Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/40/5/MRN-1008

Biosynthesis and characterization of silver nanoparticles using root extract of *Saussurea lappa* (Decne.) Clarke and their antibacterial activity

Paper received: 25.09.2018 *Revised received*: 18.12.2018 *Re-revised received*: 09.01.2019 *Accepted*: 25.03.2019

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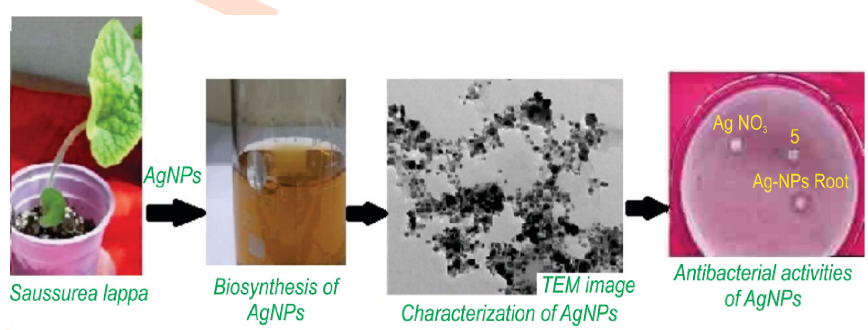
Abstract

Aim : To synthesize and characterize stable silver nanoparticles (AgNPs) from the root extract of *Saussurea lappa*, a medicinally important plant. The AgNPs was further evaluated for their antibacterial activity.

Methodology : The biosynthesised AgNPs were characterised by change in colour pattern and confirmed by UV–Visible spectroscopy, Transmission Electron Microscopy (TEM), X-Ray Diffraction (XRD) and Fourier Transform Infrared Spectroscopy (FTIR).

Results : UV-Vis spectroscopy study showed characteristic surface plasmon band (SPR) of synthesized AgNPs at 418 nm. FTIR studies revealed presence of some biomolecules responsible for reduction, stabilization and capping agents towards these synthesized AgNPs. TEM analysis revealed that the size of AgNPs ranged between 7.13 – 24.0 nm and had spherical shape. XRD data showed face-centered cubic (fcc) nature of AgNPs. The synthesized AgNPs showed significant antibacterial activity against one Gram positive (*Bacillus cereus*) and one Gram negative (*Escherichia coli*) bacterial strain.



Interpretation : The biologically synthesized nanoparticles from the root extract of *S. lappa* were stable and showed enhanced antibacterial activity against both type of bacterial strains and could be utilized for industrial and remedial purposes.

Key words: Antibacterial activity; Biosyntheis; Medicinal plant; *Saussurea lappa*; Silver nanoparticles

How to cite : Groach, R., K. Yadav, J. Sharma and N. Singh: Biosynthesis and characterization of silver nanoparticles using root extract of *Saussurea lappa* (Decne.) Clarke and their antibacterial activity. *J. Environ. Biol.*, **40**, 1060-1066 (2019). **DOI :** http://doi.org/10.22438/jeb/40/5/MRN-1008

Introduction

Saussurea lappa (Decne.) Clarke (family: Asteraceae), commonly known as Costus, is a renowned perennial herb, endemic to a geographically limited part of the Himalaya region at 2500-3500 m altitude. This critically endangered plant species is enlisted in Appendix I of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Kuniyal *et al.,* 2005). It is widely used as a traditional herbal medicine with satisfactory efficacy and no adverse effects. It has influential anti-inflammatory, suppresses hepatitis B, anticancer, antibacterial, antihepatotoxic, antiarthritic, antiviral, antifungal, antiasthma, antiproliferative, antidiarrheal, antioxidant and has inhibitory effect on gastric mucosal lesions (Arora and Bhojwani, 1989; Chena *et al.,* 1995; Hasson *et al.,* 2013). The major phytoconstituents isolated from this plant are lactone cynaropicrin, dehydrocostus, germacrene, lappadilactone (Gokhale *et al.,* 2002; Pandey *et al.,* 2007; Choi *et al.,* 2013; Zahara *et al.,* 2014). Costus oil, an essential oil extracted from the roots of this plant, is very costly and used in the preparation of high class perfumery and cosmetics. The oil is also said to be valuable in treating leprosy and other skin diseases (Chadha, 1972).

Destructive harvesting of *S. lappa* plants for commercial purposes is the major factor threatening its extinction. It can be propagated either through seed or by root cutting. Seeds have very poor viability (up to 30%). However, propagation by root cutting has its limitation because roots are exploited for commercial purposes (Johnson *et al.,* 1997). Keeping in mind the critically endangered position of this valuable medicinal plant species, a new and very effective multi-branched approach can provide solution to the problem (Kuniyal *et al.,* 2005). The modern nano-biotechnological techniques have come as a most fascinating research area in the field of biology, electronics and pharmaceutical sciences owing to the sturctural and functional aspects of matter in atomic and molecular scale (Morones *et al.,* 2005; Bagherzade *et al.,* 2017). Various physical, chemical and radiation methods are expensive to undertake and involve the use of toxic chemicals associated with risks to the natural ecosystem (Netala *et al.,* 2015).

The nanoparticles synthesized through biological synthesis are found to be more biocompatible. Recent studies focus towards ecofriendly, non-pathogenic and economical approach to develop inorganic nanoparticles with pervasive biomedical applications by utilizing biological sources (Benakashani *et al.,* 2016). Extracts from different parts of plants like roots, seeds, leaves, flowers, fruits etc., contain a broad spectrum of primary and secondary metabolites such as enzymes, amino acids, polysaccharides, organic compounds vitamins., etc. that may act as reducing and capping agents for nanoparticles synthesis by maintaining an aseptic environment (Ahmed *et al.,* 2016a). Plant based synthesis of nanoparticles has proved to be the most effective method for prospective appliance in biomedicine over other biological synthesis methods because it does not require maintenance of cell cultures (Ibrahim, 2015). Silver is a non toxic, safe inorganic metal possessing a strong inhibitory result towards many bacterial strains and microorganisms even at low concentration (Morones *et al.,* 2005). Medicinal plants with well established therapeutic importance are being widely used for shape and size controlled synthesis of AgNPs. Thus, AgNPs have emerged as a potential solution to fight against diverse pathogenic microorganisms. They have broad medical applications in the health industry, food storage, home appliances, textile coatings, water treatment and cosmetics (Bhatia *et al.,* 2016). Hence, the present study was undertaken to synthesize and characterize stable AgNPs from the root extract of *S. lappa.* The AgNPs were further evaluated for their antibacterial activity.

Materials and Methods

Preparation of aqueous root extract: Healthy plants of *S. lappa* were collected from Forest Department of Jammu and Kashmir (India) and maintained under poly house of Botany Department, Kurukshetra University (India). *S. lappa* is a perennial robust herb with erect, stout and fibrous stem having radial leaves (Pandey *et al.,* 2007). Fresh roots of *S. lappa* plant were collected and freed from extraneous matter. In order to remove the residues of adhering soil particles, the sample was thoroughly washed under running tap water, rinsed with distilled water and shade dried at room temperature. Analytical grade silver nitrate ($AgNO_3$) was purchased from Himedia Laboratories, Mumbai, India. The dried sample was pulverized to fine powder using pestle and mortar. Ten gram of air dried root powder was boiled in 100 ml of distilled water for 20 min at 70°C and then cooled to room temperature. The cooled aqueous mixture was filtered using Whatman's Filter paper no. 1. The filtrate was stored at 4°C in the refrigerator until further use.

Biosynthesis of AgNPs: AgNPs were synthesized by reacting 90 ml of 1mM $AgNO_3$ with 10 ml of aqueous plant root extract and incubated in dark at room temperature for about 48 hr.

Ultraviolet-visible spectra analysis: Biosynthesis of AgNPs were characterized by UV-vis spectrophotometer (W-2450, Shimadzu, Japan) between of 200 and 800 nm wave length after 48 hrs. After biosynthesis, the reduced solution containing AgNPs was separated by centrifugation at 12,000 rpm for 10 min at room temperature. Supernatants were discarded and AgNPs were redispersed in distilled water and purified by repeated centrifugation five times. The pellet obtained was air-dried in hot air oven to evaporate excess liquid, and was used for further characterization.

Analysis of particle size : The particle mean size distribution of AgNPs was measured at 25°C using Particle Size Analyzer by dynamic light scattering (DLS). The dried AgNPs were dissolved in double distilled water and loaded in quartz cuvette to analysis using particle size analyzer. The standard solutions were first run to know the size distribution, the detectors record the energy scattered, absorbed at particular angle and scattering patterns. The values of samples were then compared with these standard values.

Transmission Electron Microscopy Analysis: TEM (Hitachi H-7500) analysis was employed to visualize the shape, size distribution and morphology of AgNPs. In TEM technique, TEM grid was prepared by placing a drop of particle solution on a carbon-coated grid and drying under an incandescent lamp for 5 minutes. The voltage of the instrument was recorded at 100 kV and the micrographs were recorded at different magnifications from the sample.

X-Ray Diffraction Analysis: The crystalline structure of synthesized AgNPs was investigated by XRD. The angular accuracy was 0x001º and the angular resolution was 0x01º. The study was carried out to find out any phase transformation during annealing. XPERT-PRO diffractometer (45 kV, 40 mA) equipped with a Giono-meter PW3050/60 working with Cu/Kα radiation of wavelength (λ) 1.5406 ˚A was used for this purpose. The sample was scanned in the 2θ ranging between 10º to 80º with a step of 0.02º and step time 32.8 sec.

Fourier Transform Infrared Spectroscopy Analysis : AgNPs prepared with root extract were subjected to FTIR spectrophotometer (Perkin Elmer, Model RZX) in the range of 4000-450 cm^{-1}. FTIR measurements were carried out using potassium bromide pellet technique. About 2 mg of solid powder sample of AgNPs was crushed and mixed with 10 mg of Potassium bromide. This mixture was then subjected to hydraulic pressure of about 1.5 bar for few seconds and released to make a pellet. The spectrum was collected with eight scans co-added at 4 cm^{-1} in the transmission mode (4000 to 400 cm^{-1}). The detector was purged carefully using clean nitrogen gas to increase the signal level and reduce moisture. The sample discs were then introduced in the instrument and spectrum was recorded.

Antibacterial assay: The extracts of synthesized AgNPs from root extract of *S. lappa* was analyzed for antibacterial assay against *Bacillus cereus* (MTCC 430) and *Escherichia coli* (MTCC 1885) obtained from the Department of Biotechnology, Kurukshetra University (India) using agar disk diffusion method. One ml of bacterial inoculum was swabbed on the surface of nutrient agar medium plates. For this 50 µl of 50 µg ml^{-1} concentration of streptomycin, 1 mM concentration of $AgNO_3$ solution and synthesized root derived AgNPs extract were applied on sterilized paper discs (4mm diameter) and allowed to dry before placing on the surface of the inoculated medium. The agar plates were incubated for 24 hr at 37ºC. The plates were examined for evidence of zone of inhabitation (ZOI).

$$\text{Enhanced antibacterial activity of AgNPs against streptomycin} = \frac{\text{ZOI of AgNPs sample}}{\text{ZOI by streptomycin}} \times 100$$

$$\text{Enhancement antibacterial activity of AgNPs against } AgNP_3 = \frac{\text{ZOI of AgNPs sample}}{\text{ZOI by } AgNO_3} \times 100$$

The values of diameter of ZOI was measured with a meter rod and recorded as mean ± SD of triplicate experiment.

Results and Discussion

The formation of AgNPs during the reduction process was monitored by change in colour from light brown to dark brown in aqueous $AgNO_3$ solution due to surface plasmon resonance (SPR) indicating the reduction of Ag^+ to Ag^o. The synthesized nanoparticles UV absorption band occurred at 418 nm (Fig. 1). UV-Vis surface plasmon absorption band in the range of 400-460 nm wavelength is an evidence of SPR of AgNPs. The AgNPs synthesized using plant extract are surrounded by a thin layer of some capping organic material and are stable in solution even after 3 weeks of synthesis. Thus, no change in optical properties of nanoparticles solution was observed in the tested time.

The width of SPR bands is influenced by morphology, composition, dielectric environment and surrounding medium of synthesized AgNPs (Bhatia *et al.,* 2016). Synthesis of AgNPs has been reported from Annona squamosa, *Tridax procumbens* and *Phlomis bracteosa* extracts (Kumar *et al.,* 2012; Kushwaha and Malik, 2014; Anjum and Abbasi, 2016). Dynamic light scattering is a powerful technique for evaluating particle size, zeta potential and polydispersity index of nanomaterials in the solution. The mean particle size of silver particles of root derived AgNPs was found at 171.3 nm using particle size analyzer by dynamic light scattering as shown in Fig. 2. The size measured by DLS includes both the size of metallic nanoparticles and stabilizers absorbed on the surface of nanoparticles (Bhatia *et al.,* 2016). Netala *et al.* (2016) also observed the poly-dispersed nature of biosynthesized AgNPs while working on the aqueous callus extract of *Centella asiatica.* FTIR analysis was carried out for the identification of major functional groups present at different positions in the root extract of *S. lappa*.

The obtained peaks in FTIR spectrum of silver was compared for best matches with libraries of spectra that have been catalogued for known materials. The intensity peaks of root extract derived AgNPs appeared at 3399.40 cm^{-1}, 2127.76 cm^{-1}, 1645.44 cm^{-1} and 1045.69 cm^{-1}, respectively (Fig. 3). The interaction of biomolecules present in cell free extract with AgNPs may be responsible for the stability (capping material) of nanoparticles in the medium (Ahmad *et al.,* 2011). The biomolecules may be proteins, peptides, carbohydrates present in the cell free extract (Ahmad and Sharma, 2012). The intense broad line at 3,399.40 cm^{-1} is due to the stretching vibrations of hydroxyl functional group in alcohols and phenolic compounds. The peak at 2125 cm^{-1} corresponds to OH stretch COOH. Whereas at 1,645.44 cm^{-1} correspond amide II bond conjugated at C=O. The peak observed at 1045.69 cm^{-1} corresponds to C-N stretching vibration of aliphatic amines (Ramamurthy *et al.,* 2013; Rajeshkumar and Malarkodi, 2014). The TEM micrographs of root extract prepared AgNPs were more or near spherical in shape as shown in Fig. 4. It is evident from Fig. 4 that the average particle size calculated was 16.126 nm with size ranging between 7.13 nm to 24.0 nm. Electron microscopy is one of the techniques that can be applied to visualize nanoparticles and to generate

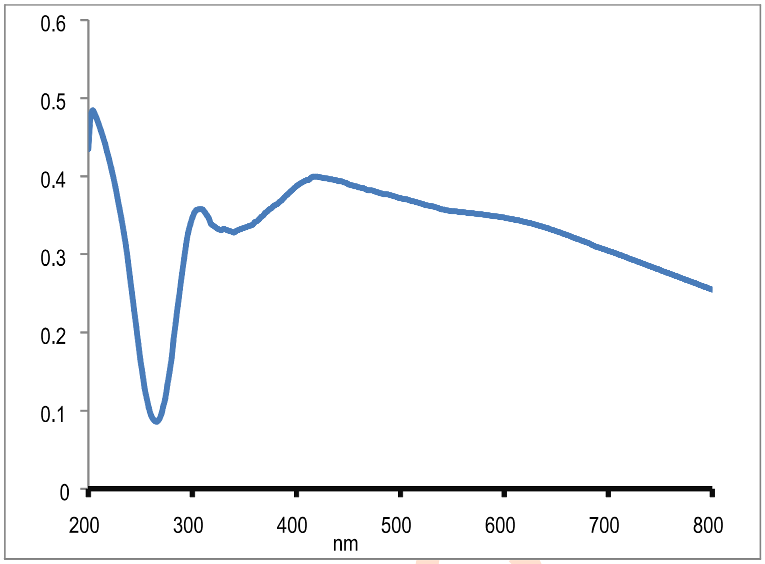
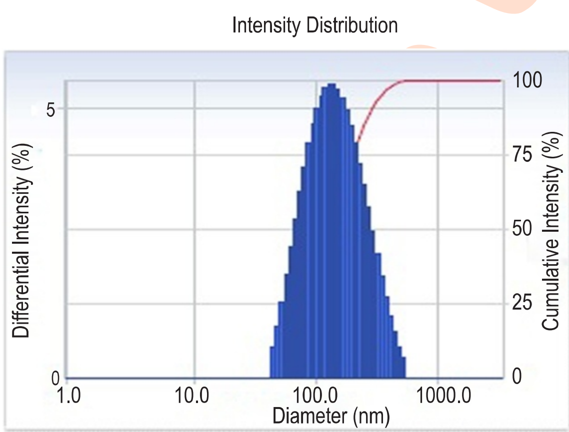


Fig. 1 : UV-VIS Absorption spectra of silver nanoparticles prepared with root extract of *S. lappa.*

useful data on the morphological properties (Bhatia *et al.,* 2016). Netala *et al.* (2015; 2016) also characterized the synthesis of Ag NPs from aqueous callus extract of *Centella asiatica* using TEM.

XRD is a non-destructive technique widely used to confirm the crystalline nature of the particles synthesized by using biological agents (Bhatia *et al.,* 2016). The X-ray diffraction profile of root derived AgNPs showed well defined diffraction peaks (Fig. 5). The four diffraction peaks at 38.19, 44.37, 64.56 and 77.47 were indexed as (111), (200), (220) and (311) planes of face center cubic (FCC) silver (Fig. 5). Appearance of these peaks was due to presence of phytochemical compounds in the root extract of *S. lappa.* Synthesis of AgNPs from aqueous extracts of *Ocimum sanctum, Malus domestica, Erythrina indica, Combretum indicum* and *Ephedra intermedia* and their characterization by UV–vis, FTIR and XRD has been reported in previous studies (Mallikarjuna *et al.,* 2011; Umoren *et al.*, 2014; Rathi Sre *et al.*, 2015; Bahuguna *et al.*, 2016 and Ebrahiminezhad *et al.*, 2017), respectively. The antibacterial effect of prepared AgNPs were investigated by growing *B. cereus* and *E. coli* colonies on nutrient agar plates (Table 1). The antimicrobial activity of AgNPs was measured in terms of diameter of inhibition zone (DIZ). The DIZ (in mm) reflects the magnitude of microorganism susceptibility. The strains susceptible to



Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	171.3	96.2
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	171.3	96.2
Residual :	1.063e-002	(O.K)

Fig. 2 : Analysis of particle size silver nanoparticles synthesized by root extract of *S. lappa.*

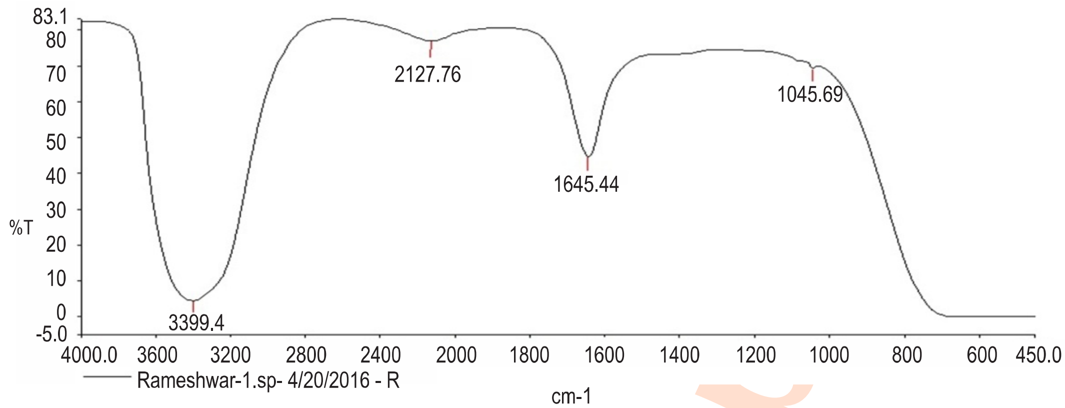


Fig. 3 : FTIR profile of AgNPs prepared from root extract of *S. lappa.*

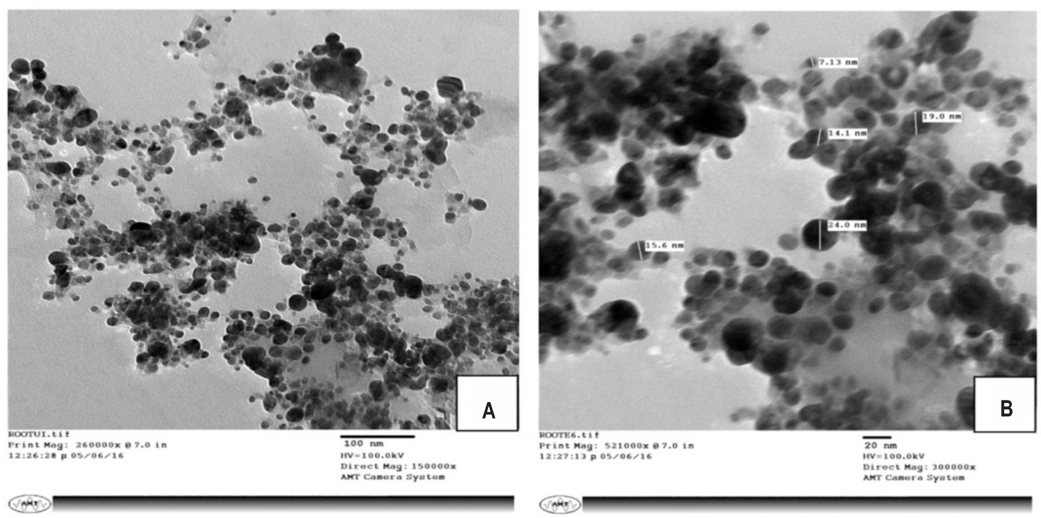


Fig. 4 : TEM images of AgNPs prepared from root extract of *S. lappa* : (A) 100 nm ; (B) 20 nm.

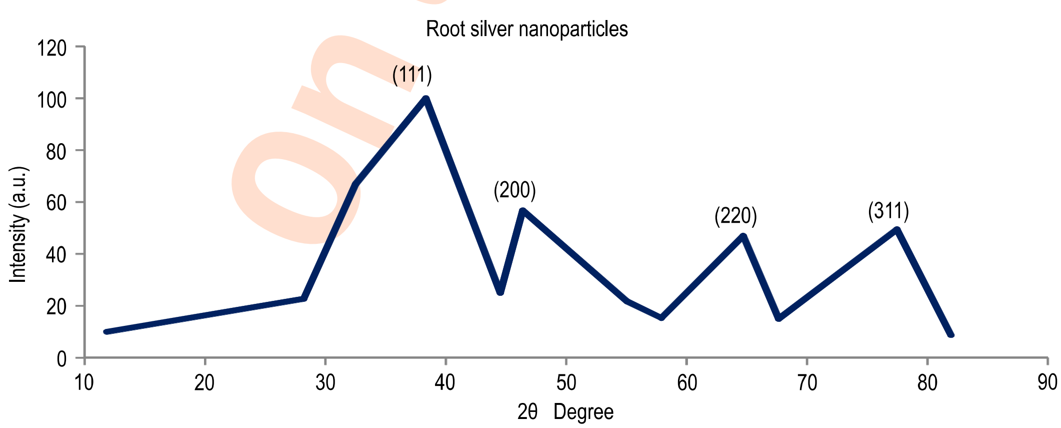


Fig. 5 : X-Ray diffraction pattern of root derived silver nanoparticles of *S. lappa.*

Table 1: Antibacterial activity of the silver nanoparticles prepared from root extract of *S. lappa*

Microorganism	Zone of Inhibition (cm)		
	$AgNO_3$	Antibiotic	Ag-NPs root
	(50 µg ml^{-1})	(50 µg ml^{-1})	(50 µg ml^{-1})
Bacillus cereus	0.9±0.1	0.7±0.1	1.5±0.1
Escherichia coli	0.8±0.1	0.7±0.1	1.2±0.1

antimicrobial agent exhibit larger DIZ whereas resistant strains exhibit smaller DIZ or no inhibition zone. The biosynthesized AgNPs have higher antibacterial activity than $AgNO_3$ against both type of bacterial strains over streptomycin at same concentration showing comparatively larger zone of inhibition due to their extreme large surface area (Table 1).

It may be due to the release of diffusible inhibitory compounds from AgNPs. It is reasonable to state that AgNPs bind to the negatively charged cell surface area causing structural changes, degradation and finally cell death by disturbing the important functions like membrane permeability, electron transport, osmoregulation and respiration (Panacek *et al.,* 2006). Recently, Mohammed (2015), Ahmed *et al.* (2016b) and Bagherzade *et al.* (2017) also reported antibacterial assay of synthesized AgNPs of *Eucalyptus camaldulensis, Azadirachta indica* and *Crocus sativus* against both Gram-positive and Gram-negative bacterial strains. This study integrates nanotechnology and bacteriology, leading to possible advances in the formulation of new types of bactericides in medical field (Hamouda and Baker, 2000). The study demonstrated a method of biosynthesis to develop AgNPs using *S. lappa* root extract act as a reducing as well as stabilizing agent. The biosynthesis of AgNPs using root extract has the potential to serve as a consistent, quick and eco-friendly alternative to hazardous physical and chemical methods. Hence, due to their benign and stable nature, the synthesized AgNPs showed a synergistic effect on the antimicrobial activity of standard antibiotic streptomycin against Gram-positive and Gram-negative bacteria, which an important aspect to be utilized for industrial and remedial purposes.

Acknowledgments

The authors are grateful to Kurukshetra University, Kurukshetra, for providing laboratory facilities and financial assistance to RG in the form of URS. Thanks are also owed to Department of Forest, Jammu and Kashmir for providing healthy plants of *S. lappa* to carry out this research.

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