Infection and Drug Resistance

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ORIGINAL RESEARCH

In vitro Antibacterial Activities of Selected Medicinal Plants Used by Traditional Healers for Treating Urinary Tract Infection in Haramaya District, Eastern Ethiopia

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Background: Despite the presence of antibacterial agents for urinary tract infection treatment, most of the uropathogenic bacteria reveal multi-drug resistance. Health and economic loss due to these represent a rising burden worldwide which necessitates serious action at regional, national and global levels. Thus, alternative approaches to overcome this problem by using bioactive compounds from traditional medicinal plants are required. This study was designed to evaluate the in-vitro antibacterial activity of *Punica granatum* fruit peels, *Nigella sativa* seeds, and *Echinops kebericho* used in the traditional treatment of urinary tract infections.

Methods: An experimental study was employed to evaluate the in vitro antibacterial activity of methanol and ethanol crude extract of *Punica granatum* fruit peels, *Nigella sativa* seeds, and *Echinops kebericho* roots of six dilutions (25, 50, 100, 125, 250, and 500) mg/mL. Disc diffusion and macro broth dilution methods were used to determine antimicrobial activity test and minimum inhibitory concentration respectively against *E. coli, P. aeruginosa, K. pneumoniae, P. mirabilis*, and *S. aureus* bacterias.

Results: Antibacterial activities of ethanol and methanol crude extract of *Punica granatum* fruit peels against *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. aureus* ATCC25923, *K. pneumoniae* UK5099 and *P. mirabilis* UK5999 had highest inhibition zones among tested plants. All tested bacteria were highly sensitive to *Punica granatum* extract. The second most active plant extract in inhibiting the growth of tested bacteria was *Nigella sativa* while *Echinops kebericho* showed the smallest efficacy against tested bacteria. The inhibition zone diameter produced by the methanol extract of each screened plant had higher inhibition zones than ethanol extract.

Conclusion: The crude extracts of *Punica granatum* fruit peels, *Nigella sativa* seeds, and *Echinops kebericho* roots have promising antibacterial activity against tested uropathogenic bacteria.

Keywords: urinary tract infections, antibacterial, medicinal plants, Haramaya

Introduction

Urinary tract infections (UTIs) are the most common bacterial infections, in which one or more parts of the urinary system become infected.[1] UTI is generally caused by Gram-Negative and Gram-Positive bacteria.[2] *Escherichia coli* (*E. coli*) is the most common causative agent of UTI followed by *Klebsiella pneumoniae* (*K. pneumoniae*),[3] *Proteus mirabilis* (*P. mirabilis), Enterococcus faecalis* (*E. Faecalis), Staphylococcus saprophyticus* (*S. saprophyticus*).[4]

A variety of antibiotics are being employed to treat UTIs according to the severity of the disease.[5] High resistance rates are reported among common uropathogens bacteria according to various studies carried out in different countries.[6]

In Ethiopia, studies showed various resistance rates among commonly isolated uropathogenic bacteria. The 66.7% of *Pseudomonas aeruginosa* (*P. aeruginosa*) strains showing resistance against Ciprofloxacin, Meropenem, Cotrimoxazole and *K. pneumoniae* strains were 100% resistant to Ceftriaxone, Ceftazidime, Ciprofloxacin, and Augmentin.[7] For *E. coli*

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high resistance rates were observed for ampicillin 83.81%, amoxicillin 75.79%, tetracycline 67.18%, trimethoprim-sulfamethoxazole 57.47%, and cephalothin 56.69%[8] and GP, *Staphylococcus aureus* (*S. aureus*) showed a high level of resistance for cotrimoxazole (53.3%), ampicillin (43%) and penicillin (36.7%).[9]

Herbal traditional medicines are effective to combat bacterial resistance of uropathogens with high efficacy, and easy availability with minimal side effects,[10] this led the researcher to find new antimicrobial agents in particular from traditional medicinal plants.[11] One of the common traditional medicinal plants reported to have therapeutic potential for the management and cure of UTIs is Cranberry (*Vaccinium macrocarpon*). As research has suggested cranberry may be active against UTIs because it prevents *E. coli*, from attaching to the walls of the bladder.[12] An extract of Uva Ursi (*Arctostaphylos uva-ursi)* is used as traditional herbal medicine in Europe and North America for the treatment of UTIs.[13] This herbal medicine is approved in Germany for bladder infection treatment; it is effective against *E. coli* in the bladder and is a urinary antiseptic for bladder and kidney infections.[14] When combined with marshmallows, it can eliminate stones from the kidney, and bladder and is also good for treating bacterial vaginosis and ulcerative cystitis.[15] Barberry (*Berberis vulgaris*) has remarkable infection-fighting properties. As studies indicated, it can kill microorganisms (*E. coli, Streptococci*) that cause UTIs.[14] Marshmallow root (*Althaea Officinalis*) inhibits bacterial growth in the urinary tract and cleans the bladder; it also soothes the urinary system and helps to treat kidney, bladder inflammations, and effectively stops bleeding in the urine.[15]

In Ethiopia, therapeutic plants have been utilized as customary medication to treat distinctive human and animal sickness by the community individuals from days of yore.[16]

Currently, antibiotics like trimethoprim, sulfamethoxazole, amoxicillin/clavulanate acid, cephalosporins, quinolone, etc. as the first choice of the drug are being used for treating UTIs worldwide. However, there was various resistance development evidence reported to these drugs from different parts of the world.[17]

Antimicrobial assessment of therapeutic plant studies is regularly significant in revealing locally accessible and important plant species, particularly for the disclosure of crude medications.[18] Ethiopia has a long history of Traditional healthcare systems, however, studies conducted on the traditional medicinal plants are restricted when compared with the multiethnic, cultural, and flora varieties.[19] In Ethiopia, studies regarding the antibacterial activities of Pomegranate, Black seed, and Costus were not available. Therefore, the main aim of this study was to evaluate the in-vitro antimicrobial activities of Pomegranate, Black seed, and Costus commonly used for UTI treatment informal among traditional healers in Haramaya District.

Materials and Method

Study Area and Period

The study was conducted in the Haramaya district of Eastern Hararghe, Oromia region. The sample of plants was collected from July 16 to 25 September 2021, whereas the laboratory tests were performed from October to November 2021. Haramaya is one of 24 districts in the East Hararghe zone which is located 520km from Addis Ababa; the capital city of Ethiopia, and 20km from the historical city, of Harar, with a total land area of 525.64 sqm.

Study Design

An experimental study was carried out to evaluate the in-vitro antibacterial activity of traditional medicinal plants against bacterial species that cause UTIs.

Collection and Identification of the Plants

Two-kilogram fresh specimens of each of the three medicinal plants were collected from Haramaya woreda, Eastern Ethiopia. These plant specimens were taken to Haramaya University, Department of Plant Science Herbarium for identification using taxonomic keys by plant taxonomic experts with their deposited voucher specimen.

The peels of pomegranate fruit, black seed, and root of kebericho were manually separated and washed with tap water first then followed by distilled water. These three plants were air-dried for 17 days in the shade and then grounded in

a mixer grinder until the powder was made. Finally, the powder was stored at 4°C in well-closed containers until extraction.

Plant Extract Preparation

The dehydrated tested plants were cut into lesser parts and ground using an electric grinder till converted into a good powder. Each powder of tested plants was sieved using a (75 µm × 20 cm) sieve. One hundred grams of powder from each tested plant was weighed and mixed with 500mL of both absolute methanol and ethanol solvent in Erlenmeyer flasks separately and then subjected to extraction by using the maceration technique.[20]

The flasks were left on a mechanical shaker at 150 rpm for 24 hrs at room temperature for three days and then filtered through Whatman No. 1 filter paper using the Buchner funnel. The procedure was repeated three times on the marc to allow the solvent to extract substantial quantities of the chemical constituents from the pounded plant materials. The extracts were further concentrated to dryness under reduced pressure at 37°C using a Buchi rotary evaporator. Three tested plants of residues were further evaporated by using a hot air oven at 40°C for 3 days to obtain dried extracts then dried extracts were stored in labelled sterile screw-capped bottles at 4 °C.

Later on, 4 g of each dried extract was dissolved in 8 mL Dimethyl sulfoxide (DMSO) to get 500gm/mL of the stock solution then different concentrations (25mg/mL, 50mg/mL, 100mg/mL, 125mg/mL and 250mg/mL) were prepared from three stock solution by two-fold dilution with dimethyl sulfoxide (DMSO) solution and used as the test extracts for antimicrobial activity. The sterility of filtered extracts was checked for further function.[21]

Test Organisms

The tested pathogenic bacteria used in this study were obtained from the laboratory of Hararghe Health Research (CHAMPS project) and these included one gram-positive bacteria, *S. aureus ATCC25923* and four gram-negative bacteria which were *K. pneumoniae UK5099, P. mirabilis UK5999, E. coli ATCC25922* and *P. aeruginosa ATCC27853*.

Antibacterial Activity of Medicinal Plants

The screening of the ethanol and methanol crude extracts of these plants for antibacterial activity was performed using the disc diffusion and Minimum Inhibitory Concentration method to compare their effectiveness against the antimicrobial activity.

Inoculum Preparation

For performing both disc diffusion and minimum inhibitory concentration method, bacterial suspensions were prepared for all tested bacteria. To form bacterial suspensions, tested bacteria were inoculated and spread on prepared agar plates using inoculating wire loop following aseptic condition. Three to five overnight well-isolated colonies of the same morphological type were selected from an agar plate culture. The top of each colony was touched with a sterile wire loop and suspended in a tube containing sterile normal saline. The turbidity of bacterial suspensions was adjusted with sterile saline to achieve turbidity equivalent to that of a 0.5 McFarland standard.[22]

Determination of Antibacterial Activity Test

The antibacterial activity of three plants was performed by Kirby-Bauer's disc diffusion method. Susceptibility of all tested bacteria was performed according to the method reported by the National Committee for Clinical Laboratory Standards.

To perform the disc diffusion test, Mueller–Hinton agar was prepared and autoclaved for 15 min at 121 °C then sterilized Mueller–Hinton agar (MHA) was poured into each Petri dish (90 mm diameter) and allowed to solid. From overnight bacterial cultures, the suspensions of each test organism were prepared and adjusted to 0.5 McFarland turbidity standard. After adjusting the inoculum to a 0.5 McFarland standard, a sterile cotton swab was dipped into the inoculum and rotated against the wall of the tube above the liquid to remove excess inoculum then after the surface of the MHA plate was streaked in three directions rotating at 60° to ensure the spread of the tested microbes on the surface of the plate completely.

Diffusion discs of approximately 6mm diameter were prepared from Whatman no. 1 filter paper and sterilized by autoclave then dried in a hot oven. A hundred microliters of serially diluted concentration of crude extracts were impregnated on each disc using sterile micropipette tips and then air-dried. Impregnated discs with Dimethyl Sulfoxide (DMSO) were used as a negative control whereas 30µgm per disc of Chloramphenicol was used as a positive control. All impregnated discs and positive control discs were placed aseptically at appropriate places on the inoculated plates. Plates were kept for 15 minutes at room temperature (25 °C) for pre-diffusion and then were incubated at 35 °C for 24 hours.

The experiment was carried out in triplicates and the diameter of inhibition zones for the extract against each test organism was measured and recorded in mm using a calliper. Results were presented as the mean ± standard deviation (SD) from three replicates. Finally, Based on CLSI guidelines, the inhibition zone diameters less than 10 mm were considered weak activity, above 10 and less than 13 mm were considered moderate activity, and 13 mm and above are considered as high antibacterial activity. The ZOI ≥ 10 mm diameters around the plates were regarded as significant susceptibility of the organisms to the extract of tested medicinal plants.[23]

Minimum Inhibitory Concentration

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the extracts that inhibit the growth of organisms. In this experiment, the crude extract of medicinal plants, which showed a zone of inhibition (ZOI) ≥ 10 mm considered active and was further subjected to determine the MIC of tested plants. The MIC of plant extraction was determined by the macro broth dilution method using nutrient broth as the medium. This was carried out using the lowest concentration of crude extracts (25 mg/mL) of tested plants which exhibited antimicrobial activities against one or several pathogenic bacteria and were further serially diluted get concentrations of (25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39) mg/mL.

In the tube dilution test, 2 mL diverse concentration of each extract (25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39) mg/mL were additional to tubes having 2 mL nutrient broth and typical 100µL of overnight bacterial suspension cultures matched to 0.5 McFarland standards was inoculated. Two types of controls were also run with this experiment, Control I which served as the negative control, contained nutrient broth with extract in serial dilutions in the absence of bacterium, whereas control II was regarded as positive control and contained nutrient broth with respective bacterium and without extracts for comparing the turbidity status.

All tubes were incubated for 24 hrs at 36 °C then observed for the presence or absence of turbidity. The absence of growth was confirmed by the absence of turbidity and by sub-cultured on MHA plates and incubated at 35±2°C for 18–24 hrs. The tubes with a minimum concentration of extract in which the growth was completely checked were noted as the MIC of the plant extract (Gurmachhan et al, 2019). These were done in triplicate and reported as mean ± SD.

Quality Control

All culture media were prepared by following the manufacturer's instructions and their sterility was checked by incubating 3–5% of the batch at 37 °C overnight and observing for growth. Culture media, which showed any growth, was rejected and replaced by a new sterile batch. Its sterility was checked by incubating 3–5% of the batch at 37 °C overnight and observed for growth. Culture media, which showed any growth, was rejected and replaced by a new sterile batch. Each extract of methanol and ethanol was tested for the growth of bacteria. This was carried out by inoculating 0.5 mL of each of them on sterile Mueller Hinton Agar and incubating at 37 °C for 18–24 h. The plates were observed for growth. If no growth in the extracts after incubation indicated that they were sterile and later used for antibacterial activity as described by (Sahu et al, 2018) guideline. To keep the quality of antimicrobial agents, *S. aureus* (ATCC 25923) against Chloramphenicol and *Escherichia coli* (ATCC 25922) against Chloramphenicol was used to observe the inhibition zone and compare with the pre-seated standard zone of inhibition.[24]

Statistical Analysis

Data obtained from the experiment were analyzed using SPSS, version 26. The statistical differences of the mean zone of inhibition of extract for individual bacterium were carried out by employing ANOVA followed by Tukey's Post Hoc Multiple Comparison tests at a significance level of $P < 0.05$. MIC was analyzed by using descriptive statistics.

Ethical Considerations

The study protocol was reviewed and approved by Haramaya University, College of Health and Medical Sciences Institutional Health Research Ethics Review Committee (Ref. no. IHRERC/042/2021). An official letter of support was obtained from the post graduate directorate office.

Results

General Description of Tested Plants

Three types of traditional medicinal plants (*Punica granatum* fruit peels, *Nigella sativa* seed, and *Echinops kebericho* roots) used against bacterial species that cause UTIs were collected from Haramaya woreda.

Two-kilogram fresh specimens of each tested plant (*Punica granatum* fruit peels, *Nigella sativa* seeds, and *Echinops kebericho* roots) were collected. After the identification of these medicinal plants was performed by Herbarium then these were dehydrated by air in the shade for 17 days. Three dehydrated tested plants were grounded in a mixer grinder until the powder was made. One hundred grams of powder from each plant was taken and made extraction with two solvents by maceration technique. The extract percentage (% yield w/w) of medicinal plants was calculated by dividing the weight of the dried extract by the weight of the plant powder used for extraction multiplied by 100. In the following table, some base information about scientific names, local names, Voucher numbers, used parts and yield percentage of tested medicinal plants were tabulated in Table 1.

Determination of Antibacterial Activity

In the present study, the in-vitro antibacterial property of each plant extract against 5 uropathogenic organisms (*E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis*) was carried out at six concentrations (25, 50, 100, 125, 250 and 500 mg/mL) for each crude extract and the results were measured in mm then expressed with mean ± SD (n = 3). The result of antibacterial potentials varies depending upon the concentration and solvent used for extraction.

Antibacterial Activity of *Punica granatum*

Antibacterial Activity of Methanol Extract of *Punica granatum*

As the result indicated in Table 2, and Figure 1, the methanol extract of *Punica granatum* had the highest efficacy against *E. coli* which is the most common bacteria responsible for UTIs. As the result displayed in Table 2, *E. coli, S. aureus, P. aeruginosa, P. mirabilis, and K. pneumoniae* showed sensitivity from higher to lower for methanol extract of *Punica granatum* respectively.

Table 1 Characteristic of the Traditional Medicinal Plants Used for Antibacterial Activity

Scientific Name	Common Name	Local Name	Used Parts	Voucher No	(%yield w/w)
Punica granatum	Pomegranate	Rumana	Fruit peels	HUHE0000014717	28% for methanol
					27% for ethanol
Nigella sativa	Blackseed	Habasudda	Seed	HUHE0000014841	30% for methanol
					29% for ethanol
Echinops kebericho	Costus	Qabarush	Root	HUHE0000004074	17.2% for methanol
					10% for ethanol

Note: Mean ± SD (n = 3).
Abbreviations: DMSO, dimethyl sulfoxide; Chlor, Chloramphenicol.

Table 2 Antibacterial Activity of Methanol Extract of *P. granatum* by Disc Diffusion

Plant Extracted	Concentration in mg/mL/Zone of Inhibition in mm							Control	
	Tested Organisms	500	250	125	100	50	25	Chlo 30µg/Disc	DMSO
P. granatum	*E. coli*	36±2.02	32.5±0.29	30.33±0.50	25.33±0.76	24±1.89	18±1.26	22.7 ±15.5	0±0.00
	P. aeruginosa	29.33±2.47	26 ±1.04	22.33±1.06	20.5±1.32	18±1.0	17 ±0.76	17 ± 1.6	0±0.00
	S. aureus	33±3.50	28.5±0.87	26.5±0.87	25 ±01.26	21.5±0.87	19.5±0.50	30 ±1	0±0.00
	K. pneumoniae	22.3±0.29	19.2±1.04	17.5±0.50	16±0.50	15±0.76	14±1.52	6 ±5.7	0±0.00
	P. mirabilis	25.33±1.76	22.33±1.04	21±1.04	19 ±1.53	17±0.50	14.2±0.76	12 ± 2.3	0±0.00

Note: Mean ± SD (n = 3).
Abbreviations: DMSO, dimethyl sulfoxide; Chlor, Chloramphenicol.

Antibacterial Activity of Ethanol Extract of *Punica granatum*

The ethanol extract of *P. granatum* was most effective against *E. coli* then followed by *P. aeruginosa* which is the most common cause of UTIs respectively. Ethanol extract of *P. granatum* showed less efficacy against *P. mirabilis* among tested bacteria.

The result obtained from the experimental study showed that the highest *P. granatum* fruit peel activity was recorded against *E. coli* at the concentration of 500 mg/mL for both solvent extractions. *P. mirabilis* displayed the smallest inhibition zone diameter among the tested organisms for ethanol extract and *K. pneumoniae* had the smallest inhibition zone diameter for methanol extract. The active crude extracts of *P. granatum* fruit peels displayed greater ZOI results when compared with the positive controls (Chloramphenicol 30µg/disc) where negative control (DMSO) demonstrated no visible activity at all.

Antibacterial Activity of *Nigella sativa*

Antibacterial Activity of *Nigella sativa* with Methanol Extract

As the results displayed in Table 3 and Figure 2, *K. pneumoniae* had the highest sensitivity for methanol extract of N. *sativa* among tested bacteria while *E. coli* had the lowest sensitivity for N. *sativa* methanol extract. The ZOI observed at the lowest concentration (25mg/mL) against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* were 10,11,10,12, and 11.5 mm, respectively.

Antibacterial Activity of Ethanol Extract of *Nigella sativa*

As antibacterial activity of ethanol extract of *N. sative* displayed the highest inhibitory effect against *P. aeruginosa* among tested organisms. The inhibition zone for *E. coli, S. aureus, K. pneumoniae*, and *P. mirabilis* was almost similar at

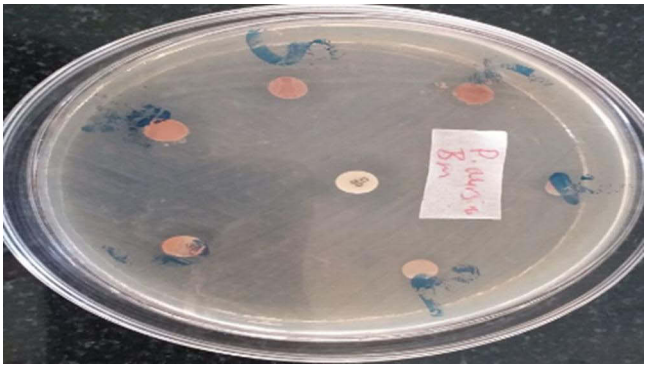

Figure 1 Methanol extract of *Punica granatum* against *P. mirabilis* where Pm is methanol extract of *Punica*.

Table 3 Antibacterial Activity of Methanol Extract for *N. sativa* by Disk Diffusion Method

Plant Extracted	Tested Organisms	Concentration in mg/mL/Zone of Inhibition in mm						Control	
		500	250	125	100	50	25	Chlor 30µg/Disc	DMSO
N. sativa	*E. coli*	18±0.29	15.5±1.26	14±1.0	13.5±0.76	12 ± 1.04	10±0.73	22.7 ±15.5	0±0.00
	P. aeruginosa	20±1.04	17±0.29	16±1.04	14.5±0.50	13±0.76	11±0.00	17 ± 1.6	0±0.00
	S. aureus	19±0.80	17.5 ± 0.29	15.5±0.29	14±0.58	12±0.29	10 ±0.29	30 ±1	0±0.00
	K. pneumoniae	21±0.29	18.5±0.50	17±0.50	16±0.29	14.2±0.76	12±0.29	6 ±5.7	0±0.00
	P. mirabilis	20±1.00	18±0.76	16.5±0.50	15±0.76	13±1.15	11.5±6.08	12 ± 2.3	0±0.00

Note: Mean ± SD (n = 3).
Abbreviations: DMSO, dimethyl sulfoxide; Chlor, Chloramphenicol.

Table 4 Antibacterial Activity of Methanol Extract of *E. kebericho* by Disk Diffusion Method

Plant Extracted	Tested Organisms	Concentration in mg/mL/Zone of Inhibition in mm						Control	
		500	250	125	100	50	25	Chlo 30µg/Disc	DMSO
E. kebericho	*E. coli*	21 ±0.58	18.2±0.50	17.5±0.29	16.5±0.76	15 ±1.26	9.5 ±1.26	22.7 ±15.5	0±0.00
	P. aeruginosa	20±0.76	18±0.29	17±0.29	16±0.76	14±0.00	8.5±0.50	17 ± 1.6	0±0.00
	S. aureus	19±0.50	17±1.00	16.2±0.76	15±1.00	13.33±0.50	7.5±0.60	30 ±1	0±0.00
	K. pneumoniae	18±0.00	16±0.00	15.3±1.04	14.5±1.50	12.2±2.25	7±1.04	6 ±5.7	0±0.00
	P. mirabilis	19.5±0.50	17±0.00	16.2±0.76	15 ±1.26	13.2±0.73	8±1.32	12 ± 2.3	0±0.00

Note: Mean ± SD (n = 3).
Abbreviations: DMSO, dimethyl sulfoxide; Chlor, Chloramphenicol.

the highest concentration (500mg/mL) of extract used. The inhibitory effect of ethanol extract of *N. sative* against *K. pneumoniae* and *P. mirabilis* was almost similar throughout all used concentrations.

Antibacterial Activity of *Echinops kebericho*

Antibacterial Activity of Methanol Extract of *E. kebericho*

As a result, as observed from Table 4, and Figure 3, the methanol extract of *E. kebericho* against *E. coli and P. aeruginosa* had almost equal inhibition zone for all concentrations. It had a less inhibitory effect against *K. pneumoniae* among tested organisms.

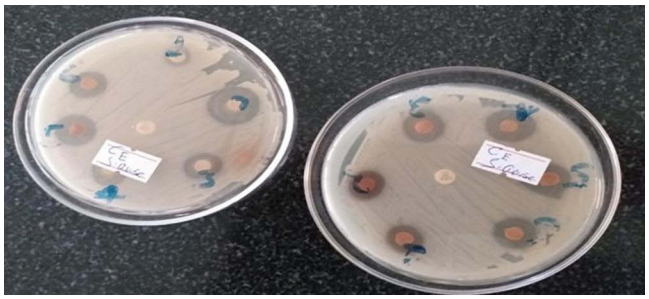

Figure 2 Methanol extract of *Nigella sativa* against *P. mirabilis*. Where Bm is methanol extract of black seed.

Figure 3 Ethanol extract of *Echinops kebericho* against *S. aureus*, where CE is ethanol extract of Costus.

Antibacterial Activity of Ethanol Extract of *Echinops kebericho*

Results shown in Table 5, indicated that the inhibition zone for ethanol extract of *E. kebericho* against *E. coli* was smallest when compared with another tested organism. The inhibition zone diameter revealed by this plant against *P. aeruginosa* was the highest among tested bacteria.

Table 5 Minimum Inhibitory Concentrations Values of *P. granatum* Peels and *N. sativa* Seeds by Macro Broth Dilution Method

Solvent Used	Tested Plants	Tested Organisms	Concentration (mg/mL)							
			50	25	12.5	6.25	3.12	1.56	0.78	0.39
Ethanol	*P. granatum*	*E. coli*	+	+	+	–	–	–	–	–
		P. aeruginosa	+	+	+	–	–	–	–	–
		K. pneumoniae	+	–	–	–	–	–	–	–
		P. mirabilis	+	–	–	–	–	–	–	–
		S. aureus	+	+	+	_	_	–	–	–
Methanol	*P. granatum*	*E. coli*	+	+	+	–	–	–	–	–
		P. aeruginosa	+	+	+	–	–	–	–	–
		K. pneumoniae	+	+	+	–	–	–	–	–
		P. mirabilis	+	+	+	–	–	–	–	–
		S. aureus	+	+	+	–	–	–	–	–
Methanol	*N. sativa*	*E. coli*	+	–	–	–	–	–	–	–
		P. aeruginosa	+	+	–	–	–	–	–	–
		K. pneumoniae	+	+	–	–	–	–	–	–
		P. mirabilis	+	–	–	–	–	–	–	–
		S. aureus	+	+	–	–	–	–	–	–

Notes: Mean ± SD (n = 3), Positive "+" Presence of activities, Negative "–" Absence of activities.

Minimum Inhibition Concentration

Minimum inhibition concentration tests for *P. granatum and* N. *sativa* were performed against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis*. The crude extract of medicinal plants, which showed ≥10 ZOI at 25g/mL, was subjected to a two-fold serial with a macro dilution method to determine their MIC value. The results of ethanol and methanol extracts of *P. granatum* and methanol extract of *N. sativa* are shown in Table 3. As the result indicated in Table 5, the ethanol extract of *P. granatum* MIC value against *E. coli, P. aeruginosa*, and *S. aureus* was 12.5 mg/mL respectively. Whereas the MIC value for *K. pneumoniae* and *P. mirabilis* was 50mg/mL. Similarly, for the methanol extract of *P. granatum* MIC value against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* was 12.5mg/mL respectively. Similarly, as Table 3, displayed, the methanol extract of *N. sativa* for MIC value against *P. aeruginosa, S. aureus*, and *K. pneumoniae* was 25mg/mL respectively, and for *E. coli* and *P. mirabilis* was 50mg/mL.

Discussion

In this study, the in-vitro antibacterial activity of ethanol and methanol of *Punica granatum* peels, *Nigella sativa* seed, and *Echinops kebericho* root extract against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* were evaluated by disc diffusion method using six dilutions (25, 50, 100, 125, 250 and 500) mg/mL.

The antibacterial activities of tested medicinal plants against tested bacteria showed different results of inhibition zone with remarkable antibacterial activities of methanol extract of *Punica granatum* peels observed. Methanol extract of *P. granatum* had high efficacy against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* at all concentrations. These results are consistent with previous findings of (Borikar et al, 2018) where *Punica granatum* fruit peels of methanol extract against *S. aureus* and *P. aeruginosa* at (100 mg/mL) concentration was 22mm and 26mm respectively. This result also agreed with (Mishra et al, 2017) where methanol extracts of *P. granatum* fruit peels against *S. aureus, E. coli, K. pneumoniae, P. mirabilis*, and *P. aeruginosa* were 26,25,18,17 and 24 respectively. But disagrees with a study by[25] where *Punica granatum* peels had a low ability to overcome the growth of *P. aeruginosa, P. mirabilis, S. aureus, E. coli, K. pneumoniae* and had 3 mm, 10 mm, 7 mm, 4 mm, 9 mm inhibitions zone respectively.

Ethanol crude extract of *Punica granatum* peels against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* showed remarkable efficacy at all concentrations. This study resembles that of (Das et al, 2018) where *Punica granatum* peels of ethanol extract against *S. aureus* and *P. aeruginosa* at (100 mg/mL) concentration was 21mm and 24mm respectively. These results also consist of[26] who reported that ethanol extract of *P. granatum* peels against *K. pneumoniae* with four concentrations of the extract (25,50, 100, 150) mg/mL gave (12,13,15,16) mm inhibitory results respectively. Similarly, these results also agreed with the study of (Al-Wazni and Hadi, 2016) at a concentration of 25mg/mL, 50mg/mL, and 100 mg/mL. However, this result disagreed with reports by,[25] who reported that the *Punica granatum* Peels of ethanol extract against *P. aeruginosa, P. mirabilis, S. aureus, E. coli, and K. pneumoniae* was 3 mm, 10 mm, 7 mm, 4 mm and 9 mm respectively. This difference in activity may be explained by many reasons such as extraction method difference, susceptibility of bacterial strain and used different procedures.

Based on the findings of the current study, the second most active plant extract in inhibiting the growth of tested bacteria was ethanol and methanol extracts of *N. sativa*. The result of the present investigation revealed the strong antibacterial activity of *N. sativa* seeds ethanol extracts against all studied bacteria at all concentrations. Present data were similar to previous reports of[27] who reported that ethanol extract of *N. sativa* effectiveness against *S. aureus, E. coli*, and *K. pneumoniae* with 18mm of inhibition zone. These results are also comparable to the previous findings of (Ali et al, 2020) and[28] where the antibacterial activity of ethanol extract *N. sativa* against *S. aureus* and, *E. coli* at a concentration of 25, 50 and 100mg/mL, and against *K. pneumoniae* and *P. aeruginosa* was 14mm and 11mm respectively at concentrations 50 mg/mL.

Similarly, it was also observed that methanolic seed extract of *N. sativa* exhibited higher antibacterial activity towards all the strains of studied bacteria than ethanol extract for all used concentrations. This result was in agreement with previous reports by[29] who reported that the antibacterial activity of the methanol extract of this plant was most effective against tested bacteria. But this result disagreed with the report of[30] who reported that *P. aeruginosa* and *S. aureus* were inhibited with 1% concentration which was the smallest concentration when compared with the present concentration.

Variations in the results may be due to the variation in the method of antibacterial activity, extraction method, and the difference between environment and soil.

The other effective medicinal plant against the tested organism was *Echinops kebericho* in this study. As this study demonstrated, methanol and ethanol extract of *E. kebericho* against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* for the highest concentration (500mg/mL). This was consistent with the results of a previous study done by[31] against *S. aureus* (ATCC 29213), *K. pneumoniae* (ATCC 700603), *P. aeruginosa*, ATCC 27853, and *E. coli* (ATCC 25922). This result was also consistent with the previous study by Ameya et al.[32]

In the present study, the antibacterial activity of the methanol extract of *Punica granatum* fruit peels is relatively higher than ethanol extracts against tested bacteria. The antibacterial activity of the methanol extraction of *E. kebericho* roots had more efficacy against these organisms than ethanol extract. Similarly, the antimicrobial activity of the methanol extract of *N. sativa* seed was more active against tested bacteria than ethanol extraction. As available literature indicates, the possible reason for the decreased activity of the plant's extract may be due to the slower release of active compounds from the plants. This is because of the relative releasing potential of two solvents since extraction of medicinal plants with different solvents may show different results based on the potential of the solvents used to extract the biologically active constituents so this might be that methanol extracted tested plant's constituents were released more quickly than ethanol extracted plant's constituents.

In this experimental result, we have observed that *E. coli* and *S. aureus* were susceptible to Chloramphenicol (control), *P. aeruginosa* was intermediate for Chloramphenicol, while *K. pneumoniae* and *P. mirabilis* were resistant to Chloramphenicol. The Chloramphenicol control against *E. coli* was (22.7 ±15.5) mm, while the antibacterial activity of *P. granatum* peels against *E. coli* was greater than Chloramphenicol for methanol and ethanol extract. When active crude extracts of *N. sativa* seed were compared with this antibiotic control, methanol and ethanol active crude extracts exhibited better antimicrobial activity than Chloramphenicol. Similarly, methanol and ethanol extracts of *E. kebericho* revealed better antimicrobial activity against tested bacteria.

From this current study, the MIC value for the methanol extract of *N. sativa against tested organisms* was comparable to the previous findings of[33] where the MIC of ethanolic plant extract against *S. aureus*, and *E. coli* was 25 and 50 mg/mL, respectively. However, these results disagreed with the finding of[34] where the *N. sativa* methanol extract had shown a 25 mg/mL MIC value against *P. aeruginosa, S. aureus*, and *K. pneumoniae* while 50mg/mL for *E. coli* and *P. mirabilis*. These results also contrast with that of[35] who reported that the MIC value against *P. mirabilis* was 1.5 mg/mL, *P. aeruginosa* was 3.125 mg/mL, *K. pneumoniae* was 6.25 mg/mL and *E. coli* was 6.25 mg/mL.

As it showed the result that the MIC of *P. granatum* fruit peels were less than *N. sativa*, and this means that *P. granatum* fruit peels are more effective when compared to the effect of *N. sativa* extract. This indicated that the active substances present in the *P. granatum* fruit peels plant extract are more effective against tested bacteria compared to the active substances present in *N. sativa* seeds extract.

Strength of the Study

In this study, the in-vitro antibacterial activity of crude extract of three medicinal plants was performed against common urinary tract infection-causing bacteria.

Limitations of the Study

This study focused on the antibacterial activity of crude extract of selected medicinal plants with five reference strains bacterial only other bacteria, such as *K. oxytoca, Enterococcus faecalis*, and *Citrobacter freundii*, were not included in this study.

Conclusion

This study demonstrated the antibacterial activity of methanol and ethanol extract of *Punica granatum, Nigella sativa* and *Echinops kebericho* against *E. coli, P. aeruginosa, S. aureus, K. pneumoniae*, and *P. mirabilis* of uropathogenic bacteria. The result showed that all three tested plant extracts inhibited the in-vitro growth of at least one or more tested organisms. The *P. granatum* fruit peels extract displayed the maximum inhibitions zone against tested bacteria among tested plants then followed by *N. sativa* extract. The mean diameter of inhibition zones of tested bacteria increased proportionally when the

concentration tested medicinal plant was raised. The *P. granatum* extracts had the lowest MIC value against tested bacteria. These tested traditional plants can be the best candidate for further studies in the development of a new antimicrobial agent against uropathogenic bacteria after the isolation and characterization of their active compounds.

Recommendations

For traditional users of the plants, *Punica granatum* fruit peels, *Nigella sativa* seeds, and *Echinops kebericho* roots of medicinal plants are effective against uropathogenic bacteria. Among tested medicinal plants, *Punica granatum* fruit peels are the most effective against tested UTI bacteria. Due to this, using *Punica granatum* fruit peels for treating UTIs is more important. Methanol solvent is more effective in extracting tested medicinal plants, so using methanol for extract is important.

For a researcher, in this study, the antibacterial activity of three medicinal plants testing was conducted on a limited number of bacteria. Because of this, the same work should be carried out on a large variety of bacterial strains to have a clear picture of the spectrum of antimicrobial activity of these herbal medicinal plants. Similarly, studies on several clinically isolated bacteria are necessary for further analysis to be able to standardize the effective inhibitory action of *Punica granatum, Nigella sativa*, and *Echinops kebericho*, and further studies are also required to investigate the active compound of each part of these plants.

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Disclosure

The authors report no conflicts of interest in this work.

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