

## Evaluation of antibiofilm activity for siderophore produced from rhizospheric *Acinetobacter baumannii* against of some respiratory tracts bacteria

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### Abstract:

Siderophore was extracted from *Acinetobacter baumannii* isolated from rhizosphere region in Baghdad City . The results appeared the ability of isolate (*Acinetobacter baumannii* 5) to produce Siderophore with diameter of (15) mm and the condition for production was temperature reached 30 degrees, and the optimum pH was 7 . Purification of siderophore through : Ion exchange chromatography for purify more stable form as ferric-siderophore complex and final step through gel filtration. The activity of purified siderophore as antibacterial agent was tested against 13 different local clinical bacterial as follows (*Klebsiella pneumoniae* ,*Pseudomonas aeruginosa* ,*Acinetobacter baumannii* , *Escherichia.coli* ,*Streptococcus pyogenes* ,*Staph. aureus* ,*Enterobacter cloacea*, *Enterobacter aerogenes* ,*Enterococcus faecalis* ,*Burkholderia cepacian* ,*Serratia fiaria* ,*S. marcescens* ,*Klebsiella oxytoca* ) isolates at concentration 20 mg/ml of free iron siderophore and the results showed that highest activity of siderophore was reported on *Streptococcus.pyogenes* with inhibition zone (26)mm, and on *Acinetobacter baumannii*, *Serratia.marcescens* and *Escherichia.coli* the inhibition zone was (20)mm while the lowest effects were on *Serratia. fiaria* and *Klebsiella .oxytoca* with inhibition zone (16mm). As well as, the purified siderophore showed good activity as antibiofilm of pathogenic bacteria. The highest antiadhesive percentage was observed for *Escherichia.coli* by percentage 64.4%.

**Keywords:** Siderophore, CAS agar, *A. baumannii*, Biofilm, Anti-biofilm ,ion exchange , gel filtration .**Note:** The research is based on an MSc thesis.

## 1.Introduction:

Acinetobacter spp. are free-living saprophytic organisms and widely distributed in different environments including soil, water, wastewater, vegetables, and skin of animals and humans (Asif et al.,2018 Tawfeeq et al. 2022).It resists many classes of antibiotics by virtue of chromosome-mediated genetic elements on one hand, while it can also persist for a prolonged period in harsh environments (walls, surfaces, and medical devices) in the hospital settings on the other hand( Mussa et al ,2020 a).One mechanism of microorganisms and plants to surveil is to produce low molecular (500–1000 Daltons) iron chelators termed siderophores to enhance the acquisition of iron from the soil environment, particularly under the Fe-limited circumstances, which selectively complex iron (III) with very high affinity. Siderophores are high-affinity iron chelator proteins which compete with host cells for iron , Iron is an essential nutrient for nearly all life on Earth (Ilbert &Bonney, 2013). Bacterial siderophores have essential and many applications in wide types of fields, including agriculture, disease treatment, bacteria culture, plant growth promotion, phytopathogen management, and heavy metal pollution detoxification . Pulmonary infection is the leading cause of death in developing countries and the third leading cause of death worldwide). Siderophores can be employed as a "Trojan Horse Strategy" in the medical profession, forming a complex with antibiotics and delivering them to the appropriate places, notably in antibiotic-resistant bacteria. (Prabhakar, 2020). The **aim** of this study investigated the purified siderophore produced from *Acinetobacter baumannii* isolated from soil on some pathogenic bacteria from lower respiratory tract.

## 2.Materials and Method

### 2.1 Collection and isolation *A. baumannii*

The rhizosphere soil samples were taken from 10 randomly selected wetlands rhizosphere zones from various plants growing in different places in the city of Baghdad. one gram of soil samples was dissolved in 9 ml sterile distilled water and shaken vigorously for 2 min. (Omolola 2007) After wards, 1 mL of the solution was diluted into 9 ml of sterile distilled water to make  $10^{-2}$  soil dilution. Serial dilution was repeated from ( $10^{-1}$  ,  $10^{-2}$  ,  $10^{-3}$  ,  $10^{-4}$  ,  $10^{-5}$  ).0.1 ml taken from ( $10^{-3}$  - $10^{-5}$  ) soil dilution straking on Nutrient agar. The isolates were growing on CHROM agar as a selective medium for *A. baumannii* ,along with the other media namely Blood agar, MacConkys agar.

## 2.2 Detection of *A. baumannii*

Detection of *A. baumannii* for **siderophore production** was done by using two methods. First method detection of siderophore by using a (CAS) agar test, the second method using a spectrophotometric analysis to detect siderophore (Ali and Mussa ., 2020)

**2.3 Extraction of siderophore:** The siderophore was extracted According to (Cody & Gross, 1987) as following SM broth inoculated with *A. baumannii* , incubated in 200 rpm Shekar for 48 hour in 30°C , FeCl<sub>3</sub> was added then Centrifugation at 10000 rpm for 20 min to removal the bacterial cell after. The protein in supernatant was precipitated with saturated ammonium sulphate in 25%, 50% and 100% saturation .Followed extraction with equal volume of chloroform-phenol (1:1 v/wt). Organic phase was mixed with 3 volumes of diethyl ether then partitioned into deionized water until color could no longer be extracted from organic phase. The aqueous extract was washed with chloroform to remove phenol then dried by lyophilization.

## 2.4 Purification of siderophore :- Ion exchange chromatography

According to (Cody & Gross, 1987) the crude extract was dissolved in 50mM pyridine-acetate pH 5. The dissolved sample was added to the column of carboxymethylethyl (CM) Sephadex C-25 (1.5 by 80) cm. The column was eluted with the same buffer and the eluents were collected in 5-ml in each tube and read fraction at (405 nm).

**-Gel filtration chromatography:** The free iron siderophore was prepared by add same volume of (1 M) of EDTA with siderophore for 30 min obtained from previous step then passthrough gel filtration column (Sephadex G-25). The eluted with D.W and the elution was collected 5 ml for each fraction read at (405 nm) (Mussa et al ., 2020 ) The fraction tubes with siderophore was dried by using lyophilization method .

## 2.5 Biofilm formation bacteria

Isolation and Identification of respiratory tracts bacteria: Bacteria were isolated from sputum samples collected from local hospitals in Baghdad city), Identification of isolates were done according to the morphological, biochemical test (Khazzal et al ., 2022) and VITEK 2 system were used to confirmed the identification of all isolates . Microtiter Plate method was used to assess the formation of biofilms as mentioned by Babapour et al. (2016), the steps as follow: 20 µl of bacterial suspension equal to 0.5 McFarland standards tube are used to inoculate microtiter wells containing 180 µl of B.H.I. broth with 2 % sucrose. Each well consist of 200 µl (isolated bacteria+

media), In triplicate for each isolate. incubation at 37°C for 24 hrs. 200µl of crystal violet solution was added, washed wells to remove the unbounded dye, allowed to dry at room temperature. At 630 nm, the absorbance of each well was measured using an ELISA reader, the O.D value for well control has been deducted.

## 2.6 Antibiofilm activity of siderophore on pathogenic bacteria

Inhibition of biofilm formation by purified siderophore against some pathogenic bacterial isolated from respiratory tract system including: E.coli, K.pneumoniae, P. aeruginosa, S.marcescens, A.baumannii, S.aureus and Stre. pyogens were studied. after cultured in trypticase soy broth supplemented by 2% of glucose for 24 hr /37°C according to Gudina et al., (2010), Aliquots of 100 µL of the bacterial culture and 50 µL of purified siderophore was loaded to 96-well flat-bottomed microplates and incubated at 37 °C for 24 hours. Control wells contained 100 µl of the culture media and 50 µl of bacterial suspension. Unattached bacterial cells was washed by Normal Saline, 200 µl of crystal violet solution percentage 1% was loaded to the wells for 15 min. the stained attached bacterial cells was extracted by adding 160µl of 95% ethanol, and the absorbance was measured at 630 nm by using ELISA Reader (HS-Human, Germany). The inhibition of Biofilm percentage of the siderophore against pathogenic bacteria were calculated equal described by.

$$\% \text{ Inhibition of biofilm formation} = \frac{\text{OD control} - \text{OD treatment}}{\text{OD control}} \times 100$$

## 3. Results and Discussion

### 3.1 Cultural and Microscopic Examination of A.baumannii

According to the gram stain, the Acinetobacter isolates appeared gram-negative, coccobacilli bacteria and arranged as diplococcic. A.baumannii isolates were appeared as small, smooth, and pale (non-lactose fermentative) on MacConkey agar. While, appeared as convex, opaque, white, and with no hemolysis area around colonies (non blood hemolytic) on Blood agar. For further identification and purification, the isolates were cultured on CHROM agar, which is considered a selective media for A. baumannii and multidrug resistance bacterium. After adding the supplement that allows the growth of only isolates of Acinetobacter, all the colonies appeared Purple (Raut et al., 2020).

### 3.2 CAS agar medium

The results appeared that isolate of *A.baumannii* produce siderophore . Figure(1). through convert the media to yellow color indicate presence siderophore . Because of the action of Hexadecyl Trimethyl Ammonium bromide (HDTMA) as indicators, CAS agar, in addition to being a minimal medium, has some toxicity against fungi and gram-positive bacteria, which would further reduce microbial development on the agar plates . These results were in agreement with other studies reported that siderophore could be produced by numerous microorganism found in soils, Ferreira et al.,(2020) making a comparison of five isolates of bacteria isolated from soil in production siderophore, such as *Azotobacter* spp., *B cereus*, *B subtilis* Pantoea spp. and *Rhizobium* , and isolate *B subtilis* produced highly amount of siderophore. CAS agar a colorimetric approach in which the color of colonies changes to orange or yellow after incubation period because of elimination of iron  $Fe^{+}$  from the dye, indicating the potential of *A. baumannii* isolates to produce siderophores. The blue dye can turn brown, red brown, orange, or yellow depending on the type of siderophore (hydroxamate, chatechole, carboxylate) (Kuzyk et al., 2021).



**Figure (1) CAS agar assay for siderophore detection from isolate of *A.baumannii* AB5**

**3.3. Analysis for Spectrophotometric siderophore production:** The isolate was read on UV/VIS'S spectrophotometer at 405 nm to determine the absorbance of siderophore (Table 1) show the isolate production of siderophore under UV-light appeared fluorescent. Thes result agreement with Kang et al., (2018) .

**Table (1) Spectrophotometric Analysis for siderophore production from *A.baumannii***

| Isolate No. | Absorbance at 405 nm | Absorbance at 600nm | OD 405/OD 600 |
|-------------|----------------------|---------------------|---------------|
| A.B5        | 1.86                 | 0.78                | 2.38          |



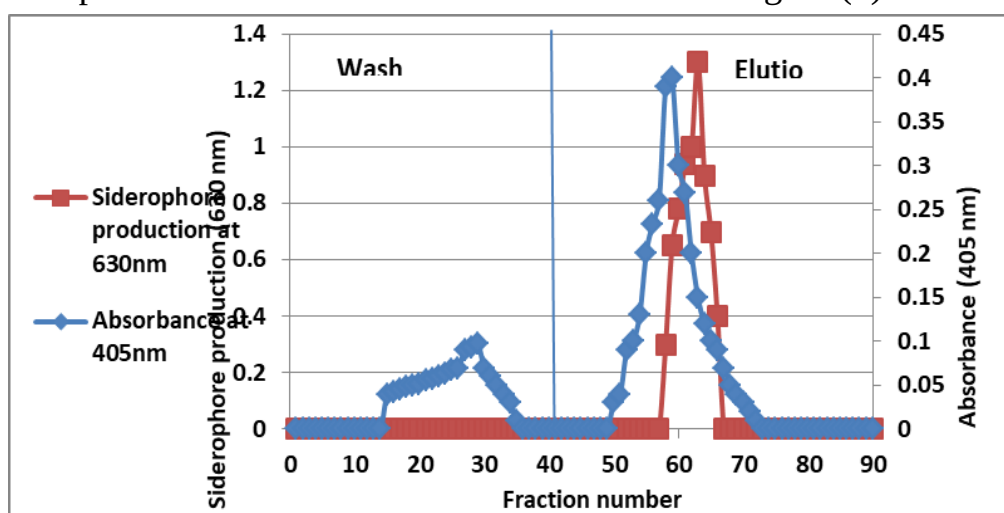
The bacterial isolate was qualitatively and quantitatively for siderophore production. the isolate give positive on media CAS agar consider as a positive siderophore production. In addition, cell-free supernatant of culture was applied to the well of CAS agar plate, a yellow to orange halo was observed around the wells and measure the diameter in mm. This isolate appeared highly production of siderophore with diameter of zone about (15) mm .This result was agreed with the results recorded by Chowdappa et al., (2020). Who declared that different strains of bacteria appeared variations in production of siderophore due to genome regulation.

**3.4 Extraction of siderophore:** After incubation of *A.baumannii* AB5 in modified succinate broth (SM) extract was washed three time with chloroform to eradicate phenol. Presence of siderophore checked by CAS assay to avoid losing of siderophore in discarding and extraction steps . then dehydrated by lyophilization .

### 3.5 Purification of siderophore

#### 1- ion exchange chromatography

The lyophilized crude extract siderophore was subjected to ion exchange chromatography and The result appeared two peaks from tube no. 15 to 35 and other peak from tube no. 50 to 73. As mention in figure (2).

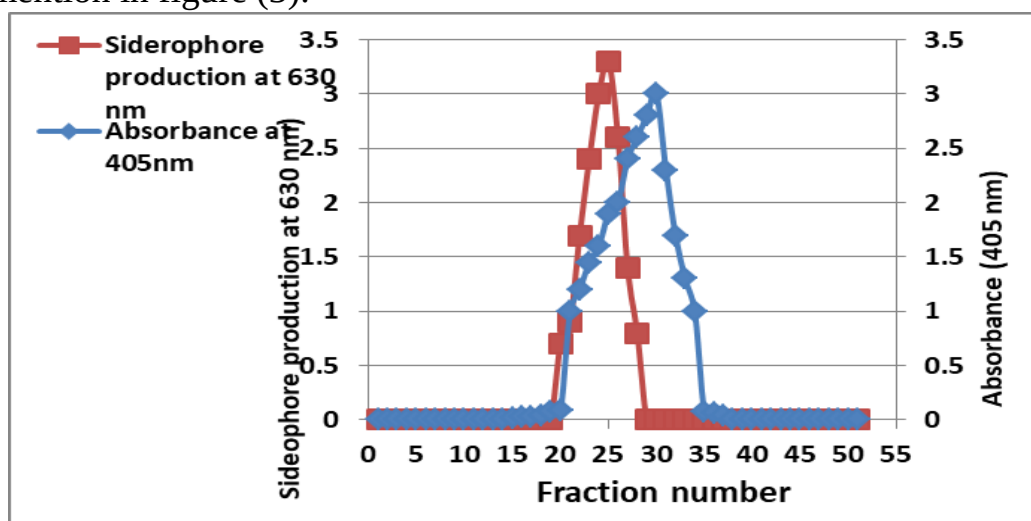


**Figure (2) Purification of ferric siderophore by using CM-Sephadex C-25 chromatography with pyridine acetate buffer pH 5 (0.05M), column (1.5 by 80) flow rate 1 mL/min; detection wavelength: 405 nm**

All the fractions obtained were tested by CAS reagent. The fractions, which appeared yellow to brown in color, showed presence siderophore , were

pooled together and concentrated in rotary evaporator. The dried sample was re-dissolved in 2 ml ddH<sub>2</sub>O and stored at 4 C till further use.

**2-Purification of free iron siderophore :** Gel filtration chromatography technique was the final step in the purification of free iron siderophore ,the results appeared single peak when read at 405 nm in fraction 20 to 35 with more yellowish color and analysis CAS agar determination of siderophore. As mention in figure (3).



**Figure 3 Purification of free siderophore by using sephadex G-25 gel filtration chromatography, column (60×1.5 mm); flow rate: 1 mL/min; detection wavelength: 405 nm; The eluted with D.W.**

The current results disagreed with Khazaal et al., (2009) who obtained four peaks after purification of siderophore by using ion exchange CMC22 and with Cox and Adams, (1985) who obtained three peaks after purification of siderophore. The different in number of peak could be return to variation in method used in purification , as well as , *Acinetobacter baumannii* and other organisms produce more than one category of siderophore (Schalk et al.,2020) . **Penwell and his team** proved in their research that *P. aeruginosa* and plenty of other *Acinetobacter baumannii* produce siderophore under iron limitation and culture supernatants resulted in the isolation of two fractions, which contained only hydroxamates ( baumannoferrin A and baumannoferrin B), which differ only by a double bond and the ability to growth under iron limitation depends on produce siderophore to play a role in virulenc (**Penwell et al., 2015**) .

### 3.6 Biofilm formation of clinical isolation bacteria

The formation of biofilms in 38 pathogenic bacterial isolated from respiratory tract was investigated. This assay involved quantitation of the biofilm biomass that attaches to the walls of the microtiter plate. IN our study, 71.05% (n = 27) were biofilm producers while 28.94% (n = 11) were biofilm nonproducers. Of the total biofilm producers, 39.47% (n = 15) were strong producers, and 21.05% (n = 8) were moderate producers while 10.52% (n = 4) were weak producers as show in the table . These findings closely resembled the study of Gupta et al. (2015) in which 40.6% of the gram-negative isolates were biofilm producers and all of them were MDR. Our study complies well with the study of Mishra et al. (2015) in the same setting in which 46.3% of the total isolates obtained from various clinical specimens were found to be biofilm producers.

**Table (2) Biofilm activity of pathogenic bacteria**

| <b>Biofilm formation</b>      |                        |                     |                        |                            |                          |
|-------------------------------|------------------------|---------------------|------------------------|----------------------------|--------------------------|
| <b>Bacterial isolates</b>     | <b>No. of isolates</b> | <b>Non adherent</b> | <b>Weakly adherent</b> | <b>Moderately adherent</b> | <b>Strongly adherent</b> |
| <b>P.aeruginosa</b>           | 6                      | 0(0%)               | 0(0%)                  | 2(33.3%)                   | 4(66.7%)                 |
| <b>A.baumannii</b>            | 4                      | 1(25%)              | 0(0%)                  | 1(25%)                     | 2(50%)                   |
| <b>K.pneumonia</b>            | 13                     | 2(15.38%)           | 2(15.38%)              | 5(38.46%)                  | 4(30.76%)                |
| <b>E.coli</b>                 | 1                      | 0(0%)               | 0(0%)                  | 0(0%)                      | 1(100%)                  |
| <b>S.aureus</b>               | 1                      | 0(0%)               | 0(0%)                  | 0(0%)                      | 1(100%)                  |
| <b>S.pyogenes</b>             | 1                      | 0(0%)               | 0(0%)                  | 0(0%)                      | 1(100%)                  |
| <b>Enterobacter cloacea</b>   | 2                      | 2(100%)             | 0(0%)                  | 0(0%)                      | 0(0%)                    |
| <b>Enterobacter aerogenes</b> | 1                      | 0(00%)              | 1(100%)                | 0(0%)                      | 0(0%)                    |
| <b>Enterococcus faecalis</b>  | 1                      | 1(100%)             | 0(0%)                  | 0(0%)                      | 0(0%)                    |
| <b>Burkholderia cepacia</b>   | 2                      | 1(50%)              | 0(0%)                  | 0(0%)                      | 1(50%)                   |
| <b>Serratia marcescens</b>    | 2                      | 0(0%)               | 1(50%)                 | 0(0%)                      | 1 (50%)                  |
| <b>Serratia fiaria</b>        | 1                      | 1(100%)             | 0(0%)                  | 0(0%)                      | 0(0%)                    |
| <b>K.oxytoca</b>              | 3                      | 3(100%)             | 0(0%)                  | 0(0%)                      | 0(0%)                    |
| <b>Total</b>                  | 38                     | 11                  | 4                      | 8                          | 15                       |



The results revealed that four isolates from *P.aeruginosa* and *K.pneumonia*, two isolates *A.baumannii* and one isolate from each of the bacteria (*E.coli*, *S.aureus*, *S.pyogenes*, *B.cepacia* and *S.marcescenes* ) the adherence property of biofilm producers was graded as strong, moderate and weak in this study. Multidrug-resistant *P. aeruginosa* has been reported worldwide and it has now been recognized to cause one of the healthcare-associated infections which are most difficult to control and treat. We evaluated biofilm formation capability of originate from the sputum of the patients of lower respiratory tract infection (LRTI). The number of isolates which showed strong biofilm formations was 4(66.7%) and weak biofilm producers were 0%.. The current results showed that 25 % of *A. baumannii* isolates were non adherent, 25 % were moderate and 50 % were strong adherent isolates this may be related to reason that among the outer membrane proteins identified in *A. baumannii* (Stowe et al., 2011). According to these results, the ability of *K. pneumoniae* strains to form biofilms may be high than other strains, the isolates of *K. pneumoniae* (38.46% moderate, 30.76% strong adherent and 15.38% weakly ) were biofilm producer the current results were not close with study by Seifi et al., (2016) revealed that 5.9 % were weak adherent, 29.4 % were moderate and 64.7 % were strong,. In our study we found 100% of isolate was strong biofilm producing (n=1). Bridier et al. (2010) reported that *S. aureus* strains from different sources produced biofilms with high biovolumes.

**3.7 Antibiofilm activity of Siderophore :** As shown in Table (3) the antibiofilm activity of siderophore was evaluated pathogenic bacteria. The siderophore showed antiadhesive activity against all tested bacteria. The highest antiadhesive percentage was observed for *E. coli* by percentage 64.4%. Positive percentage indicates the reduction in bacterial adhesion when compared to the control and negative percentage indicates an increase in bacterial adhesion. Adhesion is the first stage in the production of a biofilm. Another study on *Pseudomonas aeruginosa*, which causes cystic fibrosis, found that biofilm development necessitates a higher quantity of iron than growth (Bouvier, 2016).

**Table (3): antibiofilm percentage of Siderophore against test bacteria isolates**

| Bacterial isolates | OD of Control | OD of Siderophore | Antibiofilm percentage% |
|--------------------|---------------|-------------------|-------------------------|
| E. coli            | 0.413         | 0.147             | 64.4                    |
| S. marcescens      | 0.452         | 0.243             | 46.2                    |
| A. baumannii       | 0.392         | 0.144             | 63.2                    |
| P. aeruginosa      | 0.505         | 0.270             | 46.5                    |
| K.pneumoniae       | 0.591         | 0.253             | 57.1                    |
| S. aureus          | 0.372         | 0.223             | 40.1                    |
| Strept. pyogens    | 0.421         | 0.311             | 26.1                    |

addition, Ishida et al., (2011) demonstrated that a cyclic trihydroxamate siderophore, desferrioxamine E, derived from Actinomycete MS67, inhibits the biofilm formation of Mycobacterium smegmatis and Mycobacterium bovis by removing iron from the medium, which is an essential substance for M. smegmatis and M. bovis biofilm formation (Jiang et al. 2020). Tobramycin in conjunction with deferoxamine or deferasirox, a siderophore, was also found to reduce biofilm biomass and prevented P.aeruginosa biofilm development on cystic fibrosis airway cells.

#### 4.Conclusion

Siderophores have a direct impact on host function through change cellular iron balance, so offering a way for resident bacteria to control local environment at the virulence host's microbial .here we demonstrated the purified siderophore by the rhizospheric A.baumannii showed significance role as antibacterial agent and in inhibition biofilm of pathogenic bacteria isolated from respiratory tract infection.

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**تنقية siderophore المنتجة من جذور *Acinetobacter baumannii* والتحقيق في نشاط المضادات الحيوية على بعض البكتيريا المسببة للأمراض المعزولة من الجهاز التنفسي**  
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**المستخلص:** تم استخلاص Siderophore من *Acinetobacter baumannii* المعزولة من ريزوسفير جذور النبات في مدينة بغداد. أظهرت النتائج قدرة العزلة (A.B5) على إنتاج Siderophore بقطر (15) ملم وكانت ظروف الإنتاج المثلى عند درجة الحرارة 30 درجة مئوية ، وكان الرقم الهيدروجيني الأمثل 7. تمت عملية تنقية siderophore من خلال: كروماتوغرافيا التبادل الأيوني لتنقية الشكل الأكثر استقراراً كمركب حديد - siderophore والخطوة النهائية من خلال الترشيح الهلامي. تم اختبار فعالية siderophore المنقى كعامل مضاد للبكتيريا ضد 13 عزلة بكتيرية سريرية محلية مختلفة بتركيز 20 ملغم / مل من siderophore الحر ، كذلك أظهر حامل الحديد المنقى نشاطاً جيداً كمضاد حيوي للبكتيريا المسببة للأمراض ومنع الالتصاق وتكوين الغشاء الحيوي وكانت أعلى نسبة لمنع تكوين الغشاء الحيوي في بكتيريا *E. coli* بنسبة 64.4%.

**الكلمات المفتاحية:** Siderophore, CAS agar, *A. baumannii*, Biofilm , Antibiofilm , ion exchange , gel filtration

**ملاحظة:** البحث مستل من رسالة ماجستير.