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Antibacterial Activity of Chalcone Compound against *Pseudomonas aeruginosa* Isolated from Different Sources

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Abstract: Natural products are used as complementary or alternative treatment, there are chemical substance found in nature and manufactured by a living organism that have biological activity. The *Pseudomonas aeruginosa* is an opportunistic pathogen that involved in different diseases globally and locally, the current study was done in Baqubah teaching hospital; one hundred and twenty seventh clinical and environmental isolates were collected from April 2021 to October 2021. The results were in range of thirty-two 32/127(25.2%) isolates belong to *P. aeruginosa*, bacterial isolates were primarily identified by using routine laboratory methods (macro and microscopic examination), then by specific biochemical tests and confirmed the diagnosis by using VITEK-2 compact system and molecular study included detected virulence gene *toxA* by conventional PCR method were used for diagnosis that offer a time saving and accurate method of detection bacterial species. The results lead to conclude that the Minimum Inhibitory Concentration value of chalcone against *P. aeruginosa* isolates at (156.25µg/ml), furthermore the Minimum Bactericidal Concentration value were at (315.5µg/ml).

Keywords: *Pseudomonas aeruginosa*; Chalcone; Virulence genes.

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Introduction

Many Natural products are used as complementary and alternative treatment some bacterial infections, it contains chemical substance found in nature and could manufactured by the living organism that have biological or pharmacological activity. Living organisms synthesized many primary and secondary biological metabolites (1).

Chalcone is one of the major classes of flavonoids across the whole plant kingdom. Chalcone is on open chain precursors for biosynthesis of iso flavonoids and flavonoids and occur in the main as polyphenolic compounds (2). It considered as a good inhibitor and bactericidal for most Gram-negative and Gram-positive bacterial strains, as well as it have a lower cytotoxic effect which reflect their therapeutic possible in antimicrobial agents field (3).

Pseudomonas aeruginosa is a gram negative, motile, a heterotrophic, facultative aerobe bacterium that grows under aerobic and anaerobic respiration, rod shaped about (1–5) µm long and (0.5–1.0) µm wide. It is considered an opportunistic pathogen. Typically, can grow on a Low salts growth medium with single of carbon and energy source (4). These characteristics are important for allow it to attach and a long survive on medical tools and on other hospital surfaces, which causes the beginning of infections in immune-compromised patients (5).

Pseudomonas aeruginosa involved in many infections worldwide, it caused many infections for human such as respiratory infections, urinary tract infections, nosocomial infection, wound and burn infections and bacteremia in immunocompromised patients. It considers as multidrug-resistant and can cause many infections in many countries, including Iraq (6).

Materials and methods

Samples

The bacterial isolates were collected over period from April 2020 to October 2020, a 127clinical and environmental swabs were collected from patients and different environmental sources in Baqubah Teaching Hospital.

Bacterial isolation and identification

All the bacterial isolates were cultured by routine laboratory methods using different types of general and selective bacterial medium included Blood agar, MacConkey agar, Muller Hinton broth and KigA agar.

The positive isolates bacteria were identified by primarily routine lab. Tests (macro and micro characteristics and selective media) then it was confirmed by applying the conventional biochemical tests and confirmed by using VITEK 2 compact system.

Determination minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Chalcone against *P. aeruginosa* isolates

The minimal inhibitory concentration (MIC) value is the lowest concentration of chalcone which prevent bacterial growth. Broth micro dilution method was used to determine the MIC. according to CLSI guidelines. Each one of the 96-well micro-titer plates received 50µl of MHB, whereas the 10th well received 100µl of MHB (sterile control) and the 12th well was treated with Mueller Hinton Broth (MHB) and 5% DMSO (growth control).

Chalcone extracted from *Acacia.nilotica* were prepared by dissolved in 5% DMSO in concentrations ranging from 9.76-5000 µg/ml.

According to CLSI recommendations and the modified Wiegand technique, a serial two-fold dilution was performed by transferring 50µl of the suspension to the successive wells until the eighth well (7). To achieve a final inoculum size of 5 x 10^5 CFU per ml, around 0.5 McFarland broth inoculum was diluted in a 1:100 ratios and distributed to 1 to 8th wells (8).

Molecular detection of the bacterial strain

The bacterial cultures were inoculated in (10 ml) of the Nutrient broth medium and incubated to overnight at 37°C.

Genomic DNA was extracted from isolates using extraction Kits of Genomic DNA, Purification depending on instruction of manufacturing company (Promga USA).

The primer used in this study is given in the Table (1). The primers *toxA* specific to *P. aeruginosa* were chosen from the published sequence and reference *fbp* gene.

Table (1): The Primer used for Detecting *P. aeruginosa*

Primer	Sequence 5" to 3"	Annealing Temp. (°C)	Reference
***toxA*-F**	GGAGCGCAACTATCCCACT	50	Sabharwal *et al*., 2014
***toxA*-R**	TGGTAGCCGACGAACACATA		
***fbp*-F**	CCTACCTGTTGGTCTTCGACCCG	58	Liu *et al*., 2000
***fbp*-R**	GCTGATGTTGTCGTGGGTGAGG		

Amplification condition

A gradient PCR is often done to optimize PCR protocol and to figure out what annealing temperatures work best. PCR conditions were optimized by repeated changing annealing temperatures (from 48Cº to 60Cº according to primers) and number of cycles (30) according to current study till being fixed at the conditions listed in Table (2). This method offers significant time-savings and minimizes reagent use, relative to a standard PCR optimization protocol.

Table (2): Programs of PCR thermo cycling conditions

PCR Program			
Steps	**°C**	**m:s**	**Cycle**
Initial Denaturation	95	5 min	1
Denaturation	95	30sec	30
Annealing	50 or58	30sec	
Extension	72	30 min	
Final Extension	72	7 min	1
Hold	10	10 min	

Results and discussions

Isolation and identification of *Pseudomonas aeruginosa*

Out of one hundred twenty-seven clinical and environmental isolates, detected thirty-two *P. aeruginosa* isolates

By the Microscopically examination was achieved for the target isolates using of differentiated dye by Gram stain method, the bacterial isolates had been tinted with the pink color referred to Gram negative type, and its appeared as straight or slightly rods in shapes, this was compatible with what referred by The Standards Unit of Microbiology Services (9).

At first the (32) Gram negative bacterial isolates of *P. aeruginosa* cultured in specific media included by King A and then subjected to the IMVIC test, triple sugar iron (TSI) test. The *P. aeruginosa* bacteria given the Blue - greenish of (Pyocynine) color on the King A agar medium. This medium promotes of the production of Pyocyanin dye, a blue-green pigment which oxidizes to brown, the pigment diffuses throughout the medium and the blue color is observed (10), as indicated in Figure (1).

Figure (1): *P. aeruginosa* spp. Colonies on king A agar.

Biochemical tests

All the *P. aeruginosa* bacterial isolates did not change the color of bottom and slant remained pink, not appeared any black color of the media and un damaging in the structure of the agar the template refer to non-ferment any sugar, without the H2S production, and no gas produced. These results were similitude to the (11) for the determination of *P. aeruginosa* species. All the *P. aeruginosa* isolates showed negative results for the indole, methyle red and the Voges - proskauer tests while it was positive for citrate test according to (12) as shown in Table (3).

Table (3): Biochemical tests and their results of the *P. aeruginosa* bacteria

Test	*P.aeruginosa*
Gram staining	Gram negative/rod shaped
King A agar	Blue- greenish (Pyocynine) color
Catalase	+
Oxidase	+
Indole	-
Methyle red	-
Voges-proskauer test	-
Citrate utilization test (Simmon's Citrate slant)	+
Triple sugar iron	Alk / No change, No H_2S, No gaseous

(+) and (-) denote to positive and negative results, respectively.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the chalcone against the *P. aeruginosa* isolates

Bacteria development multi drug resistance as results to the misuse and the overuse of the antimicrobials by the patients, so the natural antimicrobial used as an alternative approach to the overcome the restrictions of traditional synthetic of the antimicrobial agents. The Chalcone was selected as the alternative of antibiotics to detect the (MIC and MBC) activity against all the *P. aeruginosa* bacterial isolates depended on the (CLSI) recommendations and the the modified Wieg and its technique (7).

The (MIC) and (MBC) detection was performed by the utilizing of Muller Hinton broth medium in two-fold serial of micro dilutions method. The MIC values for each isolate were detected by the selecting of the lowest concentrations that can inhibit of the visible growth. After the (MIC) of the chalcone was detected, (MBC) values was detected by the sub-culturing of bacteria on Muller Hinton agar the of each tube and incubation it at 37 °C for 24 h, when do not observe any of bacterial growth at the lowest concentration of the chalcone, it is called (MBC) endpoint. Table (4) showed that chalcone MIC for *P. aeruginosa* was (156.25 µg / ml), furthermore the (MBC) value was (315.5 µg / ml). While another study was showed a different result which recorded that the higher (MIC) value against the Gram negative was (0.125 mg / ml) and for Gram positive bacterial type (MICs) was between 0.008 mg / ml to 0.063 mg / ml (13). The results refer to that newly developed compound would be important

antimicrobial agents in the treatment of the infections from resistant bacteria.

Table (4): Growth value of *P. aeruginosa* isolates (no=7) with different chalcone concentration

Concentration (µg/ml)	P.1	P.2	P.3	P. 4	P.5	P.6	P.7
Control negative	1.112	1.634	1.917	3.598	1.523	2.687	1.806
Control positive	0.602	1.412	0.889	2.303	0.501	1.301	0.667
9.76	0.484	1.15	0.542	1.126	0.362	1.13	0.431
19.53	0.367	0.631	0.518	0.59	0.234	0.421	0.36
39	0.191	0.009	0.314	0.244	0.171	0.007	0.122
78.125	0.004	0.225	0.148	0.117	0.153	0.113	0.126
156.25	0.139	0.393	0.002	0.076	0.125	0.162	0.043
315.5	0.142	0.125	0.077	0.005	0.122	0.103	0.029
625	0.342	0.394	0.095	0.055	0.121	0.073	0.033
1250	0.342	0.164	0.139	0.016	0.132	0.127	0.013
2500	0.198	0.541	0.028	0.046	0.176	0.321	0.014
5000	1.349	1.18	1.113	0.308	1.125	1.15	1.105
LSD value	0.327 **	0.502 **	0.371 **	0.487 **	0.363 **	0.449 **	0.532**
** ($P \leq 0.01$).							

Molecular study

The molecular study results were showed that 10 from 13 isolated species (76.9 %) were *P. aeruginosa* showed that expected amplicons size 150 bp for *toxA* gene, Figure (2) showed shine bands of the positive isolates compared with 100bp. DNA ladder. That the results differ with the study by (14) when appeared all the isolates have showed presence of *toxA* gene by 100 % within the genetic structure of the isolates of the *P. aeruginosa*, also the study in Kirkuk city by (15) detection *toxA* gene by 100 % of the *P. aeruginosa* genetic structure, and the study by (16) detected that 40 % of the tested isolates have *toxA* gene.

The genotypic analysis via the PCR reaction with the specific *toxA* gene, which ended to confusion in the diagnosis of confirmation process, although the VITEK 2 system proved that all the isolates were with in the *P. aeruginosa*. The accurate conclusive outcome from the PCR - technique detection through the target gene *toxA* confirmed that all isolates belong to the species of the *P. aeruginosa*. So, there is no clear difference between VITEK 2 system test and the molecular analysis. Polymerase Chain Reaction is one of a better alternative than conventional methods, as its targets the (DNA) of an organism. The conventional methods were used for diagnosis that offer a time saving and accurate method of detection bacterial species and laboratory intensive and it can be used as confirmation to the selective medium and the biochemical tests (17).

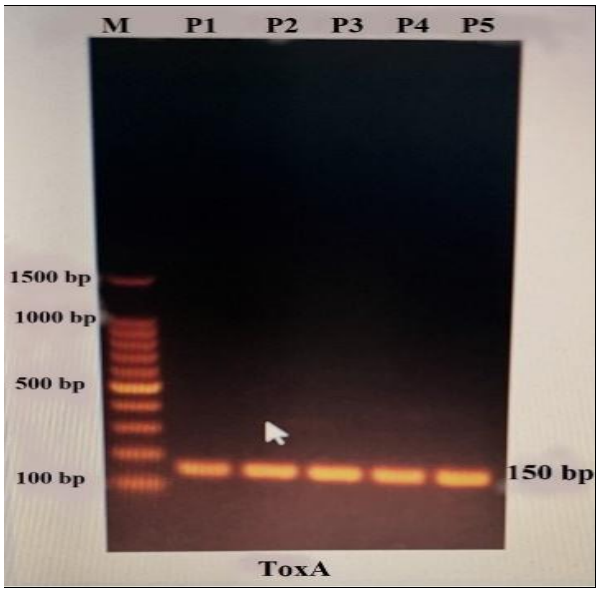

Figure (2) Agarose gel electrophoresis of *toxA* gene of *P. aeruginosa* isolates (150bp amplicon). Electrophoresis was run at (1% agarose, 7v/cm2 for 60min), Lane L DNA Ladder.

Analysis of gene expression of *toxA* gene by using the Quantitative Real Time qRT - PCR Technique

The experiment of the quantitative RT - PCR reaction was completed by using *P. aeruginosa* isolates from different sources to measure that the expression of *toxA* virulence gene. The results were presented when the isolates were treated with the Chalcone using a concentration that was below the dose of the sub (MIC) for each sample and the Ct value of *toxA* in the current study is shown in Table (5).

Table (5): The Ct Value of *toxA* gene of *P. aeruginosa* before and after Treatment with chalcone

No. of isolates	Ct value of *toxA* before treated	Ct value of *toxA* after treated with chalcone (156.25µg/ml)	χ 2 (P-value)
1	32.66	21.12	0.0001 **
2	33.79	21.35	0.0001 **
3	33.62	18.33	0.0001 **
0.147 NS		0.0483 *	---

The expression of *toxA* showed average 1 as level of folding before treated with subMIC of chalcone, while the expression of *toxA* showed average 0.16 as level of folding in the case of the isolates that treated with chalcone at the concentration of 156.25µg/ml. Treated with chalcone that lead to the decrease in the value of gene expression in all four isolates. This due to effectiveness exposure of 156.5µg/ml chalcone which highly effected on expression of both *toxA* and *Fbp* genes in selected target isolates Table 6, also there were non-significant differences according to χ^2 values among target isolates in Mean Δ CT(Treated), while shown there were a significant difference according to χ^2 values at $p<0.05$ among target isolates Mean ΔCT (Untreated), ΔΔCT, and $2^{-\Delta\Delta CT}$ fold change of qRT-PCR results Table (5).

Table (6): Ct values and fold of gene expression of *toxA* and *fbp* genes of *P. aeruginosa* that was treated with chalcone

Groups	No. of isolate	Mean Ct of reference *Fbp*	Mean Ct of target *toxA*	ΔCt	ΔΔCt	(Folding)= $2^{-\Delta\Delta Ct}$	Average of folding
Untreated	1	25.39	32.66	7.27	0.00	1.00	1
	2	26.05	33.79	7.74	0.00	1.00	
	3	27.20	33.62	6.41	0.00	1.00	
Treated with chalcone (156.25µg/ml)	1	15.74	21.12	5.39	1.88	0.27	0.16
	2	16.85	21.35	4.50	3.24	0.11	
	3	15.20	18.33	3.13	3.28	0.10	

In Iraq the study by (6) showed which highly effected of ZnO NPS on the (*MexY*) gene expression, and the study by (18) appeared Chalcone significantly inhibits the pyocyanin production, swarming and the swimming motility of the *P. aeruginosa*. The current study concluded that chalcone compound were active against the *P. aeruginosa* isolates and give low level of expression of *toxA* gene after the treatment with the Chalcone, this means that it has ability of action as antimicrobial agents (19).

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