Green Synthesis, Characterization of Gold Nanoparticles from *Cymbopogon Flexuosus* and Its Effect on Anti-Cancer Activity

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**Abstract:**
The biomolecules present in the Cymbopogon flexuosus plant induced the reduction of Au3+ ions from HApCL4, resulted in the formation of Gold nanoparticles. The formation of gold nanoparticles can be visually observed by a color change from yellowish to wine red. The growth of nanoparticles was monitored by UV-Vis spectrophotometer that demonstrated a peak at 540 nm. Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) were used to find the size and shape of the Gold nanoparticles. Gold nanoparticles were 20-110nm in size and it is spherical. X-ray Diffraction analysis (XRD) studies corroborated that the biosynthesized nanoparticles were crystalline gold. Energy dispersive, spectroscopy (EDAX) confirmed an elemental gold signal. Fourier transform-infrared (FTIR) spectroscopy analysis revealed that biomolecules were of the synthesis and capping of gold nanoparticles. In vitro studies, the IC50 of A549 cells was found to be 60µg/ml and 3T3 normal cell line is nontoxic up to 80µg/ml confirmed by MTT assay. Further, apoptosis in A549 was analyzed by fluorescence microscopy. The results suggested that the gold nanoparticles of C. Flexuosus extract clearly show toxic on normal cells but toxic in cancer cells. The observations in this study show that this can be developed a promising nanocarrier in pharmaceutical and healthcare sector.

**Keywords:** Biosynthesis, Gold Nanoparticles, Cymbopogon flexuosus, Transmission electron microscopy, MTT assay, Apoptosis.

1. INTRODUCTION

Nanotechnology grasps the assembling and utilization of physical, substance and natural frameworks at scales running from particular iotas or particles to submicron measurements, just as the consolidation of the subsequent nanostructures into more noteworthy frameworks [1]. Science and innovation inquire about in nanotechnology confirmations advancements in such territories as materials and assembling, nanoelectronics, drug and medicinal services, vitality, biotechnology and data innovation [2]. Nanoparticles are commonly alluded to as particles in the scope of 1 – 100 nm. Nanoparticles show totally new or improved properties paralleled to bigger particles of the mass material and these novel properties are coming about because of the variety in specific attributes, for example, size, scattering and morphology of the particles [3,4]. In expansive, metal nanoparticles are arranged and stabilized by physical and chemical methods [5,6]. They are costly, need high weight, vitality and temperature and involve the utilization of some lethal synthetic compounds for the adjustment of the nanoparticles, prompting unfriendly impacts when they are being valuable in the medicinal and pharmaceutical applications [7]. In this way, there is a combined requirement for the improvement of an eco-accommodating procedure for the synthesis of nanoparticles. The biological synthesis of nanoparticles have interested noteworthy consideration as they are modified to meet their specific necessities [9]. Among the noble metal nanoparticles, silver nanoparticles and gold nanoparticles are estimated to be of vast hugeness. These nanoparticles have a wide range of utilizations: combating microbes, drug delivery, catalysis [10], waste purification [11], treatment of ecological waste [12], food industry [13], material industry, bio-labelling in the treatment of malignant growth [14]. Biosynthesis of gold nanoparticles utilized differing natural causes like plant tissues and smaller scale life forms. It is the best stage for synthesis of gold nanoparticles, being free from toxic chemicals as well as providing natural capping agents for the stabilization of gold nanoparticles which focuses towards a green methodology [15]. The therapeutic and diagnostic application of nanoparticles should not be toxic and biocompatible. Cytotoxicity of AuNPs has been examined by various examination gatherings, in this way everything is poisonous at a high portion. The important question is AuNPs are toxic at the concentration at which they will be used, believed to be in the range of 1 to 100 nm [16]. The inert element like gold becomes highly active in nanometer dimensions. Nanotoxicological investigations are intended to decide if and to what sum these benefits may represent a hazard to the earth and individuals. Cell culture studies confirm the toxicity of engineered nanoparticles reporting cytotoxicity, decreased cell viability and the production of pro-inflammatory agents. Data on metal based engineered nanomaterials are few and there are some reports focussing on genotoxicity. Characterization of in vivo toxicity has been daunting task as nanomaterials are quite complex and conflicting studies have led to different views of their use and safety. This makes it difficult to evaluate, generalize and predict important aspects of toxicity. Toxic effects are highly dependent on the target organisms, emphasizing that toxicity testing should be performed on a
wide variety of organisms [17]. In this study, synthesis, characterization of synthesized gold nanoparticles and their invitro cytotoxicity effect on normal 3T3 cell line and A549 cancer cell line has been studied. Consequently, that the gold nanoparticles induces apoptosis in A549 cell line.

2. EXPERIMENTAL

2.1 Materials

2.1.1 Chemicals and Reagents

Cell culture Dulbeco's modified eagle medium (DMEM) and Fetal Bovine Serum (FBS), 0.25% trypsin-EDTA were obtained from the Gibco (Grand Island, NY, USA). Streptomycin, penicillin, Dimethyl sulfoxide (DMSO), (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) (MTT), Phosphate support and fluorescent stains, for example, Acridine orange and Ethidium bromide (AO/EB) with 98% virtue were bought from the Sigma-Aldrich (St Louis, MO, USA). Chloroauric corrosive/gold chloride (HAuCl4) was acquired from the Himedia Lab Pvt. Ltd. (Mumbai, India). New leaves of C.flexuosus plant were gathered from home nursery garden, Chennai, Tamil Nadu. The plant has been affirmed by a plant taxonomist from the Plant Anatomy Research Centre(PARC), Tambaram, Chennai, India. Further chemicals utilized in this analysis were of diagnostic evaluation.

2.2 Methods

2.2.1 Preparation of the plant extracts

Well and new leaves were washed with refined water to wipe out earth and debasements. For about a month, the leaves were dull dried at room temperature. Subsequent to drying, the leaves were crushed coarsely and sieved utilizing mesh (60µm) to get an unvarying size range. 10 g of leaf fine particles was added to 100 ml of sterile refined water and bubbled for 20 min. These concentrates were then separated twice through utilizing Whatman No.1 filter paper which was then kept in the cooler (4°C) and utilized for further analyses.

2.2.2 GC-MS examination of leaf extract

GC-MS investigation was done on Shimadzu 2010 or more containing an AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument utilizing the accompanying conditions: section RTX 5Ms (Column distance across is 0.32 mm, segment length is 30 m, segment thickness 0.50 µm), working in electron sway mode at 70 eV; Helium gas (99.99 %) was utilized as bearer gas at a consistent progression of 1.73 ml/min and an infusion volume of 5 µl was utilized (split proportion of 10:1), injector temperature 270 ºC; particle source temperature 200 ºC. The broiler temperature was customized from 40 ºC (isothermal for 2 min), with an expansion of 8ºC/min, to 150ºC, at that point 8ºC/min to 200 to 800 nm utilizing UV-Vis HITACHIU-2900.

2.2.3 Synthesis of Gold Nanoparticles

Synthesis of gold nanoparticles were performed by following strategy for Mubarakali et al.[18]. One ml of the leaf watery concentrate of C.flexuosus was taken and included 9.0 ml of 1.0 mM of HAuCl4 independently. The response was performed in dim at room temperature for 24hrs in a static condition. The synthesis of gold nanoparticles was learned outwardly by shading change from yellowish to wine red.

2.2.4 UV-Visible Spectroscopy

The bio-reduction of AuCl4 particles in arrangement was inspected by intermittent testing of aliquots (2ml) and surveying the UV-VIS spectra of the arrangement in quartz cuvettes. The development of wine red AuNPs was confirmed as they were bio-nanoparticles. The biosynthesis of Au nanoparticles were filtered in the wavelength extending from 200 to 800 nm utilizing UV-Vis HITACHIU-2900.

2.2.5 Characterization of gold Nanoparticle

2.2.5.1 Transmission Electron Microscope(TEM)

TEM estimations were accomplished on a JEOL model 1200 EX instrument worked at a quickening voltage of 120 kV. Tests for TEM exams were set up by inclusion a drops of the gold nanoparticle arrangements in tests 1-4 on carbon covered TEM lattices. The films on the TEM grids were admissible to dry in air for 2 min following which the extra solution was removed by utilizing a blotting paper.

2.2.5.2 X-ray Diffraction measurements

X-ray diffraction (XRD) investigation of drop covered films on glass substrates from the AOT-topped gold nanoparticles in test 1 was completed on a Phillips PW1830 instrument usable at 40 kV and a 30 mA with CuKa radiation.

2.2.5.3 Energy Dispersive X-ray Analysis (EDX)

EDX investigation was led in above instrument appended with thermo EDX to confirm the presence of different elemental composition of the sample. The synthesized AuNPs were isolated by centrifugation for 20 min at 10,000 rpm. The pellets were gathered and dried in the oven at 50 ºC to remove any overabundance water, cooled to room temperature and watched for EDXanalysis.

2.2.5.4 Dynamic Light Scattering zeta potential measurements

Zeta potentials were determined with a Zetaphoremeter IV (CAD, France), which subjects a sample to an applied electric field and uses digital image analyzing software to measure the resulting particle travel distances as electrophoretic mobility (EM). The software uses EM to calculate the zeta potential according to the Smoluchowski equation, which is valid when κa>>1, as is the case with most microalgae (Henderson, 2008). If necessary, samples were diluted with filtered media to obtain an analyzed particle number of 30-200. A minimum of three replicate measurements were performed for each sample.

2.2.5.5 Particle Size Analyser

The range of particle size of the nanoparticles was controlled by utilizing particles analyser (Malvern Zetasizer nanosizer). Particle size was arrived based on measuring the dependent fluctuation of laser light by the nanoparticles.

2.2.5.6 Fourier Transform Infrared Spectroscopy (FTIR)

Samples were estimated by shimadzhu 8400s and utilizing spectral range of 4000-400 cm-1 with resolution of 4 cm-1 control tests for the FTIR was arranged similarly concerning
powder diffraction estimations. The FTIR spectra of leaf extracts taken before the synthesis of AuNPs were analysed. This investigation shows the presence of functional groups in this extract, which helps in the formation of AuNPs.

2.2.6 Cell Culture Maintenance
3T3 fibroblast normal cell line, A549 adenocarcinoma cancer cell line were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in the log arithmic phase of growth in DMEM medium supplemented with 10% (v/v) heat inactivated foetal bovine serum, 100 U/ml penicillin in, 100 μg/ml streptomycin. They were maintained at 37°C with 5% CO2-95% air humidified incubator.

2.2.7 Effect of synthesis of sized Gold Nanoparticles on Cytotoxicity of Cell Lines – MTT Assay
The cytotoxic impact of synthesized AuNPs from the watery leaf extract of C.flexuosus affirmed against both 3T3 ordinary cell line and cancer cell lines such as A549 by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test [19]. Briefly, the cell lines were independently seeded in 96-well microplates (1 x 10^6 cells/ml) and incubated at 37 °C for 24 h with 5% CO2 incubator and enabled them to develop to 90% confluence. At end of the incubation, the medium was placed and the typical cells were treated with AuNPs at various concentrations of 20, 40, 60, 80 and 100 μg/ml. In this way cancer cell lines were treated with AuNPs at same concentrations. Then these samples were incubated for 24 h. The cells were then washed with phosphate-buffered saline (PBS, pH-7.4) and included 20 μl of (MTT) arrangement 5 mg/ml to each well and permitted to represent 37 °C in dark for extra 4 h. After that 100 μl DMSO was included and broke down the formazan crystals. The absorbance was read by spectrophotometry at 570 nm utilizing ELISA plate reader.

2.2.8 Analysis of Morphological changes
The concentration that restrained half of cell development were referred as IC50 value, which was utilized as a factor for cytotoxicity study. The morphological changes of control and the cells treated at IC50 were saw under bright field magnifying instrument, after 24 h and shot. The cell lines 3T3 treated with synthesized AuNPs showed the IC50 value of 80 μg/ml while A549 cells were of 60 μg/ml at 24 h.

2.2.9 Assesment of Apoptosis
2.2.9.1 Propidium Iodide (PI) Nucleic Acid Stain
A549 cells were plated at 5 x 10^4 cells/well into a six well chamber plate. At 90% confluence, the cells were treated with synthesized AuNPs for 24 h. The cells were washed with PBS fixed in methanol: acidic acid (3:1, v/v) for 10 min and stained with 50 μg/ml propidium iodide (PI) for 20 min. Atomic morphology of apoptotic cells was seen underneath Floid Cell Imaging fluorescent microscopy.

2.2.9.2 Acridine Orange(AO)/Ethidium Bromide (EB) Dual Staining
Cell passing were controlled by a technique for Jeyaraj et al. [20]. The A549 cells were seeded in 6-well plate sand treated with IC50 concentration of AuNPs for 24h. For then clear analysis is, the monolayer of cells was washed with PBS and stained with 5μl of acridine orange (100μg/ml) and 5μl of ethidium bromide (100μg/ml).The morphological changes in the stained cells, the apoptotic nuclei were observed by FLoid Cell Imaging floures cent micros copy.

2.2.10 Statistical Analysis
All the assembled information were surveyed utilizing PASW measurements 18 programming. Theory testing technique included as One-path analysis of variance (ANOVA) followed by significant difference test. P< 0.05 was considered to indicate factual significance. Every one of the outcomes were expressed as mean ± standard deviation (SD) for each test.

3. RESULTS AND DISCUSSIONS
3.1 Characterization of gold Nanoparticles
Absorption spectra of gold nanoparticles synthesized from C.flexuosus is shown in fig. 1. The Reduction of gold iron into Au particles through exposure to the plant extracts could be tracked by a colour change. Au nanoparticle show wine red colour in aqueous solution due to surface plasmon resonance. UV- vis spectra of the colloidal solution of gold nanoparticles has been recorded. Absorption spectra of Au nanoparticles made in the reaction media has absorbance peak at 540.

![Figure.1a](http://ijesc.org/)  
(a) UV—vis spectrum of Au NPs synthesized from C.flexuosus us. b) synthesis of AuNPs

3.2 Analysis of gold nanoparticles by Transmission Electron Microscopy: TEM investigation is used to classify the large population of poly dispersed gold nanoparticles containedspherical and triangular shapes. Figure 2(a) and (b) shows the morphologies of the spherical nano gold particles under 50 and 100 nm.
3.3 XRD Analysis of Gold Nanoparticles

XRD patterns gained for gold nanoparticles synthesized from C. flexuosus is shown in fig.3(a). The experimental data found from X-ray diffraction (XRD) of very small particle is pretty difficult to examine. Nanoparticles in XRD patterns shows several different size dependent structures leading to anomalous peak position height and width. XRD analysis is mainly used to study the crystalline nature of gold nanoparticles. A number of Bragg reflections equivalent to the (111), (200), (220), (311) and (222) sets of lattice planes are observed. The XRD pattern thus evidently shows that the gold nanoparticles formed by the reduction of HAuCl₄ by leaves extract are crystalline in nature.

3.4 Analysis of Energy Dispersive X-ray (EDX)

EDX analysis is used for identification of the presence of the elemental gold, which showed the reduction of gold ions to gold nanoparticles (fig.3 b). The EDX profile indicates a strong gold signal along with weak oxygen, which may have initiated from the biomolecules bound to the surface of gold nanoparticles. It has been stated that nanoparticles synthesized using plant extracts are enclosed by a thin layer of some capping organic material from the plant leaf broth and stable in solution after synthesis.

3.5 Dynamic light Scattering zeta Potential Measurement

The Figure 4(a) displays the zeta potential (ζ) is a measure of the electrostatic potential on the surface of the nanoparticle and is related to the electrophoretic mobility and stability of the suspension of nanoparticle. The complete absorbance of Zeta Potential shown the incipient instability nature happened in this sample. In the present study, AuNPs were negatively charged with a zeta potential of -9.24 mV, which evidences that the particles were dispersed in the medium, evidencing the judgement that they are stable.

3.6 Particle Size Analyser

The Figure 4(b) shows the particle size of the nanoparticle samples. The particle size of AuNPs was noticed by intensity and laser diffraction, which are poly dispersed in blend solution. The size of synthesized AuNPs ranges from 20 to 110nm. Conversely, beyond 100 nm range the percentage of nanoparticle is very less. The highest portion of AuNP present in the solution was of 93.43 nm. From the plot it was evident that the solution consisted of nanoparticle having various sizes.

3.7 Analysis of Fourier Transform Infrared spectroscopy

FTIR band of Au nanoparticle is used to detect the probable biomolecules answerable for capping and effective stabilization of the Au nanoparticle synthesized by plant leaf extract. The peaks perceived (Figure 5) for Au nanoparticle formed through reduction by Cymbopogan flexuosus at 3553 cm⁻¹ (Alcohol, Phenol), 2133 cm⁻¹ (Aromatic), 1640 cm⁻¹ (Amide) 1368 cm⁻¹ (Aromatics) and 1215 cm⁻¹ (Alkyl halides ) propose the presence of flavonoids and phenols adsorbed on the surface of Au nanoparticles. The analysis of IR spectrum also providing an idea about biomolecules posture different functionalities which are present in the underlying system. The results of FTIR analysis confirmed the presence of phenol, alkanes, aliphatic amine, secondary alcohol, alkynes and aromatic amines compound.
3.8 Identification of Bioactive Compounds in *Cymbopogan flexuosus* leaves Extract by GC-MS Analysis

The presence of compounds in the leaf extract were identified by GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) are presented in fig 6. The principal compounds were oxirane, 2,3- dimethyl trans, 1-heptanol, dimethyl phthalate, 9- eicosane, 1- tridecyl 4-ol, DOP. This study discovers the goodness of the leaves of the plant *Cymbopogan Flexuosus* which has a estimable sense of purpose and can be advised as a plant of phytopharmaceutical importance.

3.9 Analysis of Cytotoxicity and Morphology against 3T3 (Normal) Cell Line

Gold nanoparticles plays a most important role in industrial, pharmaceutical and biomedical applications. Development of drug delivery systems that can efficiently cause the intended therapeutic effect in patients with minimal antagonistic reactions has been broadly explored in recent years [21]. The cytotoxicity of AuNPs under in vitro conditions on fibroblast cells were studied in terms of effect of nanoparticles on cell proliferation by the MTT assay. The effect of synthesized AuNPs at different concentrations such as 20, 40, 60, 80 and 100 μM nanoparticles on cell viability of 3T3 normal cells was made at 24 h, 48 h, 72 h and 96 h. The cells showed 85–90% viability to the concentrations up to 100 μM. In this study the cytotoxicity effects of different concentration of synthesized AuNPs under in vitro conditions on fibroblast cells (normal cell line) was observed by the MTT assay. AuNPs exhibited a maximum IC50 value of 80 μg/ml (Fig.7A). AuNPs tested against 3T3 cells showed better biocompatibility and Significantly losses the cell viability in treated concentrations when related to control (p < 0.05). The cell viability assay made in the current attempt shown that AuNPs are less toxic than rest. The bio-compatibility of AuNPs was progressively increased at all concentrations by time. There are two prospects reported for the interaction and metabolism of AuNPs, one is adsorption source to delay in growth, next is metabolism or internalization fallouts the viability of cell and strong growth. Here I believed that, AuNPs are not chronically toxic to the cell growth or for their viability. The 3T3 fibroblast cells treated for 24 h with the particular IC50 concentration of AuNPs revealed that the cells converted into round, shrink and lose their contact with neighboring cells. The images (Fig.7B) confirmed the toxic effect the samples tested against 3T3 fibroblast cells.
3.10 Cytotoxicity and Morphology for synthesized AuNPs against Cancer Cell Lines

In the current study the anti-proliferative activities of AuNPs were made on A549 cancer cell lines, it can be assessed by MTT assay. Dose-dependent inhibition of AuNPs on A549 cells was detected at different concentrations: 20, 40, 60, 80 and 100 μg/ml. The IC50 value for A549 cells was recorded as 60 μg/ml at 24 h (Fig. 8A). There was a significant reduction in the cell viability in treated concentrations when related to control (p< 0.05). These outcomes revealed that AuNPs mediated concentrations dependent increase in the toxicity of all cancer cell lines. The A549 cells treated with IC50 60 μg/ml of AuNPs for 24 h (Fig. 8B), after that it was found irregular confluent aggregates, rounded and polygonal. The cells showed numerous cell surface protuberances. The plasma membrane was found condensed. A dense mass of accumulation of nuclear chromatin was detected below the nuclear membrane. Gold ions released from gold nanoparticles can act together with phosphorus moieties in DNA, resulting in inactivation of DNA replication or reacting with sulfur containing proteins, foremost to the inhibition of enzyme functions which results in loss of cell viability and finally resulting in cell death [22]. Similarly, results of the present study the A549 cell line treated with the synthesized AuNPs at 60 μg/ml showed cell death up to 51%. The cells treated at the above condition showed cell shrinkage.
3.11 Morphological Features of A549 Cells Stained with Propidium Iodide under Fluorescence Microscope
The cytotoxic effect encouraged by biosynthesized AuNPs encompasses apoptotic changes and nuclear condensation were proved by the PI staining method. In the case of cells treated at IC50 concentration of AuNPs at 24 h showed a progressive rise in the number of Propidium Iodide positive cells (Fig 10). It was recommended that nanoparticles can make cell death in A549 cells through the reactive oxygen species mediated apoptotic process. The increased ROS levels and following loss of mitochondria membrane potential might be the reason for improved apoptotic morphological changes in nanoparticles treated cells [23].

3.12 Morphological Features of A549 Cells Double Stained with AO/EB under Fluorescence Microscopy
Initiation of apoptosis was examined by finding of nuclear and cytoplasmic condensation with blebbing leading to the formation and discharge of apoptotic bodies. The detection and quantification of apoptosis and necrosis were also assessed and compared by AO/EB double staining for fluorescence microscopy [24]. The AO is taken up by both viable and nonviable cells and emits either green fluorescence as a result of intercalation into double-stranded nucleic acids (mainly DNA), or red fluorescence as a result of binding to single-stranded nucleic acids (mainly RNA). The EB is taken up only by nonviable cells, and emits red fluorescence by intercalation into DNA. Thus a viable cell holds a uniform bright green nucleus and orange cytoplasm. An early apoptotic cell, whose membrane is still undamaged but has started to cleave its DNA, still has a green nucleus, but the chromatin of the cell becomes visible resulting from condensation in the form of bright green patches. A late apoptotic cell shows bright orange areas of condensed chromatin in the nucleus and a necrotic cell shows a uniform bright orange nucleus. In present study, after 24hrs the A549 cells were treated with IC50 concentration of AuNPs stained with AO/EB fluorescence staining. It showed the morphological apoptotic changes when related to untreated control cells (Fig.11 a &b). The control cells had presented uniform bright green nuclei and cytoplasm, Whereas the cells treated with AuNPs displayed characteristic change of apoptosis i.e. cell shrinkage, nuclear condensation and fragmentation and formation of apoptotic bodies. They were witnessed as orange coloured bodies. The necrotic cells were witnessed as red colour fluorescence due to their loss of membrane integrity when viewed under fluorescence microscope [25].

4. CONCLUSION
Green chemistry and bioapproach has leads to develop rapid method for the synthesis of gold nanoparticles. In the present study, the synthesized gold nanoparticles from C. flexuosus leaf has occurrence of flavonoids act as reducing agent and the amino group as a stabilizing agent in the synthesis of AuNPs.
In pharmacology aspects, the gold nanoparticles are non-toxic in normal 3T3 fibroblast cells at maximum concentration whereas A549 adenocarcinomic alveolar cell line was toxic at lower concentration. However, the exact mechanism behind the in vitro and in vivo anticancer properties of synthesized gold nanoparticles needs to be studied.

5. REFERENCES


