

Optimization of Parameters for Biosynthesis of Silver Nanoparticles Using Leaf Extract of *Aegle marmelos*

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ABSTRACT

The aim of this study was to optimize the biosynthesis of silver nanoparticles using leaves of *Aegle marmelos* as the primary source. The optimal reaction medium comprised 2:1 concentration of leaf extract and 6mM concentration of silver nitrate solution (pH 7). The biosynthesized silver nanoparticles were confirmed by UV-Vis spectroscopy at 420 nm, XRD and FTIR analysis. The antimicrobial properties of silver nanoparticles were confirmed with *Bacillus subtilis* and *Pseudomonas aeruginosa*.

Key words: AgNO₃, *Aegle marmelos* leaf, UV-Vis spectroscopy, XRD, FTIR

INTRODUCTION

Bael (*Aegle marmelos* (L.) Corr.) is Indian medicinal plant belonging to Rutaceae family and commonly known as wood apple, which has enormous traditional values against various diseases. From this plant, many bioactive compounds have been isolated (Badam et al. 2002; Gupta and Tondon 2004). It is used in the treatment of asthma, anaemia, fractures, healing of wounds, high blood pressure (Saswati Parichha 2004) and diabetes (Kar and Bandhopadhyay 2003). It has hepatoprotective activity (Singanan et al. 2007), anti-inflammatory & antipyretic activity (Shankaranath 2007), antifungal activity (Patil 2009), antibacterial activity (Maheshwari 2009) and many others. The phytoconstituents of *A. marmelos* leaf comprise skimmianine, aegelic, lupeol, cineol, citral, citronella, cuminaldehyde, eugenol and marmesinine (Maity et al. 2009).

Nanotechnology is one of the fast developing frontiers in the field of research and technology (Mc Donnell et al. 1999) and the synthesis of nanoparticles through green synthesis is gaining

importance all over the World (Govindaraju et al. 2010). Silver, which is a very common and well-known metal, has gained importance due to its wide range of applications. Among the nanoparticles, silver nanoparticles have several important applications in the field of bio-labelling, sensors, antimicrobial agents and filters (Pal et al. 2007). It is also capable of destroying pathogenic bacteria by altering the cell membrane structure and functions as pesticide degrading agent. It also purifies drinking water (Liu et al. 1994; Sondi et al. 2004).

The importance of biological synthesis of nanoparticles has been realized all over the world since chemical methods are capital intensive and toxic. Thus, the need for clean, ecofriendly, cost-effective, and biocompatible synthesis of metal nanoparticles encouraged the researchers to exploit the biological sources as nanofactories (Rai et al. 2009; Seshadri et al. 2011; Wei et al. 2012). There is a need to develop eco-friendly processes (Vikas et al. 2014).

The ability of plant extracts to reduce the metal ions has been known since the early 1900s,

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although the nature of the reducing agents involved was not well understood. In view of its simplicity, the use of live plants or whole plant extract and plant tissue for reducing metal salts to nanoparticles has attracted considerable attention within the last 30-years (Beattie and Haverkamp 2011; Park et al. 2011; Gan and Li 2012; Kandasamy et al. 2012).

Compared with the use of whole plant extracts and plant tissue, the use of plant extracts for making nanoparticles is simpler. Plant extract mediated synthesis is an increasing focus of attention (Babu and Prabu 2011; Castro et al. 2011; Kaler et al. 2011; Lee et al. 2011; Baskaralingam et al. 2012; Daisy and Saipriya 2012).

The approach of biological synthesis of nanoparticles is not dependent on specific conditions and the rate of reaction of plant extract remains high (Bianchini et al. 2002). Silver nanoparticles are highly stable for which they are used in electronic devices to biological tools (Morgan et al. 2005). To attain greater stability, maximum yield and aggregation of particles with controlled size, it is imperative to optimize the different parameters such as the concentration of leaf extract, silver nitrate, pH and time (Morgan et al. 2005).

Thus, the aim of the present study was to optimize the synthesis of silver nanoparticles by altering the plant extract ratio with that of silver nitrate and to characterize the silver nanoparticles using UV-spec., XRD, FTIR and antimicrobial property against the pathogenic bacteria, *Bacillus subtilis* and *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

The healthy leaves of *Aegle marmelos* (Fig. 1) were collected from the campus of VIT University, Vellore, Tamil Nadu, India.

Synthesis of Silver Nanoparticle

Healthy leaves of *Aegle marmelos* were washed with tap water, dried and powdered by crushing in mortar and pestle. The powdered leaves were heated with 100 mL of Milli-Q water at 65°C for 5 min and the resulting extract was filtered. From the filtrate, 10 mL was mixed with 10 mL of 1 mM silver nitrate solution and the mixture was incubated in dark at 37°C on incubator shaker. A control mixture was made by 10 mL *A. marmelos* leaf extract without silver nitrate solution.



Figure 1 - Showing the photograph of *Aegle marmelos*.

Optimization of Nanoparticles Synthesis

Concentration Ratio of Leaf Extract and Silver Nitrate Solution

The concentration ratio of leaf extract and silver nitrate was optimized with the increase in concentration of leaf extract (10, 20, 30 mL) in 10 mL of 1 mM silver nitrate (ratio- 1:1,2:1,3:1). After two days of incubation, the absorbance of the resulting solution was measured spectrophotometrically.

The concentration ratio of leaf extract and silver nitrate was optimized with the increase in concentration of silver nitrate solution (2, 4, 6 and 8 mM) in constant volume of leaf extract (20 mL). Reaction mixture was incubated for two days and the absorbance of the resulting solution was measured spectrophotometrically.

Effect of the pH

The pH of the reaction was optimized by using different pH where the reaction pH was maintained at 2.0, 5.0, 7.0 and 9.0. The pH was adjusted by using 0.1 N HCl or 0.1 N NaOH. After two days of incubation, the absorbance of the resulting solution was measured spectrophotometrically.

Characterization of Silver Nanoparticles

Synthesis of silver nanoparticles was confirmed by taking the absorbance in UV-Vis spectra at a range 200-800 nm at a resolution of 1 nm. The lyophilized powdered sample was used for XRD (X-ray Diffraction) and FTIR (Fourier transform infrared spectroscopy) analysis. The XRD patterns were collected on broker AXS D8 Advanced X-ray diffractometer with Cu K α radiation of wavelength 1.541 \AA and scanning angle 2 θ over the range of 10-80°. FTIR was used to characterize the nanoparticles using the lyophilized sample by KBr pellet technique in the range of 400-4000 cm^{-1} .

Antibacterial Activity

Using the leaf extract of *A. marmelos*, synthesized silver nanoparticles were tested for antibacterial activity.

Well Diffusion Method

Bacillus and *Pseudomonas* sp. was cultured in nutrient broth for 24 h and lawn culture was made on Mueller Hinton agar (MHA) plates. Lyophilized AgNP was dissolved in sterile distilled water and sonicated. Three wells each of 5 mm diameter were made on each plate and the synthesized AgNP solution at a concentration of 60, 80 and 100 $\mu\text{g mL}^{-1}$ was loaded in each well. The plates were then incubated at 37°C for 24 h and the zone of inhibition was calculated.

Determination of Growth Curve

Test *Mycobacterium* sp. was inoculated in Lowenstein-Jenson medium prepared in two side arm flask. One flask was kept as control without silver nanoparticle solution and the other with 500 μL silver nanoparticle solution. OD was checked at a gap of 1 h at 600 nm.

RESULT AND DISCUSSION

Synthesis of Silver Nanoparticles

The synthesis of the silver nanoparticles was confirmed by the characteristic color change to brown (Fig. 2) that was found in the solution containing AgNO_3 . The nature of the plant extract, its concentration, the concentration of the metal salt, the pH, temperature and contact time are known to affect the rate of production of the nanoparticles, their quantity and other characteristics (Dwivedi and Gopal 2010). The synthesis of silver nanoparticles using a leaf extract of *Polyalthia longifolia* was reported by Prasad and Elumalai (2011). Silver and gold ions could be reduced to nanoparticles using a leaf extract of *Cinnamomum camphora* (Huang et al. 2007). The reduction was ascribed to the phenolics, terpenoids, polysaccharides and flavones compounds present in the extract.

During the synthesis of silver nanoparticles using *Annona reticulata* the color of the reaction mixture, after 20 min, at room temperature, changed to dark brown, indicating the formation of AgNPs (Sivakumar and Vidyasagar 2014). The color of the *Chrysanthemum morifolium* extract

solution containing AgNO_3 changed from light yellow to yellow brown confirming the synthesis of silver nanoparticles (Yan et al. 2013).

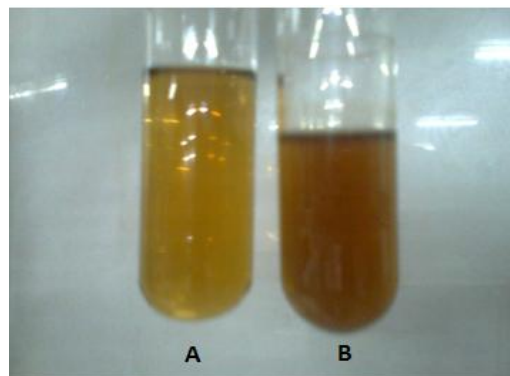


Figure 2 - (A) Leaf extract without Silver nitrate. (B) Leaf extract with Silver nitrate.

Characterization of Silver Nanoparticles

The UV-visible spectroscopy is a commonly used technique (Pal et al. 2007). Light wavelengths in the 300–800 nm are generally used for characterizing various metal nanoparticles in the size range of 2 to 100 nm (Feldheim and Foss 2002). Spectrophotometric absorption measurements in the wavelength ranges of 400–450 nm has been reported by Huang and Yang (2004). Characterization of silver nanoparticles was confirmed by the formation of peak between 400–420 nm range in UV-Vis spectroscopy Fig. 3) in the present study. A single Surface Plasmon Resonance (SPR) band corresponds to the spherical nanoparticles, whereas two or more SPR bands correspond to the anisotropic molecules (Krishnaraj et al. 2010). In this study, a single SPR band was exhibited by the reaction mixture, which revealed the spherical shape of the AgNPs. A characteristic peak for silver nanoparticle has been reported at 420 nm by Sivakumar and Vidyasagar (2014).

XRD is used for the phase identification and characterization of the crystal structure of the nanoparticles (Sun et al. 2000). X-rays penetrate into the nanomaterial and the resulting diffraction pattern is compared with standards to obtain structural information. The crystalline nature of AgNPs was confirmed by the X-ray diffraction studies (Fig. 4).

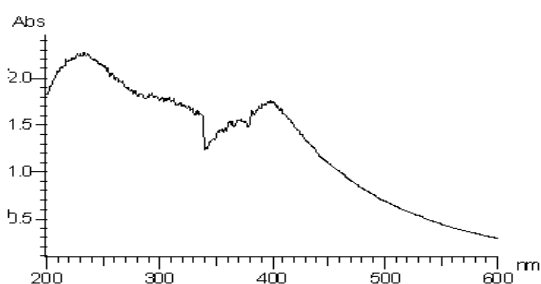


Figure 3 - Characterization of AgNPs with a peak at 400 – 420 nm.

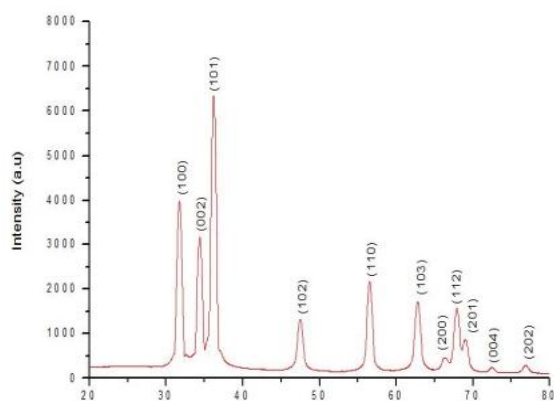


Figure 4 - XRD image of silver nanoparticles synthesized from *Aegle marmelos* leaf extract.

FTIR spectroscopy is useful for characterizing the surface chemistry (Chithrani et al. 2006). Organic functional groups (e.g., carbonyls, hydroxyls) attached to the surface of nanoparticles and the other surface chemical residues are detected using FTIR. FTIR spectrum of AgNPs is shown in the Figure 5. It revealed the possible biomolecules present in the leaf extract, which was accountable as the reducing agent for the silver ions and its interaction with the AgNPs. The IR spectrum showed an intense band at 3441.01 cm^{-1} , which corresponded to the strong stretching vibrations of hydroxyl group (-OH) of phenolic compounds (Yan et al. 2013).

Optimization of Silver Nanoparticle Synthesis Concentration of Leaf Extract

Different concentration of leaf extract was optimized and the maximum silver nanoparticle synthesis occurred in 2:1 ratio, which was further confirmed by the formation of highest peak in spectroscopy and brown color formation. Thus,

this ratio was considered as optimum and the next parameter was performed based on this ratio and color change (Fig. 6). The ratio varied from plant to plant; for example, in the leaf of *P. guajava* plant, a combination of 1.0 mL + 9.0 mL showed highest peak at 440 nm (Vikas et al. 2014). In the synthesis of silver nanoparticles using a geranium (*Pelargonium graveolens*) leaf extract, the particles formed rapidly and a stable size of 16–40 nm was achieved (Shankar et al. 2003).

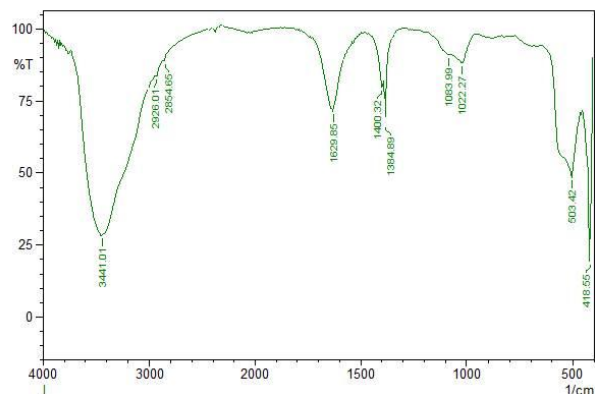


Figure 5 – FTIR image of silver nanoparticles synthesized from *Aegle marmelos* leaf extract.

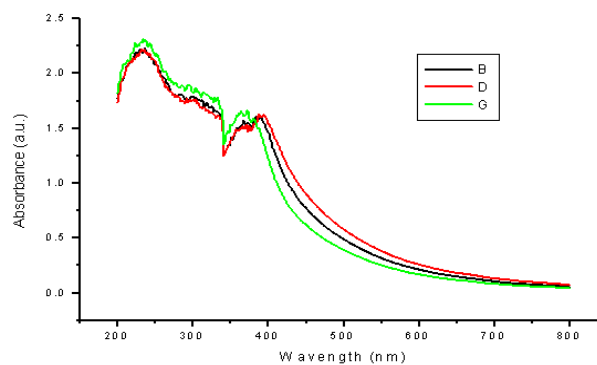


Figure 6 - UV-vis spectroscopy results showing concentration of leaf extract Vs. silver nano particles synthesis. (G-2:1, D-1:1, B-3:1).

Concentration Ratio of Silver Nitrate Solution

Results showed that 6 mM concentration of silver nitrate gave the maximum formation with the absorbance peak at 400-420 nm (Fig. 7) and color turned brown (Fig. 2) after 48 h incubation. At 1 mM, silver nitrate gave characteristic absorption peak at 440 nm in the UV-vis spectrum for *P. guajava* leaf extract (Vikas et al. 2014). The

intensity of absorption spectra of AgNP increased with increasing the concentration of AgNO₃ (1 to 5 mM); 4 mM concentration of AgNO₃ solution showed narrow size distribution of AgNPs using *O. tenuiflorum* leaf extracts as a reducing agent (Dulen 2014).

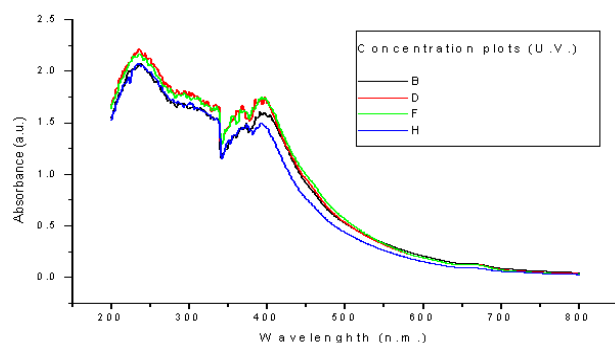


Figure 7 - UV-vis spectroscopy results showing concentration of silver nitrate Vs. silver nano particles synthesis (F-6 mM, D-4 mM, B-2 mM).

pH

pH is considered as important parameter in silver nanoparticle synthesis. The solution was adjusted in different pH and the concentration was kept at 6 mM in 2:1 ratio. Minimum silver nanoparticles formation was found at acidic pH (2.0 and 5.0) and alkaline pH (9.0). pH 7.0 was optimum with maximum nanoparticle synthesis. Absorbance peak was highest at 400-420 nm (Fig. 8). Similarly in the study of Vikas et al. (2014), the AgNP synthesis using *P. guajava* leaf extract, pH 7.0 was optimum for the synthesis of the nanoparticles.

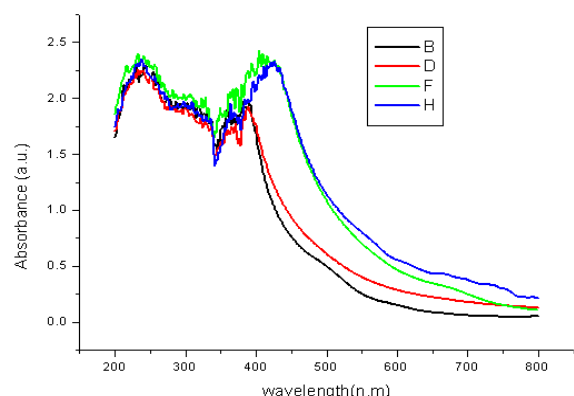


Figure 8 - UV-vis spectroscopy results showing different pH Vs. silver nano particles synthesis. (F-7, H-5, D-9, B-2).

Antibacterial Activity

Silver nanoparticles have been commonly found to have broad spectrum antimicrobial activity against human and animal pathogens (Arulkumar and Sabesan 2010; Ali et al. 2011). The antimicrobial activity of synthesized silver nanoparticles was confirmed by relative zone of inhibition (Table 1, Figs 9 and 10). The antimicrobial activity of the silver nanoparticle synthesized from *A. marmelos* had sensitivity against *E. coli*, *P. aeruginosa* and *S. aureus* (Yamini et al. 2014). The impregnated disc with *A. marmelos* AgNPs exhibited lesser inhibition compared to that of the direct addition into the well. The AgNPs synthesized from the stem bark of *Adansonia digitata* (L.) showed highest inhibiting effect on *P. vulgaris*, followed by *E. coli*, *P. aeruginosa*, *K. pneumonia*, *S. typhimurium*, *B. subtilis* and *S. aureus* as confirmed by the diameter of zone of inhibition (Maruti et al. 2015). Patil et al. (2012 a,b) produced highly stabilized silver nanoparticles (25–40 nm) using a leaf extract of *O. tenuiflorum*. Krishnaraj et al. (2010) synthesized silver nanoparticles (20–30 nm) using a leaf extract of *Acalypha indica*. The nanoparticles showed antimicrobial activity against water-borne pathogens such as *E. coli* and *Vibrio cholerae*. Spherical silver nanoparticles (40–50 nm) have been produced using a leaf extract of *Euphorbia hirta* (Elumalai et al. 2010). Silver nanoparticles produced using peel extract of *Citrus sinensis* showed a broad spectrum antibacterial activity (Kaviya et al. 2011b). The particles formed at 60°C had an average size of around 10 nm but reducing the reaction temperature to 25°C increased the average size to 35 nm (Kaviya et al. 2011b). Gram positive bacterium *S. aureus* showed highest activity compared to Gram negative bacterium *Aeromonas hydrophila* in silver nanoparticles synthesized by *P. guajava* leaf extract (Vikas et al. 2014). The silver nanoparticles causes an increase in cell membrane permeability and results in cell death (Guzman et al. 2008).

Table 1 - Showing zone of inhibition for two organisms.

Concentration	Zone of inhibition (mm)	
	<i>Bacillus</i>	<i>Pseudomonas</i>
60µL	4	8
80µL	9	11
100µL	13.5	14

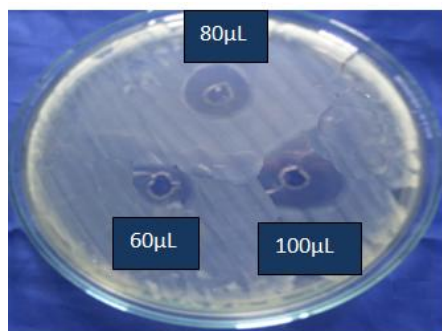


Figure 9 - Zone of inhibition (*B.subtilis*).

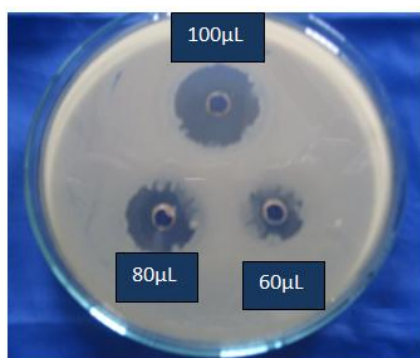


Figure 10 - Zone of inhibition (*P.aeruginosa*).

Determination of Growth Curve

Antibacterial activity of silver nanoparticles was determined using the growth curve method, which showed relative decrease in OD containing AgNPs reducing the growth of both *B. subtilis* and *P. aeruginosa*; in the absence of AgNPs, the OD of both bacterial cultures increased steadily, indicating rapid bacterial growth (Fig. 11).

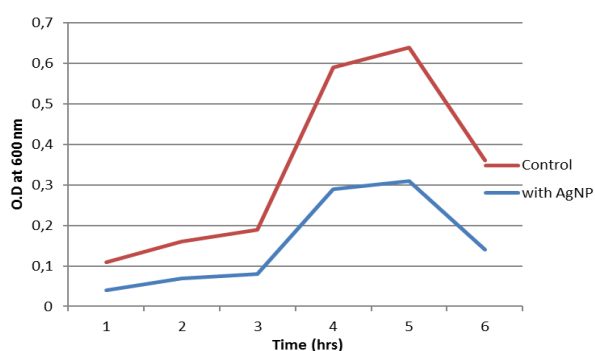


Figure 11 - Growth curve showing antibacterial activity of AgNPs.

Similar study with *Vitis vinifera* was carried out where the presence of AgNPs reduced the growth of *B. subtilis* and *E. coli* indicated by the decreased OD value (Kaushik et al. 2013). The optimum

harvest time for the cells of *Fusarium oxysporum* in the presence of the enzyme inducer AgNO_3 (0.1 mM) was between 28 to 32 h (Korbekandi et al. 2013).

CONCLUSION

In this work, silver nanoparticles were synthesized with greater stability using simple low cost and eco-friendly biological approach. This procedure could be highly suitable for large-scale production as it is very rapid and shortens the duration of many bio-based applications. The use of plant extracts for making metallic nanoparticles is inexpensive, easily scaled-up and environmentally benign. It is especially suited for making the nanoparticles that must be free of toxic contaminants as required in therapeutic applications. The plant extract-based synthesis can provide the nanoparticles of a controlled size and morphology. Thus, silver nanoparticles could become one of the most common metals used in medical treatment.

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