Development of antibiotics is one among the greatest achievements of medicine in human history (Yoneyama and Katsumata 2006). Antibiotics literally “against life” are chemical compounds produced by Actinomycetes, Fungi (or) Bacteria that interfere with some bacterial structure (or) function with no effects on eukaryotic host having the infectious agents (Walsh, 2003a). Semi synthetic modifications of natural products also resulted in the emergence of varieties of antibacterial agents such as β-lactam and macrolides, which is now included in the definition of antibiotics (Mascaretti, 2003). The global public health problem has been caused by emerging infections not yet recognized, reemerging infections experienced previously that have reappeared in more virulent forms and antimicrobial resistant bacterial infections (Hirakata et al., 1998 and Faush, 2001). Many new resistance mechanisms are increasingly reported in many countries (Pfeifer et al., 2010).

Antibiotics can be classified based on different classification schemes, but the most useful ones are on the basis of their chemical structure and mode of action. Based on the chemical structure, they are mainly classified as β-lactams, macrolides, fluoroquinolones, tetracyclines and aminoglycosides. Among this, β-lactam antibiotics is one of the largest group of antibiotics which includes, penicillin (sulphur containing penam), cephalosporin (sulphur containing cepham), natural and synthetic monocyclic β-lactams, carbapenems, oxapenams, carbacephems and oxacephams (Walsch, 2003a).

Penicillin is the first naturally occurring β lactam antibiotic to be used for the treatment of bacterial infections. Penicillin G (or) benzyl penicillin contains phenyl acetic acid and penicillin–V contains phenoxy acetic acid. Next to penicillin, cephalosporin was introduced. Cephalosporin C has a structure in its nucleus similar to that in penicillin with the exception that, the thiazolidine ring of the penicillin nucleus undergoes a specific ring expansion to form dihydrothiazine ring. Cephalosporin C itself is not a useful antibacterial drug, chemical modification of the acetoxy group (cephaloridine, cephalexim, cefexime) makes it more active (Franklin and Snow 2005a).

Although varieties of antibiotics are classified, their targets are limited. They are generally classified on the basis of their chemical structure and mode of action.

The main classes that inhibit five classical targets are:
i. Cell wall biosynthesis (β-lactams, glycopeptides)

ii. Protein biosynthesis (aminoglycosides, tetracyclines, macrolides)

iii. DNA & RNA biosynthesis (quinolones)

iv. Membrane function (lipopeptides, polymyxin)

v. Antimetabolites (sulphonomides)

Among different targets, inhibition of cell wall biosynthesis is more commonly prescribed for antimicrobial therapy since it is non toxic, relatively inexpensive and does not target the metabolic process of humans. Peptidoglycan layer is a part of cell wall component of both gram negative and gram positive bacteria. Peptidoglycan undergoes cross linking of the glycan strands by the action of transglycosidase and of the peptide strands by the action of transpeptidase (Walsh, 2003b). Bifunctional enzymes possessing both transglycosidase and transpeptidase activities are the target sites of penicillin and cephalosporins collectively known as β-lactams. β-lactams attack and acylate the active site of transpeptidase leading to the inactivation of enzymes and thus inhibiting cell wall biosynthesis (Spartt and Cromie 1998).

Although the era of antibiotics led to the optimism that infectious diseases can be controlled and prevented, infectious diseases are still the second leading cause of death worldwide and the third leading cause of death in developed countries, due the emergence of the phenomenon called antimicrobial resistance (Fauch, 2001 and Nathan 2004). Antimicrobial resistance was recognized soon after the deployment of sulphonamides in 1930’s and penicillin in 1940’s and it now appears that the emergence of antibiotic resistance bacteria is inevitable to almost every drug (Cohen, 2000). Emerging and increasing resistance to antibiotics is a global phenomenon in both developed and developing countries.

Antimicrobial resistance continues to emerge and proliferate at new sites due to the competition of bacteria with other bacteria for the same ecological niche (Palumbi, 2001). Consequently there remains a strong need for new antibiotics, particularly those directed against Multidrug resistant (MDR) gram negative bacteria in hospitals. Already some non fermenters like Acinetobacter spp. and Pseudomonas spp. are resistant to all good antibiotics and many enterobacteriaceae are resistant to all except carbapenems. It is now discovered that clinical significant resistance to all antibiotics has appeared within months to years soon after
the antibiotics is introduced into medical use (Walsch, 2000 and Livermore, 2004) with an
only exception to vancomycin which took 30 years to develop resistance (Murray, 1997).
Health care associated infections due to resistance of gram negative bacteria are the most
threatening all over the world.

Antimicrobial resistance has to be dealt with a clear understanding of its biochemical
and molecular mechanism. In general antibiotic resistance can be divided into natural or
acquired resistance. Natural resistance includes the bacteria which are intrinsically resistant
and acquired resistance is often caused by mutations in chromosomal genes, or by the
acquisition of mobile genetic elements, such as plasmids or transposons which carry
antibiotic resistance genes.

Different mechanisms of antibiotic resistance includes,

i. Reduced permeability or uptake

ii. Enzymatic inactivation

iii. Enhanced efflux

iv. Alternation or over expression of target

v. Loss of enzymes involved in drug metabolism.

Among different biochemical mechanisms of resistance, enzymatic inactivation plays
a prominent role. The destruction of penicillins, cephalosporin and carbapenems by bacteria
that produce β-lactamase is one of the most wide spread and serious forms of antimicrobial
resistance. Bacterial resistance mechanism with respect to β-lactam antibiotics is divided
between those that occur at the level of primary metabolism (altered and acquired proteins
and enzymes ) and those that occur at secondary level of metabolism (biosynthesis of altered
β-lactams that are better antagonists to altered proteins (Bentley, 1997).

β-lactamase is the enzyme that opens the β-lactam ring, inactivating the antibiotics
predate. Penicillin binding proteins and β-lactamases may have evolved from same ancestral
protein. The first plasmid mediated β-lactamase in gram negative bacteria was discovered in
Greece in 1960’s. It was named TEM after a patient Temoniera, subsequently a closely
related enzyme as discovered and named TEM-2. It was identified with biochemical
properties more common to TEM-1, but difference in single amino acid substitution with a
resulting change in isoelectric point. These two enzymes are effective against penicillin and narrow spectrum cephalosporin, such as cephalothin and cefaxolin. However, they are not effective against higher generation cephalosporin with oxyimino side chains, cefotaxime, ceftazidime, ceftriaxone (or) cefepime. A related but less common enzyme was termed SHV, because sulphhydril reagents had variable effect on substrate specificity. Spontaneous mutations lead to the progressive modifications of β-lactamases, enabling them to cope with many novel chemical variants of β-lactam (Franklin and Snow 2005c).

The complex outer envelope of gram negative cells makes them intrinsically less sensitive to many β-lactams. Both Penicillin Binding Protein (PBP) and β-lactamases are present in the periplasmic space of gram negative bacteria. In gram positive bacteria (Which lack the outer membrane) the PBPs are located on the outer surface of cytoplasmic membrane and the β-lactamases are either excreted or bound to the cytoplasmic membrane (Nielson and Lampen 1982). Many β-lactamase in E.coli are found to be non inducible and are expressed constitutively. In E. coli β-lactamase is encoded by chromosomal blaampC gene which is expressed constitutively at low levels. bla ampG and blaampR genes are inducers of bla ampC gene. There is another gene bla ampD which encodes amidase that hydrolyses the activating peptidoglycan fragment. Mutations that inactivate bla ampD result in high level constitutive β-lactamase synthesis (Negative regulation) (Franklin and Snow 2005c).

To combat the menace of gram negative β-lactamase, compounds were needed that resist both β-lactamase attack and penetrate effectively to the penicillin binding protein in the cytoplasmic membrane. The number of β-lactamase produced by different bacteria is astonishing. More than 340 such enzymes have been identified so far, with a wide range of substrate preferences for penicillin, cephalosporins and carbapenems. Clinical isolates of bacteria commonly express several different β-lactamases, thus providing a formidable array of defenses against many different antibiotics.

The classification of this wealth of enzymes is a major challenge and there are two current schemes.

(I) Ambler classification based on similarities in amino acid sequences (Ambler, 1980 and Bush and Jacoby, 2010)

Class A: Penicillinases and Cephalosporinases usually found on plasmids or transposons.
Class B: Metallo β-lactamases

Class C: Chromosomal Cephalosporinases

Class D: Oxacillinas

(II) Bush Jacoby medeiros scheme based on substrate and inhibitor profiling (Bush et al., 1995)

Group I: Chromosomally encoded cephalosporinases, poorly inhibited by clavulinic acid.

Group II: Penicillinases, cephalosporinases and carbapenemases both chromosomal and plasmid encoded that are inhibited by clavulinic acid and other active site directed inhibitors of β-lactamases.

Group III: Metallo β-lactamases unaffected by all conventional β-lactamase inhibitors.

Group IV: Limited number of penicillinases that are uncharacterized and not inhibited by clavulinic acid.

Extended Spectrum β-lactamases that confer resistance to most of β-lactam antibiotics were discovered in 1983 in *Klebsiella* spp. and are now increasing in number and variety in almost all groups of Enterobacteriacae (Dubios et al., 1995 and Jeong et al., 2004). Unfortunately following the introduction of novel β-lactams, plasmid borne enzymes TEM-1 & TEM-2 in *E.coli* underwent mutations near the active center that markedly increased their ability to hydrolyze several of these variable agents. *bla SHV*-1 which originated in *Klebsiella* spp. conferred resistance to ampicillin, eventually transferred to *E.coli* and evolved to hydrolyze novel β-lactams. More than 90 Extended Spectrum β-lactamases (ESBL) with 70 in TEM family and 20 SHV groups have been evolved with slight modifications. For example TEM-26 has two critical amino acid replacements, serine for arginine in 164th position and lysine for glutamic acid in 104th position. These changes dramatically enhance the hydrolytic efficiency of the enzyme against drugs of major importance, such as ceftazidime and cefuroxime (Spanu et al., 2002).

β-lactam carbapenems in which sulphur atom of β-lactam fused ring system is replaced by a carbon atom (Meropenam) have good stability to serine active β-lactamases and various forms of *bla TEM*. However, nowadays these carbapenems are hydrolyzed by some
unusual serine active site enzymes. Next to bla TEM and blaSHV genes that code for β-lactamases another variant called bla CTX-M is found in some gram negative bacteria. bla CTX-M type is the newly emerged ESBL and it is the predominant form in Enterobacteriaceae (Rajesh et al., 2010). ESBL producers may also carry resistance genes for other resistance enzymes such as amino glycoside transferase (Spanu et al., 2002). Recently, mechanism of resistance due to the production of cephalosporinases with broadened substrate activity has been reported among clinical enterobacterial isolates. Those extended-spectrum AmpC β-lactamases (ESACs) confer reduced susceptibility to all cephalosporins including not only ceftazidime but also cefepime and cefpirome. These enzymes are structurally related to cephalosporinases by amino acid insertions, deletions, or substitutions (Mammeri and Nordmann 2007).

Antibacterial drug resistance is generally thought to have emerged by mutations during the modern era of chemotherapy. In addition to point mutations, deletions, and insertions there is also the remarkable phenomenon of mosaic genes which arise by interspecific recombination. Genes for drug resistance are carried by composite transposon (Tn)-Tn9-chloramphenicol acetyl transferase. Genes for chloramphenicol, streptomycin and sulphonamide resistance have a combined molecular mass of 12x10^6 KDa. Genes called regulators exert transcriptional control over several chromosomal genes that confer resistance to many antibiotics by restricting access to molecular targets, MarA locus in E.coli, ramA in Klebsiella pneumoniae. Genetic loci resembling marA and ramA are wide spread among gram negative bacteria, and the regulation of Multidrug resistance by these operons is likely to be significant contribution to the overall problem of resistance in gram negative bacteria (Franklin and Snow 2005b). The genetic environment of plasmid-mediated cephalosporinase genes has remained unclear (Doi et al., 2002).

Sharing of genetic material among microbial population is relatively facile. Gram negative bacteria can indeed transfer drug resistance not only to cells of same species but also to bacteria of different species and genus. For many years, the movement of genes among plasmids and chromosomes are believed to result from classic recombination dependent on the product of bacterial recA gene and the reciprocal exchange of DNA in regions of considerable genetic homology. This permits the exchange of genetic information only between closely related genomes. Replicons, known as transposons, can insert themselves into varieties of genomic sites that often have little (or) no homology with the inserting
sequence, although such transposition events are rare, one in $10^5 - 10^7$ cells per generation (Franklin and Snow 2005b).

Mobilization of class-I transposons along with their complement of drug resistance genes occurs by non replicative transposition. Class–II transposons are mobilized by replicative process. In some transposons, drug resistance genes are arranged within structure called integrons. Both transposons associated and independent integron and containing resistance gene cassettes exchange and capture cassettes by site specific recombination. There are also conjugate transposons which are discrete DNA elements normally integrated into bacterial chromosome which encode proteins that enable excision of transposon from the chromosome and its transfer to recipient bacteria by intercellular conjugation. Conjugative transposon has been reported in *Proteus* spp. Example Tn16 – Carrying Tetracycline resistance (Franklin and Snow 2005b).

Conditions that give rise to increased number of resistance plasmid may sometimes give rise to enhanced resistance. Thus the resistance genes can be transferred through,

- Conjugate transfer (conjugation)
- Non conjugate transfer (Transduction, Transformation)

Cellular conjugation mediated by R plasmids is the major mechanism for the spread of drug resistance through gram negative bacterial populations. A variety of R-plasmids have been described which carry various combinations of drug resistance genes. R-plasmid may sometime dissociate to form conjugate and resistance determinants. This is more common in *Proteus mirabilis* and *Salmonella typhimurium* rather than in *E.coli*. The larger the R-plasmid, the more the limited number of copies per chromosome will be in *E.coli*, whereas in *Proteus mirabilis* the number is much more variable and even varies during growth cycle. The adverse contribution of R-plasmid mediated drug resistance to human morbidity and mortality is undeniable (Franklin and Snow 2005b).

Genetic information of antibiotic resistance is also transferred by phage particles from one bacterium to a related phage susceptible cell. Although transduction of drug resistance determinants is readily demonstrated under laboratory conditions, its contribution to the spread of drug resistance is natural and clinical settings are difficult to quantify. Under certain conditions most genes of bacteria can absorb, integrate and express fragments of naked DNA containing intact genetic information including that for drug resistance. Only
bacteria in a state of competence are able to absorb and integrate exogenous DNA into their own genome. The complexity and diversity of the transformation system indicates its evolutionary importance in the exchange of genetic information in the bacterial world. A specific example with the relevance of transformation to drug resistance is illustrated by the existence of mosaic genes for penicillin binding proteins with diminished affinity for β-lactam antibiotics (Franklin and Snow 2005b).

The problem of antimicrobial resistance is encountered more in two infections, namely, nosocomial infections and urinary tract infections. A urinary infection is a bacterial infection in any part of the urinary tract. Although urine contains a variety of fluids, salts and waste products it does not usually have bacteria in it. Urinary tract infections have been reported to affect up to 150 million individuals annually. Urinary tract infections result in approximately 80 million physician visits every year and more than 100,000 hospital administrations per year in United States (Sahm et al., 2001). Urinary tract infections (UTIs) are one of the most frequent bacterial infections in industrialized countries (Simon et al., 2006). The pathogens causing UTI are almost predictable with *E.coli* among the primary etiological agent among both outpatients and inpatients (Zhanel et al., 2000).

Despite the availability of antibiotics urinary tract infections remain the most important ones in human population (Sharma, 1997). Antibiotics are given empirically before the laboratory results of urine culture are available. For appropriate therapy current knowledge of the organisms that cause infections and their antibiotic susceptibility is mandatory (Greenberg, 1984).

Urinary pathogens are increasing its resistance pattern now and then with the emergence of Multidrug resistance isolates. The prevalence of *E.coli* resistance to SXT worldwide varies considerably in published reports ranging from approximately 18-20% (Jones et al., 1997, Winstanley et al., 1997 and Sahmet al., 2001). Resistance to SXT may complicate the management of urinary tract infection (Raz et al., 2002). James et al. (2003) observed that trimethoprim – sulfamethoxazole (SXT) acts as initial therapy for females with acute uncomplicated bacterial cystitis in settings where the prevalence of SXT resistsancedoes not exceed 10 to 20%. In vitro surveillance data published from countries across United States and Canada indicate that approximately 10-20% of urinary tract isolates of *E.coli* from female outpatients are resistant to trimethoprim sulphmethoxole (SXT) (Zhanel et al., 2005b).
Regional variability in resistance to single and multiple antimicrobial agents increase over time and the predictability of the organisms causing UTI, in vitro susceptibilities play an important role in therapy of UTIs through local, regional and national surveillance programs. Routine monitoring of antibiotic resistance provides data for antibiotic therapy and resistance control participation programs making policy decisions and accessing the effectiveness of both (Omigie et al., 2006). Emergence of antibiotic resistance in bacterial pathogens should be followed with the discovery of novel antibiotics and new strategies for extending the life of antibiotics such as new combinations. These challenges can be made only by a clear understanding of fundamental biology of pathogens, their resistance mechanism and host pathogen interactions (Yoneyama and Katsumata 2006).

Different strategies have been developed to overcome antibiotic resistance. One among them is the development of β-lactamase inhibitors. Some of early β-lactamase stable β-lactams like cloxacillin have a degree of inhibitory activity against β-lactamases. Naturally occurring inhibitors of these enzymes are clavulanic acid, sulbactam and tazobactam that opened the way of effective synergism with β-lactamase susceptible drug like amoxicillin and ampicillin. Although clavulanic acid is a remarkable effective inhibitor of many β-lactamases of gram positive and gram negative bacteria, it reacts irreversibly with the active site serine β-lactamase to form stable enzymatically inactive complexes and bacterial evolution is again proving equal to the challenges of inhibitors of β-lactamase. Mutant forms of enzymes are available which are highly potent. Numerous inhibitor resistance forms of TEM β-lactamase have been identified. For example, replacement of methionine at position 69 by the aliphatic amino acids isoleucine, leucine (or) valine and arginine at position 244 by serine (or) cysteine caused marked increased resistance to inhibitors (Franklin and Snow 2005c).

Other bacteria resistant to combination of β-lactamase inhibitors with susceptible β-lactams are characterized by over production of β-lactamase which overwhelms the protective capacity of inhibitors. The clinical threat posed by the metallo β-lactamase continues to devise inhibitors of these enzymes. Unfortunately at present these are no useful inhibitors of metallo β-lactamases, although there are some promising developments of using plant secondary metabolites at the laboratory stage. Natural flavanoids in plant metabolites can be used to inhibit β-lactamase in clavulanic acid resistant E.coli (Naoualet et al., 2011).

The use of botanicals and nutritional supplements for medicinal purposes has been increased dramatically in the last two decades (Shahnaz et al., 2009). Screening of plants for
phytochemicals such as alkaloids, terpenoids and flavanoids have been accelerated to discover novel antimicrobial drugs (Pathmanathan et al., 2010). Allium vegetables, particularly garlic exhibits a broad antibiotic spectrum against MDR gram positive and gram negative bacteria (Sivam, 2001). A striking aspect of the activity of garlic is their apparent inability of most bacteria to develop resistance to it because its mode of action is completely different from that of other antibiotics. It has been proposed that the development of resistance to β-lactam antibiotics is 1000 fold easier than the development of resistance to allicin making garlic a prime candidate for therapeutic use (Jabar and Mossawi 2007).

The Indian scene is particularly grim and there is little control in the use of antibiotics. In the absence of central monitoring agency, the national scene in India with regard to antimicrobial resistance is not known. Unfortunately, majority of the doctors’ prescriptions are more generalized as they are based by the information gathered from the representatives of pharmaceutical companies, especially for the newer antibiotics. Community awareness of the issues involved in antibiotic therapy is poor and this is compounded by over the counter availability. Continued surveillance for antibiotic resistance at all levels- the hospitals, city / region, country and supra national level should be introduced. Such mechanisms are in position in the industrialized countries but the developing world is yet to invest in building them (Raghunath, 2008).

There is a need to keep a check and control on the prescription of any newly introduced broad spectrum antibiotics (or) those that target highly resistance organism, both in indoor and outdoor practices. There should be refined regulations of antibiotic registration for use with central prescribing and advertising restrictions (Raghunath, 2008; Vashishtha, 2010). A new Medical Council of India (MCI) rule that doctors must attend 30 hours of continuing medical education every five years to maintain their licenses will help encourage such courses (Sinha et al., 2007). In the absence of strict antibiotic control policy, the number of ESBL producers may exceed the non ESBL producers and complicate the therapeutic regimen (Ndugulile et al., 2005 and Spellberg et al., 2008).

The recent advance in bacterial genomics has changed the antibacterial therapeutic environment from target poor to target rich. Apart from the five basic targets in use, bacterial genomics introduces new molecular targets like aminoacyl t-RNA synthesis, Fatty acid synthesis, isoprenoid synthesis, protein secretion. Studies of the resistance mechanism in antimicrobial resistant organisms seem to be worrying and the data are not sufficient to
clearly delineate trends for specific organisms or specific antibiotics, but clearly outline resistance (Ganguly et al., 2011).

The present work is aimed to study the antimicrobial resistance in urinary bacterial isolates and analyze the β-lactamase enzyme & associated genes with the following objectives:

- To isolate and identify the bacterial isolates associated with urinary tract infection.
- To select multidrug resistant isolates based on their antimicrobial resistance pattern
- To measure the kinetic parameters of the partially purified β-lactamase of selected MDR urinary isolates.
- To screen the antimicrobial effect of selected plants against chosen MDR uropathogens
- To study the phytochemical profile of selected plants
- To analyze the role of the plant extract on the kinetic properties of β-lactamase.
- To assess the transferring ability of β-lactam resistance
- To analyse the sequence of β-lactamase specific genes
- To submit the antibiotic resistance blaTEM and blaSHV gene sequences in NCBI.
- To compare the blaTEM and blaSHV gene sequences with other sequences in databases and analyzing these gene sequences and their translated products using bioinformatic tools.