زانکۆی سه لاحه دین- هه ولیر

Salahaddin University-Erbil

Antibacterial Effect of Biosynthesized Silver Nanoparticles against *Staphylococcus aureus* Isolated from Date Fruit in Erbil City

Research Project
Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of BSc. in Biology

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Supervisor Certification

I certify that this research project was prepared under my supervision at the Department of Biology, College of Science/ Salahaddin University- Erbil in partial fulfilment of the requirements for the degree of BSc. in **Biology**.

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Dedication

This research is dedicated to:

This Research specialized for those who supported us during four years of studying in order to achieve the meaning of success on the whole of life.

(Aiesha & Soleen)

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(Aiesha & Soleen)

Abstract

Foodborne diseases are the illnesses contracted from eating contaminated food with contaminants, such as pathogenic microorganisms, microbial and nonbacterial toxins, chemicals, or other substances. Illnesses include foodborne intoxications and infections. *Staphylococcus aureus* (*S.aureus)* is capable of becoming resistant to all classes of antimicrobials clinically available. Nowadays nanoparticles use as an alternative method for eliminate bacteria instead of antibiotics. The objectives of this study were to estimate the prevalence of *S.aureus* in date palm fruit in Erbil city, and comparing effect of antimicrobials and silver nanoparticles (AgNPs) on these multi-drug resistance (MDR) bacteria. For this, during November 2023 to January 2024, 50 date palm fruit samples were collected from markets in Erbil city. The samples were analyzed for the presence of *S.aureus*. Afterwards, the antimicrobial susceptibility and resistance of the isolates was evaluated on 6 different antimicrobials using Kirby-Bauer test. Then effect of AgNPs was determined by well diffusion method. According the result (13) 26% of samples were positive for *S.aureus.* The results of antibiogram test indicated that among utilized antimicrobial agents, amikacin and vancomycin were more effective against *E. coli* isolates. The highest resistance was shown toward amoxicillin + clavulanate acid by 100% of isolates and against Levofloxacin and azithromycin (92%). The all 13 isolates were sensitive for AgNPs, which produce inhibition zones with a mean of 14mm. The results revealed that the date fruits contaminated by MDR *S.aureus* in Erbil city, therefore serious measures are needed to control and prevent the spread of this pathogen.

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Chapter One: Introduction

Foodborne diseases are caused by contamination of food and occur at any stage of the food production, delivery and consumption chain. Over 200 diseases are caused by eating food contaminated with bacteria, viruses, parasites or chemical substances such as heavy metals (WHO, 2022).

Date fruit is a rich source of vitamins, minerals, dietary fibers, energy, and easily digestible and absorbable sugars that instantaneously replenish and revitalize the body specially after fasting condition (Al-Okbi, 2022). The microbial load in date fruit is dependent on water activity (a*w*) (Piombo *et al.*, 2020). Bacterial growth is facilitated due to the high moisture content of date flesh, fresh dates with microbial load can cause food poisoning or even foodborne intoxication (Zamir *et al.*, 2018).

Staphylococcus aureus is a major human pathogen that produces a wide array of toxins, thus causing various types of disease symptoms (Hasan and Hoshyar, 2019). About 25% of people and animals have *S.aureus* on their skin and in their nose (Hasan and Hoshyar, 2019). Staphylococcal enterotoxins (SEs), are a leading cause of gastroenteritis resulting from consumption of contaminated food (Hasan and Hoshyar, 2019).

The resistance of many pathogenic bacteria to antimicrobials has increased rapidly during the last decades (Shaaban *et al.*, 2023). Multidrug-resistant bacteria (MDRB) or “super bugs” are exceptionally treacherous and pose a serious threat to global health systems as they can survive an attack from drug. Adaptive living style of bacteria with antimicrobials causes them to modify their genetic makeup to emerge resistance against known antimicrobials and their combinations (Irfan *et al.*, 2021).

The appearance of nanoparticles as new antimicrobial agents has boosted up the research for tackling these superbugs. As nanoparticles target the bacterial cell through multiple pathways, it becomes difficult for bacteria to escape from these magical agents (Irfan *et al.*, 2021). The matter is considered at the nanoscale when its size ranges from 1 to 100 nm.

Nanoparticles have a large surface area due to their nanoscale size, which contributes to their enhanced physical and chemical properties, which are useful in a variety of fields such as antimicrobial properties (Shaaban *et al.*, 2023).

Silver has been adopted as an antimicrobial material and disinfectant that is relatively free of adverse effects. Silver nanoparticles possess a broad spectrum of antibacterial, antifungal and antiviral properties. Silver nanoparticles have the ability to penetrate bacterial cell walls, changing the structure of cell membranes and even resulting in cell death. Their efficacy is due not only to their nanoscale size but also to their large ratio of surface area to volume. They can increase the permeability of cell membranes, produce reactive oxygen species, and interrupt replication of deoxyribonucleic acid by releasing silver ions(Yin *et al.*, 2020).

As mentioned above knowing the prevalence and detection of super bug bacteria *S aureus* contaminated date fruit samples in Erbil city is crucial and could help intervention and prevention of serious food borne diseases. Therefore, the present study aimed at the followings:

- To estimate the presence of *S aureus* in Date fruit samples sold in Erbil city. (Determine quality of date fruit).
- To determine the antimicrobial resistance of the isolates.
- To determine effect of biosynthesized Silver nano particle against isolates.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Laboratory Equipment's and Apparatus

The instrument and their manufactures used in this study are summarized in table (2.1).

Table 2.1 equipments and instruments, company and origin

No.	Equipment	Company	Country
1	Autoclave	hirayama	Japan
2	Balance	kern	Germany
3	Disposable Petri plat	Afco-Dispo	Jordan
4	Incubator	Memmrt	Germany
5	Light microscope	Olympus	Japan
6	Oven	Memmert	Germany
7	Refrigerator	Beko	Turkey

2.1.2 Culture Media:

Ready media which were used listed in table (2.2). Prepared and sterilized according to the manufacturing information on the container.

Table 2.2 Media with their manufacturing company

No.	Culture media	Company/Origin
1	Mannitol Salt Agar	Oxoid, UK
2	Nutrient Agar	Oxoid, UK
3	Muller Hinton agar	Oxoid, UK
4	Nutrient Broth	Oxoid, UK.

2.1.3 Chemicals:

The chemical substances that were used in this study are shown in the following table (2.3).

Table 2.3: Chemicals, company, and origin

No.	Chemicals	Company/Origin
1	Ethanol	Scharlau/spain
2	Gram stain	Atom scientific/ UK
3	Antimicrobial discs	Oxoid / UK
4	Silver Nitrate powder	Sigma/USA
5	0.5 McFarland	Atom Scirntific/UK

2.1.4 Antimicrobial discs:

The antimicrobial discs that were used in this study are shown in the following table (2.4).

Table 2.4: Antimicrobial discs, dose, and code.

No.	Antimicrobial discs	Dose mcg/disc	code
1	Levofloxacin	5	LEV
2	Amoxicillin+Clavolanic acid	30	AMC
3	Amikacin	30	AK
4	Ciprofloxacin	5	CIP
5	Vancomycin	10	VA
6	Azithromycin	15	AZM

2.2 The Methods

2.2.1 Specimen Collection

A total of 50 date fruit samples were collected randomly during November and December 2023 in Erbil city. Samples were directly transported to the laboratory in a sample container for analysis(Tripathi and Sapra, 2024)**.**

2.2.2 Isolation of *S.aureus*

The specimens were initially cultured onto Mannitol Salt agar medium (Oxoid, UK) and incubated for 24 h at 37 °C. The individual small circular golden colonies were identified as *S.aureus* by various biochemical and conventional diagnostic tests (FDA, 2019).

2.2.3 Identification of *S.aureus*

2.2.3.1 Microscopic Examination

2.2.3.1.1 Gram Staining Method

All the bacteria isolated were examined for gram reaction. A small quantity of bacterial suspension from the 18 hours pre-grown colony incubated at 37°C was spread on a glass slide, and a thin smear was made, the film air-dried and then fixed using the gas flame of benzene burner. The fixed smear stained with Gram stain and examined under microscope oil immersion lens (Tripathi and Sapra, 2024).

2.2.3.2 Cultural Identification

Each sample inoculum was streaked (swap) on Mannitol Salt Agar (MSA), a differential medium and small golden colonies appeared after incubation at 37ºC for 24 hours.

2.2.3.3 Identification Based on Biochemical Tests

Biochemical identification of suspected colonies was carried out according to the methods recommended by FDA (2023) employing the following tests: catalase, coagulase, DNase, mannitol fermentation.

2.2.4 Antimicrobial Susceptibility Testing by Kirby-Bauer Disc Diffusion Method

An antimicrobial sensitivity test was performed according to the clinical and laboratory standards institute guidelines for *S.aureus* (CLSI, 2020)The colonies were suspended in saline. Then inoculum adjusted to a turbidity equivalent to a 0.5 McFarland standard after that, they were plated on MHA plates. The inoculum was well spread over the agar surface with the help of sterilized swab. Plates were allowed to dry for 10 minutes, and antimicrobial disks were then carefully placed on the surface with enough space around each disk for the antimicrob to spread. Plates were incubated for 24 hrs at 37c and the organism growth inhibition zone around each disk was measured in mm and interpreted in accordance with CLSI guidelines (CLSI, 2018).

2.2.5 Biosynthesis of Biogenic Silver Nanoparticles

A loopful of the *P. aeruginosa* inoculum was placed in 500 ml of sterile NB and incubated overnight at 37ºC. Following incubation, the broth was centrifuged for 10 minutes at 6000 rpm to obtain a cell-free broth. The later was mixed with 500 ml of 1mM silver nitrate and incubated in the dark for 72 hours at 60 ºC. After the incubation period, a change in the color to dark brown was regarded as the initial indicator for AgNPs synthesis(Yang *et al.*, 2020)**.**

2.2.6. Determination of Antimicrobial effect of AgNP on *S.aureus*

Well diffusion method was employed to evaluate the antimicrobial activity of the PA-AgNPs. Overnight bacterial cultures adjusted to the turbidity of 0.5 McFarland were swabbed onto the surface of MHA plates containing 6mm bored wells. Then, 150 µl of AgNPs solution was added into the wells and incubated overnight at 37 ºC. Inhibitions formed around the wells were measured by a ruler (Hetta *et al.*, 2021).

Chapter Three: Results and Discussion

The inactivation and eradication of multidrug-resistant bacteria by conventional antimicrobials and drugs have not been effective. The hindering of these infections caused by Gram-positive bacteria, particularly strains of *S. aureus.* Bare and functionalized metal and metal oxide nanoparticles (NPs) specifically silver (Ag) NPs have shown significant antibacterial activities (Alavi and Ashengroph, 2023).

3.1 Prevalence of *S.aureus* in Date fruit Samples

In this study, a total of 50 date fruit samples have been collected and screened for the presence of *S.aureus*, this comprised of 13 samples positive, the percentages of positive sample with *S.aureus* were 26% samples; the percentages of negative samples were 74% samples, based on microbiological and biochemical examination as shown in table (3.2). The presence of *S.aureus* in date fruit samples could be attributed to high moisture content of date flesh , Date samples with less or no processing is responsible for this contamination (Zamir *et al.*, 2018).

Table 3.2 The incidence of *s.aureus* in date fruit samples in Erbil city

Sample	Sample size	No(%) of sample Positive for *s.aureus*	No(%) of sample negative for *s.aureus*
Date fruit	50	13 (26%)	37 (74%)

3.2 Identification and characterization of *S. aureus*

Identification of *S.aureus* isolates include some morphological, cultural and biochemical tests which are summarized in table (3.1). On the basis of conventional microbiological and biochemical examination out of the total 50 date fruit samples taken, 13 isolates of *S.aureus* were recovered as indicated in table (3.2). Date fruit samples were cultured onto Mannitol Salt Agar (MSA). All 13 isolates were found to be able to ferment mannitol (change the medium color from pink to yellow) and produce golden colonies on MSA agar as clear in figer (3.1). These characteristics come in accordance with the corresponding cultural characteristic of *S.aureus* that mentioned by (Wang *et al.*, 2020).

Regarding microscopical examination and biochemical characteristics, pure colonies were identified according to their gram staining and other microscopically characteristic. The bacterial isolates were gram positive cluster arranged cocci in shape. Bacterial isolates suspected to be *S.aureus* according to microscopical characteristics were subjected to the related biochemical tests. Results illustrated in Table (3.1) and fig (3.2) showed that all the 13 isolates were had given positive test for catalase, DNAase, coagulase, and mannitol fermentation (FDA, 2023).

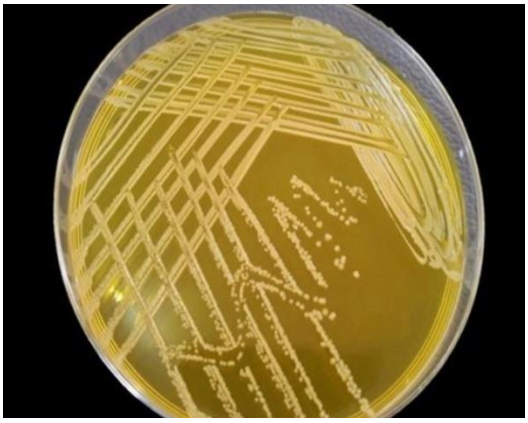
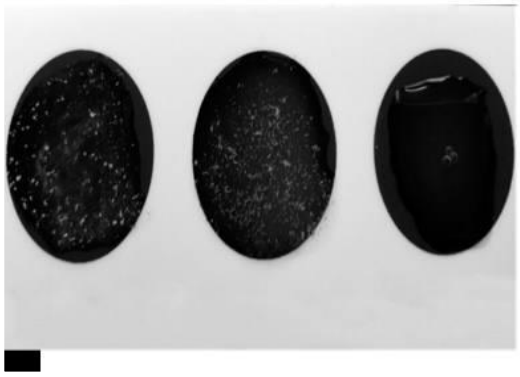


Figure 3.1 Growth of *S.aureus* on Mannitol salt agar

Table 3.1 Some physiological and biochemical properties of 13 *S.aureus* isolates from date samples.

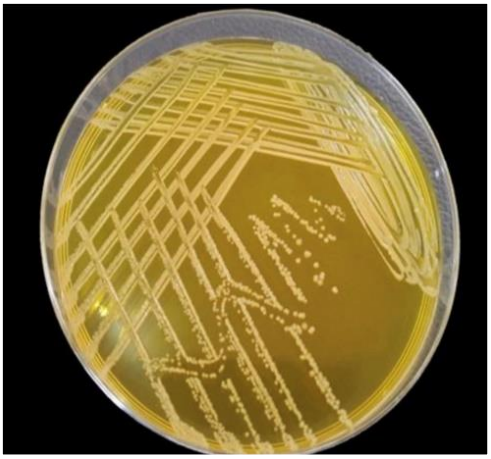
Physiological properties	**Positive isolate**	
	NO	**%**
Coagulase	13	100
Catalase	13	100
DNAase	13	100
Mannitol fermentation	13	100



Coagulase Test / +ve test

DNAase Test /+ve test

Catalase Test/ +ve test



Mannitol fermentation /+ve

Figure 3.2 Some biochemical tests of *S.aureus* isolates

3.3 Antimicrobial Susceptibility Test of *S.aureus* Isolates

Currently, antibiotic resistance is recognized as a serious economic, social, and community health problem worldwide (Madani *et al.*, 2022). In the present investigation, the antimicrobial susceptibility test was performed manually to all 13 *S.aureus* isolates according to Kirby- Bauer disk diffusion method against a panel of 6 antimicrobial agents as displayed in table (3.3).

Table (3.3) illustrates that the susceptibility of *S.aureus* isolates were variable to antimicrobes, the highest percentage of resistance to Amoxicillin+clavulanic acid was (100%), Levofloxacin and Azithromycin was (92%), Ciprofloxacin was (46%), to Vancomycin was (8%), to Amikacin was (0%).

The same table (3.3) and figure (3.3) demonstrate the resistance percentages of *S.aureus* isolates, the sensitivity for Amikacin was highest (85%), and for Vancomycin was (84%), and (23%) for Ciprofloxacin**.** The primary mechanism of antibiotic resistance in *S. aureus* is the acquisition of resistance genes, like the *mecA* gene that confers methicillin resistance. Other mechanisms include the synthesis of efflux pumps, beta-lactamase enzymes, modifications to drug targets, and decreased drug permeability due to modifications in the bacterial cell wall (Abebe and Birhanu, 2023).

Table 3.3 Antimicrobial susceptibility exhibited by *s.aureus* isolates obtained from Date fruit sample.

NO	Antimicrobials	Resistant isolates		Intermediate isolates		Sensitive isolates	
		NO	%	NO	%	NO	%
1	Levofloxacin	12	92	1	8	0	0
2	Amoxicillin+clavulanic acid	13	100	0	0	0	0
3	Amikacin	0	0	2	15	11	85
4	Ciprofloxacin	6	46	4	31	3	23
5	Vancomycin	1	8	1	8	11	84
6	Azithromycin	12	92	0	0	1	8

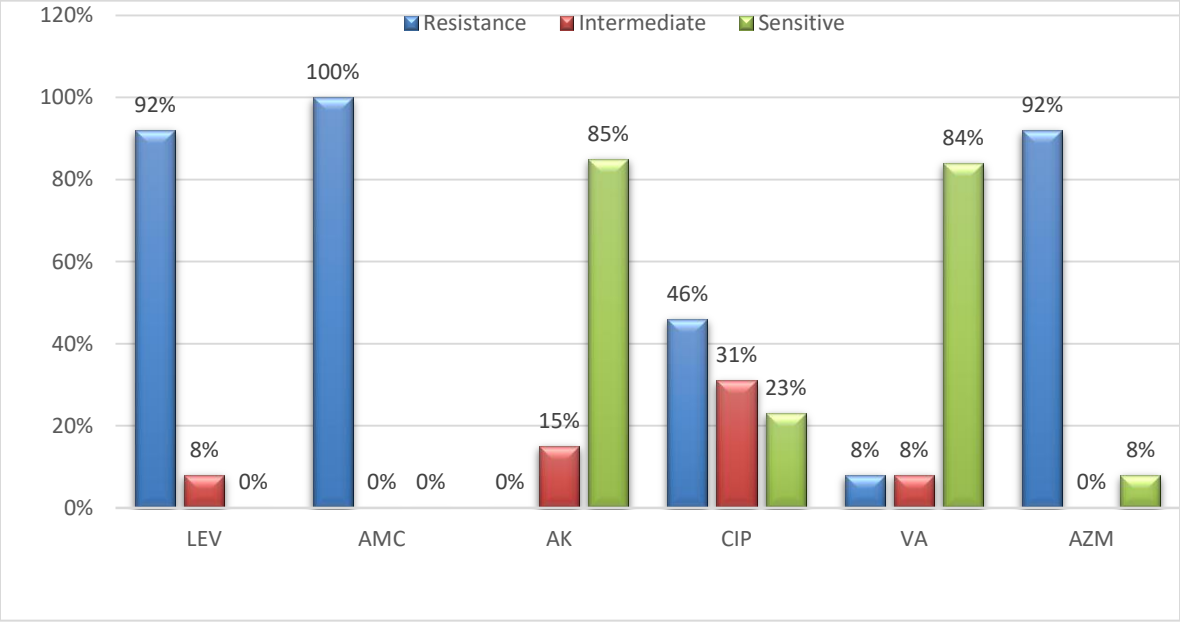


Figure 3.3 Antimicrobial resistance of *S.aureus* isolates.

3.4 Screening of Antibacterial Efficacy of the Bacteriogenic AgNPs

The inhibitory effect of AgNPs on bacterial growth was evaluated using the well diffusion method. The 13 isolates were exposed to 150 µl of AgNPs. The inhibition zones formed around the wells was calculated. As shown in figure (3.4) marked inhibition zones with a mean of 14mm were observed following overnight incubation at 37 ºC. Similar results obtained by (Hamida et al., 2020, Sivalingam and Pandian, 2024). The test of AgNPs' ability to inhibit bacterial growth produced the greatest results for S.aureus bacteria (Hamida et al., 2020, Ibraheem et al., 2023). Silver nanoparticles can continually release silver ions, which may be considered the mechanism of killing microbes (Hamida *et al*., 2020).

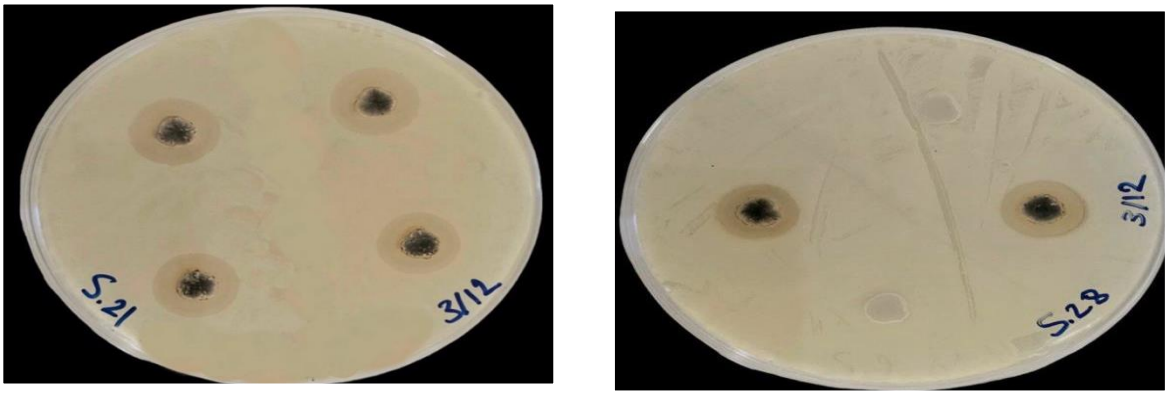


Figure 3.4 Effect of AgNP *on S.aureus* isolates.

Conclusions and Recommendations

Conclusions

From the present data it can be concluded that the date palm fruit represents a potential hazard for consumers, due to the presence of *S.aureus* as well as there is neglected sanitary measures adopted during storage, handling and sailing of this fruit. Furthermore, according the results AgNPs can be use as alternative against bacteria in place of antimicrobials, specially against MDR bacteria like *S.aures* .The presence of these bacteria in date palm fruit samples seemed to be related to the unhygienic transportation and storage conditions. Therefore, food specialists should design comprehensive programs and implement of Hazard Analysis, and consumers of this fruit should properly wash the date fruit before eat to ensure the freedom of such foods from these pathogens.

Recommendations

Further studies are suggested to investigate the occurrence of other microorganism in date fruit samples that consumed in Erbil city. And differently work on specific part of date fruit sample like seed or epicarp (skin), for microbial isolation.

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