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BACTERIA ASSOCIATED WITH URINARY TRACT INFECTIONS AND THEIR SUSCEPTIBILITY TO HERBAL EXTRACTS

MIR NAIMAN ALI[1*] AND MOHAMMED MAZHARUDDIN KHAN[2]

[1]Department of Microbiology, Mumtaz Degree & P.G.College, Hyderabad, India
[2]Center for Environmental Science, College of Natural Sciences, Addis Ababa University, Ethiopia

ABSTRACT

In the present study 500 urine samples were collected from different diagnostic laboratories of Hyderabad, India. 350 urine samples were scored as positive UTI and a total of 211 bacterial cultures were isolated belonging to 5 species: *Escherichia coli* (43%); *Klebsiella pneumoniae* (23%); *Pseudomonas aeruginosa* (19%); *Enterobacter faecalis* (10%) and *Proteus mirabilis* (4%). Three selected plant extracts (*Allium sativum, Mentha piperita,* and *Zingiber officinale*) were used to test their antibacterial activity activity by agar well diffusion assay and Minimum inhibitory concentration. Aqueous, ethanolic and methanolic extracts were used; highest antibacterial activity was recorded with ethanolic extracts of *Zingiber officinale* on *E. coli* and least against *K. pneumoniae* with diameter of inhibition zones (DIZ) of 20.0 ± 0.60 and 9.0 ± 1.0 mm respectively. Phytochemical analysis revealed the presence of active compounds such as phenolics, tannins, alkaloids and flavonoids. The results clearly demonstrated strong antibacterial activity of extracts on all UTI isolates than with standard antibiotics.

KEYWORDS: **Urinary tract infection, Antibacterial activity, Phytochemicals, Minimum inhibitory concentration.**

MIR NAIMAN ALI
Department of Microbiology, Mumtaz Degree & P.G.College, Hyderabad, India

***Corresponding author**

INTRODUCTION

Urinary tract infections (UTI) are among the most common bacterial infections which are prevalent extraintestinal and affecting people of all ages from neonates to geriatric age group.[1] Worldwide, about 150 million people are diagnosed with UTI each year and it is estimated that about 35% of healthy women suffer with UTI infection at some stage in their life. The incidence of UTI is greater in women than men, which may be either due to anatomical predisposition or urothelial mucosal adherence to the mucopolysaccharide lining or other host factors.[2] A urinary tract infection (UTI) is a bacterial infection, when it affects the lower urinary tract it is known as a simple cystitis (a bladder infection) and when it affects upper urinary tract it is known as pyelonephritis (a kidney infection). UTI has become the most common hospital-acquired infection, accounting for as many as 35% of nosocomial infections, and they are the second most common cause of bacteremia in hospitalized patients.[3] The most common cause of UTI is Gram negative bacteria that belong to the family Enterobacteriaceae. Members of these families include *E.coli*, *Klebsiella, Enterobacter* and *Proteus*. Also Gram positive Staphylococcus sp. plays a role in the infection.[4] *E.coli* is one of the most common bacteria capable of causing urinary tract infections.[5] The frequency of *E.coli* in urine samples varies in different studies from 32, 40 and 75%.[6-8] Nowadays, drug resistance is a huge growing problem in treating infectious diseases like malaria, tuberculosis, diarrheal diseases, urinary tract infections etc. The improper and uncontrolled use of many antibiotics resulted in the occurrence of antimicrobial resistance, which became a major health problem worldwide.[9] In the last 3 decades, there have been a lot of reports in the scientific literature on the inappropriate use of antimicrobial agents and the spread of bacterial resistance among microorganisms causing UTIs.[10,11] Natural products have been used in traditional medicine all over the world for thousands of years and predate the introduction of antibiotics and other modern drugs. The antimicrobial efficacy attributed to some plants in treating diseases has been beyond belief. It is estimated that local communities have used about 10% of all flowering plants on earth to treat various infections, although only 1% have gained recognition by modern scientists.[12] Plants were rich in a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found in vitro to have antimicrobial properties.[13] Owing to their popular use as remedies for many infectious diseases, search for plants containing antimicrobial substances is frequent.[14] A number of phytotherapy manuals have mentioned various medicinal plants for treating infectious diseases due to their fewer side effects and reduced toxicity.[15] There are several reports on the antimicrobial activity of different herbal extracts.[16,17] Many plants have been found to cure gastrointestinal disorders, respiratory diseases and cutaneous infections.[18,19] According to WHO, medicinal plants would be the best source for obtaining variety of drugs.[19] These evidences contribute to support and quantify the importance of screening natural products. Keeping in view the growing problem with UTIs and drug resistance, the present study was carried out with an objective to detect more efficient antibacterial agents of plant origin.

MATERIALS AND METHODS

(i) Sample Collection

A total of 500 urine samples were collected from different diagnostic laboratories in Hyderabad Metropolis, India. Samples were transported to laboratory in an ice cold condition by adding boric acid at a final bacteriostatic concentration of 1.8% without delay.[20]

(ii) Colony count of urine samples

Each urine sample was subjected to colony count by Urine Dip Slide method (VWR International BVBA, Leuven). The dip slide is immersed in urine sample so that both of the agars are completely covered by the sample; slide is removed from the sample, drained to remove any excess sample and incubated at 35 ± 2^0 C for 16 - 24 hrs. After incubation the dip slide is compared with the comparison chart provided. Equal or more than 10^4 CFU/ml of a single potential or two potential pathogens interpreted as positive UTI and a result of 10^2 – 10^4 CFU/ml was repeated. A less than 10^2 CFU/ml was interpreted as negative UTI.

(iii) Isolation and identification UTI bacterial pathogens

For isolation of UTI bacterial strains, loop full of urine samples were streaked on Mac Conkey agar, Blood agar and Nutrient agar plates (Hi Media, India & Merck, Germany) and incubated at 37± 2^0 C for 24 hrs. After incubation colonies were selected and characterized on the basis of morphological, cultural, physiological and biochemical characteristics.[21] A presumptive identification was performed by Gram staining, oxidase activity, motility, catalase production, acid production in glucose, oxidation-fermentation (OF) test (glucose lactose and sucrose fermentation), Indole test, Voges-Proskauer test (VP) and hydrogen sulfide production. The bacterial isolates were identified with the help of Bergey's Manual of Systematic Bacteriology [22] and PIB computer kit.[23]

(iv) Plant material

A total of 3 plants and their parts: *Allium sativum* (bulb), *Mentha piperita* (leaf), and *Zingiber officinale* (rhizome) were collected from local market in Hyderabad, Telangana, India. All specimens were identified in Dept. of Botany, Mumtaz College, and voucher specimens **(Voucher Specimen No: MCBDA-20/06/12-17)** have been maintained in Department of Microbiology, Mumtaz Degree and P.G College, Hyderabad, India.

(v) Preparation of extracts

The plant parts were washed with distilled water, dried in shade, grinded to fine powder and stored in airtight containers at room temperature in dark until used. The

powdered samples were subjected to extraction by the following method of Gupta et al.[24]

(a) Aqueous extraction

For aqueous extraction 10g of air dried powder was mixed well in 100ml distilled water with constant stirring for 30 minutes. The solution was kept at room temperature for at least 24h and then filtered using muslin cloth. The filtrate was centrifuged at 5000 rpm for 15 minutes. The supernatant was again filtered using Whattman's Filter No. 1 under strict aseptic conditions. The filtrate was collected in fresh sterilized glass tubes and stored at 4°C until use. Aqueous extract was prepared in final concentration of 100 mg/ml.

(b) Extraction using Organic Solvents

10g of air dried powder was thoroughly mixed with 100ml organic solvent (ethanol and methanol). The mixtures thus obtained were filtered through muslin cloth and then re-filtered by passing through Whattman's filter No. 1. The filtrates were then concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts were prepared by mixing well the appropriate amount of dried extracts with appropriate solvent to obtain a final concentration of 100 mg/ml. Each solution was stored at 4°C in sterilized glass tubes until use.

(vi) Antibacterial Susceptibility Assay

To evaluate the antibacterial potential of plant extracts Agar well diffusion assay was followed.[25] Extracts were first sterilized by sterile membrane syringe filter (pore size 0.45 µm, manufactured by Pall Life Sciences). Petri dishes (100mm) containing 18ml of Mueller Hinton Agar were seeded with approximately 100µl inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 10^8 CFU/ml). Media was allowed to solidify. Wells of 6mm diameter were cut into solidified agar media using a sterilized cup-borer. 100µl of each extract was poured in the respective well and the plates were incubated at 37°C overnight. The experiment was performed in triplicate under strict aseptic conditions to ensure consistency of all findings. The antibacterial activity of each extract was expressed in terms of the mean of diameter of inhibition zone (DIZ) in mm ± SD, produced by each extract at the end of incubation period. Organic solvents used in preparation of extracts were also used as negative controls during the study. Ten commercially available standard antibiotics commonly used for UTI treatment were also used in the present study for testing the susceptibility of isolated UTI pathogens.

(vii) Assessment of Minimum Inhibitory Concentration

MIC (minimum inhibitory concentration) of methanolic and ethanolic extracts was further examined by standard two-fold microdilution broth method.[26] A stock solution of each extract was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml. A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately 5×10^5 CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain using reflective viewer.

(viii) Phytochemical Analysis

Phytochemical analysis of the extracts was carried out by the methods described by Harborne[27] and Kolkate et al.[28] By this analysis, the presence of several phytochemicals like phenolics, alkaloids, flavonoids, tannins, saponins, steroids and glycosides were tested.

(ix) Statistical Analysis

Results obtained were analyzed statistically and values were expressed as Mean ± SD.

RESULTS

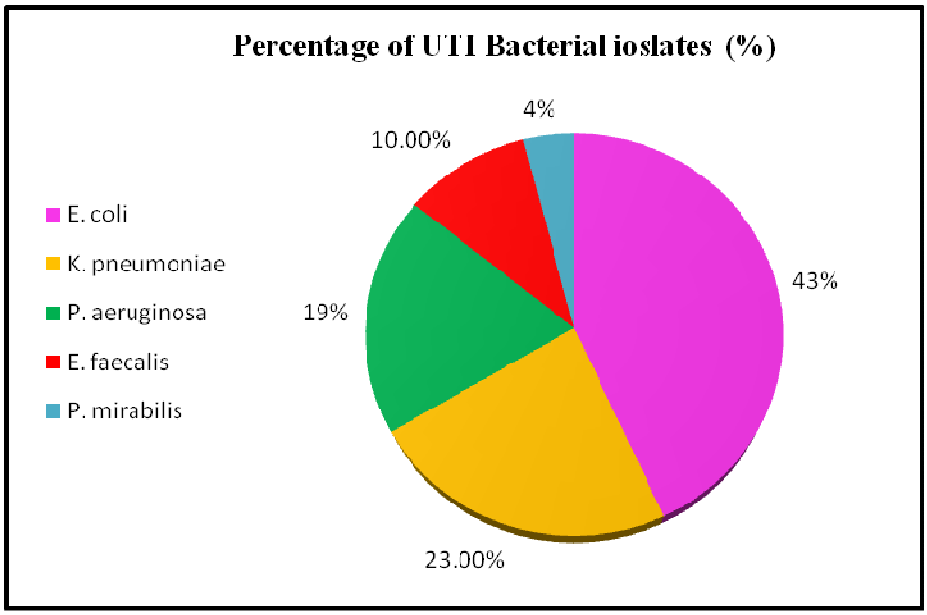
In the present study 350 urine samples were shown to be urine culture positive as their colony count was equal or more than 10^4, remaining 150 samples were scored as negative for UTI. 211 bacterial cultures were isolated, characterized and identified by studying different biochemical properties as mentioned in materials and methods. The identification characteristics were cross checked with those of standard manuals.[21-23] The identification characteristics revealed that, these isolates belong to 5 species (Table-1). *Escherichia coli* was the predominant species isolated (43%); *Klebsiella pneumoniae* (23%); *Pseudomonas aeruginosa* (19%); *Enterobacter faecalis* (10%) and *Proteus mirabilis* (4%) as depicted in Figure-1.

Table 1
Biochemical Characteristics for Identification of UTI Bacterial isolates

Characteristics		Identity of Isolates				
		E.coli	K.pneumoniae	P.aeruginosa	Enterobacter faecalis.	P. mirabilis
Grams Nature		-	-	-	-	-
TSI	Slant	-	+	-	+	+
	Butt	-	+	+	-	+
Mannitol		Acid	Acid	Acid	Acid	-
Motility		+	+	+	+	+s
Indole test		+	-	-	-	-
Methyl red test		+	-	+	-	+
V.P. test		-	+	-	+	-
Citrate test		-	+	-	+	+
Urease test		-	+	+	-	+
Oxidase test		-	-	+	-	-
Catalase test		-	-	+	-	-
H_2S production		-	-	-	-	+

TSI- Triple sugar iron test; '+' : positive '-' : negative 's': swarming motility.

Figure 1
Pathogenic Bacteria isolated from positive UTI samples



Antibiotic susceptibility testing results revealed that all bacteria isolated from UTI showed highest degree of resistance to gentamycin, nalidixic acid, trimethoprim-sulphamethaoxazole, clotrimazole and cefotaxime which are commonly prescribed drugs for UTI treatment (Table-2). The antibiotics which inhibited bacterial growth up to some extent were ampicillin, norfloxacin, nitrofurantoin and tetracycline.

Table 2
Antibacterial susceptibility of isolated bacterial UTI pathogens

	Diameter of Inhibition Zone in mm				
Standard Antibiotics	**E. coli**	**K.pneumoniae**	**P.aeruginosa**	**E.faecalis**	**P.mirabilis**
Ampicillin	+	-	-	-	+
Ciprofloxacin	+	+	-	-	+
Gentamycin	+	+	+	+	+
Norfloxacin	-	-	-	+	-
Nitrofurantoin	+	+	-	+	-
Nalidixic acid	+	+	+	+	+
Trimethoprim-sulphamethaoxazole (SXT)	+	+	+	+	+
Clotrimazole	+	+	+	+	+
Cefotaxime	+	+	+	+	+
Tetracycline	+	+	-	-	-

+ Resistant – Sensitive

Results obtained for antibacterial studies reveal following findings. Aqueous, ethanolic and methanolic extracts of plants exhibited antibacterial activity towards all five isolated UTI pathogens, with more activity observed with ethanolic extracts. There was significant variation in the antibacterial activities (DIZ values) of different plant extracts. The aqueous extracts have shown moderate antibacterial effect on isolated UTI pathogens except *Allium sativum* and *Mentha piperita* which have shown high antibacterial activity compare to other plants with DIZ values in range of 14.0 ± 0.30 and 16.0 ± 0.20 mm; 5.0 ± 0.10 and 15.03 ± 0.15 mm respectively. The aqueous extracts of *Zingiber officinale* have shown less antibacterial activity (Figure-2). The methanolic extracts of all the plants have shown good antibacterial effect against the UTI isolates. The most effective antibacterial activity was recorded for *E. coli* with maximum inhibition by *Zingiber officinale* (17.0 ± 0.60), followed by *Allium sativum* (16.03 ± 0.057) and *Mentha piperita* (11.03 ± 0.15). There was a slight variation in results obtained for remaining four UTI isolates (Figure-3). The extracts of *Zingiber officinale* expressed next higher effect against *P. aeruginosai* (16.0 ± 0.60), *P. mirabilis* (11.66 ± 0.57), and *E. feacalis* (9.67 ± 0.58); least antibacterial effect was observed against *K. pneumoniae* (7.67 ± 0.58). On contrary, the extracts of *Allium sativum* expressed more effect on *P. mirabilis* (15.06 ± 0.30), followed by *E. faecalis* (13.03 ± 0.251) and then *P. aeruginosa* (12.03 ± 0.251); similar to *Zingiber officinale*, least effect was observed against *K. pneumoniae* (10.06 ± 0.057). Methanolic extracts of *Mentha piperita* in general expressed less antibacterial effect compared to other two plants, at the same time it expressed highest antibacterial effect towards *P.aeruginosa* with a DIZ value of 18.0 ± 0.50.

Figure 2

Antibacterial activity of Aqueous Plant Extracts on Bacterial UTI Isolates

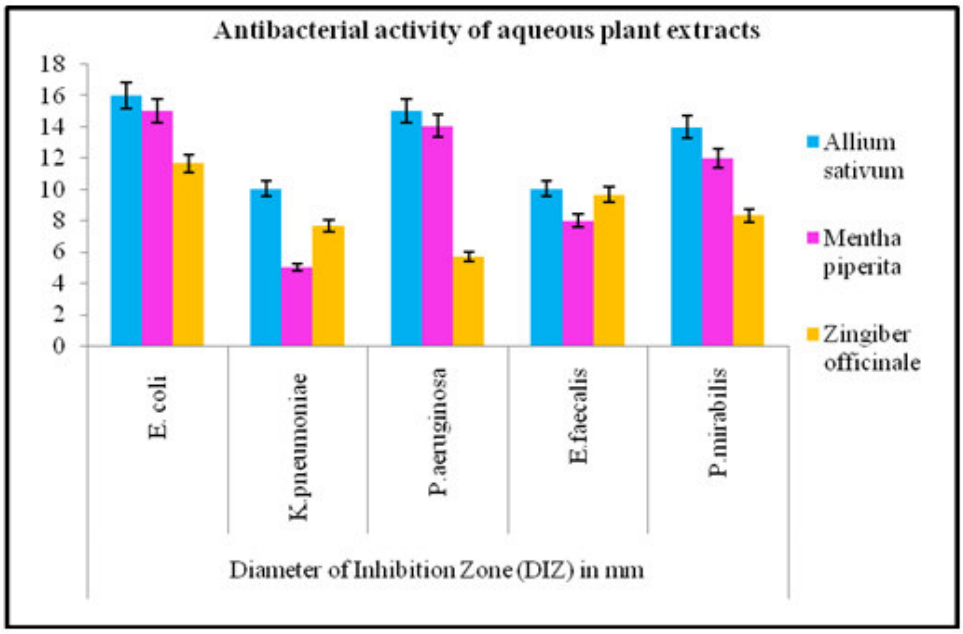
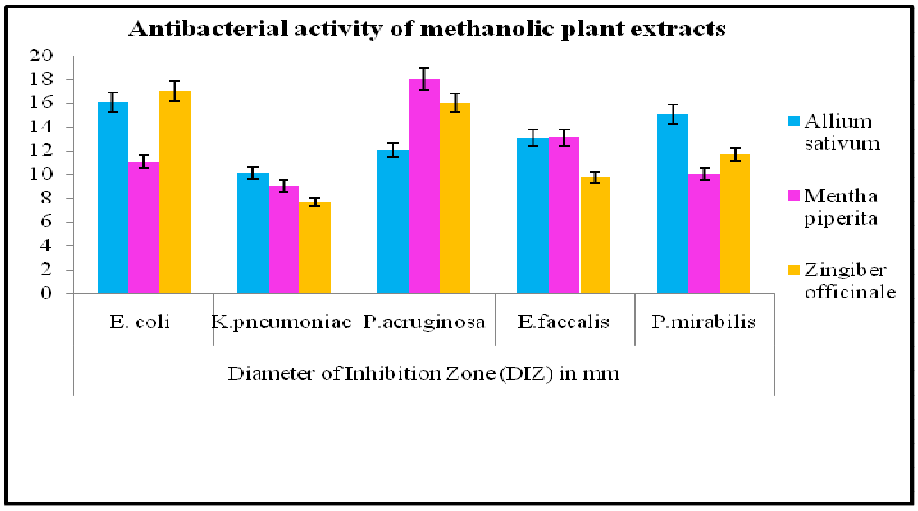
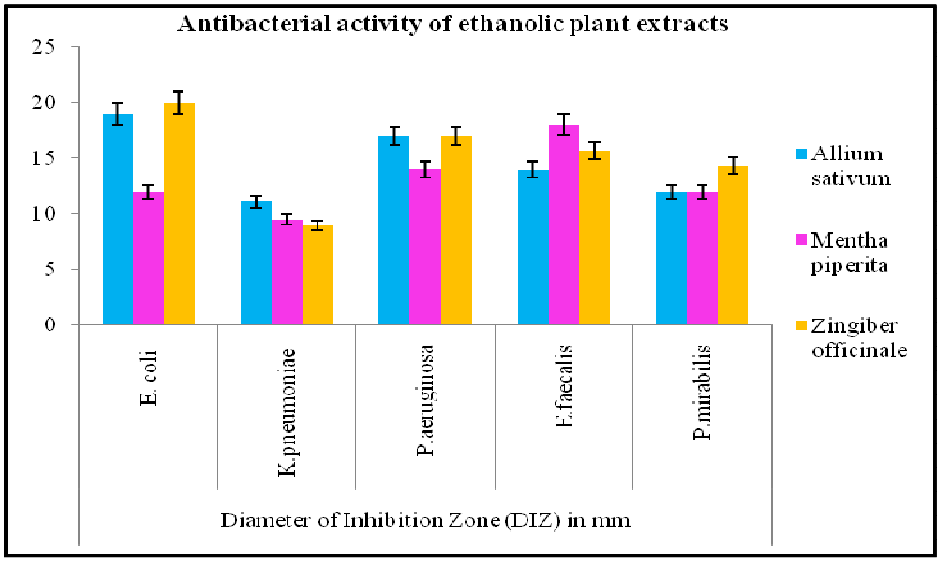


Figure 3
Antibacterial activity of Methanolic Plant extracts on Bacterial UTI Isolates



In the present study highest antibacterial activity was exhibited by ethanolic plant extracts. Among all plant extracts highest DIZ values were recorded for *Zingiber officinale* in the range of 20.0 ± 0.60 and 9.0 ± 1.0 mm against UTI bacterial isolates (Figure-4). Highest antibacterial effect was observed against *E. coli* (20.0 ± 0.60 mm), followed by *P. aeruginosa* (17.0 ± 0.60 mm), *E. faecalis* (15.7 ± 0.58 mm), *P. mirabilis* (14.33 ±0.57) and least for *K. pneumoniae* (9.0 ± 1.0 mm). The pattern of antibacterial activity shown by other ethanolic plant extracts was similar to that of methanolic extracts where in, next higher activity was observed for *Allium sativum* followed by *Mentha piperita* but with higher DIZ values than obtained for methanolic extracts. Among ethanolic extracts lowest antibacterial effect was observed for *Mentha piperita* against all UTI isolates except *E. faecalis* against which it has expressed strong antibacterial effect with a DIZ values 18.06 ± 0.40 mm.

Figure 4
Antibacterial activity of Ethanolic Plant Extracts on Bacterial Isolates



The phytochemical studies reveal that flavonoids, phenolics, alkaloids and tannins are present in all these selected plants. Steroids are present in all three plant extracts. Results of other phytochemical constituents are shown in Table-3. Quantitative evaluation of antibacterial activity (MIC) was carried out by microdilution method for methanolic and ethanolic extracts. Figure-5 shows the MIC of plant extracts on five bacterial UTI isolates. A wide range of MIC values were recorded depending on the microbial strain.

Table 3

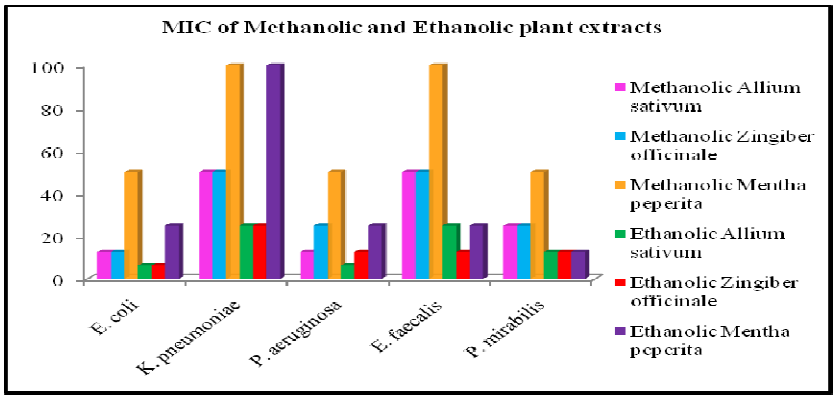
Phytochemical Analysis of Selected Plants

PLANT	TAN	ALK	FLAV	SAP	GLY	STER	PHEN
Allium sativum (bulb)	+	+	+	+	+	-	+
Zingiber officinale (rhizome)	+	+	+	+	+	-	+
Mentha piperita (leaf)	+	+	+	+	+	-	+

TAN: Tannins; ALK: Alkaloids; FLAV: Flavonoids; SAP: Saponins; GLY: Glycosides; STER: Steroids; PHEN: Phenolics "+": Presence "-": Absence

Figure 5

MIC of Methanolic and Ethanolic Plant extracts on UTI Bacterial isolates by Micro dilution method (mg/ml)



DISCUSSION

The emergence of drug resistance with patient's poor compliance, drugs adverse effects and the higher cost of therapy combinations, indicates a strong need for a therapy regimens with higher antibiotics beneficial properties but with better adverse effects profiles. Urinary tract infection is an ever increasing problem that continues to present new challenges due to change in the etiology of UTI and the antimicrobial resistance of urinary pathogens over the years. Factors such as the change in patient population and extensive use of antimicrobial agents could contribute to changes in the microbial profile of urinary tract isolates.[29] Results obtained in the present study presents a strong antibacterial activity for clinical isolates, and indicate the superiority of the antibacterial activity of plant extracts compared to standard antibiotics. Antibacterial activity of aqueous, methanolic and ethanolic extracts of three plants: *Allium sativum* (bulb), *Zingiber officinale* (rhizome), and *Mentha piperita* (leaves) wese tested on five bacterial clinical isolates: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Enterobacter faecalis* and *Proteus mirabilis* from urine samples with UTI. Similar UTI pathogens have been reported by Tabassum et al.[30], Amit Kumar et al.[31] and Fuad et al.[32] The antibacterial potency was initially determined by the agar well diffusion method (as shown in Figures-2, 3 and 4) followed by quantitative evaluation of antibacterial activity by MIC method (Figure-5). Ethanolic extracts of all the plants exhibited higher antibacterial effect than aqueous and methanolic extracts. Among all ethanolic extracts, *Zingiber officinale*– rhizome exhibited highest antibacterial activity which inhibited all five bacterial UTI isolates in following order- *E. coli* > *P. aeruginosa* > *E. faecalis* > *P. mirabilis* > *K. pneumonia* (Figure-4). The antibacterial effectiveness of ethanolic extracts on UTI pathogens was also reported by Anjana et al.[33] Among water, acetone and ethanol extracts, highest antibacterial effect was recorded for ethanolic extracts. The order of antibacterial activity observed was: *P. aeruginosa* > *E. coli* > *K. Pneumoniae*. There was no inhibitory effect observed for all the three extracts against *P. mirabilis* and *E. faecalis*. The results obtained in our study are superior to these previous reports and clearly confirm the effectiveness of *Zingiber officinale*– rhizome extracts on inhibition of bacterial activity. Next to *Zingiber officinale*, strong antibacterial effect was recorded for ethanolic extracts of *Allium sativum*- bulbs. The order of inhibition followed same pattern exhibited by *Zingiber officinale- E. coli* (19.0 ± 0.40) > *P. aeruginosa* (17.0 ± 0.20) > *E. faecalis* (14.0 ± 0.15) > *P. mirabilis* (12.0 ± 0.25) > *K. pneumoniae* (11.13 ± 0.057). Efficacy of *Allium sativum* extracts against UTI isolates was studied by Anandharaj and Saju varghese.[34] They have reported highest antibacterial effect against *E. coli* with a DIZ value of 16 mm and least against *K. pneumoniae* (12 mm). The results obtained in our study are in accordance with this report and also gives a confirmation that *Allium sativum* extracts exerts highest antibacterial effect against *E.coli* and least against *K. pneumoniae*. Antimicrobial effect of methanolic bulb extracts of *Allium sativum* was also studied by Ameh et al.[35] where in a strong antimicrobial effect was reported on six microorganisms including fungi. In the present study *Mentha piperita* leaf extracts expressed lower antibacterial effect against the tested UTI bacterial isolates when compared with *Zingiber officinale* and *Allium* sativum. As observed with other plant extracts the inhibition pattern of *Mentha piperta* extracts was quite different (Figure-5). The highest antibacterial effect was recorded against *E. faecalis* (18.06 ± 0.40), followed by *P. aeruginosa* (14.03 ± 0.15); *E. coli* and *P. mirabilis*

were equally inhibited with same DIZ values of 12.0 ± 0.50 and 12.0 ± 0.20 respectively. The lowest effect was against *K. pneumoniae* (9.53 ± 0.057). Antibacterial activity of *Mentha piperita* leaf, stem and root extracts on pathogenic bacteria was studied by Sujana et al.[36] High antibacterial activity was reported in ethanolic extracts (7.2 – 15.3 mm) against *E. coli, K. pneumonia, P. vulgaris, S. aureus* and *S. pneumoniae*. Comparative analysis of leaf and stem extracts of *Mentha piperita* was reported by Saeed and Tariq.[37] The study reported strong antibacterial effect of leaf extracts when compared with stem extracts on 56 bacterial isolates. These results are giving a confirmation that ethanolic leaf extracts of *Mentha piperita* has strong antibacterial effect. The results obtained for the plant extracts in the present study showed significant antibacterial activity against the five pathogenic UTI isolates tested. Among all the tested bacteria *K. pneumoniae* is the least affected organism giving a clue that capsule and other determinant factors such as enzyme may be responsible for decreasing the effectiveness of the components present in plant extracts. MIC by micro dilution showed good results compared to well diffusion method as there may be a problem with the diffusion of the biological component into the agar. The hydrocarbon components either remain on the surface of the medium or evaporate.[38] That could be the reason for the better results obtained by the microdilution method. Broth method has advantage of lower workloads for a large number of replicates and the use of small volumes of the test substance and growth medium.[39] The difference in the antibacterial activity with the same source when extracted with different solvent has proven that not all phytochemicals that are responsible for antibacterial activity are soluble in a single solvent. Hence solvents of different polarity should be employed as discussed in this study (polar and nonpolar). In the present study ethanolic extracts of selected plants expressed highest and broad spectrum antibacterial activity to pathogenic UTI isolates. Two possibilities that may account for this higher antibacterial activity of ethanolic extracts are the nature of biological active components (alkaloids, flavonoids, essential oils, terpenoids, tannins etc.), which may be enhanced in the presence of ethanol; and stronger extraction capacity of ethanol that may have yielded a greater number of active constituents responsible for antibacterial activity.[40]

CONCLUSION

Based on the results obtained we it can be concluded that all selected plants used in this study expressed broad spectrum antibacterial activity on bacterial UTI isolates with highest activity recorded for ethanolic extracts. The data obtained for antibacterial activity of 10 standard antibiotics commonly used for UTI treatment concludes that, most of the antibiotics were ineffective in inhibiting the growth of these bacterial isolates, on the other hand methanolic and ethanolic extracts exerted good antibacterial activity. Importantly the results suggest that these plants contain active ingredients which qualify them for medicinal use. The presence of phytocompounds in the extracts including phenols, tannins and flavonoids as major constituents may be responsible for the antibacterial activity.

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