



doi: 10.4103/2221–1691.245969

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Enhanced cytotoxic effect on human lung carcinoma cell line (A549) by gold nanoparticles synthesized from *Justicia adhatoda* leaf extract

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ARTICLE INFO

Article history:

Received 18 July 2018

Revision 15 August 2018

Accepted 31 October 2018

Available online 27 November 2018

Keywords:

Justicia adhatoda

Leaf extract

AuNPs

Cytotoxicity

A549 cell line

ABSTRACT

Objective: To synthesize bio-inspired gold nanoparticles (AuNPs) using the leaf extract of *Justicia adhatoda* and evaluate the anti-cancer activity on human lung cancer cell line (A549).

Methods: Synthesis of AuNPs was done using an aqueous leaf extract of *Justicia adhatoda* as a green route. The bio-synthesized AuNPs were confirmed and characterized by using various spectral studies such as UV-Vis spectrum, Scanning Electron Microscope with EDAX, Transmission Electron Microscope, Fourier Transmission Infrared Spectroscopy analysis and Surface Enhanced Raman Spectroscopy. The cell viability was determined by MTT reduction assay. In addition, cytomorphology and the nuclear morphological study of A549 cell line was observed under fluorescence microscope. **Results:** UV-Vis spectrum showed surface plasmon resonance peak at 547 nm, scanning electron microscope and transmission electron microscope studies showed the monodispersed spherical shape and its average size in the range of 40.1 nm was noticed. Fourier Transmission Infrared Spectroscopy analysis confirmed that the C=O group of amino acids of proteins had strong ability to bind with the surface of nanoparticle. Interestingly, our results also demonstrated inhibited proliferation of A549 cell line by MTT (IC₅₀ value: 80 µg/mL). Cell morphology was observed and cell death was caused by apoptosis as revealed by propidium iodide staining. **Conclusions:** The current study proves the anticancer potential of bio-synthesized AuNPs. Thus, synthesized AuNPs can be used for the treatment of human lung cancer cell (A549) and it can be exploited for drug delivery in future.

1. Introduction

Nanotechnology is expected to be a frontier technology for all sciences due to extensive applications in all fields. Accordingly related research and developments are growing rapidly in this field. Interdisciplinary nature of green chemistry proposes the environmentally benign materials and process. Now a major health

issue around the world is cancer caused by an unusual growth of cells[1,2]. International Agency for Research on Cancer has reported that approximately 7.6 million deaths arise due to cancer and

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How to cite this article: Latha D, Prabu P, Arulvasu C, Manikandan R, Sampurnam S, Narayanan V. Enhanced cytotoxic effect on human lung carcinoma cell line (A549) by gold nanoparticles synthesized from *Justicia adhatoda* leaf extract. Asian Pac J Trop Biomed 2018; 8(11): 540-547.

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12.7 million cancer patients are diagnosed every year[3]. Cancer diagnostics and treatment costs are very high and also have side effects. Researchers have strived to attain a novel therapy and drugs for treatment of cancer. Antibiotics can kill many diseases instigated by some organisms meanwhile nanomaterials can be able to kill 650 kinds of cells, consequently nanomaterials are called as “a Wonder of Medicine”[4]. In past decades, nanomaterials were acting as a good anticancer agent[5]. In recent years, AuNPs were used for anticancer therapy for different kinds of cancers such as Hep2 cell[5,6], breast cancer (MCF-7)[7] and prostate cancer (PC-3)[8]. Almost 60% of anticancer agents are reported to be from the plant materials[9]. Many types of respiratory disorders are characterized by inflammatory reaction in air way. In humans, many causative agents are responsible for formation of lung cancer. Most of the lung cancer are carcinomas and 31% of cancers are adenocarcinomas[10]. It is the acronym of human lung adeno carcinoma epithelial cell (A549).

In this work, the synthesis of AuNPs was conducted by using *Justicia adhatoda* (*J. adhatoda*) (Acanthaceae family) leaf extract. Previous studies reported that the leaf contains phenols, tannins, alkaloids (vasicine, vasicinol, vasicinone, adhasavinone, adhatonine and peganine), anthraquinones, saponins, flavonoids (apigenin, astragalol, kaempferol, quercetin, vitexin), reducing sugars and vitamin C[11–13]. Vasine and vasinone are the major alkaloids in *J. adhatoda* leaf extract. Chanu and Sarangthem reported the phytochemical components in leaves of *J. adhatoda*, which contains total carbohydrate (16.1 mg/g), soluble proteins (7.8 mg/g), phenols (32.1 mg/g), flavonoids (37.9 mg/g) and alkaloids (1.09 mg/g)[14]. Various phytochemicals such as alkaloids, polyphenols and flavonoids have been proven to fight against various types of human diseases[15,16]. Flavonoids can prevent the activities of enzyme which are involved in breaking the carbohydrate into sugar and few flavonoids show cytotoxicity in various cancer cells. The polyphenols can maintain the level of glucose in blood by numerous mechanisms. Phenolic compounds might act as an antioxidant agent by scavenging free radicals[17]. Earlier reports have focused on biological activities of *J. adhatoda* such as antibacterial, bronchodilator, anti-asthmatic, anti-tubercular, and anti-inflammatory activities[12,15]. However, none of them have tested anticancer effect of *J. adhatoda* mediated gold nanoparticles (AuNPs) on A549 cell line. Therefore, we designed to explore the anticancer efficiency of AuNPs by measuring cytotoxicity through MTT assay and apoptosis by propidium iodide (PI) staining. To the best of our knowledge, this is the pioneer report of cytotoxicity of AuNPs synthesized from *J. adhatoda* leaf extract against lung cancer (A-549) cell line.

2. Materials and methods

2.1. Materials

Tetrachloroauric acid ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$) Dulbecco's Modified Eagle's medium (DMEM), Fetal Bovine Serum, trypsin, streptomycin, MTT, and dimethyl sulfoxide were purchased from Hi Media and Sigma Aldrich. Human lung cancer carcinoma cell lines (A549) were

obtained from Cancer Institute (Chennai, Tamilnadu).

2.2. Collection and preparation of leaf extract

Fresh leaves of *J. adhatoda* were collected from agriculture farms in Cumbum valley, at Theni district, Tamilnadu, India. Collected leaves were washed and shade dried for 15 d and ground into coarse powder. Ten mg of coarse powder was taken in a 250 mL beaker along with 120 mL of double distilled water. The mixture was digested for 20 min at 60–70 °C and then the extract was filtered with Whatman No.1 filter paper. This filtrate was called as aqueous extract, and then it was stored in the refrigerator for further experiments.

2.3. Synthesis of AuNPs

2.3.1. Effect of time on synthesis of AuNPs

One mL of leaf extract was added to 7 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ solution. Within 20–30 min pink-ruby red color appeared due to reduction of Au^{3+} ions to Au^0 . The formation of color was monitored by visual appearance.

2.3.2. Effect of temperature on formation of AuNPs

The effect of temperature on the synthesis of AuNPs was studied by 1 mL leaf extract from *J. adhatoda* added to 7 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1 mM) followed by the reaction in a water bath at 30, 50 and 70 °C. The formation of AuNPs was monitored as a UV-Vis spectrophotometer.

2.3.3. Effect of pH on formation of AuNPs

The effect pH was experimented by 1 mL leaf extract from *J. adhatoda* added to 7 mL of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (1 mM) at different pH (4, 7 and 11). The formation of AuNPs was monitored by UV-Vis spectrophotometer.

2.4. Characterization of AuNPs

A UV-Visible spectrophotometer technique is used to ascertain stability and formation of nanoparticles. UV-Vis spectra were recorded using a Perkin-Elmer Lambda-45 spectrophotometer. Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) were performed on Hitachi S-3400N and TEM-JEOL model 1200EX. Fourier Transmission Infrared Spectroscopy (FTIR) measurements were carried out using Perkin-Elmer in the range of 400 to 4000 cm^{-1} by using KBr pellets. Surface enhanced Raman spectrum measurements (SERS) were analyzed by Nano photon-Raman 11 spectrometer.

2.5. In vitro anticancer studies

2.5.1. Cytotoxicity assay

Lung cancer (A549) cells were grown in DMEM containing 10% fetal bovine serum, penicillin and streptomycin, and subsequently incubated

at 37 °C in a 5% CO₂. Cultured cells (1 × 10⁵ per well) were seeded into a 96-well plate with 1 mL of DMEM medium. Various concentrations of the AuNPs (20, 40, 60, 80, 100 and 120 µg/mL) were added to each well and incubated for 24, 48 and 72 h at 37 °C in a CO₂[18]. Control cells were also maintained without adding AuNPs. After the incubation period, the medium was removed and fresh medium was introduced with 10 µL of MTT solution and incubated for 4 h. The formation of purple colored formazan crystal was dissolved with dimethyl sulfoxide (DMSO), and then spectrophotometric absorbance was measured using an Elisa-Reader at 570 nm. The effect of biosynthesized AuNPs on the proliferation of A549 cells was expressed as percentage of cell viability.

$$\text{Percentage of viability} = \frac{\text{OD value of experimental samples}}{\text{OD value of experimental controls}} \times 100$$

2.5.2. Cytomorphological observation

The cells were seeded in a six well plate at 1 × 10⁵ cells/well and incubated overnight. Thereafter the cells were maintained in DMEM medium. Biosynthesized AuNPs of IC₅₀ (80 µg/mL) were added and then incubated for 24, 48 and 72 h. The cytomorphology of the exposed cells was monitored under fluorescence microscope.

2.5.3. PI staining

A549 cells were seeded into a six well plate at 5 × 10⁴ cells/well. After that when it reached more than 90% confluence, the cells were treated with AuNPs at 80 µg/mL concentration (IC₅₀ value) for 24–72 hours of incubation time. The cells were washed with PBS and CH₃OH:CH₃COOH (3:1) for 10 min and stained with 50 µg/mL PI for 20 min. Nuclear morphology of apoptotic cells with condensed or fragmented nuclei was observed by fluorescence microscope[19].

3. Results

3.1. UV-Visible spectroscopy studies

3.1.1. Effect of contact time

The present study was aimed to monitor the contact time of the reaction mixture in synthesis of AuNPs by UV-Visible spectroscopy. The *J. adhatoda* plant contains alkaloids, flavonoids, glycosides, poly phenolic compounds, reducing sugar *etc.*, Such kind of phytochemicals are beneficial to reducing the metal and also act as a capping agent as well as stabilizing agent. The surface plasmon resonance (SPR) peak of nanoparticles occurred at 547 nm within 25 min, which was steadily increasing with reaction time and without any shift in wavelength (Figure 1).

3.1.2. Effect of reaction temperatures

Several factors are responsible for synthesis of AuNPs, amongst them temperature and pH occupy important role in a reaction

process. Consequently, the first factor is optimal temperature requisite to synthesis of AuNPs. Thus, the reaction mixtures were prepared and kept at various temperature *viz.*, 30, 50 and 70 °C which were examined under UV-Visible spectrum. As shown in Figure 2, with increasing temperature, the SPR band sharpness also increased which indicated temperature was one of the factor for affecting synthesis of AuNPs. When the temperature increased, the rapid color change of mixture was noticed.

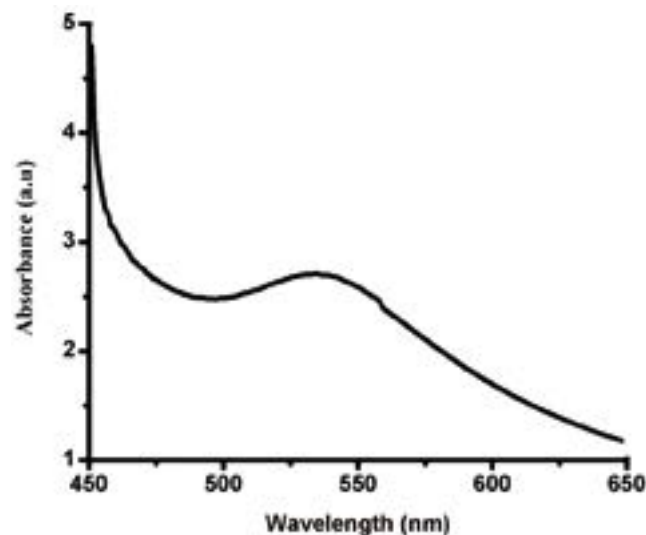


Figure 1. UV-Vis spectra of AuNPs (*J. adhatoda* leaf extract added with H₂AuCl₄·3H₂O).

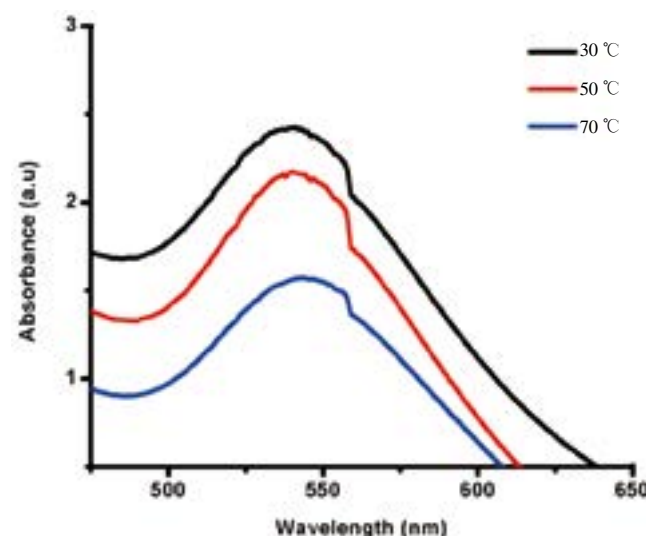


Figure 2. UV-Vis spectra of AuNPs by the addition of leaf extract with chloroauric acid at different reaction temperatures (30, 50, 70 °C).

3.1.3. Effect of pH on stability of nanoparticles

After that, the another factor is pH which acts as a vital role in the formation of AuNPs. As above mentioned, the experimental set up was maintained in synthesis of nanoparticle with varying pH of the mixture *viz.*, 4, 7 and 11. The rate of AuNPs formation increased with increasing pH, which was visually monitored by immediate

dark color and a red shift was observed in UV-Vis. The absorption spectra showed the peaks at 538 nm (pH-4), 541 nm (pH-7) and 545 nm (pH-11). The pH 7 was the most favourable for synthesis of AuNPs (Figure 3).

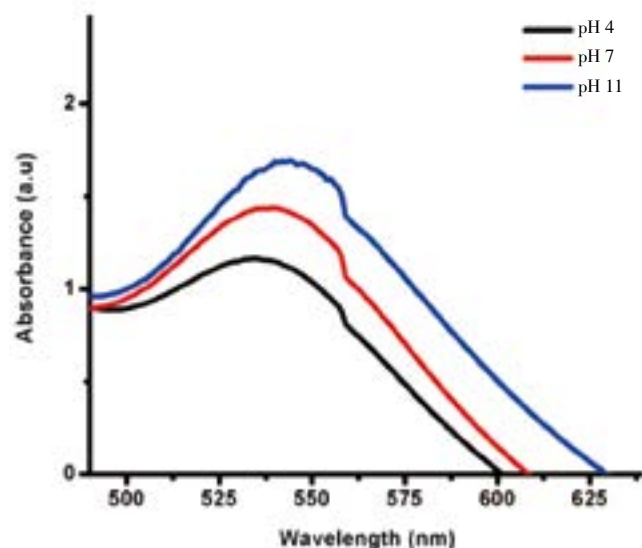


Figure 3. UV-Vis spectral studies of AuNPs at different pH values.

3.2. Morphological studies

3.2.1. SEM analysis

The SEM images of AuNPs synthesized from *J. adhatoda* leaf extract were shown in Figure 4 (a-b). SEM photographs described the morphology and distributions of AuNPs. EDAX pattern exhibited the strong signals for AuNPs (Figure 4c). The optical peak was noted at 2.3 KeV which denoted for the crystalline nano gold due to SPR.

3.2.2. TEM studies

TEM analysis discussed the morphology of biosynthesized AuNPs as shown in Figure 5. It showed that the TEM images of AuNPs were spherical shape and the average size of the AuNPs was observed at the range of 40.1 nm (Figure 5).

3.3. FTIR analysis of AuNPs

FTIR analysis stands for the molecular absorption and transmission bands or peaks which is due to the vibration frequencies between the bonds of atoms. FTIR was revealed to identify the functional groups of biomolecules that were occupied on the surface of synthesized AuNPs. The FTIR analysis of synthesized AuNPs was examined under the peaks at 3419, 2927, 1631 and 1401 cm^{-1} (Figure 6).

3.4. SERS

Figure 7 showed the Raman peak intensities were at 269, 447, 503, 588, 663, 768, 774, 1012, 1154, 1398, 1444 and 1609 cm^{-1} . The

synthesized AuNPs have shown a strong SERS effect band 447 cm^{-1} with a shoulder peak at 503 cm^{-1} due to the skeletal deformation vibration of C-N-C and C-S-C. The small peaks were observed at 774 and 1012 cm^{-1} which correspond to C-N-C and C-S-C skeletal deformation as well as N-CH₃ stretching.

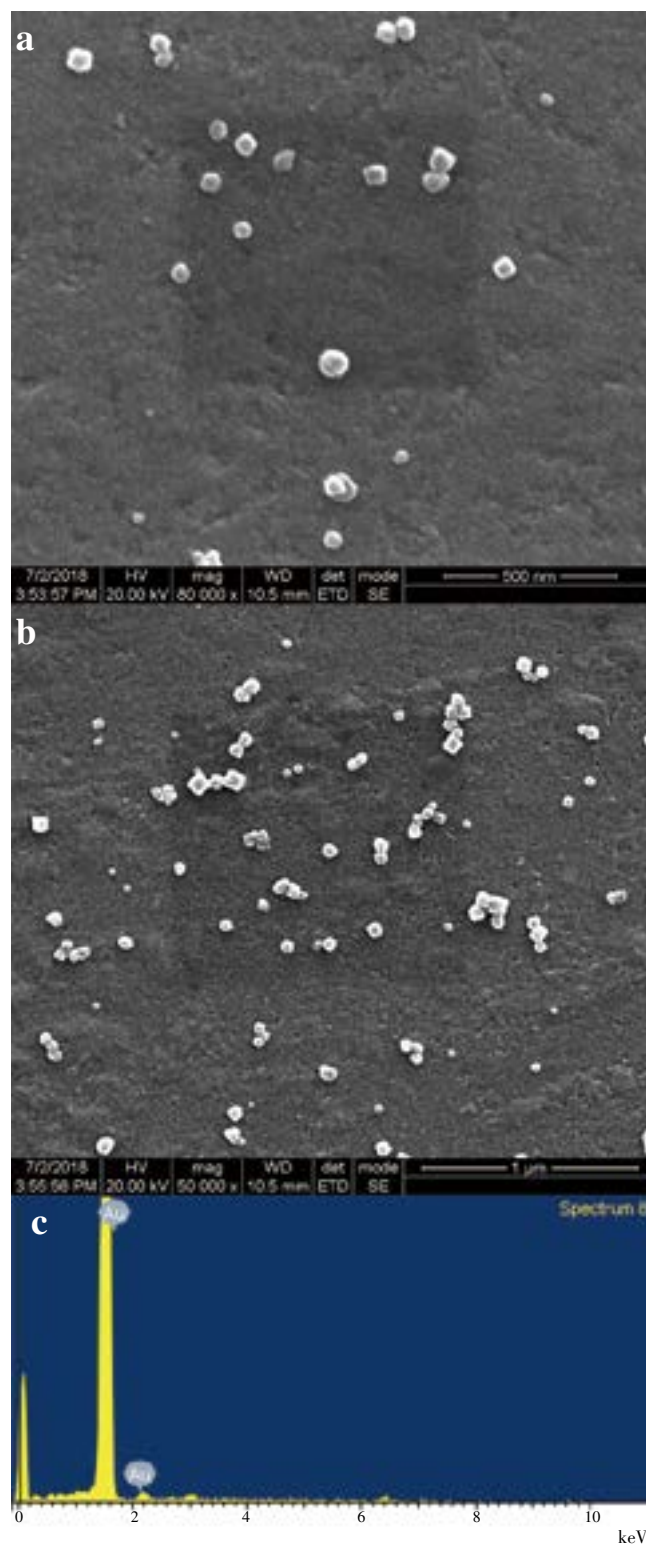


Figure 4. SEM images of AuNPs (a-b) from *J. adhatoda* leaf extract and EDAX of AuNPs (c).

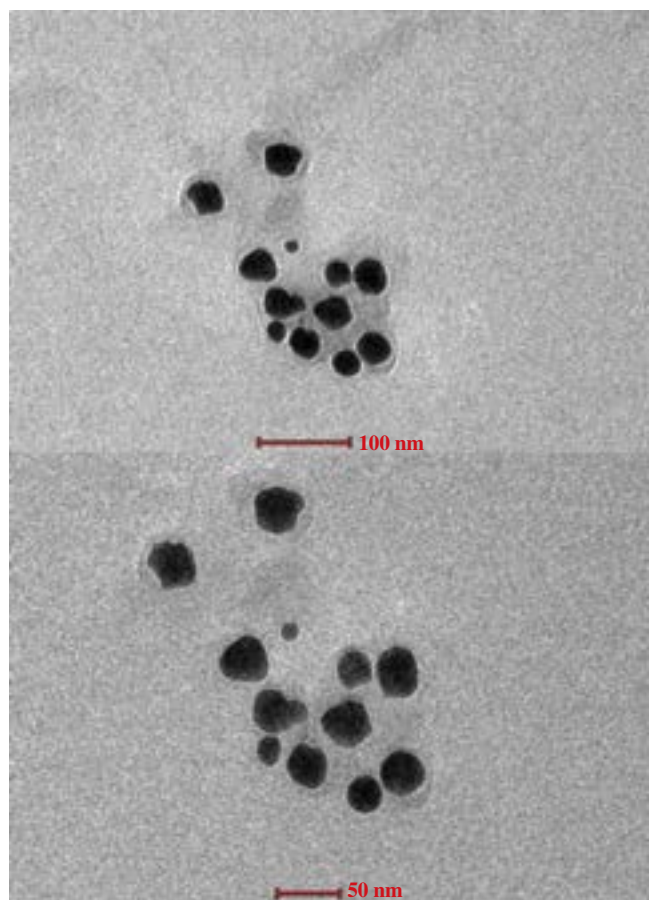


Figure 5. TEM images of AuNPs from *J. adhatoda* leaf extract.

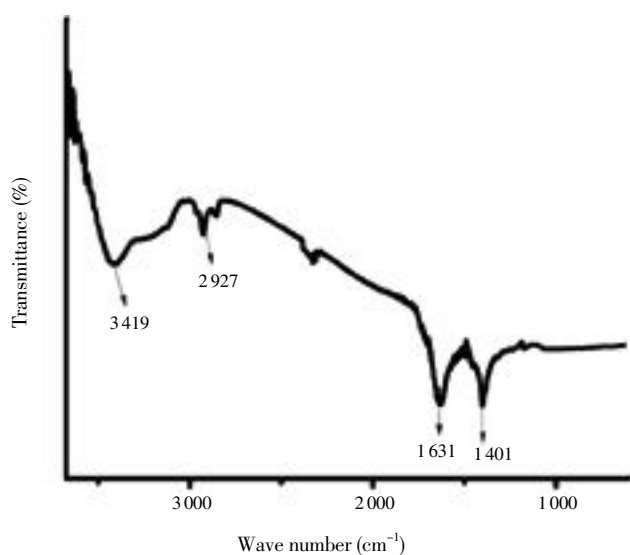


Figure 6. FTIR analysis of AuNPs from *J. adhatoda* leaf extract.

3.5. Study of anticancer activity

3.5.1. Cell viability assay

The cytotoxic effect of AuNPs was determined against A549 cancer cell line. Synthesized AuNPs were added with A549 cell line and later were monitored for their cell viability using the MTT. There was low rate of metabolism which occurred in cells treated by

AuNPs compared with untreated cells (control). The viability of cells depended upon the dosage of nanoparticles and incubation period (24–72 h). Significantly, reduction of viability was observed by the concentration of AuNPs and incubation period (20–120 $\mu\text{g/mL}$ & 1–3 days) which was shown in Figure 8.

3.5.2. Cytomorphological observation

Human lung cancer cells were treated with freshly synthesized AuNPs at the IC_{50} (80 $\mu\text{g/mL}$) concentration for 24–72 h incubation period (Figure 9). Cytomorphology of treated cells clearly exhibited the apoptotic mechanism leading to cell death which was observed at 24 h incubation. The apoptotic cells increased with increasing incubation time. Morphological changes including uniform detachment of cells from 6-well plate, inhibition of cell growth, membrane integrity and cell shrinkage were observed (Figure 9). The results indicated that the biosynthesized AuNPs treated A549 cells underwent cell death compared to untreated cells. Treated cells showed distinct cellular morphological changes.

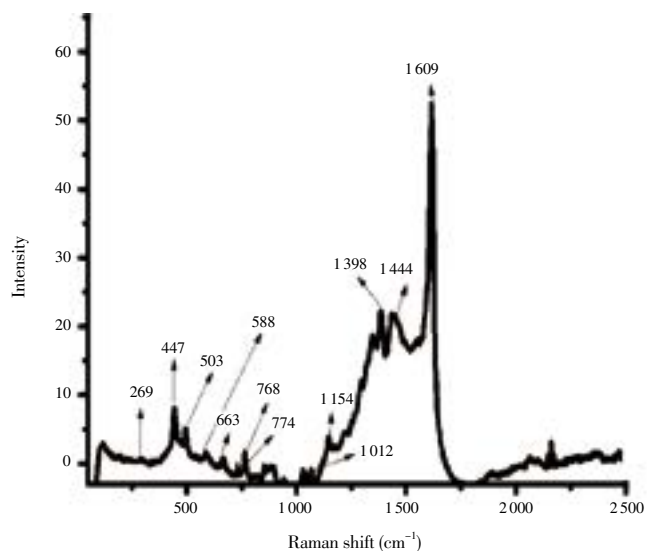


Figure 7. SERS analysis of green synthesized AuNPs using *J. adhatoda* leaf extract.

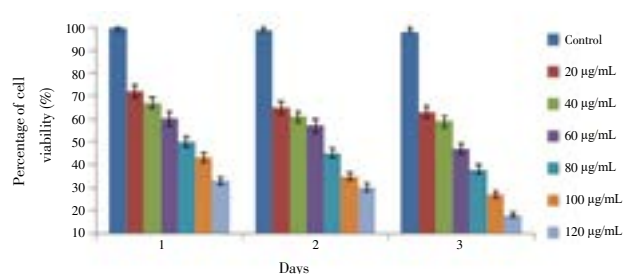


Figure 8. *In vitro* cytotoxicity of AuNPs against A549 cell line.

3.5.3. PI staining

To confirm the cytotoxic effect encouraged by biosynthesized AuNPs from leaves extract of *J. adhatoda*, apoptotic changes were monitored. In control cells, negligible number of PI positive cells was observed. In the case of treated cells at 80 $\mu\text{g/mL}$ concentration

(IC₅₀) of biosynthesized AuNPs with exposure period of 24-72 h, an increase in the number of PI positive cells was also monitored during increasing incubation period (Figure 10). The results were shown in Figure 10. Nuclear compactness and apoptotic cell changes were identified by the PI staining method.

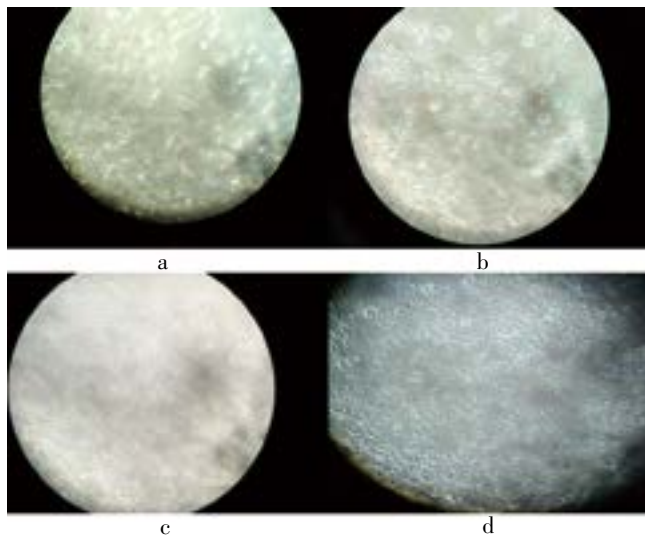


Figure 9. Morphology of control and AuNPs treated A549 cells (a) Control; (b) (IC₅₀: 80 µg/mL) 24 h; (c) 48 h; (d) 72 h.

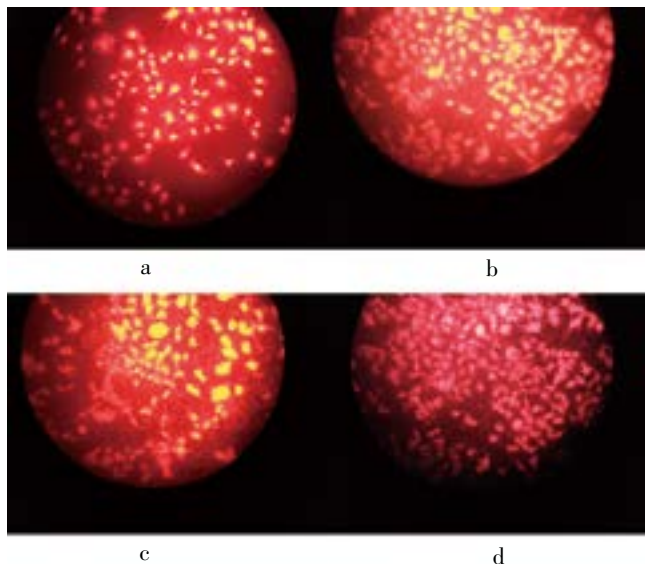


Figure 10. PI staining of A549 cells treated with AuNPs (a) Control; (b) 24 h; (c) 48 h; (d) 72 h.

4. Discussion

In recent years, the application of metallic nanoparticle is increasing due to their economy, growing interest of biological field and eco-friendly benign method of their production. Phytochemical compounds of *J. adhatoda* are the foremost sources for several biological activities. Synthesis of AuNPs using *J. adhatoda* leaf was conducted in this study, and it was identified by the color changes

of mixture from pale yellow to pink-ruby red color. The color of the nano gold is ascribed to the combined oscillation of free electron induced by electromagnetic field that is called as SPR[20,21]. Previous reports demonstrated the SPR band of AuNPs appeared almost in this region (500-600 nm), but the position of SPR range may vary which depends on the size as well as shape of the nanoparticles[16]. Lokina *et al.* also reported that the sharpness of the peak of SPR elevates with the increase of reaction time[16]. The formation of stable aggregation of the AuNPs in a solution or anisotropy nature of particles was pointed out by shoulder peak of SPR[22].

At high temperature, faster rate of the reduction enhances the nucleation rate, utmost number of nanoparticles is formed, thereby stopping the secondary reduction process and providing minimal possibility for particle size-growth[23,24]. Interestingly, at low temperature, the particle size is large due to overgrowth of nuclei which is possible to form the anisotropic nature of nanoparticles. With increasing temperature, the SPR band sharpness also increased, the same result was also observed in the *Coleus aromaticus* leaf[23]. Above 30 °C, SPR peak decreases due to overgrowth of crystal around the nucleus[25,26]. Therefore the optimal temperature for AuNPs synthesis was preferred to be 30 °C which is the best result compared with other results[26-28].

In pH parameter, the absorption band shows the red shift due to localized SPR of nanoparticles. In general, acidic medium suppresses the growth of nano particles but the basic condition boosts the growth of nanoparticles[26,27]. At lower pH, larger size of nanoparticle may be possible to form this that is due to over nucleation, besides higher pH, utmost number of functional groups is bound on surface of nanoparticle; as a result, maximum number of nanoparticle is obtained with small size[24]. At lower pH, synthesized AuNPs were bound with -SH group (cysteine), and -NH₂ (glutamic acid) groups which is confirmed by FTIR analysis[28]. Verma *et al.* also reported that the red shift (longer wavelength) of SPR indicates that size of the nanoparticle increases along with increasing pH[28]. The optimal pH for AuNPs synthesis was preferred to be pH 7 which is in agreement with the available literature[26]. SEM and TEM studies revealed spherical shape of AuNPs without aggregation. It was obviously illustrated that a spherical and triangle shape of the nanoparticle was capped with the biomolecules. The capping and stabilization of AuNPs are due to the *J. adhatoda* leaf extract which contains flavonoids, glycosides and proteins. The broad band at 3419 cm⁻¹ is due to the O-H stretching vibration[22]. The band at 2927 cm⁻¹ corresponds to C-H groups[22]. A sharp peak at 1631 cm⁻¹ is attributed to primary amide bond I in FTIR analysis[29].

FTIR analysis showed that the C=O group of amino acids of proteins, these molecules had strong capability to bind with AuNPs and it also acted as a capping agent for AuNPs. Hence, the capping agents can prevent agglomeration and thereby stabilize the AuNPs. The biological molecules are reducing and stabilization agents of AuNPs.

SERS is a very efficient technique to determine the enhanced effect of surface metal nanoparticles which is associated with efficient capping biomolecules. A small peak that appeared at 269 cm^{-1} due to vibrations between Au⁰ and Cl, S, N or C confirms that the biomolecules are at the surfaces of AuNPs[30]. Nuntawong *et al.*, have also reported that the SERS intensity increases with decreasing the concentration of methylene blue[31]. Several Raman bands are found and in some peaks are splitting in SERS[32].

The splitting and shifting of few Raman bands pointed out that the methylene blue molecule is chemisorbed on AuNPs[32]. This study also revealed that various peaks were found in SERS spectrum, such as 1 012 and 1 398 cm^{-1} which correspond to histidine and cysteine respectively[33]. In general, such kind of protein molecules can bind on the surface of AuNPs. SERS analysis also verified that the protein molecules acted as a capping agent with the AuNPs.

The percentage of cell viability decreased by increasing concentration of AuNPs as well as incubation period. The synthesized AuNPs has shown 50% of cell death at 80 $\mu\text{g/mL}$ concentration[16] which is considered as IC_{50} value, while at 120 $\mu\text{g/mL}$, concentration of nanoparticle, cell viability reduced to 33% at 24 h. Cell viability became almost 20% for maximum concentration at 72 h. Similar results were observed by Pernodet *et al.*, in AuNPs on dermal fibroblasts[34]. Control cells were shown regular shape and AuNPs treated cells exhibited spherical shape and cell spreading patterns compared to control cells[16]. Biosynthesized AuNPs can travel into the ion channels through cell membranes and interact with DNA molecules, impeding replication and other nuclear works thus suppressing the metabolic activity[35–37]. The PI staining method has shown that plant mediated AuNPs caused cell death which occurred by apoptosis.

In conclusion, the AuNPs were synthesized from *J. adhatoda* leaf extract in this study. The UV-Visible spectroscopy, SEM with EDAX, TEM, FTIR and SERS confirmed the monodispersed AuNPs which were capped with protein molecules. The synthesized AuNPs showed a potent anti-proliferation effect on the A549 lung cancer cell. Thus, the present study highlighted the biomedical properties of *J. adhatoda* mediated synthesis of AuNPs, which could form significant baseline information for development of a new nano-anticancer drug.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgements

The authors would like to thank to University of Madras and TNAU for providing facilities.

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