DISCUSSIONS

The present research work was taken up with a view to ascertain characters of the multiple antibiotic resistant uropathogenic gram negative bacteria especially *E. coli* with respect to various molecular and functional aspects which contribute for their virulence, atypicalness and multiple antibiotic resistant nature especially for beta lactam antibiotics by production of Extended Spectrum Beta Lactamases i.e. ESBLs. Multiple antimicrobial drug resistance has often been reported among the *E. coli* isolated from epidemics and pandemics. This character is mostly coded by transferable plasmids. These plasmids may also carry certain genes for various other factors as colicin, toxin, aerobactin production etc. which might contribute directly in the degree of virulence of the organism. A number of *E. coli* serotypes have been identified for host related diseased state caused by this organism, although a wide variation in serotypes among the isolates have been reported with emerging trend of transmissible nature of the virulent character, the serotype related host specificity becomes a lesser important feature (Arya, 2005). Hence an attempt is made in the present work to relate to various characters of *E. coli* as a pathogen and also to come across some wet lab and Insilico remedial approach towards the burning problem which is presented in the following discussion.

The clinical isolates in our study show fastidious type of growth and thus are suppose to be highly communicable. The tendency of occurrence of UTI was observed in community patient rather than hospitalized patients. The existence of UTI was more prominent in age group of 21-40 years. More no. of female patients
was located with positive urine infection. *E. coli* is found to be the most occurring pathogen in this study which was also observed by Sayah (2005), Khan (2006), Rai (2008), Kannan (2008) and Mashouf (2009). The high drug resistance was also reported by Sayah R. as 21% of isolates exhibited resistance to more than one antimicrobial agent. The drugs to which the clinical isolates under our study show maximum resistance were all beta lactams especially Ampicillin, Carbencillin, Pipracillin, Ciprofloxacin, Vancomycin, Co Trimaxazole and lower degree to antibiotics like Lomfloxacin, Sparfloxacin, Chloramphinicol and Nitrofurantoin. The similar findings were obtained by Rai in 2008 while working with *E. coli* isolated from pediatric UTI patients. The study by Khan has also observed the maximum (90%) resistance towards Ampicillin. Rammazanzadeh in 2010 observed the high degree of resistance towards Co-Trimaxazole amongst *E. coli* from UTI which was similar to our study. Tembekar has found the higher MARI indexed *E. coli* isolates amongst the clinical isolates in their study.

Minimum inhibitory concentration when determined for these 15 selected isolates, the majority of isolates show high MIC for Ampicillin and Amoxicillin. When MIC for Ampicillin was determined, three clinical isolates show lower range of MIC (0-100µg), nine cultures show moderate range of MIC (100-200µg) and three cultures show higher range (200-300µg) of MIC. When amoxicillin was considered 10 clinical isolates show lower range of MIC, four cultures show moderate range of MIC and only one MDR uropathogenic *E. coli* show MIC in higher range. Siu *et al.* in 1999 while studying bacteremic infections due to Extended Spectrum Beta Lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*
have been observed the higher level MIC for broad spectrum cephalosporins whereas Koler et al. in 2010 have found higher MIC for Fluoroquinolones and Tetracyclines whereas the less MIC was exhibited by Aminoglycosides, Meropenam, Tigecycline and Colistin when working with the clinical isolates belonging to family Enterobacteriaceae obtained from poultry. Jitsurong et al. in 2006 in his study has found higher resistance towards ceftazidime using E-strip.

The apparent virulence associated with certain groups may be mediated through other virulence factors like P fimbriae, haemolysin, serum resistance, which is commonly associated with UTI associated strains as described by Jonson in 1991. Naveen in 2005 had studied the existence of p-fimbre, type 1 fimbriae and haemplysin in uropathogenic E. coli and get the higher occurrence of p-fimbre and hemolysin production in antenatal and postnatal women than in urologic abnormalities.

Results in our study showed that a large number of urinary isolates had exhibited more than one virulence markers. Our experimental data revealed that higher Serum resistance (more than 75%) and production of hemolysin were exhibited by 46 % isolates wherein alpha hemolysin was given by 33% isolates and beta hemolysin was given by 67% isolates. The both virulence factors are also found to be correlated as observed by Siegfried in 1995 along with the higher bactericidal activity for serum. Martin et al. in 2006 has found hemolysin as the maximum occurring (96%) virulence factor. In our study haemagglutination was exhibited by 75% isolates, cell surface hydrophobicity was exhibited by 33 % isolates. This type of result was also observed in 92% of uropathogens under study of
Siegerifed. In present work, colicin production was given by 20% isolates which suppose to contribute for their virulence whereas Colicin production was observed in 21 % uroisolates was observed by Siegfried et al. in 1994. Wanderley et al. in 1996 have also observed this result for eight isolates in his study. Mendonca in 1996 has observed hemolysin production and haemagglutination production in 10 isolates amongst 18 in the study whereas he has observed 8 colicin producers amongst those 18 selected E. coli strains.

Siegfried has observed while working with E.coli in children suffering from UTI. He has observed the MRHA human erythrocytes were found in 43%, MRSH in 14% and no agglutination was found in 43% of isolates. The presence of above virulence characters is found to be alike of our results. Hemoglobin protease was produced by 33% selected MAR E. coli isolates which was also observed by Otto and Doron in 1998.

Urease activity was determined by using Urea agar which was exhibited by 40% of uropathogenic isolates. 20 isolates in the study of Arya were able to produce urease enzyme. Production of H₂S was shown by 30% of pathogenic E. coli which was also observed in the study done by Arya in 2005. Ability to utilize citrate is an atypical character which was observed by 33% uropathogens, adonitol utilization by 33% of analyzed most drug resistant isolates, dulcitol utilization by 26% of clinical isolates and Raffinose fermentation was observed by 40% of isolates. These findings are similar to that by Arya in her work related to isolation and identification of E. coli from diarrhoeic calf faeces.
Instead of doing the wet lab serotyping in the present study the biotype and serotype of the uropathogen were correlated as per the references and findings of Arya (2005) in her compilation submitted for Masters Degree. The correlation between biotype and serotype was determined for our study and the major serotypes of urinary tract infections were O157, 0128, O131, O171, 086, 076, 015, 0128, O101, O161, O162, O172 etc. The most occurring serotype was found to be O86. Siegerifed et al. had worked on 168 strains of *E. coli* isolated from cases pyelonephritis and lower urinary tract infection. Of these 168 *E. coli* strains investigated, 129 were typable with 52 monovalent O antisera. Within these 129 typable strains serogroups O6 was detected most frequently amongst 21 strains.

Double disc synergy method was used and the increased zone size around the augmentin disc was found as was observed by Tankhiwale in her study in 2004 and Sinha *et al.* in 2007. Vrábelová *et al.* had observed the major occurrence of ESBL in the clinical isolates as our study founds. ESBL determination was done by using various methods to reconfirm the ESBL production by uroisolates under the study. The standard disc diffusion method was used initially to detect the ESBL production. Phenotypic confirmatory method was also carried out and the increase in zone size around the disc containing Clavulunic acid was observed as reported by Jitsurong *et al.* in 2006. The modified disc synergy method was used where the zone shape elongation was considered as an indicator for ESBL production which was also observed by Kolar in 2010. The Disk approximation method was considered as the inhibition zone flattening of indicator disc towards the inducer disc was observed similarly like Ghatole *et al.* in 2004. The Amp C confirmation which
was done by three dimensional extract method gives the zone of inhibition in presence of crude enzyme extract as observed by Taneja 
et al. in 2008.

Vrábelová had worked on the nosocomial plasmids responsible for multiresistance of bacterial isolates from inpatient cases. In his study nitrocefin was used for the detection of beta lactamases enzyme. All isolates show production of enzyme beta lactamases as was observed in present study. Sawai et al. (1978) has worked on the iodometric Assay Method for Beta-Lactamases detection. In his study he has used various beta lactam antibiotics for enzyme Quantitation. They have analyzed the enzyme kinetics in terms of hydrolysis of various 6APA and 7APA with enzyme and alkali. The enzymatic hydrolysis gives the similar results observed in our study as enzyme stability analysis. The calculus given by Sawai et al. was used to determine the residual antibiotic in our study. Very less work has been done on beta lactamases in E. coli where as only the increased production under anaerobic conditions was studied by Rashtchian in 1979. The analysis for beta lactamases produced by Bacillus fragilis was analysed by Britz and Wilkinson in 1978. Here the extraction and purification method for the enzyme was studied. A method for the purification of the beta lactamase from strain AM78 is described using selective column chromatography. The specific activity of that purified enzyme from AM78 was found to be 3,424 U/mg i.e. about 3,000-fold that of the crude cell-associated enzyme. In our study, after the ammonium sulphate precipitation the enzyme activity was found to be 5.8 EU at 70% saturation of ammonium sulphate. In our study pooled ammonium sulphate activity was found to be 5.2 EU (1.5 fold purification). Further when we have dialyzed the enzyme
the EA was found to be 5.6 i.e. 2.2 fold purification. After desalting and vacuum concentration steps the enzyme activity was found to be 5.8 EU i.e. 4 fold purification as our experimental data suggests. Yoshiaki Fujii-Kuriyama while working on *Proteus morganii* has observed a single polypeptide of molecular weight 38,000 to 40,000 band of enzyme using sodium dodecyl sulfate-acrylamide gel electrophoresis after gel filtration on Sephadex G-100. He observed its isoelectric point as pH 7.2. No cysteine residue was found in its amino acid composition. The specific activity was 190 µmol/min per mg of the purified enzyme protein for the hydrolysis of cephaloridine; the optimal pH was about 8.5 which was found to be 8 in our study. Mayers and Shaw in 1989 has worked on New methods for the production of consistently high levels of metal-dependent/\beta-1\-lactamases (\beta-1\-lactamhydrolase, (EC 3.5.2.6) from strains $69/H/9$ and $/B/6$ of *Bacillus cereus* are described which have significant advantages over those reported previously.

The efflux of the drug was also been considered. Elizabeth and Jean-Marie in 2002 had considered the AcrAB -TolC Efflux Pump Contributes to Multidrug Resistance in the Nosocomial Pathogen *Enterobacter aerogenes*. The efflux action was monitored by considering the alterations in the drug (Amoxicillin) conc. inside and outside of the cell of Y3. The Amoxicillin conc. inside and outside was determined by HPLC and the flow of antibiotic was determined as the measure of efflux pump activity exhibited by Y3.

After performing plasmid curing by heat denaturation alteration of resistance was observed for Amoxicillin, Mithicillin, Ceftazidime and Ciprofloxacin. There is no change in sensitivity pattern for antibiotics like Ampicillin, Pipracillin, Carbancillin,
Nalidixic acid, Cefuroxime and Norfloxacin. Noor et al. in 2004 has analyzed split role of plasmid genes and the location of the pesticide resistance marker were detected by ETBR and Acridine orange plasmid curing method. The bacterium lost the property to grow on the nutrient agar containing 10mg/mL “chlorpyrifos” after acridine orange mediated curing. Noor et al. in 2004 has analysed the Gentamicin, kanamycin and streptomycin resistance markers were cured after Acridine orange curing in one urinary tract infection isolate while chloramphenicol and kanamycin resistance was lost in another isolate. However, ampicillin and tetracycline resistance determinants were not lost in any of the selected isolates.

We have used the alkaline lysis method for isolation & purification of extra chromosomal genetic material of Y3. This was then subjected to restriction digestion using enzymes like as Eco RI, Hind III, and Bal HI. Three fragments were generated when the plasmid was digested with EcoRI and two fragments were observed for Hind III whereas there is no fragmentation was observed for Bal HI.

After performing RFLP analysis of the plasmid with RFLP kit GeNei, it was observed that the banding pattern of plasmid was very much similar to lanes I and 6 as well as test plasmid from the kit in the lane numbers 4 and 5. The pattern of plasmid bands from lane 3 showed different banding patterns which were similar to that of cured plasmid. Naiemi et al. (2006) had obtained the similar results when pattern was obtained by digestion with EcoRI and 1.0% Agarose Tris-borate-EDTA gel electrophoresis. Plasmids from all strains isolated from an outbreak from ICU in Amsterdam had identical EcoRI RFLP patterns.
The other molecular markers for specific gene coding region were detected by using Polymerase Chain Reaction. Pérez-Pérez and Hanson in 2002 has confirmed the plasmid mediated expression of Amp C type of beta lactamases by PCR. Amongst various characters in our study, integrase activity was able to find the annealing site on plasmid whereas primers coding for Class I beta lactamases and Aerobactin production were unable to show their presence. Detection of integrone coding region, aerobactin production and coexistence of class A ESBL along with class C ESBL were determined by sequence annealing with respect to palindromic sequence was done using PCR. In their study, Trac et al. in 2005 have confirmed the plasmid born AmpC nature of E. coli using RT PCR and found that cefoxitin resistance in test strain was due to over expression of Amp C caused by an increase in promoter strength. Al agamy et al.in 2009 has confirmed the high rate of ESBL in K. pneumoniae clinical hospitals in Riyadh using PCR.

Determination of transferable drug resistance was done by Transformation where transformed cell of Recipient DH5α show various characters of donor i.e. multiple drug resistant clinical isolate. The transformation efficiency using Am plates was 3.469, where as 0.036% was the percentage efficiency of CaCl₂ transformation. Corliss et al. in 1981 had worked on R plasmid transfer from E. coli using K-12 as recipient strain at frequencies of $5 \times 10^{-7}$ or less. Every transformant in our study show an acquisition of Am resistance (50 µg /ml). Similar type of study was carried out by Noor N. in 2004 and the transformation efficiency observed for E. coli plasmid was $230 \times 10^{-7}$. 


Various plant parts were analysed for their antimicrobial and enzyme inhibition activity. They were *Terminilia chebula, Terminilia belirica, Acorus calamus, Morjnga olifera, Terminelia cordifoliea, Aegle marmelos, Himidesmus indicus, Terbulus terreutris, Andrographis panicalata*. In their study Sharma *et al.* (2009) have analysed the antimicrobial activity of *Corriander sativum, Abutilon indicum, Boerhavia diffusa Andrographis paniculata, Plantago ovata, Bacopa monnieri, Bauhinia variegata, Flacouratia ramontchi, Embelia tfsgerium, Euphorbia ligularia, Zinziber officinale, Terminalia chebula, Azadirachta indica, Ocimum sanctum* and *Cinnamomum cassia* against UTI isolates including *Proteus mirabilis, Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Enterobacter cloacae, Providencia pseudomallei, Pseudomonas aeruginosa* and *Klebsiella oxytoca* by disc diffusion method. Their studies concluded that crude extracts of the selected plants especially the acetone and ethanol extracts exhibited significant activity against UTI pathogens.

The plants selected in our study were authenticated, processed and their crude extracts using various organic and inorganic solvents were obtained in the dried form as were obtained by Chattopadhyay *et al.* in 2009. DMSO is used as an inert solvent which was also used by Ahmad in 2007 while working against ESBL producing enteric bacteria. In our study these crude extracts were then analysed against the Multiple Antibiotic Resistant isolate *E.coli* Y3 for obtaining their antimicrobial activity.

Amongst the plants selected, *Aegle marmelos* is found to be antimicrobial in the form of water and acetone extract against Y3 in this study giving (28%) of activity. Venkateshan *et al.* in 2009 has
observed the antimicrobial activity for ethanol extract of this plant against NCIM 2065 *E. coli*. Rajasekeran in 2008 has obtained the chloroform extract as the most effective against the selected gram negative and positive isolates under his study.

When *Terbulus terreutris* is considered, in our study it gives moderate activity i.e. only the IPA extract is able to show antimicrobial activity. In our study *E. coli* Y3 shows higher degree of sensitivity towards various extracts obtained from *Terminalia chibula*, *Tinospora cordifolia* and *Andrographis paniculata*. The study carried out by Sharma *et al.* (2009) has showed the antimicrobial capacity of *Andrographis paniculata* as an acetone extract against the *E. coli* under their study. This finding was similar to our results where the acetone as well as water and ethyl acetate extract exert their antimicrobial activity. *Tinospora cordifolia* (43%) show moderate antimicrobial activity as observed by Ghosh *et al* in 2003.

Ethanol, acetone, IPA and Ethyl acetate extracts of *Acorus calamus* shows the antimicrobial activity. Its invitro antifungal activity was detyermined by Begem *et al.* in 2004 against *Macrophomina Sp.* Petroleum ether, ethyl acetate and chloroform extract of *T. belirica* has antimicrobial activity. Elizabeth in 2005 has observed antimicrobial potential against human pathogens and found the methanol extract as the potential antimicrobial potion. *Hemidesmus indicus* has shown the antimicrobial for ethanol extract as observed by Iqbal *et al.* in 2007.

None of the extract obtained from *Morjinga olifera* seeds show antimicrobial activity against ESBL positive *E. coli* Y3. Whereas
Rahman in 2009 has found the effectivity of leaf ethanol extract against gram negative and gram positive organisms (collected from ICDDR Bangladesh) under their study except *S. aureus*.

Sharma *et al.* in 2009 has also observed the good antimicrobial activity for *Terminilia chebula* i.e. hirda/ Haritaki against their test *E. coli* as observed in our results. The similar analysis was when done by Chattopadhyay *et al.* in 2009, the alcohol extract was found to be the most effective whereas water extract has least value against trimethoprim-sulphamethaxazole resistant uropathogenic *E. coli* results. Chang and Lin in 2012 have analysed the phytochemical Composition, Antioxidant Activity, and Neuroprotective Effect of *Terminalia chebula* Retzius. Aneja (1993) has analysed the antimicrobial activity of Hirda against dental carries and found the antimicrobial potential of ethanolic, hot aqueous, cold aqueous and methanolic extracts of Haritaki. The plant Hirda (*Terminilia chebula*) in our study was selected for further analysis as it yields maximum antimicrobial activity in the individual extracts and with the antibiotic Amoxicillin it show major activity. The aqueous and petroleum ether extract of *Terminilia chebula* were came up as potent bioactive extracts as combinational therapy since they give improved antimicrobial activity in presence of Amoxicillin giving comparable zone of inhibition to that with Amoxicillin Clavulanic acid which can be an non conventional way to tackle the problem. The extracts were also investigated for their activity in presence of the extracted beta lactamases enzyme from clinical isolate Y3.

The fruit of Hirda was analysed for the phenotypic inspection and was found to be having characteristic odour, yellowish brown color, ovoid shape, 3.5-4.0 cm length, 1.5-2.0 width, astringent-bitter
taste and longitudinally wrinkled surface. The standardization of Hirda fruit powder was also carried out for ash value as well as for phytochemicals.

The extracts were then investigated by HPLC analysis and wet lab chemical identification. Above results indicates the presence of Gallic acid as one of the component of Hirda extract. The enzyme inhibition activity of herbal extract was found to be comparable with Clavulunic acid, the available marketed inhibitor when estimated with HPLC analysis. Further the extract formulation study in the form of cream and ointment as on the lab experimental level was done. The MIC for the formulation against the selected isolate Y3 under laboratory conditions was calculated for both cream and ointment. Cream was found to be more effective (no viable growth with 175μg/ml water extract cream formulation + 200 μg/ml Amoxicillin) whereas for ointment only the depletion of growth was observed even at 200μg/ml water extract cream formulation+ 200 μg/ml Amoxicillin.

When an in-silico analysis was applied in our study it was of interest to see whether Gallic acid molecule competes with Amoxicillin molecule for active site of Class C β-lactamase. This could be thus the novel approach to come up the problem of ESBL exhibiting urinary infections.