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THE PHENOLIC COMPOUNDS EXTRACTED FROM *Rosmarinus officinalis L*. AND EFFECT OF ON THE BIOFILM GENES IN *Pseudomonas aeruginosa*

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Abstract

The emergence of antibiotic resistance has made microbial infections one of the biggest public health issues in the world, and this has prompted researchers to look into the antibacterial properties of medicinal plants. Therefore, this study aimed to extract phenolic compounds from *Rosmarinus officinalis* and study their effect on some virulence genes responsible for biofilm formation in *Pseudomonas aeruginosa.* Ten *p. aeruginosa* isolates were collected from the Genetic Engineering and Biotechnology Institute/University of Baghdad labs. The isolates were cultured on cetrimide agar and identified using the VITEK-2 system. Aqueous and methanolic extracts of *Rosmarinus officinalis* leaves were made using the maceration technique and the Soxhlet equipment, respectively. Several experiments were conducted on the extracts including high performance liquid chromatography (HPLC), biofilm formation using the micro-titter plate method, and the expression of *algD* and *pelA* genes by comparing the isolates treated with methanolic and aqueous extracted with untreated isolates. The HPLC results showed four phenolic acids (gallic, rosmarinic, ferulic and caffeic) were found by comparing them to the retention times of the reference standards. The result of biofilm formation in *P. aeruginosa* revealed that 100 % of the isolates were strong biofilm formation. In addition, the treatment with the aqueous extract of the *Rosmarinus officinalis* inhibits the biofilm formation in 16 mg/ml, while the biofilm formation was inhibited by the methanolic extract in 64 mg/ml. The virulence genes *algD* and *pelA* responsible for biofilm formation in *P. aeruginosa* were investigated. The results showed the presence of these genes in all isolates. The gene expression results showed low levels of *algD* and *pelA* genes after being treated with the aqueous and methanolic extracts compared to the untreated isolates.

Keywords: *P. aerugenosa, R. officinales*, phenolec compounds, antibiofelm, *pelA*, *algD*.

1. Introduction

Bacterial and fungal resistance to antibiotics is one of the issues in biomedical and medical sciences; some of these bacteria are more than 90% resistant to chemical treatments (Jardak *et al*., 2017). In the 1950s, many dangerous bacteria have displayed antibiotic resistance, and this resistance is continually growing as new compounds and antibiotics are produced. These medications also progressively started to have negative side effects (Jafari-Sales *et al*., 2017). Because of their excessive usage, most bacteria now have higher levels of drug resistance to many antibiotics (Mobaiyen *et al*., 2016). This is one of the causes of the rising popularity of using herbs instead of synthetic antibiotics to treat bacterial infections since they are low-risk, cheap, and affordable natural substances. Natural products are increasingly being used in the drug-making process. Not only when bioactive compounds are used directly as therapeutic drugs but also when they are used as a raw material in the drug-synthesis process or as a starting point for the creation of novel biologically active compounds (AL-Azawi, 2017; Jafar-sales and Shadi-Dizaji, 2019; Alhan and AL-Azawi, 2022). As a result, the use of herbal medicines as an alternative to chemical medications and antibiotics was considered.

Rosmarinus officinalis is a Lamiaceae family plant that is widely used as a spice and a medicinal herb in many nations. It can help fight cancer and bacteria since it has the highest levels of antioxidants. The antioxidant activities of *Rosmarinus officinalis* extracts vary depending on genetic and growing circumstances, location, geographic origin, weather, extraction technique, primary plant characteristics, and harvest date (Andrade *et al*., 2018). The rosemary plant has significant

immune-boosting abilities, as well as the ability to fight against bacterial illnesses, particularly those that affect the stomach. Additionally, rosemary is linked to preventing *staphylococcus* infection which threatens thousands of lives each year (Habtemariam, 2016).

2. Materials and Methods

2.1 Reagents for Chemistry

The reagents chemical and pure methanol were bought from (BDH, England). Cetrimide agar, Nutrient agar, (Himedia, India) and violet crystal from (Pro-Lab, Canada).

2.2 Bacterial Isolates

Ten *P. aeruginosa* isolates were obtained from the Institute of Genetic Engineering and Biotechnology - University of Baghdad which was previously collected from multiple sources from Baghdad city hospitals and was diagnosed by the chemical and molecular tests. The diagnosis was confirmed by culturing the isolates on cetrimide agar for 18-24h at 42°C and using VITEK-2 System. The isolates were activated by re-culture on the Nutrient agar medium and incubated aerobically at 37°C for 24 hours.

2.3 Assessment of biofilm formation

Quantification of biofilm formation by *P. aeruginosa* was assessed as described by Patel *et al*. (2016); in Brain Heart Infusion Broth, all isolates were cultivated over the course of one night at 37 °C. Each isolate was added to tryptic soy broth (TSB) that contained 1% glucose, and the mixture was well mixed by pipetting. The bacterial isolate's suspension was calibrated to the 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. 200 µl of 0.1% crystal violet were poured into each well and left there for 15 minutes to stain the adherent cells. After the staining process was finished, additional stain was removed by repeatedly washing with distilled water (2–3 times). The plate was dried by leaving it 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 by using an ELISA auto reader at a wavelength of 630 nm. The average of sterile medium OD values was computed and subtracted from all test readings. The cut off value (ODc) was computed which can help isolates be classified as biofilm producers or not (Kirmusaoglu, 2019).

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

According to the cutoff value (ODc) calculation, the biofilm detection result is as follows:

ODc ≥ OD (no biofilms are produced).

2 × ODc ≥ OD > ODc (biofilm production weak).

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

OD > 4× ODc (strong biofilm production).

2.4 Collect of *Rosmarinus officinalis L.*

The specialist at the Department of Biology, College of Science, University of Baghdad, identified the *Rosmarinus officinalis* collected from the plantation in Baghdad city as (*Rosmarinus officinalis L*.). The leaves were cleaned with water, air dried, powdered in a grinder, and then kept at 4^{o}C for subsequent study.

2.4.1 Preparation of *Rosmarinus officinalis* extracts

Firstly, in order to defat the leaves, 400 grams of *Rosmarinus officinalis* leaves powder was macerated with 2 liter of petroleum ether solvent. The residue was collected, air-dried, and separated into two batches. Each batch of the defatted plant leaves was individually extracted with hot water and methanol to prepare aqueous and methanolic extracts according to N'Guessan *et al*. (2007) and AACC (1984) respectively.

2.5 High-Performance Liquid Chromatography (HPLC)

Methanolic and aqueous extracts of *Rosmarinus officinalis* preparation were identified by (HPLC) according to Radovanovic *et al*. (2015).

2.6 Study the antibiofilm activity of *Rosmarinus officinalis* extracts

The antibiofilm activity of the methanolic and aqueous extracts of *Rosmarinus officinalis* was evaluated using the 96-well microtiter plate. working solution of the plant extracts was prepared at 128 mg/ml for the methanolic and aqueous extract to make the concentrations (128-1) mg/ml. The first wells in row A were filled with 200 µl of each sample, columns had 100 l of the broth in just rows B through H. Using a micropipette, twofold serial dilutions were performed methodically down the columns (from rows A-H). 100 µl was withdrawn from the starting concentrations in row A and transferred to the next row with the 100 µl broth which was appropriately mixed, and the operation was repeated until the last row (H) and the last 100 µl was discarded. Except for the negative control, 100 µl of the 1106 CFU/ ml bacterial inoculum was added to each well. The same procedure was done as indicated in paragraph (**Assessment of biofilm formation**).

2.7 Molecular detection5 of Virulence Genes Using Polymerase Chain Reaction (PCR)

2.7.1 Extraction of Genomic DNA

DNA was extracted from *P. aeruginosa* bacteria using a commercial purification system (Genomic DNA Extraction Mini Kit (iNtron®, Korea).

2.7.2 Molecular Detection of *algD* and *pelA* Gene

This stage involves doing the reaction using the optimal PCR conditions for the gene as shown in PCR conditions for *algD* gene (Mitov *et al*., 2010) and PCR conditions for *pelA* gene (Maita and Boonbumrung, 2014) which includes adding 12.5 µl from OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1 µl 10 pmol/µl from each primer and 7.5 µl of free-nuclease water.

2.8 Gene Expression Analysis Using qRT PCR Technique

To assess the effect of the *Rosmarinus officinalis* extract on the gene expression of *algD* and *pelA* related to biofilm formation, measurement of the gene expression of 2 genes in the resistant isolates was done before and after the treatment with the methanolic and aqueous extract.

RNA was extracted by using TRIzol™ Reagent according to the the protocol described by the manufacturer. In order to assess the gene expression of *algD* and *pelA* gene, primers are listed in Table 1; the reaction mixture was summarized in Table 2. Moreover, after several trials, the thermo cycler protocol was optimized and the protocol is listed in Tables 3.

The qRT-PCR data findings were computed based on a direct comparison of the Ct values between the target and reference (housekeeping) genes. The genes were analyzed by the relative quantification of gene expression levels (fold change) using the ΔΔCt method described by (Schmittgen *et al.*, 2008).

Table (1): The Primers used in this Study

Primer name		Sequence (5´-3´)	Production size	Reference
PelA	F	CCTTCAGCCATC CGTTCTTCT	118 bp	Colvin *et al.*, 2011
	R	TCGCGTACGAAG TCGACCTT		
algD	F	GAGGAATACCAG CTGATCCGG	190 bp	Newly Designed
	R	CACCGAGTTCAA GGACCTGAA		
House Keeping gene	F	GTGCTATACCGC TGGGATCAA	238 bp	Newly Designed
	R	GGTTCTATTTGC TGTGAATCC		

Table (2): Volumes and concentrations of qRT-PCR reaction mix

Component	Volume (µl)
Universal Luna qPCR Master Mix	10
Primer forward (10 µM)	1
Primer reverse (10 µM)	1
DNA template	5
Free-Nuclease Water	3
Total	**20**

Table (3): qRT-PCR Cycling Program

Cycle Step	Temperature	Time	Cycles No.
Initial Denaturation	94 °C	55 seconds	1
Denaturation	94°C	18 seconds	40
Annealing	59 °C	35 seconds	
Melt Curve	59-94 °C	40 minutes	1

3. Results and Discussion

3.1 Detection of biofilm Formation

The quantitative production of biofilms is detected using the microtiter plate technique, with the absorbance being measured at 630 nm by an ELISA reader. Isolates have an average optical density of 0.435 0.287. (in the range 0.215 – 0.622). The results indicated that as mentioned in (Table 4), all isolates were 100% vigorous biofilm formation. *P. aeruginosa* ability to build biofilm is a crucial component of bacterial pathogenicity which promotes bacterial survival in a variety of conditions including burn wounds, and ultimately leads to chronic infections (De

Almeida Silva *et al*., 2017). Previous research revealed a link between *P. aeruginosa* ability to produce biofilms and its multidrug resistance phenotype (Yekani *et al*., 2017). Bacalso *et al*. (2011) emphasized the significance of biofilm and its part in the development of high antibiotic resistance by several bacterial species. Bacteria ability to create biofilms facilitates their attachment to host cells (Ramos *et al*., 2013). The results of the current study agree with Al-Nuaimi (2015) findings, which determined that 100% of isolates are capable of forming biofilm.

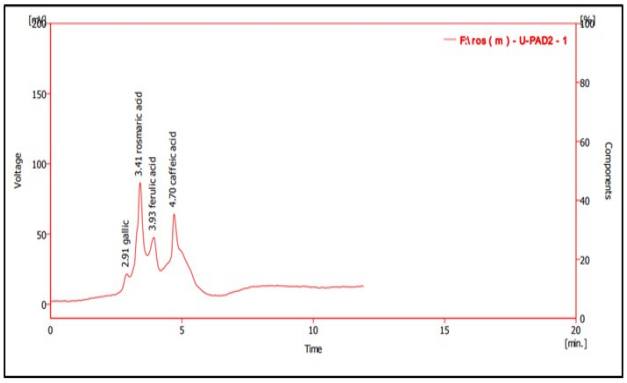
Table (4): Biofilm forming of *P. aeruginosa* isolates

P. aeruginosa isolates	Source of isolate	Biofilm formation
P_1	Wound	Strong
P_2	Wound	Strong
P_3	Burn	Strong
P_4	Sputum	Strong
P_5	Urine	Strong
P_6	Burn	Strong
P_7	Ear	Strong
P_8	Wound	Strong
P_9	Burn	Strong
P_{10}	Urine	Strong

(P): *P. aeruginosa* isolate, Control negative (cut off) =0.149

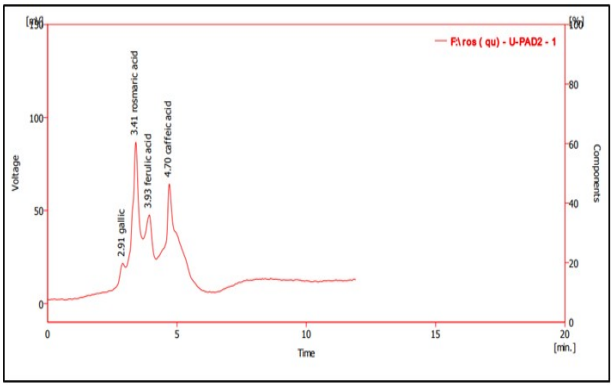
3.2 High-performance Liquid Chromatography (HPLC)

According to Radovanovic *et al*. (2015) individual phenolic contents of Rosemary officinalis were examined using the HPLC technique. In this study, 4 phenolic compounds (gallic acid, rosmarinic acid, ferulic acid and caffeic acid) were detected in methanolic and aqueous extracts (Figures 1 and 2) respectively, when compared with standard compounds (Figure 3). A quantitative analysis of *Rosemary officinalis* extracts showed different quantities in the methanolic and aqueous extract as shown in Table (5). In this study, the quantity of phenolic compounds in the aqueous extract was higher than in methanolic extract. This may be due to the use of hot water in the extraction, and this result was in agreement with Parejo *et al*. (2002) who noted that plant material subjected to hydro-distillation contained more phenolic compounds than plant material not subjected to distillation. When exposed to heat, some bonded phenolics or phenolics found in cell walls may release, produce additional phenolics that to be extracted (Bubonja-Sonje *et al*., 2011; Jordan *et al*., 2013). In previous reports for Wojdyło *et al*. (2007) the phenolic compounds determined in this study were approached in content and concentration, they mention that the *Rosemary officinalis* extracts contain rosmarinic acid, carnosic acid, caffeic acid and ferulic acid.



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.908	32.791	4.094	5.8	5.5	0.16	gallic
2	3.408	247.992	34.870	44.2	46.8	0.12	rosmaric acid
3	3.932	88.907	10.806	15.8	14.5	0.16	ferulic acid
4	4.704	191.980	24.750	34.2	33.2	0.13	caffeic acid
	Total	561.671	74.521	100.0	100.0		

Figure (1): HPLC of phenolic compounds in *Rosemary officinalis* methanolic extract



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.908	57.214	5.612	2.7	4.5	0.20	gallic
2	3.408	699.427	56.982	32.5	45.5	0.18	rosmaric acid
3	3.932	227.001	18.187	10.5	14.5	0.22	ferulic acid
4	4.704	1168.684	44.405	54.3	35.5	0.22	caffeic acid
	Total	2152.326	125.185	100.0	100.0		

Figure (2): HPLC of phenolic compounds in *Rosemary officinalis* aqueous extract

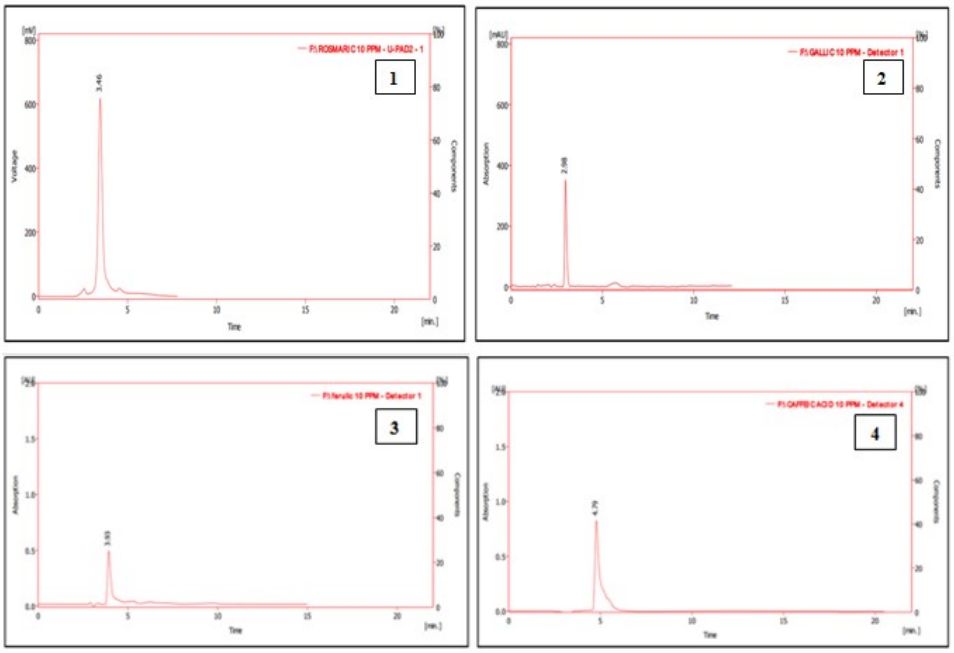


Figure (3): HPLC of phenolic compound standards (1): gallic acid, (2): rosmarinic acid, (3): ferulic acid, (4): caffeic acid

Table (5): Quantitative analysis of *Rosemary officinalis* leaves extracts

Phenolic compound	Methanolic extract (mg/200 g)	Aqueous extract (mg/200 g)
Gallic acid	3.86	6.73
Rosmarinic acid	48.74	137.47
Ferulic acid	9.55	24.39
Caffeic acid	32.45	197.69

3.3 Anti-Biofilm Activity of *Rosemary Officinalis* Extracts

A tightly packed group of microbial cells known as a biofilm attaches to and develops either on live or inanimate surfaces, and it surrounds itself with polymers secreted. Because of multidrug resistance, infections linked with biofilms are sometimes challenging to treat (Kumar *et al*., 2013). So, finding novel and potent compounds that inhibit the growth of bacterial biofilms is crucial.

Rosemary officinalis aqueous leaves extract inhibited 100% of the biofilm formation of *P. aeruginosa* in 16 mg/ml, as shown in (Table 6). While the antibiofilm activity of the methanolic extract on *P. aeruginosa* isolates was inhibited 100% in 64 mg/ml, as shown in (Table 7).

The ability to inhibit biofilm of the phenolic compounds was demonstrated by the inhibition or decrease of biofilm development in a concentration-dependent method. It is likely that flavonoids promote bacterial aggregation by partially lysing them. This results in membrane fusion, which limits the active nutrient absorption through a smaller membrane surface (Awolola *et al*., 2014). Numerous mechanisms of phenolics antibacterial activity have been identified including interactions with bacterial proteins and cell walls, damage to cytoplasmic membranes, decreased fluidity of membranes, and inhibition of the production of nucleic acids, or synthesis cell walls, or energy metabolism (Daglia, 2012). On the other hand, research on plant phenolics anti-biofilm activity has shown that, in addition to their harmful effects on bacteria, they also have "softer" effects that suppress biofilms by affecting bacterial regulatory systems like quorum sensing or other global regulator systems, without having any impact on bacterial growth (Silva *et al*., 2016). Furthermore, Liu *et al.* (2017) discovered that gallic acid killed *S. aureus* by penetrating the biofilm. Alama *et al*. (2020) revealed that phenolic compounds extracted from *B. ciliata* could be a potential candidate for drug discovery to treat *P. aeruginosa* PAO1, induced infectious diseases especially for its biofilm treatment. Ivanov *et al*. (2022) state that Rosmarinic acid was shown to have potential anticandidal and antibacterial action, and it was able to prevent cell attachment and remove existing biofilm by reducing mitochondrial activity, altering membrane integrity, and somewhat inhibiting protease synthesis.

Table (6): Biofilm formation of *P. aeruginosa* before and after treatment with *Rosmarinus officinalis* aqueous leaves extract

Isolates	Before Treatment (Control)	After Treatment Concentration (mg/ml)							
		1	2	4	8	16	32	64	128
P_1	Strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_2	Strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_3	strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_4	strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_5	Strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_6	Strong	Strong	Strong	Moderate	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_7	Strong	Strong	Strong	Moderate	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P_8	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P9	strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm
P10	strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm	No Biofilm

(P): *P. aeruginosa* isolate, Control negative (cut off) =0.11

Table (7): Biofilm formation of *P. aeruginosa* before and after treatment with *Rosmarinus officinalis* methanolic leaves extract

Isolates	Before Treatment (Control)	After Treatment Concentration (mg/ml)							
		1	2	4	8	16	32	64	128
P_1	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_2	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_3	strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_4	strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_5	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_6	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_7	Strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P_8	Strong	Strong	Moderate	Moderate	Weak	Weak	Weak	No Biofilm	No Biofilm
P9	strong	Strong	Strong	Moderate	Moderate	Weak	Weak	No Biofilm	No Biofilm
P10	strong	Strong	Moderate	Weak	Weak	No Biofilm	No Biofilm	No Biofilm	No Biofilm

(P): *P. aeruginosa* isolate, Control negative (cut off) =0.11

3.4 Molecular detection of genes responsible for biofilm formation in *P. aeruginosa*

3.4.1 Detection of *algD* and *pelA* Genes

In this study, the PCR technique was used to detect the *pelA* and *algD* genes that are responsible for the biofilm formation in *P. aeruginosa*. Gel electrophoresis results indicated the size of the amplicons as 118 bp for *pelA* and 190 bp for *algD*, compared with the DNA ladder; the results showed that all isolates of *P. aeruginosa* contain *algD* and *pelA* genes, as shown in (Figures 4 and 5). The *algD* and *pelA* genes encode for the synthesis of the alginate layer, an

Exopolysaccharide that has a high viscosity and protects the bacterial cell from host defense factors such as phagocytic cells and complement system components (Tan *et al*., 2014), This alginate layer is necessary for biofilm production and so provides protection when exposed to antibiotics (Lamppa and Griswold, 2013). The results of this experiment were in agreement with those of Wolska and Szweda (2009). They found that clinical isolates of *P. aeruginosa* possessed *algD* gene. Also, Garallah (2015) found that 65.38% of the isolates of *P. aeruginosa* contain the *algD* gene. A study by Elmaraghy *et al*. (2019) detected *algD* and *pelA* gene in 42 isolates (89.4%). Moreover, Al-Dahmoshi (2013) found that every isolate carried the *algD* gene and had a strong potential for the development of alginate biofilms which interfered with the *P. aeruginosa* isolates ability to respond to antibiotics.

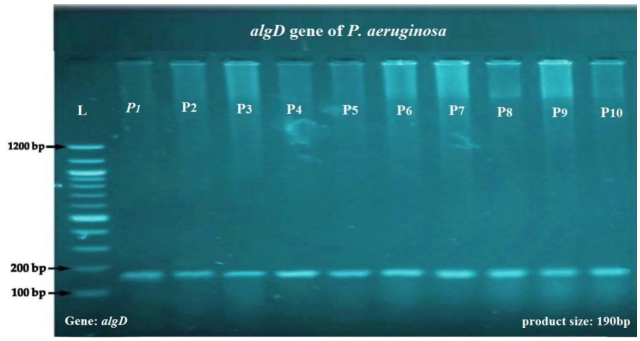


Figure (4): Gel electrophoresis of amplified *algD* (190 bp), from *P. aeruginosa* using conventional PCR. Agarose 2% stained with Ethidium bromide dye DNA ladder 100-1200 bp and visualized on a UV transilluminator

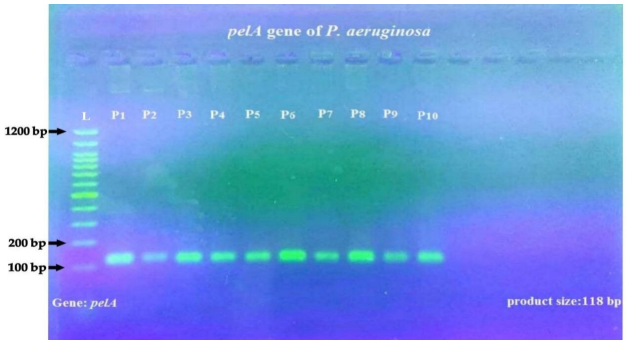


Figure (5): Gel electrophoresis of amplified *pelA* (118 bp), from *P. aeruginosa* using conventional PCR. Agarose 2% stained with Ethidium bromide dye DNA ladder 100-1200 bp and visualized on a UV transilluminator

3.5 Gene expression of *algD* and *pelA* genes

In this study, the quantitative RT-PCR assay analyzed the mRNA expression of *algD* and *pelA* genes by comparing untreated isolates with the isolates treated with the *Rosemary officinalis* methanolic and aqueous leaves extract (Tables 8, 9, 10 and 11). The results indicated a decrease in gene expression in *algD* and *pelA* genes. The reliability of qRT-PCR findings is heavily reliant based on the reference genes used (Huang *et al*., 2013) which is required for the accurate use of qRT-PCR to assess the target gene expression changing (Liu *et al*., 2016). Many previous studies conducted locally (AL-Mousawi *et al*., 2019; Ibraheem *et al*., 2019) used 16SrRNA gene as housekeeping gene. The amplification was recorded as Ct value (cycle threshold) indicating that high Ct values indicate low gene expression and low Ct value indicates a high gene expression. The housekeeping gene is used in molecular studies due to the fact that its expression remains constant in the cells or tissues and under different conditions (Schmittgen *et al*., 2008). In addition to anti-virulence mechanisms, phenolic compounds can exert antibacterial activity through a variety of mechanisms, including disruption of the cytoplasmic membrane, inhibition of nucleic acid synthesis, inhibition of energy metabolism, inhibition of folic acid synthesis, inhibition of cell membrane synthesis, and function (Al-Snafi, 2018; Al-Kamel and Al-Snafi, 2019). As a result, the antibacterial and down-regulation effects of phenolic extract in our investigation might be attributable to all of these pathways. Many researchers studied the effect of plant extracts on gene expression. For example, Pangastuti *et al*. (2020) used an ethyl acetate extract of *Curcuma aeruginosa* rhizomes and demonstrated that the plant extract could reduce the expression of *P. aeruginosa* virulence factors controlled by quorum sensing. Moreover, Al-Bayati (2021)

demonstrated that remarkable down-regulation effect of *Ficus carica* extracts on the *lasA* and *lasB* genes for 80% of *P. aeruginosa* isolates.

Table (8) Gene expression results for *algD* before and after treatment with *Rosmarinus officinalis* aqueous extract

Group	Sample	Ct reference gene *16S rRNA*	Ct target gene *algD*	ΔCT	ΔΔCt	Fold of gene Expression
Before treated (control)	C_1	38.5	13.13	-25.37	0	1
	C_2	37.76	13.12	-24.64	0	1
	C_3	33.17	18.34	-14.83	0	1
	C_4	32.81	15.81	-17	0	1
	C_5	39.7	13.33	-26.37	0	1
	C_6	37.94	14.51	-23.43	0	1
	C_7	39.15	20.69	-18.46	0	1
	C_8	39.5	12.01	-27.49	0	1
	C_9	34.61	13.97	-20.64	0	1
	C_{10}	38.86	14.89	-23.97	0	1
After treated	P_1	32.21	17.64	-14.57	10.8	0.00056089
	P_2	31.42	18.86	-12.56	12.08	0.00023097
	P_3	28.26	21.15	-7.11	7.72	0.00474295
	P_4	32.1	19.28	-12.82	4.18	0.05516894
	P_5	31.73	15.62	-16.11	10.26	0.00081552
	P_6	36.49	19.05	-17.44	5.99	0.01573368
	P_7	35.09	21.42	-13.67	4.79	0.03614651
	P_8	36.33	18.73	-17.6	9.89	0.00105393
	P_9	32.91	17.94	-14.97	5.67	0.01964083
	P_{10}	34.56	29.48	-5.08	18.89	2.0585 E-06

(P): *P. aeruginosa* isolate, **(C):** Control

Table (9) Gene expression results for *pelA* before and after treatment with *Rosmarinus officinalis* aqueous extract

Group	Sample	Ct reference gene *16S rRNA*	Ct target gene *pelA*	ΔCT	ΔΔCt	Fold of gene Expression
Before treated (control)	C_1	38.5	14.26	-24.24	0	1
	C_2	37.76	13.72	-24.04	0	1
	C_3	33.17	19.42	-13.75	0	1
	C_4	32.81	17.37	-15.44	0	1
	C_5	39.7	14.25	-25.45	0	1
	C_6	37.94	14.3	-23.64	0	1
	C_7	39.15	20.21	-18.94	0	1
	C_8	39.5	13.24	-26.26	0	1
	C_9	34.61	14.73	-19.88	0	1
	C_{10}	38.86	15.79	-23.07	0	1
After treated	P_1	32.21	19.29	-12.92	11.32	0.00039115
	P_2	31.42	19.86	-11.56	12.48	0.00017504
	P_3	28.26	22.34	-5.92	7.83	0.00439476
	P_4	32.1	20.9	-11.2	4.24	0.05292158
	P_5	31.73	17.68	-14.05	11.4	0.00037005
	P_6	36.49	19.5	-16.99	6.65	0.0099575
	P_7	35.09	21.86	-13.23	5.71	0.01910375
	P_8	36.33	18.63	-17.7	8.56	0.00264962
	P_9	32.91	18.53	-14.38	5.5	0.02209709
	P_{10}	34.56	30.92	-3.64	19.43	1.4158 E-06

(P): *P. aeruginosa* isolate, **(C):** Control

Table (10) Gene expression results for *algD* before and after treatment with *Rosmarinus officinalis* methanolic extract

Group	Sample	Ct reference gene *16S rRNA*	Ct target gene *algD*	ΔCT	ΔΔCt	Fold of gene Expression
Before treated (control)	C_1	38.5	13.13	-25.37	0	1
	C_2	37.76	13.12	-24.64	0	1
	C_3	33.17	18.34	-14.83	0	1
	C_4	32.81	15.81	-17	0	1
	C_5	39.7	13.33	-26.37	0	1
	C_6	37.94	14.51	-23.43	0	1
	C_7	39.15	20.69	-18.46	0	1
	C_8	39.5	12.01	-27.49	0	1
	C_9	34.61	13.97	-20.64	0	1
	C_{10}	38.86	14.89	-23.97	0	1
After treated	P_1	34.98	11.4	-23.58	1.79	0.28917205
	P_2	31.06	16.08	-14.98	9.66	0.00123609
	P_3	28.12	16.64	-11.48	3.35	0.09807301
	P_4	31.99	19.85	-12.14	4.86	0.03443453
	P_5	37.75	13.26	-24.49	1.88	0.27168372
	P_6	38.21	15.78	-22.43	1	0.5
	P_7	33.77	15.79	-17.98	0.48	0.71697762
	P_8	36.79	12.22	-24.57	2.92	0.13212726
	P_9	31.1	19.47	-11.64	9	0.00195313
	P_{10}	35.78	13.41	-22.37	1.6	0.32987698

(P): *P. aeruginosa* isolate, **(C):** Control

Table (11) Gene expression results for *pelA* before and after treatment with *Rosmarinus officinalis* methanolic extract

Group	Sample	Ct reference gene *16S rRNA*	Ct target gene *pelA*	ΔCT	ΔΔCt	Fold of gene Expression
Before treated (control)	C_1	38.5	14.26	-24.24	0	1
	C_2	37.76	13.72	-24.04	0	1
	C_3	33.17	19.42	-13.75	0	1
	C_4	32.81	17.37	-15.44	0	1
	C_5	39.7	14.25	-25.45	0	1
	C_6	37.94	14.3	-23.64	0	1
	C_7	39.15	20.21	-18.94	0	1
	C_8	39.5	13.24	-26.26	0	1
	C_9	34.61	14.73	-19.88	0	1
	C_{10}	38.86	15.79	-23.07	0	1
After treated	P_1	34.98	12.76	-22.22	2.02	0.24655818
	P_2	31.06	16.32	-14.74	9.3	0.00158643
	P_3	28.12	17.67	-10.45	3.3	0.10153155
	P_4	31.99	20.93	-11.06	4.38	0.04802735
	P_5	37.75	13.21	-24.54	0.91	0.53218509
	P_6	38.21	15.66	-22.55	1.09	0.46976137
	P_7	33.77	15.79	-17.98	0.96	0.51405691
	P_8	36.79	13.04	-23.75	2.51	0.17555561
	P_9	31.1	19.14	-11.96	7.92	0.00412898
	P_{10}	35.78	14.46	-21.32	1.75	0.29730178

(P): *P. aeruginosa* isolate, **(C):** Control

4. Conclusion

This study concluded that the phenolic compounds extracted from *Rosmarinus officinalis* leaves displays a high antibiofilm agent on *p. aeruginosa* and can down-regulation the *algD* and *pelA* genes despite the bacterial isolates' strong biofilm formation.

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