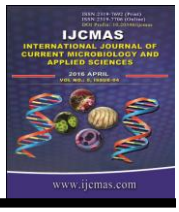
Int.J.Curr.Microbiol.App.Sci (2016) *5*(4): 226-236

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 5 Number 4 (2016) pp. 226-236**
Journal homepage: http://www.ijcmas.com



Original Research Article http://dx.doi.org/10.20546/ijcmas.2016.504.028

Antibacterial Activity of Silver Nanoparticles Synthesized by *Sidr (Ziziphus spina- Christi)* Leaf Extract against Pathogenic Bacteria

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Keywords

Silver nanoparticles; Antibacterial activity; *Ziziphus spina*-Christi; UV-Vis Spectrophotometer, FE-SEM, XRD.

Article Info

Accepted:
15 March 2016
Available Online:
10 April 2016

A B S T R A C T

This study aimed to synthesize silver nanoparticles using aqueous sidr leaves extract and to evaluate the antibacterial efficacy of sidr leaves extract, silver nitrate and silver nanoparticles against gram positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria using agar well diffusion technique. The synthesis of silver nanoparticles using sidr leaves extract was successfully carried out. UV-Vis Spectrophotometer, Field Emission Scanning Electron Microscope (FE-SEM) and X-ray diffraction (XRD) analysis confirmed synthesis of silver nanoparticles. The UV–visible spectra showed that the absorption peak was existed at 442nm. FE-SEM analysis demonstrated that the synthesized AgNPs were ellipsoidal and spherical in shape, loosely bound with no agglomeration and had an average size of 30-70 nm. The XRD results indicated that the synthesized product showed typical crystalline peaks for the face-centered cubic metallic Ag. The results showed that the water sidr leaves extract at the concentration used for synthesis of silver nanoparticles (7.5 mg/ml) did not exhibit any growth inhibitory effect against the tested microorganisms. Silver nanoparticles and silver nitrate inhibited the growth of all gram positive and gram-negative bacteria nevertheless; Silver nanoparticles had superior antibacterial activity than silver nitrate. *K. pneumoniae* was the most sensitive organism to silver nanoparticles with IZD 15.67 ±0.33. The present study showed that AgNPs might be a promising antimicrobial agent for successful treatment of bacterial infection and limitation of drug resistance.

Introduction

The field of nanotechnology is one of the most active researches nowadays in modern material science and technology. Nanoparticles are fundamental building blocks of nanotechnology.

The most important and distinct property of nanoparticles is their large surface area to volume ratio (Leela and Vivekanandan, 2008). There is a commercial demand for nanoparticles due to their wide applicability

in various areas such as electronics, catalysis, chemistry, energy and medicine (Veerasamy *et al.,* 2011). An eco-friendly green mediated synthesis of inorganic nanoparticle is a fast growing research in nanotechnology (Sathya *et al.,* 2012). The most important application of silver and AgNPs is in medical industry such as topical ointments to prevent infection against burn and open wounds (Muhammad *et al.,* 2012). The biosynthesis method employing plant extracts have drawn attention as a simple and viable alternative to chemical procedures and physical methods. Bio reduction of silver ions yields metal nanoparticles using living plants (Savithramma *et al.,* 2011). Bacterial resistance develop and spread and the effectiveness of the antibiotics is diminished. Antibiotic resistance creates a challenge for developing new drugs to overcome infections (Lia *et al.,* 2015).

This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics, including the major last-resort drugs, as the frequencies of resistance are increasing worldwide (Levy and Marshall, 2004; Mandal *et al.,* 2009). Discovery of new antibacterial agent represents a revolution in the world of antimicrobials to face increase of bacterial resistance to conventional antibiotics (Gouda and Masoud, 2014).

Plants are considered an important potential source of new antibacterial agents. *Zizyphusspina-christi* is a scientific name of a plant where its common name is Sidr, Nebeq or Nabg in Saudi Arabia. The genus Zizyphus belong to the family Rhamnaceae. The plant is mentioned in the holy Quran many times and very much used in Arab countries. Sidr plants are widely distributed in Saudi Arabia. All parts of this plant are used by local Arab people to help maintain a healthy lifestyle. *Zizyphusspina-christi* has been used in folk medicine as a demulcent, depurative, anodyne, emollient, stomachic, for toothaches, astringent and as a mouth wash (Nazif, 2002). The fruits are edible and nutritive, leaves used for chest and stomach problems, bleeding of uterus and for hair and skin care. In different folk medicine the plant is used for scorpion stings, cough, constipation, pneumonia, headache, dysentery, intestinal worms, fever, eye diseases and inflammations of throat and pharynx. Different extracts and fractions of the leaves, fruits and seeds of Zizyphus showed antiviral, antifungal and antibacterial activities (Shaht *et al.,* 2001). Silvernano particles (AgNPs) have become an attractive alternative to antibiotics due to their broad-spectrum antimicrobial activity. Synthesized silver nanoparticles from plant species are toxic to multi drug resistant microorganisms. It shows that they have great potential in biomedical applications. More over the silver nanoparticles enhance the therapeutic efficacy and strengthen the medical values of herbal plants (Savithramma *et al.,* 2011).The aim of this study was to synthesize silver nanoparticles using aqueous sidr leaves extract and to evaluate the antibacterial efficacy of sidr leaves extract, silver nitrate and silver nanoparticles against pathogenic bacteria.

Materials and Methods

Collection of Sidr Leaves

Healthy leaves of Sidr *(Ziziphusspina-Christi)* were collected from sidr trees distributed in Najran region (Figure 1a, 1b), Saudi Arabia. Najran is a region of Saudi Arabia, located in the south of the country along the border with Yemen. It has an area of 119,000 km^2. Its capital is Najran.

Preparation of Sidr Leaves Extract

Collected leaves were washed thoroughly 4 times in running tap water followed by distilled water. Leaves were shade dried at room temperature for 10 days, then powdered using kitchen blender. 750 mg ofsidr leaves powder was weighed and mixed in 100 ml of distilled water and the mixture was boiled for 5 minutes then filtered through Whattman filter paper No.1.The filtrate was collected and stored at 4˚C for further use (Figure 2a). The plant extract was prepared atMicrobiology laboratory, Community College, Najran University.

Preparation of 1 mM$AgNO_3$ Solutions

0.0424 gms$AgNO_3$(Sigma grade,USA) was dissolved in 250ml of Milli Q water and stored in an amber colored bottle to avoid auto oxidation of silver ions (Figure 2b).

Synthesis of Silver Nanoparticles

For synthesis silver nanoparticles, 10 ml of sidr leaves extract was added to 90 ml of 1 m$MAgNO_3$ solution in a 250 ml flask with vigorous shaking for the bioreduction of Ag+ ions. The color change indicated preliminary confirmation for the formation of silver nanoparticles (Figure 2C).The Mixture was incubated in the dark at 37°C for 24 hours.

Characterization and Confirmation of Silver Nanoparticles Synthesis

The characterization and confirmation of silver nanoparticles synthesis was conducted at Advanced Materials and Nano scale Research Laboratory**,** Najran University, Saudi Arabia.

UV-Vis Spectrophotometer

UV–vis spectral analysis was conducted using a Perkin Elmer-Lambda 950 – UV-visiblespectrometer. UV–vis spectra of AgNPs and Ag ions were recorded in the range from 200 to 800 nm.

FE-SEM Analysis of Silver Nanoparticles

Field Emission Scanning Electron Microscope, FE-SEM (JSM-7600F-JEOL) was usedto examine the morphology of synthesized silver nanoparticles.

X-ray Diffraction

XRD patterns were taken on a Bruker AXS D4 Endeavour X diffractometerusing Cu $K\alpha_{1/2}$, $\lambda\alpha_1$=154.060 pm, $\lambda\alpha_2$=154.439 pm radiation.

Source of Bacterial Isolates and Culture Media

The bacterial isolates used in this study were *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. All the isolates were obtained from the Microbiology laboratory, King Khalid Hospital, Najran region, Saudi Arabia. The organisms were identified by an automated system (Micro Scan Walkaway, Siemens) and the results were confirmed (Koneman *et al.,* 1992).The isolates were subcultured on appropriate agar plates 24 hours before any antibacterial test. Brain Heart Infusion broth (Oxoid, England), Mueller Hintonagar (Oxoid, England) and Nutrient agar media (Oxoid, England) were used in this investigation.

Antibacterial Activity

The growth inhibitory effect of sidr leavesextract,1mM silver nitrate and synthesized silver nanoparticles was determined by the agar well diffusion method according to NCCLS (1993). The

bacterial cultures were grown in Brain Heart Infusion broth for 4 hours at 37 °C. Each microorganism, at a concentration of 1.5 × 10^6 cells/ml (matched to 0.5 McFarland turbidity standards) was streaked over the surface of Mueller Hinton agar plates by using swab. The inoculum was allowed to dry at room temperature, the medium was punched with six millimeters diameter wells. The growth inhibitory effect was checked by introducing 50 µl of sidr leaves extract, 1 mM silver nitrate and synthesized silver nanoparticles into the wells and allowed to diffuse at room temperature for 20 minutes. The plates were incubated at 37 °C for 24 hours and inhibition zones diameter (IZD) were measured (mm). Tests were done in triplicate and the growth inhibitory effect was recorded. Amikacin (Oxoid) antibiotic disc (30 µg) was used as a positive control.

Statistical Analysis

The results of antibacterial activity for Sidr leaves extract, silver nitrate and silver nanoparticles against tested bacteriawere expressed as means ± S.E. (Standard Error) and differences between means were analyzed statistically by analysis of variance (ANOVA) according to Tukey's HSD test via Statistical Package for the Social Sciences(SPSS) 15.0 software package in Microsoft Windows 7.0 operating system. Differences are considered significant when $p \leq 0.05$.

Results and Discussion

Synthesis of Silver Nanoparticles using Sidr Leaf Extract

The synthesis of silver nanoparticles is an advanced technique in modern nano biotechnology and is evolving as an important branch of nanotechnology (Koyyati *et al.,* 2014).In this study, the synthesis of silver nanoparticles using sidr leaves extract was successfully carried out, as the characteristic reddish brown color of the mixture was observed within 3 minutes after addition of 10 ml of water sidr leaves extract into 90 ml of 1mM silver nitrate with vigorous shaking (Figure2c). Formation of reddish brown coloration indicated the ability of sidr leaves extract for biochemical reduction and capping of silver nitrate and synthesis of silver nanoparticles due to excitation of surface plasmonvibrations. The reduction rate was increased by incubation the mixture at37 °C for 24 hours. These findings were supported by the results previously recorded (Lalitha *et al.,* 2013; Reiad *et al.,* 2013; Satyavani *et al.,* 2013; Muniyappanand Nagarajan, 2014; Sundararajan and Kumari, 2014; Ibrahim, 2015).

UV-Vis Spectrophotometer Analysis

The reduction process was further monitored via the measurement of the UV–visible spectra. Figure3 shows the UV-visible absorption spectra recorded for Ag ions and AgNPs. A broad surface plasmon resonance (SPR) absorption peak for silver nanoparticles was noticed at wavelength around 442 nm, owning to the dipole resonance of conducting electrons on the surface of AgNPs. The inset UV-vis spectrum was related to the Ag ions before the bio-reduction event, in which an absorption band appeared at 290 nm.The appearance of the above SPR peak at 420 nm, along with the absence of 290 nm absorption band is an indication of the successful synthesis of AgNPs under the current experimental conditions. Such a characteristic SPR peak for AgNPs has been reported to predominantly appear in the range of 400-500 nm (Kaur *et al.,* 2012; Venugopal and Mitra, 2013; Judita *et al.,* 2014; Salem *et al.,* 2014; Ajitha *et al.,* 2015). Additionally, a similar absorption peak for AgNPs synthesized using a coffee

extract is recently reported (Dhand *et al.*, 2016).

FE-SEM Analysis of Silver Nanoparticles

Field Emission Scanning Electron Microscope as a powerful tool used for investigation the distribution and morphology of the synthesized silver nanoparticles. Fig.4 shows the FE-SEM image of synthesized AgNPs. The shapes of AgNPs proved to be ellipsoidal and spherical. Owning to the sonication treatment applied during the separation of AgNPs from the liquor, the SEM micrograph revealed loosely bound particles with almost no agglomerates. SEM analysis demonstrated that the particle sizes located in the range of 30–70 nm. Similar phenomenon has been reported in previous studies (Sukirtha *et al.*, 2011; Jeeva *et al.*, 2014; Koyyati *et al.*, 2014).

X-ray Diffraction

The XRD pattern was depicted in Fig.5 for the as-synthesized AgNPs. The results indicated that the synthesized product showed typical crystalline peaks for the face-centered cubic metallic Ag (JCPDS no.04-0783); at 2θ = 38.28, 44.48, 64.68 and 77.22 corresponding, respectively to (111), (200), (220) and (311) facets of Ag. The appearance of the Ag-related peaks in the diffraction pattern clearly indicated the formation of crystalline AgNPs. Unidentified peaks were showed in the XRD studies. Appearance of these peaks may be attributed to phytochemical compounds present in sidr leaves extract. The present results were consistent with the findings previously observed by (Rajakumar and Abdul Rahuman,2011; Bhuvaneswaria *et al.*, 2014; Latha *et al.*, 2015; Remya *et al.*, 2015).

Antibacterial Activity

The growth inhibitory effect of sidr leaves extract, 1mM silver nitrate and silver nanoparticles against bacteria were presented in Table1.The results showed that thewater extract of sidr leaves at the concentration used for synthesis of silver nanoparticles (7.5mg/ml) did not exhibit any growth inhibitory effect against all the tested microorganisms with IZDs 00.00±0.00. These results may be attributed to use a low concentration of aqueous sidr leaves extract. El-Kamali and Mahjoub (2009) stated that aqueous extract of *Ziziphus spina-christi* at a concentration of 100 mg/ml had antibacterial activity against selected bacteria.1 mM$AgNO_3$ solution inhibited the growth of tested bacteria and *Strept. Pyogenes* was the most sensitive organism with IZD 12.67±0.33. This result was consistent with the results previously cited by (Najah, 2012).

Table.1 The Growth Inhibitory Effect of Sidr Leaves Extract, Silver Nitrate, Silver Nanoparticles and Amikacin (30 µg) against Bacteria (all values in mm)

	Bacteria	Sidr extract	$AgNO_3$	AgNPs	Amikacin(30 µg)
1	*S.aureus*	00.00±0.00	11.00±0.00^{ab}	14.00 ±0.00^{ab}	24.00 ±0.00
2	*Strept. Pyogenes*	00.00±0.00	12.67 ±0.33^{c}	14.33 ±0.33^{b}	29.00 ±0.00
3	*E. coli*	00.00±0.00	11.33 ±0.33^{ab}	13.00 ±0.00^{a}	23.00 ±0.00
4	*P. aeruginosa*	00.00±0.00	10.33 ±0.33^{a}	13.33 ±0.33^{ab}	23.00 ±0.00
5	*K. pneumonia*	00.00±0.00	12.00 ±0.00^{bc}	15.67 ±0.33^{c}	22.00 ±0.00
F- value		-	12.167	16.167	-

-Values are the mean of three replicates ± S.E. - In the same column, means followed by the same letters are not significantly different ($p \leq 0.05$) as analyzed by Tukey's HSD test. F-value is significant at $p \leq 0.001$

Fig. 1: (a) sidr tree; (b) sidr leaves

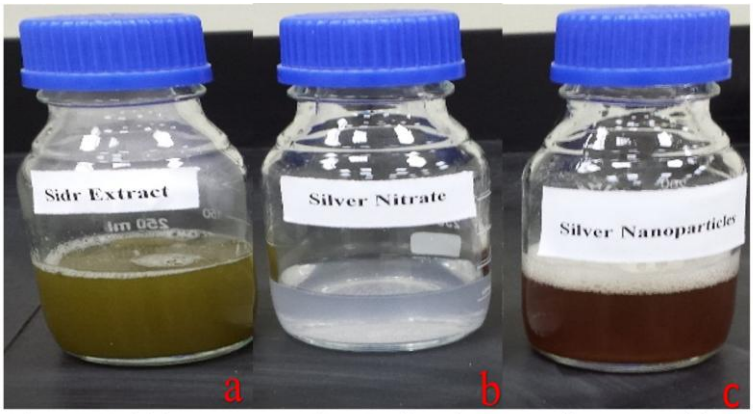

Figure 2:(a) sidr leaves extract; (b) 1mM silver nitrate; (c) silver nanoparticles

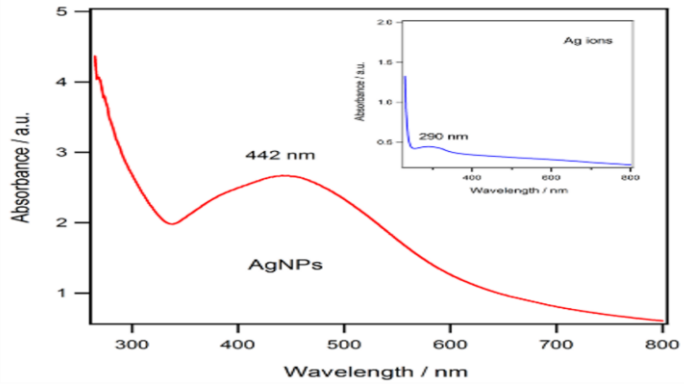

Figure 3: UV-vis spectra of AgNPs synthesized by sidr leaves extract. The inset shows the absorbance spectrum measured for Ag ions before the bio-reduction process.

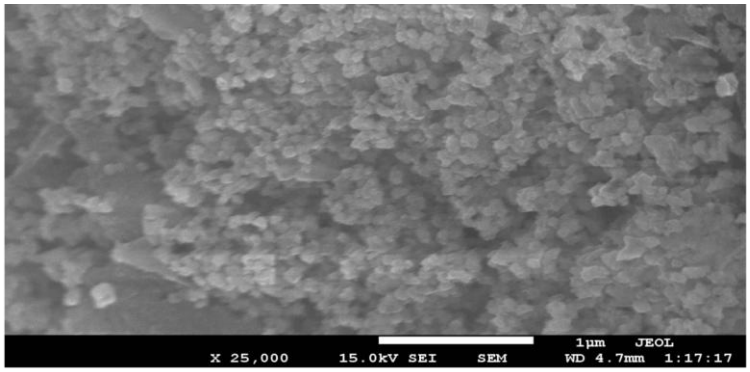

Figure 4: FE-SEM image of silver nanoparticles synthesized by sidr leaves extract.

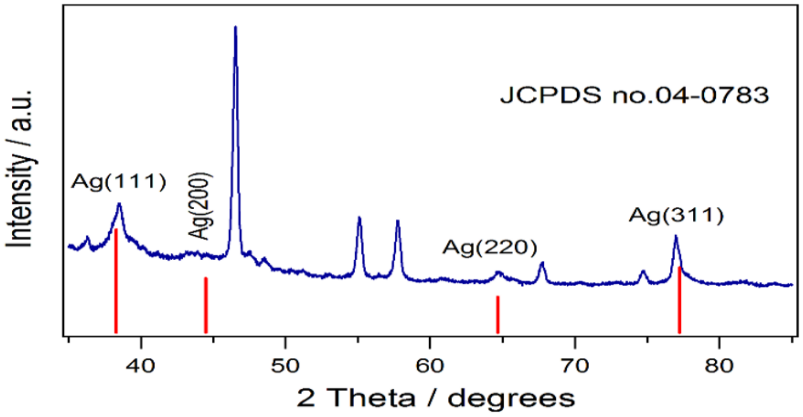

Figure 5: XRD pattern of as-synthesized AgNPs

In the present investigation, silver nanoparticles inhibited the growth of all gram positive and gram-negative bacteria and exerted the highest growth inhibitory effects compared to sidr leaves extract and 1 mM silver nitrate. *K. pneumoniae* was the most sensitive microorganism to silver nanoparticles with IZD 15.67 ±0.33. Our findings agree with other observations (Okafor *et al.,* 2013; Prasad and Swamy, 2013). Silver nanoparticles exert efficient growth inhibitory effect due to their extremely large surface area, which provides better contact with microorganisms. The nanoparticles release silver ions in the bacterial cells, which enhance their antibacterial activity (Morones *et al.,* 2005; Muthukrishnan *et al.,* 2015). Bactericidal activity of silver nanoparticles are influenced by the particles size, the smaller the particles, the greater the antibacterial efficacy. This suggests that the AgNPs may penetrate inside the bacteria causing damage by interacting with electron phosphorous and sulphur containing compounds such as DNA (Baker *et al.,* 2005).The synthesized silver nanoparticles exhibited significant antibacterial effect on both Gram classes of bacteria (Kora *et al.,* 2010; Rhim *et al.,* 2013). Inhibition zones of Amikacin antibiotic discs were in between 29.00 ±0.00 mm for *Strept. Pyogenes* and 22.00 ±0.00 mm for *K. pneumoniae*.The statistical analysis revealed that there were significant differences in the values ($P \leq 0.05$) for silver nitrate and silver nanoparticles among the investigated organisms. No significant differences in the values ($P \leq 0.05$) were observed for sidr leaves extract and amikacin (30 µg) on bacteria.

In conclusion, this study concluded that sidr leaves extract was successfully used for synthesis of silver nanoparticles and it can be used for synthesis of metal nanoparticles in nanotechnology industry. Formation of silver nanoparticles was confirmed using UV-Vis Spectrophotometer, FE-SEM and XRD analysis. Silver nanoparticles inhibited the growth of gram positive and gram-negative bacteria and *K. pneumoniae* was the most sensitive organism. Preparation of nanoparticles drugs as effective antimicrobials synthesized by plant extract being simple, fast, eco-friendly, low cost and presented the ideal attitude for treatment of bacterial infection and limitation of drug resistance.

Competing Interest

The authors declare that they have no competing interests.

Funding and Policy

This study was sponsored by Deanship of scientific research, Najran University, Kingdom of Saudi Arabia (Grant Code: NU/ESCI/14/010).

Acknowledgment

The authors thank Najran University, Saudi Arabia for its financial support of this study. Special thank for Prof. FaridHarraz for helping and advising during confirmation of silver nanoparticles synthesis. The authors are thankful to prof. Mohammed Ammar for helping in data statistical analysis.

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How to cite this article:

Masoud E. A., A. Al-Hajry M and Al-Marrani. A. 2016. Antibacterial Activity of Silver Nanoparticles Synthesized By *Sidr (Ziziphus spina- Christi)* Leaf Extract against Pathogenic Bacteria. *Int.J.Curr.Microbiol.App.Sci.* 5(4): 226-236.
doi: http://dx.doi.org/10.20546/ijcmas.2016.504.028