ORIGINAL ARTICLE

Possible antivirulent activity of some agents against clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background*: Pseudomonas aeruginosa has developed different mechanisms of resistance against antibiotics and became one of the most life-threatening pathogens. Fighting against its virulence Factors are an alternative therapeutic target.* ***Objective:*** *This study was directed towards the investigation of anti-quorum sensing activity and inhibitory action on virulence factors of different agents including antibacterial agents to which Pseudomonas aeruginosa isolates are resistant and non-antibacterial agents.* ***Methodology:*** *Anti-quorum sensing activity of ceftriaxone, ceftazidime (CAZ), cefepime (FEP), vancomycin (VA), paracetamol (PA), and pheniramine maleate (PHE) investigated as well as their ability to reduce other virulence factors including protease, hemolysin, and pyocyanin production.* ***Results****: This study showed that 3rd and 4thgenerations cephalosporins could be used as anti-quorum sensing agents effectively in the treatment of Pseudomonas aeruginosa infections, however, vancomycin, paracetamol, and pheniramine maleate had no effect on inhibiting the studied virulence factors.* ***Conclusion****: From our study we conclude that although cephalosporins at the used concentrations did not show anti-pseudomonal activity they were effective as anti-virulent agents that could be utilized in therapeutically in controlling Pseudomonas aeruginosa infections.*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and is considered one of the most life threatening microbes, especially in patients with chronic diseases like cystic fibrosis or for immune-compromised patients like cancers patients[1]. The resistance of *Pseudomonas aeruginosa* against antimicrobial agents has been significantly escalating even against "last resort" drugs like colistin [2].*P.aeruginosa* pathogenicity is attributed to an arsenal of virulence factors including proteolytic enzymes like protease, elastase and hemolysin[3] and other virulence factors such as pyocyanin which reduce host immunity response[4]. However, the most powerful mechanism of resistance of *P.aeruginosa* is forming a polysaccharide biofilm that protects bacteria from the antimicrobial agents[5]. A regulatory system called quorum sensing, which is consisting of diffusible molecules called autoinducers have been showed to be the controller of these virulence factors[6]. Today, pharmaceutical companies are no longer directing their investments towards manufacturing new antibiotics, as a result of the accelerated bacterial resistance[7] , therefore this study aims at redeploying of different therapeutic agents such as cephalosporin antibiotics ,vancomycin, and non-antibacterial drugs paracetamol and pheniramine maleate as anti-virulence factors against *P.aeruginosa*.

METHODOLOGY

Ethical statement:

This work was approved by the Ethical Committee of Faculty of Pharmacy, Mansoura University, Egypt.

Bacterial isolates:

Six *P.aeruginosa* clinical isolates were collected from burn center of Mansoura University Hospital. The isolates were identified by using MacConkey's agar (Oxoid, UK) as a Gram negative pathogen differentiating media and then one colony of each isolate was streaked on cetrimide agar as a selective media for *P.aeruginosa*. Microscopic examination of isolates was done in addition to several biochemical tests including catalase and oxidase tests [8].

Antibiotic susceptibility testing of the clinical isolate:

To determine the susceptibility pattern of the clinical isolates, antibiotic sensitivity test was carried out using disc diffusion technique on Muller Hinton agar (Oxoid, UK)[9], where antibiotic disks (Oxoid,UK) were selected to be representative to different antibiotics classes.

Minimal inhibitory concentration determination:

Minimum inhibitory concentration (MIC) of tested antibiotics used in the study was determined by using broth micro-dilution method[9] for all of the clinical isolates. MIC is identified as the concentration that prevents the visible growth of pathogen under the specified conditions, where cultures were overnight incubated in nutrient broth at 37 C°.

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***In vitro* bacterial growth conditions for assessing anti-virulent activity:**

For assessment of antivirulent activity, all clinical isolates were cultured overnight with shaking at150 rpm at 37 C° in tryptic soy broth (TSB; Oxoid).[10] Agents with anti-virulent activity were added to the corresponding media at sub-inhibitory concentrations, where were chosen to be equal to their breakpoints[9], in case of tested antibiotics, ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP) and vancomycin (VA) were used at concentrations of 64 ug/ml, 32 ug/ml, 32 ug/ml, and 6 ug/ml, respectively, the tested non-antibacterial agents including paracetamol (PA) and pheniramine maleate (PHE)were tested at concentration of 4mg/ml within their therapeutic doses.

The effect of used concentrations on bacterial growth:

Optical densities at 600nm (OD_{600nm}) of the treated and untreated cultures were determined following overnight incubation to evaluate the effect of tested agents on bacterial viability[11].

Protease production assessment:

Using 10% skimed milk agar plates, the proteolytic activity of the tested isolates was assessed[12]. Quantitative evaluation of anti proteolytic activity of the tested agents was carried out by incubation 0.5ml of cell free culture supernatant (following overnight incubation of treated and untreated isolates) with 1ml of 1.25% skimed milk solution for 20 mins at 37 C°[13]. OD_{600nm} was measured for each tube using spectrophotometer.

Evaluation of pyocyanin production

Anti Pyocyanin activity of the tested agents against *P.aeruginosa* isolates was evaluated as following; isolates were incubated in King A media (peptone 20gm/L, K_2SO_4 10gm/L and MgC_{l2}14gm/L) for 48 hours with shaking at 200 rpm at 37C°.Total pyocyanin was extracted to the acidic phase using chloroform and 0.2N HCL, respectively. Optical density of treated and untreated isolates was measured at 520nm, then the obtained values were multiplied times 17.072 to obtain pyocyanin concentrations[14].

Hemolysin assay:

To determine the influence of tested agents on hemolysin enzyme inhibition, which is produced by *P.aeruginosa*, 100ul of treated and untreated bacterial supernatant were mixed to 100ul solution of2%v/v red blood cells (RBCs) in tris-buffered saline. Following incubation at 37°C for one hour, released hemoglobin was detected spectrophotometrically at 540 nm, after centrifugation. For calculation the percent lysed cells, the following equation was used[15]

% = [(X-B)/ (T-B)] ×100.

B is a negative control, T is positive control refers to total cells lysed by 0.1% sodium dodecyl sulfate solution (SDS), X is the OD value of tested sample.

Anti-Quorum sensing activity detection:

To investigate the possibility of anti-quorum sensing properties of tested agents, the pigmentation of *Chromobacterium violaceum* was used as indicator. Luria-Bertani agar plates, (LB; Tryptone10 g /1, Yeast extract 5 g /1 and NaCl 10 g /1) were seeded with overnight culture of *Chromobacterium violaceum.* Cups were made in agar plates filled with tested agents and then incubated at 30C° for 24h. Appearance of colorless growth zone around cups indicates anti-quorum sensing activity[16].

Statistical analysis:

The Student's (t-unpaired test) was performed for statistical analysis using GraphPad Prism software (version 5.01) where $P<0.05$ was considered statistically significant. Each experiment was repeated three times and the mean was used to represent the results.

RESULTS

Antibiotic resistance pattern:

Using disc diffusion antimicrobial susceptibility testing, we found that 66.6% of *P.aeruginosa* isolates were multi drug resistant (MDR) according to CDC definitions of different type of resistant pattern[17]. MDR isolates (P1, P2, P3, and P6) showed different antimicrobial susceptibility patterns (table, 1). P1 isolate was resistant to all tested antibiotics, except amikacin and pipracillin, while P2 was resistant to all tested antibiotics, except amikacin. Furthermore, P6 exhibited resistance to all antimicrobials with the exception of gentamicin. However, two isolates (P4 and P5) were susceptible toward more than two antimicrobial classes. All tested clinical isolates were colistin sensitive.

Table 1: Antibiotic resistant pattern of clinical isolates of *P. aeruginosa*

	Antibiotic										
Clinical isolates	**AK**	**CN**	**LVX**	**CIP**	**IPM**	**MEM**	**ATM**	**CRO**	**CAZ**	**FEP**	**CAR**
P1	I	R	R	R	R	R	R	R	R	R	R
P2	S	R	R	R	R	R	R	R	R	R	R
P3	I	R	R	R	S	R	R	R	R	R	R
P4	S	R	R	R	S	S	R	R	R	R	R
P5	S	S	S	S	S	S	S	R	R	R	S
P6	I	S	R	R	R	R	R	R	R	R	R
Clinical isolates	**PRL**	**AMC**	**CLR**	**CEC**	**VA**	**OX**	**TE**	**SXT**	**LZD**	**CFR**	**CT**
P1	I	R	R	R	R	R	R	R	R	R	S
P2	R	R	R	R	R	R	R	R	R	R	S
P3	R	R	R	R	R	R	R	R	R	R	S
P4	R	R	R	R	R	R	R	R	R	R	S
P5	S	R	R	R	R	R	R	R	R	R	S
P6	R	R	R	R	R	R	R	R	R	R	S

- amikacin (AK), ceftriaxon (CRO), sulfamethaxzol(SXT), ciprofloxacin, cefadroxil (CFR), colistin (CT), meropenem (MEM), cefepime (FEP), gentamicin (CN), carpenicillin (CAR), aztreonam[18], amoxacillin and clavulanic acid (AMC),clarithromycin (CLR), cefaclor (CEC), vancomycin (VA), oxacillin (OX), pipracillin (PRL), ceftazidime (CAZ), imipenime (IPM), tetracycline (TE), levofloxacin (LVX), linezolide (LZD).

Minimum inhibitory concentrations for the isolates

The MICs of cefepime, ceftazidime, ceftriaxone and vancomycin for *P.aeruginosa* clinical isolates were determined and mentioned in (table 2). All isolates were resistant to tested antibiotics based on the MIC level. MIC values ranged from 0.5-127 fold increase of cephalosporins breakpoint concentrations and 31-680 fold increase of vancomycin breakpoint based upon CLSI interpretive breakpoints against *Staphylococcus aureus*.

Table 2: Minimum inhibitory concentration (ug/ml) of tested antibiotics against *P.aeruginosa* clinical isolates

Clinical isolates	MIC (µg/ml)			
	FEP	**CAZ**	**CRO**	**VA**
P1	96	48	384	192
P2	>3072	>4096	>4096	>4096
P3	96	1536	1536	192
P4	>4096	>4096	>4096	3072
P5	48	48	384	3072
P6	>3072	768	>4096	1536

Effect of breakpoint concentration on the bacterial growth

The possible anti-virulent activity of the tested agents on *P.aeruginosa* clinical isolates could be due to their effect on bacterial growth and to preclude this possibility, the effect of breakpoint concentration of each of the tested agents on bacterial viability was investigated. No statistically significant difference in bacterial growth was found in the presence or absence of each tested agent (figure 1).

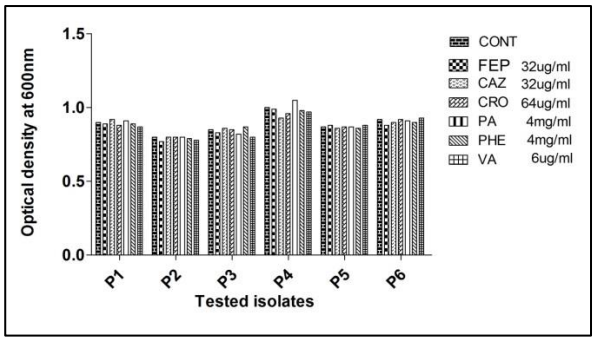

Fig. 1: Growth of *P.aeruginosa* clinical isolates treated with sub-inhibitory concentrations of cefepime (FEP), ceftazidime (CAZ), ceftriaxone (CRO), paracetamol (PA), pheniramine maleate (PHE), and vancomycin (VA) compared to control untreated cultures (CONT), where no statistically significant difference in bacterial growth was found in the presence or absence of each tested agent.

Effect on protease production

Protease production by *P. aeruginosa* clinical isolates was qualitatively determined on skimmed milk agar plates; all isolates were protease positive causing protein hydrolysis as shown in (figure 2).

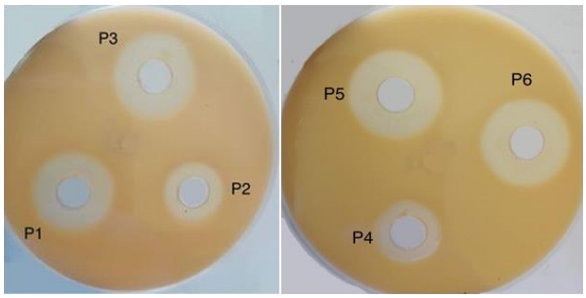

Fig. 2: Proteins hydrolysis by *P. aeruginosa* clinical isolates. P1, P2, P3, P4, P5, and P6, where the hydrolyzed zones are representative for the corresponding proteolytic activity of each isolate.

Our results showed that the tested 3rd and 4th generation cephalosporins (CRO, CAZ and FEP) had a significant inhibitory effect on protease production by all isolates (P<0.05). However, vancomycin, paracetamol and pheniramine maleate did not possess any anti-protease activity compared with control untreated isolates (figure 3).

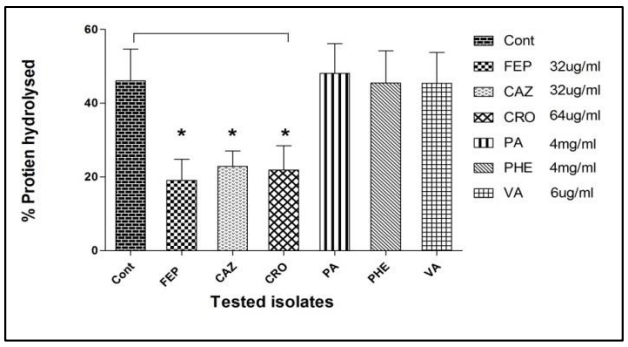

Fig. 3: Anti- protease activity of the six tested agents. Cephalosporins significantly reduced protease production. Control untreated isolates (Cont), cefepime treated isolates (FEP), ceftazidime treated isolates (CAZ), ceftriaxone treated isolates (CRO), paracetamol treated isolates (PA), pheniramine maleate treated isolates (PHE) and vancomycin treated isolates (VA), (*) P<0.05. Data shown represents means of results from 3 independent experiments ± SEM.

The effect on pyocyanin and hemolysin production

Sub-inhibitory concentrations of ceftriaxone, ceftazidime and cefepime decreased pyocyanin production significantly (P<0.05). However, paracetamol, pheniramine maleate and vancomycin were inefficient in reducing pyocyanin production (figure 4). On the other side, hemolytic activity was observed in three of six *P.aeruginosa* clinical isolates, which was further assessed in the presence of sub-inhibitory concentrations of the tested agents. Ceftazidime and cefepime decreased hemolysin production significantly (P<0.05), while ceftriaxone reduced hemolysin activity, but the reduction was insignificant (P= 0.08). However, vancomycin, paracetamol and pheniramine maleate did not show any anti-virulent effect (figure 5).

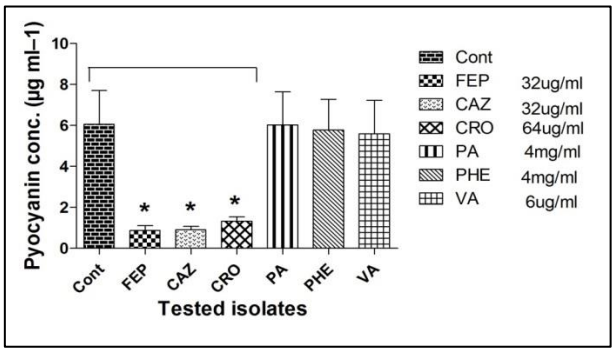

Fig. 4: The effect of tested agents against pyocyanin production. Cephalosporins significantly reduced pyocyanin production when compared to control untreated isolates (Cont). Cefepime treated isolates (FEP), ceftazidime treated isolates (CAZ), ceftriaxone treated isolates (CRO), paracetamol treated isolates (PA), pheniramine maleate treated isolates (PHE), and vancomycin treated isolates (VA), (*) P<0.05. Data shown represents means of results from 3 independent experiments ± SEM.

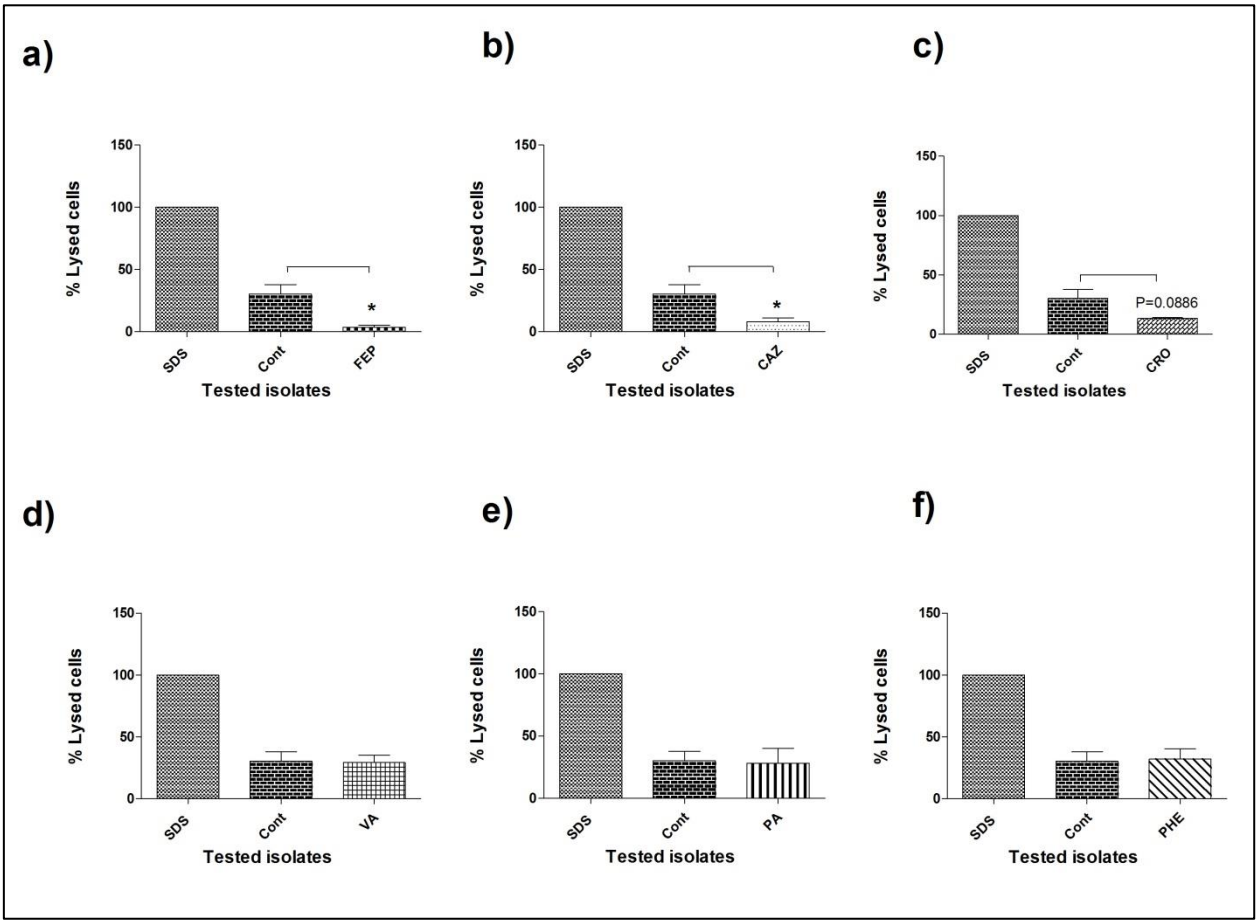

Fig. 5: Effect of tested agents on haemolytic activity of *P.aeruginosa* clinical isolates. Sodium dodecyl sulfate (SDS) solution (0.1%) used as positive control. Cefepime treated isolates (FEP), and ceftazidime treated isolates (CAZ) showed a significant reduction in hemolysin production compared to control untreated isolates (Cont). Ceftriaxone treated isolates (CRO), paracetamol treated isolates (PA), pheniramine maleate treated isolates (PHE), and vancomycin treated isolates (VA). (*) $P<0.05$. Data shown represents means of results from 3 independent experiments ± SEM.

The effect on quorum sensing system:

To identify the anti-quorum sensing activity of tested agents using *Chromobacterium violaceum*, violacein pigmentation inhibition zone around each tested agents was measured. Violacein production in *Chromobacterium violaceum* was greatly inhibited by cefepime (inhibition zone of 21-23mm) (figure 6a) followed by ceftazidime (16-18mm inhibition zone) (figure 6b) and ceftriaxone (12-14mm inhibition zone) (figure 6c). On the other side, VA, PA, PHE did not show anti-quorum sensing activity (figure 6d, 6e and 6f).

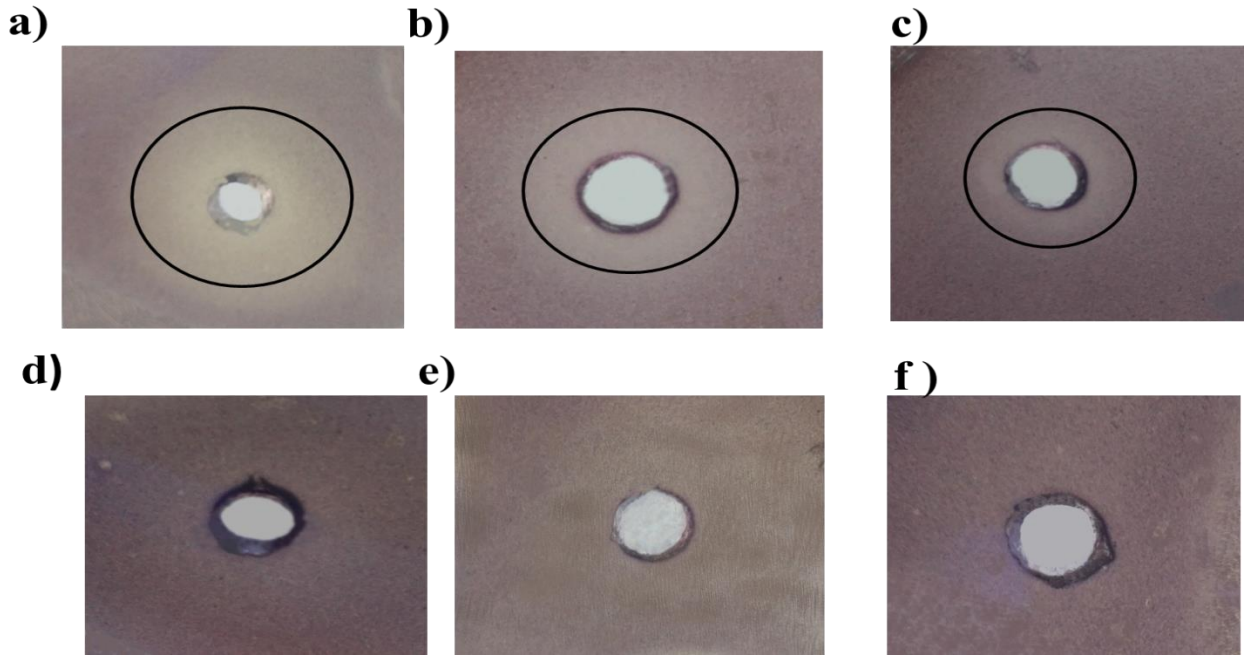

Fig. 6: Anti quorum sensing activity of some tested agents. *Chromobacterium violaceum* violacein was inhibited by **a)** cefepime, **b)** ceftazidime, **c)** ceftriaxone, while **d)** vancomycin**, e)** paracetamol, and **f)** pheniramine maleate did not show inhibitory action, where (circle) represents pigmentation inhibition zone.

DISCUSSION

Infectious disease management is becoming more and more difficult with each passing year. This is especially true for infections caused by *P.aeruginosa*, with its ability to rapidly develop resistance to different classes of antibiotics[19]. Resistant to multiple antimicrobial agents was triggered by *P.aeruginosa* either through genetic mutation or acquisition of resistance genes on mobile genetic elements [20]. Biofilm formation, exotoxin secretion, hydrolytic enzyme production like (protease, elastase and hemolysin) and pigment release as pyocyanin, all of these are representing *P.aeruginosa* weapons against antibacterial agent and host immunity[21]. Furthermore, quorum sensing system helps *P.aeruginosa* in controlling its virulence phenotypes through small autoinducers molecules including 4-quinolones and N-acylhomoserine lactones (AHLs)[22]. The prevalence of antibiotic resistance in the Middle East is increasing significantly compared to other regions, for example the resistant of *P.aeruginosa* against ciprofloxacin have been raised to 97.4% in the United Arab Emirates[23], furthermore, in Tanta University Hospital 19.6% of *P. aeruginosa* clinical strains isolated from catheter associated urinary tract infection were found to be meropenem resistance [24]. However, there is a serious therapeutic challenge, which is the prevalence of MDR *Pseudomonas aeruginosa* strains that exhibit resistance to three or more classes of antibiotics. Therefore, it is essential to search for new strategies to face this phenomenon. One of the novel methods is targeting bacterial virulence factors for decreasing its pathogenicity and facilitating its killing [25]. Development of QS inhibition therapies are extensively being investigated to improve our ability in controlling infectious diseases [26]. Thus, we studied the influence of different compounds on some virulence factors of *P.aeruginosa*, as well as its quorum signaling system.

Ceftazidime at sub-inhibitory concentration significantly reduced the production of protease, pyocyanin and hemolysin by *P.aeruginosa* clinical isolates. This was in accordance with another study, where ceftazidime at sub-inhibitory concentration (0.25 ug/ml) reduced the production of protease, elastase and hemolysin[27] which also suggested that CAZ has the ability for interfering with the QS system and hindering its signals.

Furthermore, a recent study confirmed that cefepime at sub-inhibitory concentrations MIC_{90} = 8 mg/L, and $MIC_{12.5}$ = 1 mg/L acts as potent anti-virulent agent reducing the production of pyocyanin, protease and biofilm formation [28]. Taking into consideration our results, it is highly confirmed that cefepime is an anti-virulent agent with the ability to reduce protease, hemolysin, and pyocyanin production.

In a similar study[29], cephalosporin antibiotics (CRO, CAZ and FEP) were considered as effective quorum sensing quenching agents in *Chromobacterium violaceum* and all of them proved their ability to reduce pyocyanin production and biofilm formation in *P.aeruginosa* in addition to the synergistic effect of FEP with aminoglycosides in reducing MICs remarkably. In agreement with the previous mentioned study, our data gives additional evidence of the success of cephalosporins as anti-virulent agents, especially 3rd and 4th generations, where all of these agents caused significant decrease in *P. aeruginosa* virulence factors.

Furthermore, the antimicrobial activity of several approved drugs for different noninfectious diseases were investigated and summarized previously[30]. In this study, the effects of paracetamol and pheniramine maleate were assessed in addition to vancomycin, which was assayed as a representative of Gram-positive antibacterial class. A new research found that H1-antihistamines could decrease the MICs of different antibacterial agents in the treatment of *Escherichia coli*[31]. Another study suggested that there is an insignificant effect of paracetamol on the activity of ciprofloxacin against different Gram-positive and Gram-negative bacteria[32]. Likewise, our study investigated the effect of paracetamol, pheniramine maleate, and vancomycin on protease, elastase and hemolysin activity of *P.aeruginosa* and anti-quorum sensing property against *Chromobacterium violaceum*. None of these tested drugs had anti-virulent activity on tested clinical isolates of *P.aeruginosa*.

CONCLUSION

In this study, we report that 3rd and 4thgenerations of cephalosporins have proved their ability to fight against multidrug-resistant *P.aeruginosa* isolates, even if the isolates were resistant against them through disrupting QS signals and reducing other virulence factors. However, molecular and *in-vivo* studies are still needed to improve their utilization as anti-virulent agents. On the other side, investigating the activity of PA, PHE, and VA against *Pseudomonas aeruginosa* isolates virulence factors was not promising, therefore more drugs are in need to be investigated in the future for their possible anti-virulent activities.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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