

Research Article

Green Synthesis of Silver Nanoparticles using *Cinnamomum Zylanicum* and their Synergistic Effect against Multi-Drug Resistance Bacteria

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Abstract

Multidrug - resistant (MDR) bacteria are considered life-threatening and need fast identification and antibiotic sensitivity testing to overcome this problem. In the current article, we highlighting the potential of developing silver Nanoparticles (AgNPs) using *Cinnamomum zylanicum* bark extracts as a promising approach. UV, SEM, TEM and FT-IR analysis were carried out to characterize the biosynthesized AgNPs. UV-visible spectroscopy showed the presence of characterized peak at (420 nm), TEM showed spherical shaped and monodispersed nanoparticles of size range 10 to 78.9 nm and FT-IR spectrum confirmed the presence of various functional groups in the biomolecules which serve as a capping agent for the nanoparticles. Biosynthesized (AgNPs) have been evaluated as an antibacterial against MDR gram-negative bacteria *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* strains and gram-positive bacteria *Staphylococcus aureus*. The results showed that obtained silver Nanoparticles are efficient in inhibiting both gram-positive and gram-negative bacteria when compared with antibiotic giving a zone of inhibition of 25 mm against *S. aureus*, 24 mm against *K. pneumoniae*, and *P. aeruginosa* and 22 mm against *A. baumannii* respectively. Furthermore, the effectiveness of AgNPs against these test strains was assessed with multiple broad spectrum antibiotics. The results demonstrated that the incorporation of antibiotics with AgNPs has amazing antibacterial effects. The highest extent was observed with gentamycin against *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*, respectively. The minimum inhibitory concentrations (MICs) of AgNPs were also determined using microdilution assay. This study gives encouragement that AgNPs can be used to improve the effectiveness of the current antibiotics against MDR bacteria.

Keywords: AgNPs; *Cinnamomum zylanicum*; UV-Visible spectroscopy; TEM; FT-IR; MDR bacteria; MICs

1. Introduction

Nanotechnology is the potential future solution for the elimination of antibiotic-resistant microbes, which can stimulate the creation and synthesis of a new generation of antibiotics [1]. Nanotechnology has an important role in promoting the treatment of highly virulent bacterial diseases, especially those that resistant to antibiotics and are difficult to control with traditional treatments [2-6]. The antibiotic resistant bacteria has increased rapidly in the last few years, and become a growing problem that has impacted the world and brought about the beginning of the end for the old generation of antibiotics [7]. The miss-use of traditional antibiotics to combat bacterial infection has led to the current and major challenge of resistant bacteria, especially the multi-resistant bacteria (MDR) such as Methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumonia*, and *Neisseria meningitides* [8]. It is known that silver is capable of killing 650 microbial germs that harm the human body [9]. Extensive studies have been carried out in evaluating antibacterial potentials of Nano particles. The antibacterial effects exhibited by NPs accentuate the potential of their advancement into future generation of antimicrobial agents [10]. NPs derived from plants that manifest promising antibacterial activities have high potential to be developed into future antibacterials mainly due to their low toxic effects [11].

NPs have been previously reported to inhibit gram-positive bacteria such as *Staphylococcus spp* [11, 12] *Streptococcus spp.* [13], and *Bacillus spp* [14, 15], and gram-negative bacteria such as *Escherichia spp* [16], *Pseudomonas spp* [17], *Salmonella spp* [18], *Shigella spp* [17, 21], *Proteus spp.* [21, 22], and *Vibrio spp.* [21, 23]. More promising, NPs have also shown potential to inhibit antibiotic-resistant bacteria such as Methicillin-resistant *S. aureus* (MRSA) [24, 25] and drug-resistant *E. coli* [26]. Previous studies have also manifested the diverse mechanisms for the silver Nanoparticles' bactericidal effect. Besides that AgNPs interact with the surface of the membrane, they can also penetrate into the bacterial cell membrane [27]. Moreover, silver Nanoparticles can attach to the DNA inside the bacterial cells, suppressing its replication or interaction with the bacterial ribosome [28]. It has been observed that silver Nanoparticles have the ability to damage the structure of the bacterial cell membranous enzymes, which cause bacterial death eventually [29]. The goal of this study is to develop new therapeutic antibacterial drug using silver Nanoparticles to combat the growing threat of antibiotic-resistant bacteria.

2. Materials and Methods

2.1 Bacterial identification

Nutrient broth and Nutrient agar, Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), silver nitrate, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The *Staphylococcus aureus*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* mutants strains used in the present study were collected from King AbdulAziz hospital, Mekkah, KSA. All four strains were routinely grown in nutrient agar at 37°C. The characterization of bacteria was performed as outlined by Kalimuthu et al. [30]. The morphological characterization was carried out according to the methods indicated in Bergey's Manual. Molecular characterization was conducted using the 16s rRNA technique. The nucleotide sequences of resistant strains of *P. aeruginosa* and *K. pneumoni* have been submitted to GenBank under accession no. MN121254, MN121255, MN121256, and MN121250 respectively.

2.2 Preparation of aqueous extract of *C. zylanicum*

10 gm of Barks of *C. zylanicum* were used for biosynthesis of the AgNPs due to its cost effectiveness, ease of availability and medicinal properties. *C. zylanicum* was homogenized into fine powder and boiled with 100 mL distilled water for 30 min at 60°C and the extract was obtained after filtration through Whatman No. 1 filter paper and was used for biosynthesis experiments [31].

2.3 Biosynthesis of AgNPs using *C. zylanicum* aqueous extracts

10 mL of aqueous extract of *C. zylanicum* was mixed with 1 mM of silver nitrate solution (100 mL) in the dark condition for 24 h. The synthesis of silver nanoparticles was done at 60°C by adding the aqueous extract of *C. zylanicum* drop by drop on magnetic stirrer. A change in the color of the solution from light yellow to dark brown indicated the synthesis of AgNPs. The obtained yield of AgNPs was calculated according to Kalishwaralal et al. [32].

2.4 Characterization of synthesized silver nanoparticles

The biosynthesized AgNPs were characterized according to the method described by Gurunathan et al. [33]. UV-vis spectra were measured using a (UV-2450, Shimadzu, Tokyo, Japan). The shape and size of silver nanoparticles were determined by TEM. For TEM, a drop of aqueous silver nanoparticles sample was fixed on a carbon coated grid, and let dry in room temperature; the micrographs were acquired using TEM. The particle sizes were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Fourier transform infrared spectroscopy (FT-IR) (Thermo Scientific Smart iTR™) was used to characterize the changes and the composition on the surface of the synthesized nanoparticles.

2.5 Determination of the minimum inhibitory concentrations (MICs) of AgNPs

Two-fold broth microdilution of AgNPs and different antibiotics were carried out in 96-well microtiter plates using MHB according to the Clinical and Laboratory Standards Institute (CLSI 2005). To examine AgNP MICs, *S. aureus*, *A. baumannii* and *K. pneumoniae*, and *P. aeruginosa* mutants strains were exposed to 0-10 µg/mL AgNPs prepared in phosphate-buffered saline (PBS). Each concentration of AgNP solution was mixed with 1 mL of the bacterial suspension in MH media until the final concentration of bacteria is $\sim 10^5$ - 10^6 (CFUs)/mL O/N. Then, 10-fold dilution has been done and 100 µL of which was cultured on MH agar media. The viability loss was estimated by colony counting method and compared with those on control plates (AgNP free MH media). All experiments were performed independently in triplicate. The MIC was defined as the minimum concentration of AgNPs that inhibit the growth of bacteria. Control tests were carried out with solutions containing all the reaction components except for AgNPs. In addition, different concentrations of antibiotics alone, AgNPs alone, and/or a combination of both were carried out in different microtitre plates under the same conditions. The optical density of each well was measured at 600 nm by using ELISA reader. All samples were repeated trice and the average values were calculated independently.

2.6 Agar diffusion assay

The agar diffusion assay was accomplished as illustrated by Shahverdi et al. The bactericidal activity of antibiotics in combination with or without AgNPs was tested against bacterial strains on Mueller Hinton Agar plates. Broad-spectrum antibiotics were selected to analyze the combined effect of antibiotics and AgNPs. According to CLSI standard, antibiotics were used at appropriate concentrations as follows: amoxicillin (10 µg/mL), cefatoxime (25 µg/mL), sulbactam (15 µg/mL), gentamicin (20 µg/mL), and flucoxacin (30 µg/mL). Each filter disk was soaked with the minimum inhibitory concentration of AgNPs for each bacterial strain. A single colony of each test strain was grown overnight in MHB on shaker with (200 rpm) at 37°C. 0.5 McFarland standard was composed by diluting the overnight bacterial cultures with 0.9% NaCl and then plated on MH agar plates together with the prepared disks containing several antibiotics. Similar experiments were executed with AgNPs alone. After incubation at 37°C for 24 h, a zone of inhibition (ZOI) was measured. The assays were completed in triplicate. The increment in antibacterial activity of different antibiotics has been calculated by the formula $(B - A)/A \times 100$, where A and B are the ZOI for antibiotic and antibiotic+AgNPs, respectively [34].

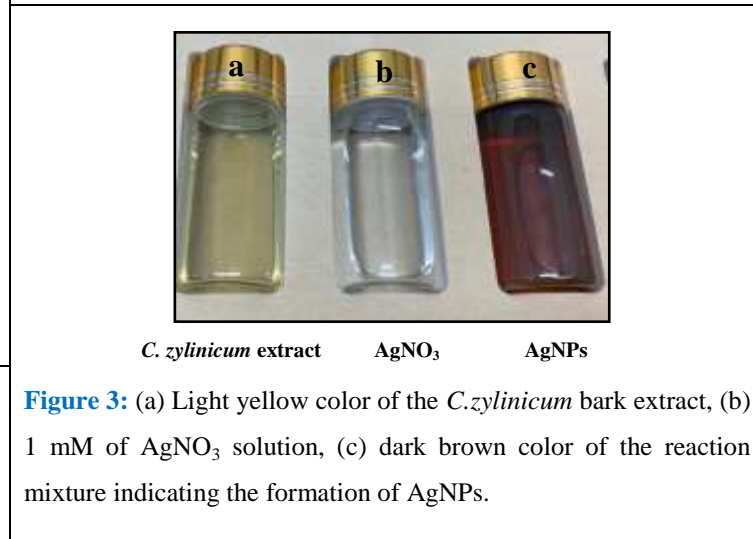
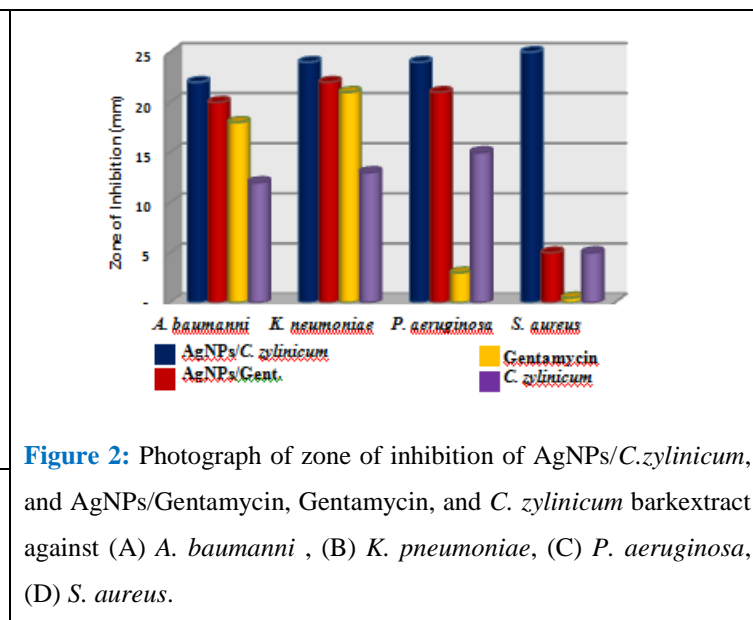
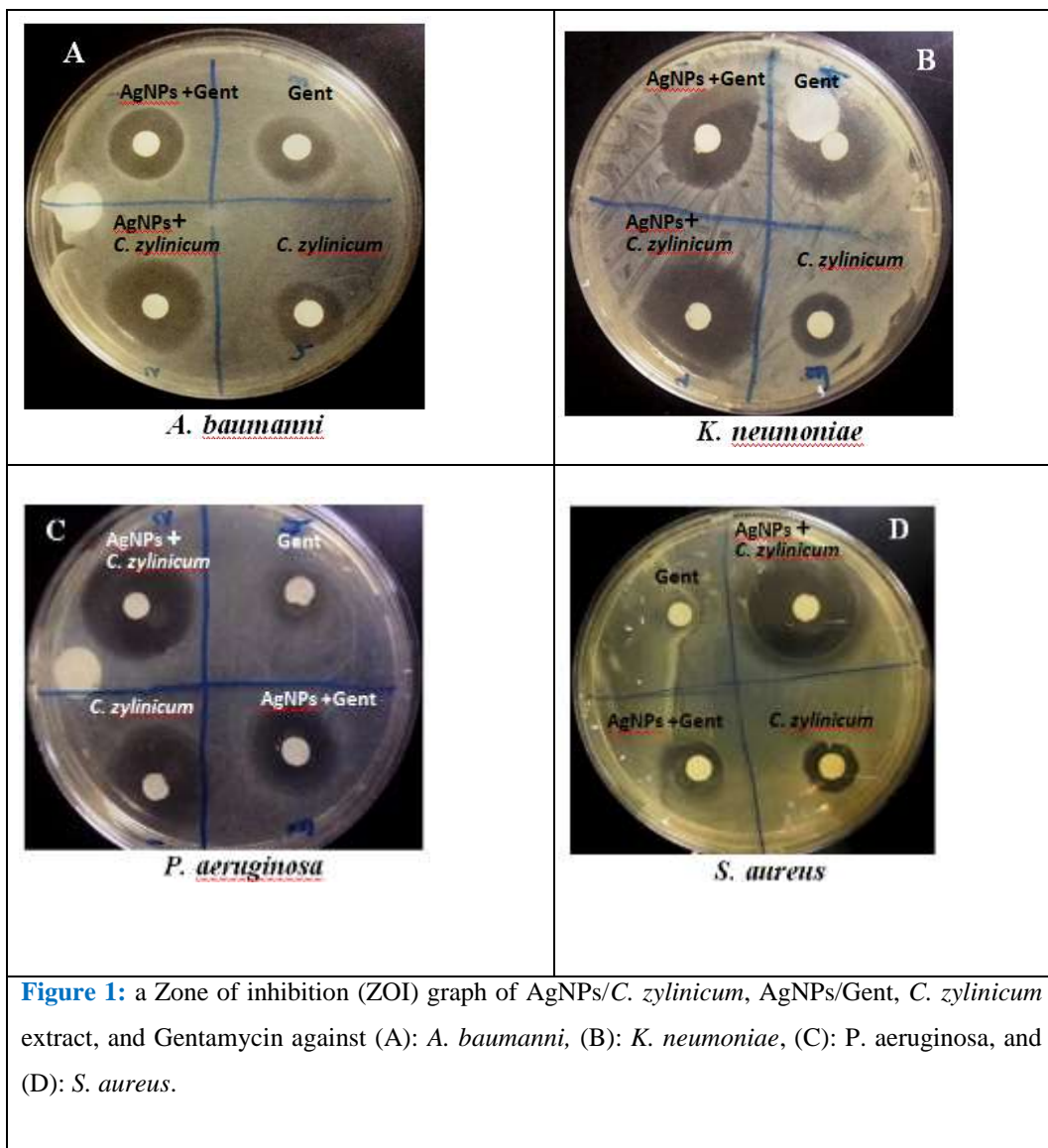
2.7 Disk diffusion assay to evaluate synergistic effect

To evaluate the synergistic effects of green synthesized silver nanoparticles with various antibiotics were studied according to [35] against MDR strain on MH agar plates using disk diffusion method. The antibiotic discs used were amoxicillin (10 µg/mL), cefatoxime (25 µg/mL), sulbactam (15 µg/mL), gentamicin (20 µg/mL), and flucoxacin (30 µg/mL). The bacterial inoculum was diluted in 5 ml of NaCl to obtain 0.5McFarland and cultured on the plate. Each disc was soaked in 5µL of different concentration (10, 20, 40, 60, 80, and 100) µg/ml of AgNPs, and then the plates were incubated at 37°C overnight. The inhibition zones were measured as described by Fayaz et al. [35] and compared with those obtained from antibiotics which tested in Antibiotic susceptibility. Statistical analysis was performed using all the assays were performed in triplicate and the results are presented as means ± SD.

3. Results and Discussion

3.1 UV-Vis spectroscopy

Addition of bark extract of *C. zylanicum* into the aqueous solution of silver nitrate produced a color change in the mixture to brown (Figure 3) duration the reaction period due to silver nanoparticles' Surface plasmon resonance (SPR) excitation [36]. When 5 ml of bark extract of *C. zylanicum* was added to 1 mM of silver nitrate solution (10 ml), the color of the solution changed from faint light to colloidal brown suggesting the formation of silver nanoparticles. UV spectra of Plasmon resonance band was observed at 420 (Figure 4) according to those reported by [37]. The UV-Visible spectra recorded after 1 h from the initiation of reaction. The results of the UV-vis spectra recorded, suggested that the bioreduction was achieved using *C. zylanicum* bark extract as reducing agent and that formation of silver nanoparticles occurred rapidly within 1h. These results were in agreement with that reported by Ahmed et al. who found that *A. indica* leaf extract was the most rapid reducing agent for silver nitrate solution and the UV-vis spectra was detected rapidly within 15 min indicating the formation of silver nanoparticles.



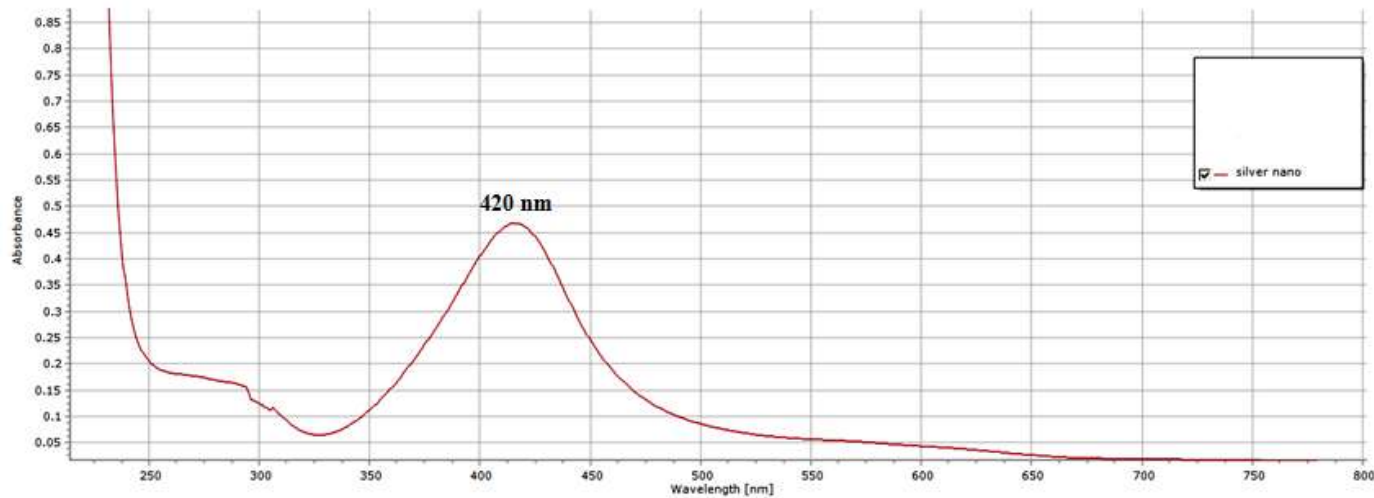


Figure 4: UV-visible absorption spectrum of biosynthesized AgNPs by the reduction of AgNO₃ solution with the *C. zylanicum* after 1h.

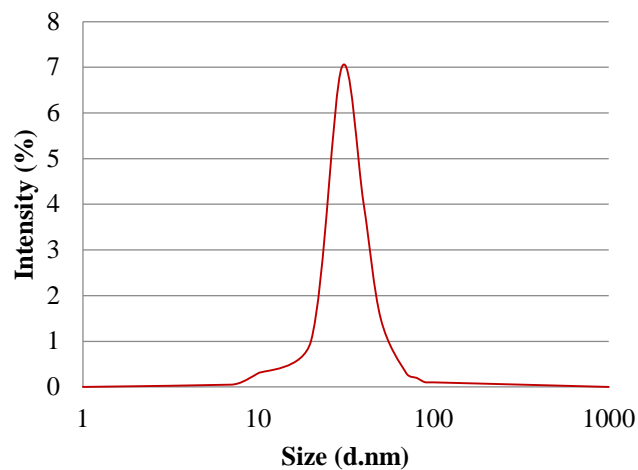


Figure 5: Graph representing the distribution particle size of biosynthesized AgNPs.

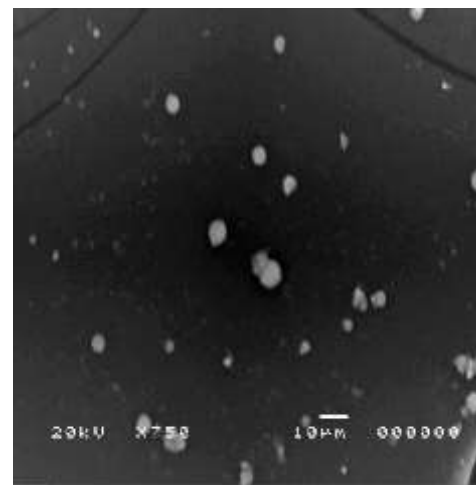


Figure 6: SEM micrograph of synthesized silver nanoparticles using *C. zylanicum* bark extract magnified 750x.

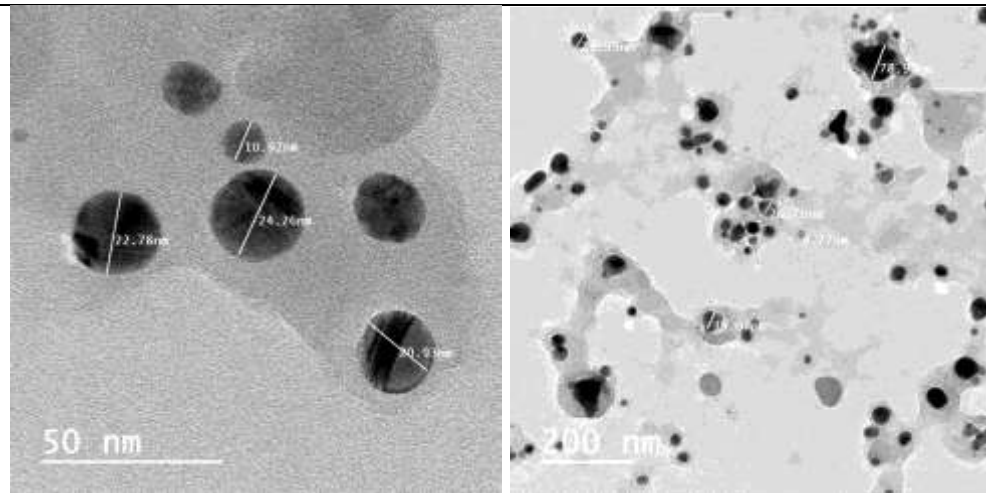


Figure 7: Transmission electron microscopic (TEM) images of the biosynthesized AgNPs.

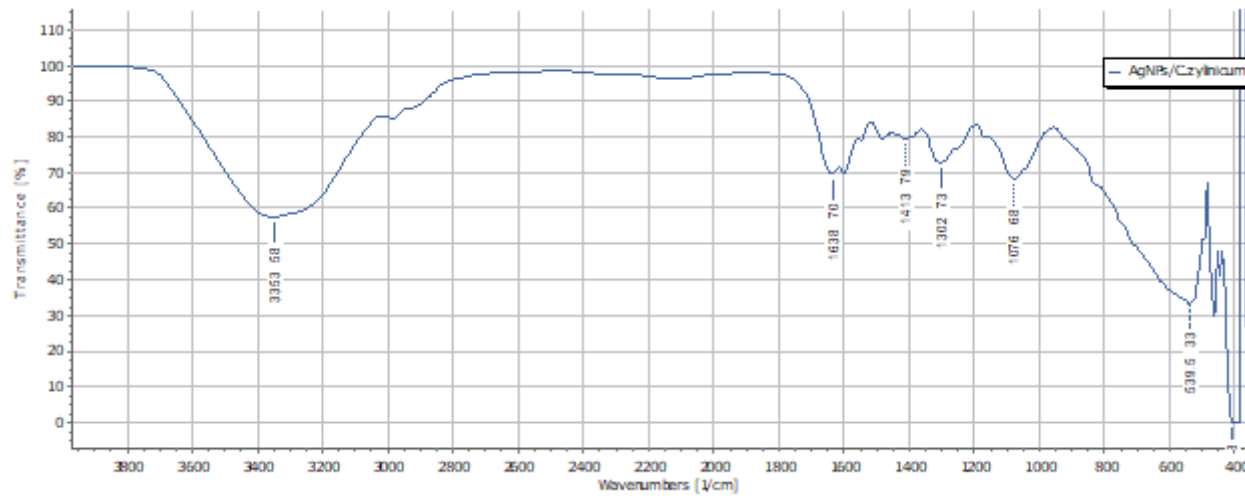


Figure 8: FTIR analysis of biosynthesized AgNPs/C.zylanicum.

Tested strains	Gentamycine (Standard antibacterial agent)	*Diameter of inhibition zone (mm) produced by AgNPs (Mean \pm SD)	*Diameter of inhibition zone (mm) produced by AgNPs + antibiotic (Mean \pm SD)	MICs of AgNPs (μ g/ml)	<i>p</i> -value
<i>Staphylococcus aureus</i>	0.46 \pm 0.06	25 \pm 0.20	4.8 \pm 0.2	4.5 \pm 0.04	0.096
<i>Acinetobacter baumannii</i>	17.3 \pm 0.48	22 \pm 0.21	19.9 \pm 0.4	5.7 \pm 0.02	0.057
<i>Pseudomonas aeruginosa</i>	2.8 \pm 0.20	24 \pm 0.14	20.8 \pm 0.3	3.1 \pm 0.01	0.072
<i>Klebsiella pneumonia</i>	20.7 \pm 0.32	24 \pm 0.19	21.5 \pm 0.6	2.8 \pm 0.03	0.01

*Three repeats were performed for each tested strain

p-value significant <0.05

p-value non-significant >0.05

AgNPs (20 μ g/ml), concentration of antibiotics (20 μ g/ml)

Table 1: The antimicrobial activity (inhibition zone in mm) and the minimum inhibitory concentration values of the biosynthesized AgNPs against different bacterial strains.

3.2 SEM, TEM and particle size

The dynamic light scattering (DLS) profile of the synthesized AgNPs using *C. zylanicum* aqueous bark extract is shown in (Figure 5). The shape of the synthesized silver nanoparticles was analyzed by SEM magnified at 750x is shown in (Figure 6). The image obtained by the SEM showed spherical nanoparticles and some particles of irregular shape. As well as, the biosynthesized silver nanoparticles were studied by TEM. The image obviously showed that particles size were in the range from 10 to 78.9 nm (Figure 7). The nanoparticles are spherical shaped and homogeneous which comply with the shape of SPR band in the UV-visible spectrum.

3.3 FTIR analysis of AgNPs

FTIR analysis of silver nanoparticles was conducted to confirm the role of the plant extract as a capping and reducing agent and existence of specific chemical groups responsible for the characteristic chemical reactions of biosynthesized AgNPs (Figure 8). The broad band at 3353 cm^{-1} is due to stretching vibration of bonded and non-bonded –O–H groups. The band at 1638 cm^{-1} are attributable to stretching vibration of carbonyl group which is characteristic for the secondary amides and other compounds containing C=O group [38]. The strong bands at 1413 cm^{-1} correspond to the bending vibrations of CH which is indicative for the lignins presence. The observed peak at 1302 denote C(O)–O stretching vibrations and –OH in plane vibrations/amide III (e.g. in aromatic ethers). The absorption bands at 1100-1000 cm^{-1} in the fingerprint region suggest several modes such as C-H deformation or C-O or C-C stretching, pertaining to carbohydrates. The C-O-C groups exhibit strong bands at 1076 cm^{-1} [39]. The

absorbance bands at 539 cm^{-1} represent C-O-O and P-O-C bending of aromatic compounds (phosphates). The phytochemical constituents of *C. zylanicum* bark extract are expected to interact with metal salts through these functional groups and facilitate their reduction to nanoparticles [40].

3.4 Antimicrobial activity of silver nanoparticles

Silver nanoparticles exhibiting antimicrobial activity against multidrug resistant bacteria, with different extents, as showed by the diameter of inhibition zone (Figure 1(A)-(B)-(C)). The Gram negative bacteria (*A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*) strains exhibited larger inhibition zones, compared with the Gram positive bacteria (*S. aureus*) (Figure 1D), that may vary because of the structure of the cell wall. The composition of Gram- positive cell wall contained a dense peptidoglycan layer composed of linear polyaccharide chains crossed through brief peptides forming a stiffer framework that makes silver nanoparticles hard to penetrate while there is a thin layer of peptidoglycan in Gram negative bacteria the cell wall [41]. The results of antibacterial activities of biosynthesized silver nanoparticles evaluated from the agar well diffusion method are given in (Figure 1). *C. zylinica* extract mediated silver nanoparticles revealed that the AgNPs showed great antibacterial activity against both gram negative and gram positive bacterial strain where the inhibition zone diameter were (25, 22, 24, and 24 mm) for *S. aureus*, *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*, respectively (Table 1). However, gentamycine and *C. zylanicum* bark extract alone showed very low antibacterial activity because of the water medium of extraction as well as lower concentration used in the experiment. Although, it is to be presumed that, the tested bacterial strains were supposed to be resistant for gentamycine, it shows very low activity against *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, while it has no activity against *S. aureus*. Based on inhibition zone assay, biosynthesized silver nanoparticles combined with gentamycine antibiotic showed a great synergistic antibacterial activity against all tested bacterial strains (Figure 1).

MIC was used to analyze the antimicrobial activity of silver nanoparticles. The technique which used to detect the MIC of silver nanoparticles is broth microdilution methods. The MIC is defined as the lowest concentration of the antimicrobial compound that inhibits the growth of a microorganism [42]. After 24 h of incubation at 37°C , no growth of *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa*, and *S. aureus* in the microtitre plate supplemented with 5.7, 2.8, 3.1 and 4.5 $\mu\text{g/ml}$ of silver nanoparticles was observed, and the optical density was 0.041, 0.017, 0.038 and 0.021, respectively. Therefore, the MICs were 5.7, 2.8, 3.1 and 4.5 $\mu\text{g/ml}$, respectively (Table 1). The biological characteristics of silver nanoparticles against microorganisms are underpinned by several mechanisms. Firstly, silver nanoparticles bind to negatively charged cell surfaces, alter cell membrane and cell wall physical and chemical characteristics and interfere with key functions such as permeability, osmoregulation, electron transport and respiration [27, 43, 44, 45]. Second, silver nanoparticles can interact with DNA, thiol group of L-cysteine protein and other phosphorus- and sulfur-containing cell constituents causing enzymatic dysfunction [43, 45, 46, 47]. Finally, the silver nanoparticles cause damage on proteins and DNA via release of reactive oxygen species (ROS) [48]. Third, Silver nanoparticles release silver ions, which generate a size and dose-dependent enhanced biocidal impact [45, 49].

4. Conclusions

Green synthesis of silver nanoparticles has a promising antibacterial action and has a great synergistic effect in enhancing the efficacy of antibiotics. There is now a huge effort in the production of extremely powerful and robust NPs for clinical use.

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