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Mulberry leaf extract mediated synthesis of gold nanoparticles and its anti-bacterial activity against human pathogens

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Mulberry leaf extract mediated synthesis of gold nanoparticles and its anti-bacterial activity against human pathogens

K Adavallan and N Krishnakumar

Department of Physics, Annamalai University, Annamalainagar 608 002, Tamil Nadu, India

E-mail: nskumarphyamu@gmail.com

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Abstract

Gold nanoparticles (Au-NPs) were synthesized at room temperature using Morus alba (mulberry) leaf extract as reducing and stabilizing agent. The development of plant mediated synthesis of nanoparticles is gaining importance due to its simplicity, low cost, non-toxicity, eco-friendliness, long term stability and reproducible aqueous synthesis method to obtain a self-assembly of nearly monodispersed Au-NPs. The formation and morphology of biosynthesized nanoparticles are investigated with the help of UV-Vis spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), atomic force microscopy (AFM), x-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FT-IR) techniques. Au-NPs formation was screened by UV-Vis spectroscopy through color conversion due to surface plasmon resonance band at 538 nm for Au-NPs. DLS studies revealed that the average size of Au-NPs was 50 nm. TEM studies showed the particles to be nearly spherical with few irregular shapes and particle size ranges 15−53 nm. The AFM image clearly shows the surface morphology of the well-dispersed Au-NPs with less than 50 nm. The high crystallinity of nanoparticles is evident from bright circular spots in the selected area electron diffraction (SAED) pattern. X-ray diffraction pattern showed high purity and face-centered cubic structure of Au-NPs. The FT-IR results indicate the presence of different functional groups present in the biomolecule capping the nanoparticles. Further, biosynthesized Au-NPs show strong zone of inhibition against Vibrio cholera (gram-negative) and Staphylococcus aureus (gram-positive) whereas, chemically synthesized Au-NPs and mulberry leaf extract exhibit a fair zone of inhibition.

Keywords: gold nanoparticles, biosynthesis, mulberry leaf, anti-bacterial activity

Classification numbers: 2.05, 4.02

1. Introduction

Nanotechnology is emerging as a rapidly growing field with its applications in industrial, biomedical and electronic applications. Nanoparticles of noble metals belong to the most extensively studied colloidal systems in the field of nanoscience and nanotechnology. Noble metals such as Au, Ag, Pd, Pt and Cu have been widely used for the synthesis of stable colloids which are useful in the areas of optoelectronics [1], catalysis [2], photothermal therapy [3], surface enhanced Raman scattering (SERS) detection [4] and biological labeling [5]. Among several metal nanoparticles, gold nanoparticles (Au-NPs) have been considered an important area of research due to their unique and intense plasmon resonance in the visible range and their applications in biomedical field. Synthesis of Au-NPs by chemical methods leads to the presence of some toxic chemical species adsorbed on the surface that may have adverse effects in medical applications [6]. Due to the obvious disadvantages of the chemical reduction method, a biological synthesis method has been developed to obtain biocompatible, inexpensive and eco-friendly size controlled nanoparticles [7]. Biological route synthesis of
nanoparticles has received much focused attention from researchers in order to elucidate the mechanism of synthesis. Nowadays bioreduction methods based on fungi, micro-organisms and plant extracts are being attempted due to the ease of synthesis, environmentally benign nature and greater stability of nanoparticles [8]. In recent years, plant materials have been of special interest to the scientific community due to their eco-friendliness and are advantageous over other biological processes because they eliminate the elaboration of the process of maintaining cell structures and can also be suitably scaled up for large scale synthesis of nanoparticles [9]. Reduction of Au3+ using plant extracts is advantageous since the phytochemicals have several medicinal properties which may aid in therapy and may be superior to polymer capped Au-NPs. Recently, the synthesis of Au-NPs has been reported using plants such as, *Chenopodium album* [10], *Ocimum sanctum* [11], *Cassia auriculata* [12], *Rosa hybrida* [13], *Crocus sativus* [14], *Rosa damascena* [15], *Thuja orientalis* [16], *Terminalia chebula*. [17] Ankanwar et al [18] reported the synthesis of gold nanotriangles using tamarind leaf extract and studied their potential applications in vapor sensing. Govindaraju et al [19] demonstrated the β-glucosidase assisted biosynthesis of Au-NPs and this report also focused on the newly formed Au-NPs application on promoting the defensive mechanism of silkworm *Bombyx mori*. Banker et al [20] investigated banana peel extract mediated Au-NPs displaying efficient anti-microbial activity towards most of the tested fungal and bacterial cultures. Daizy et al [21] reported that *Cassia fistula* stem bark mediated Au-NPs are better hypoglycemic agents in the treatment of diabetes mellitus and its associated complications. Mukherjee et al [22] proposed that green synthesized gold nanobioconjugates using *Olax Scandens* leaf (Au-NPs-OX) could be used as alternative diagnostic and therapeutic approaches for cancer diseases. Kumar et al [23] reported that the physicochemical properties, biostability and blood compatibility evaluation of Au-NPs prepared from *Zingiber officinale* extract support its usage as vectors for the applications in drug delivery, gene delivery or as biosensors, where a direct contact with blood occurs. Fayaz et al [24] have reported that the synthesized gold nanotriangles using *Maduca longifolia* leaf extract can be easily coated in the glass windows which are highly efficient in absorbing IR radiations.

Genus *Morus* (mulberry) is one such example that consists of over 150 species; among them *Morus alba* L. is dominant [25]. Mulberry leaf (the leaf of *Morus alba*) commonly used in the silkworm diet, has been used in edible foods. Dietary mulberry (*Morus alba*) leaf exhibits a wide range of pharmacological effects such as anti-microbial antioxidant anti-inflammatory functions, activity against atherosclerosis and diabetes mellitus, neuroprotective functions and L-3,4-dihydroxyphenylalanine (DOPA) oxidase inhibition and antitryrosinase activity [26]. It has a unique nutritional profile containing proteins, phenolics, flavonoids and anthocyanins that enhances its significance as promising nature’s functional tonic [27]. The polyphenols contained in mulberry leaf also show the ability to inhibit cancer cell proliferation, invasion, and metastasis [28]. Also, mulberry leaves are rich in iminosugars such as the glucose analogue 1-deoxynojirimycin (DNJ), N-methyl-DNJ, and 2-O-α-D-galactopyranosyl-DNJ, DNJ being the most abundant and accounting for 50% of the mulberry iminosugars [29]. Since DNJ is believed to be the most bioactive agent (R-glucosidase inhibitor), dietary mulberry DNJ might be beneficial for suppressing abnormally high blood glucose levels, thereby helping to prevent diabetes mellitus. At present, various food-grade mulberry products (i.e., teas, powders, and tablets) have been made commercially available in Japan and many other countries [30]. So far, there is no report on the synthesis of Au-NPs by utilizing the aqueous leaf extract of *Morus alba*. Hence, the present study involves the synthesis and characterization of mulberry leaf (*Morus alba*) mediated Au-NPs and evaluating the anti-bacterial effect of biosynthesized Au-NPs in comparison to chemically synthesized Au-NPs and plant extract against human pathogens such as *Staphylococcus aureus* (gram-positive) and *Vibrio cholera* (gram-negative) bacteria.

2. Materials and methods

2.1. Materials

The mulberry (*Morus alba*) leaves (figure 1) were obtained from Sericulture Farmers Training Centre at Jayankondampatinam, Tamilnadu, India. Hydrogen tetra chloroaurate (III) hydrate (HAuCl₄, 3 H₂O) was purchased from Sigma-Aldrich Chemicals, Bangalore, India and used as-received. Nutrient agar for bacterial culture and Muller–Hinton broth and agar for anti-bacterial activity were purchased from Hi-Media, Mumbai, India. All other reagents used in the reaction were of analytical grade with maximum purity. All aqueous solutions were prepared using de-ionized water. All glasswares were cleaned with chromic acid followed by thorough washing with de-ionized water and then acetone for prior use.
2.5.1. UV–Vis spectral analysis. The reduction of pure Au$^{3+}$ ions was routinely monitored by visual inspection as well as the optical absorption spectra of biosynthesized Au-NPs. UV-Vis absorption spectrum of the biosynthesized Au-NPs was done in UV-Vis spectrophotometer (Shimadzu UV-1650) in a wave length range from 200 to 800 nm.

2.5.2. Particle size analysis and zeta potential measurements. Particle size (hydrodynamic diameter) and size distribution measurements of the biosynthesized and chemical synthesized Au-NPs were carried out using a Zetasizer, version 6.32 (Malvern Instruments Ltd) based on dynamic light scattering. Stability of biosynthesized nanoparticles was also determined by means of zeta potential analyser using a Zetasizer, version 6.32 (Malvern Instruments Ltd). The measurement of zeta potential is based on the direction and velocity of particles under the influence of known electric field.

2.5.3. Transmission electron microscopy. The size and shape of the particles were measured with high resolution transmission electron microscope (HR-TEM) using Phillips Technai G2 Fei 12 Model equipped with selected area electron diffraction pattern (SAED) operating at an accelerating voltage of 200 kV. A specimen for HR-TEM sample was made by placing a drop of suspension on a carbon coated copper grid and the excess solution was removed by tissue paper and allowed to air dry at room temperature for overnight.

2.5.4. Atomic force microscope. Atomic force microscope (AFM) was used to observe the surface morphology and size of the resultant Au-NPs. The sample was dropped onto new cleaved mica slices and dried overnight. AFM study of the Au-NPs deposited on mica slices was performed in a microscope AGILENT-N9445A series 5500 AFM.

2.5.5. X-ray diffraction. The crystalline characterization of the biosynthesized Au-NPs was conducted with on Bruker AXS D8 Advance X-ray diffractometer operating at a voltage of 40 kV and a current of 30 mA With Cu-Kα radiation (λ = 1.5420 Å). The scanning range (2θ) was selected from 30° to 80° at a 0.045° min$^{-1}$ continuous speed. The crystallite domain size was calculated through the Debye–Scherrer’s formula.

2.5.6. Fourier transform infrared spectroscopy. FT-IR spectroscopy measurements were carried out to identify the possible functional groups of leaf extracts for nanoparticles synthesis and stabilization. FT-IR spectra of the leaf extract and dried biosynthesized Au-NPs were mixed with KBr pellets and recorded with an Avatar 330 FT-IR spectrometer in the range of 4000–400 cm$^{-1}$.

2.5.7. Screening of anti-bacterial activity. The anti-bacterial activity of biosynthesized Au-NPs, chemically synthesized Au-NPs and crude leaf extract were evaluated against human pathogens such as Staphylococcus aureus (gram-positive) and Vibrio cholera (gram-negative) by well diffusion method. The selected bacteria were maintained on nutrient agar

**Figure 2.** Photograph of colloids (a) Morus alba leaf extract, (b) $10^{-4}$ M HAuCl$_4$ metal ion solution and (c) purple pink color indicating the formation of Au-NPs.
media. Approximately 7 mm diameter of well was made on Muller Hinton Agar plate with the help of gel puncture. The cultures were swabbed on test media with sterile cotton swab. 50 μL of both chemical and biosynthesized Au-NPs were inoculated to the well, and then the plates were incubated in incubator for 37 °C for 24 h; the zones of inhibition were discussed.

3. Results and discussion

3.1. UV-Vis spectra of Au-NPs

UV-Vis absorption spectroscopy is an important technique to determine the formation and stabilization of biosynthesized Au-NPs in aqueous solution. Colloidal solutions of biosynthesized Au-NPs show a very intense color, which is absent in the bulk material as well as for individual atoms. Color of gold colloid is attributed to surface plasmon resonance (SPR) arising due to the collective oscillation of free conduction electrons induced by an interacting electromagnetic field [32]. Figure 3 shows the UV-Vis spectra of Au-NPs formation using a constant HAuCl$_4$ concentration (2×10$^{-4}$ M) with various concentrations of mulberry leaf extract of (gm$_1$) 0.2 mL, (gm$_2$) 0.4 mL, (gm$_3$) 0.6 mL, (gm$_4$) 0.8 mL and (gm$_5$) 1 mL of Au-NPs in solution. Initially, the maximal SPR peaks of Au-NPs showed a broadening in bandwidth of the peak with the decreased concentration of leaf extract (gm$_1$ is 0.2 mL and gm$_2$ is 0.4 mL). At higher concentrations, the SPR peak is shifted towards shorter wave length region which shows a decrease in particle size [33]. The broadening in SPR at lower concentrations was probably due to the damping of the SPR caused by the combined effect of an increase in particle size and shape of the Au-NPs in colloidal solutions. Also, at lower concentrations of the leaf extract, nanoparticles syntheses are not greatly favored due to the absence of sufficient biomolecules responsible for capping and efficient stabilization. Further, it is observed that SPR band becomes narrower and finally a sharp absorption peak occurs at 538 nm, which is characteristic of spherical nanoparticles. Thus, from the results it can be inferred that the concentration of extract plays an important role in determining the size distribution of Au-NPs.

Figure 4 shows the UV-Vis spectra of the HAuCl$_4$ solution after the addition of 1 mL (gm$_5$) of mulberry leaf extract to 10$^{-4}$ M aqueous HAuCl$_4$ resulting in the color change to purple-pink color after 4 h of reaction. The color change in the solution indicates the formation of Au-NPs. Further, it can be observed that the reduction of gold ions reaches saturation after 24 h of reaction, and after that, only slight variations can be noted in the intensity of SPR bands. This result indicates the stability of the nanoparticles for 24 h.

3.2. Particle size and zeta potential measurements

The dynamic light scattering (DLS) analysis is used to measure the shell thickness of a capping or stabilizing agent
enveloping the metallic nanoparticles along with the actual size of the metallic core. Figure 5(a) shows the particle size distribution of the biosynthesized Au-NPs using DLS measurements. The average particle size of the Au-NPs was found to be around 50 nm. DLS analysis showed the size distribution of chemical synthesized Au-NPs with maximum intensity at 23 nm (figure 5(b)).

Zeta potential (ZP) values reveal information regarding the surface charge and stability of biosynthesized Au-NPs. Figure 5(c) shows the corresponding average ZP value (~16 mV) suggesting higher stability of colloidal Au-NPs. The rich source of proteins in the mulberry leaf extract may possibly be responsible for reduction of metal ions and efficient stabilization of biosynthesized nanoparticles [34].

3.3. Transmission electron microscope (TEM) analysis

The size and shape of the biosynthesized Au-NPs was further confirmed by TEM analysis. Figures 6(a) and (b) showed that two different magnifications of nearly spherical Au-NPs were synthesized along with a few irregular shaped particles. The particle sizes distributed in the range of 15–53 nm, with an average particle size of 35 ± 6 nm synthesized (figure 6(c)). This large variation in particle size was due to the presence of

Figure 5. (a) DLS pattern of biosynthesized Au-NPs and (b) of chemical synthesized Au-NPs, (c) zeta potential distribution of biosynthesized Au-NPs.
a few irregular shaped particles. The nanoparticles appear to be considerably smaller than the average particle size observed with the DLS analyzer, presumably arising from the dry state of the TEM measurements. Also, the large size of particles observed by DLS is due to the fact that the measured size also includes the bio-organic compounds enveloping the core of the Au-NPs. Crystalline nature of the Au-NPs is confirmed by the selected area electron diffraction (SAED) pattern (Figure 6(d)) with bright circular spots corresponding to (111), (200), (220) and (311) planes of the fcc lattice of gold [12].

3.4. Atomic force microscopy (AFM) analysis
AFM is an important biophysical technique for studying the morphology of nanoparticles and biomolecules. Figures 7(a) and (b) show the atomic micrograph of biosynthesized Au-NPs with aerial and 3D topographical view of the topological structures. The particle size is in the range below 50 nm. From the topographical view, it is evident that most of the nanoparticles are spherical and have regular shapes. The results observed in TEM images (figure 6) quite agree with the AFM observations results.

3.5. X-ray diffraction (XRD) studies
The crystalline nature of biosynthesized Au-NPs was further confirmed by XRD measurements. Figure 8 shows a representative XRD pattern of the Au-NPs synthesized by the mulberry leaf extract after the complete reduction of Au$^{3+}$ to Au$^{0}$. The intense diffraction peak was observed at 2θ values of 38.3° which was indexed to the (111) planes of face-centered cubic (fcc) gold crystals, respectively (JCPDS no. 04-0784). The absence of any other crystallographic impurities and peak broadening in XRD spectrum has confirmed the high purity of synthesized Au-NPs. The peak widths and shapes describe the deviation from a perfect crystal and make clear about the crystallite size if it is less than roughly 100−200 nm. The width of the most intense reflection (111) peak was employed to calculate the average crystallite size using Debye−Scherrer equation. From the equation, the average crystallite size of Au-NPs found to be around 14 nm. The XRD pattern thus clearly shows that the Au-NPs formed by
the reduction of \( \text{AuCl}_4^- \) ions by \textit{Morus alba} leaf extract are crystalline in nature.

3.6. \textit{Fourier transform infrared spectroscopy (FT-IR) spectroscopy}

FT-IR measurements were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the Au-NPs synthesized using mulberry leaf extract. Curves \( a \) and \( b \) in figure 9 show the FT-IR spectra of biosynthesized Au-NPs and leaf extract of \textit{Morus alba}, respectively. The biosynthesized Au-NPs show intense bands at 3402 cm\(^{-1}\), 2920 cm\(^{-1}\), 1636 cm\(^{-1}\), 1385 cm\(^{-1}\), 1078 cm\(^{-1}\), and 669 cm\(^{-1}\). This represents different functional groups of adsorbed biomolecules on the surface of the nanoparticles and also indicates the influence of organic moieties on the formation of Au-NPs and for stabilization in the aqueous medium. The strong band observed at 3402 cm\(^{-1}\) corresponds to the amine group stretching vibrations super imposed on the side of hydroxyl group [14]. The peak at 2920 cm\(^{-1}\) could be assigned to C–H stretching vibrations of methyl, methoxy and methylene groups [20]. The band observed at 1636 cm\(^{-1}\) is identified as the amide I and arises due to the carbonyl stretch vibrations in the amide linkages of the proteins. [32, 33] The band observed at 1385 cm\(^{-1}\) was assigned to C–N stretching [20]. The peaks at 1078 cm\(^{-1}\) and 1023 cm\(^{-1}\) are the characteristics of C–OH vibrations of proteins and −C–O–C bending mode, respectively. [33, 35] The band at 669 cm\(^{-1}\) might be the plane bending vibrations N-H groups of proteins [36]. The presence of secondary metabolites such as flavonoids, phenols, aminoacids and anthocya- nins was reported in \textit{Morus alba} leaf extract [27]. Figure 9 showed variations in the band position and band intensity due to the reduction of \( \text{Au}^{3+} \) ions to \( \text{Au}^0 \). Curve \( a \) in figure 9 shows slight shifts along with increase in the intenses at 3402 cm\(^{-1}\) and 1636 cm\(^{-1}\), respectively, and reveals the binding of a (NH)C=O group with Au-NPs. Curve \( a \) also shows increased band intensities at 1023 cm\(^{-1}\) and 669 cm\(^{-1}\) as compared to curve \( b \) in figure 9 and suggests that synthesized Au-NPs were stabilized by negatively charged aminoacid molecules.
3.7. Antibacterial activity of gold nanoparticles

In the present study, antibacterial activity of biosynthesized Au-NPs, chemical synthesized Au-NPs and aqueous leaf extract were tested against two human pathogens such as *Staphylococcus aureus* (gram-positive) and *Vibrio cholera* (gram-negative) at the concentration of 50 µl by well diffusion method (figures 10(a) and (b)). The diameter of inhibition zones (mm) around each well with biosynthesized Au-NPs, chemical synthesized Au-NPs and aqueous leaf extract were represented in table 1. The Au-NPs synthesized by *Morus alba* leaf extract were found to be highest antibacterial activity against *Vibrio cholera* and moderate inhibition against *Staphylococcus aureus*. Chemical synthesized Au-NPs exhibit very fair activity against *Staphylococcus aureus* and *Vibrio cholera*. The lesser anti-bacterial activity of aqueous leaf extract was found against *Staphylococcus aureus* and *Vibrio cholera*. The accumulation of gold ions on the negatively charged cell membrane of *Vibrio cholera* leads to conformational changes in cell membrane, which loses permeability control which in turn causes the cell death [37]. This result is possible due to difference in the structure of the cell wall between gram-positive and gram-negative bacteria. The cell wall of the gram-positive bacteria is composed of a thick layer of peptidoglycan, consisting of linear polysaccharide chains cross-linked by short peptides thus forming more rigid structure leading to difficult penetration of the Au-NPs compared to the gram-negative bacteria where the cell wall possesses a thinner layer of peptidoglycan [38]. Biosynthesized Au-NPs, showed efficient anti-bacterial activity compared to chemical synthesized Au-NPs due to their capping agents (mulberry proteins) and it has great potential to kill the pathogens.

From the present results, it is clearly evident that the higher inhibitory action of biosynthesized Au-NPs depends not only on size of the nanoparticles, but also on the capping agent (proteins) of the nanoparticles. The surface charge and chemical properties of NPs are determined by capping agents, which play an important role during NPs and bacterial interactions.

4. Conclusion

The present work reports a simple, novel and successful synthesis of gold nanoparticles using *Morus alba* leaf extract as a novel reducing and stabilizing agent of gold salts. UV-Vis spectral analysis confirmed the surface plasmon resonance of biosynthesized Au-NPs. The DLS HR-TEM and AFM images studies had shown that the synthesized Au-NPs have a size below 60 nm. Zeta potential value for biosynthesized Au-NPs was -16 mV indicating the stability of the nanoparticles. Crystalline nature of the nanoparticles is evident from bright circular spots in the SAED pattern and characteristic peak in the XRD pattern. From FT-IR spectra, it was found that biomolecules responsible for capping and stabilization of Au-NPs were water soluble proteins present in the mulberry leaf extract. Moreover, anti-bacterial activity of biosynthesized Au-NPs showed better activity towards gram-

### Table 1. Inhibitory action of control, *Morus alba* leaf extract, chemically synthesized Au-NPs and biosynthesized Au-NPs against human pathogenic bacteria.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Control</th>
<th>Leaf extract (30 µm)</th>
<th>Au-NPs (chem.) (50 µm)</th>
<th>Au-NPs (chem.) (50 µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio cholera</em></td>
<td>—</td>
<td>6</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>—</td>
<td>5</td>
<td>11</td>
<td>13</td>
</tr>
</tbody>
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Figure 10. Anti-bacterial assay of *Morus alba* extract, biosynthesized Au-NPs and chemical synthesized Au-NPs using well diffusion test against *Staphylococcus aureus* (a) and *Vibrio cholera* (b).
negative *Vibrio cholera* than gram-positive *Staphylococcus aureus* compared to chemically synthesized Au-NPs. The development of such plant materials mediated synthesis of Au-NPs can be used as a good therapeutic agent against human and veterinary pathogens and also for the successful development of drug delivery in the near future.

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Antioxidant and Antifungal Potential of Morus Alba Leaf Extract Mediated Synthesis of Gold Nanoparticles

K. Adavallan, N. Rajendra Prasad, N. Krishnakumar

Abstract

Biosynthesis of gold nanoparticles (AuNPs) using plant extract have been suggested as possible eco-friendly alternatives to chemical and physical methods. Present study reports a green chemistry approach for the synthesis of AuNPs using the aqueous leaf extract of mulberry (Morus alba L.). The synthesized AuNPs were characterized by UV–Vis spectroscopy, high-resolution transmission electron microscopy (HR-TEM) and selected area electron diffraction (SAED) analyses. UV–visible spectroscopic studies confirm the formation of AuNPs through color conversion due to surface plasmon resonance band at 537 nm. The results obtained from HR-TEM revealed that the synthesized AuNPs were in the size range of 15-53 nm. Further, SAED pattern clearly show the pure crystalline nature of the synthesized AuNPs. Furthermore, biosynthesized AuNPs nanoparticles exhibited strong antioxidant activity such as DPPH radical and hydroxyl radical scavengers compared to the mulberry leaf extract alone. In addition, the biosynthesized AuNPs shows good antifungal activity against human pathogenic fungi (Candida albicans and Candida glabrata). Therefore, the present study thus suggests that the preparation of AuNPs using mulberry leaf extract provides strong antioxidant and antifungal activity and it has great potential in the preparation of drugs for various diseases.

Keywords: Biosynthesis; Mulberry leaf extract; Gold nanoparticles

Introduction

Noble metal nanoparticles such as gold, silver and platinum are particularly interesting due to their size and shape dependent unique optoelectronic properties [1]. Among these, gold nanoparticles are the most extensively studied materials due to their unique and tunable surface plasmon resonance (SPR) and potential applications in nonlinear optics, catalysis, electronics and other domains of high technology and medicine [2-4]. The controlled synthesis of gold nanoparticles of well-defined size, shape and composition, to be used in biomedical field is a big challenge [5]. The most common synthesis of AuNPs is the chemical reduction of a ionic gold in aqueous phase by a chemical reducing agent such as NaBH₄, citrate, and ascorbate [6]. But hazardous effects of such reducing agents applied for synthesis of AuNPs on environment, encouraged researchers to develop eco-friendly methods for preparation of gold nanoparticles [7, 8]. Recently, a number of researchers are focusing towards the biosynthesis methods, which can offer a reliable, non-toxic and environmentally benign alternative to chemical and physical methods. Among the biosynthesis methods, plant mediated synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness because it does not require the elaborate process of maintaining cell cultures. The plant mediated synthesis of AuNPs attracts an increasing interest due to their ease synthesis, simplicity, eco-friendliness and novel physico-chemical properties as compared with those of bulk particles, that allow AuNPs in various biomedical applications such as, treatment of cancer and diabetes and also used as good source of antioxidant and antimicrobial agents [9-12].
In the present study, single step synthesis of the AuNPs is presented by reduction of chloroauric acid (HAuCl₄) at room temperature with mulberry (Morus alba L.) leaf extract. Mulberry leaves are rich in many nutritional components including flavonoid, which is known as a powerful polyphenol and antioxidant [13]. In addition, chemistry of mulberry leaves enumerate that it contains some antimicrobial agents like kuwanon G and leachianone etc [14]. In previous reports, different parts of mulberry from the root bark to the leaves have been extensively investigated for their health benefits, including anti-oxidative, hypo-lipidaemic, anti-hyperglycemic, anti-atherogenic, anti-viral, and anti-microbial and neuro-protective effects [15]. Hence, the present study involves with the synthesis and characterization of Mulberry leaf mediated synthesis of AuNPs and evaluating the antioxidant (DPPH and hydroxyl radical scavenging assay) and antifungal effect of biosynthesized AuNPs against human pathogenic fungi such as Candida albicans and Candida glabrata in comparison to the mulberry leaf extract.

Materials and Methods

Materials

The mulberry (Morus alba L.) leaves were obtained from Sericulture Farmers Training Centre at Jayankondapattinam, Tamilnadu, India. Hydrogen tetra chloroaurate (III) hydrate (HAuCl₄·3H₂O) was purchased from Sigma-Aldrich Chemicals, Bangalore, India and used as-received. Potato dextrose agar (PDA) for anti-fungal activity was purchased from Hi-Media, Mumbai, India. All other reagents used in the reaction were of analytical grade with maximum purity. All aqueous solutions were prepared using de-ionized water.

Preparation of Leaf Extracts

The fourth and fifth leaves from the apex of the healthy plants were plucked and washed with de-ionized water until no foreign material remained. The leaves were shade-dried for 5 days and ground into fine powder using an electrical blender. The powdered samples were stored in an air tight container and protected from sunlight for further use. 10 g of leaf powder was taken and mixed with 100 ml of de-ionized water and kept in a boiling water bath at 60 °C for 15 min. The extracts were filtered with Whatman filter paper no. 1. The filtered extract was stored in a refrigerator for further experiments as reducing agent and stabilizer.

Biosynthesis of Gold Nanoparticles

For the biosynthesis of AuNPs, mulberry leaf extract (1 mL) was added to a vigorously stirred 10 mL aqueous solution of 2×10⁻⁴ M HAuCl₄·3H₂O and stirring continued for 1 min. Reduction takes place slowly at room temperature and the reaction rate was completed in 4 h as shown by stable purple-pink color of the solution. Appearance of purple-pink color (Fig.1) in the reaction confirmed the formation of AuNPs and there was no further color change.

Characterization of AuNPs

UV-Vis Spectral Analysis

The reduction of pure Au³⁺ ions was routinely monitored by visual inspection as well as the optical absorption spectra of biosynthesized AuNPs. UV-Vis absorption spectrum of the biosynthesized AuNPs was done in UV-Vis spectrophotometer (Shimadzu UV-1650) in a wave length range from 200 nm to 800 nm.

Transmission Electron Microscopy

The size and shape of the particles were measured with high resolution transmission electron microscope (HR-TEM) using PHILLIPS TECNAI G2 FEI 12 Model equipped with selected area electron diffraction pattern (SAED) operating at an accelerating voltage of 200 kV. A specimen for HR-TEM sample was made by placing a drop of suspension on a carbon coated copper grid and the excess solution was removed by tissue paper and allowed to air dry at room temperature for overnight.

Free Radical Scavenging Activity

1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

Biosynthesized AuNPs and mulberry leaf extract were tested for the scavenging effect on DPPH radical according to the method of Blois [16]. Different concentrations (50, 100 and 150 μL) of mulberry leaf extract and biosynthesized AuNPs were added, in equal volume, to 0.1 mM methanolic DPPH solution. The reaction mixture was incubated for 30 min at room temperature under shaking condition and the absorbance was recorded at 517 nm. The synthetic antioxidant
butyl hydroxyl toluene (BHT) was used as positive control. All determinations were performed in triplicate. The DPPH radical scavenging activity (RSA) was expressed in percentage of inhibition using the following formula

\[
\text{RSA} (%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

**Hydroxyl Radical Scavenging Assay**

Hydroxyl radicals were generated by a Fenton reaction system (Fe\(^{3+}\)-ascorbate -EDTA H\(_2\)O\(_2\)) and the scavenging capacity towards the hydroxyl radical was measured by using a deoxyribose method (Halliwell) [17]. The reaction mixture contained 0.8 mL of phosphate buffer solution (50 mmol L\(^{-1}\), pH 7.4), 0.2 mL of a sample of different concentrations (50, 100 and 150 µL), 0.2 mL of EDTA (1.04 mmol L\(^{-1}\)), 0.2 mL of FeCl\(_3\) (1 mmol L\(^{-1}\)), and 0.2 mL of 2-deoxyribose (60 mmol L\(^{-1}\)). The mixtures were kept in a water bath at 37 °C and the reaction was started by adding 0.2 mL of ascorbic acid (2 mmol L\(^{-1}\)) and 0.2 mL of H\(_2\)O\(_2\) (10 mmol L\(^{-1}\)). After incubation at 37 °C for 1h, 2 mL of cold thiobarbituric acid (10 g L\(^{-1}\)) was added to the reaction mixture followed by 2 mL of HCl (25%). The mixture was heated at 100 °C for 15 min and then cooled down with water. The absorbance of the solution was measured at 532 nm with a spectrophotometer. The hydroxyl radicals scavenging capacity were evaluated with the inhibition percentage of 2-deoxyribose oxidation on hydroxyl radicals. The scavenging percentage was calculated according to the following formula.

% Scavenging = \left[ \frac{A_0 - (A_1 - A_2)}{A_0} \right] \times 100

where \(A_0\) is the absorbance of the control without a sample, \(A_1\) is the absorbance after adding the sample and deoxyribose and \(A_2\) is the of the sample without deoxyribose.

**Antifungal Activity**

The antifungal activity of biosynthesized AuNPs and aqueous leaf extract were evaluated against two human pathogenic fungi (Candida albicans MTCC 227 and Candida glabrata MTCC 3019) by disc diffusion method [18]. Potato dextrose agar (PDA) plates were prepared, sterilized and solidified. After solidification fungal cultures were swabbed on these plates. The discs of AuNPs (100µL), aqueous leaf extract (100µL) and standard drug (Amphotericin-B) (100µL) were placed on the PDA plates. Amphotericin-B was used as positive control to compare the results. PDA plates were subsequently kept at 30 °C for 48 hours. The antifungal activity was evaluated by measuring the zone of growth inhibition surrounding the discs with the help of an antibiotic zone reader.

**Statistical Analysis**

Data were analyzed by one-way analysis of variance (ANOVA) and significant difference among treatment groups were evaluated by Duncan’s multiple range test (DMRT) by using statistical package of social science (SPSS) version 17.0 for windows. Values were considered as statistically significant when \(p < 0.05\).

**Results**

**Characterization of Biosynthesized Gold Nanoparticles (AuNPs)**

A band observed in UV–visible spectrum (Fig. 2) corresponding to the surface plasmon resonance occurs at 538 nm and clearly indicates the formation of AuNPs. The size and morphology of the as-synthesized AuNPs were analyzed by transmission electron microscope (TEM), which shows nearly spherical particles and few irregular shaped particles with a diameter of around 15-53 nm (Fig. 3 (a)). SAED pattern shows (Fig 3 (b)) the diffraction ring from inner to outer which can be indexed as (1 1 1), (2 0 0), (2 2 0) and (3 1 1) reflections respectively of face centered cubic (fcc) gold.

**Free Radical Scavenging Activity**

**DPPH Radical Scavenging Activity**

In the present study, the DPPH radical scavenging assay was used to evaluate the anti-oxidant potential of biosynthesized AuNPs and mulberry leaf extract in a dose-dependent manner. Percent of inhibition for DPPH radical scavenging activity is presented in Fig. 4a. The results obtained in the DPPH assay showed maximum percent of (69 %) free radical inhibition by the biosynthesized AuNPs, whereas mulberry leaf extract exhibit (46 %) less inhibition.

**Hydroxyl Radical Scavenging Activity**
The dose-dependent inhibition of site specific hydroxyl (•OH) radical-induced deoxyribose degradation was observed in Fig. 4b. The data obtained in the •OH scavenging activities were 23 ± 1.86, 35 ± 3.46 and 49 ± 9.74 % in 50, 100 and 150 µL for mulberry leaf extract, whereas they were 37 ± 2.65, 50 ± 3.65 and 65 ± 6.53 % in 50, 100 and 150 µL for AuNPs, respectively.

**Antifungal Activity**

The antifungal effect of biosynthesized AuNPs and aqueous leaf extract in comparison to the standard drug (Amphotericin-B) were investigated against human pathogenic fungi such as *Candida albicans* and *Candida glabrata* (Table 1). Further, biosynthesized AuNPs show strong zone of inhibition against *Candida albicans* and *Candida glabrata* whereas, crude leaf extract exhibit a fair zone of inhibition (Fig 5(a) and (b)).

**Discussion**

In recent years, plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness. Interaction between organic ligands and the surface of an inorganic nanoparticle paves the way for the coupling of biomolecular recognition systems to generate novel materials [19]. Based on the capping agents, environmentally benign AuNPs using biological components will have excellent effect in biomedical applications.

UV-Visible absorption spectroscopy is an important technique to determine the formation and stabilization of biosynthesized AuNPs in aqueous solution. The UV–Vis absorption spectra recorded from the gold colloid after 24 h of reaction showed a SPR band at about 538 nm confirm the formation and stability of AuNPs. The TEM studies showed the particles to be nearly spherical with few irregular shapes and particle size ranges from 15-53 nm. Reactive oxygen species (ROS) are often simply called “free radicals” because their majority is characterized by at least one unpaired electron in their outer orbitals [20]. The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical [21]. Overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans [22, 23]. To avoid the oxidative damage, antioxidant defences have evolved to remove most of these free radicals. 1′-Diphenyl-2-picrylhydrazyl (DPPH) and Hydroxyl radical (•OH ) scavenging method is widely used to evaluate the free radical scavenging ability of antioxidants. DPPH is a stable nitrogen-centered free radical. In the present study, the antioxidant activity of leaf extract and AuNPs reacts with DPPH free radical solution and the violet color of the DPPH radical was reduced to yellow colored diphenylpicrylhydrazine radical. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [24].

Hydroxyl radical is an extremely reactive free radical formed in biological system and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule, proteins, DNA, unsaturated fatty acids and lipids in almost every biological membranes found in living cells [25-27]. In this study, the •OH radical scavenging assay was used to evaluate the antioxidant potential of biosynthesized gold nanoparticle in comparison to the leaf extract. When mulberry leaf extract and AuNPs were added to the reaction mixture they removed hydroxyl radical and prevented the degradation of 2-deoxy-2-ribose. Over all, biosynthesized gold nanoparticles (AuNPs) showed higher antioxidant activity than the leaf extract in DPPH and •OH radical scavenging assays, whereas various phytochemicals (quercetin, rutin and isoquercitrin) present in the mulberry leaf extract might get adsorbed onto the active surface of the AuNPs [14]. It is an obvious indication that the resulting enhanced antioxidant activity of the AuNPs is due to the capping agent (due to adsorbed antioxidant moiety onto the surface) and particle size (high surface area to volume ratio of nanoparticles). Similar observations with enhanced DPPH and Hydroxyl radical scavenging activity of biosynthesized AuNPs have been reported previously [11, 28].
Fungal infection diseases are more rapid and severity increases in patients with compromised immune function. *Candida* species are one of the most important fungal pathogens which are responsible for variant life-threatening disorders [29]. The infection caused by *Candida* fungi are more common in people who have underlying risk factors such as cancer, leukemia, diabetes mellitus, long-term antibiotic and corticosteroid treatment, human immune deficiency virus (HIV), pregnancy, scorch, and transplant [30]. In the present study, antifungal activity of biosynthesized AuNPs, and aqueous leaf extract were tested against two human pathogenic fungi such as *Candida albicans* and *Candida glabrat*a at the concentration of 100 µl by Kirby–Bauer disc diffusion method. The AuNPs synthesized by mulberry leaf extract were found to be highest antifungal activity against *Candida albicans* and moderate inhibition against *Candida glabrata*. Leaf extract exhibit lesser antifungal activity against *Candida albicans* and *Candida glabrat*a. Biosynthesized AuNPs, showed efficient antifungal activity compared to leaf extract. It is due to their particle size and capping agents, which have great potential to kill the pathogens. The possible mechanism of their antifungal activity suggested that the smaller gold nanoparticles might have diffused easily through the cell membrane to the inside of the cell. Since gold being a soft acid might have interacted strongly with the soft bases like sulphur containing proteins in the membrane or phosphorus containing bases in the DNA, thus retarding their normal functions like synthesis, repair and replication leading to the cell death [31]. Recently, many reports are available in the literature on the good antifungal activity of biosynthesized AuNPs [12, 32].

**Conclusion**

Mulberry leaf extract is found to be an efficient candidate for biosynthesis of AuNPs due to its controlled reducing power as well as presence of capping molecules. The UV-visible spectrum of the synthesized gold nanoparticles showed a surface plasmon resonance (SPR) around 538 nm after 24 h. The results obtained from HR-TEM revealed that the synthesized AuNPs were in the size range of 15–53 nm. Crystalline nature of NPs is evident from bright circular spots in the SAED pattern. Further, mulberry mediated synthesis of AuNPs showed a higher antioxidant and antifungal activity. Biosynthesis of AuNPs has encouraged scientist to look into discover novel drugs for various biomedical applications.

**References**


**Author’s Affiliation**

1Department of Physics, Annamalai University, Annamalainagar, Tamilnadu- 608 002, India

2Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar, Tamilnadu- 608 002, India


**List of Figures**

Figure 1: Photograph of colloids (a) *Morus alba* leaf extract, (b) $10^{-4}$ M HAuCl$_4$ metal ion solution and (c) purple pink color indicating the formation of Au-NPs.
Figure 2: UV-Vis spectra of aqueous solution of chloroaucic acid with *Morus alba* (mulberry) leaf extract at 24h.

Figure 3: (a) TEM images of gold colloid (b) selected area electron diffraction (SAED) pattern.

Figure 4: Antioxidative properties of *Morus alba* leaf extract and gold nanoparticles (AuNPs): a: 1’1’-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay; b: hydroxyl radical scavenging assay.

Figure 5: Anti-fungal assay of *Morus alba* leaf extract and biosynthesized AuNPs using disc diffusion test against *Candida albicans* (a) and *Candida glabrata* (b).
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**TABLE 1**: Inhibitory action of control, *Morus Alba* leaf extract and biosynthesized Au-NPs against human pathogenic fungi.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Inhibition zone (mm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf extract (100µl)</td>
<td>AuNPs (100µl)</td>
<td>Standard Antibiotic Disk* (100µl)</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>12</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
<td>11</td>
<td>16</td>
<td>19</td>
</tr>
</tbody>
</table>

*Amphotericin-B*
ANSWERS FOR VIVA-VOCE QUESTIONS

INDIAN EXAMINER

Question 1
Being a physics student, why did you choose a complete biological work?
Answer:

This work is not a complete biological work. In this study, various techniques based on the physical concept were used to study the particle size distribution, morphological analysis, surface charge determination, structural confirmation and functional group analysis. Moreover, the present work utilized different spectroscopic methods to study tissues optical properties and provides important biochemical information at the molecular level during diabetic induced tissues.

Question 2
Why did you choose Au as your base materials? Why didn’t you try any other cost effective materials for the same applications?
Answer:

Gold nanoparticles have advantages over other metallic nanoparticles due to their unique properties such as small size, good biocompatibility, stability, low toxicity, simple surface chemistry and easy surface modification, making them promising candidates for biomedical applications. Hence, gold has been selected as the base material in the present work.

Question 3
In chapter 3, the results and discussion were given separately and this leads to the bit confuse to readers, comment on it.
Answer:

To signify the results obtained through various techniques and to compare the important findings with earlier works, the results and discussion were given separately.

Question 4
The UV results in Fig 3.2, shows the reaction of choloroaauric acid with mulberry leaf extract with different time intervals. It confirms the formation of Au nanoparticles occurs at 3h and further it increases little for 4 and 24 h. Have you done any reaction with intermediate time? What is reason for making 24 h reaction?
Answer:

No significant changes in the SPR band during intermediate time 4-24 hours. Moreover, it has been observed that the reduction of Au ions reaches saturation after 24 h of the reaction. This means that maximum reduction takes place within 24 h of reaction.
Question 5
Why there is only one peak in the XRD pattern for Au in Fig 3.6 what happened to the other planes.
Answer:

The planes (2 0 0), (2 2 0), (3 1 1) and (2 2 2) is also present but it is very weak when compared with more intense plane (1 1 1).

Question 6
In the FTIR- spectra in Fig 3.7, why there is no difference between two spectra?
Answer:

It was observed from the Fig 3.7 that there is a variations in the peak positions and intensities between FTIR spectra of leaf extract alone and biosynthesized AuNPs.

Question 7
Why the results of chemically synthesized sample was not provided and compared?
Answer:

Chemical synthesized AuNPs exhibit very lesser antibacterial activity than biosynthesized AuNPs. Moreover, the synthesis of AuNPs using chemical methods may lead to the presence of some toxic chemical species being adsorbed on the surface of nanoparticles, which can cause adverse effects on their biomedical applications. Hence, chemically synthesized sample was not provided and compared.

Question 8
In Table 3.1, a comparative study has been done for control leaf extract, chemically synthesized Au, biosynthesized Au, However, there is no comparison with standard antibiotics, where as table 4.1 it is used and the control was not taken for comparison, Explain.
Answer:

Initially, comparative study has been done only to identify the efficacy of drug between control, leaf extract alone, chemically synthesized and biosynthesized AuNPs and the results were given in Table 3.1. Therefore, standard antibiotics were not included. Moreover from Table 3.1, the biosynthesized AuNPs were found to be highest anti-bacterial activity than others. In addition, control group shows no antibacterial effect. Hence in Table 4.1, control sample was not included and standard antibiotics were taken and compared with leaf extract alone and biosynthesized AuNPs.