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Original Article

Antimicrobial susceptibility of Extended Spectrum *β*-Lactamase (ESBL) producing Uropathogens isolated from ICU patients

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Abstract

Urinary tract infection (UTI) is one of the most common domiciliary and nosocomial bacterial infections prevalent in both males and females. Normally, the urinary tract proximal to the distal urethra is sterile, but it is constantly challenged by infectious pathogens fighting to gain access. . In the present study, two hundred Urine samples were collected from ICU patients suspected to have urinary tract infection at the discretion of the provider from a multispeciality hospital from Coimbatore. Isolation and identification was done based on their microscopic, cultural and biochemical characteristics..The most prevalent organism *Escherichia coli* and *Klebsiella pneumoniae* were chosen and subjected to antibiogram. Virulence factors like hemolysin production , serum inactivation, gelatinase, beta lactamase, cell surface hydrophobicity were analysed for their presence.Antimicrobial activity of medicinal plants *Euphorbia heterophylla* and *Acalypha indica* were assesed for ESBL positive uropathogens. Phytochemical screening of medicinal plants have been done with the plant extracts and the results are interpreted.
Keywords: ESBL, Urinary tract infection, Virulence factors, Medicinal plants

Introduction

The incidence of UTI is higher among females, in whom it commonly occurs in an anatomically normal urinary tract. Conversely, in males and children, UTI generally reveals a urinary tract lesion that must be identified by imaging and must be treated to suppress the cause of infection and prevent recurrence. UTI can be restricted to the bladder (essentially in females) with only superficial mucosal involvement, or it can involve a solid organ (the kidneys in both genders, the prostate in males).

Multiple drug resistance has significantly increased in recent years. The existence of enzymes of Extended-Spectrum β-Lactamases (ESBLs) producing organism that are resistant to virtually all β-lactam antibiotics have been reported (Philippon *et al*.,1989). ESBLs are plasmid-mediated class A enzymes commonly found in the family Enterobacteriaceae, mainly *Klebsiella pneumoniae* and *Escherichia coli.*

Infections caused by ESBL-producing bacteria often involve immune-compromised patients, making it difficult to eradicate these organisms in high-risk wards, such as intensive care units (Bonnet *et al*., 2000). It is necessary to investigate the prevalence of ESBL positive strains in hospitals so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher (Mathur and Kapil,2002).

The present increase in resistance to second and third general cephalosporins observed in medical institutions as a result of the acquisition and expression of extended-spectrum β-lactamase enzymes among Enterobactericeae has posed a serious public health problem. Theclinical implications are extremely serious and lack of sensitive diagnostic method needed to guide therapy, monitor resistance developments and implementing intervention strategies have complicated the problem (Bradford,2001; Sturenburg and Mack,2003).

The ESBL producing bacteria are increasingly causing urinary tract infections (UTI) both in hospitalized and outpatients. The increase of drug resistance among these organisms has made therapy of UTI difficult and has led to greater use of expensive broad spectrum antibiotics such as third generation of cephalosporin. Detection of ESBLs using conventional antimicrobial susceptibility methods and delay in the detection and reporting of ESBL production by gram-negative bacilli is associated with prolonged hospital stay, increase morbidity, motility and health care costs (Mehrgan and Rahbar,2008).

As the threat towards multidrug resistance organisms are high among all over the world especially hospitals, the recent study was focussed on ESBL positive multidrug resistant organisms and its sensitiveness towards medicinal plants. 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.

Materials and Methods

Sample Collection

A prospective study was conducted over a period of months from September 2009 to September 2010 at the Laboratory of the Department of Microbiology, at Dr.N.G.P. Arts and Science College, Coimbatore, Tamil Nadu. Two hundred Urine samples were collected from ICU patients suspected to have urinary tract infection at the discretion of the provider at Kovai Medical Centre and hospital [KMCH], A Multispeciality Hospital, Coimbatore.

Isolation and Identification of Bacterial strains

Preliminary isolation and identification was based on the microscopic, cultural characteristics and other standard biochemical analysis (Siegfried *et al*.,1994).

Antibiotic Sensitivity Test (Ast)

The most prevalent organisms were chosen and subjected to antimicrobial sensitivity test. The AST for each isolate was carried out on Muller-Hinton agar by Kirby-Bauer disc diffusion technique (Bauer *et al*.,1966). The microorganism suspensions used for inoculation were prepared at 10^7 cfu (colony forming units)/ml by diluting fresh cultures at McFarland 0.5 density (10^8 cfu/ml) (Philippon *et al*.,1989). 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 comparision of inhibition zones with published criteria for zone diameters (Wayne, 2004).

Double-Disc Synergy Test

0.1ml of the multidrug resistant organisms esp 3GC resistant organisms was inoculated on the surface of the Mueller-Hinton agar plate using a sterile swab sticks. A combination disc containing (amoxicillin, 20 mg and clavulanic acid 10 mg) was placed at the centre of the petri-dish and ceftazidime (30 mg) and cefotaxime (30mg) were placed 15 mm apart center to center on the plates. This was incubated at 37^0C for 18 - 24h. An enhanced zone of inhibition between any one of the beta-lactam discs and the amoxicillinclavulanic acid disc was interpreted as presumptive evidence for the presence of ESBL (Iroha *et al*.,2008).

Characterization of isolates

There are several virulence factors produced by the different microorganisms. Some common virulence factors are hemolysin production (Siegfried *et al*.,1994), serum resistance (Timmis,1979), gelatinase test (Collee *et al*.,1993), beta lactamase (Lateef *et al*.,2004), cell surface hydrophobicity (Siegfried *et al*.,1994; Raksha *et al*.,2003).

Collection of the plant material

The fresh plants *Euphorbia hirta, Phyllanthus amarus* from the surrounding areas of Dr.N.G.P. Arts and Science college, Coimbatore and the farm field in Idappadi, salem and the medicinal plants confirmation by Tamil Nadu Agricultural University.

Preparation of the extracts

10g of the dried powdered sample was soaked in 100 ml of solvents (water, Acetone and PET ether / Petroleum ether) and the mixture was left to stand overnight (24 h) in a shaking water bath maintained at 40°C and the filtrate was taken with whatman filter paper. The filtrate was then evaporated to dryness.

Preliminary Phytochemical Analysis:

Phytochemical screening was carried out on the powdered plant material based on standard protocol (Emeruwa,1982; Trease and Evans, 1996).

Determination of the Antimicrobial Activity:

From the dry material, the preparation of dilutions of crude extracts for antibacterial assay was prepared by reconstituting the extracts in the respective extracting solvents and further diluted to obtain 100mg/ml. The modified agar well diffusion method (Collins *et al*.,1995) was employed to determine the antimicrobial activities of plant extracts. Three different extractions (Water/Aqueous, Acetone, PET ether/ Petroleum ether) were taken. In Agar well diffusion method, 100µl in the concentration of 100mg/ml taken as the standard concentration for all plant extracts. The mean of triplicate results were taken (Junaid *et al*.,2006).

Results and Discussion

Isolation and Identification of Uropathogens

From 200 urine samples (100 samples from male and 100 from female), 90 isolates were obtained from 100 female samples where as 64 isolates were obtained from male urine samples. Totally among 200 samples, 154 isolates (77%) were obtained. As it is well known, females are more susceptible to UTI than males (Fig.1). In the present study, it revealed that most of the isolates from patients of the age group 17-30 years, 56(36.4%) (Fig. 2).

The microorganisms were confirmed as *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* and *Pseudomonas aeruginosa* by standard confirmatory tests. In the present study *Escherichia coli* 103(66.9%), and *Klebsiella pneumoniae* 38(24.7%) are the most prominent organisms isolated from UTI patients(Fig.3). Similar study was done by Amali *et al*., (2009) reported that 20- 29 age group shows most positive numbers 168(96.5%) among 213 samples. In case of Jha and Bapat (2005) study, they confirmed in all the hospitals where they conducted their study shows that the percentage was 53-80% for females and 20-47% in males. Similar study was conducted among 1020 pathogen from 11308 urine sample, revealed that 620 strains(60.78%) of *E. coli* and 115 strains(11.27%) of *K. Pneumoniae* (Ava Behrooozi *et al*.,2010) were isolated from urine specimens of patients correlates our present study.

Antibiotic Sensitivity Test

The foremost pathogen *Escherichia coli* and *Klebsiella pneumoniae* were taken for the antibiogram. Among 103 isolates of *Escherichia coli,* 40 isolates were multidrug resistance and in case of 38 isolates of *Klebsiella pneumoniae* 9 isolates were multidrug resistance especially to 3GC (3rd generation) cephalosporins. (Fig.4,5&6;Plate 1& 2). Similar study in 300 *Klebsiella* spp isolates were found to be resistant to ciprofloxacin (31.2%), cefotaxime (74%), ceftazidime (69%) and imipenem (0%) and gentamicin (92.6%). During the past decade, ESBL producing *K. pneumoniae* have emerged as one of the major multi-drug resistant organisms. The incidence of ESBL producing *Klebsiella* isolates in the Unites States has been reported to be 5%. In France and England 14 to 16% ESBL producers among clinical *Klebsiella* isolates has been reported (Romanus *et al*.,2009).

Double Disc Synergy test

Among 40 multidrug resistant isolates 0f *E. coli,* 35(87.5%) are ESBL positive and in case of 9 multidrug resistant isolates *K. pneumoniae*, 6 (66.7%) were ESBL positive. It was confirmed by the enhancement of zone around Amoxiclav and any one of the adjacent cephalophorins. (Fig.7; Plate-3 & 4). Earlier, Neelam Taneja *et al*.,(2008) reported that ESBL producers could explain only 36.5 % of HDRU (HIGHLY DRUG RESISTANT UROPATHOGENS) in their study. Mustafa Onur Aladag and Durak,(2009) conducted a study on *K.pneumoniae* isolated from urinary tract infection. Among 125 isolates of *K.pneumoniae*, 45 strains (36%) produced ESBL and 80(64%) are non- ESBL producing strains (Aladag and Durak *et al*.,2009; Dharmadhikari *et al*.,2009).

Characterization for Esbl Producers

From the existing results on present study it showed the highest presence of virulence factors was found in *β*-lactamase(100%) and secondly to Serum inactivation (74.2% of *E. coli* and 100% of *K. pneumoniae*). The least virulence factors of Cell surface hydrophobicity was observed in 42.8% *E.coli* and 50% of *K. pneumoniae* (Fig. 8 & 9)(Plates: 5 - 12). Raksha *et al*.,(2003), study showed serum resistance in 32.7% of *E.coli* isolated from urine. In case Siegfried *et al.,*(1994) revealed that 68% of the urinary isolates were resistant to serum bactericidal activity which is comparable to our results (Raksha *et al*.,2003; Dharmadhikari and Peshwe,2009) We concluded that serum resistance also supported the bacterial resistance to different drugs and also they shows 2% serum resistance (Yusha'u *et al*.,2008).

Analysis of Phytochemical Analysis

The results of *Euphorbia heterophylla* phytochemical analysis showed the presence of steroids, tannins, alkaloids, saponins, terpenes and absence of catachol, anthraquinones, cardiac glycosides , triterponoids. *Acalypha indica* phytochemical analysis showed the presence of tannins, steroids, saponins, cardiac glycosides, terpenes, alkaloids and absence of anthraquinones, Catachol, triterponoids. Results were interpreted in table-1. Phytochemical study was studied by many researchers previously reported (Jigna Parekh and Chanda, 2007;Edeoga *et al*.,2005;

Adegoke *et al*.,2010a,b; Omale James *et al*.,2010; Pavithra *et al*.,2010) in *Euphorbia heterophylla* was studied.

Antimicrobial Activity of Medicinal Plants

From the results, acetone is the first highest antimicrobial activity of *E. heterophylla* (0-10mm) where as second the highest antimicrobial activity of *E. heterophylla* was shown by Aqueous extract (0-7 mm) and the least activity shown by PET ether extract(0-6 mm) (Table -2; Plates 3).

Table-1: Phytochemical analysis of *Euphorbia sp*

S.No	Constituents	Medicinal plants					
		Euphorbia heterophylla			*Acalypha indica*		
		W	A	P	W	A	P
1	Alkaloids (Picric acid)	-	+	+	+	+	+
2	Tannins	-	+	+	+	+	-
3	Saponins	+	+	+	+	+	+
4	Terpenes	+	+	+	+	+	+
5	Steroids	+	+	+	+	+	+
6	Triterponoids	-	-	-	-	-	-
7	Cardiac glycosides	-	-	-	+	+	+
8	Catachol	-	-	-	-	-	-
9	Anthraquinones	-	-	-	-	-	-

W- Water; A-Acetone; P- PET ether (Petroleum ether).

Table- 2: Antimicrobial activity of *Euphorbia heterophylla* and *Acalypha indica* against *Escherichia coli* in mm

Isolates	*Euphorbia heterophylla*			*Acalypha indica*		
	Aqueous (100mg/ ml)	Acetone (100mg/ ml)	PET ether (100mg/ ml)	Aqueous (100mg/ ml)	Acetone (100mg/ ml)	PET ether (100mg/ ml)
E1	4	8	5	15	11	11
E7	3	8	6	16	10	11
E16	5	8	-	10	9	9
E17	7	8	6	12	10	9
E18	4	7	-	13	12	11
E19	5	7	6	16	9	9
E20	4	8	5	14	10	7
E26	7	10	3	12	11	7
E28	4	8	-	10	9	8
E38	5	8	6	14	9	11
E39	6	7	5	15	11	10
E42	4	7	6	10	11	9
E46	5	9	5	11	9	8
E49	4	8	-	13	12	10
E50	6	9	6	10	9	7
E51	5	7	3	14	10	14
E52	4	8	-	12	11	10
E55	7	7	5	13	12	9
E59	2	8	-	15	10	9
E62	4	9	6	14	10	9
E63	-	8	-	10	9	9
E64	7	10	4	16	11	10
E65	4	8	-	14	8	7
E67	2	9	-	11	9	7
E75	-	7	-	12	11	9
E79	-	6	-	10	10	11
E84	-	8	-	10	9	9
E91	-	9	3	11	10	8
E92	5	6	-	16	12	10
E93	7	-	-	9	12	9
E94	4	7	-	10	8	10
E95	-	9	-	11	10	9
E96	-	10	-	13	12	9
E97	5	-	5	15	11	10
E103	4	6	-	10	8	7

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Table- 3: Antimicrobial activity of *Euphorbia heterophylla* and *Acalypha indica* against *Klebsiella pneumoniae* in mm

Isolates	*Euphorbia heterophylla*			*Acalypha indica*		
	Aqueous (100mg/ ml)	Acetone (100mg/ ml)	PET ether (100mg/ ml)	Aqueous (100mg/ ml)	Acetone (100mg/ ml)	PET ether (100mg/ ml)
K10	-	-	5	10	6	5
K29	-	10	-	13	10	11
K33	6	-	-	9	6	5
K35	7	6	-	8	7	6
K37	-	8	-	14	12	10
K38	-	7	6	7	9	6

Table -4: Minimal Inhibitory Concentration of *Acalypha indica* (*Escherichia coli & K.pneumoniae*)

S.No:	Isolates	Extracts (mg/ml)		
		Aqueous	Acetone	PET ether
1	E1	20	20	20
2	E7	20	20	20
3	E18	20	20	10
4	E39	20	20	30
5	E49	20	20	20
6	E52	10	20	20
7	E64	20	20	30
8	E79	20	20	30
9	E92	30	20	20
10	E97	10	20	20
11	K29	20	20	30
12	K32	20	20	30

E1 – E97 = *Escherichia coli*; K29- K32 = *Klebsiella pneumonia*

Acetone is the first highest antimicrobial activity of *Euphorbia heterophylla* (0-10 mm)where as second highest antimicrobial activity of *Euphorbia heterophylla* is shown by Aqueous extract (0-7 mm)and the least activity shown by PET ether extract(0-6 mm).(Table 3 & Plate 14).In Falodun study the aqueous extract showed significant activity ($P < 0.001$) comparable to the reference drug used. Among different dose range used (50, 100 and 150 mg/kg), 100 mg of aqueous extracts shows high inhibition rate than methanolic extracts (Falodun *et al*.,2006 a,b)

Escherichia coli

Aqueous is the first highest antimicrobial activity of *A. indica* (9-16 mm) where as second highest antimicrobial activity *A. indica* is shown by Acetone extract (8-12 mm) and the least activity shown by PET ether extract (7-11mm). (Table-2; Plate-15). Sumathi and Pushpa(2007) obtained aqueous extracts and tested against different bacterial pathogens and the aqueous extracts of *A. indica* shows 9 mm inhibition zone to *E. coli* and in our present study of aqueous extract it shows 16 mg/ml at the concentration of 100 mg /ml. In case of Jayakumari *et al*.,(2010) *E. coli* shows 30mm in 100mg/ml of ethanol extracts.

Klebsiella pneumoniae

Aqueous is the first highest antimicrobial activity of *A. indica* (7-14 mm) where as second highest antimicrobial activity *A. indica* is shown by Acetone extract (6-12 mm)and the least activity shown by PET ether extract(5-11 mm). (Table 3; Plate -16).

From our present study it reveals, all the extract of *A.indica* shows highest antimicrobial activity than any other extracts of other medicinal plants. The maximum zone measured as 14 mm in Aqueous extract.

Minimal Inhibitory Concentration

From the 2 selected plants, *A. indica* shows more resistance against ESBl positive uropathogens, *E. coli* and *K. pneumoniae* and hence from the results, *A' indica* is choosen for MIC (Minimal Inhibitiory Concentration) with highly susceptible ESBL positive uropathogens.

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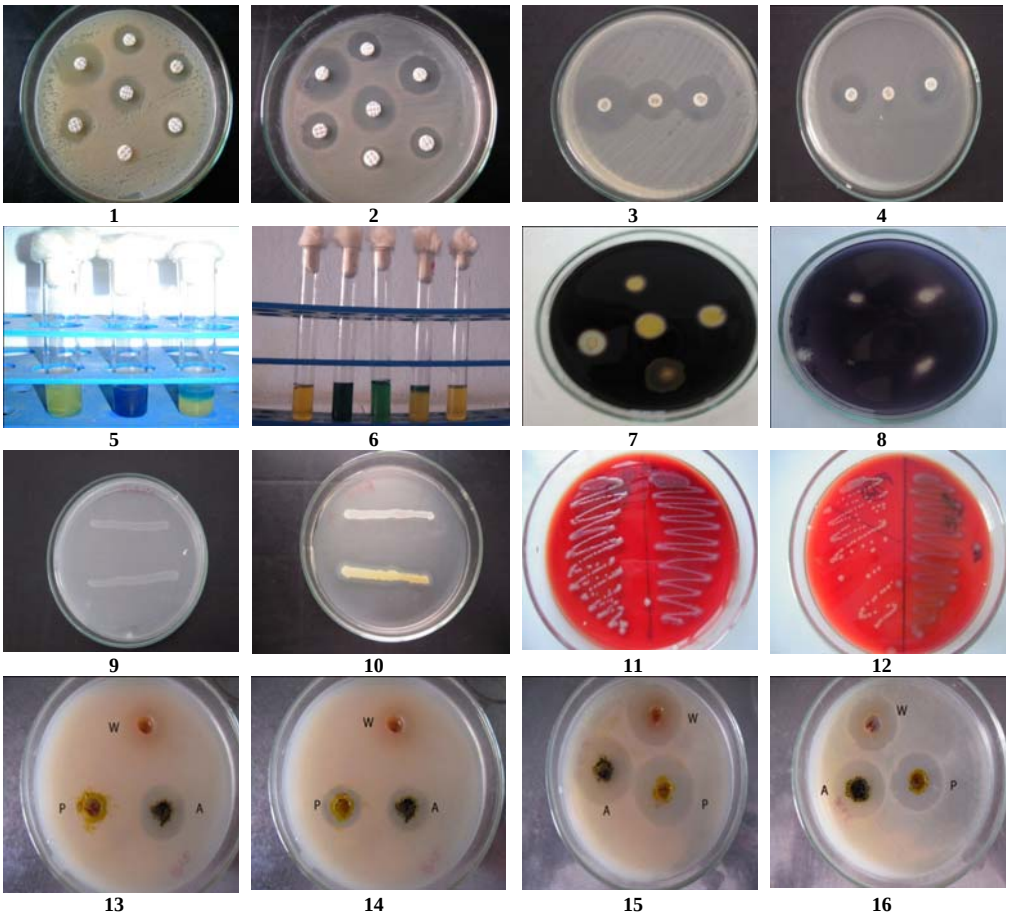
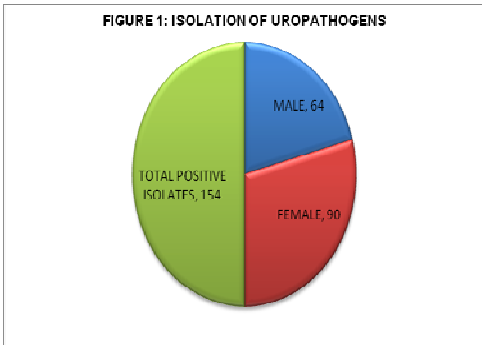
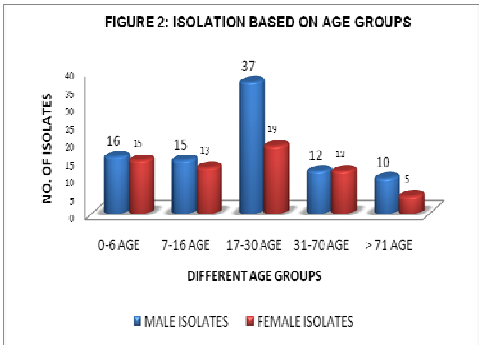


Plate 1 & 2: Antibiogram of *Escherichia coli* & *Klebsiella pneumoniae.*; Plate 3 &4: Double Disc Synergy test (ESBL positive &ESBL negative).; Plate 5 & 6: Serum resistance of *Escherichia coli* and *Klebsiella pneumoniae;*Plate 7& 8 : Beta lactamase of *Escherichia coli* and *Klebsiella pneumoniae* ; Plate 9 & 10 : Gelatinase negative *Escherichia coli* & positive *Klebsiella pneumoniae;* Plate 11& 12 : Hemolysin of *Escherichia coli* and *Klebsiella pneumoniae;* Plate 13 &14 : Antimicrobial activity of *Euphorbia heterophylla* against *Escherichia coli* & *Klebsiella pneumoniae;* Plate 15 & 16 : Antimicrobial activity of *Acalypha indica* against *Escherichia coli* & *Klebsiella pneumoniae.*





Among 35 ESBL positive *E. coli* isolates, 10 isolates showed maximum sensitivity and Among 6 ESBL positive *K. pneumoniae* isolates, 2 isolates showed maximum sensitivity for the 3 extracts used and the zone ranges above 10mm for all the 3 extracts. *A. indica* MIC was assessed for these 12 isolates.

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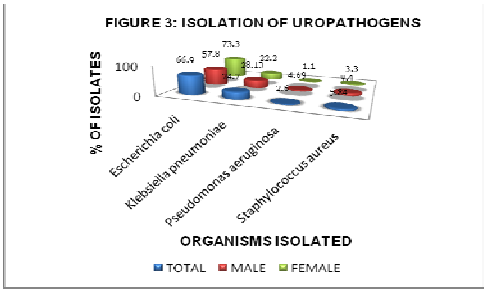
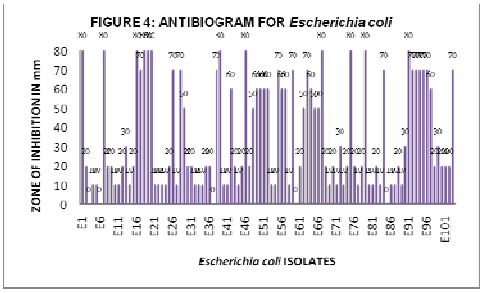
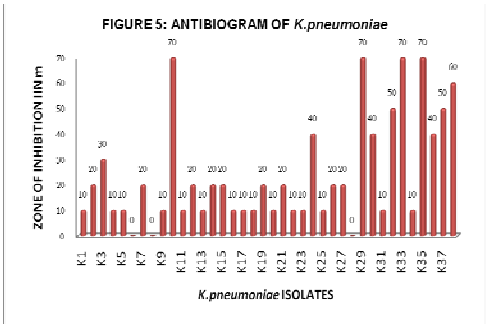
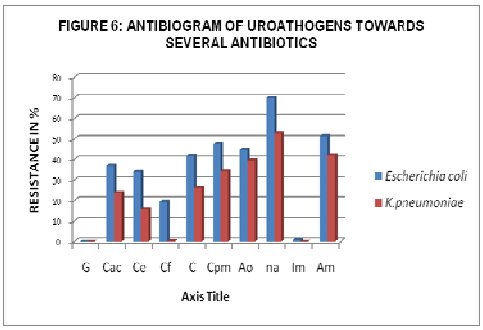
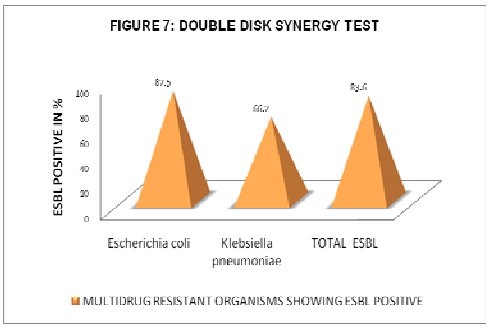
The MIC values of *A. indica* against *Escherichia coli* observed for aqueous extract was between 10 and 30 mg/ml. Acetone extracts exhibited MIC values was 20 mg/ml and for PET ether extract MIC values was between 10 and 30 mg/ml for *E. coli* ESBL positive isolates. The MIC values of *A. indica* against ESBL positive isolates of *K. pneumoniae* observed for aqueous extract was 20mg/ml.

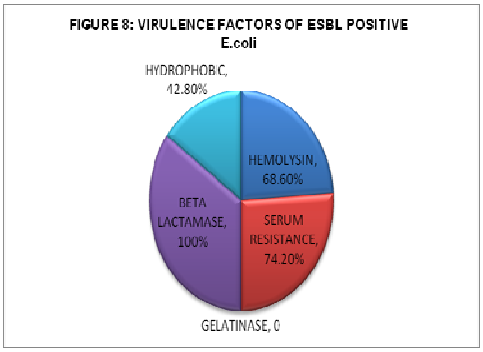


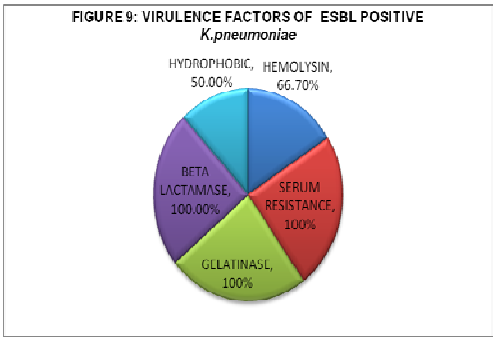












Acetone extracts exhibited MIC values was 20 mg/ml and for PET ether extract MIC values was 30 mg/ml. (Table 4).Babu uma *et al*.,(2009) observed the MIC values for methanol extract was between 1.25 and 2.5mg/ml. Chloroform extracts exhibited MIC values was 2.5 mg/ml and for aqueous extract MIC values was 5 mg/ml. ESBL positive multidrug resistant organisms are highly pathogenic to human beings. 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 , PET ether) & 2 medicinal plants (*Euphorbia heterophylla* and *Acalypha indica*),water extract of Acalypha indica shows maximum activity against multidrug resistant ESBL uropathogens. As the multidrug resistance to antibiotics is high now a days, herbal plants 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 positive uropathogens isolated from ICU patients .As water crude extracts exhibited an antibiotic potential against multidrug resistant ESBL positive uropathogen especially isolated from ICU patients, it proved that traditional use of *Acalypha indica* has scientific basis. Further investigation is necessary to find out the active compounds and to confirm their bioactive principles.

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