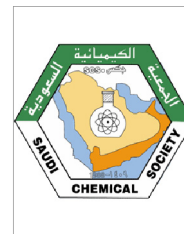




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ORIGINAL ARTICLE

Green synthesis of silver nanoparticles using olive leaf extract and its antibacterial activity



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Abstract The silver nanoparticles (AgNPs) synthesized using hot water olive leaf extracts (OLE) as reducing and stabilizing agent are reported and evaluated for antibacterial activity against drug resistant bacterial isolates. The effect of extract concentration, contact time, pH and temperature on the reaction rate and the shape of the Ag nanoparticles are investigated. The data revealed that the rate of formation of the nanosilver increased significantly in the basic medium with increasing temperature. The nature of AgNPs synthesized was analyzed by UV–vis spectroscopy, X-ray diffraction, scanning electron microscopy and thermal gravimetric analysis (TGA). The silver nanoparticles were with an average size of 20–25 nm and mostly spherical. The antibacterial potential of synthesized AgNPs was compared with that of aqueous OLE by well diffusion method. The AgNPs at 0.03–0.07 mg/ml concentration significantly inhibited bacterial growth against multi drug resistant *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*). This study revealed that the aqueous olive leaf extract has no effect at the concentrations used for preparation of the Ag nanoparticles. Thus AgNPs showed broad spectrum antibacterial activity at lower concentration and may be a good alternative therapeutic approach in future.

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1. Introduction

Biosynthesis of nanoparticles as an emerging highlight of the intersection of nanotechnology and biotechnology has received

increased attention due to growing need to develop environmentally benign technologies in material synthesis (Bhattacharya and Gupta, 2005). A great deal of effort has been put into the biosynthesis of inorganic material, especially metal nanoparticle using microorganisms and plants (Mohanpuria Rana et al., 2007; Farooqui et al., 2010).

Nanosilver has many important applications. It is used as an antimicrobial agent; it is applied in textiles, home water purification systems, medical devices, cosmetics, electronics, and household appliances (Wijnhoven et al., 2009). Besides their antimicrobial features, silver nanoparticles exhibit strong optical features making the nanoparticles suitable for

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biological sensing and imaging (Jain et al., 2008). Due to their high conductivity, silver nanoparticles are applied in conductive inks, adhesives and pastes for a range of electronic devices (Park et al., 2008). Silver nanoparticles are also used as catalysts in several chemical reactions such as the oxidation of styrene (Jiang et al., 2005; Xu et al., 2006).

Various strategies are employed for synthesis of silver nanoparticles (Tolaymat et al., 2010). Silver nanoparticles are synthesized by reduction in solutions (Guzmán et al., 2009), thermal decomposition of silver compounds (Navaladian et al., 2007), microwave assisted synthesis (Sreeram et al., 2008), laser mediated synthesis (Zamiri et al., 2011) and biological reduction method (Sastry et al., 2003). The latest is the most preferred way for the synthesis of nanoparticles as it offers one step, eco-friendly way of synthesis of nanoparticles.

A survey of earlier literature suggests that leaf extracts from various plants such as *Azadirachta indica* (Shankar et al., 2004), *Aloe vera*, (Chandran et al., 2006), *Bryophyllum* sp., *Cyperus* sp., *Hydrilla* sp. (Jha et al., 2009), *Gliricidia sepium*, (Raut et al., 2009), *Rosa rugosa* (Dubey et al., 2010), *Chenopodium album* (Dwivedi and Gopal, 2010), *Cycas* (Jha and Prasad, 2010), *Acalypha indica* (Krishnaraj et al., 2010), *Cassia fistula* (Lin et al., 2010), *Hibiscus rosa sinensis*, (Philip, 2010), *Ipomoea aquatica*, *Enhydra fluctuans*, *Ludwigia adscendens* (Roy and Barik, 2010), *Psidium guajava* (Raghunandan et al., 2011), *Garcinia mangostana* (Veerasamy et al., 2011), *Ocimum sanctum* (Philip, 2010), *Krishna tulsi* (*Ocimum sanctum*) (Philip and Unni, 2011), *Cocos nucifera* coir (Roopan et al., 2012), etc. have been explored for the synthesis of silver and gold nanoparticles.

The rate of reduction of metal ions using plants has been found to be much faster as compared to micro-organisms and stable formation of metal nanoparticles has been reported. The shape and size of the nanoparticles synthesized using plants can be controlled and modulated by changing the pH (Gardea-Torresedey et al., 2003).

The antibacterial effects of Ag salts have been noticed since antiquity and Ag is currently used to control bacterial growth in a variety of applications, including dental work, catheters, and burn wounds. In fact, it is well known that Ag ions and Ag-based compounds are highly toxic to microbes, showing strong biocidal effects.

The olive plant has been an important source of nutrition and medicine. The first formal report of medicinal use was made in 1854, when olive leaf extract (OLE) was reported to be effective in treating fever and malaria (Hanbury, 1854). OLE contains compounds with potent antimicrobial activities against bacteria, fungi, and mycoplasma (Juven and Henis, 1970; Aziz et al., 1998; Bisignano et al., 1999; Furneri et al., 2002). In addition, OLE has antioxidant (Ziogas et al., 2010; Caruso et al., 1999; Lee et al., 2009; Benavente-García et al., 2000) and anti-inflammatory (Visioli et al., 1998; de la Puerta et al., 2000) activities. Also, it was found that OLE inhibits acute infection and cell-to-cell transmission of HIV-1 and also inhibits HIV-1 replication (Lee-Huang et al., 2003).

The major active components in olive leaf are known to be oleuropein and its derivatives such as hydroxytyrosol and tyrosol, as well as caffeic acid, p-coumaric acid, vanillic acid, vanillin, luteolin, diosmetin, rutin, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside (Bianco and Uccella, 2000; Farag et al., 2003).

In the present work, we investigated the synthesis of stable silver nanoparticles with the bioreduction method using aqueous olive leaf extract and evaluated their antibacterial activity against drug resistant bacterial isolates. The work adds to the confirmation of previous reports on biosynthesis of nanometals using plant leaf extracts.

2. Experimental

2.1. Materials

Silver nitrate AgNO_3 was obtained from Sigma–Aldrich chemicals and used as received. Deionized water was used throughout the reactions. All glass wares were washed with dilute nitric acid HNO_3 and distilled water, then dried in hot air oven. 2.0 g of olive leaf broth was boiled for 15 min, filtrated and completed to 100 ml to get the extract. The filtrate used as reducing agent was kept in the dark at 10 °C to be used within one week. A stock solution of AgNO_3 2×10^{-2} M was prepared by dissolving 0.34 g/100 ml de-ionized water.

2.2. Instrumentation

The UV–vis spectra were recorded at room temperature using a λ -Helios SP Pye-Unicam spectrophotometer. Photoluminescence spectra were recorded on a Perkin Elmer LS 50B luminescence spectrophotometer. Transmission electron microscopy (TEM) studies were performed using a JEOL JEM 1200 electron microscope operating at an accelerating voltage of 90 kV. For the TEM measurements, a drop of a solution containing the particles was deposited on a copper grid covered with amorphous carbon. After allowing the film to stand for 2 min, the extra solution was removed by means of blotting paper and the grid allowed drying before the measurement. Fourier transform infrared (FTIR) spectra were recorded at room temperature on a Nicolet 6700 FTIR spectrometer. For the FTIR measurements of capped silver nanoparticles, a small amount of silver nanoparticles (0.01 g) dried at 60 °C for 4 h was mixed with KBr to form a round disk suitable for FTIR measurements. To obtain the FTIR spectrum of the extract, an appropriate amount of the extract was mixed with KBr. Thermogravimetric analyses were carried out with a heating rate of 10 °C/min using a Shimadzu DT-50 thermal analyzer. X-ray diffraction (XRD) pattern was obtained using a Shimadzu XRD-6000 diffractometer with Cu K α ($\lambda = 1.54056 \text{ \AA}$) to confirm the biosynthesis of AgNPs. Atomic absorption was used to confirm the amount of AgNPs formed for the concentrations used in antimicrobial assay.

2.3. Synthesis of silver nanoparticles

For the synthesis of the silver nanoparticles, a certain volume of the olive leaf extract (0.2–9) ml was added to the AgNO_3 solution and the volume was adjusted to 10 ml with de-ionized water. The final concentration of Ag^+ was 1×10^{-3} M. The solution was stirred for 2 min. The reduction process Ag^+ to Ag^0 nanoparticles was followed by the color change of the solution from yellow to brownish-yellow to deep brown depending on parameters studied such as the extract concen-

tration, temperature and pH. The nanoparticles were prepared at different pH values, the pH of the solutions was adjusted using 0.1 N H_3PO_4 or 0.1 N NaOH solutions.

2.4. Antibacterial assay

2.4.1. Clinical isolates

Three identified clinical isolates namely *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*) were supplied from Microbiology department, Faculty of Science, Ain Shams University, Cairo, Egypt. All bacterial clinical isolates were maintained routinely on nutrient agar slants (Oxoid) at 4 °C.

2.4.2. Preparation of Ag-Nps

Extraction of olive leaves was carried out using different amounts of the OLE (0.5, 1, 3, 4 and 5 ml) added to 0.5 ml of 3.4 mg/ml Ag^+ to form Ag-Nps and the solution stands for 24 h. The samples referred as Ag NP-1, Ag NP-2, Ag NP-3 and Ag NP-4 vs 0.5, 1, 3, 4 and 5 ml OLE, respectively.

2.4.3. Preparation of bacterial inoculum

A twenty-four hour nutrient broth culture of tested bacteria was grown in an orbital shaking incubator, centrifuged, washed twice with PBS and then standardized to approximately 10^6 CFU ml^{-1} using broth medium.

2.4.4. Bacterial sensitivity test

Standard well agar diffusion method was carried out to detect the activity of Ag-Nps against the clinical bacterial isolates according to Cheesbrough (2000). For antibacterial activities of the compounds, wells were made in plates containing nutrient agar medium seeded with 100 μl of 24 h of each clinical isolate. From each solution (1–5), that contains both Ag and olive leaves extracts (OLE), as well as the control, 100 μl was placed in separate wells. The plates were left in refrigerator for 2 h then, incubated at 37 °C for 24 h. The diameter of inhibition zones was measured and tabulated.

3. Results and discussion

3.1. UV-visible and TEM of Ag nanoparticles formed at room temperature

In order to monitor the formation and stability of silver nanoparticles, the absorption spectra of the synthesized silver nanoparticles were recorded against water. Fig. 1 shows the UV-visible spectra of silver nanoparticle formation using constant AgNO_3 concentration (1×10^{-3} M) with different extract concentrations at room temperature after 24 h. The color of the solutions changed from pale yellow to yellowish brown to deep brown depending on the extract concentration indicating silver nanoparticle formation as the color change observed is due to excitation of surface Plasmon vibration in the silver nanoparticles. It can be seen that the surface plasmon resonance (SPR) of AgNPs is 440–458 nm.

As the concentration of the olive leaf extract increases, the absorption peak gets more sharpness and blue shift was observed from 458 to 441 nm. This blue shift indicates a reduction in the mean diameter of the silver nanoparticles. The

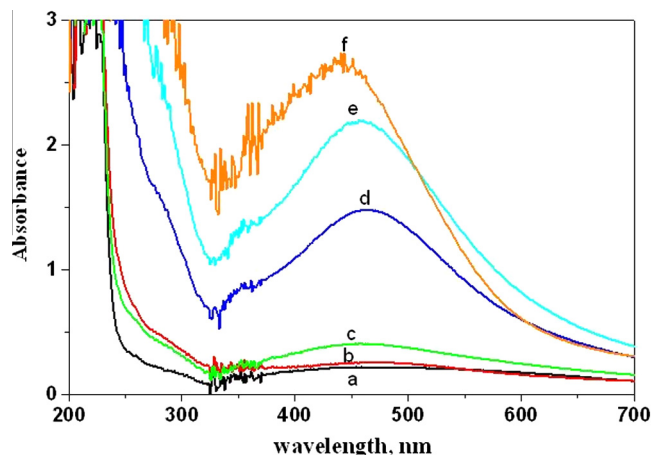


Figure 1 UV-vis spectra of silver nanoparticles at different concentrations of olive leaf extract (a, b, c, d and f refer to 0.2, 0.5, 3, 5 and 7 ml, respectively).

blue shifted and sharp narrow shape SPR band indicating the formation of spherical and homogeneous distribution of silver nanoparticles was observed. This was further confirmed by TEM images of leaf extract mixed samples using 1 and 5 ml extract at room temperature after 24 h incubation, Fig. 2. The results indicate that the average particle size of the synthesized silver nanoparticles is highly influenced by the concentration of leaf broth. Increasing leaf extract concentration in the reaction mixture decreases the particle size. At lower extract concentrations (1 ml extract), quasi spherical nanoparticles were formed with an average size of 30 ± 6 nm (Fig. 2a), together with some small particles in the range of 7–15 nm. On the other hand, at higher extract concentrations (5 ml), the majority of the Ag nanoparticles were in the range of 8–15 nm (Fig. 2b). This indicates that low quantities of the extract can reduce silver ions, but do not protect most of the quasi-spherical nanoparticles from aggregating because of the deficiency of biomolecules to act as protecting agents. On the other hand, at higher extract concentrations the biomolecules act as reducing agent and cap the nanoparticle surfaces protecting them from aggregation. Similar studies showed that a comparatively higher extract ratio is responsible for the synthesis of symmetrical nanoparticles (Sosa et al., 2003).

3.2. Effect of contact time at room temperature

The reaction between Ag^+ and the reducing material in the extract was followed for one week. Fig. 3 shows the UV-visible spectra of Ag nanoparticles as a function of time after addition of 3 ml olive leaf extract addition. Increasing the reaction time resulted in gradual increasing of absorbance spectrum with SPR at 446 nm and the color intensity increased with the duration of incubation. The intensity of the SPR peak increased as the reaction time increased, which indicated the increased concentrations of the silver nanoparticles. This result implies that the silver nanoparticle prepared by this green synthesis method is very stable without aggregation. After one week, the absorbance slightly decreased. It is pertinent to note that in previous studies the time span required for reduction of silver ions ranged from 24 to 48 h (Chandran et al., 2006; Lin et al.,

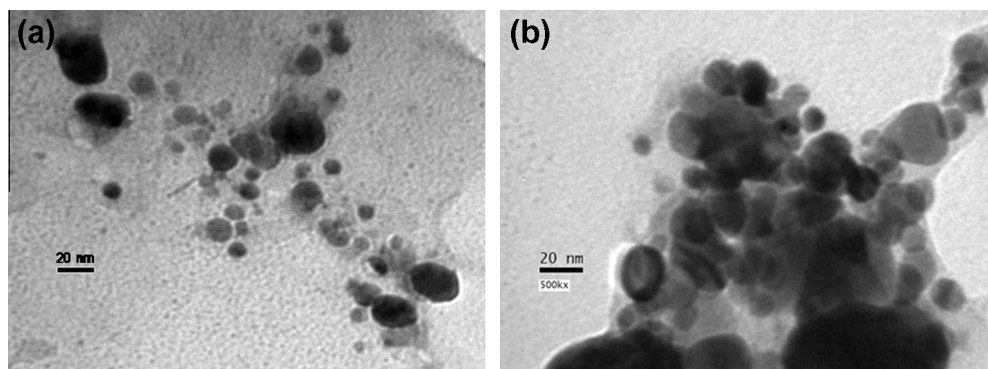


Figure 2 TEM micrograph of the silver nanoparticles: (a) the scale bar corresponds to 10 nm (inset: SAED pattern), and (b) the scale bar corresponds to 20 nm.

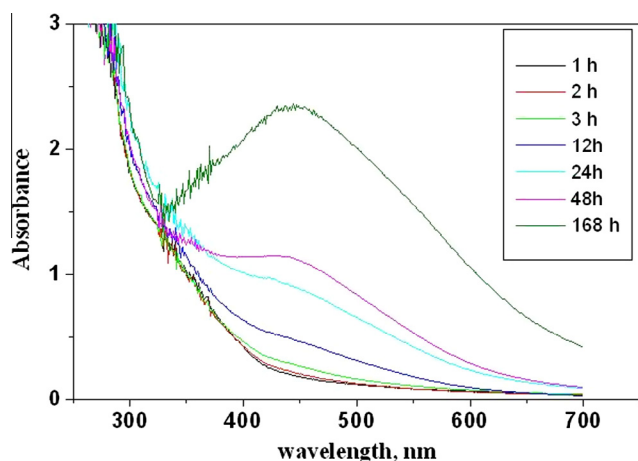


Figure 3 UV-visible spectra of Ag nanoparticles as function of time at room temperature (10^{-3} M AgNO_3 and 3 ml olive leaf extract).

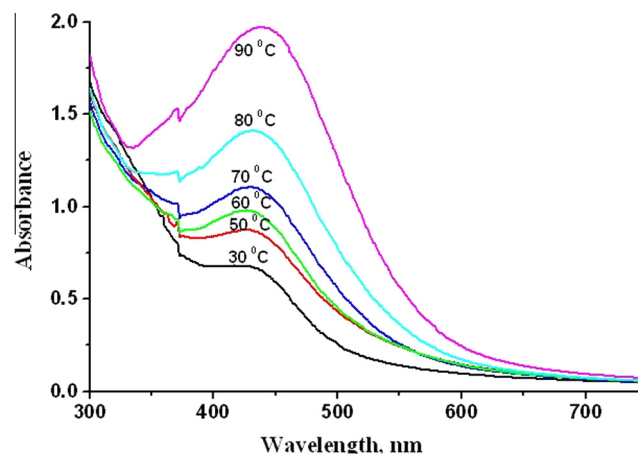


Figure 4 UV-vis spectra of Ag NPs as a function of temperature (10^{-3} M AgNO_3 and 3 ml olive leaf extract).

2010) or longer time as one week (Dipankar and Murugan, 2012; Bindhu and Umadevi, 2013).

3.3. Effect of temperature

Fig. 4 shows UV-visible spectra of the Ag NPs prepared at different temperature. It can be seen that the absorbance increases with increasing temperature. This experiment suggests that the slow rate of Ag NPs at room temperature can be accelerated by increasing temperature of the reaction mixture.

Increasing of the reaction temperature led to a rapid reduction rate of the Ag^+ ions and the subsequent homogeneous nucleation of silver nuclei-allowing for the formation of AgNPs with small size.

3.4. Effect of pH

Fig. 5 shows the effect of pH on formation of silver nanoparticles. It can be seen that absorbance increases with increasing pH from 2 to 8 and then decreases. Furthermore, it is observed that the brown color of the nanoparticles appeared shortly after mixing the AgNO_3 with the extract. In previous studies, it was shown that the size and shape of

biosynthesized nanoparticles could be manipulated by varying the pH of the reaction mixtures. A major influence of the reaction pH is its ability to change the electrical charges of biomolecules which might affect their capping and stabilizing abilities and subsequently the growth of the nanoparticles. The particle size is expected to be larger in acidic medium than in basic medium. This result was confirmed by the TEM measurement carried out at pH 3 and 8, Fig. 6. The size of the particles at pH 3 was larger than those at pH 8 with regular spherical shape in both cases. The alkaline pH environment enhanced the reducing and stabilizing capability of the antioxidants in the olive leaf extract. Since the reaction was faster at pH 8, it was necessary to follow the rate of the Ag NPs at pH 8 at room temperature, Fig. 7. It is clear that the rate of reaction increased and the reduction of Ag^+ to Ag^0 was completed in 52 min. The extract at pH 8 mediates number of the nuclei and thus the size of the resulting silver nanoparticles (LaMer and Dinegar, 1950; Goia, 2004). The number of the nuclei increased with elevated pH due to the promoted reactivity of the olive leaf extract reductant, thus absorption peaks of the products underwent blue-shift with increased pH as shown in Fig. 5 attributed to the decreased sizes of the silver nanoparticles.

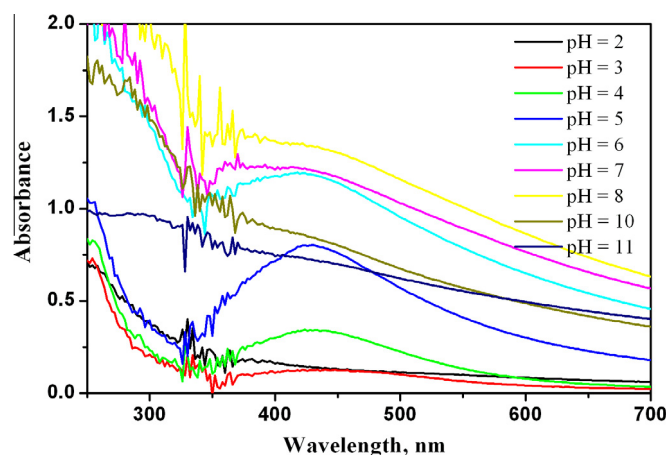


Figure 5 Effect of pH on the formation of Ag NPs at room temperature.

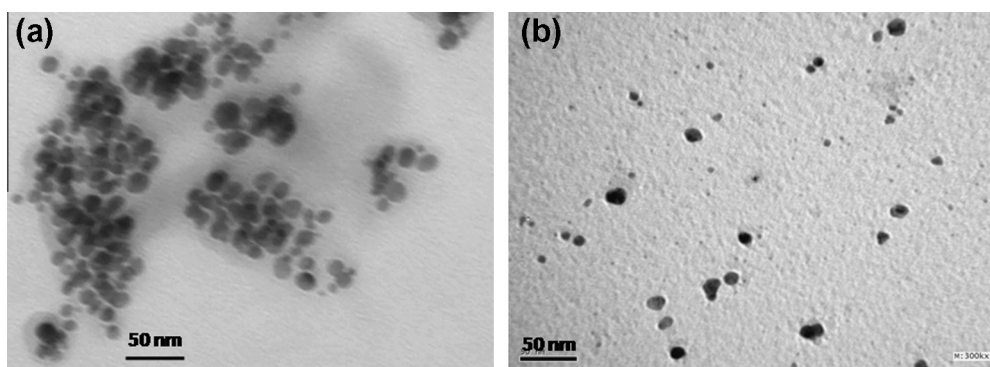


Figure 6 TEM micrograph of the silver nanoparticles: (a) at pH 3, the scale bar corresponds to 100 nm and (b) at pH 8, the scale bar corresponds to 50 nm.

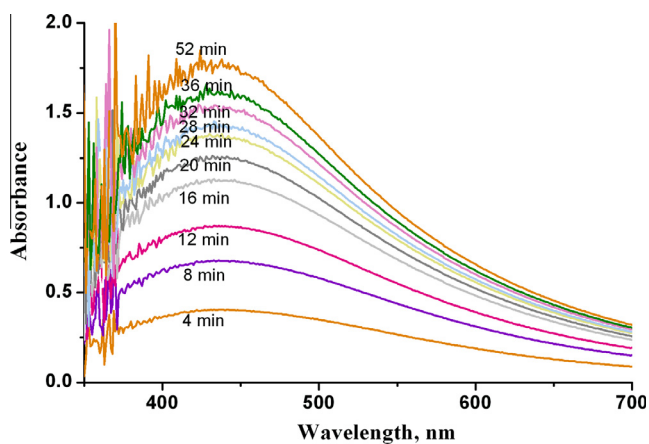


Figure 7 UV-visible spectra of Ag nanoparticles as function of time at room temperature (10^{-3} M AgNO_3 and 3 ml olive leaf extract, pH 8).

3.5. X-ray diffraction (XRD)

Fig. 8 shows the X-ray diffraction (XRD) patterns of dried silver nanoparticles synthesized using olive leaf extract at room temperature. The XRD patterns of Ag/extract indicated that

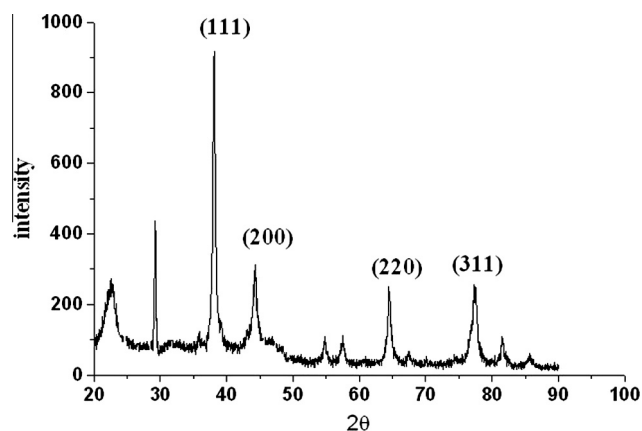


Figure 8 X-ray diffraction pattern of Ag nanoparticles prepared with aqueous olive leaf extract.

the structure of silver nanoparticles is face-centered cubic (fcc) (Shameli et al., 2010). In addition, the XRD peaks at 2θ of 38.17° , 44.31° , 64.44° , 77.34° and 81.33° could be attributed to the 111, 200, 220, 311 and 222 crystallographic planes. A peak was also observed at 2θ equal 22 suggesting that the crystallization of bio-organic phase occurs on the surface of the silver nanoparticles (Sathyavathi et al., 2010). Hence from

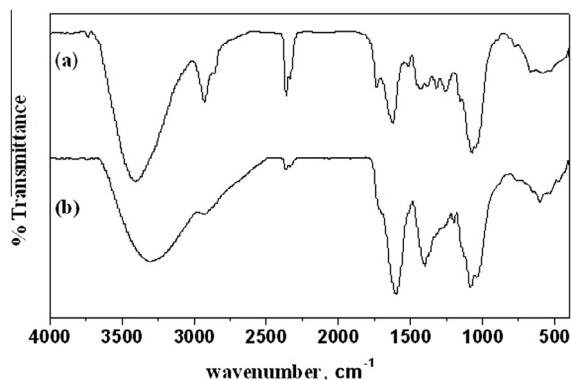


Figure 9 FTIR spectra of (a) a plain olive leaf and (b) capped AgNPs.

the XRD pattern it is clear that AgNPs formed using *olive leaf* broth were essentially crystalline.

The average nanocrystalline size has been estimated by using well known Debye–Scherrer formula, $D = k\lambda/\beta\cos\theta$, where D is particle diameter size, k is a constant equals 1, λ is wavelength of X-ray source (0.1541 nm), β is the full width at half maximum (FWHM) and θ is the diffraction angle corresponding to the lattice plane (111). The average crystallite size according to Debye–Scherrer equation calculated is found to be 51 nm slightly higher compared to the particle size obtained from the TEM image of AgNPs. This can be attributed to the slight deviation of the spherical shape of the particles that is required for the Debye–Scherrer formula.

3.6. Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of the olive leaf and Ag nanoparticles synthesized using olive leaf extracts was shown in (Fig. 9a and b). FTIR measurements were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized by leaf broth. The peak IR bands (Fig. 9a) observed at 3409 and 1733 cm^{-1} in dried olive leaf are characteristic of the O–H and C=O stretching modes for the OH and C=O groups possibly of oleuropein, apigenin-7-glucoside and/or luteolin-7-glucoside present in the olive leaf (Khalil et al., 2012). The medium band at 1624 cm^{-1} corresponds to amide I arising due to carbonyl stretch in proteins. The strong peak at 1077 cm^{-1} corresponds to C–N stretching vibration of the amine. The peak near 651 cm^{-1} is assigned to CH out of plane bending vibrations of substituted ethylene systems $-\text{CH}=\text{CH}-$.

In the case of nanoparticles, a large shift in the absorbance peak with decreased band intensity was observed from 3436 to

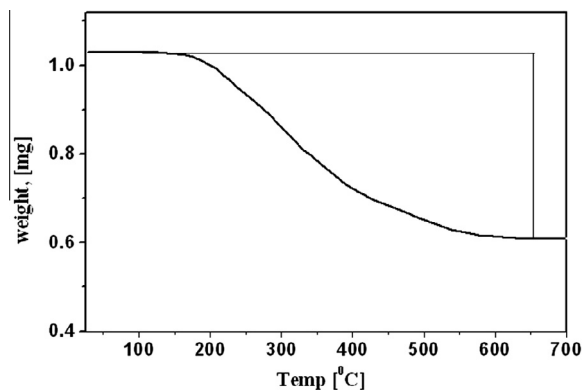


Figure 10 TGA of capped Ag NPs prepared using an olive leaf extract.

3395 cm^{-1} and 1420 to 1454 cm^{-1} , implying the binding of silver ions with hydroxyl and carboxylate groups of the extract (Khalil et al., 2012). The spectra also illustrate a prominent shift in the wave numbers corresponding to amide I (1651–1630 cm^{-1}) and amide II (1520–1537 cm^{-1}) linkages, validates that free amino ($-\text{NH}_2$) or carboxylate ($-\text{COO}^-$) groups in compounds of the olive leaf extract have interacted with AgNPs surface making AgNPs highly stable.

3.7. Thermal gravimetric analysis

The TGA plot of the capped Ag NPs prepared using 5 ml olive leaf extract (Fig. 10) showed a steady weight loss in the temperature range of 160–600 $^{\circ}\text{C}$. The weight loss of the nano powder due to desorption of bioorganic compounds in the AuNPs was 42.3%.

3.8. Antimicrobial assay

Silver ions as well as Ag Nps were known to have strong antimicrobial activities (Furno et al., 2004). The antibacterial activity of different solutions containing Ag Nps demonstrated that both Gram positive and Gram negative bacteria were inhibited by different solutions with different extents. The results of the antibacterial assay are depicted in Figs. 11 and 12. These results agreed with previous work carried out by Kim et al. (2007), Li et al. (2010, 2011) and Bindhu and Umadevi, (2013). The activity of these solutions was mainly due to the different amounts of Ag-Nps formed upon addition of different concentrations of OLE. This was confirmed by the UV as well as atomic absorption results. Atomic absorption measurements of the five solutions revealed that conversion values

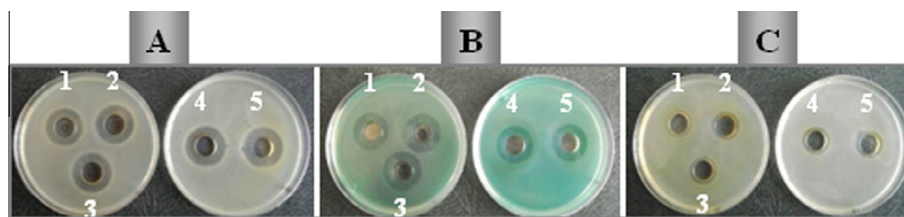


Figure 11 Antimicrobial activities of Ag-Nps against A: *S. aureus*, B: *P. aeruginosa* and C: *E. coli*. 1, 2, 3, 4 and 5 are solutions Ag-NPs1, Ag-NPs2, Ag-NPs3, Ag-NPs4 and Ag-NPs5, respectively.

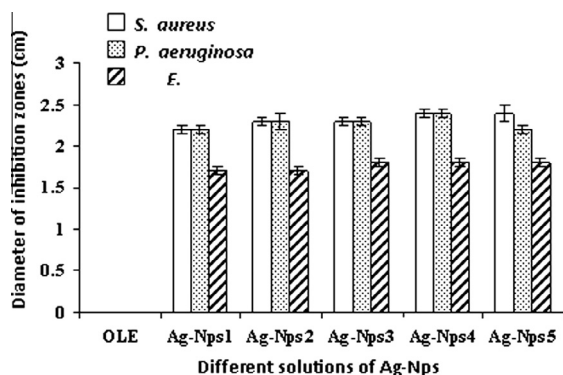


Figure 12 Antimicrobial activities of Ag-Nps from different solutions (1–5) against three clinical isolates. OLE: olive leaf extract and Ag-Nps: silver nanoparticles.

of Ag^+ ions to Ag^0 were 52%, 78%, 91.9%, 98% and 100% at 0.5, 1, 3, 4 and 5 ml OLE, respectively. The Gram negative bacteria *E. coli* was less sensitive to Ag Nps compared with *S. aureus*. This was similar to those found by Sondi and Salopek-Sondi (2004); however, Kim et al. (2007) showed that *S. aureus* was less affected by Ag Nps compared with *E. coli* even in high concentrations. This was due to the characteristics of certain bacterial species (Kim et al., 2007). Maximum activity on all bacteria was detected with solution 4 that showed highest UV absorption indicating formation of high amounts of Ag-Nps. The difference in sensitivity of Gram positive and Gram negative bacteria to Ag-Nps was due to the difference in thickness and constituents of their membrane structure (Kim et al., 2007).

Several studies propose the mechanism(s) of the bactericidal action of Ag-Nps. Kvitek et al. (2008) suggested that Ag-Nps may attach to the surface of the bacterial cell membrane via interacting with sulfur containing proteins (Feng et al., 2000), disturbing permeability and respiration functions of the cell resulting in cell death. In addition, the more bactericidal effect of solutions (Ag-OLE) containing higher amounts of OLE could be explained on the basis of smaller Ag-Nps having extremely large surface area that provides better contact and interaction with bacterial cells than the larger ones (Kvitek et al., 2008). This explanation was supported by the TEM results obtained in this work. It is also possible that Ag-Nps not only interact with the surface of membrane, but can also penetrate inside the bacteria (Morones et al., 2005). The action of Ag-Nps on the bacteria was also due to the interaction with thiol group compounds found in the respiratory enzymes of bacterial cells thus inhibiting the respiration process in bacteria (Klasen, 2000, Li et al., 2010, 2011). Moreover, Li et al. (2011) showed that Ag-Nps entered into bacteria cells and condensed DNA as a result preventing DNA from replication and cells from reproduction.

4. Conclusions

Quasi-spherical silver nanoparticles were synthesized using olive leaf extract as reductant and stabilizer. Average size of the silver nanoparticles was tunable by simply changing the extract concentrations used and pH of the reactions. Quantitative analyses indicated that reduction of the silver precursor was

promoted at elevated pH due to increased activity of olive leaf extract constituent. As a result, the number of nucleus and thus size of the silver nanoparticles decreased with increased pH of the reactions. The silver particles became more spherical-like in shape.

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