CHAPTER - 4

IN VITRO ANTIOXIDANT AND ANTICANCER ACTIVITIES OF MYCOSYNTHESIZED SILVER AND GOLD NANOPARTICLES
4.1. Introduction

An important area of research in nanotechnology deals with the synthesis of nanoparticles of different chemical compositions, sizes and controlled monodispersity. Indeed, nanoparticle shape control is a recent addition to the list of demands being made of newly emerging synthesis methods. Currently, there is a growing need to develop environmentally benign nanoparticle synthesis processes that do not use toxic chemicals in the synthesis protocol (Kanchana et al., 2010).

Nanotechnology has received considerable awareness in advanced biomedical science over the past decade. Nanoparticles can be engineered to have specific or multiple functions and can be used for investigating and pursuing an in-depth understanding of the mechanisms involved in biochemical processes. In clinical research, the drug is dissolved, entrapped, encapsulated or attached to a nanoparticle matrix. Nanoparticles with unique optical properties, facile surface chemistry and appropriate size scale are generating much interest in clinical diagnostics. Gold nanoparticles have huge potential in cancer diagnosis and therapy due to their surface plasmon resonance (SPR) property with enhanced light scattering and absorption. Conjugation of AuNPs to ligands specifically targets the biomarkers of cancer cells which helps in molecular-specific imaging and detection of cancer. Currently, nanotechnology is described as revolutionary discipline in terms of its possible impact on industrial applications. Nanotechnology offers potential solutions to many problems using emerging nanotechniques. Depending on its strong interdisciplinary character, many research fields and several potential applications are involved in nanotechnology (Chandra et al., 2010).

Cancer is a major cause of death in the modern world. Effective treatment of cancer is most readily accomplished following early detection of malignant tumours. Having fought cancer for decades, medical doctors and researchers still face brick walls blocking further progress such as when attempting to find and precisely track cancer cells, especially at an early stage (Faloppi et al., 2011). Metal nanoparticles have attracted increasing interest in diagnosis and therapy of disease due to the strong and size-tunable surface plasmon resonance (SPR), fluorescence and easy-surface functionalization (Gamaleia et al., 2010).

Dependence of human life on nanotechnology was emerged naturally from ayurveda, a 5000-year-old system of Indian medicine. Though the modern science has
started exploring the term ‘Nano’ in 21\textsuperscript{st} Century, ayurvedic medicinal systems have used noble metals such as gold, silver, etc. in nanoform as basmas for various medical applications (Sukirtha \textit{et al.}, 2012). Since nanoparticles are more biocompatible than the conventional therapeutics, they are exploited for drug encapsulation and delivery (Wang \textit{et al.}, 2008). It has been stressed over the years that size reduction of nanoparticles play an important role in improving their bioavailability as well as compatibility for therapeutical applications in diseases like cancer (Kim \textit{et al.}, 2007).

The toxicity is determined by \textit{in vivo} using many parameters including dose, route of exposure, metabolism, excretion, and immune response. The toxicological profiles of nanomaterials might also be determined by nanomaterial chemical composition, size, shape, aggregation and surface coating (Kim \textit{et al.}, 2009; De Jong \textit{et al.}, 2008). The size-dependent organ distributions of gold nanoparticles have been investigated and the results showed that smaller gold nanoparticles of 5–15 nm had wider organ distribution than that of larger sized gold nanoparticles of (50–100 nm) and liver and spleen were the dominant targeted organs. Meanwhile, it has been found that gold nanoparticles with a long blood circulation time can accumulate in the liver and spleen and have obvious effects on gene expression (Zhang \textit{et al.}, 2009). Furthermore, the toxicity and biodistribution of polyethylene glycol (PEG)-coated gold nanoparticles have also been investigated. Interestingly, 20 nm sized gold nanoparticles coated with TA-terminated PEG5000 were found to be more stable and had lower toxicity than the larger sized AuNPs coated with same material. These in vivo results clearly demonstrated that the possible toxicity of gold nanoparticles could occur in the body over short- and middle-term injection and necessitated further toxicological investigations (Balasubramanian \textit{et al.}, 2010; Cho \textit{et al.}, 2009; Chen \textit{et al.}, 2009). The present chapter deals with the \textit{in vitro} antioxidant and anticancer activities of \textit{A. terreus} synthesized AgNPs and AuNPs against different cancerous cell lines.
4.2. Materials and methods

4.2.1. Chemicals

The chemicals doxorubicin hydrochloride, 5-fluorouracil, propidium iodide, ethidium bromide, acridine orange hemi (zinc chloride) salt, 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from the Sigma Chemical Company (St. Louis, MO, USA), Dulbecco's modified eagle medium (DMEM), minimum essential medium (MEM), Roswell Park memorial institute (RPMI-1640) medium, fetal bovine serum, penicillin streptomycin-neomycin (psn), trypsin phosphate versene glucose (TPVG, 1X) solution were purchased from HiMedia Laboratories, India and other chemicals were purchased from SRL Pvt. Ltd. unless otherwise specified.

4.2.2. Antioxidant activity of mycosynthesized silver and gold nanoparticles

4.2.2.1. DPPH scavenging assay (Wang et al., 2008)

The free radical scavenging activity of mycelial free extract of A. terreus, mycosynthesized silver and gold nanoparticles was evaluated in vitro by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay with minor modifications (Yang et al., 2011). For each sample, different concentrations of mycelial free extract (100 to 1000 μg/mL), mycosynthesized silver and gold nanoparticles (10 to 100 μg/mL) were prepared with methanol. The test was carried out in 96-well plates with a reaction mixture consisting of test sample (100 μL) and DPPH radical (100 μL, 0.2 mM) dissolved in methanol. The reaction mixture was properly stirred and incubated at room temperature for 15 min in dark condition. After incubation, the absorbance was recorded at 517 nm against appropriate blank and the percentage of scavenging ability of each test sample was determined. The percentage scavenging effect was calculated as,

\[
\text{Scavenging rate} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100\%
\]

Where,
A1- absorbance in the presence of test sample
A2- absorbance of the sample without DPPH radical
A0 - absorbance of the control (without test sample)
The scavenging ability of tested samples was expressed as IC$_{50}$ value, which is the concentration required to scavenge 50% of DPPH radicals and was calculated from the relationship curve of scavenging activities (%) versus concentrations of respective test sample.

### 4.2.2.2. ABTS radical cation decolourisation assay (Chun et al., 2005)

The ABTS radical scavenging activity of mycelial free extract of *A. terreus*, mycosynthesized silver and gold nanoparticles was carried out *in vitro* via ABTS cation radical decolorization with slight modifications (Yang et al., 2011). For ABTS assay, the samples were prepared as described in the DPPH assay method.

The ABTS cation radical solution was prepared by dissolving 7 mM of ABTS in double distilled water (15 mL) and then mixed with 140 mM of potassium persulphate (264 L). The preparation was kept as such in dark at room temperature for 16 h prior to use. Before the assay, the prepared ABTS mixture was diluted with methanol to attain an absorbance reading of 0.70 ± 0.02 at 734 nm. The diluted ABTS mixture was kept at room temperature for few minutes and used as working solution for further experiment. The ABTS assay was conducted in 96-well plates with a reaction volume consisting of 50 L of different test samples and 100 L of ABTS working solution. The reaction mixture was stirred properly and incubated at dark condition for 10 min at room temperature. After incubation, the absorbance was measured at 734 nm against a blank and the percentage of ABTS scavenging was determined.

The antioxidant capacity was given as percent (%) ABTS scavenging and calculated as:

\[ \text{Scavenging rate} = \left[ 1 - \frac{(A1 - A2)}{A0} \right] \times 100\% \]

Where,

- A1 - absorbance in the presence of test sample
- A2 - absorbance of the sample without ABTS working solution
- A0 - absorbance of the control (without test sample)

The scavenging ability of the tested samples was expressed as IC$_{50}$ value, which is the concentration required to scavenge 50% of ABTS radicals and was calculated from the relationship curve of scavenging activities (%) versus concentrations of respective test samples.
4.2.3. Anticancer activity of mycosynthesized silver and gold nanoparticles

4.2.3.1. Cell culture

The following cell lines were procured from the cell repository facility of the National Centre for Cell Science (NCCS), Pune, India.

1. Vero Cell line (African monkey kidney, normal)
2. HepG2 (hepatocellular carcinoma)
3. A549 (lung cancer)
4. MCF-7 (breast cancer)
5. HCT 15 (colon cancer)
6. A375 (skin cancer)
7. HeLa (cervical cancer)

4.2.3.2. Cell culture maintenance

The cell lines HepG2, A549 and A375 were grown in 25 cm² culture flasks containing Dulbecco's modified eagle medium (DMEM) supplemented with glucose (4.5 g/L), 1 mM sodium pyruvate, 4 mM L-glutamine, 1.5 g/L of sodium bicarbonate, 1% penicillin-streptomycin-neomycin (PSN) and 10% fetal bovine serum (FBS) and then incubated at 37 °C in 5% CO₂ atmosphere.

The cell lines Vero, MCF-7 and HeLa were grown in 25 cm² culture flasks containing minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acid (NEAA) and 1.5 g/L of sodium bicarbonate, 1% penicillin-streptomycin-neomycin (PSN) and 10% fetal bovine serum (FBS) and then incubated at 37 °C in 5% CO₂ atmosphere.

The cell line HCT 15 was grown in 25 cm² culture flask containing Roswell Park memorial institute (RPMI-1640) medium supplemented with glucose (4.5 g/L), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, 1.5 g/L of sodium bicarbonate, 1% penicillin-streptomycin-neomycin (PSN) and 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO₂ atmosphere.
4.2.3.3. MTT assay of mycosynthesized silver and gold nanoparticles

The present study was designed to investigate the cytotoxic effect of mycosynthesized AgNPs and AuNPs against seven different cell lines viz., Vero, HepG2, A549, MCF-7, HCT 15, A375, HeLa by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Mosmann (1983) with slight changes.

4.2.3.4. Cell toxicity analysis against Vero cell line

It is essential to determine the drug compatibility and dosage on normal cell lines before studying the antiproliferative effect against any cancer cell line. In this present study, different concentrations of mycelial free extract of A. terreus, silver nitrate, gold chloride, doxorubicin, mycosynthesized AgNPs and AuNPs were treated against normal Vero cells to study the cytotoxic nature.

The viable Vero cells (1.5×10^4) were separately seeded into sterile 96-well flat bottom plates and were incubated in BOD incubator at 37 °C for 24 h with 5% CO₂ in order to reach a confluence of more than 90%. At the end of the incubation, the medium was replaced and the cells were treated with different concentrations of mycelial free extract (100 to 1000 µg/ mL), silver nitrate (10 to 100 µg/ mL), gold chloride (10 to 100 µg/ mL), doxorubicin (10 to 100 µg/ mL), mycosynthesized AgNPs and AuNPs (10 to 100 µg/ mL). The plates were incubated in BOD incubator at 37 °C for 24 h with 5% CO₂. The cells were then washed with phosphate-buffered saline (PBS, pH-7.4) and 20 µL of MTT reagent (5 mg/ mL in PBS) was added to each well and the plates were incubated for another 4 h. Then, 100 µL of dimethyl sulfoxide (DMSO) was added and the resulting purple colored formazan crystals were observed by measuring the absorbance at 570 nm using microplate ELISA reader (Curcic et al., 2012).

4.2.3.5. Cell toxicity analysis against cancerous cell lines

Similarly, viable cancer cells (1.5×10^4) were separately seeded into sterile 96-well flat bottom plates and then incubated at 37 °C for 24 h with 5% CO₂ atmosphere as described above. After incubation, the medium was refreshed and the cells were treated with different concentrations of mycelial free extract (100 to 1000 µg/ mL), silver nitrate (10 to 100 µg/ mL), gold chloride (10 to 100 µg/mL), doxorubicin (10 to 100 µg/ mL), mycosynthesized AgNPs and AuNPs (10 to 100 µg/ mL). The plates were incubated in
BOD incubator at 37 °C for 24 h in the presence of 5% CO₂ atmosphere. Twenty microlitre of MTT reagent (5 mg/mL in PBS) was added to each well and the cells were incubated for another 4 h prior to washing with PBS buffer (PBS, pH-7.4). Then, 100 µL of dimethyl sulfoxide (DMSO) was added to each well and the absorbance was measured at 570 nm using microplate ELISA reader. The percentage of cell viability was calculated using following formula,

\[
\text{Cell viability (\%)} = \left( \frac{A_{450 \text{ of treated cells}}}{A_{450 \text{ of control cells}}} \right) \times 100
\]

The concentration that inhibits 50% of cell growth was referred as IC₅₀ value, which was used as parameter for cytotoxicity and was calculated by Graph Pad Prim 6 software. The morphological changes of untreated (control) as well as treated cells were observed under inverted bright field microscopy at 20x magnification after 24 h. The maximum activity (lesser IC₅₀ value) of AgNPs and AuNPs was observed against MCF-7 and HepG2 cells and hence, these cell lines were chosen for further studies.

### 4.2.3.6. Acridine orange-ethidium bromide (AO-EB) staining

The extent of apoptosis/ necrosis of tumor cells caused by nanoparticles was assessed microscopically by acridine orange and ethidium bromide (AO/EB) dual staining method (Darzynkiewicz et al., 1994). The tumor cell lines, MCF-7 and HepG2, were grown in 24-well plates (1 × 10⁵ cells per well) as described above and then treated with IC₅₀ concentration of AgNPs and AuNPs separately. After 24 h, the cells were washed with phosphate buffered saline (PBS, pH 7.2) and the mixture of acridine orange and ethidium bromide (1 mg/mL AO and 1 mg/mL EB in PBS) stain was added and then incubated for 5 min. The cells were washed again with PBS buffer to remove the excess stain and were visualized using FLoid® cell imaging station (Life Technologies) with an excitation filter of 482 nm (Jeyaraj et al., 2013).

The treated cell lines showing fragmented, shrunken nuclei and brightly fluorescent were considered as apoptotic cells and the necrotic cells were observed with red fluorescence due to ethidium bromide.

### 4.2.3.7. Nuclear staining with DAPI fluorescent dye

The 4’, 6-diamidino-2-phenylindole (DAPI) staining assay was performed to detect apoptotic nuclei of the nanoparticles-treated cells. The MCF-7 and HepG2 cells
were grown in 24-well plates (1 × 10^5 cells per well) as described earlier and then treated with IC_{50} concentration of AgNPs and AuNPs separately. After 24 h, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at 4 °C followed by 5 min at room temperature. The cells were again washed with PBS and treated with Triton-X 100 (0.4% in PBS buffer) for 20 min at room temperature. Then the cells were stained with DAPI (1 μg/mL in PBS buffer) and allowed to stand for 5 min in dark condition at room temperature. The images of the stained cells were captured using FLoid® cell imaging station (Life Technologies) with an excitation filter of 390 nm (Dolatabadi, 2014).

4.2.3.8. Cell cycle analysis (Chang et al., 2014)

Flow cytometric analysis was performed to analyze cell cycle distribution percentage and to measure the DNA content of the untreated and treated cells. The MCF-7 and HepG2 cells were seeded (1 × 10^5 cells per well) in 75 cm^2 culture flasks separately and incubated for 24 h. The cells were then treated with IC_{50} of AgNPs and AuNPs against MCF-7 and HepG2 cells, respectively. After treatment, the floating cells along with attached cells were harvested by trypsinization, washed twice with PBS and then slowly fixed with 70% ethanol in PBS and incubated at -20 °C for 1 h. The cells were then centrifuged at 1500 rpm for 5 min and the pellet was washed with PBS. The fixed cells were then suspended in 1 mL of PBS buffer containing Propidium iodide and RNAse (PI-50 μg/mL, EDTA - 5 mM and RNAase - 5 μg/mL, pH - 7.4). The stained cells were incubated for 30 min at 37 °C and were analyzed by FACS Calibur flow cytometry (Becton Dickinson System) at an excitation wavelength of 488 nm and emission wavelength at 610 nm. The cell cycle histograms were analyzed by cell Quest Software.

4.2.4. Statistical analysis

All the experiments were performed in triplicate and the data were expressed as means ± standard deviation.
4.3. Results and discussion

The most commonly used methods for antioxidant activity are the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assays. The DPPH assay measures the ability of a substance to scavenge the DPPH radicals by reducing it to hydrazine, thereby change in violet to pale yellow color is observed. In case of ABTS, the radical is generated in stable form when reacted with antioxidant substances leading to blue/ green color formation by reacting with the existing potassium persulfate (Blois, 1958). Both tests are of spectrophotometric based assay where the color formation can be measured and the percentage of inhibition is calculated (Formagio et al., 2014; Sanna et al., 2014).

4.3.1. Antioxidant activity of mycosynthesized silver and gold nanoparticles

4.3.1.1. DPPH free radical scavenging activity of mycosynthesized AgNPs and AuNPs

The antioxidant activity of mycosynthesized AgNPs and AuNPs along with mycelial free extract was determined using DPPH assay with gallic acid as a standard. The DPPH is a stable and well described synthetic radical ion used for the evaluation of antioxidant property of various potential compounds. The present results revealed that the increase in AgNPs and AuNPs concentration increases the scavenging activity, similar to earlier reports. The change in blue to yellow color after the addition of nanoparticles into the methanolic DPPH solution confirmed their antioxidant property (Dipankar and Murugan, 2012; Amarowicz et al., 2004). The maximum inhibition of 92.31% and 89.39% was observed when reacted with 100 µg/ mL of AgNPs and AuNPs, respectively. However, the standard antioxidant, gallic acid showed 97.58% inhibition at the lowest test concentration (20 µg/ mL). The IC_{50} value of AgNPs, AuNPs, mycelial free extract and gallic extract was found to be 28.23, 44.20, 75.12, 4.90 µg/ mL, respectively (Figs. 4.1a and b). The obtained results clearly revealed that AgNPs show better antioxidant activity compared to the AuNPs; however, greater antioxidant activity was found in gallic acid.

Niraimathi and co-workers (2013) have investigated the antioxidant activity of green synthesized AgNPs using DPPH scavenging assay and observed increased scavenging activity with increasing concentration of AgNPs up to 500 µg/ mL, where
62% of inhibition was recorded. Antioxidant potential of biologically synthesized AgNPs was evaluated using DPPH assay, which showed 65.43% of inhibition when treated with 20 mg/L of AgNPs, thus exhibiting fairly lower activity compared to the present study (Abdel-Aziz et al., 2014). Similar kind of results were also reported by various researchers who have found that the increase in AgNPs concentration increases the free radical scavenging activity (Kiran Kumar et al., 2014; Kumar et al., 2014; Sreekanth et al., 2014).

Similarly, Ramamurthy et al. (2013) have also reported the antioxidant activity of both AgNPs and AuNPs by monitoring the quenching ability of synthetic stable DPPH radicals into non-radical form. Recently, the synthesized AuNPs showed more than 80% inhibition in DPPH scavenging assay when treated with a concentration of 100 g/mL, which correlates to our present study showing an inhibition percentage of 89.39% with the same concentration of AuNPs (Balasubramani et al., 2015).

4.3.1.2. ABTS scavenging activity of mycosynthesized AgNPs and AuNPs

Antioxidants are reportedly protecting the cells against severe damages due to the various reactive oxygen species which neutralize the free radicals thereby causing detrimental effect to the specific cells in biological system (Halliwell, 1995). Both the mycosynthesized AgNPs and AuNPs along with mycelial free extract showed comparable scavenging effects on ABTS (Figs 4.2a and b). The mycelial free extracts were very less effective, showing an inhibition percentage of 62.32% when treated with 100 μg/mL. In case of nanoparticles, AgNPs showed 91.48% scavenging activity at 100 μg/mL concentration compared with AuNPs, which exhibited 85.39% activity at the same concentration (100 μg/mL). The IC50 value of mycelial extract was found to be 80.65 g/mL whereas those of AgNPs and AuNPs were 32.46 and 44.81 g/mL, respectively. Gallic acid was used as a standard and its IC50 value was found to be 5.99 g/mL. The result also clearly implies that both AgNPs and AuNPs inhibit ABTS radicals in a dose dependent manner, similar to other reports investigated by several researchers (Ramamurthy et al., 2013; Raghunandan et al., 2011; BarathManiKanth et al., 2010).

The antioxidant potential of mycosynthesized AgNPs was studied using ABTS assay by Nagajyothi et al. (2014). They have found a maximum inhibition of 76.57% when treated with 1 mM concentration of AgNPs compared to 0.125 mM, which showed 60.98% inhibition. Very recently, Kokila et al. (2015) have determined the antioxidant
potential of biologically synthesized silver nanoparticles using ABTS assay. Interestingly, more than 90% scavenging activity was recorded at 250 µg/mL of AgNPs.

Dauthal and Mukhopadhyay (2012) have studied the in vitro free radical scavenging activity of AuNPs and AgNPs using ABTS assay with different concentrations such as 2.5, 5, 7.5, 10 and 12.5 mg. ABTS scavenging percentage was increased up to 97.23% and 84.71% when treated with 12.5 mg of AuNPs and AgNPs, respectively, with an IC$_{50}$ value of 3.4 mg for AuNPs and 7.12 mg for AgNPs.

4.3.2. Anticancer activity of mycosynthesized silver and gold nanoparticles

4.3.2.1. Cytotoxicity analysis of mycosynthesized AgNPs and AuNPs by MTT assay

The cytotoxic effect of silver nitrate, gold chloride, AgNPs, AuNPs and mycelial free extract of A. terreus against normal cells (Vero cell lines) was performed using MTT assay to determine the biocompatibility. The figures 4.3a and b depict the percentage of cell viability at different concentrations of silver nitrate, gold chloride, AgNPs, AuNPs and mycelial free extract against Vero cells. Among them, the mycelial free extract showed maximum IC$_{50}$ value of 146.9 µg/mL followed by AuNPs, AgNPs, gold chloride, silver nitrate and doxorubicin with an IC$_{50}$ value of 85.83, 31.23, 21.71, 17.98 and 8.36 µg/mL, respectively. The results clearly indicate that the toxicity of silver nitrate and gold chloride showed a greater reduction in the cell viability percentage of normal cells at the lower concentration; in addition, the standard drug, doxorubicin showed lesser IC$_{50}$ value against normal Vero cells. Among the nanoparticles tested, AuNPs showed greater biocompatibility when compared to AgNPs, which showed greater inhibition of control cells (Vero). However, the mycelial free extracts showed very less inhibitory activity.

Figure 4.4 showed the morphological changes occurred in Vero cells during the treatment of silver nitrate, gold chloride, mycelial free extract, doxorubicin, AgNPs and AuNPs along with the untreated (control) Vero cells. These images clearly showed the toxic effects of various test samples with their respective IC$_{50}$ values.
4.3.2.2. Cell toxicity analysis of mycosynthesized AgNPs and AuNPs against cancerous cell lines

Figures 4.5a-d summarize the percentages of cell viability at different concentrations (1 - 100 μg/mL) of AgNPs, AuNPs, doxorubicin and mycelial free extract of A. terreus against six different cancer cell lines namely, HeLa, MCF-7, HepG2, A549, A375 and HCT15 at 24 h (Figs. 4.6a-f). AgNPs and AuNPs showed significant antiproliferative activity against all the tested cell lines, with an IC<sub>50</sub> value ranging from 2 to 36 g/mL. In case of AgNPs, the maximum inhibitory activity was found when treated against MCF-7 cells with an IC<sub>50</sub> value of 2.90 g/mL. Similarly, for AuNPs, the greater antiproliferative activity was observed with an IC<sub>50</sub> of 25.79 g/mL against HepG2 cells. In addition, AgNPs also showed good antiproliferative activity against other tested cell lines with an IC<sub>50</sub> value of 5.57, 6.64, 5.18, 5.44 and 5.68 μg/mL for HeLa, HepG2, A549, A375 and HCT15, respectively. Interestingly, the mycosynthesized AuNPs also exhibited significant dose-dependent cytotoxicity against other tested cancer cell lines, showing an IC<sub>50</sub> value of 32.81, 31.44, 35.94, 30.91 and 30.24 μg/mL for HeLa, MCF-7, A549, A375 and HCT15, respectively. Doxorubicin was used as a standard anticancer drug. The IC<sub>50</sub> value of doxorubicin against the tested cell lines was found to be 2.72, 1.91, 2.92, 2.73, 1.92 and 3.41 μg/mL for HeLa, MCF-7, HepG2, A549, A375 and HCT15, respectively. In addition, the mycelial free extract was also tested for cytotoxic activity and the maximum inhibitory activity was found against HCT15 cell with an IC<sub>50</sub> value of 72.36 μg/mL followed by A549 cells with IC<sub>50</sub> of 84.86 μg/mL.

In vitro cytotoxicity of mycosynthesized silver nanoparticles (50, 250, 5000 and 1000 μg/mL) was investigated by Syed et al. (2013) against mouse embryonic fibroblast cell line (NIH3T3) and human breast carcinoma cell line (MDA-MB-231). The maximum reduction of cell viability percentage was found to be 20.83% in case of NIH3T3 and 42.18% for MDA-MB-231 cell lines when treated with 1000 μg/mL of AgNPs.

Park et al. (2011) have compared the effects of silver nanoparticles of different sizes (20, 80 and 113 nm) using in vitro cytotoxicity against RAW264.7 (murine peritoneal macrophage cell line), D3 (murine embryonic stem cell line) and L929 (mouse fibroblasts). They also concluded that for all the toxicity endpoints, AgNPs of 20 nm size were more toxic than larger nanoparticles especially in L929 fibroblasts but not in RAW
264.7 macrophages. Similarly, Manivasagan and co-workers (2013) have studied the cytotoxic property of biologically synthesized silver nanoparticles against human cervical cancer cell line (HeLa) by dose response activity. The IC$_{50}$ value was 200 µg/ mL, comparatively higher than the present results.

The cell viability assay is one of the most important tools used for toxicology analysis of various cells and helps to study the cellular response of different toxic materials and also provides information related to cell death and metabolic activities (Asha Rani et al., 2009). Piao et al. (2011) have observed the dose dependent cytotoxicity of AgNPs and AgNO$_3$ against human chang liver cells and reported that AgNPs had higher toxicity compared to AgNO$_3$ which is contrary to our results.

Gurunathan et al. (2013) have treated different concentrations (0–25 g/ mL) of AgNPs against MDA-MB-231 cells for 24 h and found that AgNPs had reduced the cell viability at concentration more than 10 g/ mL. This is in good agreement with our present results. Few other researchers have also reported the cytotoxicity of AgNPs against various cancer cell lines such as human IMR-90, U251 cells, HeLa cells and MDA-MB-231 cells (Asha Rani et al., 2009; Oves et al., 2013). Kanipandian et al. (2014) have investigated the cytotoxic effect of silver nanoparticles against A549 cells and the IC$_{50}$ value was 30 µg/ mL. They also analyzed the toxicity level of AgNPs against normal cell line HBL 100, which exhibited significant toxicity at 60 µg/ mL, similar to our findings. On the contrary, Ahamed et al. (2011) have studied the exposure of AgNPs against A549 cells at different concentrations and reported less toxicity with a cell viability percentage of 92% and 90% at 24 h and 48 h, respectively, at the test concentration of 50 g/ mL.

Similar to our study, Suman et al. (2013) have also demonstrated the cytotoxicity of silver nanoparticles against HeLa cell lines. During their investigation, they found that LC$_{50}$ value at different concentrations showed the cell inhibition of 60.6%; further, 100% cell death of HeLa cells was observed when treated with 100 µg/ mL of AgNPs displaying their potential ability against cancer cells. The cytotoxic ability may be due to the formation of reactive oxygen species or due to the increased intracellular oxidative stress including apoptosis and necrosis process (Nel et al., 2006).

Vivek et al. (2012) have also demonstrated the antiproliferative activity of biologically synthesized AgNPs against MCF-7 and HBL 100 cells at different
concentrations ranging from 10 to 100 µg/ mL. Cell death of fifty percentage (IC$_{50}$) was observed against MCF-7 cells when AgNPs were maintained at 50 g/ mL for 24 h and 30 g/ mL for 48 h. Similarly, for normal HBL 100 cells, the IC$_{50}$ value was recorded with 80 g/ mL at 24 h and 60 g/ mL at 48 h. Silver nanoparticles have also revealed to have important antiangiogenic properties against various cancer cells (Safaepour et al., 2009; Foldbjerg et al., 2009; Franco-Molina et al., 2010).

Mishra et al. (2011) have synthesized biogenic gold nanoparticles using *Penicillium brevicompactum* and analyzed their cytotoxic effects on C$_2$C$_{12}$ cells (mouse mayo blast cancer cells) at different concentrations. Interestingly, gold nanoparticles showed a killing rate of 21.42%, 41.92% and 69.21% at 200, 1,000 and 2,000 ng/ mL, respectively, at 24 h. The authors have concluded that the survival ability of cancer cells was found to be decreased with increasing incubation time as well as the concentration of nanoparticles.

Pan et al. (2007) have investigated the cytotoxicity of gold nanoparticles against SK-Mel-28 (human melanoma), HeLa, L929 (mouse fibroblasts) and J774A1 cells (mouse macrophages). Gold nanoparticles were found to be more sensitive to connective tissue fibroblasts, epithelial cells, macrophages and melanoma cells, showing an IC$_{50}$ value ranging between 30 to 56 M. Interestingly, Sathishkumar and his co-workers (2015) have compared the cytotoxic activity of chemically synthesized AuNPs with that of biologically synthesized nanoparticles against human epithelial lung cells (A549). The cell viability percentage was found to be 65.7% and 72.3% when cells exposed to chemically synthesized AuNPs using sodium citrate and sodium borohydride, respectively, at the maximum dosage of 200 nM compared with the biologically synthesized AuNPs, which showed 80.2% of cell viability.

Recently, Chuang et al. (2013) have observed that the size of the nanoparticles plays a major role in cellular uptake and intracellular distribution that leads to improved cytotoxicity against cell lines such as AGS (human gastric adenocarcinoma cells), A549, NIH3T3 (mouse embryonic fibroblast), PK-15 (porcine kidney), MRC5 (human normal lung tissue) and Vero cells. Some researchers have also investigated the effect of gold nanoparticles against various cell types and suggested the involvement of clathrin-mediated endocytosis and phagocytosis due to gold nanoparticle internalization (Johnston et al., 2010; Mironava et al., 2010; Rejman et al., 2004).
The cytotoxicity of gold nanoparticles has been well studied and researchers have concluded that the antiproliferative activity of AuNPs against target cells was depended much on several factors such as surface charge, coating and size of the nanoparticles. Among them, the particle size of gold nanoparticles has been emerged as an important factor that has a major impact on cytotoxic property (Sonavane et al., 2008; Pan et al., 2007; Connor et al., 2005).

4.3.2.3. Acridine orange-ethidium bromide (AO-EB) staining

The apoptotic property of AgNPs and AuNPs against MCF-7 and HepG2 cells, respectively, was assessed using acridine orange and ethidium bromide dual staining. The MCF-7 and HepG2 cells were exposed to AgNPs and AuNPs nanoparticles, respectively, at 24 h as shown in Figs. 4.7a and b. The AgNPs treated MCF-7 cells showed the early apoptotic cells with a characteristic appearance of bright green dot of nuclei due to nuclear fragmentation and chromatin condensation. The orange-colored cells indicated the late apoptotic cells due to the condensation of nuclei; nuclear shrinkage and blebbing were also observed in AgNPs treated MCF-7 cells, thus confirming their apoptotic activity. These results correlate with the earlier findings (Pillai et al., 2014; Nair K et al., 2011; Cui et al., 2006). Similarly, AuNPs treated HepG2 cells also showed early apoptotic features with brightly fluorescent green colored cells and appearance of orange-colored cells due to the late apoptotic process. Presence of necrotic cells with red colored fluorescence was also observed in AgNPs and AuNPs treated MCF-7 and HepG2 cells, respectively. On comparison, the untreated (control) cells showed green color when stained with AO-EB, indicating their viability nature.

4.3.2.4. Nuclear staining of mycosynthesized AgNPs and AuNPs with DAPI fluorescent dye

The DAPI staining of AgNPs and AuNPs treated MCF-7 and HepG2 cells, respectively, was shown in Figs. 4.8a and b. The DAPI staining is a popular nuclear staining method and is used to confirm the intracellular localization of nanoparticles. The DAPI stained cells showed bright and strong blue color fluorescence after binding with DNA (Mathew et al., 2012; Shao et al., 2011).

The apoptotic nuclei with bright blue color fluorescence were observed in both AgNPs and AuNPs treated MCF-7 and HepG2 cells, respectively, due to either the
condensed or fragmented chromatin. On the other hand, the untreated MCF-7 and HepG2 cells showed normal smooth nuclei when stained with DAPI. The above staining technique clearly revealed the nuclear morphology of nanoparticles treated cells with characteristic features of apoptosis such as condensation of chromatin and nuclear fragmentation. Similar findings were also reported by Vivek et al. (2012), Zhang et al. (2012) and Pujalté et al. (2011).

Interestingly, earlier studies have discussed the nuclear fragmentation of AuNPs treated tumor cells and clearly indicated the important role of DAPI staining to study the photodynamic effect at the nuclear level (Guo et al., 2010; Kang et al., 2010; Dhas et al., 2014).

4.3.2.5. Cell cycle analysis of AgNPs and AuNPs treated cancerous cell lines

In order to determine whether the apoptosis was induced in nanoparticles treated cancer cells was accompanied by alterations in the cell cycle; the distribution index of the treated cells was analyzed by flow cytometer with PI staining. In this study, cell cycle analyses of mycosynthesized AgNPs and AuNPs treated MCF-7 and HepG2 cells, respectively, were performed by treating the cancer cells with their IC₅₀ dosage for 24 h and analyzed using flow cytometry. The AgNPs treated MCF-7 cells resulted in the increased number of cells at Sub G₀-G₁ phase (40%) whereas the control cells showed lesser number of cells (0.33%). Moreover, a decrease in cell number (55%) was observed at G₀-G₁ phase in AgNPs treated MCF-7 cells compared to the control cells (69%), suggesting the induction of apoptosis in MCF-7 cancer cells (Figs. 4.9a and b). Interestingly, AuNPs treated HepG2 cells with IC₅₀ also showed similar trend in the cycle analysis, showing an increased amount of cells in Sub G₀-G₁ population (27.76%) compared to the untreated (control) cells showing 4.58%. In addition, the G₀-G₁ phase also showed a decreased number of cells in AuNPs treated HepG2 cells with 21% compared to the untreated cells (80%), thus confirming the apoptotic induction of AuNPs (Figs. 4.10a and b).

The present findings highly correlate with the investigation of Choudhury et al. (2013), who have confirmed the induction of apoptosis by cell cycle arrest at G₀/G₁ phase during the treatment of functionalized AuNPs against A549 cells. Recently, Huang et al. (2014) have also examined the anticancer activity of PEG-AuNPs against human K562 (chronic myeloid leukemia) cells. PEG-AuNPs exhibited good cytotoxicity against
K562 cells in cell viability assay and showed a marked increase at sub G1 population in cell cycle analysis, thus revealing their apoptotic activity against leukemia cells. Similar reports were also shown by various researchers who have investigated the apoptotic induction of AgNPs and AuNPs using cell cycle analysis against different cancer cell lines (Gajendran et al., 2014; AshaRani et al., 2009; Rejeeth et al., 2014; Roy et al., 2012).

4.4. Summary

1. The mycosynthesized AgNPs and AuNPs were evaluated for their potent antioxidant activity.

2. In the DPPH free radical scavenging assay, the mycosynthesized AgNPs and AuNPs showed good antioxidant activity with an IC$_{50}$ value of 28.23 and 44.20 µg/ mL, respectively.

3. Similarly, in the ABTS scavenging assay, the mycosynthesized AgNPs and AuNPs showed significant antioxidant activity with an IC$_{50}$ value of 32.46 and 44.81 µg/ mL, respectively.

4. The mycosynthesized AgNPs and AuNPs were investigated for their potential anticancer activity against different cancer cell lines such as HeLa, MCF-7, HepG2, A549, A375 and HCT15.

5. Among different cancer cell lines tested, the mycosynthesized AgNPs showed excellent anticancer activity against MCF-7 cancer cells with an IC$_{50}$ value of 2.90 µg/ mL. In case of AuNPs, the maximum activity was observed in HepG2 cell lines with an IC$_{50}$ of 25.79 µg/ mL.

6. The AgNPs and AuNPs treated MCF-7 and HepG2 cancer cells, respectively, showed nuclear condensation, membrane blebbing and apoptotic bodies in acridine orange-ethidium bromide (AO-EB) dual staining.

7. The DAPI staining also showed apoptotic nuclei with bright blue color fluorescence in AgNPs and AuNPs treated MCF-7 and HepG2 cells, respectively.
8. Interestingly, AgNPs treated MCF-7 cells recorded increased number of cells at Sub G0-G1 phase (40%) while a decrease in cell number (55%) was observed at G0-G1 phase, suggesting the induction of apoptosis.

9. Similarly, AuNPs treated HepG2 cells also showed similar trend in the cycle analysis, showing an increased amount of cells at Sub G0-G1 population (27.76%). A decrease in cell number (21%) was observed at G0-G1 phase, thus confirming the apoptotic induction of AuNPs.
4.5. References


Fig. 4.1a: *In vitro* antioxidant activity of mycosynthesized AgNPs and AuNPs using DPPH free radical scavenging assay

*In vitro* antioxidant analysis using DPPH free radical scavenging method. The values represent mean ± standard deviation of three independent experiments.

![Graph showing percentage inhibition vs concentration for mycelial extract, AgNPs, and AuNPs](image)

Fig. 4.1b: *In vitro* antioxidant activity of standard antioxidant (Gallic acid) using DPPH free radical scavenging assay

*In vitro* antioxidant analysis using DPPH free radical scavenging method. The values represent mean ± standard deviation of three independent experiments.

![Graph showing percentage inhibition vs concentration for Gallic acid](image)
Fig. 4.2a: *In vitro* antioxidant activity of mycosynthesized AgNPs and AuNPs using ABTS scavenging assay

In *vitro* antioxidant analysis using ABTS scavenging method. The values represent mean ± standard deviation of three independent experiments.

Fig. 4.2b: *In vitro* antioxidant activity of standard antioxidant (Gallic acid) using ABTS scavenging assay

In *vitro* antioxidant analysis using ABTS scavenging method. The values represent mean ± standard deviation of three independent experiments.
Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against Vero (African monkey kidney cell) cell lines; the cell lines were treated with Mycelial free extract, Silver nitrate, Gold chloride, AgNPs and AuNPs for 24 h and the cell viability was determined by MTT assay. Data represent mean ± standard deviation of three independent experiments.

Cytotoxic effects of Doxorubicin against Vero (African monkey kidney cell) cell lines; the cell lines were treated with Doxorubicin for 24 h and the cell viability was determined by MTT assay. Data represent mean ± standard deviation of three independent experiments.
Fig. 4.4: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against Vero cell line

Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against Vero (African monkey kidney cell) cell lines; the images are the representation of (i) untreated (control) cells; cells treated with IC$_{50}$ of (ii) Mycelial free extract, (iii) Doxorubicin, (iv) Silver nitrate, (v) Gold chloride, (vi) AgNPs and (vii) AuNPs at 24 h; the images were captured at a magnification of 200X.
Fig. 4.5a: *In vitro* cytotoxic activity of *A. terreus* mycelial extract against different cancer cell lines

Cytotoxic effects of *A. terreus* mycelial extract against different cancer cell lines. The values represent mean ± standard deviation of three independent experiments; the different colors of the bars represent the different cell lines (HeLa, MCF-7, HCT15, HepG2, A375, A549).

Fig. 4.5b: *In vitro* cytotoxic activity of *A. terreus* synthesized AgNPs against different cancer cell lines

Cytotoxic effects of *A. terreus* synthesized AgNPs against different cancer cell lines. The values represent mean ± standard deviation of three independent experiments; the different colors of the bars represent the different cell lines (HeLa, MCF-7, HCT15, HepG2, A375, A549).
Fig. 4.5c: *In vitro* cytotoxic activity of *A. terreus* synthesized AuNPs against different cancer cell lines

Cytotoxic effects of *A. terreus* synthesized AuNPs against different cancer cell lines. The values represent mean ± standard deviation of three independent experiments; the different colors of the bars represent the different cell lines (HeLa, MCF-7, HCT15, HepG2, A375, A549).

Fig. 4.5d: *In vitro* cytotoxic activity of Doxorubicin against different cancer cell lines

Cytotoxic effects of Doxorubicin against different cancer cell lines. The values represent mean ± standard deviation of three independent experiments; the different colors of the bars represent the different cell lines (HeLa, MCF-7, HCT15, HepG2, A375, A549).
Fig. 4.6a: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against HeLa cell line

Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against HeLa (cervical cancer cells) cell lines; the images are the representation of (i) untreated (control) cells; cells treated with IC$_{50}$ of (ii) AgNPs, (iii) AuNPs, (iv) Mycelial free extract and (v) Doxorubicin at 24 h; the images were captured at a magnification of 200X.
Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against MCF-7 (breast cancer cells) cell lines; the images are the representation of (i) untreated (control) cells; cells treated with IC\textsubscript{50} of (ii) AgNPs, (iii) AuNPs, (iv) Mycelial free extract and (v) Doxorubicin at 24 h; the images were captured at a magnification of 200X.
Fig. 4.6c: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against HepG2 cell line

Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against HepG2 (hepatocellular carcinoma) cell lines; the images are the representation of (i) untreated (control) cells; cells treated with IC$_{50}$ of (ii) AgNPs, (iii) AuNPs, (iv) Mycelial free extract and (v) Doxorubicin at 24 h; the images were captured at a magnification of 200X.
Fig. 4.6d: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against A479 cell line

Cytotoxic effects of *A. terreus* synthesized AgNPs and AuNPs against A479 (lung cancer cells) cell lines; the images are the representation of (i) untreated (control) cells; cells treated with IC$_{50}$ of (ii) AgNPs, (iii) AuNPs, (iv) Mycelial free extract and (v) Doxorubicin at 24 h; the images were captured at a magnification of 200X.
Fig. 4.6e: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against A375 cell line

In *vitro* cytotoxic analysis of mycosynthesized AgNPs and AuNPs against A375 (skin cancer cells) cell lines. The images are the representation of A479 cells treated with IC$_{50}$ of AgNPs (ii); AuNPs (iii); Mycelial free extract (iv); Doxorubicin (v), for 24 h incubation time. The untreated control cells (i) are also shown; the images were captured at a magnification of 200X
Fig. 4.6f: *In vitro* cytotoxic activity of mycosynthesized AgNPs and AuNPs against HCT15 cell line

In *vitro* cytotoxic analysis of mycosynthesized AgNPs and AuNPs against HCT15 (colon cancer cells) cell lines. The images are the representation of A479 cells treated with IC_{50} of AgNPs (ii); AuNPs (iii); Mycelial free extract (iv); Doxorubicin (v), for 24 h incubation time. The untreated control cells (i) are also shown; the images were captured at a magnification of 200X.
Fig. 4.7a: Acridine orange-ethidium bromide dual staining of AgNPs treated MCF-7 cells

Morphological evidence of apoptosis by AO-EB dual staining. Fluorescent microscopic images of AgNPs treated MCF-7 cells showing apoptotic bodies and fragmented nuclei. Blue arrows indicate live cells; white arrows indicate early apoptotic cells; red arrows indicate late apoptotic cells; the images were captured at a magnification of 200X.
Fig. 4.7b: Acridine orange-ethidium bromide dual staining of AuNPs treated HepG2 cells

Morphological evidence of apoptosis by AO-EB dual staining. Fluorescent microscopic images of AuNPs treated HepG2 cells showing apoptotic bodies and fragmented nuclei. Blue arrows indicate live cells; white arrows indicate early apoptotic cells; red arrows indicate late apoptotic cells; the images were captured at a magnification of 200X.
Fig. 4.8a: Nuclear staining (DAPI fluorescent dye) of AgNPs treated MCF-7 cells

The image is the representation of AgNPs treated MCF-7 cells stained with DAPI and is captured at a magnification of 200X.

Fig. 4.8b: Nuclear staining (DAPI fluorescent dye) of AuNPs treated HepG2 cells

The image is the representation of AuNPs treated HepG2 cells stained with DAPI and is captured at a magnification of 200X.
Fig. 4.9: Cell cycle analysis of AgNPs treated MCF-7 cells

(a)

Untreated MCF-7 cells were stained with propidium iodide with RNAase and analyzed for cell cycle analysis.

(b)

AgNPs treated MCF-7 cells were stained with propidium iodide with RNAase and analyzed for cell cycle analysis.
Fig. 4.10: Cell cycle analysis of AuNPs treated HepG2 cells

(a) Untreated HepG2 cells were stained with propidium iodide with RNAase and analyzed for cell cycle analysis.

(b) AuNPs treated HepG2 cells were stained with propidium iodide with RNAase and analyzed for cell cycle analysis.