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Antibacterial activity of bark extracts of *Terminalia arjuna* (*Roxb*.) against Extended Spectrum β-Lactamase producing multi drug resistant Bacteria from Urinary Tract Infections

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Abstract

Urinary tract infections (UTIs) are most common in both community and hospital settings and affect both male and females. The production of Extended Spectrum β-Lactamases (ESBL) is an important mechanism for resistance to the 3rd generation cephalosporins in Enterobacteriaceae causing UTIs. Hence an attempt is made in this study to determine the effect of Terminalia arjuna(Roxb.) against UTIs by ESBL producing bacteria. A study was conducted on 94 bacterial isolates from 162 urine samples. ESBL production was identified by using Cefotaxime and Ceftazidime by Combination Disc Method (CDM). The antibacterial activity of the bark extract of Terminalia arjuna against ESBL producing bacteria was determined by Disc diffusion and Minimum Inhibitory Concentration (MIC). A total of 52 isolates were identified as ESBL positive (26 were Escherichia coli, 15 Klebsiella pneumoniae, 8 Pseudomonas aeruginosa, 2 Proteus vulgaris and 1 was Enterobacter sp.).The results of antibacterial activity of bark extract of Terminalia arjuna(Roxb.) against ESBL producing isolates revealed that ethanol is showing the maximum antibacterial property followed by aqueous extract. From this study, it was found that Terminalia arjuna(Roxb.) has the potential for the production of drug for the treatment of UTIs caused by ESBL producing bacteria. This might be the first report of antimicrobial activity of bark extract of Terminalia arjuna (Roxb.) against ESBL producing human pathogenic bacteria.

Keywords: Combination Disc Method (CDM), Extended-Spectrum β-Lactamases (ESBL), MIC, Terminalia arjuna (Roxb.).

Introduction

Urinary Tract Infections (UTIs) are the most common diseases affecting neonate to the growing age groups[1]. The Extended Spectrum β-lactamase (ESBL) producing gram negative bacteria of Enterobacteriaceae, are increasingly causing UTIs both in hospitalized patients and outpatients[2,3]. ESBL are enzymes produced by bacteria belonging to Enterobacteriaceae, most commonly *Escherichia coli* and *Klebsiella pneumoniae*[4]. ESBL are plasmid mediated enzymes showing resistance to Oxyimino-cephalosporins and monobactams[5,6]. The increasing multi drug resistances among these UTIs causing bacteria are due to uncontrolled usage of broad spectrum antibiotics and it has made treatment to very difficult [7]. ESBL producing organisms are capable of hydrolyzing β-lactam antibiotics including 3rd generation cephalosporins and monobactams. Clavulanic acid, sulbactam and tazobactam generally inhibit ESBL production by bacteria[8]. It is increasingly being reported that ESBL are acquiring a transmissible form of antibiotic resistance. This creates that penicillins and cephalosporins which are used for many years found no more effective against ESBL producing bacteria[9]. New antibiotics or new combinations of various antibiotics would be required to overcome the current threat posed by ESBL producing bacteria [10]. The resistance problem demands that a renewed effort be made to screen various medicinal plants for their potential antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. The bioactive phytocompounds in medicinal plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body[11]. *Terminali arjuna* (Roxb.) belongs to family Combretaceae and it is a large and evergreen tree (common name: Arjuna tree). Ayurvedic physicians found that *Terminalia arjuna* has antibacterial activity[12,13]. In this study, an attempt has been made to screen ESBL production among bacterial isolates and also to find out the antibacterial activity of bark extracts of *Terminalia arjuna (Roxb.)* against ESBL producing bacteria.

Material and Methods

Clinical samples: Midstream Urine samples were collected from Urinary Tract Infected patients using a clean container and immediately transported to the laboratory.

Isolation and identification of UTI isolates: For the isolation of UTI causing bacterial strains, loop full of urine samples were streaked onto nutrient agar plate and incubated at 37°C for 24hours. Isolated colonies were selected and identified based on the standard morphological, cultural and biochemical

characteristics. To check morphological characteristics: gram-staining, capsule staining and motility test were performed. To check the growth pattern, different culture media including MacConkey's agar, EMB agar were used. For biochemical characterization TSI, IMVIC (Indole, MR, VP and Citrate), nitrate test and sugar fermentation (Lactose, glucose, mannitol, maltose, sucrose and xylose) were performed[14].

Combination Disc Method (CDM): ESBL production among gram negative bacteria was detected by the Combination disc method according to CLSI guidelines using both cefotaxime and ceftazidime, alone and in combination with clavulanic acid. In this test, an overnight culture suspension of the test isolates was adjusted to 0.5 McFarland's standard. Lawn culture was made on the surface of Mueller Hinton Agar (MHA) plate. The Cefotaxime (30 µg) and cefotaxime-clavulanic acid (30 µg/ 10 µg) discs were placed 20 mm apart on the agar surface. Similarly, the ceftazidime (30 µg) and ceftazidime-clavulanic acid (30 µg/ 10 µg) ((Himedia Laboratories, Mumbai) discs were also placed. After incubating overnight at 37^{o}C, a ≥ 5mm increase in the zone diameter was interpreted as positive for ESBL production[15]. The quality control strain used for this study is *E. coli* ATCC 25922 as a negative control (figure 1).

Antibiotic Sensitivity Test: The Antibiotic susceptibility of the ESBL producing bacteria was detected using the following antibiotics namely Amikacin (30mcg), Ampicillin (10mcg), Ciprofloxacin (5mcg), Doxycycline (30mcg), Gentamycin (10mcg) and Tetracycline (30mcg) (Himedia Laboratories, Mumbai). Antibiotic sensitivity tests were done on MHA plates by the Kirby-Bauer disc diffusion method according to the CLSI guidelines[15].

Collection of Arjuna bark: Arjuna bark was purchased from Perumal Chetti Store, Parrys Corner, Chennai, Tamil Nadu, India.

Preparation of extracts: Twenty grams of *Terminalia arjuna* stem bark powder was well dissolved in 100 ml of double distilled water (ratio 1:5). The suspension was filtered by using a Seitz filter of pore size 0.2 µm.

The sterile extract was then transferred to lyophilization flask and kept in a deep freezer at– 80°C for 4 hours. The frozen extract was then loaded onto the Lyophilizer. The lyophilized powder was then transferred to sterile 5ml vials and stored for further use. Then the ethanol extract was prepared by using 100 ml of 70% of ethanol instead of 100 ml of double distilled water. The other steps were same as given in the aqueous extract preparation.

Antibacterial Studies: Disc Diffusion Method: Antibacterial activities of *Terminalia arjuna* bark extracts were studied by using agar disc diffusion method according to Bauer *et al*.[16]. A stock solution (1mg/ml) of the extracts and the dilutions of the stock solution containing 0.5, 1.0, 1.5 and 2.0µg/ml were prepared in dimethyl sulfoxide (DMSO). The inoculum was prepared and adjusted to the Mc Farland's standard 0.5 Scale. Lawn culture was made on Muller Hinton agar (MHA) plates. Prepared extract loaded discs were placed on the swabbed plates and incubated at 37˚C for 48 hours. After the incubation the zone of inhibition was measured in mm and compared with the standard antibiotic discs.

Minimum Inhibitory Concentration (MIC): Minimum inhibitory concentration (MIC) was determined by the microdilution method [17] using Mueller Hinton broth (MHB). A stock solution (1mg/ml) of the extracts was prepared in DMSO and the dilutions of the stock solution containing 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml were prepared in MHB. 100µl of each dilution was loaded into the respective wells and 100µl MHB as control in the microtitre plate. Loop full of broth culture was inoculated into each well. Chloramphenicol and Gentamycine were used as standard reference drugs (100 µg). The microtitre plates were incubated at 37°C for 18-24 hrs. The lowest dilutions that showed no growth were termed as inhibitory activity.

Statistical Analysis: The Chi - square test was used for determination of significance of association. The *P* value ≤ 0.05 was considered as statistically significant. All Statistical analysis was done with SPSS software version 15.0.

Results and Discussion

A total of 94 isolates were obtained from 162 urine samples collected from various Hospitals in Chennai. Among these, 33 (35.10%) were from males and 61 (64.89%) from females (table 2). The percentage wise distribution of various bacterial isolates has been given in figure 1. *E.coli* was found to be the predominant uropathogen causing UTI followed by *K. pneumoniae*. A total of 52 (55.31%) isolates were identified as ESBL positive by using Combination Disc Method and it was found that, 26 were *E. coli* (50.00%), 15 *K. pneumoniae* (28.84%), 8 *P.aeruginosa* (15.38%), 2 *Proteus vulgaris* (3.84%) and 1 *Enterobacter* sp. (1.92%) (table 1 and figure 2). Sex wise and age wise distribution of ESBL positive isolates have been depicted in table 2. Females (69.23%) were found to have more ESBL isolates when compared to those in males (30.76%). More ESBL isolates (69.23%) were found to be in the age group 20-40. ESBL producing isolates showed multi drug resistance against ≥3 antibiotics. All the 52 ESBL producing isolates from UTIs showed the highest degree of resistance to ampicillin (45, 86.53%) followed by doxycycline (33, 65.38%). Out of 52 ESBL producing isolates 42 (80.76%) were susceptible to amikacin and 34 (63.46%) to gentamycin. Among 26 *E.coli* isolates 24 (92.30%) showed resistance against ampicillin, 16 (61.53%) against ciprofloxacin and tetracycline, 13 (50.00%) against doxycycline and 11(42.30%) against gentamycin. Among 15 *K. pneumoniae* 13 (86.66%) showed resistance against ampicillin, 10 (66.66%) against doxycycline, 8 (53.33%) against ciprofloxacin, 7(46.66%) against tetracycline,

4(26.66%) against amikacin and 3(20.00%) against Gentamycin. A total of 8 *P. aeruginosa* 8 (100%) isolates showed resistance against doxycycline and tetracycline and against ampicillin. All the 2 *Proteus vulgaris* showed 100% resistance to Ampicillin and Doxycycline. *Enterobacter* sp. isolates showed 100% resistance to Ampicillin, Ciprofloxacin and Gentamycin (table 3). Out of above isolates from 5 bacterial genera, *E.coli* showed maximum resistance against ampicillin, ciprofloxacin, doxycycline, gentamycin and tetracycline and *K. pneumoniae* showed maximum against amikacin.

Table-1
Various clinical isolates and their ESBL Positivity

S.No	Clinical Isolates	Total Isolates (N=94)	ESBL Positive (N=52)	%	*p* value
1	*E.coli*	42	26	50.00	0.123
2	*K. pneumoniae*	30	15	28.84	1.000
3	*P. aeruginosa*	15	8	15.38	0.796
4	*P. vulgaris*	4	2	3.84	1.000
5	*Enterobacter* spp.	3	1	1.92	0.564

Table-2
Sex wise and Age wise distribution of ESBL positive isolates

S.No	Age	Clinical Isolates				Total %
		Male	%	Female	%	
1	1 – 20	0	-	1	1.92	1.92
2	21 – 40	10	19.23	26	50.00	69.23
3	41 – 60	4	7.69	6	11.53	19.23
4	61 – 80	2	3.84	3	5.76	9.61
Total		16	30.76	36	69.23	100

Table-3
Antibiotic susceptibility of ESL producing isolates from Urinary Tract Infection

Organisms	Interpretation	Antibiotics					
		Amikacin (30mcg)	Ampicillin (10mcg)	Ciprofloxacin (5 mcg)	Doxycycline (30mcg)	Gentamycin (10mcg)	Tetracycline (30mcg)
E.coli(26)	Sensitive (S)	26 (50.00%)	1(1.92%)	9(17.30%)	8(15.38%)	15(28.84%)	5(9.61%)
	Intermediate (I)	-	1(1.92%)	1(3.84%)	5(9.61%)	-	5(9.61%)
	Resistant (R)	-	24(46.15%)	16(30.76%)	13(25.0%)	11(21.15%)	16(30.76%)
K. pneumoniae (15)	Sensitive (S)	9(17.30%)	-	5(9.61%)	2(3.84%)	11(21.15%)	3(5.76%)
	Intermediate (I)	2(3.84%)	2(3.84%)	2(3.84%)	3(5.76%)	1(1.92%)	5(9.61%)
	Resistant (R)	4(7.69%)	13(25.0%)	8(15.38%)	10(19.23%)	3(5.76%)	7(13.46%)
P. aeruginosa (8)	Sensitive (S)	4(7.69%)	-	8(15.38%)	-	6(11.53%)	-
	Intermediate (I)	3(5.76%)	2(3.84%)	-	-	-	-
	Resistant (R)	1(1.92%)	6(11.53%)	-	8(15.38%)	2(3.84%)	8(15.38%)
P. vulgaris (2)	Sensitive (S)	2(3.84%)	-	1(1.92%)	-	2(3.84%)	1(1.92%)
	Intermediate (I)	-	-	-	-	-	-
	Resistant (R)	-	2(3.84%)	1(1.92%)	2(3.84%)	-	1(1.92%)
Enterobacter spp. *(1)*	Sensitive (S)	1(1.92%)	-	-	1(1.92%)	-	1(1.92%)
	Intermediate (I)	-	-	-	-	-	-
	Resistant (R)	-	1(1.92%)	1(1.92%)	-	1(1.92%)	-

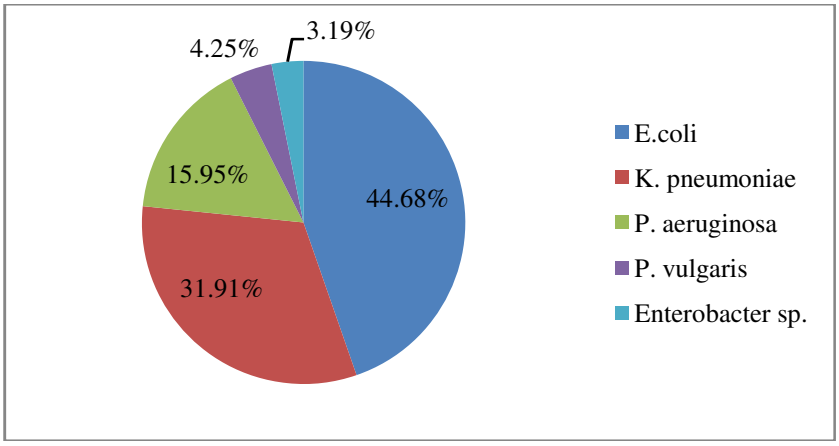

Figure-1
The percentage wise distribution of various bacterial isolates

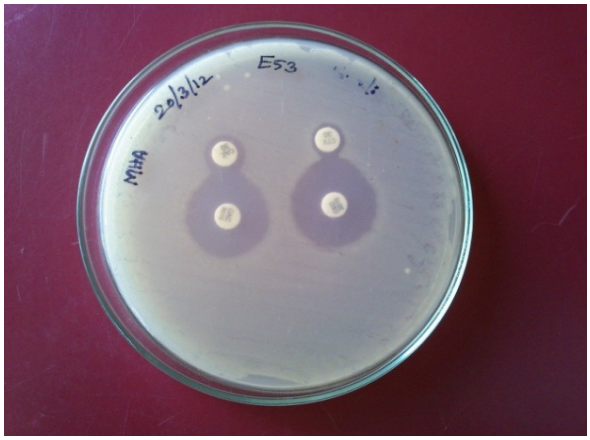

Figure-2
Plate showing ESBL production by Combination Disc Method(CDM)

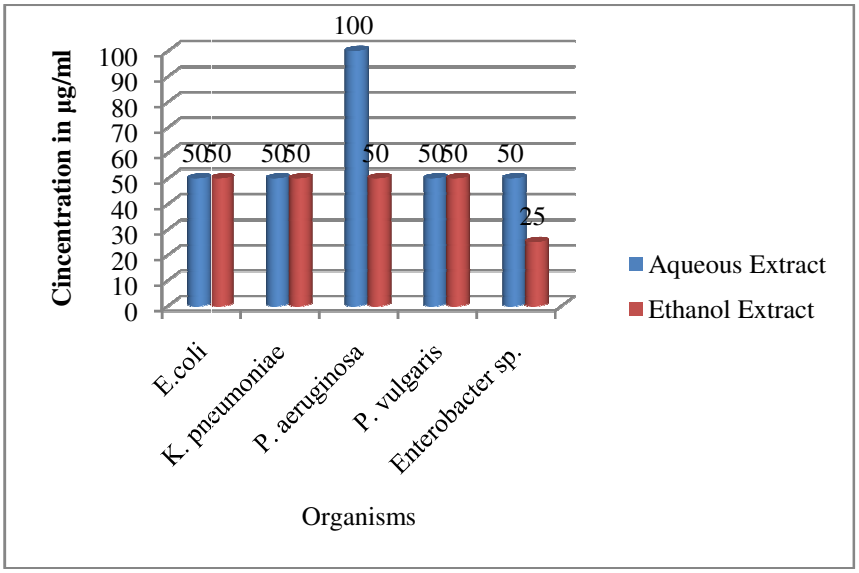

Figure-3
Effect of aqueous, ethanolic extracts of bark of *Terminalia arjuna (Roxb.)* against ESBL producing organisms by Broth Microdilution method

Agar Disc Diffusion Assay: Zones of inhibition of 11, 12, 13 and 15mm against *Escherichia coli*, 10, 12,13 and 15 mm against *Klebsiella pneumoniae*, 11, 12, 14 and 15mm against *Pseudomonas aeruginosa*, 11,12,14 and 16 mm against *Proteus* spp. and 10,11,13 and 15 mm against *Enterobacter* spp. were obtained at 0.5,1.0, 1.5 and 2.0 mg respectively for Aqueous extract. Zones of inhibition of 13, 14, 16 and 18mm against *Escherichia coli*, 12,13,15 and 16 mm against *Klebsiella pneumoniae*, 12, 13, 14 and 16mm against *Pseudomonas aeruginosa*, 11,14,16 and 17 mm against *Proteus* spp. and 12,14,15 and 17 mm against *Enterobacter* spp. were obtained at 0.5,1.0, 1.5 and 2.0 mg respectively for Ethanol extract. Ethanolic extract from bark of *T. arjuna(Roxb.)* showed better antibacterial activity when compared to aqueous extract against tested ESBL producing bacteria.

Minimum Inhibitory Concentration Assay (MIC): In MIC assay aqueous extract from *T. arjuna*(Roxb.) showed inhibitory activity against *Escherichia coli, Klebsiella pneumoniae*, *Proteus vulgaris* and *Enterobacter sp*. at 50 µg/ml and against *Pseudomonas aeruginosa* at 100µg/ml and ethanol extract exhibited inhibitory activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa and Proteus vulgaris* at 50µg/ml and *Enterobacter* spp. at 25µg/ml. Studies of MIC assay reveals that *Enterobacter* sp. showed lesser MIC value (25µg/ml) as compared to other ESBL bacterial isolates (figure 3).

Discussion: In recent years, ESBL production among UTI causing bacterial isolates creating the major clinical therapeutic problem. These organisms show resistance to most of the antibiotics currently being used. This study provides the predominant uropathogen, antibiotic susceptibility pattern of UTI causing ESBL producing bacterial pathogens and antimicrobial property of arjuna extracts against ESBL producing isolates. All isolates were screened for ESBL production by CDM and multi drug resistance of ESBL producing isolates were identified by disc diffusion method. In this study, the UTI causing organisms include *E.coli*, *K. pneumoniae*, *P. vulgaris* and *Enterobacter sp*. and the results are in close agreement with that Ali[18]. The predominant uropathogen in this study was *E.coli*. Previous studies have also demonstrated that *E.coli* is the most frequent etiological agent causing community and hospital acquired UTIs[19-22]. ESBL production was found in more than 50% of all isolates with the highest incidence in *E.coli* (50%) and lower incidence in *Enterobacter sp.* (1.92%). 68% ESBL production was reported by Mathur *et al*.[23] among gram negative bacteria from a tertiary care hospital. Tankhiwale *et al.* [24] reported 48.3% of urinary isolates as ESBL producers in their study. Most of the isolates in this study showed resistance to more than 3 antibiotics. Resistance was very high against ampicillin and lowered against amikacin. This study is comparable with other studies in India reported by Rameshkumar *et al.,* 2012 [25]. Who reported that all isolates from their study showed high resistance to Ampicillin and lower to amikacin.

In the present study the ethanol extract from bark of *T. arjuna* showed maximum antibacterial activity as compared aqueous extract against the ESBL producing bacteria tested by Disc diffusion and Microdilution methods. A zone of inhibition of bacterial growth of more than 10mm by the plant extract indicated significant inhibitory activity[26]. In this study also most of the organisms showed zone of inhibition of more than 10mm. Our results agree with Morshed *et al*, 2011[27]. Who reported that 18mm of zone of inhibition against *E.coli* and *P.aeruginosa* by ethanol extract of bark of *T. arjuna*.

Berghe *et al*. [28] reported that if an inhibition is obtained by 1-10 mg plant extract/ml test solution, the extract can be considered worthy for further investigations. In the present study ethanol extract showed minimum inhibitory concentration ranging from 25-100µg/ml and thus the arjuna plant product was found to have posses significant antibacterial activity. The active principle in this plant product can be further studied before using it for effective control of important clinical bacteria.

Conclusion

In conclusion the ESBL producing bacteria are a breed of multidrug resistant pathogens that are increasing rapidly and becoming a major problem in clinical therapeutics. It is essential to report ESBL production along with the routine antibiotic sensitivity reporting, which will help to treat the MDRs organisms causing infections with proper antibiotics. Plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by resistant microorganisms. Based on our results, it is concluded that *Terminalia arjuna* (*Roxb.*) has the potential for the production of drug for the treatment of urinary tract infections caused by ESBL producing pathogenic bacteria.

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