Biosynthesis, characterization and antimicrobial action of silver nanoparticles from root bark extract of *Berberis lycium* Royle

Ansar Mehmood*[1], Ghulam Murtaza[1], Tariq Mahmood Bhatti[2], Rehana Kausar[1] and Muhammad Jamil Ahmed[1]

[1]Department of Botany, City Campus, University of Azad Jammu and Kashmir, Muzaffarabad, Pakistan
[2]Department of Chemical Engineering, Pakistan Institute of Engineering and Applied Sciences (PIEAS), Islamabad, Pakistan

Abstract: Various biological methods are being recognized for the fabrication of silver nanoparticles, which are used in several fields. The phytosynthesis of nanoparticles came out as a cost effective and enviro-friendly approach. When root bark extract of *Berberis lycium* was treated with silver ions, they reduced to silver nanoparticles, which were spherical, crystalline, size ranged from 10-100nm and capped by biomolecules. Synthesized silver nanoparticles were characterized by UV-visible spectroscopy, Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDX), Transmission Electron Microscopy (TEM), X-Ray Diffraction (XRD) and Fourier Transform Infra Red Spectroscopy (FTIR). The plant mediated synthesized silver nanoparticles showed pronounced antimicrobial activities against both Gram negative bacteria (*Escherichia coli, Klebseilla pneumoniae, Pseudomonas aeruginosa*) and Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*). The plant mediated process proved to be non-toxic and low cost contender as reducing agent for synthesizing stable silver nanoparticles.

Keywords: Antimicrobial, biomolecules, silver nanoparticles, SEM, TEM.

INTRODUCTION

Nanotechnology deals with the materials smaller than 1 µm on nanoscale (Singh *et al.,* 2011). It is a new branch of science, which deals with the examination of materials ranging from 1-100nm. Nano materials have new substantial, optical, chemical and biological properties as compared to bulk materials (Roco, 1998). The objectives of the nanoscience is to manipulate matter at atomic or molecular level to produce nano-scale materials with novel physico-chemical properties that enhance conductivity, potency, stability, reactivity and other properties of products and applications (Powell *et al.,* 2008).

Metal nanoparticles are synthesized by using different physical and chemical methods. Although chemical method is most popular technique for the production of nanoparticles but it may cause drastic effects in the field of medical applications as it requires many toxic chemicals for synthesis process and also it has low yield. Biological synthesis of silver nanoparticles is proved to be more advantageous as compared to physical and chemical synthesis because it is cost-effective, eco-friendly, rapid and allows large scale synthesis of nanoparticles. Biological methods include use of bacteria (Minaeian *et al.,* 2008), fungi (Mukherjee *et al.,* 2001), enzymes (Willner *et al.,* 2007) and bark (Ankanna *et al.,* 2010; Sathishkwar *et al.,* 2009) and leaf extracts (Priya *et al.,* 2011; Prasad *et al.,* 2011; Mehmood *et al*., 2014a) of plants. Among the biological methods, plants have got enormous attention because they eliminate the need of sustaining cell cultures and equally well suited for large scale production of nanoparticles under non-toxic environment.

Biological applications of nanoparticles include target drug discharge, gene therapy, identify affective tissues, biological molecules and cells separation and purification, bio-labeling, repair of damage tissues by tissue engineering, DNA probing and microsurgical techniques (Salata, 2004). Silver nanoparticles can be used in water purification process because they have pronounced antimicrobial activity (Revina and Egorova, 1998). Silver is considered to be good antibacterial and antimicrobial agent and is found in medical and industrial processes (Jiang *et al.,* 2004; Mehmood *et al*., 2014b).

In this study, rapid synthesis of silver nanoparticles from silver ions was carried out by using root bark extract of *Berberis lycium,* which is readily available plant. It is the first report of using root bark of plant for reduction of silver ions. The root bark is selected because of local uses like cure of wounds, broken bones, healing piles and unhealthy ulcers. Root bark is also used as bitter tonic astringent, diaphoretic and febrifuge. Besides the production of silver nanoparticles, applications of AgNPs have got great consideration now days. They are known to be excellent antimicrobial agents because of their tremendous antimicrobial property (Rai *et al.,* 2009). Therefore, to explore the applications of silver nanoparticles in the formation of antibacterial products, the antibacterial efficacy of phyto-synthesized AgNPs was screened out by using agar disc diffusion method against

**Corresponding author:* e-mail: ansar.mehmood321@gmail.com

Gram-negative bacteria (*Escherichia coli, Klebseilla pneumoniae, Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus* subtili). This study may provide a significant tool for bio-fabrication and antibacterial applications of silver nanoparticles.

MATERIALS AND METHODS

Plant materials and preparation of extract

The fresh root bark of *Berberis lycium* (Family Berberidiaceae) was collected and washed for removing any dust particle. Five-gram root bark of *Berberis lycium* was cut into small (about 1mm) pieces and put into 500 ml Erlenmeyer flask with 300ml de-ionized water and boiled for 10min. The boiled extract was allowed to cool at ambient temperature and filtered by using What man filter paper No. 1.

Synthesis of silver nanoparticles

The procedure developed for synthesis of AgNPs by mixing 100ml of 4mM silver nitrate ($AgNO_3$) solution with 100ml of root bark extract in 250ml Erlenmeyer flask at room temperature. The conversion of silver ions to silver nanoparticles was monitored by colour change, periodic FAAS and UV-visible spectrum analysis.

Flame atomic absorption spectrometry (FAAS)

The rate of reduction of silver ions to silver nanoparticles in the reaction solution was observed by FAAS analysis. The reaction samples were taken at 0, 1, 2, 3, 4, 5 and after 6 hours of reaction and centrifuged at 14000 rpm for 4 mins. The supernatant was subjected to FAAS (Varian 30/40 Model) analysis for the presence of silver ions in the solution.

Yield of silver nanoparticles

The yield of silver in percentage was obtained by using the following formula

$$\text{Yield} = \frac{\text{mas of Ag obtained}}{\text{mass of Ag used}} \times 100$$

UV-visible Spectroscopy

The synthesis of silver nanoparticles in the reaction solution was monitored by using UV-visible spectroscopy carried out on PerkinElmer Lambda 950 UV/Vis Spectrometer. The small aliquot of the sample was taken after zero and 6 hrs of reaction in Quartz cuvette with water as a reference and then analyzed for absorption spectra between 350-700nm wavelengths at room temperature.

Characterization of silver nanoparticles

After 6 hrs the solution containing silver nanoparticles was centrifuged at 14000rpm for 4mins and the pellet was re-dispersed in distilled water. The process of centrifugation and re-dispersion in distilled water was repeated three times and finally washed with acetone. The purified silver nanoparticles were characterized by using following techniques.

Scanning electron microscopy and energy dispersive X-ray analysis

The samples for SEM and EDX analysis were sonicated for 5 min to make suspension of silver nanoparticles in distilled water and then a drop of suspension was placed on double carbon coating conductive tape and allowed to dry under lamp. The SEM and EDX analysis was carried out on same instrument named JEOL JSM-6490A Analytical Scanning.

Transmission electron microscopy

The shape and size of silver nanoparticles was determined by TEM. The sample was prepared by sonicating the pellet of centrifuged silver nanoparticles in distilled water. A drop of suspension was placed on a carbon coated copper grid and allowed to complete dry under lamp. TEM analysis was performed at JEOL JEM-1010 (accelerating potential 80 KV) at magnification of X15 k, X40 k and X 250 K.

X-Ray diffraction analysis

The purified silver nanoparticles were allowed to freeze dry and then analyzed for XRD. Dried mixture of silver nanoparticles was used for the study of synthesis of silver nanoparticles on Bruker D8 Diffractometer at a wavelength of 1.54 nm with Cu K-α radiation. The size of the silver nanoparticles was calculated by using Scherrer's equation.

Fourier transform infra-red spectrometry

It is used to determine the possible phytochemicals responsible for the reduction of silver ions to silver nanoparticles. The sample was prepared by freeze-drying the purified pellet and then analyzed using PerkinElmer Spectrum 100 FT-IR spectrometer.

Antimicrobial assay

Antimicrobial activity of different concentrations of synthesized silver nanoparticles (10µg/ml, 5µg/ml, 2.5 µg/ml and 1.25µg/ml) and root bark extract (10µg /ml, 5 µg/ml and 2.5µg/ml) was determined by using paper disc diffusion method (Arya *et al.,* 2010) against common selected pathogens (*Escherichia coli, Klebseilla pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis*). $AgNO_3$ (4mM) was used as positive control. Fresh culture of *Escherichia coli, Klebseilla pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* was used to form the inoculums, poured into sterilized Petri plates along with sterilized nutrient agar medium (28g dehydrated nutrient agar in 1000 ml distilled water), and allowed to solidify. The sterilized filter paper discs (6 mm) were impregnated with silver nanoparticles and plant

extracts, placed in agar plates and incubated at 37°C for 24hrs. The inhibitory activity was determined by measuring the zones of inhibition around the discs. The experiment was performed in 3 replicates and mean diameter of zones of inhibition plus standard error of means (SEM) mean was presented.

RESULTS

When silver nitrate solution was added to root bark extract, the color of the reaction fusion changed from yellowish to yellowish brown (fig. 1a). The formation of silver nanoparticles in the colloidal solution of silver nitrate and root bark extract was further analyzed by UV-visible spectroscopy. It demonstrated an absorbance peak at 422nm after 6 hrs (fig. 1b). The absorption peak was not observed for $AgNO_3$ solution, bark extract and at zero time in the range of 350-700nm.

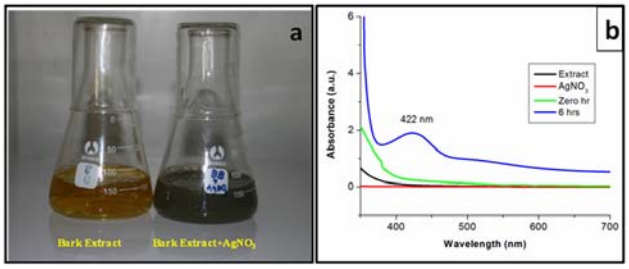

Fig. 1: Color of plant extract before and after adding AgNO3 solution (a) and (b) UV-visible spectrographs of silver solution containing root bark extract of *Berberis lycium*

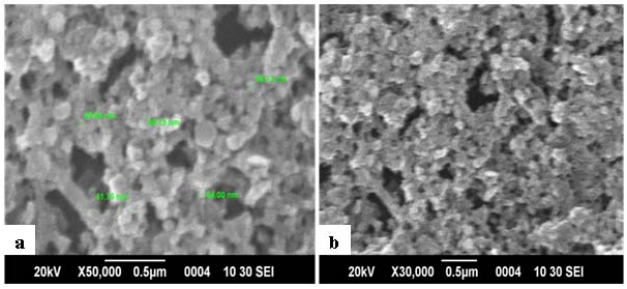

Fig. 2: SEM images of silver nanoparticles at (a) X50,000 and (b) X30,000

The rate of reduction of Ag^+ to AgNPs in reaction mixture was monitored by FAAS analysis. The reaction mixture (216ppm Ag) contained silver nitrate solution and plant extract was used for FAAS analysis. The concentration of silver ions was recorded as 216, 134, 64, 45, 33, 28 and 27ppm at 0, 1, 2, 3, 4, 5 and 6 hrs, respectively.

Total yield of silver nanoparticles obtained after six hrs was calculated by using formula. At the time of beginning the reaction solution was containing 216ppm silver and it was reduced to 27 ppm after six hrs.

Silver obtained = 216 ppm - 27 ppm = 189 ppm

So,

$$\text{Total yield} = \frac{\text{Silver obtained in ppm}}{\text{Silver used in ppm}} \times 100$$

$$\text{Total yield} = \frac{189}{216} \times 100 = 87\%$$

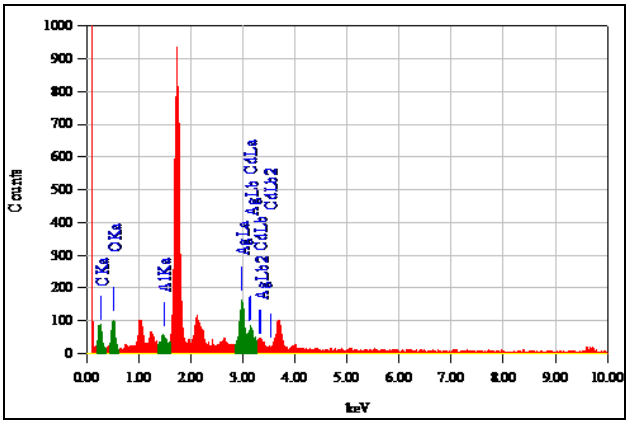

Fig. 3: EDAX spectra of silver nanoparticles

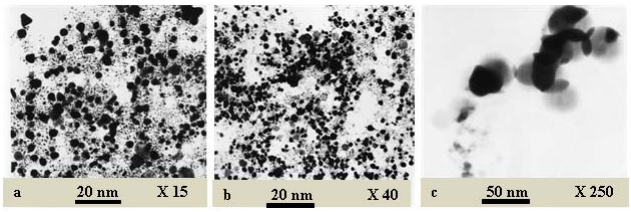

Fig. 4: TEM micrographs of silver nanoparticles at (a) X 15k, (b) X 40k and (c) X 250k

The SEM micrograph of silver nanoparticles showed spherical and some undefined shapes particles with mean size of 54nm (fig. 2). The formation of silver nanoparticles was further confirmed by EDX, which showed that there was strong signal in the silver region. Fig. 4 revealed the TEM micrograph of AgNPs. The size ranged from 10-100nm was observed at magnification of X 15K, X 40K and X 250K. The particles were mostly spherical. Although some other shapes were also observed.

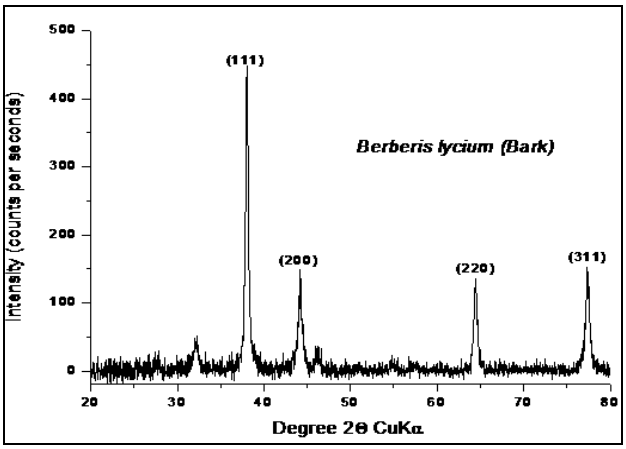

Fig. 5: XRD pattern of silver nanoparticles suggested crystalline nature

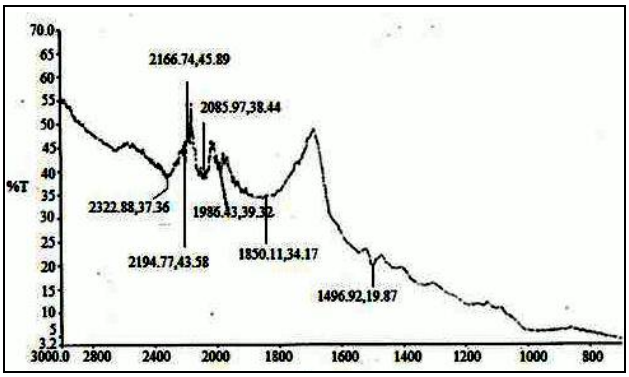

Fig. 6: FTIR spectrum of silver nanoparticles showed biosynthesized nanoparticles.

XRD image of AgNPs demonstrated number of Bragg's reflections at 38.0375, 44.2419, 64.4173 and 77.3023 corresponding to (111), (200), (220) and (311) that could be attributed to the crystalline face-centered cubic structure of silver nanoparticles and range in size from 24.93-39.67 nm (table 1). XRD pattern demonstrated the crystal structure of AgNPs (fig. 5).

For the determination of the compounds responsible for reducing and capping of silver nanoparticles synthesized from plant extract, FTIR was carried out at a resolution of 4 cm^{-1}. The bands at 2322.88, 2194.77, 2166.74, 1986.43, 1850.11 and 1496.92 cm-1 were observed in FTIR spectrum, which were specified for to C-H stretching of aldehyde, 2194.77 cm-1 and 2166.74 cm^{-1} were assigned to carbon carbon triple bond of alkynes or N-H stretching of free amino group, 1986.43 cm-1 for C=C asymmetric stretch, 1850.11 cm^{-1} for C=O stretching of aldehyde and the band at 1496.92 cm^{-1} was matched to C-C stretch (in-ring) or C-N of aromatic amino group or C=C of aromatic ring (fig. 6).

The antimicrobial activity results revealed that phyto-synthesized silver nanoparticles were very active against Gram-negative bacteria (*E. coli, K. pneumoniae, P. aeruginosa)* and Gram-positive bacteria *(S. aureus* and *B. subtilis)*. Table 2 showed the zones of inhibition of nanoparticles (10, 5, 2.5 and 1.25µg/ml), plant extract (10, 5 and 2.5µg/ml) and positive control (4mM $AgNO_3$). Silver nanoparticles showed highest activity against *K. pneumoniae* (14±0.58mm). The lowest antibacterial activity was reported against *S. aureus* (7.67±0.33). In case of minimum inhibitory concentration it was observed that silver nanoparticles with 10µg/ml concentration showed highest activity while 2.5µg/ml silver nanoparticles showed least inhibitory activity. However, 1.25 µg/ml silver nanoparticles did not show any inhibitory activity. Aqueous bark extract (10µg/ml) showed highest activity against *E. coli* and *P. aeruginosa* with inhibition zone of 10.67±0.33mm and 10.67±0.67 mm respectively. Aqueous extract of 10µg/ml displayed highest activity and 5µg/ml had minimum activity while aqueous extract of 2.5 did not inhibit the growth of any bacteria.

DISCUSSION

It is well known that excitation of surface Plasmon resonance in AgNPs shows yellowish - brown colour in aqueous solution (Thirumurgan *et al.,* 2010). The emergences of yellowish-brown colour in the reaction solution indicate the synthesis of AgNPs (Shankar *et al.,* 2004). The change in colour with the passage of time was due to conversion of silver ions to AgNPs, which was further confirmed by different techniques. Three different ways were suggested by Ahmad *et al*. (2011), which can participate in the reduction of silver ion in plant extract. At first place, the presence of secondary metabolites in the plant extract may be the reason of reduction of silver ions into silver nanoparticles. Second mechanism suggested that Nicotinamide adenine dinucleotide (NAD+), carrying electrons from one reaction to another. It is an oxidizing agent. It accepts electron from other molecules and becomes reduced. This reaction forms NADH, which can donate electrons. These electron transfer reactions are the main function of NAD:

$AgNO_3 \rightarrow Ag^+ + NO_3$
$NAD^+ + e^- \rightarrow NAD$
$NAD + H^+ \rightarrow NADH + e^-$
$e^- + Ag^+ \rightarrow Ag^0$

NAD^+ keeps on getting deoxidized and gets continuously renewed due to redox reactions. This might have reason of transformations of Ag^+ to Ag^0. The third mechanism is releasing of an electron as a result of formation of ascorbate radicals from ascorbate reduces the silver ions. FTIR studies support these results that chemical compounds present in root bark extract were reducing silver ions to silver nanoparticles.

UV-visible spectrum showed an absorbance peak for silver nanoparticles after 6 hrs which was due to excitation in surface Plasmon resonance of silver nanoparticles, which indicates the reduction of silver ions to silver nanoparticles. The broadening of the absorbance peak after 6 hrs indicated that silver nanoparticles were polydispersed. The decrease in silver ion concentration (FAAS analysis) indicated the conversion of silver ions into AgNPs. The studies of Singhal *et al*. (2011) indicated that the conversion of silver ions into AgNPs was completed in almost 8 min but in present work it was comparatively slow and took about six hours. It was also observed that rate of reduction of silver ions was very high during first two hours of reaction and then became slow. It is suggested that high reduction rate during initial hours was due to availability of bioactive components of extract. As the reaction proceeded, the bioactive components used led to a decrease in reduction rate. It can

Table 1: Size of silver nanoparticles obtained from bark extract of *Berberis lycium* calculated by Scherer's equation

Peak	2-Theta	d-spacing (Å)	FWHM*	Relative peak intensity (%)	Particle size (nm)
111	38.0375	2.36573	0.2214	100.00	39.67
200	44.2419	2.04730	0.3444	29.03	26.03
220	64.4173	1.44640	0.3936	28.71	24.93
311	77.3023	1.23331	0.3600	30.80	29.54

*Full-Width Half-Maximum

Table 2: Antimicrobial activity of bark extract, silver nanoparticles and positive controls

Bacterial species						
Zones of inhibition-mm (Means+ SEM)						
Agents	Conc. (µg/ml)	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*S. aureu*	*B. subtilis*
Bark extract	10	10.67±0.33	7.67±0.33	10.67 ±0. 67	9. 33±0. 67	9. 33±0.33
	5	8.33±0.33	--	8.67±0.33	7.33±0.67	7.67±0.33
	2.5	--	--	--	--	--
AgNPs	10	13.67±0.33	14±0.58	12.33±0.33	11.67±0.33	12.33±0. 33
	5	10.67±0.33	11.67±0.33	10.67±0.33	9.33±0.33	10.33±0.33
	2.5	8.33±0.33	8.67±0.33	9±0.58	7.67±0.33	8±0.58
	1.25	--	--	--	--	--
$AgNO_3$	4mM	14.67±0.33	13.33±0.33	13.67±0.67	14.33±0.67	13±0.58

also be suggested that biomolecules are involved in the capping of nanoparticles, so it might be the reason of slow reduction rate of silver ions after 3 hrs.

The SEM showed spherical and some undefined shapes particles, which is similar to previous work reported (Sathishkwar *et al.,* 2009). It was evident that Metallic silver nanocrystals usually show typical visual absorption peak in the region of 3 keV owing to surface Plasmon resonance (Magudapatty *et al.,* 2001). It has been noticed that other EDX peaks like Cd and Al were also formed in EDX spectrum (fig. 3). It is suggested that these peaks arose due to mixed precipitates in bark extract. This investigation demonstrated that the nano-structures were formed exclusively of silver. The present work is an agreement with earlier work done on silver nanoparticles synthesized from leaf extract of *Saururus chinenis* (Nagajyoti *et al.,* 2011). The results of TEM image were supported by Mahitha *et al.* (2011).

FTIR stretching vibrations showed that the bio-molecules such as alkaloids, flavonoids and phenols in bark extract were responsible of reducing, capping and stabilizing of silver nanoparticles. Present results are in accordance with the earlier study stating that the biological molecules played role in forming and stabilizing of silver nanoparticles (Sathyavathi *et al.,* 2010).

Biosynthesis of nanoparticles is a conventional process and the exploitation of extracts from plants has a new attentiveness for manage of disease, in addition being secure and no phytotoxic effects (Gardea-Torresdey *et al.,* 2003). It is already known that *Berberis* bark contains an alkaloid namely Berberine which has great antimicrobial potential. It clearly shows that increase in concentration of silver nanoparticles and bark extract also increases the inhibitory activity. Increase antimicrobial activity of AgNPs was due to large surface area to volume ratio. Higher activity of AgNPs than aqueous extract was due to composite of biomolecules with AgNPs. Silver nitrate demonstrated higher activity than AgNPs due to presence of ionic form of silver (Ag+). Silver ions are greatly inhibited the bacterial growth (Liau *et al*., 1997). Previous studies also confirmed that AgNPs synthesized by using plant extract had pronounced antibacterial activities (Reddy and Gandhi, 2012). It is interested to see that silver nanoparticles obtained from bark of *Berberis lycium* showed more antibacterial activity than aqueous extract of root bark of *Berberis lycium*. Silver nanoparticles anchor and penetrate to cell wall of bacteria, causing changes in the permeability of cell membrane leading to cell death. There is development of pits and accumulation of nanoparticles on the surface of the cell (Sondi and Sondi, 2004). Another mechanism of death of cells is considered to be formation of free radicals by the silver nanoparticles. Electron spin resonance spectroscopy suggested that when silver nanoparticles are in contact in bacteria they form free radicals. These free radicals change the cell membrane by making it porous. These changes in cell membrane ultimately lead to death of cells (Danilcauk *et al.,* 2006; Kim *et al.,* 2007). Recently silver nanoparticles are widely used in coatings, textiles and wood flooring as antibacterial agents. These biogenic synthesized silver nanoparticles showed high antibacterial activity, which may be used in these materials.

CONCLUSION

Nanotechnology is a rapid growing discipline not only in physics and chemistry but also in the field of biology. The

synthesis of AgNPs by using plant material is rapid, large scale and size and shape controlled process. It is concluded that the compounds present in root bark of *Berberis lycium* has the ability to reduce silver ions into stable silver nanoparticles at room temperature and synthesized AgNPs were very active against pathogens. This research throws light on future study on synthesis of silver nanoparticles from bark of *Berberis lycium* on the road to biomedical applications.

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