Antibacterial activity of silver nanoparticles synthesized by *Cinnamon zeylanicum* bark extract against *Staphylococcus aureus*

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Abstract

Nanoparticles are a special group of materials with unique features and extensive applications in diverse fields. The use of nanoparticles of some metals is a viable solution to stop infectious diseases due to the antimicrobial properties of these nanoparticles. In this work, antibacterial potential of silver nanoparticles synthesized by extract of *Cinnamon zeylanicum* plants barks on pathogenic *Staphylococcus aureus* bacteria was investigated. 1ml of *Cinnamon zeylanicum* plants barks extract was added to 50 ml of 1mM aqueous silver nitrate (AgNO3) solution at the room temperature for 1-8hrs to produce silver nanoparticles. After 8 hrs, the color intensity was higher. Separation by centrifugation, characterization using UV-VIS spectrophotometry and electron microscopy analysis were performed. The UV-Vis spectral analysis showed silver surface plasmon resonance band at 400 nm. Electron microscopy showed that the particles were crystalline in nature, most of the nanoparticles were roughly spherical or circular in shape, while the average size of the nanoparticles ranged between 8 and 20 nm. Antimicrobial activity against pathogenic *Staphylococcus aureus* was tested using agar well diffusion method and minimum inhibitory concentration (MIC). The synthesized silver nanoparticles efficiently inhibited this pathogenic organism in a dose dependent when compared with reference antibiotic cefotaxime. The approach of green synthesis seems to be cost effective, eco-friendly and easy alternative to conventional methods of silver nanoparticles synthesis. The powerful bioactivity demonstrated by the synthesized silver nanoparticles leads towards the clinical use as antimicrobial.

Keyword*:* *Staphylococcus aureus*, *Cinnamon zeylanicum*, silver nanoparticles synthesized
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التأثير المضاد البكتيري لأجسام الفضة النانوية المصنعة بواسطة مستخلص نبات القرفة ضد المكورات العنقودية الذهبية

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الخلاصة

ان الاجسام النانوية هي مجموعة خاصة من المواد ذات المواصفات الفريدة والتطبيقات الواسعة في المجالات المختلفة. لقد وجد ان استعمال الاجسام النانوية لبعض المعادن هو الحل الامثل لايقاف الامراض المعدية نتيجة للخواص الضد ميكروبية التي تمتلكها هذه الأجسام النانوية. تم في هذه الدراسة بحث الفعالية المضادة للبكتريا لدقائق الفضة النانوية المتشكلة بواسطة مستخلص نبات القرفة على المكورات العنقودية الذهبية المرضية. اذ تم مزج 1 مل من المستخلص النباتي مع 50 مل من محلول نترات الفضة المحضرة بتركيز 1 ملي مولاري بدرجة حرارة الغرفة ولمدة 1–8 ساعات لانتاج دقائق الفضة النانوية حيث لوحظ زيادة كثافة اللون بعد 8 ساعات. لفصل الدقائق المتشكلة تم استعمال الطرد المركزي أما تشخيصها فتم باستخدام المقياس الطيفي والمجهر الالكتروني. ظهرت حزمة التصوير الطيفي عند القراءة 400 نانوميتر واظهر المجهر الالكتروني بان جزيئات الفضة كانت بتركيب كروي دائري الشكل وبمعدل حجم يتراوح بين 8–20 نانوميتر. كما درست الفعالية الضد بكتيرية على المكورات العنقودية الذهبية المرضية باستخدام طريقة الانتشار بالحفر واقل تركيز مثبط للبكتريا(MIC). واظهرت

جزيئات الفضة النانوية المتشكلة تثبيطاً للبكتريا الممرضة اعتماداً على تركيزها وعند مقارنتها مع السيفوتكسام، بذلك يعد تصنيع دقائق الفضة النانوية بهذه الطريقة البايولوجية الصديقة للبيئة سهلا وغير مكلفا ويقود نحو استخدامها سريريا كمضادات مايكروبية.

Introduction

Nanotechnology is the newest and one of the most promising areas of research in modern medical science. Nanoparticles are usually a cluster of atoms ranging between 1-100 nm in size and exhibit new and improved properties based on size, distribution and morphology than larger particles of the bulk materials from which the nanoparticles are made (1). Silver nanoparticles have found wide applications in the area of catalysis, optoelectronics, detection and diagnostic, antimicrobials and therapeutics (2). Silver has long been recognized as an effective antimicrobial agent that exhibits low toxicity in humans and has diverse in vitro and in vivo applications (3). Recently, silver-based topical dressings are widely used to treat infections in open wounds and chronic ulcers (4). Several approaches are out there for the synthesis of silver nanoparticles for example, chemical reduction (5), photochemical (6), thermal decomposition (7), radiation assisted (8), electrochemical (9), and recently via green chemistry method (10). Biological method of nanoparticles synthesis using microorganisms (11), enzyme, and plant or plant extract offers numerous benefits over chemical and physical method (10). Among the various known synthesis methods, plant mediated nanoparticles synthesis is preferred as it is cost-effective, environmentally friendly, and safe for human therapeutic use (12). Many reports are available on the biogenesis of silver nanoparticles using several plant extracts (13). *Cinnamomum zeylanicum* is a small, tropical, evergreen tree most noted for its bark, which provides the world with the commonly known spice, cinnamon.*C. zeylanicum* bark is rich in terpenoids, including linalool, eugenol and methyl chavicol and in chemicals, including resinous compounds(14), cinnamaldehyde, ethyl cinnamate and caryophyllene(15), Cinnamic acid, L-borneol, L-bornyl acetate, E-nerolidol, and cinnamyl acetate which contribute to its distinct aroma (16). In addition, some protein is also present in the bark (17). Terpenoids are believed to play an important role in silver nanoparticle biosynthesis through the reduction of silver (Ag) ions (18). It has been reported that silver nanoparticles (SNPs) are non-toxic to body cells and most effective against bacteria, virus and other eukaryotic micro-organism at low concentrations and without any side effects (19). In small concentrations, silver is safe for cells, but lethal for microorganisms. Antimicrobial capability of SNPs allows them to be suitably employed in numerous household products such as textiles, food storage containers, home appliances and in medical devices (20). With the emergence and increase of microbial organisms resistant to multiple antibiotics, and the continuing emphasis on health-care costs, many researchers have tried to develop new, effective antimicrobial reagents free of resistance and cost (21)**.**Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface (22). The most important application of silver and SNPs is in medical industry such as tropical ointments to prevent infection (23). Biomaterials are commonly applied in regenerative therapy and tissue engineering in bone, and have been substantially refined in recent years. In bone, locally applied nanoparticles may be suitable for numerous potential uses with respect to the improvement of tissue regeneration, the enhanced osseointegration of implants, and the prevention of infections (24).

This work was aimed to Biosynthesis of silver nanoparticles using plants, and Iinvestigate the antibacterial activity of the silver nanoparticles which are obtained by *C. zeylanicum* bark extracts in-vitro.

Materials and Methods

- **Sterilization Methods:**

All sterilization methods were done according to (25).

1. **Sterilization by moist heat (autoclave):** Culture media and solution used in the study were sterilized in the autoclave for 15 minutes at 121 C^o under the pressure 1.25kg/cm^2.The pressurized steam lead to destroy microorganisms and are the most dependable systems available for the decontamination of laboratory waste and sterilization of laboratory glassware and reagent (26).
2. **Dry heat sterilization (Oven):** This method was used to sterilize glassware,metal surgical instruments and caps that used in the study, commonly, a temperature of 160 C^o was maintained for 3 hours.
3. **Direct flaming (heating):** Inoculating loop, point of forceps and spatulas were sterilized by holding them on the flame of gas burner until they were seen to be red.

- **Preparation of Normal Physiological Saline Solution:** This solution was prepared by adding 9 g of sodium chloride (NaCl) in 1 L of distilled water, then sterilized by autoclaving, cooled and stored in a refrigerator at 4C^o until used (27).
- **Preparation of Cultures Media:** Cultures media were prepared according to the manufacture company (oxoid).
- **Activation and Maintenance of Bacterial Isolates:** Bacterial cultures were activated in screw capped tubes containing 10 ml of brain heart infusion agar slants and incubated for 24 hours at 37C^o. For maintenance of isolates, nutrient and brain heart infusion agar were stored at 4C^o, and were sub -cultured once every two-weeks (25).
- **Plant Materials:** *Cinnamon zeylanicum* barks that were collected from local market in Baghdad. The plant classification was done in the Ministry of Agriculture/ State Board for Seeds Testing and Certification S.B.S.T.C in Abu Graib /Baghdad at certificate No. 1077 in 26/3/ 2014. The plant were dried naturally in room temperature at shade for a week for complete moisture removal**.** The barks were cut into small pieces, crushed to a fine powder by an electrical grinder then sieved using a 20-mesh sieve to get uniformed size range. The final sieved powder was used for all further studies (28).
- **Biosynthesis of Silver Nanoparticles Using *Cinnamon zeylanicum* Plants:**

a. **Preparation of *Cinnamon zeylanicum* Plants Barks Extract:** The extract was produced by adding 2.5gm of *Cinnamon zeylanicum* plants barks powder into 100 ml of distilled water and boiled for 5 minutes in 500 ml flask and after cooling was filtered using whatman No.1 filter paper. The final extract was kept at 4◦C (29).

b. **Preparation of 1mM Silver Nitrate Solution:** For the preparation of 1mM Silver nitrate ($AgNO_3$) 0.0421gms of $AgNO_3$ was dissolved in 100 ml of double distilled water. The solution was mixed thoroughly and stored in a yellow colored bottle to prevent auto oxidation of silver (30).

c. **Synthesis of Silver Nanoparticles using *Cinnamon zeylanicum* Plants Barks Extract:** One (ml) of *Cinnamon zeylanicum* plants barks extract was added to 50 ml of 1mM aqueous silver nitrate ($AgNO_3$) solution and kept at room temperature for 8hrs to produce silver nanoparticles (28). The solution initially appeared yellowish in color and upon reduction of nitrate from silver Ag+ to free reduced form change to dark color. The changing of color solution was measured every 1hrs. The changing in the color intensity after the reduction of Ag+ to silver nanoparticles by *Cinnamon zeylanicum* plants barks extracts with increasing time of reaction was recorded.

- **Separation and Identification of Silver Nanoparticles:**

1. **Spectrophotometry:** Formation and stability of silver nanoparticles in sterile distilled water is confirmed with UV-vis spectrophotometer in a range of wavelength from 100 to 700 nm. For the UV-Vis spectral analysis. (1ml *Cinnamon zeylanicum* plants barks extract treated with 50 ml of 1mM aqueous silver nitrate). This solution was kept under UV light at 250 nm for 1-8hrs. Absorbance was measured at 1hr interval and the changing in the color was observed gradually as it turned dark brown at the end of 8hrs of exposure to UV radiation (31).
2. **Centrifugation:** Silver nanoparticles solution which was obtained from (1ml *Cinnamon zeylanicum* plants barks extract /50 ml of 1mM aqueous silver nitrate) was centrifuged at 10,000 rpm for 30 min. The pellet was washed three times with 20 ml of distilled water, and finally dried at 60°C, to get rid of the free proteins/ enzymes that are not capping the silver nanoparticles. The centrifugation process were done at time (1-8hrs). This was done in order to know the effect of time incubation on amount of silver nanoparticles synthesized (32).
3. **Scanning Electron Microscopy (SEM):** Huang *et al.*, (33) reported that there was no marked difference in the shape and size of SNPs at various initial biomaterial concentrations, therefore the samples for electron microscopy were prepared from the concentration (ml of extract/50ml of 1mM aqueous silver nitrate) and various time of reaction. The biomass had settled at the bottom of the conical flasks and the suspension above the precipitate was sampled for scanning electron microscopy (SEM) observation. Scanning electron microscopy samples of the aqueous solution of silver nanoparticles prepared by placing a drop of the solution on carbon-coated copper grids and the films on the SEM grids allowed standing for 2 min, after which the extra solution removed using a blotting paper and drying the grid. The size distribution of the resulting nanoparticles estimated on the basis of SEM micrographs (34).

- **Identification of Bacteria by Morphological Examination and Cultural Characteristics**: Pathogenic *staphylococcus aureus* isolate was obtained from the College of Veterinary Medicine/ University of Diala. These isolate was identified by studying morphological and some biochemical characteristics as the following (Table 1):

Table (1) Morphological and Biochemical Test to *Staphylococcus aureus*

Bacterial spp.	Morphological examination	Biochemical tests
Staphylococcus aureus	Mannitol salt agar Gram stain Staph 110 agar Blood agar	Catalase test Coagulase test Oxidase test

Biochemical tests (catalase test, coagulase test and oxidase test were done according to (25 and 35).

- **In vitro Antibacterial Activity of SNPs and Antibiotic:**
- **Preparation of Standard Bacterial Suspension:** The average number of viable *Staphylococcus aureus* cell per ml of the stock suspension was determined by means of the standard McFarland solution No.0.5. By taking 1 ml from overnight culture (brain heart infusion broth) of *Staphylococcus aureus* suspension mixing with 9 ml of pepton water, then taking 1 ml of this suspension and making serial ten-fold dilution. Standard McFarland solution No.0.5 was prepared according to (35) as follows:

- **Solution (A),** this was prepared by dissolving 1.175 g of barium chloride ($BaCl_2.2H_2O$) in 100 ml distilled water.
- **Solution (B),** this was prepared by adding 1 ml of concentrated sulfuric acid (H_2SO_4) in 100 ml distilled water.

The two solutions were mixed by adding of 0.5 ml from solution A to 99.5 ml from solution B. The prepared solution was used to compare turbidity of bacterial suspension to obtain an approximate cell density of 1.5×10^8 cell/ml. Spectrophotometer was used to measure the turbidity of the *Staphylococcus aureus* suspension to reach an appropriate optical density (O.D), whereas the percent of focus transmission was 25% at a wave length of 450 nm, after the instrument has been blanked on the cleaned (without bacterial growth), nutrient broth with the same wave length and transmission 100% (25).

- **Preparation of Different Concentrations of Antibiotic:** Stock solution of cefotaxime was prepared by mixing 0.1 gram of cefotaxime with10 ml of distilled water (10mg/ml), then concentrations of (10, 15, 25, 50 and 100µg/ml) were prepared by mixing known volume from the stock solution with distilled water (36).
- **Preparation of Different Concentrations of SNPs:** Stock solution of silver nanoparticles was prepared by mixing 0.1 gram of SNPs with 10 ml of distilled water (10 mg/ml), then concentrations of (10, 15, 25, 50 and 100µg/ml) were prepared by mixing known volume from the stock solution with distilled water. These concentrations of silver nanoparticles and cefotaxime were used in sensitivity test to determine the *Staphylococcus aureus* sensitivity to SNPs and cefotaxime (31).
- **Sensitivity Test:** Sensitivity test of the nanoparticles which obtained by plant compared with cefotaxime. The agar well diffusion method was adopted according to (37). For assessing the antibacterial activity of the prepared SNPs, 0.5 ml of standardized bacterial stock suspensions (1.5×10^8 cfu/ml) of *Staphylococcus aureus* was thoroughly mixed to 500 ml of sterile Mueller Hinton agar. Fifteen (15) ml of the inoculated Mueller Hinton agar was distributed into sterile Petri dishes of each. The agar was left to set for 10 minutes to allow solidifying the agar and making wells in each of these plates, 6 mm in diameter were cut using a sterile Pasteur pipette and the agar discs were removed by sterile forceps, after that the wells were filled with 55 microliter of each concentrations of silver nanoparticles (10, 15, 25, 50 and 100µg/ml) using micropipette. The plates were then incubated in the upright position at 37 C^o for 24 hours. Three replicates were carried out for each concentration of SNPs and the activity was determined by measuring the diameter of inhibition zone around each well by millimeter against *Staphylococcus aureus*. Simultaneously, addition of respective solvent (distilled water) instead of SNPs was carried out as controls. The results and standard errors meanes values were tabulated.
- **In vitro Antibacterial Activity of Standard Antibiotic:** Cefotaxime was used as a reference antibiotic to determine the sensetivity test for *Staphylococcus aureus*. The same technique which was used for silver nanoparticles antibacterial sensetivity was used for determination of cefotaxime by using the concentrations (10, 15, 25, 50 and 100µg/ml) with 15 microliter of sterlized distilled water was served as control.
- **Assaying the Minimum Inhibitory Concentration of SNPs Against *Staphylococcus aureus*:** The minimum inhibitory concentration (MIC) of Ag-NPs was determined using the plate count method (38 and 39). The SNPs powder was sterilized with UV radiation for 1 hr, and the weight of the powder was then measured under aseptic conditions. Further, Mueller-Hinton broth containing 1.5 x 10^8 CFU/ml of bacterial cells was used as a culture medium.

The final concentrations of SNPs were 0, 2.5, 5, 10, 15, 25, 50 and 100 µg/ml. The medium was cultured in incubator at 37^{o}C for 24hrs, and the cultured media (100 µl) was spread onto Muller-Hinton agar and incubated at 37^{o}C for 24 h. After incubation, the number of colonies grown on the agar was counted (38).

- **Statistical analysis:** Data were analyzed statistically using the Microsoft Program (SPSS). Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). Differences were determined using least significant differences (LSD) as described by (40).

Results and Discussion

- **Synthesis of Silver Nanoparticles Using *Cinnamon zeylanicum* Plants Barks Extract:** For the green synthesis of silver nanoparticles by *C. zeylanicum* plant extracts were carried by adding 1 ml of extract to 50 ml of 1mM $AgNO_3$ solution. We adopted a simple procedure to synthesize silver nanoparticles from cinnamon bark extract. On mixing the plant extract of *C. zeylanicum* with silver nitrate solution (1mM), the color of the reaction mixture started changed to yellowish within 1hr and to dark brown after 8 h, indicating the generation of silver nanoparticles, due to the reduction of silver metal ions Ag+ into silver nanoparticles via the active molecules present in the *C. zeylanicum* plant extracts. Changing in color after the reduction of Ag+ to silver nanoparticles is shown in (Fig. 1). The reduction rate and formation of nanoparticles can be increased further by increase in incubation time.

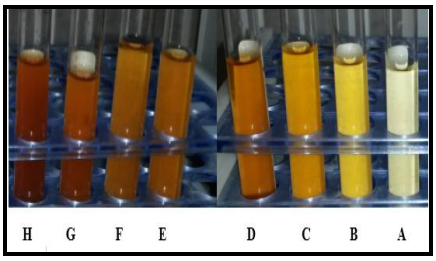

Fig. (1) Change in color after the reduction of Ag+ to silver nanoparticles by *Cinnamon zeylanicum* plants barks extracts 1ml(CBPE) /50 ml(AgNO3), at: (A) 1hrs, (B) 2hrs, (C) 3hrs, (D) 4hrs, (E)5hrs, (F)6hrs, (G)7hrs and (H)8hrs.

- **Separation and Identification of Silver Nanoparticles:**

- **Spectrophotometry:** The formation of silver nanoparticles was monitored with color change and UV-Vis spectrum. The color of the reaction mixture started changing to yellowish brown within 1hr and to dark brown after 8 hr. This color is attributed to the excitation of surface plasmon resonance. The absorption spectra of silver nanoparticles solution consists a single sharp surface plasmon resonance band at 405 nm (Fig. 2). The most characteristic part of silver solution is a narrow plasmon absorption band observable in the 350-600 nm regions. The distinct visible peak was observed at 405nm which is an indication of reduction of silver. This indicates that by UV method, silver gets reduced in a faster way than the conventional method. Control silver nitrate solution neither developed the dark brown color nor did they display the characteristic band, indicating that abiotic reduction of silver nitrate did not occur under the used conditions.

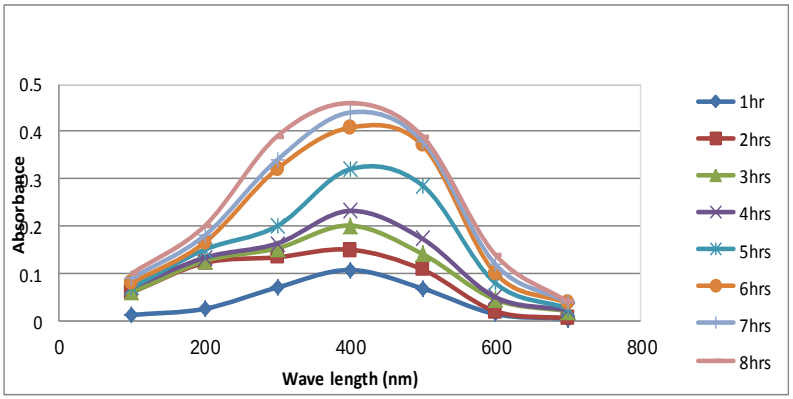

Fig. (2) UV-Visible absorption spectra of synthesized silver nanoparticles, showing the surface plasmon resonance peak of silver nanoparticles at 425 nm, showing the color change upon formation of silver nanoparticles at different incubation time.

- **Centrifugation**: After the centrifugation of silver nanoparticles solution at different incubation time interval (1-8hrs), the samples were dried and weighing after washed three times with 20 ml of de-ionized water to get rid of the free proteins/ enzymes that are not capping the silver nanoparticles. After 1hs and 2hrs of incubation time, there was a decrease in pellet weight when they were compared with 6, 7 and 8 hrs of incubation time. 1 ml of *C. zeylanicum* plant extract mixing with 50 ml of 1mM $AgNO_3$ solution, gave a deep dark color pellet (Fig. 3).
- **Scanning Electron Microscopy (SEM):** Electron Microscopy images of silver nanoparticles solution are shown in (Fig. 4). These observations indicate the adsorption and/or deposition of silver nanoparticles onto the surface of roughly sphere-shaped polydispersed particles. The Ag-nanoparticles that emerged in the images have variety of shapes: spherical, triangle and irregular. As can be seen in (Fig. 4) typical example, presence of rings patterns in the selected area electron diffraction reveals the single face-centered cubic crystalline nature of the spherical nanoparticles with a preferential growth direction along the Ag. The shape evolution of photomorphic silver nanoparticles (SNPs) was noticed in electron microscope images of samples prepared at various times. The average size of the nanoparticles ranged between 8 and 20 nm for *C. zeylanicum* bark extract, with a few larger particles exceeding 60 nm only in the case of *C. zeylanicum* bark extract at the longer reaction time.

Fig. (3) Appearance of silver nanoparticles after centrifugation

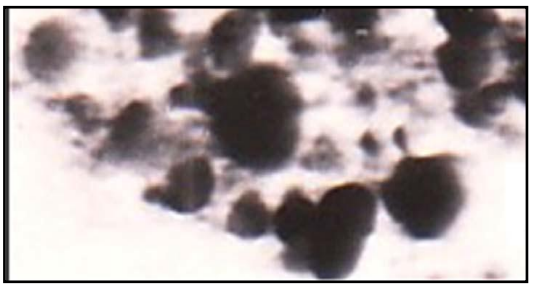

Fig. (4) Crystalline clusters of silver nanoparticles (obtained by SEM)

- **Identification of *S.aureus***
- **Morphological Examination and Culture Characteristics:**

a. Gram Stain: Gram stain was shown spread of bacteria as spherical single cocci, diplococci, or quadrates, but the predominant shape was grape-like clusters of blue color under light microscope, and those mentioned features were characteristics for Staphylococci (Fig. 5).

b. Blood Agar: After 37 ºC incubation for 24 hrs on blood agar, colonies appeared as golden yellow, round, smooth and glistening β or α hemolytic 1 mm in diameter.

c. Staph 110 Agar: Growth of pathogenic Staphylococci produced golden yellow to orange-pigmented colonies on Staph 110 agar (Fig. 6 and Table: 2).

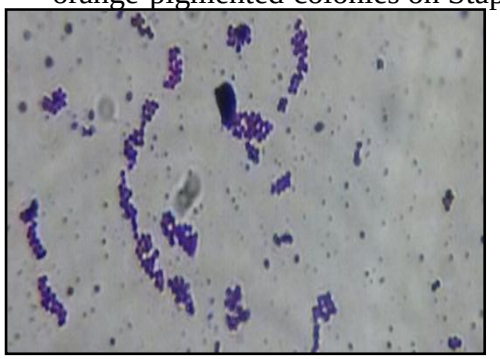

Fig. (5) Shows microscopic appearance of *S.aureus* after Gram staining (100X)

Fig. (6) Shows growing of *S.aureus* on Staph 110 agar

Table (2) Morphological tests and microscopic examination used to identify *Staphylococcus aureus.*

morphological tests	Results
Gram stain	G +ve cocci
Staph 110 agar	golden yellow to orange colonies
Blood agar	golden yellow ,round, smooth colonies
Brain heart agar	golden yellow colonies

- **Biochemical Characteristics:**

a. Mannitol Salt Agar: Mannitol Salt Agar (MSA) is the selective media usually used to detect the *S. aureus*. Suspected *S.aureus* was subcultured on mannitol salt agar for purification, the colonies appeared as rounded, and smooth convex colonies yellowish in color disseminated to the background of the agar indicated fermentation of mannitol sugar. The color of mannitol media was converted from rosy to yellowish color (Fig. 7).

b. Slide Catalase Test: The test showed appearance of air bubbles directly after mixing 3% hydrogen peroxide with the suspected colonies (Fig. 8). *S. aureus* isolates would produce bubbles when H_2O_2 3% added.

c. Coagulase Test (**Slide method**): It is an important test to differentiate between pathogenic and non-pathogenic staphylococci, only pathogenic one gave positive results. The results showed appearance of agglutination particles rapidly in 90 second (positive result) after the adding of bacterial suspension into rabbit plasma (Fig. 9).

Fig. (7) Shows growing of *S.aureus* on mannitol salt agar.

Fig. (8) Appearance of air bubbles after mixing H_2O_2 with suspension of *S. aureus*.

Fig. (9) Appearance of agglutination particles after mixing the suspension of *S. aureus* with rabbit plasma

d. Oxidase Test: The results showed that the test microorganisms are absolutely negative for this test (Table 3).

Table (3) Biochemical tests used to identify *Staphylococcus aureus*.

Biochemical tests	Results
Mannitol salt agar	**+ve** ,change from rosy to yellowish color
Catalase test	**+ve**
Coagulase Test (Slide method)	**+ve**
Oxidase test	**-ve**

After gram staining the predominant shape was grape-like clusters of blue color under light microscope, and those mentioned features were characteristics for *Staphylococci.* This description agrees with (41). Colonies appeared as golden yellow, round, smooth due to β or α hemolytic 1 mm in diameter on the blood agar. That is in agreement with (41). While on the staph 110 agar produced golden yellow to orange-pigmented colonies and this is in agreement with (42). On the mannitol salt agar the colonies of *S.aureus* appeared as rounded, and smooth convex colonies yellowish in color disseminated to the background of the agar indicated fermentation of mannitol sugar. The color of manitol media was converted from rosy to yellowish color, this may be attributed to the ability of *S.aureus* for fermentation in the presence of phenol red indicator in the medium that converted to yellowish color when the medium becomes acidic by fermentation, and this fits the description of (42). In the catalase test the appearance of air bubbles directly after mixing 3% hydrogen peroxide with the suspected colonies. *Because S. aureus* isolat able to produce enzyme catalase that breakdown hydrogen peroxide to water and oxygen and this in agreement with (43). While in the oagulase test (Slide method) the appearance of agglutination particles rapidly in 90 second after the adding of bacterial suspension into rabbit plasma attributed to that pathogenic bacterial isolates are able to cause coagulation of plasma (43). The microorganisms is absolutely negative for oxidase test, this resembles the description of *staphylococcus aureus* mentioned by other researchers (41).

- ***In vitro* Antibacterial Activity of Silver Nanoparticles and Cefotxime against *s.aureus*:** Different concentrations of silver nanoparticles and Cefotxime were used in agar well diffusion assay, caused different degrees of zones of inhibition against *staphylococcus aureus*. The size of inhibition zones were different according to concentration of the silver nanoparticles and antibacterial agent, the size of inhibition zone proportionally increased with increasing of concentration of the agents, table (4), Fig. (10, 11, 12 and 13). The results showed that *staphylococcus aureus* was sensitive significantly ($P<0.05$) to silver nanoparticles (SNPs) more than Cefotxime in the concentrations (50 µg/ml and 100 µg/ml) for both. In this concentrations there was a significant increase ($P<0.05$) in diameter of zone of inhibition in *S. aureus* growth as compared with zone of inhibition of Cefotxime. Distilled water was used as control, it did not give any noticed zone of inhibition, distilled water was used as a solvent for silver nanoparticles in vitro studies, it is considered as one of the solvents that can be used for screening the antimicrobial activity because of its 100% biologically inert substances. The results of inhibitory zone diameter indicated the sensitivity of *S. aureus* after 8hrs of incubation, towards different tested concentrations. Silver nanoparticles and Cefotxime antibacterial activities were observed to be concentration dependent and this appeared significantly in the concentrations 25, 50 and 100 µg/ml.

Table (4) In vitro antibacterial activity of silver nanoparticles and reference antibiotic (cefotaxime) in different concentrations on *S. aureus* (diameter of inhibition zone in mm.)

Conc. µg/ml	Zone of growth inhibition(mm) M±SE		
	Cefotaxime	SNPs	D.W.
10	11.0±0.13 Ac	11.0±0.13 Ac	0.0±0.00 B
15	12.0±0.13 Ac	13.0±0.15 Ac	0.0±0.00 B
25	13.0±0.15 Ac	14.0±0.18 Ac	0.0±0.00 B
50	15.0±0.16 Bb	18.0±0.18 Ab	0.0±0.00 C
100	18.0±0.20 Ba	21.0±0.19 Aa	0.0±0.00 C

***Values represent mean ±S.E**

***Different capital letters mean significant (P<0.05) results between different concentrations.**

***Different small letters mean significant (P< 0.05) results between SNPs and cfotaxime.**

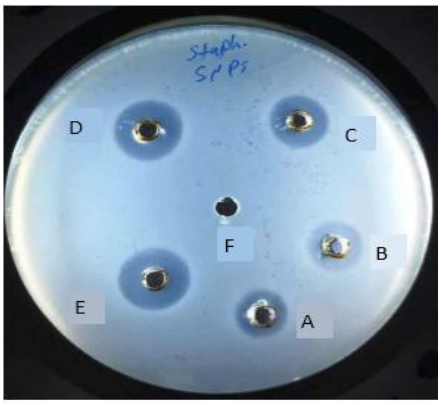

Fig. (10) Sensitivity of *staphylococcus aureus* to SNPs in different concentrations (A=10µg/ml; B=15µg/ml; C=25µg/ml; D=15µg/ml; E=100µg/ml; F=D.W.)

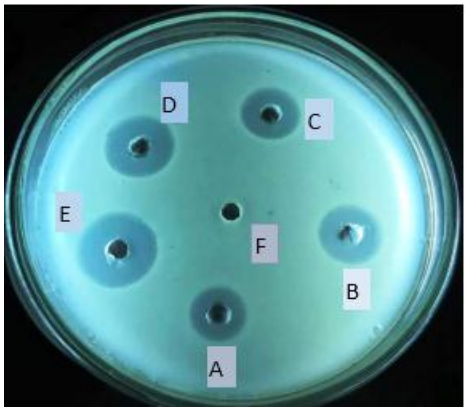

Fig. (11) Sensitivity of *staphylococcus aureus* to cefotaxime in different concentrations (A=10µg/ml; B=15µg/ml; C=25µg/ml; D=15µg/ml; E=100µg/ml; F=D.W.)

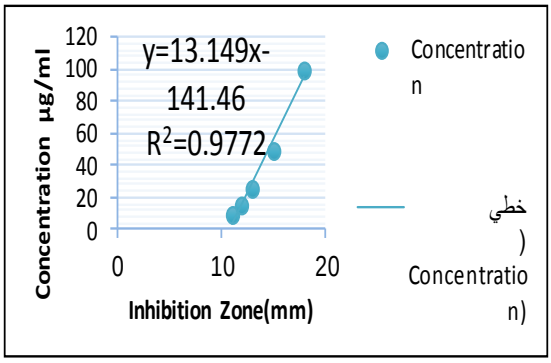

Fig. (12) Proportional relationship between concentrations of SNPs and mean diameter zone of inhibition (mm.) against *staphylococcus aureus*

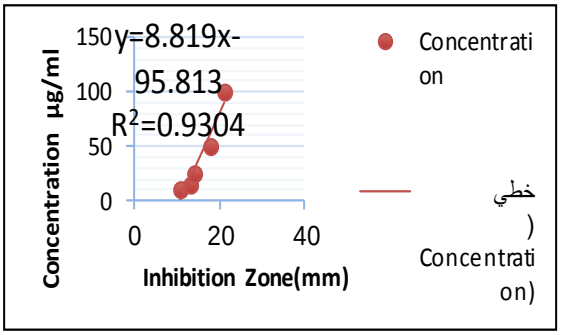

Fig. (13) Proportional relationship between concentrations of cefotaxime and mean diameter zone of inhibition (mm.) against *staphylococcus aureus*

- **Minimum inhibitory concentration of SNPs:** To determine the lowest concentration that completely inhibited visible growth, the minimum inhibitory concentration (MIC) was used. The MIC of SNPs against *S. aureus* was 10µg/ml. When 15, 25, 50 and 100µg/ml SNPs powder were used, growth was inhibited; however, when 5µg/ml Ag-NPs was used, growth was only slightly inhibited (Fig.14).

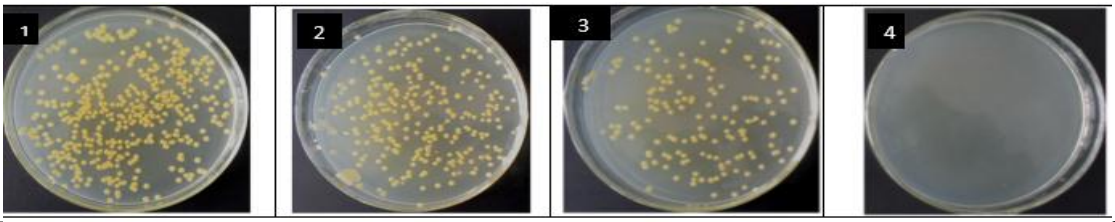

Fig. (14) Minimum inhibitory concentration (MIC, µg/ml) of SNPs (1=0µg/ml, 2=2.5µg/ml, 3=5µg/ml, 4=10-100µg/ml).

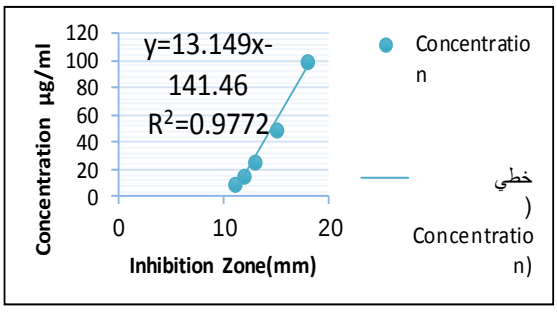

Fig. (15) Proportional relationship between concentrations of SNPs and mean diameter zone of inhibition (mm.) against *staphylococcus aureus*

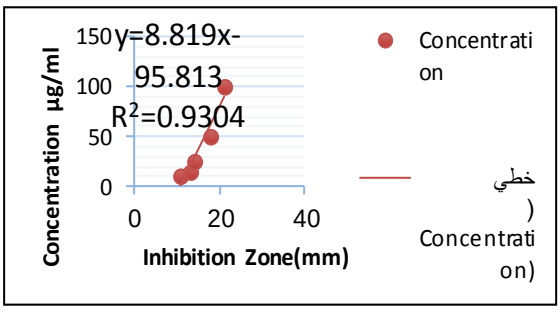

Fig. (16) Proportional relationship between concentrations of cefotaxime and mean diameter zone of inhibition (mm.) against *staphylococcus aureus*

Clear zone around the well reflects the bacterial sensitivity towards antibiotics (44). The results showed that SNPs biosynthesized using *Cinnamon zeylanicum* plants barks extract showed good inhibition against the *Staphylococcus aureus* and this in agreement with (45). From this study, it is clear that the synthesized SNPs were found to be bactericidal against *Staphylococcus aureus* (46). It has been reported the greater the zone of inhibition, greater the antibacterial properties of the SNPs and that antibacterial effect was concentration dependent in agreement with (47) and (48). This fact was also proved by (49) that showed that the inhibition zone increased as the concentration of AgNPs increased. But, (50, 51) showed that silver nanoparticles exhibited more activity than that showed in this study. This may be explained by the low concentration of Ag-NPs used in this work. Silver nanoparticles exerts their antibacterial effects by anchoring to- and penetrating the bacterial cell wall and modulating cellular signaling by dephosphorylating putative key peptide substrates on trypsin residues, this main mechanism by which silver nanoparticles exhibit antibacterial properties .Silver binding to plasma binding protein (PBP) lead to inhibiting transpeptidation and formation of the bacterial cell wall, leading to cell lysis and death. It is clear that treated bacteria also show significant changes, damage to membranes and formation of "pits" on their surfaces. A similar effect was described by (52). A bacterial membrane exhibits a significant increase in permeability, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane and, finally, causing cell death (53). Silver nanoparticles showed excellent antibacterial activity against *Staphylococcus aureus* by producing lactate dehydrogenase enzyme which leads to damage in the cell membrane (54). Another proposed mechanism involves the association of silver with oxygen and its reaction with sulfhydryl (–S–H) groups, the reaction of monovalent silver with sulfhydryl groups produces a much more stable -S-Ag group only on the bacterial cell wall surface thereby blocking respiration and causing cell death (55). Silver ions may not be capable of permeating through cell membranes to react with the interior -S-H groups to form R–S–S–R bonds, rendering silver relatively nontoxic to humans and animals (56). Silver nanoparticles bind to sulphur and phosphorous containing compounds like DNA and damage them. It is believed that DNA loses its replication ability and cellular proteins become inactivated (57). In addition, it was also shown that Ag+ binds to functional groups of proteins, resulting in protein denaturation (58). Silver nanoparticles modulated phosphotyrosine profile of bacterial peptide that in turn affects signal transduction and inhibited growth of micro-organisms. In addition, silver ions can interact with nucleic acids (59), they preferentially interact with the bases in the DNA rather than with the phosphate groups and DNA loses its replication ability and expression of ribosomal subunit proteins as well as some other cellular proteins and enzymes essential to ATP production becomes inactivated (60). The possibility of free-radical involvement in the antibacterial activity of silver nanoparticles (SNPs) has been previously reported by (61).The relation between reactive oxygen species (ROS) and bacterial cell death was revealed by (62). According to the study, bacterial DNA or mitochondria can be affected by ROS. (63) used electron spin resonance spectroscopy to investigate the effect of silver nanoparticles on microbes and found that they exert their effect by generation of free radicals. It is believed that cellular proteins become inactive after treatment with silver nanoparticles (64). It is also believed that silver nanoparticles after penetration into the bacteria have in- activated their enzymes, generating hydrogen peroxide and caused bacterial cell death (65),and react with proteins, therefore they bind protein molecules; as a result cellular metabolism is inhibited causing death of microorganism (64). (66) also investigated the antibacterial activity of silver nanoparticles on *Staphylococcus aureus* and suggested that concentration of silver nanoparticles above 8ug/ml resulted in release of muramic acid (MA) into the medium which causes cell wall distraction. From these results, we can conclude that the silver nanoparticles (SNPs) have highly antibacterial activity against pathogenic *Staphylococcus aureus*.

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