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BIOGENIC SYNTHESIS BY *SPHEARANTHUS AMARANTHOIDS*; TOWARDS THE EFFICIENT PRODUCTION OF THE BIOCOMPATIBLE GOLD NANOPARTICLES

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Engineered nano materials are at the leading edge of the rapidly developing nanosciences and have found important classes of new materials with specific physiochemical properties different from the bulk materials with the same composition. Since the concept of nanotechnology was first formed, the possible medical application of nano-scale materials and devices has drawn considerable interest. Increasing environmental concerns over chemical synthesis routes have resulted in attempts to develop nanoparticles through biogenic synthesis. One of them is the synthesis using plant parts, which eliminates the elaborate process of maintaining the microbial culture and often found to be kinetically favourable than the other bioprocesses. In this paper we have reported the green synthesis of gold (SA-AuNPs) nanoparticles by reduction of chloroauric acid solution, using leaf extract of *Sphearanthus amaranthoides.* The process for the synthesis of gold nanoparticles (AuNPs) is rapid, novel and ecofriendly. Formation of the AuNPs was confirmed by surface plasmon spectra using UV–Vis spectrophotometer and absorbance peak at 525 nm. Their morphology and elemental composition were determined by Scanning Electron Microscopy (HR-TEM), Energy Dispersive X-Ray spectroscopy (EDX). The average size of SA-AuNP was in the range of 39±5 nm and the observed morphology was spherical. A viability assay of SA-AuNPs showed biocompatibility with African green monkey kidney (Vero) cells. Accordingly this eco-friendly process for the bio-mimetic production of SA-AuNP is nontoxic in nature; consequently, it will find potential application in nano-biotechnology.

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1. Introduction

Nanotechnology (Feynman 1991) is a rapidly expanding field, encompassing the development of man-made materials in nanometer size range. In a broad sense it is the science and engineering concerned with the design, synthesis, characterization and application of materials and devices that have functional organization in atleast one dimension on the nanometer (i.e, one billionth of a meter) scale, ranging from a few to about 100 nanometer. Nanoparticles appeal to scientists across many disciplines due to the opportunity to engineer many particles that might otherwise be in compatible on a single device. Nanomaterials are being viewed as the future material and for various diverse applications in the areas such as biomedical science, optics, mechanics, magnetic, catalysts, biosensors and energy science (Wang 1991; Salta 2004; Alivisatos

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1996). A decade ago nanoparticles were studied because of their size dependant physical and chemical properties (Murray et al., 2000). Now they have entered commercial explosion period (Mazzola 2003; Paull et al., 2003). Medical therapies have become more tailored to specific disease and patients in recent years. Although ultraviolet irradiation, aerosol technologies, lithography, laser ablation, ultrasonic fields, and photochemical reduction techniques have been used successfully to produce nanoparticles, they remain expensive and involve the use of hazardous chemicals (Esumi et al., 2001; Feitz and Waite 2004; Lin and O'Connor 2001). If the manufacturing process is reinvestigated and eco friendlier processes are adapted, then nanotechnology would be a technology which performs miracles in life. In the era of growing awareness about increasing population and global warming, there is a quest to develop new methods for the synthesis of nanomaterials with green technologies.

This quest for an environmentally sustainable synthesis process has led to few biomimetic approaches which involve the bioreduction processes employing different microorganisms (Mohanpuria et al., 2008; Sastry et al., 2003; Verma et al., 2011; Narayanan and Sakthivel 2010). But sometimes the synthesis of nanoparticles utilizing plants/parts of plants could prove advantageous over others biological processes by eliminating the elaborate process of maintaining the microbial culture. It is also important to recognize that various herbs, spices, and plant source occlude powerful antioxidants as phytochemical constituents in seeds, stems, fruits and in leaves (Deorukhkar et al., 2007; Nishino et al., 2007; Johnson 2007; Kwon et al., 2007; Kaur 2007). While exploring the natural secrets for the synthesis of nanoparticles by plants/parts of plants, which are regarded as potent eco-friendly green nano-factories, scientist have discovered gold nanoparticles from live Alfalfa plants (Gardea et al., 2002), *Terminalia catappa* leaf extract (Ankamwar 2010), tamarind leaf extract (Ankamwar et al., 2005) *Pelargonium graveolens* leaf extract (Shankar et al., 2003), neem leaf extract (Tripathy et al., 2010), pear extract (Ghodake et al., 2010), tea leaves (Nune et al., 2009), cumin seeds (Katti et al., 2009) and *Cinnamomum camphora* leaf extract (Hung et al., 2007). The utility of plant based phytochemicals in the overall synthesis and architecture of nanoparticles and various nanoparticle embedded products is highly attractive as it brings an important symbiosis between natural/ plant sciences and nanotechnology (Siddiqui et al., 2009; Matthew and Albrecht 2007; Roy 2006). This connection between nanoscience and nanotechnology provides an inherently green approach to nanotechnology referred to as green nanotechnology (Hutchison 2008; Hutchison 2009; Vigneshwaren et al., 2006). The interaction between metals and various phytochemicals in plants have been exploited for various biological applications in the field of biomedicine. And the plant mediated synthesis have been emerged as a promising field of research as Nanobiotechnology interconnecting nanotechnology and biotechnology.

Sphearanthus amaranthoides, a reservoir of phytochemicals like flavonoids, carbohydrate, tannins, saponins, steroids, glycosides, terpenoids and alkaloids is a small procumbent herb, with stems rooting and pubescent with appressed hairs, leaves palmately 3-foliolate. This plant is well known for its medicinal value for the treatment of eczema, blood disorders, stomach worms, filaria, fever and as a remover of kapha, vata and piles. It is also known to cure skin diseases (Kirtikar and Basu 1971).

Inspite of the above credentials the synthesis of nanoparticles from this plant have not yet been explored to the best of our knowledge. Hence in this study, we synthesized gold nanoparticles using screened dry leaf extract of the *Sphearanthus amaranthoides* (SA-AuNP). Fourier-transform infrared spectroscopy (FTIR) analysis was used to identify the biomolecules responsible for reducing chloroaurate ions and stabilizing the gold nanoparticles formed.

2. Experimental section

Chloroauric acid and other chemicals unless specified were purchased from Sigma (St.Louis, Mo). Dry leaf powder of *Sphearanthus amaranthoids* used in the synthesis of gold nanoparticles was procured from organic grocery sources.

2.1. *Sphearanthus amaranthoids* leaf extract mediated biosynthesis of gold nanoparticles (SA-AuNp)

In this study the dry leaf extract of *Sphearanthus amaranthoides* is used to obtain phytochemically derived reducing agents for the generation and stabilization of gold nanoparticles. SA-AuNPs were prepared by (Nune et al., 2009) method with slight modifications. To a 25 ml vial was added 20 ml of doubly ionized water (DI), followed by the addition of 90 mg *Sphearanthus amaranthoides*. The reaction mixture was stirred continuously. To the stirring mixture was added 300 µl of 0.1 M $HAuCl_4$ solution (in DI water). The color of the mixture turned purple-red from pale yellow within 5 minutes of the addition, indicating the formation of gold nanoparticles. The reaction mixture was stirred for an additional 15 minutes. The gold nanoparticles thus formed were separated from residual *Sphearanthus amaranthoides* immediately using a 5 micron filter and were characterized by UV-Vis absorption spectroscopy HR-TEM, EDX and FTIR analysis.

2.2. Characterization of gold nanoparticles

The stability and the identity of the biocompatible gold nanoparticles were measured by recording UV absorbance after 30 min. A 0.5 ml of aliquot of reaction mixture was harvested after 30 min, diluted with same volume of water and monitored with a UV-visible spectrophotometer Hewlett-Packard diode array spectrophotometer (model HP-8452) using 10 mm optical path-length quartz cuvette operated at a resolution of 2 nm. Samples for high-resolution transmission electron microscopic (HR-TEM) analysis were prepared by drop coating Au nanoparticles solutions onto carbon coated copper TEM grids. The films on the TEM grids were allowed to stand for 2 min following which the extra solution was removed using a blotting paper and the grid is allowed to dry, prior to the measurement. HR-TEM measurements were performed on a JEOL TEMSCAN2000EX instrument operated at an accelerating voltage at 80 keV. Elemental analysis was performed using energy dispersive X-Ray spectroscopy (EDX). FTIR spectroscopy measurements were carried out to recognize the bio-groups that bound distinctively on gold surface and involved in the synthesis of these nanoparticles. For FTIR spectroscopy measurements the drop coated samples on Si (III) wafers were prepared. After complete reduction of $AuCl_4$ ions by *Sphearanthus amaranthoides* leaf extract the gold nanoparticles thus formed was centrifuged at 9000 rpm for 10 min to isolate the gold nanoparticles from free proteins or other compounds present in the solution. The gold nanoparticle pellets obtained after centrifugation were redispersed in water prior to FTIR analysis centrifuged again at 9000 rpm for 10 min to isolate gold nanoparticles from trace of free proteins or other compounds present in the solution if any. The FTIR measurement of SA leaf extract reduced gold nanoparticles were carried out on a Brukere Tensor 27 FTIR spectrometer in attenuated total reflection mode using the spectral range of 4000-400cm^{-1} with the resolution of 4 cm^{-1}.

2.3. *In vitro* stability of gold nanoparticles

The *in vitro* stability of SA-AuNPs was determined by observing the consistency of SPR band when the nanoparticle solution was combined with NaCl, Bovine serum albumin (BSA), cysteine and histidine. In brief, 0.5 ml of gold nanoparticles was added to aqueous solutions of 10% NaCl, 0.5% cysteine, 0.2 M histidine and 0.5% BSA. The stability with respect to the SPR band was determined by recording the UV-visible absorbance.

2.4. Cell culture and viability test

The VERO cells were maintained in Dulbecco's modified eagle's medium (DMEM;Invitrogen) supplemented with 12.5% horse serum, 2.5% fetal bovine serum, 50 U/ml penicillin, and 5 mg/ml streptomycin - at an incubator setting of 5% CO_2 and 37°C. All the experiments were carried out 24-48h after cells were seeded. The cells were routinely harvested by trypsinization 0.25% when the cells approached sub confluent stage and were plated on 25-cm culture flasks split into 1:6.

2.5. Determination of cellular viability

The cell viability was determined using a modified MTT assay as described previously (Yamamoto et al., 2000). In brief, VERO cells were seeded in collagen coated 96 well plates at a density of 1×10^6 cells/ml. The cultures were grown for 48h, then the medium was changed to that containing various concentrations of SA-AuNPs (25 µg/ml, 50 µg/ml and 100 µg/ml). After incubation for up to 48h, MTT solution (5mg/ml in DMEM) was added to 96 well plates and the cells were allowed to incubate for 4h at 37°C. After the medium had been removed, the cell and the dye crystals were solubilised by adding 200µl of dimethylsulfoxide (DMSO), and the absorbance was measured at 570nm (540nm as reference) with a model 550-microplate reader (Biorad). Viable cells exhibiting normal growth as that of control were considered to be 100% viable.

3. Results and discussion

We initiated studies to address the possible application of leaf extract of *Sphearanthus amaranthoides* for the synthesis of gold nanoparticles. The exploitation of gold nanoparticle in biomedicine to treat various fatal diseases has inspired us to pursue the application of an assortment of phytochemicals in various vectors from plant kingdom for the synthesis of gold nanoparticles. The outstanding prerequisite for applying gold nanoparticles in *in-vivo* imaging and therapy is that the nanoparticles should be produced in biologically providential media (Siddique et al., 2009; Shankar et al., 2004; Mohanpuria et al., 2007). The leaf extract of *Sphearanthus amaranthoides* is a resovior of phytochemicals including flavonoids, carbohydrate, tannins, saponins, steroids, glycosides, terpenoids and alkaloids (Swarna Latha and Neelakanta Reddy 2009). In addition the presence of saccharides in the extract provides synergestic reducing power for the rapid transformation of chloroaurate ions to gold nanoparticles. The preliminary objective of this study is to synthesize gold nanoparticles under 100% green chemistry processes and to confirm the nontoxic nature of green nanoparticles of size ranging from 39.1 to 46.4 nm in *in-vitro* system.

3.1. Characterization of gold nanoparticles

Simple mixing of the leaf extract of *Sphearanthus amaranthoides* with 0.1 M $HAuCl_4$ at room temperature promptly initiated the appearance of a purple red colour, indicating the formation of gold nanoparticles (Figure 1 insert). It is well known that the optical absorption spectra of the metal nanoparticles are dominated by the surface Plasmon resonance (SPR). The change in colour was due to the collective coherent oscillation of conduction electrons at the surface of gold nanoparticles. When these particles interact with the oscillating electric field of the incident light, a phenomenon called surface Plasmon resonance results. This change in colour indicates that the reduction in $HAuCl_4$ ions take place. When this reaction was traced with UV-Visible spectroscopy, the presence of characteristic SPR bands observed at *ca.* 525 nm (Figure 1) which further confirmed the presence of gold nanoparticles.

UV visible spectroscopy is a valuable tool for structural characterization of gold nanoparticles. The optical properties of metal nanoparticles are dominated by surface Plasmon resonance (SPR), which shifts to longer wavelength with increasing particle size (Brause et al., 2002). The position and shape of Plasmon absorption of gold nanoparticles are strongly dependent on particle size, dielectric medium and surface adsorbed species (Kreibig and Vollmer 1995; Mulvaney 1996). According to Mie's theory (Mie G 1908) spherical nanoparticles have strong absorption at 520 nm with almost no absorption after 600 nm with only a single SPR band in adsorption spectra, however, the triangular shape has absorption at 540 which extends well in near infra red region, whereas anisotropic particles could give raise to two or more Surface Plasmon Resonance band depending on the shape of the particle. The number of SPR peaks increases as the symmetry of the nanoparticle decreases (Sosa et al., 2003). Thus spherical nanoparticles, disks and triangular nanoparticles of gold show one, two or more peaks respectively. The absorption

measurement of the biologically synthesized gold nanoparticles [SA-AuNP] indicated that the Plasmon resonance wavelength, λ_{max} is at 525, nm (Figure 1). This spectral characteristic of nanoparticles interprets that the biologically synthesized gold nanoparticles were spherical in shape. The surface Plasmon band in the SA-AuNPs remains close to 520 nm throughout the reaction period, suggesting that the particles are dispersed in the aqueous solution with no evidence of aggregation.

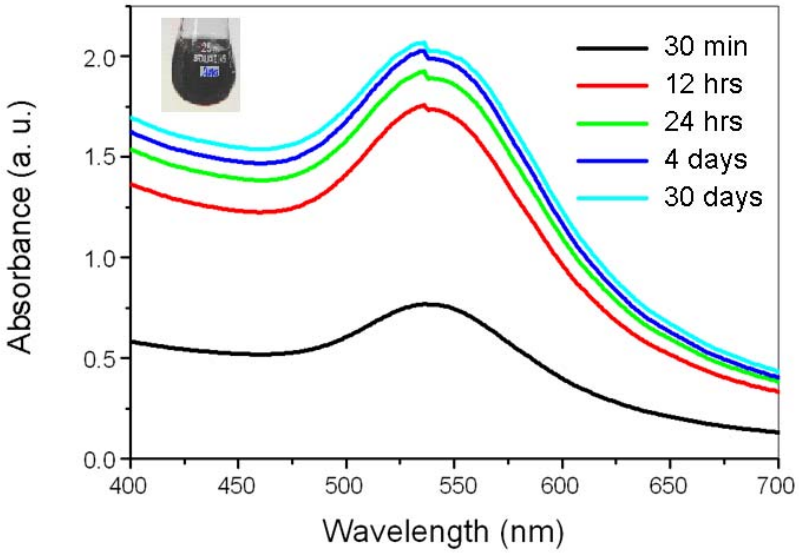

Fig. 1. Absorption spectra obtained after 30 min, with or without dilution with the same volume of water, of Sphearanthus amaranthoides dry leaf extract and $HAuCl_4$ incubated for 12hrs, 24hrs, 4days and 30days. The SA-AuNPs were stable for over a month with no signs of aggregation. The insert shows conical flak containing SA-AuNP after reaction with 0.1 M $HAuCl_4$ aqueous solutions.

After completion of the reaction the nanoparticles were tested for their stability. It was observed that the nanoparticle solution of gold was extremely stable for more than a month with no sign of aggregation even at the end of this period (Figure 1). The particles are thus stabilized in the solution by capping agent that is likely to be the polyphenols present in the leaf extract. The leaf extract of *Sphearanthus amaranthoides* thus performed well as a bioresource for initiating the reduction of $HAuCl_4$, resulting into rapid production of gold nanoparticles.

Our process for the production of gold nanoparticles uses the direct interaction of sodium tetrachloaurate with dried leaf powder of *Sphearenthus amarenthoides* without the intervention of any toxic reducing chemical reagents. Therefore, this reaction pathway satisfies all the conditions of a 100% green chemical process (Hung et al., 2007; Jorge et al., 2003; Gardea et al., 2002). Gold nanoparticles produced by this process did not require any external chemicals for the stabilization of the nanoparticulate matrix. The current innovation on the unique chemical power of phytochemicals in *Sphearenthus amarenthoides* in initiating nanoparticle formation is of overriding importance in the context of production of gold nanoparticle for medical and biological application under non-toxic conditions. It has been reported that the synthesis of gold nanoparticles using geranium extract required more time to initiate and was complete in 48 h (Joerger et al., 2000). Biosynthesis of silver and gold nanoparticles using bacteria (Klaus et al., 2001; Nair and Pradeep 2002; Ahmad et al., 2003; Mukherjee et al., 2001), and fungi (Huang et al., 2007), has been reported, and the time required for completion of the reaction range from 24-120 h. Intracellular synthesis, prolonged synthesis, multiple purification steps and the maintainance of cell cultures are the drawbacks of microbial procedures (Kumar and Yadav 2009). A simple, efficient, green approach to the production of biocompatible gold nanoparticles using coffee, tea and soy beans has also been described (Nune et al., 2009; Song et al., 2009). In addition *Cinnamonum camphora* leaf extract has been identified as an efficient reducing and stabilizing agent for the production of gold as well as silver nanoparticles (Ankamwar et al., 2005). The

extracellular synthesis of highly stable Ag and Au nanoparticles has also been achieved using *Emblica officinalis* fruit extract (Jain 2007). Recently, the biosynthesis of biocompatible gold nanoparticles using essential nutritious phytochemicals as well as their possible application in molecular imaging and therapy have been reported (Nune et al., 2009, Song et al., 2009, Ankamwar et al., 2005, Santra et al., 2005)

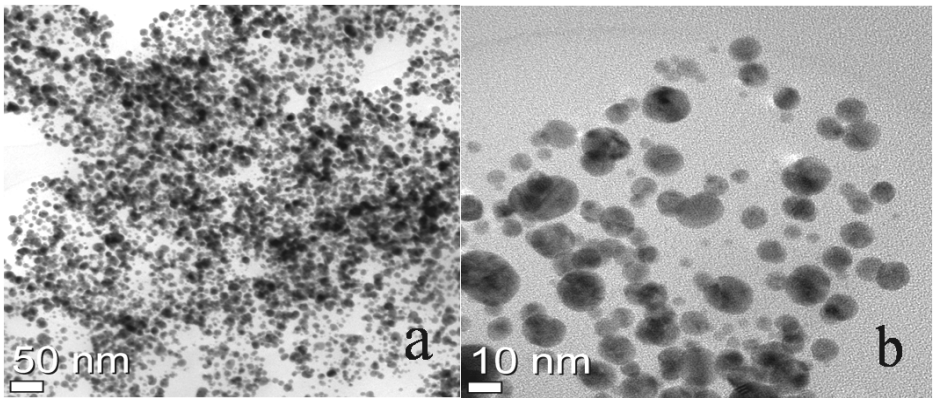

Fig. 2. HR-TEM images of gold nanoparticles synthesized using Sphearanthus amaranthoides dry leaf extract at 30 min.

A representative HR-TEM pictures recorded from the SA-AuNP film deposited on a gold HR-TEM grids is shown in Figure 2. The picture shows individual gold particles as well as a number of aggregates. The gold nanoparticles formed were polydispersed, predominantly spherical with uniform size distribution in the regime of 39-47 nm (Figure 3). Although the actual value of the mean size might vary slightly from each preparation, the size distribution was found to be always about 10% standard deviation. Size distribution analysis clearly showed that nearly 90% of the particles reside within their size range. The nanoparticles were not in direct contact even within the aggregates indicating stabilization of the nanoparticles by a capping agent. The capping agent may be various phytochemical constituents present in *Sphearenthus amarenthoides.* These phytochemicals are presumably responsible for the creation of a robust coating on gold nanoparticles and thus rendering the nanoparticles stable against agglomeration. The results obtained in the synthesis and characterization of the green synthesized nanoparticle is strongly supported by previously published reports on synthesis of gold nanoparticles using phytochemicals (Nune et al., 2009, Katti et al., 2009). The compositional analysis by EDX illustrated the purity of the gold, with spectra showing a strong AU signal and Na signal from the grid on which the nanoparticle is coated (Figure 3).

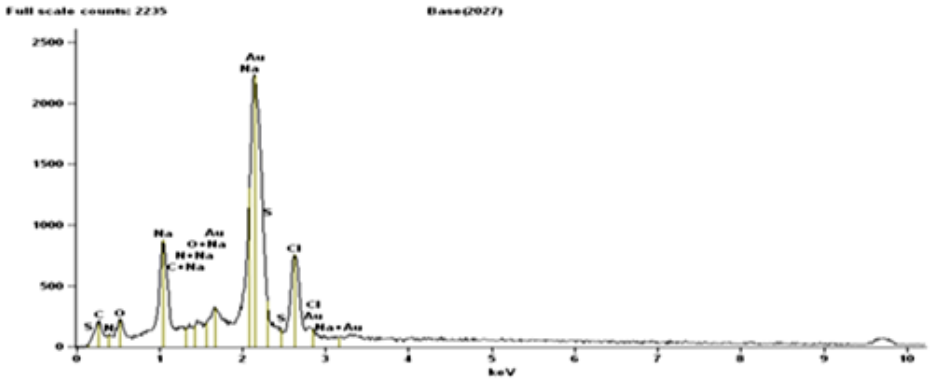

Fig. 3. Composition of gold nanoparticles as determined by EDX.

FT-IR absorption spectra can provide the information about the chemical change of the functional groups involved in bioreduction. Figure 4 shows FT-IR absorption spectra of SA extract

before and after bioreduction. To a large extent, the band at 1101 cm^{-1} might be contributed by the —C—O groups of the polyols, such as flavones, terpenoids and polysaccharides in the biomass (Kang et al., 2008; Shankar et al., 2003). The disappearance of band at 1101 cm^{-1} after bioreduction suggested that the polyols might be partly responsible for the reduction of chloroaurate ions. FT-IR analysis of bioextract before and after the addition of gold solution also revealed the strong bands at 1021, 1443, 1634 and 3428 cm^{-1}. The band at 1021 cm^{-1} corresponds to C—N stretching vibrations of amine and at 1443 cm^{-1} corresponds to C—H and OH bending and 3428 cm^{-1} is characteristic of —NH stretching of amide (II) band. The weaker band at 1634 cm^{-1} corresponds to amide I, arisen due to carbonyl stretch in proteins.

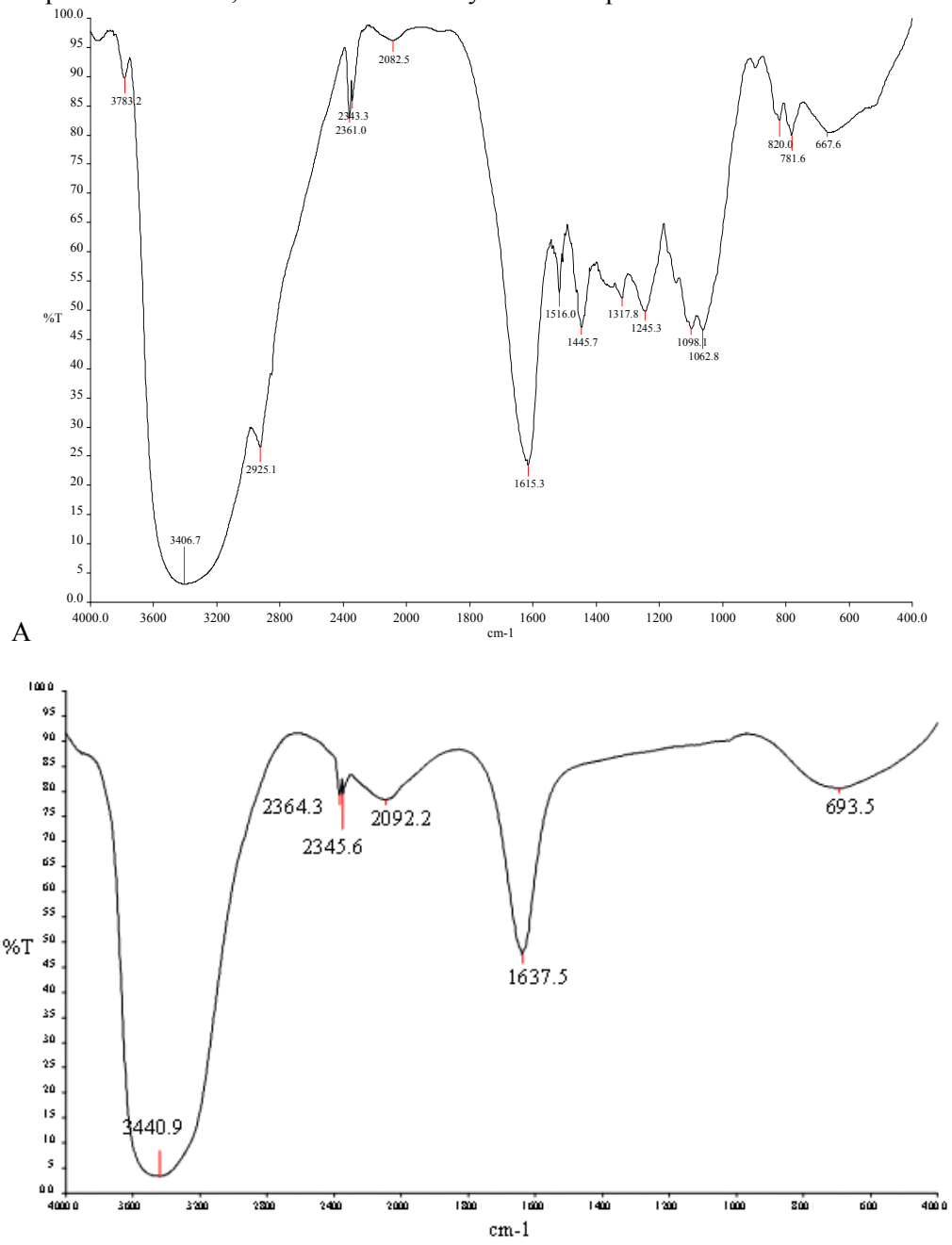

Fig. 4. FTIR spectra of Sphearanthus amaranthoides extract (A) and gold nanoparticles generated from Sphearanthus amaranthoides (B).

3.2. *In vitro* stability of gold nanoparticles

An important concern regarding the biomedical application of nanoparticles is their stability in aqueous environment over a reasonable period of time. Therefore the *in vitro* stability of SA-AuNPs was assessed. The absorbance in aqueous mixtures changed by approximately 10 nm under all experimental conditions upon combining nanoparticle solution with 10% NaCl, cysteine, histidine and Bovine Serum Albumin (BSA) solutions that mimic biological environments. The stability and the identity of SA-AuNPs were measured by recording UV absorbance after 24 h, as shown in Figure 5. The plasmon resonance band at ~535 nm confirmed the retention of nanoparticulates in all the above mixtures. This retention indicates that the AuNPs are intact, and thereby demonstrate excellent *in vitro* stability in biological fluids.

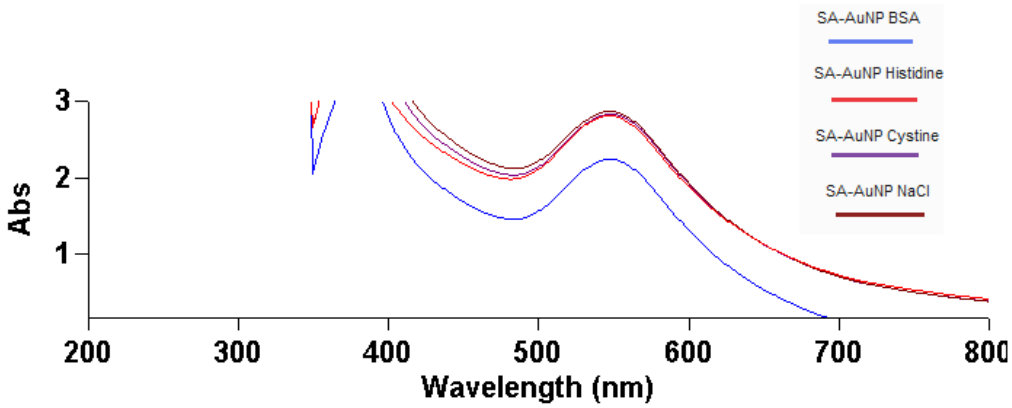

Fig. 5. In vitro stability of SA-AuNPs in various biological fluids. The figure shows the absorption spectra obtained after the reaction of SA-AuNP with BSA, Histidine, Cystine and NaCl.

3.3. Cell viability of gold nanoparticles

The key parameters in evaluating the biocompatibility of gold nanoparticles are cytotoxicity and cell viability. The cytotoxicity of SA-AuNPs under *in vitro* conditions in Vero cells was examined in terms of effect of gold nanoparticles on cell proliferation by the MTT assay. We examined the effect of SA-AuNPs on cell viability by subjecting Vero cells to the concentration of 10, 50, 100, 150 and 200 µM nanoparticles for 24h, 48 hr, 72 hr and 96 hrs. The cells showed more than 85-90% viability to the concentrations up to 150 µM (Figure 6). As the incubation time increased, biocompatibility increased, reaching 95 - 99% in all studied concentrations, indicates the adaptation of the Vero cells to the SA-AuNP environment. Therefore the cell viability assay indicated that SA-AuNPs are nontoxic. The bio-compatibility of AuNPs was increased gradually at all concentrations by time, because the regulation of cell metabolism to new environment and their healthy acceptance as incubation increases. Here there are two possibilities about the interaction and metabolism of SA-AuNPs, one is adsorption source to delay in growth, next is metabolism or internalization results viability of cell and healthy growth. Here we belive that, AuNPs prepared by this method are not chronically toxic to the cell growth or for their viability. The capability of SA-AuNP phytochemicals to effectiently reduce chloaurate ions to biocompatible gold nanoparticles has now been demonstrated. This single-step green method uses *Sphearenthus amaranthoides* extract for both the production and subsequent nontoxic biomimetic coating of gold nanoparticles. This approach could find continuing application in diagnostics and therapeutics as previously reported (Albrech et al., 2006, Nune et al., 2009, Ankamwar et al., 2005, Lewinski et al., 2008).

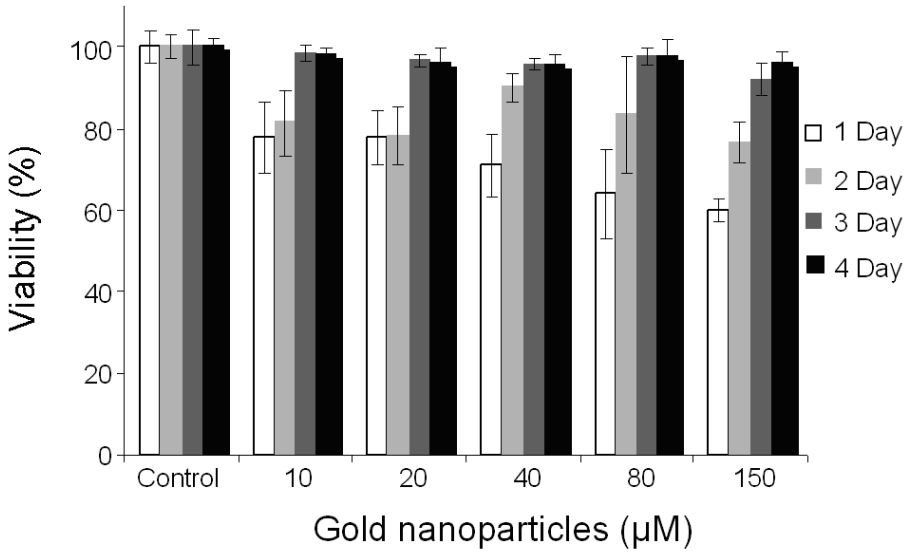

Fig. 6. Viability of Vero cells treated with 10, 50, 100, 150, 200 µM gold nanoparticles and measured after incubation for 24h, 48 h, 72 h and 96 h.

4. Conclusion

Increasing awareness towards green chemistry and biological processes has led to a desire to develop spanking new and simple method for the synthesis of non-toxic gold nanoparticles. This gold nanoparticle offers a valuable contribution in the area of green synthesis and nanotechnology without adding different physical and chemical steps. Unlike other processes in physical and chemical methods, which involve hazardous chemicals, phytochemical mediated biosynthesis of nanoparticles is cost-effective and eco-friendly approach. Therefore, plant mediated synthesis of nanoparticles has been emerged as an important branch of Nanobiotechnology. Due to their rich diversity of phytochemicals, plants have the innate potential for the synthesis of nanoparticles and they could be regarded as potential biofactories for nanoparticles synthesis. Our results have demonstrated the unique kinetic propensity of phytochemicals present in dry leaf powder of *Sphearanthus amaranthoides* to reduce gold metal to the corresponding gold nanoparticle. The versatile phytochemical mediated green nanotechnological process has been shown to be effective in the generation and stabilization of non-toxic gold nanoparticles for their direct application in myriad of therapeutics and diagnostics. Occlusion of disease fighting phytochemicals in various plant specious and their future utility in the development of gold nanoparticles will provide unprecedented opportunity towards the design and development of functional gold nanoparticles that can be safely produced, stored and shipped worldwide.

Competing interests

No competing interests

Authors' contributions

Both the authors contribute equally to this work in co-ordinating and performing the experiments, writing the manuscript, design, interpretation and data analysis.

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