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Antimicrobial Effect of Eco- Friendly Silver Nanoparticles Synthesis by Iraqi Date Palm (*Phoenix dactylifera*) on Gram-Negative Biofilm-Forming Bacteria

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Abstract:

Date palm silver nanoparticles are a green synthesis method used as antibacterial agents. Today, there is a considerable interest in it because it is safe, nontoxic, low costly and ecofriendly. Biofilm bacteria existing in marketed local milk is at highly risk on population health and may be life-threatening as most biofilm-forming bacteria are multidrug resistance. The goal of current study is to eradicate biofilm-forming bacteria by alternative treatment green synthesis silver nanoparticles. The biofilm formation by bacterial isolates was detected by Congo red method. The silver nanoparticles were prepared from date palm (khestawy) fruit extract. The formed nanoparticles were characterized with UV-Vis and AFM. The antibacterial activity of synthetic silver nanoparticles was evaluated by agar well diffusion method. Gram-negative bacteria isolates were *E. coli* in 3 isolates and *Klebsiella pneumoniae* in 5 isolates and all are biofilm producer. The size of synthetic green silver nanoparticles is 18 nm and the generation of silver nanoparticles was confirmed by change of date extract color from yellow to brown with an absorption maximum at 410 nm. Highly antibacterial activity of silver nanoparticles was recorded in comparison to plant extract and silver nitrate against gram-negative biofilm-forming bacteria. From this study, the antibacterial activity of date palm silver nanoparticles was more efficient to eradicate gram negative biofilm-forming bacteria isolated from marketed local milk.

Keywords: Biofilm bacteria, Date palm, Milk, Multidrug resistant, Silver nanoparticles.

Introduction:

Nanoparticles are a small object that have size ranging from 1-100 nm ($\text{nm}=10^{-9}$ m)(1). Silver Nanoparticles (AgNPs) are applied vastly today in medicine, health care, and environmental field. In medicine, the antimicrobial activity of silver nanoparticles is the ability to destroy wide spectrum of pathogens and multidrug-resistant bacteria, especially biofilm forming pathogens (2). Many methods are used in the synthesis of nanoparticles such as physical, chemical and biological or green synthesis methods. The safer one is the green synthesis, especially plant synthesis method because

it's applied on non-toxic materials in addition to being, eco-environmental friendly, less costly and they utilize renewable materials. So recently, the green synthesis nanoparticles have gained considerable attention. Different plants are used in silver nanoparticles synthesis such as *Petalium murex* (3), *Mimusops elengi* (4), date palm (*Phoenix dactylifera*) (5) and others. The Date palm tree (*Phoenix dactylifera*) (Fig. 1) is a tropical and subtropical tree, the cultivation of this plant is famous in the country of Mesopotamia (Iraq) and begins in Babylon 4000 years ago BC (6).



Figure 1. Date palm tree in Iraq and date palm fruit

The date palm fruit contains many phytochemicals like carbohydrates and sugars, phenolics, flavonoids, sterols and carotenoids(7). Most of the biomolecules can act as a capping and reducing agent. Then, the Date fruits extract that are inherently rich in these phytochemicals could be used as a novel reducing agent for green synthesis AgNPs in large scale production (5).

Biofilm bacteria in dairy milk

Biofilm is an aggregation of bacteria that attach to biotic and abiotic surface and its protected by an extracellular polymer matrix composed of polysaccharides and extracellular DNA(8). Growth of biofilms in milk processing environments leads to increase the opportunity for microbial contamination of processed dairy products. On its journey from the farm to the consumer, milk comes into contact with the walls of the equipment in which it is being processed and transported (9). Bacteria have ability to attach and adhere on stainless steel equipment's surface to form biofilm which lead to contaminate the dairy products (9). Biofilm is a protective mode of bacteria to prevent and evade phagocytosis and antibiotics action (10) and almost biofilm-forming bacteria have the ability to resist antibiotics more than planktonic bacteria to be multidrug resistance (MDR) bacteria. Eradication of biofilms often requires an antibiotic dose up to 1000 times higher than the lethal dose for planktonic bacteria(11). So, it needs an efficient and alternative way to eradicate or eliminate biofilm (MDR) bacteria by novel antimicrobial like silver

nanoparticles synthesis by a safe method (green synthesis) to evade the properly any toxic materials that may be present during the synthesis by using date palm fruit extract. Very rare number of studies have been found about the antimicrobial activity of silver nanoparticles synthesized by date palm fruit extract. Besides, the influence of the silver nanoparticles on milk-isolated bacteria has not been studied properly. Accordingly, the aim of the study is to eradicate biofilm-forming bacteria by alternative treatment green synthesis silver nanoparticles.

Materials and methods:

Bacterial isolates:

The bacterial isolates were isolated from marketed milk as follow:

1. Collection of milk samples:

The milk samples were collected from local markets and stored in 10 ml in sterile tube.

2. Culturing and diagnostic:

The milk samples were cultured on routinely culture media (nutrient, blood, and MacConkey agar) and incubated at 37°C for 24hrs. Later biochemical test, staining, and microscopic examination were done to positive growth culture (12). Bacterial isolates identified by the automated system VITEK 2 to confirm final diagnostic and identification.

Biofilm detection: Congo red method (13) was achieved for the identification of biofilm formation isolates. Only gram-negative bacterial isolates showed positive results.

Antibiotic sensitivity test: The test was done using VITEK 2 AST system: The test was done according to the manufacturer's instructions as diagnostic VITEK 2 system with antimicrobial susceptibility testing cards for Enterobacteriaceae containing more than 15 antibiotics. The results were shown digitally on monitor connected to VITEK system apparatus.

Synthesis of AgNPs:

The synthesis of AgNPs was done using date palm (*Phoenix dactylifera*) fruit extract as following:

1. Collect and extract date palm fruit:

The date palm fruits (khestawy) were purchased from local market, a 15 gm of date were washed, cut into a small pieces and mixed with 100ml of deionized water in blender until the mixture was homogenized. The homogenized solution was filtered using filter paper No. 1 to get clear solution (5).

2. Synthesis of AgNPs:

Two ml of (0.1 M) of AgNO_3 was added to three ml of date palm extract solution. Next, the total volume of 10 ml, was completed by adding deionized water. All the mixture was mixed in

beaker (250ml) on magnetic stirrer at (400 rpm for 30 min in dark condition and heated to 40C), until the change in the color of the solution was observed.

Characterization of silver nanoparticles product:

The synthesized AgNPs were characterized using UV. Spectrophotometer and AFM (atomic force microscope). The characterization was carried out at Veterinary College of Al-Qasim Green University and Science College of Babylon University.

a) Measuring the Surface Plasmon Resonance (SPR) by UV-visible spectroscopy: The SPR of silver nanoparticles was measured using UV-visible spectroscopy at wavelength ranging from 300-500 nm according to (14).

b) Atomic force microscope (AFM)

AgNPs were first diluted in deionized water to an appropriate concentration and then ultra sonicated for 15 - 30 minutes. The prepared sample was drop coated on a glass substrate, then left over night to get dried to form a thin film of silver nanoparticles. The test was done according to (15). The test was done to evaluate and measure the size of tested nanoparticles.

Antimicrobial activity testing for AgNPs:

The antimicrobial activity of green synthesis AgNPs was tested by agar well diffusion method.

The selected bacterial inoculum 1.5×10^8 CFU/ml was seeded on Muller Hinton agar plate by streaking using sterile swab then let it to set for 10 min. Later, 3 wells were made by cork borer 8mm in width. The wells were filled with 100 µl of filtered synthetic AgNPs solution, plant extract and AgNO₃ in each well.

All plates were incubated at 37c for 24hrs with dark conditions. The diameter of inhibition zone was measured using a ruler then record the results value.

Statistical analysis:

Statistical analysis was done using SPSS Statistics 24.0 software.

Results and Discussion:

The 3 isolates of *E. coli* and 5 isolates of *Klebsiella pneumoniae* were selected among other bacterial groups which are biofilm formation bacteria isolated from marketed milk. The biofilm forming by bacteria was confirmed by culturing bacterial isolate on congo red media (Fig.2) where the black color indicated the ability of Congo red dye to stain the polysaccharide matrix which was formed during the biofilm forming process (16).

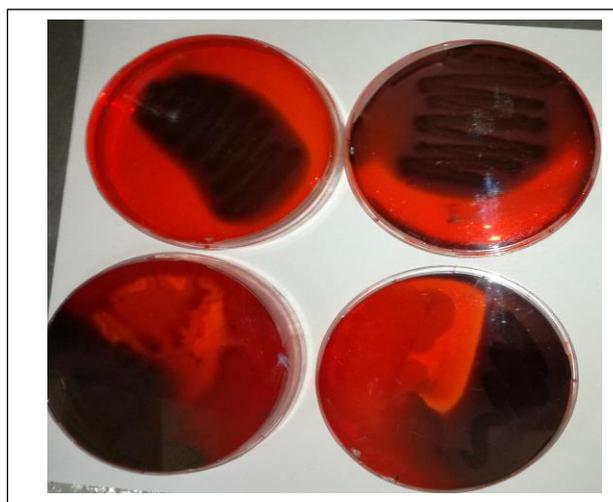


Figure 2. Congo red agar indicating the biofilm formation bacteria.

The biofilm is a community of bacteria which attach and aggregate on biotic or abiotic surface (8). The formation of the biofilm in the local marketed milk can be attributed to the adhesion ability of the bacteria to the milk stainless steel container (17). Ksontini and co author, pointed that dairy biofilm formation depends on the types of surface material and bacterial community that investigated the biofilm formation based on thermodynamic approach(17).

Antibiotic susceptibility profile by Vitek AST to *K. pneumoniae* and *E. coli* (Table 1) revealed that both biofilm forming bacteria were resistant to most antibiotics used in this test which was confirmed by the previous studies (18, 19).

Table 1. Antibiotics provide by VITEK AST card for Enterobacteriaceae with MIC breakpoints.

Antibiotics	<i>K. pneumoniae</i>		<i>E.coli</i>	
	MIC(µg/ml)	Interp.	MIC(µg/ml)	Interp.
ESPL	Negative		Negative	
Ampicillin	≥ 32	R	≥ 32	R
Ampicillin/ clavulanic	≥ 32	R	≥ 32	R
Cefazolin	≥64	R	≥64	R
Ceftazidime	≥64	R	≥64	R
Ceftriaxone	≥64	R	≥64	R
Cefepime	≥64	R	16	R
Imipenem	≥2	I	≥ 16	R
Gentamicin	≥ 16	R	≥ 16	R
Tobramycin	≥ 16	R	≥ 16	R
Ciprofloxacin	2	I	2	I
Levofloxacin	1	S	4	I
Nitrofurantain	256	R	128	R
Trimethoprim/s ulfamithaxzol	≥ 320	R	≥320	R

The MIC breakpoints according to M100 (20)

The biofilm-forming bacteria displayed antibiotics resistant patterns. Many studies approved that biofilm bacteria were multi-drug resistance bacteria(18, 19, 21, 22). The reasons behind their resistance were antibiotics penetration difficulty(23), extracellular matrix act as shield and chelating agent (24) and low bacterial metabolic state(25). The Table above also shows that *E. coli* is more resistance than *K. pneumonia*, whereas *K. pneumonia* is sensitive to one antibiotic (Levofloxacin).

Silver nanoparticles green synthesis:

The green synthesis of AgNPs by date palm fruit extract is characterized as rapid, simple, non-costly, safety and eco-friendly method. Date palm fruit extract is considered a novel natural reducing and stabilizing agent (5) because it contains a various secondary metabolites necessary to synthesis a metallic nanoparticles (26).

Silver nanoparticles synthesis characterization:

The visual observation of synthesized AgNPs shows a change in the color of date palm extract from yellow to brown after adding AgNO_3 solution due to excitation of surface Plasmon resonance (SPR) vibrations in Ag NPs (27) which mean converting Ag^+ to Ag^0 and indicating AgNPs production (Fig. 3), instead of colorless silver nitrate (AgNO_3) solution.

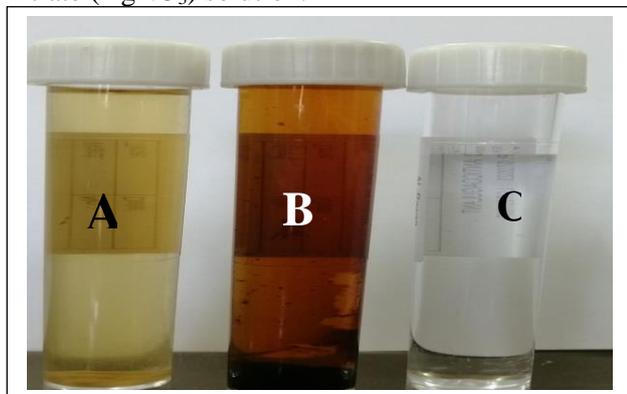


Figure 3. Visual observation of color changing A= date palm extract, B= synthesized AgNPs, C= AgNO_3 .

UV-Vis spectroscopy:

The absorption peak of synthesized AgNPs was strong with narrow surface peak reaching to 410 nm (Fig. 4). Narrow peak indicates to narrow size range of nanoparticles less than 100 nm (28).

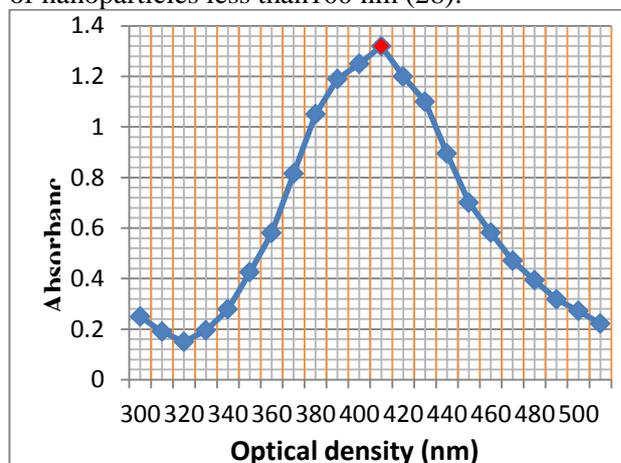


Figure 4. UV-Vis spectrophotometer analysis of synthesis AgNPs.

Atomic force microscope (AFM):

Using the Atomic Force Microscope (AFM), individual particles and aggregated particles can be resolved and unlike other microscopy techniques, the AFM offers visualization and analysis in three dimensions (15). The particles distribution of green synthesis AgNPs shows the most of the particles have grain size distribution (15-20nm) maximum number of 18 nm (Fig.5).

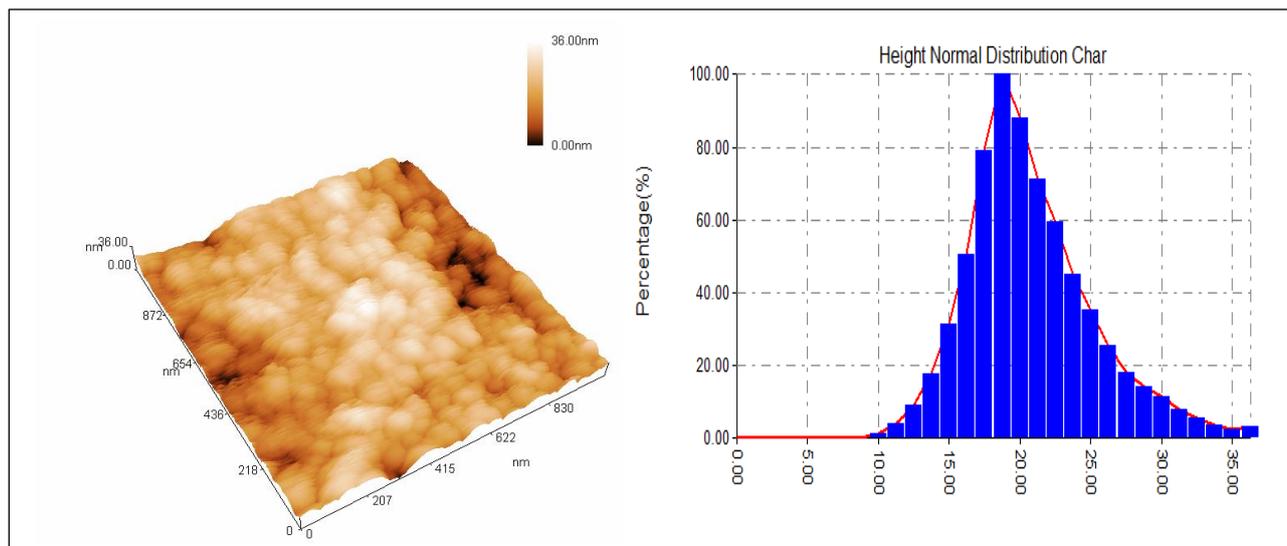


Figure 5. AFM results of synthesized AgNPs size at 18 nm.

Antimicrobial effects of AgNPs on biofilm forming bacteria: The test was done by agar well diffusion method. The results showed the antibacterial effect of green synthesis AgNPs was more effective than date

palm fruit extract and AgNO_3 solution against gram-negative biofilm-forming bacteria, *K. pneumoniae* (Fig. 6) and *E. coli* (Fig. 7).

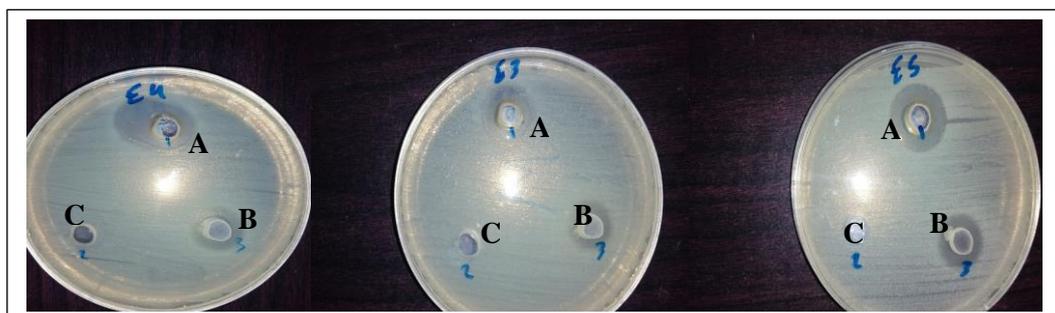


Figure 6. The inhibition zone of biofilm *E. coli* growth, A= AgNPs, B= date palm extract, C= AgNO_3 .

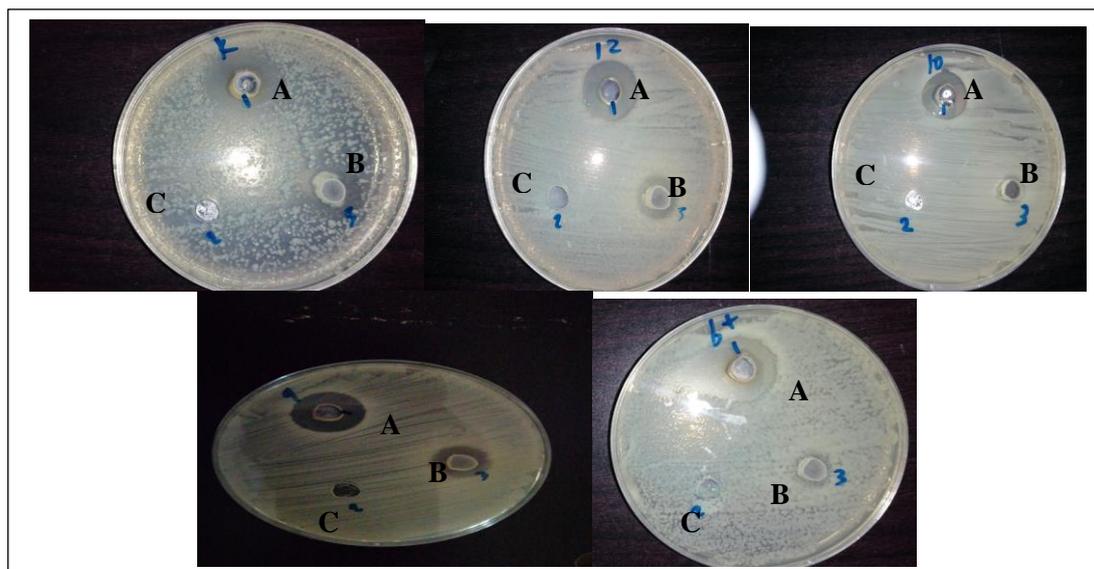


Figure 7. The inhibition zone of biofilm *K. pneumoniae* growth, A= AgNPs, B= date palm extract, C= AgNO_3 .

Other researcher results revealed that date palm AgNPs have good antibacterial activity against

E. coli and *K. pneumoniae* which found that inhibition zone reach to 11mm to each one (5, 29).

But there is no study about date palm AgNPs antibacterial effect on biofilm-forming bacteria. Many studies about anti-biofilm activity of AgNPs synthesis by other methods showed a high response to be anti-biofilm (30-32). Antibacterial activity of green synthesis AgNPs in the present study may be related to attach to the surface of the cell membrane disturbing permeability and respiration functions of the cell (33). It is also possible that silver and silver nanoparticles not only interact with the surface of membrane but can also penetrate inside the bacteria(34). Mahltig *et al.*, pointed that Ag⁺ ions interact with the thiol groups in bacteria proteins, affecting the replication of DNA(35).

The antibacterial activity of AgNPs is more effective than AgNO₃ (Fig. 6 and 7) due to their small size and larger surface area, probably. These two characteristics lead to the increase of the permeability of the membrane and cell destruction (36).

The results revealed that biofilm forming *K. pneumonia* were more susceptible than *E. coli* (Fig. 8) with no significant differences ($p=0.11$) (Table 2). That may be related to the susceptibility of *K. pneumonia* to antibiotics more than *E.coli* according to (Table 1) which mean that *K. pneumonia* were more susceptible to any antimicrobial substance and *E. coli* seem to be more virulent and may carry antibiotic resistant gene more than *K. pneumonia*.

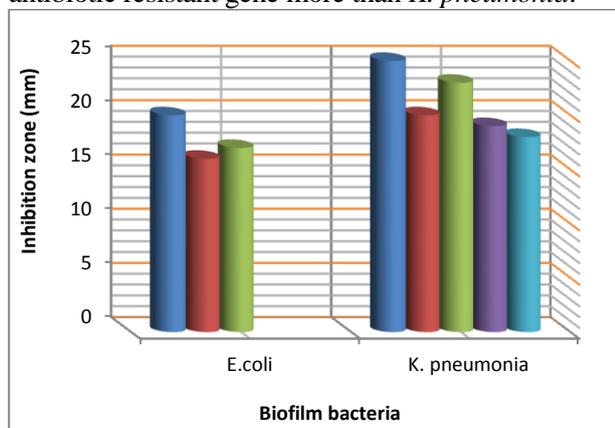


Figure 8. Zone of inhibition (mm) growth of gram-negative biofilm-forming bacteria by green synthesis AgNPs by date palm.

Table 2. Statistical analysis of green silver nanoparticles antibacterial effect on biofilm, forming bacteria.

Bacteria	N	IZ Mean \pm SE	P value
<i>E. coli</i>	3	17.6 \pm 1.2	0.11
<i>K. pneumonia</i>	5	21 \pm 1.3	

IZ= inhibition zone, SE= standard error.

Conclusion:

The green synthesis AgNPs by date palm extract reveals highly antibacterial activity against gram-negative multi-drug resistance biofilm bacteria especially *Klebsiella pneumonia* isolated from milk which is considered the most population consumed fluid.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Al-Qasim Green.

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تأثير المضاد الجرثومي لدقائق الفضة النانوية صديقة البيئة المصنعة من خلاصة تمر النخيل العراقي ضد البكتريا السالبة لصبغة كرام والمكونة للغشاء الحيوي

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الخلاصة:

يعد تصنيع دقائق الفضة النانوية بواسطة التمر بالطريقة الخضراء او النباتية واستخدامه كمضاد بكتيري. في الوقت الحاضر حصلت هذه الطريقة على اهتمام الباحثين لانها امينة وغير سامة وقليلة التكلفة وصديقة للبيئة. تشمل البكتريا المكونة للغشاء الحيوي والموجودة في الحليب المحلي المتوفر بالاسواق على خطورة عالية على صحة المجتمع بسبب ان اغلب البكتريا المكونة للغشاء الحيوي تكون مقاومة للمضادات الحيوية. الهدف من الدراسة هو القضاء على البكتريا المكونة للغشاء الحيوي والموجودة بالحليب المحلي باستخدام علاجا بديلا بتحضير دقائق الفضة النانوية من التمر. حيث كشفت قابلية البكتريا المعزولة من الحليب المحلي على تكوين الغشاء الحيوي باستخدام طريقة صبغة الكونغو الحمراء. حضرت دقائق الفضة النانوية باستخدام خلاصة التمر. حيث تم فحص دقائق الفضة المصنوعة بواسطة جهاز الاشعة فوق البنفسجية ومجهز القوة الذرية. تم تقييم فاعلية دقائق الفضة المصنوعة المضادة للبكتريا بواسطة طريق الانتشار الحفر بالأكار. اظهرت النتائج ان البكتريا المعزولة من الحليب والمنتجة للغشاء الحيوي هي الاشرشيا القولونية *E. coli* بعدد 3 وال *Klebsiella pneumoniae* بعدد 5 عزلات ضمن العزلات السالبة لصبغة غرام. حجم النانو المحضر كان 35 نانوميتر حيث تم الكشف عن تكونه بواسطة التغيير اللوني للمستخلص النباتي من اللون الاصفر الى البني وقمة امتصاص عند 410 نانوميتر. كذلك اظهرت النتائج عن الفاعلية العالية لدقائق الفضة في القضاء على البكتريا السالبة لصبغة غرام و المنتجة للغشاء الحيوي. نستنتج من هذه الدراسة ان دقائق الفضة المصنعة من مستخلص التمر ذو كفاءة عالية في القضاء على البكتريا السالبة لصبغة غرام والمنتجة للغشاء الحيوي.

الكلمات المفتاحية: البكتريا المكونة للغشاء الحيوي، تمر النخيل، دقائق الفضة النانوية، حليب، المقاومة للعديد من الادوية