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Impact of Chalcone in Biofilm Formation of Clinical and Environmental Antibiotic Resistant *Pseudomonas aeruginosa* Isolated in Kut City

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Abstract: Objectives: *Pseudomonas aeruginosa* continues to be the most common pathogen in the nosocomial settings. This organism shows the high level of resistance against various groups of antibiotics. Therefore, this study was conducted to find out the antimicrobial resistance effects of *Pseudomonas aeruginosa* and the role chalcone in the biofilm formation. Materials and Methods: A total isolates of *P. aeruginosa* were collected from burn, wound, sputum and Patient room VITEK-2 System, a standard technique, was used to perform antibiotic susceptibility testing. The broth microdilution method was used to determine the MIC of the chalcone using the 96-well microtiter plate. Results: Cetrimide agar used to isolate and diagnose of *P. aeruginosa*, among 111 (61.78%) clinical isolation and 7 (35%) environmental samples, then full identification of *P. aeruginosa* using the conventional biochemical tests. The antibiotic susceptibility test for thirteen antibiotics was performed by the standard disk diffusion method, and the results showed that 48 isolate of *p. aeruginosa* was multi-drug resistance with (70-100) %, 53 isolates (50-70) %, and 17 isolates with (30-50) % resistance. Ten multi-drug resistant isolates of *P. aeruginosa* were used to examine their ability in biofilm formation Microtiter plate methods. The results showed that all the isolates had strong biofilm production except isolates No. 4, 7, and 9 which was moderate. The broth microdilution method was used to determine the MIC of the chalcone using the 96-well microtiter plate. The result showed that the MIC values of the chalcone compound on all *P. aeruginosa* isolates were 1000 mg\ml except for isolates No.4, 7, and 9 which were 500 mg\ml. Furthermore, chalcone inhibited 100% of the biofilm formation of *P. aeruginosa* isolates in 500 µg/ml, and reduction the biofilm formation in concentration 32.25 µg/ml.

Keywords: *Pseudomonas aeruginosa*, Antibiotics resistant, Chalcone.

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Introduction

The diverse opportunistic pathogen Pseudomonas aeruginosa (*P. aeruginosa*) is thought to be a major factor in an increasing number of different life-threatening infections (1). Multidrug-resistant *P. aeruginosa* strains, in particular, have caused major issues in a number of nations, such as Iraq. Antimicrobial therapy is a difficult challenge due to the rising incidence of nosocomial infections caused by strains of *P. aeruginosa* that are multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) (2). Because of both acquired and intrinsic resistance to various effective groups of antibiotics, *P. aeruginosa* infections are problematic. Limited permeability regarding the outer membrane, the generation of inducible β-lactamase, and the

Multidrug Efflux System are the causes of intrinsic Mara (3). *P. aeruginosa* was the 3rd most frequent cause of nosocomial urinary tract infections (UTIs), the 2nd most frequent cause of nosocomial pneumonia, and the seventh most frequent cause of nosocomial bacteremia (4).

P. aeruginosa is one of the virulent agents with a propensity to the diverse opportunistic pathogen *Pseudomonas aeruginosa* (*P. aeruginosa*) is thought to be a major factor in an increasing number of different life-threatening infections (1). Multidrug-resistant *P. aeruginosa* strains, in particular, have caused major issues in a number of nations, such as Iraq. Antimicrobial therapy is a difficult challenge due to the rising incidence of nosocomial infections caused by strains of *P. aeruginosa* that are multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) (2). Because of both acquired and intrinsic resistance to various effective groups of antibiotics, *P. aeruginosa* infections are problematic. Limited permeability regarding the outer membrane, the generation of inducible β-lactamase, and the Multidrug Efflux System are the causes of intrinsic (3). They could create pigments like pyocyanine (green blue) and pyoverdin (yellow green) fluorescence and are nonspore-forming (5). Since P. aeruginosa is a very common cause of wound and burn infections, it is considered to be one of the curses of burn units (6). *P. aeruginosa* frequently thrives in clinical settings, and there are numerous reports regarding multiple resistant strains of P. aeruginosa being discovered from hospital bed rails, basins, floors, and the hands of medical personnel. This poses one of the biggest challenges in treating infections caused by this bacterium. Because of patient-to-patient transfers, multidrug-resistant clones could persist in hospitals for several years (7). This is one of the primary etiologies behind the high patient morbidity and mortality rates (8). P. aeruginosa represents one of the Gram- negative opportunistic pathogens that had demonstrated multi-resistant infections in both the hospital and community environment (9). Nosocomial UTIs, wounds, burns, endocarditis, and otitis externa are all frequently caused by P. aeruginosa. These sites are also where P. aeruginosa could invade the bloodstream, causing bacteremia, a systemic infection (10, 11).

Materials and methods

This prospective study was conducted between 17/10/2021 to 5/2/2022.

VITEK-2 System

The bacteria were put into Mannitol salt agar plates, and they were after that incubated at a temperature of 37 Celsius overnight. Then, a single colony was removed and placed in solution. By using VITEK (Densichek/BioMerieux), the bacterial suspension's turbidity was brought into compliance with the McFarland 0.50 standard in 0.45 % sodium chloride. Bacterial suspension tubes as well as the VITEK 2 ID-Gp (Gram Positive) card were after that manually put into VITEK-2 system. The next software procedures were carried out in accordance with the directions provided by the manufacturer (BioMerieux, France) (12).

Isolation of bacteria

All the clinical samples were inoculated on MacConkey, cetrimide and blood agar. The isolates were recognized using the standard

laboratory protocol for the identification of *P. aeruginosa*).

Determination of minimum inhibitory concentration (MIC) of chalcone

The broth microdilution method was used to determine the (MIC) of the Chalcone using the 96-well microtiter plate. The working solution of the Chalcone was prepared at 2000 µg/ml in broth and serial two-fold dilutions of Chalcone were prepared directly on the plate to make the concentrations 2000-15.62 µg/ml. 100 µl of the prepared chalcone was introduced into the first wells in row A. Rows B-H in columns had 100 µl of the broth alone. Twofold serial dilutions using micropipette were done systematically down the columns (from rows A-H). 100 µl was removed from the starting concentrations in row A and transferred to the next row with the 100µl broth, properly mixed, and the procedure was repeated up to the last row (H) where the last 100µl was discarded. This brings the final volume in all the test wells with the Chalcone to 100 µl except the column which had 200 µl of the broth that served as sterility control. 100µl of the 1×10^6 CFU/ ml bacterial inoculum was transferred into all the wells except the negative control.

Microtiter plates were incubated at 37oC for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for 30 minutes to observe any color changes. The Minimum Inhibitory Concentrations were determined visually in broth micro dilutions as the lowest concentrations of the extracts at which no color changed from blue to pink in the resazurin broth assay (13).

Assessment of biofilm formation

Quantification of biofilm formation by *P. aeruginosa* was assessed as described by Patel *et al*. (14); all isolates were grown over night in Brain Heart Infusion Broth at 37°C. Each isolate was transferred to tryptic soy broth (TSB) containing 1% glucose and mixed well by pipetting. A suspension of the bacterial isolate was adjusted to McFarland No. 0.5 turbidity standard.

A volume (200 µl) of each isolates culture was added, in triplicate, to a sterile 96 wells microtiter plate with a flat bottom. The plate was covered with their lids and incubated under aerobic conditions at 37°C for 24h. After the incubation period, the planktonic cells were rinsed twice with distilled water to remove the unattached bacteria. The adhering bacterial cells in each well were fixed with 200 µl of absolute methanol for 20 min at room temperature. The adhering cells were stained by adding 200 µl of 0.1% crystal violet to each well for 15 min. Once the staining reaction has completed, the excess stain was removed by repeated washing (2-3 washes) with distilled water. The plate was dried by leaving them at room temperature for approximately 30 min to ensure they were completely dry, finally, 33% acetic acid was added to fix the stain.

Optical density (OD) readings were determined using an ELISA auto reader at a wavelength of 630 nm. Average of OD values of sterile medium were calculated and subtracted from all test values. Cut off value (ODc) was calculated, which can provide categorization of isolates as biofilm producer or not (15).

ODc: Average OD of negative control + (3 × standard deviation (SD) of Negative control), **OD isolate:** Average OD of isolate – ODc.

By the calculation of cutoff value (ODc), the result of biofilm detected as below:

OD ≤ ODc (no biofilm production).

ODc < OD ≤ 2 × ODc (weak biofilm production).

2 × ODc < OD ≤ 4 × ODc (moderate biofilm production).

4× ODc < OD (strong biofilm production).

Result and discussion

Antibiotic susceptibility testing

In the present study, the antibiotic susceptibility test of the 118 isolates (111 clinical and 7 environmental) of *P. aeruginosa* was performed on 13 antibiotics represented by Ticarcillin (TIC), Piperacillin (PRL), Imipenem (IMI), Tobramycin (TOB), Piperacillin /Tazobactam (PRZ), Ticarcillin /Clavulanic acid (TIV), Ceftazidime (CAZ), Gentamicin (GN), Cefepime (CFM), Meropenem (MER), Colistin (CO), Amikacin (AK) and Ciprofloxacin (CIP) using the disc diffusion method.

The antibiogram of the studied isolates revealed that 48 isolate of *p. aeruginosa* was multi-drug resistance with (70-100) %, 53 isolates (50-70) %, and 17 isolates with (30-50) % resistance. Furthermore, the isolates were resistance 100% to (TIC and PRL), 95.67, 94.91, 90.67, 80.50 and 74.57% to (IMI, CAZ, TOB, CFM and GN) respectively, followed by 64.40, 55.08 and 47.45% to (TIV, PRZ and MER) respectively, while the resistance were 32.20, 30.50 and 25.42% to (CIP, AK and CO) respectively.

The result showed, the isolates were less resistance to Colistin, and the susceptibility rate was 74.45%. This finding was disagreement with previous studies by Burgh *et al*. (16) in Kurdistan, Iraq; Izadi Pour Jahromi *et al.* (17) in Iran and Hussein *et al*. (18) in Wasit, Iraq, which the susceptibility rate was (96%), (97.5%), and (98.03%) respectively, while Khudair and Mahmood (19) in Baghdad, Iraq and Najeeb *et al.* (20) in Baquba City, Iraq reported that the Colistin was susceptible to *P. aeruginosa* isolates in (60%) and (65%) respectively.

This ability is either normal or acquired through mutations in their genetic material or through the horizontal transference of genes (21). P. aeruginosa is considered a major cause of hospital-acquired infections due to its high antibacterial resistance (22).

Aminoglycosides, such as gentamicin are multifunctional hydrophilic sugars that possess several amino and hydroxyl functionalities that can inhibit prokaryotic protein synthesis in a bacterial cell by binding to 30S subunit of the ribosome, and changing the conformation of the A site to one that resembles the one induced by an interaction between cognate tRNA and mRNA (23). The reason behind the bacterial resistance to these antibiotics is due to the production of β-lactamase enzymes, which acted to destroy the β-lactams ring thereby led to modification of antibiotics structure and spoilage their effects (24).

P. aeruginosa resistance to numerous antibiotics, resulting from excessive antibiotic administration, is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of multi-drug resistant MDR forms of *P. aeruginosa* (25). In the present study, out of the total *P. aeruginosa* isolates, ten isolates (8 clinical and 2 environmental) with multi-drug resistance are selected for further experiments, as shown in Table (1).

Table (1): Antibiotic susceptibility test of ten *P. aeruginosa*

Antibiotic / Isolate		TIC	PRL	IMI	TOB	PRZ	TIV	CAZ	GN	CFM	MER	CO	AK	CIP	Percentage of Resistance
		Clinical isolates													
P_1	P_4	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_2	P_8	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_3	P_{11}	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_4	P_{24}	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_5	P_{27}	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_6	P_{50}	R	R	R	R	R	R	R	R	R	R	R	R	R	100%
P_7	P_{60}	R	R	R	R	R	R	R	R	R	R	S	R	R	92.30%
P_8	P_{95}	R	R	R	R	R	R	R	R	R	R	R	R	S	92.30%
Environmental isolates															
P_9	P_{117}	R	R	R	R	R	R	R	R	R	R	S	S	S	76.92%
P_{10}	P_{119}	R	R	R	R	R	R	R	R	R	S	S	S	R	76.92%
Percentage ofResistance		100%	100%	100%	100%	100%	100%	100%	100%	100%	90%	20%	80%	80%	

(P): *Pseudomonas aeruginosa*, **(TIC):** Ticarcillin, **(PRL):** Piperacillin, **(IMI):** Imipenem, **(TOB):** Tobramycin, **(PRZ):** Piperacillin /Tazobactam, **(TIV):** Ticarcillin /Clavulanic acid, **(CAZ):** Ceftazidime, **(GN):** Gentamicin, **(CFM):** Cefepime, **(MER):** Meropenem, **(CO):** Colistin, **(AK):** Amikacin, **(CIP):** Ciprofloxacin

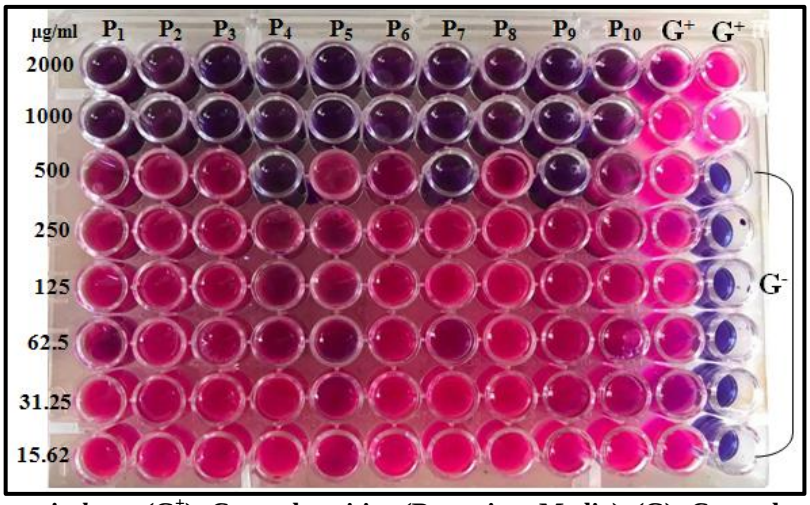
Determination the (MIC) of the chalcone on *P. aeruginosa* isolates

The broth microdilution method was used to determine the MIC of the chalcones using the 96-well microtiter plate. A method using the oxidation-reduction colorimetric indicator resazurin has determined the MIC of the antimicrobial agents against *P. aeruginosa*. Resazurin, which is blue in its oxidized state, turns pink when reduced by viable cells and can easily be detected with the naked eyes, and the MIC is determined even without the aid of a spectrophotometer (26).

The result showed that the MIC values of the chalcone compound on all *P. aeruginosa* isolates were 1000 mg\ml except for isolates No.4, 7, and 9 which were 500 mg\ml, as shown in (Table 2) and (Figure 1).

Chalcone is an antibacterial agent with moderate to high activity due to the presence of the reactive αB-unsaturated system. Their flexibility to change their structure by incorporating different types of substituent groups into the aromatic ring can potentially achieve a higher potency, lower toxicity, and a wider spectrum of antibacterial activity (27).

Another study tested chalcones for their antibacterial activity against Gram-positive strains *Bacillus subtilis* and *Staphylococcus aureus* and Gram-negative strains *P. aeruginosa* and *Salmonella typhimurium* (28). Chalcone exhibited antibacterial actions via various targets, which mainly include efflux pump inhibitory, interfering DNA replication, and filamentous temperature-sensitive mutant Z. (29). Dhaliwal *et al*. (30) found that chalcone compounds can inhibit diverse targets of antibiotic-resistance development pathways; therefore, they overcome resistance, and bacteria become susceptible to antibacterial compounds.



(p): *P. aeruginosa isolates*, (G^+): Control positive (Bacteria + Media), (G): Control negative (Media only).

Figure (1): MIC of chalcone on *P. aeruginosa* isolates.

Table (2): MIC of chalcone on *P. aeruginosa*

Isolate	Chalcone (µg/ml)
	MIC
P_1	1000
P_2	1000
P_3	1000
P_4	500
P_5	1000
P_6	1000
P_7	500
P_8	1000
P_9	500
P_{10}	1000

(P): *P. aeruginosa* isolate.

Biofilm of *Pseudomonas aeruginosa*

The microtiter plate method was used to determine the biofilm formation of multi-drug resistant isolates. The results showed that all the isolates had strong biofilm production except isolates No. 4, 7, and 9 which was moderate as shown in Table (3). Biofilms are of utmost medical relevance with biofilms being abundantly present in sputum samples of individuals with cystic fibrosis, pacemakers, diabetic wounds, and in many other infections. However, most of the early conventional medical microbiological work has been based on planktonic bacteria. The last two decades clearly suggest that nearly 80% of disease progression is caused by biofilms (31). *P. aeruginosa* pathogen is known to form successful biofilms and these biofilms are often the root of serious medical complications (32). Al-Saray(33) reported that 95.56% of *P. aeruginosa* isolates have the ability to form biofilm.

Table (3): Biofilm formation of *P. aeruginosa* using the microtiter plate method

Clinical isolate	Source of Isolate	Biofilm Production
P_1	Burn	Strong
P_2	Burn	Strong
P_3	Wound	Strong
P_4	Wound	Moderate
P_5	Wound	Strong
P_6	Wound	Strong
P_7	Wound	Moderate
P_8	Wound	Strong
P_9	Wound	Moderate
P_{10}	Wound	Strong

(P): *P. aeruginosa* isolate.

Antibiofilm activity of chalcone in *P. aeruginosa* isolates

Biofilm is a densely packed community of microbial cells that attach and grow on living or nonliving surfaces and surround themselves with secreted polymers. Biofilm associated infections are often difficult to treat because of multi drug resistance, so it is important to identify new and effective molecules against bacterial biofilm formation (34).

In this study, chalcone inhibited 100% of the biofilm formation of *P. aeruginosa* isolates in 500 µg/ml, and reduction the biofilm formation in concentration 32.25 µg/ml, as shown in Table (4). ESKAPE pathogens, includes *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp., have received wide attention due to their immense clinical importance. Several studies indicate that the biofilm mediated infections associated with these bacteria serve to increase their resistance towards antibiotics, necessitating the urgent need to develop more efficacious and safer drugs. Naturally occurring chalcones, which are precursors of flavonoids, have received wider attention for their potent antibacterial and antibiofilm activity(35). Dusan *et al*. (36) demonstrated the antibacterial activity of chalcone molecules and proved their ability to act inhibitory or bactericidal on some multi-resistant clinical isolates of *A. baumannii* and *P. aeruginosa*, in a dose-dependent manner. Besides, these compounds were able to significantly inhibit some of the important virulence factors when applied at sub-inhibitory concentrations, such as biofilm production and motility of *A. baumannii* and *P. aeruginosa.* Pereira *et al*. (2022) (37) showed the potential of chalcone compounds regarding the reversion of bacterial resistance and showed a higher ability than reserpine to inhibit biofilm formation of resistant *P. aeruginosa*. Recently, as a result of the search for new antimicrobial adjuvants, some chalcones have demonstrated the ability to potentiate the antimicrobial effect, as well as on biofilm inhibition (38,39). Favonoids cause bacterial aggregation by their partial lysis, which leads to membrane fusion, and consequently reduces the active nutrient uptake via a smaller membrane area, the anti biofim activity of chalcone attributed to the forming a cage-like structure around the cell wall to allow the entry of the active moiety of the drug into the cell (40). On the other hand, Lima *et al.* (41) showed comparatively better antibiofim activity against *P. aeruginosa* with MIC 256 µg/ml.

Table (4): Biofilm formation of *Pseudomonas aeruginosa* isolates before and after treatment with chalcone.

Clinical isolatee	Source of isolate	Before treatment	After Treatment Concentration (µg/ml						
			32.25	64.5	125	250	500	1000	2000
P1	Burn	Strong	Moderate	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm
P2	Burn	Strong	Moderate	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm
P3	Wound	Strong	Moderate	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm
P4	Wound	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P5	Wound	Strong	Moderate	Moderate	Week	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P6	Wound	Strong	Moderate	Moderate	Week	Moderate	No Biofilm	No Biofilm	No Biofilm
P7	Wound	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P8	Wound	Strong	Moderate	Moderate	Week	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P9	Wound	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P10	Wound	Strong	Moderate	Moderate	Week	Week	No Biofilm	No Biofilm	No Biofilm

Conclusion

The isolates were resistance 100% to (TIC and PRL), 95.67, 94.91, 90.67, 80.50 and 74.57% to (IMI, CAZ, TOB, CFM and GN) respectively, followed by 64.40, 55.08 and 47.45% to (TIV, PRZ and MER) respectively, while the resistance was 32.20, 30.50 and 25.42% to (CIP, AK and CO) respectively. Chalcone display a high antibacterial agent on *P. aeruginosa* despite the fact that bacterial isolates were resistant to commonly used antibiotics. Furthermore, chalcone had an excellent reduction of biofilm formation in *P. aeruginosa*.

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