In vitro Antibacterial Activity of Aqueous and Ethanol Extracts of *Aristolochia indica* and *Toddalia asiatica* Against Multidrug-Resistant Bacteria

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Bacteria have developed multidrug resistance against available antimicrobial agents. Infectious diseases caused by these multidrug-resistant bacteria are major causes of morbidity and mortality in human beings. Synthetic drugs are expensive and inadequate for the treatment of diseases, causing side effects and ineffective against multidrug-resistant bacteria. The medicinal plants are promising to have effective antimicrobial property due to presence of phytochemical compounds like alkaloids, flavanoids, tannins and phenolic compounds. The present study aimed to find the antimicrobial activity of medicinal plants against multidrug-resistant bacteria. Multidrug-resistant bacteria were identified by Kirby-Bauer disc diffusion method. Production of β-lactamases (extended spectrum β-lactamases, metallo β-lactamase and AmpC β-lactamase) were identified by combination disc method. Antibacterial activity of aqueous and ethanol extract of *Aristolochia indica* and *Toddalia asiatica* were detected by agar well diffusion assay and minimum inhibitory concentration. All bacteria used in this study showed antibiotic resistance to ≥3 antibiotics. *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Vibrio cholerae* were found to be positive for β-lactamase production. Ethanol extract of *Aristolochia indica* showed more significant antibacterial activity against multidrug-resistant bacteria than *Toddalia asiatica*. Ethanol extracts of *Aristolochia indica and Toddalia asiatica* showed minimum inhibitory concentration values of 50-100 µg/ml and 100-200 µg/ml, respectively against multidrug-resistant bacteria. From this study, it was concluded that *Aristolochia indica* has more potential to treat multidrug-resistant bacteria than *Toddalia asiatica*.

Key words: Multidrug-resistant bacteria, combination disc method, *Aristolochia indica*, *Toddalia asiatica*, minimum inhibitory concentration, extended spectrum β-lactamases

Infectious diseases caused by the multidrug resistant bacteria are a major cause of morbidity and mortality worldwide[1]. In the recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented[2]. These multidrug-resistant bacteria have also created clinical therapeutic problems in cancer and immune compromised patients. Methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE), extended spectrum β-lactamases (ESBLs) producing bacteria in enterobacteriaceae and multidrug resistant *Mycobacterium tuberculosis* (MDR-TB) are major important multidrug resistant bacteria in global scale[3,4]. The reduced susceptibility of multidrug-resistant bacteria to available antibiotics is continuously increasing and it is due to uncontrolled usage of broad spectrum antibiotics. Synthetic antimicrobial drugs are expensive, inadequate for the treatment and also produce side effects. This situation provided the need to the search for new antimicrobial agents from medicinal plants[5].

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Accepted 25 November 2015
Revised 17 February 2015
Received 07 August 2014
Indian J Pharm Sci 2015;77(6):788-791

Plant extracts have great potential as antimicrobial compounds against microorganisms and they can be used in the treatment of infectious diseases caused by drug resistant microorganisms[6]. Medicinal plant extracts have been used for centuries for treating many health disorders. The medicinal property of plants is due to the presence of phytochemicals. The most important bioactive compounds are alkaloids, flavanoids, tannins and phenolic compounds[7]. The phytochemicals were used to cure the infectious diseases in herbal medicine. India is one of the country that has richest flora of medicinal plants of 120 families comprising 130,000 species. The use of about 2400 of these plants are mentioned in traditional system of Indian medicine for treating ailments such as wounds, leprosy, skin disorders, diarrhoea, dysentery, jaundice, cough and cold[8].

In this present work, aqueous and ethanol extracts of whole plant of *Aristolochia indica* and *Toddalia asiatica* were evaluated for their antibacterial property against multidrug-resistant β-lactamases producing bacteria.

Multidrug-resistant bacterial strains were obtained from Post Graduate and Research Department of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai, India. The antibiotic susceptibility of strains were done on Mueller Hinton Agar plates using Kirby-Bauer disc diffusion method according to CLSI guidelines (CLSI, 2012)[9] using antibiotics namely amikacin, ampicillin, ciprofloxacin, gentamicin, doxycycline, tetracycline, cefotaxime, ceftazidime, imipenem, chloramphenicol, nalidixic acid and co-trimoxazole (Himedia, Mumbai).

β-lactamases production among gram negative bacteria was detected using combination disc method according to CLSI guidelines[9]. 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 for detection of extended spectrum β-lactamase production. After incubating for overnight at 37°, a ≥5 mm increase in the zone of inhibition diameter was measured and interpreted as positive for ESBL production. The quality control strain used for this study is *E. coli* ATCC 25922 as a negative control and *K. pneumoniae* ATCC 700603 as a positive control.

Metello β-lactamase production was identified using imipenem (10 µg) alone and in combination with EDTA (750 µg) and for AmpC β-lactamase production using cefoxitin (30 µg) alone and in combination with cloxacillin (200 µg).

The whole plants of *A. indica* and *T. asiatica* were collected from Ooty hill areas in Nilagiris District, Tamil Nadu, India. The whole plant was air dried and powdered. For the preparation of aqueous and ethanol extracts, 20 g of whole plant powder was well dissolved in 100 ml of double distilled water and ethanol, respectively (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° for 4 h. The frozen extract was then loaded onto the lyophilizer. The lyophilized powder was then transferred to sterile storage vials and stored for further use.

Antibacterial activities of *A. indica* and *T. asiatica* plant extracts were studied by agar well diffusion method according to Bauer *et al*.[10]. A stock solution (1 mg/ml) of the extracts and the dilutions of the stock solution containing 0.5, 1.0, 1.5 and 2.0 µg/ml were prepared in dimethyl sulfoxide (DMSO). The inoculum was prepared and adjusted to the McFarland's standard 0.5 scale. Lawn culture was made on Muller Hinton agar (MHA) plates. Prepared extract were loaded in well on the swabbed plates and incubated at 37° for 48 h. After the incubation the zone of inhibition was measured in mm and compared with the standard antibiotic discs. Minimum inhibitory concentration (MIC) was determined by the microdilution method[11] using Mueller Hinton broth (MHB). A stock solution (1 mg/ml) of the extracts was prepared in DMSO and the dilutions of the stock solution containing 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml were prepared in MHB. 100 µl of each dilution was loaded into the respective wells and 100 µl MHB for control in the microtitre plate. Loop full of broth culture was inoculated into each well. Ciprofloxacin and gentamicin were used as standard reference drugs (100 µg). The microtitre plates were incubated at 37° for 18-24 h. The lowest dilutions that showed no growth were termed as minimum inhibitory activity.

All bacteria used in this study were drug resistant to ≥3 antibiotics. Antibiotic resistance and β-lactamase production of the bacteria were shown in Table 1. Ethanol extract of *A. indica* shows significant activity against gram positive and gram negative bacteria followed by ethanol extract of *T. asiatica*. Zone of inhibition obtained was comparable with the standard bacterial antibiotics. In aqueous extracts, *A. indica* shows better activity against both gram positive and gram negative bacteria than aqueous extract of *T. asiatica*. The results for the agar well diffusion were represented in Table 2. Ethanol extract of *A. indica* shows better MIC value of 50-100 µg/ml against bacteria tested than other extracts of both *A. indica* and *T. asiatica*. In aqueous extracts *A. indica* and *T. asiatica* showed MIC range of 100-400 µg/ml and 200-400 µg/ml, respectively. MIC values of *A. indica* and *T. asiatica* against multidrug-resistant bacteria were shown in fig. 1. Antimicrobial activity of these plants might be due to the phytochemical components.

Shafia *et al.*[12] have reported that the essential oil of *A. indica* showed good antimicrobial activity against *P. aeruginosa*, *B. substilis*, *S. aureus* and *E. coli* ranging at 50 mg/ml. Our study also revealed that the ethanol extract of whole plant of *A. indica* showed antimicrobial activity ranging from 50 to 100 µg/ml against all bacteria tested.

Kar *et al.*[13] reported that the aqueous extract of stem bark of *T. asiatica* showed 7 and 5 mm of

TABLE 1: ANTIBIOTIC DRUG RESISTANCE PROFILE OF BACTERIA

Organisms	Resistance to antibiotics	β-lactamase production
Escherichia coli	AMP, CTX, CAZ, COT, NA, TE, CPD, DO, CX	ESBL and AmpC
Klebsiella pneumoniae	AK, AMP, CTX, COT, GEN, NA, TE, CPD, IMP, CX	ESBL, MBL and AmpC
Pseudomonas aeruginosa	AMP, CTX, COT, NA, TE, IMP	MBL
Proteus mirabilis	AMP, CTX, COT, NA, TE	ESBL
Enterobacter aerogenes	AMP, C, CTX, COT, NA, TE	-
Enterococcus faecalis	AMP, CTX, NA, TE	-
Vibrio cholerae	AMP, C, CTX, COT, NA, CX	ESBL
Staphylococcus aureus	AMP, NA, TE	-
Staphylococcus epidermidis	AMP, CTX, COT	-
Acetobacter sp.	CTX, COT, NA, TE	-
Bacillus substilis	NA, TE, COT	-

Multidrug resistance and ESBLs, MBL and AmpC β-lactamases production of bacteria. AMP: Ampicillin, CTX: cefotaxime, CAZ: ceftazidime, COT: co-trimoxazole, NA: nalidixic acid, CPD: cepodoxime, DO: doxycycline, CX: cefoxitin, GEN: gentamicin, TE: tetracycline, C: chloramphenicol, AK: amikacin, IMP: imipenem, ESBLs: extended spectrum β-lactamases, MBL: metallo β-lactamase

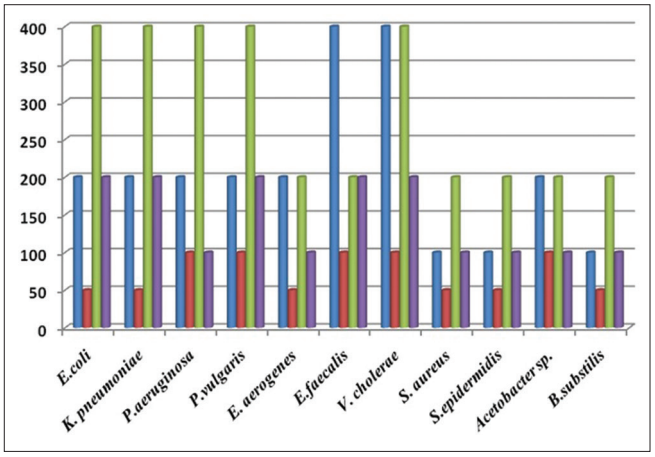

Fig. 1: Minimum inhibitory concentration of aqueous and ethanol extracts of *Aristolochia indica* and *Toddalia asiatica* against multi drug resistant bacteria by microdilution.
■ **Aqueous extract of *A. indica,* ■ ethanol extract of *A. indica* ■ aqueous extract of *T. asiatica* ■ ethanol extract of *T. asiatica***

TABLE 2: ZONE OF INHIBITION OF AQUEOUS AND ETHANOL EXTRACTS OF *ARISTOLOCHIA INDICA* AND *TODDALIA ASIATICA*

Organisms	*Aristolochia indica* (zone of Inhibition in mm)								*Toddalia asiatica* (zone of inhibition in mm)							
	Aqueous extract (mg)				Ethanol extract (mg)				Aqueous extract (mg)				Ethanol extract (mg)			
	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Escherichia coli	11	13	14	15	13	15	16	18	-	-	10	11	12	13	15	16
Klebsiella pneumoniae	09	10	12	13	12	14	16	17	-	-	9	10	09	11	13	15
Pseudomonas aeruginosa	10	12	13	14	15	16	18	19	-	-	8	10	09	10	12	14
Proteus mirabilis	10	11	13	14	13	14	15	17	-	-	-	11	12	14	16	17
Enterobacter aerogenes	11	13	15	16	14	16	17	18	-	-	-	10	09	11	13	15
Enterococcus faecalis	11	12	14	15	13	15	15	16	-	-	9	11	10	11	13	14
Vibrio cholerae	09	11	13	14	12	14	15	15	-	10	11	12	11	13	14	15
Staphylococcus aureus	13	14	15	16	13	15	16	19	9	11	12	13	13	14	15	16
Staphylococcus epidermidis	11	12	14	16	14	15	17	18	8	10	11	13	10	12	14	15
Acetobacter sp.	13	15	17	15	13	14	16	18	-	9	10	12	13	15	16	17
Bacillus substilis	09	11	13	15	14	15	16	18	10	11	13	14	13	14	16	16

Zone of inhibition of aqueous and ethanol extracts of whole plant of *Aristolochia indica* and *Toddalia asiatica* against multidrug-resistant bacteria by Agar well diffusion method

zone of inhibition against *S. aureus* and *E. coli,* respectively. Ethanol extract showed 10 and 6 mm against *S. aureus* and *E. coli,* respectively. In this study, whole plant ethanol extract of *T. asiatica* showed 13 and 11 mm against *S. aureus* and *E. coli*, respectively and aqueous extract showed 16 mm against both *S. aureus* and *E. coli* at a concentration of 2 mg/ml.

Ali *et al*.[14] reported phytochemical, pharmacological and toxicological properties of *A. indica* showed antimicrobial activity against *Enterococcus faecalis*. It exhibits effective antimicrobial activity against *E. faecalis*. Gopinath and Prakash[15] reported that the ethanol extract of *A. indica* showed highest antimicrobial activity of 19 mm against clinically isolated multidrug-resistant *Enterococcus faecalis.* In our study the ethanol extract of *A. indica* showed 18 mm against multi drug resistant *E. faecalis*.

Yadav and Khan[16] reported that *T. asiatica* showed promising activity against tested microorganisms. The tested plant extracts were most active against gram positive microorganisms than the gram negative microorganisms. In this study also *T. asiatica* exhibited significant antibacterial activity against gram positive bacteria than gram negative bacteria.

Poor hygienic conditions and uncontrolled used of antibiotics in both hospital and community settings are the major reasons for emergence of multi drug resistant bacterial infections. These bacteria are making available antibiotics into ineffective compounds. Plant extracts have great potential as antimicrobial compounds against multidrug resistant bacteria. This study emphasizes the importance of *A. indica* and *T. asiatica* and active compounds purified from these plants can be used as effective antimicrobial agents against multi drug resistant resistant β-lactamases producing bacteria.

Financial support and sponsorship:
Nil.

Conflicts of interest:
There are no conflicts of interest.

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