Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 1, January – March 2014, p. 325 - 332

GREEN SYNTHESIS OF SILVER NANOPARTICLE USING *MYRISTICA FRAGRANS* (NUTMEG) SEED EXTRACT AND ITS BIOLOGICAL ACTIVITY

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Green nanotechnology is generating attention of researchers toward ecofriendly biosynthesis of nanoparticles. In this study, the possible role of nutmeg extract in reducing silver nitrate into silver nanoparticle is highlighted. These biosynthesized nanoparticles were characterized with the help of UV-visible spectrophotometer, atomic absorption spectroscopy, X-ray diffraction, fourier transform infrared spectroscopy and transmission electron microscopy. It was observed that *Myristica fragrans* seed extract can reduce silver ions into silver nanoparticles within 12 min of reaction time. The surface plasmon resonance peak, centered near 410 nm, affirmed the reduction of Ag^+ to Ag^0. Stability of synthesized silver nanoparticles was analyzed using UV-visible absorption spectra. Transmission electron microscopy images show the presence of silver nanoparticles in the size range 7–20 nm. These biosynthesized silver nanoparticles were found to be stable for 3 months. Rapid and ecofriendly biosynthesis of stable silver nanoparticles was observed in this study. The crystalline nature of these biosynthesized silver nanoparticles was analyzed by X-ray diffraction studies. With the help of fourier transform infrared spectroscopy analysis the possible role of proteins and phenols as a reducing and capping agent was inferred. These silver nanoparticles synthesized from biological route also showed promising antibacterial effects against test microorganisms.

(Received January 8, 2014; Accepted February 28, 2014)

Keywords: Antimicrobial, Silver nanoparticles, *Myristica fragrans*, UV-visible spectrophotometer, Biosynthesis

1. Introduction

Metal nanoparticles have a high specific surface area and a high fraction of surface atoms. Because of the unique physicochemical characteristics of nanoparticles, including catalytic activity, optical properties, electronic properties, antibacterial properties, and magnetic properties, they are gaining the interest of scientist for their novel methods of synthesis [1]. Some of the engineered nanoparticles are being developed for various applications to replace the use of more hazardous chemicals [2]. Along with the increasing industrialization of engineered nanoparticles, the risks to human health and environment have also been a major concern. Researchers are now focusing on development of sustainable nanotechnology in terms of high benefits, low risk and social acceptance [3]. Silver nanoparticles can be synthesized using various approaches including chemical, physical, and biological routes. Although chemical method of synthesis requires short period of time for synthesis of large quantity of nanoparticles still this method has a disadvantage that it also requires capping agents for size stabilization of the nanoparticles. Thus, there is need to

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develop environmental friendly methods which are free from the use of toxic chemicals. Moreover, nanocrystalline silver colloids produced by such aqua-chemical routes exhibit aggregation with time, thereby compromising with the size factor upon storage [4]. Thus, there is an increasing demand for ‘‘green nanotechnology” for synthesis of stable nanoparticles. Microorganisms have already been reported as efficient bioreducing agent for metal nanoparticles [5]. Researchers are now focusing on use of plants and plant parts for green synthesis of nanoparticles considering the lower cost of production and easy handling as compared to the chemical routes. Using plant parts also reduces the issues related to elaborate microbial culture handling used in microbe mediated biosynthesis of silver nanoparticles [6]. Various biomolecules present in a wide variety of plant extracts have been found to be involved in the bioreduction of silver nanoparticles from Ag^+ ions like reductase enzymes present the cell of *Fusarium oxysporum* [7], proteins [8], terpenoids present in neem leaf extract [9], ascorbic acid present in leaf of *Ocimum sanctum* [10] and polyphenols [11].

Silver is well known for possessing an inhibitory effect toward many bacterial strains and microorganisms commonly present in medical and industrial processes [12]. In the field of medicines, silver and silver nanoparticles have a wide application including skin ointments and creams for infection on burns and open wounds, medical devices and implants with silver-impregnated polymers [7]. Silver has been used as an antimicrobial agent for over ages [13], and the increased surface area offered by the nanoparticle form of the metal offer great resistance to microbial growth [14]. In textile industry, silver-embedded fabrics are now used in sporting equipment [15]. Today, manufacturers add silver nanoparticles to hundreds of consumer products, including food storage containers, clothing, computer keyboards, cosmetics, pillows, cell phones, and medical appliances. The properties of these consumables can be further improvised on by using silver nanoparticles synthesized via the ecofriendly green routes.

Myristica fragrans, commonly known as nutmeg, belongs to the Myristicaceae family. *Myristica fragrans* is a common flavoring agent in Indian cooking. It also possesses medicinal and aromatic properties. It serves as an antidiarrhoeal, stomachic stimulant, carminative, intestinal catarrh and colic to stimulate appetite. The chemical composition of the seed of *Myristica fragrans* contains sugars, phenols, proteins, etc. [16]. Many bioactive compounds including camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, safrol, myristic acid, myristicin, and lignan were found in *Myristica fragrans.* The present study focuses on the use of the seed extract of medicinally important plant, *Myristica fragrans,* as a template for silver nanoparticles synthesis and to exploit their medicinal importance in terms of antimicrobial activity.

2. Materials and methods

Synthesis of silver nanoparticles

$AgNO_3$ was purchased from Qualigens fine chemicals, Mumbai, India. Fresh nut of M*yristica fragrans* (nutmeg) was collected from Amity University Uttar Pradesh, Noida, U.P., India; it was dried and powdered for subsequent extract preparations. 8 g of the dried nut powder was added to 100 mL of distilled water and stirred at 95ºC for 1 h. The solution was then cooled and filtered using Whatman paper No. 1 (pore size 25 μm) and then centrifuged at 8000 rpm for 10 min. The supernatant solution obtained after centrifugation was further used as the *Myristica fragrans* seed extract throughout. 5 mL of the extract was added to 45 mL of 10^{-3} M $AgNO_3$ solution for bioreduction process at 50ºC.

Characterization of silver nanoparticles

UV-visible spectroscopy

UV-visible spectroscopy was used to detect and confirm the presence of various ions in a given sample based on their optical absorbance peaks. As the size of the nanoparticles decreases, the band gap increases and thus the optical absorbance increases as compared to that of the bulk particles and therefore their color changes. To study the optical behavior of the biosynthesized silver nanoparticles, aliquots of samples were periodically analyzed every 2 min through UV-visible spectroscopic measurements (ELICO U.V. 165) at room temperature operated at a resolution of 1 nm between 250 nm and 800 nm.

Atomic absorption spectroscopy

Atomic absorption spectroscopy (AAS) was used to analyze the varying concentration of Ag^+ ions in the solution over a period of time (GBC 932 AA). The conversion of Ag^+ to Ag^0 can be inferred with this measurement. During the course of the reaction at regular intervals, the aliquots of samples were withdrawn and centrifuged at 14,000–15,000 rpm so that the supernatant solution would contain the unreacted silver nitrate (Ag^+ ions) for the reason that Ag^+ ions are much smaller than Ag^0 and the pellets will contain the Ag nanoparticles (Ag^0). The supernatant solution was then analyzed by AAS to detect the amount of Ag^+ ions. The rate of decrease in the concentration of the Ag^+ ions depicts the conversion of Ag^+ to Ag^0. Deionized water was used in this procedure as the precipitation of silver is highly sensitive to the presence of Cl^-.

Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out to study the size distribution and the shape of the nanoparticles based on the phenomena of transmittance of the electron beam through an ultra thin specimen. The transmitted electrons carried an image of the specimen which was further focused to analyze the shape and the size of the specimen. TEM measurements were performed on Philips model CM 200 instrument operated at an accelerating voltage at 200 kV with the samples loaded on carbon coated copper grids after sonication for 10 min.

X-ray diffraction measurements

X-ray diffraction (XRD) measurement was carried out to determine the crystallographic structure and crystallite size (grain size) based on the angle of diffraction of the X-ray beam by the atoms in the crystalline planes. For sample preparation the Ag nanoparticle solution was centrifuged at 14,000 rpm for 10 min and the pellets were redispersed in distilled water. The nanoparticles were then casted onto glass slides. XRD was performed on a eMMA diffractometer operating at a voltage of 40 kV and current of 20 mA with Cu K(a) radiation of 1.54187 nm wavelength. The scanning was done in the region of 2θ from 20° to 80° at 0.02°/min and the time constant was 2s.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) measurement was carried out to recognize the interaction between the Ag nanoparticles and the capping agent, from the nutmeg extract. For this, the biosynthesized Ag nanoparticle solution was centrifuged at 14,000 rpm for 15 min in order to remove any free biomass residue or compound that is not the capping ligand on the nanoparticles. The Ag nanoparticle pellet obtained after centrifugation was re-dispersed in water and washed with distilled water thrice. The dried samples were grinded with KBr pellets and analyzed on a Nicolet IR 200 (Thermo Electron Corp.) model.

Antimicrobial studies

Antimicrobial potential of biosynthesized silver nanoparticles was analyzed against gram negative *E. coli* and gram positive *S. aureus*. The biosynthesized silver nanoparticles were dispersed in autoclaved deionized water by ultrasonication and their various concentrations (20 µg/mL, 50 µg/mL and 100 µg/mL) were made. Active cultures of *E. coli* and *S. aureus* were prepared on Luria bernati (LB) medium. Bacterial inocula were added to 25 mL of LB broth containing different concentrations of silver nanoparticles in each flask kept on incubator cum shaker on rotation of 150 rpm. Flasks were maintained at 37°C and their bacterial growth was observed. $AgNO_3$ and seed extract of *Myristica fragrans* were also tested for antimicrobial activity. The growth of bacterial culture was measured using UV-visible spectrophotometer (ELICO 165) at 600 nm from 0 h up to 24 h. Further, minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of silver nanoparticles were also measured on *E. coli* and *S. aureus*. The MIC was determined as the lowest concentration that inhibited the visible growth of the used bacterium and MLC was determined as the lowest concentration that is lethal for the bacterium. Ciprofloxacin was taken as standard antibiotic in this study. The experiment was done in triplicate to maintain the accuracy of the results.

3. Results and discussion

UV-visible absorbance studies

It is generally recognized that UV-visible spectroscopy could be used to examine size and shape controlled nanoparticles in aqueous suspensions [17]. Here, seed extract of *Myristica fragrans* changed the color of silver nitrate solution from transparent to dark yellow brown due to the reduction of Ag^+ ions to Ag^0 within 12 min of the commencement of the reaction. These color change arise because of the excitation of surface plasmon vibrations with the silver nanoparticles [18]. The surface plasmon resonance (SPR) peak centered near 410 nm affirmed the reduction of Ag^+ to Ag^0. Similar SPR peak was also obtained from chemically synthesized polyvinyl alcohol capped silver nanoparticles reported by Patil *et al*. (2012) [19]. UV-visible absorbance of reaction mixture was taken after 12 min of the reaction commencement which further remained constant (Fig. 1a).

UV-visible absorbance of *Myristica fragrans* seed extract also showed absorbance at about 240 nm and 320 nm indicating the presence of proteins and phenols in the extract respectively (Fig. 1b). Absorption peak at around 320 nm (shown in Fig. 1b) disappeared during the reaction (shown in Fig. 1a) which indicates the involvement and role of phenols in the reaction.

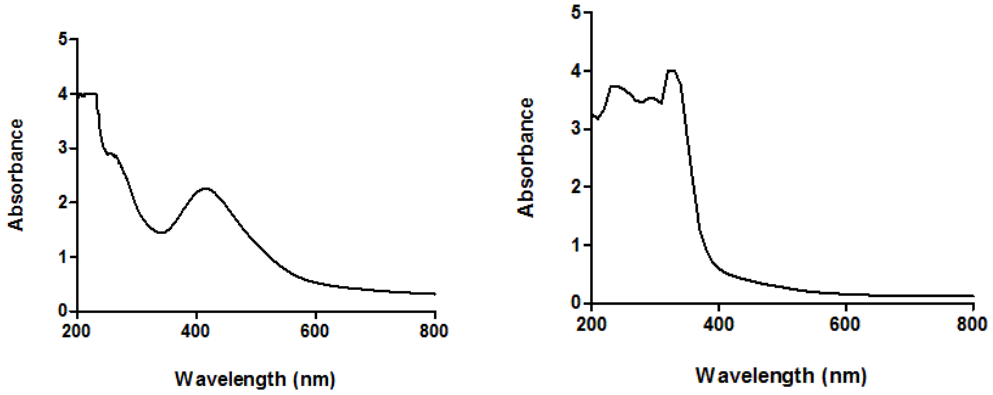

Fig. 1. UV-visible spectra of (a) aqueous solution of 10^{-3} M $AgNO_3$ with the Myristica fragrans extract (b) Myristica fragrans seed extract

AAS analysis

AAS analysis for the reacting solution done at regular intervals of time showed the conversion of Ag^+ ions to Ag^0. Initially, standard solution of 5.5 ppm of $AgNO_3$ was prepared and analyzed with AAS at 0 min. Now, Ag ion concentration in the reaction solution, after adding seed extract, was monitored at regular time intervals. The result showed decrease in concentration of Ag ions (5.5, 4.01 3.33, 2.91, 1.93, and 0.06 ppm at 0, 2, 3, 5, 8, 12 min, respectively) indicating the conversion of Ag^+ to Ag^0 (Fig. 2).

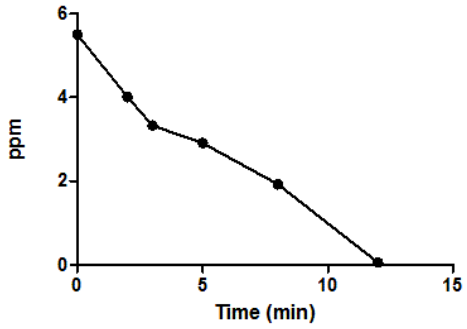

Fig. 2. Atomic absorption spectroscopy graph of Ag^+ concentration in reaction mixture

TEM analysis

The TEM images of the so prepared silver nanoparticles at 5 nm and 20 nm scales are shown in the Figure 3a and Figure 3b respectively. It was observed that Ag nanoparticles were predominantly spherical in shape with maximum particles in the size range 7–20 nm. Lanje *et al*. (2010), also showed the chemical synthesis of silver nanoparticles of 15.0 nm in size which served as an efficient antimicrobial agent [20]. Thus, our results indicate that the efficiency of seed extract of *Myristica fragrans* to synthesized silver nanoparticles in non toxic way is comparable to chemical synthesis route. TEM images also show even distribution of silver nanoparticles in the sample.

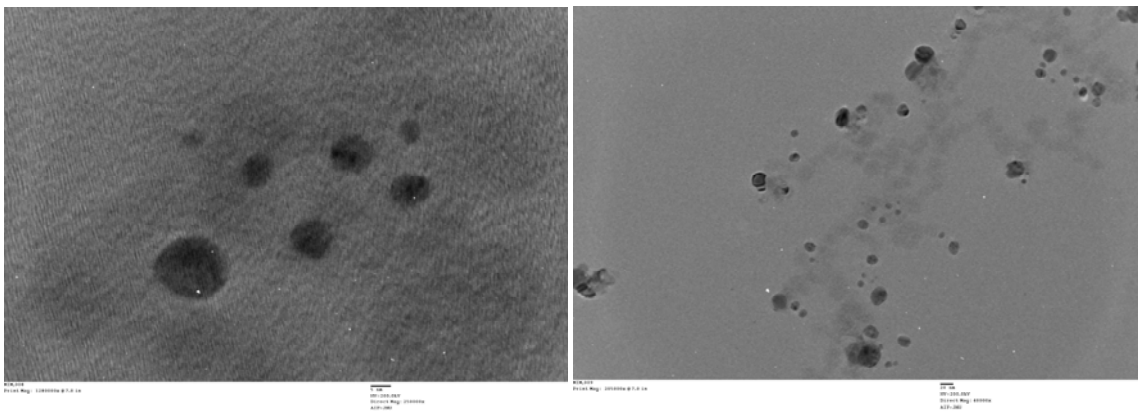

Fig. 3. TEM image of biosynthesized silver nanoparticles using Myristica fragrans seed extract (a) at 5 nm scale (b) at 20 nm scale.

XRD analysis

Intense peaks were observed at 38.18°, 44.38°, 64.48°, and 77.38° (Fig. 4), which corresponds to 111, 200, 220, and 311 Bragg's plane thus concluding that the silver crystallites are fcc structured (JCPDS card file no. 04-0783). Sharp peak of (111) with high intensity was observed depicting thin film formation on the substrate. The crystallite domain size was calculated from the width of the XRD peaks using the Scherrer's formula [21].

$$D = 0.94\,\lambda / \beta \cos\theta$$

where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle. The broadening of the Bragg peaks indicates the formation of nanoparticles. The average particle size estimated was 15 nm.

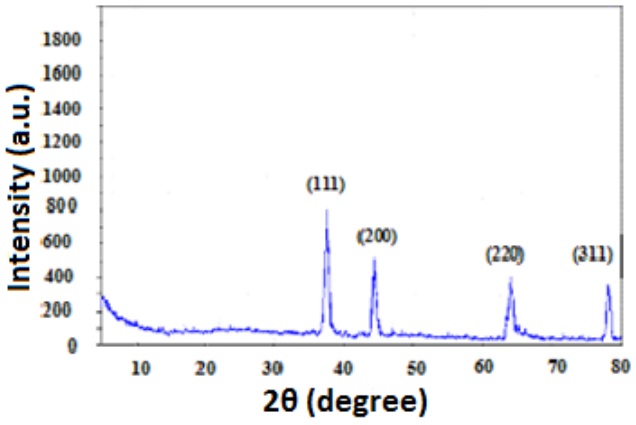

Fig. 4. X-ray diffraction pattern of biosynthesized silver nanoparticles using Myristica fragrans

FTIR Analysis

The FTIR measurements of biosynthesized silver nanoparticles were carried out to identify the possible interaction between protein and silver nanoparticles. Results of FTIR study showed sharp absorption peaks located at about 1635 and 3430 cm^{-1} (Fig. 5). Absorption peak at 1635 cm^{-1} may be assigned to the amide I bond of proteins arising due to carbonyl stretch in proteins, and peaks at 3430 cm^{-1} are assigned to OH stretching in alcohols and phenolic compounds [22]. The absorption peak at 1635 cm^{-1} is close to that reported for native proteins [23]. This evidence suggest that proteins are interacting with biosynthesized nanoparticles and also their secondary structure was not affected during reaction with Ag^{+} ions or after binding with Ag nanoparticles [24]. These IR spectroscopic studies confirmed that carbonyl group of amino acid residues have strong binding ability with metal suggesting the formation of layer covering metal nanoparticles and acting as capping agent to prevent agglomeration and providing stability to the medium [25]. Phenolic compounds belonging to the lignans group have been earlier reported to be capable of chelating with metallic elements to form complexes [26]. Thus, it can be concluded that hydroxyl and carboxyl groups present in phenolic compounds of the *Myristica fragrans* seed extract may inactivate silver ions by chelating and additionally suppressing the superoxide driven reaction, which is believed to be the most important source of reactive oxygen species (ROS). These results confirm the presence of phenols and proteins which may act as reducing and stabilizing agents for silver nanoparticles.

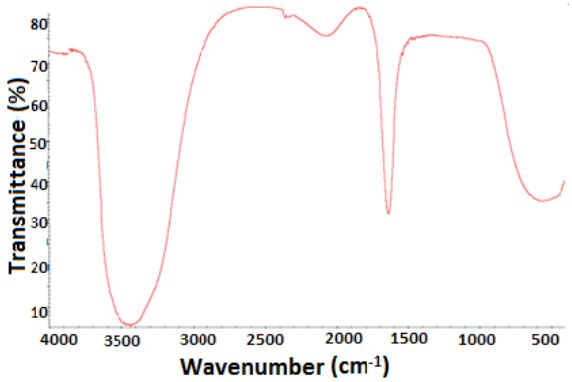

Fig. 5. FTIR spectrum recorded by making KBr disc with as synthesized silver nanoparticles prepared from the Myristica fragrans seed extract

Antimicrobial activity analysis

Antimicrobial activity of biosynthesized silver nanoparticles was analyzed against both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria at different concentrations. Both the test microorganisms were found to be resistant for the aqueous extract of *Myristica fragrans.* Earlier studies done by Munir *et al*. [27] also reported that the aqueous extract of *Myristica fragrans* showed no growth inhibition at any concentration on *E. coli* and *S. aureus*. Results showed that these silver nanoparticles reveal a strong dose-dependent antimicrobial activity against both gram negative and gram positive microorganisms (Fig. 6a and Fig. 6b). It was observed that the microbial growth decreases with the increase in concentration of biosynthesized silver nanoparticles. The MIC and MLC of silver nanoparticles against *E. coli* and *S. aureus* is shown in Table 1. It was clearly observed that the minimum inhibitory concentration and minimum lethal concentration of biosynthesized silver nanoparticles was significantly low as compared to standards like silver nitrate and standard antibiotic indicating stronger antimicrobial activity of biosynthesized silver nanoparticles which may be due to their increased surface area. Therefore, these silver nanoparticles can be used in low doses for antimicrobial treatment in comparison to standard antimicrobial agents. Also, because of the biological reducing and capping agents these silver nanoparticles are also environment friendly and non toxic in comparison to chemically synthesized silver nanoparticles.

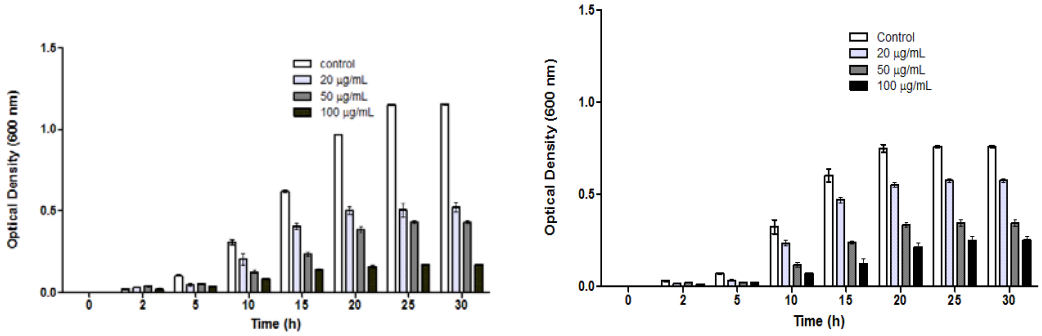

Fig. 6. Antimicrobial activity of silver nanoparticles prepared from the Myristica fragrans seed extract against (a) E. coli (b) S. aureus.

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC) of biosynthesized silver nanoparticles against E. coli and S. aureus

Microorganisms	Silver nanoparticles (µg mL^{-1})		Silver nitrate (µg mL^{-1})		Standard antibiotic (µg mL^{-1})	
	MIC	MLC	MIC	MLC	MIC	MLC
E. coli	0.628	1.25	2.5	2.5	1.25	2.5
S. aureus	0.314	1.25	2.5	2.5	1.25	2.5

4. Conclusion

This study shows that seed extract of *Myristica fragrans* can serve as a suitable reducing agent for biosynthesis of silver nanoparticles within 12 min of reaction time. The size of these nanoparticles was between 7 and 20 nm. Furthermore, the possible role of phenolic compounds and proteins are also concluded in bioreduction and capping process rendering them stability. Although this is an ecofriendly approach which can eliminate other methods of nanoparticle synthesis which involves toxic chemicals in the process, the mechanistic approach for nanoparticle synthesis is still unknown and needs to be explored further.

In this study the presence of phenolic compounds in association with silver nanoparticles are reported which indicate their possible role in the bioreduction mechanism. These bioreduced silver nanoparticles were found to be stable for 3 months which is a major advantage of using biological approach for nanoparticle synthesis. From FTIR spectra it was found that biomolecules responsible for capping and stabilization of silver nanoparticles are proteins present in the extract. This study also showed that biosynthesized silver nanoparticles using seed extract of *Myristica fragrans* have potent antimicrobial activities against *E. coli* and *S. aureus* cells. The slight variation in the number of cell deaths in case of *E. coli* and *S. aureus* is due to the fact that *E. coli* being a gram negative bacterial has higher amount of membranes as a barrier to be disrupted by Ag nanoparticles compared to that of *S. aureus*. Thus, this approach can be applied for rapid, cost effective and ecofriendly green synthesis of silver nanoparticle for industrial and medical application.

Acknowledgement

This research was supported by basic science research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, (2011-001-4792), grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea, (A121370), and by Hallym University Research Fund (HURF-2013-28). We also heartly acknowledge Dr. A. K. Chauhan for their support and providing facilities for the fulfillment of this study.

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LIST OF ACRONYMS

AAS	:	Atomic Absorption Spectroscopy
XRD	:	X-ray diffraction
FTIR	:	Fourier Transform Infrared Spectroscopy
TEM	:	Transmission Electron Microscopy
MIC	:	Minimum inhibitory concentration
MLC	:	Minimum lethal concentration