INTERNATIONAL RESEARCH JOURNAL OF PHARMACY

www.irjponline.com

ISSN 2230 – 8407

Research Article

BIOGENIC SYNTHESIS, CHARACTERIZATION OF SILVER NANOPARTICLES FROM *CARISSA CARANDAS* LEAF EXTRACT AND ITS PHARMACOLOGICAL ACTIVITIES

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Article Received on: 13/12/20 Approved for publication: 31/12/20

DOI: 10.7897/2230-8407.1112107

ABSTRACT

A Plant-mediated synthesis of nanoparticles is considered as a representative approach to environment benignity in material synthesis to avoid chemical toxicity. In this present study, we report an eco-friendly technique for the preparation of silver nanoparticles (AgNPs) from aqueous leaves extract of *Carissa carandas* Linn, and the evaluation of their pharmacological activities. The formation of AgNPs is confirmed by UV-Visible spectrum which peak highest at 436nm that corresponds to surface plasmon resonance of AgNPs. The shape and size of synthesized AgNPs were determined by HR-TEM and FEG-SEM which indicated well-dispersed nanoparticles with size ranging from 20nm-40nm. The functional groups responsible for reduction of silver ions are demonstrated by FTIR. XRD reported the crystalline nature of AgNPs. DPPH assay shows AgNPs has better antioxidant potential than its aqueous leaves extract. Antibacterial activity of AgNPs using resazurin dye against gram-positive and gram-negative bacteria with minimum inhibition concentrations (MICs) was found to be in the range from 31.5 µg/mL to 125 µg/mL. This biogenic synthesis provides an environment friendly, clean, cost-effective, and easy method for synthesis of AgNPs with enhanced pharmacological activities.

Keywords: *Carissa carandas*; green synthesis; silver nanoparticles; antioxidant potential; antibacterial activity.

INTRODUCTION

The particles with a nanometer size of 1–100 nm are represented as nanoparticles. The nano scale material has new, discrete and advantageous physio-chemical properties related to its bulk structure due to an increased ratio of surface area per volume of particles[1]. While the particle size reduces, not only does the surface-to-volume ratio changes but also the physical, chemical and biological properties of the particles vary from their bulk counterparts[2,3]. Despite the numerous metals that exist in nature, only a few of them are synthesized and studied extensively in nanostructured forms, such as gold (Au)[4], silver (Ag)[5,6], palladium (Pd)[7] and platinum (Pt)[8-10]. Recently, silver nanoparticles (AgNPs) have gained more importance because of its versatile potentials, advantageous properties such as antimicrobial, antioxidant, anti-inflammatory, anti-angiogenic, anti-cancer, wound healing and pharmaceuticals activity, etc[11]. Nanoparticles are synthesize using various physical and chemical methods. However, the uses of non-biodegradable compounds as reducing agents in the nanoparticles synthesis are highly expensive, potentially toxic to the environment and detrimental to biological system. Therefore, the biological approach which utilizes non-toxic reactants derived from biological sources ranging from single-cell organisms to multicellular higher plants for the synthesis of nanoparticles becomes essential. Metal ions reduction is much faster and stable nanoparticles are produced from plant extract than other biological processes[12]. Biomolecules such as protein, phenols and flavonoids found in plants reduce ions to nano size and also plays a significant role in the capping of nanoparticles[13]. Silver nanoparticles prepared using plant materials are small in size and have high surface area[14]. The biosynthesis of AgNPs using different plants namely *Azadirachta indica*[15], *Parthenium hysterophorus*[16], *Memecylon edule*[17], *Callicarpa maingayi*[18], *Artocarpus heterophyllus*[19] and *Tribulus terrestris*[20] have been reported.

The use of medicinal plants is beneficial as their medicinal properties are added during synthesis onto the surface of nanoparticles forming a capping layer that control the size and shape of nanoparticles[21,22]. *Carissa carandas* Linn. belongs to the family *Apocynaceae*. It is a large dichotomously branched evergreen shrub with a short stem and strong thorns in pairs. It is commonly known as Karaunda. The plant is very valuable as medicine particularly in Indian Ayurveda system[23]. Different parts of *C. carandas* plant like roots, leaves, barks, and fruits are used in the treatments of various human diseases such as anorexia, intermittent fever, stomachic, mouth ulcer and sore throat, syphilitic pain, burning sensation, diarrhoea, scabies, and epilepsy[24]. It has pharmacological activities such as hepatoprotective, neuropharmacological, anticancer, antioxidant, anticonvulsant, antiulcer, anthelmintic, analgesic, cardiovascular, anti-diabetic, antipyretic, cardiotonic, antibacterial, antiviral, diuretic, etc[25].

In this paper, we present a simple, low-cost, plant-mediated synthesis of stable AgNPs by reduction of silver nitrate ($AgNO_3$) solution using *C. carandas* leaf extract. The characterization of the AgNPs was performed by UV-Vis spectroscopy, HR-TEM, FEG-SEM, FTIR and XRD. We have also evaluated the antioxidant potential and antibacterial activity was studied against both gram-positive and gram-negative bacteria.

MATERIALS AND METHODS

Collection of *C.carandas* leaves

The Leaves of *C. carandas* were collected from the Mahad region of Maharashtra. The leaves were thoroughly washed with distilled water to remove any dust particles present and dried in oven for 3-4 days at 37°C.

Preparation of *C. Carandas* leaves extract

The dried leaves were grounded to fine powder and stored in airtight container. 5% of powdered leaves (5g dissolved in 100ml MilliQwater) was boiled for 30 min. After cooling, the mixture was filtered through Whatman filter paper no. 42. and further with 0.22 µm syringe filter. This filtrate was used for further study.

Synthesis of silver nanoparticles

20 ml of 5% leaf extract was added into 180 ml of 2mM silver nitrate ($AgNO_3$) solution in 500ml Erlenmeyer flask at room temperature. The mixture was stirred continuously on magnetic stirrer for 15 min. After 30 min, colour of the mixture changed to dark brown colour which indicated the reduction of $AgNO_3$ to AgNPs. After completion of the reaction, the solution was purified by repeated centrifugation at 10,000 rpm for 15 min at 4°C and the pellet was resuspended in MilliQ water.

CHARACTERIZATION OF SILVER NANOPARTICLES

UV-Vis Spectroscopy studies

The formation of AgNPs was observed through visual colour changes of the solution. The reduction of $AgNO_3$ to AgNPs was periodically monitored by UV–Vis spectroscopy (Shimadzu UV-1800) at a wavelength range of 200-900 nm. An UV–Vis spectrograph of the AgNPs was recorded against MilliQ water as a reference.

High resolution transmission electron microscopy (HR-TEM)

High resolution transmission electron microscopy (HR-TEM) was used to determine the shape and size of the nanoparticles (Tecnai G2, F30 model at 300 kV). Suspension of AgNPs was first sonicated for 10 min, then 1-2 drops of the sonicated suspension was placed on a carbon-coated copper grid and dried for 30 min under Infra-Red (IR) lamp.

Field emission gun scanning electron microscopy (FEG-SEM)

The morphology of the AgNPs was determined by Field emission gun scanning electron microscopy (FEG-SEM) equipped with Energy-dispersive X-ray spectroscopy (EDX). Briefly, the sample was sonicated for 5 min, a drop of the sample was added on double-side coated carbon stubs, dried and observed at different magnifications (JEOL JSM-7600F at a voltage of 0.1-10 kV).

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared (FTIR) analysis was performed to identify the potential biomolecules found in plant that are responsible for reducing silver ions (Ag) to AgNPs. The synthesized AgNPs were centrifuged at 12,000 rpm for 10 mins. Pellet was dissolved in MilliQ water and dried. The purified pellet was mixed thoroughly with potassium bromide (KBr) and then exposed to IR subjected to FTIR (3000 Hyperion Microscope with Vertex 80 FRIT System Bruker, Germany).

X-ray diffraction (XRD)

The crystalline size of the synthesized AgNPs was measured using 40 kV voltage and 40 mA current X-ray diffractometer (D8 Discover) with CuKα radiation (1.54 Å).
The crystalline size was measured with the following Debey-Scherrer equation 1.

$$D = \frac{k\lambda}{\beta \cos\theta} \qquad (1)$$

Where D is the crystalline size, *k* is the shape factor i.e 0.94, λ is the X-Ray wavelength (0.154), β is Full Width at Half Maximum (FWHM) in radians and θ is the Bragg angle.

PHARMACOLOGICAL APPLICATIONS OF SYNTHESIZED SILVER NANOPARTICLES

Antioxidant activity by free radical scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant activity of both synthesized AgNPs and aqueous leaves extract was determined by the stable DPPH radical. 100µL of different concentrations (20, 40, 60, 80, 100µg/mL) of samples (AgNPs and aqueous leaves extract respectively) was mixed with 600µL of freshly prepared 0.1mM DPPH solution. A control without sample was run simultaneously with the test. Ascorbic acid was used as standard. The reaction mixtures were vortex and kept in dark for 30 mins at room temperature. The Absorbance was taken at 517 nm against methanol as blank.
The free radical scavenging activity was measured as a reduction in DPPH absorption and determined from equation 2.

$$\text{Scavenging effect } (\%) = \frac{(Ac - As)}{Ac} \times 100 \qquad (2)$$

Where, the absorbance of control is A_C and of sample or standard is A_S respectively .

Antibacterial activity by Minimum Inhibitory Concentration (MIC)

The MIC test was performed with slight modifications in a 96-well round-bottom microtiter plate using a standard broth microdilution method[26]. The antimicrobial activities of the synthesized AgNPs against two gram-positive strains *Staphylococcus aureus*-ATCC 25923 & *Bacillus subtilis*-ATCC 6633 and two gram-negative strains *Escherichia coli*-ATCC-25922 & *Salmonella typhi*-ATCC 23564 were investigated. Briefly, overnight bacterial cultures in sterile nutrient broth were used in the experiment. 50 µL of sterile nutrient broth was added in respective wells. Two-fold serial dilution was performed with 50 µL of AgNPs and *C. carandas* aqueous leaves extract (5%) with concentrations ranging from 7.812 µg/mL to 1000 µg/mL and further inoculated with 50 µL of bacterial cultures in respective wells. Ampicillin (4mg/mL) was used as positive control. After proper mixing, the plate was sealed with parafilm and incubated at 37°C for 24 hr. 10 µL of the resazurin solution (0.02 mg/mL) was added post incubation and incubated at 37°C for 30 min. The purple colour of resazurin indicator reduce in the presence of living bacteria. Blue/Purple colour indicates no growth of bacteria while pink/colourless indicates growth of bacteria. The MIC value is the lowest concentration at which colour change occurs.

RESULTS AND DISCUSSIONS

Synthesis and characterization of silver nanoparticles

UV-Visible spectroscopy analysis

The UV-Visible spectroscopy is very reliable and effective preliminary technique for the characterization of AgNPs, also used to monitor the synthesis, structure and stability of the AgNPs[27,28]. On addition of *C.carandas* leaves extract (Figure 1A) to $AgNO_3$ solution (Figure 1B) the colour gradually changed to dark brown colour (Figure 1C). This colour change confirmed the reduction of silver ions (Ag^+) to a silver atom (Ag^0)[29] and was observed by UV-Visible Spectrophotometer (Figure 1). The UV–Visible spectrograph of synthesized AgNPs showed distinct maximum peak at 436 nm which showed the formation of AgNPs. This peak was because of surface plasmon resonance. Most of the research papers reported absorption peak of AgNPs to be in the range of 430-450 nm[30-32]. AgNPs strongly interacts with specific wavelengths of light because of unique optical properties. The conduction band and valence band in AgNPs lie very close to each other where electrons freely move. Due to the collective oscillation, these free electrons give rise to an absorption band of surface plasmon resonance in resonance with the light wave[33,34].

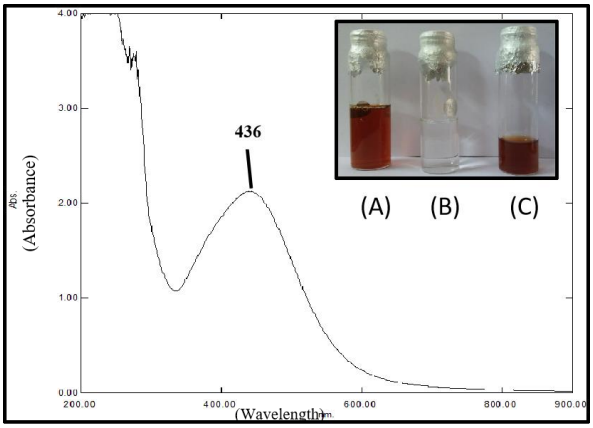

Figure 1: UV-Visible spectrum of synthesized AgNPs from leaves extract from *C. carandas* (A) *C.caradas* leaves extract. (B) Silver nitrate solution (C) Synthesized AgNPs.

HR-TEM analysis

The morphology of AgNPs produced by *C. carandas* was displayed by HR-TEM micrograph in Figure 2. The analysed sample revealed variations in particle size (Figure 2A). The shape of synthesized AgNPs were mostly spherical (Figure 2D) and uniformly distributed (Figure 2B). Selected area electron diffraction (SAED) pattern image (Figure 2C) showed concentric rings [111, 200, 220, 311] with bright spots due to Braggs reflection. These bragg reflection from separate crystals confirmed the crystallinity of the AgNPs. A similar result was reported on AgNPs synthesized from *Elephantopus scaber* leaf extract[35].

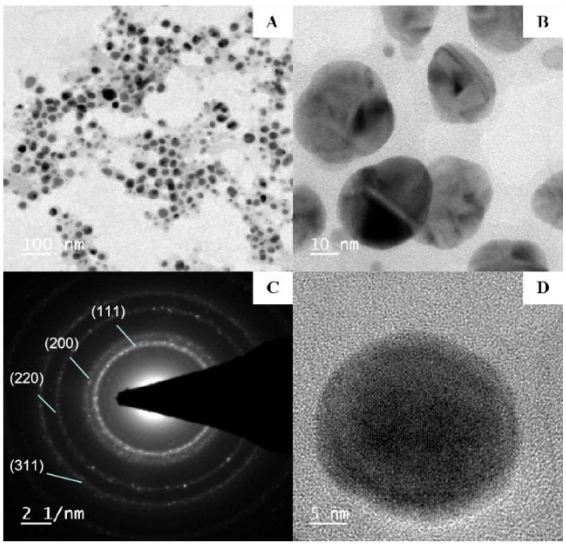

Figure 2: HR-TEM images of AgNPs at various magnifications (A) 100nm range (B) 10nm range, (D) 5nm range (C)SAED patterns of the AgNPs exhibit concentric rings.

FEG-SEM analysis

Morphology and shape of AgNPs were identified by FEG-SEM micrograph presented in Figure 3A. The resulting synthesized AgNPs were predominately in spherical shape and the size between 20nm-40nm range. Chemical characterization or qualitative and quantitative elemental analysis of the sample is measured by EDX[36]. The Energy dispersive spectrum confirmed the presence of nanocrystalline elemental silver by the optical absorption peak at 3 keV (Figure 3B). Metallic AgNPs normally have a relatively high signal peak at 3 keV because of surface plasmon resonance[37]. Similar results were also reported for AgNPs synthesized from *Pedalium murex* leaf extract where particle size range from 20 to 50nm[38].

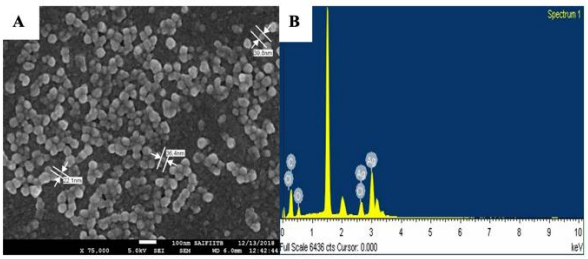

Figure 3: (A) FEG-SEM image of synthesized AgNPs using leaf extracts of *C. carandas*. (B) EDX graph.

FTIR analysis

FTIR analysis of AgNPs confirmed the role and presence of certain functional groups of the plant leaves extract as reducing and capping agent responsible for reduction and capping of silver ions. The FTIR spectra of *C. carandas* leaves extract and synthesized AgNPs are presented in Figure 4. The peaks observed in leaf extract are at 3250 cm^{-1},1639 cm^{-1}, 1411 cm^{-1}, 1289 cm^{-1}, 1172 cm^{-1}, 1041 cm^{-1}, 780 cm^{-1}, respectively. After adding silver nitrate the peaks are shifted at 3224 cm^{-1}, 1762 cm^{-1}, 1614 cm^{-1}, 1422 cm^{-1}, 1314 cm^{-1}, 1108 cm^{-1}, 824 cm^{-1}. The strong spectral peak at 3250 cm^{-1} and 1639 cm^{-1} in leaf extract which mainly corresponds to O-H stretching of alcohol or carboxylic acid and NO_2 gets reduced to 3224 cm^{-1} and 1614 cm^{-1} respectively are mainly responsible for the reduction of AgNPs. A prominent peak of 1411cm^{-1} of plant extract is bioreduced to 1422 cm^{-1}

corresponds to S=O and O-H bending respectively. A peak of plant extract at 1289 cm^{-1} bioreduced to 1314 cm^{-1} and 1172 cm^{-1} bioreduced to 1108 cm^{-1} are attributed to C-O stretching. Absorbance band at 780 cm^{-1} in the extract is bioreduced to 824 cm^{-1} due to C-H bending. The additional band in AgNPs at 1760 cm^{-1} corresponds to C=O stretching. Some peaks appeared in the FTIR spectrum of leaf extract but disappeared in FTIR spectrum of synthesized AgNPs. The disappearing of peaks suggest that the phytochemicals present in the leaf extract are involved in the reduction of AgNPs[39].

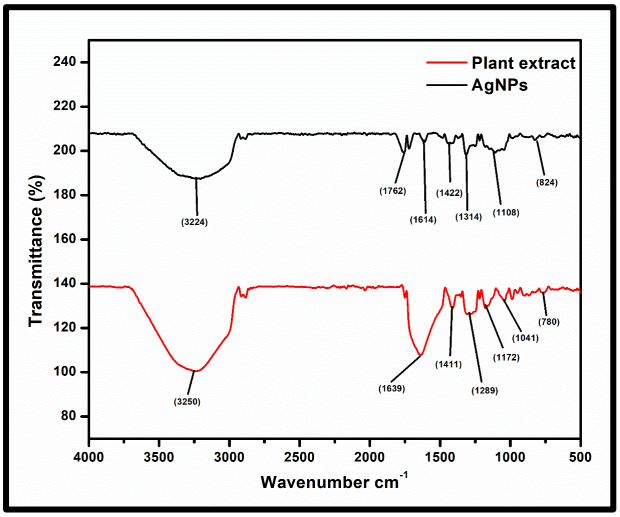

Figure 4: FTIR Spectrum of *C. carandas* leaves extract and AgNPs.

XRD analysis

X-ray Diffraction pattern study confirmed the presence of crystalline nature of synthesized AgNPs (Figure 5). The Bragg reflection peak of AgNPs is observed at 2Θ values of 37.93º, 43.78º, 64.32º, 76.99º which can be indexed to (111), (200), (220) and (311) crystal plane orientations. XRD spectra of crystalline silver structures are in accordance with the Joint Committee on Powder Diffraction Standards (JCPDS file no. 00-001-1167). It is observed that the typical pattern of synthesized AgNPs had the face-centred cubic (fcc) structure[31,40]. A few additional peaks were noticed at 2Θ values 27.63 º, 32.05 º, 46.05 º, 54.70 º, 57.30 º corresponds to (111), (200), (220), (311) and (222) of cubic phase AgCl crystal which resemble with reference value of JCPDS file no. 00-031-1238. These bragg peaks might be due to the capping agents which stabilize the nanoparticles. The crystallization of the bio-organic phase usually occurs on the surface of the AgNPs[41]. The mean size of the AgNPs was calculated from Debye-Scherrer's equation 1 and found to be 17 nm.

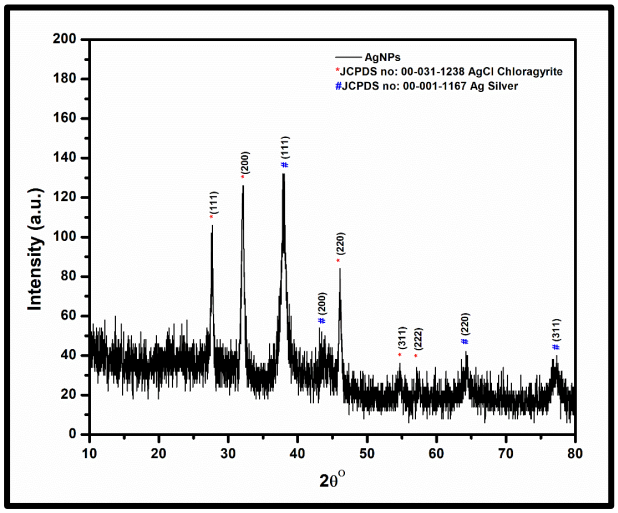

Figure 5: XRD graph represents crystalline nature of synthesized AgNPs using *C. carandas* leaves extract.

Antioxidant activity by free radical scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Molecules that are capable of preventing the oxidation of other molecules are known as antioxidants. The antioxidants defend cells from oxidative stress by scavenging free radicals. These molecules play very important role in the treatment of various diseases[42]. The antioxidant property of *C.carandas* leaves extract and its synthesized AgNPs were assessed against free radical, DPPH and compared with standard ascorbic acid (Table 1). The colour of freshly prepared DPPH solution was dark purple with maximum absorbance at 517 nm. The discolouration of DPPH solution on addition of aqueous leaves extract and synthesized AgNPs respectively indicated the scavenging of free radical which might be due to antioxidants present in the medium[43,44]. Interestingly, the AgNPs showed better scavenging activity for DPPH, with 50% inhibition (IC_{50}) at 46.01µg/mL compared with standard ascorbic acid. However, the IC_{50} of aqueous leaves extract was at 86.52 µg/mL. These antioxidant properties might be due to the presence of the functional groups on the surface that are responsible for capping of AgNPs[45,21].

Table 1: Inhibitory concentration (IC_{50}) for antioxidant activity using DPPH assay.

Test samples	% Inhibition IC_{50} (µg/mL)
Ascorbic acid	40.66µg/mL
Aqueous leaves extract	86.52µg/mL
AgNPs	46.01µg/mL

Antibacterial activity by Minimum Inhibition Concentration

Silver is a naturally occurring, non-toxic element that does not accumulate in the body or produce harmful effects and is considered to be environmentally safe. For the viability of mammalian cells and bacteria, the resazurin provides a straightforward, fast and delicate measurement. In living cells, oxidoreductases converts the non-fluorescent resazurin salt (blue) to fluorescent resorufin (pink)[46]. The MIC value for different samples was taken at the lowest concentration at which colour change[47]. As showed in Table 2, the MIC of AgNPs against *Staphylococcus aureus* (Figure 6A) and *Bacillus subtilis* (Figure 6B) was 125 µg/mL and 62.5 µg/mL respectively (Table 2). On the other hand, the MIC of AgNPs against *Escherichia coli* (Figure 6C) and *Salmonella typhi* (Figure 6D) was 31.5 µg/mL

and 62.5 µg/mL respectively (Table 2). The sterility control and solvent control wells for tested organisms remained blue. The least MIC value of AgNPs than $AgNO_3$ may be due to the smaller size of the nanoparticles. Our findings showed that antibacterial activity of AgNPs was found to be significant against both gram-positive and gram-negative bacteria. However aqueous leaves extract and ampicillin showed no activity. A similar result was reported of AgNPs synthesised from *P. amarus and T. cordifolia* which inhibit *E. coli* at the low concentration compared with *S. aureus*[48]. Another finding on the effect of AgNPs was tested against *E. coli* and *S. aureus* pathogens where *S. aureus* had less antibacterial effect compared with *E. coli*[49].

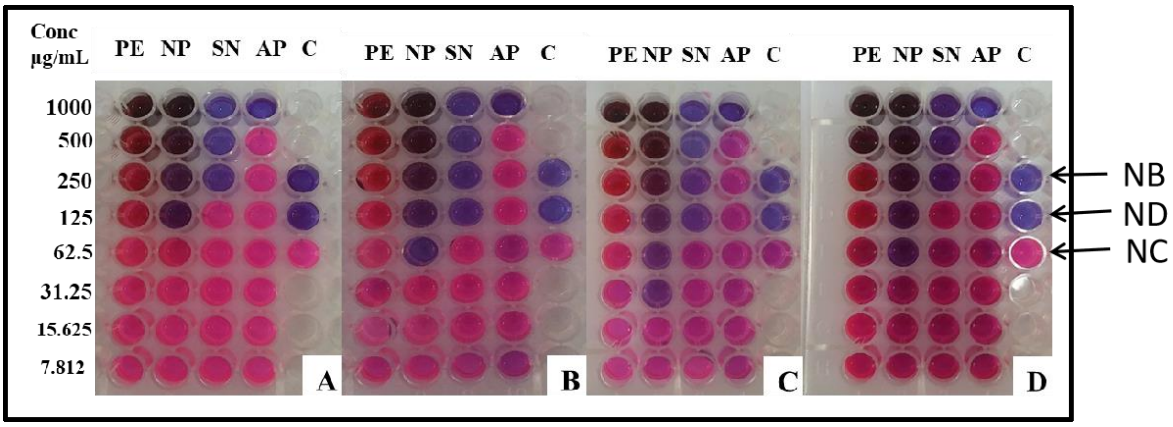

Figure 6: Plates after 30 mins of adding resazurin; the test organism was (A) *Staphylococcus aureus*, (B) *Bacillus subtilis*, (C) *Escherichia coli*, (D) *Salmonella typhi*; PE- Plant extract, NP-Silver nanoparticle, SN-Silver nitrate, AP-Ampicillin, C-Control, NB- only Nutrient broth, ND- Nutrient broth + D/W, NC-Nutrient broth + culture.

Table 2: MIC against gram-positive and gram-negative bacteria.

	Gram positive bacteria (MIC)		Gram negative bacteria (MIC)	
Bacterial strain	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*	*Salmonella typhi*
Aqueous leaves extract	ND	ND	ND	ND
Silver nanoparticles	125 µg/mL	62.5 µg/mL	31.5 µg/mL	62.5 µg/mL
Silver nitrate	250 µg/mL	125 µg/mL	125 µg/mL	250 µg/mL
Ampicillin	1000 µg/mL	1000 µg/mL	1000 µg/mL	1000 µg/mL

Note* ND- Not detected

CONCLUSION

The present study reported that silver nanoparticles can be synthesized in an eco-friendly, non-toxic, low-cost and simple method using *Carissa carandas* leaf extract. The UV-Vis spectrograph showed a highest peak at 436 nm. The particles showed crystalline nature and were found to be in spherical shape ranging from 20nm-40nm. AgNPs showed better antioxidant potential and antibacterial activity compared to leaf extract. The advantage of using plant extract for the synthesis of nanoparticles is energy-efficient, protects environment and human health that leads to less wastage and safer products. This environment friendly approach can be a competitive alternative to the conventional physical and chemical methods used for the formation of AgNPs. Silver nanoparticles can play major roles in the biologicals and medical applications in near future.

ACKNOWLEDGMENTS

We thank research staffs of Department of Life Science, University of Mumbai; Sophisticated Analytical Instrument Facility (SAIF)-IIT Bombay for accessing instruments facility.

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Cite this article as:

Steffi S. Madari *et al*. Biogenic synthesis, characterization of silver nanoparticles from *Carissa carandas* leaf extract and its pharmacological activities. Int. Res. J. Pharm. 2020;11(12):46-52.
http://dx.doi.org/10.7897/2230-8407.1112107

Source of support: Nil, Conflict of interest: None Declared