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

Shaimaa Obaid Hasson\textsuperscript{1} Sumod Abdul kadhem Salman\textsuperscript{1} Shurooq Falah Hassan\textsuperscript{2}
Shatha Mohammed Abbas\textsuperscript{3}

\textsuperscript{1}Department of Microbiology, College of Veterinary, Al-Qasim Green University, Babylon, Iraq.
\textsuperscript{2}Department of Genetic Engineering, Faculty of Biotechnology, Al Qasim Green University, Babylon, Iraq.
\textsuperscript{3}Corresponding author: shaimaaobaid@vet.uoqasim.edu.iq, sumodkadhem8@gmail.com, shuroofalah@gmail.com, shathaabbas@gmail.com

Received 17/6/2019, Accepted 5/1/2020, Published Online First 30/4/2021, Published 1/12/2021

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 eco-friendly. 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 (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 Pedalium murex (3), Minusops 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).
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 synthetized 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 manufacturers instructions as diagnostic VITEK 2 system with antimicrobial susceptibility testing cards for Enterobactericeae 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 AgNO₃ 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–visible 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 x 10⁵ 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 37°C 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).

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. pneumonia* 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).

![Figure 2. Congo red agar indicating the biofilm formation bacteria.](image)

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

| Antibiotics          | *K. pneumonia* | *E. coli* |
|----------------------|----------------|-----------|
|                      | MIC(µg/mL)     | Interp.   | MIC(µg/mL)     | Interp.   |
| ESPL                 | Negative       | Negative  | Negative       |           |
| Ampicillin           | ≥ 32           | R         | ≥ 32           | R         |
| Ampicillin/clavulanic| ≥ 32           | R         |               | R         |
| Cefazolin            | ≥ 64           | R         | ≥ 64           | R         |
| Ceftriazone          | ≥ 64           | R         | ≥ 64           | R         |
| Ceftazidime          | ≥ 64           | R         | ≥ 64           | 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         | 1              | I         |
| Nitrofurantain       | 256            | R         | 128            | R         |
| Trimethoprim/sulfamithaxzol | ≥ 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 at 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 (Levofoxacin).

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 AgNO₃ solution due to excitation of surface Plasmon resonance (SPR) vibrations in Ag NPs (27) which mean converting Ag⁺ to Ag⁰ and indicating AgNPs production (Fig. 3), instead of colorless silver nitrate (AgNO₃) solution.

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).

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).
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).

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 11 mm 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). Mahlig 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.](image)

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

| Bacteria   | N | IZ Mean ± SE | P value |
|------------|---|--------------|---------|
| E. coli    | 3 | 17.6 ± 1.2   | 0.11    |
| K. pneumonia | 5 | 21±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 republication attached with the manuscript.
- **Ethical Clearance:** The project was approved by the local ethical committee in University of Al-Qasim Green.

**References:**

1. Anselmo AC, Mitragotri S. A review of clinical translation of inorganic nanoparticles. The AAPS journal. 2015;17(5):1041-54.
2. Abinaya C, Mayandi J, Osborne J, Frost M, Ekstrum C, Pearce JM. Inhibition of growth of S. epidermidis by hydrothermally synthesized ZnO nanoparticles. Mat Res Exp. 2017;4(7):075401.
3. Anandalakshmi K, Venugobal J, Ramasamy V. Characterization of silver nanoparticles by green synthesis method using Pedalium murex leaf extract and their antibacterial activity. Appl. Nanosci. 2016;6(3):399-408.
4. Prakash P, Gnanaprakasam P, Emmanuel R, Arokiyaraj S, Saravanam M. Green synthesis of silver nanoparticles from leaf extract of Minusops elengi, Linn. for enhanced antibacterial activity against multi drug resistant clinical isolates. Colloids Surf B Biointerfaces. 2013;108:255-9.
5. Farhadi S, Ajerloo B, Mohammadi A. Green biosynthesis of spherical silver nanoparticles by using date palm (phoenix dactylifera) fruit extract and study of their antibacterial and catalytic activities. ACTA CHIM SLOV. 2017;64(1):129-43.
6. Tengberg M. Beginnings and early history of date palm garden cultivation in the Middle East. J ARID ENVIRON. J of Ari Env., 2012;86:139-47.
7. Jahromi MAF, Moien MR, Mollaei M. Volatile Constituents and Antioxidant Activity of Spathes from Five Un-common Varieties of Phoenix dactylifera L. Trends Pharmacol Sci. 2018;4(4).
8. Neethirajan S, Clond MA, Vogt A. Medical biofilms—nanotechnology approaches. J. Biomed. Nanotech. 2014;10(10):2806-27.
9. Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Comp Rev Food Sci F. 2012;11(2):133-47.
10. Gula G, Dorotkiewicz-Jach A, Korzekwa K, Valvano MA, Drilis-Kawa Z. Complex Signaling Networks...
Controlling Dynamic Molecular Changes in Pseudomonas aeruginosa Biofilm. Curr Med Chem. 2019.
11. Anes J, Sivasankaran SK, Muthappa DM, Fanning S, Srikumar S. Exposure to Sub-inhibitory Concentrations of the Chemosensitizer 1-(1-Naphthylmethyl)-Piperazine Creates Membrane Destabilization in Multi-Drug Resistant Klebsiella pneumoniae. Front Microbiol. 2019;10(92).
12. MacFaddin JF. Biochemical Tests for Identification of Medical Bacteria. 3rd ed: Williams and Wilkins. Baltimore, USA; 2000.
13. Freeman D, Falkiner F, Keane C. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol. 1989;42(8):872-4.
14. Karthik C, Radha K. Biosynthesis and characterization of silver nanoparticles using Enterobacter aerogenes: a kinetic approach. Dig J Nanomater Biostruct. 2012;7:1007-14.
15. Rao A, Schoenenberger M, Gnecce E, Glatzel T, Meyer E, Brändlin D, et al., editors. Characterization of nanoparticles using atomic force microscopy. Journal of Physics: Conference Series; 2007: IOP Publishing. pp. 971-976.
16. Bose S, Khodke M, Basak S, Mallick S. Detection of biofilm producing staphylococci: need of the hour. J Clin Diagn Res.2009;3(6):1915-20.
17. Ksontini H, Kachouri F, Hamdi M. Dairy biofilm: impact of microbial community on raw milk quality. J Food Quality. 2013;36(4):282-90.
18. Al-Azawi IH, Al-Hamadani AH, Hasson SO. Association between Biofilm Formation and Susceptibility to Antibiotics in Staphylococcus Lentus Isolated from Urinary Catheterized Patients. Nano Biomed Eng. 2018;10(2):97-103.
19. Hasson SO. Phenotypic and Genotypic Detection of Biofilm Formation Pseudomonas oryzihabitance and Susceptibility to Antibiotics. Nano Biomed Eng. 2019;11(1):11-7.
20. CLSI. Performance standards for antimicrobial susceptibility testing. M100, Clinical and Laboratory Standards Institute, Wayne, PA. 2017.
21. Ebrahimi A, Hemati M, Shabanpour Z, Dehkordi SH, Bahadoran S, Lotfalialian S, et al. Effects of benzolkonium chloride on planktonic growth and biofilm formation by animal bacterial pathogens. Jundishapur J Microbiol. 2015;8(2).
22. Hasson SO, Al-Awady MJ, Al-Hamadani AH, Al-Azawi IH, Ali AI. Boosting Antimicrobial Activity of Imipenem in Combination with Silver Nanoparticles towards S. fonticola and Pantoecia sp. Nano Biomed Eng. 2019;11(2):200-14.
23. Bodie KB. Effects of triclosan exposure on nitrification in activated sludge, biofilms, and pure cultures of nitrifying bacteria: Montana State University-Bozeman, College of Engineering; 2016.
24. Wang S, Zhou C, Ren B, Li X, Wein MD, Masri RM, et al. Formation of persisters in Streptococcus mutans biofilms induced by antibacterial dental monomer. J Mater Sci Mater Med 2017;28(11):178.
25. Band VI, Weiss DS. Heteroresistance: A cause of unexplained antibiotic treatment failure? PLoS pathogens. 2019;15(6):e1007726.
26. Kuppusamy P, Yusoff MM, Maniam GP, Govindan N. Biosynthesis of metallic nanoparticles using plant derivatives and their new avenues in pharmacological applications–An updated report. Saudi Pharm J. 2016;24(4):473-84.
27. Kumar B, Smita K, Cumbal L, Angulo Y. Fabrication of silver nanoparticles using Nephelium lappaceum (Rambutan) peel: a sustainable approach. J. Mol. Liq. 2015;211:476-80.
28. Shahverdi AR, Minaeian S, Shahverdi HR, Jamalifar H, Nohi A-A. Rapid synthesis of silver nanoparticles using culture supernatants of Enterobacteria: a novel biological approach. Process Biochem. 2007;42(5):919-23.
29. Al-Awady MJ, Balakit AA, Al-Musawi S, Altsultani MJ, Kamil A, Alabbas M. Investigation of Anti-MRSA and Anticancer Activity of Eco-Friendly Synthesized Silver Nanoparticles from Palm Dates Extract. Nano Biomed. Eng. 2019;11(2).
30. Mathur T, Singhal S, Khan S, Upadhayay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. 2006;24(1):25.
31. Guzmán MG, Dille J, Godet S. Synthesis of silver nanoparticles by chemical reduction method and their antibacterial activity. Int J Chem Biomol Eng. 2009;2(3):104-11.
32. Hasson SO, Al-Awady, M.J., Al-Hamadani, A.H. and Ibtsisam Habeeb Al-Azawi. . Boosting Antimicrobial Activity of Imipenem in Combination with Silver Nanoparticles towards S. fonticola and Pantoecia sp. Nano Biomed Eng. 2019;under press.
33. Kvitík L, Panaček A, Soukupova J, Kolář M, Večeřová R, Prucek R, et al. Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). J Phsc Chem C. 2008;112(15):5825-34.
34. Norouzzadeh Helali Z, Esmaizadeh M. A comparative study of antibacterial effects of mouthwashes containing Ag/ZnO or ZnO nanoparticles with chlorhexidine and investigation of their cytotoxicity. Nanomed. J. 2018;5(2):102-10.
35. Mahlig B, Grethe T, Haase H. Antimicrobial Coatings Obtained by Sol-Gel Method. Handbook of Sol-Gel Science and Technology: Processing, Characterization and Applications. 2018:3461-87.
36. Vijayan SR, Santhiyagu P, Ramasamy R, Arivalagan P, Kumar G, Ethiraj K, et al. Seaweeds: A resource for marine bionanotechnology. Enzyme Microb Technol. 2016;95:45-57.
تأثير المضاد الجرثومي لدقائق الفضة النانوية صديقة البيئة المصنعة من خلاصة تمر النخيل العراقي ضد البكتيريا السالبة لصبغة كرام والمنكوبة لغشاء الحيوي

شيماء عبيد حسون1 صموئيل الكاظم سلمان1 شروق فلاح حسن2 شذى محمد عباس1

1فرع الأحياء المجهرية، كلية الطب البيطري، جامعة القاسم الخضراء، بابل، العراق.
2قسم الهندسة الوراثية، كلية التقانات الأحيائية، جامعة القاسم الخضراء، بابل، العراق.

الخلاصة:
بعد تصميم دفاتق الفضة النانوية بواسطة التمر بالطريقة الخضراء أو النباتية واستخدامه كمضاد بكتيري. في الوقت الحاضر حصلت هذه الطريقة على اهتمام الباحثين لأنها أمنة وغير سامة وقليلة التكلفة وصديقة للبيئة. تشتهر البكتيريا المكونة للغشاء الحيوي والموجودة في الحليب المحلي المتوفّر بالأسواق على خطورة عالية على صحة المجتمع بسبب مقاومة البكتيريا المكونة للغشاء الحيوي والموجودة بالحليب المحلي. النتائج تشير إلى كفاءة الفضة النانوية المصنعة من الحليب المحلي. حيث كشفت قابلية البكتيريا المعزولة من الحليب المحلي على تكوين الغشاء الحيوي باستخدام فعالية عالية. في البكتيريا K. pneumoniae باستخدام طريقة فحص دفاتق الفضة النانوية واستخدام خلاصة التمر. حيث تم فحص دفاتق الفضة المصنوعة بواسطة جهاز الإشارة فوق البنفسجية ومجهر الفوهة الذرية. تم تقسيم فاعلية دفاتق الفضة النانوية المصنوعة للمضاد البكتيري تطبيق طريقة الانتشار الحفر بالأكث. أظهرت النتائج عالياً فعالية دفاتق الفضة النانوية في القضاء على البكتيريا السالبة لصبغة غرام. حيث تم الكشف عن كفاءة الفضة النانوية في القضاء على البكتيريا السالبة لصبغ غرام. نستنتج من هذه الدراسة أن دفاتق الفضة النانوية مصنوعة من مستخلص التمر ذو كفاءة عالية في القضاء على البكتيريا السالبة لصبغ غرام والمنكوبة لغشاء الحيوي.

الكلمات المفتاحية: البكتيريا المكونة للغشاء الحيوي، حليب، تمر النخيل، دفاتق الفضة النانوية، حليب، المقاومة للعديد من الادوية.