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The Effect of Ciprofloxacin and Resveratrol Combination on Expression of *Pela, Psld, Mexa Genes* of *Pseudomonas Aeruginosa* Isolates from Lower Respiratory Tracts

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Abstract

A total of 200 sputum samples were collected from the patients infected with lower respiratory tract (LRT) infection attending at Baqubah Teaching Hospital and Chest Diseases Centre Baqubah City/Iraq. The study aimed to investigate the effect of resveratrol as an efflux pump inhibitor on the MIC of ciprofloxacin and to compare the effect of two agents on the expression of pelA, pslD and mexA genes for *P. aeruginosa* isolates, all samples were inoculated onto appropriate media for isolation and identification of bacteria by morphological and biochemical tests and molecular diagnosis using polymerase chain reaction. The results showed that the overall prevalence of *Pseudomonas aeruginosa* among patients was 24 (12%) isolates were identified .The percentages of the phenotypic detection haemolysin test and pyocinin pigment were 100 % and 95.83% respectively. Our study reveal that sub-MIC of Resveratrol causes a reduction in MIC of Ciprofloxacin for most isolates .The combination effect of Resveratrol and Ciprofloxacin on the expression of Biofilms formation genes *pelA* , *psID* , and efflux pump gene *mexA* revealed a synergistic impact were cause a reduction in a mean of gene expression of this three genes(0.45 , 0.60,. 0.90) respectively in three MDR *P. aeruginosa* isolates.

Keywords: *P.aeruginosa*, *pelA*, *pslD*, and *mexA* genes, Biofilom, Resveratrol, Ciprofloxacin.

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Introduction

Pseudomona aeruginosa is recognized as the leading cause of infections obtained in hospitals due to its innate and plasmid-mediated resistance to multiple antibiotics, resulting in a multi-drug-resistant (MDR) pathogen [1]. Bacteria possess several virulence factors, include secreted quorum sensing ,enzymes , toxins, the formation of biofilms which increase their ability to cause potentially fatal infections [2]. *P. aeruginosa* represents one of the most commonly isolated pathogens in people with non-CF bronchitis, and it is linked to deteriorating pulmonary function and higher mortality rates [3, 4]. *P. aeruginosa* is one of the most common biofilm-forming pathogens causing cystic fibrosis (CF) infections in the lung. In the biofilm state, *P. aeruginosa* develops resistance to antimicrobials, making it difficult to treat [5]. Bacteria produce a large number of pigments. One of the most noticeable is the blue - green pyocyanin pigment, which appears on the surface of every plate grown and is recognized as blue pus. Pyorubin pigment is red, while pyomelanin pigment is black [6].

Efflux pumps are membrane proteins that transport toxic compounds from the bacterial cell to the environment.Efflux pump genes are present in all bacterial species and can be found on bacterial chromosomes or mobile genetic components such as plasmids [7].As mentioned above, efflux pumps play an important role in the emergence of multidrug resistance in bacteria. extrude toxic molecules resulting in reduced susceptibility to antibiotics [8]. In addition, overexpression of these multidrug efflux pumps in *P. aeruginosa* is directly associated with resistance to many anti- pseudomonal drugs [9]. Efflux pumps are essential for bacterial biofilm formation, andresearch studies show that efflux pump expression is increased in biofilms resulting in increased antibiotic resistance [10]. Several studies has demonstrated that Resveratrols as efflux inhibitor are capable of changing bacterial virulence, reducing the integrity of membranes, and preventing biofilm formation [11, 12]. Other studies reported that resveratrol displays activity against various infections that affect humans[13].It can greatly improve the impact of aminoglycoside antibiotics (such as Gentamicin, Tobramycin, Netilmicin, and Amikacin on *P. aeruginosa* biofilms [14] .The current study suggested to investigating the prevalence of *P. aeruginosa* among lower Respiratory Tracts(LRT) patients

in Baqubah City /Iraq ,the effect of Resveratrol on Ciprofloxacin MICand to investigate the synergistic effect of Ciprofloxacin and Resveratrol combination on the expression of Biofilms formation genes *pelA*, *psID* , and efflux pump gene *mexA* gene.

Materials and Methods

Collection of Specimens and *P. aeruginosa* Diagnosis:

In the current study approximately 200 sputum specimens were collected from patients admitted to Baqubah Teaching Hospital and Chest Diseases Center with a lower respiratory tract infections during the period 1st of January to the 30th March 2024. Sputum specimens were inoculated on MacConkey agar and incubated aerobically for 24 hours at 37 °C. To confirm identification of the expected *pseudomonas* isolates, it was sub- cultured on Cetrimide agar medium and incubated in the same condition. Bacteria were diagnosed using standard methods including colony characteristics,microscopic examination [15]. and Biochemical tests [16]. Finely identification confirmed by molecular method via polymerase chain reaction.

Determination of Minimum Inhibitory Concentration (MIC) for Ciprofloxacin and Resveratol

Minimum inhibitory concentration (MIC) of Ciprofloxacin (CIP), and Resveratrol for all the 24 *P.aeruginosa* isolates' was determined using the serial dilution method in Mueller-Hinton broth. The test was performed in concentrations range from 2 to 1024 µg/mL. A bacterial suspension with 1.5×10^8 CFU/ml prepared by using 0.5 MacFarland standard solution , Bacterial growth transferred to tubes containing this concentrations that incubated for 18–24 hours at 37°C. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a substance that prevents the growth of bacteria [17].

Detection of virulence factors

Haemolysin production: Haemolysin test is done to investigate the ability of isolate for enzyme production. The bacteria were inoculated into a blood agar medium and incubated for 24 hours at 37 °C. If clear zone of hemolysis formed around colonies this indicate a positive result for the test [18] .

Pyocyanin pigments production: Pigment production was observed utilizing Cetrimide agar and Nutrient agar medium, inoculated with bacterial isolates, and incubated at 37 °C for 18 to 24 hours for detection of the pigment production.

Molecular Study

Detection of *16S rRNA* , *pelA, pslD,* and *mexA* genes By PCR

The Polymerase chain reaction was used to detect the *16S rRNA* gene as well as the *pelA, pslD,* and *mexA* genes for (15) *P. aeruginosa* isolates, primer was used in the current study illustrated in Table (1), and the Programs of PCR thermocycling conditions for each primer of genes Table (2) .

Primers Preparation

The primers used in this study were synthesized according to the instructions supply by the manufacturer, Macrogen Company. These primers were supplied in lyophilized form. The lyophilized primers were reconstituted in nuclease-free water to obtain a stock solution with a final concentration of 100 pmol/µl. To prepare a functional solution of these primers, 10µl of primer stock solution (stored in a freezer at -20 C) was combined with 90µl of nucleasefree water. This resulted in a working primer solution with a concentration of 10 pmol/µl. The reaction conditions for molecular detection are shown in Table 2.

Table 1: The primers used to identify the genes in the present study

Primer Name	`Sequence 5`-3	Annealing Temp. (°C)	Size (bp)	Reference
PelA –F	CCTTCAGCCATCCGTTCTTCT	45	118	[19]
PelA –R	TCGCGTACGAAGTCGACCTT			
PslD –F	FTGTACACCGTGCTCAACGAC	56	369	[20]
PslD –R	RCTTCCGGCCCGATCTTCATC			
mexA1- F	CGACCAGGCCGTGAGCAAGCAGC	57	316	**[21]**
mexA2- R	GGAGACCTTCGCCGCGTTGTCGC			
Pseu_16s-F	CCTTCAGCCATCCGTTCTTCT	55	104	[22]
Pseu_16s-R	TCGCGTACGAAGTCGACCTT			
fbp-F	FTGTACACCGTGCTCAACGAC	58	284	**[23]**
fbp-R	RCTTCCGGCCCGATCTTCATC			

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Table 2: Programmes of PCR thermocycling conditions for each primer of the genes

Steps	°C	m: s	Cycle
Initial Denaturation	95	05: 00	1
Denaturation	95	00:30	30
Annealing	55,57,45,56	00:30	
Extension	72	00:30	
Final extension	72	07:00	
Hold	4	10:00	1

Gel Electrophoresis

Agar gel electrophoresis was used to verify the presence of amplification following PCR amplification. The reliabilit of PCR was entirely dependent on the specific parameters of the isolated DNA [24].

Agarose preparation

A flask was filled with 100 ml of 1X TAE. A total of 2 grams of agarose, or 2% concentration, was added to the buffer solution. This solution was then heated to its boiling point using a microwave until all gel particles were completely dissolved. Comma 1 microlitre of ethidium bromide (10 mg/ml) added to the agarose. The agarose was agitated to ensure thorough mixing and prevent the formation of bubbles. The solution was allowed to cool to a temperature range of 50-60°C.

The horizontal agarose gel

The horizontal agarose gel was cast by pouring the agarose solution into the gel tray prepared by covering both edges with cellophane tape. The agarose was then allowed to solidify at room temperature for approximately 30 minutes. The combining tool was carefully removed and the gel was placed in the gel tray. The tray was filled with 1X TAE electrophoresis buffer to a level of approximately 3-5 mm above the surface of the gel.

DNA loading

5 µL of the PCR products for all samples were immediately added to each well. A current of 100 volts per metre ampere was applied for 60 minutes. DNA moves from the negatively charged cathode toward anod poles with a positive charge. The bands visualised with ethidium

bromide were further analysed using an ultraviolet (UV) transilluminator equipped with a gel imaging device and a digital camera.

Gene expression for *pelA, pslD,* and *mexA* by qRT-PCR program

To detect the gene expression of biofilm formation and efflux pump genes (*pelA, pslD,*and *mexA*) of three multidrug resistance isolates (PA3, PA6, PA9),detection was done with three treatments included subculture of isolates in the media: the first treatment with sub- MIC of Ciprofloxacin, the second treatment with sub-MIC of Resveratrol, the third treatment with combinations of sub-MIC for Ciprofloxacin and Resveratrol. Gene expression levels for one or more genes are analyzed and calculated from the concentration of RNA or mRNA after transformation into cDNA. The entire procedure includes total RNA purification, qPCR amplification, and data analysis. The Gene 9600 quantitative PCR instrument was used for this experiment and the primers are shown in Table (1). Differences in gene expression levels were calculated using the following equation [25].

ΔCT =CT target gene - CT House Keeping gene, ΔΔCT = ΔCT Treated - ΔCT Control

Folding = 2-ΔΔCT

RNA extraction and purification

The RNA extraction and purification process involved isolating RNA from the sample using the TRIzol™ Reagent procedure, which consisted of the following steps: Example of cell lysis: Cells were cultured in liquid medium, for pellet cells, 1.4 mL of cell culture was separated by centrifugation at 13000 rpm for 2 minutes. The liquid above the pellet was then removed, and 0.5 mL of TRIzol™ Reagent was added to the pellet. The lysate was homogenized by repeated pipetting up and down. In the process of separating three phases: 0.2 mL of chloroform was added to each tube containing the lysate, and the tube was securely closed. The mixtures were incubated for 2-3 minutes and subjected to centrifugation at a 12,000 rpm for 10 minutes. This process the separated of the mixture into three distinct phases: a lower organic phase, an intermediate phase, and an upper aqueous phase that was colourless. The solution containing the RNA the aqueos phase was transferred to another tube. To precipitate RNA, 0.5 mL of isopropanol was added to the aqueous phase and incubated for 10 minutes. The mixture was

then centrifuged at 12,000 rpm for 10 minutes. After precipitation, the total RNA solidified and a white gelatinous pellet formed at the bottom of the tube. The liquid portion was then discarded. To wash the RNA, 0.5mL of 70% ethanol was added to each tube. The mixture was vortexed and centrifuged for at 10000 briefly for 5 minutes. The pellet was subsequently aspirated with ethanol and the nair-dried. To increase the solubility of the RNA, the pellet was rehydrated in 50 µl of Nuclease free water and then incubated in a water bath or heating block set at a temperature range of 55-60°C for 10-15 minutes. The concentration of extracted RNA was determined using the fluorescence method (Quantus Fluorometer) to assess the quality of the samples for subsequent applications. A mixture was prepared by combining 1 µl of RNA of diluted QuantiFluor dye. RNA concentration measurements were identified after a 5-minute incubation at room temperature in a dark location.

Results and Discussion

Isolation and identification of *P. aeruginosa*

The percentage of identified *P. aeruginosa* isolates was identified 24 (12 %), diagnosis were done according to standard methods , the positive growth of bacterial isolates form pale yellow, small, smooth, round colonies on Mac Conkey agar medium. colonies on cetrimide agar on other hand, were slimy and smooth in a shape with flat edges and a higher centre, creamy in colour and fruity in odour [26]. 21 (95.83 %) of *P. aeruginosa* isolates produce blue-green pyocianin which is a combination of two metabolites of *P. aeruginosa* pyocyanin (blue) and pyoverdine (green), which give the characteristic color of cultures [27].. Figure (1). All the 24 (100%) of isolates on Blood agar medium yield large, flat colonies with grape-like smell show β-hemolysis after 24 hours of incubation .This result agrees with the study conducted by [28] .The microscopic examination of twenty-four isolates stained with gram stain revealed that the cells of this bacteria emerged in the form of small bacilli with pink colour indicating that this bacteria is negative for gram stain as stated by [16]. The results of biochemical tests showed that Simmons citrate, oxidase, and catalase give positive reaction in all bacterial isolates. Whereas negative results in all the isolates for Voges-Proskauer (VP), Methyl red (MR), and

Indole production[29].Results also showed that all the isolates could grow at 4 and 42°C, which is an result consistent with [30]

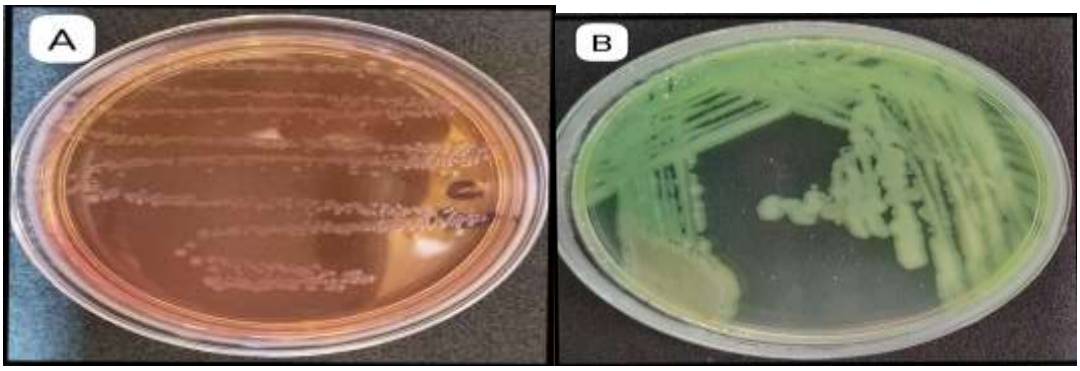

Figure 1: *P.aeruginosa* colonies (A) on MacConkey agar (B) on Cetrimide agar.

Molecular detection of *P.aeruginosa* using the *16S rRNA* Gene

The results of the identification by molecular and extraction of genomic DNA were satisfactory, and the quantification of DNA concentration was performed immediately using the Quantum Fluorometer. DNA concentration was obtained from 15 isolates. The PCR results under investigation were verified and examined by horizontal gel electrophoresis in a 1.5% agarose gel for a duration of 90 minutes. The gel was subjected to an electric field of 100 v/mA and then exposed to UV light, causing the DNA to appear as condensed bands. Analysis showed that all 15 isolates (100%) were identified as *Pseudomonas aeruginosa*, with estimated amplicons of 104 bp for the 16S rRNA. Figure (1) shows the bright bands of the positive isolates compared to the 100 bp marker.16SrRNA sequencing has traditionally served as a reliable benchmark for determining phylogenetic relationships among bacteria.

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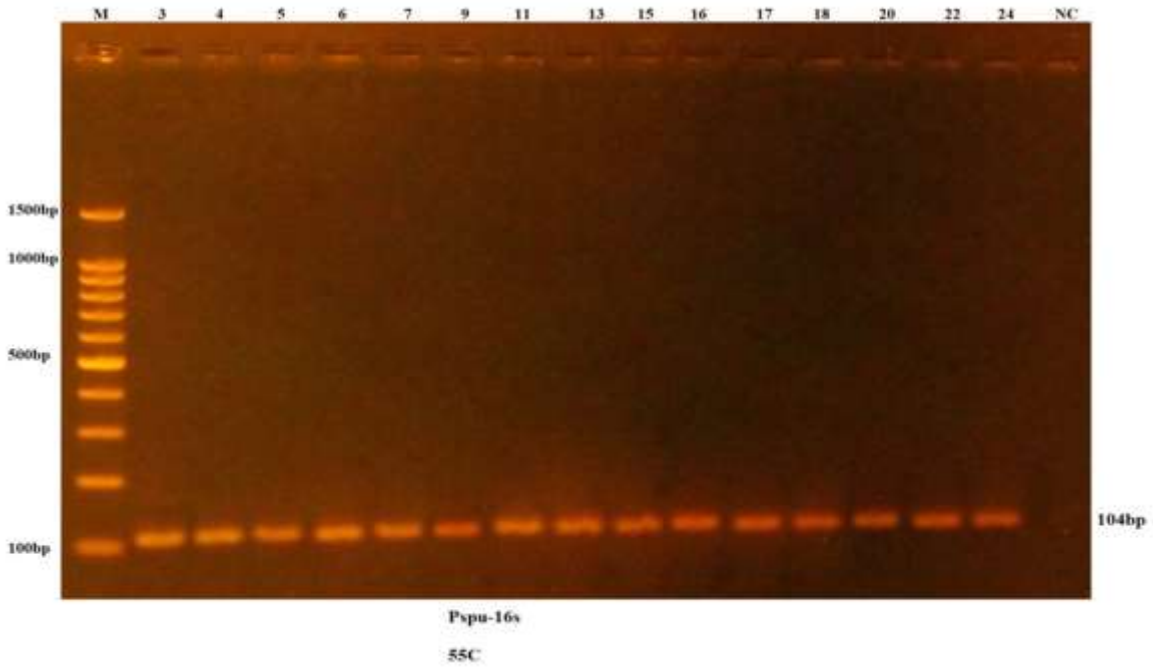

Figure 2: Amplification of the *P. aeruginosa's Pspu-16S rRNA* gene was fractionated on 2% agarose gel electrophoresis and stained with an Eth.Br. M: 100bp ladder marker. Lanes (3, 4, 5, 6, 7, 9, 11, 13, 15, 16, 17, 18, 20, 22, and 24) look like 104bp PCR products.

Effect of resveratrol on MIC of ciprofloxacin:

MIC of ciprofloxacin were determined for twenty-four clinical isolates of P. aeruginosa obtained from patients with lower respiratory tract infections. The test was performed by broth dilution using a series of microdilution tubes with concentrations of (2, 4, 8, 16, 32, 64, 128, 256, 512 and 1024) µg/mL. The value of ciprofloxacin MIC without treatment with resveratrol for all *P.aeruginosa* isolates ranged from (8 - 128 µg/ml). While the results showed high reduction in value of Ciprofloxacin MIC after treatment for most isolates were the MIC value ranging become from(2-16 µg//ml), table(3).The prevalence of MDR isolates is increase primarily by active efflux mechanisms so that inhibiting these pumps with effective EPIs could slow the rise of MDR. Plants are a rich source of bioactive molecules, including potential EPIs, making them a promising alternative for the discovery new antimicrobial agents [32].

Antibacterial agents when used in mixtures enhance the ability of plant-derived compounds to overcome or contribute to overcoming the resistant of bacteria, for instance [33]. Plant bioactive compounds have combined or synergistic effect against multiple targets within the bacterial cell. As a result, we expect anticipate that bacterial resistance to such synergistic combinations to develop at a much faster rate than resistance to single chemical agents such as antibiotics [34] Resveratrol is one of many medicinal plants with antibacterial properties that have been reported to be used as efflux pump inhibitors [35].

Table 3: Effect of sub-MIC resveratrol on ciprofloxacin MIC for (24) isolates of *P. aeruginosa.*

Isolates Series	**Before treatment**	**After treatment**	**Isolates Series**	**Before treatment**	**After treatment** (24) isolates of *P.aeruginosa* **After treatment**
Breakpoint	**≤1(S)/ ≥ 4(R)**		**Breakpoint**	**≤1(S)/ ≥ 4(R)**	
PA1	32	4	**PA13**	32	4
PA2	32	4	**PA14**	64	16
PA3	16	8	**PA15**	16	4
PA4	128	8	**PA16**	32	8
PA5	32	16	**PA17**	8	4
PA6	8	8	**PA18**	8	8
PA7	128	16	**PA19**	64	4
PA8	32	8	**PA20**	16	8
PA9	32	16	**PA21**	128	16
PA10	32	8	**PA22**	32	8
PA11	16	8	**PA23**	8	2
PA12	16	8	**PA24**	16	8

PA = *P . aeruginosa*, S = Sensitive , R = Resistance

Molecular detection of biofilms formation genes (*pelA, pslD*):

A total of 15 *P. aeruginosa* isolates were subjected to DNA extraction to detect the presence of two Biofilm formation genes (*pelA* and *pslD*), using conventional PCR technique was used with primers designed as shown in Tables (1). The detection assay was performed on 15 *P. aeruginos*a isolates. The amplified products were resolved by 1.5 percent agarose gel

electrophoresis in the presence of a molecular marker DNA ladder (100–1500 bp). The result of the genes detection in the current study showed 15 (100 %) isolates were carried *pelA* and *pslD* genes ,showed the bands (118bp) of DNA for *pelA* and bands (369 bp) of DNA for *pslD* .Figure (3) and Figure (4) This result almost agrees with the results of [36] who stated that the percentage of *pelA* and *pslD,* was 97% and 95.9% respectively ,were found in all clinical isolates of *P. aeruginosa*. The results of our research found awhole hundred percent occurrence of *pelA gene* which is higher than the rates reported by [37] They record that *PelA* gene was detected in (80%) of *P. aeruginosa* , the biofilm producing isolates and those associated with the polysaccharide stage of biofilm development . Our result nearly in close to finding of Badal *et al.,(*2020) [38]were he detect *pslD* gene among21 isolates of 23

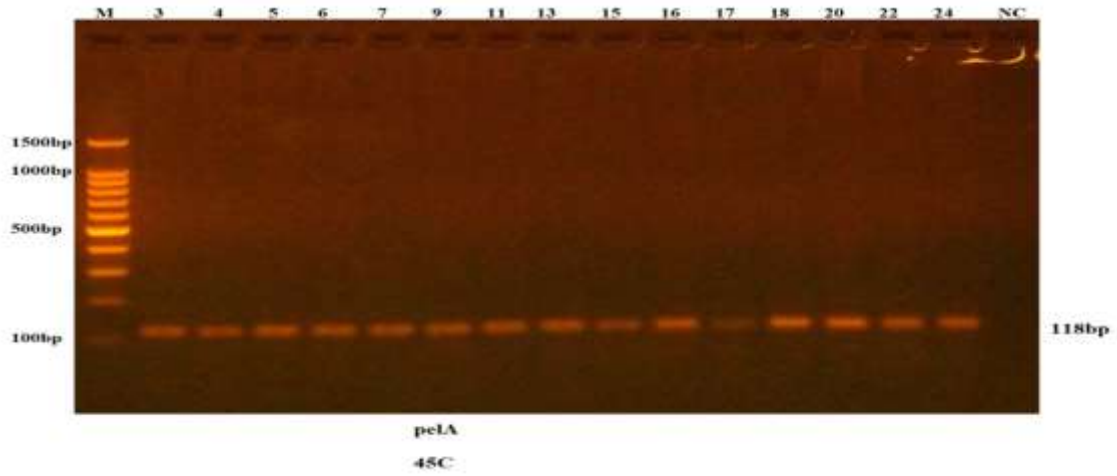

Figure 3: Amplification of *P. aeruginosa pelA* gene was fractionated on 2% agarose gel electrophoresis and stained with an Eth.Br. M:100bp ladder marker. Lanes (3, 4, 5,6, 7, 9, 11, 13, 15, 16, 17, 18, 20, 22, and 24) look like 118bp PCR products.

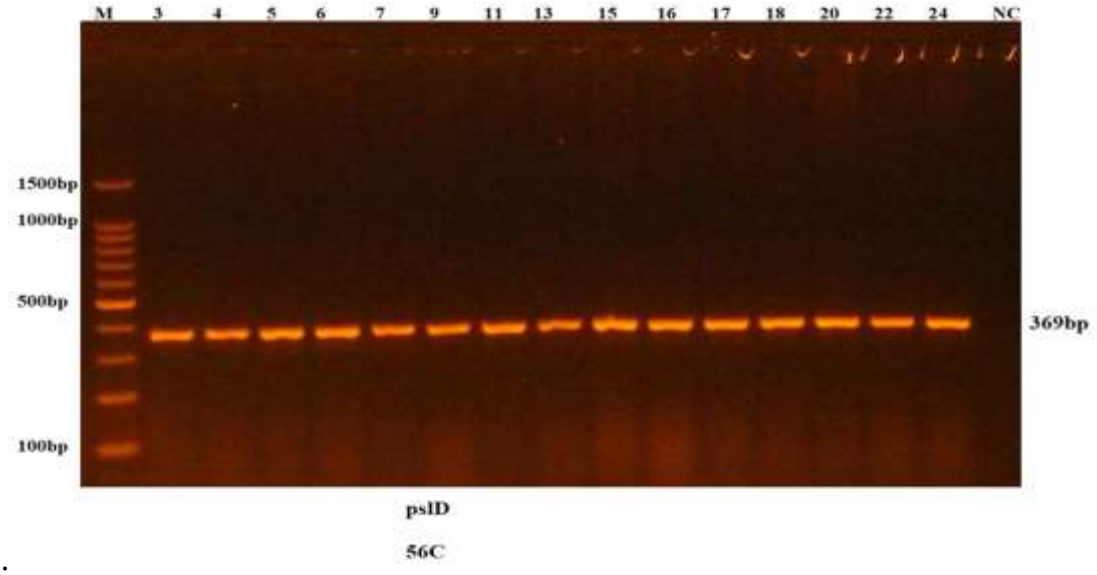

Figure 4: Amplification of *P. aeruginosa pslD* gene was fractionated on 2% agarose gel electrophoresis and stained with an Eth.Br. M:100bp ladder marker. Lanes (3, 4, 5, 6, 7, 9, 11, 13, 15, 16, 17, 18, 20, 22, and 24) look like 369bp PCR products.

Molecular detection of efflux pump *mexA* gene:

The detection of efflux pump *mexA* gene in all the (24) *P.aeruginosa* isolates were done by molecular technique the conventional PCR technique. A total of 15 bacterial isolates were subjected to DNA extraction with primers designed as shown in Tables (1). The present study showed that the gene of efflux pump in *P. aeruginosa* were distributed globally and found in about 100 percent of clinical isolates this indicate 15 isolates carried the bands (316 bp) of DNA for *mexA*. Figure(5) These result agree with the findings of [39, 40] both researchers discovered that the gene efflux pump *mexA*, were the most abundant and found in almost every isolate (100%). This result is similar to that of [41, 42], who indicate the presence of *mexA*, in all strains of *P. aeruginosa* by 90–100%. result is in agreement with the findings of [43] in Iraq,who found that all isolates (100%) the exhibited efflux pump *MexA*, but the result was higher than the findings of Mansoura University Hospital in Egypt [44] who found the presence of efflux pump genes in 66.03%.

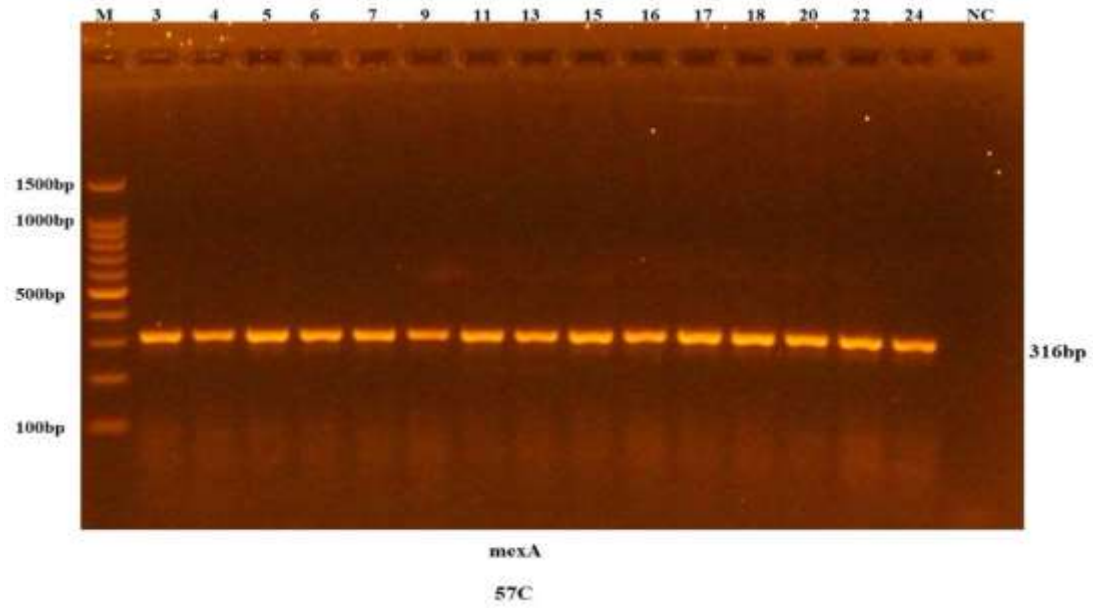

Figure 5: Amplification of *P. aeruginosa mexA* gene was fractionated on 2% agarose gel electrophoresis and stained with an Eth.Br. M:100bp ladder marker. Lanes (3, 4, 5, 6, 7, 9, 11, 13, 15, 16, 17, 18, 20, 22, and 24) look like 316bp PCR products.

Gene expression study by quantitative RT-PCR:

The quantitative RT-PCR reaction of three MDR resistant isolates *of P. aeruginosa*. The isolates weresubjected to three treatments to study the effect of Ciprofloxacin and Resveratrol as EPIs and their combination on the expression of *pelA,pslD and mexA*. The treatments included, sub-MIC Ciprofloxacin (8 , 4 , 16 µg/ ml) sub-MIC Resveratrol (32, 256, 32 µg /ml) and a combination of the two agents for each isolate .

Effect of ciprofloxacin and resveratrol on expression of *pelA* gene

Quantitative changes in the mRNA expression levels were determined using the comparative threshold cycle (Ct) method (2-ΔΔCt). A few changes in the expression fold of each isolate after exposure to Ciprofloxacin alone, when the average of treated isolates reached 1.08, resulted in a noticeable attempt by the bacteria for survive and grow. In the treatment of isolates with sub-MIC of resveratrol, and mixture of sub - MIC of two agents the results showed decreased in the

expression of *pelA* with a low level of folding where mean after treatment become 0.67 and 0.45 respectively, Table(4).

This result is beneficial for changing antibiotic resistance because Resveratrol increases Ciprofloxacin susceptibility **[45]**.The results of the current study show that the combination of Ciprofloxacin and Resveratrol was far better than either alone drug in reducing the expression of the *pelA* gene and, consequently, the ability of the drugs to kill the bacteria, or achieving a synergistic effect greater than the antibiotics when used alone [46].Bacterial biofilms are a characteristic of bacteria that enhance the bacterium's ability to resist unfavorable environmental conditions, like antibiotics. Plant extracts are able to control the biofilm formation and growth in *P. aeruginosa* as their active constituents can affect pre-formed biofilms and prevent the formation of new ones [47]. Several plant extracts have shown antibiofilm activity, affecting existing biofilms and preventing the formation of new ones. The anti-biofilm inhibitory effect occurs through various mechanisms, such as disruption of cell-to-cell communication, inhibition of cell mobility, stimulation of bacterial dispersal, and others [48]. According toastudy conducted by [12] state that resveratrol can alter bacterial virulence, as decrease membrane integrity and prevent biofilm formation.

Table 4: Ct values and fold of expression for *pelA* of *P. aeruginosa* treated with Ciprofloxcin and Resveratrol.

Isolate	*fbp*	*pelA*	ΔCT	ΔΔCT	Folding	Mean
PA 3	19.21	20.80	1.59	0.00	1.00	1
PA 6	19.50	21.17	1.67	0.00	1.00	
PA 9	22.45	26.43	3.97	0.00	1.00	
PA 3 C	23.79	27.64	3.84	2.25	0.21	1.08
PA 6 C	18.38	20.14	1.75	0.08	0.95	
PA 9 C	20.74	23.66	2.92	-1.06	2.08	
PA 3 RES	22.77	26.67	3.90	2.31	0.20	0.67
PA 6 RES	20.54	22.60	2.06	0.39	0.77	
PA 9 RES	21.84	25.74	3.90	-0.07	1.05	
PA 3 C + RES	21.24	24.14	2.91	1.32	0.40	0.45
PA 6 C + RES	17.20	19.57	2.37	0.70	0.61	
PA 9C + RES	19.05	24.52	5.47	1.50	0.35	

PA =*Pseudomonas aeruginosa*, C = Ciprofloxacin, RES = Resveratrol

Effect of Ciprofloxacin and Resveratrol on expression of *pslD* gene

Quantitative changes in mRNA expression levels were determined using the comparative threshold cycle (Ct) method (2-ΔΔCt). Few changes occur in the expression folds of *pslD* gene for isolates after exposure to sub MIC of Ciprofloxacin and sub MIC of Resveratrol alone, when the mean of folding all isolates reached 0.98 and 1.04 respectively resulting in a noticeable attempt by bacteria for survival and growth. Whereas in the treatment of isolates with combination of Ciprofloxacin and Resveratrol the results s high reduction in the expression of *pslD* with low mean of folding 0.61 Table(5). results show of the current study show that the combination of Ciprofloxacin and Resveratrol was far better than a single drug in lowering expression of the *pslD* gene and beneficial for changing antibiotic resistance because Resveratrol increases Ciprofloxacin susceptibility **[45]**, consequently, the ability of the drugs to kill the bacteria, or get synergistic effect greater than the antibiotics if when used alone [46]. Plant-derived compounds showed significant anti-biofilm activity against *P. aeruginosa* [49]. The finding of [12], other results was similar to our finding when they concluded that Resveratrol are capable of preventing biofilm formation by affecting bacterial virulence and lowering the integrity of membrane [14] reported that Resveratrol can significantly improve the effect of aminoglycoside antibiotics on *Pseudomonas aeruginosa* biofilms.

Table 5: Ct values and fold of expression for *pslD* of *P.aeruginosa* treated with Ciprofloxcin and Resveratrol

Isolate	*fbp*	*pslD*	ΔCT	ΔΔCT	Folding	Mean
PA 3	19.21	20.56	1.35	0.00	1.00	1
PA 6	19.50	20.71	1.21	0.00	1.00	
PA 9	22.45	23.88	1.43	0.00	1.00	
PA 3 C	23.79	24.66	0.87	-0.48	1.40	0.98
PA 6 C	18.38	19.99	1.61	0.40	0.76	
PA 9 C	20.74	22.49	1.74	0.31	0.80	
PA 3 RES	22.77	24.13	1.37	0.02	0.99	1.04
PA 6 RES	20.54	21.92	1.38	0.17	0.89	
PA 9 RES	21.84	22.95	1.12	-0.32	1.24	
PA 3 C + RES	21.24	22.67	1.43	0.08	0.94	0.61
PA 6 C + RES	17.20	18.75	1.56	0.35	0.79	
PA 9C + RES	19.05	23.65	4.61	3.17	0.11	

PA =*Pseudomonas aeruginosa*, C = Ciprofloxacin, RES = Resveratrol

Effect of Ciprofloxacin and Resveratrol expression of *mexA* gene

The Ct values of the mexA gene amplification were recorded using the qRT-PCR software in a single step. The positive results showed amplification at the Ct (threshold cycle) value of the mexA gene under this study. After treatment of isolates by sub MIC of ciprofloxacin resulted in the noticeable effort of bacteria for survival and growth, while the decrease of mean fold after exposure to sub MIC of resveratrol as efflux pump inhibitor and combination of two agents to 0.88 and 0.90, respectively. 90 respectively, this result benefit for changing the antibiotic resistance Tables(6).

The results of the current study show that the combination of ciprofloxacin and resveratrol was far better than a single drug in lowering the expression of the mexA gene and, consequently, the ability of the drugs to kill the bacteria, or obtain a synergistic effect greater than the antibiotics when used alone [46].The average fold change in the expression of the *mexA* gene decreased after treatment with Ciprofloxacin and Resveratrol, both individually and in combination, for each of the three *Pseudomona. aeruginosa* isolates with resistance. The fold changes were 1.02, 0.88, and 0.90, respectively. The efflux pump transporter plays a crucial part in this process. These membrane proteins that are always present ensure that the amount of antibiotic inside the bacteria is kept at low level. This allows the bacteria to take longer-term protective measures against the antibiotic, such as changing the expression levels of the right genes or developing mutations [50].The expression is reduced after treatment with resveratrol at a concentration of 0.88.This suggest that resveratrol has the potential to increase the sensitivity of multi drug susceptibility *Pseudomonas aeruginosa* and enhance its bactericidal efficacy. These results are useful in using the Resvratrol in combination to reduce the antibiotics resistance and increase the activity of antibiotic in treatment of bacterial diseases [45]. Some plant-derived compounds, such as polyphenolic molecules, the attach directly to the protein structures of the efflux pump can, causing channel and preventing the substance from being eliminated [51] .(EPIs) that inhibit the activity of drug efflux pumps can limit the build-up of antibiotics needed to eliminate germs, thereb lowering resistance and reversing multidrug resistance (MDR) [52].

Table 6: Ct values and fold of expression for *mexA* gene of *P.aeruginosa* treated with Ciprofloxcin and Resveratrol.

Isolate	*fbp*	*mexA*	ΔCT	ΔΔCT	Folding	Mean
PA 3	19.21	20.00	0.78	0.00	1.00	1
PA 6	19.50	19.76	0.26	0.00	1.00	
PA 9	22.45	24.18	1.73	0.00	1.00	
PA 3 C	23.79	24.87	1.08	0.30	0.81	1.02
PA 6 C	18.38	18.52	0.14	-0.13	1.09	
PA 9 C	20.74	22.23	1.49	-0.23	1.18	
PA 3 RES	22.77	24.29	1.52	0.74	0.60	0.88
PA 6 RES	20.54	21.26	0.71	0.45	0.73	
PA 9 RES	21.84	23.18	1.34	-0.39	1.31	
PA 3 C + RES	21.24	22.42	1.18	0.40	0.76	0.90
PA 6 C + RES	17.20	16.58	-0.62	-0.88	1.84	
PA 9C + RES	19.05	23.88	4.84	3.11	0.12	

PA =*Pseudomonas aeruginosa*, C = Ciprofloxacin, RES = Resveratrol

Conclusion

The prevalence of *P. aeruginosa* among patients with lower respiratory tract diseases in Baquba city were (12%).Resveratrol reported a high effect in reduction of the Ciprofloxacin MIC of most isolates. The results of molecular detection showed that all *P.aeruginosa* isolates contain *pelA* & *pslD* of biofilm genes and *mexA* efflux pump gene, indicating that efflux pumps and biofilm are very important to confer antibiotics resistance in *P. aeruginosa* isolates. The investigation of gene expression by qRT-PCR after treatment of three resistance isolates with sub–MIC of resveratrol as natural source (EPIs) , comparison effect of resveratrol and Ciprofloxacin combination with that of each agent when used as alone cause more reduction. On gene expression of *pelA*, *pslD*, and *mexA* genes. This result useful in using the resvratrol in combination with antibiotics to reduce the antibiotic resistance and increase the activity of antibiotic in the treatment of bacterial diseases.

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References

[1] R. Farhan, A. Hanora, M. M. Azab, S. Solyman, Detection of resistant and biofilm forming Pseudomonas aeruginosa isolated from burn patients in Ismailia governorate, Records of Pharmaceutical and Biomedical Sciences, 7(2),143-51(2023), DOI(https://doi.org/10.21608/rpbs.2023.210346.1226)

[2] D. Reynolds, M. Kollef, The epidemiology and pathogenesis and treatment of Pseudomonas aeruginosa infections: an update, Drugs, 81(18),2117-31(2021), DOI(https://doi.org/10.1007/s40265-021-01635-6)

[3] D. Bilton, Update on non-cystic fibrosis bronchiectasis, Current opinion in pulmonary Medicine,14(6), 595-9(2008), DOI(https://doi.org/10.1097/mcp.0b013e328312ed8c)

[4] M. Loebinger, A. Wells, D. Hansell, N. Chinyanganya, A. Devaraj, M. Meister, Mortality in bronchiectasis: a long-term study assessing the factors influencing survival, European Respiratory Journal, 34(4), 843-9(2009), DOI(https://doi.org/10.1183/09031936.00003709)

[5] DL. Ferguson, ES. Gloag, MR. Parsek, DJ. Wozniak, Extracellular DNA enhances biofilm integrity and mechanical properties of mucoid Pseudomonas aeruginosa, Journal of Bacteriology, 205(10), e00238-23(2023), DOI(https://doi.org/10.1128/jb.00238-23)

[6] S. Basak, S. Das, Pseudomonas aeruginosa, the Superbug, (Cambridge Scholars Publishing, 2022)

[7] MA. Webber, LJ. Piddock, The importance of efflux pumps in bacterial antibiotic resistance, Journal Of Antimicrobial Chemotherapy, 51(1), 9-11(2003), DOI(https://doi.org/10.1093/jac/dkg050)

[8] AL. Colclough, I. Alav, EE. Whittle, HL. Pugh, EM. Darby, SW. Legood, RND efflux pumps in Gram-negative bacteria; regulation, structure and role in antibiotic resistance, Future Microbiology, 15(2),143-57(2020), DOI(https://doi.org/10.2217/fmb-2019-0235)

[9] G. Cabot, AA. Ocampo-Sosa, F. Tubau, MD. Macia, C. Rodríguez, B. Moya, Overexpression of AmpC and efflux pumps in Pseudomonas aeruginosa isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study, Antimicrobial Agents and Chemotherapy, 55(5), 1906-11(2011), DOI(https://doi.org/10.1128/aac.01645-10)

[10] FF. Tuon, LR. Dantas, PH. Suss, VS. Tasca Ribeiro, Pathogenesis of the Pseudomonas aeruginosa biofilm: a review, Pathogens, 11(3), 300(2022), DOI(https://doi.org/10.3390/pathogens11030300)

[11] Z. He, Z. Huang, W. Zhou, Z. Tang, R. Ma, J. Liang, Anti-biofilm activities from resveratrol against Fusobacterium nucleatum, Frontiers in Microbiology, 7, 1065(2016), DOI(https://doi.org/10.3389/fmicb.2016.01065)
[12] DA. Rasko, V.Sperandio, Anti-virulence strategies to combat bacteria-mediated disease, Nature Reviews Drug discovery, 9(2),117-28(2010), DOI(https://doi.org/10.1038/nrd3013)
[13] M. Vestergaard, H. Ingmer, Antibacterial and antifungal properties of resveratrol, International Journal of Antimicrobial Agents, 53(6),716-23(2019), DOI(https://doi.org/10.1016/j.ijantimicag.2019.02.015)
[14] J. W. Zhou, T. T. Chen, X. J. Tan, J. Y. Sheng, A. Q. Jia, Can the quorum sensing inhibitor resveratrol function as an aminoglycoside antibiotic accelerant against Pseudomonas aeruginosa, International journal of Antimicrobial Agents, 52(1), 35-41(2018), DOI(https://doi.org/10.1016/j.ijantimicag.2018.03.002)
[15] P. Tille, Bailey & Scott's diagnostic microbiology-E-Book: Elsevier Health Sciences,(2015)
[16] JG. Cappuccino, N. Sherman Microbiology: a laboratory manual: Pearson Higher Ed, (2013).
[17] DI. Andersson, D. Hughes, Microbiological effects of sublethal levels of antibiotics, Nature Reviews Microbiology, 12(7), 465-478(2014), DOI(https://doi.org/10.1038/nrmicro3270)
[18] F. MKK, R. MA, S. Rashid, N. MHM, Detection of virulence factors and beta-lactamase encoding genes among the clinical isolates of Pseudomonas aeruginosa, arXiv preprint arXiv:190202014(2019), DOI(https://doi.org/10.31838/ijpr/2019.11.01.031)
[19] KM. Colvin, VD. Gordon, K. Murakami, BR. Borlee, DJ. Wozniak, G. C.Wong, The pel polysaccharide can serve a structural and protective role in the biofilm matrix of Pseudomonas aeruginosa, PLoS Pathogens, 7(1), e1001264(2011), DOI(https://doi.org/10.1074/jbc.M115.674929)
[20] M. Banar, M. Emaneini, M. Satarzadeh, N. Abdellahi, R. Beigverdi, WB. Leeuwen, Evaluation of mannosidase and trypsin enzymes effects on biofilm production of Pseudomonas aeruginosa isolated from burn wound infections, PloS One, 11(10), e0164622(2016), DOI(https://doi.org/10.1371/journal.pone.0164622)
[21] B. Ozer, N. Duran, Y. Onlen, L. Savas, Efflux pump genes and antimicrobial resistance of Pseudomonas aeruginosa strains isolated from lower respiratory tract infections acquired in an intensive care unit, The Journal of Antibiotics, 65(1), 9-13(2012), DOI(https://doi.org/10.1038/ja.2011.102)

[22] Q. Chang, T. Amemiya, J. Liu, X. Xu, N. Rajendran, K. Itoh, Identification and validation of suitable reference genes for quantitative expression of xylA and xylE genes in Pseudomonas putida mt-2, Journal of Bioscience And Bioengineering, 107(2), 210-4(2009), DOI(https://doi.org/10.1016/j.jbiosc.2008.09.017)

[23] S. Chakravarty, CN. Melton, A. Bailin, TL. Yahr, GG. Anderson, Pseudomonas aeruginosa magnesium transporter MgtE inhibits type III secretion system gene expression by stimulating rsmYZ transcription, Journal of Bacteriology, 199(23),10.1128/jb. 00268-17(2017), DOI(https://doi.org/10.1128/jb.00268-17)

[24] MR. Green, J. Sambrook, Molecular cloning, A Laboratory Manual, 4th ed., 448(2012)

[25] D. Parai, M. Banerjee, P. Dey, A. Chakraborty, E. Islam, SK. Mukherjee, Effect of reserpine on Pseudomonas aeruginosa quorum sensing mediated virulence factors and biofilm formation, Biofouling, 34(3), 320-34(2018), DOI(https://doi.org/10.1080/08927014.2018.1437910)

[26] K. Carroll, J. Hobden, Pseudomonads and Acinetobacter, Jawetz, Melnick, & Adelberg's Medical Microbiology, (McGraw-Hill Education, New York Carroll KC JA Hobden, 2016), 245-251

[27] KK. Aryal, S. Mehata, S. Neupane, A. Vaidya, M. Dhimal, P. Dhakal, The burden and determinants of non communicable diseases risk factors in Nepal: findings from a nationwide STEPS survey, PloS one, 10(8), e0134834(2015), DOI(https://doi.org/10.1371/journal.pone.0134834)

[28] M. Zainab, Detection of CTX-M-1 and CTX-M-3 genes in virulent, multidrug resistant Pseudomonas aeruginosa isolates: M. Sc. Thesis, College Of Education For Pure Science/Diyala University,(2017)

[29] K. Todar, Pseudomonas and related bacteria, Todar's online text book of bacteriology, (2004)

[30] NMH. Al-mamari, Isolation and Identification of Pseudomonas aeruginosa from Some Clinical And Environmental Samples And Study It, s Activity for The Production of Pyocyanin and Protease, Journal of Education and Science, 28(4), 93-107(2019), DOI(https://doi.org/10.33899/edusj.1970.163328)

[31] T. Spilker, T. Coenye, P. Vandamme, JJ. LiPuma, PCR-based assay for differentiation of Pseudomonas aeruginosa from other Pseudomonas species recovered from cystic fibrosis patients, Journal of Clinical Microbiology, 42(5), 2074-2079(2004), DOI(https://doi.org/10.1128/jcm.42.5.2074-2079.2004)

[32] AJ. Seukep, V. Kuete, L. Nahar, SD. Sarker, M. Guo, Plant-derived secondary metabolites as the main source of efflux pump inhibitors and methods for identification,

Journal of Pharmaceutical Analysis, 10(4), 277-90(2020), DOI(https://doi.org/10.1016/j.jpha.2019.11.002)

[33] M. Stavri, LJ. Piddock, S. Gibbons, Bacterial efflux pump inhibitors from natural sources, Journal of Antimicrobial Chemotherapy, 59(6), 1247-60(2007), DOI(https://doi.org/10.1093/jac/dkl460)

[34] PD. Gupta, TJ. Birdi, Development of botanicals to combat antibiotic resistance, Journal of Ayurveda and Integrative Medicine, 8(4), 266-75(2017), DOI(https://doi.org/10.1016/j.jaim.2017.05.004)

[35] R. Kumar, S. Pooja Patial, A review on efflux pump inhibitors of gram-positive and gram-negative bacteria from plant sources, Int J Curr Microbiol Appl Sci., 5(6), 834-55(2016), DOI(https://doi.org/10.20546/ijcmas.2016.506.092)

[36] P. Maita, K. Boonbumrung, Association between biofilm formation of Pseudomonas aeruginosa clinical isolates versus antibiotic resistance and genes involved with biofilm, (2014)

[37] I. Sharma, D. Choudhury, Detection of Pel A gene in P. aeruginosa from clinical samples using polymerase chain reaction with reference to biofilm production in NE India. Ind J Res. 4, 119-21(2015)

[38] D. Badal, AV. Jayarani, MA. Kollaran, A. Kumar, V. Singh, Pseudomonas aeruginosa biofilm formation on endotracheal tubes requires multiple two-component systems, Journal of Medical Microbiology, 69(6), 906-19(2020), DOI(https://doi.org/10.1099/jmm.0.001199)

[39] G. Wareth, C. Brandt, LD. Sprague, H. Neubauer, MW. Pletz, WGS based analysis of acquired antimicrobial resistance in human and non-human Acinetobacter baumannii isolates from a German perspective, BMC Microbiology, 21, 1-13(2021), DOI(https://doi.org/10.1186/s12866-021-02270-7)

[40] M. Ali, S. Suhad, Molecular detection for efflux pump adeIJK genes of multi-drug resistance Acinetobacter baumannii isolated from different clinical sources, Mcs thesis, (2021)

[41] C. Xu, S. Bilya, W. Xu, adeABC efflux gene in Acinetobacter baumannii, New microbes and new infections, 30, 100549(2019), DOI(https://doi.org/10.1016/j.nmni.2019.100549)

[42] R. Kaviani, I. Pouladi, M. Niakan, R. Mirnejad, Molecular detection of adefg efflux pump genes and their contribution to antibiotic resistance in Acinetobacter baumannii clinical isolates, Reports of Biochemistry & Molecular Biology, 8(4), 413(2020)

[43] T. Al Jumaily, RB. Rose'Meyer, A. Sweeny, R. Jayasinghe, Cardiac damage associated with stress hyperglycaemia and acute coronary syndrome changes according to level of

presenting blood glucose, International Journal of Cardiology, 196, 16-21(2015), DOI(https://doi.org/10.1016/j.ijcard.2015.05.143)

[44] MH. Zaki, T. Sayed, Automated analysis of pedestrian group behavior in urban settings, IEEE Transactions on Intelligent Transportation Systems, 19(6), 1880-9(2017), DOI(https://doi.org/10.1109/TITS.2017.2747516)

[45] L. Liu, C. Beck, K. Nøhr-Meldgaard, A. Peschel, D. Kretschmer, H. Ingmer, Inhibition of the ATP synthase sensitizes Staphylococcus aureus towards human antimicrobial peptides, Scientific Reports, 10(1), 11391(2020), DOI(https://doi.org/10.1038/s41598-020-68146-4)

[46] CB. Landersdorfer, NS. Ly, H. Xu, BT. Tsuji, JB. Bulitta, Quantifying subpopulation synergy for antibiotic combinations via mechanism-based modeling and a sequential dosing design, Antimicrobial Agents and Chemotherapy, 57(5), 2343-51(2013), DOI(https://doi.org/10.1128/AAC.00092-13)

[47] Z. Lou, KS. Letsididi, F. Yu, Z. Pei, H. Wang, R. Letsididi, Inhibitive effect of eugenol and its nanoemulsion on quorum sensing–mediated virulence factors and biofilm formation by Pseudomonas aeruginosa, Journal of Food Protection, 82(3), 379-89(2019), DOI(https://doi.org/10.4315/0362-028x.jfp-18-196)

[48] S. Gutierrez, A. Morán, H. Martínez-Blanco, MA. Ferrero, LB. Rodríguez-Aparicio, The usefulness of non-toxic plant metabolites in the control of bacterial proliferation, Probiotics and Antimicrobial Proteins, 9, 323-33(2017), DOI(https://doi.org/10.1007/s12602-017-9259-9)

[49] J. Rajkumari, S. Borkotoky, A. Murali, K. Suchiang, SK. Mohanty, S. Busi, Attenuation of quorum sensing controlled virulence factors and biofilm formation in Pseudomonas aeruginosa by pentacyclic triterpenes, betulin and betulinic acid, Microbial Pathogenesis, 118, 48-60(2018), DOI(https://doi.org/10.1016/j.micpath.2018.03.012)

[50] Y. Shuster, S. Steiner-Mordoch, N. Alon Cudkowicz, S. Schuldiner, A transporter interactome is essential for the acquisition of antimicrobial resistance to antibiotics, PLoS One, 11(4), e0152917(2016), DOI(https://doi.org/10.1371/journal.pone.0152917)

[51] LJ. Piddock, MI. Garvey, MM. Rahman, S. Gibbons, Natural and synthetic compounds such as trimethoprim behave as inhibitors of efflux in Gram-negative bacteria, Journal of Antimicrobial Chemotherapy, 65(6), 1215-23(2010), DOI(https://doi.org/10.1093/jac/dkq079)

[52] G. Spengler, A. Kincses, M. Gajdács, L. Amaral, New roads leading to old destinations: efflux pumps as targets to reverse multidrug resistance in bacteria, Molecules, 22(3), 468(2017), DOI(https://doi.org/10.3390/molecules22030468)