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Green synthesis of silver nanoparticles using *Lonicera quinquelocularis* leaf extract exhibits antibacterial and antioxidant activities

Arshya Iqbal[1], Farmanullah Khan[1], Syed Badshah[1], Masood Afzal[2], Liqun Zhang[3], Wang Zhao[3,4], Kamran Tahir[1], Fawad Ali[5], Shafiullah Khan[*1]

[1]*Institute of Chemical Sciences, Gomal University, D. I. Khan, KP, Pakistan*

[2]*Department of Chemistry, University of Sargodha, Punjab, Pakistan*

[3]*State key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, PR China*

[4]*Department of Chemistry and Applied Biological Sciences, South Dakota School of Mines and Technology, United States*

[5]*Department of Chemistry, University of Peshawar, Pakistan*

Key words: *Lonicera quinquelocularis,* Green method, Silver nanoparticles, Antibacterial activity, Antioxidant activity.

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Abstract

Nanotechnology is a field that is mushrooming, having an impact in all circles of human life. Nano biotechnology represents an economic alternative for chemical and physical methods of nanoparticles formation. Presently available literature revealed that the NP synthesis using marine plants, microrganisms and algae as source has been unexplored and underexploited. The development of green processes for the synthesis of silver NP is developing into an important branch of nanotechnology. It has many benefits such as, ease with which the process can be scaled up, economic viability (Varahalarao and Kaladhar, 2014). In this study, the novel, one-step biosynthesis of AgNPs using the extract of *Lonicera quinquelocularis* at room temperature. The aim of this study is to synthesize AgNPs using a green synthesis method. A simple, ecofriendly, low cost and harmless green method have been developed to synthesized silver nanopaticles using *Lonicera quinquelocularis* leaf extract. The key points of our method were to produce highly dispersed, small size (5-12 nm) and spherical shape silver nanoparticles as compared to other methods. The biogenic silver nanoparticles exhibited maxima absorbance at 423nm due to surface Plasmon resonance which indicates the formation of silver nanoparticles. These nanoparticles were characterized by X-ray diffraction (XRD), scanning electron microscopy (SEM), UV-visible spectroscopy (UV-Vis), Energy-dispersive X-ray spectrometry (EDX), high-resolution transmission electron microscopy (HRTEM) and Fourier-transform infrared spectroscopy (FT-IR). Infrared spectral analysis confirmed that *Lonicera quinquelocularis* leaf extract contains active functional groups which work both as a reducing and stabilizing agent. The strong antioxidant and antibacterial activities of synthesized nanoparticles make them a lead source of therapeutic agent with broad spectrum biological activities. The considerable activities are attributed to the small size, high dispersion of silver nanoparticles and the active constituents of *Lonicera quinquelocularis* extract.

* **Corresponding Author:** Shafiullah Khan ✉ s.khan@gu.edu.pk

Introduction

The development of green synthesis of nanoparticles is evolving into an important branch of nanotechnology. The synthesis of nanoparticles using various plants can be advantageous over other biological synthetic approaches like fungi and bacteria. The latter in particular may involve complex procedures for maintaining the cultures and can be scaled-up for large scale production under non aseptic environments, as well. Lonicera Japonica (Nagajyothi *et al.*, 2012), Dioscorea batatas (Nagajyothi *et al* 2011), Magnolia kobus (Abelev *et al.*, 2013), *Cinamomum camphora* (Mubayi *et al.*, 2012) and Geranium species (Shankar *et al.*, 2004) are some of the plants used to synthesize nanoparticles. Since solvent toxicity is one of the major drawbacks in the preparation of silver nanoparticles, the new plant extract could reduce the metal ion, as it had been exploiting and reducing as a capping agent for the nanoparticles synthesis. *Lonicera quinquelocularis* is a large shrub or small tree up to 6 m tall, with hollow pith and pubescent to villous branches. One of the commonest and largest species with a wide distribution, growing in dry sunny places between 750-3000 m in N.W. Himalaya, Suliman range and Safed Koh. The subject of interest is green synthesis of Ag nanoparticles using medicinally beneficial plant *Lonicera*.

This *Lonicera* belonging to family Caprifoliaceae and genus *Lonicera*. It is distributed in Afghanistan, Pakistan Himalaya eastward to India, Nepal, Bhutan, Tibet and China. In Pakistan it is used for the treatment of different diseases e.g., Flowers are used in the form of syrup in disease of the respiratory tract. Nanotechnology is a rapidly growing field of science with several technological innovations being reported in recent times. The properties of nano sized particles have been widely exploited in different applications such as optical (Murphy *et al.*, 2005), magnetic (Shipway and Willner, 2000), electronic (Pankhurst and Quentin *et al.*, 2003) thermal (Berry, 2009) and catalytic activities (Moshfegh, 2009). Among the nanoparticles, silver has been known for its unique physical, chemical and biological properties which makes them the most resourceful.

Silver has been positioned as the 47th element in the periodic table, having an atomic weight of 107.8 and two natural isotopes 106.90 Ag and 108.90 Ag with abundance of 52 and 48% whereas the Colloidal silver is of particular interest because of distinctive properties such as good conductivity, chemical stability, catalytic and antibacterial activity (Frattini *et al.*, 2005.). The medicinal and preservatives properties of silver have been known for over 2,000 years. Silver is one of the basic element that and makes up our planet. It is a rare, but naturally occurring element, slightly harder than gold and very ductile and malleable (Magudapathy *et al.*, 2001).

Silver nanoparticles are extensively used in many applications like formulation of dental resin composites, disinfectants, bactericidal coatings in water filters (Phong *et al.,* 2009). It was also used as an antimicrobial agent in air sanitizer sprays, respirators, socks, wet wipes, pillows, detergents, soaps, washing machines, shampoos, toothpastes and many other consumer products. In medicinal field it is used as bone cement, inks (Zhang *et al.,* 2011), microelectronics (Thirumalai *et al.,* 2010), medical imaging (Zeng *et al.,*2012) and in wound dressing applications (Sankar *et al.,* 2014).

Synthesis of nanoparticles is mediated through a number of approaches such as chemical (Guzmán and Godet, 2009), physical (Santiago *et al.,* 2010) and biological (Mubayi *et al.,* 2012; Salam *et al.,* 2014). Indeed, most of the chemical methods used for the synthesis of silver nanoparticles are too expensive and also involves the use of toxic, hazardous chemicals which are responsible for various biological risks (Marambio-Jones and Eric, 2010).This enhances the growing need to develop environment friendly processes like green synthesis and other biological approaches. Presently available literature revealed that the NP synthesis using marine plant, microrganisms and algae as source has been unexplored and underexploited. Resistance to antimicrobial agents by pathogenic bacteria has emerged in recent years and is a major health problem. Seaweeds or benthic marine algae are the group of plants that live either in marine or brackish water environment.

The use of marine algae in the synthesis of AuNPs emerges as an ecofriendly and exciting approach. Utilizing a biological source gives an easy approach, easy multiplication and easy increase of biomass and size uniformity (Sun *et al*., 2002).

Lonicera quinquelocularis is one of the most important, easily available, highly valuable ayurvedic medicinal plants used to treat cold, cough, asthma, bronchitis and tuberculosis (Chhatre *et al*., 2014). These properties were tempted to use it in the synthesis of Ag-NPs (Kim *et al*., 2007).

The studies on the plant mediated biosynthesis of Ag-NPs using *Lonicera quinquelocularis* root extract as reducing and stabilizing agent, which were characterized using UV-vis spectroscopy, XRD, TEM, FTIR studies. This work was also involved to evaluate antioxidant activity of AgNPs against 1,1-diphenyl-2-picryl-hydrazyl and antibacterial activity against bacteria. In this study, the novel, one-step biosynthesis of AgNPs using the extract of *Lonicera quinquelocularis* at room temperature. The objective of this study is to synthesize AgNPs using a green synthesis method. Having these data in hand a try will be made to propose a model, which can explain the properties of the Silver nanoparticles using *Lonicera Quinquelocularis* up to a large extent.

Due to this high potency against aliment it is selected for synthesis of Ag nanoparticles to explore its hidden Mystery.

Materials and methods

Preparation of Lonicera quinquelocularis extract

Lonicera quinquelocularis was collected from Dera Ismail Khan KPK Pakistan and washed three times with de-ionized water before its extraction. A 35 g of this *Lonicera quinquelocularis* was shade dried, grinded it to powder. Then 18 g of it was mixed with water and stirred at 60 ^{o}C for 9 hours and then filtered to get the extract. The filtrate of *Lonicera quinquelocularis* extract was used both as stabilizer and reducing agent.

Synthesis of silver nanoparticles using Lonicera quinquelocularis extract

For silver nanoparticles synthesis, 20 ml of *Lonicera quinquelocularis* extract was added to 70 ml of 3×10^{-2} M aqueous solution in 150 ml beaker. As a visual observation aqueous solution of $AgNO_3$ stirred with the *Lonicera quinquelocularis* extract showed a color change from yellow to black within 50 minutes (Fig.1).The appearance of black color with extract is the clear indication of formation of silver nanoparticles. The silver nanoparticles suspension thus obtained was purified with the help of repeated centrifugation at 10,000 rpm for 15 minutes. Then the silver nanoparticles were freeze dried using VirTis freeze mobile 6ES freeze drier.

Characterization

The biogenic Ag nanoparticles was examined by scanning the aliquot sample in the wavelength range of 350-800 nm and recorded the absorption maxima in Shimadzo UV-2400 spectrophotometer at a resolution of 1 nm. The Wide angle X-ray diffraction (XRD) measurements were carried out on a Rigaku D/Max 2500 VBZ+/PC diffractometer using Cu K_a radiations at a scanning rate of 20 min–1 with an operating voltage of 40 kV and a current of 200 mA. High-resolution transmission electron microscopy (HRTEM) on a JEM-3010 microscope with an accelerating voltage of 200 kV was used to examine the morphologies and size of the nanoparticles. Infrared (IR) spectrum of Ag nanoparticle was obtained using the KBr pellet technique on an ABB MB3000 spectrophotometer where it was scanned between 2000 and 500 cm^{-1} at a resolution of 4 cm^{-1} in transmittance mode.

Micro organisms

Two bacterial species, *Escherichia coli* and *Staphylococcus aureus* were used in antimicrobial assay. Strains were obtained from State key laboratory of chemical resource engineering, Beijing University of chemical technology, Beijing 100029, Laboratory No 1102, PR China, where these were identified and characterized. These strains were maintained on agar slants at 4°C for antimicrobial tests.

Microorganisms were incubated overnight at 37°C in Mueller-Hinton Broth (Oxoid) at pH 7.4. The antibiotic, cephalexin 50µl of 8mg/ml was used as reference.

Screening for antibacterial activity by agar well diffusion method

For determination of antibacterial activity of AgNPs, the agar well diffusion method was carried out (Hadacek & Harald, 2000). All bacterial strains were grown in nutrient broth at 37°C for 24 h incubated till turbidity became equal to McFarland 0.5 turbidity standard. Using a sterile swab, the inocula of the respective bacteria was streaked on to the Muller Hinton agar (Oxoid) plates in order to make sure a uniform thick lawn of growth following incubation. Using sterile cork borer, wells of 6 mm in diameter were formed on to nutrient agar plates. The wells were filled with (50 µl) of the AgNPs and the plates were then kept to stay for 2 h at 25°C. At last the plates were incubated at 37°C for 24 h and the resultant diameters of zones of inhibition were measured carefully.

Determination of minimum inhibitory concentration (MIC)

In order to determined Minimum inhibitory concentration (MIC) of the AgNPs, Serial dilution method was used. With 1mL of different concentrations of AgNPs in sterilized test tubes was mixed with 1 mL of Bacterial solution (*Staphylococcus aureus* and *Escherichia coli*) having turbidity of 0.5 McFarland turbidity standard. After mixing, these test tubes were placed in incubator at 37 °C for 24 h. A test tube having only growth media and bacteria was used as a control. 3 mg/mL to 0.125 mg/mL concentrations of AgNPs were used in this test.

The minimum concentration of the compound, which inhibited the growth of the respective organism, was considered as MIC. The assay was carried out in triplicate.

DPPH free radical scavenging assay

DPPH radical scavenging activity for silver nanoparticles was performed according to the (Mulvaney, 1999 & Choi *et al.*, 2002) with a little modification. Different concentrations (31-1000µg/ml) of silver nanoparticles were separately mixed with 0.5 ml of 1mM DPPH and incubated in dark for 30 minutes. After incubation the absorbance of the samples was determined by UV 1100 spectro-photometer (MAPADA instruments) at 517 nm against methanol as a blank. Vitamin C was used as standard and DPPH methanol reagent without sample was used as control. The percentage of inhibition was calculated by the following formula.

Percentage of inhibition= $Absorbance_{control}$-$Absorbance_{test}$/$Absorbance_{control}$

Results and discussion

UV-vis spectroscopy

As a visual observation aqueous solution of $AgNO_3$ stirred with the *Lonicera quinquelocularis* extract showed a color change from yellow to black within 50 minutes. (Fig. 1). The appearance of black color with extract is the clear indication of formation of silver nanoparticles. The UV-vis spectroscopy was used to examine the formation of silver nanoparticles. It is a very useful technique for the analysis of silver nanoparticles. At the initial stage the characteristics surface Plasmon resonance (SPR) peak was not observed but after one hour the free electrons of silver give rise to SPR peak (Mulvaney, 1996; Rai *et al.*, 2006; Noginov *et al.*, 2007) which appeared at 423 nm.

Table 1. Zone of inhibition of silver nanoparticles and standard.

Microorganisms	Zone of inhibition in mm	
	Silver nanoparticles	Standard
Staphylococcus aureus	24(±0.5)	9(±0.4)
Escherichia coli	20(±0.4)	13(±0.5)

Mm = millimeter, standard=cephalexin, 37°C, 20-24hrs, 50µl of 8mg/ml

The SPR absorbance peak depends on the shape, size, dispersion and surrounding media of silver. The biogenic silver nanoparticles will have a spherical shape if the SPR peak is in between 400-450 nm (Shameli *et al.*, 2014, Kelly *et al.*, 2003, Stepanov, 2004.

The various size, shape and dispersion of silver nanoparticles depend upon the blue and red shift in $\lambda_{max.}$

The silver nanoparticles formation was examined at different time intervals. It was noted that with the passage of time the intensity of the absorption peaks of Ag nanoparticles increases and broadness of the peaks decreases (Fig 1). The UV-vis absorption spectra were recorded after every 60 minutes. Hence it is clear from this result that the *Lonicera quinquelocularis* extract has the ability to stabilize and reduce the silver nanoparticles.

Table 2. Minimum inhibitory concentration (MIC) of silver nanoparticles against *Escherichia Coli* and *Staphylococcus aureus* in 24 h.

Bacteria	AgNPs (mg/mL)					
	3	2	1	0.5	0.25	0.125
Escherichia coli	-	-	+	+	+	+
Staphylococcus aureus	-	-	-	-	+	+

Wide angle XRD

The crystalline nature of biogenic silver nanoparticles was confirmed by wide angle XRD analysis which is shown in (Fig. 2). The diffraction intensities of biogenic Ag nanoparticles were recorded in the range of 10^{o} -70^{o}. (Fig. 2)

shows three well resolve peaks 38.3^{o}, 44.4^{o} and 64.5^{o} that can be indexed to (111), (200) and (220) reflections which confirm Bragg reflections of face centered cubic (fcc) crystal structure of silver nanoparticles while all the other peaks due to .

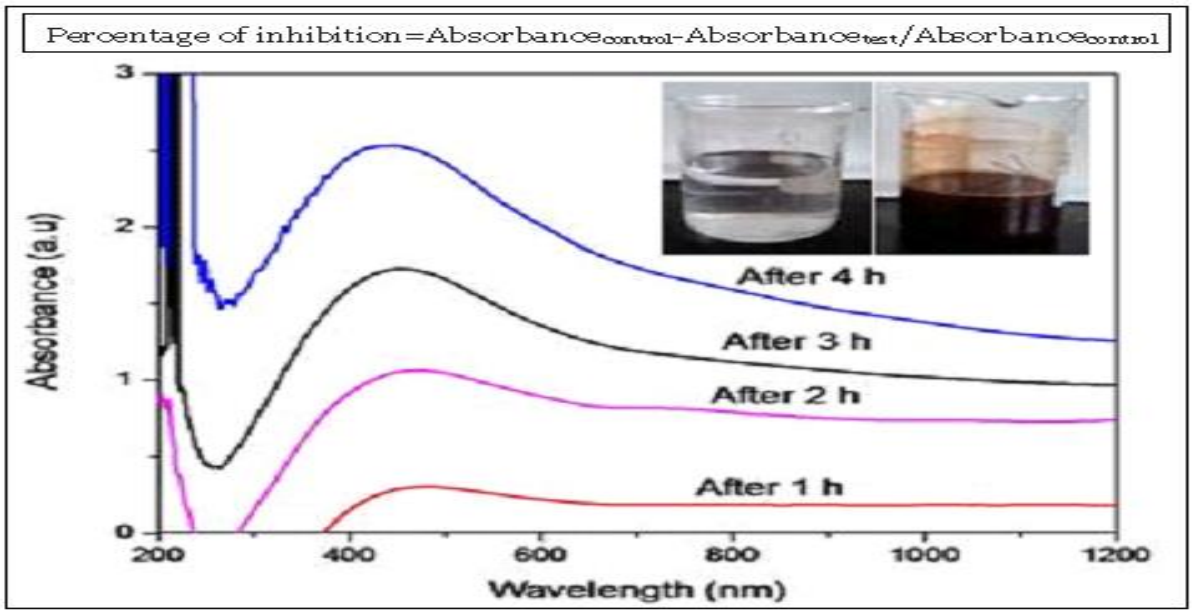

Fig. 1. UV-vis spectra of silver nanoparticles recorded at different time interval.

SEM analysis

The SEM images (Fig. 3a) show no aggregation among the silver nanoparticles and the particles have a small size.

Some particles show accumulation with each other but they are not in direct contact which assigns the stability of silver nanoparticles by phytoconstituents of *Lonicera quinquelocularis* extract.

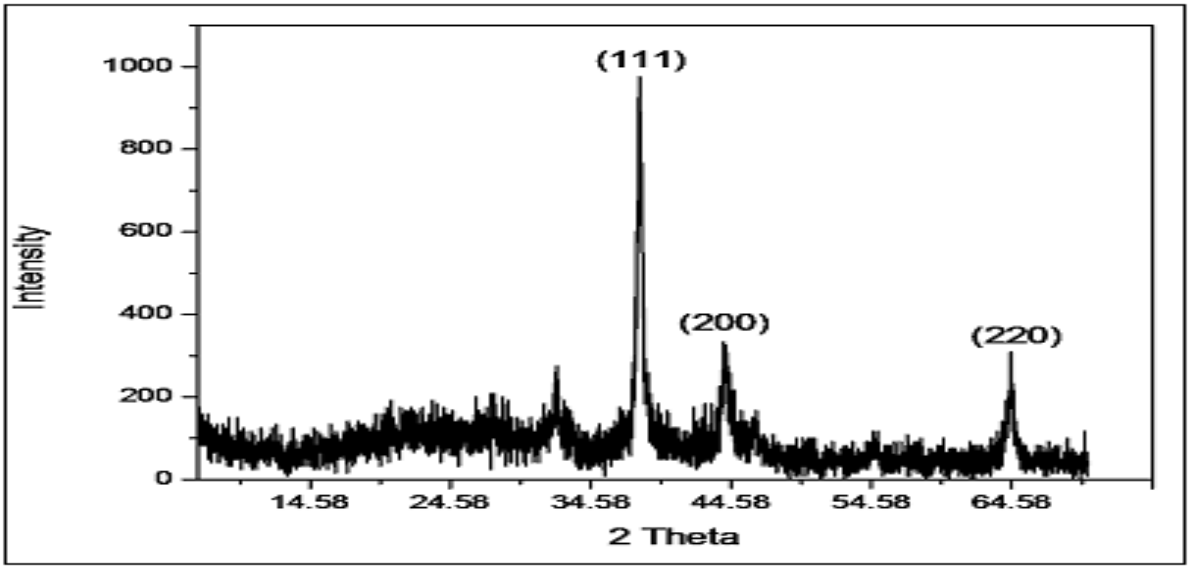

Fig. 2. XRD patterns of synthesized silver nanoparticles by *Lonicera quinquelocularis* leaf extract.

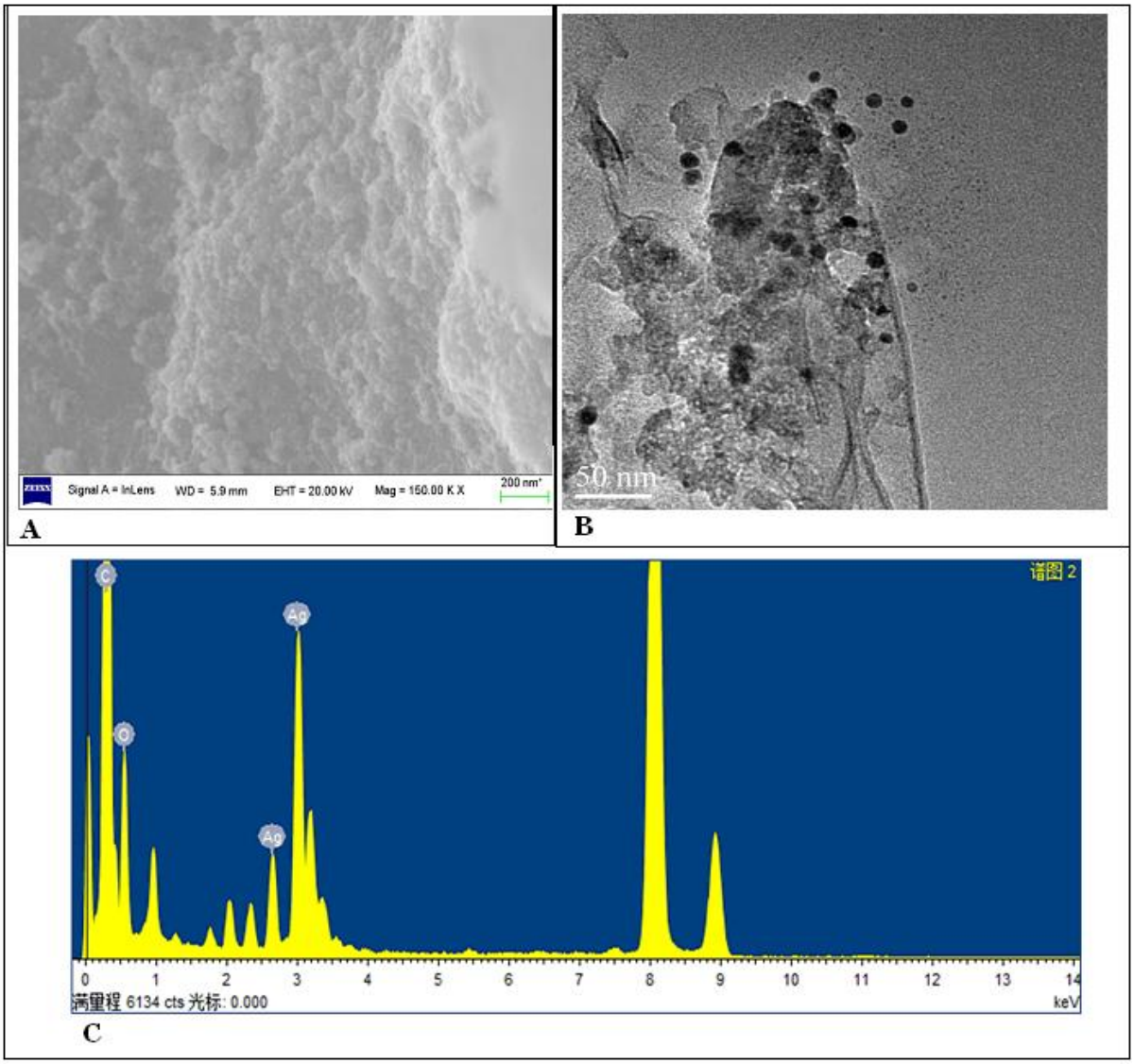

Fig. 3. (a) SEM image (b) HRTEM image and (c) EDX spectrum of green synthesized silver nanoparticles by *Lonicera quinquelocularis* leaf extract.

High resolution transmission electron microscopy (HRTEM)

The size, shape, morphology and dispersion of biogenic silver nanoparticles were confirmed by High resolution transmission electron microscopy which is shown in (Fig. 3b). It is clear from HRTEM images that silver nanoparticles have spherical shape, small size and highly dispersed. The size of silver nanoparticles is in the range of 5-12 nm which is modified from the previously reported. It is evident from HRTEM images that silver nanoparticles were surrounded by organic moiety which works both as a good reducing and stabilizing agents (Huang *et al.*,2007).

EDX analysis

EDX detector was used to determine the elemental composition of material. In EDX analysis strong peak was examined at 3keVwhich confirmed the metallic form of silver nanoparticles (Fig. 3c). The peaks for carbon and oxygen were also observed.

FTIR analysis

FT-IR spectral analysis was used to identify the phytoconstituents in the *Lonicera quinquelocularis* extract which are responsible for the reduction and stabilization of silver nanoparticles. (Fig. 4) shows FT-IR spectra of silver nanoparticles reduced and stabilized by *Lonicera quinquelocularis* extract. FT-IR spectrum shows absorption peaks at 3433, 2923, 1626, 1385, 1054, and 535in (Fig. 4).

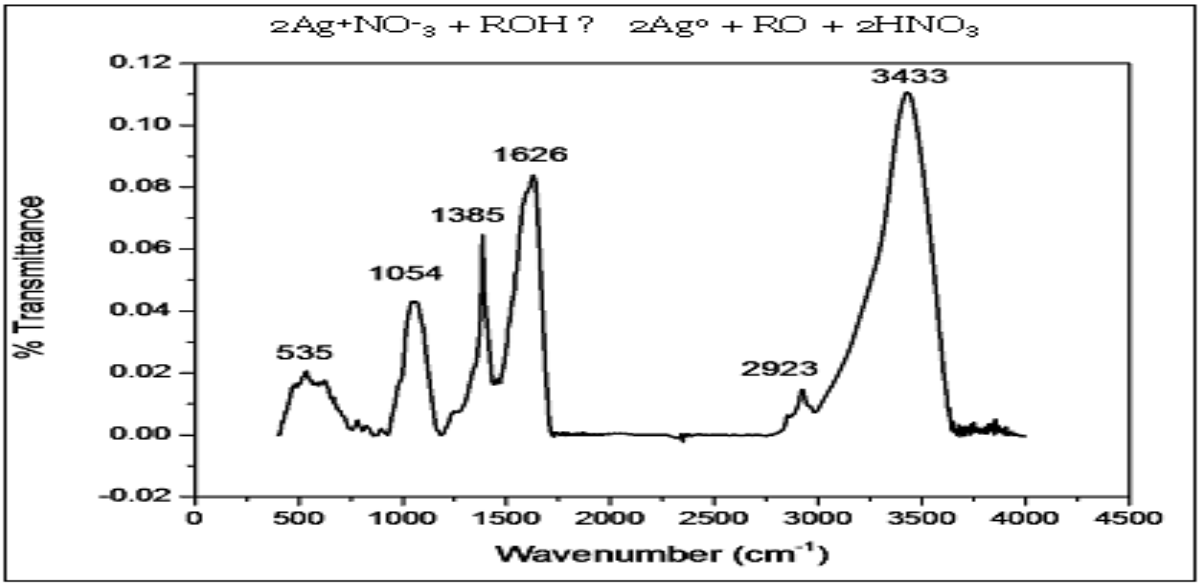

Fig. 4. FTIR spectrum recorded by making KBr pellet with synthesized AgNP.

The absorption peak at 3433showsthe O-H stretching vibration and the absorption peak at 2923 represent aldehydic C-H stretching vibration. The C=C aromatic vibration shows peak at 1626. The absorption peak at 1054 was for C-N stretching vibration of straight chain amines. The FT-IR absorption peak at528 was attributed to alkyl halides. It is clear from the FT-IR spectrum that –OH group is present in the *Lonicera quinquelocularis* extract which is responsible for the reduction of silver ions to silver metals through the oxidation of alcohol to aldehyde group. The data examined in the FT-IR absorption spectrum indicates the presence of carboxylic, amide, amino and amino acid groups in the *Lonicera quinquelocularis* leaf extract which are responsible for the silver nanoparticle synthesis. The formation of silver nanoparticles from *Lonicera quinquelocularis* extract in the form of chemical reaction can be represented as follows.

Hence it is clear from FT-IR spectral analysis that phenolic phytoconstituents are responsible for the reduction and stabilization of silver nanoparticles. This recommended that phenolic compounds could work as reducing and stabilizing agent for silver nanoparticles formation (Mondal, *et al.*, 2011; Jagtap and Bapat, 2013).

Antibacterial activity

The antibacterial activity of green synthesized silver nanoparticles was tested against *Staphylococcus aureus* and *Escherichia coli*. Clear transparent rings were obtained around the samples against the back ground of agar medium, showing the inhibition of bacterial cells. The biogenic AgNPs showed excellent antibacterial activity against the *S. aureus and E. coli*. The antibacterial activity of AgNPs was maximum against *S. aureus* as compared to *E. coli* by measure the zones of inhibition in agar plates. It may be due to the weak cell wall of *S. aureus*. (Fig. 5).

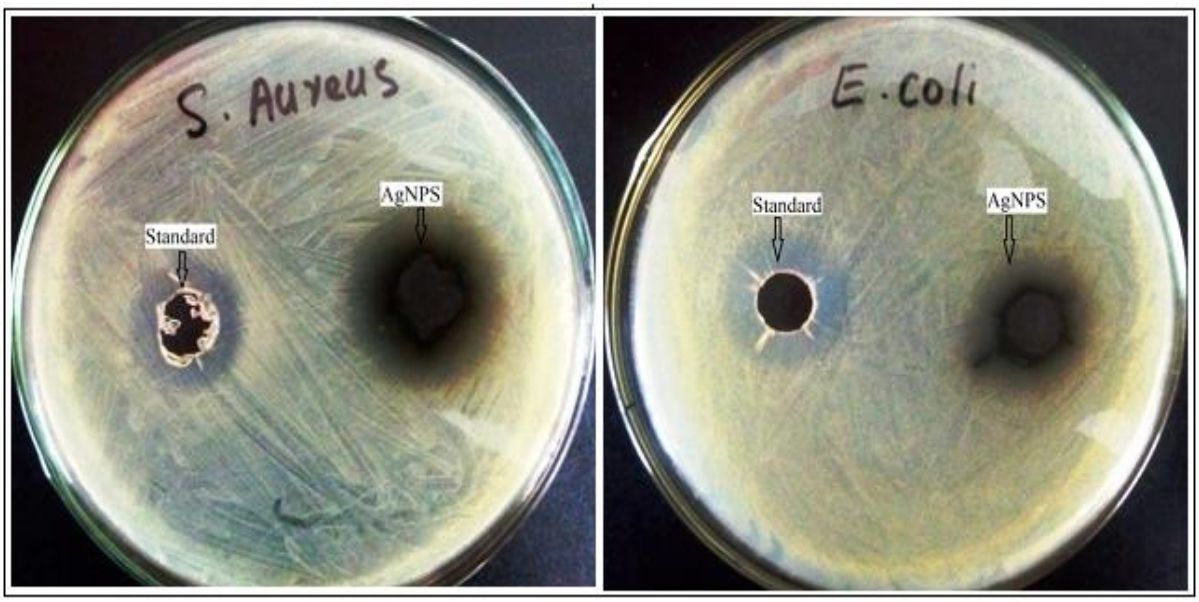

Fig. 5. Antibacterial activity of biogenic silver nanoparticles against*Staphylococcus aureus* and *Escherichia coli*.

The antibacterial activity of AgNPs was also compared with the standard (Cephalexin) and the result showed that silver nanoparticles have higher antibacterial activity than standard.

The diameter of zone of inhibition of silver nanoparticles against *S. aureus and E. coli* were 24(±0.6) mm and 20(±0.4) mm respectively (Table 1). While that of the standard Cephalexin were 9(±0.4) mm and 13(±0.5) mm against *S. areus* and *E. coli* respectively. The antimicrobial activity of silver nanoparticles against *S. aureus and E. coli* revealed that it is active antibacterial agent.

In order to determine quantitatively the antibacterial activity of AgNPs we applied the method of minimum inhibitory concentration (MIC). The MIC is the minimum concentration of antibacterial material that inhibits the growth of a pathogenic microorganism in artificial media after a specific incubation time. Similarly diluted suspensions of AgNPs were incubated with equal volumes of *S. aureus and E. coli* solutions and the bacterial growth was observed. After 24h of incubation, we found that the MIC was 2 mg/mL for *Escherichia coli* and 0.5 mg/mL for *Staphylococcus aureus* which is shown in (Table.2).

Mechanism of antibacterial activity

The average size of synthesized silver nanoparticles was from 5-12 nm. Due to its small size it easily enters into bacterial cell whose size is from 100-1000nm. Inside, the bacterial cell silver nanoparticles combined with various enzymes (protease and respiratory enzymes) and DNA to disturbed their functions which lead to death of the bacterial cell.

DPPH free radical scavenging analysis

Antioxidant activity of silver nanoparticles was evaluated by DPPH scavenging assay by using Vitamin C as standard. DPPH was a stable compound and have the ability to accept hydrogen or electrons from silver nanoparticles.

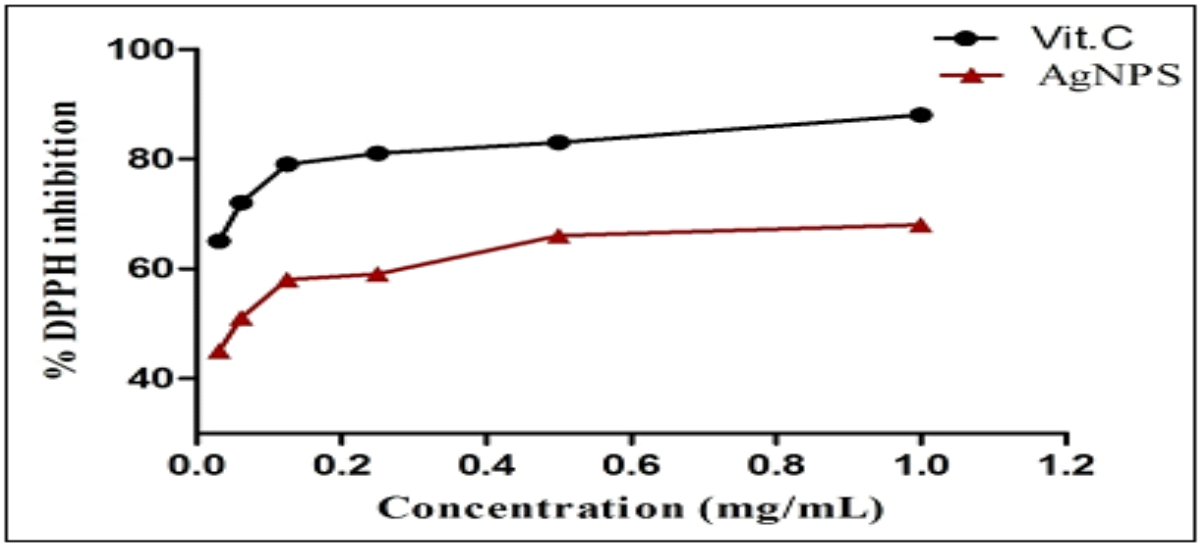

Fig. 6. DPPH free radical scavenging activity of silver nanoparticles.

The results obtained in the DPPH assay showed effective free radical inhibition by silver nanoparticles (Fig.6). Six different concentrations of silver nanoparticles were used in this study and the antioxidant activity increased with increasing concentrations of AgNPs. Similar observations with enhanced DPPH scavenging activity by selenium, platinum, silver nanoparticles (Gao and Zhang, 2002; Saikia *et al.,* 2010) and by torolex and chitosan coated gold nano particles (Nie *et al*., 2007; Raghunandan *et al.,* 2010) have been reported.

Conclusion

The rapid biological synthesis of AgNPs using extract of Lonicera *quinquelocularis* provides an environmental friendly, simple and efficient route for synthesis of benign nanoparticles. We have successfully synthesized silver nanoparticles via complete green synthetic method. During synthesis of AgNPs, *Lonicera quinquelocularis* extract were used as a reducing and stabilizing agent.

The phyto constituents of the *Lonicera quinquelocularis* extract provide a good environment for the formation of silver nanoparticles with high dispersion, uniform spherical shape and small size distribution. The spectroscopic characterizations from UV-Vis, FTIR, SEM, EDX and XRD support the formation and stability of the green synthesized AgNPs.

The antibacterial and antioxidant activities of silver nanoparticles were also evaluated. The antibacterial activity of AgNPs exhibited strong inhibition towards *Staphylococcus aureus* and *Escherichia coli*. The antioxidant activity of silver nanoparticles was compared with standard vitamin C which showed excellent result. Thus it is the cheap, nonhazardous new biological approach for the synthesis of silver nanoparticles from *Lonicera quinquelocularis* leaf extract as compared to others methods.

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