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Original Article

IN SILICO MODELLING OF β-LACTAM RESISTANT *ENTEROCOCCUS FAECALIS* PBP4 AND ITS INTERACTIONS WITH VARIOUS PHYTO-LIGANDS

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ABSTRACT

Objective: The resistance to β-lactam antibiotics is a serious problem worldwide. This resistance has emerged due to two main mechanisms: production of β-lactamases that hydrolyses β-lactam antibiotics and other is the production of low affinity mutated Penicillin Binding Proteins (PBPs) that can sustain even at the high concentration of antibiotics. The current study epitomises the identification of T(425)S mutations in PBP4 of β-lactam resistant *Enterococcus faecalis* isolated from uropathological samples of urinary tract infected (UTI) patients. Also, the effect of the this mutation was analysed by *in silico* strategies on ligand binding efficiencies of the active site of PBP4 towards selected β-lactam antibiotics as well as phytochemicals.

Methods: To study the effect of T(425)S mutation towards emergence of antibiotic resistance pattern, the structural model was generated for wild-type and mutated PBP4 of *E. faecalis* using MODELLER and further studied the interactions of PBP4 with β-lactam antibiotics along with various phytochemicals identified and purified from selected medicinal plants possessing antibacterial activity using Autodock4 suite.

Results: Based on the results of different docking parameters and a number of H-bond interactions, gallic acid, and quercetin were identified with highest binding affinity to the active site pocket of PBP4 of *E. faecalis*, compared to β-lactam antibiotics. Further, molecular simulation studies also supported this fact.

Conclusion: T (425)S mutation has been identified with a significant change in ligand binding efficiencies towards tested β-lactam antibiotics. Moreover, gallic acid and quercetin have showed the possible antibacterial agent via blocking the active site of PBP4 of *E. faecalis*. The results presented here could be useful in designing more effective phyto-ligands based therapeutic antibacterial compounds against PBP4 of *E. faecalis*.

Keywords: PBP4, *Enterococcus faecalis*, Autodock, Molecular docking

INTRODUCTION

Enterococci are normal human inhabitants of the gastrointestinal tract, however, they are also categorised as pathogens responsible for causing a wide variety of infections to humans [1]. *Enterococcus faecalis* caused 80-90% enterococcal infections while *Enterococcus faecium* accounted for 5-10% [1]. Both strains have been identified as emerging β-lactam resistant. Moreover, among UTI causing *enterococci*, multi-drug resistant *E. faecalis* such as vancomycin-resistant strains (VRE) have been reported increasingly in many countries [2].

The important mechanisms reported for the development of β-lactam resistance in *E. faecalis* are β-lactamase production, overproduction of PBP4 or PBP5 and decreased affinity for β-lactam antibiotics by point mutations in penicillin binding domains of PBPs [2]. Penicillin-binding proteins or PBPs are crucial bacterial enzymes that catalyse the synthesis of the cell wall, an important constituent, essential for their survival and reproduction [3, 4].

The urinary tract infections (UTIs) are among the very common infectious diseases occurring publicly. In spite of an enormous success of antibiotics as antibacterial agents, the widespread and uncontrolled use of antibiotics has led to the emergence of multidrug-resistant (MDR) bacteria. "Antibiotic Resistance" is the ability of the bacteria to multiply continuously in the presence of antibacterial agents, even in the presence of high concentration [5]. As antimicrobial resistance is increasing day by day leaving limited treatment options, it is essential to study the antibiotics resistance pattern among the bacteria to prescribe correct and effective antibiotics.

As bacteria have emerged multidrug resistant (MDR), that has put all human beings with a risk of limited treatment options along with increased mortality. Hence, there is an urgent need to search for a new antibacterial agent. Moreover, the medicinal plants have been used since ancient times for the treatment of various infectious diseases worldwide. The potent antibacterial phyto constituents were identified and purified from the medicinal plants against *E. faecalis*.

Detailed analysis was performed for a better understanding of the molecular interactions between the ligands and target PBP4 (wild type and mutant) of *E. faecalis* with the help of *in silico* molecular docking tools. The molecular docking programs usually used to establish new ligands/inhibitors for the selected target receptor protein from the different available databases based on their efficiency to bind the active sites on the receptor. The docking exhibited different binding poses out of which the one with minimum binding energy was selected. The favourable conformation was analysed with hydrogen bonding with the active site residues of PBP4.

MATERIALS AND METHODS

Isolated strains from clinical urine specimens

In total, 100 urine samples were collected from the suspected UTI patients from different pathology labs in Nagpur region, Maharashtra, India. It was observed that 86% of collected urine samples were UTI positive, and 35.16% of the patients were found to be infected with *E. faecalis*, confirmed with 16s rRNA sequencing.

Antibiotic sensitivity

The antibiotic sensitivity for isolated *E. faecalis* was checked for following β-lactam antibiotics using 4X-concentration of reported MIC (minimum inhibitory concentration) by Kirby-Bauer method [6]. The β-lactam antibiotics used were *Cefpodoxime* (16µg), *Cephalexin* (4.0µg), *Cefuroxime* (16µg), *Cefixime* (4.0µg), *Ceftazidime* (2.0µg), *Cefazoline* (4.0µg), *Cefotaxime* (32µg), *Ceftriaxome* (2.0µg), *Cefaclor* (32µg), *Feropenem* (8.0µg) and *Cefepime* (32µg). The β-lactam resistant *E. faecalis* strains were selected for further molecular analysis.

Amplification and sequencing of PBP4 of *E. faecalis*

PBPs are encoded by the bacterial genomic DNA (gDNA). The gDNA was extracted using following method: The bacterial cells were pelleted by centrifugation at 3000rpm for 5 min and washed with milliQ water. The pellet was suspended in water and boiled for 10 min at 100 °C. This lysate was centrifuged, and the supernatant was collected. This supernatant was used directly as a template for the amplification of *PBP4*. The primer set used were Forward (5'-CTG TGG ATA CTC TCC CGC AC-3') and Reverse (5'-CAG CAG AAT ACC CAC CAG CA-3'). Thermal cycler (PeqLab) was used to amplify PBP4 under following conditions: Initial denaturation at 95ºC for 5 min, followed by 30 cycles of 1 min at 94ºC, 1 min of annealing at 52.2ºC and 1.5 min at 72ºC and finally 10 min at 72ºC. PCR amplicons were detected electrophoretically in 1% agarose gel using 1X TAE, visualized by staining with 0.5 µg/ml ethidium bromide, examined in UV light and photographed by Molecular Imager Gel Doc XR (Biorad Laboratories).

DNA sequencing for mutational analysis

Amplified PCR products were purified using Qiagen purification kit (Germany) and bidirectional sequencing was outsourced to Xceleris Labs, India. The mutations were analysed with public access software (www.ncbi.nlm.nih.gov). Also, the data was analysed with Chromuslite, SIFT and PROVEAN PROTEIN.

Phytochemical extraction, purification, and identification

The antibacterial activity was studied in the crude extract of *Andrographis paniculata* Burm. f. Wall. Ex. Nees *(Ap), Astercantha longifolia* (L.) Nees *(Al), Bixa orellana* L. *(Bo), Gardenia resinifera* Roth, *(Gr), Pongamia pinnata* (L.) Pierre *(Pp), Psoralea corylifolia* L. *(Pc), Sphaeranthus indicus* Linnaeus *(Si), Solanum trilobatum* L. *(St), Soyamida febrifuga* (Roxb.) Juss *(Sf)* and *Thespesia populnea* (L.) Sol. Ex. Correa *(Tp)*. These plants were collected from Nagpur city, Maharashtra, India. All plants were identified by a taxonomist at the Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur. The herbarium were deposited in the department of botany with the following voucher numbers 9038 (*Andrographis paniculata*), 9039 (*Astercantha longifolia*), 9041 (*Bixa orellana*), 10012 (*Gardenia resinifera*), 10037 (*Pongamia pinnata*), 10038 (*Psoralea corylifolia*), 10039 (*Sphaeranthus indica*), 10041 (*Solanum trilobatum*), 10042 (*Soyamida febrifuga*) and 10043 (*Thespesia populnea*).

These plants have shown a significant antibacterial activity against isolated MDR *E. faecalis* and hence the phytochemicals were extracted from these medicinal plants by Soxhletion (hot extraction) and cold maceration for analysis of antibacterial compounds. It was observed that the phytochemicals extracted through cold maceration using 50% aqueous methanol (V/V) showed significant phytochemicals yield and highest antibacterial activity against MDR *E. faecalis* isolates compared to the Soxhlet extracts. Hence, the cold extracts of all plants were used for HPLC analysis [7] to identify and purify the probable phytoconstituents accountable for giving the antibacterial activity by Reverse phase C-18-aminopack zorbax eclipse-AAA column with SPD 10 AVP pump. Methanol: water (90:10 v/v) was utilized as the mobile phase.

***In silico* studies**

In silico studies by molecular docking is an important tool to study the interaction of ligands with active site residues of the receptor [8, 10]. The docking involves the use of sampling algorithm and a scoring function to evaluate the proper orientation and pose of ligand molecule in relation to the binding energy. The correct identification of this binding pose of one or more related ligands is important in establishing a structure-activity relationship in lead optimization. The second use of scoring functions is to rank different ligands to predict their relative experimental activity [10-12].

The molecular docking simulations were carried out using Windows 8,1 professional, 64 bit Intel core M-5Y10c CPU at 1.00 GHz, 4 GB RAM) using the Auto dock program (v1.5.6) which defines the binding site in terms of grid of interaction points, structural homology models were generated for mutant protein by Modeller, simulation, and salvation studies were carried out by Gromacs.

Ligand preparation

The phytochemicals that exhibited prominent antibacterial activity towards isolated multidrug-resistant bacteria were selected for molecular docking analysis. The structure of these phytochemicals was downloaded from PubChem online portal and drawn in Chemsketch. For auto dock tool, all ligand molecules were in PDB format, hence these drawn ligand structures in MDL-mol format were converted to pdb format by Open Babel GUI software.

Homology model generated for PBP4

The biological crystal structure of PBP4 is still not available on protein data bank (PDB); hence to get structural information the homology model was generated for both wild type and mutant protein using Modeller, Swiss model and Phyre2. PBP4 was found to have 36% identities and 56% positives with PBP2a from *methicillin*-resistant *Staphylococcus aureus* (PDB ID: 1VQQ_A) with resolution 1.8A ° and this structure was used for the preparation of model for pbp4 of *E. faecalis*. The prepared models were further validated by Ramachandran plot with the help of PROCHECK and the best model was used for energy minimization and optimisation by SPDBV. Gromacs was used to analyse the energy minimization and Procheck was used to study the Ramachandran plot of minimised structure. These models were used further to analyse and compare the effect of the mutation on the binding efficiency of PBP4 towards different β-lactam antibiotics as well as purified phytochemicals.

Virtual screening by Auto Dock

The catalytically important serine residue of the active site of PBP4-Ser (232) was defined as the center of the grid with grid box 60×60×60A ° having a grid spacing of 0.375A °, which covered all the active site residues and allowed flexible rotation of the ligand. The number of generations, energy evaluations, and individuals in the population are set to 27000, 5×106, and 150 respectively. The Lamarckian genetic algorithm was adopted for sampling ligand conformations.

The default parameters of free energy scoring function were used for the docking studies. The best scoring and lowest RMSD solutions from 50 runs were considered as the predicted binding conformers. The RMSD values of each docked conformer were calculated from the corresponding crystal structure as a reference to access the accuracy of poses with respect to their binding energy. The H-bond interactions were analysed in Chimera.

RESULTS

The β-lactam resistant *E. faecalis isolates*

It was found that 35% of the UTI patients were infected with multi drug resistant *E. faecalis.* The other predominant bacteria isolated were *E. coli (26%)* and *Pseudomonas aeruginosa* (36%). *E. faecalis* were found highly resistant to *Cephalexin* (1µg) followed by *Cefpodoxime* (16µg) *Cefotaxime* (32µg). Also, they showed emerging resistant towards other tested β-lactam antibiotics.

PCR amplification of *pbp4*

The PCR amplification of PBP-4 gene in *E. faecalis* was standardised at 52.2 °C primer annealing temperature. The amplification was seen in five isolates. *E. faecalis* strains numbers that showed amplifications were 3, 5, 6, 9 and 30 (data shown in the following fig.).

Mutation in PBP-4 of *E. faecalis*

The point mutation observed in PBP-4 was T(425)S which means that, at position 425, Threonine (T) was replaced by Serine (S). These sequence data have been submitted to NCBI gene bank and following accession numbers have been allotted.

Purified phytochemicals by HPLC

The following phytochemicals were identified and purified by HPLC as described in fig. and table.

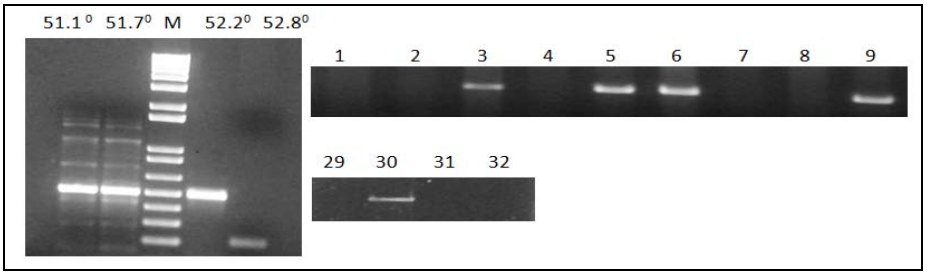

Fig. 1: Amplification of PBP-4from *E. faecalis* on 1%agarose gel

Table 1: Accession numbers for *E. faecalis* MDR isolates

S. No.	*E. faecalis* MDR isolate number	Accession number
1	EF 2	KR422418
2	EF 5	KR422419
3	EF 6	KR422420
4	EF 9	KR422421
5	EF 30	KR422422

☐EF: *Enterococcus faecalis*

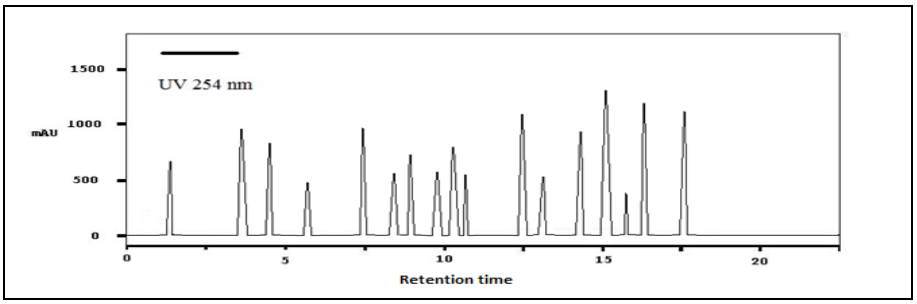

Fig. 2: HPLC chromatogram of phytochemicals

Table 2: Identified phytochemicals by HPLC

S. No.	Retention time in min	Peak area	Phytochemical
1	1.25	625	*Tannic acid*
2	4.44	854	*Ellagic acid*
3	3.02	958	*Quercetin*
4	10.02	758	*Chlorogenic acid*
5	15.00	1235	*2-Furaldehyde,5 (hydroxy methyl)*
6	3.89	558	*Naringenin*
7	10.98	501	*Theophylline*
8	5.82	425	*Betulinic acid*
9	12.50	1001	*Resorcinol*
10	7.48	1001	*Catechol*
11	17.50	1123	*Salicylic acid*
12	8.54	625	*Vanillin*
13	16.23	1234	*Hexadecanoic acid*
14	13.02	526	*3-o-methyl glucose*
15	4.62	802	*Gallic acid*
16	8.95	596	*Squalene*
17	14.25	977	*Pyrogallol*

Antibacterial activity of phytochemicals

The antibacterial potency of these purified phytochemicals has been analysed, as described in the following table.

Structure model for wild-type and mutant PBP4

After the homology search, PBP4 of *E. faecalis* was found to have 36% identities and 56% positives with the crystal structure of PBP2a of *methicillin*-resistant *Staphylococcus aureus* (PDB ID: 1VQQ_A) with resolution 1.8A°. Based on the available structure information of PBP2a of *S. aureus*, it was used as a template and homology model was generated for Wild type and mutant PBP4 of *E. faecalis* as shown in the following fig.

The prepared chimera model for pbp4 of *E. faecalis* was validated by Ramachandran plot. The amino acids found in the white area were PRO 602, ASP 81, ASP 159, ASP 630, ILE 507 and few Glycine. These amino acids were not interfering with the substrate binding region, but the structure was refined further.

Ligand docking and binding energy calculations for PBP4

The comparative analysis of ligand binding energy for wild-type and mutant PBP4 is shown in the following table:

Table 3: **Zone of inhibition (mm) for phytochemicals against MDR *E. faecalis***

S. No.	Phytochemical (1µg)	Antibacterial activity
		Zone of inhibition (mm)
		Mean
1.	*Catechol*	16
2.	*Chlorogenic acid*	14
3.	*Ellagic acid*	10
4.	*Gallic acid*	19
5.	*Naringenin*	14
6.	*Pyrogallol*	12
7.	*Quercetin*	17
8.	*Resorcinol*	<10
9.	*Salicylic acid*	17
10.	*Tannic acid*	20
11.	*Theophylline*	14
12.	*Vanillin*	16

Data represents mean of triplicate

Table 4: Comparative analysis of ligand binding energy for wild type and mutant PBP4

Ligands	Minimum binding energy (Kcal/mol)	
	Wild type PBP4	Mutant PBP4
Chlorogenic acid	-42.742	-43.589
Quercetin	-43.921	-47.396
Cephalosporin	-31.662	-29.874
Methicillin	-30.859	-29.114
Bocillin	-41.082	-38.112
Penicillin	-37.712	-37.643
Mezlocillin	-38.551	-32.767
Ampicillin	-36.433	-35.116
Ellagic acid	-35.884	-35.731
Naringenin	-40.117	-39.792
Gallic acid	-49.512	-41.766
Pyrogallol	-33.129	-24.458
Amoxicillin	-30.131	-28.747
Catechol	-30.794	-30.721
Vanillin	-21.089	-32.299
Salicylic acid	-29.254	-29.391
Theophylline	-19.006	-19.203
Resorcinol	-28.741	-28.796

The interactions of two standard antibiotics, cephalosporin, and penicillin along with two phytochemicals with highest binding affinity, *gallic acid* and *quercetin* are shown in the following table.

Table 5: Interaction of ligand with active site residues of wild type and mutant PBP4

Ligand	Interaction of ligand with wild-type PBP4	Interaction of ligand with mutant PBP4
Penicillin	ASN 163 (H-bond, 1.86447A °)	LYS 88 (H-bond, 2.21943A °)
	ASN 163 (H-bond, 2.24557A °)	ASN 78 (H-bond, 1.86247A °)
	ASN 78 (H-bond, 1.88405A °)	ASN 163 (H-bond, 2.10571A °)
	LYS 88 (H-bond, 2.2124A °)	ASN 163 (H-bond, 2.12828A °)
	LYS 88 (π-cation, 4.82044A °)	
Cephalosporin	LYS 88 (Salt bridge, 2.92706A °)	ASP 164 (H-bond, 1.54807A °)
	LYS 88 (Salt bridge, 4.36444A °)	ASP 325 (H-bond, 1.78339A °)
	ASN 163 (H-bond, 2.07318A °)	LYS 77 (H-bond, 1.77396A °)
	LYS 88 (H-bond, 1.952A °)	LYS 152 (H-bond, 1.63866A °)
	ASP 164 (H-bond, 2.48168A °)	LYS 152 (Salt bridge, 4.33472A °)
	ASP 326 (H-bond, 1.71764A °)	SER 161 (H-bond, 2.58339A °)
Quercetin	LYS 88 (π-cation, 3.78364A °)	ASP 326 (H-bond, 1.74624A °)
	ASP 326 (H-bond, 2.55525A °)	LYS 88 (π-cation, 3.83474A °)
	ILE 166 (H-bond, 1.90599A °)	ASN 78 (H-bond, 2.03053A °)
	ASN 78 (H-bond, 2.23008A °)	LYS 77 (H-bond, 1.91414A °)
		ASN 163 (H-bond, 2.31256A °)
Gallic acid	ASP 164 (H-bond, 1.88277A °)	ASP 164 (H-bond, 1.66558A °)
	ASP 164 (H-bond, 1.87262A °)	ASP 164 (H-bond, 1.93206A °)
	LYS 88 (π-cation, 4.18453A °)	LYS 88 (π-cation, 4.4133A °)

From above docking analysis, it has been observed that T(425)S point mutation in *E. faecalis* has changed its antibiotic affinity towards β-lactam antibiotics. The minimum binding energy required to bind the active site of PBP4 has increased for *cephalosporin, methicillin, ampicillin* and *amoxicillin.* This means the affinity to bind the active site has been decreased after mutation, and it could be one of the possible reasons to develop resistance or increased MIC towards these antibiotics.

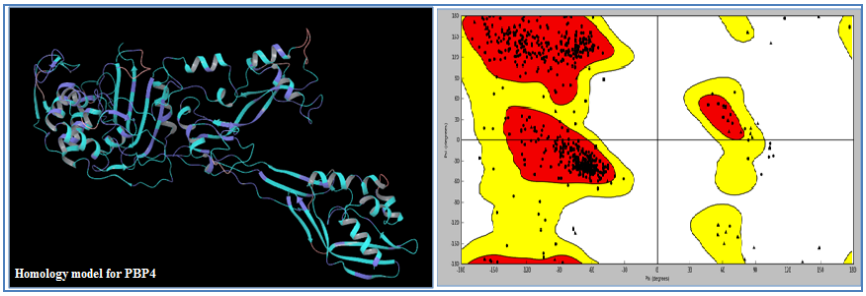

Fig. 3: Chimera model for wild type PBP4 of *E. faecalis* and its ramachandran plot

On the other hand, compared to these β-lactam antibiotics, *gallic acid* and *quercetin* have showed very less binding energy in wildtype and mutant form. This docking analysis opens new avenues to design and synthesise new antibacterial agent based on phytoconstituents structures, especially gallic acid, and quercetin.

DISCUSSION

Many researchers have shown the emergence of multidrug resistance in *Enterococci faecalis* to all clinically useful antibiotics [13] and in the current study, it was identified that *E. faecalis* have developed resistance towards 3rd and broad spectrum antibiotics, more specifically towards β-lactam antibiotics. The resistance towards β-lactam antibiotics has already been reported by overproduction of penicillin binding protein 4 (pbp4) [1]. PBP4 plays an important role in synthesis and turnover of peptidoglycan. In the current study, pbp4 have been identified with T(425)S point mutation. Further analysis was carried out to see the effect of this mutation on its ligand (antibiotics) binding efficiency and it was confirmed with the *in silico* analysis that mutation has decreased the binding efficiency for *cephalosporin, methicillin, ampicillin* and *amoxicillin*. Further, to propose the possible antibacterial agent, phytochemicals were purified from the medicinal plant. *Catechol, chlorogenic acid, gallic acid, naringenin, pyrogallol, quercetin, salicylic acid, tannic acid, theophyllin* and *vanillin* have shown the significant antibacterial activity against isolated MDR E. faecalis strains. The molecular docking was carried out to check the efficiency of this phytochemicals to bind the active site of pbp4 in wildtype and mutant form. Among all these phytochemicals, *gallic acid* and *quercetin* have shown the significant potential to bind the active site of pbp4.

CONCLUSION

From the above results, it is clear that the purified phytochemicals (*catechol, chlorogenic acid, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, tannic acid, theophylline* and *vanillin*) from selected medicinal plants (viz., *Andrographis paniculata* Burm. f. Wall. Ex. Nees *(Ap), Astercantha longifolia* (L.) Nees *(Al), Bixa orellana* L. *(Bo), Gardenia resinifera* Roth, *(Gr), Pongamia pinnata* (L.) Pierre *(Pp), Psoralea corylifolia* L. *(Pc), Sphaeranthus indicus* Linnaeus *(Si), Solanum trilobatum* L. *(St), Soyamida febrifuga* (Roxb.) Juss *(Sf)* and *Thespesia populnea* (L.) Sol. Ex. Correa *(Tp)* have shown significant antibacterial activity against isolated β-lactam resistant *E. faecalis* isolates.

Further part of the study dealt with the mutation in the pbp4 gene of *E. faecalis* and T(425)S point mutation was detected in the pbp4 gene. To analyse the effect of this mutation on the binding efficiency towards β-lactam resistance, the structural information should be available. But unfortunately, its crystal structure is not available in protein data bank. Hence, the homology model was generated for both wildtype and mutant protein using Modeller, Swiss model and Phyre2. PBP4 was found to have 36% identities and 56% positives with PBP2a from methicillin-resistant *Staphylococcus aureus* (PDB ID: 1VQQ_A) with resolution 1.8A ° and this structure was used for the preparation of model for pbp4 of *E. faecalis*. The prepared models were validated by Ramachandran plot, and molecular docking was performed.

It is clear from molecular docking analysis that T(425)S point mutation of pbp4 in *E. faecalis* has decreased the binding affinity for *cephalosporin, penicillin, methicillin, ampicillin, amoxicillin.*

Moreover, the phytochemicals with antibacterial activity were analysed for their binding efficiency to the active site of pbp4 of *E. faecalis*. Among all tested phytochemicals, *gallic acid* and *quercetin* were found with highest binding affinity to the ligand binding pocket of pbp4 of *E. faecalis* than tested β-lactam antibiotics. Hence, *gallic acid and quercetin* could be possible antibacterial agents for PBP4 mediated multidrug resistance of *E. faecalis*. More studies on molecular dynamics and simulation are needed for corroborating the role of *quercetin* and *gallic acid* as antibacterial agents to treat MDR *E. faecalis* mediated uropathological infections.

ABBREVIATION

PBP-Penicillin Binding Proteins, T-Threonine, S-Serine, *E. faecalis* (EF)-*Enterococcus faecalis*, UTI–Urinary Tract Infections, VRE-vancomycin-resistant *Enterococci*, MDR–Multi Drug Resistant, gDNA–Genomic DNA, HPLC–High-Performance Liquid Chromatography, PDB–Protein Data Bank

CONFLICT OF INTERESTS

Declared none

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