Inhibition of Biofilm Formation in Some Pathogenic Gram Negative Bacteria by Anti-quorum Sensing Compounds

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Abstract:

The present study included collecting of 125 clinical bacterial isolates from different sources such as pus, sputum, nasal polyps, urine and blood. The collected bacterial isolates were preliminary identified by biochemical tests as 44 *Pseudomonas* spp., 35 *Klebsiella* spp., 18 *E. coli*, 17 *Acinetobacter* spp., 8 *Proteus* spp. and 3 *Enterobacter* spp., The test for biofilm formation in these isolates was carried out by using three different methods that finally resulted in 27 positive biofilm forming isolates. Production of quorum sensing signal molecules (acyl homoserine lactone) was detected in 20 isolates using gas chromatography mass spectrum (GC/MS); These isolates were 3 *Pseudomonas* spp., 6 *Klebsiella* spp., 8 *E. coli* and 3 *Enterobacter* spp., The anti-biofilm activity of some plant extracts was carried out using tube method. It showed that *Syzygium aromaticum* was the most effective one and inhibited biofilm formation in all isolates followed by *Allium sativum. Syzygium aromaticum* extract also inhibited quorum sensing production in the selected 20 bacterial isolates. From the above results, *Syzygium aromaticum* extract was recommended to be used as an active antibacterial agent against multidrug resistant bacteria due to its ability to inhibit biofilm formation and quorum sensing signal production. Finally, the most potent bacterial isolates were genetically identified by 16S rRNA gene sequencing.

Keywords: Quorum sensing, Acyl homoserine lactone, Gram Negative bacteria & Biofilm.

1. Introduction

Biofilm is an exopolysaccharide, a slime matrix around multiple layers of cells. Biofilm formation is considered to be two-step process in which the bacteria first adhere to a surface by production of exopolysaccharides, followed by multiplication to form multilayered biofilm [1,2]. Within a biofilm, bacteria communicate with each other by production of chemotactic particles or pheromones, a phenomenon called quorum sensing [3]. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold. [4]. Quorum sensing (QS) is a complex environmental sensing system employed by bacteria to communicate among themselves and thereby regulate their population activities in response to various stimuli. The QS mechanism depends on the synthesis and release of chemical signals into the environment and on the detection of these signals as a function of cell population density.

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2. Materials and Methods

Such group behavior results in altered gene expression that drives the activities of the bacteria in a coordinated manner [5, 6]. Bacteria synthesize chemical signals that include a wide variety of small molecules [7]. Of these, the N-acylhomoserine lactones (AHLs) are the most commonly used by Gram-negative bacteria for bacterial communication. The biosynthesis and effects of AHLs depend primarily on the activity of the LuxI and LuxR protein families, respectively. After AHLs are produced by LuxI enzymes (AHL synthases), they diffuse across bacterial membranes and accumulate externally until reaching high local concentrations. At a given threshold intracellular concentration, the AHL binds to a LuxR response regulator forming a complex that regulates gene expression [8, 9] such as biofilm formation. Therefor targeting inhibition of quorum sensing resulted in inhibition of biofilm formation and stop pathogenicity and resistance of pathogenic gram negative bacteria against the common used antibiotics.

The aim of this work was to inhibit biofilm formation via targeting quorum sensing system in some pathogenic Gram negative bacteria by using some medicinal plants.

2.1. Bacterial isolates

A total number of 125 clinical isolates were collected from microbiology laboratory in El-Demerdash hospital. All of these isolates were Gram negative bacteria.

2.2. Plant materials

Twelve plant species were exploited in this study some of them were collected from the garden of Botany Department, Faculty of Science, Ain Shams University and the others were purchased from local commercial markets in Cairo city, Egypt.

2.3. Preliminary Identification of clinical isolates

Preliminary screening was carried out according to Bergey's Manual of Determinative Bacteriology [10].

2.4. Detection of biofilm formation

Biofilm formation was detected by three different methods; Congo Red Agar method according to [11], Micro-titer plate assay [12] and tube method [13].

2.5. Inhibition of biofilm formation

The antibacterial activity of some plant extracts was detected using the standard well agar diffusion method [14] and also tested for their ability to inhibit biofilm formation in the selected bacterial isolates using tube method [13].

2.6. Antibiotic susceptibility test

The selected bacterial isolates were tested for their resistance and sensitivity to 13 different antibiotics using disc diffusion method and the inhibition zone was measured according to Clinical Laboratory Standard Institutes guidelines [15].

2.7. Detection of quorum sensing signal production

Production of quorum sensing signal molecules (acyl homoserine lactones) was detected using gas chromatography mass spectrum (GC/MS) before and after treatment with *Syzygium aromaticum* extract. Acyl homoserine lactone was extracted from the bacterial culture as described in [16].

2.8. Identification of the selected bacterial isolates by 16S rRNA gene sequencing

The most potent bacterial isolates were genetically identified by 16S rRNA gene sequencing at Sigma Scientific services Co., El Giza, Egypt.

2.9. Data analysis

Means and standard deviations of the studied variables were calculated. The differences among the means of the studied variables were tested using F-test. In addition, after testing the data for normality, one way and two way analysis of variance (ANOVA) was used to assess the significance of variations among the different variables using post hock according to SPSS software [17].

3. Results and discussion

3.1. Preliminary Identification of clinical isolates

Table (1): Biochemical tests for identification of the clinical isolates.

Identification of clinical isolates	fermentatio	fermentatio	fermentatio	Oxidase test	Citrate test	Motility test	Indole test	Urease test	Haemolytic activity	Nitrate reductase test
Pseudomonas spp. (44)	-	-	-	+	+	+	-	+	+ α/β	+
Klebsiella spp. (35)	+	+	+	-	+	-	V	+	+ α	+
E. coli (18)	+	+	+	-	-	+	+	-	V	+
Acinetobacter spp. (17)	-	-	-	-	V	V	-	V	-	-
Proteus spp. (8)	+	-	+	-	+	+	-	+	+	+
Enterobacter spp. (3)	+	+	+	-	+	+	-	+	+ α	+

+:positive result - : negative results Variable (V): some isolates produced positive results others produced negative ones.

3.2. Detection of biofilm formation

Twenty seven isolates were positive biofilm forming in the three different methods of biofilm detection (Table 2), these isolates were tested for quorum sensing signal production.

Table (2): The percentage of positive biofilm forming isolates using different methods.

Isolates	Biofilm production						
	CRA method		Microtiter plate assay (ELISA)	Tube method			
	After 24h	After 48h		Weak	Moderate	Strong	Total no.
Ps spp.	12	12	11	0	1	5	6
K spp.	14	14	15	2	2	4	8
E. coli	7	7	8	3	2	3	8
Ac spp.	6	6	6	1	0	1	2
Pr spp.	2	2	1	0	0	0	0
En spp.	2	2	3	2	0	1	3
Total no.	43	43	44	8	5	14	27
%	34.4		35.2	21.6			

The previous results reported that using of more than one method was required for the test of biofilm formation; these results were correlated with [18, 19, 20 &21].

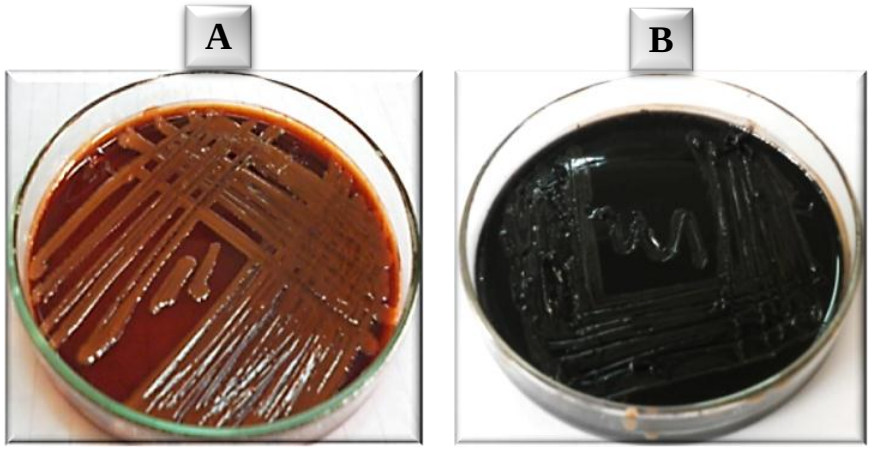

Figure (1): Biofilm formation test by different bacterial isolates using CRA medium. A: -ve biofilm formation (red color), B: +ve biofilm formation (black color).

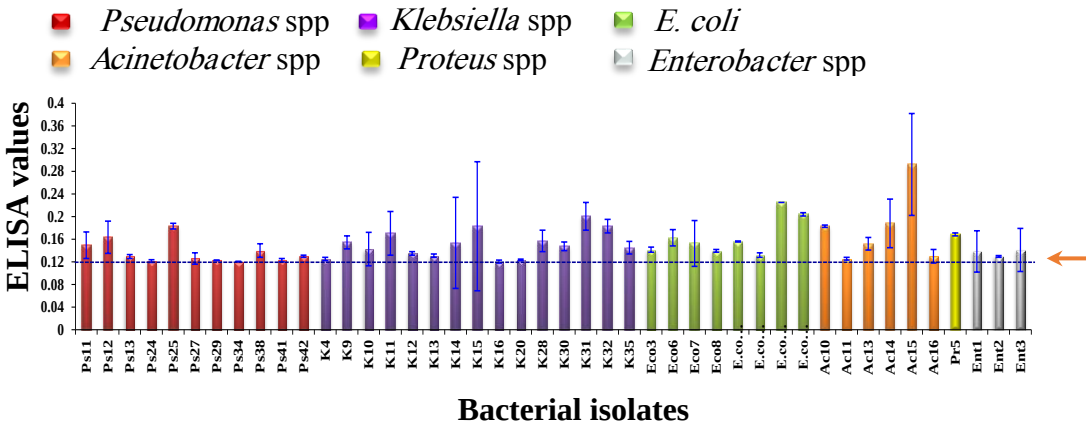

Figure (2): Bacterial isolates showing positive biofilm formation by ELISA reader

Ps: Pseudomonas; K: Klebsiella; E. coli: Escherichia coli; Ac: Acinetobacter; Pr: Proteus and *En: Enterobacter*. The arrow on the right side of the figure indicates the level of positive biofilm production (absorbance value >0.12).

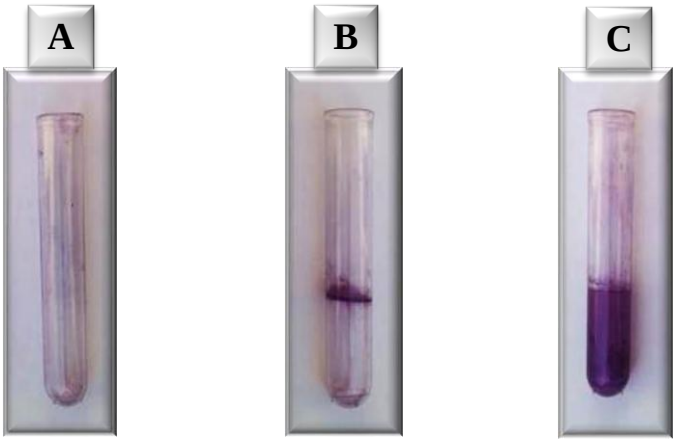

Figure (3): Positive and negative biofilm forming isolates using tube method.

A and B: -ve biofilm formation; C: +ve biofilm formation

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3.3. Antibiotic susceptibility test

Sensitivity of the identified 20 clinical isolates towards different standard antibiotics was carried out to detect the multi-drug resistance of these isolates. Table (3) represented the susceptibility of the selected 20 clinical isolates to 13 different antibiotics. The sensitivity and resistance of the isolates were performed using the disk diffusion method. From the statistical analysis by F test, it showed that there is a highly significant difference between different 20 bacterial isolates with each other by the value F=2337***, F= 100798*** between different antibacterial agents (12 plant extracts and 13 antibiotics) with each other and F=773*** between different antibacterial agents (12 plant extracts and 13 antibiotics) and different 20 bacterial isolates at the probability P<0.001. From the previous results, *Pseudomonas* sp41

was the most resistant one against the all tested antibiotics followed by *Pseudomonas* sp42. For *Klebsiella* spp, the isolate *K*35 was more resistant than *K*28. For *E. coli*, the isolate *E. coli*13 was the most resistant one followed by *E. coli*16 and *E. coli*14. Finally the isolate *Enterobacter* sp3 was more resistant than *En*1 and *En*2 to the tested antibiotics.

The results obtained were confirmed by [22 & 23], who found that erythromycin had the highest overall resistance and also most of the selected isolates (*Pseudomonas* spp., *Klebsiella* spp., *E. coli* and *Enterobacter* spp.) showed highly resistance against commonly used antibiotics.

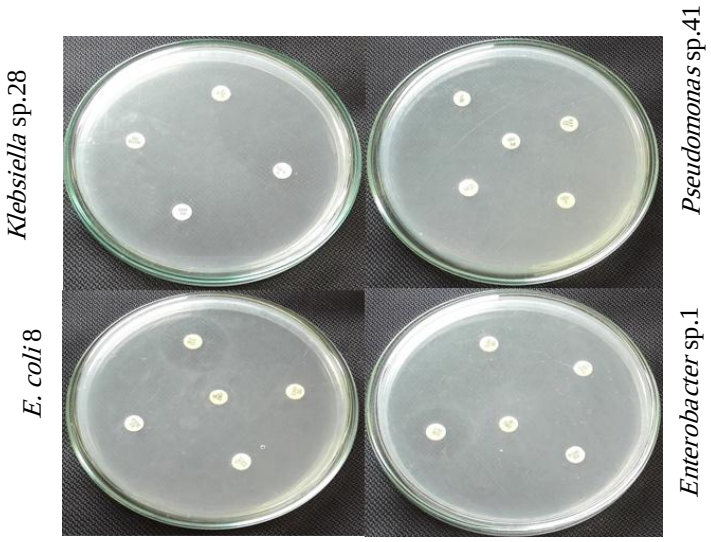
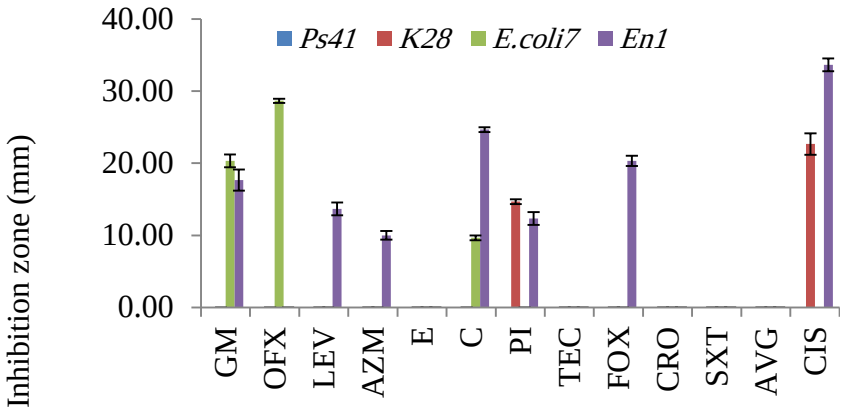


Figure (4): Effect of different antibiotics on some selected bacterial isolates.



Figure(5): Antimicrobial activity of different antibiotics on *Pseudomonas* sp.41, *Klebsiella* sp.28, *E. coli* 7 and *Enterobacter* sp.1.

Table (3): Effect of different antibiotics on the biofilm forming bacterial isolates.

Isolate	Inhibition zone (mm)												
	GM	OFX	LEV	AZM	E	C	PI	TEC	FOX	CRO	SXT	AVG	CIS
*Ps*25	15±0.6	28±2.0	30±1.5	0	0	0	13±0.6	0	15±0.6	16±1.0	0	12±0.6	21±1.5
*Ps*41	0	0	0	0	0	0	0	0	0	0	0	0	0
*Ps*42	11±0.6	20±0.6	39±1.7	0	0	12±0.6	16±1.0	0	0	0	16±1.5	0	12±1.5
*K*9	0	0	9±0.6	0	0	16±0.6	10±0.6	0	0	0	0	0	11±1.5
*K*12	20±0.6	0	10±0.6	0	0	20±2.0	11±0.6	0	0	0	0	0	11±0.6
*K*13	0	26±0.6	0	0	0	22±1.5	0	0	0	0	0	0	0
*K*16	18±0.6	0	13±1.5	0	0	20±0.6	11±0.6	0	0	0	0	0	24±1.5
*K*28	0	0	0	0	0	0	15±0.6	0	0	0	0	0	23±2.5
*K*35	0	0	0	0	0	0	14±1.2	0	0	0	0	0	20±2.0
*E.coli*3	0	0	10±1.2	11±0.6	0	20±3.5	0	0	14±2.5	0	0	0	12±0.6
*E.coli*6	22±0.6	30±1.5	31±2.3	0	0	18±2.5	17±2.5	0	0	13±1.5	24±1.5	0	28±1.5
*E.coli*7	20±1.5	29±0.6	0	0	0	10±0.6	0	0	0	0	0	0	0
*E.coli*8	21±1.2	30±2.5	29±1.2	0	0	17±1.5	16±1.5	0	0	10±0.6	24±0.6	0	24±0.6
*E.coli*13	14±0.6	0	0	0	0	20±0.6	0	0	0	0	0	0	0
*E.coli*14	18±1.0	0	0	0	0	19±1.2	0	0	0	0	0	0	0
*E.coli*15	15±0.6	0	0	0	0	15±3.1	0	0	13±0.6	0	14±1.5	0	13±1.2
*E.coli*16	16±2.5	0	0	0	0	20±2.5	0	0	0	0	0	0	0
*En*1	18±2.5	0	14±1.5	10±1.0	0	25±0.6	12±1.5	0	20±1.2	0	0	0	34±1.5
*En*2	16±0.6	0	11±0.6	0	0	20±2.5	9±0.6	0	12±0.6	0	0	10±1.5	16±0.6
*En*3	19±1.2	0	0	10±0.6	0	21±1.2	0	0	16±0.6	0	0	0	13±1.0

3.4. Plant extracts and inhibition of biofilm formation

3.4.1. Antibacterial activity of some plant extracts

Ethyl acetate extracts of 12 plant species were tested against the 20 bacterial isolates that showed both biofilm and QS signal production, using the standard well agar diffusion method.

Each bacterial isolate was inhibited by certain plant extracts, then the most effective plant extracts were tested for their anti-biofilm activity on each bacterial isolate using tube method. Data in table 9 showed that, *Syzygium aromaticum* extract was the most effective one against almost of the tested selected bacterial isolates, followed by *Allium sativum*, but *Ocimum* sp. extract showed highly activity against isolates *Ps*41, *K*9 and *E. coli6* than *Syzygium aromaticum* one. Also *Illicium verum* was more active against the isolate *Ps*41 than *Syzygium aromaticum*.

The results of the present study were in harmony with the study carried out by [24 & 25] in which Clove extract exhibited antibacterial activity against all tested bacterial isolates.

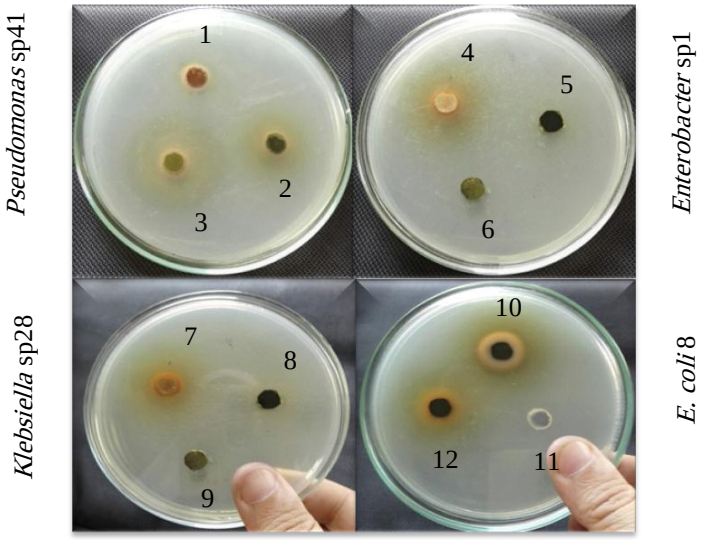


Figure (6): Effect of plant extracts on the selected bacterial isolates.

1: *Illicium* sp.; 2: *Callistemon* sp.; 3: *Ocimum* sp.; 4: *Syzygium* sp.; 5: *Moringa* sp.; 6: *Clerodendrum* sp.; 7: *Syzygium aromaticum*; 8: *Moringa* sp.; 9: *Clerodendrum* sp.; 10: *Callistemon* sp.; 11: DMSO and 12: *Melaleuca sp*

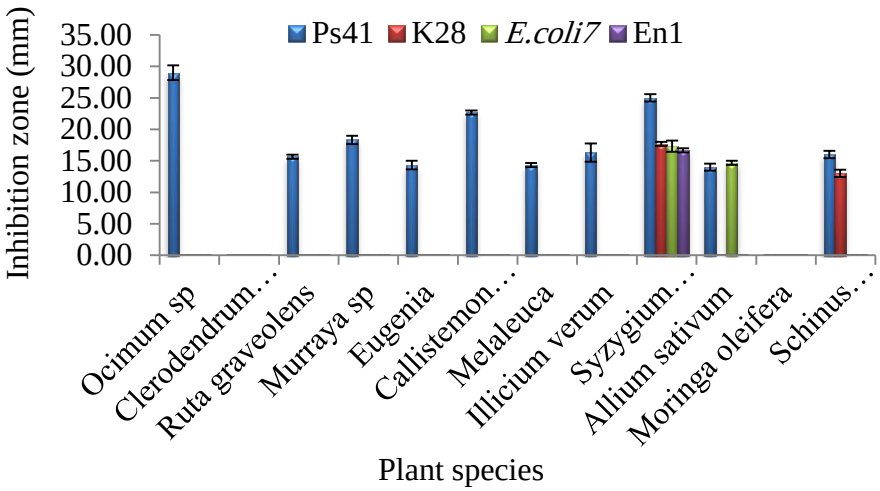


Figure (7): Antimicrobia activity of different ethyl acetate plant extracts on *Pseudomonas* sp.41, *Klebsiella* sp.28, *E. coli* 7 and *Enterobacter* sp.1.

3.4.2. Plant extracts and inhibition of biofilm formation

The most effective two plant extracts against each bacterial isolate as antibacterial agent were selected and tested for their antibiofilm activity against this biofilm forming and quorum sensing producing bacterial isolate using tube method. This selection was according to the previous results obtained in Table (4). Five plant extracts were selected for this test as shown in Table (5).

Figure (8): Antibiofilm activity of *Dianthus* sp. and *Allium sativum* extracts on *Pseudomonas* sp. isolate no. 25 A: +ve biofilm (before treatment), B: -ve biofilm after treatment with *Syzygium aromaticum* extract, C: -ve biofilm after treatment with *Allium sativum* extract.

Table (4): The antibacterial activity of each ethyl acetate plant extract against twenty clinical isolates

Isolate	Inhibition zones (mm)											
	***Ocimum* sp**	***Clerodendrum inerme***	***Ruta graveolens***	***Murraya* sp**	***Eugenia***	***Callistemon viminalis***	***Melaleuca***	***Illicium verum***	***Syzygium aromaticum***	***Allium sativum***	***Moringa oleifera***	***Schinus terebinthifolius***
*Ps*25	0	0	0	0	0	0	0	14±1.0	18±0.6	21±0.6	0	0
*Ps*41	29±2.0	0	16±0.6	18±1.2	14±1.2	23±0.6	14±0.6	16±2.5	25±1.0	14±1.0	0	16±1.0
*Ps*42	14±0.0	11±1.0	11±1.2	12±0.6	12±0.6	13±1.0	12±1.0	18±0.6	15±1.5	12±1.0	0	13±1.5
*K*9	22±2.0	0	12±0.6	12±0.6	12±1.0	15±2.5	11±1.2	15±1.0	16±0.6	12±2.0	0	14±1.5
*K*12	13±0.6	12±0.6	0	0	0	14±1.5	12±2.5	14±0.6	17±1.0	15±1.5	0	12±1.0
*K*13	14±1.0	0	0	0	13±1.5	14±1.0	13±1.5	14±1.5	16±2.5	15±0.6	0	13±0.6
*K*16	0	0	0	0	0	12±1.5	12±0.6	0	18±1.5	19±1.5	0	14±1.5
*K*28	0	0	0	0	0	0	0	0	18±0.6	0	0	13±1.0
*K*35	0	0	0	0	12±0.6	12±1.2	12±1.0	0	16±1.0	20±1.0	0	14±1.0
*E.coli*3	0	0	0	0	0	12±1.5	11±1.2	0	16±0.6	20±1.5	0	14±0.6
*E.coli*6	22±1.2	0	15±0.6	13±1.0	0	14±0.6	0	15±0.6	17±0.6	14±1.5	14±0.6	0
*E.coli*7	0	0	0	0	0	0	0	0	17±1.5	15±0.6	0	0
*E.coli*8	23±0.6	0	0	0	18±1.5	22±0.6	18±0.6	0	34±1.5	25±1.5	14±1.5	20±2.5
*E.coli*13	0	0	0	0	0	15±0.6	15±0.6	0	17±0.6	18±0.6	0	15±1.0
*E.coli*14	0	0	0	0	0	14±1.5	12±1.0	0	16±2.0	18±1.2	0	13±0.6
*E.coli*15	15±1.0	0	0	16±0.6	15±1.2	15±0.6	15±0.6	13±0.6	20±1.5	26±0.6	0	15±1.5
*E.coli*16	12±2.5	0	0	13±1.5	12±1.5	12±1.0	12±1.0	0	17±0.6	18±0.6	0	12±1.5
*En*1	0	0	0	0	0	0	0	0	17±0.6	0	0	0
*En*2	14±1.5	0	0	0	0	14±1.5	12±2.0	13±1.0	18±0.6	15±1.0	0	13±0.6
*En*3	0	0	0	0	0	11±1.2	12±0.6	12±1.0	17±1.0	19±1.5	0	14±1.5

Ps: Pseudomonas; K: Klebsiella; E. coli: Escherichia coli and *En: Enterobacter*

Table (5): Activity of plant extracts on biofilm production in each bacterial isolate.

MO	Plant extract and biofilm production																								
	Ocimum sp					*Illicium verum*					*Syzygium aromaticum*					*Allium sativum*					*Schinus terebinthifolius*				
	0.05 ml	0.1 ml	0.2 ml	0.5 ml	1 ml	0.05 ml	0.1 ml	0.2 ml	0.5 ml	1 ml	0.05 ml	0.1 ml	0.2 ml	0.5 ml	1 ml	0.05 ml	0.1 ml	0.2 ml	0.5 ml	1 ml	0.05 ml	0.1 ml	0.2 ml	0.5 ml	1 ml
*Ps*25											+	-	-	-	-	+	-	-	-	-					
*Ps*41	+	+	+	-	-						+	-	-	-	-										
*Ps*42						+	-	-	-	-	+	-	-	-	-										
*K*9	+	+	+	-	-						+	-	-	-	-										
*K*12											-	-	-	-	-	+	+	-	-	-					
*K*13											+	-	-	-	-	+	+	-	-	-					
*K*16											+	-	-	-	-	+	-	-	-	-					
*K*28											-	-	-	-	-						+	-	-	-	-
*K*35											-	-	-	-	-	+	+	-	-	-					
*E*3											+	-	-	-	-	+	+	-	-	-					
*E*6	+	+	-	-	-						-	-	-	-	-										
*E*7											-	-	-	-	-	+	-	-	-	-					
*E*8											-	-	-	-	-	+	-	-	-	-					
*E*13											+	-	-	-	-	+	+	-	-	-					
*E*14											-	-	-	-	-	+	-	-	-	-					
*E*15											+	-	-	-	-	+	-	-	-	-					
*E*16											-	-	-	-	-	+	-	-	-	-					
*En*1											-	-	-	-	-										
*En*2											+	-	-	-	-	+	-	-	-	-					
*En*3											-	-	-	-	-	+	+	-	-	-					

MO: microorganism; *Ps: Pseudomonas; K: Klebsiella; E: Escherichia coli* and *En: Enterobacter*

Syzygium aromaticum extract was the most effective one as a biofilm inhibitor by the concentration ranged between 0.05-0.1 ml against the all twenty selected bacterial strains, followed by *Allium sativum* which was effective by the concentration ranged between 0.1-0.2 ml. Then *Syzygium aromaticum* extract was tested for inhibition of quorum sensing signal (AHLs) production in the selected twenty bacterial strains.

3.5. Quorum sensing signal production before and after treatment with Clove extract

Quorum sensing signal (AHLs) production was inactivated by *Syzygium aromaticum* extract in the selected bacterial strains, and so no lactone signal was detected using GC/MS. The concentration of *Syzygium aromaticum* extract was 20 µl/ ml.

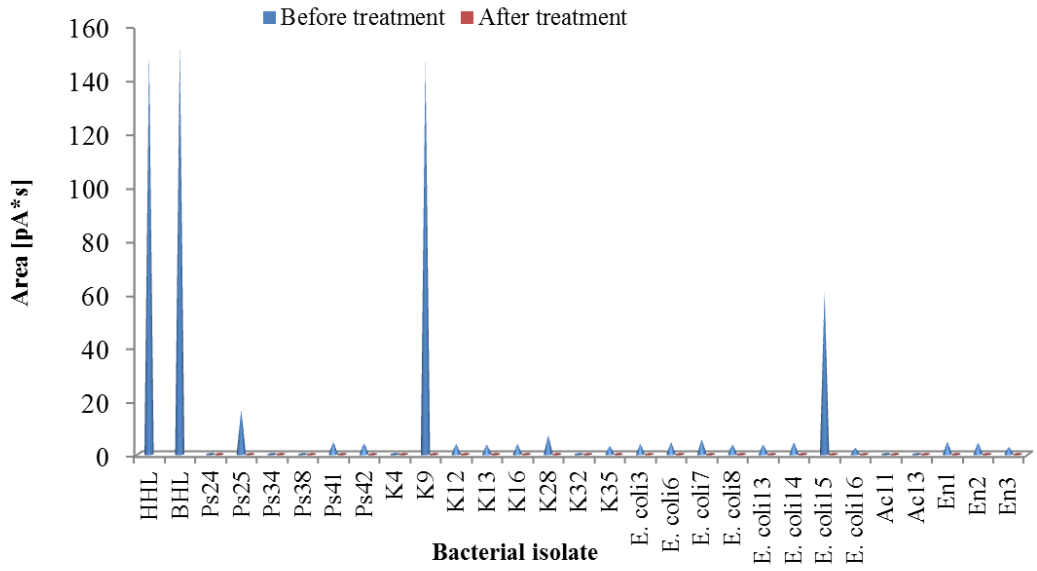


Figure (9): GC/MS of Q.S signal (AHLs) production before and after treatment with *Syzygium aromaticum* extract.

3.6. Molecular analysis

The most virulent bacterial isolates were genetically identified by 16S rRNA gene sequencing. The identified strains were presented in (Table 6).

Table (6): 16S rRNA gene sequencing of 4 selected bacterial isolates

Isolate No.	Blast identity	NCBI accession No.
FS7	*Escherichia coli* FS7	MF197876
FPF28	*Klebsiella pneumonia* FPF28	MF197877
FS1	*Enterobacter cloacae* FS1	MF197878
FS41	*Pseudomonas aeruginosa* FS41	MF197879

4. Conclusions

The results reported that using of some medicinal plants is safer and more effective than using agents of chemical origin to inhibit quorum sensing and biofilm formation in the studied pathogenic Gram negative bacteria. These bacteria not only are the common cause of nosocomial infection but also are considered to be multi-drug resistant bacteria as a result of formation of biofilm. Clove extract showed highly antibacterial activity and inhibit both biofilm formation and quorum sensing system in the studied bacterial isolates.

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الملخص باللغة العربية

تثبيط تكوين الغشاء الحيوى فى بعض البكتريا الممرضه السالبه لصبغة جرام باستخدام مركبات مضاده للحس العددى

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فى هذه الدراسة تم تجميع ١٢٥ عزله بكيترية من معمل الميكروبيولوجى بمستشفى الدمرداش بمحافظة القاهرة.
وقد تم عزل هذه البكتريا من مصادر مختلفه مثل الصديد ، اللعاب ، افرازات الانف ، الدم والبول.

تبعاً لصفات الشكل والنمو على الوسط والتشخيص الكيمو حيوي تم تعريفهم إلى ٤٤ عزله ينتمون لجنس السيدوموناس ، ٣٥ عزله ينتمون لجنس الكليبسيلا و ١٨ عزله لجنس الإيشيرشيا كولاى ، ١٧ عزله ينتمون لجنس اللاسينيتوباكتر ، ٨ عزلات ينتمون لجنس بروتيس ، ٣ عزلات ينتمون لجنس الانتيروباكتر.

وتم تعيين قدرة هذه العزلات البكتيريه على تكوين الغشاء الحيوى بثلاث طرق مختلفه وكانت النتيجه وجود ٢٧ عزله مكونه للغشاء الحيوى. تم اكتشاف انتاج اشارات وجزيئات نظام الحس العددى فى ٢٠ عزله من هذه العزلات البكتيريه وذلك باستخدام جهاز الغاز الكروماتوجرافى المعتمد على الكتله والطيف ، وكانت العزلات المنتجه للحس العددى كالتالى ٣ عزلات من جنس السيدوموناس ، ٦ عزلات من جنس الكليبسيلا ، ٨ عزلات من جنس الإيشيرشيا كولاى و ٣ عزلات من جنس الانتيروباكتر.

تم اجراء اختبار النشاط المضاد لتكوين الغشاء الحيوى لبعض المستخلصات النباتيه باستخدام طريقة الانابيب حيث أظهرت النتائج ان مستخلص نبات القرنفل هو الاكثر فاعليه فى تثبيط تكوين الغشاء الحيوى فى العشرين عزله يليه مستخلص نبات الثوم. ايضا وجد ان مستخلص القرنفل ثبط انتاج اشارات وجزيئات نظام الحس العددى فى كل من العشرين عزله.

من النتائج الموضحه اعلاه، تم ترشيح مستخلص نبات القرنفل لاستخدامه كماده فعاله وعامل مضاد للبكتريا ضد البكتريا المقاومه لكثير من المضادات الحيويه الشائع استخدامها نظرا لقدرته على تثبيط تكوين الغشاء الحيوى وانتاج اشارات وجزيئات نظام الحس العددى . وفى النهايه تم تعريف العزلات البكتيريه الاكثر ضراوه جينيا.