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IN-SILICO DOCKING STUDIES OF PHYTO-LIGANDS AGAINST *E. COLI* PBP3: APPROACH TOWARDS NOVEL ANTIBACTERIAL THERAPEUTIC AGENT

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Keywords:

Penicillin Binding Protein (PBP), Chlorogenic acid, Antibiotic Resistance, Phytochemicals, Molecular Docking, Bocillin Assay

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ABSTRACT: Emergence of antibiotic resistance has become a serious problem worldwide. The extensive and uncontrolled use of antibiotics increases the number of multi drug resistant (MDR) bacterial strains. The major mechanisms that bacteria evolve to develop resistance against β-lactam class of antibiotics are the production of β-lactamases and expression of low affinity penicillin binding proteins (PBPs). These PBPs are involved in the final stages of peptidoglycan synthesis. Hence, PBPs are the primary targets for developing antibacterial agents as its inhibition leads to irregularities in cell wall structure and eventually cell death. Bioactive phytochemicals from medicinal plants have been proven to reveal important pharmacological and therapeutic properties for developing novel antibacterial agents. The current study epitomizes the binding of phytochemicals to the transpeptidation (TP) domain of PBP3 of *E. coli, a* major causative agent of bacterial infection. The phytochemicals exhibiting antibacterial activities were selected as ligands for docking studies employing Schrödinger Suite (Maestro, LLC, New York, NY, 2015). Amongst all the phytochemicals studied, chlorogenic acid (CGA) was found to have highest affinity towards PBP3 TP domain. The bocillin binding assay corroborated the docking analysis. The blocking of active site of PBPs by phytochemicals opens up new avenues for screening and developing new phytochemical-based therapeutic antibacterial agents.

INTRODUCTION: Bacterial murein sacculus, also known as peptidoglycan orthe cell wall, represents essential cell stress-bearing and shape-maintaining entity [1]. The synthesis of this murein sacculus is catalysed by various penicillin binding proteins (PBPs) [2]. One of them is PBP3; a membrane bound mono functional protein with C-terminal domain having transpeptidase activity [3, 4], encoded by *ftsI* gene and is essential for cell division [3], a process that involves more than 10 essential cell division (Fts) proteins which assemble to form a ring-like structure at the division site [4] via its N-terminal region and dependent on the presence of FtsW [5].

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All β-lactam antibiotics share the same mechanism to inhibit peptidoglycan synthesis by binding to transpeptidase domain of PBPs. They form a stable covalent adduct with the active site serine residue of PBPs [6]. So, PBPs remain attractive targets for developing novel antibacterial agent. But widespread use of β-lactam antibiotics has led to the worldwide appearance of drug-resistant bacterial strains through expression of low affinity PBPs.

Due to emergence of multi drug resistance in bacteria, there is an urgent need to search for new antibacterial agents. Medicinal plants reveal important pharmacological activities that can be applied to developing novel therapeutic agents. The phytoconstituents purified from plants were investigated for their antibacterial efficacy and potent antibacterial agents were docked *in silico* with the PBP3 from *E. coli* to analyse their interactions with active site residues of transpeptidase domain. The phytochemicals

analysed were chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin. Further characterization of their interaction provided catechol, chlorogenic acid, gallic acid, salicylic acid and vanillin as potent antibacterial molecule. Overall results open new avenues to design and synthesize phytochemical based antibacterial agents to fight against serious bacterial infections.

The aim of this study was to identify the mutations in PBP3 from multidrug resistant *E. Coli* and analysing the effects of the mutation(s), on the binding efficiency of antibiotics as well as phytochemicals to the active site of PBP3 in wildtype and mutant forms.

Since, the multidrug resistant *E. coli* were isolated, identified and characterized from uropathological samples from UTI infected patients, hence, no ethical approval was required for this study.

MATERIALS AND METHOD:

Isolation and identification of β-lactam resistant *E.coli* isolates:

Twenty four *E. coli* isolates were obtained from 100 UTI (urinary tract infection) patients by streaking uropathological samples on LB agar plate (Himedia DT001) and then identified by Gram's staining and various biochemical tests(Himedia KBM001, KB002, and KB003) [7].

The antibiotic sensitivity for these isolates was checked with following β-lactam antibiotics using 4X-concentration of the reported MIC (minimum inhibitory concentration) by Kirby-Bauer method [8]. The β-lactam antibiotics used were Cefpodoxime (16µg), Cephanexin (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. coli* strains were selected for further molecular analysis.

Amplification and Sequencing of PBP3:

The bacterial cells were pelleted by centrifugation at 3000rpm for 5mins and washed with milliQ water. The pellet was suspended in water and boiled for 10 min at 100^0C. This lysate was centrifuged and the supernatant was used directly as a template for PCR amplification.

The primer was designed using NCBI primer blast tool (www.ncbi.nlm.nih.gov) for amplification of PBP3 (encoded by *ftsI* gene) was Forward (5'-ACT GGT GCT GGA GCG AGA TGC-3') and Reverse (5'- AGC TAC AAA GAG ATC GCC CG -3'). Thermal cycler (Peq Lab) was used to amplify *ftsI* under following conditions: Initial denaturation at 95ºC for 5min, followed by 30 cycles of 1min denaturation at 94ºC, 1 min of annealing at 58.2ºC and 1.5min at 72ºC and finally 10min at 72ºC [9].

PCR amplicons were detected electrophoretically in 1% agarose gel using 1X TAE, visualized by staining with 0.5µg/ml ethidium bromide, examined in UV light and photographed by Molecular Imager Gel Doc XR (Biorad Laboratories).The purified PCR products were sequenced for mutational analysis.

Purification and identification of phytochemicals:

The antibacterial activity was studied for the extracts of following ten medicinal plants, obtained by 50% aqueous-methanol (v/v) solution with cold maceration; *Andrographis paniculata (Ap), Astercantha longifolia (Al), Bixa orellana (Bo), Gardenia resinifera (Gr), Pongamia pinnata (Pp), Psoralea corylifolia (Pc), Sphaeranthus indicus (Si), Solanum trilobatum (St), Soyamida febrifuga (Sf)* and *Thespesia populnea (Tp)*. All these extracts showed significant antibacterial activity, hence were utilized for identification of phytochemicals by HPLC [8].

Antibacterial activity of purified phytochemicals against *E. coli* isolates:

The phytochemicals identified and purified were betulinic acid, chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin. These purified phytochemicals were studied for their antibacterial activity against *E. coli* [8].

Purification of PBP3

The PCR amplicon was purified by QIA quick purification kit (Qiagen) and cloned into pCR 2.1-

TOPO vector. These were digested with XhoI and Bam HI and cloned into the expression vector pET15b (Novagen) at the same restriction sites. BL21 (DE3) *E. coli* host cells were transformed with expression vectors. These cells were grown at 37^0C till OD_{600} reached 0.6. 1mM IPTG was used as an inducer. The bacterial cells were pelleted down at 5000rpm/10min/4^0C.

These cells were suspended in 20mMTris (pH 8.0) and 300mMNaCl and broken by sonication. Recombinant protein containing an N-terminal (His)6 purified by nickel chelation chromatography in the presence of 1 mM DDM. The N-terminal (His)6 tag was removed by thrombin cleavage (Sigma–Aldrich) at room temperature overnight. PBP3 was further purified by Superdex 200 size-exclusion column (GE Life Sciences). Peak fractions were collected and were exchanged to PBS using Amicon Ultra filter units (Millipore).

Bocillin assay:

The purified PBP3 was assayed for its binding with phytochemicals in presence of bocillin. Bocillin is a fluorescent analogue of penicillin V and it binds to the transpeptidase domain of all PBPs. In the current study, binding efficiency of catechol, chlorogenic acid (CGA), gallic acid, salicylic acid and vanillin was analysed to TP domain of PBP3. The purified PBP3 was incubated with 20, 50 and 100µM of respective phytochemicals in bocillin binding assay [10]. CGA showed the highest binding efficiency to TP domain of PBP3.

AMES test for CGA mutagenicity testing:

AMES test was performed to check the mutagenicity of CGA with Bio Era's kit method (BTK 150905, India) containing mutant *Salmonella typhimurium* oxotrophic bacterial cell. These bacteria contain mutation in a gene required for histidine synthesis. This mutation prevents translation of functional enzyme and thus cell cannot synthesize histidine. These are histidine-dependent mutants because they depend on external source of histidine in medium.

S. typhimurium was revived in 10ml of sterile LB broth (2gm of dehydrated LB broth powder in100ml distilled water) and incubated at 37^0 in incubator for 48 hours. Minimal agar plates were prepared by dissolving 1.2gm of dehydrated media in 400ml distilled water; pH was adjusted to 7.0 with 1N NaOH and then added 2gm agar powder. This was sterilised and poured in petriplates. The soft agar was prepared by adding 0.1gm of agar powder in 10ml minimal media and sterilised.

10mg of chlorogenic acid was dissolved in 1ml sterile distilled water. The concentration of CGA tested for mutagenicity varied from 10µg to 1mg. In 1ml of soft agar, added required concentration of CGA and 50µl of bacterial culture (McFarland's turbidity standard of 0.5 which in turn was equal to 1.5×10^8cells/ml), 200µl of this mixture was spotted on minimal agar plate. The plates were incubated at 37^0 for 72 hrs. The number of visible colonies was counted.

Cytotoxicity testing for CGA:

Reduction of 3-(4,5-dimethylthiazol-2-yl) – 2,5 – diphenyltetrazolium bromide (MTT) by CPR (a microsomal enzyme, NADPH-cytochrome P450 reductase) was used in measuring the cell cytotoxicity. Blood lymphocytes were used for cytotoxicity assay (HiSep LSM). The reduced was measured spectroscopically at 610nm. The concentrations of CGA tested for mutagenicity were 10µg and 1mg. The detailed methodology has been described by Sung-Kun Yim *et. al.* [11].

***In Silico* studies:**

The computational studies were carried out using Windows 8.1 professional, 64 bit Intel core M-5Y10c CPU at 1.00 GHz, 4 GB RAM) using the Schrödinger 2015 suite (Maestro 10.1, Schrödinger, LLC, New York, NY, 2015).

Ligand preparation:

The phytochemicals showing prominent antibacterial activity against MDR *E. coli* (chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin) were sketched in maestro 10.1 (Maestro, Schrödinger, LLC, New York, NY, 2015), and prepared by ligprep (LigPrep, version 3.3, Schrödinger, LLC, New York, NY, 2015) with OPLS 2.1 force field to rectify the molecular geometries and get least energy conformations.

Selection and preparation of PBP3 structure;

E. coli PBP3 is encoded by *ftsI* gene and plays an important role in catalysing the cell wall peptidoglycan during cell division. PBP3 protein structure was downloaded from protein data bank (PDB ID:4BJP, http://www.rcsb.org/pdb/. The PBP3 structure crystallised by Souvage *et. al.* 2014 was selected for analysis as it belongs to the same species of bacteria i.e *E. coli* with the resolution $2.50A^{0}$ [12].

The protein was prepared and minimised by protein preparation wizard with OPLS 2005 force field in maestro 10.1 (Maestro, Schrödinger, LLC, New York, NY, 2015). The sitemaps were generated by grid based method (Glide, sitemap, Schrödinger, LLC, New York, NY, 2015) to locate primary ligand binding site in the receptor. The sitemaps were analysed by Site Score, D score, balance of hydrophobic-hydrophilic characters of the site and H-bond donor/acceptor ratio of ligand. The grid was specified by Glide receptor grid generation using centroid of crystal native ligand for ligand docking. The reported conserved region in the active site of penicillin binding proteins is Gly-Ser-X-X-Lys-Pro. The catalytically important active Serine in PBP3 is S(307). This information was used to locate and confirm the active site region and set up a grid for ligand docking.

Homology model preparation for mutant PBP3:

The sequencing analysis has shown the alteration of N(104)I in PBP3 in the form of a point mutation in *E. coli*. The homology model for mutant PBP3 was built in Prime (Prime, Homology model, structure prediction wizard, Schrödinger, LLC, New York, NY, 2015) by using wild type structures of respective proteins with 99% homology. The built model was validated by Ramachandran plot. The homology modelling includes protein structure prediction from the template, alignment and model building. The built models were again prepared in protein preparation wizard (Maestro 10.1, Schrödinger, LLC, New York, NY, 2015) and site map were generated to predict the ligand binding site to compare with the wild type data in further calculations. The MM-GBSA method was used for predicting binding energy in protein-ligand complex.

Molecular Docking:

Molecular docking was carried out by glide docking (Glide, version 6.0, Schrödinger, LLC, New York, NY, 2015)using an extra precision mode, which utilizes as an input the prepared ligand and generated grid. The ligand sampling was selected as flexible for docking against the biological target. Here the interaction pattern between ligands and protein, glide energy and docking score were studied. It was observed that among all the tested phytochemicals, chlorogenic acid showed most prominent results.

RESULTS AND DISCUSSION:

Selection of β-lactam resistant *E. coli* isolates:

We have found that 26% of the UTI patients were infected with multi drug resistant *E. coli.* The other predominated bacteria isolated were *Pseudomonas aeruginosa* (36%) and *Enterococcus faecalis* (35%). *E. coli* were found completely resistant to Cefpodoxime (16µg), Cephalexin (4.0µg), Cefuroxime (16µg) and Cefazoline (4.0µg). Also, they showed emerging resistant towards Cefixime (4.0µg), Ceftazidime (2.0µg) and Ceftriaxome (2.0µg).

The PCR amplification of *ftsI* gene in *E. coli* was standardised at 58.2^{0}C primer annealing temperature. The amplification was seen in nineteen *E. coli* isolates out of twenty four. The *E. coli* strains numbers that showed amplifications were 1, 2, 4, 5, 6, 11, 12, 13, 14,1 5, 16, 17, 18, 19, 20, 21, 22, 23 and 24 (data shown in **Fig.1**).

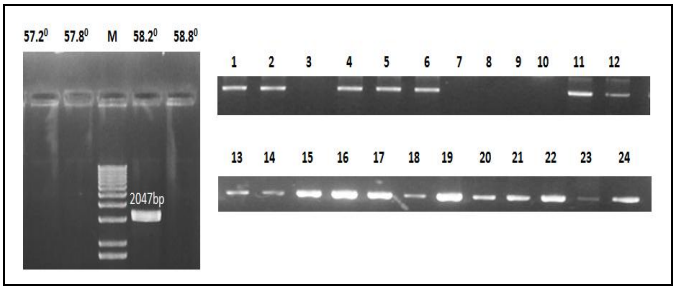

FIG. 1: AMPLIFICATION OF PBP-3 (*FTSI* GENE) FROM *E. COLI* ON 1%AGAROSE GEL

Mutation in PBP-3 of *E. Coli:*

The point mutation observed in PBP-3 gene was N(104)I. At position 104, Aspargine (N) is replaced by Isoleucine (I). These sequence data have been submitted to NCBI gene bank as shown in **Table 1**.

TABLE 1: ACCESSION NUMBERS OF *FTSI* GENE

S. No.	*E. coli* MDR isolate number	Accession Number
1	EC 3	KR186013
2	EC 11	KR186014
3	EC 14	KR186015
4	EC 15	KR186016
5	EC 21	KR186017

EC: *E. coli*

Purified phytochemicals by HPLC:

The phytochemicals identified by HPLC were tannic acid, ellagic acid, gallic acid, quercetin, chlorogenic acid, naringenin, theophylline, betullinic acid, resorcinol, catechol, salicylic acid, vanillin, squalene and pyrogallol [8].

Antibacterial activity of phytochemicals:

Though MDR *E. coli* isolates have emerged resistant against commonly prescribed β-lactam antibiotics, they are still susceptible to many selected phytochemicals. These phytochemicals have shown significant antibacterial activity against MDR isolates. The antibacterial potency of these purified phytochemicals has been analysed, as described in **Table 2**.

TABLE 2: ZONE OF INHIBITION (MM) FOR RESPECTIVE PHYTOCHEMICALS AGAINST MDR *E. COLI*

S.No.	Phytochemical (1mg)	Antibacterial activity MDR *E. coli* isolates
1.	Catechol	17
2.	Chlorogenic acid	11
3.	Ellagic acid	10
4.	Gallic acid	15
5.	Naringenin	12
6.	Pyrogallol	15
7.	Quercetin	10
8.	Resorcinol	<10
9.	Salicylic acid	<10
10.	Tannic acid	17
11.	Theophylline	10
12.	Vanillin	10

Protein structure:

The crystal structure of wild type PBP3 was downloaded from PDB. The homology model for mutant PBP3 was prepared based on wild type sequence as template and both wild type as well as mutant PBP3 structures were validated by Ramachandran plot, as shown in the following **Fig.2**.

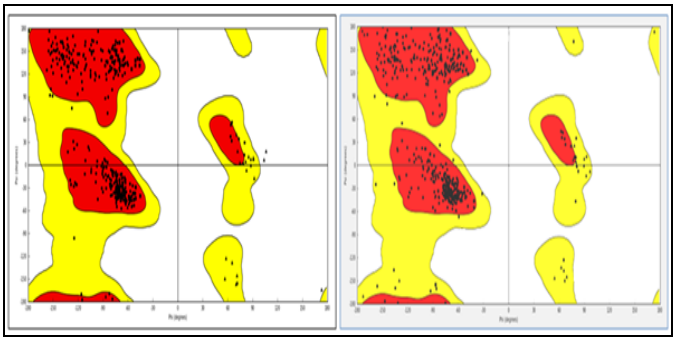

FIG. 2: RAMACHANDRAN PLOT OF WILD TYPE AND MUTANT PBP3 RESPECTIVELY

Effect of N(104)I mutation on active site of PBP3

It was found that N(104)I mutation is not in the active site cavity of PBP3 but still have significant impact on active site. The mutation has increased the site score, size, drug ability score and volume as observed from sitemap analysis. Moreover, ligand interaction surface has increased as evidenced from increased hydrogen bond donor/acceptor area, hydrophobic as well as hydrophilic area of the active site. The data is shown in following **Table 3**, **Fig.3** and **Table 3**.

TABLE 3: SITEMAP COMPARISON BETWEEN WILDTYPE AND MUTANT ACTIVE SITE

Active site	Site Score	Size	D score	Volume
PBP3_wild	0.913	68	0.916	302.869
PBP3_mutant	1.035	101	1.065	449.673

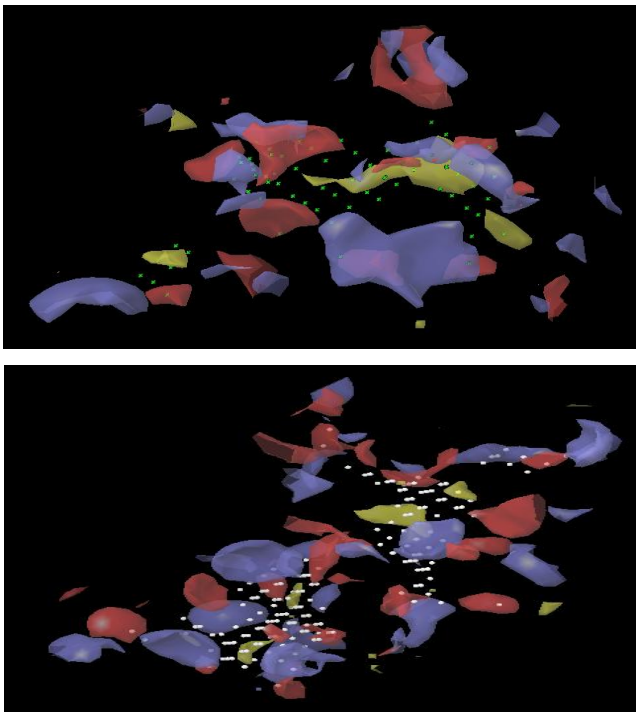

FIG. 3: SURFACE ANALYSIS OF ACTIVE SITE OF PBP3 IN WILDTYPE AND MUTANT PROTEIN RESPECTIVELY

TABLE 4: ACTIVE SITE SURFACE ANALYSIS

PBP3 Active Site Surface	Surface Area	
	Wild type PBP3	Mutant PBP3
H-bond acceptor (red)	423.012	618.982
H-bond donor (blue)	428.698	1089.330
Hydrophobic (yellow)	64.361	102.300
Hydrophillic	853.820	1730.746

TABLE 5: COMPARATIVE ANALYSIS OF DOCKING DATA BETWEEN WILD TYPE AND MUTANT PBP3

Ligands	Wildtype PBP3		Mutant PBP3	
	Glide score	Binding energy	Glide score	Binding energy
Chlorogenic acid	-9.04	-50.577	-8.94	-64.504
Quercetin	-8.05	-40.372	-8.24	-55.862
Cephalosporin	-7.28	-40.250	-8.78	-39.963
Methicillin	-6.87	-40.405	-4.33	-36.243
Bocillin	-6.85	-9.664	-5.65	-7.131
Penicillin	-6.74	-31.652	-4.44	-50.866
Mezlocillin	-6.72	-49.640	-6.49	-54.034
Ampicillin	-6.59	-20.873	-4.49	-25.005
Ellagic acid	-6.49	-41.263	-5.94	-41.343
Naringenin	-5.36	-34.355	-6.03	-46.306
Gallic acid	-5.35	-27.267	-7.01	-28.440
Pyrogallol	-5.19	-28.693	-5.71	-22.639
Amoxicillin	-5.23	-35.277	-5.63	-24.029
Catechol	-4.97	-27.043	-5.74	-27.475
Vanillin	-4.81	-23.612	-4.85	-29.744
Salicylic acid	-4.44	-19.245	-4.53	-22.567
Theophylline	-5.40	-22.359	-3.84	-29.463
Resorcinol	-3.77	-19.313	-3.80	-27.953

Binding energy: Kcal/mol

From the above table for docking analysis, it is clear that, the N(104)I point mutation in PBP3 has lead to noticeable changes in respect to its binding affinity towards tested β-lactam antibiotics as well as towards phytochemicals.

The interactions of bocillin, penicillin, cephalosporin, chlorogenic acid, quercetin, ellagic acid and gallic acid with the residues of ligand binding pocket of wildtype and mutant PBP3 is shown in the following **Fig.4**.

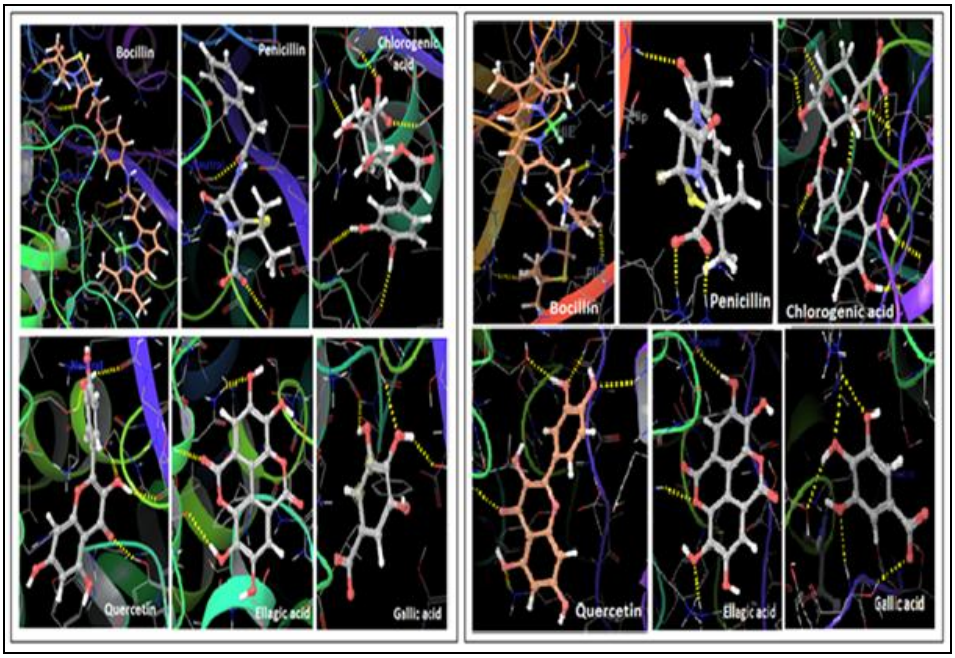

FIG. 4: DOCKED POSE OF LIGANDS TO LIGAND BINDING POCKET OF WILDTYPE AND MUTANT PBP3 RESPECTIVELY

The interactions of various ligands with active side residues of PBP3 are shown in the following **Table 6.**

TABLE 6: INTERACTION OF LIGAND WITH ACTIVE SITE RESIDUES OF WILDTYPE AND MUTANT PBP3

Ligand	Interaction of ligand with active site residues of wild type PBP3	Interaction of ligand with active site residues of mutant PBP3
Bocillin	LYS 358 (H-bond, 1.7856A^0)	GLY 542(H-bond, 2.06613A^0)
	TYR 348 (H-bond, 2.05977A^0)	THR 497 (H-bond, 2.55801A^0)
	SER 307 (H-bond, 2.76096A^0)	THR 497 (H-bond, 2.17004A^0)
		LYS 499 (Salt bridge, 3.60317A^0)
		LYS 499(H-bond, 2.17102A^0)
Penicillin	SER 359 (H-bond, 2.09756A^0)	GLY 542 (H-bond, 2.39749A^0)
	TYR 419 (H-bond, 1.75585A^0)	THR 497 (H-bond, 2.26763A^0)
		THR 497 (H-bond, 2.36757A^0)
Cephalosporin	THR 497 (H-bond, 2.09298A^0)	GLY479 (H-bond, 1.68185A^0)
	TYR 419 (H-bond, 2.09932A^0)	GLY 543 (H-bond, 1.99558A^0)
	LYS 499 (H-bond, 1.91674A^0)	GLY 542 (H-bond, 2.02225A^0)
	LYS 499 (Salt bridge 3.37512A^0)	THR 497 (H-bond, 2.16351A^0)
	LYS 310 (Salt bridge, 3.03825A^0)	THR 497 (H-bond, 1.9157A^0)
		SER 307 (H-bond, 2.17677A^0)
		LYS 358 (Salt bridge, 3.27551A^0)
		LYS 358 (H-bond, 2.22045A^0)
Chlorogenic acid	LYS 310 (H-bond, 2.43818A^0)	ASN 361 (H-bond, 2.11181A^0)
	SER 307 (H-bond, 1.86255A^0)	ASN 361 (H-bond, 1.8853A^0)
	TYR 419 (H-bond, 2.08417A^0)	THR 497 (H-bond, 1.85426A^0)
	GLU 340 (H-bond, 1.98435A^0)	THR 497 (H-bond, 2.03069A^0)
	LYS 342 (H-bond, 1.82931A^0)	TYR 419 (H-bond, 2.44813A^0)
		ASP 534 (H-bond, 1.93849A^0)
		ASP 534 (H-bond, 1.97045A^0)
		SER 307 (H-bond, 1.89537A^0)
		LYS 499 (H-bond, 2.58246A^0)
Quercetin	THR 497 ((H-bond, 1.93998A^0)	THR 495 ((H-bond, 1.79042A^0)
	SER 307 (H-bond, 2.33022A^0)	SER 359 (H-bond, 2.02285A^0)
	TYR 419 (H-bond, 1.87225A^0)	GLY 543 (H-bond, 2.59896A^0)
	SER 359 (H-bond, 2.289A^0)	ASN 361 (H-bond, 2.12148A^0)
	THR 495 (H-bond, 1.81335A^0)	LYS 499 (H-bond, 1.94126A^0)
Ellagic acid	ASN 361 (H-bond, 2.76686A^0)	SER 359 (H-bond, 2.19023A^0)
	LYS 342 (H-bond, 1.86866A^0)	ASN 361 (H-bond, 2.51006A^0)
	GLU 340 (H-bond, 2.38452A^0)	LYS 499 (H-bond, 2.02554A^0)
Gallic acid	SER 307 (H-bond, 2.01539A^0)	ASN 361 (H-bond, 2.39468A^0)
	THR 497 (H-bond, 1.97068A^0)	ASN 361 (H-bond, 2.64108A^0)
	PHE 417 (H-bond, 2.49363A^0)	THR 497 (H-bond, 1.96272^0)
	TYR 419 (π-π stacking, 5.47751A^0)	THR 497(H-bond, 2.48927^0)

From the docking analysis of phytochemicals, in comparison to standard antibiotics, as antibacterial agents, and also through analysis of their interaction with active site residues, it is clear that chlorogenic acid, ellagic acid, quercetin, naringenin, gallic acid, pyrogallol, catechol, resorcinol, vanillin, salicylic acid and theophylline are able to bind the active site of PBP3.

Bocillin binding assay: The docking analysis showed the highest affinity of CGA towards TP domain as compared to penicillin, bocillin, naringenin, quercetin, salicylic acid and theophylline. To corroborate this docking data, the binding analysis of bocillin in presence and absence of CGA was performed. The bocillin binds to TP domain of purified PBP3 and gives fluorescence in UV light but the incubation of protein with CGA prevents the binding of bocillin and decreases the fluorescence, as described in **Fig.4**.

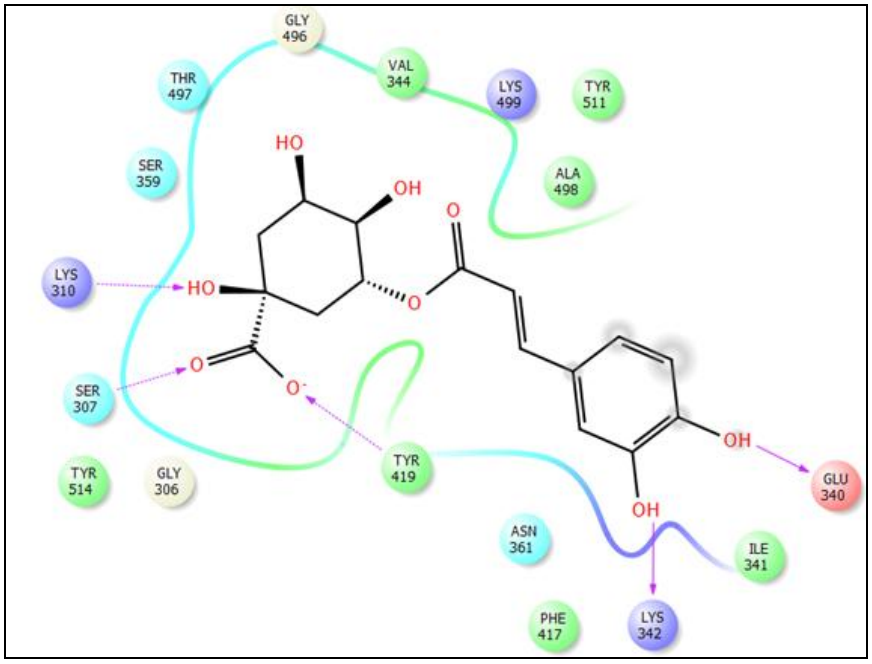

FIG. 5: INTERACTION OF CHLOROGENIC ACID TO THE ACTIVE SITE OF PBP3

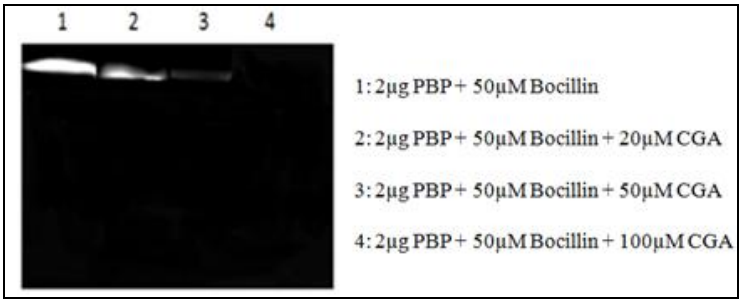

FIG. 6: CGA BINDING DECREASES BOCILLIN FLUORESCENCE

Mutagenicty and cytotoxicity of CGA: CGA did not show any indication for mutagenic and cytotoxic effect at tested concentration.

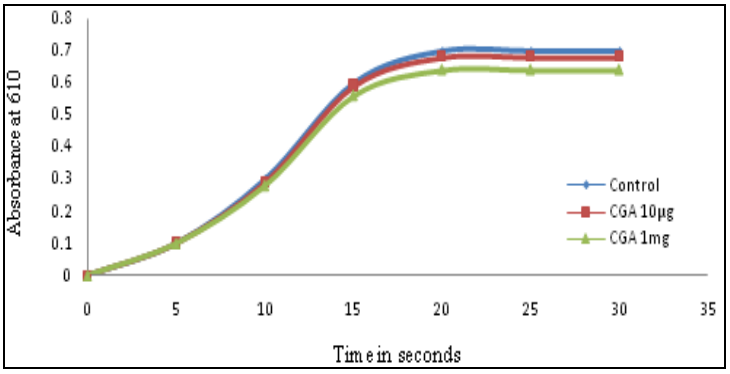

FIG.7: CYTOTOXIC ASSAY FOR CGA

CONCLUSION: PBP3 has only one active site, TP domain. The docking analysis showed decreased binding affinity after mutation towards penicillin (glide score -6.74 to -4.44), methicillin (glide score decreased from -6.87 to -4.33), mezlocillin (from -6.72 to -6.49) and ampicillin (from -6.59 to -4.49) but increased with amoxicillin (-5.23 to -5.63). This shows that these antibiotics are binding with less affinity to the mutated PBP3 and could be less effective as antibacterials except amoxicillin. But, the glide score for cephalosporin has increased from (-7.28) to (-8.78). So,

cephalosporin can still be used as a potent antibacterial agent.

Among all the phytochemicals tested, the top three molecules found to bind the active site of wild type PBP3 were chlorogenic acid (-9.04), quercetin (-8.05) and ellagic acid (-6.49). Moreover, chlorogenic acid and quercetin were found to have higher affinity than cephalosporin (-7.28). Although, in mutated PBP3, the affinity for these three phytochemicals has been found to decrease, but gallic acid (-7.01) has showed good affinity for mutated protein.

Patents filed:

The phytochemicals; chlorogenic acid, quercetin and ellagic acid have not only showed promising results in blocking the active site of PBP3, but also, they have showed significant efficacy in inhibiting β-lactamase activity (unpublished data). The following patents have been filed and published in Indian Patent Journal with following application numbers 4342/MUM/2015, 4621/MUM/2015 and 4622/MUM/2015.

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