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Isolation and identification of urinary catheter associated bacteria and study of *in vitro* antibacterial activity of methanolic and petroleum ether leaf extracts of *Ipomoea mauritiana* Jacq. against bacteria isolated from urinary catheters

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Abstract
The enormous increase in the use of urinary catheters in the hospitals has resulted in an increase in catheter associated urinary tract infections. *Escherichia coli* is the most predominant uropathogen involved in urinary tract infections in patients catheterized for long periods.
The bacteria isolated from patients catheterized for long term were identified by biochemical methods and then confirmed by molecular methods like polymerase chain reaction and sequencing of 16srRNA gene. The antibacterial activity of medicinal plant *Ipomoea mauritiana* was evaluated against bacteria isolated from urinary catheters by disk diffusion method.

Keywords: Urinary tract infections, urinary catheters, *Ipomoea mauritiana*, disc diffusion method, minimum inhibition concentration

1. Introduction

1.1 Urinary tract infections (UTIs)

Urinary tract infections (UTIs) represent the most commonly acquired bacterial infection. The prevalence of UTI is very high in both outpatient and hospital populations and are estimated to be responsible for over 100,000 hospitalizations every year [1]. According to an estimation nearly 25% to 40% of nosocomial infections are urinary tract infections [2, 3]. With the increasing use of indwelling devices such as catheters and urethral stents/sphincters, the risk of developing a UTI also increases significantly. Indwelling catheters are the primary contributing factor in the development of these infections. The use of catheters to manage urinary incontinence in nursing home and spinal cord injury patients makes these populations especially vulnerable to these infections. Urinary catheters are frequently used in hospital and community care, whereby up to 25% of hospitalized patients need urinary catheters. However, the number of days of catheterization increases, the risk of colonization by microorganisms also increases by 5-10% [4]. The catheter associated urinary tract infection (CAUTI) accounts for 80% of all UTIs in hospitals due to enormous increase in the use of catheters for hospitalized patients [5]. The effective treatement of bacterial colonization is very difficult cleared until removal of the catheter and hence catheter associated urinary tract infections accounts for enormous costs for health systems [4]. In addition, the colonized catheter is a source of potentially multiple drug resistant bacteria in an environment with susceptible hosts. The predominant pathogens in urinary tract infections are *Escherichia coli*, followed by Enterococci, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans*, *Enterobacter* sp., *Proteus mirabilis* and coagulase-negative Staphylococci [6]. These pathogens are usually present in the lower intestinal tract and can be introduced into the urinary tract via contaminated indwelling devices.

1.2 *Ipomoea mauritiana*

Infectious diseases are the main cause of mortality in developing countries [7]. The death rate is increasing throughout the world day by day due to the increasing drug resistance by various pathogenic microorganisms. Hence scientists are now more interested at the herbal medicines used in the traditional medical practices.
In India, medicinal plants are widely used for the treatment of many diseases in traditional medicinal practices like ayurveda and are also used in the pharmaceutical preparations of modern medicines.

Herbal medicines are considered to be very safe when compared to synthetic drugs. A wide range of plants are used in modern medicine for the synthesis of many important drugs [8]. Many human diseases are treated by using medicinal plants which contain many active components. Plants are rich sources of secondary metabolites, which are potent in curing different ailments [9]. Plant extracts have been used since ancient times for the treatment of many diseases in many of our traditional medicinal methods. The plant extracts often contain many numbers of small molecules called plant natural products or plant secondary metabolites, which make the plant extract a complex mixture [10]. These chemicals isolated from plants have been the source of many present day drugs. *Ipomoea mauritiana* Jacq. (also referred to as vidari or ksheera vidari) belonging to the family Convolvulaceae is one of the very commonly used plants in various ayurvedic formulations. Vidari is useful as diuretic, cardiotonic, demulcent, aphrodisiac and galactogogue [11]. In traditional medicine it is also used in the treatement of enteric fever and spermatorrhea [12]. The current study was undertaken to determine the antibacterial activity of *Ipomoea mauritiana* Jacq. against UTI causing bacteria.

2. Materials and methods

2.1 Collection and identification of plant materials

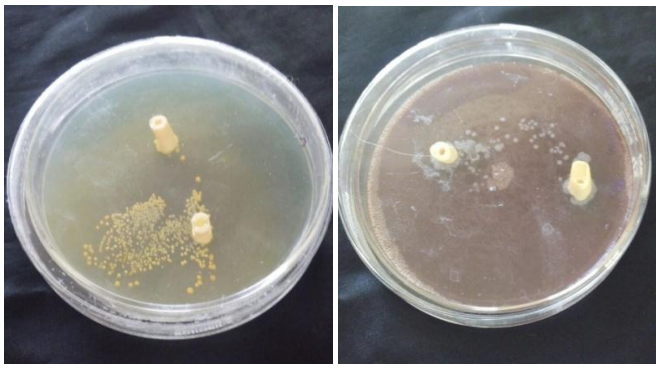
Fresh and healthy leaves of *Ipomoea mauritiana* Jacq. were collected and the taxonomic identities of the collected plants were authenticated by a plant taxonomist.

2.2 Preparation of plant extracts

The *I. mauritiana* leaves collected were thoroughly washed under running tap water, dried in shade and then powdered by using an electric grinder. These powders were stored in air sealed brown bottles at ambient temperature. The finely ground *I. mauritiana* leaf powder (30 g) was loaded in Soxhlet apparatus and were extracted with the solvents methanol and petroleum ether [13]. The solvents from the extracts were removed using a rotary vacuum evaporator (Heidolph) under reduced pressure to collect the crude extract. The yield of each extract was weighed and stored at 4 °C until used.

Antibacterial Screening

2.3 Isolation of UTI causing bacteria from urinary catheters



a. b.

Fig 1: Bacteria isolated from urinary catheters (a). Bacteria isolated on CLED agar (b). Bacteria isolated on Blood agar

Urinary catheters from hospitalized patients who were catheterized for more than 7 days were carefully collected under aseptic conditions. The catheters were transported immediately to the laboratory for analysis by placing the catheters in sterile glass bottles. The outer surfaces of the catheters were disinfected with sodium hypochlorite solution [14, 15]. It was then followed by washing the inner surface of the catheters with sterile distilled water using a syringe. The catheters were then aseptically cut into 3-4 mm thick discs. They were then placed on the surface of cysteine lactose electrolyte deficient agar and blood agar plates and incubated at 37°C for 48-72 hours (Fig. 1) [16].

2.4 Detection of uropathogens by chromogenic media

Bacteria have many enzymes for their physiological function by which they can utilize different substrates [17]. In chromogenic media, the enzyme present in the particular bacteria breaks down the chromogenic substrates specifically and hence the colour of bacterial colonies would be different for different bacteria. HiCrome UTI agar M1353R (HiMedia) is a chromogenic medium that is very much useful in rapid isolation as well as presumptive identification of most uropathogens.

In the present study bacteria isolated on CLED agar and blood agar were subcultured on Hichrome UTI agar for the presumptive identification of bacteria isolated from urinary catheters.

2.5 Identification of bacteria by PCR

The bacterial isolates presumptively identified as *E. coli*, *P. mirabilis* and *Klebsiella* Sp were subjected molecular identification by PCR. Genomic DNA was isolated by using the InstaGeneTM Matrix Genomic DNA isolation kit (Catalog # 732-6030). 16S rRNA gene was amplified using standard primers (27F – AGAGTTTGATCATGGCTCAG and 1492R – TACGGCTACCTTGTTACGACTT). Target gene fragment was amplified using Teledyne Prime Thermal Cycler (Fig 2).

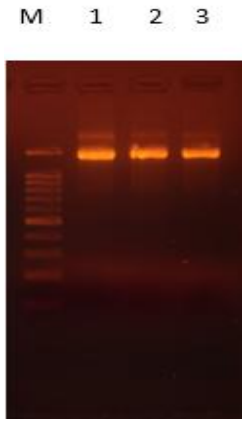


Fig 2: PCR amplification of 16S rRNA gene of *E. coli*, *P. mirabilis* and *K. pneumoniae* M-Molecular size ladder, 1- *E. coli*, 2- *P. mirabilis*, 3 – *K. pneumoniae*

2.6 Disc diffusion method [18]

Antimicrobial screening of the methanolic and petroleum ether plant extracts were carried out by disc diffusion method against *E. coli*, *P. mirabilis*, *K. pneumoniae* and *E. faecalis* isolated from urinary catheters. Cysteine lactose electrolyte deficient agar (CLED) plates were prepared as per manufacturer instructions. Overnight brain heart infusion broth (BHI) culture of the test organisms was seeded over the CLED plates by spread plate method using a sterile glass spreader so as to make lawn culture. The different concentrations of extracts (2-10 mg/disc) were loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5

minutes and the plates were incubated at 37 °C for 24 hrs. After the incubation, zones of inhibition formed around the disc were measured with transparent ruler in millimeter (Fig.3) (Table 1, 2, 3, 4 and 5).

Table 1: Antibacterial activity of *Ipomoea mauritiana* extracts against uropathogens

Organism	Extract	N	Mean (in mm)	Std. Deviation	Std. Error	F & P values
K. pneumoniae	Methanol	3	0.00	0.00	0.00	F = 6359.077; p =.000
	Pet ether	3	0.00	0.00	0.00	
	Erythromycin	3	16.6000 [b]	0.36056	0.20817	
	Total	9	5.5333	8.30196	2.76732	
E. coli	Methanol	3	13.5667[a]	0.40415	0.23333	F = 262.902; p =.000
	Pet ether	3	13.6667[a]	0.15275	0.08819	
	Erythromycin	3	18.8000[b]	0.34641	0.20000	
	Total	9	15.3444	2.60678	0.86893	
E. faecalis	Methanol	3	10.8000[b]	0.20000	0.11547	F = 1420.980; p =.000
	Pet ether	3	7.6667[a]	0.30551	0.17638	
	Erythromycin	3	17.6000[c]	0.17321	0.10000	
	Total	9	12.0222	4.40249	1.46750	
P. mirabilis	Methanol	3	0.00[a]	0.00	0.00	F = 8307.000; p =.000
	Pet ether	3	0.00[a]	0.00	0.00	
	Erythromycin	3	22.6000[b]	0.20000	0.11547	
	Total	9	7.5333	11.30044	3.76681	

Note: Mean values with different superscripts are significantly different from each other as indicated by Tukeys's HSD (alpha=0.05).

Table 2: Effect of *Ipomoea mauritiana* extracts and standard drug on *Klebsiella* Sp.

Extracts	N	Subset for alpha = 0.05	
		1	2
Methanol	3	0.00[a]	
Petroleum ether	3	0.00[a]	
Erythromycin	3		16.600[b]

Mean values with different superscripts are significantly different from each other as indicated by Tukeys's HSD (alpha=0.05).

Table 3: Effect of *Ipomoea mauritiana* extracts and standard drug on *Escherichia coli*

Extracts	N	Subset for alpha = 0.05	
		1	2
Methanol	3	13.5667[a]	
Petroleum ether	3	13.6667[a]	
Erythromycin	3		18.8000[b]

Mean values with different superscripts are significantly different from each other as indicated by Tukeys's HSD (alpha=0.05).

Table 4: Effect of *Ipomoea mauritiana* extracts and standard drug on *Enterococcus faecalis*

Extracts	N	Subset for alpha = 0.05		
		1	2	3
Methanol	3	7.6667[a]		
Petroleum ether	3		10.8000[b]	
Erythromycin	3			17.6000[c]

Mean values with different superscripts are significantly different from each other as indicated by Tukeys's HSD (alpha=0.05).

Table 5: Effect of *Ipomoea mauritiana* extracts and standard drug on *Proteus mirabilis*

Extracts	N	Subset for alpha = 0.05	
		1	2
Methanol	3	0.00[a]	
Petroleum ether	3	0.00[a]	
Erythromycin	3		22.600[b]

Mean values with different superscripts are significantly different from each other as indicated by Tukeys's HSD (alpha=0.05).

2.7 Determination of MIC against *E. coli* and *E. faecalis* by micro broth dilution technique as per NCCLS method [19]

Cell suspensions was prepared from *E. coli* and *E. faecalis* bacterial cultures grown on Trypticose soya broth were adjusted to 1-2 x 10^5cells/mL. Different concentrations of the *Ipomoea mauritiana* leaf extracts (16 - 1024µg/ml) were prepared in Muller Hinton broth. The standard drug erythromycin of different concentrations (0.5 – 32µg/ml) was prepared in Muller Hinton broth. 90 µl *I. mauritiana* plant extracts of different test concentration with 10 µl inoculum were placed in 96 well plate. Similarly, 90 µl standard drug of different test concentration with 10 µl inoculum were placed in 96 well plate. 90 µl Muller Hinton broth without drug with 10 µl inoculums placed in 96 well plate as control. The plates were then incubated at 37 ºC at 24-48 h. After incubation optical density at 600nm is measured in Tecan plate reader. Minimum concentrations of drug and plant extracts giving 50% inhibition of OD as compared with control were determined (Table 6, 7 and 8).

Table 6: Determination of Minimum Inhibitory concentration of Erythromycin against test cultures

Concentration (µg/mL)	% inhibition	
	E. coli	*E. faecalis*
0.00	0.00	0.00
0.50	18.21	25.65
1.00	33.25	41.28
2.00	56.50	63.25
4.00	78.25	82.64
8.00	91.25	95.36
16.00	96.21	95.88
32.00	99.21	99.95
MIC (µg/mL)	2.0	2.0

Table 7: Determination of Minimum Inhibitory concentration of test extracts against *E.coli*

Concentration (µg/mL)	% inhibition	
	Methanol	Petroleum ether
0	0.00	0.00
16	10.25	9.57
32	17.25	15.24
64	28.65	23.25
128	41.25	43.21
256	52.25	50.18
512	71.25	67.57
1024	81.50	71.25
MIC (µg/mL)	256	256

Table 8: Determination of Minimum Inhibitory concentration of test extracts against *E.faecalis*

Concentration (µg/mL)	% inhibition	
	Methanol	Petroleum ether
0	4.25	0.00
16	7.36	6.32
32	11.21	10.56
64	15.68	14.27
128	29.87	21.68
256	50.98	37.25
512	63.25	43.25
1024	71.25	50.15
MIC (µg/mL)	**256**	**1024**

2.8 Statistical analysis
Data were expressed as the mean ± SEM. The data were analyzed using one way analysis of variance (ANOVA) followed by Tukey's test.

3. Results
Urinary catheters used by hospitalized patients for more than five to seven days were carefully collected under aseptic conditions. The catheters were obtained from Vikram hospital, Mysore. The number of catheters used in the present study is 103.

The majority of the pathogens isolated from the catheter samples were *E. coli* (45) followed by *Proteus mirabilis* (23), *Klebsiella* pneumoniae (19), *Enterococcus faecalis* (12) and *Pseudomonas aeruginosa* (4). The bacteria isolated from catheters were sub cultured and presumptively identified by culturing on Hichrome UTI agar.

The most predominant uropathogens isolated namely *E. coli*, *P. mirabilis* and *K. pneumoniae* were further identified by PCR using 16S rRNA gene primers. DNA fragments were amplified using 1 µl of template DNA in 20 µl of total PCR reaction mixture using 27F/1492Rprimers.

The PCR product was then sequenced using the 27F/1492R primers. Sequencing reactions were performed using a ABI PRISM® Big DyeTM Terminator Cycle Sequencing Kits with Ampli Taq®DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using16S rRNA gene universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re suspended in distilled water and subjected to electrophoresis and the end products were sequenced in an ABI 3730xl sequencer (Applied Bio systems) and the sequences were compared with the sequences of National Centre for Biotechnology Information (NCBI) database. The Genbank accession numbers are MF103667, MF103668 and MF103669 for *Escherichia coli*, *Klebsiella oxytoca*., and *Proteus mirabilis* respectively.

Antimicrobial screening of the methanolic and petroleum ether crude extracts and the standard drug erythromycin were tested against the predominant uropathogens namely *E. coli*, *P. mirabilis*, *K. pneumoniae* and *E. faecalis* isolated from urinary catheters. Overnight nutrient broth culture of the test organisms was seeded over the CLED plates by spread plate method. Sterile discs impregnated with plant extracts were placed on the seeded plates. Sterile discs with standard drug erythromycin were also placed on seeded medium. The plates were then incubated at 37 °C for 24 h. after incubation, the plates were observed for clear zone around the discs impregnated with plant extracts. The diameters of the inhibition zone were measured in mm. Antimicrobial activity was shown for both the extracts against *E. coli* and *E. faecalis* but *P. mirabilis* and *Klebsiella* Sp were resistant against the plant extracts (Table 1) (Fig 3).

Since both the extracts showed antibacterial activity against only *E. coli* and *E. faecalis* the minimum inhibition concentration of the plant extracts against these two bacteria were determined. The MIC of both the methanolic and petroleum ether extract was found to be 256 µg/ml for *E.coli* whereas the MIC of methanolic and petroleum ether extracts for *E. faecalis* were found to be 256 µg/ml and 1024 µg/ml respectively.

4. Discussion
The microbial colonization of urinary bladder with uropathogens are quite high in those patients catherized for a long term. Most of the uropathogens isolated were gram negative rods with *E. coli* being the most predominant which is very much similar to the findings of Bouza *et al*. (2001) [20] and Jacobsen *et al*. (2008) [5] followed by *P. mirabilis* and *Klebsiella* species. The bacteria were presumptively identified by biochemical techniques and were further confirmed by PCR using 16S rRNA gene universal primers and they were further sequenced.

Ipomoea mauritiana belongs to the family Convolvulaceae and many of the plants belonging to genera *Ipomoea* show considerable antimicrobial activity. The antibacterial activity of *Ipomoea mauritiana* leaf extracts were tested against different uropathogens where only *E. coli* and *E. faecalis* were found to be sensitive whereas *Klebsiella* sp and *Proteus mirabilis* were resistant towards the plant extracts. Further the MIC for both methanolic and petroleum ether extracts were found to be 256µg/ml for *E. coli* and the MIC for methanolic extract was found to be 256µg/ml for E. faecalis as well which shows that the methanolic plant extract is very good antimicrobial compound against these two uropathogens.

5. Conclusion
Many types of indwelling devices like catheters, stents etc are used in hospitalized patients due to which bacteria get a surface to colonize and form biofilm and then cause secondary infections in patients. Hence the patients have to be given with antibiotics to prevent or cure the infections arising due to catheterization. With increase use of drugs, the antibiotic resistance by bacteria is also increasing. Hence herbal formulations act as a very important alternative to combat against these infection causing bacteria.

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