Synthesis of Gold Nanoparticles Using *Centella Asiatica* Extract and its In Vitro Cytotoxic Activity Against Human Breast Adenocarcinoma MCF-7 and Normal Vero Cell Line

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**Abstract:** In the past few decades, the gold nanoparticles (AuNPs) have attained much attention among all metal nanoparticles and it is greatly contributed for cancer photo-diagnostics, photo-thermal therapy, bio-labelling, drug delivery etc. In the present investigation, AuNPs were green synthesized using *Centella asiatica* leaf aqueous extract. The physicochemical properties of synthesized AuNPs were characterized using UV–visible and Transmission Electron Microscopy (TEM) analysis. Further, in vitro anticancer efficacy of biosynthesized AuNPs were assessed against human breast adenocarcinoma (MCF-7) and normal African green monkey kidney Vero cell line. Bio approach synthesized gold nanoparticles were highly stable, monodisperse particles in the size ranging <100 nm. Cytotoxic activity has been evaluated using MTT, morphological observation, Annexin-V/PI Double-Staining Assay, Comet assay for apoptosis. AuNPs were inhibited MCF7 cancer cells and were not toxic to the normal Vero cells at 48 h exposure, therefore, the present finding confirms that the bio-approach synthesized metal AuNPs possess in vitro cytotoxic effects and it may be used as an anticancer substance.

**Keywords:** *Centella asiatica*, Gold nanoparticles, MCF-7, Vero, Cytotoxicity, Apoptosis

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**Introduction**

Nanotechnology is an interdisciplinary approach to innovation of the most active areas of research in the field of modern materials science (Prashanth et al., 2011). Nanoparticles have gained significant attention due to their application in cancer therapy and imaging, angiogenesis, genetic disease and genetic disorder diagnosis, photothermal therapy and photoimaging (El-Sayed et al., 2005; Sadeghi et al., 2015). Cancer is a complicated disease which
occurs in cells of specific tissue with no longer receptive to signals that regulate cellular differentiation, survival proliferation, and death (Martin et al., 2013). Cancer is the second leading cause of death worldwide next to diabetes mellitus and responsible for 9.6 million death in 2018 (Ackermann and Mrowka, 2019). Globally, statistics for the occurrence of common cancers are lung cancer: 2.09 million, breast cancer: 2.09 million, colorectal: 1.80 million, prostate cancer: 1.28 million, skin cancer (non-melanoma): 1.04 million, and stomach cancer: 1.03 million. In particular, breast cancer is most common cancer diagnosed in women worldwide (El Hilali et al., 2019). At the present time, systematic chemotherapy combined with medical procedure is acted in emergency clinics. Though, poor biodistribution and non-specific delivery of chemotherapeutic drugs are considered to be a fundamental problem (Ud Din et al., 2017). Therefore, researchers are keen to develop such a delivery system that can efficiently penetrate both cellular and outer mitochondrial membranes in triggering apoptosis (Jeyarani et al., 2020).

Gold nanoparticles (AuNPs) can be synthesized in the lab chemically, physically and biologically methods. Among these methods the bio-approach of nanoparticles synthesis has various advantages compared to other methods such as biocompatibility and extensive application in biology and medicine, simplicity of the method, use of natural resources (microorganisms, algae, fungi, plants), non-toxic method making the synthesized NPs suitable for pharmaceutical and biomedical applications, external capping or stabilizing agents are not needed for synthesis of NPs, cost effective method because of minimum or non-requirement of energy, simplicity in scaling up for large-scale synthesis (Sperling et al., 2008; Ghosh et al., 2008; Boisselier and Astruc, 2009; Swaha et al., 2019). Plant-derived natural compounds can be used as efficient reducing and stabilizations agents which can confer stable size and agglomeration of synthesized AuNPs (Khan et al., 2019). The stabilizing ligands of nanoparticles are usually formed from alkanoids, flavonoids, alkaloids, proteins or starch (Jayaprakash et al., 2017). The medicinal values of C. asiatica are due to the presence of various phyto-constituents such as terpenes, phenols, vitamins, minerals, polyacetylene, and fatty acids (Archana et al., 2019). The herb is also known for possessing strong anti-inflammatory, anti-asthmatic, anti-oxidative, anti-cancer, hepatoprotective, and neuroprotective activities (Barnes et al., 2007; Huang et al., 2011; Bian et al., 2012; Orhan, 2012).

In this present investigation, we focused on the biosynthesis of gold nanoparticles using medicinal herb C. asiatica and to investigate the biocompatibility and in vitro cytotoxicity effect against normal green monkey kidney Vero cell line and breast cancer (MCF7) cell lines.

**Materials and Methods**

**Chemical:**

Human breast adenocarcinoma cancer cell line (MCF7) and normal green monkey kidney cell line (Vero) were obtained from National Centre for Cell Science (NCCS), Pune, India. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS), antibiotics and chloroauric acid (HAuCl₄) were purchased from HiMedia (Mumbai, India). Annexin V-FITC/ PI apoptosis detection kit was purchased from Sigma Aldrich (Saint Louis, Missouri, USA).

**Biosynthesis of gold nanoparticles:**

Biosynthesis of gold nanoparticles were performed by following method of Mubarakali et al. (2011). C. asiatica aqueous leaf extract (1 ml) was taken and added 9.0 ml of 1.0 mM of HAuCl₄ separately in conical flask. The reaction was performed in dark at room temperature for 12 h in a static condition and color changes were recorded.

**Characterization of gold nanoparticles:**

After 12 h the ruby red color formation was confirmed as gold nanoparticles. In addition to the biosynthesis nanoparticles, C. asiatica aqueous leaf
extract, chloroauric acid solutions were recorded for their absorbance from 300 to 800 nm using UV–visible spectrum (Shimadzu-1800 spectrophotometer). Size and shape of AuNPs were characterized by higher resolution transmission electron microscope (HRTEM) (FEI, TECNAI T30). Briefly, the sample 2 µl was placed on the carbon-coated copper grid then immediately transfer to the microscope.

**Cell viability assay:**

The effect of AuNPs synthesized from *C. asiatica* aqueous leaf extract on cancer cell line MCF7 and Vero cell line was determined by the MTT assay (Mossmann, 1983). Briefly, DMEM added with L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100 µg/ml), FBS (10%) was used for cell culture. Cells were cultured in 25 cm² flasks at 37ºC in a 5% CO₂ atmosphere. For further dosage studies, cells were seeded into 96 well plates (1x10⁶ cells in each well and incubated at 37ºC for 24 and 48 h. MCF 7 breast cancer cells and normal Vero cells were treated with AuNPs and HAuCl₄ alone at different concentration (5, 10, 20, 40, 80 and 160 µg/ml). Plates were incubated for 24 and 48 h with treatment followed by cell viability test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT solution (10 µl) was added to each well at concentration of 5 mg/ml, plates were incubated at 37ºC for 4 h. Formazan crystals of purple color appeared were dissolved in 100 µl of dimethyl sulfoxide (DMSO). Using a multi-well ELISA plate reader, the optical density (OD) was measured at 570 nm. Percentage of cell viability was evaluated by using the following formula:

\[
\text{Percentage cell viability} \% = \frac{OD}{OD \text{ of control}} \times 100
\]

**Cytomorphological observation:**

The MCF7 cells were seeded at 1x10⁶ cells/well into a six well chamber plate and incubated overnight. Later, the medium was replaced with maintenance DMEM with 10% FBS containing inhibitory concentration (IC₅₀) of AuNPs synthesized from *C. asiatica* aqueous leaf extract and incubated for 48 h. The cells morphology was examined under inverted light microscope.

**Apoptosis assay:**

MCF7 cells were plated at 1x10⁶ cells/well into a six well chamber plate. At >90% confluence, the cells were treated with an inhibitory concentration of AuNPs synthesized from *C. asiatica* aqueous leaf extract and control alone for 48 h. Then cells were washed with PBS, fixed in methanol: acetic acid (3:1, v/v) for 10 min and stained with 5 µl of annexin V-FITC conjugated and 10 µl of propidium iodide (40 µg/ml) for 20 min. Nuclear morphology of cells were examined under Carl Zeiss Axio vision fluorescent microscope (Software: Axiovision 4.8).

**Comet assay:**

Comet assay was performed to analyze the level of DNA damage caused due to the effect of AuNPs following method of Dhawan *et al.* (2003) with slight modifications. Briefly, cells were subjected to centrifugation, supernatants were discarded and washed with PBS to obtain the yield of 1x10⁶ cells/ml. Cells treated with AuNPs synthesized from *C. asiatica* aqueous leaf extract were mixed with LMPA (Low melting point agarose) and were spread on fully frozen microscopic slides containing NMA (Normal melting agarose). The slides were then immersed in the lysis solution for nearly 2 to 6 h at 4°C, arranged side by side on a gel electrophoresis apparatus and were allowed to sit in an alkaline buffer for around 20 min for the unwinding of DNA and the expression of alkaline labile DNA damage. Further, the electric field was applied by adjusting the current of 300 Ma and electrophoresis was performed for 30 min. Slides were washed using distilled water followed by chilled 70% ethanol and were air dried. Finally, the slides were stained with 75 μl of propidium iodide (40 µg/ml) and kept for 5 min. Further, the slides were observed under a fluorescent microscope.

**Results and Discussion**

In this study, after the addition of the solution containing Au(III) ions to the fresh *C. asiatica* aqueous leaf extracts, a color change from yellow
to ruby red was observed (Fig. 1). The colloidal dispersion of AuNPs lead to a change in the color of nanofluids from yellow to ruby red or bluish due to absorption or scattering of the light that passes through synthesized solution as previously described by Cademartiri and Ozin (2009). Figure 2 shows the UV-Visible absorption spectra of AuNPs solutions observed at different time intervals (2, 4, 6 and 8 h) and revealed a well-defined surface plasmon band centred at around 535 nm. Based on the above-stated phenomenon of absorption and scattering of the light by AuNPs, the color change of the reaction mixtures was consistent with occurrence of the maximum ($\lambda_{\text{max}}$) of the localized surface plasmon resonance (LSPR) absorption band, which for gold nanofluids typically occurs in the range of 520–570 nm (Pal and Kryschi, 2015). In order to determine the size and shape of the produced AuNPs, TEM analyses were performed. The synthesized gold nanoparticles were spherical in shapes and ranged from <100 nm as shown in Figure 3A. Selected area of electron diffraction pattern image showing of AuNPs with ring arisen due to the reflections from 111, 200, 220 and 300 and confirmed the polycrystalline nature of synthesized AuNPs (Fig. 3B). The results are in accordance with the earlier reports of synthesized gold nanoparticles using
Fig. 3: (A) TEM micrographs of gold nanoparticles. Bar represents 50 nm. (B) Polycrystalline nature of synthesized AuNPs confirmed by Selected Area Electron Diffraction (SAED) pattern.

Fig. 4A: Cytotoxicity effect of HAuCl₄ and AuNPs on normal kidney (Vero) cells at 24 and 48 h. Values are given as mean ± S.D. for each concentration. (*) represents the treated concentrations statistically significant (p < 0.05) when compared to control.

The present study evaluated the cytotoxicity and biocompatibility of synthesized gold nanoparticles against metabolically active proliferating cells. To ensure this, different concentrations (5, 10, 20, 40, 80, and 160 µg/ml) of gold nanoparticles and HAuCl₄ alone were tested against breast cancer cell line (MCF7) and normal green monkey kidney cell line (Vero). MTT is a tetrazolium salt which readily reacts with mitochondrial dehydrogenase of metabolically active cells to yield coloured formazan product. This can be measured spectrophotometrically for the determination of viable or active cells present (Mossmann, 1983). The inhibitory concentration (IC₅₀) of AuNPs to inhibit MCF7 breast cancer cells was found to be 20 µg/ml at 48 h. In contrast, inhibitory concentration of HAuCl₄ to inhibit cancer cells was found to be 160 µg/ml at 24 h. In normal Vero cells treated with AuNPs, 87.77 % of cell viability was found to be 160 µg/ml at 48 h. While the same concentration of HAuCl₄ were used to treat normal Vero cells showed only 28% of cell viability (Figs. 4A, B). Based on the above-stated phenomenon, the activity of AuNPs on Vero cell line was constantly less at experimented dilutions.

the aqueous leaf extract of Acalypha indica (Krishnaraj et al., 2014) and Biogenic gold nanoparticles using Commiphora wightii and their cytotoxic effects on MCF7 breast cancer cell line (Uzma et al., 2020).
Fig. 4B: Cytotoxicity effect of HAuCl₄ and AuNPs on MCF7 breast cancer cells at 24 and 48 h. Values are given as mean ± S.D. for each concentration. (*) represents the treated concentrations statistically significant (p < 0.05) when compared to control.

![Graph showing cell viability]  

Fig. 5: Cytomorphological effects of MCF7 cancer cells. (A) Control (untreated), (B) MCF7 treated with 20 µg/ml of AuNPs at 48 h.

as compared with cancer cell line. Also, in the different concentrations of AuNPs, MCF7 cells showed highly significant (p<0.05) reduced cell viability when compared to Vero cells. Overall, AuNPs from *C. asiatica* aqueous leaf extracts were toxic to breast cancer cells and non-toxic to normal Vero cells, respectively. Jeyarani *et al.* (2020) reported the cytotoxic effects of novel biomimetic gold nanoparticles from *Geranium pusillum* against normal (HEK-293) and cancer (MDA-MB-231) cell lines. Swaha *et al.* (2019) reported the cytotoxic effects of novel eco-friendly approach for biosynthesis of gold nanoparticles (AuNPs) using *Hygrophiila spinosa* extract against MCF-7 and MDA-B-231 (breast), SKOV-3 (ovarian) and U-87 (brain) cancer cell lines.

*C. asiatica* aqueous extract mediated AuNPs and HAuCl₄ alone used to treated and untreated MCF7 cells at 48 h and observed under inverted microscope. Untreated (control) MCF7 showed irregular confluent aggregates with round polygonal cells appeared normal (Fig. 5A). AuNPs treated MCF7 cells showed persuade cell reduction which lead to the changes in membrane integrity and cell shrinkage due to appearances of apoptotic cell demise (Fig. 5B). HepG2 cell line treated with the phytosynthesized AuNPs at 30 µg/ml showed cell death up to 51%. In addition the cells showed apoptotic lead to cell death as evidenced by showing changes in loss of membrane integrity, inhibition of cell growth and cytoplasmic condensation (Ashokkumar *et al.*, 2019).
Fig. 6: AuNPs induced apoptosis of MCF7 cells as established through phosphatidylserine (PS) translocation using a fluorescent microscope. Control cells displayed no Annexin V-FITC/PI staining. Cells treated with AuNPs (IC\(_{50}\) 20 µg/ml at 48 h) showed double staining of Annexin V-FITC/PI, indicating apoptosis.

Fig. 7: Apoptotic inducing effect of AuNPs on MCF7 cells assessed by comet assay. Control (A), MCF7 cells treated with AuNPs showing olive tail movements which indicates DNA damage (B).

Moreover, the phytosynthesized gold nanoparticles (AuNPs) showed cytotoxicity effect on three different cancer cell lines such as HepG2, HeLa and MCF7 and cytomorphological changes were observed (Balashanmugam et al., 2016).

A fluorescence-based assay was employed to test the effect of synthesized gold nanoparticles on breast cancer cells (MCF7) using annexin V-FITC/PI dual staining (Fig. 6). MCF7 untreated cells stained negative for annexin V-FITC and PI. MCF7 cells treated with IC\(_{50}\) of C. asiatica aqueous extract mediated AuNPs showed intense positive staining with annexin V and PI induces apoptosis. Recently, Mahalakshmi et al. (2019) have synthesized gold nano conjugates which showed apoptotic effects on HeLa cells upon treatment with IC\(_{50}\) concentration used with dual stain. Moreover, Prabhu et al. (2014) documented apoptotic effects on human colon cancer cell lines HCT15 and HT-29 treated with synthesized AgNPs mediated isolated pure compound from Vitex negundo leaves using annexin V-FITC/PI dual stain.

Cell death detection using comet assay allows the recognition of DNA comet tails for the detection of extant of DNA damage using the PI. An important aspect of DNA damage is the establishment of free radicals that mainly influence the formation of single-strand breaks. The comets originating from damaged cells have a distinct head with a tail (Babu et al., 2019). In our study, alkaline comet assay were used to detect
the DNA strand breaks in the MCF7 cells following exposure to the C. asiatica aqueous extract mediated AuNPs. The levels of DNA damage in the cells treated at IC<sub>50</sub> concentrations of AuNPs when compared to untreated control cells. The results revealed that the MCF7 cells treated at IC<sub>50</sub> concentrations (20 μg/ml at 48 h) showed significant DNA damage with olive moments while DNA damage was not observed in untreated control cells, as the halo surrounding cell nuclei was clearly visible (Fig. 7). Similarly, DNA damage in the MCF7 cells was noticed after treatment with different concentrations of AuNPs synthesized from Commiphora wightii aqueous extract (Uzma et al., 2020). Prabhu et al. (2013) reported apoptotic inducing effect of biologically synthesized AgNPs on HCT15 cells assessed by comet assay. Moreover, the DNA damage was induced by p-GNPs on cell lines (MCF-7 and MDA-MB-231) and drastically increased the length of comet tail at IC<sub>50</sub> concentration of p-GNPs (Uma Suganya et al., 2016).

**Conclusion**

In the present study, we established a rapid bioapproach synthesis of AuNPs using herb, C. asiatica aqueous extract. The characterization methods revealed that terpenes and phenolic compounds were involved in the C. asiatica extract-mediated synthesis of AuNPs by using UV-visible and Transmission Electron Microscope. Cytotoxicity of AuNPs synthesized using C. asiatica extract against MCF7 breast cancer adenocarcinoma cell line is evident from the reduction of cell viability in treated cells. AuNPs induced apoptosis confirmed by dual staining method and significant DNA damage was evident from the AuNPs treated MCF7 cells showed comet tails analyzed by Comet assay. Based on the *in vitro* studies, the significant cytotoxic and apoptotic effects could be proven against MCF7 cancer cell line treated with biosynthesis AuNPs.

**References**


