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Research Paper

One Step Biosynthesis of Gold Nanoparticles Using the Leaf Extract of *Gymnema sylvestre* and Study of Its In-vitro Anticancer Activity on MCF-7 Cell Lines

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Abstract: *The leaf extract of Gymnema sylvestre has been utilized for the synthesis of gold nanoparticles (AuNPs) at room temperature under a very mild condition without any additional stabilizing or capping agents. Mostly spherical shaped AuNPs of 9-11 nm average diameters were obtained. The stabilized gold nanoparticles were characterized by Surface Plasmon Resonance Spectroscopy, DLS, HRTEM, Energy dispersive X-ray spectroscopy, SAED and X-Ray diffraction studies. The antioxidant activity of the leaf extract of Gymnema sylvestre has been studied against a long lived 2,2-diphenylpicrylhydrazyl (DPPH) radical at room temperature. The in-vitro MTT assay revealed a potent, selective, dose and time dependent anticancer activity of the freshly synthesized AuNPs on MCF-7 breast cancer cell lines and showed IC_{50} at 30.47 µg/mL.*

Keywords: Key words: *Gymnema sylvestre, green synthesis, gold nanoparticles, MTT assay, anticancer.*

Introduction

Metal nanoparticles having extremely small size and high surface area to volume ratio, with at least one of their dimensions within 1-100 nm, have received tremendous attention during the last two decades due to novel chemical and physical properties[1-5]. Among various metal nanoparticles, gold nanoparticles (AuNPs) with its unique optical, electronic and magnetic properties are one of the most extensively studied and broadly used nanomaterials in biomedical applications as well as catalysis, biodiagnostics, pharmaceuticals, etc.[6-9]. Colloidal AuNPs dispersed in water and stabilized with non-toxic biomolecules are required for many of such applications for environmental reasons, biocompatibility and biodegradability[10]. Although various methods have been reported in the literature for the synthesis of AuNPs, the plant extract based reductive method, involving the reduction of Au(III) to Au(0) by the easily oxidizable phytochemicals, has gained profound significance in recent years because such methods will lead to "green" and "sustainable" developments. The renewable and non-toxic nature of the plant extracts, mild reaction conditions and eco-friendly aqueous medium make the method advantageous over other hazardous synthetic methods[11,12]. Additionally, as the phytochemicals present in the plant extract stabilize the synthesized AuNPs, no additional stabilizers or capping agents are needed for obtaining stabilized AuNPs. The green synthesis of AuNPs from the extracts of *Macrotyloma uniflorum*[13], *Trigonella foenum-graecum*[14], *Aloe-vera*[15], *Acacia nilotica* leaf[16], *Saraca indica* bark[17], *Punica granatum*[18], *Green coconut* shell[19], *Mimusops elenge*[20], *Abroma augusta*[21] *etc.* have been reported[22,23]. *Gymnema sylvestre*, popularly known as "Gurmar" is a climbing plant distributed over most of India. It is well known for its use as a traditional medicine in the treatment of

diabetes along with other diseases like dyspepsia, constipation[24], jaundice, haemorrhoids[25], renal and vesicle calculi[26], cardiopathy, asthma[27], bronchitis, amenorrhoea and leucoderma[28]. Recently, a preliminary report on the anticancer activity on HT29 cell lines has been reported utilizing the AuNPs synthesized using the leaf extract of *Gymnema sylvestre*.

The incidence of breast cancer has been increasing worldwide for many decades and efforts are ongoing to fight against this deadly disease[29]. Among various cell lines used for the study of breast cancer, MCF-7 is the most studied human breast cancer cell line in the world[30]. Additionally, the results from this cell line had a fundamental impact upon breast cancer research and patient outcomes[31]. However, according to our knowledge, there is no report in the literature of AuNPs synthesized using the leaf extract of *Gymnema sylvestre* for the anticancer activities on MCF-7 cell lines.[32] Herein, we report a very mild and environment friendly method for the synthesis of AuNPs from the leaf extract of *Gymnema sylvestre* without any additional capping or stabilizing agents. Characterization of the stabilized colloidal AuNPs were carried out by High Resolution Transmission Electron Microscopy (HRTEM), Energy Dispersive X-ray spectroscopy (EDX), Selected Area Electron Diffraction (SAED), SPR spectroscopy, X-Ray diffraction and FTIR studies. The antioxidant activity of the leaf extract of *Gymnema sylvestre* has been studied against a long lived 2,2-diphenylpicrylhydrazyl (DPPH) radical at room temperature. The *in-vitro* MTT assay revealed a potent, selective, dose and time dependent anticancer activity of the freshly synthesized AuNPs on MCF-7 breast cancer cell lines. Reactive Oxygen Species (ROS) measurement shows the involvement of apoptosis in cell death.

Material and Methods

Reagents

DPPH was purchased from Sigma-Aldrich. $HAuCl_4$ was purchased from SRL. Ferric chloride ($FeCl_3$) was procured from Himedia. All the chemicals were analytical grade and used without further purification. Double distilled water was used for the experiment.

Cell Culture and Maintenance

MCF-7 breast cancer cell line was obtained from Jadavpur University, Kolkata, India. The cell line was cultured in DMEM complete media with 10% FBS (fetal bovine serum). L-glutamine (2 mM), penicillin (100 µg/mL), streptomycin (100 µg/mL) were required for the culture of the cell. Cultivated cells were incubated under 5% CO_2 at 37°C temperature in a CO_2 incubator. Cells were grown in an exponential form until it reaches $1x10^6$ cells/mL growths.

Selection of Subjects for Lymphocytes

Lymphocytes were separated from six different human samples belonging to the same geographical area and having the same environmental condition. The subjects were devoid of any kind of drug and anti-oxidant supplementation. Written consents were provided by these patients. Total process of lymphocytes separation was abided by Helsinki[23]. Ethical committee of Vidyasagar University had approved the process.

Isolation of Human Lymphocytes

Blood samples (5 mL) were collected from six healthy persons by vein-puncture in a heparin coated Vacutainer according to the method of Hudson and Hay[24]. Blood was diluted with PBS (Phosphate Saline buffer, 1:1 v/v) and centrifuged using Histopaque 1077 (Sigma) at 1500 rpm for 40 min for separation of the layers. Then the separations of lymphocytes were carried out following the previously described method.

Au (III) solution

$HAuCl_4$ was purchased from SRL (Sisco Research Laboratory) and used without further purification. $HAuCl_4$ (36.5 mg) was dissolved in deionized water (10 mL) to obtain a 10.74 mM Au(III) stock solution.

Preparation of the leaf extract of *Gymnema sylvestre*

The leaves of *Gymnema sylvestre* were collected from the local area of Midnapore, West Bengal, India and identified at the Department of Botany and Forestry, Vidyasagar University, Midnapore. The leaves were dried in sunlight and pulverized by using a grinder. Finely powdered leaves of *Gymnema sylvestre* (5 g) was suspended in methanol (40 mL) and stirred magnetically at room temperature for 1 h and then filtered. Volatiles of the filtrate were removed under reduced pressure to get a greenish solid (0.6 g). The crude greenish solid was purified by column chromatography (Silica-gel, 100 – 200 mesh) using methanol/ethyl acetate (0-30%) as the eluent to afford a solid (0.2 g). The leaf extract of *Gymnema sylvestre* (0.05 g) was suspended in deionized water (10 mL) and sonicated in an ultrasonicator bath for 20 min to obtain a semi transparent solution (5000 mgL^{-1}).

Identification of Polyphenolic Compounds

The presence of the phenolic compounds in the leaf extract of *Gymnema sylvestre* was examined by ferric chloride test. The leaf extract of *Gymnema sylvestre* (1 mL) was mixed with ethanol (1 mL). Then two drops of concentrated $FeCl_3$ solution was added to the solution. Greenish color appeared instantly indicating the presence of phenolic compounds in the leaf extract which is confirmed by mass spectra analysis.

Synthesis of Gold Nanoparticles

Aliquots of Au (III) solution (0.16 mL, 10.74 mM each) were added drop-wise to the solution of leaf

extract of *Gymnema sylvestre* to prepare a series of stabilized AuNPs where concentration of the extract were 500, 1000, 1500, 2000, and 2500 mgL^{-1} and the concentration of Au (III) was fixed at 0.43 mM. UV-visible spectroscopy of the solutions was carried out after 24 h of $HAuCl_4$ and the leaf extract of *Gymnema sylvestre* had been mixed.

Characterization

TEM images, SAED and EDX of AuNPs were taken from Technai G2 instrument. X-ray diffraction (XRD) patterns of the stabilized AuNPs were recorded in PAN alytical X'pert Pro with Cu-Kα radiation (λ= 1.54 Å). Mass spectra were recorded in Shimadzu GCMS QP 2100 Plus. UV-visible spectra were recorded in Shimadzu 1601 spectrophotometer. FTIR spectra of samples were analyzed using a Perkin Elmer FTIR Spectrum Two model using KBr pellet. DLS Study was carried out by using Malvern Zetasizer Nano series (Model-Nano ZS90) to know the stability and size distribution of AuNPs.

Results and Discussion

The leaf extract of *Gymnema sylvestre* is a rich source of different types of plant secondary metabolites such as triterpenoid saponins, steroids, polyphenolic compounds including flavanoids, etc[25,26]. Mass spectra analysis of the leaf extract carried out in our laboratory also supported the presence most of the compounds. Evidence for the presence of phenolic compounds in ethanolic extract was also obtained from a positive ferric chloride test. In continuation of our investigations on the utilization of triterpenoids (C30s) as renewable functional nano entities[27-33], it occurred to us that the medicinally important leaf extract of *Gymnema sylvestre*, rich in polyphenolic compounds, can be utilized for the synthesis of AuNPs from[34] $HAuCl_4$. As the phenolic compounds have antioxidant properties, initially we resorted to test the antioxidant activity of the extract against a long lived 2, 2-diphenylpicrylhydrazyl (DPPH) radical at room temperature.

Determination of Antioxidant activity by DPPH Assay

The 2, 2-diphenylpicrylhydrazyl (DPPH) assay is widely used in plant biochemistry to evaluate the properties of plant constituents for scavenging free radicals. Antioxidants react with DPPH and convert it to 1,1-diphenyl-2-picryl hydrazine. The degree of decolorization indicates the scavenging potential of the leaf extract. The reducing ability of antioxidants towards DPPH radical can be evaluated by monitoring the decrease in the absorbance intensity at 517 nm in the UV-visible spectroscopy (Figure 1). The decrease in the absorption intensity of DPPH takes place because of the reaction between antioxidant (A-H) present in the leaf extract of Gurmar and DPPH radical. % radical scavenging activity was calculated to be 65.13 when the concentration of the leaves extract is 100 mgL^{-1}.

Synthesis of AuNPs and study of its Surface Plasmon Resonance spectroscopy

Antioxidants including polyphenols are well known for their use in the facile synthesis of metal nanoparticles under very mild condition[11,12]. As the leaf extract of *Gymnema sylvestre* was rich in easily oxidizable plant secondary metabolites including polyphenols, we felt that leaf extract of *Gymnema sylvestre* can be utilized for the green synthesis of AuNPs at room temperature. To test this, we treated the aqueous solutions of the leaf extract contained in vials with $HAuCl_4$ solution (Figure 2). Violet to pinkish red coloration appeared after 30 mins indicating the formation of AuNPs. The intensities of the colors increased on standing the solutions at room temperature for several hours and then remained constant and the AuNPs once formed were stable for several months at room temperature. The stability of the AuNPs could be explained by the high negative zeta potential value -11.0 mV for the AuNPs colloids synthesized by using 2000 mgL^{-1} of the leaf extract**.**

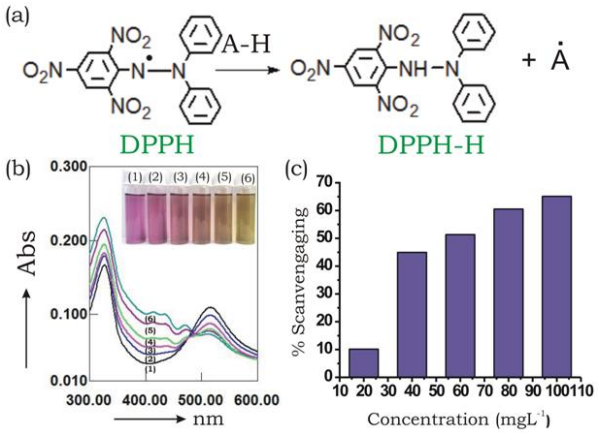

Figure 1: Antioxidant activity studies of the leaf extract of *Gymnema sylvestre*: (a) Reaction scheme showing quenching of DPPH radical by the antioxidant (A-H); (b) UV-visible spectra of DPPH and DPPH + ethanol extract of the leaves; (c) plot of % DPPH radical scavenging vs concentration of the leaf extract

The $HAuCl_4$ showed a strong peak at 221.5 nm and a shoulder peak at 293.5 nm. This was due to the charge transfer interactions between the metal and the chloro ligands (Figure 2a). The intensities of these two peaks decreased with increasing concentration of the leaf extract of *Gymnema sylvestre* and new peaks appeared around 530 nm. This is due to *surface plasmon resonance* (SPR) of the AuNPs, a phenomenon arising due to collective oscillation of the conduction band

electrons interacting with the electromagnetic component of the visible light[35,36]. With increasing the concentration of the leaf extract, a blue shift of the SPR band was observed due to the formation of smaller sized AuNPs. The shoulder peaks observed in the 270-275 nm regions of AuNPs colloids were due to the formation of quinone moiety formed by the oxidation of the phenolic compounds.

HRTEM, XRD, EDX, DLS and FTIR studies

High resolution transmission electron microscopy (HRTEM) was carried out to study the size distribution, shape and morphology of the AuNPs formed at different concentration of the leaf extract of *Gymnema sylvestre*. AuNPs of spherical, triangular, tetragonal, pentagonal and hexagonal shapes were observed. The average size of the AuNPs formed at 1000 mgL^{-1} concentration of the leaf extract was 12 nm. At a higher concentration of the leaf extract (2000 mgL^{-1}) the average particle size was 9 nm (Figure 3**).** The polyphenolic compounds, quinone and other chelating phytochemicals present in the leaf extract could effectively stabilize the smaller sized AuNPs. As the surrounding chelating ligands of the AuNPs prevent further aggregation, the size of the AuNPs is smaller at higher concentration of the leaf extract.

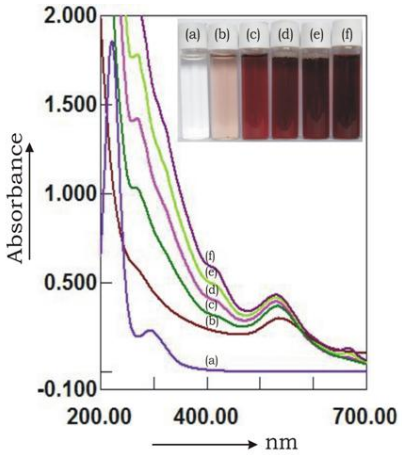

Figure 2: UV-visible spectra of (a) $HAuCl_4$ (0.43 mM), (b-f) AuNPs at 500, 1000, 1500, 2000 and 2500, mgL^{-1} concentrations of leaf extract respectively. Inset: Photograph of the vials containing (a) $HAuCl_4$ (0.43 mM) solution, (b-f) colloidal AuNPs at 500, 1000, 1500, 2000, and 2500 mgL^{-1} of leaf extract respectively (after 24 h of mixing)

X-ray diffraction analysis of the leaf extract of *Gymnema sylvestre* stabilized AuNPs is given in (Figure 4a). Crystalline nature of the metallic face centered cubic AuNPs was confirmed from the characteristic reflections of the planes (111), (200), (220), and (311) at $2\theta = 38.2^{\circ}$, 44.3°, 64.7°, and 77.8° respectively which is further confirmed by SEAD (Figure 4c). This supported the reduction of Au (III) to Au (0) by the phytochemicals present in the leaf extract. The comparatively larger peak intensity of the (111) plane indicated the predominant orientation of this plane. These values are in agreement with the reported standards JCPDS file no. 04-0784. Formation of the AuNPs was also confirmed from EDX analysis which showed the presence of Au along with C from the stabilizing organic ligands (Figure 4b).

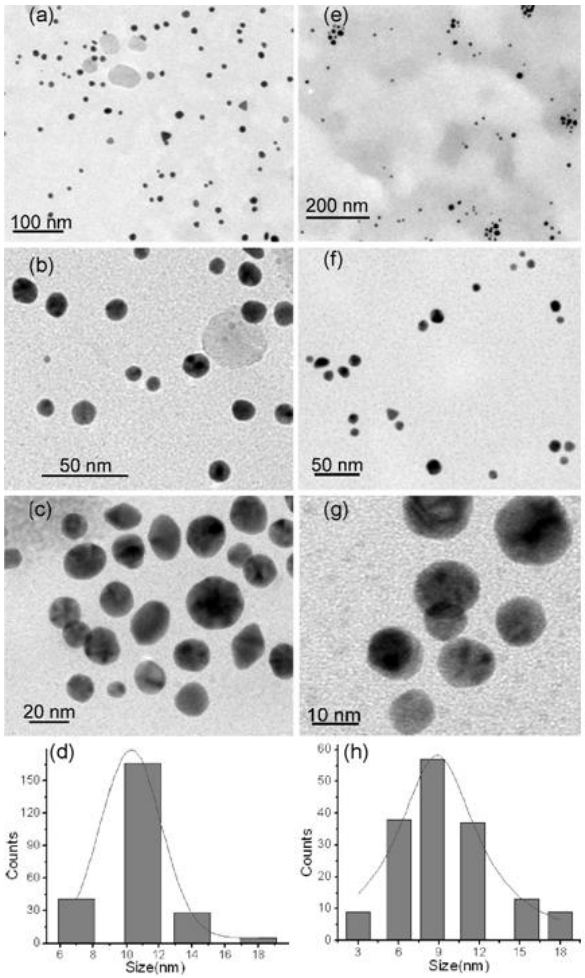

Figure 3: TEM Images of AuNPs obtained from the leaf extract of *Gymnema sylvestre* at 1000 mgL^{-1}(a,b,c) and 2000 mgL^{-1} (e,f,g); (d) Histogram at 1000 mgL^{-1} (average diameter 12 nm); (h) Histogram at 2000 mgL^{-1}(average diameter 9 nm)

The broad peak around 3411 cm^{-1} observed in the FTIR spectrum of the leaf extract of *Gymnema sylvestre* was due to the stretching vibration of the phenolic hydroxyl group (-OH) present in it. The broadness of the peak may be due to intermolecular H-bonding. However, in the FTIR spectrum of stabilized AuNPs utilizing the leaf extract of *Gymnema sylvestre*, the peak in this region became narrower at 3406 cm^{-1} probably due to the interaction of the OH groups with stabilized AuNPs**.**

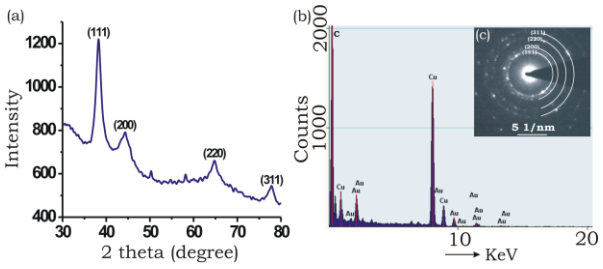

Figure 4: (a) XRD; (b) EDX (c) SEAD of stable gold nanoparticles obtained from the leaf extract of *Gymnema sylvestre*

Mechanism of the formation of Stabilized AuNPs

Leaf extract of *Gymnema sylvestre* is rich source of different types of phytochemicals including polyphenols, flavanoids, fatty acids, terpenoids, etc. The o-dihydroxy compounds present in the leaf extract can form a five member chelate ring with the Au(III) ions. Au(III) ions having a very high reduction potential can be reduced to Au(0) with concomitant oxidation of the polyphenols to corresponding quinones. The freshly generated Au(0) atoms in the reaction mixture can collide with each other forming AuNPs which are stabilized by the concomitantly formed quinones, polyphenols and other coordinating phytochemicals. The steric bulk of the backbone of the benzoquinones derivative and other phytochemicals wrapping around the nanoparticles provide robustness against further aggregation of the stabilized AuNPs (Figure 5).

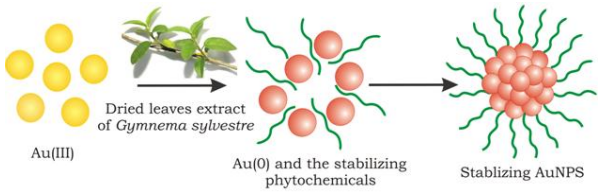

Figure 5: Mechanism of the formation and stabilization of AuNPs by the phytochemicals present in the leaf extract of *Gymnema sylvestre*

Application of Stabilized AuNPs

Application of AuNPs as Anti Cancer agents

Dose and time dependent cytotoxicity assay (MTT assay) of freshly prepared AuNPs (synthesized with 0.5 mL, 1.65 mM $HAuCl_4$) and 4000 mgL^{-1} of the leaf extract of *Gymnema sylvestre* was treated on MCF-7 cell line and viable lymphocytes. The cell viability was estimated by non-radioactive colorimetric assay system using tetrazolium salt, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenil tetrazolium bromide (MTT). The % of cell viablility was calculated by using the following equation[30].

% of cell viability = [OD sample – OD control] X 100/OD control.

The % of cell killing by the drug was 9.62, 52.88, 54.81, 56.73, 61.54 and 65.38 after applying the drug in a concentration of 1 µg/mL, 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL on MCF-7 cell. Interestingly the % of lymphocytes cell killing by the drug was 5.8, 7.4, 9.52, 10.79, 15.57, 20.53, 22.37, 26.53 and 31.13 at 1 µg/mL, 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/ml,100 µg/mL, 200 µg/mL, 300 µg/mL and 500 µg/mL respectively (Figure 6) compared with the control group ($P<0.05$). The half inhibitory concentration (IC_{50}) value was determined from the % of cell death obtained from MTT assay.

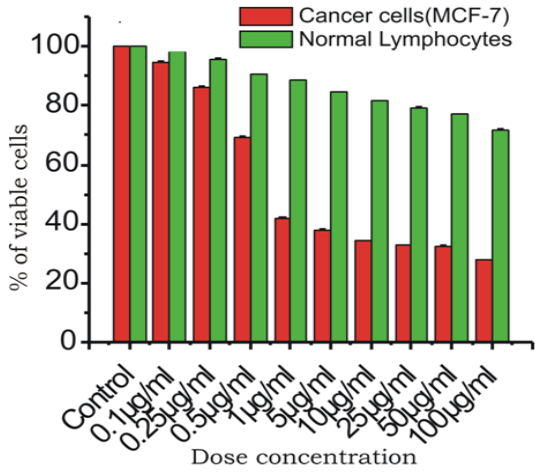

Figure 6: Dose and time dependent MTT assay on MCF-7 breast cancer cell lines and normal

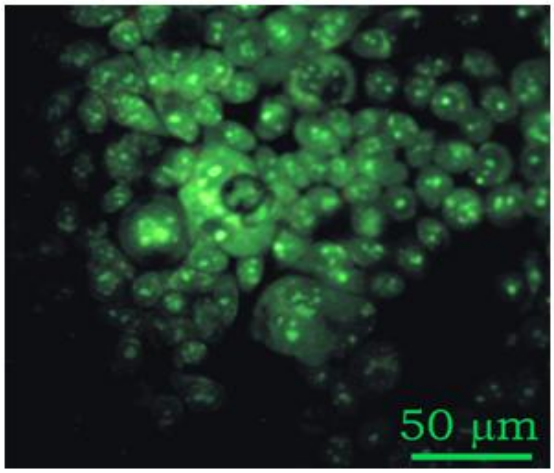

Figure 7: Effect of ROS on MCF-7 cells treated with AuNPs drug at 50 µg/mL dose

The concentration required to inhibit the 50% proliferation of MCF-7 cells and viable lymphocytes was 30.47 μgmL^{-1} and 828.97 μgmL^{-1} respectively. (Multiple linear regressions were used for comparison

of data through statistica version 5.0 (Statsoft, India) software pakage.) As the IC_{50} value of the drug in the case of lymphocytes was high, the drug may be non toxic to the normal lymphocytes at a particular range of doses. 50 μgmL^{-1} doses can be used as a biologically safe drug.

Intracellular Reactive Oxygen Species (ROS) measurement

Normal Lymphocytes and MCF-7 cells were administrated with AuNPs drug for 24 h at a particular concentration of 50 μgmL^{-1}. The AuNPs untreated cells were considered as positive control cells. After the treatment, the cells were stained with 2′,7′-dichlorodihydro fluorescein diacetate (H_2DCFDA) in the media and incubated for 30 min at 37 $^{\circ}$C temperature. Then the cells were washed three times to remove the excess stain. DCF fluorescence was measured at 485 nm excitation and 520 nm emission (Figure 7). Results showed that IC_{50} dose of AuNPs is susceptible of significantly elevating the reactive oxygen species indicating the effective involvement of apoptosis in cell death.

Conclusion

Colloidal gold nanoparticles of 9-11 nm average diameters have been synthesized using the leaf extract of *Gymnema sylvestre* in water at room temperature. The antioxidant activity of the leaf extract of *Gymnema sylvestre* has been studied against a long lived 2,2-diphenylpicrylhydrazyl (DPPH) radical at room temperature. Evidence for the presence of polyphenols has prompted us to propose a mechanism for the formation of gold nanoparticles. The *in-vitro* anti cancer activity of the biofabricated AuNPs on MCF-7 cell lines was demonstrated significantly killing the cancer cells at 50 μgmL^{-1}. The normal lymphocytes were found to be non-toxic at this dose.

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