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Isolation and Identification of Predominate Urinary Tract Infection Bacteria and Assessed Their Chemotactic Behavior in the Presence of Some Medicinal Herbal Plant Extracts

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Abstract: The Purpose of the study was the isolation and identification of Urinary tract infection (UTI) bacteria and the evaluation of their susceptibility against antibiotics. In addition, susceptibility and chemotactic behavior of isolated strains were evaluated against medicinal herbal plant extracts.

To perform the study, 200 samples were collected from the patients suffering from UTI. Then, the bacterial agents were isolated and their susceptibility were assessed against gentamicin, ciprofloxacin, nitrofurantoin, trimethoprim-sulfamethoxazole, nalidixic acid, cephalexin, cefixime and ceftriaxone by disk diffusion method. In addition, *Thymus vulgaris, Achilleamillefolium, Teucriumpolium, Origanumvulgare, Salvia officinalis, Urticadioica, Matricariachamomilla, Foeniculumvulgare, Alliumascalonicum* and *Cinnamomumverum* were extracted using boiling water and ethyl alcohol (70%) and their antimicrobial effects were tested against isolated strains by Well Agar Diffusion method. Finally, chemotaxis behavior of some isolates evaluated against sub Arbitrary concentration of effective medicinal herbal plants. The results obtained indicated that Gram negative and Gram positive bacteria were isolated with a frequency of 80% and 20% respectively. *Escherichia coli* and *Staphylococcus saprophyticus* were predominant agents. Moreover, ciprofloxacin, nitrofurantoin, and gentamicin exhibited more effectiveness. Out of all drug plant extracts, *Allium ascalonicum* and *Cinnamomumverum* had exhibiting relatively more antimicrobial effect against urinary tract infection agents. Our finding, concerning to chemotactic behavior of motile bacterial strains indicated that both herbal medicinal plants *Allium ascalonicum* and *Cinnamomumverum* eliminated chemotactic behavior of *Escherichia coli, proteusmirabilis* and *Enterobactersakazaki.* Hence, due to increasing level of antibiotic resistant bacteria, which causing UTI, some medicinal herbal plants such as *Allium ascalonicum* and *Cinnamomumverum* could be considered *as* a new source of remedy for treatment of the patients suffering from the infection.

Keywords: Urinary tract infection, chemotactic behavior, medicinal herbal plant

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1. Introduction

Urinary tract infection is an infection which could affect the kidneys, ureters, bladder or urethra and it's subjected as one of the most common infections in humans (Geetha *et al.,* 2011).). Among bacteria *Escherichia coli* follow by *Staphylococcus, Klebsiella, Enterobacter, Proteus* and *Enterococci* species are the most important infection agents responsible for all UTI cases, (Ronald, 2003). Although, UTI could affect upper and lower part of the genitourinary tract, the majority of cases are associated with lower part of the tract (Najar*et al.,* 2009). Different factors such as female gender, sexual activity, menopause, diabetes, catheter use, and urinary tract obstruction providing the risks for increasing the rate of Urinary tracts infection (Gales *et al.,* 2002). Generally, several antibiotics viz., penicillins, sulfanilamide, nitrofurantoin and cephalexin have been prescribed for treatment of UTI, however the recent reports opined that the frequency of occurrence of antibiotic resistant strains of UTI agents is increasing (Nicolle, 2002). On the other hand, medicinal herbal plants and their application as therapeutic agents instead of antibiotic therapy have been suggested for reduction of frequency of existence of antibiotic resistant bacteria. Nowadays, plants have served as a basis for development of novel drugs due to different functional groups in their structure with multiple mechanisms (Burt, 2004). Indeed, two strategies are essential in utilizing herbal medicine: the choice of herbs, and their actions (Geethaet *al*., 2011). In this case, the chemotactic behavior of bacteria could be considered as a target for elimination of UTI bacterial agents without the specific site effects. Therefore the present study was conductedto isolate different bacterial agents causing UTI and assessed their antibiotic susceptibilities against common antibiotics. In addition, antimicrobial property of different medicinal herbal plants was evaluated against the isolated strains and eventually chemotactic behavior of isolated motile strains from urinary tract infection samples were evaluated in present of sub Arbitrary Unit of medicinal herbal plant extracts.

2. Methods and Materials

Isolation and Identification of Bacteria

Totally, 200 urine samples were collected from the patients suffering from Urinary Tract Infection. Clean-catch midstream urine of the patients was collected in a sterile tube (4-5ml) and assessed immediately within 30 minute. To perform the test calibrated loopful was streak onto blood and Eosin Methylene blue agar media and incubated at 37C° for 24-48 hours. Then the number of pure bacteria equal or more than 105CFU/ml considered as potential pathogenic bacterium and recorded as positive UTI patient (Wu 2006). All bacteria isolated from the urine samples were phenotypically identified using conventional biochemical tests and Api 20 E and 20NE biomerieux kits.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing was done on Mueller Hinton agar (Merck, Germany) using disk diffusion (Kirby Bauer's) technique. The technique was carried out according to Clinical and Laboratory Standards Institute

(CLSI) guidelines to determine susceptibility of the UTI agents (CLSI 2006). The antibiotic disks (PadtanTeb, Tehran, Iran) were: Gentamicin (10µg), ciprofloxacin (5µg), nitrofurantoin (300µg), trimethoprim-sulfamethoxazole (25µg), nalidixic acid (30µg), cephalexin (30µg), Cefixime (5µg) and ceftriaxone (30µg) (Herminiaet al., 2005).

Preparation of Drug Plant Extracts

Totally ten medicinal herbal plantsViz., *Thymus vulgaris, Achilleamillefolium, Teucriumpolium, Origanumvulgare, Salvia officinalis, Urticadioica, Matricariachamomilla, Foeniculumvulgare, Allium ascalonicum* and *Cinnamomumverum* were purchased from retail shops Shiraz, Iran and they were powdered for preparing the extracts.

Drug plant extraction was performed after overflowing of 20 g of each drug plant in 200 ml boiling water and ethyl alcohol (70%) in different flasks. The flasks were plugged with cotton and kept in a rotary shaker incubator (150 rpm). After 72 hrs. the suspensions were centrifuged (3000 rpm for 10 min) and the solvent was evaporated. The extractions were stored at 4C° in airtight bottles for further studies (Gomma*et al.,* 2003; Vasu and Singara 2010).

Antimicrobial Effect of Plant Extracts

The antimicrobial effect of the plant extracts was evaluated against antibiotic resistant strains**.** At the onset the isolated strains were inoculated in nutrient broth and incubated for 24 hrs. Then the activated strains were full streaked on Muller Hinton agar and the wells were made in the medium using sterile borer (6 mm). Then 100 µl of the each extract was added to each well and the plate incubated at 37C° for 24-48 hours. After this period inhibiting growth zone around each well was measure and recorded. The experiment was performed in triplicates (Skocibusic*et al.,* 2004; Sharma *et al.,* 2011).

Determination of Arbitrary Unit (AU)

To determine arbitrary unit of each plant extract, 100 µl of various concentrations of each extracts (1/2, 1/4, 1/8, 1/16, … , 1/1024), was added into the wells, which made in cultivated Mueller Hinton Agar with sensitive microorganisms. The plates were incubated at 37 °C. AU for each extract was determined after 24 hrs. Arbitrary Unit of the extract for each sensitive bacterium was determined by the reciprocal of the highest dilution showing antimicrobial effect.

Molecular Identification of Motile Bacteria Isolated from UTI Patient

Of all isolated bacteria, three motile strains were randomly selected for molecularidentification using 16SrRNA gene. To perform the test, DNA extraction was carried out using DNA PCR kit (Roche-Germany).The extracted DNA with ratio (260/280nm) more than 1.9was used for Polymerase Chain Reaction (PCR). Amplification of 16SrRNA gene was performed using Forward and Reverse universal primers for Gram negative bacteria. Each reaction tube was containing 18 µl of water , 2.5 µl of 10×PCR buffer , 1 µl of each forward and reverse PCR primers, 0.5 µl of a 10 mMdNTPs, 0.25 µl of Smarttaq polymerase, 0.75 µl of 50mM $MgCl_2$ and 5 µl of DNA template.PCR conditions of thermocycler were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 60 s,

56°C for 45 s, and 72°C for 60 s, with a final extension at 72°C for 5 min and storage at 4°C. The PCR product was run on a 1.5% (w/v) agarose gel. PCR products was electrophoresed at 90V for 20 min and then DNA bands was virtualized after staining with ethidium bromide. Finally, the PCR product with pure DNA band has been sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing. The 16S rRNA sequenced data was subjected to BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to identify respective 16S rRNA gene amplicon.

Chemotaxis Assay

The chemotaxis assay was carried out using the method that explained by Hazelegere*t al.,* (1998). In this method 15 ml of the sterile melted SIM medium was mixed with 0.1 ml of the bacterial culture (concentration equal to 0.5 Mcfarland tube) and poured in the plate. Then two wells were made in the medium using sterile sharp borer and 100 µl of urine and urine plus subAU concentrations of herbal plant extracts added in the wells. All plates were incubated at 37 C^{o} for 24-48 h. Then, the bacterial cells density around the wells was evaluated and negative and positive chemotactic response was recorded.

3. Results

Isolation and Phenotypic Identification of UTI Agents

Microbial analysis of collected samples opined that Gram negative and Gram positive bacteria were isolated from the samples with frequency of 80% and 20% respectively. Among isolated bacteria the most common gram negative bacterium was *E.coli* (53.5%) and gram positive was *Staphylococcus saprophyticus* (13%). In contrary, *Proteus* spp. and *Enterococcus faecalis* were isolated from UTI patients with less frequency (Table 1).

Table 1 Frequency of occurrence of UTI bacterial agents

Bacterial isolates	No. of isolates	Percentage
Escherichia coli	107	53.5
Klebsiellapneumoniae	34	17
Enterobacter sp.	13	6.5
Proteus sp.	6	3
Staphylococcus aureus	11	5.5
Staphylococcus saprophyticus	26	13
Enterococcus faecalis	3	1.5

Antibiotic Susceptibility of Isolated Strains

In total most effective antibiotics against the bacteria isolated from UTI were ciprofleoxacin, nitrofurantoin, and gentamycin. However, comparatively the effect of nalidixicacid, trimethoprim-sulfamethoxazole and cefiximewas less. Furthermore, all of the isolated bacteria (except Entrococcusfaecalis) were sensitive to the antibiotics tested with different responses Table 2.

Table 2 Antibiotic susceptibility of isolated bacterial strains from UTI samples

Bacterial Strains	Antibiotics							
	Ciprofloxacin	Gentamicin	Cefexime	Nalidixic acid	Nitrofuran	Sulfometaxazole	Cephalexin	Ceftriaxon
Escherichia coli	75(70)	77(71/9)	64(59/8)	50(46/7)	92(85/9)	48(44/8)	66(61/6)	71(66/3)
Klebsiellapneumoniae	27(79/4)	23(67/6)	20(58/8)	22(64/7)	13(38/2)	26(76/4)	17(50)	24(70/5)
Enterobacter sp.	11(84/6)	8(61/5)	7(53/8)	8(61/5)	8(61/5)	7(53/8)	6(46/1)	8(61/5)
Proteus sp.	6(100)	6(100)	6(100)	6(100)	2(34)	4(66/6)	3(50)	3(50)
Staphylococcus saprophyticus	22(84/6)	18(69/2)	5(19/2)	4(15/3)	23(88/4)	13(50)	22(84/6)	20(76/9)
Staphylococcus aureus	7(63/6)	7(63/6)	2(18/1)	1(9)	8(72/7)	5(45/4)	7(63/6)	6(54/5)
Enterococcus faecalis	2(66/7)	0	1(33/3)	0	2(66/7)	1(33/3)	1(33/3)	1(33/3)

Antimicrobial Susceptibility of Drug Plant Extracts Against Resistant Isolates

The results obtained from antimicrobial susceptibility of herbal plant extracts against antibiotic resistant strains indicated that ethyl alcohol extracts of the herbal plant extracts relative showed more effective. In addition, as shown in Table 3 *Enterobacter* sp. and *Enterococcus faecalis* were relatively more resistant and *Staphylococcus aureus* and *Proteus* spp. were relatively more sensitive to the herbal plant extracts. Out of all herbal plant extracts, *Allium ascalonicum* and *Cinnamomumverum* exhibiting relatively more antimicrobial effect against urinary tract infection agents.

In addition,the result obtained from determination of Arbitrary Unit showed 32 AU as a specific Arbitrary Unit, thereforesubAU(AU64) was selected for determination of chemotacticbehavior of the isolates against herbal medicinal plant extracts.

Table 3 Antimicrobial effect of alcohol herbal plant extract to UTI isolated agents

Bacterial Strains	Herbal plant extracts						
	A. ascalonicun	*C. veron*	*U. dioica*	*O. vulgare*	*T. poliul*	*A. millefoliun*	*T. vulgaris*
Escherichia coli	15*	–	–	–	–	–	13
Klebsiellapneumoniae	14	10	–	–	–	–	–
Enterobacter sp.	12	10	–	–	–	–	–
Proteus sp.	–	12	–	6	8	8	–
Staphylococcus aureus	10	–	8	–	–	–	10
Staphylococcus saprophyticus	14	12	–	8	8	10	16
Enterococcus faecalis	14	8	–	–	–	–	–

Molecular Identification of Motile Bacteria Isolated from UTI Samples

The results obtained from alignment analysis of 16SrRNA genes of the bacterial strains showed 98%, 100% and 99% identical to *Escherichia coli, Proteus mirabilis* and *Enterobactersakazaki.* strainsrespectively (table 4).

Table 4 Alignment analysis of 16SrRNA genes of the bacterial strains isolated from UTI samples

Accession numbers	Genus/Species	NCBI Taxonomy ID
gb|CP007390.1	*Escherichia coli*	ST540
gb|HQ169118.1	*Proteus mirabilis*	FUA1240
gb|EF088349.1	*Enterobactersakazakii*	05 01 123

Evaluation of Chemotactic Behavior of UTI Agents to Drug Plant Extracts

The results obtained from evaluation of the chemotactic behavior of some UTI agents in presence of subAU concentration of *Allium ascalonicum* and *Cinnamomumverum* indicated that both herbal medicinal plants eliminated migration of the bacteria, and therefore density of the bacteria around the urine plus herbal extracts wells were less. In addition, *Allium ascalonicum* illustrated more effective in compared to *Cinnamomumverum*. Furthermore, motion of *Escherichia coli, Enterobactersakazakii* and *Proteus mirabilis* were eliminated against *Allium ascalonicum* and *proteus* was diminished against *Cinnamomumverum*.

4. Discussion

Urinary tract infections (UTI) are common conditions worldwide and the pattern of antimicrobial resistance varies in different regions. We conducted to isolate different agents of UTI and assessed their antibiotic susceptibilities. Furthermore, we describe the relationships between chemotactic behavior of the some UTI agents and their motility against drug plant extracts. The study was confined to UTIs in men as well as women adults. In addition, *E. coli* was the most predominant bacteria. There are earlier studies in agreement to present finding *Escherichia coli* recognized as most important agent follow by *Staphylococcus saprophyticus* and *Klebsiellapneumoniae*. Several reports verified our finding in case of UTI agents' detection (David and vrahas 2000; Ronald 2001; Gales *et al.,* 2002). Antibiotic susceptibility properties of our isolates indicated that most of the isolates were susceptible to ciprofloxacin fallow by nitrofuranand cephalexin. However, susceptibility to ceftriaxone and cefexime was less. In this regard, it must be noted that UTI agents' responses to antibiotics are not identical. For example, Tankhiwale and its colleagues (2004) reported that isolated a *Echerichia coli* strains from UTI were sensitive to Nitrofuran and ceftriaxone, Diass Neto and its colleagues (2003) reported impenem as more effective antibiotic against UTI agents. On the other hand, our investigationevaluated the effect of some drug extracts against resistant strains of isolated UTI agents. The results obtained indicated that of all drug plant extracts, ethyl alcohol extracts of *Allium ascalonicum* and *Cinnamomumverum* exhibited as more effectiveness. In addition, subAU concentration of these drug plant extracts eliminated chemotaxis behavior of motile UTI agents Viz., *Echerichia coli*, *Proteusmirabilis* and *Enterobactersakazakii*. It means, because of increasing level of antibiotic resistant bacteria, which causing UTI, someherbal medicinal plant extracts could be considered as new source of remedy for treatment of the patients suffering from the infection. Moreover, less concentration of these drug plant extracts could eliminate chemotatacticbehavior of these agents with less possibility for causing the site effects.

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