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Evaluation of the Efficiency of *Allium sativum* and *Alhagi maurorum* Extracts as Antimicrobial Agent in Inhibiting Biofilm Formation of some Pathogenic Bacterial Species

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تقييم كفاءة مستخلصي *Allium sativum* و *Alhagi maurorum* كعامل مضاد للاحياء المجهرية في تثبيط تكوين الأغشية الحيوية لبعض الانواع البكتيرية المسببة للأمراض

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Abstract

Background:

The ability to form biofilms by microorganisms is one of the virulence factors used by bacteria to cause disease and is measured by measuring optical density (O.D), which is a measure to estimate the concentration of bacterial species growing in the culture media.

Materials and Methods:

The bacterial cultures of six different bacterial isolates of *S. marcescens* and *Klebsilla pnemoniae* , *Enterococcus cloacea* , *Eshcherchia coli* , *Staphylococcus aureus*, *Psedomonas aeruginosa* were reconstituted from frozen stock, which diagnosed by using VITEK 2 Densi screening tool, then plant extracts of *Allium sativum* and *Alhagi maurorum* were prepared to test the effect of these extracts on the bacterial isolates under study.Optical density was measured using a spectrophotometer at a wavelength of 490 nm before and after treatment of the bacterial isolates with the two extracts *A.sativum* and *A. maurorum* .

Results:

The ability of *K. pnemoniae* , *S. marcescens* and *E. cloacea*, to form biofilm reduced with absorption value 0.52±0.01, 0.66±0.03, 0.8±0.01 respectively, and the ability to form biofilm from *E. cloacea* , *E. coli* after adding *A. maurorum* extract was decreased and the absorbance value reached 0.66±0.01,0.66±0.005 respectively after being treated with *A. maurorum* extract.

Conclusion:

Biofilm formation is one of the virulence factors that helps microorganisms resist the environment in which they exist and resist antibiotics. Therefore, medicinal plant extracts are used as an alternative, which have been shown to inhibit the growth of some pathogenic bacterial species.

Key words:

anti-biofilm; Antimicrobial agent; biofilm formation; pathogenic bacteria; plant extracts

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الخلاصة

مقدمة

ان القدرة على تكوين الأغشية الحيوية من قبل الكائنات الدقيقة حيث يعد تكوين الغشاء الحيوي احد عوامل الضراوة وتستخدمها البكتيريا لإحداث المرض ويتم قياسها من خلال قياس الكثافة البصرية ، وهو مقياس لتقدير تركيز نمو الأنواع البكتيرية في الوسط الزرعي .

طرق العمل

تم تنشيط مزارع بكتيرية لست عزلات بكتيرية مختلفة من *Serratia marcescens* S. و *Klebsilla pnemoniae* و *Enterococcus cloacea*، *Eshcherchia coli*، *Staphylococcus aureus*، *Psedomonas aeruginosa* من المخزون المجمد والتي تم تشخيصها باستخدام جهاز VITEK 2 Densi, بعدها تم تحضير المستخلصات النباتية من *Allium sativum* و *Alhagi maurorum* لاختبار تأثير هذه المستخلصات النباتية على العزلات البكتيرية قيد الدراسة . تم قياس الكثافة الضوئية باستخدام جهاز المطياف الضوئي بطول موجي 490 نانوميتر قبل وبعد معاملة العزلات البكتيرية بالمستخلصين *A.sativum and A. maurorum* .

النتائج

وجد أن قدرة *K. pnemoniae* و *S. marcescens* و *E. cloacea* على تكوين الغشاء الحيوي قد انخفضت و بلغت الامتصاصية 0.52 ± 0.01 ، 0.66 ± 0.03 ، 0.8 ± 0.01 على التوالي. ولوحظ تأثير مستخلص *A. maurorum* على *E. cloacea*، *E. coli* ، حيث انخفضت قدرتها على تكوين الغشاء الحيوي وبلغت الامتصاصية 0.66 ± 0.01،0.66 ± 0.005 على التوالي .

الاستنتاجات

يعد تكوين الأغشية الحيوية أحد عوامل الضراوة التي تساعد الكائنات الحية الدقيقة على مقاومة البيئة التي توجد فيها ومقاومة المضادات الحيوية. لذلك ، يتم استخدام المستخلصات النباتية الطبية كبديل ، والتي ثبت أنها تمنع نمو بعض أنواع البكتيريا المسببة للأمراض.

الكلمات المفتاحية : مضاد لتكوين الغشاء الحيوي, عامل مضاد للاحياء المجهرية, تكوين الغشاء الحيوي, البكتيريا المسببة للأمراض, المستخلصات النباتية.

Introduction

The formation of biofilms by pathogenic bacterial species is one of the factors of virulence possessed by these bacterial species as a way to cause disease and resistance to antibiotics used for treatment[1]. There are an opportunistic and antibiotic-resistant bacterial species that includes *P.aeruginosa* ; it is famous as the major cause of cystic fibrosis in addition to nosocomial contagions,Because of methods for acclimation, existence in addition to immovability against numerous types of antibiotics; Therefore, it is considered dangerous to public health[2]. *S. marcescens* is an opportunistic, gram negative, nosocomial bacteria, *S. marcescens* was considered a non-pathogenic, saprophytic water bacteria; it was often utilized as a biological indicator because of its readily detectable red colonies[3].

S. aureus is gram positive; it is the main reason of pathogen and infective endocarditis in addition to osteoarticular, skin and soft tissue, pleuropulmonary[4]. *Escherichia coli* is a stick-formed ,gram-negative , usually establishes in the bottom intestine of warm-blooded organisms, that is considered normal microbiota ,about

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0.1% of gut micro biota[5].Bacterial species are able to remain alive external surface of the body for a restricted period. Which makes it a source of fecal pollution of samples[6].*Enterococcus faecalis* is a gram-positive bacterium that can cause to cause a variety of nosocomial infections. The treatment of this infection is complicated and humiliating because *E. faecalis* is resistant to many drugs [7].

K. pneumoniae is an opportunistic bacterium related to community-obtained nosocomial contagions[8]. It is a gram-negative, incapable of motion. and encapsulated, which is found in the normal flora of the mouth, skin, and intestines, it is able to lead to destructive modification to human and animal lungs if breathed, precisely to the alveoli causing bloody, colored jelly similar to a mixture of saliva and mucus coughed up from the respiratory tract [9]. For a long time, medicinal plants have been used to treat diseases. These plants contain organic compounds, which have an effective role in inhibiting the growth of microorganisms.These compounds include tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids[10].

A. sativum or garlic contains many compounds that are considered antimicrobial. The occurrence of diseases which occur due to diseases is attributed to the ability of microorganisms to form biofilms, which is evidence of very high resistance to antibiotics[11]. *A. maurorum* has the ability to inhibit microorganisms because it contains active compounds which are alkaloids, flavonoids, anthraquinones, cardiac glycosides, coumarins, saponins, phlobatannins, tannins and terpenoids in leaves and roots[12,13].Therefore,this study investigates the ability of *Allium sativum* and *Alhagi maurorum* as a natural antibiotics against six pathogenic bacterial species,Particularly in relation to biofilm disruption and inhibition of the metabolic activity of biofilm cells.

Materials and Methods

Preparation of bacterial cultures

Six different clinical bacterial isolates of *S. marcescens , K. pnemoniae , E. cloacea , E. coli ,S. aureus, P. aeruginosa* were reconstituted from frozen stock; laboratory reference strains have been sub-cultured for months since their first isolation. These isolates were obtained from the refrigerator of the microbiology laboratory in the college as preserved isolates, as they were previously isolated from pathological samples of people with burns and from patients suffering from infections in the respiratory system or in the digestive system and they were previously diagnosed using the VITEK 2 Densi device [14], and kept for laboratory experiments.After lightly scratching the surface of the frozen stock, with a sterile inoculating loop, bacterial cultures were suspended in 5 mL Tryptic Soy Broth (TSB) and grown in a vibrating incubator at 37°C to 24 hours. The culture was preserved in a vibrating incubator for the period of the trials[15].

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Preparation Plant extracts

10 g of *A. sativum* and *A. maurorum* were dissolved and 200 ml of distilled water was added to it. The mixture was well mixed and the extracts were filtered using 0.1 cm diameter filter paper. Then they were transferred to centrifuge tubes and used for 2500 centrifugal cycles of five duration each minutes. The supernatant part of the extract was collected, dried and preserved until use and finally the concentrations 400 mg/ml were prepared to test the effect of these extracts on the bacterial isolates under study[16,17]. Finally, a series of concentrations 300, 200, and 400 mg/ml were prepared; A concentration of 400 mg/ml was chosen because was more effective at inhibiting the bacterial isolates under examination[16,17].

Overnight cultures of *S. marcescens , K. pnemoniae , E. cloacea , E. coli ,S. aureus, P. aeruginosa* isolates were incubated with 3 ml of fresh TSB .The bacterial count was adjusted to 2 x 10^7 CFU/ml. A 2000 µl of standardized inoculums were added to the wells of sterile flat-bottom polystyrene micro titer plates, and incubated at 37°C for 24 hours in a closed and humidified plastic tube, than a 1000 µl of standardized inoculums 2 x 10^7 CFU/ml plus 1000 µl of plant extracts were added to each of six sterile flat-bottom polystyrene tubes which have a diluted bacterial suspension, and incubated at 37°C for 24 hours in a closed and humidified plastic container to measure optical density later.

Measurement optical density of growth bacterial isolates

The wavelength of the Spectrophotometer(PD-303) was set at 490nm , the cultures were diluted in tryptic soy broth from 10^{-1} to 10^{-6}. Fourth and fifth dilutions 10^{-4},10^{-5} were used in the present study of measuring optical density before adding *A.sativum* and *A. maurorum* extract and after adding two plant extracts.

Statistical Analysis

The differences between two groups were explanation by IBM SPSS Statistics 26 and used to Mean of optical density± standard deviation and for three identical replicates from identical trials. A value of standard deviation SD was deemed statistically important.

Results and Discussion

The ability of *S. marcescens , K. pnemoniae , E. cloacea , E. coli ,S. aureus, P. aeruginosa* to form biofilm before and after treated with *A. sativum* and *Alhagi maurorum* extracts were measured by spectrophotometer (PD-303) at a wavelength of 490 nm.The current study showed that not all six bacterial isolates affected by *A. sativum* extract. A decrease in the formation of biofilms was observed in the *K. pnemoniae , S. marcescens* and *E. cloacea*, with absorption value 0.52±0.01, 0.66±0.03, 0.8±0.01 respectively. While *P. aeruginosa , S. aureus, E. coli* are showed increasing of biofilm formation when treated with garlic extract, where the value of the absorbance or optical density reached 0.98±0.02, 0.91±0.04,0.81±0.01 (table 1)

Table 1. Mean and Standard Deviation of biofilm formation in terms of optical density for *S. marcescens* , *K. pnemoniae* , *E. cloacea* , *E. coli* ,*S. aureus*, *P. aeruginosa* before and after being treated with *A. sativum* extract.
**(+/- SD + : high ; - : low).*

Bacterial isolates	**O.D ± SD***		
	without treatment	**with treatment (10^{-4}) dilution**	**with treatment (10^{-5})dilution**
Serratia marcescens	0.75± 0.05	0.68±0.03	0.66±0.03
Klebsilla pnemoniae	0.80± 0.08	0.56±0.07	0.52±0.01
Enterococcus cloacea	0.98±0.05	0.86± 0.02	0.8±0.01
Eshcherchia coli	0.703±0.005	0.74±0.005	0.81±0.01
Staphylococcus aureus	0.45±0.04	0.87±0.015	0.91±0.04
Psedomonas aeruginosa	0.66±0.005	0.95±0.05	0.98±0.02

For explanation of the results, isolates may be separated into the following groups: no biofilm producer O.D=0 ,weak biofilm producer O.D=1, moderate biofilm producer O.D=2 and strong biofilm producer O.D=3[18].In table 2, showed increasing the ability of *P. aeruginosa*, *S. marcescens*, *K. pnemoniae*, *S. aureus* to form biofilm after are treated with *A. maurorum* extract,where the value of the optical density were 0.74±0.005, 0.63±0.01, 0.52±0.02, 0.52±0.01 respectively. It depends on the size of these bacterial isolates; the reason for this dependence becomes evident bacterial isolates are approximately 600 nm in diameter according to Ref.[19]. instead of using wavelength 490nm. While the effect of *A. maurorum* extract on *E. cloacea* , *E. coli* ,were observed, their ability to form biofilm decreased and the absorbance value reached 0.66±0.01,0.66±0.005 respectively after treated with *A. maurorum* extract(table 2).

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Table 2. Mean of biofilm formation in terms of optical density for *S. marcescens* , *K. pnemoniae* , *E. cloacea* , *E. coli* ,*S. aureus*, *P. aeruginosa* before and after being treated with *A.maurorum* extract.

Bacterial isolates	**O.D ± SD***		
	without treatment	**with treatment (10^{-4})dilution**	**with treatment (10^{-5})dilution**
Serratia marcescens	0.57±0.005	0.605± 0.005	0.63±0.01
Klebsilla pnemoniae	0.49± 0.02	0.49±0.01	0.52±0.02
Enterococcus cloacea	0.85± 0.005	0.67±0.005	0.66±0.01
Eshcherchia coli	0.703±0.005	0.68±0.005	0.66±0.005
Staphylococcus aureus	0.45±0.04	0.5±0.01	0.52±0.01
Psedomonas aeruginosa	0.66±0.005	0.703±0.005	0.74±0.005

**(+/- SD + : high ; - : low).*

The results in the current study showed that most resistant of the six bacterial species when treated was *S. aureus* from the other five bacterial species, followed by *S. marcescens* and *P. aeruginosa* when treated with *A. sativum*, which biofilm strength was about 1.9,1.06,1.06 respectively,while *K. pnemoniae* showed less biofilm formation after treatment with garlic extract, followed by *E. cloacea* which biofilm strength was about 0.7,0.78 respectively, between the organosulfur components of garlic, S-allyl cysteine sulfoxide or alliin is the odorless compound. Sliced garlic is affected by the alliinase enzyme, which is the cysteine sulfoxide lyase, and converts into allicin,these substances are in control of the strong smell of garlic and have antimicrobial in addition antioxidant features[20] and remedy of bacterial infections[21].*Allium sativum* is evaluated to consist of more than two hundred chemical materials that can conserved the human organism against several illnesses according to Ref.[22].As for *A. maurorum* extract, its efficiency was less in reducing the susceptibility of the six bacterial species to biofilm formation.Whereas *S. aureus* then followed with *S. marcescens* and *P. aeruginosa*, showed a high ability to form biofilm despite being treated with *A. maurorum* extract ;where the strength of the biofilm reached 1.1,1.06,1.06 respectively. As for the bacterial species, *E. cloacea* and *E.coli* are weakened their ability to form biofilm, which affected when treated with *A. maurorum* extract, , as the strength of the a biofilm reached 0.78,0.9 respectively(fig.1).

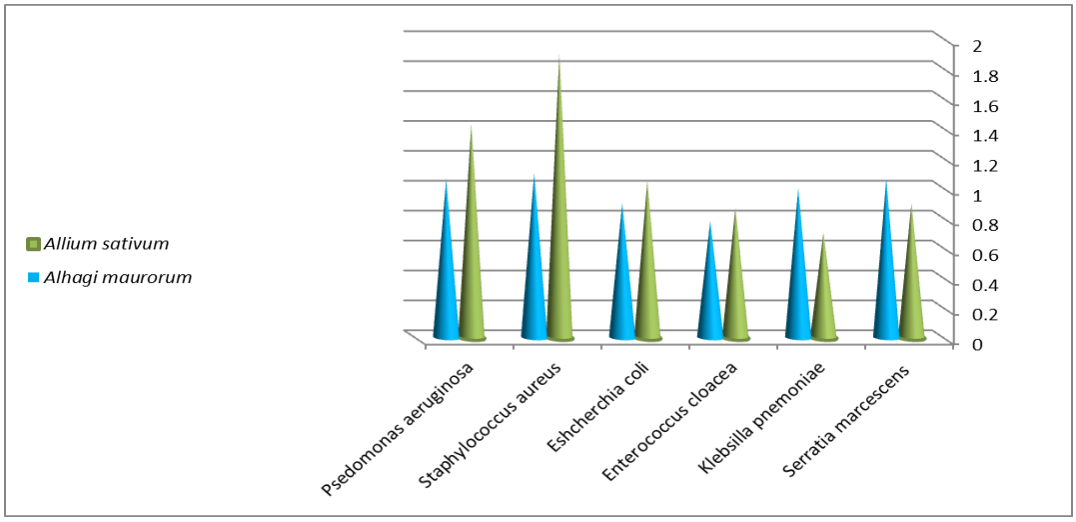

Figure 1. strength of biofilm of fourth dilution for *S. marcescens* , *K. pnemoniae* , *E. cloacea* , *E. coli* ,*S. aureus*, *P. aeruginosa* after being treated with *A. maurorum* and *A. sativum* .

In a previous study has described the medicinal properties of *A. maurorum* in communal medicines to be used as streaky, increased passing of urine, used to treat coughs, and an anti-ulcerogenic drug [23]. *A. maurorum* is rich in phenolic and flavonoid components [24], and these components have been shown to have several curative advantages such as being antibacterial and anti-inflammatory [25]. According to Figure 2, the most resistant of the six bacterial species when treated was *S. aureus*, which outperformed the other five bacterial species when treated with *Allium sativum* and *Alhagi maurorum* extracts, with biofilm strengths of about 2.02 and 1.15, respectively. While when used *Alhagi maurorum* extract, Among the other five bacterial species, *Alhagi maurorum* extract is effected on *E. cloacea*, as the biofilm strength reached 0.7.Whereas *S. aureus* and *P. aeruginosa* showed a high ability to form biofilm despite being treated with *Alhagi maurorum* extract, as the biofilm strength was reached (1.15,1.12)respectively.

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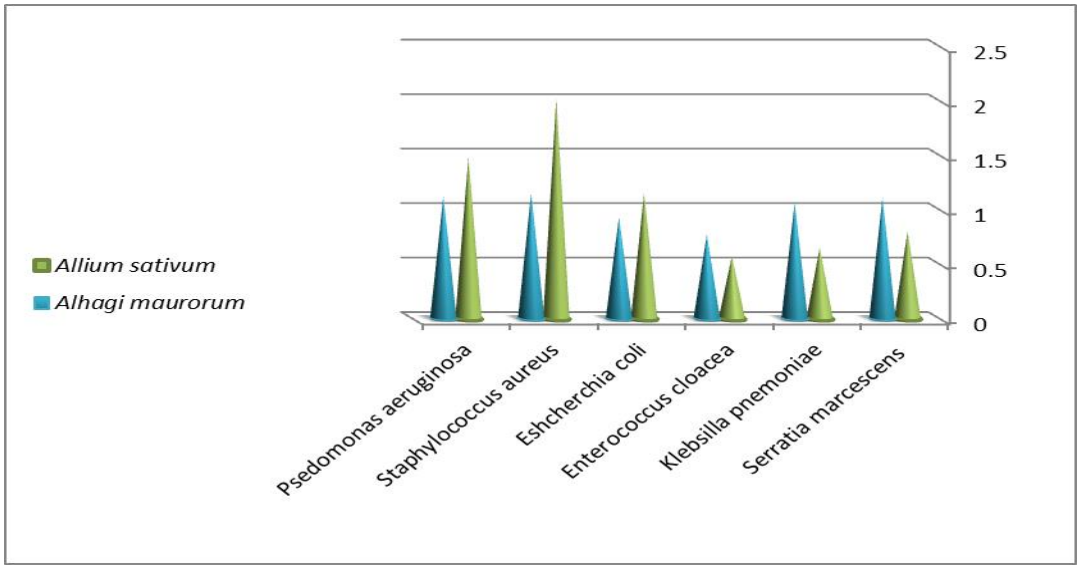

Figure 2. Strength of biofilm of fifth dilution for *S. marcescens* , *K. pnemoniae* , *E. cloacea* , *E. coli* ,*S. aureus, P. aeruginosa* after being treated with *A. maurorum* and *A. sativum* .

Conflict of interests

The authors declare that there is no competing interest.

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