IN VITRO-STUDY OF EFFECT OF HERBAL SYRUP OF MEDICINAL PLANT EXTRACT AGAINST ESBL PRODUCING KLEBSIELLA PNEUMONIA CAUSING UTI INFECTION

V. Singh*1, Sandip Patil2, Shakti Pal3 and P. K. Chauhan3

*1Dept. of Microbiology, Himachal Institute of Life Sciences, Paonta Sahib (H.P.) India.
2Dept. of Microbiology, faculty of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Solan (H.P.) India
3Dept. of Biochemistry, Himachal Institute of Life Sciences, Paonta Sahib (H.P.) India

E-mail of Corresponding Author: virender_micro83@yahoo.co.in

Abstract:
This study was carried out from 8th Jan 2010 to 8th Feb 2011. A total of 60 consecutive Klebsiella recovered during the study period in 100 urine sample of UTI patients. 22 isolates were ESBL producer and 38 isolates were non-ESBL producers. The prevalence of extended spectrum β-lactamase producing Klebsiella in urine sample of UTI patients was 22%. Detection of extended spectrum β-lactamase producing Klebsiella in urine sample of UTI patients was carried out by double disc diffusion method on Muller Hinton Agar. A susceptibility disk containing Piperacillin/Tazobactum was placed as the inhibitor of β-lactamase in the center of the plate, Piperacillin were placed 30 mm from the Piperacillin/Tazobactum disk. Enhancement of zone of inhibition of disc of Piperacillin alone towards the disc containing Piperacillin/Tazobactum, showing a figure of eight impression were considered as ESBL producer. All recovered isolates were resistant against ampicillin, amoxicillin, ceftazidime, ceftriaxone, tetracycline, chloramphenicol, gentamycin, and cefotaxime and sensitive against impenem, amikacin, and ciprofloxacin and meropenem. Antimicrobial activity of medicinal plants Euphorbia heterophylla and Acalypha indica were assessed for ESBL producing Klebsiella pneumoniae.

Key words: ESBL; UTI; β-lactamase and Antimicrobial activity

1. Introduction:
Nosocomial outbreaks are often caused by ESBL-producing isolates, particularly in intensive care, they result from the clonally transmission of epidemic isolate and/or the horizontal transfer of resistance genes1. Many and regular studies on ESBL-producing bacteria are conducted in numerous countries, whereas very few information on this issue is available in Iran. ESBLs are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime, and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephapemycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., imipenem or meropenem). These ESBLs are commonly inhibited by lactamase-inhibitors such as clavulanic acid, sulbactam and tazobactam. ESBLs were first identified in 1983.Since that time, these have been identified worldwide and have been found in a number of different organisms, including Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Morganella morganii, Serratia marcescens, Shigella dysenteriae, Pseudomonas aeruginosa, Burkholderia cepacia, Capnocytophaga, Citrobacter species, and Salmonella species. Guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) recommend screening all K. pneumoniae, K. oxytoca, and E. coli for which MICs of cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, and aztreonam are 2µg/ml. The organism may produce ESBL against anyone or more of the above antibiotics. Various conventional or automated laboratory methods are available to detect this. Among gram-negative bacteria, the emergence of resistance to extended-spectrum cephalosporin has been a major concern. Treatment of infections caused by ESBL-producers is complicated not only by resistance to extended-spectrum cephalosporin, but also because many ESBL genes are on large plasmids containing genes which also encode resistance to many other antibiotics including aminoglycosides, chloramphenicol, sulfonamides and tetracycline antibiotics. The present study made an attempt to find out the antimicrobial activity of Euphorbia heterophylla and Acalypha indica against ESBL positive
Escherichia coli and Klebsiella pneumoniae isolates of urinary tract infections collected from ICU patients.

2. Materials and Methods:
2.1 Sample Collection: Samples of urine of UTI patients were collected from Dr. Puran Chand hospital, Paonta Sahib (H.P.). The samples were collected aseptically in sterile 50 ml Oakridge tubes. The samples received were initially inoculated on Nutrient agar and MacConkey Agar. A measured amount of urine using inoculating loop was inoculated to Nutrient agar medium for colony count. Equal or more than 10⁴ CFU/ml of single potential pathogens interpreted as positive UTI. A less than 10² CFU/ml was interpreted as negative UTI.

2.2 Isolation and Identification of Bacterial strains: The isolates obtained were identified on the basis of colony morphology and biochemical reactions.

2.3 Antibiotic Sensitivity Test: The prevalence and antimicrobial sensitivity and resistance pattern of bacteria causing UTI infection was detected by Kirby Bauer disc diffusion method, on Muller Hinton Agar. The microorganism suspensions used for inoculation were prepared at 10⁷ cfu (colony forming units)/ml by diluting fresh cultures at McFarland 0.5 density (10⁸ cfu/ml). Ten several antibiotics (Hi-media) were used for the antibiotic sensitivity test. Standardisation of the technique controls variation in results and interpretation is based on comparison of inhibition zones with published criteria for zone diameters.

2.4 Double-Disc Diffusion Test: Detection of extended spectrum β-lactamase producing Klebsiella in urine sample of UTI patients was carried out by Double disc diffusion method, on Muller Hinton Agar. 0.1ml of the multidrug resistant organisms was spread on the surface of the Mueller-Hinton agar plate using a sterile swab. A susceptibility disk containing Piperacillin\Tazobactum was placed as the inhibitor of β-lactamase in the center of the plate, Piperacillin were placed 30 mm from the Piperacillin\ Tazobactum disk. Enhancement of zone of inhibition of disc of Piperacillin alone towards the disc containing Piperacillin\ Tazobactum, showing a figure of eight impression were considered as ESBL producer. All recovered isolates were resistant against ampicillin, amoxicillin, ceftazidime, ceftriaxone, tetracycline, chloramphenicol, gentamycin, cefotaxime and sensitive against imipenem, amikacin, and ciprofloxacin and meropenem. K. pneumoniae ATCC 700603 were used as control strains.

2.5 Collection of the plant material: The fresh plants leaves of Euphorbia heterophylla and Acalypha indica were collected from the surrounding areas of Paonta sahib (H.P.) and identified by Botanical survey of India, Dehradun.

2.6 Preparation of the extracts: For the preparation of plant extract the leaf of plants were dried under shade and stored into fine powder using electric blender. 50g of dried powder sample was taken and extracted by sox let apparatus using distilled water, methanol, petroleum ether and ethanol separately. The solvents were removed under reduced pressure in a rotary evaporator until they become completely dry. Filtrates were preserved at 4°c temperature.

2.7 Preliminary Phytochemical Analysis: Phytochemical screening was carried out on the powdered plant material based on standard protocol.

2.8 Determination of the Antimicrobial Activity: From the dry filtrate material, the 500 mg/ml dilutions of plant paste were prepared for antibacterial assay. The modified agar well diffusion method was employed to determine the antimicrobial activities of plant extracts. Three different extractions (Aqueous, Acetone, and methanol) were taken. In Agar well diffusion method, 100µl of the extracts (500 mg/ml) were poured into the wells. All the agar plates were incubated at 37°C. If antimicrobial activity was present on the plates, it was indicated by an inhibition zone. The diameter of the inhibition zones were measured in millimeter at 24 hours using a scale. The experiments were conducted in triplicate for each test. The mean of triplicate results were taken.

3 Results and Discussion
3.1 Isolation, Identification and Detection of ESBL producing Klebsiella pneumonia: A total of 60 consecutive Klebsiella recovered during the study period in 100 urine sample of UTI patients. 22 isolates were ESBL producer and 38 isolates were non-ESBL producers. The prevalence of extended spectrum β-lactamase producing Klebsiella in urine
sample of UTI patients was 22%. The incidence of ESBL-producing isolates varies according to countries, regions or even hospitals. The ESBLs prevalence rate in the present study was much lower than those reported from Karnataka (40.6%)\(^8\); Pune (27.8%)\(^9\); Ghana (26.3%)\(^10\), Lahore (37%)\(^11\) (2006) and Sudan (38%)\(^12\). The ESBLs prevalence rate in the present study was much higher than those reported from Tamil Nadu (21.1%)\(^13\); Iran (17.9%)\(^14\); and Egypt (16%)\(^15\). Mustafa Onur Aladag and Durak (2009) conducted a study on \textit{K. pneumoniae} isolated from urinary tract infection. Among 125 isolates of \textit{K. pneumoniae}, 45 strains (36%) produced ESBL and 80 (64%) are non-ESBL producing strains\(^16\). In France and England 14 to 16% ESBL producers among clinical \textit{Klebsiella} isolates have been reported\(^17,18\), reported that ESBL producers could explain only 36.5% of high drug resistance pathogen. Detection of extended spectrum \(\beta\)-lactamase producing \textit{Klebsiella} in urine sample of UTI patients was carried out by Double disc diffusion method, on Muller Hinton Agar. A susceptibility disk containing Piperaclln\textsuperscript{Tazobactum} was placed as the inhibitor of \(\beta\)-lactamase in the center of the plate, Piperaclln were placed 30 mm from the Piperaclln\textsuperscript{Tazobactum} disc. Enhancement of zone of inhibition of disc of Piperaclln alone towards the disc containing Piperaclln\textsuperscript{Tazobactum}, showing a figure of eight impression were considered as ESBL producer.

### 3.2 Antibiotic Sensitivity Test:

All recovered isolates were resistant against ampicillin (25mcg), amoxicillin (25 mcg), cefazidime (30 mcg), ceftriaxone (30 mcg), tetracycline (30 mcg), chloramphenicol (30 mcg), gentamycin (25 mcg), cefotaxime (30 mcg) and sensitive against imipenem (30 mcg), amikacin (30 mcg), and ciprofloxacin (25 mcg) and meropenem (10 mcg). \textit{K. pneumoniae} ATCC 700603 were used as control strains. The presence of ESBL-producing isolates is indicative of a selection pressure, the new betalactams, such as cefotaxime, are extensively used in hospital practice in our country. Most of ESBL-positive \textit{Klebsiella} spp. has been isolated from urinary tract infection and blood stream infection (6.25%). Therefore, the high rate of ESBL positive isolate with this infection might be determining the nosocomial spread of this enzyme. The antibiotics which were found to be resistant against \textit{klebsiella} were carbapenem, meropenem\(^9,19\) cephalosporin \(^21\), ampicillin\(^8,9\), ertapenem, colistin\(^19\), penicillin\(^8\), amoxicillin\(^21\), gentamicin\(^22\), ampicillin, kanamycin\(^14\), aminoglycosides\(^10\), cefoxitin\(^21\),Naldixic acid and nitrofurantoin, cefuroxime, amoxicillin, ciprofloxacin and gentamicin\(^12\) and the antibiotics which were found to be sensitive were cefotaxime, cefazidime , ceftriaxone\(^{22}\), imipenem\(^22\), tetracycline, chloramphenicol\(^19\) and amikacin\(^9\). Paterson et al. (2004) reported that ciprofloxacin resistance in 60% in ESBL positive and 13.16% in ESBL negative of \textit{Klebsiella} bacteria. There were marked geographic differences in the occurrence of ciprofloxacin resistance, resistance rates were in range of 33 to 60% of ESBL producing isolates. Ciprofloxacin resistance in \textit{K. pneumoniae} is closely associated with ESBLs. This association is of grave concern since ESBL-producing isolates are usually resistant to penicillins, cephalosporins and aminoglycosides. Therefore, ciprofloxacin resistance severely limits already restricted treatment. ESBL production rates are now very high compared with Europe Hawkey (2008) and USA Canton et al. (2008). The prevalence of ESBL producers at our study was lower in comparison to the prevalence reported from other studies. Routine detection of ESBL-producing microorganisms is required to be done by each laboratory by the standard detection methods so as to control the spread of these infections and also to institute proper therapeutic strategies.

### 3.3 Antimicrobial Activity of Medicinal Plants:

From the results of antimicrobial activity of \textit{E. heterophylla}, the range of zone of inhibition in case of acetone extract were (6-16 mm), aqueous extract (5-10 mm) and methanolic extract were (5-8 mm) as shown in slide (A). But in case of \textit{A. indica} the range of zone of inhibition in case of acetone extract were (6-13 mm), aqueous extract (9-19 mm) and methanolic extract were (5-11 mm) as shown in slide (B). In Falodun study the aqueous extract showed significant activity comparable to the reference drug used. Among
different dose range used (50, 100 and 150 mg/ml), 100 mg of aqueous extracts shows high inhibition rate than methanolic extracts\textsuperscript{24}.  

3.4 Minimal Inhibitory Concentration:  
Acalypha indica is chosen for MIC (Minimal Inhibitory Concentration) with highly susceptible to ESBL producing *Klebsiella pneumoniae*. The MIC values of *A. indica* against ESBL positive isolates of *K. pneumoniae* observed for aqueous extract was 20 mg/ml\textsuperscript{23}. observed the MIC values for methanol extract was between 1.25 and 2.5mg/ml. Medicinal plants were preferred than synthetic antibiotics due to multidrug resistance towards antibiotics. Now a day’s ESBL presence is high among worldwide especially hospitals. Among 3 solvents (water, Acetone and methanol) of medicinal plants (*Euphorbia heterophylla* and *Acalypha indica*), water extract of Acalypha indica shows maximum activity against multidrug resistant ESBL producing *Klebsiella pneumoniae*. As the multidrug resistance to antibiotics is high now days, an herbal plant plays an important role in antimicrobial activity. From the present study, it is evident that the phytoconstituents present in *Acalypha indica* were responsible for the inhibitory effect on ESBL producing *Klebsiella pneumoniae*. As water crude extracts exhibited an antibiotic potential against multidrug resistant ESBL producing *Klebsiella pneumoniae* isolated from ICU patients, it proved that traditional use of *Acalypha indica* has scientific basis.  

**Conclusion**  
From the results obtained in this work, it can be concluded that (*Euphorbia heterophylla* and *Acalypha indica*), has the potential for the production of drug for the treatment of urinary tract infections caused by antibiotic resistant *Klebsiella pneumonia*.  

After checking the toxicity of *Acalypha indica* if the toxicity value is more than the MIC value of *Acalypha indica* i.e.20 mg/ml then it will become better syrup for the treatment of urinary tract infections caused *Klebsiella pneumonia*.  

**References:**  
1. Akram Hassan Mekki, AbdullahiNur Hassan and Dya Eldin M Elsayed. Extended spectrum beta lactamases among multi drug resistant *Escherichia coli* and *Klebsiella* species causing urinary tract infections in Khartoum. Journal of Bacteriology Research 2010; 2(3): 18-21.  
2. Sasirekha B, Manasa R, Ramya P and Sneha R. Frequency and Antimicrobial Sensitivity Pattern Of Extended Spectrum Beta-Lactamases Producing E.Coli And *Klebsiella pneumoniae* Isolated In A Tertiary Care Hospital. AlAmeen Journal Medical Sciences 2010; 3 (4) :265 -271.  
3. Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. 1966. Antibiotic Susceptibility testing by a standardized single disc method. *AM J Clin Pathol.*, 45:493-6. Wayne, 2004. National committee for clinical laboratory standards. Nccls document M 100- S15. Performance standards for antimicrobial susceptibility testing. 8th ed, NCCLS.  
4. Philippon A, Labia R, Jacoby GA. Extended-spectrum beta- lactamases. *Antimicrob Agents Chemother*1989; 33:1131-6.  
5. Iroha, R., Oji, A. E. and Esimone, C. O. 2008. Antimicrobial resistance pattern of plasmid mediated extended-spectrum β-lactamase producing strains of *Escherichia coli*. *Scientific Research and Essay*, 3:215-218.  
6. Trease, G.E. and Evans, W.C.1996. Pharmacognosy, Brailar Tiriden Company, Macmillan publishers. (eds.), 56-109.  
7. Collins, C. H, Lynes, P. M. and Grange, J. M. 1995. *Microbiological Methods* (eds.),. Butter wont-Heinemann Ltd, Britain pp. 175-190.  
8. S B. Sasirekha R. Manasa, P. Ramya and R. Sneha. Frequency and Antimicrobial Sensitivity Pattern of Extended Spectrum β-Lactamases Producing E.coli And Klebsiella Pneumoniae Isolated in a Tertiary Care Hospital. Al Ameen J Med Sci (2010)3(4) : 265 - 271  
9. Amit Tagore, Sandeep Kadam, Umesh Vaidya, Jayant Deodhar, Anand Pandit Multidrug resistant *Klebsiella pneumoniae* in NICU – what next? Trend of antibiotic resistance 2010; 5: 119-124.  
10. Feglo PK, Acheampong DO, Gbedema SY. Prevalence and antibiogram of *Klebsiella* species recovered from clinical samples at the Komfo Anokye teaching hospital in Ghana. Journal of Clinical Research Letters, ISSN: 0976–7061 & E-ISSN: 0976–707X. 2010; 1(2): PP-04-08.
11. Hasan Ejaz, Aizza Zafar, Anwar N, Cheema TA, Humaira Shehzad. Prevalence of bacteria in Urinary Tract infections among children 2006; 22(19).

12. Akram Hassan Mekki, Abdullahi Nur Hassan* and Dya Eldin M. Elsayed. Extended spectrum beta lactamases among multi drug resistant *Escherichia coli* and *Klebsiella* species causing urinary tract infections in Khartoum. Journal of Bacteriology Research Vol. 2(3), pp. 18-21, August 2010

13. Lakshmi Sarayu, Shalini Maya A & Prabhakar K. A study on Prevalence and Evaluation of Clinical isolates from Community Acquired Infections using different Media in Semi-urban Areas. World Journal of Medical Sciences 2010; 5(2):49-53.

14. Ranjbar R, Hagh-Ashtiani MT, Jonaidi Jafari N, Abedini M. The Prevalence and Antimicrobial Susceptibility of Bacterial Uropathogens Isolated from Pediatric Patients Iranian Journal Public Health 2009; 38(2):134-138.

15. Tarek A Salem, Mohamed H El-Azab. Uropathogens Causing Urinary Tract Infections in Females and Their Susceptibility to Antibiotics. Uro Today International Journal 2011; Volume 4.

16. Aladag, M.O. and Durak, Y. 2009. Investigation of some antibiotic susceptibilities, plamid profiles and ESBL characteristics of *K. pneumoniae* isolated from urinary system infections. *World Applied Sciences Journal.* 6:630-636.

17. Romanus, I.I., Oji Anthonia Egwu, Afuwka Tifani Ngozi, Nwuzo Agabus Chidiebube, Ejikeegwu Peter Chika, 2009. Extended Spectrum Beta – Lactamase (ESBL) Mediated Resistance to Antibiotics Among *Klebsiella Pneumoniae* in Enugu Metropolis. *Macedonian Journal of Medical Sciences.* 2:196-199.

18. Neelam, Taneja, Pooja Rao, Jitender Arora and Ashok Dogra, 2008. Occurrence of ESBL & Amp-C b-lactamases & susceptibility to newer antimicrobial agents in complicated UTI. *Indian J. Med Res.,* 127: 85-88.

19. Mohamudha Parveen, Harish BN and Parija SC. Emerging Carbapenem Resistance among Nosocomial Isolates of *Klebsiella pneumoniae* in South India. International Journal of Pharma and Bio Sciences 2010; V1 (2).

20. Kyungwon Lee, Chang Hyun Lim, Ji Hyun Cho, Wee Gyo Lee, Young Uh, Hwi Jun Kim, DongueunYong, Yunsop Chong, and the KONSAR group. High Prevalence of Ceftazidime-Resistant *Klebsiella pneumoniae* and Increase of Imipenem-Resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. in Korea. *Yonsei Medical Journal* 2006; 47(5):634-645.

21. Shukla i, Tiwari R, agrawal A. prevalence of extended spectrum lactamas producing klebsiella pneumoniae in a tertiary carehospital. Indian Journal of Medical Microbiology, (2004) 22 (2):87-91.

22. Tarek A Salem, Mohamed H El-Azab. UIJ - Uropathogens Causing Urinary Tract Infections in Females and Their Susceptibility to Antibiotics Uro Today Int J. 2011 Feb;4(1) doi:10.3834/uij.1944-5784.2011.

23. Babu Uma, Kesani Prabhakar, and Sadayappan Rajendran, 2009. Phytochemical analysis and antimicrobial activity of *clitorea ternatea* Linn against extended spectrum beta lactamase producing enteric and urinary pathogens. *Asian Journal of Pharmaceutical and Clinical Research,* 2 (4): 94-96.

24. Falodun A, Usifoh CO and Nworgu ZAM. Smooth muscle relaxant activity of 3-carbo methoxyl pyridine from *Pyrenacantha staudeitii* leaf on isolated rat uterus. African Journal of Biotechnology Vol.5 (10), 1271-1273, May 2006.