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Green Synthesis of Gold Nano Particles from *Cassia auriculata* Leaf Aqueous Extract and Its Cytotoxicity Effect on in Vitro Cell Line

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Abstract:A facile and simple method for synthesis of gold nanoparticles is proposed in this paper. The gold nanoparticles were synthesized in an eco friendly manner using aqueous leaf extracts of *Cassia auriculata* and the extracts acts as reducing and stabilizing agents. The synthesized nanoparticles were characterized by UV – VIS, HRTEM, SAED, XRD and EDAX. The stability of the gold nanoparticles was studied by UV – VIS absorption spectroscopy. The characteristic surface plasma peak was centred at 530 nm. The HR TEM image revealed the size of nanoparticles in the range of 20-30 nm. XRD and SAED confirmed the crystalline nature of nanoparticles. The elemental composition and purity of gold nanoparticles was analysed by EDAX. The cytotoxicity of synthesized gold nano particles were examined on Vero and Hela cell lines and was found to be non – toxic and hence it can be employed for bio medical applications.
Key words: Gold nano particles, *Cassia auriculata,* Vero, Hela cell lines.

Introduction

Nanotechnology is an exquisite field in modern material sciences and it is currently an intense area of interest for research. The various products of Nanotechnology after several researches include nanocrystals, nanocomposites, nanoparticles, nanotubes, nanocatalysts, nanofilters, nano composite coatings and nanotubes. According to quantum physics, the particles with the size range of 1-100nm are said to be nanoparticles. Scientists are fascinated by the unique properties of nanoparticles that can be engineered as per the need. Nanoparticles have physical, chemical, electronic and optical properties that differ from their bulk properties [1] making it applicable in various arenas like medicine, electronics, biomaterials and energy production. As of now, metal nanoparticles are synthesized largely for various applications. The choice of metal nanoparticle depends upon the end product application. Metal nanoparticles can be synthesized by physical methods, chemical methods or by biological methods. Synthesis by physical and chemical methods is non-ecofriendly as hazardous chemicals are involved and the process is also expensive[2]. Researchers are more concerned about the safety of the environment [3] and eventually biological method of synthesis has become noteworthy. The synthesis of nanoparticles from biological sources is called "green" nanotechnology. The biological sources utilised for synthesis includes bacteria, fungi or plant parts such ac leaves, fruits, flowers etc[4]. Among these plant extracts has found to be the best choice for the synthesis of nanoparticles. Synthesis from plant extracts eliminates the difficulty in handling micro organisms which makes the process simple and facile. The widely accepted nanoparticle for the localization and treatment of cancer is gold nanoparticles.

The preliminary step in the synthesis of AuNPs is the reduction of gold ions ($Au^{3+)}$ to neutral gold atoms (Au^0). It occurs by reduction of chloroauric acid (H [$AuCl_4$]) in a solution in the presence of suitable reducing agent. The gold nanoparticles synthesized using plant extracts do not require reducing agent as the compounds which readily exists in plants acts as reducing agents as well as stabilizing agents. The synthesis of gold nanoparticles using plant extracts has been tried and it has become successful. Biosynthesis of goldnanoparticles from plants like *Hibiscus rosa*-sinensis[5], geranium[6], lemon grass[7], *Cinnamomum camphora*, *Azadirachta indica* [8], *Aloe vera* [9] has been reported. In this study, we report the plant-mediated synthesis of gold nanoparticles using the aqueous leaf extracts of *Cassia auriculata* an evergreen shrub which is found in many parts of India. The gold nanoparticles (CA-AuNPs) synthesized were characterized by UV-Vis spectroscopy, TEM, XRD, EDAX, DLS and Zeta. The cytotoxic effects of gold nanoparticles on VERO and HeLa cells were also investigated by following MTT assay[10,11].

Materials and Methods

Fresh plants of *Cassia auriculata, Cassia senna, Murraya koenigii, Mukia maderaspatana, Solanum trilobatum, Ocimum sanctum* were identified and collected.

Preparation of plant leaf powder

The fresh and healthy leaves were collected and washed with distilled water to remove dust particles. After washing the leaves were spread evenly in a clean paper and the leaves were allowed to dry in shade for about 3-4 days. When the leaves were dried completely they were finely powdered using mixer and was used for extract preparation.

Preparation of aqueous extracts

About 2g of each leaf samples were mixed with 50ml of glass distilled water and the mixture was boiled at 60°C for 20min. The mixture was brought to the room temperature and the aqueous leaf extract was collected using Whatman filter paper. It was stored in a glass bottle and kept in refrigeration for future use.

Synthesis of gold nanoparticles

1ml of aqueous leaf extract was taken and 1mM $HAuCl_4$ was added and the reaction volume was made up to 10ml by adding glass distilled water. The solution was observed for colour change from yellow to ruby red within 1 hour indicating the synthesis of gold nanoparticles. The control solution was maintained for all six extracts without adding $HAuCl_4$.

The colour change from yellow to ruby red was observed in aqueous extracts of *Cassia auriculata* within 10min and it is used for further studies.

Characterization of gold nanoparticles

The filtrate treated with chloro auric acid was observed for the colour change from yellow to ruby red in comparison to the control solution. The colour change is the visual method of detection of synthesis of gold nanoparticles. The gold nanoparicles were then characterized by using UV-Vis spectroscopy (HITACHI U-2900 UV-Visible spectrophotometer) reduction of gold ions was monitored by measuring the UV-Vis range of the reaction mixture at 1hour. The morphology of the obtained nanoparticles was characterized by using TEMSCAN2000EX a high-resolution transmission electron microscope (HR-TEM) of JEOL operated at an accelerating voltage at 80 keV. Chemical composition of the obtained nanoparticles were analysed by EDAX technique using scanning electron microscope (HITACHI SO-6600, Japan). The crystallographic structure of Gold nanoparticles and the phase properties was revealed by XRD (SEIFERT JSO- DEBYEFLEX 2002 model) measurements using an X'Pert Pro x-ray diffractometer (PAN analytical BV, The Netherlands) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in θ- 2 θ configurations. The average the crystallite size was calculated by using Scherrer's formula, $D = \frac{0.94\lambda}{\beta \cos\theta}$, where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the diffraction angle.

Stability studies

Time

The stability studies for the synthesized gold nanoparticles were performed by measuring the UV-Vis absorbance over a period of 0hour, 1min, 15mins, 5hours, 25 days and 60days. The absorbance of the sample solutions was measured spectrophotometrically.

***In Vitro* Cytotoxicity**

The toxic effect of nanoparticles is highly dependent on the organs, and more specifically the type of cell, encountered. This is due to the variation in cell physiology (e.g., epithelial or lymphoid), proliferation state (tumor or resting cells), membrane characteristics and phagocyte characteristics among different cell types. Cancer cells, for instance, are more resilient towards nanoparticles toxicity than normal cells due to an increased rate of proliferation and metabolic activity. The difference in toxic effects is even observed for nanoparticles of the same material. Therefore, selection of the appropriate cell type based on target introduction methods of nanomaterials is an important factor in cytotoxicity assays.

The cell culture used for cytotoxicity studies were VERO and HeLa cell lines. VERO cells are derived from an African green monkey. It is a normal kidney cell line. HeLa cell lines are derived from human cervical cancer cells.

Cell culture

200 µL of MEM medium is added in the required number of wells of 96 well micrometer plate equipment (Nunc, Denmark). To this 50 µL of different cell lines are added in each well. Different concentrations (10, 20, 30, 40 and 50 µL) of functionalized AuNP are added into the wells separately. The cells were grown using a medium containing 1% penicillin and streptomycin mixture and 5–10% Fetal Bovine Serum and then incubated at a temperature of 37^{o}C and 5% CO_2. 100µl of suspension containing 1 x 10^5 cells was seeded in each well of the plate and incubated overnight in an environment of 5% CO_2 at a temperature of 37^{o}C.

Preparation of Stock (Gold nanoparticles)

Confluent healthy monolayer to adsorb on the cell monolayer of Vero cell line of each 25cm^2 bottle was selected. The growth medium was decanted and the cell layer was rinsed in PBS. 100µl of known TCID 50 of gold nano particles inoculated in each bottle. The particles were allowed to adsorb on the cell monolayer for half an hour at 37ºC sufficient amount of medium was replenished. The bottles were frozen on the day 4+ CPE observed. The bottles were frozen and thawed for three consecutive times and centrifuges at 6000 rpm for 30 minutes and the supernatant was collected. The pool were estimated and stored at -20ºC.

Cell Lines

The cytotoxicity studies were performed in the normal VERO cell line and HeLa cell lines purchased from National Centre for Cell Science (NCCS), Pune, India. The Vero and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The culture mediums were supplemented with 10% Fetal Bovine serum (FBS), 1.5 g/L of Sodium bicarbonate, 100units/mL Penicillin, 100 µg/mL streptomycin, and 2 mM glutamine and the cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Actively growing cells were seeded with density around 1X10^5 cells/well in a 96-well tissue culture plate. The cells were treated with different concentrations of gold nanoparticles (10, 20, 30, 40, 50, µg/ mL) for 24 hrs. The control cells were not treated with gold nanoparticles and were kept in the same volume of phosphate buffer saline (PBS, pH-7.4) for the same period of time. After the end of the exposure time, the cell viability was checked using the 3-(4,5-dimethylazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) assay. The MTT assay is based on the optical detection of the purple coloured formation at 570 nm, which is formed by the enzymatic reduction of yellow tetrazolium MTT. All experiments were performed 3 times in quadruplets, and their average has been shown as cell viability percentage in comparison with the control experiment. The gold nanoparticles untreated controls were considered to be 100% viable.

In Vitro Cytotoxicity Assay

In vitro cytotoxicity used in the study was determined using MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) method. Vero cells were cultured in 96-well cell culture microplate. Confluent cells were treated by increasing concentration of AuNPs in triplicate wells. The final volume of cell culture medium and AuNPs in each well was 90 µl. Treated cells were incubated for 4 days at 37°C. After 4 days, 15 µl of MTT solution (Promega, WI, USA) was added to each well. The microplate was kept at 37°C for an additional 4 hrs in a humidified 5% atmosphere. Then the plates were centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded lightly. After which 100 µl of the DMSO solution was added to each well followed by gentle shaking at 37^{o}C for 10 minutes. The absorbance value of each well was measured using a 96-well plate reader (TECAN, Mannendorf, Switzerland) at 570 nm.

Cell viability was expressed as follows.

Average cell count x dilution factor x 10^4= Lakhs /ml.

Results and Discussion

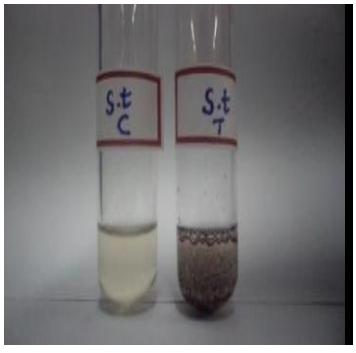
Synthesis of AuNPs from *Cassia auriculata*

In the present study, green synthesis of gold nanoparticles from aqueous extract of *Cassia auriculata* was carried out. The outstanding prerequisite for applying gold nanoparticles in cancer therapy is that the nanoparticles should be produced in biologically providential media. Usually, the plant leaves are important source for antioxidant molecules such as polyphenols and flavonoids which have ability to reduce metal ions. These antioxidant molecules produce gold nanoparticles by reduction of gold ions (Au3+) (Roy *et al.,* 2011).

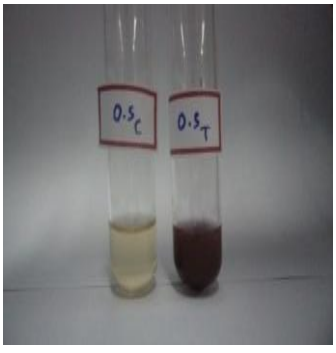
The extracts of six plants namely *Murraya koenigii, Ocimum sanctum, Solanum trilobatum, Cassia auriculata, Cassia senna, Mukia maderaspatna* were collected for screening their ability in synthesizing the gold nanoparticles. Among these, *Cassia auriculata* showed the long lasting stability while other extracts showed aggregation and precipitation. Hence *Cassia auriculata* was used for further studies. It is well known that gold nanoparticles formation is indicated by the colour change from yellow to ruby red colour in aqueous solution (Figure- 1).

Figure 1: Gold nanoparticles synthesis using leaf extracts

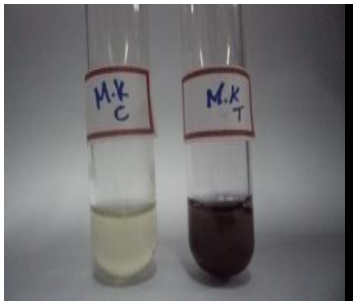
1. Using *Solanum trilobactum* extract



2. Using *Ocimum sanctum* extract

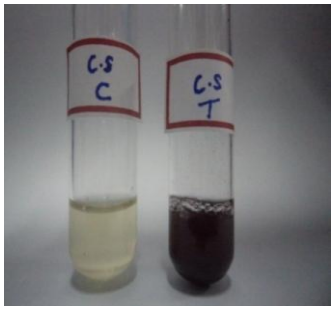


3. Using *Murraya koenigii* extract

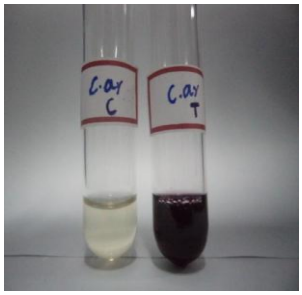


4. Using *Mukia maderaspatna* extract

5. Using *Cassia senna* extract



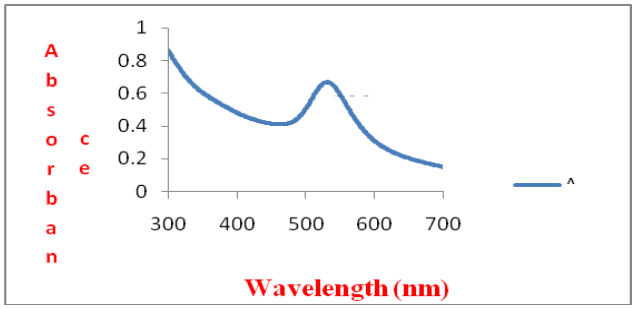
6.Using*Cassia auriculata* extract



UV-Vis spectroscopy

The synthesized gold nanoparticles were characterised using UV-visible spectroscopy, The optical properties of metal nanoparticles are dominated by surface Plasmon resonance (SPR), which shifts to longer wavelength with increasing particle size [12]. The strong surface Plasmon reference centred at 530nm is the characteristics of colloidal gold (Figure- 2).

Figure 2: UV-Vis graph



High Resolution Transmission Electron Microscope

The size distribution, shapes and morphology of AuNPs were studied by HRTEM. Nanoparticles of mostly spherical in shapes were observed in HRTEM image analysis and sizes were in the range of 1 -100nm. In this case, the sizes were observed in the range between 20nm to 30nm.

Figure 3: HRTEM image

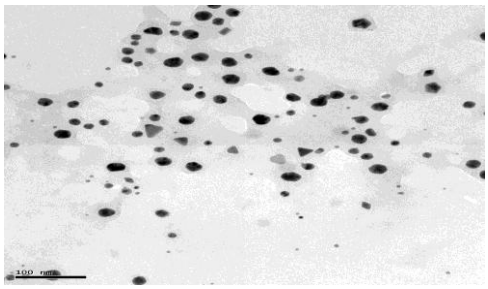
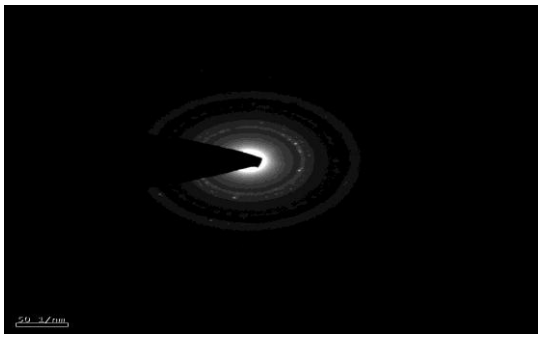


Figure 3: The HRTEM image shows gold nano particles of a maximum of 30 nm size. Mostly spherical AuNPs were observed along with certain percentage of triangular shaped particles.

Selected Area Electron Diffraction

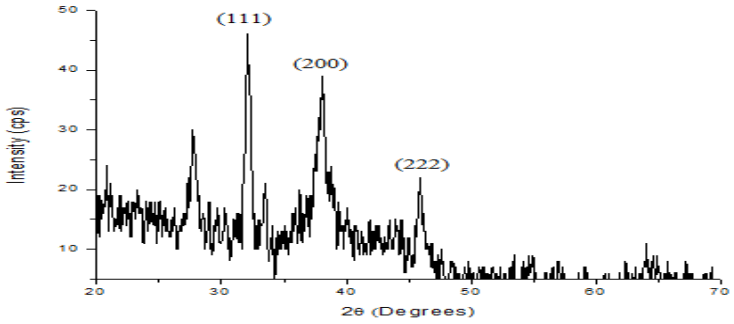
Figure 4: Selected Area Diffraction Pattern



Selected area electron diffraction (SAED) pattern obtained from a AuNPs (Figure- 4) revealed three diffraction rings from inner to outer associated with the [111], [200], and [222] planes of FCC crystalline Au indicating the formation of crystalline gold nanoparticles. The spots are indexed according to FCC structure of Au.

X-Ray Diffraction

Figure 5: X-Ray Diffraction Spectrum

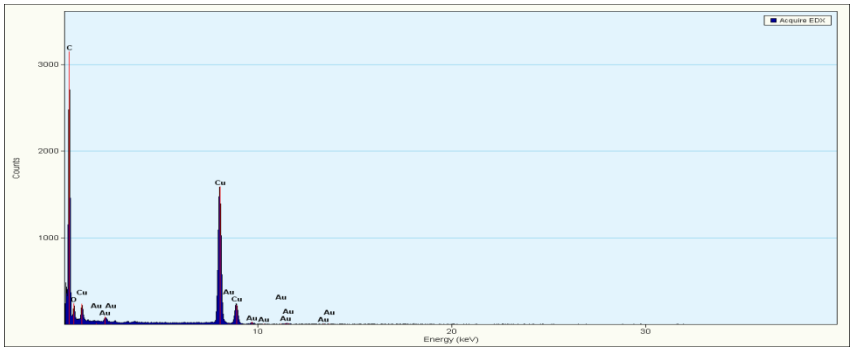


XRD analysis was designed to confirm the crystalline nature of particle. XRD analysis usually shows intense peak at various planes which corresponds to a particular 2θ value. The XRD spectra of gold nano particles exhibited diffraction peaks corresponding to (111), (200) and (222) phase (Figure- 5). The diffraction peaks corresponds to 2Θ = 38.1°, 44.5° and 81.8° angles respectively were the only characteristic observed corresponding to bulk face cubic centred gold which confirmed that the synthesized gold nanoparticles were of crystalline nature. The mean size of particles was calculated using the Debye–Scherrer's equation by determining the width of the (1 1 1) Bragg reflection.

Energy Dispersive X-Ray

The elemental composition of the synthesized AuNPs was determined by energy dispersive X-ray analysis (EDX). Area profile analysis of the synthesized nanoparticles revealed strong peaks of Au confirming the formation of AuNPs (Figure- 6). The characteristic copper peak is attained because the sample is coated over a copper grid. The freeze-dried gold nanoparticles were mounted on specimen stubs with double-sided taps, coated with copper in a sputter coater. Peak belonging to impurity was not detected.

Figure 6: EDAX profile of AuNPs



The results indicated that the reaction product was composed of highly pure gold nanoparticles. EDAX spectrometry also confirmed the presence of gold with no other contaminants. The optical adsorption peak was observed at approximately 2.30 keV, which is typical for the adsorption of gold nanocrystallites due to SPR.

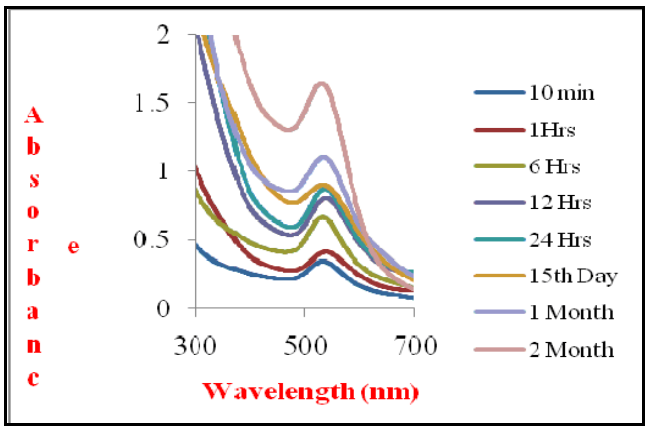
Stability of gold nanoparticles

The formation and stability of newly synthesized gold nanoparticles were regularly monitored using UV-Vis spectral analysis. To determine the status (stability) of gold nanoparticle, at a particular time aliquots a

small amount of sample from reaction mixture after completion of reaction were subjected to UV-vis spectroscopy. The stability was monitored at regular intervals of time.

Figure 7 -Shows the stability graph of AuNPs at different time interval. From this result, it has been concluded that gold nanoparticle synthesized from *Cassia auriculata* showed good stability even after 2 months.

Figure 7: Stability graph



Cytotoxicity Studies

The key parameters in evaluating the biocompatibility of gold nanoparticles are Cytotoxicity and cell viability. The cytotoxicity and cell viability can be determined by the MTT assay.

Cytotoxic Activity on Vero Cell Line

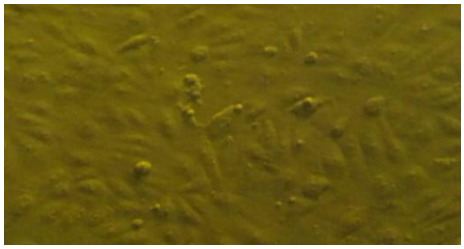
Table 1: showing stability at different time interval

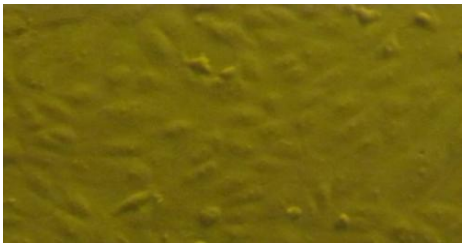
Time	Wavelength
10mins	527
1 hr	530
6 hrs	**530**
12 hrs	532
24 hrs	532
15th day	534
1 month	534
2 month	544

In vitro cytotoxic activity of the AuNPs was screened in normal Vero cell line using five different concentrations of the AuNPs of the biologically synthesized, viz., 10, 20, 30, 40 and 50 µg/mL at 1st day, 2nd day and 3rd day interval (Table- 1). At 3rd day incubation period inhibitory activity of 54.25±2.01, 32.07±0.98, and 14.78± 0.34 was recorded in 10 µg/mL, 20 µg/mL, and 30 µg/mL concentration respectively. Inhibition was not observed in last two concentrations i.e. 40 µg/mL, 50 µg/mL, based on these results 10 µg/mL; concentrations were showed non toxic effect in the normal Vero cell line (Figure- 8).

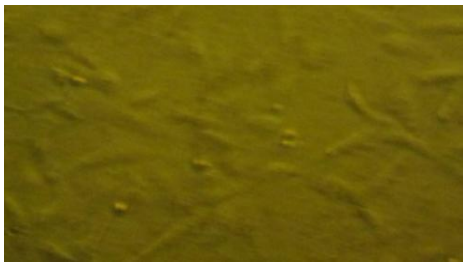
Figure 8: *In vitro* cytotoxic effect of AuNPs against Vero cell line.

1. 10 µg/mL of AuNPs **2. 20 µg/mL of AuNPs**



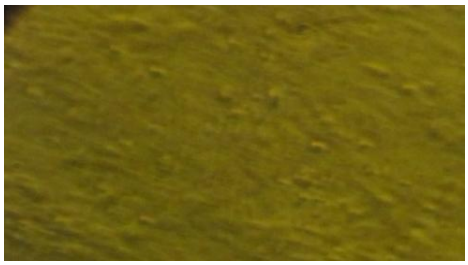


3. 30 µg/mL of AuNPs

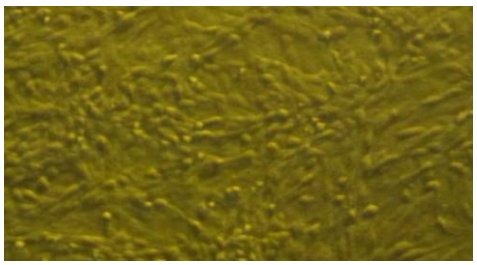


4. 40 µg/mL of AuNPs

5. 50 µg/mL of AuNPs



6. VERO cell without treat of AuNPs



Cytotoxic Activity On Hela Cell Line

Table 2: *In vitro* cytotoxic effect of AuNPs against Vero cell line

	AuNPs **µg /ml**	**1st day**	**2nd day**	**3rd day**
VERO **cell line**	10	Nd	Nd	54.25±2.01
	20	Nd	Nd	32.07±0.98
	30	Nd	Nd	14.78±0.34
	40	Nd	Nd	Nd
	50	Nd	Nd	Nd

***Nd- Not detectable**

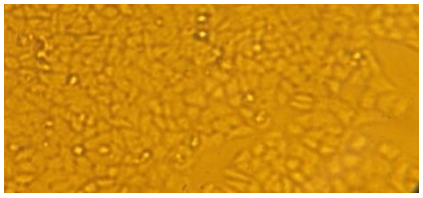
Table 3: *In vitro* cytotoxic effect of AuNPs against HeLa cell line

Cell line	AuNPs µg/**ml**	**1st day**	2nd **day**	**3rd day**
HeL**a** cell line	10	Nd	Nd	62.14±2.03
	20	Nd	Nd	43.06±0.71
	30	Nd	Nd	29.84±0.42
	40	Nd	Nd	12.63±0.36
	50	Nd	Nd	Nd

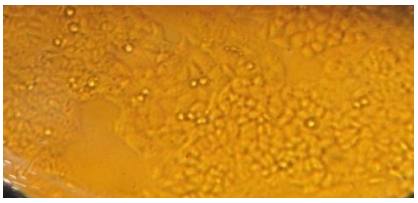
***Nd- Not detectable**

Figure 9: *In vitro* cytotoxic effect of AuNPs against HeLa cell line.

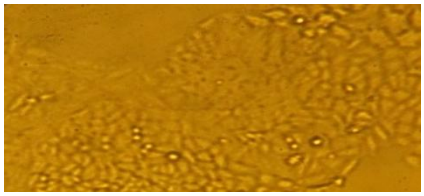
1. 10 µg/mL of AuNPs



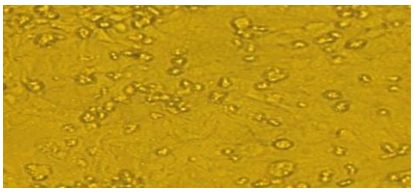
2. 20 µg/mL of AuNPs



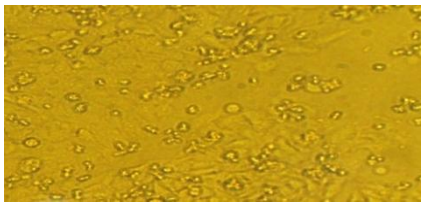
3. 30 µg/mL of AuNPs



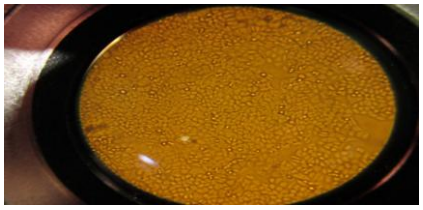
4. 40 µg/mL of AuNPs



5. 50 µg/mL of AuNPs



6. HeLa cell without treat of AuNPs



In vitro cytotoxic activity of the synthesized gold nanoparticles was also screened in HeLa cell lines. The HeLa cells were treated with increasing concentrations of gold nanoparticles (10, 20, 30, 40 and 50 µg/mL). The activity of gold nanoparticles on cell lines was observed at 1st day, 2nd day and 3rd day and interval (Table- 3). The inhibitory activity of nanoparticles in the third day was 62.14±2.03, 43.06±0.71, 29.84±0.42 and 12.63±0.36 at the concentration of 10, 20, 30 and 40µg/mL. Inhibition was not observed in the concentration 50 µg/mL. Therefore the gold nanoparticles synthesized were non toxic to humans at the concentration of 10µg/mL (Figure- 9).

Conclusion

This paper proposes a simple method to synthesize gold nanoparticles from $HAuCl_4$ using the aqueous leaf extracts of *Cassia auriculata.* The biological method of synthesis has gained importance as this method is eco-friendly and non-toxic to humans. The gold nanoparticles synthesized were characterized and the AuNPs showed excellent stability over a period of 2months. Cytotoxicity assays were carried put in VERO and HeLa cell lines and the results revealed the non-toxic nature of gold nanoparticles. The mode of action of synthesized nanoparticles should be studied before using them for biomedical applications.

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