Research Article

Molecular and Computational Analysis of β-Lactamases: Comprehensive Approach Towards Combating Drug Resistance by Chlorogenic Acid

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ABSTRACT

The multidrug resistant *E. coli* strains were isolated from Urinary Tract Infected (UTI) patients and ESBL (Extended Spectrum β-Lactamase) expressing isolates were identified. The β-lactamase (βL) activity was investigated and compared for their extracellular & intracellular activity. These isolates were also been checked for the presence of different βL by PCR and found OXA, TEM and AmpC. The sequencing and mutational analysis were done for these βL. We have also projected the use of phytochemicals identified as potential antibacterials in our lab to see whether they can inhibit βL by nitrocefin assay. Automated molecular docking with all molecules was performed by using the advanced docking program AutoDock whereas the inhibitor-enzyme interactions were estimated by the Lamarckian genetic algorithm. Both, the biological and docking data suggests the potential β-lactamase inhibitory activity of phytochemicals, specifically chlorogenic acid as it is able to bind the active site pocket of OXA, TEM & AmpC and inhibit the total βL activity as performed by nitrocefin assay.

Keywords: antimicrobial activity, β-lactam-resistant bacteria, molecular docking, multidrug resistant, β-lactamase.

INTRODUCTION

Bacterial infections have been the major cause of diseases throughout the history of human population. One of the common bacterial infection is Urinary tract infection (UTI), also called as bladder infection or acute cystitis. The causal agents include *Escherichia coli*, *Klebsiella, Proteus, Pseudomonas, Staphylococcus, Saprophyticus* and *Enterobacter.* The drug resistant bacterial strains are currently a major health concern in treating bacterial infections.[1] The β-lactam antibiotics are generally prescribed to treat UTI infections but consequent and extensive use of antibiotics has evolved multi drug resistant (MDR) bacteria. Various mechanisms have been developed by bacteria to resist the action of β-lactam antibiotics, among them the most common is the production of β-lactamases (E.C.3.5.2.6), which destroy the antibiotics before they reach the bacterial target.[2-4]

From the late 1990s, multidrug-resistant Enterobacteriaceae (mostly *Escherichia coli*) produced extended-spectrum β-lactamases (ESBLs).[5] The prevalence of antibiotic resistance among ESBL-producing *Escherichia coli* has increased markedly in recent years.[6] ESBLs appear mainly due to mutations in β-lactamases encoded by the SHV, TEM and CTX-M genes.[7,8] TEM-1 is the first plasmid mediated ESBL detected in 1965, and it hydrolyses penicillins and cephalosporins. SHV was capable of hydrolyzing oxyamino-cephalosporins.[7] OXA hydrolyses hydrolyzeoxacillin, ampicillin and cephalothin. Other plasmid-mediated ESBLs are PER, VEB, CTX-M, and IBC β-lactamases.[8] During the past decade, there has been an emergence of carbapenem-resistant *Enterobacteriaceae* that produce carbapenemases (examples are KPC, MBL, IMP, VIM, NDM, etc).[9, 10]

It has been proved worldwide that medicinal plants reveal important pharmacological activities for developing novel therapeutic antibacterial agents. The phytochemicals which exhibits antibacterial activities were selected as ligands for docking studies by using molecular docking tool autodock. The β-lactamases under this study (TEM, OXA and AmpC) have been selected as target for molecular docking simulation. The interactions between ligands and protein were observed for different poses. The potent compound having the best docking score and good interactions with the protein has been studied. Among them chlorogenic acid, a natural polyphenol present in many plants have shown best docking and drug-likeness score.

MATERIALS AND METHODS

Isolation, identification and antibiotic susceptibility testing of bacterial isolates

The bacteria were isolated from UTI patients by streaking the urine sample on LB agar plate (Himedia DT001) and then identified by Gram's staining and biochemical tests (Himedia KBM001, KB002, and KB003).[11] The antibiotic sensitivity was checked for 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) by Kirby-Bauer method.[12] The MDR *E. coli* strains were selected and further ESBL detection was done (Himedia FD278).

The antibacterial activity of betulinic acid, chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin,

pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin (purchased from SRL, India) were checked against isolated MDR *E. coli.*

β-lactamase (βL) activity assay

The presence of βL in isolated β-lactam resistant *E. coli* was detected by micro-iodometric assay.[13] The total βL activity was checked by nitrocefin assay[14,15] and was used to compare the secretory (in extracellular medium) and cellular βL (sonicated cell lysate) activity in β-lactam resistant *E. coli* isolates. Nitrocefin was purchased from BioVision (Cat No 2388-5). Stock solution of nitrocefin was prepared 5mg/ml in DMSO and the working solution 1mg/ml in PBS. The activity of the enzyme was defined as micromoles of substrate destroyed per minute per mililiter of enzyme at 37°C at pH 7.0

Statistical Analysis

Statistical analysis for activity assay was done using EndNote software. Students 't' test for two tailed probabilities assuming unequal variance was used for significant differentiation. The criterion for significant difference was 0.05.

Plasmid DNA preparation, PCR amplification and DNA sequencing

The plasmid DNA was isolated from the clinical isolates as per the guidelines (Himedia MB508). The primer pairs used for amplification of the bla_{TEM}, bla_{OXA}, bla_{SHV}, bla_{CTX-M} and bla_{PER} are shown in the table-1.[16] The primers for AmpC were designed using NCBI primer blast tool. These primers were custom made from IDT (Integrate DNA technology, India) and PCR reagents were purchased from Bioline, USA.

Table 1: PCR Amplification and Sequencing Primers Used

Target gene	Primers used	Expected amplicon size
bla_{TEM}	F-5'ATAAAATTCTTGAAGACGAAA 3' R-5'GACAGTTACCAATGCTTAATCA 3'	~1080bp
bla_{OXA}	F-5'AGC CGT TAA AAT TAA GCC C-3' R-5'CTT GAT TGA AGG GTT GGG CG-3'	~980bp
bla_{SHV}	F-5'TCGGGCCGCGTAGGCATGAT 3' R-5'AGCAGGGCGACAATCCCGCG 3'	~860bp
bla_{CTX-M}	F-5'TTAATGATGACTCAGAGCATTC 3' R-5'GATACCTCGCTCCATTTATTG 3'	~901bp
bla_{PER}	F-5' TGTGTTTTCACCGCTTCTGCTCTG3' R-5'CAGCTCAAACTGATAAGCCGCTTG 3'	~700bp
AmpC	5'- CTG TGG ATA CTC TCC CGC AC-3' 5'- CAG CAG AAT ACC CAC CAG CA-3'	~1137bp

PCR amplification of βL alleles was carried out in all twenty four MDR *E. coli* isolates.[17] A single reaction mixture contained 2ng of plasmid DNA extract for *E. coli,* 10 pmol of each primer, 2.5 µM dNTPs, 1U Taq polymerase, 10X buffer, 1mM $MgCl_2$ and reaction volume of 25 µL by double distilled water. Thermal cycler (PeqLab) was used to amplify the β-lactamase alleles under following conditions: Initial denaturation at 95°C for 5 min, followed by 30 cycles of 1min at 94°C, 1 min of annealing at 52°C (for OXA and TEM) and 57.2° (for ampC) and 1.5min at 72°C and finally 10 min at 72°C.[16] The resulting PCR products were run in 1.5% agarose gels. The purified PCR products were sequenced from Xceleris Labs, India. The chromatogram were analysed with Chromuslite, SIFT and PROVEAN PROTEIN.

Molecular docking

The crystal structures of TEM (1BTL)[18], OXA (1M6K)[19] and AmpC (1KE4)[20] were downloaded from the protein data bank (PDB). The structures were refined using OPLS force field and the energy minimized conformation was taken as starting conformation for docking studies. Automated molecular docking between all molecules was performed by using the advanced docking program AutoDock4 whereas the inhibitor-enzyme interactions were estimated by the Lamarckian genetic algorithm. A 3D grid with 60×60×60 points and a spacing of 0.375 Å was created by AutoGrid algorithm to evaluate the binding energies between proteins and ligands.

The chemical structures of the phytochemicals (betulinic caid, chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin) giving antibacterial activities against multidrug resistant *E. coli* were downloaded from PubChem. Chemsketch and OpenBabel were used for preparing ligand structures. Modeling studies had been carried out with the commercial software Autodock. The affinity and electrostatic potential grid were calculated for each type of atom in the ligand. The results were compared and validated with X-ray crystal structure of the selected protein with benzylpenicillin as a potent antibacterial compound. Chimera is used for detailed structural and interaction analysis. The physicochemical and toxicological properties of chlorogenic acid were studied

with organic chemistry program peo software. The drug likeness score was analysed by molsoft mprop tool.

RESULTS AND DISCUSSION

Bacterial identification and drug resistance profile of *E. coli*

It was found that 26% of the patients were infected with MDR *E. coli.* The other predominated bacteria isolated were *Pseudomonas aeruginosa (36%)* and *Enterococcus faecalis* (35%).[11,17] Among them, *E .coli* isolates were selected for current analysis. These *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). Also all aforementioned phytochemicals showed prominent antibacterial activity.

The βL activity in *E. coli* strains

It was found that *E. coli* isolates have significantly more extracellular βL activity (63%) in ESBL positive isolates than intracellular (P<0.05)., Fig 1 and 2.

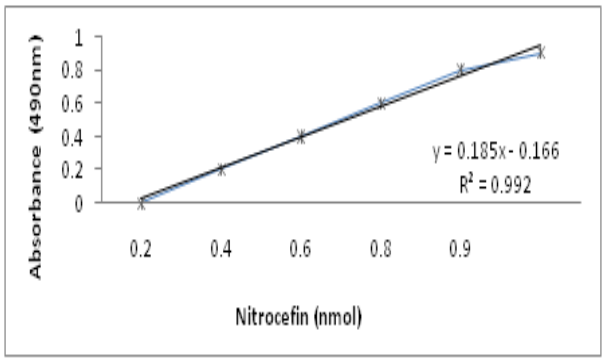

Fig1: Standard curve of Nitrocefin

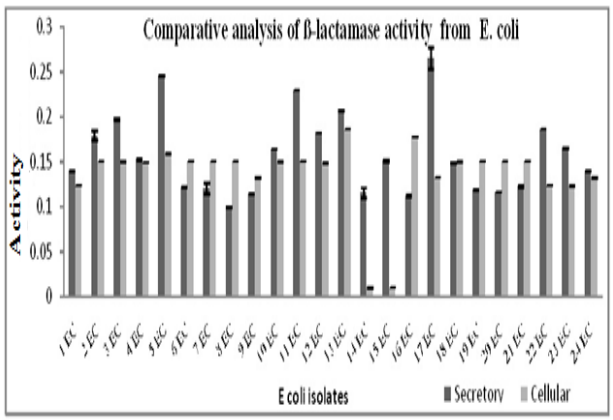

Fig 2: Comparison of secretory and cellular βL activity in *E.coli*

Detection of β-lactamase alleles in *E. coli* isolates (ESBL detection)

The ESBL expressing *E. coli* isolates were investigated for presence of β-lactamase alleles. The PCR products were electrophoresed on 1.5% agarose gel and found the amplicon size of ~980bp, ~1080bp and ~1137bp corresponded to blaOXA, blaTEM and AmpC respectively (Fig 3 and 4). The amplification of OXA and TEM were found only in ESBL positive isolates (EC1 to EC12) and not in ESBL negative isolates (EC13 to EC24).

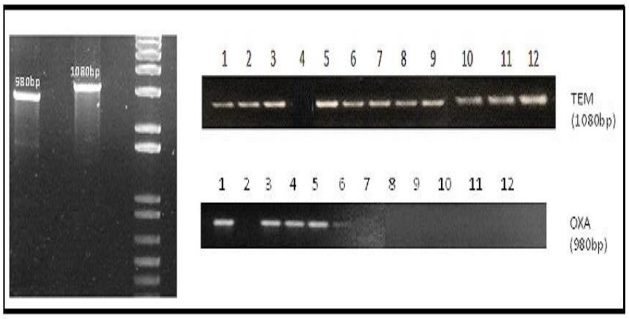

Fig 3: PCR amplification of βL gene

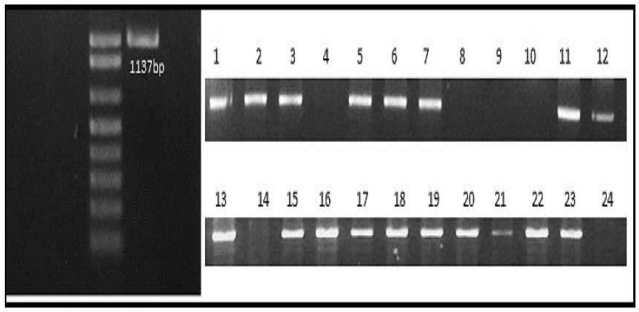

Fig 4: PCR amplification of AmpC βL gene

(Note: 1 to 12 are ESBL positive and 13 to 24 are ESBL negative *E. coli* isolates

On sequencing analysis, multiple stop codons have found in TEM due to point mutations by insertion of C(19) and nucleotide change at positions T(227)C and T(395)G. The mutation observed in OXA S(66)H and in AmpC F(57)Y are non-deleterious (Table 2 and 3).

Molecular docking

Few phytochemicals showed direct interaction with active site Serine residue of βL and their docking energy is much less comapared to standard benzylpenicillin. The phytochemicals observed to build hydrogen bond with the active site of OXA are chlorogenicacid (2.781A^0), Ellagic acid (3.225A^0), Quercetin (3.135A^0), Salicylic acid (2.697A^0), tannic acid (3.101A^0) and theophylline (3.055A^0). Catechol (2.746A^0), chlorogenic acid (2.746A^0), pyrogallol (2.849A^0), resorcinol (2.854A^0), salicylic acid (3.037A^0), tannic acid (1.850A^0), theophylline (2.862A^0) and vanilline (2.839A^0) are forming hydrogen bond with TEM active site residue. Chlorogenic acid (3.223A^0), ellagic acid (2.624A^0) and naringenin (2.966A^0) are forming the hydrogen bond with AmpC active site. The respective hydrogen bond length is shown in A^0. Based on all the results, it was found that chlorogenic acid is the most potent phytochemical able to bind the active site of all three types of βLs and showed excellent drug score (fig 5 and 6).

Table 2: Analysis of Mutations

ESBL positive isolates	ESBL β-lactamase allele	Mutation analysis	Interpretation	Accession Number
EC-1	TEM OXA	C(19) inserted; T(227)C; T(395)G No mutation	Deleterious, inserted stop codons	------- KP760834
EC-2	TEM	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	-------
EC-3	TEM OXA	C(19) inserted; T(227)C; T(395)G S(66)H	Deleterious, inserted stop codons Non deleterious	------- KP760835
EC-4	OXA	S(66)H	Non deleterious	KP760836
EC-5	TEM OXA	C(19) inserted; T(227)C; T(395)G No mutation	Deleterious, inserted stop codons --------	------- KP760837
EC-6	TEM OXA	C(19) inserted; T(227)C; T(395)G No mutation	Deleterious, inserted stop codons --------	--------- KP760838
EC-7	TEM	No mutation	--------	KP724846
EC-8	TEM	No mutation	--------	KP724847
EC-9	TEM	No mutation	--------	KP724848
EC-10	TEM	No mutation	--------	KP724849
EC-11	TEM	No mutation	--------	KP724850
EC-12	TEM	No mutation	--------	KP724851

Table 3

E. coli isolates	β-lactamase allele	Mutation analysis	Accession Number
EC-1 to EC-24	AmpC	F(57)Y	KR010370-KR010387

EC: E. coli

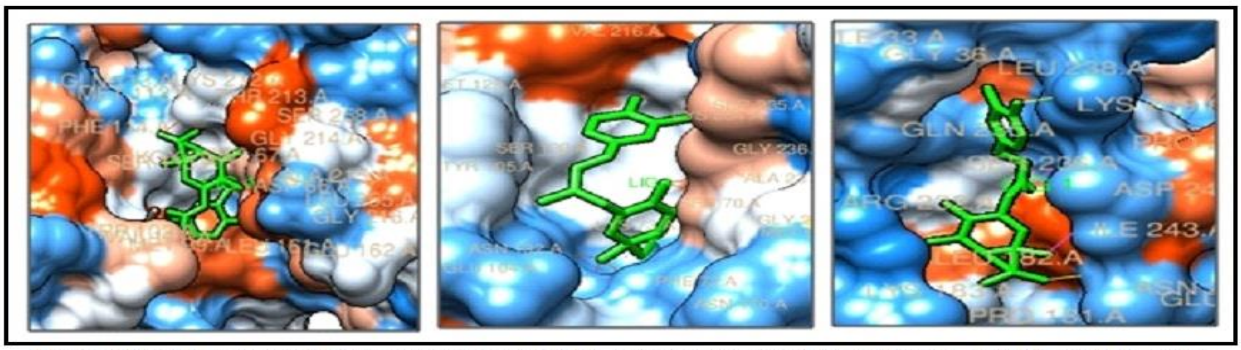

Fig5: Docking pose of chlorogenic acid to active site pocket of OXA, TEM and AmpC respectively

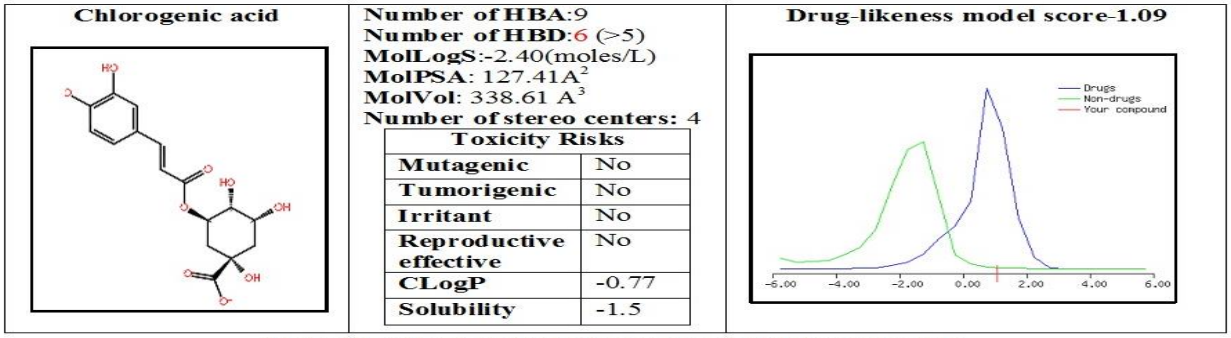

Fig6: Physicochemical properties of chlorogenic acid

HBA: hydrogen bond acceptor, HBD: hydrogen bond donor

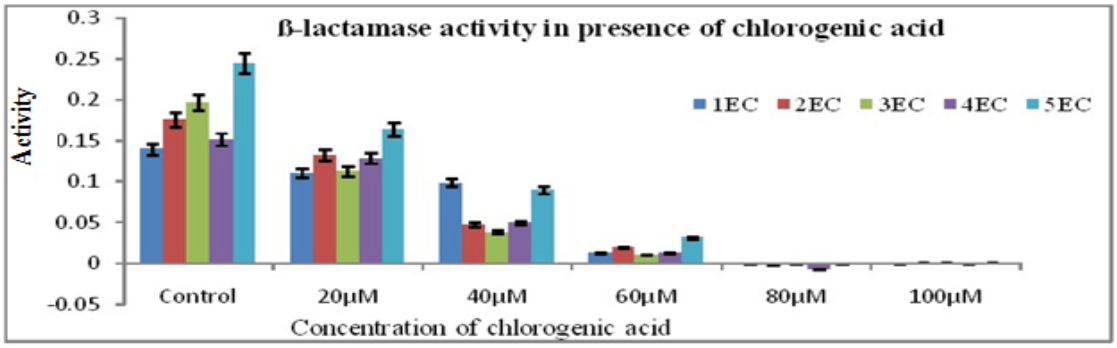

Fig7: βL inhibition by chlorogenic acid

Inhibition assay of βL in presence of chlorogenic acid

To support this docking data, the total βL activity was determined by nitrocefin assay[14] in presence of chlorogenic acid. It was found that total activity was significantly decreased at 20µM of chlorogenic acid concentration and got completely inhibited with 80µM of concentration (fig 7). This data proved the inhibition of all βL present in the sample.

CONCLUSION

UTI is a serious bacterial infection that damages kidney if untreated. The β-lactam antibiotics constitute one of the most important antibiotics families in worldwide use as antibacterial therapeutics. Hence it is important to study the antibiotic resistance pattern and possible mechanisms that bacteria uses to resist its action. In the current study, we have isolated the multidrug resistant *E. coli* and separated them into two groups based on ESBL expressing isolates. The βL activity has been investigated and it was found that ESBL positive *E. coli* isolates have significantly more secretory βL activity compared to intracellular activity, which indicates that bacteria secretes most of the βL and is the primary mode of drug resistance against β-lactam antibiotics. The presence of common ESBL alleles (TEM, OXA, CTX-M, PER and SHV) have been studied. We did not find the presence of ESBL alleles in ESBL negative *E. coli* samples but ESBL positive isolates have shown the presence of TEM and OXA. On comparison, TEM is more commonly present than OXA. But most TEM have shown frame shift mutations which have culminated into insertion of many stop codons in the reading frame, which prevent its further expression due to truncated proteins.

This study reveals the molecular allelic expressions giving rise to MDR *E. coli* strains causing UTI and also a deep insight into the molecular mechanism of emergence of MDR in UTI causative *E. coli.* This enhances the chances of developing a good drug therapy regimen accordingly for recurrent and difficult to treat MDR UTI.

The second part of this study constituted the identification of phytochemicals as possible antibacterial agents against MDR *E. coli.* We have studied the antibacterial activity of fourteen phytochemicals out of which eleven have showed the binding affinity to active site of β-lactamase by molecular docking. Chlorogenic acid, naringenin, quercetin, salicylic acid and theophylline have shown significant drug-likeness score compared to control benzyl penicillin which is a potent β-lactam antibiotic used in the treatment of bacterial infections. But the chlorogenic acid was found to be the most potent agent as it showed good binding affinity to the active site of OXA, TEM and AmpC βL and the total enzyme activity was also found to be decreased with 20µM concentration.

The future study involves the purification of each βLs from MDR *E. coli* strains and enzyme kinetics assay with the use of aforementioned phytochemicals promising significant docking and drug likeness scores comparable with benzyl penicillin, thereby paving a path for novel antibacterial drugs without side effects.

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Abbreviations: Urinary Tract Infection (UTI), β-lactamase (βL), Multi Drug Resistant (MDR), Extended-Spectrum β-lactamases (ESBLs), Chlorogenic acid (CAG).

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