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Inhibition of Biofilm Formation in *Agrobacterium tumefaciens* by Cell-Free Supernatants of *Pseudomonas aeruginosa* Analyzed by GC-MS

***Najwa Ibrahim Khaleel Al-Barhawee* *[1], *Sarah Salih Al-Rubyee*[2]**

[1]Department of Biology, College of Education for Pure Science, University of Mosul, Mosul, Iraq.
[2]Nineveh Education Directorate, Hamidiya Sharqi School for Boys, Qayarah, Nineveh Directorate of Education, Mosul, Iraq.
*Corresponding Author.

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Abstract

One of the most economically significant plant pathogenic bacteria is *Agrobacterium tumefaciens*, infects plants by exploiting biofilms it forms on their surfaces wounds. This article has been concerned with the need for new antibacterial agents due to the limitations of current treatments. The capacity of *Pseudomonas aeruginosa* cell-free supernatant to inhibit the *A. tumefaciens*-produced biofilms as well as its chemical makeup were examined in this work. Using the API 20E kit and polymerase chain reaction of the *16S rRNA* gene, *P. aeruginosa* was isolated from the soil and identified. It displayed a 93% identity with the common bacterium *Pseudomonas* sp.SeaQual P_B_845W, MT626817.1 in the GenBank. Using the microdilution method, the ability of the lyophilized supernatant was then determined at nine concentrations (10, 15, 20, 25, 30, 35, 40, 45, and 50%) of biofilm formation. The results revealed an inhibitory effect as percentages of 66, 61, 51, 27, 20, 17, and 15%,. After being injected with the GC-MC device, it was found that it consisted of 30 chemical compounds, which were identified by their names as;(Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, Hexadecanoic acid, methyl ester, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)-, 9-Octadecenoic acid (Z)-, methyl ester, and cis-13-Octadecenoic acid, methyl ester, Octadecanoic acid, methyl ester), this demonstrates that its (154, 270, 210, 296, 296, 298) Daltons and (9.38, 19.12, 6.8, 4.45, 8.33, 5.90)% of the total space. The discovery that *P. aeruginosa* cell-free supernatants include chemical compounds for the first time and have an inhibitory influence to produce biofilms by *A. tumefaciens* is the study's most significant finding.

Keywords: *Agrobacterium tumefaciens*, Biofilms, GC-MS, *Pseudomonas aeruginosa*, *16S rRNA* gene sequencing.

Introduction

A biofilm is defined as a group of microbial cells attached to surfaces by extracellular polymer materials, or what is known as a matrix (EPS), which mostly consists of polysaccharides, proteins, lipids, and DNA, knowing that among its functions is to protect microbial cells from the effect of antibiotics, it also facilitates communication between cells within the biofilm, which allows for rapid temporal adaptation, and enables bacteria to survive in conditions of food deficiency [1,2]. The development of bacterial biofilms on the surface of the plant is critical because, in some conditions, it can promote

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its growth while, in others, it can cause various diseases to it[3], *Agrobacterium* sp. is a Gram-negative, rod-shaped soil-borne plant pathogen of the Rhizobiaceae, has two unique ecological niches in nature: one is free-living, saprophytic, and non-pathogenic, while the other is pathogenic [4]. There were originally three biovars of pathogenic *Agrobacterium* based upon the host range and manner of pathogenic response in the host. Biovar I includes *A. tumefaciens*, biovar II includes *A. rhizogenes* and biovar III includes *A. vitis* [5]. As it colonizes the wounded parts of the plant and adheres to its cellulosic fibers mechanically using its biofilms, which are under the control of regulatory molecules common between the two parties [6], then it transfers its genes located on the Transfer-DNA (23kb or 15-40 kb, two classes of genes make up T-DNA: 1. Tumor formation is caused by oncogenic genes and genes that encode enzymes, which produce auxins and cytokinins. 2. the genes responsible for producing opines which are produced in either octopine or nopaline form by the majority of common *Agrobacterium* strains) of the Ti-(tumor-inducing) plasmid (140–235 kb) to the host cells and become part of their genetic material, which causes a disease crown galls in a wide range of dicotyledonous plants [7,8], estimated to be 643 out of 331 genera affected by the disease [9]. And given the fact that many of these plants are of economic importance in the world [10], so this infection causes a decrease in agricultural production, which leads to huge financial losses[11], from this standpoint it became necessary to search for ways to combat it, especially in preventing its formation of biofilms.

Agrobacterium can migrate toward the plant due to several specific proteins. When it detects that it is going in the wrong direction, it moves its flagellum randomly and swims in a straight line in this new direction until it finds the right plants for it [12] , so, the first step in combating these bacteria was to prevent them from forming unusual proteins that carry the characteristic of virulence by inhibiting the synthesis of the enzyme leucyl tRNA synthetase using the antibiotic Agrocin 84 produced by the non-pathogenic bacteria *Agrobacterium raidobacter* [13], but in some countries, the use of these was banned genetically modified bacteria that produce this antibiotic because they may also target beneficial bacterial species found in the soil [14]. This is in addition to the fact that most antibiotics are usually very expensive and have weak effects on bacteria due to their resistance when used as a treatment [15]. To effectively control crown gall disease, new strategies are needed, to stop the growth of this bacterium and prevent it from forming biofilms on the plant surface at the onset of infection[16]. In this context, Ahmed *et al.*[7] reported that at ≥150 µg/mL of trans-cinnamaldehyde and its derivatives, biofilm development was inhibited in these bacteria, and biofilm formation on nylon or polystyrene was decreased by 94–99%, which was detected using optical electron scanning and 3D spectroscopy. As for Jailani *et al*.[16], they confirmed the ability of tannic acid to inhibit the growth of bacteria and the formation of biofilms, and thus the failure of infection caused by *A. tumefaciens* colonization. In several articles, in-depth research identified specific chemicals in cell-free supernatants that have anti-QS activity [17] or alter the surface characteristics of bacterial cells [18]. On the other hand, the use of microorganisms in the field of biotechnology is one of the most prominent scientific developments used to protect economically and medically important plants from pathogens of all kinds [19], and in this context, many bacterial species were used, including the genus *Pseudomonas,* mainly isolated from the soil , where it is located near the roots of plants and releases metabolites that promote plant growth and prevent the activity of pathogenic microbes [20,21]. As well as its positive effect in reducing the levels of toxic chemicals and heavy metals in the soil [22,23]. It also contributes positively to the plant growth process by reducing the effect of growth inhibitors and enhancing the production of biological control agents [24]. On the other hand, incubating *Salmonella enterica* in Tryptic soytone broth (TSB) and Meat thawing loss broth (MTLB), in the presence of 30% and 60% CFS from *P. aeruginosa*, significantly reduced growth rates of this bacteria during the exponential phase but not during the stationary phase, and significantly inhibited biofilm development at the percentage of 70.7 and 93.3,

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respectively [25]. It turned out that it is possible to use the phage as an alternative method to eliminate the problem of its overgrowth after transformation, and the use of *Agrobacterium* in genetic transformations within the techniques of plant genetic engineering led to the emergence of a hypersensitivity reaction and turning the color of the tissue to brown, which may reduce the efficiency of transformation and regeneration of plant cells and thus to the death of the affected plant[26] .

The effects of GC-MC-lysed *Pseudomonas aeruginosa* cell-free supernatants (CFS) on wild-type *Agrobacterium tumefaciens* biofilms, which have significant virulence factors for the formation and recurrence of crown galls disease, have not been examined, despite their potential biological value as antimicrobial agents. Therefore, we wanted to study the ability of this CFS, to prevent the formation of biofilm by wild-type *Agrobacterium tumefaciens*, as well as identify its components of chemical compounds as anti-biofilm materials, using GC-MS, to propose new biocontrol agents that act as anti-biofilm agents.

Materials and Methods

From the University of Mosul garden and after removing surface plant residues, several soil samples were collected from surrounding the plant roots from the surface to a depth of 8-12 inches, mixed well to become a homogeneous sample. It was stored at a temperature of 4ºC in sealed plastic bags until use. To isolate the bacteria, 5 g of soil samples were diluted in 50 ml of phosphate-buffered saline solution and shaken for one hour. One ml of the sample was then grown in 100 mL of nutrient broth for 24 hours at 30°C and then grown on nutrient agar for 48 hours at 30°C[27]. The greenish-blue bacteria were selected and underwent microscopically examination, biochemical characterization (API 20E) kit, and *16S rRNA* gene sequencing [28], using the primer shown in Table 1, according to Edwards *et al*.,[29] the Polymerase Chain Reaction (PCR) settings were 94°C for 5 minutes of initial denaturation, 35 cycles of 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute, and 10 minutes of final extension at 72°C. *Pseudomonas aeruginosa* amplified *16S rRNA* gene was chosen for sequencing. The obtained nucleotide sequence was sent to NCBI.

Table 1. Primer using in this study.

***16S rRNA* Primer**	**Sequence (5'-3')**	**Reference**
Forward	AGAGTTTGATCCTGGCTCAG	Edwards *et al*.,[29]
Reverse	AAGGAGGTGATCCAGCCGCA	

***Agrobacterium tumefaciens* Strain**:

Obtained from postgraduate laboratories in the research unit of the Department of Biology/College of Education for Pure Sciences / University of Mosul.

CFS Preparation:

To prepare the CFS, a small modification was made to El-Mokhtar *et al*.,[30] procedure. The *P. aeruginosa,* was grown at 30°C for 18 hours in 100 mL of nutrient broth, then centrifuged to get the supernatant, ×6,000 g, for 15 min. at 4°C. A sterile filter with a 0.22 µm pore size (Sigma, Germany) was used to filter the centrifuged supernatant. The obtained filtrate was collected for freeze-drying.

CFS Lyophilized:

According to Sornsenee *et al*.,[31] the lyophilization of the CFS samples was done using Lyophilization Systems, Inc., USA, under defined conditions , at 0.2 bar pressure, and between 30 and 40°C, then the CFS was frozen at -80 °C for 24 hours. The drying process of the frozen samples was completed by powdering, after 48 hours, it was stored at -20°C until use in later experiments.

Testing for Antimicrobial Sensitivity (Kirby-Bauer Method):

Agrobacterium tumefaciens was inoculated with peptone water and incubated for 18-24 hours at 28°C using seven different antibiotics, including

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;Ciprofloxacin 10 µg, Tobramycin 10µg, Cefotaxime 30µg, Ampicillin 25µg, Gentamycin 10 µg, Amikacin 30µg, Amoxicillin 30µg, Oxoid™,Basingstoke, Hampshire, United Kingdom . They were then re-cultured in broth, and their turbidity was compared to 0.5 McFarland reference solutions. After that, Mueller-Hinton agar was swabbed with fresh cultures and incubated at 28°C for about 10-15 minutes after drying for 5-10 minutes. Interested antibiotic discs, were placed on culture plates with sterile forceps and then incubated at 28 °C for 24 hours[32].

The same procedure was used to examine the impact of various *P. aeruginosa* CFS concentrations on the development of *A. tumefaciens* in terms of the inhibitory zone's diameter (mm).

Biofilm inhibition Assay:

The method used to examine how *P. aeruginosa* CFS affected the formation of *A. tumefaciens* biofilm was modified by Yang *et al* [33]. In a nutshell, *A. tumefaciens* overnight cultures were suspended in Mueller-Hinton broth (MHB) to a cell density of 5×10^5 CFU/mL and then inoculation onto 12-well plates supplemented with different concentrations 0,10, 15, 20, 25, 30, 35, 40, 45, 50%, (v of CFS:v of medium broth) of *P. aeruginosa* CFS. Under aerobic conditions, the plates were incubated at 28 °C for 24 hours. The liquid was then removed, and phosphate buffered saline (PBS) at pH 7.4 was used to wash the biofilms three times, then fixed for 15 minutes in 200 µL of 99% (v/v) methanol. 200 µL of a 0.1% (w/v) crystal violet solution was used to stain the biofilm for 10 min. To get rid of extra color, the wells were rinsed with distilled water four times. The biofilms were dissolved in 95% (v/v) ethanol, and the absorbance was calculated at 570 nm.

Using the same methodology [33], it was possible to calculate the percentage difference between the control sample and the *Agrobacterium* biofilm formation effect of various antibiotics.

CFS GC-MS Analysis:

Lyophilized CFS was transferred to the University of Basrah to be examined for the existence of chemicals using a gas chromatograph linked to a mass spectrometer of the GC-MS QP210 ULTRA type, (Japanese Shimadzu company). These compounds were identified based on their retention periods in the GC capillary column and then computer-matched to the mass spectra using the NSTA08 library database and GC-MS Solution software[34].

Statistical Analysis:

Using the statistical tool Duncans Multiple RangeTest, the data of the antibiotics and *P. aeruginosa* CFS concentrations inhibition zone were examined.

Results and Discussion

Identification of *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa is a gram-negative bacterial rod, they are mobile, and non-spore forming, their colonies are soft, big, and irregular, and have a greenish-blue, and a grape-like odor, according to microscopic examination and morphological characteristics, while their biochemical characterization results are as shown in Fig. 1, A. To more accurately determine the genus of the isolate bacteria, the size of *16S rRNA* gene after amplification was determined (1200 bp) in Fig. 1, B; based on the fact that *16S rRNA* is a relatively stable region with a relatively slow rate of evolution, and demonstrated a 93% match with the standard bacterium *Pseudomonas sp*. strain SeaQual P_B_845W, and it is registered under the number MT626817.1 in GenBank, it was observed that some nitrogenous bases were replaced by the association of C with G in 17 sites, C with A in 4 sites, and C with T in 6 sites. Fig. 2. The great phenotypic difference shown by the isolated samples and the presence of other closely related species may lead to differences in the diagnosis of this type of bacteria when using traditional and molecular methods[35]. According to numerous studies about the identification of these bacteria [36,37], our findings supported the integration of morphological features with biochemical characteristics and the genetic sequence of the *16S rRNA* gene, confirming the diagnosis of the bacteria isolated from the soil as belonging to the genus *Pseudomonas*. This outcome is in line with what Eremwanarue *et. al.*[38] reported, who established the Polymerase Chain Reaction (PCR) approach by using the *16S rRNA* Sequencing technique was a more accurate way to identify *Pseudomonas aeruginosa*.

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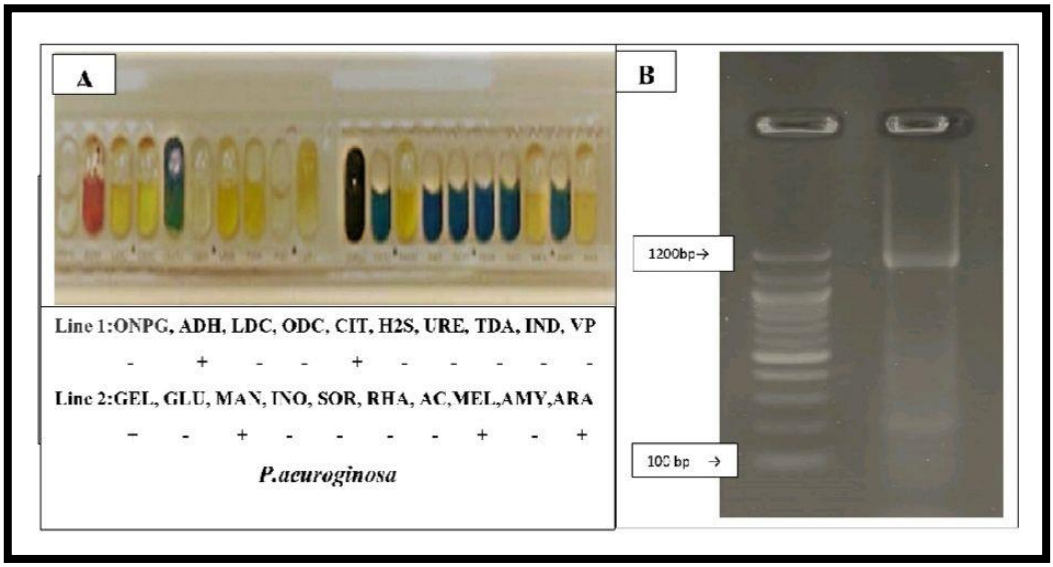

Figure 1. (A) Identification of *P. aeruginosa* by API 20E Kit, (B) Amplification of *16S rRNA* gene.

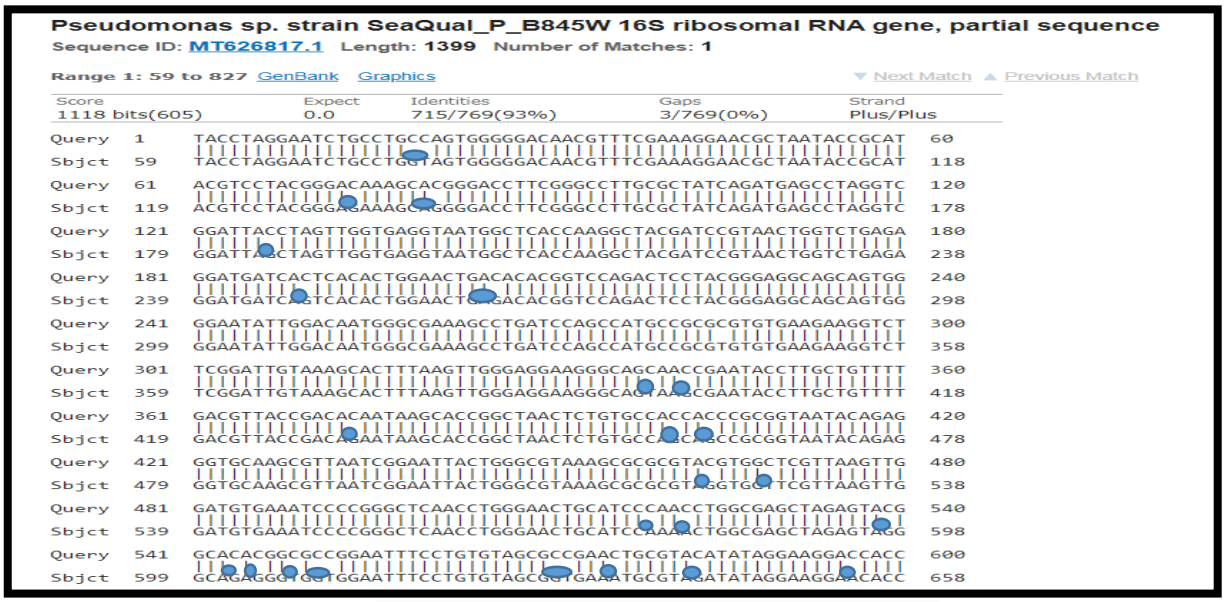

Figure 2. The sequence of *P. aeruginosa 16S rRNA* gene.

Effect of *P. aeruginosa* CFS on *A. tumefaciens* Biofilm Formation:

The results of this study showed a clear inhibitory effect when increasing the concentration of *P. aeruginosa* CFS on the formation of biofilms by the plant pathogenic bacteria *A. tumefaciens* (Table 2), in terms of the decrease in the intensity of the color of the crystal violet dye, as seen in Fig. 3. This may be primarily related to the presence of soluble substances in CFS that have an inhibitory impact on the formation of biofilms by bacteria that induce crown gall disease in plants[39]. The researcher Hibbing and Fuqua[40] demonstrated that this inhibitory effect was not brought on by nutrient reduction in the medium or a change in pH. So, this effect may be attributed to the targeting of biofilm-related proteins or biofilm-formation pathways[41]. The focus by some investigators has been on *P. aeruginosa* pathogenicity and virulence (LPS, quorum sensing, two-component systems, 6-type secretion systems, outer membrane vesicles (OMVs), CRISPR-Cas and its structure)[42], but none any of the other studies on the importance of using *P. aeruginosa* CFS in the biological control of *A. tumefaciens* biofilm.

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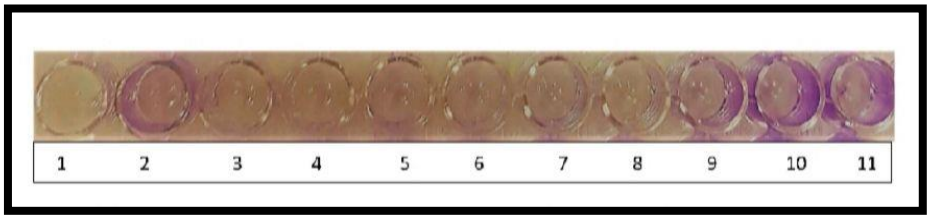
Table 2. Effect of various bacterial cell-free supernatant concentrations on *A. tumefaciens* biofilm in terms of absorbance values at 570 nm.

Concen.of CFS (V:V)	Control (-)	Control (+)	50	45	40	35	30	25	20	15	10
A.tumefacienes	0.0	0.54	0.08	0.09	0.09	0.11	0.14	0.15	0. 28	0.33	0.36



1: control(-), 2: control (+), 3-11: concentration of lyophilizared CFS; 50, 45, 40, 35, 30, 25, 20, 15, 10% (v:v)

Figure 3. Effect of various bacterial cell-free supernatant concentrations on *A. tumefaciens* biofilm formation.

Susceptibility Test of *A.tumefacienes:*

The sensitivity of *A. tumefacienes* isolate was tested against seven antibiotics as seen in Table 3 and Fig. 4. The susceptibility test was applied according to the Kirby-Baure Method (antibiotic disc diffusion method). The antibiotics had varying effects on preventing the growth of *A. tumefaciens*, particularly about the size of the inhibition zone, the two antibiotics CIP and TOB had the greatest impact (13 mm), followed by the other two antibiotics CN and AK (9 mm), with three antibiotics CTX, AMP, and AMC clearly showing resistance. The results of the statistical analysis indicate that there are significant differences in the sensitivity of bacteria to the previously mentioned antibiotics, and these results matched the results of laboratory data, as shown in the table below (Table 3). The stronger inhibitory effect of CIP and TOB antibiotics may be due to what Domalaon *et al*.,[43] indicated, through their accumulation inside the cells of Gram-negative bacteria due to their negative effect on the permeability of the outer membrane and proton-motive force disruption. As for increasing the formation of biofilms when these bacteria are resistant to antibiotics, Cefotaxime, Ampicillin, and Amoxicillin (Table, 4), it is one of the strategies used by bacteria for surviva[44].

Table 3. Effect of antibiotics on *A. tumefaciens* diameter of the inhibition zone (mm).

Antibiotics (µg)	Ciprofloxacin (CIP) 10	Tobramycin (TOB) 10	Cefotaxime (CTX) 30	Ampicillin (AMP) 25	Gentamicin (CN) 10	Amikacin (AK) 30	Amoxicillin (AMC) 30
A. tumefacienes	13 A	13 A	-	**(mm)** -	9 B	9 B	-

Data are the average of three replicates, Similar letters, no significant differences between them and the different letters there are significant differences between them.

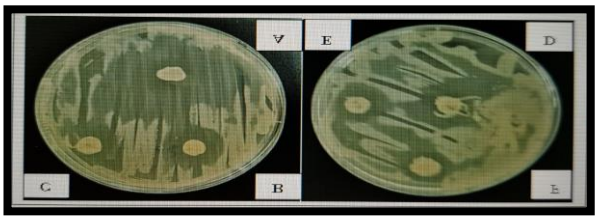


Figure 4. *A. tumefaciens* diameter of the inhibitory zone caused by various *P. aeruginosa* CFS concentrations. A: 50%, B:45%, C:40%, D:35%, E: 30%, F:25%.

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Table 4. Effect of antibiotics on *A. tumefaciens* biofilim inhibition (%) compared to the control.

Antibiotics µg	Ciprofloxacin (CIP) 10	Tobramycin (TOB) 10	Cefotaxime (CTX) 30	Ampicillin (AMP) 25	Gentamicin (CN) 10	Amikacin (AK) 30	Amoxicillin (AMC) 30
				(%)			
A. tumefacienes	-	-	54	58	15	11	55

Data are the average of three replicates.

Effect of *P. aeruginosa* CFS on *A. tumefaciens*:

Table 5, displays the action of *P. aeruginosa* cell-free supernatant based on the target bacteria's (*A. tumefacient*) biofilm production and the size of the inhibitory zone (mm). The growth of *A. tumefacienes* was inhibited at various CFS concentrations, and as indicated in Table 3, the effect became more with increasing concentrations, particularly at the concentrations between 25 to 50%, where the sensitivity ranged between weak (8 and 9 mm), medium (11, 13, and 14 mm), and strong (17 mm), while in the first three concentrations, the bacteria showed clear resistance (-).The results of the statistical analysis also indicated that there were significant differences in the effect of different concentrations of *P. aeruginosa* CFS on the diameter of the inhibition zone around the disc. These results also matched the results of laboratory data, as shown in Table 5. In contrast to the positive comparison sample, these results were accompanied by the formation of biofilms at very low rates, especially at high concentrations, but they increased when the bacteria were resistant to the first three concentrations of 10, 15, and 20%, with variations, at rates of 66, 61, and 51%, respectively, as shown in Table 6. This is because biofilms resist antimicrobials better than their floatable planktonic[45]. The findings suggest that *P. aeruginosa* culture fluids cannot encourage the formation of *A. tumefaciens* biofilms [40].

Table 5. Effect of *P. aeruginosa* CFS concentrations on *A. tumefaciens* diameter of the inhibition zone (mm) .

Concentration of CFS (V:V)	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
					(mm)				
A. tumefacienes	-	-	-	8 C	9 C	11 BC	13 B	14 AB	17 A

Data are the average of three replicates, similar letters; no significant differences between them and the different letters; there are significant differences between them.

Table 6. Effect of *P. aeruginosa* CFS concentrations on formation *A. tumefaciens* biofilm (%) compared to the control.

Concentration of CFS (V:V)	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**45**	**50**
					(%)				
A. tumefacienes	66	61	51	27	25	20	17	17	15

Data are the average of three replicates.

GC-MS Analysis:

By comparing the mass spectra, molecular weight, retention time, and chemical formula with the NIST library, GC-MS analysis of *P. aeruginosa* CFS was carried out to identify the active molecule and the existence of thirty chemicals in the sample (Fig. 5 and Table 7). Choosing six compound from them (peak 4, 10, 12, 16, 17, and 18) as shown in Fig. 6. The spectra showed a significant peak with the chemical formula $C_{17}H_{34}O_2$ (hexadecanoic acid, methyl ester) and a molecular weight of 270 Da, which covered an area of 19.12%. We focused on three molecules that are altered forms of decanoic acid: cis-13-octadecanoic acid, methyl ester, 'Octadecanoic acid, methyl ester,' and 9-octadecenoic acid (Z)-, with the chemical formula $C_{19}H_{38}O_2$, a molecular weights of 296, 296, and 298

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Da, and the amounts of the area they occupy, 8.33, 5.90, and 4.45%, respectively. The five and six molecules are Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- with the chemical formula $C_7H_{10}N_2O_2$ and $C_{11}H_{18}N_2O_2$, a molecular weight of 154 and 210 Da, which covered an area of 9.38 and 6.81%, respectively.

The decanoic acid-modified peptide demonstrated high anti-biofilm properties and had antibacterial action against germs of both the Gram-positive and Gram-negative types by modifying the permeability of cell membranes, and it also reduced the production of biofilm at low concentrations [46] Florenly *et al*.,[47] indicated that the two compounds cis-13-Octadecenoic acid and 9-Octadecenoic acid (Z)-, methyl ester occupied 40.46 and 10.93% of the area of nano-green betel leaf extracts, the difference in the area with our study is due to the difference in the source from which this compound was extracted, and their chemical composition $C_{19}H_{38}O_2$ matched the chemical composition of these two compounds extracted in this study. Gram-positive bacteria were more successfully inhibited from growing than gram-negative ones by the 9-Octadecenoic acid (Z)-methyl ester that was isolated from *Bidens bipinnata* [48]. The antibiotic Pyrrolo[1,2-a]pyraz ine-1,4-dione, hexahydro, which has been discovered in a marine bacteria named *Bacillus tequilensis* [49], effectively controls multidrug-resistant *Staphylococcus aureus*. In a different study, Rajiv Gandhi *et al*.,[50] reported that Pyrrolo [1,2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methyl propyl) isolated from endophytic actinomycetes *Nocardiopsis* sp. GRG 1 (KT235640) and analyzed in various ways, is a compound that inhibits the formation of *P. mirabilis* and *E. coli* biofilm formation and lessens the vitality of already-formed biofilms.

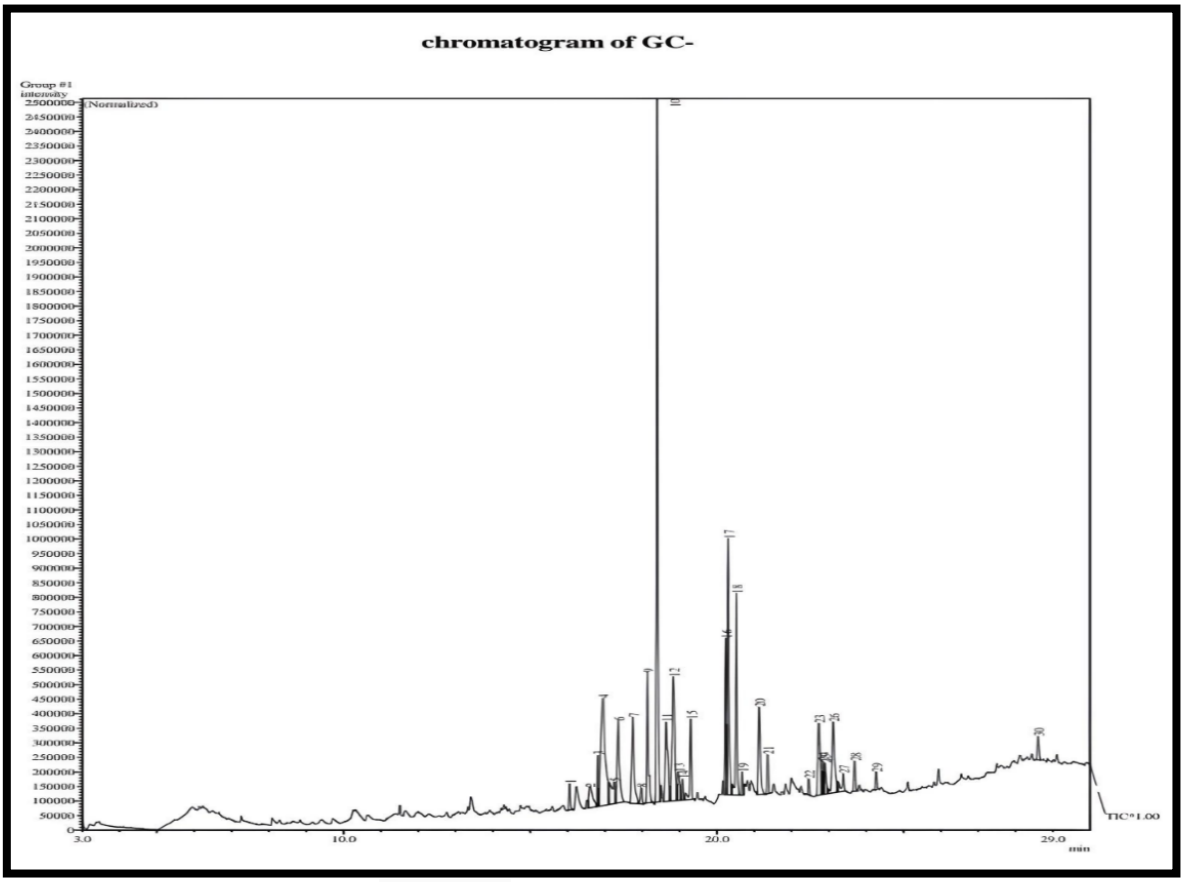

Figure 5. *P. aeruginosa* CFS's chemical composition curve.

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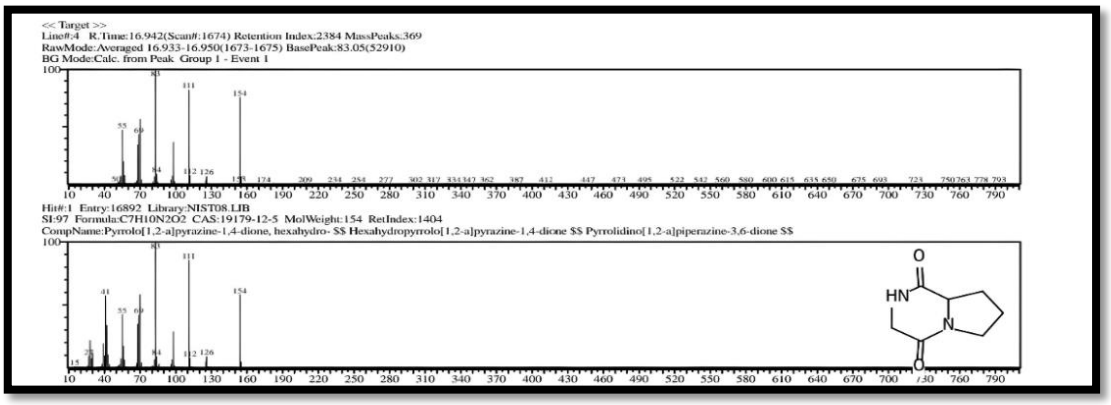
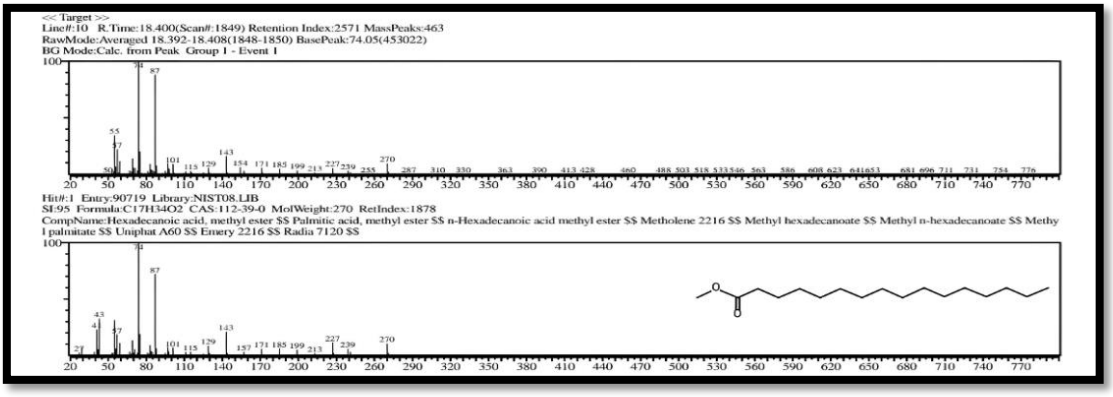
Peak#	R.Time	Area Area	%	Name
1	16.054	231056	0.72	Methyl tetradecanoate
2	16.592	528915	1.65	1-Butanamine, N-(1-propylbutylidene)-
3	16.814	424159	1.32	Pentadecanoic acid, methyl ester
4	16.946	3001690	9.38	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-
5	17.250	668365	2.09	Pentadecanoic acid, methyl ester
6	17.360	1277197	3.99	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
7	17.750	1366629	4.27	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
8	17.974	162649	0.51	Hexadecanoic acid, methyl ester
9	18.141	1293174	4.04	7-Hexadecenoic acid, methyl ester, (Z)-
10	18.399	6120966	19.12	Hexadecanoic acid, methyl ester
11	18.639	1379078	4.31	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
12	18.832	2180794	6.81	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
13	18.961	471557	1.47	n-Hexadecanoic acid
14	19.078	230756	0.72	Hexadecanoic acid, 14-methyl-, methyl ester
15	19.300	780595	2.44	Methyl 9,10-methylene-hexadecanoate
16	20.238	1425687	4.45	9-Octadecenoic acid (Z)-, methyl ester
17	20.304	2667125	8.33	cis-13-Octadecenoic acid, methyl ester
18	20.519	1888322	5.90	Octadecanoic acid, methyl ester
19	20.672	242690	0.76	Benzene, (1-methyl-1-propylpentyl)-
20	21.133	1140504	3.56	Dodecanamide
21	21.362	321826	1.01	10-Nonadecenoic acid, methyl ester
22	22.464	130946	0.41	Methyl 18-methylnonadecanoate
23	22.732	1133396	3.54	Ergotaman-3',6',18-trione, 9,10-dihydro-12'-hydroxy-2'-methyl-5'-(phenylmethyl)-, (5'.alpha.,10.alpha.)-
24	22.836	342504	1.07	9-Octadecenamide, (Z)-
25	22.894	392807	1.23	9-Octadecenamide, (Z)-
26	23.114	1216290	3.80	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-
27	23.386	312549	0.98	Isosteviol methyl ester
28	23.691	272142	0.85	Isosteviol methyl ester
29	24.265	155386	0.49	Docosanoic acid, methyl ester
30	28.606	254692	0.80	Stigmast-5-en-3-ol, oleate
		32014446	100.00	

Table 7. GC-MS analysis of *P. aeruginosa* CFS.

Peak 4



Peak 10



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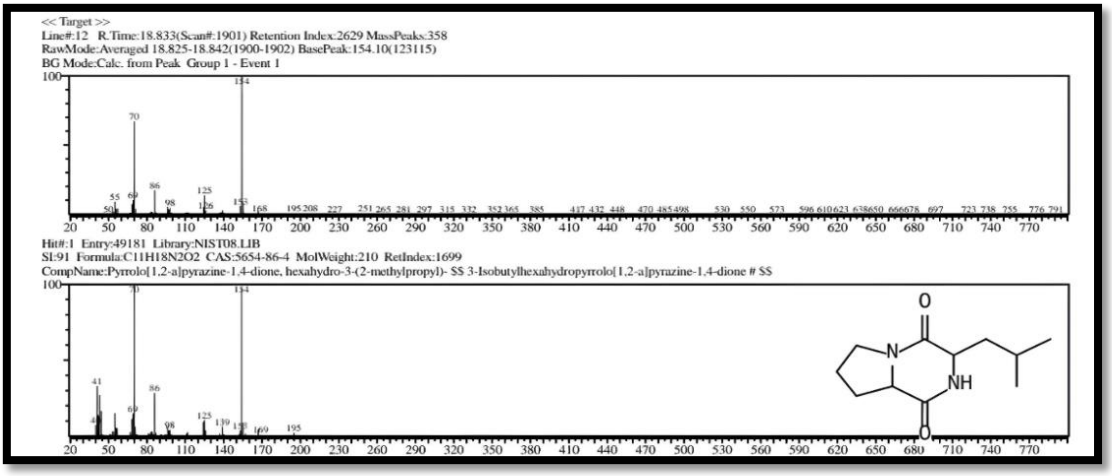
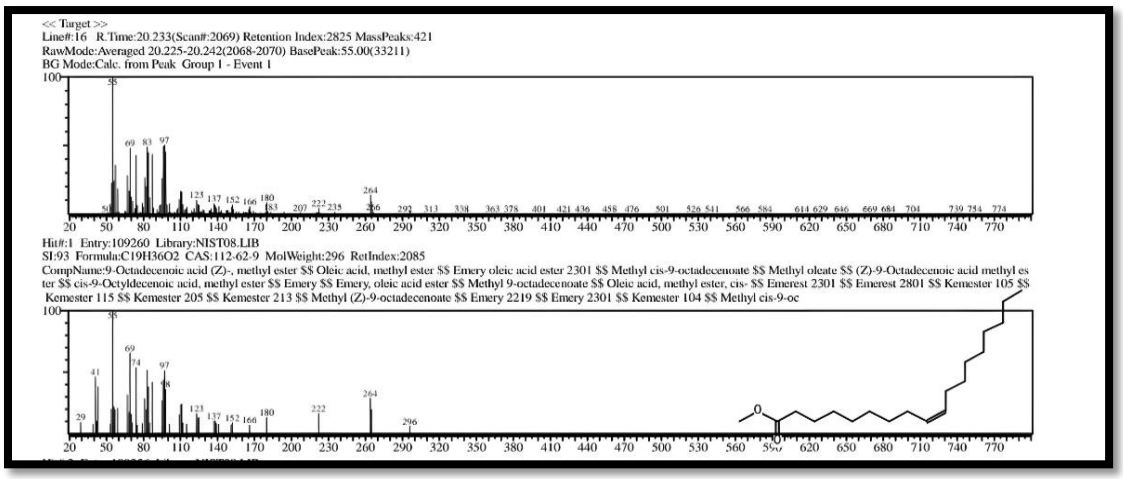
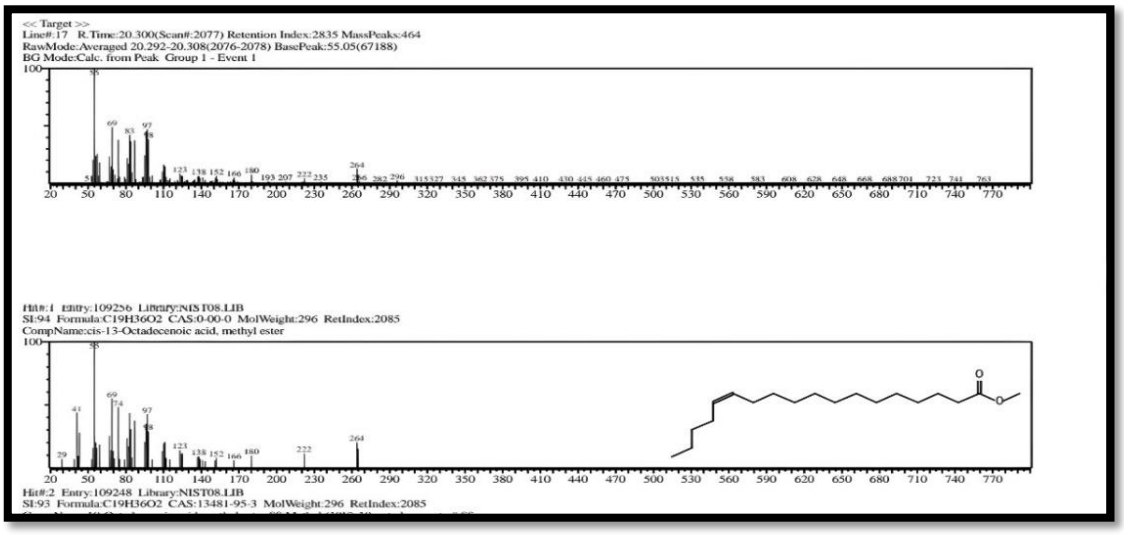
Peak 12



Peak 16



Peak 17



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Peak 18

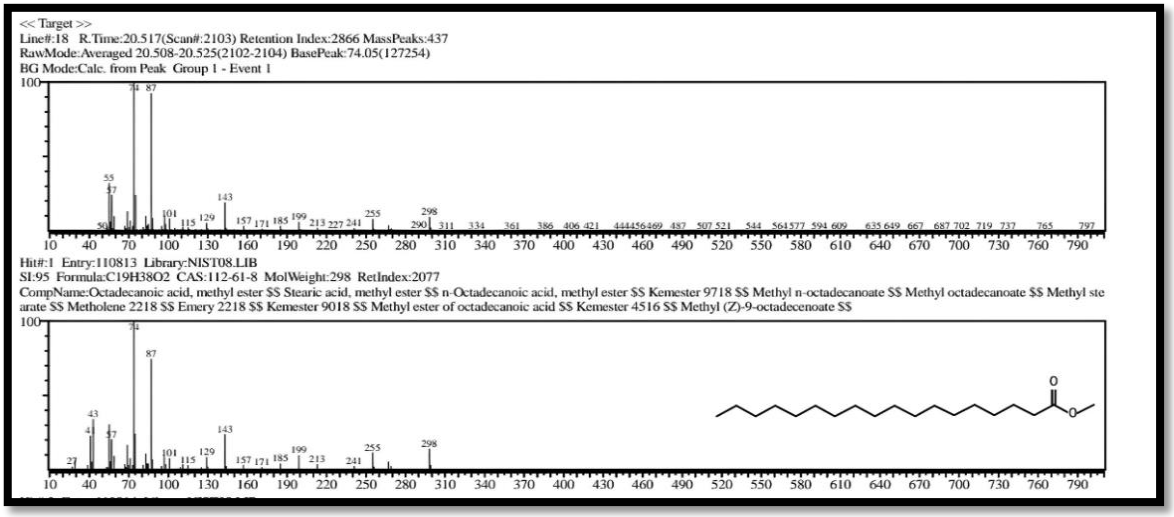

Figure 6. *P. aeruginosa* CFS's six chemical curves and their composition.

Conclusion

This study results unequivocally show that the use of DNA sequencing of the *16S rRNA* gene in the diagnosis of *Pseudomonas aeruginosa* to *Pseudomonas* sp.is more accurate than the use of conventional laboratory techniques such as the study of morphological, physiological, and biochemical features. In addition, it was shown that *P. aeruginosa* possesses extracellular compounds such as Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, Hexadecanoic acid, methyl ester, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 9-Octadecenoic acid (Z)-, methyl ester, and cis-13-Octadecenoic acid, methyl ester, Octadecanoic acid, methyl ester that have inhibitory effects on biofilm formation in *A. tumefaciens.* The detection of these chemicals for the first time by GC-MS in *P. aeruginosa* CFS is one of the most important findings of the study, which requires studying the effect of CFS on the formation of tumors *in-vivo* as a future goal of research.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Mosul.

Authors' Contribution Statement

N. I. Kh. Al. and S. S. Al. contributed to the completion of the research by participating in collecting bacterial samples, isolating and diagnosing them, and writing and reviewing the manuscript. The research tasks were distributed equally among the two authors.

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تثبيط تكوين الاغشية الحيوية في بكتيريا *Agrobacterium tumefaciens* بالمادة الطافية الخالية من خلايا *Pseudomonas aeruginosa* والمحللة بجهاز GC-MS

نجوى إبراهيم خليل البرهاوي[1]، سارة صالح الربيعي[2]

[1]قسم علوم الحياة، كلية التربية للعلوم الصرفة، جامعة الموصل، الموصل، العراق.
[2]مدرسة حميدية شرقي للبنين، القيارة، مديرية تربية نينوى، الموصل، العراق.

الخلاصة

تعد *Agrobacterium tumefaciens* من أنواع البكتيريا المسببة للأمراض النباتية المهمة اقتصاديا، وتحصل الاصابة نتيجة استخدام اغشيتها الحيوية لالصاق نفسها بالجروح المتكونة على سطح العائل النباتي، ونظرًا لمحدودية العلاجات الحالية وفعاليتها، أصبح البحث عن عوامل جديدة مضادة لهذه البكتيريا أمرا ضروريا، لذلك تم تسليط الضوء في هذه الدراسة على معرفة التأثير التثبيطي للمادة الطافية الخالية من خلايا *Pseudomonas aeruginosa* على تكوين الاغشية الحيوية من قبل *A. tumefaciens*، فضلا عن تحديد المركبات الكيمياوية المكونة لها بجهاز GC-MS. بناءا على ذلك عزلت *P. aeruginosa* من التربة وتم تشخيصها باستخدام العدة API 20 E وتفاعل البلمرة المتسلسل باستخدام الجين *16S rRNA* واظهرت تطابقا بنسبة 93% مع البكتيريا القياسية *Pseudomonas* sp.SeaQual P_B_845W, MT626817.1 في بنك الجينات. وعند الكشف عن قدرة المادة الطافية المجفدة وبتسعة تراكيز 10، 15، 20 ،25 ، 30، 35، 40، 45، 17، 17، 15 ،على التوالي. وحسب عدد القمم التي تم إنتاجها بعد حقنها بجهاز GC، تبين انها مكونة من 30 مادة كيميائية، وبعد إدخال هذه المعلومات في جهاز MS، تم تشخيصها بأسمائها ومنها(Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- ،Hexadecanoic acid, methyl ester ،Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-cis-13-Octadecenoic acid,methyl ،9-Octadecenoic acid (Z)-,methyl ester،ester (Octadecanoic acid, methyl ester)، البالغ وزنها الجزيئي(154، 270، 210، 296، 296، 298) دالتون ومقدار المساحة التي تشغلها (9.38، 19.12، 6.8، 4.45، 8.33، 5.90)%، على التوالي. أهم النتائج التي شخصتها هذه الدراسة هو تحديد المركبات الكيميائية لأول مرة للمادة الطافية الخالية من خلايا الزائفة الزنجارية وتأثيرها المثبط على إنتاج الأغشية الحيوية من قبل *A. tumefaciens*.

الكلمات المفتاحية: *Agrobacterium tumefaciens*، الاغشية الحيوية، كروموتوكرافيا الغاز المدمج بمطياف الكتلة، *Pseudomonas aeruginosa*، تسلسل الجين *16S rRNA*.