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ORIGINAL ARTICLE

A facile and rapid method for the black pepper leaf mediated green synthesis of silver nanoparticles and the antimicrobial study

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Abstract Green synthesis of nanoparticles is widely accepted due to the less toxicity in comparison with chemical methods. But there are certain drawbacks like slow formation of nanoparticles, difficulty to control particle size and shape make them less convenient. Here we report a novel cost-effective and eco-friendly method for the rapid green synthesis of silver nanoparticles using leaf extracts of *Piper nigrum*. Our results suggest that this method can be used for obtaining silver nanoparticles with controllable size within a few minutes. The fabricated nanoparticles possessed excellent antibacterial property against both Gram-positive and Gram-negative bacteria.

Keywords Biosynthesis · Silver nanoparticles · *Piper nigrum* · Reduction · Organic capping

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Introduction

Reducing the particle size of materials is an efficient tool for improving their bioactivity. A number of preparation methods have been described for the synthesis of metallic nanoparticles (Pal et al. 2007a; Rosemary and Pradeep 2003); such as, reverse micelles process (Xie et al. 2006; Maillard et al. 2002), salt reduction (Pillai and Kamat 2004), microwave dielectric heating reduction (Patel et al. 2005), ultrasonic irradiation (Salkar et al. 1999), radiolysis (Karim et al. 2007; Remita et al. 2007), solvothermal synthesis (Starowicz et al. 2006), electrochemical synthesis (Zhu et al. 2001; Liu et al. 2001) and biological techniques (Naik et al. 2002). The most widespread method of synthesis of metallic nanoparticles is based on the chemical reduction of metal salt solution by a reducing agent (Szczepanowicz et al. 2010). But the chemicals used in such processes are quite often toxic and flammable which make them unsuitable for many applications including biological applications like impregnation in wound dressings (Maneerung et al. 2008; Vivekanandhan et al. 2012) and sutures (Augustine and Rajarathinam 2012).

Biological syntheses of nanoparticles are not only a good way to fabricate benign nanostructure materials, but also to reduce the use or generation of hazardous substances to human health and the environment (Justin Packia Jacob et al. 2012). These biological methods are regarded as safe, cost-effective, sustainable and environment friendly as well as they do not require any special culture preparation and isolation techniques (Gardea et al. 2003). Biosynthesis of silver nanoparticles using microorganisms like bacteria (Saifuddin et al. 2009), fungi (Duran et al. 2009) and yeast (Kowshik et al. 2003) are already reported. However, exploitation of the plant extracts as the potential agents for the biosynthesis of nanoparticles has opened a

new way—the green synthesis of nanoparticles. Further, synthesis of silver nanoparticles using extracts of various plants like *Aloe vera* (Chandran et al. 2006), *Cinnamon zeylanicum* (Sathishkumar et al. 2009), *Stevia rebaudiana* (Varshney et al. 2010), *Papaya* (Jain et al. 2009), Geranium (Shiv Shankar et al. 2008), *Cassia angustifolia* (Peter Amaladhas et al. 2012), *Ocimum tenuiflorum* (Patil et al. 2012), lemon (Vankar and Shukla 2012), carob (Awwad et al. 2013), *Coleus aromaticus* (Vanaja and Annadurai 2012), were also reported.

Piper nigrum Linn. or Black pepper is a perennial woody climbing liana belonging to the family Piperaceae. It is native to India, Indonesia, Malaysia, South America and West Indies but is also widely cultivated in the tropical regions. It is considered as the 'King of Spices' (Srinivasan 2007; Mathew et al. 2001). The presence of a pungent alkaloid (Tripathi et al. 1996) piperine ($C_{17}H_{19}NO_3$) attributes a spicy taste to the seeds, leaves and other parts of *Piper* (Khajuria et al. 2002). It also contains small amounts of safrole ($C_{10}H_{10}O_2$), pinene ($C_{10}H_{16}$), sabinene ($C_{10}H_{16}$), limonene ($C_{10}H_{16}$), caryophyllene ($C_{15}H_{24}$) and Linalool ($C_{10}H_{18}O$) compound. The fruits have variety of activities including CNS depressant, antipyretic, analgesic, hepatoprotective (Lee et al. 1984), bioavailability enhancer (Zhao et al. 2007; Kasibhatta and Naidu 2007), antioxidant (Khajuria et al. 1997) and anti-inflammatory (Majumdar et al. 1990).

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reaction (Velioglu et al. 1998; Chanwitheesuk et al. 2005). From the *P. nigram*, a number of such compounds have been isolated and characterized. Alkaloids such as pellitorine ($C_{14}H_{25}NO$), (E)-1-[3',4'-(methylenedioxy)cinnamoyl] piperidine ($C_{15}H_{17}NO_3$), 2,4-tetradecadienoic acid isobutyl amide ($C_{16}H_{25}NO$), piperine ($C_{17}H_{19}NO_3$), cepharadione A ($C_{18}H_{11}NO_4$), piperolactam D ($C_{17}H_{13}NO_4$) and paprazine ($C_{17}H_{17}NO_3$) have been isolated from black pepper (Ee et al. 2009). Wei et al. (2004) isolated some amide-containing alkaloids from *P. nigrum*. They are Pipercycliamide ($C_{14}H_{26}NO_3$), (±)-*erythro*-1-(1-oxo-4,5-dihydroxy-2E-decaenyl)piperidine ($C_{15}H_{28}NO_3$), (±)-*threo*-1-(1-oxo-4,5-dihydroxy-2E-decaenyl)piperidine ($C_{15}H_{28}NO_3$), (±)-*threo*-*N*-isobutyl-4,5-dihydroxy-2E-octanamide ($C_{12}H_{23}NO_3Na$), 1-(1,6-dioxo-2E,4E-decadienyl)piperidine ($C_{15}H_{23}NO_2$), 1-1-oxo-3(3,4-methylenedioxy-5-methoxyphenyl)-2Z-propenylpiperidine ($C_{16}H_{19}NO_4$), and 1-1-oxo-5(3,4-methylenedioxyphenyl)-2Z,4E-pentadienylpyrrolidine ($C_{16}H_{17-}NO_3$) (Wei et al. 2004). A number of novel dimeric amide alkaloids possessing a cyclobutane or a cyclohexene ring termed as nigramides have also been isolated from the roots of *P. nigrum* (Wei et al. 2005). Shanmugapriya et al. (2012) extracted alkaloids, phenolic compounds, saponins and flavonoids from the leaves of black pepper. They have also shown the antioxidant potential of these compounds. These alkaloids may act as reducing agents, free radical scavengers or potential complexers of pro-oxidant metals (Hudson 1990). Thus as in the chemical reduction process, these natural reducing agents may act upon silver nitrate and reduce it into metallic silver nanoparticles.

Even though the biological synthesis of nanoparticles is advantageous in many aspects, the long duration needed for the formation of nanoparticles makes it inconvenient. Many workers tried to develop a method for the rapid biosynthesis of various kinds of nanoparticles but all these attempts took more than 15 min to get nanoparticles (Darroudi et al. 2010; Shenya et al. 2012; Philip 2010; Praveen Kumar et al. 2011; Bar et al. 2009). Further the controllability of the size of the nanoparticles was poor in previous reports. Thus in the present study, we report a green method for the synthesis of silver nanoparticles using an aqueous leaf extract of *P. nigram* and no toxic chemicals are used as reducing and stabilizing agents during the synthesis.

Materials and methods

Materials

Silver nitrate of analytical grade purchased from Sigma Aldrich was used as a starting material without further purification. The *P. nigram* leaves were freshly collected from the agricultural field near Kottayam, Kerala, India.

Preparation of the leaf extract

Around 5 g of freshly collected *P. nigram* leaves were grinded well using mortar and pestle. 100 ml of deionized water was added into the slurry and filtered through a cheese cloth. The filtrate was again filtered through Whatman No. 1 filter paper (pore size 25 μm). The fresh filtrate was used for the present study.

Synthesis of silver nanoparticles

1–5 mM aqueous solutions of silver nitrate ($AgNO_3$) was prepared in double distilled water and used for the synthesis of silver nanoparticles. 100 ml aqueous solution of the silver nitrate solution was taken in a round bottom flask and heated to boiling with a magnetic stirrer. The flask was covered with aluminum foil to prevent light-mediated reduction of silver nitrate. Then around 5 ml of the *P. nigram* leaf extract was added drop wise into the silver nitrate solution. During this process solution was mixed

vigorously. Within 1 min the color change was evident (pale red). Then it was removed from the heating mantle and stirred until cooled to room temperature. A portion of the obtained suspensions were used for UV–Visible spectroscopic analysis. Remaining part was centrifuged at 12,000 rpm several times in distilled water and finally in ethanol to get pure silver nanoparticles.

UV–Vis spectra analysis

The optical properties (absorbance) of colloidal solution were evaluated using a Shimadzu double-beam spectrophotometer (model 1700) at a resolution of 1 nm, between 200 and 600 nm. The reduction of pure Ag^{+} ions was monitored by measuring the UV–Vis spectrum of the reaction medium after diluting a small aliquot of the sample into distilled water.

FTIR (Fourier-transform IR) analysis

FTIR studies on the samples were carried out using Perkin Elmer, Spectrum 400 to ensure the formation of silver nanoparticles. FTIR measurements help to identify the possible interactions between silver and bioactive molecules, which may be responsible for formation and stabilization (capping material) of silver nanoparticles.

TEM analysis of silver nanoparticles

To understand the morphology and size distribution, the synthesized nanoparticles were imaged using JEOL JEM 2100 high resolution transmission electron microscope. The sample was prepared by air-drying drops of diluted solutions of the preparations on carbon films supported by copper grids. The particle size was measured using ImageJ software and the average particle size was determined.

XRD analysis

XRD was recorded in the 2θ range of 30–80° using D8-Advance of Bruker (Germany), of CuKα radiation, the energy of which was 8.04 keV and wavelength was 1.54 Å. The applied voltage was 40 kV and current was 25 mA.

Antimicrobial activity

The disc-diffusion assay Kirby–Bauer method (Pal et al. 2007b) was used by many workers to determine the growth inhibition of bacteria by the silver nanoparticles (Sadeghi et al. 2010; Augustine and Rajarathinam 2012; Amany and El-Rab 2012). The bacterial strains *Escherichia coli* (ATCC 12228) and *Staphylococcus aureus* (ATCC6538-P) were used as representatives of Gram-negative and Gram-positive bacteria, respectively. The bacteria were cultured in Mueller–Hinton Broth (MHB) at 37 °C and prepared to the turbidity equivalent to 0.5 McFarland standards (McFarland 1907). Then 100 µl of the bacterial suspension was spread on the nutrient agar. Sterile blank discs with 6 mm diameter (HiMedia, Mumbai) were impregnated with silver nanoparticles synthesized at different silver nitrate concentrations so that to get a final nanoparticle concentration of 10 µg/disc. These were then placed on the surface of the test plate. A standard antibiotic disc was used as positive control (Ciprofloxacin, 10 µg/disc). The culture plates were incubated for overnight in an incubator at 37 °C. The diameters of the inhibition zones were measured in millimeters (mm). The experiment was repeated for three times to get an average value.

Results and discussion

Color change

From the visual color change we can get preliminary information regarding the formation of silver nanoparticles. As the silver nanoparticles are formed, the color of the solution changes from white to pale yellow to brick red which is an indication of the presence of silver nanoparticles (Fig. 1). The variation of the color was due to the change in surface plasmon resonance of silver nanoparticles during the formation. At a silver nitrate concentration of 1 mM a very pale yellow color was obtained. While increasing the concentration of silver nitrate up to 5 mM, there is an increase in the redness of the solution. This is due to the fact that as the concentration of silver nitrate increases, aggregation of formed silver ions occurs and which leads to the formation of larger sized silver nanoparticles.

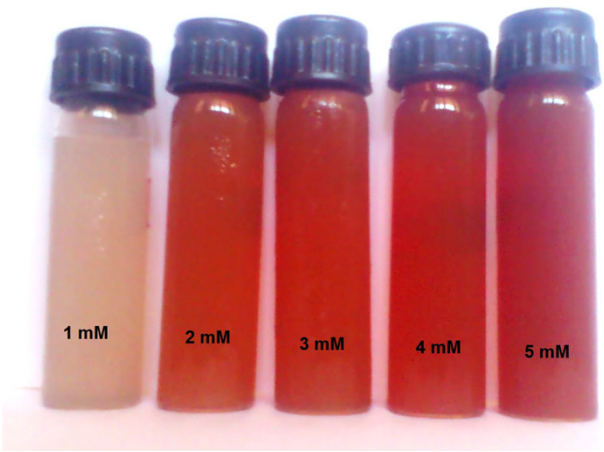

Fig. 1 Visible color change during the formation of silver nanoparticles at varying molar concentrations of silver nitrate

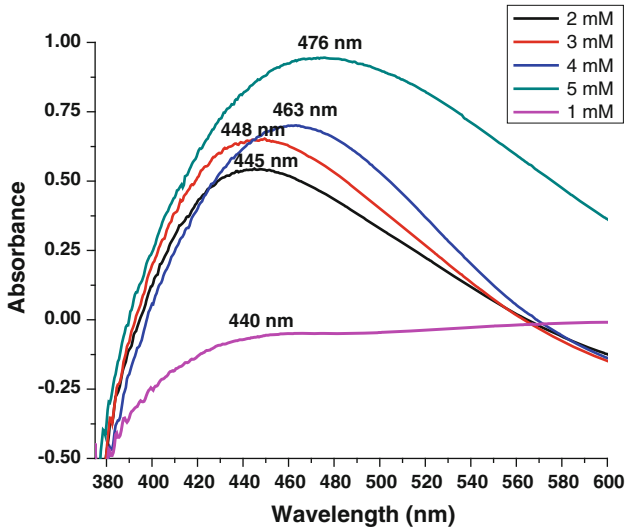

Fig. 2 UV–Visible absorption spectra of synthesized silver nanoparticles at varying silver nitrate concentrations

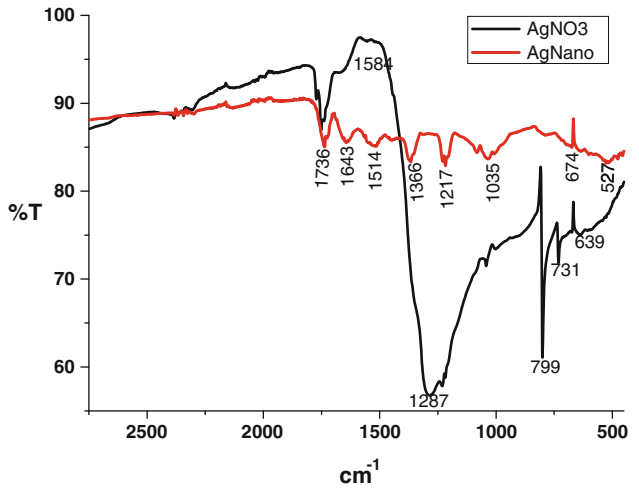

Fig. 3 FTIR spectra of silver nitrate and synthesized silver nanoparticles

UV–Visible spectrum

Characteristic absorption peaks of silver nanoparticles can be seen at around 400–475 nm. Figure 2 shows the UV–Visible spectra which are recorded after the completion of the reaction. For 1 mM solution, silver nanoparticles has shown absorbance maxima at 440 nm, 2 mM solution has peak at 445 nm, 2 mM solution has at 445 nm, 3 mM has at 448 nm, 4 mM has at 463 nm, and 5 mM solution has at 476 nm. Electrons are limited to specific vibrations modes by the particle's size and shape. The frequency and width of the surface plasmon absorption depend on the size and shape of the metal nanoparticles (Wang et al. 2007) as well as on the dielectric constant of the metal itself and the surrounding medium (Rai et al. 2006). Here the medium dielectric constant and temperature can be considered as the same but the mean diameter and morphology of the nanoparticles strongly affect the SPR band in aqueous solution (Wiley et al. 2006). The spectrum shows the red shift with increasing the molar concentration of silver nitrate. It indicates the increase of mean diameter of the silver nanoparticles as the concentration increases (Rai et al. 2006; Song and Kim 2009; Fayaz et al. 2009).

FTIR

The dried nanoparticle samples were analyzed in FTIR to identify the possible bio molecules responsible for the reduction of the Ag^+ ions by *Piper* leaf extract. The FTIR spectrum is presented in Fig. 3.

The FTIR absorption spectra of unreduced silver nitrate and silver nanoparticles after the reduction and capping by *Piper* extract were taken. While comparing the FTIR spectra of unreduced silver nitrate and biosynthesized silver nanoparticles, there is a notable reduction in certain peaks and appearance of certain new peaks was observed. Absorbance bands in pure silver nitrate were observed in the region of 450–1,750 cm^{-1} and are 1,584, 1,287, 799, 731 and 639 cm^{-1}. These absorbance bands are associated with the nitro compounds. A broad peak at 1,287 cm^{-1} which is present in the spectrum of silver nitrate is not found in the spectrum of silver nanoparticles. It indicates the loss of nitro group from silver species during the reduction process. Molecules containing NO_2 groups, such as nitro compounds, nitrates, and nitramines, commonly exhibit asymmetric and symmetric stretching vibrations of the NO_2 group at 1,660–1,500 and 1,390–1,260 cm^{-1} region. Apart from these losses of functional moieties, a number of new peaks found in the case of biosynthesized silver nanoparticles.

The FTIR spectrum of Ag nanoparticles showed distinct peaks 1,643 cm^{-1}, which represent the involvement of C–N in plane vibrations, 1,035 and 1,217 cm^{-1} shows the involvement of C–N in plane vibrations of aliphatic amines. The peak 1,514 cm^{-1} arises from N–H in-plane bending and C–N stretching modes. It may be due to the C–N bond present in the amide-containing alkaloids attached to the silver nanoparticles, which make the synthesized nanoparticles stable. Peak at 1,736 cm^{-1} is an indication of C=O Stretch and probably due to ketones. Further the presence of C–O Stretch in between 1,300 and 1,000 cm^{-1} may be due to the covalent linking of ester or ether groups to the nanoparticle. Esters show their carbonyl C=O stretch at 1,750–1,735 cm^{-1}, but also exhibit their characteristic absorption at 1,300–1,000 cm^{-1} from the couplings of C–O and C–C stretches. Thus, the peak at 1,035 cm^{-1} indicates the coupling of C–O and C–C stretches and 1,736 and

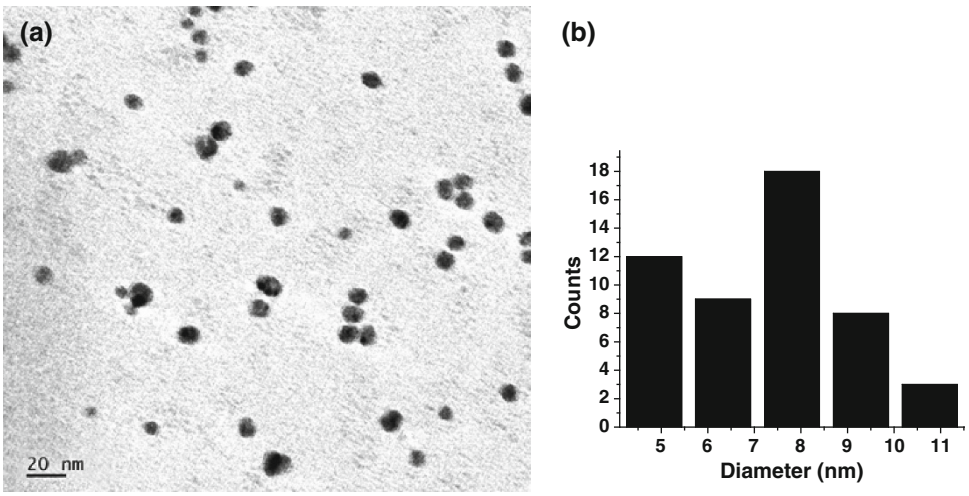

Fig. 4 TEM image of silver nanoparticles formed at 1 mM silver nitrate concentration (**a**) and the particle size distribution (**b**)

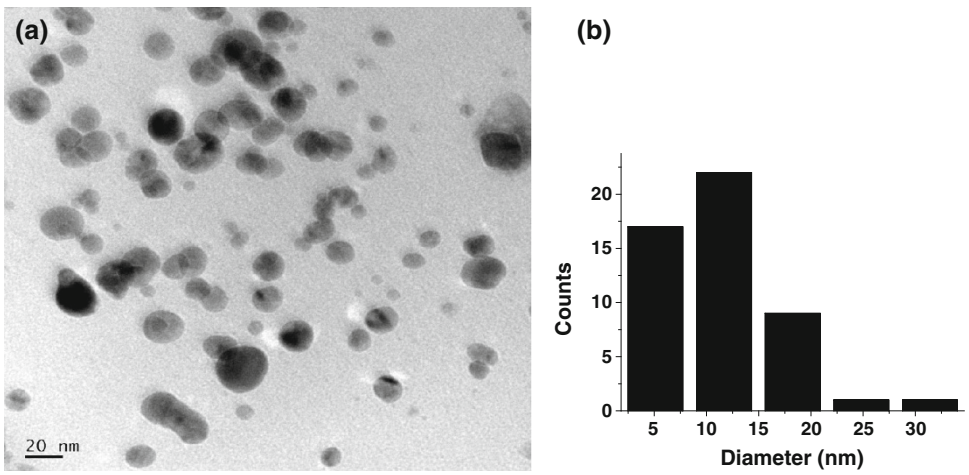

Fig. 5 TEM image of silver nanoparticles formed at 2 mM silver nitrate concentration (**a**) and the particle size distribution (**b**)

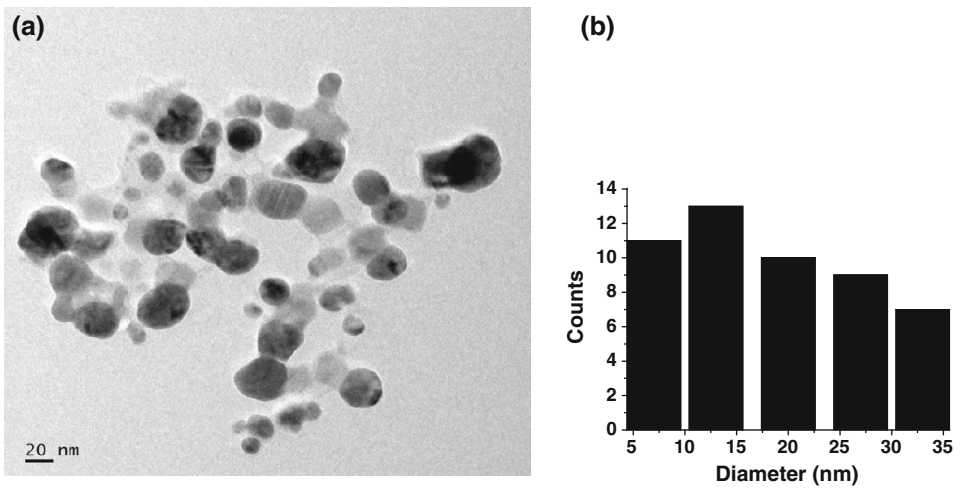

Fig. 6 TEM image of silver nanoparticles formed at 3 mM silver nitrate concentration (**a**) and the particle size distribution (**b**)

1,643 cm^{-1} indicates C=O stretch. Hence, there may be an ester link with the silver nanoparticle. These observations indicate the presence and binding of certain amide-containing compounds with silver nanoparticles. It may be due to the binding of one or more amide-containing alkaloids which is present in *Piper* like nigramides, *N*-isobutyl-4-hexanoyl-4-hydroxypyrrolidin-1-one, (±)-*erythro*-1-(1-oxo-4,5-dihydroxy-2E-decaenyl)piperidine, (±)-*threo*-1-(1-oxo-4,5-dihydroxy-2E-decaenyl)piperidine, (±)-*threo*-*N*-isobutyl-4,5-dihydroxy-2E-octanamide, 1-(1,6-dioxo-

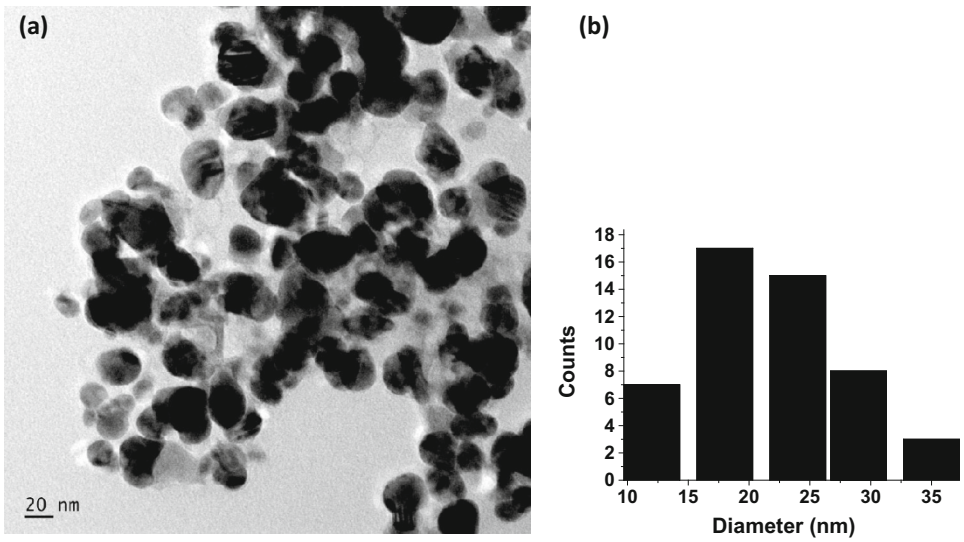

Fig. 7 TEM image of silver nanoparticles formed at 4 mM silver nitrate concentration (**a**) and the particle size distribution (**b**)

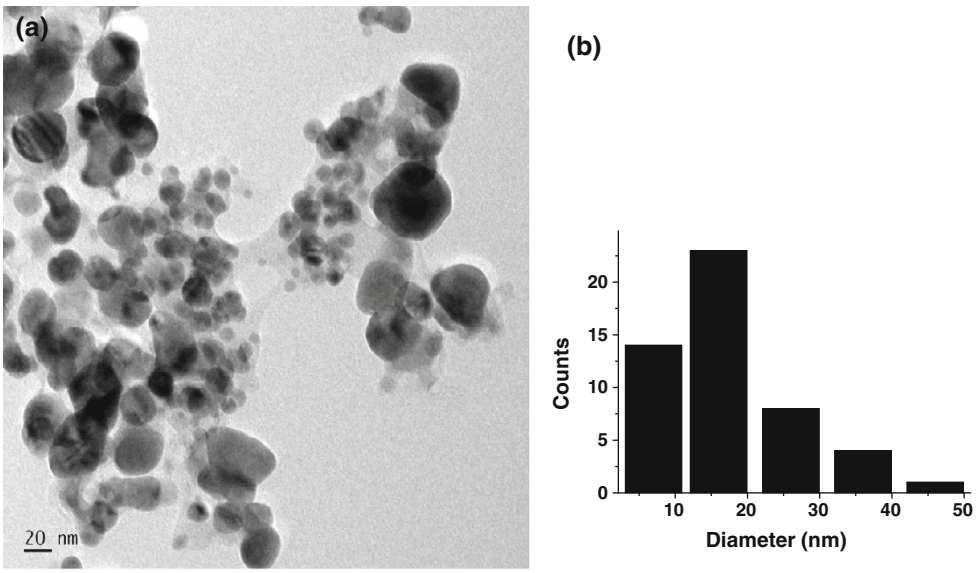

Fig. 8 TEM image of silver nanoparticles formed at 5 mM silver nitrate concentration (**a**) and the particle size distribution (**b**)

2E,4E-decadienyl)piperidine, 1-1-oxo-3(3,4-methylenedioxy-5-methoxyphenyl)-2Z-propenylpiperidine, 1-1-oxo-5(3,4-methylenedioxyphenyl)-2Z, 4E-pentadienylpyrrolidine, etc., to the synthesized silver nanoparticles. This may be the possible reason for the stability of synthesized silver nanoparticles.

TEM

Transmission electron microscopy provided a clear idea about the morphology and size of the synthesized silver nanoparticles. Comparison of TEM results has shown that the diameter of prepared nanoparticles in the solution was about 5–50 nm. Figures 4, 5, 6, 7 and 8 show the transmission electron micrograph of silver nanoparticles obtained from the proposed bioreduction method at various silver nitrate concentrations. At a silver nitrate concentration of 1 mM, silver nanoparticles with spherical morphology is obtained (Fig. 4). Each nanoparticle is individually remained without forming any agglomeration. The average particle size at this silver nitrate concentration is 8 nm. At a silver nitrate concentration of 2 mM almost similar morphology obtained as in the case of 1 mM concentration but the average particle diameter increased to 12 nm (Fig. 5). When the silver nitrate concentration was further increased, the size of the nanoparticles tends to decrease. Agglomeration of nanoparticles also observed at higher silver nitrate concentrations. At 3 mM silver nitrate concentration nanoparticles with almost spherical morphology were obtained with an average particle size of 18 nm (Fig. 6). The heterogeneity in particle size can be observed here. At 4 and 5 mM silver nitrate concentrations, the morphology of the nanoparticles becomes irregular higher tendency for agglomeration could be observed

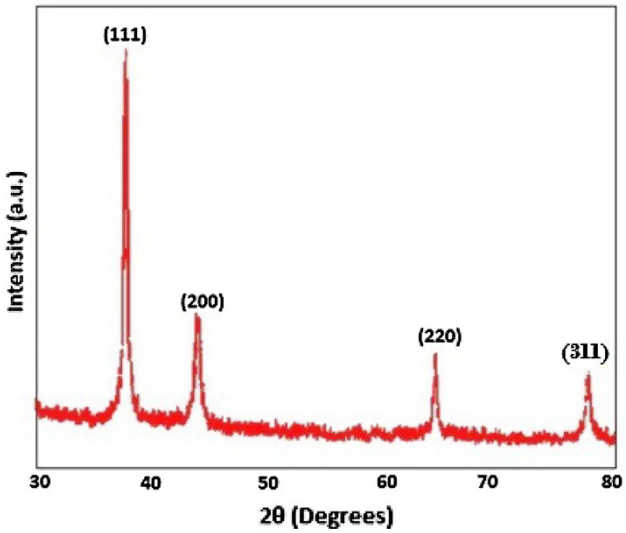

Fig. 9 Representative XRD pattern of synthesized silver nanoparticles at 2 mM silver nitrate concentration

(Figs. 7, 8). At 5 mM silver nitrate concentration the control over the particles size seemed to be completely lost and nanoparticles with varying diameters in the range of 5–50 nm obtained.

XRD

The XRD result (Fig. 9) shows that the silver nanoparticles formed are crystalline. A number of strong Bragg reflections can be seen which correspond to the (111), (200), (220), (311) reflections of face-centered cubic (fcc) silver. This is in agreement with the results obtained by other workers (Parikh et al. 2011; Theivasanthi and Alagar 2011; Martinez-Castanon et al. 2008). A sharp and strong diffraction peak centered at 38° was appeared, which can be indexed to the (1 1 1) reflection of the metallic silver with fcc structure (JCPDS File No. 04-0783). Comparably weak diffraction peaks at 44°, 66° and 77° in the pattern agree well with the (2 0 0), (2 2 0) and (3 1 1) reflections, respectively. The broadening of the peaks clearly indicates that the particles formed are in the nanoscale.

Table 1 Inhibitory zone diameter of disc diffusion assay

Samples (mM)	Inhibitory zone diameter (mm)	
	E. Coli	*S. aureus*
1	10.863	12.12
2	10.26	10.52
3	8.61	9.12
4	8.23	8.65
5	8.15	8.24
Positive control (Ciplofloxacin)	27.53	28.4

Antimicrobial activity

The disc diffusion assay or Kirby–Bauer method (Pal et al. 2007b), which is employed to assess preliminary antimicrobial activity of antimicrobial agents, was used to evaluate the antibacterial activity of silver nanoparticles synthesized at different silver nitrate concentrations. Silver nanoparticles synthesized at all the silver nitrate concentrations displayed antibacterial activity against both *E. coli* and *S. aureus* (Fig. 10). The diameter of inhibition zones around the disc containing silver nanoparticles in *E. coli* and *S. aureus* are given in Table 1. Nanoparticles prepared using 1 mM silver nitrate was the most efficient to inhibit both *E. coli* and *S. aureus*. Then there is a decrease in antibacterial activity as the concentration of silver nitrate increased. This can be correlated to the increase in particle size as the concentration of silver nitrate increased during synthesis. Antibacterial activity of silver nanoparticles was found to be dependent on the size of silver particles and as the size increases the antibacterial activity decreases (Panáček et al. 2006). All the synthesized nanoparticles have shown more antibacterial activity against *S. aureus*

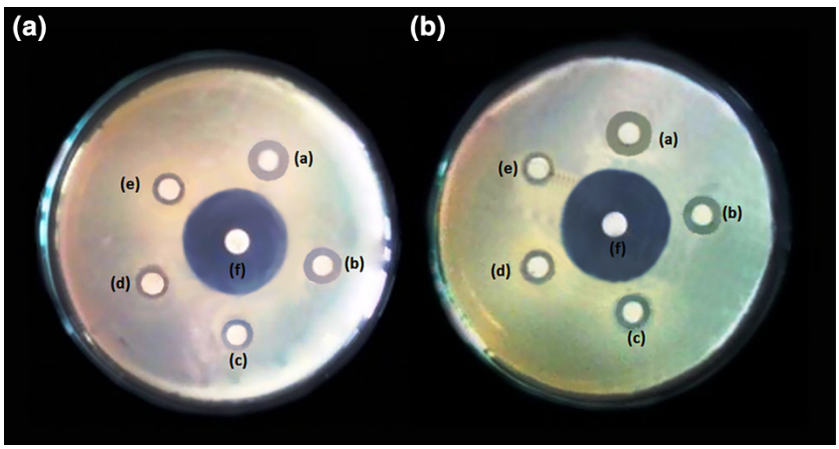

Fig. 10 Antimicrobial activity of synthesized silver nanoparticles against *E. coli* (plate **a**) and *S. aureus* (plate **b**). In both cases nanoparticles synthesized at 1, 2, 3, 4 and 5 mM silver nitrate concentrations are denoted by *a*, *b*, *c*, *d* and *e*, respectively. In both plates f denotes standard antibiotic disc (Ciplofloxacin)

than *E. coli* as reported by other workers (Sadeghi et al. 2010; Shrivastava et al. 2007), probably due to the difference in cell walls between Gram-positive and Gram-negative bacteria. Moreover, the synthesized nanoparticles enhance the therapeutic efficacy and strengthen the medicinal values of this plant. Antibacterial activity of black pepper (*P. nigrum* Linn.) and its mode of action on both Gram-negative and Gram-positive bacteria were already reported (Karsha and Lakshmi 2010).

Conclusions

A simple and facile process has been described for the preparation of silver nanoparticles starting from silver nitrate as a precursor and leaf extract of *P. nigram* as reducing as well as stabilizing agent. The characterization from UV–Visible, FTIR and TEM data supports the stability of the biosynthesized nanoparticles. The average particle size and morphology of the synthesized nanoparticles has been found to depend on the molar concentration of silver nitrate solution used. The FTIR studies indicate the capping of certain amide-containing compounds on the synthesized nanoparticles. The XRD analysis of the powder indicates that the synthesized nanoparticles are crystalline with fcc structure. The results obtained in this study are comparable with that obtained while chemical reducing agents like citrate of sodium or sodium borohydrate are used.

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