Antibacterial Activity of Aqueous Plant Extracts and Honey against UTI causing Superbugs

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Abstract
The fluent and unprescribed use of drugs has created an alarming situation of drug resistance acquired by pathogenic microorganisms. In (Urinary tract infections) UTI choice of drug is limited and increasing resistance against available antibacterial drugs made it worse than other infections. In present study urine samples from people suffering from urinary tract infections (UTI) were taken and tested for presence of highly drug resistant microorganisms by performing antibiotic susceptibility test. Out of thirty bacterial pathogens five were selected for molecular characterization and antimicrobial sensitivity testing against plant extracts. Knowing the fact that Kalonji, Methi, Neem, Jaifal and honey have great pharmaceutical potential and medical importance without any side effect, their crude extracts were used in well plate assay to determine the antibacterial activity against the UTI causing organisms. Plant extracts and honey showed remarkable antimicrobial activity against drug resistance pathogens. Another important aspect of this research is the fact that Pakistan is a developing country and most people cannot afford expensive third or fourth generation drug treatment. This scenario demands the discovery of new pharmaceutical drugs that can cope with the drug resistance problems by effectively eliminating pathogenic UTI causing organisms without causing any side effects in patients suffering from UTI.
Key words: Antimicrobial activity, Medicinal plants, Urinary tract infection (UTI), *Escherichia coli*, *Streptococcus saprophyticus*

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Introduction

A standout amongst the most common maladies experienced in the act of prescription today is brought on by the presence of microorganisms in the urinary tract [1]. Research have proposed that 95% of all urinary tract infections (UTIs) are caused by bacteria especially Gram-negative bacteria, while the other 5% are caused due to hematogenous infections. The latter is typically brought on by Gram-positive bacteria [2]. UTIs caused due to Gram negative bacteria start with the colonization of microorganisms in the periurethral area, emulated by an upward movement of microbes to contaminate the bladder. On the off chance that states of disease endure, this movement of microorganisms could proceed to the ureters lastly to the kidneys. Cystitis is the infection of urinary bladder, whereas the infection of one or both kidneys is pyelonephritis which is a more serious sort of infection that if not treated appropriately could result in kidney failure [3]. *Escherichia coli* is the most widely recognized urinary pathogen representing no less than 85% of community-acquired UTIs [4]. *E. coli* is naturally present in the large intestine of humans where they are responsible for producing vitamin K [5]. *Escherichia coli* turn into a danger when it stick to the urethra opening and begin to grow. Species of bacteria that cause UTIs other than *E. coli* include Gram-positive bacteria, for example, *Staphylococcus saprophyticus* representing 5-10% of uncomplicated cystitis, *Staphylococcus aureus*, and Gram-negative bacteria, for example, *Proteus mirabilis, Klebsiella sp, Citrobacter sp and P. aeruginosa* [6]. The fluent and uncontrolled utilization of medications and anti-infection agents created microorganisms to develop resistance against antibiotics and hence forth the pattern moved from antibiotics to utilization of natural plant extracts to cure from potentially harmful maladies. Still today 75-80% world populace hand-off on natural drug [7], primarily in view of lesser side effects. For centuries Black seed and its oil has been used to cure respiratory disorders, kidney, liver functions, cancer and disorders related to circulatory system [8]. Nigella sativa is composed of 50% thymoquinone and 40% P-Cymene, fatty acids and alcohols are also present [9]. Thymoquinone (TQ) is the main active compound present in seeds and oil of Nigella sativa as shown by GC-MS analysis [10]. TQ enhances apoptosis initiation by suppression of anti-apoptotic gene expression [11]. The thought behind the present research work was to search for an alternate and potential effective medicinal formulation to treat urinary tract infections in order to overcome the ever rising problem of multidrug resistance acquired by pathogenic UTI causing organisms.

Materials and methods

Isolation of UTI causing Bacteria:

Urine samples of patients suffering from urinary tract infections were collected in boric acid tubes and transferred to laboratory maintaining the cold chain. The urine samples were first streaked on CLED agar with a calibrated loop 1ul and then the colonies were counted after 24 hours of incubation at 37°C [12]. Number of colonies per ml of urine was counted keeping in

mind that a count of 100,000/ml or more is an indication of a significant clinical urinary tract infection. Isolated pathogens were identified and confirmed by API® identification scheme.

Antibiotics Susceptibility Test

Antibiotics susceptibility testing was done by Kirby Bauer standard agar disc (oxide) diffusion method. Zones of inhibition were measured and susceptibility of UTI causing bacteria against drugs was reported as resistant, sensitive or intermediate as per the Clinical Laboratory Standard Institute guidelines (CLSI, 2015). Controls strains of E. coli (ATCC® 25922), Pseudomonas aeruginosa (ATCC® 27853), E. faecalis (ATCC2® 9212), S. aureus (ATCC® 29213) were used as recommended by CLSI. Ampicillin (30µg), Amoxicillin (25µg), amoxicillin/clavulanic acid (30µg), ampicillin/sulbactum (30µg), cefipime (30µg), cefotaxime (30µg), cefoxitin (30µg), cefuroxime (30µg), ceftaxidime (30µg), ceftriaxone (30µg), cephalexin (30µg), cefradine (30µg), cefaclor (30µg), cefixime (30µg), imipenem (10µg), meropenem (10µg), vancomycin (30µg), amikacin (30µg), gentamycin (30µg), tobramycin (30µg), azithromycin (30µg), erythromycin (30µg), doxycycline (30µg), ciprofloxacin (10µg), levofloxacin (10µg), norfloxacin (10µg), nitrofurantoin (300µg), fosfomycin (50µg), clindamycin (10 µg), fusidic acid (50 µg), linezolid (30 µg), cefoperazone (30µg), aztreonam (30 µg), nalidixic acid (30 µg), ofloxacin (5µg), moxifloxacin (5µg), trimethoprim/sulfamethoxazole (25 µg), pipemidic acid (20 µg) cefoperazone/sulbactam (30µg), and piperacillin/tazobactam (110µg) were the antibiotics used for different pathogens isolated from UTI.

***Nigella saliva* (Kalonji) Extract:**

Nigella sativa seeds were weighed (100 gm) which were bought from a retail food store. The seeds were first surface sterilized with ethanol for 2 minutes and then washed thoroughly with autoclaved distilled water. After soaking in water, seeds were homogenized aseptically using a sterile mortar and pestle. The homogenized mixture was then centrifuged at 12,000 rpm for 15 minutes and supernatant was separated. This was considered as 100% concentration of the extract [13].

Honey Extract:

Crude honey was bought from a retail food store, was considered as the 100% concentration. Dilutions were made with appropriate amount of distilled water (50 and 75%) [14].

***Azadirachta indica* (Neem) Extract:**

Fresh and healthy leaves were collected from Neem tree. The leaves were first surface sterilized with ethanol for 2 minutes and then washed thoroughly with autoclaved distilled water and dried in shade. The leaves were weighed and using pestle and mortar, paste of Neem leaves with autoclaved distilled water (1:1 w/v) was made [15]. The homogenized mixture was centrifuged at

12,000 rpm for 15 minutes and supernatant was separated. This was considered as 100% concentration of the extract.

Trigonella foenum-graecum **(Methi) Extract:**

Fresh and healthy leaves were collected from Methi plant. The leaves were first surface sterilized with ethanol for 2 minutes and then washed thoroughly with autoclaved distilled water and dried in shade. The leaves were weighed and using pestle and mortar, paste of Methi leaves with autoclaved distilled water (1:1 w/v) was made [15]. The homogenized mixture was centrifuged at 12,000 rpm for 15 minutes and supernatant considered as 100% concentration was taken.

Myristica fragrans **(Jaiphal) Extract:**

The seeds of Jaiphal were purchased from a retail food store and then 100 gm of seeds were weighed. The seeds were first surface sterilized with ethanol for 2 minutes and then washed thoroughly with autoclaved distilled water. Weighed seeds were then soaked in water and then homogenized aseptically using a sterile pestle and mortar. The homogenized mixture was centrifuged at 12,000 rpm for 15 minutes and supernatant was separated. This was considered as 100% concentration of the extract.

Mixture of Honey and Kalonji Extract:

Honey and Kalonji extract were mixed in equal volumes (1:1 v/v) to make a mixture of 100 % honey and Kalonji extract.

Antibacterial Assay of Natural Plant Extracts by Using Agar Well Diffusion Method:

Selected UTI causing bacterial strains were inoculated in nutrient broth and incubated at 37°C for 24 hours. 50 µl of standard cultures (0.5 McFarland) was evenly spread on Mueller Hinton agar plates to obtain confluent growth. Using sterile Pasteur pipette, wells were made and 40µl of natural extracts were added to the wells by using micropipette. After 24 hours incubation at 37 C° the appearance of hollow, transparent, zones of inhibition were indicative of the antibacterial activity of extracts against UTI causing bacteria. Diameters of zones of inhibition were measured in millimeters using transparent plastic ruler. [13]

Molecular Characterization of Selected Strains:

The five selected UTI causing bacterial strains ZK 1 (*E. coli*), ZK 2 (*E. coli*), ZK 3 (*Staphylococcus saprophyticus*), ZK 4 (*E. coli*) and ZK 5 (*E. coli*) that showed susceptibility to natural extracts were inoculated in N-agar slants prepared in special sterile eppendorfs and sent to Macrogen Sequencing facility at Korea for 16S rRNA sequencing. After getting the sequences, they were blasted in NCBI and submitted to gene bank database of NCBI to get the accession numbers. By using MEGA 6 software with bootstrapping value 1000 phylogenetic tree was made. Multiple sequence alignment of five selected UTI causing strains with closely related bacterial strains was

done and then through neighbor joining method using MEGA 6, phylogenetic tree was constructed that showed molecular relationship of selected strains with closely related bacterial strains.

Statistical analysis:

Results of antimicrobial activity test of natural extracts against UTI causing bacteria were taken in triplicates and data were subjected to mean, standard error, analysis of variance (ANOVA). Data were expressed in mean ± S.E. of each replicate then means were compared by Duncan's multiple range test (DMRT).

Results

Isolation and Screening of Multiple Drug Resistant UTI Causing Bacteria:

Total 30 samples isolated and purified on CLED agar media were subjected to antibiotic susceptibility testing and the results of percentage resistance against respective drugs are given in Figure 1.

Pathogen isolated / Antimicrobial drug used	AMP	AMC	FEP	CFP	CTX	FOX	CXM	CAZ	CRO	CFM	IMP	MEM	ATM	AK	CN	TOB	E	DO	CIP	NOR	OFX	SXT	TZP	LZD	DA	FD	F	PIP	FOS
Pseudomonas	NT	NT	NT	NT	NT	NT	NT	66	NT	NT	50	50	0	66	66	66	NT	NT	66	66	66	NT	NT	NT	NT	NT	N	NT	NT
E. coli	100	100	75	90	90	NT	90	90	90	90	50	50	90	15	40	48	NT	66	78	78	78	78	15	NT	NT	NT	23	78	23
Klebsiella	100	100	100	100	100	NT	100	100	100	100	50	50	100	50	50	82	NT	50	50	50	40	82	15	NT	NT	NT	33	1OO	33
Staphylococcus	100	100	100	100	100	100	100	100	100	100	100	100	NT	75	75	90	100	100	100	100	100	100	NT	0	25	10	45	100	80
Streptococcus	50	50	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	10	NT	NT	50	100	100	100	NT	NT	0	NT	NT	50	NT	80

Figure 1: Percentage resistance of selected UTI causing *Pseudomonas*, *E. coli, Klebsiella*, *Staphylococcus* and *Streptococcus* against various antibiotics. Ampicillin (AMP), amoxicillin/clavulanic acid (AMC), cefipime (FEP), Cefoperazone (CFP) cefotaxime (CTX), cefoxitin (FOX), cefuroxime (CXM), ceftaxidime (CAZ), ceftriaxone (CRO), cefixime (CFM), imipenem (IMP), meropenem (MEM), aztreonam (ATM), amikacin (AK), gentamycin (CN), tobramycin (TOB), erythromycin (E), doxycycline (DO), ciprofloxacin (CIP), norfloxacin (NOR), ofloxacin (OFX), trimethoprim/sulfamethoxazole (SXT), piperacillin/tazobactam (TZP), linezolid (LZD), clindamycin (DA), fusidic acid (FD), nitrofurantoin (F), pipemidic acid (PIP), fosfomycin (FOS), Not tested (NT).

Morphological Characterization Based on Growth on CLED agar:

CLED Agar is a non-selective differential plating medium for the growth and enumeration of urinary tract microorganisms. Omitting sodium chloride CLED Agar inhibits the swarming of *Proteus*.[16] CLED Agar supports the growth of a great majority of the bacteria which causes urinary tract infections and is used to differentiate and identify these pathogens.

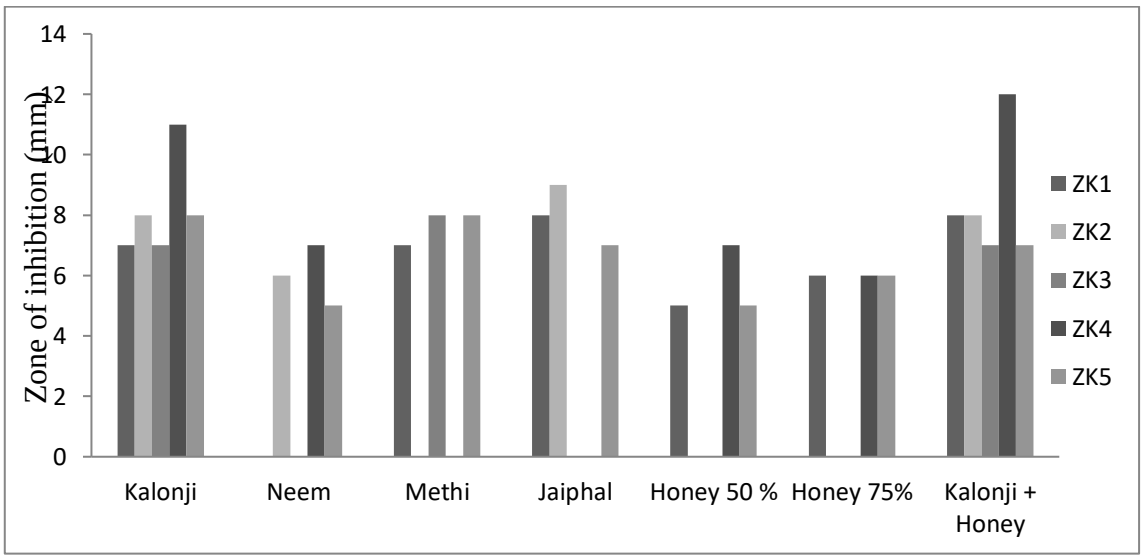
Gram Staining and Biochemical identification

Gram staining of all the 30 selected UTI causing bacteria was performed to determine whether the bacterial isolates were gram positive or gram negative and also cell shape (Cocci or Bacilli). Only 3 out of 30 strains) were gram positive cocci while the rest 27 were gram negative Bacilli. All bacterial strains were confirmed by respective biochemical and API® as per CLSI recommendation (CLSI 2015).

Antibacterial Activity Assay of Natural Extracts:

Natural extracts i.e. kalonji, methi, neem, jaiphal and honey were tested for their antibacterial effect on UTI causing organisms. Out of the 30 bacterial isolates, excellent antibacterial activity of natural extracts was seen in 5 bacterial strains. These strains were denoted as ZK 1, ZK 2, ZK 3, ZK 4 and ZK 5. Among them ZK 1, ZK 2 and ZK 3 were multidrug resistant UTI causing bacterial strains while ZK 4 and ZK 5 were drug sensitive strains used as controls along with ATCC controls. ZK1 and ZK2 were resistance all tested antibiotics except nitrofurantoin (300µg) and piperacillin/tazobactam (110µg). ZK3 is resistance to all tested antibiotics except nitrofurantoin (300µg), vancomycin (30µg) and linezolid (30 µg). The test was done in triplicates.

Kalonji as well as mixture of kalonji and honey inhibited the growth of all the 5 strains. Neem showed antibacterial activity against ZK 2, ZK 4 and ZK 5. Methi extract was affective against ZK 1, ZK 3 and ZK 5. Similarly Jaiphal showed antimicrobial activity against strains ZK 1, ZK 2 and ZK 5. Both concentrations of honey i.e. 50 and 75 % inhibited the growth of 3 bacterial strains (ZK 1, ZK 4 and ZK 5).



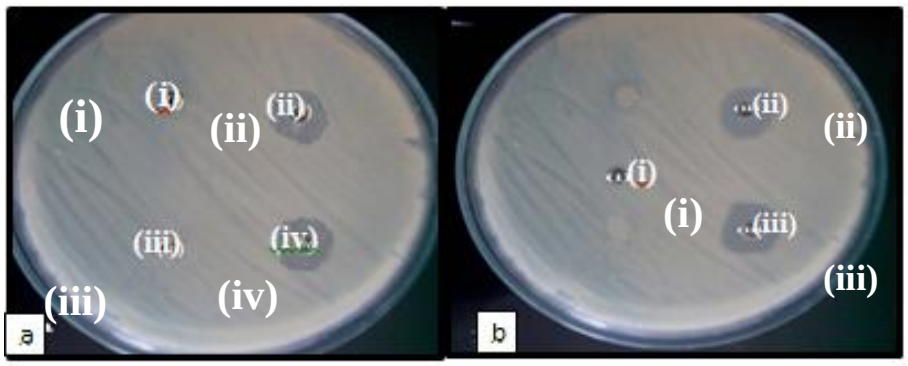


Figure 2: Antibacterial activity of natural plant extract and Honey against five selected strains of bacteria

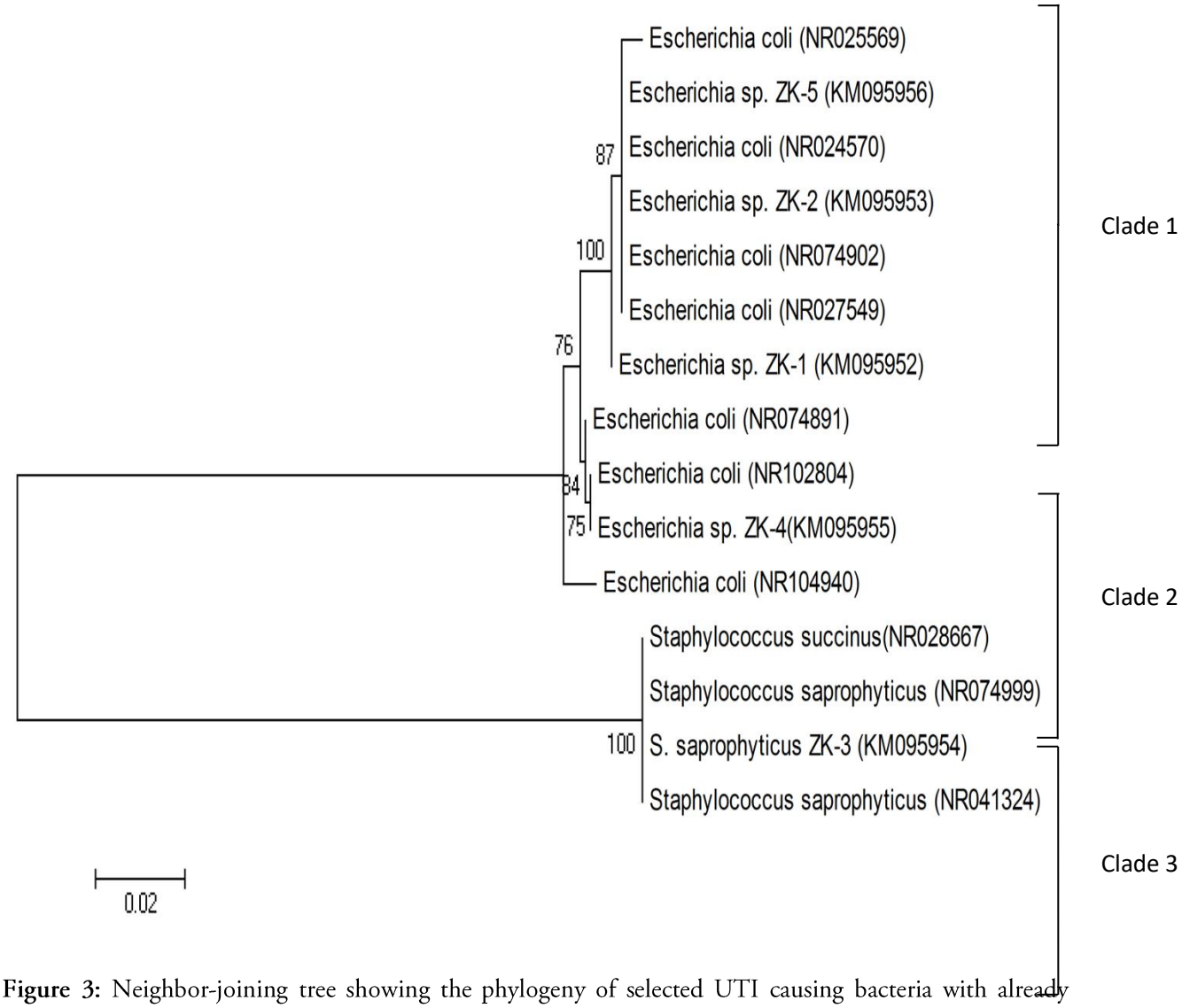


Figure 3: Neighbor-joining tree showing the phylogeny of selected UTI causing bacteria with already reported and closely related bacterial species. Scale bar represents 0.02 changes per nucleotide position.

Discussion

Urinary tract infection is second most prevalent infection worldwide and every year around 150 million people suffer from this infection [17]. It is mostly caused by gram negative bacteria but gram positive bacteria can also be involved in such infections. In case of gram negative bacteria

Escherichia species are most dominant organisms and involved in such infections but other genera are also involved which include *Klebsiella, Proteus, Pseudomonas, Enterobacter* etc. In case of gram positive bacteria mostly *Staphylococcus* species are involved in causing urinary tract infections. [18]. The frequency of gram negative enteric bacteria causing UTI is more than gram positive isolates. These bacteria causing UTI show resistant to different antibiotics and their antibiotic resistance patterns varies worldwide.

Thirty UTI causing bacterial strains were tested against 31 antibiotics and their resistance pattern was noted. Drug resistant strains were showing resistance to commercially available B-lactams, cephalosporins, tetracyclins, quinolones and fluoroquinolones. Some of those strains showed sensitivity to some antibiotics which included nitrosfurantion, fosfomycin and pipracillin etc. It is estimated that two third world populations rely on herbal medications [19]. However, only five percent of total plant species have been investigated [20]. These traditional herbal medicines were also widely putative due to their fewer side effects, relatively less expensive and better patient tolerance. Natural products and natural extracts like honey, kalonji, methi, neem and jaiphal have been long ago used for new drug discovery and development [21] to cure various diseases. Keeping in mind, formulation of different plant extracts and concentrations were used in the present study including kalonji, methi, neem, jaiphal, honey (75%), honey (50%) and combination of honey and kalonji. Out of 30 selected bacterial strains, five (ZK1, ZK2, ZK3, ZK4 and ZK5) exhibiting resistance against above prepared formulations were selected for further study. These plants extracts showed significant inhibition of these five strains. Among plant extracts kalonji was found to be most effective because it exhibited significant inhibition of growth of all 5 strains as compared to other extracts. Neem extract showed inhibition of only ZK2 and had no adverse effects on the growth of ZK-1 and ZK-3. On the other hand methi extracts were effective against ZK-1 and ZK-3 but did not inhibit the growth of ZK-2. At the same time jaiphal extracts showed significant inhibition of growth in the case of strain ZK-1 and ZK-2 and both concentrations of honey adversely effected the growth of only ZK-1 strain. In the present study it was observed that medicinal plant extracts are giving more promising positive results as compared to antibiotics. It can be easily concluded that selected plant extracts have inhibitory effect not only on drugs sensitive organism but they also show inhibitory effects on the growth of multidrug resistant pathogens. There must be some active ingredients/components present in these extracts which are responsible for such adverse effects. For example, the most important active compound present in *Nigella sativa* oil is Thymoquinone reported in various studies [22]. The present study shows that these natural products and extracts contain unrestrained fruitful applications to humans and indefinite biomedical application expedient for mankind. The different antimicrobial activity is due to different phytochemical plants properties [23]. Still further studies are needed to be done in order to understand the composition of such extracts and to find out the effective formulation of such plant products which provide them with such antimicrobial activities and can be applied in humans for medicinal purposes.

Conclusion

The results were satisfactory enough to establish that the natural extracts from plants have pharmaceutical significance against drug resistant UTI causing bacteria, in is accordance with the use of same plants in traditional medicine. But still pharmacological and toxicity studies needed before they can be used as affective treatment drugs against urinary tract infections. Results showed pharmacokinetics of investigated plant extracts were different from that of commercially available antibiotics.

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