	−27.587	−26.042	−2.319	−3.878	−2.042	25.997	−20.021
Control CID 5481173 (Ceftazidime)	−25.154	2.724	−2.076	−8.764	−0.358	14.713	−35.623

Table 4. Different energy components and net MM-GBSA binding free energy (kcal/mol) of 1YLY in complex with selected lead compounds and control.

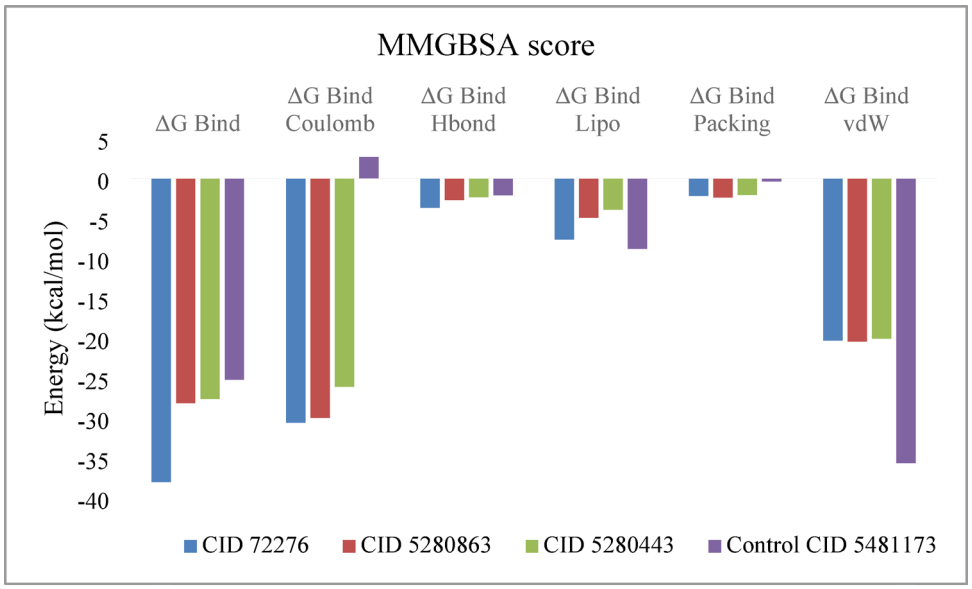

Fig. 5. Post-docking analysis of MM-GBSA ΔG binding scores of the compounds with 1YLY.

it was discovered that Epicatechin with 1YLY, was more stable due to their low RMSDs during the course of the simulation[52].

The Rg of protein–ligand complex

The radius of gyration (Rg) is the measure of compactness and overall size of a molecule, calculated based on the distribution of atoms within the molecule. In a protein-ligand complex, the Rg can provide insights into the structural changes and stability of the complex upon ligand binding. A change in the Rg may indicate conformational alterations in the protein structure induced by the ligand, affecting its overall compactness and shape[53].

Figure 6b displays the stability of CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443(Apigenin) and control CID 5481173 (Ceftazidime) in complex with 1YLY, as determined by the Rg throughout a 100 ns simulation period. Compared to the control compound Ceftazidime that exhibited a relatively expanded structure, Epicatechin revealed more compact structure with an rGyr value ranging between 3.36 Å to 3.85 Å and an average of 3.60 Å. In combination with 1YLY, Kaempferol and Apigenin revealed slightly wide ranges of Rg values at 3.9 Å to 4.09 Å (Average 4.00 Å) and 3.94 Å to 4.19 Å (Average 4.07 Å). The lower rGyr

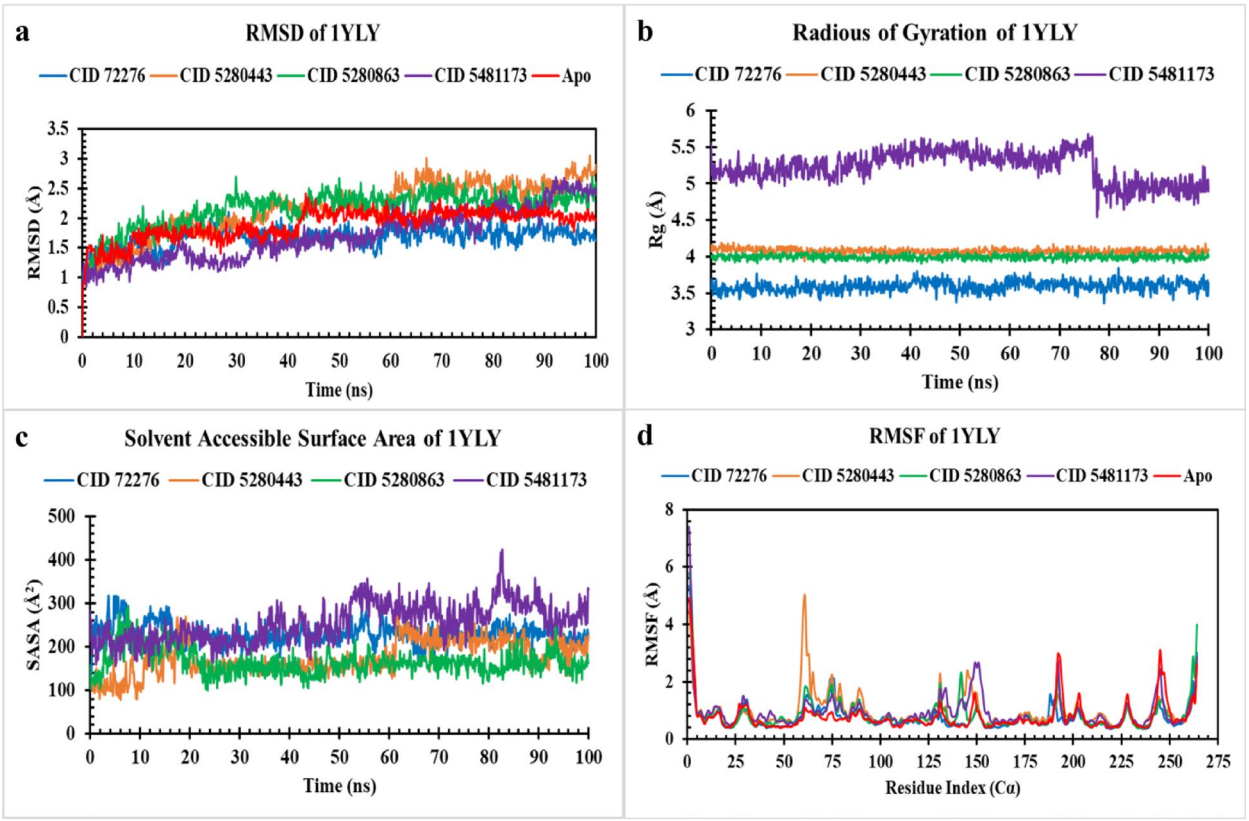

Fig. 6. Molecular dynamic simulation of the lead compounds. Here, (**a**) RMSD of 1YLY (**b**) rGyr of 1YLY, (**c**) SASA of 1YLY and (**d**) RMSF of 1YLY.

value indicates a high level of compactness, while the larger value indicates that the compounds are disassociated from the protein. So, it can be concluded that the selected three compounds (Epicatechin, Kaempferol and Apigenin) demonstrated a better rGyr value compared to that of the control.

SASA of protein–ligand complex

Solvent Accessible Surface Area (SASA) is the surface area of a molecule that can be accessed by a solvent revealing information about the folding, stability, and interactions of the molecule with its surroundings[54]. Figure 6c shows the bimolecular surface area of each complex that is assessable to solvent molecules for CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443(Apigenin) and CID 5481173 (control). During the 100ns simulation period, Kaempferol revealed the least solvent-exposed surface area with an average SASA value of 164.47 Å^2 compared to the control (255.23 Å^2), followed by Apigenin (175.48 Å^2) and Epicatechin (230.58 Å^2). Higher SASA values are indicative of higher surface area of a molecule being exposed to the solvent, resulting in a more extended or less compact structure. Therefore, it is evident that all three lead compounds maintained more compact conformations with less exposed areas compared to ceftazidime.

RMSF of protein-ligand complex

The root mean square fluctuation (RMSF) measures the flexibility and mobility of amino acid residues in a protein-ligand structure during MD simulation. It is widely accepted that the binding site is primarily composed of several key residues in the active pocket, and tracking the behavior of these residues would aid in the study of protein-ligand interactions[55]. For this purpose, RMSF of the lead compounds complexed with 1YLY was calculated in a 100 ns simulation period. Usually a significant change in the RMSF value within a range of more than 3 Å affects the flexibility of amino acid residues[56]. As depicted in Fig. 6d, when bound to the protein 1YLY, CID 72276 (Epicatechin), CID 5280863 (Kaempferol), and CID 5280443(Apigenin) and control CID 5481173 (Ceftazidime) exhibited common fluctuation profiles with the apo protein at specific amino acid residues. No major variations in the fluctuations were observed for the lead compounds during the 100 ns simulation period except Apigenin at 62th ns (5Å). Epicatechin resembled the apo protein more closely than Kaempferol and Apigenin, suggesting a binding mode aligning with the apo protein state.

Conclusion

The methanolic leaf extract of *Punica granatum* demonstrated significant antibacterial activity against multidrug-resistant *E. coli*, attributed to its phytochemical constituents. *In silico* analysis confirmed strong binding affinities and stable interactions between these compounds and CTX-M-9 beta-lactamase (PDB ID: 1YLY), an

enzyme responsible for hydrolyzing penicillins and cephalosporins. ADMET profiling identified Epicatechin, Kaempferol, and Apigenin as lead candidates with favorable pharmacokinetics and low toxicity. Molecular dynamics simulations further validated their stability and inhibitory potential, with Epicatechin emerging as the most promising. However, further *in vivo* studies and enzymatic inhibition assays are essential to confirm their efficacy in targeting CTX-M-9.

Data availability

It is only the summary of the data that is reported in this article. In case of reasonable request, datasets are available from the corresponding author.

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Author contributions

Faria Tasnim: Conceptualization; Literature Review; Methodology; Investigation; Computational analysis; Data interpretation; Data analysis; Writing (original draft). Md. Eram Hosen: Supervision, Methodology, Literature Review; Investigation; Computational analysis; Data analysis; Writing (review and editing). Al Riyad Hasan, Md. Mizanur Rahaman: Computational analysis, Data interpretation; Writing (review and editing). Md. Taohid Hasan: Data analysis, Data Curation; Writing (review and editing). Md. Aktaruzzaman: Computational analysis. Md. Tarikul Islam: Computational analysis. Md. Abdulla Al Jubayed and Magdi E. A. Zaki: Computational analysis, Data Curation; Writing (review and editing). Md. Motaher Hossain: Computational analysis, Data Curation; Writing (review and editing), Endalamaw Yihune, and Mansour Altayyar: Literature Review; Investigation; Computational analysis; Data analysis; Writing (review and editing.

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Competing interests

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Correspondence and requests for materials should be addressed to E.Y. or M.E.A.Z.