

Bioprospecting Studies of Actinomycete Bioactive Compound on *Bla* CTX-M -Extended -Spectrum Beta-Lactamase (ESBL) Producing *Escherichia Coli* and *Klebsiella* Species

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Abstract

Forty one Extended Spectrum Beta Lactamase (ESBL) producing *E. coli* and *Klebsiella* species were analyzed for the presence of *bla*_{CTX-M} genes by Uniplex Polymerase Chain Reaction (PCR) in which 15 isolates harbored *bla*_{CTX-M} genes. Transconjugation studies were carried out to demonstrate the transfer of antibiotic resistance using *E. coli* K12J62-2 (F⁻ rif^r lac⁻) strain among which UTEC31 and UTK2 strains showed successful transconjugation. Sequence analysis revealed the presence of *bla*_{CTX-M-15} genes. Antibacterial activity of *Streptomyces* sp PUT23 was evaluated against CTX-M ESBL producing *E. coli* and *Klebsiella* sp. was determined. The ethyl acetate extract showed inhibitory activity and Reversed phase thin layer chromatography (RP-TLC) analysis revealed two fractions, EF1 and EF2. Bioautogram of EF2 fraction demonstrated inhibitory activity with a minimum inhibitory concentration (MIC) value 156 µg/ml. The EF2 fraction was identified as a flavonoid upon chemical characterization. Sequencing of 16S rRNA gene and phylogenetic analysis of *Streptomyces* sp. strain PUT23 revealed its close relatedness to *S. griseus*. This study revealed the rapid spread and dominance of CTX-M producing *E. coli* and *Klebsiella* sp. in the suburban community of South Chennai, Tamil Nadu which has to be considered as a serious public health issue. There is an urgent need for newer and effective antimicrobials and bioactive compounds derived from *Streptomyces* species proved to be a promising and efficient resource. Further characterization and purification of EF2 fraction are needed and could be used in the formulation of new therapeutics against ESBL mediated infections.

Introduction

Infections caused by multidrug resistant bacterial pathogens are considered to be the important cause of morbidity and mortality [1]. Though antibacterial agents have been a major factor in treating bacterial infections, the phenomenon of microbial drug resistance has rendered most of the antibiotics useless, especially multidrug resistance among gram negative bacteria represents a unique and immediate threat [2].

The production of extended spectrum beta lactamases (ESBLs) is one of the vitriolic mechanisms of antimicrobial resistance (AMR). Reports on ESBL producing uropathogenic bacteria as a leading cause of morbidity and mortality are of major concern. Moreover, their ability to disseminate into the community is an emerging public health problem globally [3].

Broad spectrum antibiotics are frequently given to treat UTIs in place of a narrow spectrum antibiotic which may be adequate. Recent reports suggest that the global antibiotic consumption from the year 2000 to 2015 had increased by 65% while a 39% increase was reported on the rate of antibiotic consumption for defined daily doses (DDDs) per 1000 people [4].

The reasons for global antibiotic resistance are complex and multifactorial. They may be attributed to the indiscriminate and irresponsible use of antibiotics, mutations in bacteria, acquisition of resistance genes, overuse and misuse of antibiotics, selection pressure sustaining resistant bacteria and competition

among physicians prescribing antibiotics unnecessarily to retain patients, lack of awareness among people all contribute to this global problem [5]. Metagenomic analysis has revealed the widespread prevalence of genes encoding antibiotic resistance in the environment [6].

The AWaRe classification of antibiotics [7] was introduced by the WHO as a tool for antibiotic stewardship with the aim of reducing AMR and ESBL producing pathogens are included in the Priority 1 - Critical group list [8] for which the WHO cautions appropriate use of existing antibiotics and also emphasizes the need of new antimicrobials.

Actinobacteria are widely exploited for their bioactive compounds and are excellent resources for the control of ESBL producing pathogens [9]. These groups of filamentous gram positive bacteria are recognized for their pivotal role and genetic capability in the production of useful secondary metabolites like antibiotics, antitumors etc. [10].

Among many actinobacterial genera, *Streptomyces* species are renowned and eulogized for their production of diverse array of antibiotics. These accounts for over two thirds of the clinically used natural antibiotics produced by *Streptomyces* alone and about 75% of the marketable and therapeutically useful antibiotics are derived from this genus [11]. The ability of the bioactive compounds of actinomycetes in controlling multi drug resistant pathogens is well known [12]. The problem of antimicrobial resistance (AMR) is increasing while the production of newer antibiotics is slow [13]. Hence newer potential antibiotics are needed, to curb the problem of multidrug resistance, especially from natural habitats rather than their synthetic counterparts.

This study was instigated to determine the prevalence of *bla*_{CTX-M} ESBL producing *E. coli* and *Klebsiella* species in the southern semi urban regions of Chennai. This study also reported the antibacterial properties of *Streptomyces* sp. against the ESBL producing organisms.

Methods

Identification of *bla*_{CTX-M} mediated ESBL production in *E. coli* and *Klebsiella* species by uniplex polymerase chain reaction (PCR)

In this study, 41 ESBL producing bacterial cultures [14] including 27 *E. coli* and 14 *Klebsiella* species were used for the determination of *bla*_{CTX-M} genes. Plasmid DNA was isolated from the strains following the alkaline lysis method [15]. PCR amplification of *bla*_{CTX-M} alleles was carried out for all ESBL positive bacterial cultures using the set of universal primers CTX-M-F-5'-ATG TGC AGY ACC AGT AAR GT - 3' and CTX-M-R-5'-TGG GTR AAR TAR GTS ACC AGA - 3' [16]. The strain *E. coli* MTCC 443 was used as negative control. The Y, R, S represent the standard nucleotide combinations where Y is C or T, R is A or G and S is C or G. This primer combination is designed to accommodate sequence variation in CTX-M type enzymes.

Demonstration of horizontal transfer of antibiotic resistant genes by transconjugation

The isolates of *E. coli* and *Klebsiella* species that harbored *bla*_{CTX-M} genes were analyzed for capability of gene transfer in the recipient strain *E. coli* K12 J62-2 (F⁻ rif^r lac⁻). Mating experiment was carried out where the isolates and the recipient were grown in Brain Heart Infusion broth for overnight and were mixed in the ratio of 1:10 to match McFarland turbidity 0.5 [17]. They were then incubated at 37°C overnight and selected on MacConkey agar supplemented with rifampicin (2.5 mg/ml) and cefotaxime (2 µg/ml). The transconjugants were subjected to antibiotic sensitivity testing against ceftazidime disc and plasmid profile analysis.

Sequencing of *bla*_{CTX-M} plasmid DNA

The ESBL positive *E. coli* and *Klebsiella* species that demonstrated transferrable resistance were subjected to DNA sequence analysis (Beckman Coulter CEQ 8000 auto analyzer). Molecular characterization of the ESBL coding gene *bla*_{CTX-M-15} using the same set of primers mentioned above was analyzed from the selected strains UTEC31 and UTK2 [18].

The sequenced nucleotide was subjected to NCBI BLAST (Basic Local Alignment Search Tool) analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nucleotide BLAST was run using megablast (high similar sequence) mode with maximum target of 50 for confirmation of the sequence. After confirming the sequence, the sequences were submitted to GenBank/NCBI to obtain the accession number through “Sequin” submission tool. Further, the Open Reading Frame (ORF) of the sequences was predicted. The ORF and the initiation codon was predicted using tool, ORF finder (<https://www.ncbi.nlm.nih.gov/projects/gorf/>), open reading frame finding online tool. The amino acid sequence of ESBL gene was obtained from *bla*_{CTX-M-15} gene sequence by translation of nucleotide sequence using ExPASy translation tool (<https://web.expasy.org/translate/>).

Phylogenetic analysis of *bla*_{CTX-M-15} gene of *E. coli* UTEC31 and *Klebsiella* sp. UTK2 strains with other related strains was carried out to predict the evolutionary relationship with similar sequences. Dendrogram was constructed with MEGA 4.0 using neighborhood joining method from 500 resamplings [19].

In vitro testing of *Streptomyces* extract against ESBL pathogens

Description of actinobacterial strain

The actinobacterial strain PUT23, previously isolated from agricultural soil sample was obtained from the Actinobacterial Research Laboratory, Department of Microbiology, Periyar University, Salem, Tamil Nadu. Viability of the strain PUT23 was maintained on yeast extract malt extract (YEME) agar slants at 4°C as well as in 20% glycerol broth at -80°C.

Molecular analysis and taxonomy of the potential *Streptomyces* sp. PUT23

Genomic DNA was extracted from *Streptomyces* sp. PUT23 grown on YEME agar medium using the standard protocol [20]. The 16S rRNA gene of the strain PUT23 was amplified by PCR [21] using the following primers, 27f (5'-AGTTTGATCCTGGCTCAG-3') and 1492r (5'-ACGGCTACCTTGTTACGACTT-3') as forward and reverse primers, respectively.

The thermal cycling conditions included an initial denaturation of the target DNA at 94°C for 4 minutes followed by 30 cycles at 94°C for 1 minute, primer annealing at 52°C for one minute, primer extension at 72°C for one minute. The reaction mixture was then held at 72°C for 10 minutes and cooled to 4°C. The amplified product was detected by 1% agarose gel electrophoresis and was visualized by Gel Documentation System.

Sanger's Dideoxy Chain Terminator Sequencing was performed using DTCS Quick Start Dye Terminator Kit (Beckman Coulter) and Beckman Coulter CEQ 8000 auto analyzer. The cycle sequencing was carried out using the same primers used in PCR amplification. Sequence alignment was done using Clustal-X2 Version 2.1 and phylogenetic tree was constructed using MEGA Version 5.0 [19] and BLAST analysis of the strain was carried out to determine the evolutionary relationship of the strain with closely related strains derived from the NCBI database. The deduced 16S rRNA sequence of the strain PUT23 was submitted to GenBank/NCBI to get the accession number.

Production and extraction of the bioactive compounds

Extraction of the bioactive compound from the PUT23 strain was done through fermentation following the method from a previous study [22] with modifications. Briefly, pure cultures of actively growing *Streptomyces* sp. was inoculated into 100 ml of YEME broth and incubated at 30°C for 3 days. About 2% of the YMD broth culture was again inoculated in about 1 L of International Streptomyces Project 4 (ISP4) broth and incubated at 30°C for 5 days. After fermentation at 30°C for 5 days, the cell free supernatant was collected by filtration. Extraction of the crude compound from the culture filtrate was carried out by liquid-liquid extraction method with equal volumes of ethyl acetate (1:1 v/v) by vigorous shaking for 1 hour and concentrated by evaporation. The crude extract was then tested for anti-ESBL activity.

Determination of antibacterial activity

The antibacterial activity of the ethyl acetate extract against the ESBL producing *E. coli* and *Klebsiella* species was determined by agar well diffusion method [23] with ethyl acetate (50 mg/ml) and ciprofloxacin (5 µg/ml) as controls. The plates were examined for the zones of inhibition and results were compared and graded by measuring the zone diameter. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as controls. Antimicrobial efficacy was graded based on the zone diameter as high activity (≥ 15 mm), moderate activity (10-14 mm), trace activity (5-9 mm) and no activity (≤ 4 mm) [24].

Purification of the bioactive compound by reversed phase thin layer chromatography (RP-TLC)

Partial purification and characterization of the ethyl acetate extract of *Streptomyces* PUT23 was attempted by RP-TLC also known as Double Ascending TLC [25] using readymade silica gel coated TLC sheets (7.5 x 2.5 cm) (Silica Gel 60 F 254nm) using the mobile phase n-hexane: ethyl acetate: acetic acid in the ratio 7:2.5:0.5. The solvent ascent was fixed to 5.8 cm for calculating R_f values. The chamber saturation time for the mobile phase was 15 min at room temperature ($22 \pm 1^\circ\text{C}$). Double ascending TLC was performed and the plates were dried at room temperature. The spots were detected in the presence of iodine vapor. The Retardation Factor (R_f) value was calculated with values of the R_l (R_f of the Leading Front) and R_t (R_f of the Trailing Front) using the relation $R_f = 0.5 (R_l + R_t)$.

Bioautography of purified ethyl acetate extract

The anti-ESBL activity of the separated ethyl acetate fractions namely EF1 and EF2 in RP-TLC method was analyzed by contact or direct bioautography [26] by placing the chromatogram face down onto Mueller-Hinton agar seeded with *bla*_{CTX-M} producing *E. coli* and *Klebsiella* species and incubated at 37°C for 18-24 hours. The zones of inhibition on the agar surface, corresponding to the spots in chromatographic plates were indicative of the bioactive fraction.

The active fraction which showed inhibitory activity in the bioautography was further purified by preparative TLC by scrapping the fraction with silica gel in analytical TLC and repeated extraction with methanol. The purified bioactive compound present in the methanol portion was collected by centrifugation at 10,000 rpm for 10 minutes and filtration. The obtained filtrate was concentrated at 45°C using eppendorf concentrator.

Determination of MIC of the bioactive fraction against *bla*_{CTX-M} producing ESBL *E. coli* and *Klebsiella* species

The minimum inhibitory concentration (MIC) was determined for the bioactive compound using the dilutions at 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml prepared in DMSO [24]. About 50 μl of the bioactive compound from each dilution with ciprofloxacin (8 $\mu\text{g}/\text{ml}$) as control were loaded in wells of Mueller-Hinton agar plates seeded with a lawn culture of *bla*_{CTX-M} positive bacteria. Solvent controls of 1% DMSO and 1% ethyl acetate were also included to exclude the possibility of their antagonistic activity. MIC was determined after incubating the plates at 37°C for 18-24 hours by observing for the zones of inhibition.

Preliminary characterization of active compound

Appearance and solubility analysis

Colour and consistency of the bioactive fraction was determined by visual inspection. Solubility of the compound was tested by dissolving 1 mg of purified compound in the solution containing water, 2.5 M Sodium hydroxide (NaOH), 0.6 M Sodium bicarbonate (NaHCO_3), 1.5 M Hydrochloric acid (HCl) and

concentrated sulphuric acid (H₂SO₄) to resolve the functional groups present in the partially purified bioactive compound [27].

Solubility of the same was determined by dissolving 1 mg of purified compound in 10 ml of solvents such as water, methanol, acetone, chloroform, dichloromethane, diethyl ether, ethyl acetate, n-hexane, dimethyl sulfoxide.

Chemical screening of the bioactive fraction

To determine the chemical group to which the purified pigment belongs, the following qualitative biochemical reactions were performed [28]. About 10 mg/ml stock solution of the bioactive fraction was prepared using methanol for the analysis. Tests were performed to detect the presence of terpenoids, diterpenes, alkaloids, carotenoids, flavonoids, anthraquinones and glycosides.

Results And Discussion

Isolation of plasmid DNA from ESBL positive *E. coli* and *Klebsiella* species

Plasmid profile analysis of ESBL positive isolates revealed the presence of plasmids in all 27 *E. coli* and 14 *Klebsiella* sp. and few isolates harbored multiple plasmids ranging from 2 to 5 numbers. This finding is in full accordance with earlier studies reporting multiple plasmids among ESBL producing bacteria. [29], [30]. Among *E. coli* isolates, strains UTEC22, -24, -45, -48 harbored 5 plasmids ranging between 6000 bp and >1 kb and UTEC4, -5, -29, -31, -40, -53, -57 demonstrated 4 plasmids from 3000 bp to >1 kb. Eight isolates showed three plasmids and three isolates harbored two plasmids while the remaining isolates showed single plasmid.

Plasmid profile of *Klebsiella* species demonstrated the presence of 4 plasmids among five isolates namely UTK12, -21, -22, -36, -38 and three plasmids among the isolates UTK1, -2, -15, -20, and -30. Three isolates namely UTK3, -16, -29 harbored two plasmids while UTK33 showed a single plasmid. The plasmids were in the range between 4000 bp and >1 kb (Table 1 and Fig. 1).

Identification of *bla*_{CTX-M} mediated ESBL production in *E. coli* and *Klebsiella* species by uniplex polymerase chain reaction (PCR)

The CTX-M genotypes have gained clinical and epidemiological importance in the context of their ability to disseminate and causing outbreaks [31]. In India, the *bla*_{CTX-M-15} genotype is reported to be found exclusively [32]. Hence the *bla*_{CTX-M} gene mediated ESBL production among the isolates was determined by using uniplex PCR. The overall prevalence of *bla*_{CTX-M} genes in the isolates was 36.58% where 9 out of 27 *E. coli* (33.33%) and 6 out of 14 *Klebsiella* sp. (42.85%) carried the *bla*_{CTX-M} genes. The size of the amplicons ranged between 500-600 bp (Fig. 2).

The present study had identified 42.85% prevalence of CTX-M ESBLs and is in full accordance with previous report [33]. An earlier Chennai-based study [34] revealed CTX-M type ESBLs to be prevalent in 75% of the isolates followed by TEM enzymes (73%) and SHV ESBLs (42%), while another recent study reported 88% prevalence rate in Chennai [35].

This variation could be related to the shortening of the boundaries between hospitals and the community, accelerated transfer of the antibiotic resistant genes by efficient mobile elements, clonal expansion of the isolates and selective advantage for survival of the resistant isolates ideal for dissemination [32], [36].

Demonstration of horizontal transfer of antibiotic resistant genes by transconjugation

In the transconjugation analysis, 2 out of 15 donor strains (13.33%), namely, UTEC31 and UTK2 showed successful transconjugation in the recipient strain of *E. coli* K12J62-2 (F⁻ rif^r lac⁻). The recipient demonstrated acquired antibiotic resistance against ceftazidime and the acquisition of plasmids (Fig. 3). The rate of transferability was lower in this study when compared to other reports [37], [38].

It is well known that conjugative plasmids are major tools for intra- and inter- species and also inter-generic gene transfers. ESBLs encoded on plasmids isolated from clinically resistant uropathogenic bacteria are constitutively expressed. This is an important observation because plasmid mediated ESBLs possibly inhabit environments in which antibiotics are found in high concentrations [39]. It was proposed that only compatible plasmids could be rescued in transconjugation [40]. However, *bla*_{CTX-M} genes are reported to be linked to plasmids of incompatibility groups which include IncF, IncN, IncN2, IncI1, IncHI2, IncL/M, IncA/C, IncK, IncX4, IncU and RCR [32].

Determination of transferable resistance in this study emulates the dissemination of resistant genes in the environment and will be of clinical and epidemiological significance for two reasons. Firstly, the isolates belonged to community settings and hence possess the viriolic potential to disseminate within community. Secondly, they are problematic because of conferring resistance to various antibiotics providing a survival advantage under pressure of exposure to various antibiotics. This makes the treatment options not only limited but also difficult.

Sequencing of *bla*_{CTX-M} plasmid DNA of ESBL positive *E. coli* and *Klebsiella* species *Molecular characterization of ESBL coding gene*

The two isolates UTEC31 and UTK2 which showed successful transconjugation were subjected to sequencing of *bla*_{CTX-M} gene. Strains UTEC31 and UTK2 showed the amplified DNA product of *bla*_{CTX-M-15} genes of 500 and 600 bp.

Sequencing of plasmid DNA revealed the nucleotide results averaging 543 nucleotides for *E. coli* UTEC31 (GenBank Accession No. KJ746672). For *Klebsiella* sp. UTK2, it was about 568 nucleotides (GenBank Accession No. KJ746673). The BLAST results revealed that the *bla*_{CTX-M-15} sequence obtained from *E. coli* demonstrated 100% identity with *bla*_{CTX-M-15} partial sequence of *E. coli* (Genbank accession

KF723004, KF723003 etc.) and the *bla*_{CTX-M-15} sequence obtained from *Klebsiella* sp. paired 100% with *bla*_{CTX-M-15} partial sequence of *Klebsiella* species (Genbank accession JQ686199, JQ686198 etc.) under 100% query coverage.

The ORF finder online tool predicted different open reading frames but the vast size reading frame was considered as beta-lactamase ORF. The ORF consisted of 543 base pairs corresponding to 179 amino acids in *E. coli* and 568 base pairs in size coding for 156 amino acids in *Klebsiella* sp. In both the cases AUG was the initiation codon for methionine. Since there was no termination codon in this frame for both sequences, the sequence might be a partial sequence of *bla*_{CTX-M-15} gene. In order to find out the amino acid sequences, the ORF sequences were subjected to amino acid translation through ExPASy online tool. The number of amino acid sequences consisted with the results of ORF finding results.

CTX-M-15 is included under the CTX-M-3 cluster based on its amino acid identity and phylogeny. It differs from other members of CTX-M-3 groups by a single amino acid substitution, D240G, where aspartic acid is substituted with glycine at position 240 [41]. This mutation in the gene is responsible for its increased hydrolytic activity against antibiotics such as ceftazidime by the isolates [42].

Phylogenetic analysis of *bla*_{CTX-M-15} gene of *E. coli* strain UTEC31 using neighborhood joining method with a boot strap value of 500 showed similarity with *bla*_{CTX-M-15} genes of *Escherichia coli* ESBL370B15, *Escherichia coli* ESBL490B15, *Escherichia coli* ESBL360 strains. Additionally, this gene also showed similarity with *bla*_{CTX-M-15} genes of *Escherichia coli* strain C21, *E. coli* E5, *E. coli* E12, *E. coli* E11 strains. Similarly, phylogenetic analysis of *bla*_{CTX-M-15} of *Klebsiella* sp. UTK2 based on the neighborhood joining method with a boot strap value of 500 revealed that the sequence showed similarity with *bla*_{CTX-M-15} gene of *K. pneumoniae* KP34C. Additionally this gene of *Klebsiella* sp. UTK2 also showed similarity with *bla*_{CTX-M-15} genes of *K. pneumoniae* ST15, *K. pneumoniae* KP9, *K. pneumoniae* L4, *K. pneumoniae* 639 and *K. pneumoniae* 51 (Fig. 4 and Fig. 5).

Molecular analysis and taxonomy of the potential *Streptomyces* sp. PUT23 strain

The amplicon size of the 16S rRNA gene of *Streptomyces* sp. strain PUT23 was around 1000 base pairs and BLAST analysis showed 100% identity to 16S rRNA sequences of *Streptomyces griseus* strains confirming the strain PUT23 to belong to the genus *Streptomyces*. The sequence was submitted to GenBank with accession number KU356755. Based on the 16S rRNA sequence, the BLAST analysis further revealed the phylogenetic relatedness of the test strain PUT23 to its closely related strains of *Streptomyces* species obtained from the NCBI database (Fig. 6).

Determination of antibacterial activity of the bioactive compound by agar well diffusion method

Approximately, 1.94 g of crude extract was obtained in solvent extraction with ethyl acetate. Testing for anti-ESBL activity of the extract showed an inhibition zone between 13 - 17 mm (Table 2) and the result is

consistent with other studies [11], [21].

Purification of the bioactive compound by reversed phase thin layer chromatography (RP-TLC)

The RP-TLC analysis of the ethyl acetate extract resulted in the separation of 2 fractions, namely EF1 and EF2 (R_f values - 0.58 and 0.73, respectively). Double ascending TLC or Reversed Phase TLC offers added advantage in organizing hydrocarbon ligands on the silica surfaces, interaction of solvent into the bonded layers and aid in separation of pharmaceuticals like surface active pharmaceutical molecules [25].

Bioautography of purified ethyl acetate extract

In bioautography, the second fraction, EF2, (R_f value - 0.73) showed activity against the *bla*_{CTX-M} positive uropathogens tested (Fig. 7). Fraction EF1 did not show any activity against the test organisms. This technique is simple, easy to perform, inexpensive and is a useful means for the screening of compounds from natural source using developed TLC. Prediction of results is made easy indicated by the development of zones of inhibition on the agar surface near the spots in the chromatogram relative to the bioactive fractions [43]. In the preparative TLC, 1 gm of ethyl acetate extract yielded 62 mg of fraction EF2.

Determination of MIC of the bioactive fraction against *bla*_{CTX-M} ESBL uropathogens.

The MIC of the ethyl acetate extract of bioactive component was found to be 156 $\mu\text{g/ml}$ against *bla*_{CTX-M-15} producing *E. coli* and *Klebsiella* sp. which was found to increase with increasing concentrations (Fig. 8). Thus a concentration <156 $\mu\text{g/ml}$ may also be inhibitory to ESBL producing pathogens. This is higher than the findings of a previous study reporting the MIC to be as low as 25 $\mu\text{g/ml}$ against *K. pneumoniae* [44].

Determination of the functional group of bioactive fraction by chemical screening

The bioactive compound EF2 was brown powdery and was insoluble in water, 2.5 M NaOH and 0.6 M NaHCO₃ but soluble in 1.5 M HCl and concentrated sulphuric acid. The original brown colour of the compound changed to red colour upon the addition of 1.5 M HCl and concentrated sulphuric acid. This indicated that the compound EF2 might contain amine, ester, alkene and/or alcohol as functional groups. It was soluble in methanol, DMSO, less soluble in n-hexane and insoluble in water.

Chemical screening of bioactive fraction

In the various screening tests performed for the detection of chemical group, compound EF2 was found to belong to flavonoid class (Table 3). Some of the classes of flavonoids that possess antimicrobial activity include Flavones, Isoflavones, Flavonols and their glycosides, Flavan-3-ols, Flavanones, Flavans etc., [45]. The anti-ESBL activity of the ethyl acetate extract is ascribed to the presence of flavonoids as evident in the bioautography and this finding is supported by other reports [46], [47].

The inhibitory activity of the extract could be attributed to the presence of flavonoid group which has ability to complex with bacterial cell wall, extracellular and soluble proteins and probably causing membrane disruption [48]. Flavonoids conjugated with nanoparticles have been proved to be a promising alternative for antibiotics [49].

Reports on ESBL prevalence from southern suburban regions of Chennai are scarce. This study revealed the rapid emergence and dominance of CTX-M producing *E. coli* and *Klebsiella* sp. in the suburban community of South Chennai, Tamil Nadu which has to be considered as a serious public health issue. Hence the findings in this work are important due to the fact that more residential area and industrial set-ups are being laid in the region. The study had also highlighted the inhibitory activity of the flavonoid produced by *Streptomyces* PUT23 strain against ESBL pathogens and its potential to be developed into antimicrobial agent, especially in the biocontrol of multidrug resistant bacteria. To put in the words of Baltz, (2008) [50], we are in the early stages of a renaissance in the antibiotic discovery from actinomycetes.

Declarations

Ethical Approval: Ref: VISTAS-SPS/IEC/III/2018/09

Consent to Participate: Not Applicable

Consent to Publish: All authors have consented to publish this work.

Authors Contributions: GG, KK and RB contributed equally in designing the study. GG carried out the experiments. KK contributed in data analysis and manuscript preparation. RB supervised the entire study and contributed in manuscript preparation.

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Competing Interest: The authors declare that they have no competing interest.

Availability of data and materials: Available.

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Tables

Table 1. Plasmid profile of ESBL positive isolates

ESBL producing Isolates	No. of Plasmids				
	One	Two	Three	Four	Five
<i>E. coli</i> (n=27)	5	3	8	7	4
<i>Klebsiella</i> sp. (n=14)	1	3	5	5	0

Table 2. Anti-ESBL activity of *Streptomyces* bioactive compound

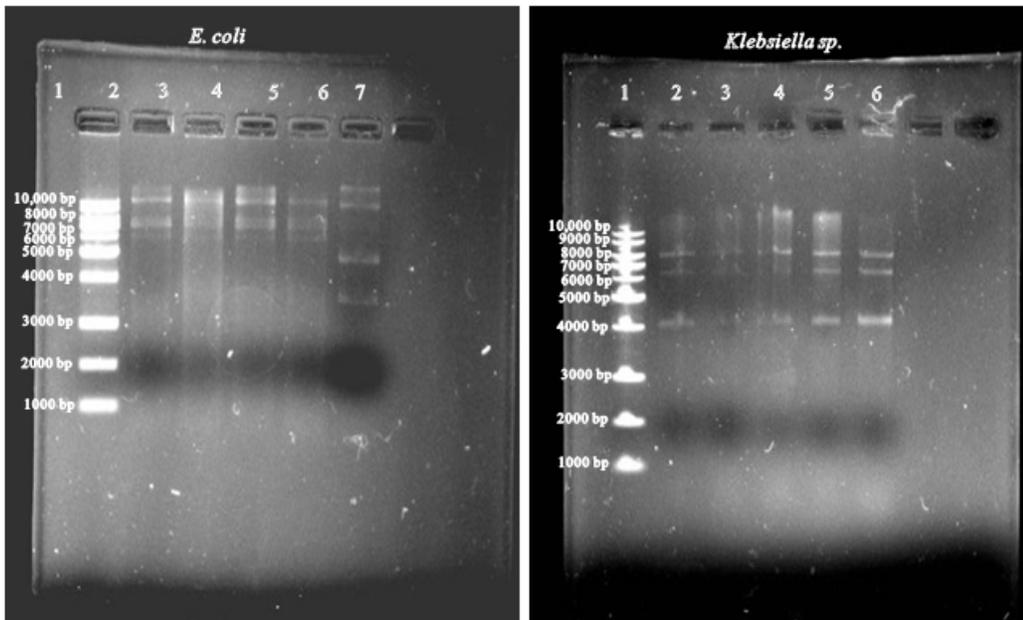
S.No	<i>bla</i> _{CTX-M} positive isolates	Diameter of zones of inhibition (mm)	
		Crude Extract	CF
1.	UTEC3	9 mm	27 mm
2.	UTEC4	10 mm	9 mm
3.	UTEC20	9 mm	NP
4.	UTEC22	11 mm	NP
5.	UTEC29	9 mm	9 mm
6.	UTEC31	NP	NP
7.	UTEC40	10 mm	NP
8.	UTEC48	9 mm	NP
9.	UTEC53	10 mm	9 mm
10.	UTK2	17 mm	13 mm
11.	UTK12	13 mm	8 mm
12.	UTK15	12 mm	8 mm
13.	UTK21	11 mm	9 mm
14.	UTK30	13 mm	8 mm
15.	UTK36	9 mm	7 mm
16.	<i>E.coli</i> ATCC 25922	16 mm	18 mm
17.	<i>K.pneumoniae</i> ATCC 700603	11 mm	10 mm

CF- Ciprofloxacin. NP-Not Predictable

Table 3. Chemical screening of the bioactive compound EF2

Biochemical analysis for	Observation	Result
Terpenoids	No reddish brown colour at the interface	Negative
Diterpenes		
Copper acetate test	No emerald green colour	Negative
Alkaloids		
Dragendorff's test	No red precipitate	Negative
Wagner's test	No brownish/red precipitate	Negative
Carotenoids	No blue colour ring at the interface	Negative
Flavonoids		
Alkaline reagent test	Development of yellow colour change upon the addition of 20% NaOH and disappearance of colour upon addition of acid. Yellow colour precipitate	Positive
Lead acetate test		Positive
Anthraquinones	No rose- pink colour	Negative
Glycosides		
Modified Borntrager's test	No red-pink colour	Negative

Figures

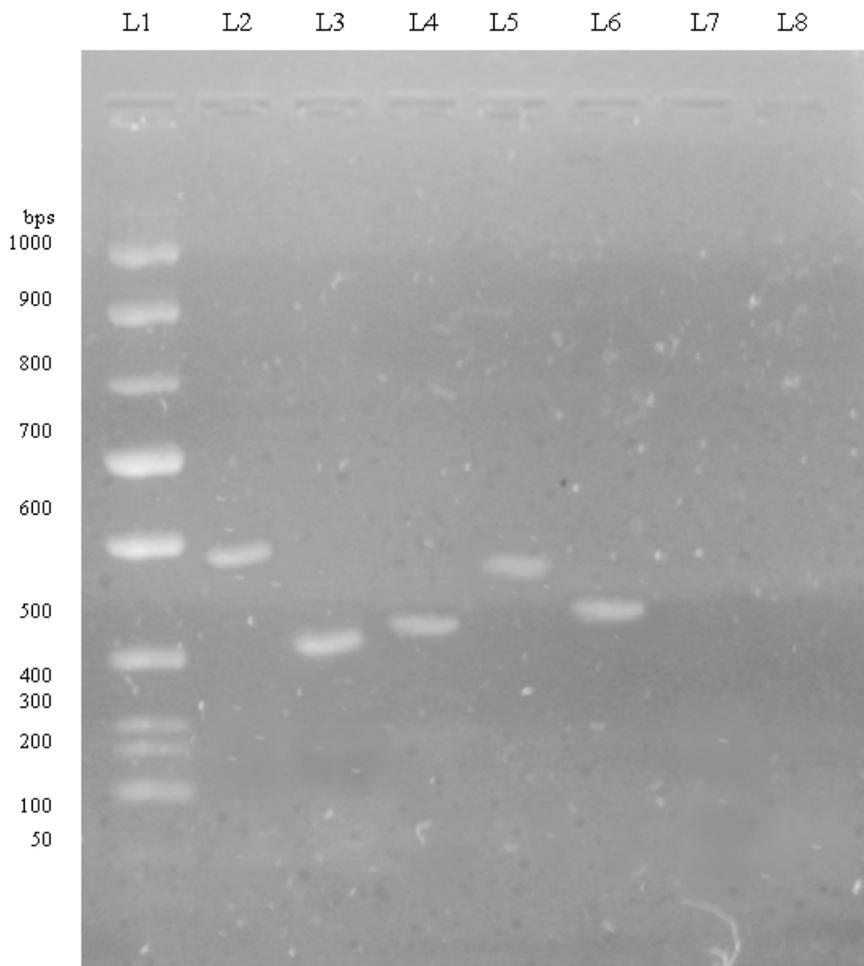


Lane-1: 1kbDNA ladder
 Lane-2: UTEC22
 Lane-3: UTEC53
 Lane-4: UTEC48
 Lane-5: UTEC57
 Lane-6: UTEC31

Lane-1: 1kbDNA ladder
 Lane-2:UTK12
 Lane-3:UTK15
 Lane-4:UTK21
 Lane-5:UTK36
 Lane-6:UTK2

Figure 1

Plasmid profile of Klebsiella species demonstrated the presence of 4 plasmids among five isolates namely UTK12, -21, -22, -36, -38 and three plasmids among the isolates UTK1, -2, -15, -20, and -30. Three isolates namely UTK3, -16, -29 harbored two plasmids while UTK33 showed a single plasmid. The plasmids were in the range between 4000 bp and >1 kb

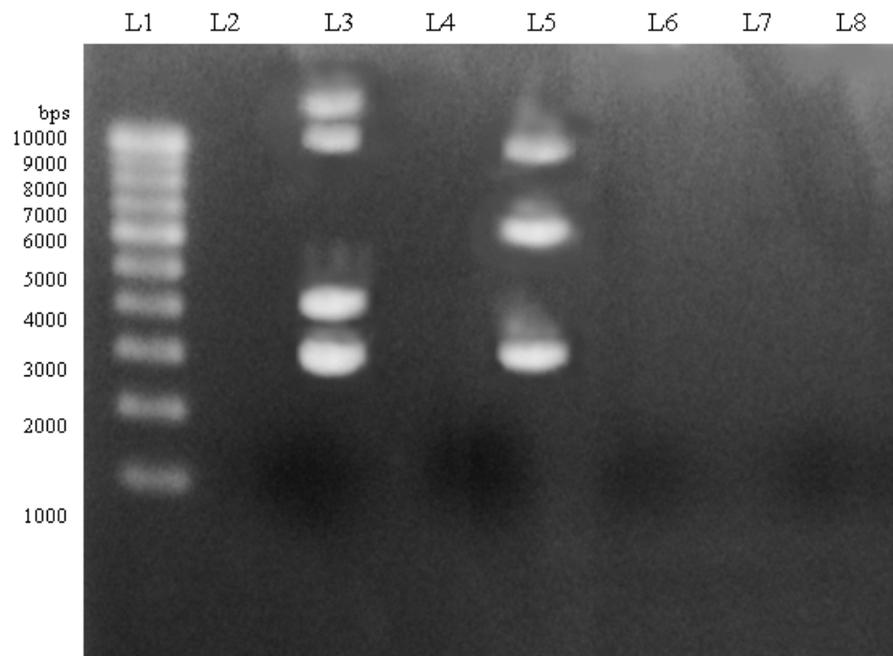


Lane 1. Marker 100bp DNA ladder (Bangalore Genei)
 Lane 2. 588bp
 Lane 3. 520bp
 Lane 4. **543bp** (GenBank Strain)
 Lane 5. **568bp** (GenBank Strain)
 Lane 6. 540bp
 Lane 7. *E. coli* MTCC443 – no amplicon
 Lane 8. PCR negative control.

Figure 2

The CTX-M genotypes have gained clinical and epidemiological importance in the context of their ability to disseminate and causing outbreaks [31]. In India, the bla CTX-M-15 genotype is reported to be found exclusively [32]. Hence the bla CTX-M gene mediated ESBL production among the isolates was determined by using uniplex PCR. The overall prevalence of bla CTX-M genes in the isolates was 36.58%

where 9 out of 27 *E. coli* (33.33%) and 6 out of 14 *Klebsiella* sp. (42.85%) carried the *bla* CTX-M genes. The size of the amplicons ranged between 500-600 bp



- Lane 1. Marker 1 Kb DNA ladder (Bangalore Genei)
- Lane 2. No plasmid
- Lane 3. Multiple plasmids - >1Kb, =1Kb, 4000bp, 3000bp
- Lane 4. No plasmid
- Lane 5. Multiple plasmids - 9000bp, 6000bp, 4000bp
- Lane 6. No plasmid
- Lane 7. No plasmid
- Lane 8. No plasmid

Figure 3

In the transconjugation analysis, 2 out of 15 donor strains (13.33%), namely, UTEC31 and UTK2 showed successful transconjugation in the recipient strain of *E. coli* K12J62-2 (F - rif r lac -). The recipient demonstrated acquired antibiotic resistance against ceftazidime and the acquisition of plasmids

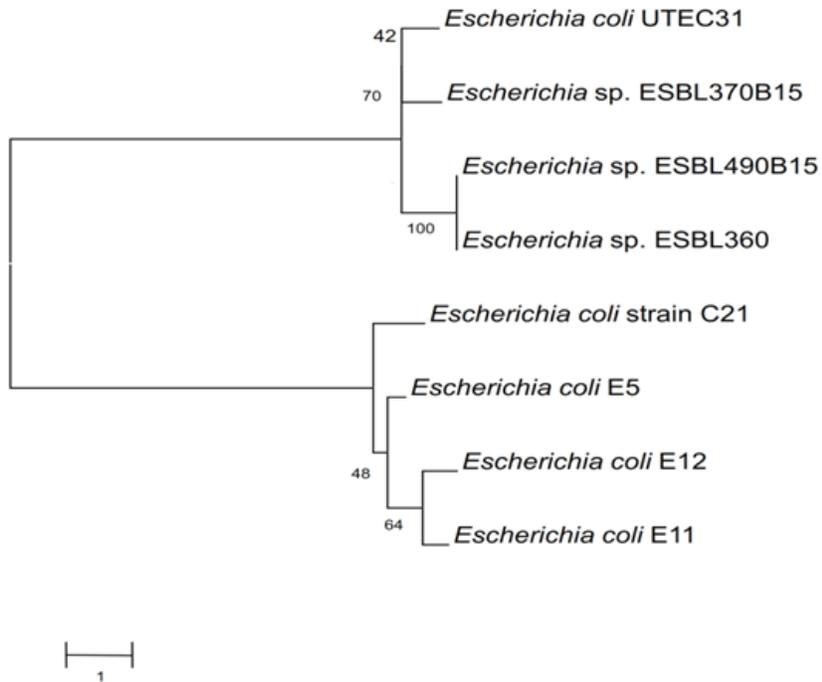


Figure 4

Phylogenetic analysis of bla CTX-M-15 gene of *E. coli* strain UTEC31 using neighborhood joining method with a boot strap value of 500 showed similarity with bla CTX-M-15 genes of *Escherichia coli* ESBL370B15, *Escherichia coli* ESBL490B15, *Escherichia coli* ESBL360 strains. Additionally, this gene also showed similarity with bla CTX-M-15 genes of *Escherichia coli* strain C21, *E. coli* E5, *E. coli* E12, *E. coli* E11 strains

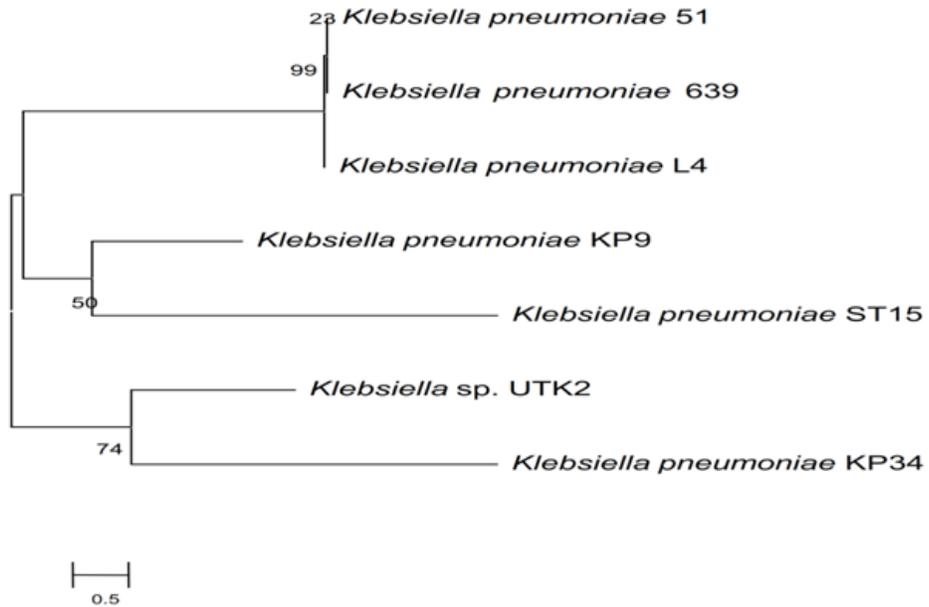


Figure 5

Similarly, phylogenetic analysis of bla CTX-M-15 of *Klebsiella sp. UTK2* based on the neighborhood joining method with a boot strap value of 500 revealed that the sequence showed similarity with bla CTX-M-15 gene of *K. pneumoniae* KP34C. Additionally this gene of *Klebsiella sp. UTK2* also showed similarity with bla CTX-M-15 genes of *K. pneumoniae* ST15, *K. pneumoniae* KP9, *K. pneumoniae* L4, *K. pneumoniae* 639 and *K. pneumoniae* 51

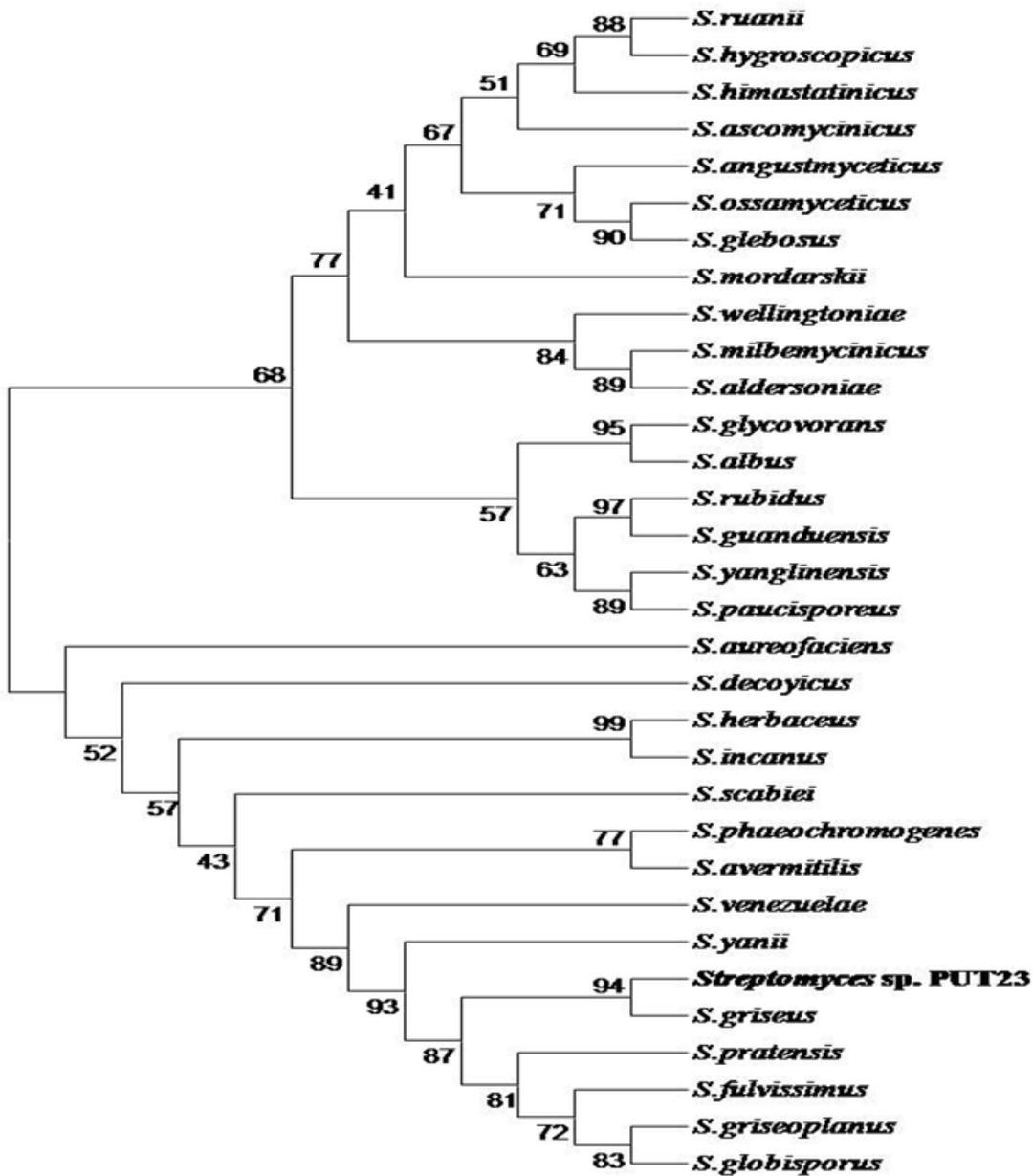


Figure 6

The amplicon size of the 16S rRNA gene of *Streptomyces* sp. strain PUT23 was around 1000 base pairs and BLAST analysis showed 100% identity to 16S rRNA sequences of *Streptomyces griseus* strains confirming the strain PUT23 to belong to the genus *Streptomyces*. The sequence was submitted to GenBank with accession number KU356755. Based on the 16S rRNA sequence, the BLAST analysis

further revealed the phylogenetic relatedness of the test strain PUT23 to its closely related strains of *Streptomyces* species obtained from the NCBI database

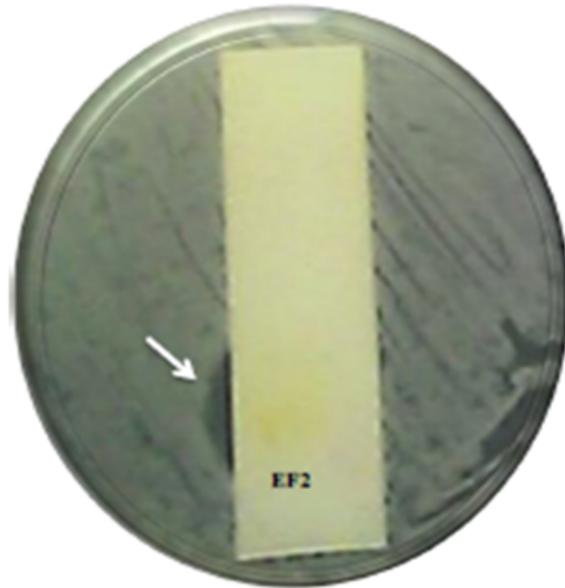
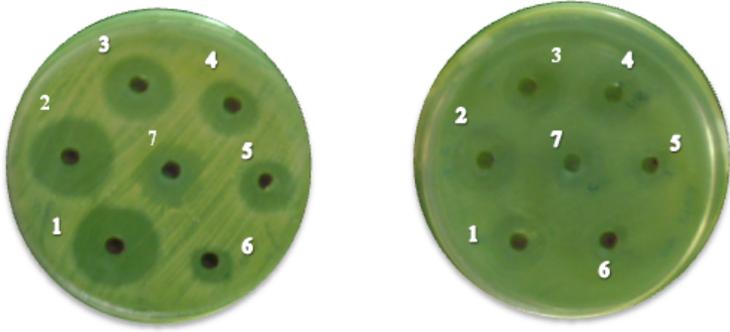


Figure 7

In bioautography, the second fraction, EF2, (Rf value - 0.73) showed activity against the bla CTX-M positive uropathogens tested



A

B



C



D

Wells 1-6: Dilutions of ethyl acetate extract of 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml respectively.

Well 7: Ciprofloxacin control (8 µg/ml)

A - *E. coli* ATCC 25922

B - *K. pneumoniae* ATCC 700603

C - *E. coli* UTEC31

D - *Klebsiella* sp. UTK2

Figure 8

The MIC of the ethyl acetate extract of bioactive component was found to be 156 µg/ml against bla CTX-M-15 producing *E. coli* and *Klebsiella* sp. which was found to increase with increasing concentrations