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THE ANALYSIS OF INHIBITORY ACTIVITY OF PHYTO-LIGANDS AGAINST TEM B-LACTAMASE FROM MULTIDRUG RESISTANT *ESCHERICHIA COLI*

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Abstract

The drug resistance is the ability of bacteria to grow even in the presence of antibacterial agents. The resistance to β-lactam antibiotics is a serious worldwide crisis that has emerged due to two main mechanisms: β-lactamase production by bacteria that hydrolyse antibiotics and other is the production of low affinity mutated Penicillin Binding Proteins (PBPs) so that the bacteria can sustain even at high concentration of antibiotics. The current study focuses on isolation of β-lactam resistant E. coli from uropathological samples and PCR amplification of TEM type of β-lactamase. The other part of the study includes purification and identification of antibacterial phytocompounds from selected medicinal plants to analyse their inhibitory activity against purified TEM from MDR E. coli. This biological data has been corroborated with insilico interaction studies using Gromacs and Autodock4 suite. The ADME toxicity analysis was also performed to select the best ligand for examining the interactions at the binding sites after inducing the salvation properties.

Key words: TEM, β-lactamase, MDR, Phytochemical, Medicinal plants, Docking.

Introduction

The infection caused by MDR bacteria leads to limited treatment options and this is one of the most serious health related issue currently the whole world is facing. Urinary tract infection (UTI) is the second most common bacterial infection after respiratory tract infection (Hoban et al. [1]) and it is more common in women compared to men (Hooton TM [2]). Approximately 75-95% of UTIs are caused by Escherichia coli but other common pathogens include Proteus mirabilis, Klebsiella pneumoniae, Enterobacter, Staphylococcus, etc (Gupta K [3]). The widespread use of β-lactam antibiotics in the treatment of UTI has led to the appearance of drug-resistant strains worldwide. All β-lactams share the same mode of action via inhibiting the bacterial cell wall synthesizing transpeptidase domain of Penicillin

Binding Proteins (PBPs) by forming a stable covalent adduct with the active site serine residue of PBPs (Papp-Wallace [4]). To tackle this, various mechanisms have been developed by bacteria to resist the action of β-lactam antibiotics (Drawz, S.M. [5]). One of the commonest is the production and secretion of β-lactamases, especially ESBLs (Extended spectrum β-lactamases) that catalyze the hydrolysis of the β-lactam ring in various 3rd and 4th generation β-lactams.

The β-lactamase (EC 3.5.2.6) enzymes are fundamentally divided into 4-classes (A-D) based on their amino acid sequences. From the late 1990s, multidrug-resistant Enterobacteriaceae produced extended-spectrum β-lactamases (ESBLs) (Johann DD Pitout and Kevin B Laupland [6]), mutant enzymes with broader spectrum activity that hydrolyze 3rd and 4th generation Cephalosporins and Aztreonam. The prevalence of antibiotic resistance among ESBL-producing Escherichia coli has noticeably increased (Ebbing Lautenbach [7]). The current article is focussed on the expression and mutational analysis of TEM β-lactamases in multidrug resistant E. coli isolated from UTI infected patients.

As bacteria have increased their capacity to develop resistance and surpass the action of β-lactams by many mechanisms, there is a need to search for new alternative antimicrobial agents with fewer side effects. The use of plant or its part in treatment of bacterial diseases has been an ancient practice and is an important component of healthcare system in India. Considerable research has been carried out on pharmacognosy, chemistry, pharmacology and clinical therapeutics of Ayurvedic medicinal plants. The active phytoconstituents (chlorogenic acid, catechol, ellagic acid, gallic acid, naringenin, pyrogallol, quercetin, resorcinol, salicylic acid, squalene, theophylline, tannic acid and vanillin) from the selected medicinal plants extracts were identified and purified to check their antibacterial activity against MDR *E. coli.* The persuasive antibacterial phytochemicals were then checked for their β-lactamase inhibitory potency in TEM among MDR E. coli clinical isolates. To corroborate this biological data, molecular docking and dynamic simulation were carried out with Gromacs and Autodock4 suite.

Experimental

Material and Methods:

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), as described in table-1.

Table 1: Biochemical tests for identification of E. coli.

S.N.	Test	Principle	Interpretation
1.	ONPG	β-galactosidase activity	-ve(EC); +ve(KP); +ve(PA)
2.	Lysine utilisation	lysine decarboxylation	+ve(EC); +ve(KP); -ve(PA)
3.	Ornithine utilisation	Ornithine decarboxylation	+ve(EC); -ve(KP); -ve(PA)
4.	Urease	Urease activity	-ve(EC); +ve(KP); +ve(A)
5.	Phenylalanine deamination	Phenylalanine deamination activity	-ve(EC); -ve(KP); -ve(PA)
6.	Nitrate reduction	Nitrate reduction activity	+ve(EC); +ve(KP);+ve(PA)
7.	H2S production	H2S production activity	-ve(EC); -ve(KP); -ve(PA)
8.	Citrate utilisation	Capability of organism to utilise citrate as a sole carbon source	-ve(EC); +ve(KP)
9.	Voges Proskauer's	Acetone production	-ve(EC); +ve(KP)
10.	Methyl red	Acid production	+ve(EC); +ve(KP);
11.	Indole	Deamination of tryptophan	+ve(EC); -ve(KP)
12.	Malonate utilisation	Capability of organism to utilise sodium malonate as a sole carbon source	-ve(EC); +ve(KP)
13.	Esculin hydrolysis	Esculin hydrolysis	+ve(EC); +ve(KP);
14.	Arabinose	Arabinose utilisation	+ve(EC); +ve(KP); -ve(PA)
15.	Xylose	Xylose utilisation	+ve(EC); +ve(KP);
16.	Adonitol	Adonitol utilisation	-ve(EC); +ve(KP)
17.	Rhamnose	Rhamnose utilisation	+ve(EC); +ve(KP);
18.	Cellobiose	Cellobiose utilisation	-ve(EC); +ve(KP);
19.	Melibiose	Melibiose utilisation	+ve(EC); +ve(KP);
20.	Saccharose	Saccharose utilisation	+ve(EC); +ve(KP);
21.	Raffinose	Raffinose utilisation	+ve(EC); +ve(KP);
22.	Trehalose	Trehalose utilisation	+ve(EC); +ve(KP);
23.	Glucose	Glucose utilisation	+ve(EC); +ve(KP); +ve(PA)
24.	Lactose	Lactose utilisation	+ve(EC); +ve(KP); -ve(PA)
25.	Sorbitol	Sorbitol utilisation	+ve(EC); +ve(KP);+ve(PA)

EC*: E. coli;* KP*: Klebsiella pneumonia;* PA*: Pseudomonas aeruginosa*

The antibiotic sensitivity for these isolates was investigated for following β-lactam antibiotics using 4X-concentration of reported MIC (minimum inhibitory concentration) by Kirby-Bauer method (Sahare and Moon 8). 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. coli strains were selected for further molecular analysis.

ESBL detection

The Extended spectrum β-lactamase (ESBL) detection was done (Himedia FD278) with 3rd generation cephalosporin antibiotics (ceftazidime, cefotaxime and ceftriazone) along with aztreonam (a synthetic monocyclin β-lactam antibiotic).

Amplification and Sequencing of TEM

Plasmid DNA was isolated as per the guidelines (Himedia MB508) from MDR E. coli ESBL positive bacteria and used for the amplification of blaTEM. The primer set used were blaTEM Forward (5'-ATAAAATTCTTGAAGACGAAA-3') and Reverse (5'-GACAGTTACCAATGCTTAATCA-3'). Thermal cycler (PeqLab) was used to amplify TEM β-lactamase under following conditions: Initial denaturation at 95ºC for 5 min, followed by 30 cycles of 1min at 94ºC, 1 min of annealing at 52ºC (for OXA) and 57.20(for AmpC) and 1.5min at 72ºC and finally 10 min at 72ºC (Parr et al. 9). 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

Amplified PCR products were purified using Qiagen purification kit (Germany) and bidirectional sequencing was performed by Xceleris Labs, India. The mutations were analysed with the public access software (www.ncbi.nlm.nih.gov).

Purification of TEM

The PCR amplicon was purified by QIAquick purification kit (Qiagen) and cloned into pCR 2.1-TOPO vector. These were digested with XhoI and BamHI 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 370C till OD600 reached to 0.6. 1mM IPTG was used as an inducer.

The bacterial cells were pelleted down at 5000rpm/10min/40C. These cells were suspended in 20mMTris (pH 8.0) and 300mMNaCl and lysed 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. The enzymes were further purified by Superdex 200 size-exclusion column (GE LifeSciences). Peak fractions were collected and exchanged to PBS using Amicon Ultra filter units (Millipore) (Ming-Ta Sung 10).

β-lactamase (βL) activity assay

The TEM activity was assayed by Nitocefin method (Ximin Zeng and Jun Lin 11, O'Callaghan 12) in E. coli strains expressing TEM. The activity of the enzyme was defined as micromoles of substrate destroyed per minute per mililiter of enzyme at 370C at pH 7.0.

Nitrocefin was purchased from BioVision (Cat No 2388-5). Stock solution of nitrocefin 5mg/ml was prepared in DMSO and the working solution 1mg/ml in PBS. 50µM of penicillin and cephalosporin (cefixime) were used as control antibiotics.

Phytochemical extraction, purification and identification

Following ten medicinal 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 herbariums were deposited in the department of botany with the following voucher numbers.

The antibacterial activity was checked in the crude extracts of Andrographis paniculata Burm. f. Wall. Ex. Nees (Ap, 9038), Astercantha longifolia (L.) Nees (Al, 9039), Bixa orellana L. (Bo, 9041), Gardenia resinifera Roth, (Gr, 10012), Pongamia pinnata (L.) Pierre (Pp, 10037), Psoralea corylifolia L. (Pc, 10038), Sphaeranthus indicus Linnaeus (Si, 10039), Solanum trilobatum L. (St, 10041), Soyamida febrifuga (Roxb.) Juss (Sf, 10042) and Thespesia populnea (L.) Sol. Ex. Correa (Tp, 10043).

These plants have shown significant antibacterial activity against isolated MDR E. coli and so the phytochemicals were extracted from these medicinal plants by Soxhletion (hot extraction) and cold maceration. 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. coli isolates compared to Soxhlet extracts. Hence the cold extracts of all plants were used for HPLC analysis 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 mobile phase (Sahare and Moon).

Effect of phytochemicals on TEM activity

The purified phytochemicals by HPLC were again investigated for confirmation of potent antibacterial compound and were also used to check their inhibitory activity against TEM β-lactamase. Clavulanic acid (50µM) was used as standard inhibitor in this assay.

***In-silico* studies**

In-silico screening of ligands for their interaction with receptor is a useful technique in drug discovery (Brooijmans and Kuntz [14], Shoichet *et al.*[15]). The docking involves the use of sampling algorithm and a scoring function to evaluate the proper orientation of ligand molecule. 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 (Kitchen *et al.* [16], Hartshorn [17], Evans and Neidle [18]). 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 Autodock program (v1.5.6) which defines the binding site in terms of grid of interaction points.

Ligand preparation

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

TEM and its preparation

The crystal structure of TEM, PDB ID: 1-BTL was downloaded from the Research Collaboratory for Structural Biology (RCSB) database (www.rcsb.org). The structural information was downloaded as PBP text and converted to pdb format by OpenBabel GUI software. This protein was energy minimised and optimised by SPDBV. Gromacs was also used to analyse the energy minimization and Procheck was used to study the Ramachandran plot of minimised structure.

Virtual screening by AutoDock

The active site residue of TEM, Ser (70) was defined as the centre of grid and the grid was made up of following grid points 6.873(X), 3.891(Y) and 36.268(Z) with grid box $60\times60\times60A^0$ having grid spacing of $0.375A^0$, 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 reference to access the accuracy of poses with respect to their binding energy. The H-bond interactions were analysed in Chimera.

Molecular dyanamics Simulation using Gromacs

The docked complexes were used as starting structures for MD simulations. The calculations were performed by using GROMACS 4.5.3 GROMOS OPLA force field (Tiwari *et al.* [19]). In this simulation, TEM was solvated using the SPC water model in a box with a 1 nm solute-wall distance. The selected ligands showing the lowest binding energy was chosen for MD simulation.

Energy minimization was performed using the steepest descent algorithm. The initial atomic velocities were generated according to a Maxwell–Boltzmann distribution at 100 K. Five 20-ps MD runs with decreasing positional restraints force constants on the solutes (The restraints force constants are equal in the x, y, z dimensions and the values decrease from 1000 to 100, 10, 0 kJ mol−1 nm−2, respectively, in every 20 ps MD) were performed to modulate the position of water, followed by two -10 ps MD runs to warm the solvents and the solute to 300K, separately. Then a 50-ns MD run was performed.

The solute, solvent, and counter ions were independently coupled to a reference temperature bath at 300K with a coupling constant τT of 0.1 ps. The pressure was maintained by weakly coupling the system to an external pressure bath at one atmosphere with a coupling constant τp of 0.5 ps.

The TEM β-lactamase complex with standard antibiotic penicillin along with tested phytochemicals that showed highest drugability score viz., Catechol, resorcinol and salicylic acid were analysed. The box dimensions ensured that any protein atom was at least $1.5A^0$ away from the wall of box with periodic boundary conditions and salvated by simple point charge (spc) water molecules.

Cl^- counterions were added to satisfy the electroneutrality condition. Energy minimisation was carried out using steepest descent method.

Results and Discussion

The β-lactam resistant *E. coli* isolates

It was found that 26% of the UTI patients were infected with multi drug resistant *E. coli.* The other predominant 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). But only

19.78% and 49.1% of E. coli have found resistant to penicillin (10 µg) and Ceftazidime (3rd generation cephalosporin, 10 µg).

ESBL expressing *E. coli*

Out of the total E. coli isolates, 50% of the bacteria were found expressing ESBL. These ESBL positive bacteria were used for TEM amplification.

Mutational analysis of β-lactamases

On sequence analysis, multiple stop codons were found in TEM due to point mutations by insertion of C(19) and nucleotide change at positions T(227)C and T(395)G. These frameshift mutations prevent the formation of an active functional enzyme. These mutations were observed in *E. coli* isolates 1-6 but 7-12 *E. coli* isolates no mutations were detected. This TEM without mutation was cloned, expressed and purified for activity assay. (Fig. 1)

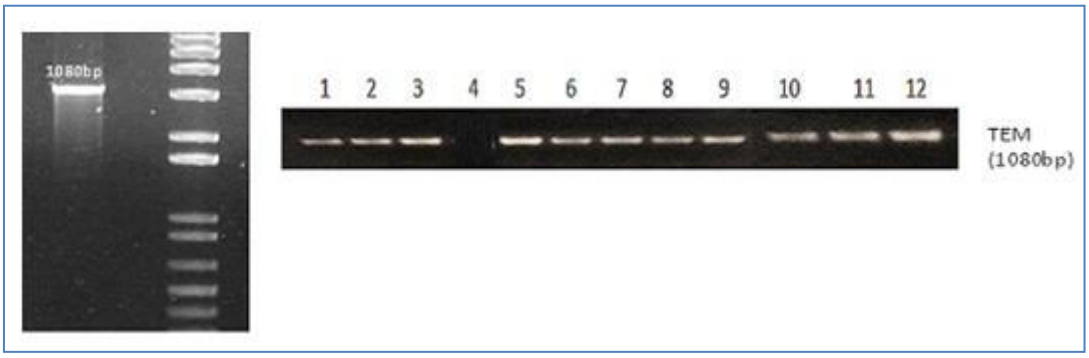

Fig 1: PCR amplicon of TEM observed in MDR *E. coli* isolates

All sequences were deposited to NCBI online portal and following accession numbers have been allotted, as described in table-2.

Table-2: Mutation analysis of TEM from *E. coli* isolates.

ESBL positive isolates	Mutation analysis	Interpretation	Accession Number
EC-1	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	--------
EC-2	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	--------
EC-3	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	--------
EC-5	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	--------
EC-6	C(19) inserted; T(227)C; T(395)G	Deleterious, inserted stop codons	--------
EC-7	No mutation	--------	KP724846
EC-8	No mutation	--------	KP724847

EC-9	No mutation	--------	KP724848
EC-10	No mutation	--------	KP724849
EC-11	No mutation	--------	KP724850
EC-12	No mutation	--------	KP724851

EC: *E. coli*

Purified phytochemicals by HPLC

The following phytochemicals were identified and purified by HPLC as described in Fig-2 and table-3.

Table-3: Identified phytochemicals by HPLC.

Sr. 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

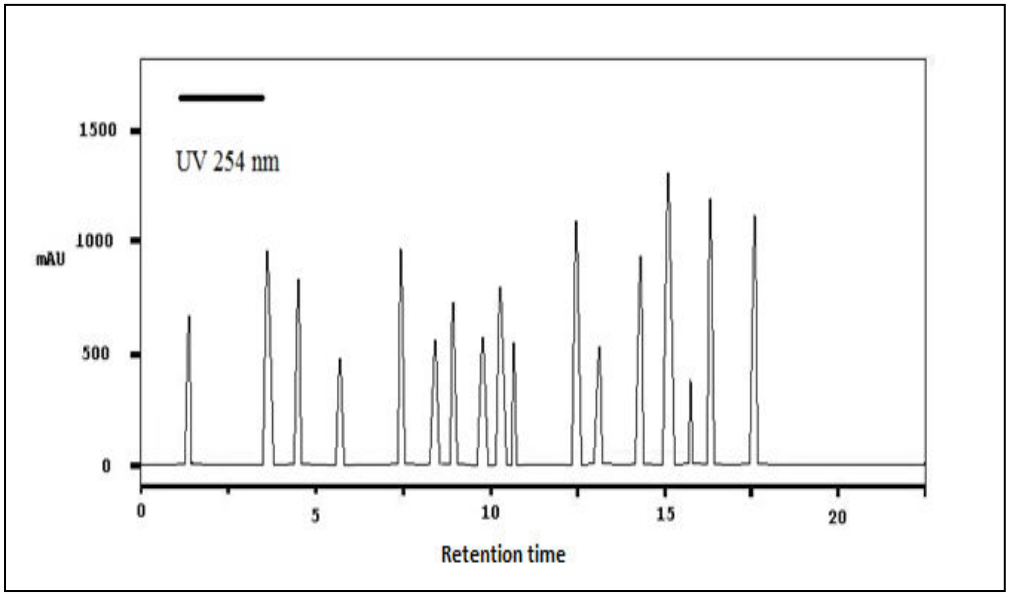

Fig-2: HPLC chromatogram of phytochemicals.

Antibacterial activity of phytochemicals

The antibacterial potency of these purified phytochemicals has been analysed, as described in table-4.

Table-4: Zone of inhibition (mm) for respective phytochemicals against MDR *E. coli.*

S.No.	Phytochemical (1mg)	Antibacterial activity
		Zone of inhibition (mm)
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

Effect of phytochemicals on TEM β-lactamases activity

Penicillin was used as a control antibiotic for the activity assay. The uropathological *E. coli* identified as penicillin resistant has gained consequence due to TEM production and secretion. Strain 8 (random selected with no mutation identified in TEM) of the isolated *E. coli* was used for this activity assay. Clavulanic acid was used as a standard inhibitor.

The phytochemicals with potent antibacterial activity were further used to check for their inhibitory efficiency towards TEM. It was found that salicylic acid, gallic acid, pyrogallol, and vanillin were significantly inhibiting the activity. The other phytochemicals i.e. chlorogenic acid and theophylline were also found to have less significant impact on the enzyme activity. Whereas betullinic acid, ellagic acid, naringenin, palmitic acid and squalene showed no effect on TEM activity of isolated E. coli.

The comparative data is shown in the following fig-3.

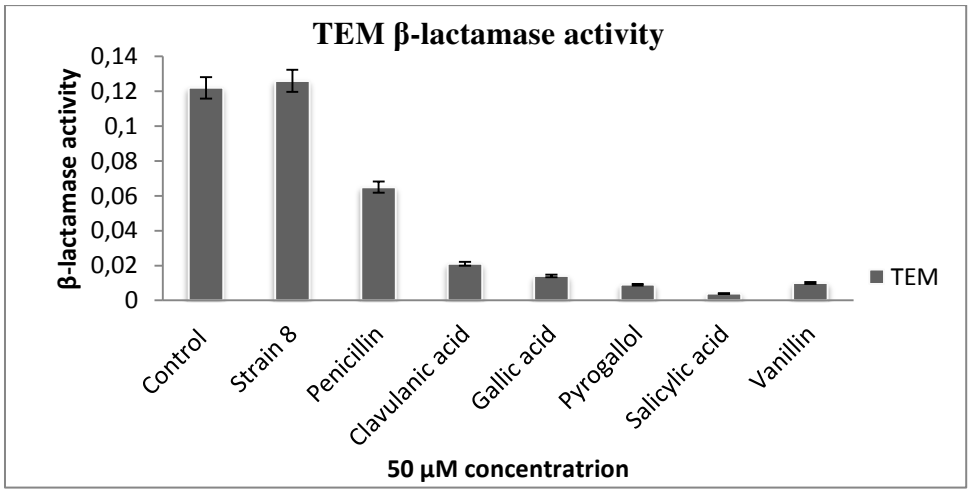

Fig 3: TEM β-lactamase activity in presence of different compounds.

Molecular Docking

Molecular docking was carried out to verify the binding of various phytochemicals to active site of TEM and to ascertain the molecules that can form H-bond with active site Ser(70). Benzylpenicillin was used as a standard antibiotic to compare docking interactions. The docking analysis data was shown in table-5.

Table-5: Molecular docking analysis of phytochemicals with active site residues of TEM.

Ligand	H-bond with active site residue	H-bond length (A^0)	Binding energy (Kcal/mol)
Benzylpenicillin	SER70.A OG--LIG 1 O LIG 1 O-- SER 130.A O ALA 237.A N--LIG 1 O	3.167 2.175 2.450	+7.26
Catechol	SER70.A N--LIG 1 O LIG 1 O--GLU166.A OE2 ASN170.A ND2--LIG 1 O ALA237.A N--LIG 1 O	3.127 3.002 2.784 3.077	- 4.36
Chlorogenic acid	SER70.A OG--LIG 1 O LIG 1 O--ASN132. A OD 1 LIG 1 O--GLU166.A OE 1 LIG 1 O--ASN170.A OD1 LIG 1 O--ALA237. A O SER235.A OG--LIG 1 O ARG244.A NH1--LIG 1 O	2.400 2.949 2.221 3.117 2.675 2.013 3.024	+10.07
Ellagic acid	SER130.A OG--LIG 1 O SER235.A OG--LIG 1 O	2.397 1.993	+16.24
Gallic acid	SER130.A OG--LIG 1 O ASN132.A ND2--LIG 1 O LIG 1 O--ALA237.A OD1	2.724 2.904 2.703	- 4.71
Naringenin	SER235.A OG --- LIG 1 O	2.103	+1.27
Pyrogallol	SER70.A N--LIG 1 O ALA237.A N--LIG 1 O LIG 1 O--GLU166.A OE2 ASN170.A ND2--LIG 1 O	2.349 2.349 3.293 2.539	- 4.74
Quercetin	LIG 1 O--GLU104.A O SER235.A OG--LIG 1 O ARG244.A NH1--LIG 1 O	3.237 2.532 2.720	+14.20
Resorcinol	SER70.A N--LIG 1 O LIG 1 O--ASN132.A OD1 LIG 1 O--GLU166.A OE1 ALA237.A--LIG 1 O	2.854 2.850 2.978 2.636	- 3.99
Salicylic acid	SER70.A N--LIG 1 O SER130.A OG--LIG 1 O ALA237.N--LIG.O	3.037 2.603 2.767	- 4.87
Theophylline	SER70.A N--LIG 1 O ASN132.A ND2--LIG 1 O LIG 1 O--ALA237.A O	2.862 2.822 2.776	- 2.19
Vanillin	SER70.A N--LIG 1 O ALA237.A N-LIG 1 O	2.839 3.058	- 4.43

Amongst all docked phytochemicals; catechol, chlorogenic acid, pyrogallol, resorcinol, salicylic acid, theophylline and vanillin were found to form H-bond with active Ser70. But, the minimum binding energy was observed for

salicylic acid (-4.87) which was lowest as compared to benzylpenicillin and other phytochemicals tested. The binding of salicylic acid and benzylpenicillin to the active site pocket of TEM is shown in Fig.4.

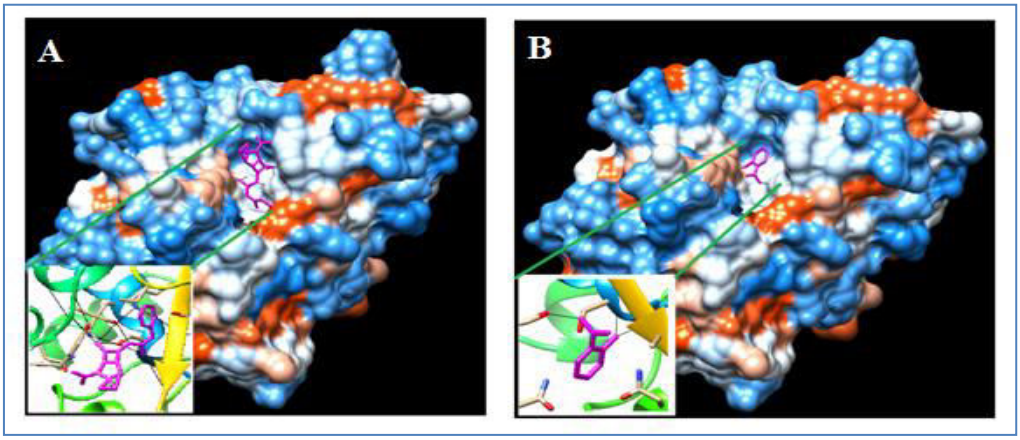

Fig 4: Binding of (A) Benzylpenicillin and (B) Salicylic acid to the active site pocket of TEM.

Molecular dynamics and simulation

In order to relax the bound TEM structure, a 50ns MD simulation was performed. The fluctuation in potential energy and the RMSD of Cα atoms from the x-ray structure were monitored during the MD simulation time. The potential energy of the whole system was stable. The RMSD values of protein backbone were calculated after fitting the ligand the initial crystal structure. The RMSD value converged after a 50ns simulation

It was noticed that most of the temperature fluctuates around the value specified 310K this indicates that the protein complexes where very stable and successfully achieved temperature equilibrium. Moreover lower total energies and potential energies confirmed the stability of structures. The data shown in table-6.

Table-6: MD simulation energy.

Energy	TEM Complex with	Average	Error Est.	RMSD	Total _drift
Potential Energy (Kcal/mol)	Penicillin	-475014	84	782.288	-492.677(kJ/mol)
	Salicylic acid	-443288	160	873.018	-1087.64 (kJ/mol)
	Pyrogallol	-442965	130	864.259	-915.855 (kJ/mol)
	Gallic acid	-441437	150	859.19	-926.047 (kJ/mol)
Total Energy (Kcal/mol)	Penicillin	-389697	86	989.093	-510.102 (kJ/mol)
	Salicylic acid	-355292	150	1068.58	-1046.15 (kJ/mol)
	Pyrogallol	-355016	130	1068.82	-923.973 (kJ/mol)
	Gallic acid	-353409	150	1074.1	-906.072 (kJ/mol)

Radius of gyration was analysed for monitoring the shape of the protein at every step while achieving equilibrium and it was concurred that the structures were stable and no major conformation occurred. The RMSDF for the derived structure also indicated stability. In conclusion the protein ligands complexes were stabilized.

Conclusions

UTI is a common, but sometimes a deadly bacterial infection. It spreads due to MDR strains making it more treacherous due to limited treatment options. Bacteria have emerged as MDR due to various mechanisms, common for β-lactam resistance is the production and secretion of ESBL. In the current study, TEM β-lactamase has been sequenced from the isolated *E. coli* of uropathological samples and some of them have been identified with no new mutations.

As medicinal plants reveal important pharmacological activities that can be used in the development of novel cost effective therapeutic antibacterial agent. The leaf extracts from the selected medicinal plants were prepared by hot and cold extraction, where later has been identified with more significant antibacterial activity. Hence this extract has been used for identification and purification of potent antibacterial and TEM inhibitory phytochemicals. On performing the activity assay, it was identified that salicylic acid, catechol, resorcinol, theophylline and vanillin with significant TEM inhibiting activity. Moreover, chlorogenic acid and pyrogallol have shown less significant impact on enzyme activity. Whereas betullinic acid, ellagic acid, gallic acid, naringenin, palmitic acid and squalene showed no effect on TEM activity of isolated *E. coli*. The molecular docking and energy minimisation was performed with Autodock4 suite and GROMACS to corroborate the biological data. It was identified that like benzylpenicillin, the identified phytochemicals catechol, chlorogenic acid, pyrogallol, resorcinol, salicylic acid, theophylline and vanillin show direct H-bond formation with active Ser70. Among these phytochemicals, except chlorogenic acid (+10.07 Kcal/mol), all others showed less docking energy as compared to benzylpenicillin (+7.26 Kcal/mol). Also, salicylic acid showed least binding energy (- 4.87 Kcal/mol) and in biological assay, it was identified with significant TEM inhibitory activity. The biological and docking research described herein signifies a promising approach and therapeutic application of phytochemicals from selected medicinal plants with prominent inhibitory activity relevant to target TEM β-lactamase. We can envision this to open up new avenues for screening and designing suitable phytoligand-based antibacterial therapeutic agents to answer blazing bacterial drug resistant issues.

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