

IN VITRO CYTOTOXICITY AND ANTIOXIDANT EVALUATION OF BIOGENIC SYNTHESIZED GOLD NANOPARTICLES FROM *MARSILEA QUADRIFOLIA* ON LUNG AND OVARIAN CANCER CELLS

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ABSTRACT

Objective: The biogenic gold nanoparticles are considered to be extremely impressive for its wide range of applications in pharmaceuticals and therapeutics. The present study was aimed at the biogenic synthesis of gold nanoparticles (AuNPs) from *Marsilea quadrifolia* aqueous extract and to investigate its antioxidant property and cytotoxic effect on human ovarian teratocarcinoma (PA-1) and lung adenocarcinoma (A549) cell lines.

Methods: The biogenic AuNPs was synthesized using an aqueous extract of *Marsilea quadrifolia*. The synthesized biogenic AuNPs were characterized by ultraviolet (UV) visible spectroscopy, transmission electron microscopy (TEM), energy dispersive X-ray analysis (EDX) and X-ray diffraction (XRD). The biogenic AuNPs was assessed for its stability over a period of time and antioxidant activity. The cytotoxicity of biogenic AuNPs against PA-1 and A549 cell lines was studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Results: The synthesized biogenic AuNPs showed peculiar ruby red color and a surface plasmon resonance (SPR) peak at 544 nm in the UV-Vis spectrum. The characterization of biogenic AuNPs by TEM, EDX and XRD revealed well dispersed spherical particles ranging from 10-40 nm and the presence of elemental gold and its crystalline nature, respectively. The AuNPs showed good stability and the scavenging activity at 50 µg/ml. The *in vitro* cytotoxicity of biogenic AuNPs against PA-1 and A549 cell lines recorded half maximal inhibitory concentration (IC₅₀) of 45.88 µg/ml and 52.015 µg/ml, respectively.

Conclusion: The biogenic AuNPs demonstrated superior antioxidant and antiproliferative activities against cancer cell lines.

Keywords: Biogenic, Gold nanoparticles, *Marsilea quadrifolia*, Antioxidant, Cytotoxicity

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INTRODUCTION

Cancer is a most highly enigmatic and complex disease responsible for the major decline in mortality. It is a multistep carcinogenesis process encompassing numerous cellular physiological systems such as cell signaling and apoptosis [1]. The early recognition and treatment of cancer remain a technological obstruction. The current diagnostic methods are insufficient to make predictions for successful treatment. Thus, there is an immediate need for a unique and comprehensive technology against cancer for its early diagnosis, personalized therapy, and medicine [2].

Nanotechnology has revolutionized the field of medicine and accelerated the growth of Nanomedicine, a new field of interest among the researchers, having a tremendous potential of enhanced bioavailability with a simultaneous reduction in toxicity and side effects of the drug [3]. Among the nanoparticles, the bio-reduced gold and silver nanoparticles have gained utmost importance in biomedical application [4]. Besides, the metallic nanoparticles have a tunable surface plasmon resonance (SPR) which enables biolabelling [5]. It is flexible for surface functionalization with various biomolecules (protein, DNA, algae, enzymes, and plant-derived bioactive compounds) [6].

The biosynthesis of metallic nanoparticles is an interesting technology as it aims to minimize the generation of a hazardous substance to health and environment. A broad classification of living organisms (cyanobacteria, bacteria, fungi, actinomycetes, biomolecules and various plant materials) are already well-known to synthesize nanostructured composites [7]. Rapid and biosynthesis methods using biological sources have shown a greater potential for nanoparticles synthesis, however, understanding the involvement of biomolecules is lacking [8]. Nanostructured materials showed many aspects of interesting characteristics, i.e., optical, catalytic, that greatly depends on the size and shape of nanoparticles.

Marsilea quadrifolia L. (Family: *Marsileaceae*), an aquatic fern is found abundantly in eastern and southern regions of India [9]. The aerial part of *M. quadrifolia* extract using chloroform and ethyl acetate has shown proven antibacterial, cytotoxic, and antioxidant effect [10]. Moreover, methanolic extract of *M. quadrifolia* fern has been reported to the higher efficiency of glucose utilization and cellular viability in 3T3-L1 adipocytes with lower toxicity [11]. Thus, in the current investigation, the gold nanoparticles (AuNPs) were synthesized by employing the aqueous leaf extract of *M. quadrifolia* as a reducing agent. The biogenic AuNPs were characterized and evaluated for its antioxidant property and cytotoxic effect on two different cancer cell lines such as human ovarian teratocarcinoma (PA-1) and lung adenocarcinoma (A549) cells under *in vitro* condition.

MATERIALS AND METHODS

Chemicals

Tetra chloroauric acid or gold chloride (HAuCl₄) was purchased from Hi-media Lab Pvt. Ltd. (Mumbai, India). Cell culture Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and 0.25 % trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). Streptomycin, penicillin, dimethyl sulfoxide (DMSO), (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) and phosphate buffer were purchased from the Sigma-Aldrich (St Louis, MO, USA).

Plant samples and preparation of plant extracts

The leaves of five different plants namely *Cassia javanica* (ABP01), *Melia azedarach* (ABP02), *Marsilea quadrifolia* (ABP03), *Stachytarpheta jamaicensis* (ABP04), *Spathodea campanulata* (ABP05) were collected from different parts of Chennai. The leaves were washed to remove impurities, air-dried in the shade and then grounded. The dried leaf powder samples (4.0 g) were taken separately and mixed with 100 ml of sterile distilled water. The

mixtures were boiled at 55 °C for 15 min and then cooled to room temperature and filtered through Whatman No.1 filter paper. The aqueous extract was collected, refrigerated and used for further experiments [12].

Biogenic synthesis of gold nanoparticles

The biogenic synthesis of AuNPs was carried out according to Balashanmugam *et al.* [13] method with modifications. Briefly, 1.0 ml of the aqueous leaf extract was added to 9.0 ml of 1.0 mmol of HAuCl₄ separately and incubated in the dark at room temperature for 24 h. The visual observation of change in color from golden yellow to ruby red color indicates the formation of AuNPs.

Characterization of biogenic AuNPs

UV-Visible Spectroscopy and stability

The formation of phytosynthesized AuNPs was confirmed by measuring the absorption spectra between 300-700 nm in PC based Systronics double beam spectrophotometer 2202. The stability of the biogenic AuNPs was recorded at different time intervals such as 6 h, 12 h, 18 h, 24 h, 10th day, 20th day, 30th day, 45th day and 60th day. The absorbance was measured using the above-mentioned instrument.

Transmission electron microscopy (TEM) and energy dispersive X-ray analysis (EDX)

The biogenic AuNPs were characterized for its morphology and size by performing TEM (TECNAI 30 G2S-TWIN, FEI Company). A drop of biogenic synthesized AuNPs was placed on the carbon-coated copper grid; the extra solution was removed using a blotting paper and then the film was dried and analyzed. The EDX analysis was conducted in the above instrument to confirm the presence of the different elemental composition of the sample [14].

X-ray diffraction (XRD)

The lyophilized biogenic AuNPs were studied by XRD measurements with the help of powder X-ray diffractometry (SEIFERT JSO DEBYEFLEX 2002). The analysis was carried out with Cu K α radiation at a wavelength of 1.5406 Å with the operating condition at 40 kV, 30 mA. The XRD pattern was scanned in the 2 θ from 30 ° to 70 ° with a step size of 0.04 ° per second [13].

Determination of free radical scavenging activity

The radical scavenging activity of biogenic AuNPs was determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay according to Rajamanikandan *et al.* [15]. Different concentrations (10, 25, 50, 100, 250 and 500 µg/ml) of biogenic AuNPs was mixed with 2 ml of 4 mmol methanolic DPPH and incubated in the dark at room temperature for 15 min. After incubation, the absorbance of the samples was measured by using UV-vis spectrophotometer at 517 nm against methanol as blank. Ascorbic acid was used as a standard,

and methanolic DPPH reagent without sample was used as a control. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}})}{\text{Absorbance}_{\text{control}}} \times 100.$$

Collection of the cell line and cell maintenance culture

The PA-1 ovarian teratocarcinoma cell line and A549 lung adenocarcinoma cell line was obtained from the national center for cell sciences (NCCS), Pune, India. Cells were maintained in the logarithmic growth phase in DMEM medium containing L-glutamine (2 mmol), sodium bicarbonate (g/l), glucose (4.5 g/l), HEPES (10 mmol) and sodium pyruvate (1.0 mmol). It was supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 100 µg/ml penicillin, 100 µg/ml streptomycin in an air humidified incubator with 5 % CO₂ at 37 °C.

Cytotoxic effect of biogenic AuNPs on cancer cell lines-MTT assay

The cytotoxicity test of biogenic AuNPs synthesized from *M. quadrifolia* leaf aqueous extract was performed against two cancer cell lines such as PA-1 and A549 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16]. Briefly, the cell suspensions were separately seeded on 96-well microplates (1 × 10⁶ cells/ml) and incubated for 24 h at 37 °C. After 90 % of cell confluence, PA-1 and A549 cells were treated with different concentrations (10, 20, 40, 60, 80 and 100 µg/ml) of biogenic AuNPs incubated for 24 h at 37 °C. The untreated cells were marked as a control. After 24 h of treatment, the medium was removed and the cells were washed with phosphate-buffered saline (PBS, pH 7.4) and incubated with 20 µL of MTT (5 mg/ml in PBS) for 4 h at 37 °C. Then 100 µL of DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance was read at 570 nm using ELISA plate reader. The cell viability percentage was expressed as follows:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100.$$

RESULTS AND DISCUSSION

Biogenic synthesis of AuNPs

The aqueous leaf extracts of five different plants namely *Cassia javanica*, *Marsilea quadrifolia*, *azedarach*, *Spathodea campanulata*, *Stachytarpheta jamaicensis* were used for the synthesis of AuNPs. Among the tested plants, the AuNPs synthesized using *M. quadrifolia* aqueous leaf extract showed ruby red color with higher stability than the other tested samples (fig. 1). Therefore, *M. quadrifolia* leaf extract was chosen for further studies. A similar observation of appearance of ruby red color that indicates the formation of AuNPs was reported using *Cinnamomum zeylanicum* [17], *Sorbus aucuparia* [18], *Rosa rugosa* [19], and *Mangifera indica* [20].

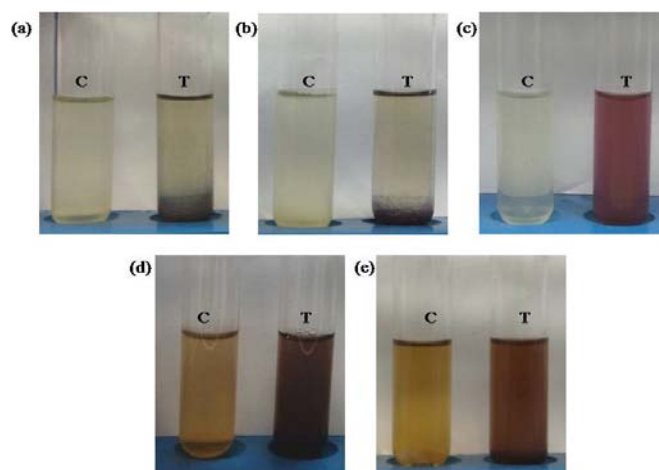


Fig. 1: Biogenic synthesis of gold nanoparticles (AuNPs) using different plants. (a) *Cassia javanica*, (b) *Melia azedarach*, (c) *Marsilea quadrifolia*, (d) *Stachytarpheta jamaicensis* and (e) *Spathodea campanulata*

Characterization of biogenic AuNPs

UV-visible spectroscopy and stability

The AuNPs synthesized using *M. quadrifolia* revealed an SPR peak at 544.8 nm in the UV-Vis spectra, confirming the formation of AuNPs (fig. 2a). Similar behavior of AuNPs has been reported by Mukundan *et al.* [21]. The stability of the biogenic AuNPs was monitored under UV-visible spectroscopy at different time

durations (6 h-2 mo). It was observed that the synthesis had started within 6 h with a peak at 538 nm and remained up to 12 h. Thereafter, there was a shift in the peak to 541 nm at 18 h. The reaction ended at 24 h with an absorption peak at 544 nm, and the two-month-old sample also exhibited a peak at 544 nm, thus indicating the higher stability of biogenic AuNPs of *M. quadrifolia* (fig. 2b). The stability of AuNPs depends on the choice of plant and the presences of phytochemicals [22].

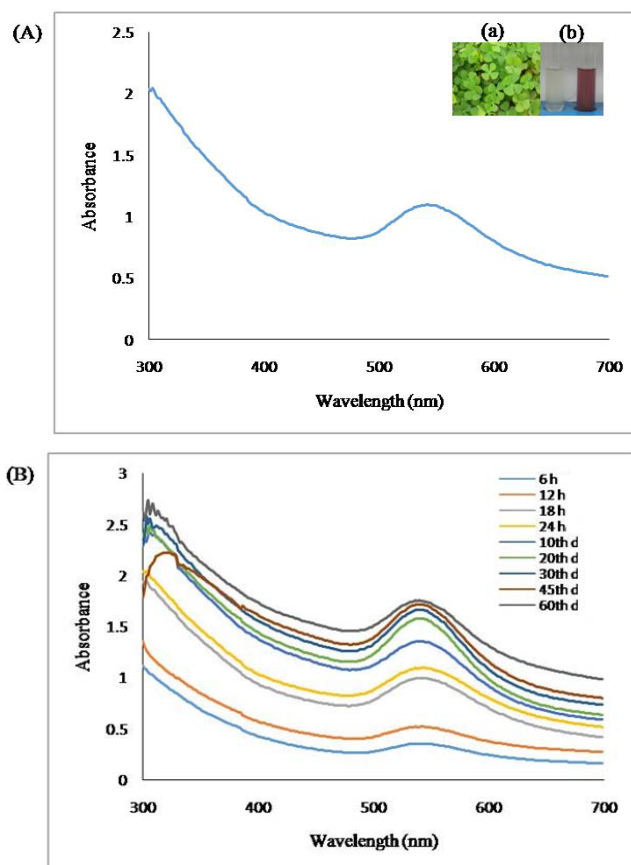


Fig. 2: (A) UV-Vis spectrum of biogenic AuNPs synthesized from *Marsellia quadrifolia* aqueous leaves extract. Inset (a) habitat, (b) biogenic AuNPs synthesized, (B) Stability of AuNPs at different time durations. Abbreviations: h (n=9)

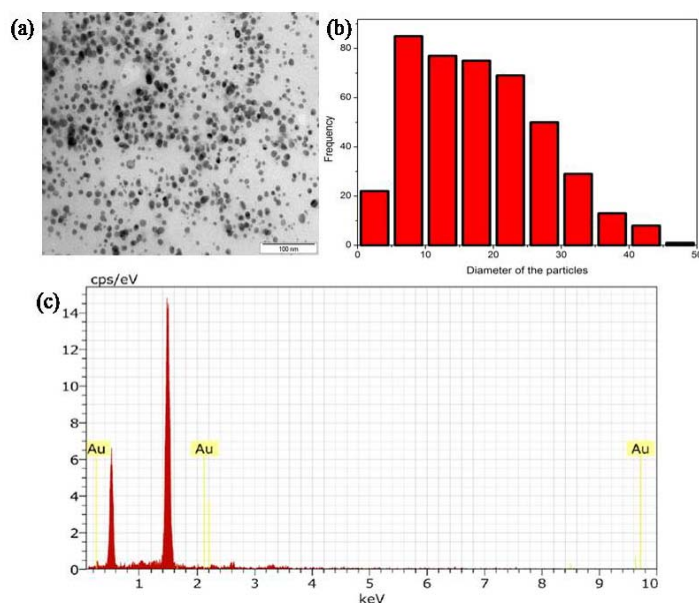


Fig. 3: Characterization of biogenic AuNPs. (a) TEM image of spherical shaped AuNPs, (b) Size distribution of particles, (c) EDX spectrum

Transmission electron microscopy and energy dispersive X-ray analysis

The TEM analysis revealed that the biogenic AuNPs were mostly spherical in shape and well dispersed in nature with a particle size range of 10 to 40 nm (fig. 3a and 3b). Similarly, Bhau *et al.* [23] reported the synthesis of spherical shape AuNPs to range from 50 nm to 80 nm using *N. Khasiana* leaf extracts. The biogenic AuNPs EDX spectrum showed a single and strong signal for gold (Au), indicating that the NPs that were neutral (fig. 3c). Similar observations were made by Elavazhagan [24] with *Mesembryanthemum edule* gold nanoparticles.

X-ray diffraction (XRD) studies

The XRD pattern revealed five distinct peaks at 38.21 °, 44.39 °, 64.62 °, 77.59 ° and 82.09 ° corresponding to (111), (200), (220), (311) and (222) planes respectively (fig. 4), representing the face-centered cubic (fcc) structure of the AuNPs. The data obtained was matched with the database of joint committee on powder diffraction standards (JCPDS) file No. 65-2870, indicating that the biogenic AuNPs are crystalline in nature. Much similar to our present report, Fazaludeen *et al.* [25] has reported the XRD patterns for gold nanoparticles synthesized using *Justicia gendarussa* leaf extract.

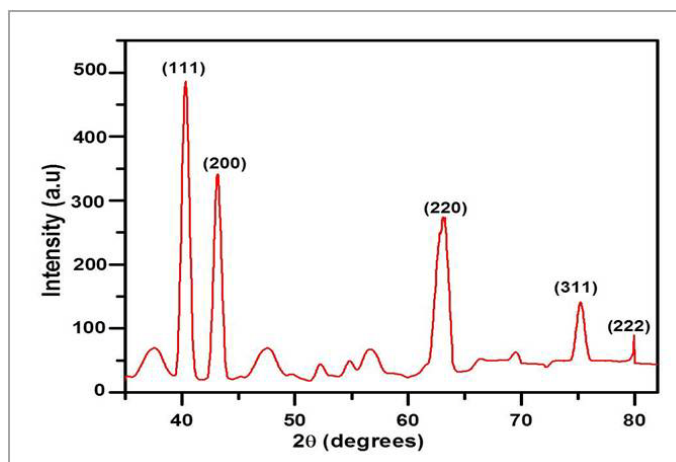


Fig. 4: XRD pattern of biogenic AuNPs

Determination of free radical scavenging activity

The free radical scavenging activity of the biogenic AuNPs was assessed by DPPH assay by using ascorbic acid as a positive control. The biogenic AuNPs were able to reduce the stable radical DPPH to the yellow-colored diphenyl-picrylhydrazine. The AuNPs showed a

dose-dependent activity, and the inhibitory concentration (IC_{50}) was found at a concentration of 50 $\mu\text{g/ml}$ whereas the standard ascorbic acid inhibitory concentration was recorded at 10 $\mu\text{g/ml}$ (fig. 5). Accordance of observations have been reported by AuNPs synthesized using *Lemna minor*, *Gymnema sylvestre*, *Sumac* with enhanced antioxidant activity [26-28].

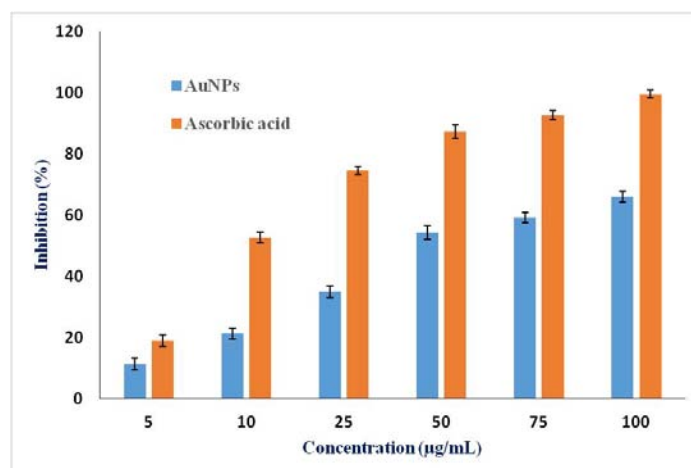


Fig. 5: Antioxidant activity of biogenic AuNPs, values are given as mean \pm SD for each concentration (n=6)

Effect of AuNPs induced cytotoxicity on cancer cell lines

The MTT assay was performed to examine the *in vitro* cytotoxic effect of biogenic AuNPs against PA-1 and A549. The viability of the PA-1 and A549 cell lines was observed after 24 h of treatment with biogenic AuNPs. A significant decrease in the cell viability was observed with increasing concentrations of the biogenic AuNPs when compared to control. The IC_{50} value for PA-1 and A549 cells was recorded at 45.88 $\mu\text{g/ml}$ and 52.01 $\mu\text{g/ml}$, respectively, at 24 h (fig. 6a). The AuNPs treated cancer cells appeared as irregular and annular-shaped

conglomerates, whereas the control cells were normal in shape (fig. 6b). Rajeshkumar [29] reported the inhibitory effect of AuNPs at 100 μg concentration of nanoparticles against A549 cells. The cytotoxicity of AuNPs is believed to be the active physicochemical interaction of gold atoms with the functional groups of the intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA [2]. The reduced cell viability of PA-1 and A549 cells observed in this study is suggestive of anticancer effects of AuNPs and further studies are required to be done to understand the process of cell death by apoptosis or necrosis pathway.

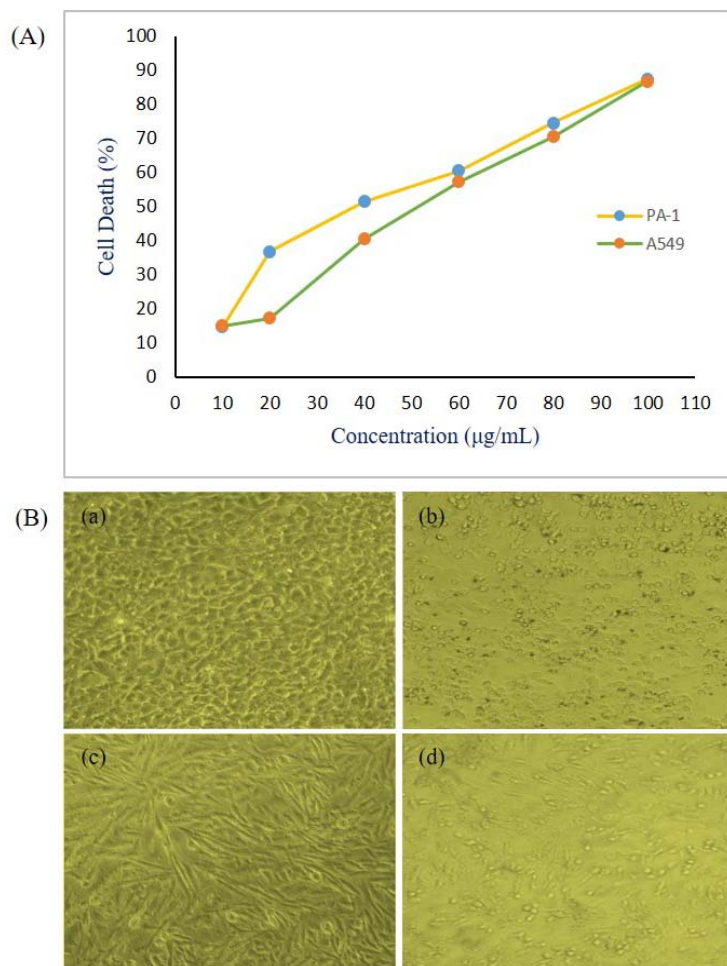


Fig. 6: (A) Cytotoxicity of different concentration of biogenic AuNPs on PA1 and A549. (B) Cytotoxic effect of AuNPs on PA1 and A549 cell line. (a) and (c) Control, (b) and (d) IC₅₀ treated cells, respectively. Values are given as mean±SD for each concentration (n=6)

CONCLUSION

The process for the synthesis of nanoparticles in large scale using the readily available plant extract may have commercial viability and to develop an interface between biology and material science. The stable bioactive AuNPs were synthesized using *M. quadrifolia* leaf extract. The phytochemicals present in the extract of *M. quadrifolia* has reduced the gold ions into metallic nanoparticles. The biogenic AuNPs exhibited a substantial *in vitro* antioxidant activity and cytotoxicity effect against both PA-1 and A549 cancer cells. Such biogenic AuNPs are expected to serve as potent anticancer agents and thus can be used in biomedical applications.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICTS OF INTERESTS

The authors report no conflicts of interest in performing this work

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