Properties and bioactivity of flavonoids and sugar induced gold nanoparticles

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The development of green method of metal nanoparticles (NPs) synthesis has become a major focus of researchers as the biogenic reduction of metals into NPs is eco-friendly, less expensive and free of hazardous chemicals. In the present study, we report the synthesis and characterization of gold (Au) NPs using aqueous Cassia fistula leaf extract. The reduction of 1 mM HAuCl4 (chloroauric acid) occurred within 7 min after addition of the leaf extract. These Au NPs were further characterized by using UV-visible spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD), dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR) analysis. MTT assay was carried out to check cytotoxicity of these Au NPs on MCF-7 cells. UV-Vis spectra confirmed the synthesis of Au NPs with SPR band obtained at 535 nm. The XRD analysis confirmed the crystalline nature of Au NPs. The average size of 27.21 nm was obtained by TEM analysis. The FTIR spectra explained the role of plant phyto-constituents in reduction and stabilization of Au NPs. We finally propose a reduction mechanism using HR-LCMS analysis of Cassia leaf extract showing the presence of flavonoids, sugar and antioxidants as supported by FTIR and NMR results. These bioreduced Au NPs showed no significant cytotoxicity to MCF-7 cells.

Keywords: Cassia fistula, FE-SEM, gold nanoparticles (Au NPs), MCF-7, reduction mechanism

Introduction

The synthesis of gold (Au) nanoparticles (NPs) has gained great significance over the last few years because of its applications in various fields like biosensors, drug delivery, cancer therapy, catalysis and electronics. Various chemical, physical and biological methods are being employed for the synthesis of Au NPs. Since chemical methods involve the use of hazardous chemicals, greener synthesis of metal nanoparticles (using plants, bacteria & fungi) is becoming popular worldwide. In green synthesis of Au NPs, plant based materials are gaining more importance as they are easily available, suitable for large scale biosynthesis and can actually yield nanoparticles of a better defined size and morphology in comparison to other existing physico-chemical processes. Many researchers have been reported the synthesis of Au NPs by using plants. The use of plant extracts of Pelargonium graveolens, Cymbopogon flexuosus, and Zingiber Officinale have been reported in the synthesis of Au NPs.

Cassia fistula L. (Family: Fabaceae) is a medicinal plant. Its various plant parts are being used in treatment of jaundice, ulcers, rheumatism, skin eruptions etc. Anticancer property of the plant has been reported on Ehrlich ascites carcinoma and oral cancer. C. fistula has earlier been reported in the synthesis Au NPs using the stem bark. In the present study, we have explored a simple and cost effective method for the synthesis of Au NPs using aqueous extract of Cassia leaves. Gold nanoparticles were further analyzed for in vitro cytotoxicity on MCF-7 cells. Also, the mechanism of reduction of Au NPs by Cassia leaf extract was studied by high resolution-liquid chromatograph mass spectrometry (HR-LCMS) analysis. This is a preliminary study towards development of gold nanoconjugates for anticancer drug delivery.

Materials and Methods

Preparation of Leaf Extract

The leaves (10 g) of Cassia fistula were cleaned, cut and crushed with liquid nitrogen, and the powder was soaked overnight in 100 mL milli-Q water. The resultant extract was filtered twice through Whatman filter paper to obtain a clear solution. Finally, a yellowish brown extract was obtained and used for the synthesis of the Au NPs. The phytochemical analysis of the aqueous leaf extract was carried out using standard tests. The protein and reducing sugar
contents were identified using the Lowry and DNSA methods.\textsuperscript{10,11}

**Synthesis of Au NPs**

The different volumes of leaf extract of *C. fistula* were mixed with 1 mM chloroauric acid (HAuCl$_4$) at room temperature to obtain the reduction of gold. After using various combinations of leaf extract and chloroauric acid, finally 50 mL of *Cassia* leaf extract and 1000 mL of chloroauric acid (1:20) was selected as the best combination for synthesis of AuNPs. The resulting reddish brown AuNP solution was purified by centrifuging at 10,000 rpm for 30 min at 20°C. After washing two times with milli-Q water, a reddish brown pellet of pure AuNPs was obtained and it was further used for characterization studies. The absorption spectrum of the pure AuNPs reconstituted in milli-Q water was recorded on Shimadzu UV-1800 UV-Visible spectrophotometer.

**Characterization of Au NPs**

TEM analysis was carried out with the Philips CM 200 TEM at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology (IIT) Bombay, Powai, India. The particle size was determined using the TEM image. The specimen for the TEM measurement was prepared by placing a drop of sample on a carbon-coated copper grid and drying it at room temperature. The morphological features of the gold particles were studied with a Field Emission Scanning Electron Microscope (FESEM) (FEI, NOVA NANOSEM 450) at Central Instrumentation Facility, Savitribai Phule Pune University (SPPU), Pune. A minimal amount of sample was placed on a silicon chip using drop cast technique for FESEM analysis. The crystalline nature and lattice structure were confirmed using X-ray diffraction analysis. The pure and dried Au NPs were studied using a Philips PW 1840 diffractometer with Cu Kα radiation at the Department of Physics, SPPU. The particle size was calculated from Full-Width at Half-Maximum (FWHM) of the diffracted lines using the Debye Scherrer formula for all the four planes and the average size was obtained. The Fourier Transform Infrared Spectroscopy (FTIR) spectrum of purified Au NPs was taken with an FTIR Bruker, Tensor37 ATR at the Department of Chemistry, SPPU. The hydrodynamic radius and surface charge of synthesized NPs was measured on Brookhaven Instrument Model 90 Plus Particle Size Analyzer at National Chemical Laboratory (NCL), Pune. The proton ($^1$H) nuclear magnetic resonance (NMR) spectroscopy of purified and lyophilized Au NPs dispersed in dimethyl sulfoxide (DMSO) was recorded on a Bruker AVANCE III HD 500MHz instrument at Central Instrumentation Facility (CIF), SPPU and scanned in the range of 0-13 ppm with and without D$_2$O exchange.

**Cell Viability Test using MTT Reagent**

The cytotoxicity of Au NPs was determined by MTT assay as described by Kadan \textit{et al.}\textsuperscript{12} along with cisplatin as a control. Briefly, MCF-7 cells were seeded in a 96 well plate at a density of $10^4$ cells/well in Dulbecco’s modified eagle medium containing 10% fetal bovine serum (FBS) and 0.1 % antibiotic solution. The cells were incubated with Au NPs and cisplatin in a dose-dependent manner for 24, 48 and 72 h, and checked for biocompatibility of the Au NPs. The samples were added at an increasing concentrations (10-50 µg/mL). The absorbance at 570 nm was measured with microplate reader. Two wells per plate without cells served as blank. All the experiments were repeated three times in triplicates. The cell viability was calculated using the following formula:-

\[
\text{Percent viability} = \frac{A_{570\text{nm}}\text{of treated sample}}{A_{570\text{nm}}\text{of control}} \times 100
\]

**Plausible Mechanism of Reduction of Au NPs**

Gel filtration chromatography was carried out for the fractionation of the *Cassia* leaf extract. For this, 5 g of Sephadex G-25 was suspended in 100 mL saline with intermediate stirring for 3 h and allowed gel particles to swell. Fine particles were removed by decantation. The column was packed and equilibrated with a physiological saline. *Cassia* leaf extract (2 mL) was loaded on G-25 Sephadex column and 2 mL of fractions were collected with 0.85% saline. The resulting fractions were checked for the ability to reduce HAuCl$_4$ to gold nanoparticles. Based on the reduction of Au NPs and their stability, fractions were further studied by High Resolution-Liquid Chromatograph Mass Spectrometer (HR-LCMS) analysis to identify possible plant constituents, which were involved in reduction of Au NPs. HR-LCMS analysis was carried out at SAIF, IIT, Bombay.

**Results and Discussion**

The preliminary phytochemical analysis of Cassia leaf extract revealed the presence of flavonoids, tannins, saponins, terpenoids, alkaloids, glycosides as
per the standard tests. The total protein content was found to be 3.48 mg/mL and reducing sugars 1.6 mg/mL. After mixing the leaf extract with chloroausreric acid, colour of the solution changed from yellow to dark brown within 7 min (Fig. 1). Au NPs showed stability for 6 months.

UV-Visible spectrum of bioreduced Au NPs is shown in Fig. 1a, while time dependant spectra of Au NPs synthesis after addition of leaf extract is shown in Fig. 1b.

**XRD Analysis**

The XRD pattern showed 4 sharp peaks in the 2θ range between 38-78°, which were indexed to the (111), (200), (220), (311) lattice planes of face-centered cubic (fcc) structure as shown in Fig. 2a. The broadening of Bragg’s reflection peaks indicated the formation of nanoparticles. The particle size was determined using the Debye Scherer equation\(^\text{13}\) by determining the average \(D_{av}\) value for the 4 planes (111), (200), (220), (311) of the Bragg’s reflection and was found to be 18.37 nm.

**TEM Analysis**

TEM analysis of Au NPs was performed and results are shown in Fig. 2b. The TEM images of gold nanoparticles in solution showed that the particles were poly-dispersed with spherical, triangular and hexagonal shapes in the size range of 11.9 to 91.3 nm with an average size of 27.21±12.03 nm. The typical selected area (electron) diffraction (SAED) pattern with bright circular rings confirmed the crystalline nature of gold nanoparticles.

**Field Emission Scanning Electron Microscopy (FESEM) and Energy Dispersive X-Ray Spectroscopy (FESEM-EDS) Analysis**

Fig. 3a shows the FESEM analysis of synthesized gold nanoparticles. The particle size ranged from 15-40 nm. The energy dispersive X-ray spectroscopy...
(EDS) pattern of gold nanoparticles in powder form is shown in Fig. 3b. The elemental analysis showed the presence of 64.38% gold, 18.99% C and 16.63% O. It also showed a strong signal for gold at 2.1 keV, a characteristic of gold nanoparticles. Dynamic Light Scattering (DLS) Analysis

The dynamic light scattering (DLS) method gives information about the hydrodynamic radii or size of AuNPs along with coated organic phase. The range of hydrodynamic diameter of Au NPs was found to be 13-64.2 nm with an average size of 32.9 nm and a low polydispersity index of 0.286 as obtained by DLS (Fig. 4). The (ξ) value of Au NPs obtained was 15.73± 0.85.

FTIR Analysis

FT-IR spectrum of Cassia leaf extract and Au NPs were analyzed for determination of functional groups of phyto-constituents involved in the bioreduction. FTIR spectra of Cassia leaf extract as well as pure Au NPs showed prominent bands at 3284, 2350, 1575.27 and 1043, and 3647, 2330, 1517 and 1078 cm⁻¹ respectively (Figs 5a & b). The broad band at 3284 cm⁻¹ (Cassia leaf extract) was due to alcohol/phenol. The decrease in intensity of absorption band with blue shift indicated the binding of alcoholic/phenolic group to Au NPs surface. The band at 2350 and 1575.27 cm⁻¹ was due to C-H stretches of aldehydes and C=C stretching of the aromatic group, respectively, which showed slight blue shift in Au NPs (2330 & 1517 cm⁻¹). The band at 1043 was due to the C-O stretch of alcohols, phenolic compounds or carboxylic acids, which was slightly red shifted and intensity of this band was decreased (1078 cm⁻¹) in case of AuNPs. The reduction in the intensity of bands and shift in positions in case of Au NPs, as compared to the bands of Cassia leaf extract, is indicative of the involvement of these functional groups, viz., –OH, –C–O and –C=C in binding to Au NPs.

¹H NMR Analysis

¹H NMR analysis of purified and lyophilized Au NPs confirmed the coatings of biomolecules on Au NPs surfaces. NMR signals at 7.1, 7.2, 7.3, 7.9 and 8.3 δ were due to the protons of aromatic ring as shown in Fig. 6a. The signal at 3.3 δ is due to protons of CH₃O group. Another signal at 9.5 δ is due to the proton of aldehyde group. Thus, the NMR results are comparable with our FTIR results. Fig. 6b shows the presence of many broad signals, owing to the
presence of –OH group, which was eliminated as seen in Fig. 6c by D$_2$O exchange and, therefore, confirmed the presence of polyhydroxy compounds on Au NPs surfaces.

**Cell Viability Assay by MTT**

MTT assay was performed to assess the effect of Au NPs on cell viability of MCF-7 cell lines (Table 1; Fig. 7). During first 24 h, per cent cell survival was found to be 40% for MCF-7 cell lines. Thereafter, the rate of cell survival for MCF-7 cells gradually increased with the increase in incubation period. Cisplatin was used as positive control against MCF-7 cell lines. Au NPs in the concentration range of 10-50 µg/mL became non-cytotoxic to MCF-7 cells after 48 h of incubation. The highest per cent cell survival of 98.26% at 48 h and 99.64% at 72 h were obtained at 10 µg/mL concentration of Au NPs, indicating less or no cytotoxicity.

**Plausible Mechanism of Reduction of Au NPs**

Fraction number 4, 5 and 6 obtained after gel chromatography exhibited colour change from yellow to dark brown when added to HAuCl$_4$ solution. The Au NPs obtained from fraction 4 were stable for longer periods. Hence, fraction 4 was further analyzed for HR-LCMS for identification of the constituents of plant extract responsible for reduction of gold. Table 2 lists the major components present in the fraction 4 of Cassia leaf extract as per HR-LCMS analysis.

The HR-LCMS showed the presence of apiiin and cosmoisin, the plant flavonoids; meglumine, the aminosugar; P azidophenacyl glutathione and xanthurenic acid, the antioxidants, as major

![Fig. 6 (a-c)—(a & b) $^1$H NMR spectra of pure gold nanoparticles in DMSO; & (c) $^1$H NMR spectra of pure gold nanoparticles in D$_2$O.](image)

![Fig. 7 (a & b)—Cell viability test using MTT assay. The per cent cell survival of MCF-7 cells obtained after incubation with different concentrations of: (a) Au NPs; & (b) cisplatin (control), for 24, 48 and 72 h.](image)

![Table 2—Major components identified in fraction 4 of C. fistula leaf extract by HR-LCMS analysis](table)
constituents of the fraction 4. Earlier, two groups independently have also reported the role of apin and glutathione in reduction of ionic gold and silver\textsuperscript{16,17}. Therefore, on the basis of present study, we propose that the flavonoids, sugar and antioxidants were responsible for reduction of ionic gold to neutral atomic state and their stabilization. Our hypothesis was further supported by the detection of –OH group of alcohols, –CO group of phenolic/carboxylic compounds and –C=C group of aromatic ring in FT-IR spectrum of Au NPs. The NMR results confirmed the presence of polyhydroxy compounds, which further supports our mechanism of reduction by the above-mentioned phyto-constituents. In our study, the results obtained from FTIR, NMR and HR-LCMS were compared, which helped us to predict the phytochemicals involved in reduction and stabilization of gold.

Conclusion

In the present study, rapid, low-cost and environmentally friendly method was developed for the synthesis of Au NPs using \textit{C. fistula} aqueous leaf extract. The biosynthesized Au NPs were thoroughly characterized by several physico-chemical techniques. These biosynthesized Au NPs were found to be nontoxic to MCF-7 cells after 48 and 72 h of incubation. A plausible mechanism of reduction has been put forward, based on HR-LCMS analysis of \textit{Cassia} leaf extract and further supported by FTIR and NMR analyses of bioreduced Au NPs. The results obtained by HR-LCMS, FTIR and NMR analyses validated the involvement of flavonoids, sugar and antioxidants in reduction of gold. The polydisperse nature of Au NPs was associated with the involvement of more than one phyto-constituents in reduction of ionic gold. In future studies, the phyto-reducing agents would be separated and used for obtaining Au NPs of uniform size and shape. Also, the nanoconjugates of Au NPs with cisplatin and other drugs will be developed to explore these nanoconjugates as better drug delivery system.

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