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Catering ESBL resistance challenge through strategic combination of Ceftriaxone, Sulbactam and Ethylenediaminetetraacetic Acid

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Abstract

Resistant development in ESBLs producing bacteria to third generation cephalosporin has emerged with alarming rapidity in recent years and become major cause of concern world wide. Therefore, in order to cater growing resistance problem, combination of third generation cephalosporin with beta-lactam inhibitors and Ethylenediaminetetraacetic acid, was studied. The in vitro antibacterial efficacy of various concentration of ceftriaxone +sulbactam and effective dose determination study of EDTA against various ESBLs producing micro organisms such as Escherichia coli (MTCC -739), Klebsiella pneumoniae(MTCC-109),Pseudomonas aeruginosa (MTCC-1688), and Staphylococcus aureus (MTCC-737) were investigated. From the AST and MIC report it was found that the 2:1 ratio has very good bactericidal activity in comparison to other ratios under study. It was found that ceftriaxone and sulbactam combination along with 3mg/ml of Disodium EDTA has significant ($p<0.001$) bactericidal activity as compared to ceftriaxone and sulbactam alone. It was concluded that the ceftriaxone +sulbactam in the ratio of 2:1 along with EDTA disodium (3 mg/ml)lowers MIC to >8 fold and possess synergy against the most ESBL producing micro organisms. The combination was found to have beneficial property against troublesome strains and might be considered as a promising therapy for severe infections to overcome resistance.

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INTRODUCTION:

Third-generation cephalosporin have a broad spectrum of activity and further increased activity

against Gram-negative organisms. They may be particularly useful in treating hospital-acquired infections, from the evidence of efficacy and safety , the third generation cephalosporin proved to be good in clinical and therapeutic conditions and mainly for the treatment of many serious infections in spite of increasing levels of extended-spectrum beta-lactamases which are reducing the clinical utility of this class of antibiotics. Resistance is a problem in the community as well as in health care settings, where transmission of bacteria is greatly amplified, in both developed and developing countries. In recent reports, 25.3–32.6% of *Enterobacter* species isolated from intensive care units in the USA and Europe were resistant to third-generation cephalosporin (TGCs) [1,2&3]. Emergence of resistance among commonly occurring bacterial pathogens has limited the utility of many penicillins, cephalosporin, and other antimicrobial classes, driving increased utilization of carbapenems for Gram-negative pathogens, and vancomycin, daptomycin, and linezolid for Gram-positive pathogens [4] .Given the recent emphasis on narrow-spectrum agents that target Gram-positive species such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Methicillin-resistant coagulase-negative staphylococci (CoNS), vancomycin-resistant enterococci, and penicillin-resistant *Streptococcus pneumoniae*, there is a paucity of broad-spectrum agents in development, which are able to simultaneously target resistant subsets of both Gram-positive and Gram-negative species[5] .The recent emergence of community-associated MRSA (CA-MRSA) is especially worrisome, given the rapidity of clonal spread that has occurred, the enhanced pathogenicity features such as necrotizing wound and pulmonary infections and the occurrence of such pathogens in populations without usual risk factors for staphylococcal infections [6,7&8] .It is well documented fact that bacterial resistance to this group of antibiotics increased parallely with increasing use of these antibiotic and created an alarming situation that currently available antibiotics are not adequate to control infection due to resistant bacteria. Streamlining empiric antimicrobial treatment has not been successful. Extended Spectrum B-lactamases (ESBLs) have more ability to deactivate antibiotics with e-lactam ring. ESBLs have serine at their active site and attack the amide bond in the lactam ring of antibiotics causing their hydrolysis. Major antibiotics included in this group are cefotaxime, ceftazidime, cefepime, cefpirome, ceftriaxone, cefpodoxime and aztreonam, but with development of resistance in bacteria they are usually inactivated [9] . Beta-lactamases open the beta-lactam ring of beta-lactam antibiotic & altered structure of drug prohibits subsequent effective binding to PBPs so that cell wall synthesis is able to continue. These can be classified into three molecular classes according to the Ambler scheme [10]. A (penicillinases, not inhibited by clavulanic acid)), B (metallo-beta-lactamases [MBLs] not inhibited by clavulanic acid)), C (not inhibited by clavulanic acid) and D (oxacillinases). The MBL variants of the types IMP, VIM, SPM-1, and GIM-1 are thus far some of the most clinically relevant, due to their ability to confer broad-spectrum B-lactam resistance, the unavailability of clinically useful inhibitors and their potential for rapid and generalized dissemination. Sulbactam act as an irreversible inhibitor of beta-lactamase activity by combining with the beta lactamase enzyme of class A & D and rendering it inactive. Therefore, beta lactam-beta lactamase inhibitor combinations offer a potential alternative to newer cephalosporin. As ESBLs are generally susceptible to available beta-lactamase inhibitors, such combinations often are seen as the only reliable antibacterials for treatment of ESBL producing bacterial infection. Such combinations have been successfully used in clinical practice to treat and manage infections, including that of resistant bacteria [11,12].MBLs(Metallo beta lactamases) are a

disparate group of proteins that make classifying and standardizing their structures virtually impossible. Attempts have been made to subdivide class B enzymes based on sequence identity and other structural features. The rationale of class B1 is that the enzymes possess the key zinc coordinating residues of three histidines and one cysteine and accommodates the transferable MBLs IMP, VIM, GIM and SPM-1. Class B2 include those possess an asparagine instead of the histidine at the first position of principle zinc binding motif, NXHXD and derive from *Aeromonas spp*. and *Serratia fonticola* enzyme SFH-1. MBL L1 is the sole occupant of class B3 enzymes, as it is singularly unique among all B-lactamases in being functionally represented as a tetramer. MBLs mediate resistance to e-lactams by cleaving the amide bond of the B-lactam ring. MBLs possess a distinct set of amino acids that define the finite architecture of active site which coordinates the zinc ions. The zinc ions in turn coordinate two water molecules necessary for hydrolysis. The principle zinc-binding motif is histidine-X-histidine-X-aspartic (HXHXD) which is common to most MBLs apart from class B2 enzymes. Without exception, the preferred metal is zinc, and while most MBLs accommodate two zinc ions in their active site, the class B2 enzymes possess just a single zinc ion. The proposed mechanism of hydrolysis suggests that active site orients and polarizes the ß-lactam bond to facilitate nucleophilic attack by zinc bound water/hydroxides. The MBLs are inhibited in vitro by $CuCl_3$, $FeCl_3$, sodium mercaptopropionic acid (MPA), 2-mercaptopropionic acid and EDTA but not by clavulanic acid, sulbactam and tazobactam. Several phenotypic methods are available for detection of MBL producing bacteria. All these are based on the ability of metal chelators, such as EDTA and thiol-based compounds, to inhibit the activity of MBLs.

Ceftriaxone is a third-generation cephalosporin which exhibits longer half life and broad spectrum of activity against Gram positive and Gram negative organisms. Sulbactam is combined with ceftriaxone or other beta lactams to enhance the spectrum of activity against ESBLs. Present study has been undertaken to compare and evaluate the efficacy and synergistic effect of a beta lactam broad spectrum third generation antibiotic ceftriaxone and a potent beta lactamase inhibitor to class A, C &D ESBLs that is sulbactam in different combination ratios and also to evaluate the effect of addition of a potent class B metallo betalactamse inhibitor that is EDTA and its salts in this potential combination, by studying antimicrobial efficacy analysis against selected organisms. Another objective of the study was to decide the most efficacious combination.

MATERIALS AND METHOD:

Bacterial Strains

The following strains obtained from Microbial Type Collection Center of Institute of Microbial Technology, Chandigarh, India were used for the study. Escherichia *coli* (MTCC -739), *Klebsiella pneumoniae(M*TCC-109*),Pseudomonas aeruginosa(*MTCC-1688*), Staphylococcus aureus(*MTCC-737*)* , MRSA used was a clinical isolate obtained from Post Graduate Institute (PGIMER) of Medical Education and Research, Chandigarh, India.

Antibiotics

The drugs Ceftriaxone and Sulbactam used in this study were provided by sponsor of the study and product manufacturer, Venus Remedies Limited, India.

Medium and reagents :

Muller- Hinton (MH) agar, Soyabean Casein Digest Broth Medium , Barium chloride and Sulphuric acid were procured from Hi – Media Laboratories Ltd., Mumbai

ANTIMICROBIAL SUSCEPTIBILITY STUDY [13,14]

Preparation of medium

Muller Hinton agar, Soyabean casein digest broth medium were prepared as per manufacturer's (Hi Media Laboratories) instructions and sterilized by autoclaving at 15 lbs pressure and 121°C for 15 minutes.

Preparation of Muller Hinton agar plates

Immediately after autoclaving of Muller Hinton agar, it was allowed to cool at about 45°C to 50°C. 20 to 25 ml of the medium were poured, into a flat-bottomed 90 mm petri dishes. The agar medium was allowed to cool to room temperature. A representative sample of each batch of plates and test tubes were examined for sterility by incubating at 35 °C for 24 hrs .

Preparation of Inoculation

At least three to five colonies of the same morphological type are selected from overnight plates cultures on non selective agar medium. The top of each colony is touched with a loop and the growth is transferred into a tube containing 10 ml of sterile Soyabean Casein Digest broth medium to produce a suspension which matches the turbidity standard of 0.5 McFarland standard.

Preparation of McFarland Standard

This standard is prepared in the nutrient broth medium. It is prepared by adding 0.5ml of 0.048 M $BaCl_2$ (1.172 % w/v $BaCl_2.H_2O$) to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) with constant stirring. Using matches cuvettes with 1 cm path length and nutrient broth medium a blank standard the absorbance in a spectrophotometer at wave length of 625nm. the acceptable range of standard is 0.08 – 0.13.

Inoculation of Test Plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This removes excess inoculum form the swab. The dried surface of a MH agar plates is inoculated with microbial culture suspension. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an ever distribution of inoculum. As a final step, the rim of the agar is swabbed. Allowed the plates to dry until there is no visible surface moisture.

Antibiotic susceptibility Testing

The comparative antibiotic Susceptibility Testing (AST) of different ratios of Ceftriaxone :Sulbactam and selected ratio of ceftriaxone:Sulbactam with various doses of EDTA were placed with the help of auto pipette. 30µl of each ratios of the combination drugs were loaded in to the well of agar plates pre-inoculated with the different test organisms. All bacterial plates and their replicates were incubated 35 °C for 24h and the lytic zone sizes were measured. Lytic zones were read with the help of antibiotic zone reader .

Minimum Inhibitory Concentration Studies

Minimum inhibitory concentration (MIC) of various ratios of Ceftriaxone and Sulbactam with and without different concentrations of EDTA and its salts against various microorganisms were studied by broth micro dilution method as per Standard National Committee for Clinical Laboratory Standards (NCCLS,1997). Overnight MH broth cultures were used to prepare inocula of 10^9 CFU/ml. The MIC was defined as the lowest concentration of antimicrobial agent that prevented turbidity after 24h of incubation at 37°C.

Statistical analysis

All values are expressed in mean SD. One- way analysis of variance (ANOVA) with student-Newman-Keuls comparison test was used to determine statistical difference between different groups under study. P values<0.05 were considered statistically significant.

RESULTS:

In antimicrobial susceptibility study, clear susceptible zone was found with all the ratios of

Ceftriaxone : Sulbactam against *K.pneumoniae, S.aureus, E.coli,* and *P.aeruginosa*. The susceptibility zones of these organisms were much higher with C:S::2:1 ratio compared with other ratios. It was observed that after keeping ceftriaxone concentration as constant and increasing the concentration of sulbactam did not show any changes in the efficacy pattern beyond a certain limit of 2:1 ratio. But, when increasing the concentration of Sulbactam alone showed more promising result in their efficacy pattern . From the observations illustrated in (Table-1)different ratios examined were C:S::1:1, 1:2, 1:3.33, 1:4, 1:6.66, 2:1, 3.33:1, 4:1 and 6.66:1 against Ceftriaxone alone, and the ratio of C:S::2: 1 is found to be the most efficacious and shows much higher zone of inhibition($p>0.001$) against all tested microorganisms. The 2:1 ratio also shows the lowest MIC values than all the other ratios compared against all microorganisms studied. At the same time when three different EDTA salts Disodium EDTA, Potassium EDTA and Magnesium EDTA were added to the most efficacious C:S::2:1 ratio and AST was observed against *K.pneumoniae,S.aureus and E.coli strains*. The Disodium EDTA was found to have bigger zone of inhibition as compared to C:S::2:1 ratio of Ceftriaxone:Sulbactam without EDTA. Result of MIC demonstrated that in all organisms studied, the ratios in which sulbactam concentration was increased beyond half the ceftriaxone concentration like C:S::1:1, 1:2, 1:3.33, 1:4 exhibited low MIC values in comparison to ratios where Ceftriaxone was increased like C:S:: 2:1, 3.33:1, 4:1, 6.66:1. Though the maximum bactericidal activity was seen in 2:1 ratio, it was apparent that increase in concentration of sulbactam or ceftriaxone beyond C:S::2:1 does not further increase in antibacterial activity of combination. When various concentration of EDTA were tested with C:S::2:1 it showed 3mg/ml dose of EDTA was found to have much better bactericidal activity against various microorganisms as compared to different concentration of EDTA used in experimentation (Table 2). Also, based on the MIC values, EDTA proved to add synergistic effect to ceftriaxone + sulbactam combination (Table 3).

DISCUSSION:

Antibacterial resistance and the worldwide spread of metallo-ß-lactamase (MBL)-producing gram-negative bacilli represents a global clinical and public health problem that has emerged with alarming rapidity in recent years and undoubtedly will increase in the near future, if appropriate measures are not taken timely. Resistant development in ESBLs producing bacteria to third generation cephalosporin has become a major cause of concern world wide. Antibiotic pressure can facilitate the selection of depressed mutant bacterial cells that produce chromosomal β-lactamases at high levels consecutively [15] .In addition, these organisms (*K pneumoniae, E coli)* possess the ability to develop resistance to the administered β-lactam antibiotic and even cross-resistance to other β-lactam antibiotics during β-lactam antibiotic therapy[16&17]. Clinical isolates of *E. coli* and some strains of *K. Pneumoniae*, *Klebsiella oxytoca*, *P. mirabilis*, and *Citrobacter freundii* are although the inhibitor-resistant TEM variants are resistant to inhibition by clavulanic acid and sulbactam alone, thereby showing clinical resistance to the beta-lactam—lactamase inhibitor combinations of amoxicillin-clavulanate (Co-amoxiclav), ticarcillin-clavulanate, and ampicillin/sulbactam. They normally remain susceptible to inhibition by tazobactam and subsequently the combination of piperacillin/tazobactam, although resistance has been described. These multi drug resistant organisms are a great threat to medical community. Therefore, in order to overcome growing resistance problem, combination of ceftriaxone+sulbactam+ EDTA is recommended for the management of ESBL infections and to overcome the resistant pattern such as with beta lactamase inhibitors including

MBL. The newest NCCLS guidelines recommend screening *Klebsiella* sp and *E.coli* isolates with a MIC greater than or equal to 2 µg/ml against cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone (order of preference for greatest sensitivity of detecting ESBL in North American strains) as potential ESBL producers [18] . It has been proved from the AST and MIC data that increasing the concentration of Sulbactam by keeping Ceftriaxone as constant does not showed any changes in efficacy pattern where as, increase in the concentration of ceftriaxone by keeping Sulbactam as constant showed drastic changes in its efficacy pattern and also among various ratios used 2:1 showed best efficacy pattern with clear and higher zone of inhibition. Further three different EDTA salts that is Disodium EDTA, Potassium EDTA and Magnesium EDTA with 2:1 ratio against *K.pneumoniae,S.aureus* and *E.coli* showed that the Disodium EDTA has very good antimicrobial pattern with 2:1 ratio at 3mg/ml . Though in the rest of the EDTA salts and concentrations used, biggest zone of inhibition was observed at higher concentrations of EDTA potassium and magnesium which is 9mg/ml , but further increase in concentration doesn't further increase the efficacy pattern and a pleatue effect is observed. EDTA above 9mg/ml leads to dissolution difficulties, hence clear zones could not be observed at higher concentrations. From this it is evident that 3 mg/ml of Disodium EDTA showed best efficacy . From our present research the efficacy of 2:1 ratio of Ceftriaxone :Sulbactam with 3 mg/ml of EDTA proved to be more potent than the 2:1 ratio of Ceftriaxone :Sulbactam alone which in turn proved its efficacy against resistant strains. Further, based on the MIC values, EDTA proved to add synergistic effect to ceftriaxone sulbactam combination in selected organisms (Table 3).

CONCLUSION:

Problems associated with ESBLs include multi drug resistance, difficulty in detection and treatment, and increased mortality. Of all available anti-microbial agents, carbapenems used to be the most active and reliable treatment options for infections caused by ESBL isolates. However, overuse of carbapenems has lead to resistance of other gram-negative organisms (MBLs). Emergence of MBL producing clinical strains emphasizes the need in clinical practice to follow antibiotic restriction policies thereby avoiding excess use of Carbapenems and other broad spectrum antibiotics. Therefore, there is need for implementation of surveillance studies in routine cases and careful selection of antibiotics in individual cases as well. The findings of current study point towards restricting combination therapies of beta lactam antibiotics alone and use of more potent combinations with EDTA as discussed above, which have potential to reduce 16 fold MIC in resistant strains of all ESBL types from molecular class A to D. In present study different ratios of ceftriaxone and sulbactam with and without EDTA were studied to find out the most promising drug concentration which is effective against all ESBLs and it was concluded that the C:S::2:1 ratio along with EDTA disodium (3 mg/ml) lowers MIC to >8 fold and possess synergy against the most ESBL producing microorganisms. From the results of MIC and AST studies against various organisms such as *K.pneumoniae, S.aureus, E.coli,* and *P.aeruginosa* clearly show that 2 : 1 ratio of ceftriaxone and sulbactam with 3 mg/ml of Di sodium EDTA gives the best antimicrobial efficacy when compared to remaining combination ratio and proved its wide spectrum of antimicrobial activity and therapeutic importance in the treatment of infections caused by ESBL producing organisms under study. To decide exact dose of EDTA disodium that would be beneficial for combination , another detailed study is required to cover different aspects of EDTA addition, hence this was considered as the limitation of current

study. Authors recommend use of Ceftriaxone+Sulbactam +Disodium EDTA to be the most synergistic combination to cater ESBL resistance to all molecular classes from A to D including carbapenem resistant metallobetalactamases and overuse of penems can be prevented.

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Table 1: AST and MIC of Ceftriaxone and various ratios of Ceftriaxone +Sulbactam

Sr. no.	Zone of Inhibition (mm) / MIC (mcg/ml)				
	Ceftriaxone		Ratio (Ceftriaxone + Sulbactam)	Ceftriaxone + Sulbactam	
	AST (30 mcg)	MIC		AST (30 mcg)	MIC
Culture:- *K.pneumoniae* (MTCC-109)					
1	22.72	32	1:1	14.98±1.23	16
2			1:2	15.02±1.04	16
3			1:3.33	15.10±2.16	32
4			1:4	15.00±2.45	32
5			1:6.66	15.35±2.62	32
6			2:1	25.21±1.52(a)	2
7			3.33:1	24.84±1.82(a)	2
8			4:1	25.06±2.45(a)	4
9			6.66:1	25.18±2.1(a)	4
Culture:- *S. aureus* (MTCC-737)					
1	19.15	16	1:1	15.67±2.62	16
2			1:2	15.88±1.58	16
3			1:3.33	15.73±2.6	16
4			1:4	15.67±1.42	16
5			1:6.66	15.81±2.12	16
6			2:1	21.98±1.18(a)	2
7			3.33:1	21.23±1.6(a)	2
8			4:1	21.36±1.42(a)	2
9			6.66 :1	21.66±2.42(a)	4
Culture:- *E. coli* (MTCC-739)					
1	21.62	8	1:1	15.12±2.72	8
2			1:2	15.14±2.32	8
3			1:3.33	15.11±2.12	8
4			1:4	15.24±1.42	8
5			1:6.66	15.18±1.67	8
6			2:1	28.15±1.46(a)	2
7			3.33:1	27.86±2.12(a)	2
8			4:1	28.06±2.42(a)	4
9			6.66:1	28.11±1.64(a)	4
Culture:-*P.aeruginosa* (MTCC-1688)					
1	21.11	64	1:1	15.45±1.43	32
2			1:2	15.66±2.55	64
3			1:3.33	15.54±2.82	64
4			1:4	15.60±1.86	64
5			1:6.66	15.71±1.23	64
6			2:1	26.96±1.10(a)	4
7			3.33:1	26.74±1.42(a)	4
8			4:1	26.68±2.42(a)	4
9			6.66 :1	26.71±2.16(a)	4

Values are presented in Mean ± SD. Where (a)= $p<0.001^{***}$ highly significant, (b) = $p<0.01^{**}$ significant , (c) = $p<0.05^{*}$ less significant, (d) = $p>0.05$ non significant

Table 2. Susceptibility of 2:1 ratio of Ceftriaxone and Sulbactam with different concentrations of different EDTAs

Culture:- *K. pneumoniae (MTCC-109)*

Sr. No.	2:1 Ceftriaxone and Sulbactam with various EDTA Concentrations	Zone diameter (Average)		
		DISODIUM EDTA	POTASSIUM EDTA	MAGNESIUM EDTA
1	As such disk	19.84±2.14	18.24±1.42	17.22±2.12
2	1mg/ml	23.95±2.26$^{(b)}$	19.76±2.23$^{(d)}$	18.25±3.41$^{(d)}$
3	3 mg/ml	**28.88±2.32$^{(a)}$**	20.26±2.42$^{(c)}$	18.53±2.86$^{(d)}$
4	5 mg/ml	28.76±1.86$^{(a)}$	20.91±2.54$^{(c)}$	19.51±3.12$^{(c)}$
5	7 mg/ml	27.87±1.82$^{(a)}$	21.11±2.73$^{(c)}$	19.72±3.21$^{(c)}$
6	9 mg/ml	27.73±2.18$^{(a)}$	24.92±1.64$^{(a)}$	20.81±2.65$^{(b)}$
7	10 mg/ml	NA	24.72±1.88$^{(a)}$	20.78±1.64$^{(b)}$
8	12mg/ml	NA	NA	NA

Culture:- *S.aureus* (MTCC-737)

Sr. No.	Solvent with EDTA Concentration	Zone diameter (Average)		
		DISODIUM EDTA	POTASSIUM EDTA	MAGNESIUM EDTA
1	As such disk	19.14±1.24	17.23±3.12	16.34±1.44
2	Solvent with 1mg/ml	19.85±1.63$^{(d)}$	18.72±3.24$^{(d)}$	17.51±1.23$^{(d)}$
3	Solvent with 3 mg/ml	25.88±1.2$^{(a)}$	20.24±2.12$^{(c)}$	17.59±2.14$^{(d)}$
4	Solvent with 5 mg/ml	25.56±2.34$^{(a)}$	20.91±2.47$^{(c)}$	18.51±3.12$^{(c)}$
5	Solvent with 7 mg/ml	25.57±2.16$^{(a)}$	21.11±1.66$^{(c)}$	19.12±1.16$^{(c)}$
6	Solvent with 9 mg/ml	24.93±2.46$^{(b)}$	22.52±1.42$^{(c)}$	19.82±1.44$^{(c)}$
7	Solvent with 10 mg/ml	NA	24.55±2.82$^{(b)}$	19.78±3.12$^{(c)}$
8	Solvent with 12mg/ml	NA	NA	NA

Culture:- *E.coli* (MTCC-739)

Sr. No.	Solvent with EDTA Concentration	Zone diameter (Average)		
		DISODIUM EDTA	POTASSIUM EDTA	MAGNESIUM EDTA
1	As such disk	26.34±2.12	20.23±2.23	20.11±3.14
2	Solvent with 1mg/ml	28.86±2.42$^{(c)}$	22.61±3.43$^{(c)}$	21.31±2.86$^{(d)}$
3	Solvent with 3 mg/ml	32.52±1.41$^{(a)}$	23.34±2.52$^{(c)}$	22.11±2.64$^{(c)}$
4	Solvent with 5 mg/ml	32.50±3.2$^{(a)}$	24.51±1.26$^{(b)}$	22.25±2.33$^{(c)}$
5	Solvent with 7 mg/ml	32.06±2.16$^{(a)}$	25.23±3.22$^{(a)}$	24.81±1.62$^{(b)}$
6	Solvent with 9 mg/ml	32.166±2.32$^{(a)}$	26.12±1.46$^{(a)}$	24.62±1.18$^{(b)}$
7	Solvent with 10 mg/ml	NA	29.51±1.82$^{(a)}$	24.82±2.42$^{(b)}$
8	Solvent with 12mg/ml	NA	NA	NA

Culture:- *P.aeruginosa (MTCC 1688)*

Sr. No.	Solvent with EDTA Concentration	Zone diameter (Average)		
		DISODIUM EDTA	POTASSIUM EDTA	MAGNESIUM EDTA
1	As such disk	16.67±1.67	15.45±1.90	15.78±2.56
2	Solvent with 1mg/ml	18.23±1.45(c)	15.89±2.24(d)	16.43±2.41(d)
3	Solvent with 3 mg/ml	23.78±1.21(a)	16.12±2.78(d)	17.21±2,.21**(c)**
4	Solvent with 5 mg/ml	23.45±1.32(a)	16.83±3.01(d)	18.12±2.77**(c)**
5	Solvent with 7 mg/ml	23.9±1.98(a)	17.84±2.67(c)	18.88±2.34(c)
6	Solvent with 9 mg/ml	23.47±2.11(a)	19.32±2.88(b)	19.76±3.12**(b)**
7	Solvent with 10 mg/ml	NA	20.33±3.34(b)	20.47±2.34(b)
8	Solvent with 12mg/ml	NA	NA	NA

Values are presented in Mean ± SD. Where (a)= $p<0.001^{***}$ highly significant, (b) = $p<0.01^{**}$ significant , (c) = $p<0.05^{*}$ less significant, (d) = $p>0.05$ non significant

Table3: MIC of 2:1 ratio of Ceftriaxone and Sulbactam with 0.01M of EDTA

Drug	E. Coli (MTCC-739) MIC	K.pneumoniae (MTCC-109) MIC	P.aeruginosa (MTCC-1688) MIC	S.aureus (MTCC-737) MIC
2:1 Ceftriaxone and Sulbactam with Disodium EDTA (0.01M)	0.25	0.06	2	1

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