

Research article

Biosynthesis and characterization of silver nanoparticles using *Cinnamomum zeylanicum* extract and a study of antibacterial effect against multi-drug resistance Gram-negative bacteria

Reyam F. Saleh, Ayad M. Gaidan

Department of Microbiology, College of Science, Tikrit University, Tikrit, Iraq

(Received: January 2021 Revised: April 2021 Accepted: April 2021)

Corresponding author: **Reyam F. Saleh**. Email: riyamf@tu.edu.iq**ABSTRACT**

Introduction and Aim: Nanoparticles for some metals can be used in the treatment of diseases caused by different pathogenic bacteria that are resistant to antibiotics due to the antibacterial properties of these nanoparticles. In the current study, the synthesis of silver nanoparticles (Ag) from Cinnamon zeylanicum bark extract was investigated.

Materials and Methods: One mL of cinnamon bark extract was added to 50 mL of 1 mM of the silver nitrate (AgNO₃). After incubation time of bark extract with AgNO₃ at room temperature for 1-18 hours, the silver nanoparticles synthesis through changed colour of the mixture to dark brown. The UV, TEM and FT-IR analysis were carried out to characterize the biosynthesized Ag-NPs, UV-Vis scan showed absorption around 435 nm while TEM showed Ag-NPs spherical shape and the sizes of the and FT-IR spectrum detected the presence of different functional groups responsible for reduction and stability of Ag-NPs in an aqueous solution.

Results: Biosynthesized Ag-NPs showed inhibitory effect against some Gram-negative bacterial species that have complete resistance to the antibiotics P, NA, VA, E, TE. The inhibition zone of Ag-NPs was determined as follows (*Citrobacter freundii* 19 mm, *Klebsiella pneumoniae* 23mm, *E.coli* 23 mm, *Enterobacter spp* 20 mm, *Acinetobacter baumannii* 20 mm. Therefore, Ag-NPs can be used as alternative treatment for some antibiotics.

Conclusion: The Ag-NPs can be successfully prepared form Cinnamomum bark plant. Besides their low cost, these NPs had an antibacterial effect especially against Gram negative bacteria which had a complete resistance to the most common antibiotics.

Keywords: Silver nanoparticles; biosynthesis; bacteria.

INTRODUCTION

The importance of antibiotic-resistant bacteria has increased rapidly in the past few years and became a health problem that affects the modern world, which may lead to complete vanishing of old generation antibiotics (1). The growing effort has begun to search for strategies that incorporate new substances that can affect multiple drug resistance (MDR) bacteria (2). Among those strategies that have attracted great interest in recent years is nanotechnology for the production of biologically effective and environmentally friendly nanomaterials that can replace conventional antibiotics and have potential applications as antibacterial materials (3). One of these techniques involves the biological methods used to synthesize mineral nanoparticles (NPs) is plant extracts, which are considered as easy, fast, environmentally friendly, and have no pathogenic effect (4). Reduction and stabilization of metal ions can be accomplished with a mixture of biomolecules such as enzymes, polysaccharides, tannins, phenols, saponins, terpenes, and other plant compounds. Although many minerals exist in nature, only a few can be used for large-scale synthesis in nano scale form such as gold, silver, palladium, and platinum (5).

Plants can produce silver ions through their primary and secondary metabolites during photosynthesis (6). Derived nanoparticles have a distinctive feature of antibacterial activity against a wide range of bacteria (7). Recently, it was found that plant bark extracts, in particular, are rich source for reducing agents used in the synthesis of the mineral NPs (8). This is mainly due to their content of phenolic compounds, from which NPs can be made while controlling the size and shape of particles, with greater stability, and more biocompatibility (9).

Cinnamomum zeylanicum is a tropical little evergreen tree known as cinnamon bark. It is widely used as a spice, while cinnamon plant extract has a long history in biomedical applications. In chemicals such as resinous compounds, Cinnamon is also wealthy in various terpenoids including eugenol, methyl chavicol and linalool (10). It's believed that Terpenoids play an important role in the silver nanoparticles biosynthesis through silver (Ag) ions reduction (11).

The present study aimed to biosynthesize Ag-NPs using *C. zeylanicum* bark extract (CBPE) and it tested it as new therapeutic antibacterial drug against some antibiotic-resistant Gram negative bacteria.

MATERIALS AND METHODS

Collection and isolation of the bacteria

Samples were collected from wound infections from inpatients at Salah al-Din General Hospital, Tikrit, Iraq during the period from October 2018 to November 2019. Bacterial identification was carried by the phenotypic characteristics of bacterial cultures (shape, color, size, edges and height of the colonies) after growing them on the culture media. Microscopic study was performed through staining the bacteria with gram dye to observe their response to the stain and to determine the shape, size and arrangement of bacterial cells. The final diagnosis was confirmed with the VITEK 2 technique (BioMerieux-France).

Antibacterial sensitivity test

The sensitivity of the bacterial isolates to antibiotics was tested according to standard method (12) was used to test the sensitivity of isolates to antibiotics discs using Muller Hinton agar medium towards 5 antibiotics: erythromycin (E), mcg/disc; tetracycline (TE), 10 mcg/disc; Vancomycin (VA), 30 mcg/disc; Penicillin (P), 10 mcg/disc; and naldixic acid (NA), 30 mcg/disc. The results were compared through measuring the areas of inhibition around the antibiotic disc.

Biosynthesis of silver nanoparticles using *Cinnamon zeylanicum* plants

Preparation of C. zeylanicum bark powder (CBP)

Cinnamon bark was obtained from *C. zeylanicum* from local markets. After a washing process with sterile distilled water to remove any impurities, it was dried naturally at room temperature in the shade for a week to remove any potential moisture. The bark was then cut into small pieces and were ground into a fine powder by an electric grinder and sieved with a fine sieve to obtain the same size as the pulverized bark particles. The final powder was used for all subsequent studies (13).

A. Preparation of Cinnamon bark extract

This extract was prepared by adding 2.5g of cinnamon bark powder to 100ml distilled water in a 500 ml Erlenmeyer flask. The mixture was boiled 5 minutes at 100° C. After cooling, the mixture was filtered Whatman No. 1 filter paper and kept in the refrigerator until be used (14).

B. Preparation of 1mM of silver nitrate

It was prepared by adding 0.0421g of AgNO₃ to 100 ml of double distilled water in a clean and sterile flask, and then the contents were mixed well stored in an opaque bottle to prevent silver auto-oxidation (15).

C. Synthesis of silver nanoparticles by using *Cinnamon zeylanicum* bark extract:

One mL of cinnamon bark extract was added to an amber glass vial containing 50mL of a 1mM aqueous silver nitrate solution (AgNO₃). The mixture was incubated at room temperature with continuous shaking stirring for 18hours to produce silver nanoparticles (16).

Characterization of AgNPs

The important characteristics of Ag-NPS have been identified to ensure the formation of silver nanoparticles, including the presence of a surface specific plasmon resonance peak, the size and shape of the particles, and the effective groups arranged by particles using the following assays (13, 16).

Spectrophotometry

Used to determine the surface specific plasmon resonance peak. It was performed by transferring 2 ml of prepared Ag-NPs to a quartz cuvette. After filtering and shaking, the absorbance was recorded at a wavelength ranging between (200-800) nanometers after calibrating the device. The absorbance of extract was also measured for the purpose of comparison with the absorbance of the nanoscale solution using a Genesys 6 spectrophotometer (Thermo Electron Corporation, USA).

Transmission electron microscopy (TEM)

A few drops of AgNPs were placed on a carbon-coated copper plate or mesh with the excess solution removed with blotting paper. The grid was allowed to dry for 2minutes, and the pattern was examined. The average size and shape of the AgNPs were evaluated at a magnification force of 130,000× and 80kV.

Fourier transmission infrared spectroscopy (FTIR)

To determine the protein functional groups associated with Ag-NPs, the sample was examined with FTIR ranging from 400to 4000cm⁻¹ and with a resolution of 4cm⁻¹, using a spectrometer. For this assay, the dried nano powder was mixed with potassium bromide in a ratio of 1: 100 and placed in a special mold in the form of a disc which was placed in the device and the sample was examined.

Antibacterial activity of silver nanoparticles

The inhibitory activity was determined by well diffusion test method. A sterile cork borer was used to make wells on a pre-prepared Mueller Hinton agar with a diameter of 6 mm and of equal dimensions. A some of 100 µl of the bacterial suspension (1 x 10⁸) was spread on the surface of plate, 100 µl of the different concentrations of Ag-NPs (100,75,50,25, 10%) were transferred to those well. One well was assigned as control in which, 100 µl of sterile distilled water were poured, while the bark extract was placed in the other wells. The plates incubated at 37°C for 18 hours, after which the inhibition zone was measured (17).

RESULTS

The color change of the mixture

The synthesis was initially confirmed in this study, when the extract was prepared under sterile conditions and used to test its ability to reduce silver

Ag ions by adding 1 mL of fresh extract to 50 mL of AgNO₃ at a concentration of 1 mM, and a color change of the reaction mixture was observed from colorless to gradually dark brown after 18 hours as shown in fig. 1.

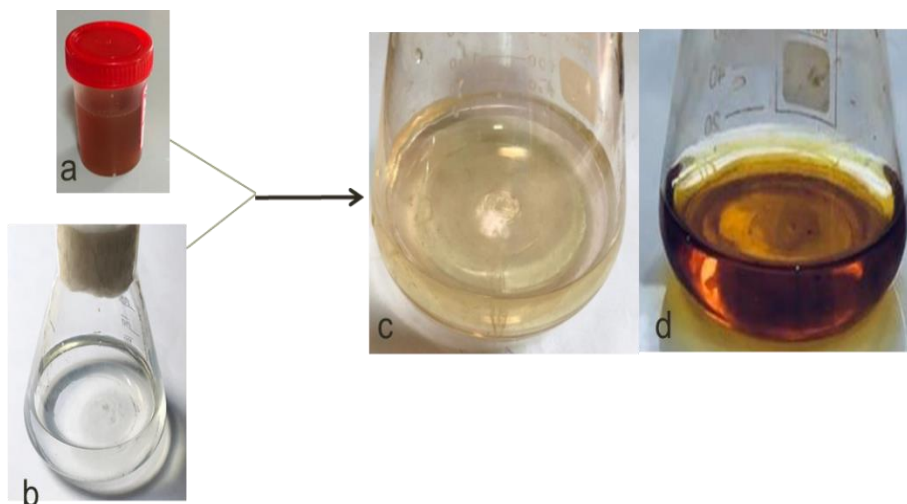


Fig. 1: Change in color after the reduction of Ag⁺ to silver nanoparticles by *Cinnamon zeylanicum* plants barks extracts 1ml (CBPE) /50 ml (AgNO₃) (a) *Cinnamon zeylanicum* plants barks extracts, (b) 1mM aqueous silver nitrate solution (AgNO₃), (c) silver nanoparticles *Cinnamon* extracts before incubation, (d) silver nanoparticles *Cinnamon* extracts after 18 h incubation.

Characterization of nanoparticles

Spectrophotometry

The reduction of silver ions to nanoparticles was primarily monitored using a spectrophotometer. The

results of the nanoscale extract showed an absorption band at a wavelength of 435 nm, which represents the absorption peak of the silver nanoparticles, and as in Fig.2, The absorption spectrum was almost bell-shaped and was an indicator of silver ions reduction.

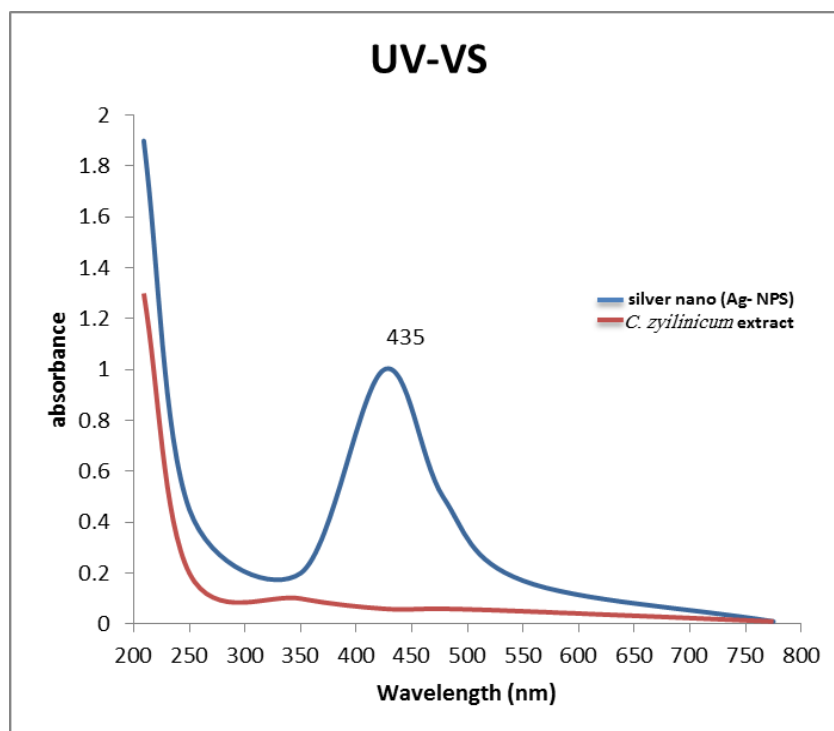


Fig. 2: Surface plasmon resonance peak of silver nanoparticles at 435 nm (UV-Visible absorption spectra of synthesized silver nanoparticles).

TEM

Fig. 3 shows the result of TEM examination of the silver nanoparticles that measured the sizes of their manufactured particles. It was observed that the

nanoparticles on the surface were almost spherical in shape and in gathered in clusters with sizes ranging from (7.55- 24.4) nm.

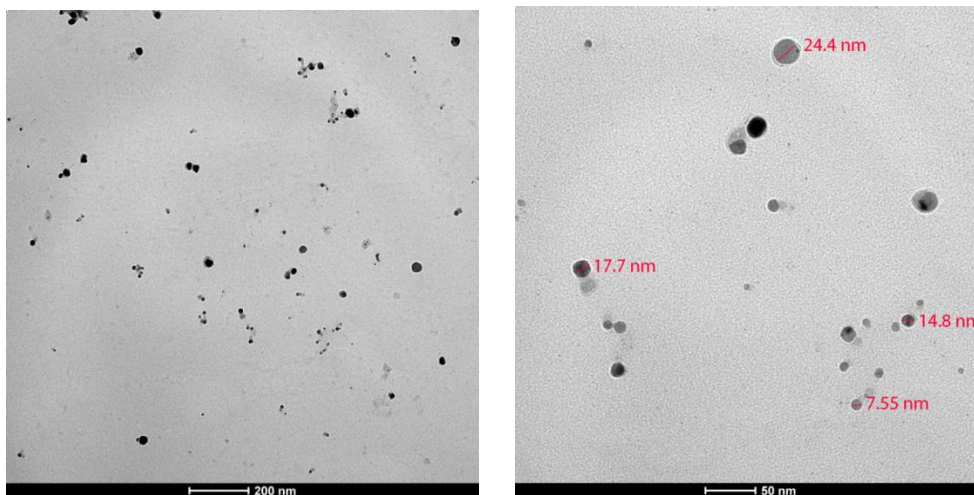


Fig. 3: Transmission electron microscopic (TEM) images of the biosynthesized Ag-NPs.

FTIR

Figure (4) shows the beams that appeared, which were between 3335-523cm-1. These beams can

represent several functional groups present in proteins that play a role in the reduction and stability of silver nanoparticles.



Fig. 4: FTIR analysis of biosynthesized Ag-NPs, *C. zylanicum*.

Antibacterial effect of silver nanoparticles

The isolated bacteria showed 100% resistance to the antibiotics used in this study. These isolates were tested for different concentrations of silver

nanoparticles, and the results showed an increase in the inhibitory activity in a concentration-dependent manner (table, figure 5).

Table 1: The antimicrobial activity (inhibition zone in mm) of the biosynthesized Ag-NPs against different MDR gram-negative bacterial strains

Bacteria	Ag NP Concentration				Antibiotics				
	25	50	75	100	TE	E	VA	NA	P
<i>Citrobacter freundii</i>	14	16	17	19	R	R	R	R	R
<i>Klebsiella pneumoniae</i>	17	19	21	23	R	R	R	R	R
<i>E. coli</i>	15	16	18	23	R	R	R	R	R
<i>Enterobacter spp.</i>	15	16	18	20	R	R	R	R	R
<i>Acinetobacter baumannii</i>	16	18	19.5	20	R	R	R	R	R

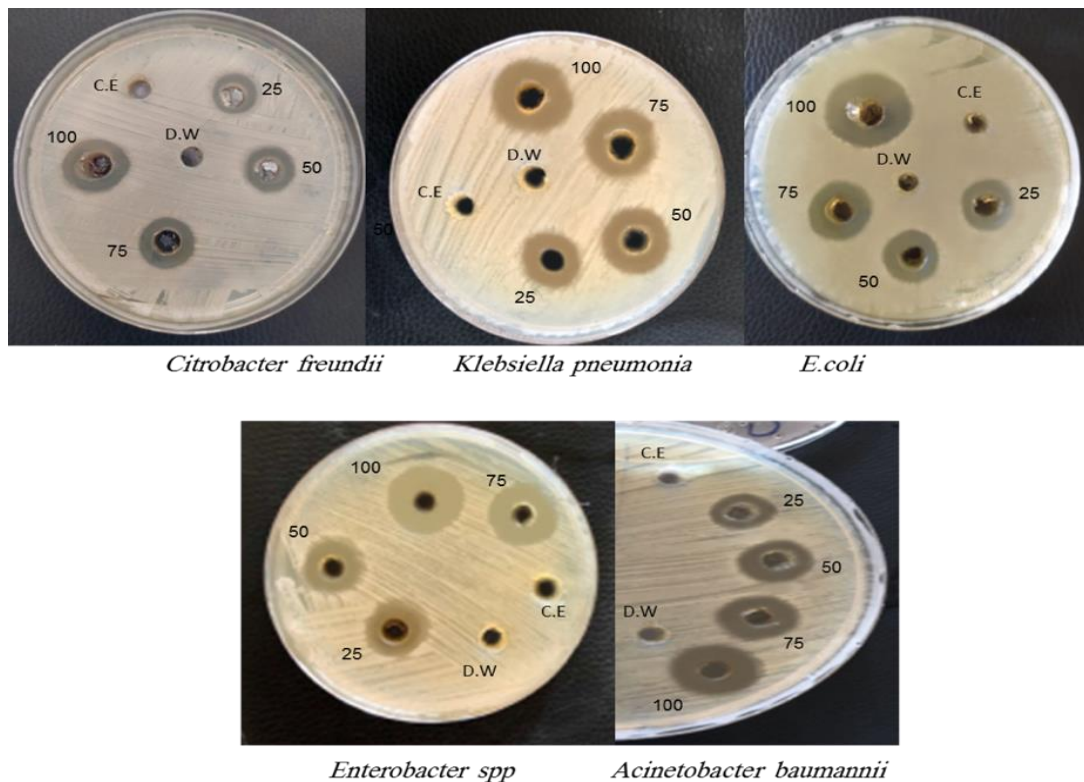


Fig. 5: Antimicrobial activity (inhibition zone in mm) of the biosynthesized AgNPs (100,75,50,25). D.W=distal water, C. E.= *Cinnamon zeylanicum* extract, against different MDR gram negative bacterial strains.

DISCUSSION

In the current study, we applied this new strategy to biosynthesis NPs by using an aqueous extract of cinnamon bark under sterile conditions. The production of silver nanoparticles was primarily monitored through the color change and ultraviolet spectrum of the reaction mixture from silver nitrate and cinnamon bark extract. It was proven through the color changes of the reaction mixture from colorless to yellow after two hours of incubation by shaking at room temperature and then gradually turning brown to become more intense and due to the reduction of silver ions into silver nanoparticles the solution color is dark brown. This result is in line with (18) who noticed that when adding the extract under investigation to the aqueous solution of AgNO₃, its color changed from colorless to brown, and the intensity of the color increased with the increased reaction time. It also agreed with (19) during the synthesis of silver nanoparticles from cinnamon bark where it was observed that the reaction mixture of silver nitrate and cinnamon extract changed to the brown color increased after that to dark brown.

This change of color refers primarily to the synthesis of silver nanoparticles, by the reduction of silver metal ions into silver nanoparticles and by the presence in the plant extract of the active particle, this color arises due to excitation of surface plasmon resonance (SPR) in the Ag-NPs. The active molecules on surface play a major role in the reduction and stabilization of the silver nanoparticles (20).

UV absorbance spectrum analysis

The ultraviolet absorption spectrum of Ag-NPs shown in the figure2 was determined by a single sharp beam at 435 nm, which is close to (19) who recorded the reading of the UV-Vis spectrum of the nanoparticle’s synthesis from the cinnamon bark through formation of the surface plasmon resonance band at 450 nm. It also agrees with (13) who noticed that the absorption beak of nanoparticles synthesis from the cinnamon barks was recorded at 435 nm and 429 nm, respectively.

TEM

The current results of TEM which determined the size of silver nanoparticles (7.55- 24.4) nm agree with the study of (18) who reported the size silver nanoparticles synthesis from bark cinnamon between 8-20 nanometers and were found to be spherical shape with smooth edges and the largest size of some of them was 60nm. Almalah (21) found that the silver nanoparticles synthesized from cinnamon bark have a size ranged from 10 to 78.9 nm.

FTIR

The present study revealed several bands from FTIR technique. The 523bands and the 1523cm-1 indicate the presence of the carbonyl group C = O, while the band 1626cm-1indicates the presence of the NH 2 it is known as an amide band, while the bands 2939and 1404cm-1 indicate the presence of the C-H and C-H 2 groups. Respectively.

The result is in accordance with Bright *et al.*, (15) detected intense bands between 514.99 to 3637.75

cm-1 which included different absorption peaks and appeared that these groups act as a reducing agent and have an important role in the stability of the Ag-NPs through the amine and carboxyl groups.

The inhibitory effect

The current results showed that the Ag-NPs had inhibitory effect against the bacterial species using different concentrations and that this inhibitory is had concentration-dependent manner compared with aqueous extract of the bark and sterile distilled water as the control sample, which did not show any inhibition effect.

These results are in line with the results of several previous studies. Almalah *et al.*, (21) noted that silver nanoparticles synthesized from the bark had an inhibition zone of 22 mm against *A. baumannii*, 24 mm against *P. aeruginosa* and 24 mm against *Klebsiella pneumonia*.

Kumaravell and Srinivasan (19) found that the inhibitory effect of nano-silver was 17 mm against *E. coli* which are similar results to our current study. More recently, Saleh *et al.*,⁽¹⁵⁾ displayed an inhibition zone for Ag-NPs of *Proteus vulgaris* 20, *Escherichia coli* 17, *Klebsiella pneumonia* 23 and *Bacillus sp* 15 mm. It was found that the inhibitory effect of silver nanoparticles determined 16mm to *Bacillus cereus* and *Citrobacter sp* 18mm (22).

Many explanations have been proposed for the effect of Ag-NPs on bacteria. It is known that the Gram-negative bacteria have the outer wall with a negative charge due to the carboxyl groups. As Ag-NPs are positively charged, it can be attached to the cell surface, which leads to its aggregate on the membrane, and modification in the chemical and physical properties of the bacterial cell wall, causing damage to it. Moreover, it is believed that Ag-NPs binds to proteins of cellular membrane and damaged them. The increase in the permeability of the membrane leads to more pronounced effects such as the loss of cellular contents, including ions, proteins and (ATP), which leads to their death (23).

In addition, Ag-NPs can also stop the process of replication of the DNA of the bacteria by preferentially attaching these nanoparticles to the nitrogenous bases of the DNA rather than the phosphate groups, so that the DNA loses the ability to replicate and express (24). There are many local articles refers to down regulation of some virulence factors caused by direct impact of Ag Nanoparticles and TiO₂ Nanoparticles against pathogenic bacteria (25).

CONCLUSION

The Ag-NPs can be successfully prepared form Cinnamomum bark plant. Besides their low cost, these NPs had an antibacterial effect especially against Gram negative bacteria which had a complete

resistance to the most common antibiotics. The antibacterial effect of the prepared Ag-NPs is concentration dependent where the higher concentration was more effective than the lower ones. Further *in vivo* studies addressing the possible side effect of these NPs are required before they can be used clinically.

CONFLICT OF INTEREST

Authors declare no conflict of interest

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