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Original Article

Isolation of ESBL-producing gram-negative bacteria and *in silico* inhibition of ESBLs by flavonoids

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الملخص

أهداف البحث: يهدف البحث لتقييم أسباب عدوى المستشفيات ل ٤٢٦ سلسلة ممتدة من بيتا-لاكتام تنتج سلالات من ٧٠٥ عزلات لتسعة أنواع من البكتيريا سالبة الغرام المسببة للأمراض في المختبر. كما تهدف هذه الدراسة لتحليل التباين الوراثي لأنواع السلسلة الممتدة من بيتا-لاكتام عن طريق بناء شجرة النشأة والتطور ولتحديد خيارات رقابة فعالة ضد أنزيمات سلسلة ممتدة من بيتا-لاكتام باستخدام فلافونويد مع الالتحام الجزيئي.

طرق البحث: تم عزل تسعة سلاسل ممتدة من بيتا-لاكتام تنتج بكتيريا من عينات البول تحديد مضاد البيوجرام بواسطة طريقة نشر القرص. وكذلك إنشاء نماذج مقارنة لتسعة إنزيمات من السلسلة الممتدة من بيتا-لاكتام باستخدام تسلسل الإشارة حسابيا والمصادق عليها من قبل موقع راماشاندران. تمت محاولات الالتحام الجزيئي مع فلافونويد ١١ ضد شكل إنزيمات السلسلة الممتدة من بيتا-لاكتام.

النتائج: كانت السلالات المعزولة لها مقاومة مبهرة متعددة للأدوية. ومن دراسة الالتحام، فإن قيمة الحد الأدنى من الطاقة المولدة لاميكاسين هي -٨.١٠٨ كيلو كالوري/مول، ضد إنزيم السلسلة الممتدة من بيتا-لاكتام لسلالة شاذة، بينما سجلت قيمة الالتحام ضد النوع المتحول من الإشريكية القولونية هي - ٧.٣٨٨ كيلو كالوري/مول. وتؤيد نتائج الالتحام التي تم الحصول عليها نتائج المختبر التي أظهرت أن المضادات الحيوية غير قادرة على السيطرة على أنواع السلسلة الممتدة من بيتا-لاكتام من السلالة المتحولة.

الاستنتاجات: أظهرت نتائج الالتحام ونتائج الأدوية المشابهة أن الفلافونويد المستخدم هو أهم بديل غير ميكروبي من العوامل المضادة للجراثيم التي يمكن أن تستخدم كعوامل مكملة، لسلالات السلسلة الممتدة من بيتا-لاكتام كاحتمالية جديدة. أوضح بناء شجرة النشأة والتطور العلاقة الوراثية للأنماط المصلية لتسعة أنواع من السلسلة الممتدة من بيتا-لاكتام.

الكلمات المفتاحية: البكتيريا سالبة الغرام؛ إنزيمات السلسلة الممتدة من بيتا-لاكتام؛ النماذج المقارنة؛ فلافونويد؛ الالتحام الجزيئي

Abstract

Objective: To evaluate nosocomial accounts of 426 extended spectrum β-lactamase (ESBL)-producing strains from 705 isolates of 9 pathogenic gram-negative bacteria *in vitro*. We analysed the genetic divergence of ESBLs by constructing a phylogenetic tree and modelled flavonoid inhibition of ESBLs with *in silico* molecular docking to determine effective control options.

Methods: Nine ESBL-producing bacteria were isolated from urine samples and their antibiograms were determined by the disc-diffusion method. Comparative models of the 9 ESBL enzymes were generated computationally using reference sequences, and validated by Ramachandran plots. Molecular docking with 11 flavonoids was conducted against the ESBL models.

Results: Isolated strains were floridly multidrug-resistant. From the docking study, the predicted minimum energy value of amikacin was −8.108 kcal/mol against the wild type TEM-1 ESBL of *Acinetobacter baumannii*, while the docking value against the mutant type *Escherichia coli* was −7.388 kcal/mol. The docking scores obtained corroborated the *in vitro* results showing that the antibiotic was incapable of controlling the ESBL of the mutant strain. Among 11 flavonoids tested against the mutant ESBL of *E. coli*, epigallocatechin 3-gallate and eriodictyol, with docking scores of −9.448 and −8.161 kcal/mol, respectively, were the most effective, with drug-likeness scores of 0.39 and 1.37, respectively, compared to 1.03 for amikacin.

Conclusion: Docking scores and drug-likeness scores indicated that flavonoids are compelling alternative antimicrobial agents that could serve as complementary therapy for newly arising ESBL-producing bacteria.

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Phylogenetic tree analysis elucidated the genetic relationship of the 9 ESBL serotypes.

Keywords: Comparative modelling; ESBL enzymes; Flavonoids; Gram-negative bacteria; Molecular docking

Introduction

Sir Alexander Fleming discovered the first antibiotic, penicillin with a β-lactam ring, in 1927. With reports of bacterial resistance to penicillin beginning around the 1940s, several chemical derivatives of penicillin have since been developed. These derivatives are extended spectrum β-lactams that include penems, cephalosporins (cephems), monobactams, and carbapenems. In the last 60 years, the β-lactam class of antibiotics constitutes approximately 60% of all antibiotics used in human and veterinary medicine to combat gram-negative bacteria.[1–3] The mechanism by which β-lactams can kill bacteria is by thwarting cell-wall synthesis. However, bacteria can survive by hydrolysing the β-lactam ring with the enzyme β-lactamase, which continually evolves, and eventually even the next generation cephalosporins, carbapenems and monobactams can be inactivated. Consequently, a spectrum of enzymes known as extended spectrum β-lactamases (ESBLs) were identified around the 1980s in gram-negative (GN) rods.[4,5] ESBLs are harboured on plasmids that are easily transmitted/transferred to other bacteria. Over time, bacterial resistance to the β-lactam group has led to the development of different antibiotics such as aminoglycosides and fluoroquinolones, which have inhibitory actions on bacterial proteins or DNA synthesis. Resistance also evolves simultaneously and independently to these classes of antibiotics in most bacteria, so that most bacteria are multidrug-resistant (MDR).[3] In spite of this, antibiotics of these classes are often used.

As increased hospitalization costs, morbidity, and proportionate mortality have been recorded,[8,9] several guidelines have been published for the control of nosocomial infections due to antibiotic-resistant bacteria.[6,7] The example of insurmountable challenges from methicillin-resistant *Staphylococcus aureus* (MRSA), as recorded from this hospital,[9] is universally reported.[10,11] The situation has gone from bad to worse with only a few pathogens, including the isolation of an antibiotic-resistant strain of the GN bacterium, *Klebsiella pneumoniae*, which has caused the fatality of a neonate in this hospital.[12]

Indeed, effective control of antibiotic-resistant bacteria, particularly the ESBL-producing cohort of GN bacteria, needs to be studied in detail because evolution in bacterial species are known to occur in a Darwinian way.[9] The nosocomial infection of patients in hospitals is due to continual amplification of reservoirs of antibiotic-resistant bacteria in healthcare settings. The use of a drug/antibiotic induces resistance to that particular drug in the target bacteria or other member(s) of the same class, which might harbour inducible resistance genes. When a patient is treated with an antibiotic, it eliminates the majority of bacteria, but a minor fraction, or even one cell, with an altered genetic makeup survives, arising from mutation or acquired from well-known genetic recombination methods. Survival and predominance of the fittest bacterium eventually spreads in the hospital as antibiotic-resistant strains. The transmission of plasmid-encoded bacterial genes allows the resistance genes to migrate to phylogenetically distant bacteria.[13]

From this perspective, development of antimicrobials derived from natural sources has been suggested in literature.[14,15] The use of crude extracts of plants, with a history of ethnomedicinal use, would be a suitable approach. Any drug-resistant microbe should not be able to survive the combined use of the plant extract with traditional antibiotics, especially in critical patients where multiple antibiotics are needed.

The present work describes nosocomial accounts of 9 ESBL-producing GN bacteria, isolated from urine samples over the course of one year in a hospital, regardless of whether the patients were suffering from urinary tract infection. Their antibiograms to commonly used antibiotics were determined. A phylogenetic tree was constructed using published reference sequences of these bacteria. Protein 3-D structures of the 9 ESBLs, including a sensitive temoneira-1 (TEM-1) ESBL variant, were generated by homology modelling and validated by Ramachandran plots. Additionally, 11 flavonoids were used in molecular docking against the modelled *Acinetobacter baumannii* ESBL protein and the TEM-1 mutant of *E. coli* obtained from Protein Data Bank (PDB).

Materials and Methods

Isolation and identification of pathogenic bacteria

A total of 1250 urine samples were collected from patients admitted to inpatient (wards, cabins and ICUs) and attending outpatient department (OPD) units of the hospital. The samples yielded 705 strains of pathogenic GN bacteria belonging to 9 bacterial species, during the span of 12 months (January to December 2013). All isolated bacterial strains were assigned to *A. baumannii*, *Citrobacter* sp., *E. coli*, *Enterobacter aerogenes, Klebsiella oxytoca, K. pneumoniae, Proteus mirabilis*, *Proteus vulgaris,* and *Pseudomonas aeruginosa* using standard biochemical tests (oxidase test, indole test, methyl red (MR) test, Voges–Proskauer test, citrate test, urease test, triple-sugar-iron test and nitrate test) and were maintained as axenic cultures in suitable media, as previously described.[9,16]

Antibiotic susceptibility test

All isolated bacterial strains, including the standard Microbial Type Culture Collection strains of each bacterium, were subjected to antibiotic sensitivity tests by the Kirby–Bauer's method, using 4 mm thick Mueller–Hinton (MH) agar (HiMedia, Mumbai) medium, with 8 high-potency antibiotic discs (HiMedia) of 15 prescribed antibiotics

within 5 different groups, following the standard antibiotic susceptibility-test chart of Clinical Laboratory Standard Institute (CLSI) guidelines.[16,17]

Computational study

ESBL-producing reference sequences of the 9 GN species were retrieved from UniprotKB sequence database. Comparative modelling was performed with MODELLER 9.10. The modelled protein was validated by PROCHECK, Ramachandran plot analysis, WHAT IF, MolProbity and ProSA-web. The energy minimization of the resulting protein model was performed by Swiss-PDB Viewer software (http://spdbv.vital-it.ch/). Protein folding illustrations and protein-ligand(s) interactions were carried out using Discovery Studio Visualizer 4.0 (http://accelrys.com/) and PyMOL (http://www.pymol.org/). The molecular protein-ligand(s) docking was performed by AutoDock Vina software.[18,19]

Comparative modelling and validation

A. baumannii TEM-1 and the 8 other ESBL protein sequences were subjected to BLASTp searches. Based on the high level of identity between the target sequence and the template structure, it was found that the "TEM-1 β-lactamase X-ray diffraction 3-D structure of *E. coli*" (PDB with ID: 1ZG4) at the 1.5 Å resolution, was the best template for *A. baumannii* TEM-1. After selecting the most appropriate template for each bacterial sequence, the MODELLER and Swiss-model software were used for homology modelling. Next, Swiss-PDB Viewer was implemented for energy minimization. The refined model was validated with both PROCHECK and Rampage to confirm that all bond lengths, dihedral angles and torsion angles attained a stable configuration.

Phylogenetic tree analysis

The analysis of phylogenetic relationships is a computational process for comparison of different variants of same or different families of genes, through a phylogenetic tree. A phylogenetic tree helps to find new or recently evolved variants, if any, by multiple sequence alignments of individual genomic or protein sequences. Phylogenetic trees are constructed by computational programs using different algorithms. Here, the phylogenetic relationships of the 9 reference ESBL protein sequences were calculated by ClustalW2 and Mega 6.06 software.

Molecular docking study

Molecular docking is a computational attempt to estimate the minimum energy generated between desired target protein and each ligand, individually. The druggable target protein is the larger molecule related to a particular phenotype and the ligand is the smaller natural or synthetic chemical that is the candidate drug, by which the activity of the target molecule is blocked after binding to the active site. The effective ligand against a targeted protein is selected by the minimum docking score between protein-ligand interactions. Here, 11 flavonoids were used as ligands against two target proteins: one was generated by homology modelling and other was retrieved from PDB. The first target protein, wild type TEM-1 of *A. baumannii* and the second target protein, a mutant TEM-12 from *E. coli* (PDB ID: 1ESU), were subjected to docking analysis. Moreover, the controlling capacities of 11 individual flavonoids were predicted against wild type and mutant TEM variant proteins, with the antibiotic amikacin used as the reference.

Results

Isolation and identification of pathogenic bacteria

We isolated 705 strains of 9 species of GN bacteria, with numbers as specified: 75 strains of *A. baumannii*, 38 strains of *Citrobacter* sp., 44 strains of *E. aerogenes*, 235 strains of *E. coli*, 139 strains of *K. pneumoniae,* 13 strains of *K. oxytoca,* 36 strains of *P. mirabilis*, 17 strains of *P. vulgaris,* and 108 strains of *P. aeruginosa*. Thus, *E. coli* was the most frequently isolated species, followed by *K. pneumoniae*, *P. aeruginosa*, *A. baumannii, E. aerogenes, P. mirabilis, P. vulgaris* and *K. oxytoca* (Table 1).

A. baumannii was identified by colony characteristics on nutrient agar (NA), MacConkey (MC) agar, cysteine-lactose-electrolyte-deficient (CLED) agar, and with results obtained from adopted biochemical procedures: it grew as colourless, smooth, opaque, raised-pinpoint colonies on NA and as non-lactose-fermenting (NLF) colonies on MC agar; it was positive for catalase, Voges–Proskauer (VP), and citrate tests; and it was negative for oxidase, indole, MR and nitrate tests. The other bacterial isolates were similarly identified.

Antibiotic susceptibility tests

Among the β-lactams, the average percent resistance to amoxyclav (30 μg/disc) were: *A. baumannii*, 50; *Citrobacter freundii*, 25.7; *E. aerogenes*, 52.7; *E. coli*, 77; *K. oxytoca*, 61; *K. pneumoniae,* 31; *P. mirabilis*, 38.3; *P. vulgaris*, 33.3; and *P. aeruginosa*, 65.3. The average percent of resistance to ampicillin (10 μg/disc) were: *A. baumannii*, 71.7; *C. freundii*, 58.3; *E. aerogenes*, 69.7; *E. coli*, 70.3; *K. oxytoca*, 78;

Table 1: Bacteria isolated from urine samples from January–December 2013 in each four month period (phases, I, II and III).

Bacteria	Phase I		Phase II		Phase III		Total
	CA	HA	CA	HA	CA	HA	
Acinetobacter baumannii	17	11	11	09	12	15	75
Citrobacter freundii	10	02	05	08	04	09	38
Enterobacter aerogenes	11	07	03	04	13	06	44
Escherichia coli	35	47	38	29	39	47	235
Klebsiella oxytoca	03	0	05	01	0	04	13
Klebsiella pneumoniae	26	21	23	28	25	16	139
Proteus mirabilis	07	03	–	08	11	07	36
Proteus vulgaris	02	01	03	01	06	04	17
Pseudomonas aeruginosa	21	14	17	18	15	23	108
Grand total	132	106	123	106	125	131	705

HA, hospital acquired; CA, community acquired.

Table 2: Percent resistance of bacterial isolates to β-lactam and cephalosporin antibiotics (μg/disc).

Bacteria	β-lactams									Cephalosporins								
	Amoxyclav 30			Ampicillin 10			P/T 100/10			Cefepime 30			Ceftazidime 30			Ceftriaxone 30		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
A. baumannii	47	52	51	69	77	69	62	67	74	50	56	62	69	77	69	52	55	59
C. freundii	25	24	28	54	59	62	56	54	56	37	38	38	54	59	62	27	34	41
E. aerogenes	45	52	61	65	69	75	52	61	63	72	77	79	65	69	75	52	57	53
E. coli	72	78	81	62	71	78	74	78	79	75	80	82	62	71	78	79	79	78
K. oxytoca	55	61	67	74	79	81	51	59	62	71	72	75	74	79	81	66	78	77
K. pneumoniae	27	31	35	74	79	83	60	68	72	32	39	42	74	79	83	54	59	63
P. mirabilis	34	39	42	32	41	43	31	45	47	27	31	34	32	41	43	34	43	45
P. vulgaris	25	34	41	34	39	48	35	37	43	38	46	47	34	39	48	36	41	43
P. aeruginosa	56	69	71	55	54	67	79	81	78	67	76	82	55	54	67	49	61	68

Table 3: Percent resistance of bacterial isolates to aminoglycoside antibiotics (μg/disc).

Bacteria	Aminoglycosides								
	Amikacin 30			Gentamicin 10			Tobramycin 10		
	I	II	III	I	II	III	I	II	III
A. baumannii	33	41	48	62	67	74	47	56	62
C. freundii	23	27	34	56	54	56	48	51	58
E. aerogenes	36	39	45	52	61	63	55	61	66
E. coli	68	71	78	74	78	79	82	84	90
K. oxytoca	66	69	73	51	59	62	75	79	81
K. pneumoniae	47	49	52	60	68	72	71	75	81
P. mirabilis	37	34	35	31	45	47	27	32	38
P. vulgaris	34	43	45	35	37	43	31	38	44
P. aeruginosa	75	77	83	79	81	78	64	69	77

Table 5: Percent resistance of bacterial isolates to stand-alone antibiotics (μg/disc).

Bacteria	Stand-alones					
	Co-trimoxazole 25			Nitrofurantoin 300		
	I	II	III	I	II	III
A. baumannii	58	63	68	42	48	53
C. freundii	45	52	57	37	41	44
E. aerogenes	51	59	62	38	42	56
E. coli	62	70	72	65	70	76
K. oxytoca	75	82	88	61	67	78
K. pneumoniae	43	51	53	75	82	87
P. mirabilis	64	65	71	29	35	40
P. vulgaris	35	32	36	32	41	52
P. aeruginosa	45	38	44	38	42	49

K. pneumoniae, 78.7; *P. mirabilis*, 38.7; *P. vulgaris*, 40.3; and *P. aeruginosa*, 58.7. Finally, the average percent of resistance to piperacillin/tazobactam (100/10 μg/disc) were: *A. baumannii*, 67.7; *C. freundii*, 55.3; *E. aerogenes*, 58.7; *E. coli*, 77; *K. oxytoca*, 57.3; *K. pneumoniae*, 66.7; *P. mirabilis*, 41; *P. vulgaris*, 38.3; and *P. aeruginosa*, 79.3 (Table 2). Similarly, resistance patterns to cephalosporins (Table 2), aminoglycosides (Table 3), fluoroquinolones (Table 4) and stand-alone antibiotics (Table 5) were recorded.

Discussion

It was evident that currently or commonly used antibiotics were ineffective against a number of the bacterial isolates. Antibacterial resistance has arisen due to rapid changes in bacterial biochemical levels, target modification, target group bypass and alterations in efflux pumps, due to genetic alterations (e.g., mutation and horizontal gene transfer). ESBLs are inhibited by antibiotics and β-lactamase

Table 4: Percent resistance of bacterial isolates to fluoroquinolone antibiotics (μg/disc).

Bacteria	Fluoroquinolones											
	Gatifloxacin 5			Levofloxacin 5			Ciprofloxacin 5			Ofloxacin 5		
	I	II	III	I	II	III	I	II	III	I	II	III
A. baumannii	62	67	74	55	64	67	38	42	49	52	55	61
C. freundii	56	54	56	27	35	39	29	35	40	47	52	51
E. aerogenes	52	61	63	54	59	62	32	41	52	52	57	63
E. coli	74	78	79	55	67	71	58	62	69	79	79	88
K. oxytoca	51	59	62	66	69	78	54	67	71	59	61	65
K. pneumoniae	60	68	72	33	35	42	35	41	47	54	59	63
P. mirabilis	31	45	47	27	31	35	27	31	35	34	43	45
P. vulgaris	35	37	43	25	32	39	34	39	42	36	41	43
P. aeruginosa	79	81	78	63	68	72	75	79	81	49	61	68

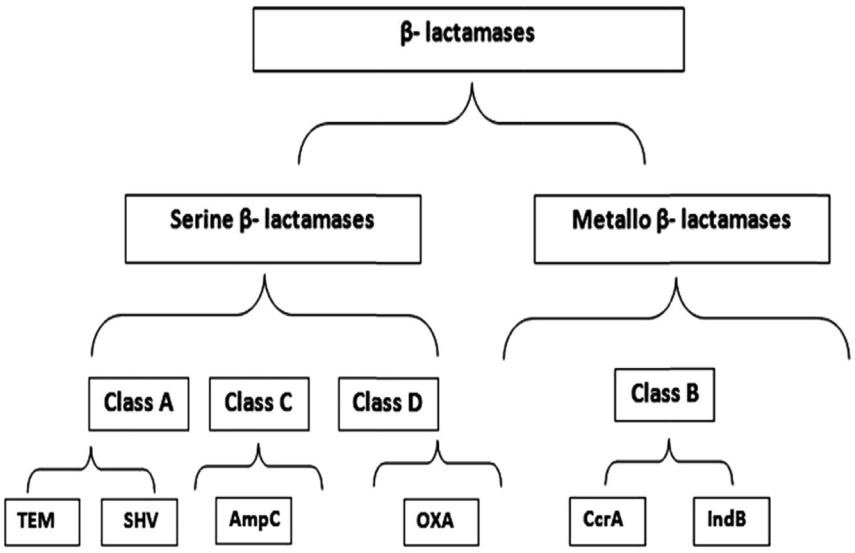


Figure 1: Classification of β-lactamase and ESBL genes. Class A and D genes are responsible for antibiotic resistance by hydrolysing the β-lactam ring.

inhibitors (e.g., clavulanic acid and tazobactam/sulbactam); eventually the bacteria are controlled. However, ESBL genes are transmissible between bacteria and remain peripatetic, mainly among gram-negatives.[20,21]

β-lactamases are broadly categorized into two types, serine β-lactamases and metallo-β-lactamases. In the former, there are three proposed classes, class A, C and D.[22,23] Among these, class A consists of TEM and sulfhydryl variable (SHV) variants of ESBLs with corresponding resistances, while class D consists of cloxacillin and oxacillin (OXA) variants. Classes A and D constitute all detected variants of ESBLs, whereas class C consists of a non-ESBL variant commonly isolated from extended-spectrum cephalosporin-resistant GN bacteria with AmpC (Figure 1, Table 1).

The OXA-type β-lactamase emerged with a narrow spectrum in the co-evolution with TEM and SHV,[24] occurring predominantly in *P. aeruginosa* strains.[25] The other types of ESBLs, cefotaximase-Munich (CTX-M), *Pseudomonas* Extended resistance (PER-1), Brazilian Extended Spectrum β-lactamase (BES-1), *Chryseobacterium meninggosepticum* (CME-1), Vietnamese Extended Spectrum β-lactamase (VEB-1) and *Serratia fonticola* (SFO-1), TLA, and a Mexican group (TLA-1) have been described in detail elsewhere.[26] The Guyana Extended-Spectrum β-lactamases (GES) belonging to class A have hydrolysing activity against penicillin and extended-spectrum cephalosporins but are sensitive to general inhibitors of β-lactamase.[27] These types of ESBL variants have evolved mainly with genetic changes from TEM and SHV sub-types, and bear 25–27%

```
Query       MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
Template    MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
            ************************************************************

Query       EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
Template    EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
            ********************:***************************************

Query       CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
Template    CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
            ************************************************************

Query       PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
Template    PVAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
            *.***********************************************************

Query       RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW 286
Template    RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW 286
            **********************************************
```

Figure 2: Sequence-structure alignment of the query and template. The alignment of the *A. baumannii* TEM-1 sequence (query) with the structure of 1ZG4 (template) was performed.

Table 6: Retrieved β -lactamase protein sequences of 9 bacterial ESBLs and their 3-D protein structures by homology modelling.

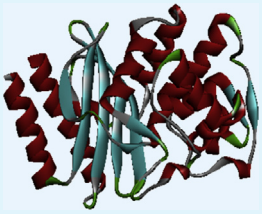
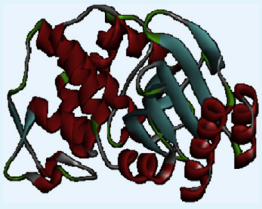
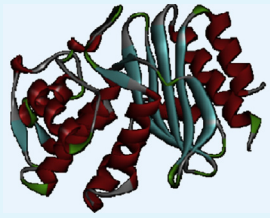
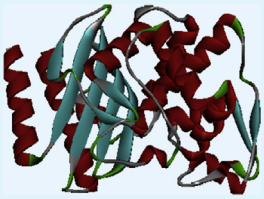
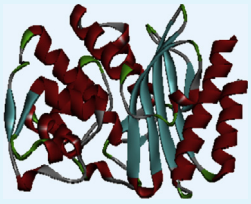
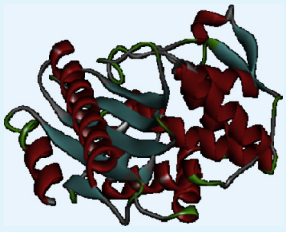
Retrieved β -lactamase protein sequence	Generated 3D structure
>Q6WZD4|Beta-lactamase OS=*Acinetobacter baumannii* GN = blaTEM-1 MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>Q7B3X5|Beta-lactamase OS=*Citrobacter freundii* GN = blaTEM1 MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>Q6W7F6|Beta-lactamase OS = *Enterobacter aerogenes* GN = blaTEM-121 MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDKLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVKYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDSWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGTGKRGS SGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>P62593|Beta-lactamase TEM OS=*Escherichia coli* GN = bla MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>Q48406|Beta-lactamase TEM-12 OS=*Klebsiella oxytoca* GN = blaT-12b MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDSWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>I7ANY8|Beta-lactamase OS=*Klebsiella pneumoniae* GN = TEM-1 MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIXLDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAXXXXSLIKHW	

Table 6 (*continued*)

Retrieved β -lactamase protein sequence	Generated 3D structure
>B9DR46|Beta-lactamase OS=*Proteus mirabilis* GN = blaTEM MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNIGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	
>B3VI32|Beta-lactamase OS=*Proteus vulgaris* PE = 3 SV = 1 MNVIIKAVVTASTLLMVSFSSFETSAQSPLLKEQIESIVIGKKATVGVAVWGPDDLEPLL INPFEKFPMQSVFKLHLAMLVLHQVDQGKLDLNQTVIVNRAKVLQNTWAPIMKAYQGDEF SVPVQQLLQYSVSHTDNVACDLLFELVGGPAALHDYIQSMGIKETAVVANEAQMHADDQV QYQNWTSMKGAAEILKKFEQKTQLSETSQALLWKWMVETTTGPERLKGLLPAGTVVAHKT GTSGIKAGKTAATNDLGIILLPDGRPLLVAVFVKDSAESSRTNEAIIAQVAQTAYQFELK KLSALSPN	
>Q6LBN9|Beta-lactamase OS=*Pseudomonas aeruginosa* GN = blatem-1A MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW	

homology with TEM- and SHV-type ESBLs.[28,29] However, these variants of ESBLs have a unique characteristics, individually and in different bacterial species.

Computational analyses

The ESBL protein models of were generated by the homology modelling protocol. The sequence-structure of *A. baumannii* TEM-1 was aligned with the structure of *E. coli* TEM-1 (PDB ID: 1ZG4) (Figure 2). Similarly, for of the remaining ESBLs, alignments were performed between template structures with individually retrieved target sequences (Table 6). After modelling of *A. baumannii* TEM-1, a 3-D structure was created (Figure 3), which was analysed by Ramachandran plot analysis and the PROCHECK program (Figure 4 and Figure S1). It was found that the phi−psi angles of 97.5% of the residues were in the most favoured regions, while 2.5% residues were in additional allowed regions, but no residues fell in the disallowed regions (Figure 4), confirming the reliability of the modelled structure. Unavailable in PDB, the generated model was used as a target herein, concomitantly that it would lend itself to future antibacterial drug development efforts.

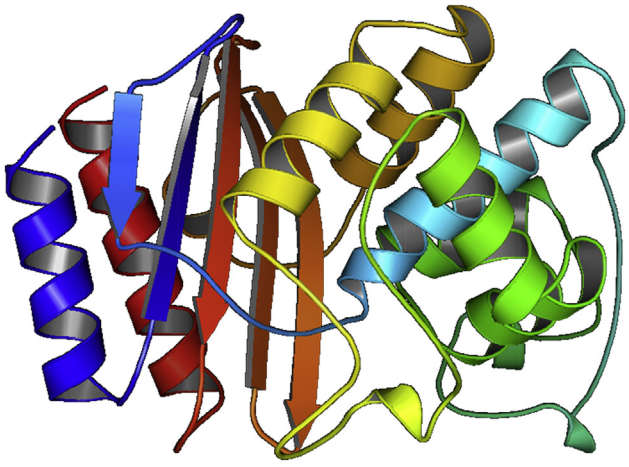


Figure 3: Ribbon representation of *A. baumannii* TEM-1 protein 3-D structure by PyMOL software.

Phylogenetic trees were constructed though the maximum likelihood method (Figure 5). ClustalW2 was used for multiple sequence alignments among ESBL reference sequences. The constructed phylogenetic tree presents the genetic distance of the 9 ESBL variants. For example, between the reference sequence of SHV-11 and that of blaSHV-12 of *E. coli,* the alignment showed the amino acid residues at positions at 234 and 235 positions were different (Figure S4). Furthermore, multiple sequence alignments of 3 variants of *K. pneumoniae*, blaSHV-40, blaSHV-41 and blaSHV-42 have changes at amino acid positions that probably occurred over several generations.

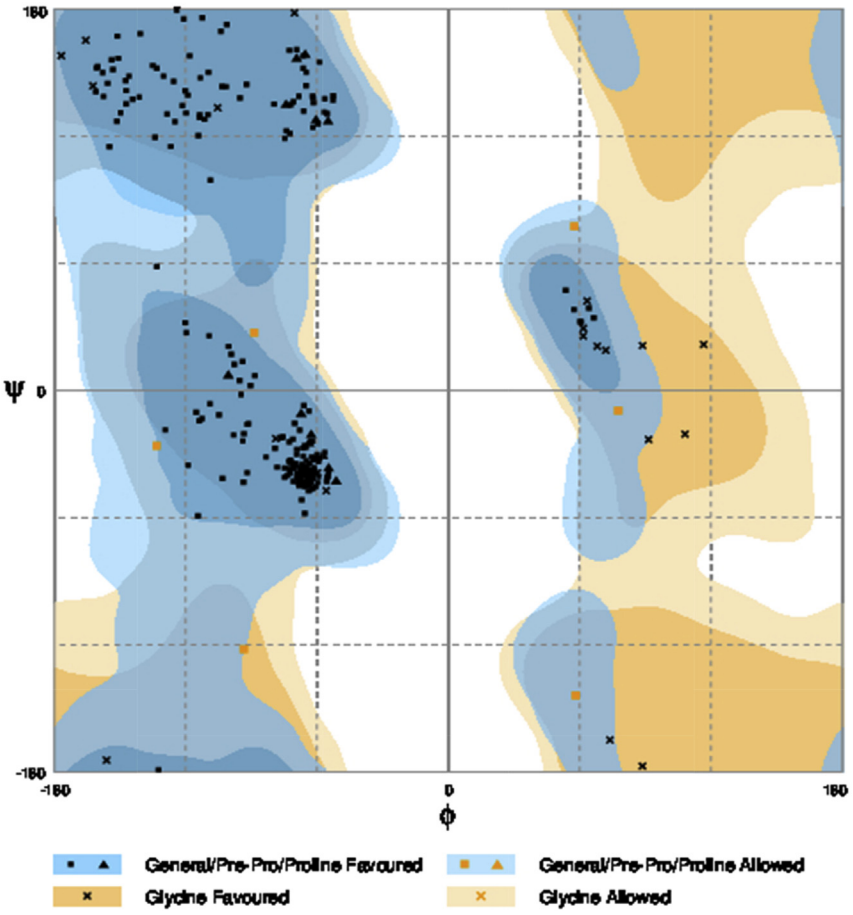


Figure 4: Ramachandran plot of *A. baumannii* TEM-1 model. The graph was created with the Ramachandran plot analysis program, with 97.5% of the amino acids in the favoured region, 2.5% in the allowed region, and none in the outlier region.

Such mutations directly affect the antibiotic resistance[30–32] (Figure S5).

The phylogenetic trees were in accordance with nosocomial data that the main cause of antibiotic resistance was due to modifications of bacterial genomes. Therefore, we performed molecular docking studies of flavonoids against two ESBLs, a wild type and a mutant target. The docking score with amikacin as the ligand

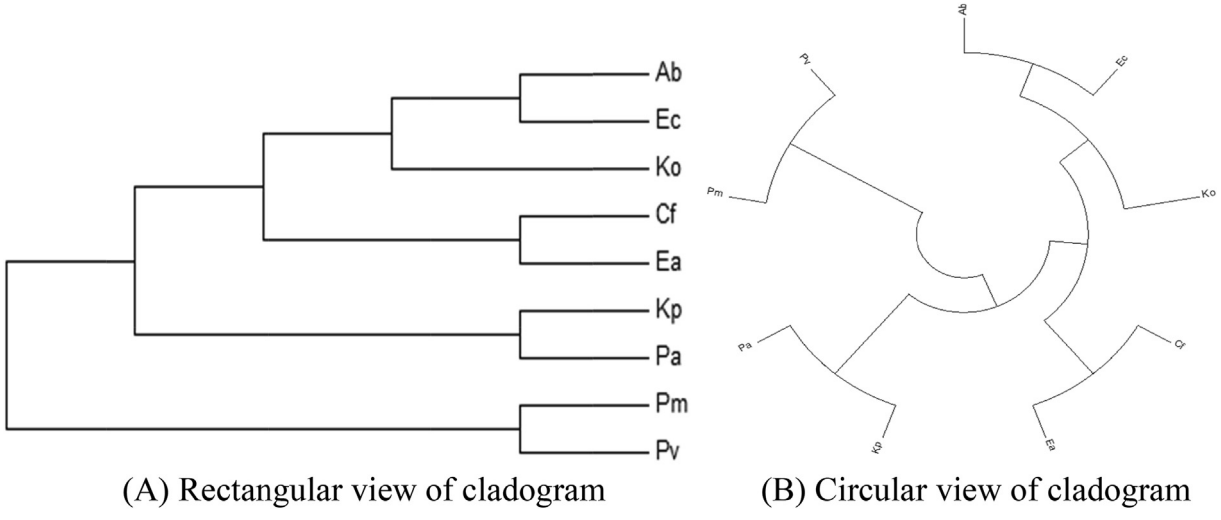


Figure 5: Phylogenetic tree of 9 species of bacteria based on reference ESBL sequences retrieved from UniprotKB database constructed using the ClustalW2 and MEGA 6.06 software; A and B are two different views of the phylogenetic tree.

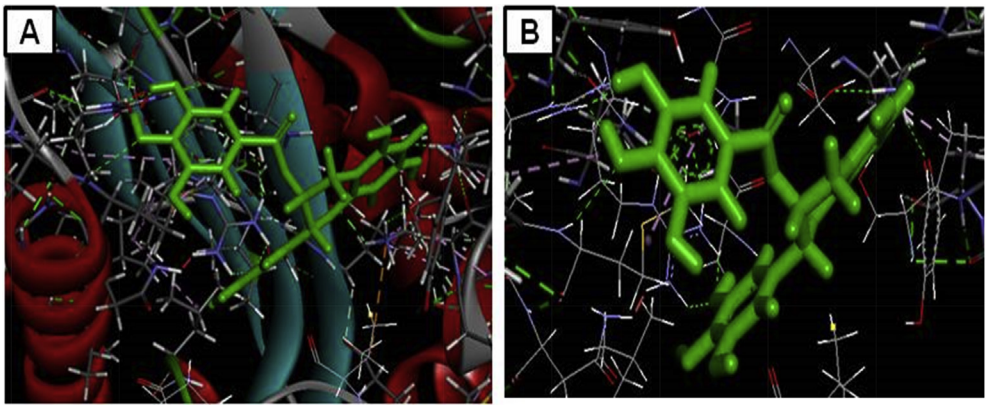


Figure 6: **A**, Three-dimensional structure of the protein-ligand interaction of epigallocatechin 3-gallate with the target surface of the *E. coli* TEM-1 mutant model (PDB ID: 1ESU); **B**, Structure of the same protein-ligand, amino acids of the target protein interacting with the ligand epigallocatechin 3-gallate were predicted with the Discovery studio Visualizer 3.1 software.

was −8.108 kcal/mol against the wild type TEM-1 of *A. baumannii*, while the docking score against the mutant *E. coli* type was −7.388 kcal/mol. The docking scores predicted that amikacin would be ineffective for controlling the mutant strain in comparison to the wild type bacterial strain (Figure 6).

Drug-likeness scores of individual flavonoids and amikacin were calculated using Molsoft (http://molsoft.com/mprop/), to predict the effectiveness as an inhibitor. The decreasing orders of flavonoids with drug-likeness scores were: eriodictyol (1.37) > naringenin (1.13) > quercetin (0.93) > catechin (0.92) > hesperetin (0.88) > luteolin (0.86) > apigenin and kaempferol (0.77) > isorhamnetin (0.67) > epigallocatechin 3-gallate (0.39) > theaflavin (0.31), and the drug-likeness score of amikacin was 1.03, based on canonical simplified molecular-input line-entry system (SMILES) of each chemical using Molsoft (Table 7). The drug-likeness score of each individual flavonoid indicated their suitability for consideration as a drug. The 11 flavonoids were individually docked against the same wild type target of TEM-1 and mutant TEM-12. A minimum (more negative) binding affinity or docking score (or energy value) in protein-ligand interactions indicates a more effective chemical compound. The docking scores of 11 flavonoids against of the wild type *A. baumannii* are listed in decreasing order: epigallocatechin 3-gallate (−9.665) > naringenin (−9.658) > catechin (−8.870) > hesperetin (−8.456) > eriodictyol (−8.432) > apigenin (−8.245) > luteolin (−8.226) > kaempferol (−8.219) > quercetin (−8.209) > theaflavin (−7.989) > isorhamnetin (−7.809). By comparison the values against the *E. coli* mutant were: epigallocatechin 3-gallate (−9.448) > naringenin (−9.206) > hesperetin (−8.335) > catechin (−8.353) > luteolin (−8.177) > eriodictyol (−8.161) > apigenin (−8.142) > quercetin-(7.965) > kaempferol (−7.954) > theaflavin (−7.918) > isorhamnetin (−7.742) (Table 8). The docking scores and drug-likeness scores imply that these flavonoids are suitable alternative antimicrobial agents, which could be used as complementary/supplementary agents, for controlling drug-resistant bacterial strains.

In the advanced drug discovery process, the Lipinski rules of five (RO5) or Pfizer's rule of five are considered to have important roles in the selection of a possible drug candidate/lead compound.[33] According to the RO5, a chemical/drug candidate would be ideal when it has a molecular weight in the range 180−500 g/mol, has 10 or less H-bond acceptors, has 5 or less H-bond donors, the XLogP3 (octanol/water partition coefficients) should be between −0.4 and 5.6, and the topological polar surface area (TPSA) should be not more than 140 $Å^2$.

Two out of 11 flavonoids used, epigallocatechin 3-gallate and theaflavin, and the antibiotic amikacin do not follow the RO5. The criteria laid down in the RO5 are advanced concepts for the selection of suitable intended agents,[33] nevertheless it is not universally followed with respect to the activity of a compound. As we have demonstrated, amikacin and the most effective flavonoid, epigallocatechin 3-gallate, overrule the RO5. Thus, flavonoids used in this study could be promoted as new antibacterials against ESBL.

Conclusions

The present work describes the relationships of 9 types of ESBL enzymes that were ascertained through the constructed phylogenetic tree, using multiple sequence alignments of protein sequences of ESBL variants. Stable configurations and validations of the 9 ESBL protein structures were modelled and analysed with several tools, such as SAVES (PROCKECK, ERRAT, Verify), as well as WHAT IF, MolProbity and ProSA. It was evident that the structures generated were geometrically and stereochemically acceptable. For example, 97.5% of residues of the *A. baumannii* TEM-1 were in the most favoured regions, while the remaining 2.5% residues were in additional allowed regions. No residues fell in the disallowed regions. Here, the ESBL structures generated were aptly druggable candidates as targets for future study, including identification of suitable inhibitor(s) for ESBLs. The 3-D structures of 9 ESBLs have not been reported in PDB; these models may be used in drug discovery efforts in the future. Moreover, these 11 flavonoids

Table 7: Information and 3-D structure of 11 flavonoids and an antibiotic.

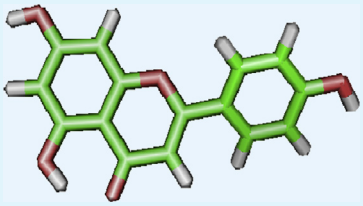
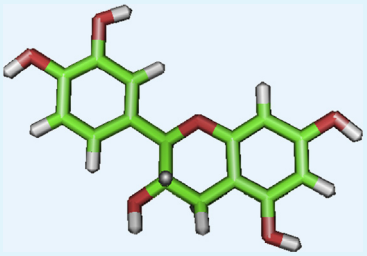
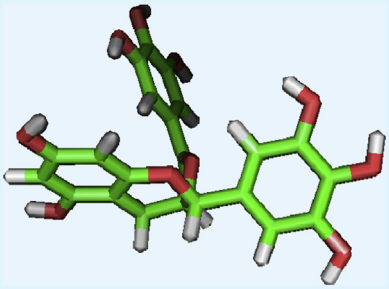
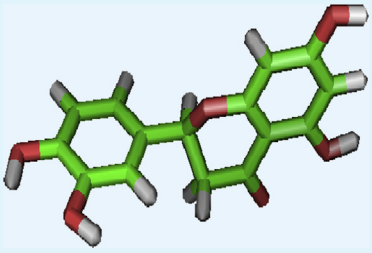
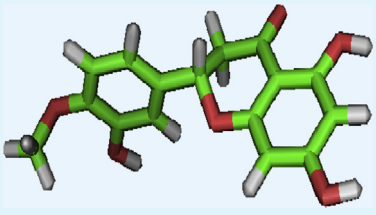
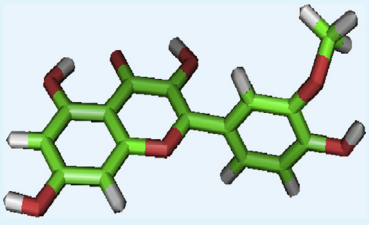
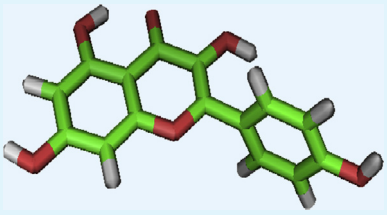
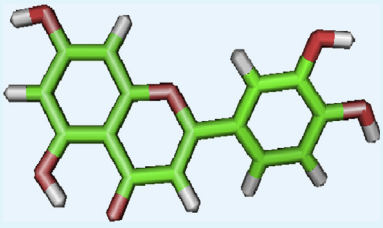
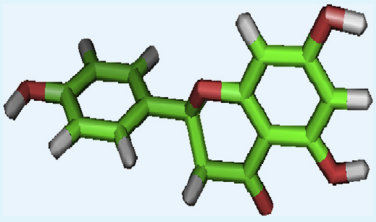
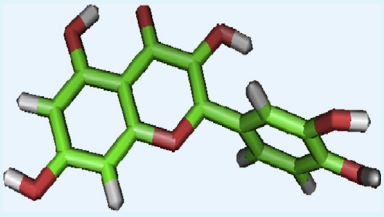
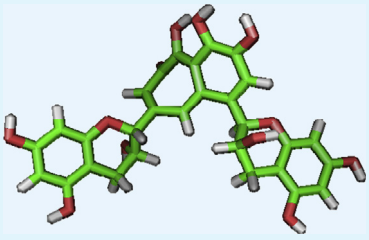
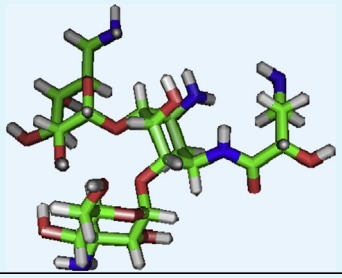
Flavonoids	Information	3D-structure	Drug-likeness score
Apigenin	MF: $C_{15}H_{10}O_5$ MW: 270.2369 g/mol H-bd: 3 H-ba: 5 XLogP3: 1.7 TPSA: 87		0.77
Catechin	MF: $C_{15}H_{14}O_6$ MW: 290.26806 g/mol H-bd: 5 H-ba: 6 XLogP3:0.4 TPSA: 110		0.92
Epigallocatechin 3-gallate	MF: 458.37172 g/mol MW: C22H18O11 g/mol H-bd: 8 H-ba: 11 XLogP3: 1.2 TPSA: 197		0.39
Eriodictyol	MF: $C_{15}H_{12}O_6$ MW: 288.25218 g/mol H-bd: 4 H-ba: 6 XLogP3:1 TPSA: 107		1.37
Hesperetin	MF: $C_{16}H_{14}O_6$ MW: 302.27876 g/mol H-bd: 3 H-ba: 6 XLogP3: 2.4 TPSA: 96.2		0.88
Isorhamnetin	MF: $C_{16}H_{12}O_7$ MW: 316.26228 g/mol H-bd: 4 H-ba: 7 XLogP3: 1.9 TPSA: 116		0.67

Table 7 (*continued*)

Flavonoids	Information	3D-structure	Drug-likeness score
Kaempferol	MF: $C_{15}H_{10}O_6$ MW: 286.2363 g/mol H-bd: 4 H-ba: 6 XLogP3: 1.9 TPSA: 107		0.77
Luteolin	MF: $C_{15}H_{10}O_6$ MW: 286.2363 g/mol H-bd: 4 H-ba: 6 XLogP3: 1.4 TPSA: 107		0.86
Naringenin	MF: $C_{15}H_{12}O_5$ MW: 272.25278 g/mol H-bd: 3 H-ba: 5 XLogP3: 2.4 TPSA: 87		1.13
Quercetin	MF: $C_{15}H_{10}O_7$ MW: 302.2357 g/mol H-bd: 5 H-ba: 7 XLogP3: 1.5 TPSA: 127		0.93
Theaflavin	MF: $C_{29}H_{24}O_{12}$ MW: 564.49366 g/mol H-bd: 9 H-ba: 12 XLogP3: 0.6 TPSA: 218		0.31
Amikacin[a]	MF: $C_{22}H_{43}N_5O_{13}$ MW: 585.60252 g/mol H-bd: 13 H-ba: 17 XLogP3: −7.9 TPSA: 332		1.03

[a] Antibiotic; H-bc, H-bond acceptor; H-bd, h-bond donor; mf, molecular formula; mw, molecular weight; TPSA, topological polar surface area.

Table 8: Docking scores of 11 flavonoids and an antibiotic against 2 ESBL target proteins.

Flavonoids	Docking score values (kcal/mol)	
	A. baumannii (TEM-1 wild, generated model by homology modelling)	*E. coli* (TEM-1 mutant, retrieved model, PDB ID: 1ESU)
Apigenin	−8.245	−8.142
Catechin	−8.870	−8.353
Epigallocatechin 3-gallate	−9.665	−9.448
Eriodictyol	−8.432	−8.161
Hesperetin	−8.456	−8.335
Isorhamnetin	−7.809	−7.742
Kaempferol	−8.219	−7.954
Luteolin	−8.226	−8.177
Naringenin	−9.658	−9.206
Quercetin	−8.209	−7.965
Theaflavin	−7.989	−7.918
Amikacin[a]	−8.108	−7.388

[a] Antibiotic.

are predicted to be effective as inhibitors of ESBLs, with epigallocatechin 3-gallate and eriodictyol being the most suitable agents based on both docking and drug-likeness scores.

Contributions

SS Swain conducted the experiments under the supervision of RN Padhy. SS Swain prepared the draft manuscript, and RN Padhy edited it.

Ethical statement

Not required.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

RNP conceived and designed the study, SSS collected bacteria from clinical samples and grew *in vitro* growth, and SSS recorded and organized data. SSS analyzed and interpreted data. SSS wrote initial and final draft of the article, and provided logistic support from bioinformatics tools. Both authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtumed.2016.03.007.

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