MOLECULAR INVESTIGATION OF GENE EXPRESSION OF BETA-LACTAMASES ENZYMES GEN FOR *PSEUDOMONAS AERUGINOSA* BACTER

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Abstract
The present study included the collection of 144 samples from various clinical and environmental sources to investigate the presence of *P.aeruginosa*. 45 isolates were identified as *P.aeruginosa* based on morphological and biochemical tests in addition to molecular diagnostics used of 16S rRNA. This diagnosis proved that all isolates belong to *P. aeruginosa*. All isolates selected susceptibility toward 11 antibiotics using disc diffusion method. The results showed a high resistance among isolates against Tetracycline, Cefixim, Cefotaxim, Amoxicillin, Erythromycin, Methicillin, Cloxacillin and Naldixic acid, and moderate resistance towards Meropenem and low resistance towards Imipenem and Ciprofloxacin. To determine the types of some genes responsible for Extended Spectrum Beta-Lactams (ESBLs) in *P. aeruginosa* using polymerase chain reaction (PCR) was used for detecting genes, (OXA-10), (OXA-4) and (VEB-1). The results showed that two isolate positive to (OXA-10), (OXA-4) and (VEB-1), while 43 isolates were negative to (OXA-10), (OXA-4) and (VEB-1). Detection of gene expression was performed by Quantitative Real Time PCR technique after RNA was extracted from isolate treated with plant extract of *Thymus vulgaris* and Amoxicillin. The result showed that gene expression was low expression after treatment with plant extract and Amoxicillin.
Gene expression, Beta-Lactamases.

Key Words:Molecular Investigation
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التحري الجزيئي عن التعبير لجينات انزيمات البيتا–لاكتاميز واسعة الطيف لبكتريا ***Pseudomonas*** **aeruginosa**

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المستخلص
تضمنت الدراسة الحالية جمع 144 عينة من مصادر بيئية وسريرية للتحري عن وجود بكتريا *P.aeruginosa* ، 45 عزلة تعود لبكتريا *P.aeruginosa*، واعتمادا اعتماد على نتائج التشخيص المجهري والكيموحيوي بالإضافة الى التشخيص الجزيئي لجين 16S rRNA. أجري فحص الحساسية لجميع العزلات البكتيرية المنتخبة والبالغ عددها 45 عزلة تجاه 11مضادا حيويا بطريقة الانتشـــار على الاطباق، واظهرت النتائج مقاومة عالية للمضادات Tetracycline، Cefixim، Cefotaxim، Amoxicillin، Erythromycin، Methicillin، Cloxacilli و Naldixic acid ، بينما كانت المقاومة متوسطة للمضاد Meropenem بينما كانت المقاومة ضعيفة للمضادات Imipenem, Ciprofloxacin. لتحديد بعض الجينات المسؤولة عن انزيمات بيتا–لاكتاميز واسعة الطيف لبكتريا *P.aeruginosa* بواسطة طرائق البيولوجي الجزيئي فقد استخدم تفاعل البلمرة المتسلسل (PCR) للكشف عن جينات (OXA–10)، (OXA–4) وجين (VEB–1) واظهرت النتائج بان عزلتين تمتلك جينات (OXA–10) ، (OXA–4) و (VEB–1) ، بينما 43 عزلة لا تمتلك تلك الجينات .تم تحديد التعبير الجيني باستعمال تفاعل البلمرة اللحظي (q RT–PCR) ، بعد استخلاص الحامض النووي RNA من العزلة البكتيرية المعاملة مع المستخلص النباتي لنبات الزعتر البري والمضاد الحيوي Amoxicillin و اظهرت النتائج انخفاض التعبير الجيني بعد المعاملة مع المستخلص النباتي والمضاد الحيوي Amoxicillin .

الكلمات المفتاحية: التحري الجزيئي، التعبير الجيني، البيتالاكتاميز.
مستل من رسالة ماجستير للباحث الاول

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INTRODUCTION

Pseudomonas aeruginosa is one of the opportunistic human pathogens that preferentially infects patients with cancer , AIDS, immunocompromised patients by surgery, cytotoxic drugs or burn wounds, people with cystic fibrosis, eye, ear and urinary tract infection (17), The success of *P.aeruginosa* as a pathogens is largely due to its distinct mechanisms used for antibiotic resistance in *P. aeruginosa* may be mediated via several distinct mechanisms including modification of site-targeted drugs or outer membranes, β-lactamase production, and efflux pumps. The increase in antibiotic resistance is mostly due to extensive misuse of antibiotics such as ciprofloxacin, β-lactamase and aminoglycosides in burn centers as well as non-availability and high costs of other effective drugs (19). Generally, ESBLs are not carried on the bacterial chromosome, rather they are found on an independent element of DNA called a plasmid. Plasmids can carry many different genes on them and have the ability to transfer a replica of themselves to other bacteria. This can be very serious for a number of reasons (16). These enzyme(OXA) are named OXA because they preferentially hydrolyze oxacillin and cloxacillin. These enzymes confer resistance to ceftazidime and are poorly inhibited by clavulanic acid (20). The OXA-10 and OXA-4 enzymes are highly homologous: there are only two amino acid differences, with the OXA-4 enzyme having Aspartic acid 48 Valine and Aspartic acid 207 Glutamic acid substitutions relative to the OXA-10 sequence. OXA-4 *β*-lactamase is generally plasmid- mediated, the chromosomal DNA of these isolates, but not their plasmids, hybridized with the OXA-4 gene amplified by the PCR method (2), VEB-1 (for Vietnamese extended spectrum beta – lactamase), VEB-1 has highest amino-acid identity with Pseudomonas Extended Resistance (PER-1) and (PER- 2) (38%), and confers high-level resistance to Ceftazidime, Cefotaxime and Aztreonam (1). The main Aimed to measure the gene expression of the OXA genes and compare the gene expression in the present of the antibiotic, plant extracts and in the absence of it in order to improve the role of these genes in the resistance of ***P.****aeruginosa* to β- lactamase

MATERIALS AND METHODS

Isolation and Identification of Bacteria: The clinical and environmental isolates of *P. aeruginosa* were collected from laboratories of some hospitals in Baghdad and AL-Ramadi city, for the period October 2017, till the end of January 2018. After the collection, all isolates obtained were cultured directly on MacConkey agar and Blood agar media and, incubated aerobically at 37°C for 24 hr. Identification was conducted using Biochemical tests in addition to molecular diagnostics via the use of 16S rRNA

Antibiotics sensitivity test

All isolates were tested for antimicrobial susceptibility depending on the CLSI criteria by disc diffusion method(12).

β-Lactamase production test

Iodometric method was employed for the detection of isolates producing β-Lactamase according to Collee (5).

Biofilm Production by *P. aeruginosa*

A suspension of bacterial isolate is equivalent to the McFarland No(0.5) turbidity standard which was inoculated in Brain – Heart infusion broth and incubated for 18-24 hours at 37ºC, 200µl of Brain – Heart infusion broth containing *P. aeruginosa* were added to individual wells of sterile polystyrene, 96-well, flat-bottomed . Each plate was covered with the lid supplied by the manufacturer. Subsequently, inoculated assay plates were transferred to an incubator set at 37°C for 18–24 h. Negative control wells contained sterile Brain – Heart infusion broth. After incubation, assay plates were uncovered and liquid culture was removed from each well, and non-adherent bacteria were removed by washing each well 2-3 times with D.W. Fixation of adherent cells was accomplished by methanol (200 µl) for 10 min . Biofilms were stained by adding 200 µl of 0.1% crystal violet to each well for 15minutes. After the staining reaction has been completed, excess stain was removed by repeated washing (2-3 washes) with D.W. as described above. Afterwards, 200µl of 95% methanol was added to each well for 10 minutes. All assays were done in triplicates. The amount of crystal violet was extracted by the methanol in each well and directly

quantified spectrophotometrically by measuring the optical density (OD) at630nm using amicroplate reader, The results were calculated according to following equation: Capacity of biofilm formation = Absorption of the sample test - absorption for control (3).

DNA extraction

Bacterial DNA was extracted according to Genomic DNA mini Kit was provided by Promega Company

PCR detection of ESBLs genes

Polymerase Chain Reaction (PCR) was used for the detection of ESBLs genes in all isolated of *P. aeruginosa* (clinical and environmental isolates), which were (*bla* OXA-10), (*bla* OXA-4) and (*bla* VEB-1). The primers sequence for ESBL genes are shown in Table (1). Lyophilized forward and reverse primers were suspended with suitable volume of TE buffer as recommended by Bioneer Corporation protocol. Lyophilized primers were dissolved in deionized water to give a final concentration of (100 pM/µl) (stock solution); to prepare 10µM concentration as work primer solution then 10 pM/µl was re-suspended in 90µl of deionized water to reach a final concentration of 10 µM.

Table 1. The sequence of forward and reverse primers of *bla*OXA-4, *bla*OXA-10 , *bla*VEB-1, 16srRN and gyrB gene

Primer Name	5' – Sequence - 3'	Product size	References
OXA-4 (F)	TCA ACA GAT ATC TCT ACT GTT	216bp	Kenji *et al*. (1999)
OXA-4 (R)	TTT ATC CCA TTT GAA TAT GGT		
OXA-10 (F)	TCA ACA AAT CGC CAG AGA AG	277bp	Poirel *et al*. (2001)
OXA-10 (R)	TCC CAC ACC AGA AAA ACC A		
VEB-1 (F)	CGA CTT CCA TTT CCC GAT GC	643bp	Bachvarova *et al*. (2005)
VEB-1 (R)	GGA CTC TGC AAC AAA TAC GC		
16srRNA (F)	GG GGG ATC TTC GGA CCT CA	956 bp	Spilker *et al*. (2004)
16srRNA (R)	TCC TTA GAG TGC CCA CCC G		
gyrB (F)	GGC GTG GGT GTG GAA GTC	190 bp	Aghamollaei et (al., 2015)
gyrB (R)	TGG TGG CGA TCT TGA ACT TCT T		

The PCR mixtures were performed in a total volume of 20µl consisting of the followings : 15µl of distilled water, lyophilized of PCR master mix (Bioneer Corporation) was dissolved by vortexing , and 2µl of each primer forward and reverse (10 pM each), master mix(5 µl) final 3µl of DNA (total volume, 25µl). Amplification was included in every set of PCR reactions, the reaction mixtures following a "hot start" were subjected to empirically optimized thermal cycling program.

Preparation of *Thymus vulgaris*

Thyme oil is extracted by the Soxhlet device. 50 g of leaves powder mixed with 500 ml of methanol in soxhlet device with temperature (60°C) for 12 hours. and the stock solution of Thyme oil concentration prepared by dissolved 4g of Thyme powder in 10ml of dimethyl sulfoxide(DMSO), then the extracted was filtered by using whatman.

Determination of gene expression

Quantitative Real time PCR (q RT-PCR) was used for the detection of gene expression of ESBLs genes which were *bla* OXA-10, *bla* OXA-4 with House-keeping gene which was gyrB as a control. Primers were prepared according to the company provided . The measurement of gene expression of the three genes in the resistant isolate was done before and after treatment with the antibiotic and Thymus vulgaris extract. The concentrations of antibiotic (Amoxicillin) and Thymus vulgaris extract used in the treatment were in the dose under the MIC value to allow the bacterial growth with induction of resistance.

One-Step Quantitative Real-time PCR Assay (QRT-PCR) reaction

Amplification of fragment of mRNA was performed with the following master amplification reaction with the program of One-Step RT-PCR.

After treated with antibiotic and Thymus vulgaris extract

Nutrient broth tubes were prepared with the appropriate concentration of Thymus vulgaris extract (250µl), antibiotic (Amoxicillin) and a mixture of antibiotic with *Thymus vulgaris* extract. The Negative control was *P. aeruginosa* in nutrient broth . Then and incubated at 37°C for 24 hours to monitor the bacterial growth in media. After growth, RNA was extracted using Total RNA Extraction by Accuzol Reagent method as suggested by the manufacture's instruction. Then Use same primers, RT master mix and programs that used before add antibiotic and plant extract.

Delta delta Ct (ΔΔCt) method

This method is the simplest one, as it is a direct comparison of Ct values between the target gene and the reference gene. Relative quantification involves the choice of a calibrator sample. The calibrator sample can be the untreated sample, Firstly, the ΔCt between the target gene and the reference gene is calculated for each sample (for the unknown samples and also for the calibrator sample). ΔCt = Ct target – Ct reference gene. Then the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value: ΔΔCt = (Ct target – Ct reference) sample – (Ct target – Ct reference) calibrator. The normalized target amount in the sample is then equal to $2^{-\Delta\Delta Ct}$ and this value can be used to compare expression levels in samples (11)**.** The relative changes in mRNA expression levels were determined using comparative threshold cycle (CT) method ($2^{-\Delta\Delta Ct}$ **)** between the samples exposed to Amoxicillin, Thymus vulgaris extract, antibiotic impregnated with Thymus vulgaris extract and samples non-exposed.

RESULTS AND DISCUSSION

Approximately, 31.25% (45 isolates) out of 144 isolates were identified as *P.aeruginosa* using the morphological, cultural, biochemical and genetic diagnostics test

Sensitivity of antibiotics test

Table 2. Percentage of *P. aeruginosa* susceptibility to antibiotics

Antibiotics	Code	*P. aeruginosa* isolates (Number =45)					
		Resistant		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Cefixim	CFM	35	77.7%	4	8.8%	6	13.3%
Cefotaxim	CTX	33	73.3%	10	22.2%	2	4.4%
Tetracycline	TE	38	84.4%	3	6.6%	4	11.1%
Amoxicillin	AMC	37	82.2%	3	6.6%	5	11.1%
Erythromycin	E	36	80%	9	20%	0	0%
Ciprofloxacin	CIP	0	0%	5	11.1	40	88.8%
Methicillin	ME	35	77.7%	4	8.8%	6	13.3%
Cloxacillin	CX	35	77.7%	6	13.3%	4	8.8%
Meropenem	MEM	6	13.3%	9	20%	30	66.6%
Naldixic acid	NA	33	73.3%	9	20%	3	6.6%
Impienem	IPM	0	0%	3	6.6%	42	93.3%

P.aeruginosas isolates showed high resistance to most antibiotics used in the present study, The antibiotics must pass across cell membrane to reach their target. Bacteria have ability to prevent accumulation of these antibiotics by chromosomally encoded efflux pump system(7) . Also the outer membrane of the *P. aeruginosa* bacteria is poorly permeable to antibiotics and many classes of compounds with a permeability rate from 10 to 500 times lower than permeability rate of *E.coli* (15). As well as the presence of resistance genes, and addition to that presence of R-plasmid gives *P. aeruginosa* more resistant to many antibiotics (13). In the current study, it is found that there is a difference in resistance and sensitivity percent of local isolates towards antibiotics, where the results of the study agreed with many local studies, while they are different with some international studies. This may be considered normal due to environmental conditions and health care among the region and others, as well as the random use of antibiotics. Finally the results show that the best effective antibiotics towards *P.aeruginosa* isolates are Imipenem and Ciprofloxacin and this corresponds to most of the internationally

known results as an effective treatment against *P.aeruginosa* bacteria.

β-Lactamase production test

The result showed most the isolate was produced of β-Lactamase as showing in Table(3) **.** The rate of ESBL production in bacteria differs greatly all over the world, and it has been changing rapidly. These results showed that there is a high percentage of isolates under study were producing ESBLs enzymes, which is an indication of possess of those isolates to a high resistance to many of antibiotics and therefore difficult to treat infections caused by these bacteria that causing a threat to the patients and increase the morbidity and mortality.

Table 3. Number and Percentage of *P. aeruginosa* produced of ESBLs

Source	Number of isolates	Isolates that positive to β-Lactamase	%
Burn	15	13	%86.6
Wound	13	13	%100
Ear	5	5	%100
cystic fibrosis	4	4	%100
Urine	2	2	%100
Water	4	3	%75
Soil	2	1	%50
Total	45	41	%91.1

Biofilm Production by *P.* aeruginosa

The results (table 4) showed that 39/45 (86.6%) of isolates produce biofilm, but which varying degrees compared to negative control. These results nearly agrees with many local studies such as (8). This high productivity of biofilm formation may be back to sensitivity of (MTB) method to measure the few quantities formed, and considered important method in studying the early stages of biofilm formation because it uses constant conditions and it can be effective in studying many of virulence factors to form biofilm such as pilli and flagella. this difference in results may be due to the type of media used or the laboratory conditions that accompanied the detection of biofilm formation among our results and disagrees results in some previous study. From the other hand, The variation in the ability of isolates to form biofilm back to association of production with its ability to produce ß-Lactamase, where the isolates produced of multiple types of enzyme were produce a strong biofilm, compared with isolates that produce one type of enzyme, while the isolates that do not produce this enzyme do not form biofilm(9).

Table 4. ability of *P.aeruginosa* isolates to biofilm formation

No. of isolates	Value biofilm formation	No. of isolates	Value biofilm formation	No. of isolates	Value biofilm formation
PS 1	0.44	PS 17	0.32	PS 33	0.33
PS 2	0.55	PS 18	0.0	PS 34	0.32
PS 3	0.32	PS 19	0.29	PS 35	0.37
PS 4	0.36	PS 20	0.34	PS 36	0.44
PS 5	0.35	PS 21	0.39	PS 37	0.0
PS 6	0.33	PS 22	0.31	PS 38	0.41
PS 7	0.26	PS 23	0.37	PS 39	0.42
PS 8	0.32	PS 24	0.34	PS 40	0.34
PS 9	0.0	PS 25	0.61	PS 41	0.37
PS 10	0.36	PS 26	0.0	PS 42	0.34
PS 11	0.42	PS 27	0.41	PS 43	0.35
PS 12	0.30	PS 28	0.39	PS 44	0.43
PS 13	0.46	PS 29	0.0	PS 45	0.0
PS 14	0.35	PS 30	0.49		
PS 15	0.0	PS 31	0.34		
PS 16	0.39	PS 32	0.32		

Molecular Detection of Some Genes Responsible for ESBL In *Pseudomonas aeruginosa* Isolates: ESBLs genes OXA-10, OXA-4 and VEB-1 was screened by PCR technique for the forty-five isolates. The results of gel electrophoresis for PCR product by using specific primers for this genes showed that one isolate were positive for this genes as shown in Figure (1, 2, 3) , While 43 /45 (95.5%) of isolates were Negative for this

genes. These results nearly agrees with many local studies such as study of Hussein (2013) who referred to the low prevalence of OXA gene, another study of the most middle east countries also showed the low prevalence of this genes in isolates of *P.aeruginosa* . In addition to that, some isolates were have more than one genes of ESBLs**,** These results nearly agrees with many local studies such as A study in Ilam (Iran) noticed that among (70) isolates of *E.coli* collected from hospitalized patients, two isolates (85%) were positive for *blaCIT* (10).

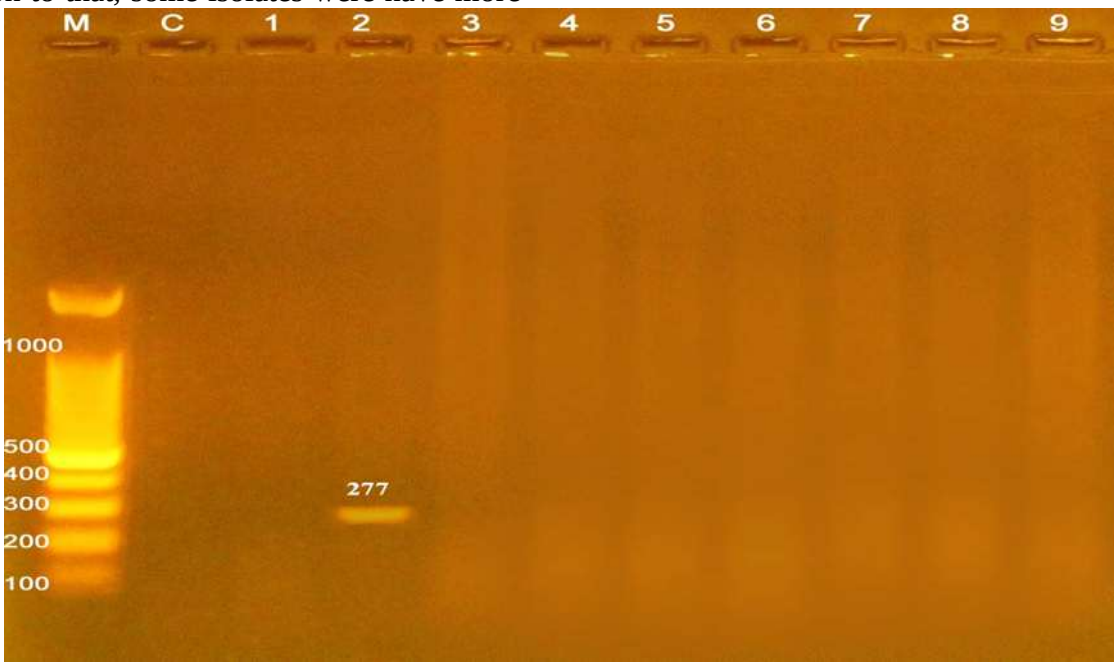

Figure 1: Gel Electrophoresis of PCR Product for Detection of ESBL blaOXA-10 Gene (277bp) Using 1% Agarose for 60 min. at 70 V\Cm))
1-M: Marker DNA ladder Size (1500bp)
2- Lanes (2) positive for blaOXA-10 (277 bp)
3-C: Negative control

Figure 2: Gel Electrophoresis of PCR Product for Detection of ESBL blaOXA-4 Gene (216bp) in *pseudomonas aeruginosa* Using 1% Agarose for 60 min. at 70 V\Cm
1-M: Marker DNA lader Size (1500bp)
2-Lanes (2) positive for blaOXA-4 (216 bp)
3-C: Negative control

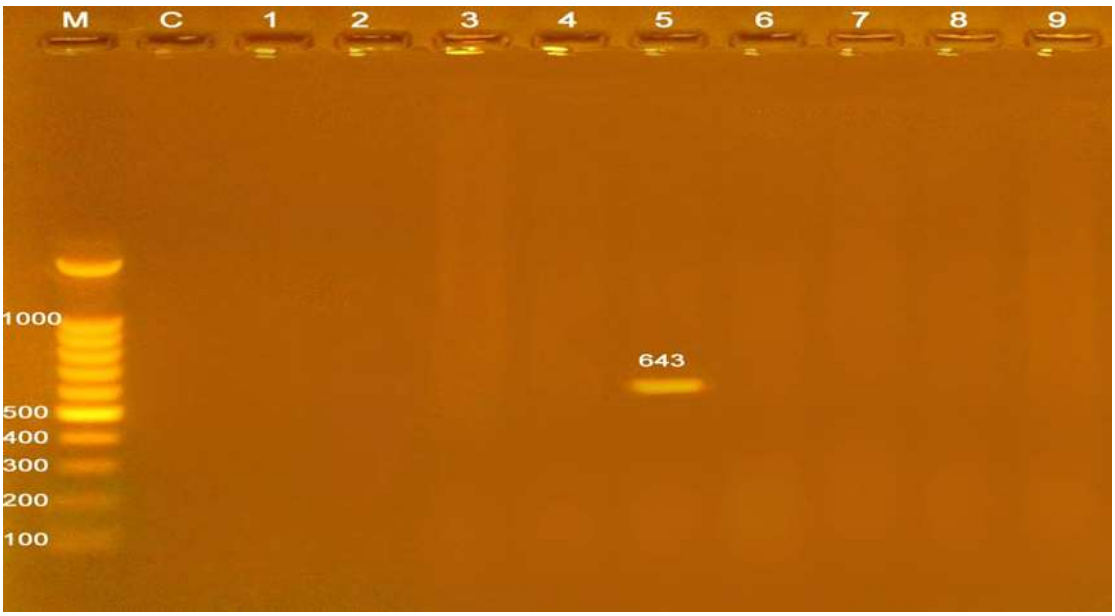

Figure 3: Gel Electrophoresis of PCR Product for Detection of ESBL *bla*$_{VEB-1}$ Gene (643bp) in *pseudomonas aeruginosa* Using 1% Agarose for 60 min. at 70 V\Cm

1- M: Marker DNA lader Size (1500bp).

2- Lanes (5) positive for *bla*$_{VEB-1}$ (643 bp).

3- C: Negative control

Determination of gene expression

Reverse transcription quantitative PCR (RT-qPCR) is distinguished from other methods for gene expression because of accuracy, sensitivity and fast results. This technology has established itself as the golden standard for gene expression analysis. It is important to realize that in a relative quantification study, the experiments are usually interested in comparing the expression level of a particular gene among different samples**.** The gene expression of qRT-PCR was measured for *P. aeruginosa*, specifically for the OXA-10 and OXA-4 genes responsible for the production of beta lactamase enzymes with Housekeeping gene(H.K) as a control , The results showed a decrease in gene expression after treated with plant extracts and antibiotics as shown in the table (5,6).

Table 5. Genetic expression values for OXA-10 gene after treatment

Sample	Housekeeping gene	OXA-10	CtΔ	CtΔΔ	Folding
Plant extract	27.2007	32.26842	5.067721	2.485991	0.178502
Plant extract +Antibiotic t	18.8377	35.3654	16.5277	13.94598	0.000063
Antibiotic	27.57332	35.77462	8.201302	5.619573	0.020339
Control	25.95764	28.53937	2.581729	0	1

Table 6: Genetic expression values for OXA-4 gene after treatment

Sample	Housekeeping gene	OXA-4	CtΔ	CtΔΔ	Folding
Plant extract	27.2007	31.06378	3.86308	1.533983	0.345323
Plant extract +Antibiotic t	18.8377	29.6023	10.7646	8.435507	0.002888
Antibiotic	27.57332	31.06675	3.493425	1.164328	0.446172
Control	25.95764	28.28674	2.329097	0	1

According to the results of the present study, Concluded that Thyme oil were active against *P. aeruginosa*

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