Green synthesis, characterization and evaluation of gold nanoparticles using aqueous extract of *Ganoderma lucidum*

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**Abstract**

The current work describes a reliable green chemistry process for gold nanoparticles synthesis by using *Ganoderma lucidum* extract. The different characterization techniques used for synthesized nanoparticles were UV-Vis spectroscopy, X-Ray Diffraction, Fourier Transform Infra-Red Spectroscopy (FTIR) and Transmission Electron Microscopy (TEM). The nanoparticles showed purple color with a characteristic SPR crest at 541 nm. Polydispersed nanoparticles irregular in shape and size range from 5 to 30 nm were revealed by TEM. The average crystalline size estimated by the Debye-Scherrer’s method was found to be ~ 10.47 nm. The functional groups responsible for bioreduction are O-H bond in alcohols, phenols, triterpenes and capping agents can be C-H stretching alkyl group and aldehydes, Amide I, O-H Bonding polysaccharides determined by FTIR study. The synthesized nanoparticles showed potent antibacterial activity against the pathogenic bacteria, as evidenced by their zone of inhibition.

**Keywords:** *Ganoderma lucidum*, gold nanoparticles, anti-microbial, UV-Vis spectroscopy

**Introduction**

Nanoparticles have gained utmost significance due to their unique physical and chemical properties such as higher surface area, lower melting point, specific magnetic property, mechanical strength and specific optical properties [1]. Due to their large bioavailable surface, gold nanoparticles allow easy functionalization and have applications in microbiology, medicine, environmental sensing, and electronics. Gold nanoparticles have applications in colorimetric biosensors [2], drug delivery systems [3], cancer treatment [4], nanomedicine [5], removal of pollutants [6] and sensors for detecting chemical pollutants [7]. Several chemical and physical methods are available that are costly; require high energy and toxic chemicals. Green synthesis being an ecologically effective and cost effective process involves the synthesis of nanoparticles using different biological agents like bacteria, fungi, yeast, plants and algae [8, 9]. The use of microorganisms particularly the fungi is potentially exciting as they produce large amount of enzymes and biomass which catalyses reduction reaction for biosynthesis of nanoparticles very efficiently and are also simple to carry. Such characteristics of fungi make them a perfect living organism for the synthesis of different metallic nanoparticles. Relatively, the higher fungi like mushrooms in this regard have not obtained the considerable recognition. Lately, gold nanoparticles have been prepared using fungi such as, *Rhizopus oryzae*, *Aspergillus clavatus*, *Epicoccum nigrum*, *Fusarium oxysporum* and edible mushrooms. Mushrooms are the fleshy fruiting bodies of the basidiomycetes fungi, typically found above ground on soil, rotten woods or trees. These fungi comprise of a very approving object of nanobiotechnological studies, as they have a high biomass yield and can accumulate large amounts of reduced nanoparticles in their mycelium [10]. *Ganoderma lucidum* (Curtis)P. Karst. (Class: Agaricomycetes, Order: Polyporales, Family: *Ganodermataceae*) is generally known as Lingzhi or Reishi, well-known across the world as an oriental mushroom with medicinal properties for over 2000 years and its existing effects have been recognized in ancient scripts. *G. lucidum* contains mainly protein, fat, carbohydrate, and fiber. *G. lucidum* contains around 400 various biologically active components that mainly consist of polysaccharides, fatty acids, triterpenoids, nucleotides, sterols, proteins, steroids, peptides, and trace elements [11, 12, 13].
**G. lucidum** exhibits antitumor, immunomodulatory, cardiovascular, respiratory, antiviral, antibacterial, antioxidant and antineopatotoxic effect. Phenol capped gold nanoparticles have been synthesized using *G. applanatum* [14].

In the present study, synthesis of gold nanoparticles is reported by bioreduction of *Ganoderma lucidum* extract using chloroauric acid at room temperature. The synthesized nanoparticles were then characterized and evaluated for antimicrobial and antioxidant activity.

**Materials and methods**

**Materials**

Pure culture of *Ganoderma lucidum* was taken from Department of Biotechnology, DCRUST, Murthal. Bacterial strains were obtained from culture collection, department of Biotechnology, DCRUST, Murthal, which in turn was purchased from Institute of Microbial Technology (IMTECH), Chandigarh and maintained constantly on nutrient agar for further use as per the condition given on the MTCC protocol. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, Hydrogentetrachloroaurate (III) hydrate HAuCl₄·3H₂O (99.9%), Potassium persulfate, and 2,2-Diphenyl-1-picrylhydrazyl were bought from Sigma-Aldrich, India. Glucose, Yeast extract, Malt extract, Peptone, Potato dextrose agar and Nutrient agar were purchased from HiMedia. All aqueous solutions were composed using double distilled water.

**Culturing and maintenance of Ganoderma lucidum**

The culture was collected and maintained as reported in preceding studies, with preferable adjustments [15, 16]. The mycelia were cultivated on potato dextrose agar and incubated at 28°C ± 2°C for 7 days. Thereafter, it was relocated on to glucose yeast malt peptone broth (GYMP). This medium was incubated at 28°C ±2°C and stirred at 150 rpm for 5 days. Following the stirring, medium was allowed to stand still at 28°C ± 2°C for another 5 days. A mat of mycelium was then observed. After incubation for 5 days, the mycelia were collected, doused with double distilled water which was later used for aqueous extract preparation. Our group has also reported the purification of laccase from *G. lucidum* using the same cultivating procedure [17].

**Preparation of mycelia aqueous extract**

The production of extract of mushroom was followed as stated in a process reported previously [15, 16], with appropriate modifications. Briefly, 10g (wet weight) mycelia was immersed in 100ml double distilled water. It was then incubated at shaking conditions at 28°C ±2°C for 72 hours. After 72 hours, it was strained through Whatman filter paper No. 1 and the filtrate was accumulated. This filtrate/aqueous extract was kept at 4°C in closed containers. The mycelial extract was utilized for AuNP synthesis due to its stabilizing and reducing properties.

**Stock preparation**

The Gold (III) chloride hydrate chemical was obtained from the Sigma Aldrich. The 1 mM solution was formulated by dissolving in distilled water and stored at room temperature for the further uses.

**Biosynthesis of AuNPs**

Gold nanoparticles synthesis was done by the procedure discussed previously [18]. The nanoparticle synthesis was optimized using three parameters i.e. ratio (mycelia extract: precursor solution), time, temperature. After the optimization, the best results were used for analysis. In a typical reaction, 4ml aqueous mushroom mycelia extract was added to 6ml of an aqueous solution of 1 mM chloroauric acid solution and stored in dark at normal room temperature kept for 24 h. Biosynthesis was noted utilizing ultraviolet visible spectroscopy at a wavelength range of 200—800 nm and a color change from pale yellow to purple.

**Characterization of gold nanoparticles**

**UV-Vis Analysis**

The optical property and nanoparticles formation was determined by UV-Vis spectrophotometer (LabIndia 3092). After the addition of HAuCl₄ to the mycelia extract, the spectra were studied after 24 hours in the range of 200 nm to 800 nm.

**FTIR analysis**

The biomolecule accountable for the reduction and stabilizing of gold nanoparticles was studied using FT-IR spectrometer. The reaction mixture was rotated in a centrifuge at 10,000 rpm for 10 minutes and doused four times with deionised water to separate gold nanoparticles from the solution. The gold nanoparticles pellets acquired after centrifugation were dried at 45°C and mixed with potassium bromide (KBr). The prepared sample was analyzed by Perkin Elmer Frontier FT-IR Spectrometer and characterized in the range 4000- 400 cm⁻¹.

**X ray diffraction**

In order to obtain/acquire the structural information of the product, the crystallographic structure of nano particles was examined by X-ray power diffraction (XRD). The crystallographic analysis of specimens in diffraction patterns were noted from 30° to 80° with a Rigaku Ultima IV X-Ray Diffractometer.

**TEM**

Transmission electron microscope was utilized for the description of shape as well as size of the prepared nanoparticles. Firstly, the sonication of sample was done for 15 min. Single drop of nanoparticle suspension was added to the copper grid. Thereafter, this solvent was enabled to vaporize in presence of Infrared light for 25 min. The measurements were executed on Tecnai with iCorr TEM instrument runned at an accelerating voltage at 200 kV.

**Evaluation study of synthesized gold nanoparticles**

**Antibacterial activity assay**

The biologically synthesized AuNPs were trialled to check the antibacterial activity counter to Gram-positive (*Staphylococcus aureus*, MTCC 133 and *Bacillus subtilis*, MTCC 70) and gram-negative (*Escherichia coli*, MTCC 249 and *Salmonella typhimurium*, MTCC 3224) bacteria by the Kirby-Bauer test. The bacteria were cultivated in Luria Bertani liquid medium for 24 h. With the help of spreader, 100 μL of bacterial culture were outspread on Nutrient agar followed by 10 minutes of drying. Whatman filter paper discs consisting the nanoparticles (Test control) at 25 μL volume were later on appended to agar plates with discs containing kanamycin (Positive control), chloauric acid (AA) and distilled water (Negative control) which followed by incubation for 24 h at 37 °C. The zone of inhibition was quantified at the culmination of incubation with transparent ruler in millimeter. The minimum inhibitory concentration
(MIC) was decided using the procedure followed by Filipa et al., 2013 [19] with slight modification. In brief, bacterial cultures were grown overnight in nutrient broth at 37°C. The bacterial suspension was then altered by spectrophotometer to concentration of \(1 \times 10^5\) CFU/ml. For broth dilution assay, 2 ml media was taken and diluted 2X with equal volume of nanoparticles in serially numbered tubes. 100 µl of diluted bacterial culture was then inoculated. Absorbance of the samples was then recorded at 625 nm using UV-Vis spectrophotometer. The lowest concentration that retarded the growth of bacteria was identified as MIC.

**Free radical scavenging activity**

Two assays were performed for measuring this activity which are ABTS assay and DPPH assay. (6-hydroxy-2, 5, 7, 8-tetramethylcroman-2-carboxylic acid; Sigma Aldrich), worked as an antioxidant standard for both assays.

**ABTS radical scavenging assay**

A mixture of ABTS (6 mM) and potassium persulfate (2.3 mM) was incubated for a period of 16h at room temperature. Thereafter, it was used for the production of ABTS cation. Different concentrations (10µl, 20µl, 40µl, 60µl, 80µl, 100µl, 120µl) of gold nanoparticles were prepared and using the above aliquots at 0.5-2.5ml of gold sample poured into 2ml of 80% ethanolic ABTS solution [20]. The absorbance was computed at 734 nm in UV-Vis Spectrophotometer. The percentage inhibition was figured out from the given equation:

\[
\% \text{ inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{\text{Absorbance control}} \times 100.
\]

**DPPH radical assay**

The antioxidant activity of gold nanoparticles was examined by DPPH assay. Various concentrations (10µl, 20µl, 40µl, 60µl, 80µl, 100µl, 120µl) of gold nanoparticles were prepared and using the above aliquots at 0.5-2.5ml of gold sample were added to 1ml of 2 mM DPPH suspension in methanol making up final volume 4ml using distilled water. The components of suspension were thoroughly vortexed and let it stand for 30 min at 30°C and the optical density was recorded at 517 nm [20]. The DPPH radical scavenging activity (RSA) was demonstrated in percentage of inhibition using the given formula:

\[
\% \text{ inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of test sample}]}{\text{Absorbance control}} \times 100.
\]

**Result and Discussion**

The whole investigation of gold nanoparticles synthesis was done utilizing aqueous extract of *Ganoderma lucidum*. Addition of *G. lucidum* mycelia extracts to 1 mM gold (III) chloride hydrate suspension at normal room temperature in the ratio of 4:6 showed color change in the solution from dull yellow to violet and finally to purple within 24 hours, suggesting the synthesis of gold nanoparticles. Changes in the color of the solution are the common indication of the nanoparticles formation.

**UV-Vis. Spectrophotometer analysis**

UV-Visible spectroscopy is an important technique to examine the formation and stability of nanoparticles. Color change is observed because of excitation of surface plasmon resonance (SPR). The location of the SPR peak in the UV-Vis spectra is approximated to size, shape, refractive index and the extent of charge movement amidst medium and the particle [21]. Figure 1 shows the UV-Vis spectra of gold nanoparticles genesis using HAuCl₃ and mycelia extract. It can be seen from the UV-Vis spectrum that AuNPs exhibited a sharp SPR crest at 541 nm after 24 h of the reaction, which is a characteristic band for gold. No other peak was observed in the spectrum, so it is reasonable to conclude that the formation of the synthesized product is gold nanoparticles only.

**FTIR analysis**

The gold nanoparticles synthesized by *G. lucidum* were exposed to FTIR characterization to find the biologically active molecules responsible for maintaining nanoparticles in solution. The AuNPs obtained from mushroom extract showed strong peaks at 1404.02, 1633.89, 2854.39, 2925.01, 3195.11 and 3415.19 cm⁻¹ (Figure 2). These peaks resemble the amide bonds of polypeptides and are in accordance with previous reports [22, 23].

The sharp band absorbance at 3415.19 cm⁻¹ is the feature of the hydroxyl group of alcohols and phenols. The peak at 1633.89 cm⁻¹ can be allocated to the amide I bond of the polypeptides present in *Ganoderma lucidum*. The bands at 1404.02 cm⁻¹ corresponds to the O-H bonding of polysaccharides. The peak detected at 2925.01 cm⁻¹ can be allocated to the C-H bonds of alky group. On the other hand, 2854.39 cm⁻¹ is identical to C-H stretching of aldehydes. The bands at 1633.89 and 1404.02 cm⁻¹ demonstrates that the AuNPs are perhaps bound to the polypeptides by carboxylate groups. These polypeptides behave as surfactant to bind to the exterior of AuNPs and preserve them by electrostatic stabilization. The terpenoids, phenols and proteins [24] constituents of *G. lucidum* extract behave as reducing agents, that probably be accountable for the reduction of chloroauric acid. Regarding the biosynthesis mechanism, it is demonstrated in case of *Fusarium Oxysporum* that reduction takes place because of release of NADH reductase [25].
Fig 2: FTIR spectra of gold nanoparticles synthesized using *G. lucidum* extract.

**X-ray diffraction analysis**
The XRD analysis shows major peaks were observed at 20 value 38.2°, 44.6°, 64.8°, 77.5° and small peaks at 24.27° as shown in Figure 3. Four major peaks at 38.2°, 44.6°, 64.8° and 77.5° corresponding to 111, 200, 220 and 311 Bragg’s reflection, respectively match with the JCPDS 76-1393. The crystalline behaviour of AuNPs synthesized by Au\(^{3+}\) ions reduction utilizing *G. lucidum* extract was inferred by well-defined and intensified XRD pattern. The proportion amidst the (200) and (111) diffraction peaks was computed as 0.32 for AuNPs. This peak intensity ratio was lower compared to the standard bulk intensity i.e., 0.52 which means that the (111) plane was in principal inclination. The average crystalline size was estimated by the Debye-Scherrer’s formula by finding the thickness of the (111) diffraction peak. It was estimated as ~ 10.47 nm. This also matched with size determined by the TEM examination. Few unknown peaks were detected, indicating crystallization of biological components onto the surface of AuNPs.

Fig 3: X-Ray diffraction patterns of AuNPs synthesized *G. lucidum* extract (* indicates the non-specific peaks due to biomass residues)

**Transmission Electron Microscopy study**
The powdered form of gold nanoparticles was analysed under the TEM for the sizes and morphology characterization. The sample was analyzed at 200 - 50 nm scales (Figure 4). It was observed that some gold nanoparticles were almost circular and some were irregular in shapes and distributed throughout. The maximum particles size range is 13.74-23.01 nm.

Fig 4: Transmission electron microscope image showing gold nanoparticles synthesized from *Ganoderma lucidum* extract
Antimicrobial activity of biologically synthesized gold nanoparticles

It has been reported that the gold nanoparticles exert the antibacterial activity by two means. Firstly, they reduce the metabolism process by changing the membrane potential and thus reducing the activity of adenosine triphosphate (ATP) synthase. Secondly, they collapse the biological mechanism by inhibiting ribosome-tRNA binding. In mammalian cells, they have shown less toxicity. Small size of AuNPs leads to direct interaction with the microorganism and thus better contact with the bacteria which in turn enhances the antibacterial activity of the NPs. NPs disturb the normal functions of the bacterial proteins of cytoplasm as well as cell wall and ultimately cause cell death. They attack thiol groups of enzymes like, nicotinamide adenine dinucleotide (NADH) dehydrogenases and stop the release of oxygen species by interrupting their electron transport chains resulting in oxidative stress. This leads to substantial destruction of cell structures ultimately causing cell death \[26\]. Antibacterial activity of nanoparticles depends on their concentration being used \[27\]. It has been determined that the catalytic as well as antibacterial properties of gold NPs rise with a decline in average size \[28\]. The antibacterial activities of the gold nanoparticle were evaluated against two Gram positive and two Gram negative bacteria. Four discs were put on each culture, which were positive control, negative control, test sample and chloroauric acid. 30µl of the test sample was pipetted in each disk. The zone of inhibition was measured after 24 hours of incubation (Table 1). The AuNPs showed medium antibacterial activity against gram positive and gram negative strains and zone of inhibition ranged from 11.7 mm to 20.03 mm (Fig.5 A, B, C, D). The present results were compared with standard antibiotic kanamycin and the nanoparticles showed fairly good antibiotic activity. No zone of inhibition was observed in case of chloroauric acid. It can be inferred from the results that Salmonella typhimurium is the most sensitive and showing lowest MIC value and the highest MIC value was observed for Bacillus subtilis. Shamaila et al., 2016; Morales et al., 2017 and Mohamed et al., 2017 \[29, 30, 31\] recently reported antibacterial potential of gold nanoparticles on different bacteria. Thus, these nanoparticles can potentially be used as antibacterial agents to cure various bacterial diseases.

![Fig 5: Plates showing Zone of inhibition of (A) E. coli (B) S. typhi (C) B. subtilis (D) S. aureus](image)

**Table 1**: Zone of inhibitions of biologically synthesized Au NPs and standard antibiotic (in mm) in different microbial cultures

| Bacterial strain | AuNPs synthesized using *Ganoderma lucidum* extract (mm) | Antibiotic (mm) |
|------------------|----------------------------------------------------------|-----------------|
| *Salmonella typhimurium* (–ve) | 18 | 23 |
| *Staphylococcus aureus* (+ve) | 20 | 25 |
| *Bacillus subtilis* (+ve) | 12 | 23 |
| *Escherichia coli* (–ve) | 15 | 20 |

Free radical scavenging activity of biologically synthesized gold nanoparticles

Gold nanoparticles have the capacity of stopping the free radical creation by the cellular materials.

**DPPH radical scavenging activity**

DPPH is a well-known free radical, reduced by receiving either electrons or hydrogen ions and is generally used as indicator to determine the radical nature of reaction. After the change of color of DPPH from purple to yellow, the reducing activity of gold nanoparticles was determined by spectrophotometer. The calculations were done using the absorbance of control and that of AuNPs to determine DPPH scavenging activity or the percent inhibition for various AuNPs concentrations. The radical scavenging activity is presented on Graph and has increased with increase of nanoparticles concentration (Figure 6A). The DPPH radical scavenging activity were 17.59%, 24.07%, 31.01%, 50.57%, 64.35%, 76.96% and 82.98% in 10, 20, 40, 60, 80, 100 and 120 µl respectively (Table 2). The synthesized gold nanoparticles exhibited inhibition on DPPH radical with IC\(_{50}\) equal to 1.67 µg/ml. Similar results showing increasing DPPH scavenging activity by silver nanoparticles synthesized by *Ganoderma lucidum* extract have been reported \[32\].
Fig 6: (A) DPPH scavenging activity of AuNPs synthesized using G. lucidum extract (B) ABTS scavenging activity of AuNPs synthesized using G. lucidum extract (RSA: Radical Scavenging Activity)

Table 2: DPPH scavenging activity for different concentrations of gold nanoparticles synthesized using G. lucidum extract

| S.NO. | Concentration of AuNPs synthesized using Ganoderma lucidum extract (µl) | DPPH Scavenging (% inhibition) |
|-------|---------------------------------------------------------------|-------------------------------|
| 1     | 10                                                            | 17.59                         |
| 2     | 20                                                            | 24.07                         |
| 3     | 40                                                            | 31.01                         |
| 4     | 60                                                            | 50.57                         |
| 5     | 80                                                            | 64.35                         |
| 6     | 100                                                           | 76.96                         |
| 7     | 120                                                           | 82.98                         |

ABTS radical scavenging activity
To determine free radical scavenging activity of chain breaking and hydrogen donating antioxidants, the ABTS radical obtained its oxidation from potassium persulfate is the perfect means. The ABTS scavenging activity for different concentrations of Gold nanoparticles was calculated. It was found that by enhancing gold nanoparticles concentration, the ABTS antioxidant activity also increased (Figure 6B). The radical scavenging activity were found to be 15.97%, 25.00%, 36.68%, 42.71%, 56.15%, 68.21% and 76.50% in 10, 20, 40, 60, 80, 100 and 120 µl respectively (Table 3). The synthesized gold nanoparticles exhibited inhibition on ABTS radical with IC₅₀ equal to 1.74 µg/ml. Similar observations showing enhanced ABTS scavenging activity with increasing gold nanoparticles concentration have been reported [33].

Table 3: ABTS scavenging activity for different concentrations of gold nanoparticles synthesized using G. lucidum extract

| S.NO. | Concentration of AuNPs synthesized using Ganoderma lucidum extract (µl) | ABTS Scavenging (% inhibition) |
|-------|---------------------------------------------------------------|-------------------------------|
| 1     | 10                                                            | 15.97                         |
| 2     | 20                                                            | 25                            |
| 3     | 40                                                            | 36.68                         |
| 4     | 60                                                            | 42.71                         |
| 5     | 80                                                            | 56.15                         |
| 6     | 100                                                           | 68.21                         |
| 7     | 120                                                           | 76.5                          |

Conclusion
The current research demonstrated the synthesis of gold nanoparticles by employing Ganoderma lucidum extract as reducing as well as stabilizing agent. Different ratios of mycelia extract and precursor solution were taken and the best results were observed in case of 4:6 ratios i.e. 4 ml mycelia and 6 ml precursor solution. At this ratio, AuNPs displayed a sharp SPR crest at 541 nm within 24 h of the reaction. To determine the thickness of peak, the Debye-Scherrer’s equation was used and mean crystalline size was found to be ~ 10.47 nm. Functional groups responsible for bioreduction are O-H bond in alcohols & phenolic compounds, triperterenes and capping agents can be C-H stretching alkyl group, C-H stretching aldehydes, Amide I (Protein C=O stretching), O-H Bonding polysacharides. The synthesized nanoparticles have the hidden capacity as an antioxidant and antibacterial compound. Apart from medical applications, the synthesized gold nanoparticle could possibly be utilized in numerous products for the benefit of mankind, in nanomedicines, to treat environmental pollution and drug delivery.

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