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Synthesis and characterization of silver nanoparticles with natural carbohydrate capping using *Zataria multiflora*

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ABSTRACT

For the first time, green synthesis of silver nanoparticles was developed by treating Ag^+ ions with *Zataria multiflora* leaf extract. Leaf extract quantity, $AgNO_3$ concentration and reaction temperature were determined as significant factors in the bioreduction reaction. Carbohydrates of the leaf extract were identified as the effective compound for reduction and capping of AgNPs. Also, TEM micrographs illustrated that micro and nano scale carbon-based materials act as structural scaffold for nucleation and growth of particles. Oxygen-bearing functional groups were identified as active groups for reduction of Ag^+ ions. The prepared particles were spherical from 16.3 nm to 25.4 nm with mean particles size of 20.3 nm. Copyright © 2016 VBRI Press.

Keywords: Biosynthesis; bioreduction; carbohydrate; plant extract; protein; silver nanocrystals.

Introduction

Metal nanoparticles have found widespread technological applications. Among all of these nanomaterials, silver nanoparticles (AgNPs) have attracted increasing interest of scientist and technologists. There are a lot of commercially available products which contain silver nanoparticles, ranging from burn treating materials to antimicrobial wearing and paints. AgNPs are recently used to treat multidrug resistant microorganisms that are among the major challenges for the healthcare industry. These nanoparticles have a synergistic antimicrobial activity with common antibiotics and can impair the resistant mechanism of microbial cells **[1]**.

Silver nanoparticles have been synthesized by a number of chemical and physical methods. However, these methods are employing toxic chemicals, organic solvents and non-biodegradable materials which are potentially dangerous to environment and human health **[2]**. Therefore, there is a demand for the development of reliable, biocompatible, clean, ecofriendly and economic process for synthesis of silver nanoparticles. In recent years, green synthesis has received more attention as a novel method to turn researchers toward green chemistry **[3-7]** in order to development and implementation of chemical processes which reduce or eliminate the use of hazardous substances. Particularly in the synthesis of silver nanoparticles via reduction of Ag^+ ions; choice of solvent, the reducing agent employed and the capping (stabilizing) agent used are the major environmentally critical points.

There is increasing interest for biosynthesis of AgNPs using microorganisms or plant extracts. Biosynthesis of AgNPs has so many advantages than traditional chemical methods. Chemically synthesized nanoparticles are not colloidal stable in aqueous media and capping agents must be used to increase the particles stability. In addition, use of detrimental solvents and harsh alkaline pH for reduction of Ag^+ ions to AgNPs has been linked to the significant health and environmental issues. Biosynthesis of metal nanoparticles eliminates all of these concerns.

Microbial cells secrete carbohydrates and proteins to the extracellular environment which can be effective in reduction of Ag^+ ions **[8-15]**. Likewise, biologic compounds in the aqueous plant extracts reduce the Ag^+ ions in physiologic pH without the use of any harsh or toxic chemicals **[16-19]**. Also, biochemical species attach on the surface of nanoparticles as capping agent and stabilize the produced particles in an environmental friendly one-put reaction.

In comparison with microbial synthesis, biosynthesis of AgNPs using plant extract has more benefit of ease handling, rapid synthesis and more economic process. AgNPs have been synthesized using leaf extract of various plants such as black tea, *Lippia citriodora* (Lemon Verbena), maple (Acer sp.), *Lantana camara*, *Artemisia annua* and eucalyptus **[16, 18, 20-23]**. Also,

other parts of plants have been used such as *Piper longum* and *Crataegus douglasii* fruit extract, coffee extract, orange peel extract, C*hrysanthemum morifolium* Ramat. extract, *Medicago sativa* and *Sterculia foetida* seed exudate, sorghum bran extract and *Cinnamon zeylanicum* bark extract **[5, 17-19, 24-27]**.

Zataria multiflora is a member of the *Lamiacea* family and its areal parts have been used in the traditional medicine as well as pharmaceutical and food industries **[28, 29]**. The antibacterial activity of the extract is also well determined against multidrug resistant microorganisms **[28]**. This may also result in synergism activity with AgNPs and enhanced germicidal properties. In the present study, for the first time, the potential of *Z. multiflora* for the green synthesis of AgNPs was investigated. Also, the effects of various parameters such as leaf extract quantity, concentration of silver nitrate and reaction temperature on the AgNPs production were evaluated.

Experimental

Materials

Silver nitrate was purchased from Merck chemicals. All glass-wares have been acid washed and then rinsed with deionized water. Dried leaves of *Z. multiflora* (**Fig. S1**, Supplementary materials) were obtained from a local market. All chemical reactions were done using Millipore water (resistance >18 MΩ cm^{-1}).

Preparation of leaf extract

Dried leaves of *Z. multiflora* were initially washed with deionized water to remove the dust and possible mods. The plant leaf aqueous extract was consequently prepared by taking 2.5 g of dried leaves in a 250 mL Erlenmeyer flask with 100 mL deionized water. The prepared mixture was boiled for 15 min and was filtered and stored at -20°C.

Biosynthesis of AgNPs

Plant leaf extract was used as reducing agent for reduction of Ag^+ ions to Ag^0 and production of AgNPs. Several sets of experiment were conducted using different leaf extract quantities and silver nitrate concentrations at 28°C. The leaf extract quantities were varied from 0.5 to 10 mL in 10 mL total volume reaction containing $AgNO_3$ (1mM) as a metal precursor. Impact of various concentrations of silver nitrate was also investigated from 0.1 to 10 mM. The effect of reaction temperature on silver ions reduction was evaluated at 15, 28, 50 and 75°C. All the reactions were followed for 24h.

UV-vis spectroscopy

Spectral analysis for the development of AgNPs at different reaction conditions was analysed by ultraviolet and visible absorption spectroscopy (T80+ UV/VIS Spectrometer PG Instruments Ltd) operated at a resolution of 1 nm within the range of 300-700 nm. In each analysis, 0.1 mL of the sample was diluted to 1 mL with deionized water **[25]**.

Characterization of AgNPs

For characterization purposes, AgNPs were synthesised while each factor was set at the optimal level. In practice, 1 mL leaf extract was added to 9 ml $AgNO_3$ solution (5 mM final concentration) and the reaction was stirred for 24 h at 28°C. Prepared AgNPs were characterized by Transmission Electron Microscopy (TEM, Philips, CM 10; HT 100 Kv), X-ray powder diffraction patterns (XRD, Siemens D5000) and Fourier Transformed Infrared spectroscopy (FTIR, Bruker, Vertex 70, FT-IR Spectrometer). Particle size distribution and zeta potential analysis were performed by dynamic light scattering (DLS) studies on a Malvern, ZS3600 instrument**.**

Determination of carbohydrate content in leaf extract

The suspension of nanoparticles was centrifuged at 16,000 g for 30 min. to sediment all of the AgNPs and the obtained clear supernatant was used as treated leaf extract. The phenol-sulfuric acid assay was used for total carbohydrate content determination in untreated and treated leaf extract **[30-32]**. In this order, 100 µL of 5% phenol in water was mixed with 100 µL of leaf extract. Subsequently, 400 µL of concentrated sulfuric acid was injected to the mixture and the tubes mixed quickly. The reaction was allowed to reach the maximum temperature and stand for 10 min. Then, the tubes were incubated in water bath at room temperature for 30 min. Light absorbance was read at 490 nm on a UV-vis photometer (Eppendorf, BioPhotometer plus). The carbohydrate content was determined as equal glucose concentration by reference to a standard curve previously constructed for D-glucose.

Determination of protein content in leaf extract

The amount of protein present in the leaf extract is determined by performing a colorimetric reaction and comparing the results with those obtained from standard amounts of albumin protein. The Bradford micro-assay test was used which is generally applied for dilute protein solutions. In practice, 20 µL of the concentrated dye reagent was added to 80 µL of the leaf extract. The mixture was shaken and incubated at room temperature for 5 min. Finally, light absorbance at 595 nm is read using a UV-vis photometer (Eppendorf, BioPhotometer plus). The dye was prepared by dissolving 5 mg Coomassie Brilliant Blue G-250 in 0.5 ml 95% ethanol. Then, 1 ml 85% (w/v) phosphoric acid was added and the solution was dilute to 10 mL.

Results and discussion

Biosynthesis of AgNPs

The solution changed from colour less to brown during the formation of AgNPs **(Fig. S2)**, which arises due to excitation of SPR in the silver nanocrystals. AgNPs have a typical surface plasmon band (SPR) absorption at about 400-450 nm. The UV-vis spectroscopy, therefore, can be used as an indirect method to examine the formation of AgNPs **[3-5, 16, 25]**. The UV-vis spectra of AgNPs prepared in different amounts of leaf extract are presented in **Fig. 1**. These spectra show that the amount of leaf extract plays a significant role in the silver reduction reaction. Hyperchromic shift was occurred with reduction in the amount of leaf extract from 10 to 1 ml. However, using leaf extract amount below 1 mL was resulted in the

hypochromic shift. As leaf extract amount reduced to below 5 mL, a hypsochromic shift in the SPR band was also observed. The shift to lower wavelengths can be justified by a decrease in the particles size and particles aspect ratio **[16]**. Synthesis of metal nanoparticles is conducted in two main steps including (a) nucleation, and (b) growth of nanocrystals. Presence of organic compounds in the reaction mixture has an inhibitory effect on the particles growth **[33, 34]**. On the other hand, in the biosynthesis of AgNPs, biological compounds are playing the key role in Ag^+ ions reduction. Thus, an optimal value of the leaf extract is required for reduction of Ag^+ ions to AgNPs.

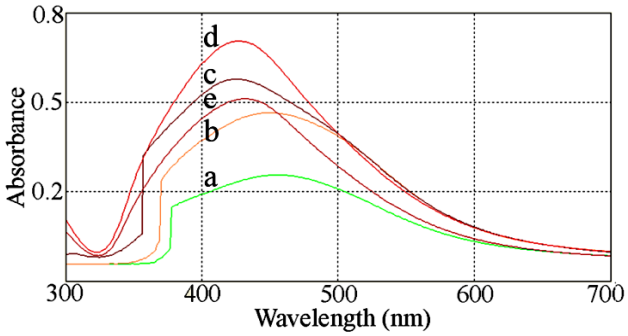

Fig. 1. The UV-vis spectra of AgNPs prepared in different amounts of leaf extract, a: 10, b: 5, c: 2.5, d: 1 and e: 0.5 mL leaf extract.

The UV-vis spectra of the prepared AgNPs in different concentrations of silver nitrate are shown in **Fig. 2**. Based on the results, intensity and sharpness of the absorption peak increases at higher $AgNO_3$ concentration up to 5 mM, whereas a hypochromic shift was found at 10 mM $AgNO_3$. Increase in peak intensity and sharpness with increase in silver ion concentration from 0.1 to 5 mM was also reported while using leaf extract of *Rosa rugosa* **[3]**.

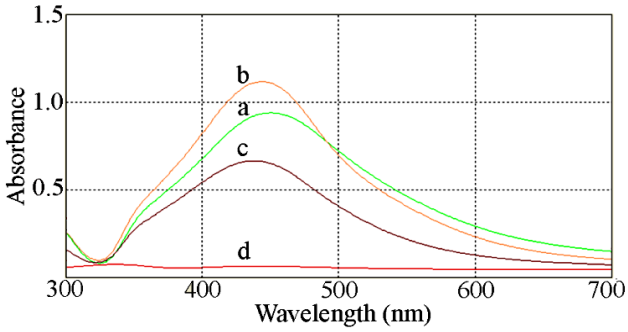

Fig. 2. The UV-vis spectra of AgNPs prepared in different concentrations of $AgNO_3$, a: 10, b: 5, c: 1 and d: 0.1 mM.

Reduction of Ag ion using *Z. multiflora* leaf extract was conducted at various temperatures. Based on the UV-vis spectroscopy analysis, the best temperature for the reaction found to be 28°C which can result the AgNPs suspension with highest SPR absorption peak **(Fig. 3)**. Better reduction of silver ions in physiological temperatures confirms the key role of biochemical species in the synthesis of AgNPs. However, it has been reported that the peak sharpness enhances with an increase in the reaction temperature up to 150 °C using *Sorbus aucuparia* leaf extract in development of silver and gold nanocolloids **[35]**. This contradictory data can be due to different bioactive compounds in various plants which take part in Ag^+ ions reduction.

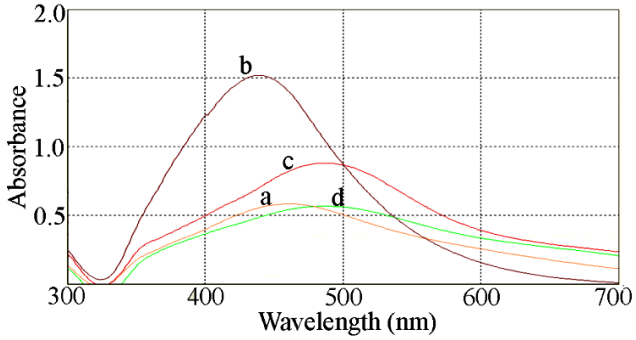

Fig. 3. The UV-vis spectra of AgNPs synthesised in different temperatures, a: 15°C, b: 28°C, c: 50°C and d: 70°C.

Characterization of AgNPs

TEM micrographs show silver nanoparticles to be attached to the micro scale pieces of bark or trapped in a biologic matrix (**Fig. 4)**. These observations clearly reveal the role of organic compounds in the leaf extract for reduction of AgNPs. The most of prepared silver nanoparticles were spherical in shape. However, anisotropic particles were also observed **(Fig. S3)**.

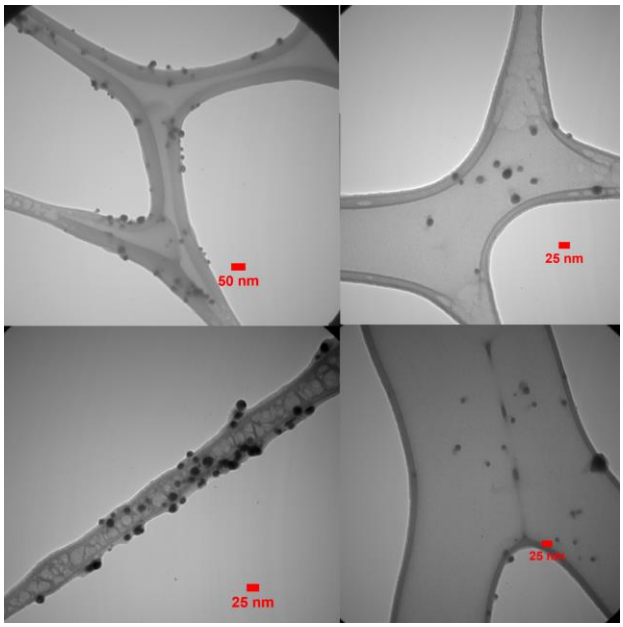

Fig. 4. TEM micrographs of silver nanoparticles.

Particles size evaluations were conducted using Image J software version 1.47v, an image analysis software developed by the NIH (http://imagej. nih. gov/ij/), and mean particles size was measured to be 20 nm.

The powder diffraction pattern of the AgNPs is depicted in **Fig. 5**. Four main characteristic diffraction peaks for Ag were observed at 38.1°, 44.2°, 64.5° and 77.4° 2θ values due to reflection from the crystal facet of (111), (200), (220) and (311), respectively **[5, 20, 25]**. The FWHMs of the four main peaks were used for size evaluation by employing the Scherer calculator tool on the X'Pert

HighScore software, version 1.0 d, 2003 (PANalytical B.V., Almelo, The Netherlands). The average crystallite size was found to be 27 nm which is fairly similar to the TEM results confirming that the biosynthesized AgNPs are single crystalline.

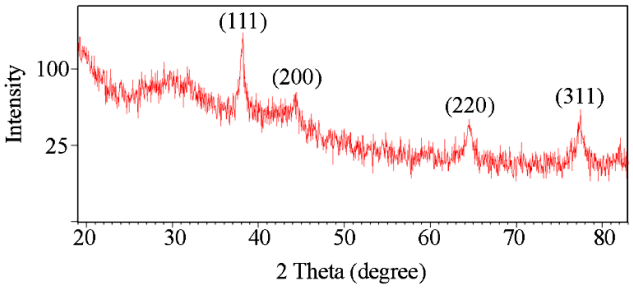

Fig. 5. XRD pattern of AgNPs, indicating main characteristic peaks of silver.

TEM and XRD evaluations were conducted on dry samples. Accordingly, DLS analysis was performed to gain a better understanding regarding the size and charge characterization in an aqueous medium. The DLS results for AgNPs are presented in **Table 1**. Particle size distribution and zeta potential plots are also depicted in **Fig. S4**. Mean particle size measured by DLS was 20.3 nm with particle size distribution of 16.3-25.4 nm indicating that the prepared AgNPs are uni-dispersed in aqueous matrices.

Table 1. DLS data for biosynthesised silver nanoparticles.

Size distribution (nm)	Mean size (nm)	Zeta potential (mV)	Mobility (µmcm/Vs)	pH	Temperature (°C)
16.3-25.4	20.3	-28.6	-2.24	7.4	25

The FTIR analysis was performed to identify the functional groups of biological compounds in the *Z. multiflora* leaf extract responsible for reducing Ag^+ ions. As shown in **Fig. 6**, the bands at 1061 cm^{-1} and 1271 cm^{-1} are from C−O and C−C stretching vibrations, respectively. A peak with medium intensity at 1406 cm^{-1} can be due to C−H bending vibrations. Stretching vibrations of aliphatic C−H absorbed IR radiation at about 2900 cm^{-1}. The absorption peak from carbonyl groups was appeared at 1599 cm^{-1}. A weak peak at 1576 cm^{-1} indicates IR absorption by C=C bonds. The broad absorption peak of hydrogen bonds from O−H groups can be seen at 3400 cm^{-1} which can overlaps with the absorption from N−H bonds **[36]**. By reduction of Ag^+ ions significant reduction in the intensity of the peaks from C−O bonds, carbonyl groups stretching vibrations and C−H bending vibrations was observed **(Fig. 6b)**, which indicates the significant role of oxygen bearing functional groups in the reduction of Ag^+ ions. Compared to untreated leaf extract, shortening of the carboxyl group's peak in AgNPs spectra **(Fig. 6c)** is due to interaction with the surface of the nanoparticles **[33, 34, 37-41]**. It is widely believed that oxygen-contain functionalities are necessary for anchoring of metal nanoparticles, and silver ions can easily oxidize these groups as Eq. (1) and (2) **[34, 42]**.

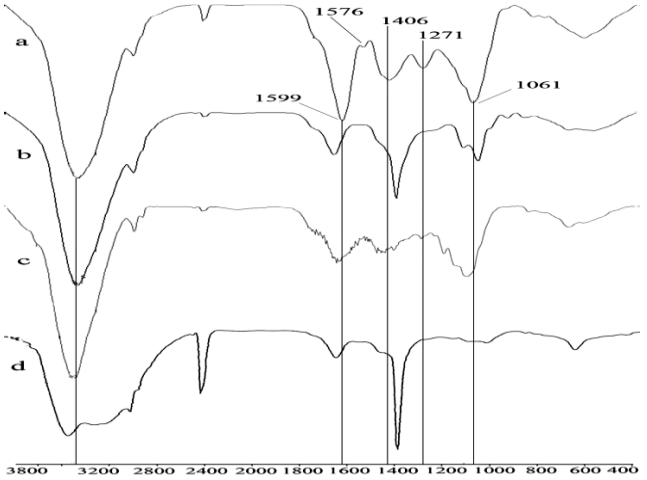

Fig. 6. FTIR spectra of (a) untreated leaf extract, (b) treated leaf extract, (c) AgNPs and (d) silver nitrate.

Scheme S1, in the supplementary material, is illustrated the possible interactions of biochemical species with AgNPs via oxygen bearing functional groups **[34]**. Also, by reduction of Ag^+ to Ag^0, absorption peaks from carbon–carbon single and double bonds was eliminated from 1271 cm^{-1} and 1576 cm^{-1}, respectively **[5]**. The intense absorption band at 1380 cm^{-1} is due to the NO_3^- from $AgNO_3$ **(Fig. 6b & d)**.

$$R - CH_2OH \xrightarrow[Ag^+]{} R - CHO \xrightarrow[Ag^+]{} R - COOH \quad (1)$$

$$R_2 - CHOH \xrightarrow[Ag^+]{} R_2 - CO \quad (2)$$

Carbohydrate and protein content of leaf extract

The Uv-vis spectra of the untreated and treated leaf extracts are shown in **Fig. S5**. The untreated extract showed a broad light absorption peak at 325 nm, which represent the typical absorptions of an aromatic pi system **[43]**. Interestingly, the leaf extract became significantly transparent to the UV-vis irradiation after Ag^+ ions reduction. The carbohydrate and protein content of untreated and treated leaf extract is showing in **Fig. 7**. About 8.5 times reduction in the carbohydrate content of leaf extract was occurred by reduction of Ag^+ ions to AgNPs. Meanwhile, just about two folds reduction in protein content of leaf extract was observed, which indicates the more effective role of carbohydrates in reduction and capping of AgNPs. Polysaccharides extracted from biological sources have electron-donating capability for direct reduction of Ag^+ ions to Ag^0 **[42]**. As illustrated in **Fig. 4**, the micro and nano scale carbon-based materials (CMs) can act as structural scaffold whose peripheral charges help to stabilize the metal particles in aqueous solution. Meanwhile, soluble polysaccharides serve as a capping agent to maintain AgNPs stable and prevent their aggregation. CMs are abundant with oxygen-bearing functional groups, such as hydroxyl, carboxylic, carbonyl and phenolic groups. Silver ions are attracted to the surface of CMs via electrostatic or coordination interactions with

these functional groups. The interfacial junction allows electrons to pass to the conductive Ag^+ ions resulted in nucleation and growth of AgNPs.

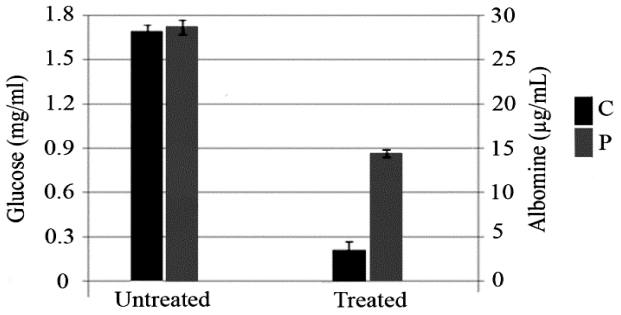

Fig. 7. Carbohydrate (C) and Protein (P) contents of untreated and treated leaf extract.

Also, proteins are effective biochemical compounds for reduction and capping of AgNPs. Proteins can provide the dual function of Ag^+ ion reduction and shape controlled synthesis of AgNPs. Hydroxyl groups in Tyr residues and carboxyl groups in Asp and Glu residues were identified as the most active functional groups for Ag^+ ion reduction and for directing the growth pattern of AgNPs, respectively. So, chemistry of the proteins are the other important factor, Tyr content (the reduction source) and the content of Ag complexers (the reaction inhibitors, *e.g.*, His and Cys) in the protein molecules are affecting the Ag^+ ions reduction kinetics **[15]**.

Conclusion

Colloidal stable AgNPs were successfully fabricated by *Z. multiflora* leaf extract without the use of any additional reducing or capping agents. The leaf extracts quantity, $AgNO_3$ concentration and reaction temperature, were found to be the key factors in the biosynthesis reaction. Carbohydrates and to some extend proteins in the *Z. multiflora* leaf extract were identified as effective biologic compounds for reduction and capping of AgNPs. Oxygen bearing functional groups act as the key sites for anchoring and reduction of metal nanoparticles.

Acknowledgements

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Supporting Information

Fig. S1. Dried leaves of Z. multiflora.

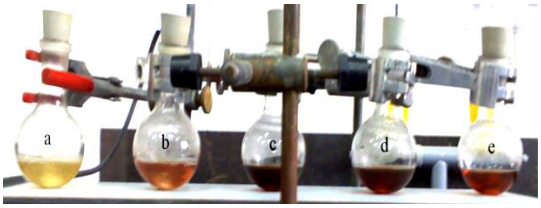


Fig. S2. A brown-yellow colour appearance after 1.5h reaction at various leaves extract quantities, a: 10, b: 5, c: 2.5, d: 1 and e: 0.5 mL leaf extract in a 10 mL reaction containing AgNO3 (1mM).

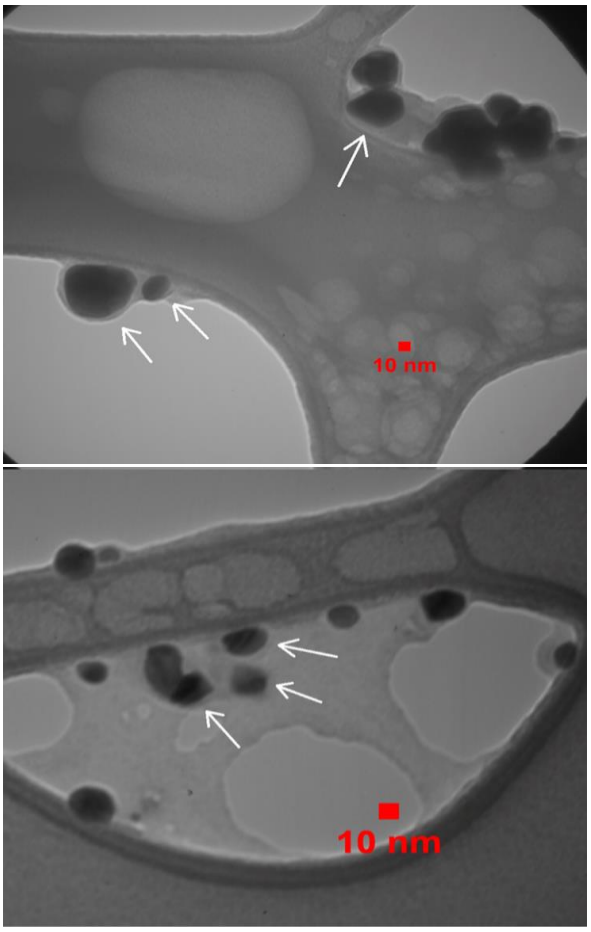


Fig. S3. TEM micrographs of the anisotropic AgNPs.

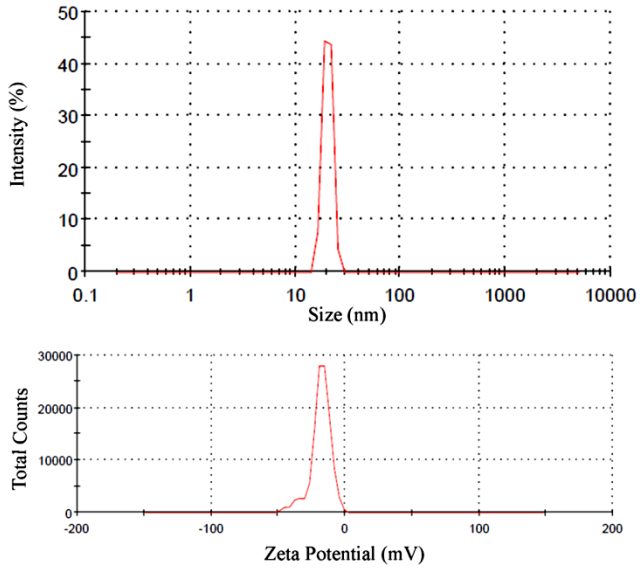
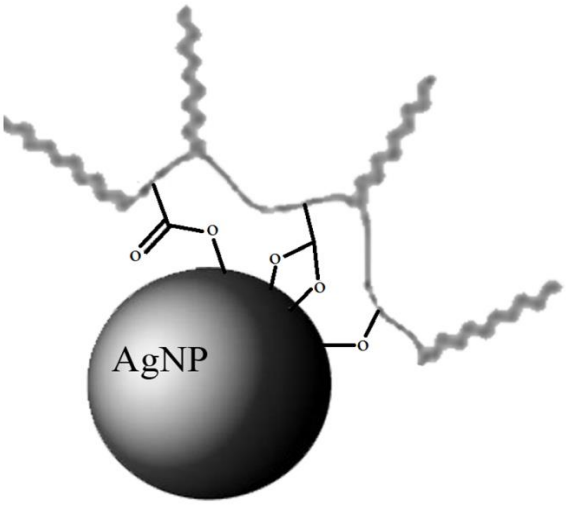


Fig. S4. Particle size and zeta potential distribution plots of biosynthesized AgNPs.



Scheme S1. The probable interactions of biochemical species with AgNPs via oxygen bearing functional groups.

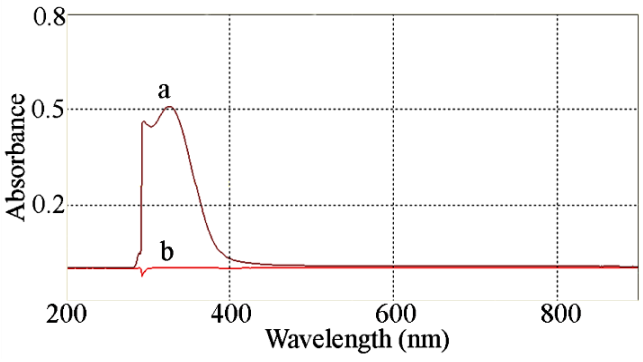


Fig. S5. Uv-vis spectra of the untreated (a) and treated (b) leaf extract.