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Screening of quorum sensing and biofilm inhibitors among pseudomonas aeruginosa clinical isolates

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***Abstract*---**Quorum sensing (QS) is a process that enables bacteria to communicate using secreted small pheromone-like signaling molecules called autoinducers (AIs). This process enables a population of bacteria to regulate gene expression collectively. These quorum sensing systems regulates biofilm formation, virulence factors expression, bioluminescence, motility patterns, exopolysaccharide production, antifungal or antibiotic production, endoglucanase production, pigmentation, competence, plasmid conjugal transfer and cross-signaling between strains and species. P. aeruginosa is an important and very dangerous opportunistic pathogen causing many fatal infections in patients with serious medical conditions. P. aeruginosa is associated with nosocomial infections mainly in immunocompromised patients, and it is rated the third-most-common microorganism associated with hospital-acquired infections representing about 10-15% of nosocomial infections recorded globally. Moreover, qRT-PCR revealed a significant reduction in expression of quorum sensing genes in virulence inhibitors-treated P. aeruginosa in comparison with untreated bacteria. Virulence inhibitors could play a role in reduction of Pseudomonas quorum sensing-dependent

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virulence factors production such as biofilm production, and therefore affect its pathogenesis in the host.

Keywords---Quorum sensing; Autoinducers; Pseudomonas aeruginosa; Quantitative Real Time Polymerase Chain Reaction.

Introduction

P. aeruginosa is a prevalent opportunistic human pathogen and considered a major cause of many hospital-acquired infections. P. aeruginosa could give rise to serious diseases such as urinary tract infections (UTIs), bacteremia, burn-wound infections, and cystic fibrosis [(1)]. P. aeruginosa virulence potential is essentially attributed to secretion of various virulence determinants such as proteases, elastase, pyocyanin, rhamnolipids, and biofilm [(2)]. Antibiotics abuse besides their improper usage resulted in a considerable increment in microbial resistance to the commonly prescribed antibiotics [(3)]. Quorum sensing inhibitors (QSIs) are drugs that interfere with bacterial cells communication system known as quorum sensing (QS) which controls the expression of many virulence factors [(4)]. Quorum sensing in P. aeruginosa is controlled mainly by secretion of different autoinducers. Numerous natural compounds were tested for their QS inhibitory activity, and some of them showed reasonable activity such as furanone compounds extracted from algae Delisea pulchra. Moreover, many chemically synthesized compounds showed potent QS inhibitory activity such as phenylpropionyl-homoserine lactones, phenyloxyacetyl- homoserine lactones, and halogenated furanones [(5)]. The present study aims to investigate the possible QS inhibitory effect of nifuroxazide, thymol and acylase on P. aeruginosa and their effect on biofilm formation. Current study could provide a valuable way to control the problem of antibiotic resistance in P. aeruginosa.

Method

Bacterial identification, standard strain and culture conditions:

One hundred and ninety specimens were collected from different sources from patients admitted to Egyptian hospitals; these isolates were collected from Kasr El-Aini University hospital and Al-Hussein University hospital during the period from May 2018 to December 2019. In addition, *P. aeruginosa* PAO-1 wild-type standard strains were used in the present study as a positive control for quorum sensing and virulence regulating genes [(6)]. Different clinical specimens included sputum, urine and wound & burn swabs were collected using proper sampling techniques and aseptically [(7)]. *P. aeruginosa* isolates were isolated from all clinical samples and were identified using conventional microbiological methods including Gram staining, growth characteristics on nutrient agar, cetrimide agar, king A agar and biochemical tests such as catalase and oxidase followed by identification by automated method using VITEK ® 2 Compact System (BioMèrieux, France).

Antimicrobial susceptibility of bacterial isolates [8]:

The antimicrobial susceptibility testing for the isolates was carried out using the disc diffusion method according to Kirby-Bauer protocol described by Clinical and Laboratory Standard Institute [9]. Antimicrobial agent used in this study were Pipracillin, Pipracillin/Tazobactam, Ceftazidime, Ceftriaxone, Cefotaxime, Cefepime, Imipenem, Meropenem, Colistin sulphate, Azetreonam, Gentamicin, Amikacin, Levofloxacin, Norfloxacin and Ofloxacin.

Identification of isolates and detection of resistance pattern by VITEK® 2 Compact System (BioMèrieux, France):

Multi-drug resistant isolates (20 isolates) were chosen to perform identification and detection of their MIC by VITEK® 2 Compact System according to manufacturer's instructions were ID GN Cards used for Identification of *P. aeruginosa* while AST-N222 card used for Antibiotic susceptibility for *P. aeruginosa.*

Determination of minimum inhibitory concentration (MIC) of the tested potential virulence inhibitors:

The minimum inhibitory concentrations (MIC) of the potential virulence-inhibitors, (nifuroxazide, thymol and acylase) were determined using the broth microdilution method. A single colony of the tested isolates was incubated overnight in Mueller-Hinton broth then the bacterial suspensions were diluted with Mueller-Hinton broth to reach the cell density of 10^6 CFU/mL. The tested chemical substances were used to prepare 2-fold serial dilution solutions in LB broth after dissolving the tested chemicals in dimethyl sulfoxide. One hundred µl from the prepared diluted potential inhibitors solutions were inoculated with 100 µl of bacterial suspensions in microtiter plates. After overnight incubation at 37ºC, the MIC values were calculated as the lowest concentration of the tested chemical substances that inhibited the visible growth of bacteria. The MIC of nifuroxazide, thymol and acylase were 1 mg/mL, 2 mg/mL and 1 mg/mL respectively.

Effect of sub-inhibitory concentrations of different potential virulence inhibitors on bacterial growth:

The effect of sub-inhibitory concentrations of different chemical substances, (1/4 MIC) of nifuroxazide, thymol and acylase (250µl, 500µl and 250µl) on the growth of *P. aeruginosa* isolates was evaluated [10]. LB broth was inoculated with overnight cultures of the tested isolates in the presence and absence of sub-inhibitory concentrations of different tested chemical substances and incubated overnight at 37°C. The turbidities of inhibitor-treated and untreated isolates were measured at 600 nm using spectrofluorometer.

Quantitative detection of biofilm formation using microtiter plate assay [11].
Microtiter plate assay has been used to determine slime production and biofilm formation. The optical densities of bacterial suspension were adjusted in LB broth medium to OD_{600} (0.2-0.25).Then, 100 µl aliquots of each suspension were

inoculated into the wells of 96-well sterile U-Shaped polystyrene microtiter plate and incubated at 37 °C for 24 hrs without shaking. The content of each well was aspirated using Pasteur pipette and wells were washed three times with 200 µl of physiological saline to remove free cells. The plate were vigorously shaken in order to remove all non- biofilm forming cells. The attached biofilm was fixed with 150µl absolute ethanol, kept for 15 mints, and then stained with 1% crystal violet (150 µl for each well) for 20 mints. Excess dye was rinsed off by placing the plate under running tap water. Dye taken up by the attached biofilm was solubilized with 150 µl of ethanol. Biofilm forming capacity was calculated at OD $_{540}$ nm using microtiter plate reader. Then taken value was the mean of three readings for each clinical tested isolates.

Polymerase Chain Reaction and qRT-PCR for detection of QS gene (*lasI* gene) in *P. aeruginosa* isolates:

RNA extraction kits:

RNA of treated and untreated isolates was extracted using GeneJET RNA Purification Kit, Thermoscientific, UK, which contained, proteinase K, lysis buffer, wash buffer 1, wash buffer 2, nuclease-free water and GeneJET RNA purification columns pre-assembled with collection tubes.

Real-Time PCR kits:

Amplification was performed using SensiFAST™ SYBR® Hi-ROX One-Step Kit with components illustrated in Table (1).

Table 1
SensiFAST SYBR® Hi-ROX One-Step Kit components

Reagent	Quantity
SensiFAST™ SYBR® Hi-ROX One-Step mix	5 x 1 ml
RiboSafe RNase inhibitor	1 x 200 µL
Reverse transcriptase	1 x 100 µL
Diethyl pyrocarbonate water	2 x 1.8 ml

DNA Molecular weight Marker (Ladder): One hundred basepair (100-1500bp) plus DNA ladder was used (Fermentas) in this study

Taq Master Mix: Dream Taq DNA polymerase, optimized Dream Taq Green buffer, magnesium chloride and deoxy Ribonucleotide Tri Phosphates (dNTPs)

Table 2
Primers used in PCR and qRT-PCR in the current study

Gene name	Primer	Primer sequence	Amplicon size
ropD	Fw	5'-CGAACTGCTTGCCGACTT-3'	131
	Rev	5'-GCGAGAGCCTCAAGGATAC-3'	
lasI	Fw	5'-CGCACATCTGGGAACTCA-3'	176
	Rev	5'-CGGCACGGATCATCATCT-3'	

Preparation of DNA:

Fresh colonies were suspended in 70 µl of sterile nuclease free water in 0.2ml sterile PCR tube. Heat block was performed in thermocycler at 95°C for 10 minutes. The obtained DNA was stored at -20°C [12].
Polymerase Chain Reaction protocol and cycling conditions [13]:

PCR analysis of tested isolates using primers of QS genes (*lasI*) using the following reaction mixture: 12.5 µl Dream Taq Green PCR Master Mix (2X), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 1 µl of bacterial DNA template and 9.5 µl of nuclease free water were added for a total of 25 µl per reaction. Negative control reaction (without bacterial DNA templates) was also performed. The cycling conditions incorporated; initial denaturing at 95°C for 5 minutes, then (denaturation at 95°C for 30 seconds, annealing for 30 seconds and extension at 72°C for 1 minute) for 40 cycles and final extension cycle at 72°C for 5 minutes.
Quantitative real-time polymerase chain reaction (qRT-PCR) assay:

The highest five Multi-Drug resistant *P. aeruginosa* isolates in addition to standard strain *P. aeruginosa* PAO-1 were chosen for further confirmation of the inhibitory effect of different tested chemical agents, nifuroxazide, thymol and acylase by using qRT-PCR. The effect of sub-inhibitory concentrations of different potential inhibitors (nifuroxazide, thymol and acylase) on the expression level of *lasI* genes which regulate virulence factors production such as biofilm production in *P. aeruginosa* was assessed.

Total RNA extraction for qRT-PCR:

Total RNA of inhibitors-treated and untreated *P. aeruginosa* isolates was extracted using GeneJET RNA Purification Kit (Thermoscientific, USA) according to the manufacturer procedures.

Quantitative RT-PCR:

The relative expression levels of QS genes such as *lasI* gene in both inhibitors-treated and untreated isolates were analyzed by qRT-PCR. The relative expression level of (*lasI*) gene was normalized to a housekeeping gene (*ropD*), a gene for which the expression levels remained unchanged in both treated and untreated isolates.

The analysis was conducted following the protocol described in SensiFAST™ SYBR® Hi-ROX One-Step Kit (Bioline, UK). The qRT-PCR was performed using StepOne Real-Time PCR system. Specific PCR amplification was confirmed by agarose gel electrophoresis of products according to the manufacturer recommendation. The comparative threshold cycle ($\Delta\Delta C_t$) method was used to calculate the relative gene expression for each tested gene [14].

Statistical Analysis: Data were analyzed using the GraphPad Prism 8 software package. The effect of different tested chemical substances on virulence factors production in *P. aeruginosa* was compared by One Way ANOVA according to Dunnet's or Tukey's Multiple Comparison Tests at $P < 0.05$ for significance. Results were expressed as the means ± standard errors of three biological experiments with three technical replicates.

Results

Clinical isolates were confirmed as *P. aeruginosa* as they were Gram-negative short rods, motile, able to grow at 42 °C with green pigmentation on cetrimide agar, king A agar and gave positive citrate utilization test.

Antimicrobial susceptibility of bacterial *P. aeruginosa* isolates:

P. aeruginosa isolates showed the highest resistance against Ceftriaxone (89%) followed by Cefotaxime (64.2%), Ofloxacin (62.7%), Ceftazidime (58.4%), Cefepime (54.3%) and Carbencillin (51.1%). Isolates of *P. aeruginosa* exhibited highest susceptibility to each of Meropenem (70%), Colistin sulphate (65.8%), Imipenem (63.8%) and levofloxacin (63.1%) as showed in Figure (1).

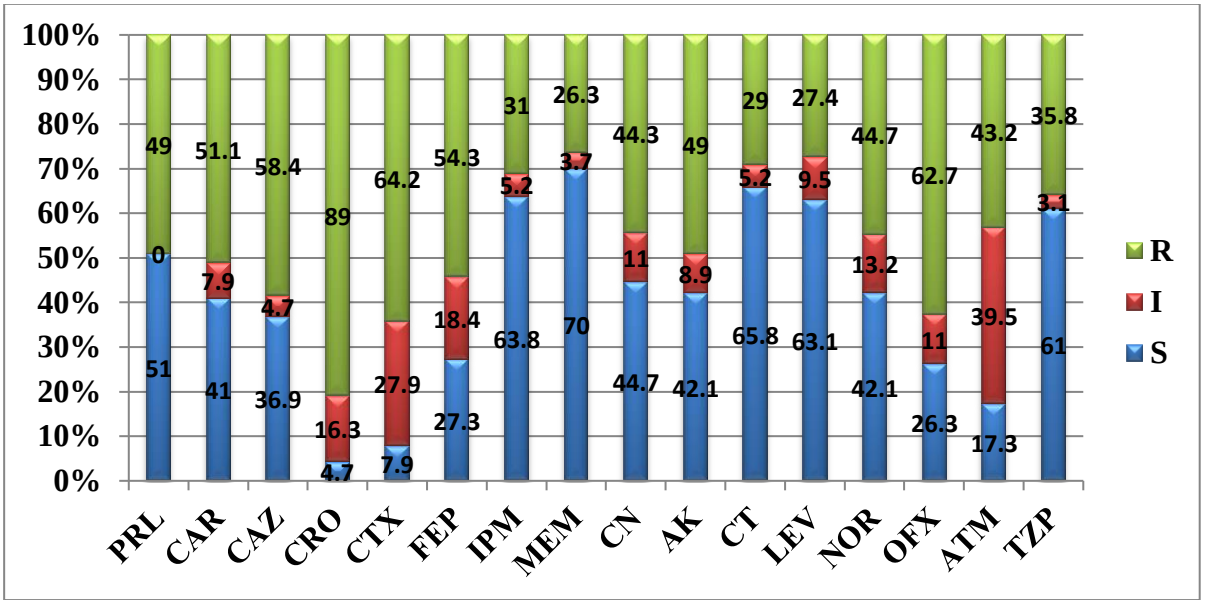

Figure (1): Frequency of antimicrobial susceptibility of *P. aeruginosa* isolates.

Isolates were collected from patients admitted in hospitals suffering from urinary tract infections, respiratory tract infections and wound infection, the prevalence of *P. aeruginosa* among different clinical samples were 66 urine isolates, 41 sputum isolates and 88 wound isolates as showed in Figure (2).

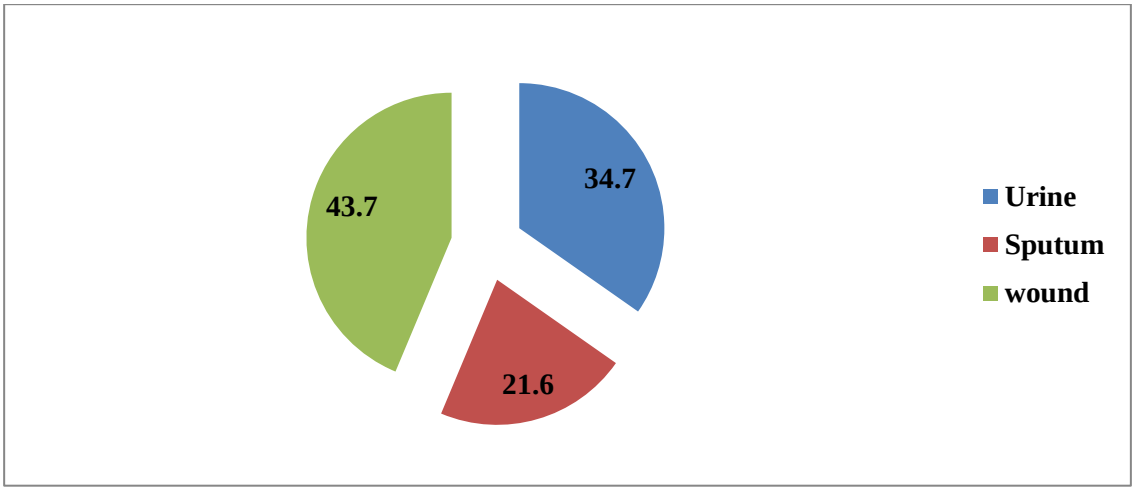

Figure (2): Frequency of *P. aeruginosa* among different bacterial specimens

Quantitative detection of biofilm formation using microtiter plate assay:

The modified microtiter plate test was used to determine the biofilm-forming capacity of tested isolates (20 MDR *P. aeruginosa* isolates) through measuring OD at 540 nm. All isolates produced biofilm compared with PAO-1 strain.

Determination of MIC and growth inhibition activity of tested virulence inhibitors:
First, the MIC of nifuroxazide, thymol and acylase against tested *Pseudomonas* isolates was determined. The QSI effect of nifuroxazide, coumarin and thymol and virulence factors inhibition in *P. aeruginosa* was estimated using a concentration of 1/4 MIC. The inhibitory effect on *Pseudomonas* isolates biofilm production by tested virulence inhibitors may be attributed to a bactericidal action of these chemicals on bacteria. To exclude this possibility, the effect of 1/4 MIC of tested chemicals on bacterial growth was determined. No significant change was observed in the growth of both treated and untreated isolates, proposing that a concentration of 1/4 MIC of tested chemicals has no bactericidal effect on bacterial viability.

Influence of tested virulence inhibitors on the production of biofilm among *P. aeruginosa* isolates:

The effect of tested chemicals on the production of QS-dependent biofilm by *P. aeruginosa* isolates was evaluated at 1/4 MIC of nifuroxazide, thymol and acylase. Biofilm production was significantly reduced in treated isolates compared to untreated isolates in presence of positive control PAO-1 strain. Nifuroxazide treated isolates significantly reduced biofilm production in *P. aeruginosa* isolates between 10% and 36% as showed in Figure (2). Thymol treated isolates showed

significant decrease of biofilm production in 19/20 tested *P. aeruginosa* isolates between 11% and 72%. Non-significant reduction caused by thymol was observed in P55 as showed in Figure (3). Acylase treated isolates showed significant decrease of biofilm production in all the tested *P. aeruginosa* isolates between 10% and 88% as showed in Figure (4).

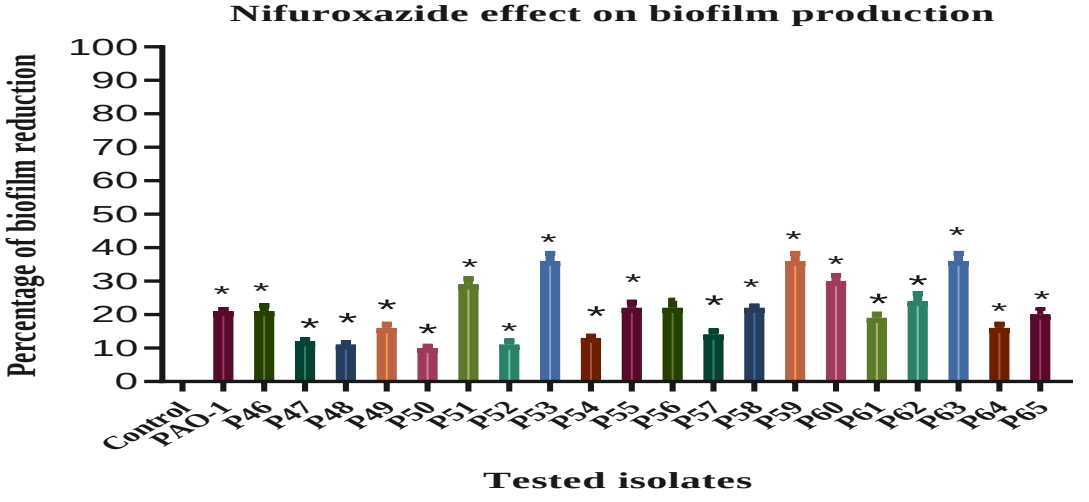

Figure (3): Effect of sub-MIC of nifuroxazide on the biofilm production of the isolates at OD_{540} nm in comparison with PAO-1 strain.

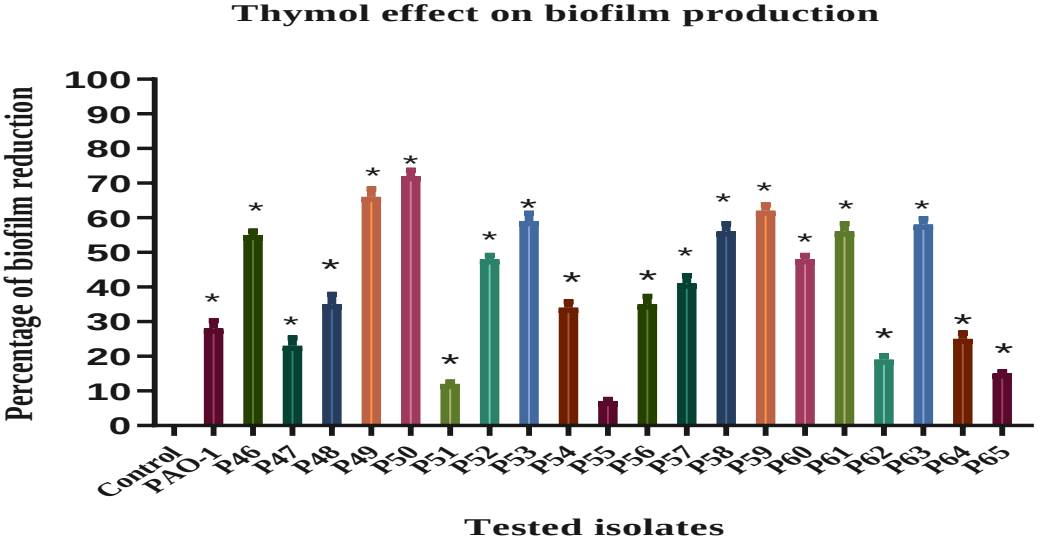

Figure (4): Effect of sub-MIC of thymol on the biofilm production of the isolates at OD_{540} nm in comparison with PAO-1 strain.

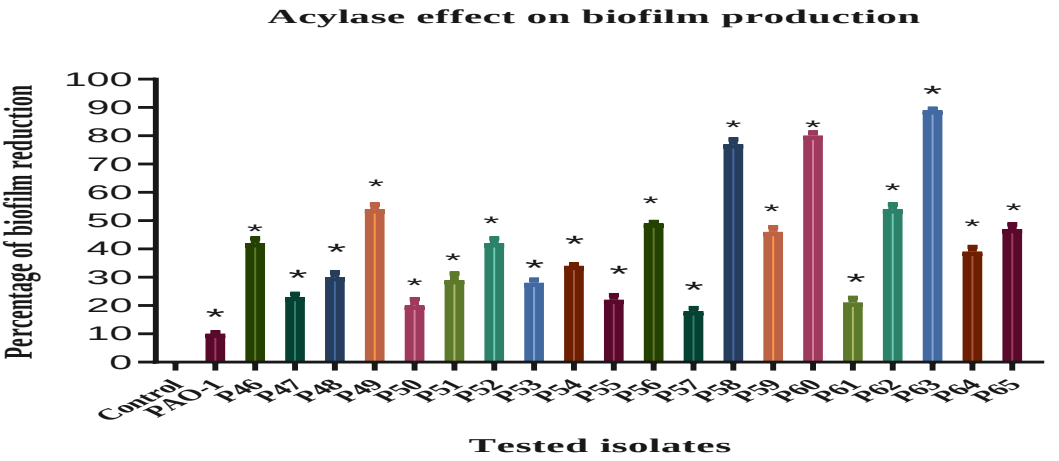

Figure (5): Effect of sub-MIC of acylase on the biofilm production of the isolates at OD_{540} nm in comparison with PAO-1 strain.

Polymerase Chain Reaction for detection of QS gene (*lasI* gene) in *P. aeruginosa* isolates:

The cycling conditions incorporated; initial denaturing at 95°C for 5 minutes, then (denaturation at 95°C for 30 seconds, annealing for 30 seconds and extension at 72°C for 1 minute) for 40 cycles and final extension cycle at 72°C for 5 minutes with amplicon size 176bp as showed in figure (5 & 6).

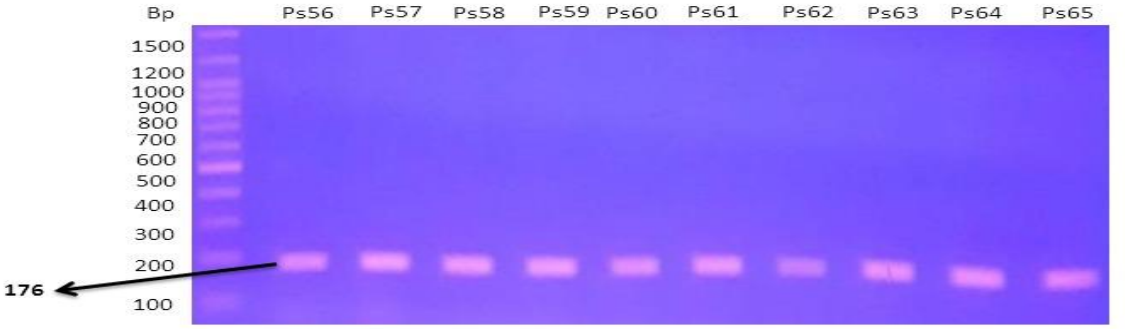

Figure (6): Polymerase Chain Reaction for detection of QS genes (*lasI* gene) in *P. aeruginosa* isolates.

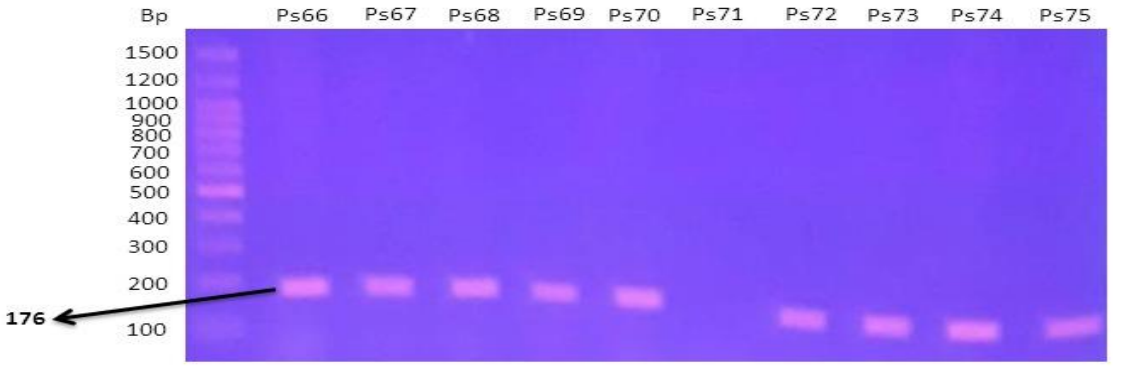

Figure (7): Polymerase Chain Reaction for detection of QS genes (*lasI* gene) in *P. aeruginosa* isolates**.**

The effect of the virulence factor inhibitors (nifuroxazide, thymol and acylase) on the expression of *lasI* gene using qRT-PCR:

Depending on the resistance profile, the highest 5 MDR *P. aeruginosa* isolates in addition to *P. aeruginosa* PAO-1 standard strain as positive control were further tested using qRT-PCR to determine the effect of different tested chemical agents (nifuroxazide, thymol and acylase) on biofilm formation. Nifuroxazide produced a significant reduction in RQ values of *lasI* gene from 100% in untreated (control) isolates to between 35% and 75% as showed in Figure (7). Thymol exhibited a significant decrease in RQ values of *lasI* gene from 100% in untreated (control) isolates to between 49% and 73% as showed in Figure (8). Acylase showed a significant reduction in RQ values of *lasI* gene from 100% in untreated (control) isolates to between 6% and 46% as showed in Figure (9).

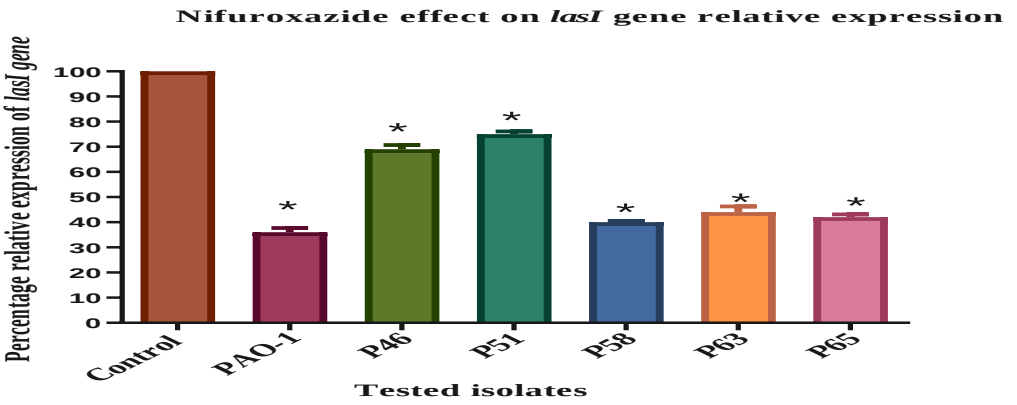

Figure (8): Relative expression of *lasI* gene determined by qRT-PCR under the effect of nifuroxazide

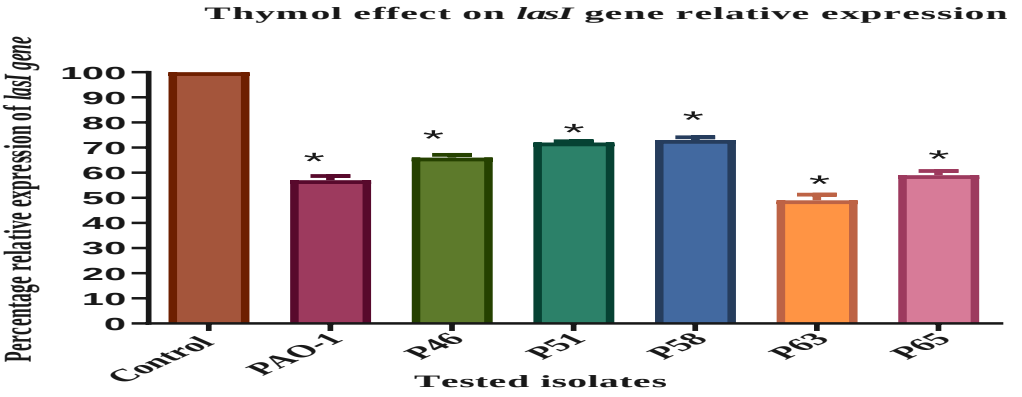

Figure (9): Relative expression of *lasI* gene determined by qRT-PCR under the effect of thymol.

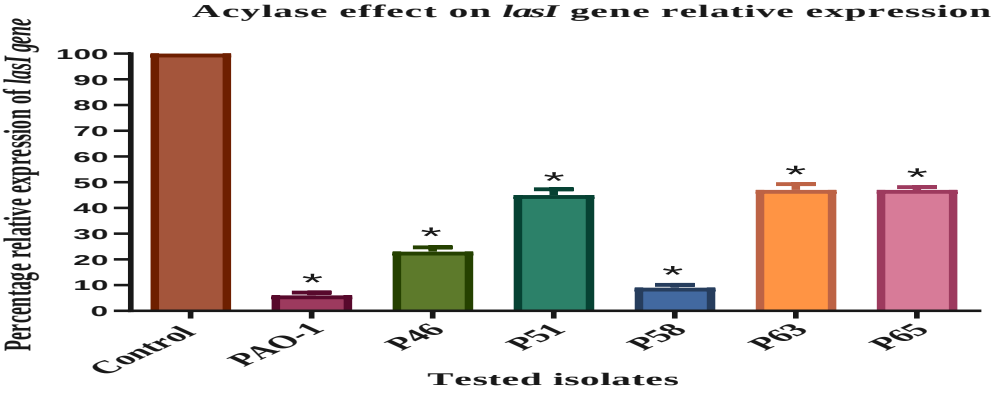

Figure (10): Relative expression of *lasI* gene determined by qRT-PCR under the effect of acylase.

Discussion

P. aeruginosa is a universal versatile Gram-negative pathogen that is normally present in aquatic and terrestrial environments. It is considered one of the most notorious and leading microorganisms causing diseases among immunocompromised patients with severe burns, wounds and CF, where it established chronic infections. *P. aeruginosa* infections are in an increase both in the community and in hospitals as its adaptability to survive in the environment and in substrates such as soaps and disinfectants make it a pathogen of interest in hospital-acquired infections [15]. In the current study, the resistance rate of the isolates observed against different antibiotics tested was variable. *P. aeruginosa* isolates in this study showed the highest resistance against Ceftriaxone (89%) followed by Cefotaxime (64.2%), Ofloxacin (62.7%), Ceftazidime (58.4%), Cefepime (54.3%) and Carbencillin (51.1%). In our study regarding susceptibility against different tested antibiotics, Isolates of *P. aeruginosa* exhibited highest susceptibility to each of Meropenem (70%), Colistin sulphate (65.8%), Imipenem

(63.8%) and levofloxacin (63.1%). The high susceptibility results of *P. aeruginosa* mainly against Colistin could be attributed to the restrictions and limited use of Colistin in the treatment of *P. aeruginosa* except in emergency conditions [16]. The widespread use of antimicrobials resulted in the emergence of MDR strains which are not easy to be treated. For this reason, studies should focus on the idea of finding new targets for treating diseases caused by *P. aeruginosa*. Recently, novel approaches focused on targeting *P. aeruginosa* virulence factors to reduce its pathogenicity without induction of microbial resistance have been investigated [17]. Multiple drug resistance by *P. aeruginosa* could be due to several mechanisms including synthesis of enzymes which degrade antibiotics, the presence of efflux pumps that deport the antimicrobials from the bacterial cells, so antimicrobial susceptibility testing is conclusive in clinical practice and remains the ideal approach to nominate the most suitable antibiotics for treating *P. aeruginosa* infections in order to control it and decrease the mortality rates and to determine the control policies should be followed to combat this pathogen[18]. Multiple resistance of *P. aeruginosa* to various antimicrobials is a major problem. Targeting virulence factors using FDA approved drugs is an innovative approach widely investigated to find a possible effective solution to combat the emergence of resistance to antibiotics without any effect on bacterial viability [19]. *P. aeruginosa* uses QS systems to regulate the expression and the production of a myriad of virulence factors. QS Systems are organized in a multi-layer hierarchy composed of three interconnected circuits *las, rhl*, and PQS under the control of *lasI/R* system [20]. The main QS-controlled virulence factors produced by *P. aeruginosa* are biofilm, pyoverdin, rhamnolipids, pyocyanin, elastase, proteases, and hemolysins. These virulence factors are responsible for *Pseudomonas* host-colonization, and the establishment of infections [21]. In the current study, the antibacterial activity of different potential virulence factors inhibitors (nifuroxazide, thymol and acylase) was determined by evaluation of MIC. Furthermore, the inhibitory effect of the tested potential inhibitors on the production of virulence factors (biofilm) was investigated by using sub-MIC of different potential inhibitors. To compare the inhibition activity of different tested potential inhibitors on the production of biofilm in tested isolates, the number of isolates showing significant biofilm reduction was calculated and compared with POA-1 strain. In the current study, acylase and nifuroxazide exhibited the highest inhibition activity on biofilm production in the tested isolates (all the tested isolates (100%) showed a reduction) followed by thymol. In summary, from the phenotypic results of virulence factors inhibition, we can conclude that thymol was the most powerful inhibitor of biofilm production followed by coumarin and nifuroxazide, respectively. Based on the current results and as previously mentioned in this study that no significant changes in cell growth and viability was observed from any of the potential inhibitors corresponding to virulence factors production, the anti-QS effect might be the most plausible hypothesis of virulence factors inhibition in *P. aeruginosa*, and so further investigations including determination of QS-regulatory genes inhibition by different potential inhibitors was carried out. In the present study, the relative expression of QS-regulatory genes (*lasI* gene) that control virulence factors production such as biofilm in *P. aeruginosa* was examined using qRT-PCR. Nifuroxazide, thymol and acylase showed lower down-regulation effect on *lasI* gene by ≥ 50%, coumarin was the most powerful inhibitor of *lasI* relative expression followed by nifuroxazide then thymol.

Conclusion

The spread of MDR strains of *P. aeruginosa* is of a particular concern causing healthcare-associated infections and increasing the challenge to hospitals, both in the clinical treatment of patients and in the prevention of the cross-transmission of this problematic pathogen. QS Controls the production of many of virulence factors in *P. aeruginosa* and plays an essential role in antimicrobial resistance. The uncontrolled overuse of antibiotics increased the problem of MDR *P. aeruginosa* to many of the antibiotics available leading to limitation of the options of treatment in many infectious diseases. This study highlighted the positive significant effect of anti-virulence agents (nifuroxazide, thymol and acylase) to combat multi-drug resistance to antimicrobial therapy. Treatment of *P. aeruginosa* with coumarin, nifuroxazide and thymol, resulted in a significant down-regulation in the relative expression of all QS-regulatory genes such as (*lasI* gene) with subsequent reduction in production of biofilm and also resulted in decreasing the pathogenesis of *P. aeruginosa*.

Declarations:

Consent for Publication: I confirm that all authors accept the manuscript for submission

Availability of data and material: Available

Competing interests: None

Funding: No fund

Conflicts of Interest: The authors declare no conflicts of interest regarding the publication of this paper.

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