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Evaluation of Some Material to inhibit Biofilm Formed by *Acinetobacter baumannii* Isolates

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ABSTRACT

The study has included the ability of *Acinetobacter baumannii* (isolated from clinical infections and hospital environment) to form a biofilm and investigated the possibility of inhibition this biofilm by using some materials: EDTA, five amino acids and five plant extracts.
The results showed that all isolates of *A.baumannii* had a good ability to form the biofilm (100%). Four isolates which had strong former of biofilm which were selected as well as a standard isolate (*A. baumannii* ATCC19606). The inhibitory effect on biofilm formation was investigated. EDTA had a good inhibitory effect, also, all amino acids showed good inhibitory activity , with the best inhibition in Glutamic acid at a concentration of 50 mM, while plant extracts varied in their inhibition ratio to inhibit the biofilm, turmeric, cloves and rosemary had a good inhibitory effect, Bay leaf has the ability to inhibit the biofilm formation but less than others, while the plant extract of the Myrtle did not show any inhibition activity.

Introduction

Acinetobacter baumannii (A.baumannii) is a Gram-negative bacteria, coccobacilli in shape, non –lactose fermenter, growing at 44 °C of temperature, which distinguishes it from other species. Acinetobacter has the ability to live in diverse environments, isolated from soil, water and many cases of diseases. It is also, isolated from non-living surfaces especially from the hospital environment, characterized by long-term survival on the dry surfaces, which contributed to the transition between the various environments. The high tolerance to drought led to being a successful pathogen causing hospital infections and the reason for its resistance to drought due to the formation of the biofilm and its growth temperature of 44 ° C and the surface of the acinetobacter.spp is a hydrophobic and is an important feature of bacterial adhesion [1,2].
Biofilms are the important feature for *A.baumannii* on living and non-living surfaces, which is a group of bacteria embedded in a mold produced by itself is easy to stay in the environment and this mold consists of living surfaces and non-living, may be living tissue or medical devices such as catheter and valves [3].There are several factors that affect in the formation of this biofilm, such as: availability of nutrients, the presence of proteins in the outer membrane, the presence of pili, secretion of large molecules such as extracellular DNA (eDNA), the presence of metal ions, including iron, which is a nutrient necessary for bacteria [4].
Biofilms community is more resistant to antibiotics and allow pathogenic bacteria to survive and invade a new host, thus pathogenicity of bacteria was increased, therefore scientists looking at antimicrobial and antibiofilm compounds that inhibit or control the process of forming the biofilm; such as: Ethylene di-aminetetraacetic acid (EDTA) material, amino acids and plant extracts [5].
The study aimed to investigate biofilm formation in *A.baumannii* isolates and to test the ability of some material to inhibit this biofilm, their were: EDTA, plant extracts and amino acids.

Materials and methods

1- Bacterial isolates: Nineteen *A.baumannii* isolated from clinical cases (wounds, urine, respiratory infections, burns, CSF) and hospital environment, {have been identified by routine and molecular methods based on identified gene ($bla_{oxa\text{-}51\ like}$)}, were used in this study. As well as, standard strain of *A.baumannii* ATCC19606 which has been obtained from Media centre /Irbil city.

2- Biofilm Formation:

The biofilm formation on non-living surfaces was determined quantitatively by the microtiter plate assay, All bacterial isolates were grown in Luria-Bertani broth medium for 24 h at 37 ° C, after that the suspension diluted until the concentration reached 0.003 cell / ml using the spectrophotometer at 600 nm, then 200 µl of this suspension was transferred to a microtiter plate wells in triplicate for each isolate, a negative controls were used. A microtiter plate was incubated at 37 ° C for 24, After the incubation period, the wells components were removed and washed once gently with deionized water. The holes were then dyed with 1% violet crystal for 30 minutes ,then were washed by distilled water three time, the adherent dye was removed by adding 200 µl of 100% methanol to each well. The absorbance was measured at 545 nm wavelength using an American ELIZA reader, the experiment was done according to [6].

Inhibition of biofilm formation:

In order to treat the problem of biofilm formation produced by the *A.baumannii*, several experiments were conducted in which different natural and chemical agents were used to compare the most effective agent to inhibit the biofilm formation: EDTA, five amino acids and a number of common plant extracts.

The study deals with four isolates selected from the bacteria under study, with the highest rate of biofilm formation (by analyzed them in statistical analysis to determine the highest rate of formation), as well as the use of standard isolate (ATCC19606), which was also efficient in biofilm formation. The steps of the experiments of inhibition were carried out as follows:

• **Use of EDTA**

EDTA was used in three different concentrations (100 mg / L, 125 mg / L, 150 mg / L).The LB broth medium was prepared and EDTA was added in the three concentrations on the medium, each separately, and then the medium was sterilized by the autoclave, and distributed into sterile tubes. Selected isolates were cultured in EDTA-containing medium, with the use of control, all were incubated for 24 hours at 37 ° C [7]. 200 µl of each control tube and isolates tube were transferred to the microtiter plate in duplicate, incubation for 24 hours at 37 ° C was done then the crystal violet assay was examined [6].

• **Use of amino acids:**

Five amino acids (Glycine, Phenylalanine, Glutamic acid, Methionine, Leucin) were used in three different concentrations of each one (50,100, 500 mM),The same previous steps were done as the EDTA experiment in terms of culturing, incubating and transferring bacterial isolates into a microtiter plate and conducting crystal violate assay.

• **Use of plant extracts**:

The plants described in Table (1) were collected from the markets of Mosul city in a dried form (turmeric, cloves, Bay leaf). The leaves of the Myrtle plant and the rosemary were collected from their areas, dried naturally in the laboratory and away from sunlight for five days and to eliminate from soil and impurities until they are used, They were then crushed into powders and stored in clean containers at room temperature.

Table (1): Plants used and their scientific names.

Plants	scientific name
Turmeric	*Curcuma longa*
Clove	Syzygium aromaticum
Bay leaf	*Laurus nobilis*
Myrtle	*Myrtus communis*
Rosemary	*Rosmarinus officinalis*

Preparation of the water plant extract:

Plant extracts were prepared based on [8], One gram of each plant powder was weighed and placed in a flask, 10 ml of distilled water was added to cover the powder, The flask was covered with aluminum foil and incubated at a temperature of 45 °C in a shaking water bath for 3 hours, the extract was filtered using filter paper Whatman No.1., Extraction was repeated twice on residue by using 5 ml of distilled water. Filtrates were collected and dried and save at a temperature of 4°C, and thus obtained a dry plant extract.

The experiment of inhibiting the biofilms of these extracts was then carried out by the following steps:

• One gram of dry plant extract was weighted and dissolved it 5 ml of distilled water to obtain a final concentration of 200 mg / ml sterilized by filtration with 0.45 µm membrane filters, 100 µl of bacterial growth were added to the wells and incubated for 4 hours at 37°C to allow cell adhesion. After incubation, 100 microliters of each plant extract were added with the previously prepared concentration to the same previous well containing 100 µl of bacterial growth. 200 µl of bacterial growth was added after comparing the mixture with the standard McFarland tube 0.5 to the microtiter plate a positive control, a replicate for each isolate was done and incubated at a temperature of 37 C for 24 hours.

• After the incubation, a crystal violet test was performed for the plate.

• The statistical analysis was performed using the SPSS.18 program.

Results and discussion

Biofilm Formation:

All of the *A.baumannii* isolates showed the ability to biofilm formation, including the standard isolate by rate 100%. As shown in Figure (1)

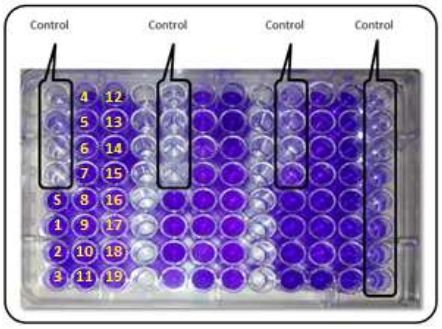

Figure (1): Biofilm formation in *A.baumannii* isolates.

After statistical analysis using SPSS-18 to confirm the efficiency of these isolates in biofilm formation, the results showed a significant difference compared to the control samples at the level of P ≤0.05, ie, the ability to form the biofilm by these isolates was 100% as shown in table (2).

Table (2): Statistical analysis for the testing of the biofilm formation of *A.baumannii* isolates.

Samples	mean ± standard deviation	Extreme values	Sig.
Isolates	2.2083± 0.5055	1.46 – 3.04	0.000
Control	0.2633± 0.2257	0.02- 0.74	0.000

The results of the study were consistent with the local studies of [9], where the percentage of biofilm formation was 100% for this bacterium in their studies, while the results of the study of [10], had a rate of 81.2% in the ability of this bacterium to form the biofilm.

Also, the results were higher than [11] with a biofilm formation percentage was 75%.

The cause of the differences in the ratios of the biofilm formation is due to several reasons, including the difference in the number of isolated isolates from different sources, the environmental factors also affect the ratios of the biofilm, including temperature, humidity, oxygen and others [12].

The ability of *A.baumannii* to survive in dry environments is due to its formation of the biofilm, as well as the presence of many resistance genes in the bacteria, making these bacteria a successful pathogen among the nosocomial bacteria [13].

Inhibition of biofilm formation:

Bacteria producing biofilms are responsible for many diseases that are difficult to treat because of the difficulty of penetrating the antibiotic of this biofilm [14].

So, the study tackled many materials, to inhibit the biofilm and finding a solution to its formation problem. Such as:

1- Inhibition by using EDTA

The results of our study showed that EDTA had an effective role in inhibiting the biofilm formation as shown in Figure(2).

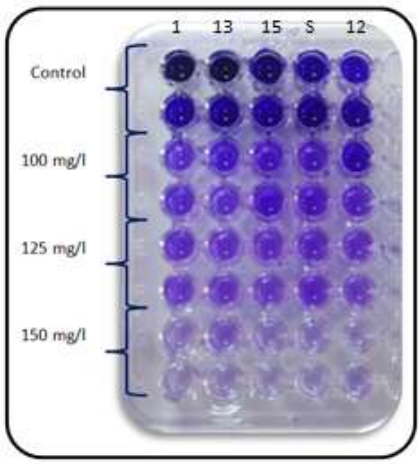

Figure (2): Biofilm inhibition in *A.baumannii* isolates by using of EDTA.

As shown in table (3) the statistical analysis showed that there were significant differences in the three concentrations used in the study (100, 125, 150 mg / ml) was 0.001 for the first concentration and 0.000 at the concentrations of 125 and 150 mg / ml, Thus it was possible to use this material for industrial purposes.

Table (3): Statistical analysis to test the inhibition of *A.baumannii* isolates using EDTA.

Objects	mean ± standard deviation	Extreme values	Sig.
Control	1.7410±0.53096	0.96_2.51	
EDTA 100 mg/ L	0.9690±0.26539	0.50_1.74	0.001
EDTA 125 mg/ L	0.6140±0.14849	0.45_0.90	0.000
EDTA 150 mg/ L	0.2730±0.43110	0.08_0.49	0.000

EDTA is a metal chelator, its high efficacy in inhibiting the formation of biofilm which was confirmed in many bacteria such as Staphylococcus spp. Pseudomnas spp. And Candida spp. [15].

Our results agreed with the study of [16], which showed the role of EDTA in inhibiting the first step of the process of biofilm formation.

The cations (Ca, Mg, Fe and Ba) are essential for the stability of the biofilm matrix, When EDTA is linked to it, a chelation agent will release LPS from the outer membrane of the cell and thus secrete cells from the biofilm [17].

2- Inhibiting by using amino acids:

The results showed that all amino acids have the ability to inhibit biofilm formation in selected isolates as shown in the Fig. (3). There is an inverse relationship between the biofilm formation and the concentration of the amino acid in the medium. At 50 µl concentration for glycine, phenylalanine, methionine, leucine and glutamic acid ,a decrease in the percentage of the biofilm formation was observed but in a small amount, with a rate of absorption between (0.320_1.948) except in the amino acid Glutamic acid, the concentration of 50 µl was significantly inhibited the biofilm formation, the absorption rate of this concentration was 0.320 as shown in table (4).

The results of the study coincided with the study of researcher [18], who showed a consensus in terms of reducing the biofilm formation by increasing the concentration of amino acid.

A study by [19] suggested that there are several factors that lead to the inhibition of the biofilm by amino acids, including that the amino acid is associated with the chains and bridges with the peptidoglycan in bacterial cell wall, leading to the disintegration of the wall and decay.

The presence of amino acids also stimulates the production of other proteins in the bacterial cell during metabolic processes, such as protease production; a virulence factor for bacteria, which inhibits the biofilm formation [20].

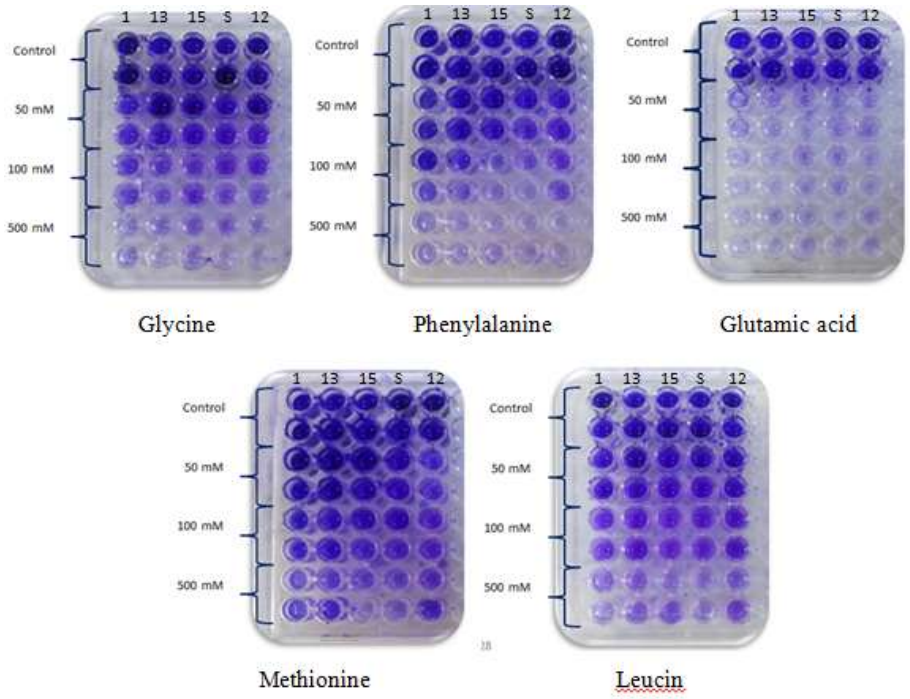

Figure (3): Biofilm inhibition in *A.baumannii* isolates by using of amino acids.

Table(4): Statistical analysis to test the inhibition of *A.baumannii* isolates using amino acids.

Amino acids	mean ± standard deviation	Extreme values	Sig.
Control	2.2830±0.40453	1.74_2.80	
Glycine 50 mM	1.6810±0.19313	1.40_1.92	0.000
Glycine 100 mM	0.6170±0.19816	0.27_0.99	0.000
Glycine 500 mM	0.1710±0.05486	0.09_0.25	0.000
Phenylalanine 50 mM	1.4740±0.31987	0.95_2.01	0.000
Phenylalanine 100 mM	0.5700±0.33902	0.22_0.91	0.000
Phenylalanine 500 mM	0.1460±0.04477	0.10_0.21	0.000
Glutamic acid 50 mM	0.3200±0.11245	0.19_0.51	0.000
Glutamic acid 100 mM	0.1740±0.2633	0.13_ 0.21	0.000
Glutamic acid 500 mM	0.1140±0.2171	0.08_0.15	0.000
Methionine 50 mM	1.6300±0.34150	0.91_2.12	0.002
Methionine 100 mM	1.0800±0.26204	0.61_ 1.44	0.000
Methionine 500 mM	0.3330±0.16242	0.11_0.63	0.000
Leucin 50 mM	1.9480±0.12453	1.75_2.11	0.023
Leucin 100 mM	1.1340±0.25400	0.89_ 1.62	0.000
Leucin 500 mM	0.3230±0.13598	0.17_0.60	0.000

3- Inhibiting by using plant extracts:

The plant extracts used in the study differed in their ability to inhibit the biofilm formation formed by our local isolates of *A.baumannii*. Turmeric, cloves and rosemary had a good inhibitory effect on the production of the biofilm as shown in figure (4).

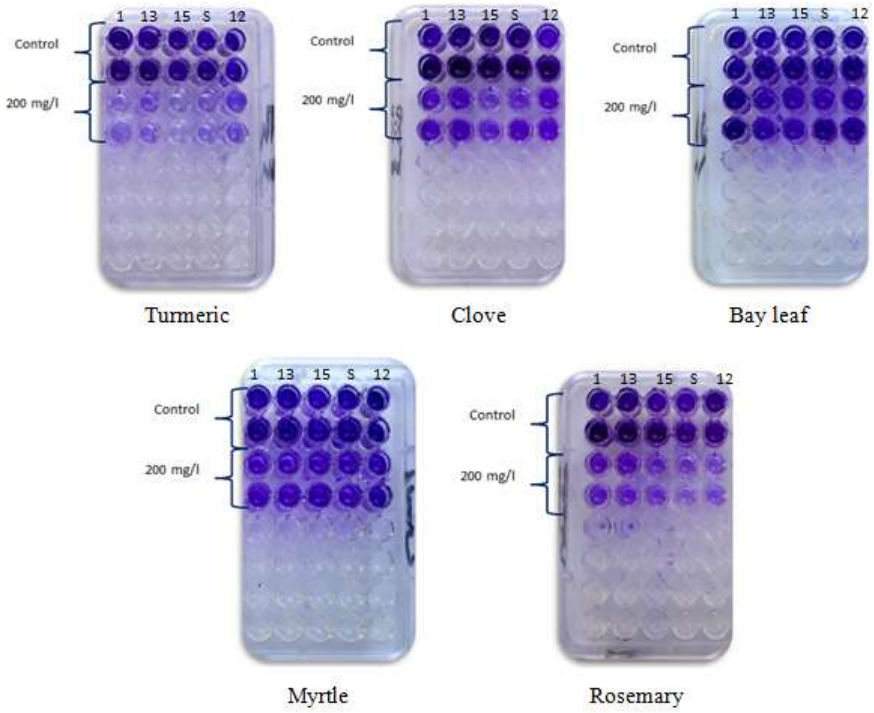

Figure (4): Biofilm inhibition in *A.baumannii* isolates by using of plant extracts.

After the statistical analysis of isolates, it was found that the plant extract of Turmeric, clove and rosemary showed a significant difference of 0.000. This is a proof of the efficiency of these extracts in inhibiting the biofilm formation. The plant extract of the Bay leaf showed a significant difference of 0.005 as it has the ability to inhibit the biofilm formation but less than other, while the plant extract of the Myrtle did not show any significant differences, the value of the mean difference was 0.10 and this value was greater than the value of $P \leq 0.05$ as shown in table (5).

Table(5): Results of statistical analysis to test the inhibition of *A.baumannii* isolates using plant extracts.

Plant extracts	mean ± standard deviation	Extreme values	Sig.
Control	2.0170± 0.14863	1.85 – 2.33	
Turmeric	0.2470 ± 0.11314	0.07- 0.4	0.000
Clove	0.5150 ± 0.21563	0.23- 0.9	0.000
Bay leaf	1.3940 ± 0.32308	0.96 – 1.98	0.005
Myrtle	1.6110 ± 0.34952	0.98 – 1.96	0.10
Rosemary	0.5970 ± 0.25096	0.2 – 0.92	0.000

The rate of absorbance for each extract showed that the water extract of turmeric has the highest activity to inhibit the biofilm with an absorption rate of 0.247, while the value of 0.515 and 0.597 for the water extract of clove and rosemary, respectively. The absorption rate of the water extract of the Bay leaf was 1.394 .This high percentage in inhibiting the biofilm formation of the plant extract of turmeric, cloves and rosemary has agreed with the study of [8]. Our study was also compatible with the study of [21] where it was found that the water extract of turmeric had a high inhibitory effect against the biofilm formation. The plant extracts contain many substances such as phenol derivatives, terpens, flavonoids, alkaloids, etc. the main cause of this inhibitory activity, as these substances inhibit microbial cell adhesion and membrane growth [22].

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تقييم بعض المواد في تثبيط الغشاء الحيوي المتكون من قبل عزلات *Acinetobacter baumannii*

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الملخص

تضمنت الدراسة قابلية عزلات *A.baumannii* المعزولة من الاصابات السريرية وبيئة المستشفيات على تكوين الغشاء الحيوي والتحري عن إمكانية تثبيط هذا الغشاء الحيوي باستخدام بعض المواد كالـ EDTA وخمس أحماض أمينية وخمسة مستخلصات نباتية.

أظهرت النتائج أن جميع عزلات *A.baumannii* لديها قدرة جيدة على تكوين الأغشية الحيوية بنسبة 100٪.

انتخبت أربع عزلات من البكتيريا التي أظهرت أعلى نسبة تكوين للغشاء الحيوي فضلاً عن استخدام العزلة القياسية وتم التحري عن تثبيط الغشاء الحيوي فيها، اظهر EDTA تأثير تثبيطي جيد، كما أظهرت جميع الأحماض الأمينية فعالية تثبيطيه جيدة في تثبيط الغشاء الحيوي مع أفضل تثبيط في حامض الغلوتاميك بتركيز 50 mM، في حين تباينت المستخلصات النباتية في نسبة تثبيطها للغشاء الحيوي، اذ اظهر كل من الكركم والقرنفل وإكليل الجبل تأثير تثبيطي جيد، بينما كان لاوراق الغار قدرة تثبيطيه قليلة، في حين أن المستخلص النباتي لنبات الاس لم يظهر أي فعالية تثبيطيه.