Effect of alcoholic extracts of *Punica grantum* and *Trigonella foenum* on *E. coli* isolated from UTI

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Abstract

This study was conducted to evaluate the inhibitory effects of 10 antibiotics and two types of alcoholic plant extracts (*Punica grantum* and *Trigonella foenum*) on *E. coli* bacteria, where 100 samples were isolated and diagnosed during the period from July 2021 to December 2022 from urinary tract infections of incoming patients. To Salah El-Din General Hospital, all bacterial isolates were planted on nutrient and selective culture media, then they were diagnosed by IMVC biochemical tests, and to confirm the diagnosis, don by Vitik device. *E. coli* isolates showed 100% absolute resistance to RA and were 88.5% resistant to AK, F, TE (85.7%), GN (80%), NA (65.7%), CIP (57.2%) and TM (51.42). %) and C (25.7%) and The MEM showed high sensitive (100%), *E. coli* samples were exposed to alcoholic plant extracts of pomegranate peel and fenugreek plant to examine the biological activity on the dish and note the inhibition. Several dilutions of the extracts were made to obtain the minimum inhibitory concentration (MIC).

Keywords: *E. coli*, vitik device, *Punica grantum*, *Trigonella foenum*

Introduction

Urinary Tract Infection (UTI) is one of the most common and important diseases in the societies of developing and developed countries, where the largest proportion of them constitute bacterial pathogens, especially in women, children and those with renal insufficiency.

The number of people infected with urinary tract infections is estimated at about 250 million each year (Ahmed *et al*., 2015) [1]. (UTI) is one of the health problems that most countries suffer from in the world, as it ranks first in Iraq in terms of total bacterial infections with a percentage of (23%) (AL-Karawyi *et al*., 2013).

This infection constitutes 46% of community-acquired infections and accounts for 80% of chronic Bacterial Prostatitis cases and 90% of Pyelonephritis cases. It also includes infections of the bladder (Cystis) and Urethritis (Urethritis). The causative agents of this disease are bacteria and fungi, and rarely, parasites and viruses are involved in infection (Schollum and Walker, 2012) [3]. *E. coli* bacteria are characterized by multing resistance (MDR) (Laird, 2016) [4]. Where this resistance resulted through its possession of resistance enzymes, including β-lactamases, which confer resistance against β-lactams, and other enzymes that confer resistance to anti-aminoglycosides and anti-quinolones. In addition to its possession of other resistance mechanisms such as changing the permeability of the cell membrane, changing the target site, inhibiting protein synthesis and having efflux pumps, as it confers resistance to antibiotics such as Microlides, Novobiocin and Rifamcin groups (Kapoor *et al*., 2017) [5] The increasing use of antibiotics has led to the emergence of resistant strains, which prompted researchers to find safer and more effective alternatives against microorganisms. Medicinal plants contain substances that have antimicrobial activity, the most important of which are alkaloids, glycosides, essential oils, resin, Resin, Tannin, Gum and phenols. Phenoles, light fats, and carbohydrates (Al Kubaisi *et al*., 2013) [3]. Due to the prevalence of urinary tract infections in the world in general and in Iraq in particular, as indicated by local studies, and because urinary tract infection (UTI) can spread among all ages and races. In view of the increase of antibiotic-resistant bacterial species, this study was carried out, which aimed to isolate and diagnose the bacteria that cause urinary tract infection from urine samples using classical methods based on microscopy, culture characteristics, biochemical tests and confirm the diagnosis using the Vitek 2 compact system.

Study of the sensitivity of bacterial isolates to antibiotics and alcoholic extracts (*Punica grantum* and *Trigonella foenum*).

Materials and Methods

Samples Collection

Collected (200) urine samples from patients suffering from Urinary Tract Infections (UTI) in Salah El-Din General Hospital, of both sexes and of different ages for the period from July 2021 to December 2022 and information about the patients was recorded (Cheesbrough, 2012) [7]. In terms of gender, age, clinical symptoms, and others, a special form prepared for this purpose was made, and phenotypic, microscopic, and culture samples were examined.

Culture and isolation of bacteria

Urine samples were cultured after microscopic examination of urine, as a centrifugation process was carried out for each sample and it was sown directly by taking a drop using the Loop Full on the media of blood agar, Maconkey agar and

saline mannitol agar, then the dishes were incubated in aerobic conditions at a temperature of 37°C for 24 One hour after the initial culture, the isolates were purified by sub culturing to isolate UTI-causing bacteria according to (Levinson and Jawetz, 2011) [8], after which bacterial colonies were identified by studying their phenotypic and biochemical characteristics (Leboffem and Pierce., 2011) [9].

Isolates Identification

Phenotypic and microscopic diagnosis

The phenotypic characteristics of the developing colonies on different media such as color, size, shape, texture, edge shape and height were studied. Then all isolates were stained with gram stain and examined by light microscope using X100 oil lens in order to know the response of the cells to the dye, their shape and arrangement, in addition to observing the size of the cells (Wanger *et al*., 2017) [10].

Biochemical Tests

These tests were conducted according to what was mentioned in (Tille, 78 2017) [11], and they are as follows:

Catalase Test, Oxidase Test, Goagulase Test

Do the test in two ways:

1- The linked clotting test (slide test)

2- Free coagulation enzyme (tube test)

Indol Test, Methyl Red Test, Vogus proskaur Test, Citrate Utilization Test, Iron Agar Kliglers Test, Urea production test, Hemolysin Production Test, Eosine Methylene Blue (EMB), Detection of Lactose Fermentation, Use acar medium and Mannitol Salt Agar Fermentation Test.

Diagnostics using modern methods using the vitek 2 compact system

Antibiotics Susceptibility Test

The sensitivity of all bacterial isolates under study against a number of antibiotics was tested by the disc diffusion method using the Kirby-Bauer method, which is described by laboratory and clinical research (CLSI, 2015) [12].

Plant extracts

In this study, two alcoholic extracts of *Punica grantum* and *Trigonella foenum* were used. The ability of the two extracts to inhibit *E. coli* isolates was tested and the MIC (Minimum Inhibitory Concentration) was determined.

Preparation of alcoholic extracts

The alcoholic extract of *Punica grantum* and *Trigonella foenum* was prepared by washing them with distilled water, then with 1% sodium hypochloride solution and washed again with sterile distilled water.

20 g of *Punica grantum* were weighed and placed in a food perocessor, then 100 ml of 95% ethanol was placed on it and mixed for 2-3 minutes and left in the flask on a shaker for 24 hours to dissolve in ethanol and then filtered Using several layers of gauze to get rid of the suspended plant parts and the remaining fibers, then filtered again using a Millipore microfiltration unit with a diameter of 0.45 mm to prevent the passage of germs from the filter. Then the mixture was placed in an electric oven at 40°C until all the alcohol had evaporated. Then the dry extract, which became in powder form, was placed in sealed sterile glass bottles and kept in the refrigerator at 4 °C until use in the experiments (Al-kareemi, 2012) [13].

As for fenugreek seeds, 20 gm of them were weighed and ground with an electric grinder, 100 ml of ethanol alcohol was added at a concentration of 95% and mixed with a shaker for 24 hours, after which the same previous steps were followed to prepare the alcoholic pomegranate peel extract (Al-Thuwaini, 2008) [14].

Sterlization of alkohol extract and prepration of stock and dilution

Take 1 g of the powder of both alcoholic plant extracts was added to 5 ml of ethanol to obtain a stock solution of 100 mg/ml and the mixture was sterilized by Pasteurisation, then the remaining dilutions were prepared 75%, 50%, 25% mg/ml stock solution for the plant extracts By taking 25 ml of the storage solution of the extract and adding 75 ml of sterile water to it, so that the concentration becomes 25%, and the 50% concentration was prepared by taking 50 ml of the storage solution of the extract was prepared by taking 50 ml of the storage solution of the extract and adding to it 50 ml of sterile water, and the concentration of 75%.

The MIC (Minimum Inhibitory Concentration) was determined, which is the lowest concentration of the extract that prevented the growth of the bacteria under study. It was confirmed that the extract was not contaminated by planting 0.1 ml of it on agar nutrient medium and incubated at 37°C for 24 hours (Sabah, 2011) [15].

Test the inhibitory efficacy of plant extracts

Doe by Agar-well diffusion method was used to test the inhibitory activity of the extracts on the growth of microorganisms. This was done by pouring 20-25 ml of agar medium into each Petri dish. After solidification of the medium, the dishes were incubated for 24 hours at 37 °C to ensure that no acar contamination. The medium was inoculated using a sterile cotton swab, where a swab was taken of the bacterial suspension and spread evenly on Muller-Hinton Agar medium and then left for 15 minutes at room temperature for the purpose of absorbing the vaccine, then 5 holes were made in each dish by means of a cork punch. Each hole represents a concentration Specific where 50 µl was added, From each of the four concentrations, it was placed in the hole designated for it, and at the same time 50 microliters of distilled water was added to one of the pits instead of the plant extract as a control sample for the purpose of comparison with the inhibitory activity of each extract, then the dishes were incubated for 24 hours at 37°C, Then the results were read using a ruler to measure the rates of the inhibition diameters for each concentration, which represents the area of no bacterial growth around each hole (Rana *et al*., 2014) [16].

Result and Discussion

Isolation and Identification

Isolation

The samples were cultured on selective and differential culture media (blood acar medium, Maconkey agar medium, mannitol acar medium). (1-1), the absence of growth is due to several reasons, including the frequent abuse of antibiotics by patients or as a result of other causes of infection such as fungi, viruses or anaerobic bacteria, which are difficult to isolate by the same methods used to isolate aerobic bacteria because they require special media and conditions for isulation.

Table 1: shows the number of isolated samples and their percentage

The total number of samples	Growth negative		Growth positive	
100	The number	The ratio	The number	The ratio
	65	65%	35	35%

E. coli bacteria isolated and dominant bacterial species in urinary tract infections, as it was isolated by 35%, and it was similar to a number of studies, including the results obtained by (Al-Mazroui, 2017) [17], when it was isolated by 30%, among the bacteria that cause urinary tract infections, respectively.

Identification

Isolates were initially diagnosed based on microscopic diagnosis by staining with Grams Stain to observe the cells' response to the dye, their shapes, sizes and assemblies.

After that, all isolates underwent biochemical diagnosis and the interpretation of the diagnostic results was based on comparing them with Brown and (Brown and Smith, 2017) [18] and the diagnosis was confirmed using the special Vitek Compact System 2.

Table 2: Shows the biochemical tests for *E. coli* bacterial isolates

Tests / Bacterial isolates	Catalase	Oxidase	Indole	Methyle red	Voges Proskaure	Citrate	Urease	Triple – Sugar Iron			
								Slop	Butt	H_2S	Gas
E. coli	+	-	+	+	-	-	-	A	A	-	+

Bacterial resistance to antibiotic

Sensitivity assay was conducted for 100 bacterial isolates using 10 antibiotics that are most commonly used to treat urinary tract infection, including (MEM), (CIP), (TM), (AK), (RA), (NA), (CL), (TE), (GN) and (F) and the results were interpreted according to what was mentioned in (CLSI, 2018) [19].

E. coli isolates showed 100% absolute resistance to RA and were 88.5% resistant to AK, F, TE (85.7%), GN (80%), NA (65.7%), CIP (57.2%) and TM (51.42). %) and C (25.7%) and The MEM showed high sensitive (100%), and the results agreed with (Heilan, 2019) [21] and Janssen and his group (2018). They found that it is 100% sensitive to MEM and it was similar to the results of (Patel and his group, 2019) as it was sensitive to 91.89%, and it also agreed with the results of Chowdhary and Parial (2015), as they found that it is sensitive to MEM at 99.9%.

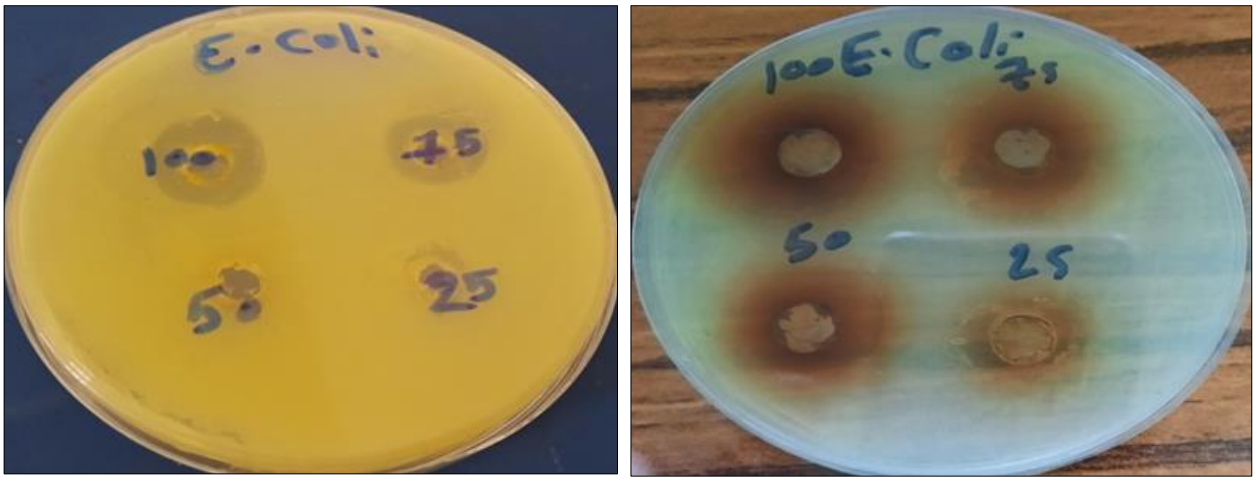
Table 3: Shows the sensitivity of isolated bacteria to antibiotics

Bacteria	TMP	CIP	MEM	TE	C	RA	NA	GN	F	AK
E. coli	48.5%	42.8%	100%	14.2	74.2	0	34.2	20	11.4	11.4

Inhibitory effect of alcoholic extracts on *E. coli*

The drilling method was used to test the biological activity of the plant alcoholic extracts (*Punica grantum* and *Trigonella foenum*), by spreading bacteria on the surface of Muller henton acre, then holes were made using a cork drill with 4 holes in each plate, each hole indicates a specific concentration of the extract, and ML 50 of each concentration was placed. Using a micropipette, the dishes were then incubated in the incubator for (18-24) hours at a temperature of 37°C. Then the average inhibition diameters of each concentration were recorded by measuring the diameter of the damping area using a graduated ruler.

The results showed that the highest inhibition of *E. coli* was at the 100% concentration of alcoholic pomegranate peel extract, as the diameter of inhibition was 25 mm as shown in Figure A (1), while for the concentrations of 75%, 50%, and 25%, the diameters of inhibition were in the amount of 22 mm, 20 mm, 14 mm respectively. While the concentration was 100% and 16 mM, while *E. coli* bacteria, were at 50% and 25% concentration, as in Figure B (1).



A- Inhibitory effect of fenugreek alcoholic extract, B- Inhibitory effect of alcoholic pomegranate peel extract

Fig 1: Inhibitory effect of plant extracts

The minimum inhibitory concentration (MIC) was determined by making several dilutions for each of them (12.5%, 25%, 50%, 75%, 100%, 200%), as it was found that the minimum inhibitory concentration of *Trigonella foenum* and *Punica grantum* is 100% and this agrees with the researcher's study (Wafaa and Bashair, 2015) [22], as their study showed that the minimum inhibitory concentration of pomegranate peel was at 100% concentration.

The effectiveness of Punica *grantum* against urinary tract pathogens is due to the fact that it contains effective antioxidant chemical compounds, which are flavonoids and phenolic compounds It also contains alkaloids, the most important of which is Granatin, Gallotannin, Pelletierine and a bitter substance called Punicine, as well as tanning materials (Orak *et al*.,2011) [23]. The tannins in the pomegranate peel interfere with the structure of the cell wall and form hydrogen bonds with proteins, which leads to their failure to build and thus inhibit the growth of bacteria (Hajoori *et al*., 2014) [24]. As for the inhibitory activity of the extract of fenugreek seeds, the reason is due to the fact that it contains many medically important active substances. The substances are mainly divided into three groups: Steroidal sapogenins, isoleucin and Galactomannans that work together in synergy to achieve the nutritional and health benefits of the fenugreek plant. It also contains a high percentage of phenolic compounds, which It plays an important role in the oxidative activity, including the compound &-amylase), which is an inhibitory factor present in the extract that may bind with the active sites of enzymes of a special type and thus prevent their work, which leads to the inhibition of bacterial growth (Dubey *et al*.,2010) [25].

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