

Research article

## Biosynthesis of Silver Nanoparticles and Evaluation of its Antimicrobial Activity

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### Abstract

The current approach involves a novel green synthesis of silver nanoparticles by means of *Ocimum sanctum* and *Clitoria ternatea* leaf extract. Silver nanoparticles (AgNPs) were synthesized only in the aqueous leaf extract of *Ocimum sanctum*. The synthesized nanoparticles were characterized by UV-Visible Spectroscopy, XRD, SEM, TEM, and FTIR. TEM analysis showed that the size of the nanoparticles was in the range of 30-50 nm. The antimicrobial activity of green synthesized nanoparticles against different bacterial and fungal stains was assessed by Disc diffusion method. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* sp. and *Proteus* sp. was effectively inhibited by the silver nanoparticles but *Staphylococcus aureus* was not inhibited by nanoparticles. Antifungal activity of nanoparticles against *Candida albicans*, *Mucor* sp. and *Aspergillus* sp. showed minimal levels of inhibition. The study indicated that plant resources can be efficiently used in the production of silver nanoparticles and it could be utilized in various biotechnological and pharmaceutical fields.

**Keywords:** Silver nanoparticles, *Ocimum sanctum*, *Clitoria ternatea*, characterization, antimicrobial activity.

### Introduction

Silver is a soft, white, lustrous transition metal possessing high electrical and thermal conductivity. It has been known longer than the recorded history due to its medical and therapeutic benefits before the realization that microbes are agents for infections. It is used in many forms as coins, vessels, solutions, foils, sutures, and colloids as lotions, ointments, and so forth. It is the foremost therapeutic agent in medicine for infectious diseases and surgical infections. The benefits of silver are more than the risk factors (Alexander, 2009). Nanoscience is a new interdisciplinary subject that depends on the fundamental properties of nano size objects (Abou El-Nour et al., 2010). Nanoparticles possess wondrous optical, electronic, magnetic, and catalytic properties than the bulk material owing to their high surface area to volume ratio (Poulose et al., 2014). Metal nanoparticles like silver and gold show different colors due to their Surface Plasmon Resonance (SPR) phenomenon. It is a collective oscillation of free electrons of the metal nanoparticles in resonance with the frequency of the light wave interactions causing the SPR band to appear in the visible and infrared region (Parsons et al., 2007). Metallic nanoparticles are produced by various methods, the more common ones being chemical and physical methods. The aforesaid methods produce pure and well-defined nanoparticles, but the chemicals used in the synthesis are toxic, energy consuming, expensive, and not suitable for biological applications.

The syntheses of metal nanoparticles are coveted in the past three decades, but research on plant extract based nano synthesis mushroomed only in the last decade (Faramarzi and Sadighi, 2013; Ghaffari et al., 2014). Silver nanoparticles have received attention due to their physical, chemical, and biological properties that attributed to the catalytic activity and bactericidal effects and found applications in nanobiotechnological research (Sharma et al., 2009). They are used as antimicrobial agents in wound dressings, as topical creams to prevent wound infections (Tian and Wong, 2007) and as anticancer agents (Kaur and Tikoo, 2013).

An aromatic plant, the tulsi grows like a branched shrub with tender and oval leaves. *Ocimum sanctum* can grow up to 75 cms in height. The plant in India is available in two varieties; one having green leaves called the Lakshmi tulsi and the other with purple leaves, known as Krishna Tulsi. The plant's flowers bare a purple shade and its fruit resemble yellowish/reddish seeds. The leaves of this plant have an astringent/peppery taste and are hence also used for culinary purposes. *Tulasi* (Sanskrit:Surasa) has been used for thousands of years in Ayurveda for its diverse healing properties. *Tulasi* extracts are used in ayurvedic remedies for a variety of ailments. Traditionally, *tulasi* is taken in many forms: as herbal tea, dried powder, fresh leaf or mixed with *ghee*.

Essential oil extracted from *Karpoora tulasi* is mostly used for medicinal purposes and in herbal cosmetics. Some of the active chemical constituents of *tulasi* are oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool,  $\beta$ -caryophyllene (about 8%). *Tulasi* essential oil has been found to consist mostly of eugenol (70%)  $\beta$ -elemene (11.0%),  $\beta$ -caryophyllene (8%) and germacrene (2%), with the balance being made up of various trace compounds, (mostly terpenes).

*Clitoria ternatea*, also referred to as the butterfly pea, is a cognitive enhancer used in Ayurveda that is also known as *Shanka Pushi*. It is currently grown as a mixed ornamental, fodder, and medicinal plant. *Clitoria ternatea* is a tropical twining herb, growing wild and also in gardens, bearing white/pink blue flowers resembling a conch-shell (Rai, 2002). The roots, leaves, and stems are all frequently used in Ayurveda, but for slightly different purposes. The roots are most widely used and are bitter, refrigerant, laxative, intellect promoting, diuretic, anthelmintic and tonic and are useful in dementia, hemicrania, burning sensation, leprosy, inflammation, leucoderma, bronchitis, asthma, pulmonary tuberculosis, ascites and fever. The seeds are cathartic, while the leaves are used in otalgia and hepatopathy. The objective of present work was the biogenic synthesis of AgNPs using leaf extract of *Ocimum sanctum* and *Clitoria ternatea* and evaluation of their potential antimicrobial properties against various bacterial and fungal clinical isolates.

### Materials and methods

**Collection of plant leaves:** Healthy and bright greenish (*Clitoria ternatea* and *Ocimum sanctum*) leaves were collected for the separation of silver nanoparticles. These plants were collected from Biotechnology botanical garden of Arignar Anna College, Krishnagiri (Fig. 1 and 2).

**Optimization of silver nanoparticle production:** The synthesis of silver nanoparticles was optimized by using different concentration of  $\text{AgNO}_3$  solution viz., 1, 3 and 5 mM. The concentration of culture broth (1 and 5 mL) was also optimized.

**Synthesis of silver nanoparticles:** About 10 mL of the leaf extract was added to 100 mL of 1 mM  $\text{AgNO}_3$  solution and kept in room temperature up to the changing of color into reddish brown. Bioreduction of silver ions in the solution was monitored by measuring UV-VIS spectra of the solution at periodic intervals. The nanoparticle synthesized was confirmed by UV-VIS spectra plasma curve (Kutt et al., 2006).

**Recovery of silver nanoparticles by centrifugation:** After bioreduction, the solution consisting of hydrosols of silver

nanoparticles was subjected to centrifugation at 5000 rpm for 20 minutes and the supernatant was discarded. The pellet formed was dissolved in 0.1 mL of deionized water and air dried (Kutt et al., 2006).

Fig. 1. *Clitoria ternatea* in its natural habitat.



Fig. 2. *Ocimum sanctum* in its natural habitat.



**UV-VIS spectra analysis:** The bioreduction of  $\text{Ag}^+$  in aqueous solution was monitored by periodic sampling of aliquots (0.2 mL) of the suspension, then diluting the samples with 2 mL deionized water and subsequently measuring UV-Vis spectra, at the wave length of 300 to 700 nm. UV-Vis spectra were recorded at initial, 1 to 5 h.

**XRD measurement:** The air dried nanoparticles were coated onto XRD grid and analyzed for the formation of Ag nanoparticles by Philips X-Ray Diffractometer with Philips PW 1830 X-Ray Generator operated at a voltage of 40 kV and a current of 30 mA with Cu K $\alpha$  radiation. The diffracted intensities were recorded from 10° to 80° of 2 $\theta$  angles.

**SEM analysis:** After synthesis of nanoparticles, the sample was filtered through millipore filters of 0.2  $\mu\text{m}$  pore size, to remove any contaminants interfering with the SEM images. About 25 mL of the sample was pipetted out and loaded on a stub provided for SEM analysis. The stub is made of copper, in the shape of a small cylinder about the size of 1 cm dia. One side of the stub was stuck with double sided carbon material.

After loading the sample on the carbon material, the stub was fixed to a holder. The holder accommodates about 4 samples at a time.

**TEM analysis:** Appropriate sample preparation to obtain well-dispersed, isolated particles is a well-recognized, crucial step in the TEM analysis process. TEM analyses of samples were prepared and performed on a Philips CM-12 TEM with a 70 m lens operating at 100 kV and with a 2.0 Å point-to-point resolutions (Echlin *et al.*, 2011). Typically, TEM pictures of each sample were taken at 3 different magnifications (100, 430, and 580 K) in order to obtain information about the sample in general (100 K), plus a closer visualization of the clusters (580 K). A number of control experiments were performed previously which provided good evidence that the results are truly representative of the sample and that the sample is not otherwise perturbed by application of the TEM beam or depositing the sample as a drop and letting it dry did not change the results; controls showing that changing the beam voltage from 40 to 100 kV, or changing the exposure time (seconds vs. minutes), did not change the images; other controls have been done as well.

**FTIR analysis:** The dried Ag nanoparticles were subjected to FTIR analysis by KBr pellet (FTIR grade) method in 1:100 ratios and spectrum was recorded in Nicolet Impact 400 FT-IR Spectrophotometer using diffuse reflectance mode operating at a resolution.

**Antimicrobial activity of silver nanoparticles:** Silver nanoparticles synthesized using *Clitoria ternatea* and *Ocimum sanctum* samples were tested for its potential antibacterial and antifungal activity against few human pathogens i.e. Bacteria namely *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. Fungus namely *Candida albicans*, *Mucor sp.* and *Aspergillus sp.* were used as the test organisms. The paper disc (No. 1 Whatmann) was cut down into small discs (6 mm dia) and sterilized at 180°C for 30 minutes in hot air oven (Maheshwari and Dubey, 2002). After sterilization, the discs were impregnated with silver nanoparticles and the standard antibiotic solution. The disc was left standing for 1-4 h at room temperature for drying. The dried discs were placed on the surface of the pathogenic bacterial and fungal swabbed culture medium and incubated for about 18-24 h at 37°C. After incubation, the diameter of the circular inhibition zones was measured.

## Results and discussion

**Synthesis of silver nanoparticles by leaf extracts:** Addition of *Ocimum sanctum* and *Clitoria ternatea* biomass to a silver nitrate solution led to the appearance of yellowish brown color in the solution in 24-28 h, indicating the formation of silver nanoparticles (Fig. 3).

Fig. 3. Silver nanoparticles synthesized in *Ocimum sanctum* sample (A-*C. ternatea*, B-*O. sanctum* and S-Silver nitrate solution).

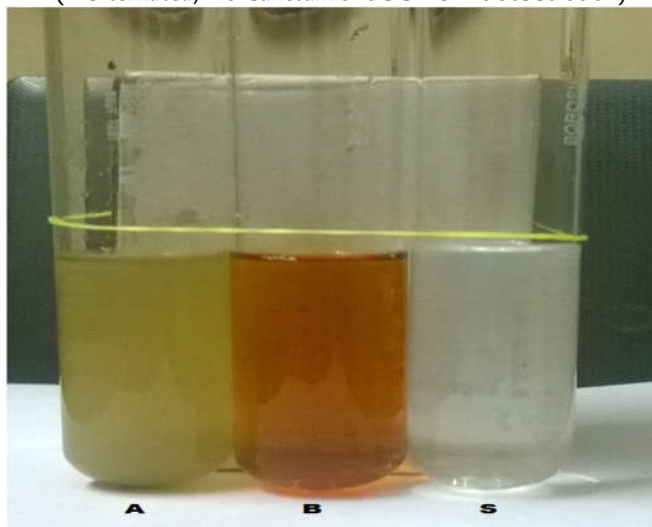


Fig. 4. UV-Vis spectra recorded after one week for the reaction mixture prepared using 1 mM silver nitrate and leaf extract of *O. sanctum*.

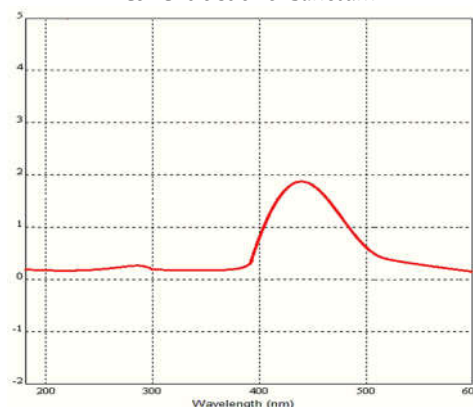
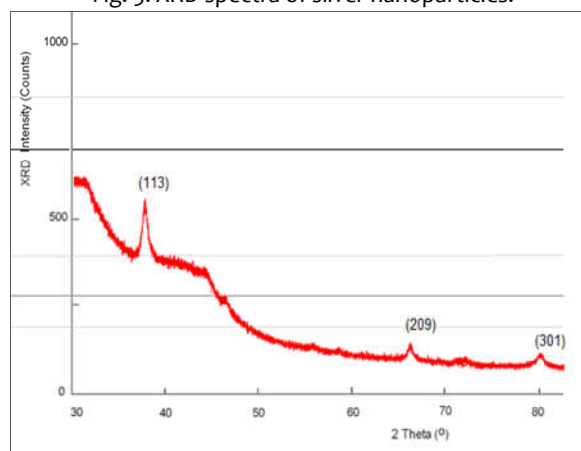


Fig. 5. XRD spectra of silver nanoparticles.





Unfortunately *Clitoria ternatea* did not produced any color formation indicating no synthesis of silver nanoparticles. Hence in this process, *O. sanctum* (B) tube was used for further analysis. The addition of leaf extract to aqueous solution of silver nitrate resulted in the color change of the solution and the color changed into dark cherry or dark yellow red due to the reaction of plant extract and silver nitrate solution. Reduction of silver ions into aqueous solution of silver nanoparticles was observed by UV-Vis spectroscopy (Garima *et al.*, 2011). This one is used to examine the concentration of the sample. Observed that the appearance of absorbance peak increased in intensity with time and also observed that the formation of silver nanoparticles in the sample. UV-Vis absorption spectra were monitored at 438 nm as shown in Fig. 4.

**XRD of silver nanoparticles:** The diffraction patterns presented in Fig. 5 correspond to the amorphous structure of samples. However, a number of Bragg's reflections corresponding to the fcc structure of silver are also seen here. Specifically, the XRD pattern shows three characteristic peaks corresponding to the (113), (209) and (301) sets of lattice planes. The XRD pattern thus clearly shows that the silver nanoparticles formed by the reduction of Ag<sup>+</sup> ions by *Ocimum sanctum* are crystalline in nature. It should be noted that the relative crystalline silver content of the *O. sanctum* biomass was not high, not more than 1%, which was at the sensitivity limit of the XRD analysis. This result conformed that silver nanoparticles were produced extracellularly.

**SEM of silver nanoparticles:** The cells of *Ocimum sanctum* were imaged by the SEM after the reaction with the silver nitrate solution for one week. The SEM images (Fig. 6) illustrate that most of the particles are spherical-like and do not create big agglomerates. The SEM results indicate that the process of formation of silver nanoparticles takes place on the surface of the cells. Similar phenomenon was reported by Yogeswari *et al.* (2012) as the SEM image showed relatively spherical shape nanoparticle formed with diameter range 0 to 50 nm. Reduction and surface accumulation of metals may be a process by which microorganisms protect themselves from the toxic effects of metal ions. In the plants, the metal components were used as induce metabolic reactions in the plants and plant leaves.

**TEM of silver nanoparticles:** Under TEM, the silver nanoparticles synthesized by *Ocimum sanctum* plant leaf extract were observed to have an average mean size of 30-50 nm corroborating well the DLS pattern (Sathyavathi *et al.*, 2010). One or two nanoparticles were appearing in the size of nearby 70 nm. The particles appeared to be spherical in shape with weak crystalline structure (Fig. 7).

Fig. 6. SEM image of nanoparticle produced by *Ocimum sanctum* (white color dots).

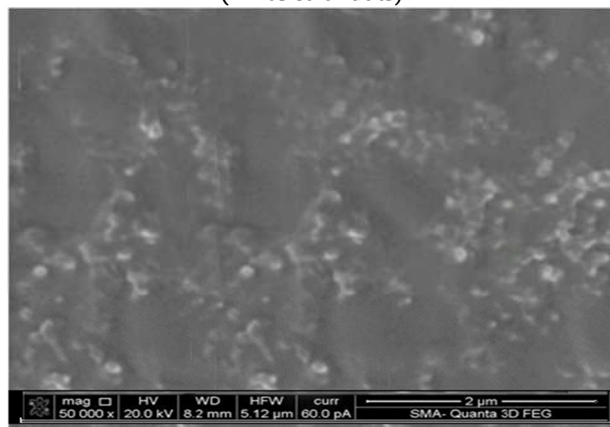
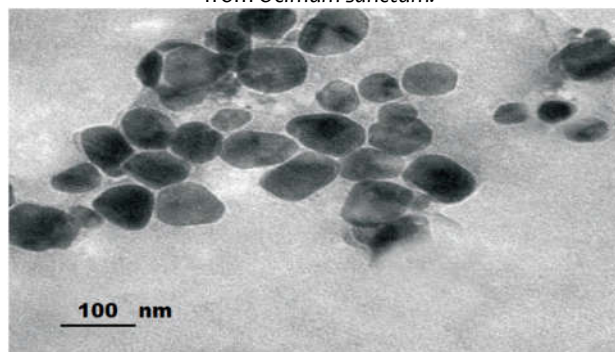


Fig. 7. TEM image of silver nanoparticles from *Ocimum sanctum*.



**FTIR spectral analysis:** The infrared spectra are recorded on Fourier Transform Spectrometer in the mid-infrared region (MIR) within the range (500-3500 cm<sup>-1</sup>). The purified nanoparticles exhibited absorption peaks at 712, 1058, 1322, 1640, 2598 and 3270 respectively cm<sup>-1</sup> due to cyclic C-O-C, C=O and OH functional groups, respectively. Thus, the nanoparticles were stabilized. FTIR shows the purified nanoparticles exhibited absorption peaks at 712, 1058, 1322, 1640, 2598 and 3270 respectively (Fig. 8).

Fig. 8. FTIR spectra of the *Ocimum sanctum* silver nanoparticles.

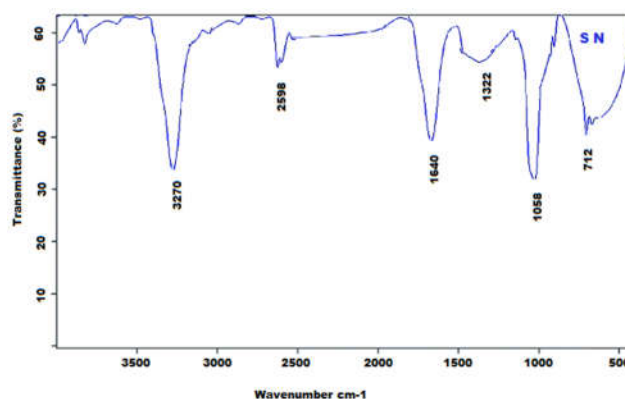


Table 1. Antimicrobial activity (zone of inhibition in mm) of silver nanoparticles from *O. sanctum* leaf extract.

Organism	Amoxyclave(A)	Amikacin(B)	Nanoparticles (SN)	Control (S)
<i>Escherichia coli</i>	30 mm	26 mm	27 mm	Nil
<i>Proteus sp.</i>	26 mm	23 mm	24 mm	Nil
<i>Pseudomonas aeruginosa</i>	22 mm	20 mm	19 mm	Nil
<i>Salmonella sp.</i>	24 mm	20 mm	21 mm	Nil
<i>Staphylococcus aureus</i>	15 mm	20 mm	15 mm	Nil

**Antimicrobial activity of silver nanoparticles:** The biologically synthesized AgNPs inhibited different pathogenic microorganisms. The resulting zones of inhibition formed were mainly due to the destabilization of the outer membrane of microbes by the silver nanoparticles. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella sp.* and *Proteus sp.* were effectively inhibited by the silver nanoparticles but *Staphylococcus aureus* was not inhibited by nanoparticles (Table 1; Fig. 9). According to the mechanism behind the bactericidal effect of the silver nanoparticles against bacteria is not well known. It has been proposed that AgNPs act similarly to the antimicrobial agents used for the treatment of bacterial infection by different mechanisms.

The sizes of zone inhibition are given below:

***Escherichia coli*:** Silver nanoparticles (Sn) produced 27 mm size of zone, Amoxyclave (A) produced 30 mm size of zone and Amikacin (B) produced 26 mm size of zone and control didn't produce any zone of inhibition.

***Proteus sp.*:** Silver nanoparticles (Sn) produced 24 mm size of zone, Amoxyclave (A) formed 26 mm of zone of inhibition and Amikacin (B) produced 23 mm mm size of zone and control didn't produce any zone of inhibition.

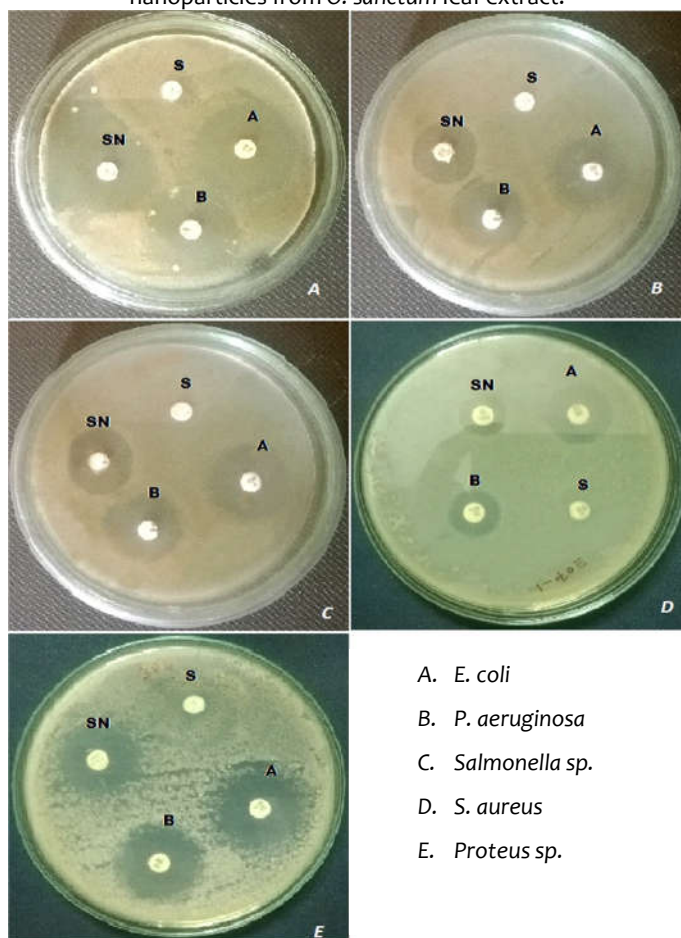
***Pseudomonas aeruginosa*:** Silver nanoparticles (Sn) produced 19 mm size of zone, Norflaxacin (A) formed 22 mm of zone of inhibition and Amikacin (B) produced 20 mm mm size of zone and control didn't produce any zone of inhibition.

***Salmonella sp.*:** Silver nanoparticles (Sn) produced 21 mm size of zone, Ampicillin (A) formed 24 mm of zone of inhibition and Tetracycline (B) produced 20 mm mm size of zone and control didn't produce any zone of inhibition.

***Staphylococcus aureus*:** Silver nanoparticles (Sn) produced 15 mm size of zone, Ceftazidime (A) formed 15 mm of zone of inhibition and Amikacin (B) produced 20 mm mm size of zone and control didn't produce any zone of inhibition.

**Antimicrobial activity of nanoparticles against fungal pathogens:** Fungus namely *Candida albicans*, *Mucar sp.* and *Aspergillus sp.* were used as test organisms for the evaluation of antifungal activity of silver nanoparticles.

Fig. 9. Antimicrobial activity (zone of inhibition in mm) of silver nanoparticles from *O. sanctum* leaf extract.



- A. *E. coli*
- B. *P. aeruginosa*
- C. *Salmonella sp.*
- D. *S. aureus*
- E. *Proteus sp.*

Silver nanoparticles recorded 24 mm of zone of inhibition against of *Candida albicans* and Clotrimazole (B) recorded 21 mm size of zone of inhibition. There is no zone of inhibition against *Aspergillus* and *Mucar sp.*

### Conclusion

This study showed that the examined leaf extract of *Ocimum sanctum* effectively produced silver nanoparticles when exposed to the silver compounds at specific concentrations.

The majority shapes of the silver nanoparticles were spherical and one or two appeared in big size. The size of nanoparticles was in the range of 30-50 nm. The described biosynthesis of nanoparticles was simple, economically viable and an eco-friendly process. The silver nanoparticles exhibited antimicrobial activity against human pathogenic bacteria but it does not act well against human pathogenic fungus except *Candida albicans*. The synthesized silver nanoparticles may be used in future to cure some specific diseases in people.

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