The Effect of Aqueous Extract of *Quercus infectoria* Plant on *Pap E* gene Expression of Uropathogenic *Escherichia coli*

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Abstract

One hundred twelve urine samples were collected from Baghdad hospitals and examined by different identification techniques. Seventy isolates (62.5%) were diagnosed as *Escherichia coli* after microscopic and cultural identifications. The result of PCR product electrophoresis on the isolates showed that thirteen isolates (18.57%) have *Pap E* gene which are uropathogenic *E. coli*. Antibiotic susceptibility test was done, and four high resistant strains were mixed with aqueous extract of *Quercus infectoria* plant in 96 well ELISA plate and incubated for different times. After 0, 6, and 12 hr. of incubation, the effect of the plant extract on the bacterial growth was determined by ELISA reader, and the effect on the expression of *Pap E* gene was examined by real-time PCR. The results were showed that the higher effect of the extract was on the E10 strain growth that dropped from 3.184 at zero time of incubation to 2.378 and 2.281 after 6, and 12 hrs. of incubation respectively. Also, shown a downregulation in the expression of *Pap E* gene of the isolates at different times of incubation. The E10 strain shows decrease in the fold from 1 before the treatment with the extract to 0.076 at zero time of incubation with the extract, after 6 and 12 hrs. of incubation it shows a total down-regulation of the gene.

Keywords: *uropathogenic Escherichia coli, Pap E gene, Quercus infectoria*

Introduction

Urinary tract infection (UTI) is the occurrence of a certain bacterial number in the urine (generally more than 10^5/ml) and symptomatic UTI was classified according to severity as: a- urosepsis syndrome, b- pyelonephritis (or upper UTI, with kidney infection) and c- cystitis (or lower UTI, with bacteria into the bladder) [(1)].

Uropathogenic *Escherichia coli* (UPEC) is estimated to responsible for approximately 80 percent of all UTIs [(2, 3)]. This high frequency of infections linked with UPEC can be related to the group of virulence reasons that each UPEC strain possesses [(4)].

Uropathogenic *Escherichia coli* are a diverse collection of *E. coli* isolates which can cause urinary tract infection in animals and humans; and are just one of several available groups of pathogenic *E. coli*, characterized by a pathotypic grouping of virulence factors and special pathology [(5)].

Despite a variable developmental background, UPEC is described as containing a determined basal number of virulence factors; P pili adhesins and type I, and an array of siderophores (iron acquisition), and different cytotoxins like cytotoxic necrotizing factor 1 (CNF1). These make up the UPEC pathotype [(6)] despite the point to which the different markers are found can vary amongst variable UPEC strains. Pyelonephritis-associated pili (*pap*) or P fimbriae are individual of the best well-studied types of fimbria in *E. coli*. Deferent epidemiological researches have shown a positive correlation between UPEC genomes having the *pap* operon and the development of more severe clinical outcomes during UTI, notably pyelonephritis [(7)].

Quercus infectoria is a small tree or a shrub of the Fagaceae (Quercaceae) family. It is present in Persia, Turkey, Cyprus, Syria, and Greece[(8)]. The different species of Quercus originated in Iraq, Turkey, and Iran, but are now widespread and specifically common in Asia Minor, North Africa, and Europe[(9)]. For medicinal

properties, the plant parts used of *Q. infectoria* are mainly leaf, seed, stem or bark, valonia-type fruit, root, and nut/apple galls[10]. It has been estimated in terms of its pharmacological actions and it was found that it had anti-inflammatory, anti-parkinsonian, antidiabetic, anti-tremorine, and antioxidant effects[11, 12] Traditionally, galls are used in the practice of postpartum[13] and diarrhea, hemorrhage, and skin disease treatments[14] Extracts of *Q. infectoria* are known to carry a wide antimicrobial activity against Gram-positive strains of bacteria. Studies have also appeared good antimicrobial activity of *Q. infectoria* against different other dental pathogens in both aqueous, acetone, and methanol extracts[15].

Materials and Methods

Sample collection

One hundred twelve urine samples were collected between September to November 2019, from hospitals of Baghdad city. A general urine examination (GUE) was done on these samples. Uropathogenic isolates of *E. coli* were kept for additional identifications, such as catalase, oxidase, and other biochemical tests.

Bacterial Identification

Cultural identification

Samples were inoculated on various culture media including Blood agar, MacConkey agar, and HiChrome UTI agar. The media were incubated at 37˚C for 24 hrs., suspected colonies were identified morphologically and biochemically.

Microscopic characteristics

The supposed colonies were tested by Gram staining, to identify its reaction with crystal violate-iodine complex.

Biochemical examination

Catalase production test [16], oxidase test [16], and indole test [17] were done for bacterial isolates identification.

Identification of Uropathogenic *E. coli* with *Pap E* gene

DNA extraction

DNA extraction was done by using a wizard purification kit of genomic DNA (Promega, USA) according to the manufacturer's instructions [18].

PCR amplification

The PCR amplification mixture used for detection of each gene was include FIREPol® Master Mix 1X (5 µl), Forward primer 1Mm (1 µl), reverse primer 1 µM (1 µl), DNA template 25 ng (2 µl), and Nuclease free dH2O (11 µl). The final volume for the mixture was 20 µl.

After the reaction volume preparing in the PCR tube, it was spin down, then the PCR tube placed in the PCR thermocycler and the amplification reactions was started according to the program in the table (2):

Table (2): PCR program

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial denaturation	95°C	3 min.	1 cycle
2-	Denaturation	95°C	45 sec	30 cycle
3-	Annealing	94 oC	45 sec	
		55 oC		
4-	Extension	72°C	50sec	
5-	Final extension	72°C	10 min.	1 cycle

Agarose gel was mixed with 5µl red safe stain. DNA band was visualized by electrophoresis and captured by gel documentation system to the observed band. Agarose gel electrophoresis was done as mentioned by [19].

Plant extract preparation

The fresh plant was collected from the local market. They were cleaned, crushed, and powdered in an electric grinder[20]

The modified method of Rashan for aqueous extraction was used. (100g) of plant, samples were weighed and added to a flask containing 400 ml of hot sterile distilled water. After that, they were added to mix well in the shaker for 15 minutes and then filtered by gauze and the extract was put in test tubes. The test tubes were put in a centrifuge (6000 rpm for 5 min.). Finally, the supernatant was filtered through filter paper and placed in Petri dishes in an incubator at 37°C for 72hr. to dry out, and then the powder was collected and preserved in plastic containers[21].

The plant extract effect on the expression of *Pap E* gene

The effect of plant extract was tested on the expression of the *Pap E* gene. Serial dilutions were prepared from the plant extract. The plant extract are mixed with isolates in a multi-well plate by mixing 180 µl of the extract and 20 µl from bacterial broth. The effects of the extracts were tested after 0 hr., 6 hr. and 12 hr. and notice the changes in the reading of results by ELISA reader.

RNA extraction

Extraction of RNA was done by using the triazole protocol [22].

cDNA synthesis

The cDNA synthesis reaction was done according to the SOLIScript® (Estonia) company instructions [23].

qPCR Master Mix:

5x HOT FIRE Pol® Eva Green® qPCR Super mix is an optimized ready to use solution for real-time quantitative PCR analyses, incorporating Eva Green® dye. It contains all the required components without the template and primers to achieve highly sensitive qPCR. The qPCR master mix components include 5x HOT FIREPol® EvaGreen® qPCR Supermix (4 µl), Primer Forward (10 pmol/µl) (0.5 µl), Primer Reverse (10 pmol/µl) (0.5 µl), DNA template2 (2 µl), and H2O PCR grade (13 µl).

HOT FIRE Pol® DNA polymerase was activated by incubation at 95°C for 12 minutes. The hot start mechanism inhibits the extension of nonspecifically annealed primers and primer dimers that formed at low temperatures in qPCR system. This kit was purchased from Solis Bio Dyne/ Europe.

Protocol

The qPCR amplification mixture used for detection of each gene includes 5x HOT FIRE Pol® Eva Green® qPCR Supermix (4 µl), 3 µl of cDNA template, 1 µl (1 mM) of each forwarded and reversed primers, and 11 µl of nuclease-free water to complete the amplification mixture to 20µl.

After the reaction volume was prepared in the qPCR tube, the mixture was spun down, then the qPCR tube was placed in the qPCR thermo cycler applied bio system and the amplification reactions were started according to the program in table (4).

Table (3): real-time PCR program

Cycle step	Temp.	Time	Cycles
Initial activation	95 oC	12 min.	1
Denaturation	95 oC	15 sec.	40
Annealing	60 oC	1 min.	
Elongation	72 oC	20 – 30 sec.	

Result and Discussion

Identification of bacteria

Isolation and identification of clinical bacterial isolates

One hundred twelve urine samples were collected from Baghdad hospitals between September and November 2019. Seventy-one samples were from women and forty-one samples were from men.

The preliminary identification of *E. coli* was carried out on different culture media. Seventy isolates (62.5%) (Forty-eight from women and twenty-two from men) were chosen for more identification which had the characteristics of *E. coli* growth.

Identification of uropathogenic *E. coli* by using *PapE* gene

From the results of gel electrophoresis figure (1), there are thirteen clinical isolates were considered as uropathogenic *E. coli*, nine isolates were from women and four isolates were from men. These isolates were had a band size of 545 bp.

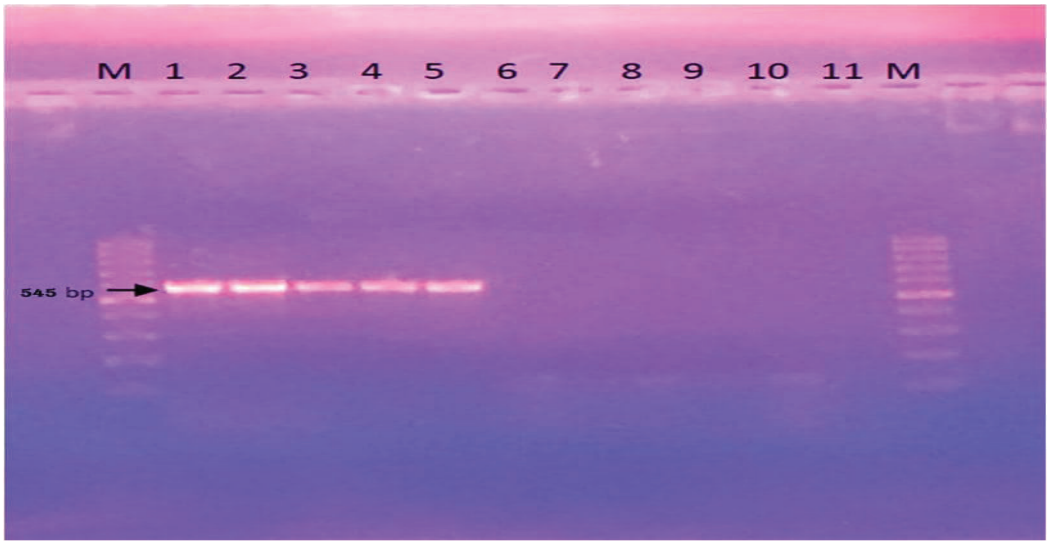

Figure (1): results of gel electrophoresis for uropathogenic *E. coli*

The effect of plant extracts on bacterial growth

Four high resistant strains were mixed with the plant extract in multi-well plates and its absorbance was read at zero, 6, and 12 hr. by ELISA reader. The results of the plant extract against bacterial growth were listed in table (5).

From table (5), the effect of the plant extract on the growth of bacterial strains was nearly close to each other. The stock is the only concentration that affects the isolates growth, while the other concentrations did not show an obvious effect on the growth of the isolates. The effect of the extract was higher on the E10 strain and the lower effect was shown on the E8 strain.

At the stock concentration, the higher effect on isolate growth was on E10 strain that dropped from 3.184 at zero time of incubation to 2.378, and 2.281 after 6, and 12 hrs. of incubation respectively. The second higher effect was shown on the E3 strain that dropped from 2.587 at zero time of incubation to 2.530, and 2.333 after 6, and 12 hrs. of incubation respectively. E1 strain growth was dropped from 2.458 at zero time of incubation to 2.422, and 2.252 after 6, and 12 hrs. of incubation. Lastly, the E8 strain growth was dropped from 2.440 at zero time of incubation to 2.284, and 2.271 after 6, and 12 hrs. of incubation respectively.

Table (4): the effect of plant extract on bacterial growth

Concentration	E1 strain			E3 strain			E8 strain			E10 strain		
	Exposure time (hr.)			Exposure time (hr.)			Exposure time (hr.)			Exposure time (hr.)		
	0	6	12	0	6	12	0	6	12	0	6	12
Stock	2.458	2.422	2.252	2.587	2.530	2.333	2.440	2.284	2.271	3.184	2.378	2.281
10-1	2.057	2.051	2.207	2.240	2.238	2.224	2.160	2.175	2.067	2.168	2.106	2.378
10-2	1.426	1.516	1.459	1.209	1.498	1.441	1.326	1.462	1.483	1.194	1.395	1.695
10-3	0.610	0.825	0.894	0.554	0.863	0.885	0.556	0.802	0.916	0.518	0.873	1.124
10-4	0.226	0.681	0.762	0.213	0.572	0.769	0.199	0.392	0.682	0.255	0.718	1.039
Control 1	0.038	0.039	0.037	0.040	0.050	0.171	0.036	0.038	0.039	0.331	0.287	0.088
Control 2	0.037	0.039	0.036	0.040	0.041	0.105	0.035	0.036	0.037	0.053	0.046	0.089
LSD value	0.412 *	0.472 *	0.358 *	0.377 *	0.452 *	0.490 *	0.428 *	0.466 *	0.471 *	0.385 *	0.398 *	0.466 *
* (P≤0.05).												

Control 1= bacteria+ media control 2= media

Gene expression

RNA extraction

Two strains E8 and E10 were chosen to study gene expression. RNA was extracted from them and then converted to cDNA as described above. The RNA extracts were preserved at – 20 ˚C.

Gene expression with plant extract

RNA extracted from isolates that grow in the same media but with different times of incubations with the stock concentration of aqueous plant extract. These were used to determine the effect of plant extracts on *Pap E* gene expression by using quantitative real-time PCR.

The results of qRT-PCR with the housekeeping gene primer for the 16sRNA gene showed pure product for this gene that exhibited by melting point value that seems very close (with one peak) to each other's in all cases of different times, which is 82.04 ˚C.

The results of melting point for *Pap E* gene product exhibited one peak in melting point curve in all processes that meant it was pure in all of them; the melting point values were also closed to each other. It was 83.65 ˚C.

From table (6), the quantification cycle (Cq) of the 16sRNA exhibited a slight difference among all processing, it ranged from 16.2 to 33.5 cycles. Also, the quantification cycle (Cq) of the *Pap E* gene exhibited a slight difference among all processing, it ranged from 17.9 to 31.8 cycle.

The results showed a downregulation of the *Pap E* gene of both isolates in different incubation times. E8 strain shows a decrease in the fold from 1 fold before the treatment with the extract to 0.016 fold and 0.012 fold at zero and 6 hrs. of incubation, while after 12 hrs. of incubation it shows a total down-regulation of the gene, while the Cq of the 16sRNA was changed from 16.2 before treatment to 18.9, 23.6, and 33.5 after 0,6, and 12 hrs. of incubation with the extract which indicate a down regulation in the expression of 16sRNA gene. Also the E10 strain shows a decrease in the fold from 1 before the treatment with the extract to 0.076 at zero time of incubation with the extract, after 6 and 12 hrs. of incubation it shows a total down-regulation of the gene, while the Cq of the 16sRNA was changed from 18.6 before treatment with the extract to 27.6 at zero time of incubation, and it shows a total downregulation of the gene after 6, and 12 hrs of incubation with the extract.

According to the result, we conclude that aqueous extract of *Quercus infectoria* plant affects *Pap E* gene expression, so it can affect the bacterial pathogenicity and prevent the adhesion and in response the invasion of bacteria to the surfaces and decrease or prevent the infection.

Table (5): the effect of plant extract on *Pap E* gene expression

Strain	Treatment	16SRNA	Pap E	ΔCT	ΔΔCT	Fold ± SD	T-test
E8	Before	16.2	17.9	1.7	0	1 ± 0.00	--
	W(0)	18.9	26.5	7.6	5.9	0.016 ±0.005	0.317 **
	W(6)	23.6	31.6	8	6.3	0.012 ±0.002	0.355 **
	W(12)	33.5	Total downregulation	Total downregulation	Total downregulation	Total downregulation	--
E10	Before	18.6	19.1	0.5	0	1 ± 0.00	--
	W(0)	27.6	31.8	4.2	3.7	0.076± 0.004	0.278**
	W(6)	Total downregulation	Total downregulation	Total downregulation	Total downregulation	Total downregulation	--
	W(12)	Total downregulation	Total downregulation	Total downregulation	Total downregulation	Total downregulation	--
** (P≤0.01).							

Conclusion

The results of our study showed that aqueous extract of *Quercus infectoria* plant can down regulate the *Pap E* gene expression that responsible for the adhesion of the uropathogenic *E. coli* to the surfaces and cause urinary tract infection, and can decrease or prevent bacterial pathogenicity. As a result, this herbal extract can be used as alternative therapy for the treatment or prevent of UTI.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

Funding: Self-funding

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