CODEN (USA): IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

Available online at: http://www.iajps.com Research Article

AN INVESTIGATION ON THE CYTOTOXICITY AND APOPTOTIC EFFECT OF BIOLOGICALLY SYNTHESIZED SILVER NANOPARTICLES ON MCF-7 AND A549 CELL LINES USING WEED *SETARIA VERTICILLATA* L.

A. Prabhu[1], K. Shankar*[2], P. Muthukrishnan[3], A. Kathiresan[4] & P.Prakash[5]

[1] Research scholar [1,2,3,4]Department of Chemistry, Karpagam Academy of Higher Education, Coimbatore -641021, Tamilnadu, India.

[5]PG & Research Department of Chemistry, Thiagarajar College, Madurai, Tamilnadu, India.

Abstract:

Novel utilization of weed synthesized nanoparticles plays major role in treatment against many cancer cells and pathogenic microbes. Green synthesis is more economical, ecofriendly and large scale production of nanoparticles. This study investigates on cytotoxic and apoptotic effect of setaria verticillata synthesized silver nanoparticles against Breast cancer cell (MCF-7) and Lung cancer cell (A549) lines. The synthesized silver nanoparticles were characterized by High resolution-transmission electron microscopy (HR-TEM). The optical property of silver nanoparticles was determined by selected area electron diffraction (SAED). The invitro cytotoxicity and apoptotic effect of synthesized silver nanoparticles was evaluated against Breast cancer cells (MCF-7) and lung cancer cells (A549) by MTT assay, Trypan blue dye assay and DNA fragmentation assay. The size of silver nanoparticles was spherical with average size of 12±4 nm. Electron diffraction pattern shows the silver nanoparticles were crystalline in nature with Face centered cubic (FCC) structure. The MTT assay shows the % CTC_{50} is 897.05µg/ml for MCF-7 and 402.75 µg/ml for A549 cell lines respectively. Trypan blue dye assay indicates cytotoxic effects increased with increase in concentration of silver nanoparticles upto 1000 µg/ml. The DNA fragmentation assay of silver nanoparticles treated MCF-7 and A549 shows the double strand breaks shows formation of DNA ladders. The invitro cytotoxic effect of setaria verticillata synthesized silver nanoparticles was toxic against Breast cancer cell (MCF-7) and lung cancer cell (A549).

Keywords: *Silver nanoparticles, MCF-7, A549, HR- TEM, MTT assay.*

Corresponding author:

K.Shankar,

Karpagam Academy of Higher Education,
Coimbatore -641021,
Tamilnadu, India.
Email : drkshankar@gmail.com
Telephone : +919791802836

QR code

Please cite this article in press as Shankar et al, ***An Investigation on the Cytotoxicity and Apoptotic Effect of Biologically Synthesized Silver Nanoparticles on MCF-7 and A549 Cell Lines Using Weed Setaria Verticillata L****, Indo Am. J. Pharm. Sci, 2016; 3(1).*

INTRODUCTION:

Nanoparticles play the major role in chemistry, catalysis [1], medicine [2], biology [3] biotechnology, electronics [4], optics [5] and physics. Nanoparticles can be synthesized by using chemicals [6] and a plant extracts [7]. Phytochemicals present in plants acts as reducing and stabilizing agents [8] in the synthesis of nanoparticles. The plant-mediated synthesis of nanoparticles is one of the efficient and eco-friendly methods in the manufacture of silver nanoparticles. In recent years many plants such as *Canthium coromandelicum[9]* , *Ocimum sanctum[10]* , *gliricidia sepium* [11], *cinnamum camphora* [12], and *Calophyllum inophyllum* [13] are used in synthesis of nanoparticles. Silver is considered to be non-toxic and nonallergic to all living things. It is effective in the treatment of wound dressings [14], creams and antibiotic coating in medical devices. The antimicrobial property of silver is excellent against harmful microorganisms such as fungus, bacteria and viruses [15].

Cancer is one of deadliest diseases in the world. Cancer is the uncontrolled growth of abnormal cells in our body. It divides and spread to adjacent tissues and creates tumours. There are nearly hundreds of cancer types out of this lung and breast cancer is important one. Most of the cancer patients are affected by breast and lung cancer. Nowadays chemotherapeutic drugs are high-cost, high toxicity and many side effects. Bio synthesized nanoparticles are the very important drug for cancer and variety of diseases. Treatments like chemotherapy[16] and radiation therapy [17] kills both normal and cancer cells, even chemotherapeutic agents can be combined with nanomaterials for cancer therapy. Researchers around the world are working to find out the novel tools for cancer therapy. SERS-encoded magnetic silver nanoparticles was produced and tested for multifunctional tags for cancer cell target[18]. In recent years, many new anticancer drugs were identified and tested against different cancer cell lines. The silver nanoparticles have best cytotoxic effect against many cancer cells. *Setaria verticillata* is one of the most important weed plant grown around the world. It consists of phytochemicals such as alkaloid, phenol and tannin [19]. Corrosion studies are carried out by using *setaria verticillata* as a corrosion inhibitor[19] (Muthukrishnan et al., 2013). In our present study, we reported the characterization of silver nanoparticles by HR-TEM (High-Resolution Transmission Electron Microscope) and SAED (Selected Area Electron Diffraction).The invitro cytotoxic effect of *setaria verticillata* synthesized silver nanoparticles was tested against MCF-7 (breast cancer cell line) and A549 (lung cancer cell line) by MTT assay, Trypan blue dye assay and DNA fragmentation assay.

MATERIALS AND METHODS:

Synthesis of *Setaria Verticillata* Mediated Silver Nanoparticles [20]

The *setaria verticillata* plants were collected from agricultural land in Coimbatore, Tamilnadu, India. Analytical grade silver nitrate was purchased from Sigma-Aldrich Chemicals, India. The silver nanoparticles were prepared by mixing 50 ml of *Setaria verticillata* extract with 250 ml of 1mM aqueous $AgNO_3$ solution under constant stirring. The mixture of the solution was stirred at a temperature of 100 ^oC for 8 hours and then, the product formed is filtered and stored in a container for further analysis.

Characterization of Silver Nanoparticles by Electron Microscopy

The synthesized nanoparticles were characterized by High-Resolution Transmission Electron Microscope (HR-TEM) and selected area electron diffraction (SAED) by JEOL JEM 2100 High-Resolution Transmission Electron Microscope (HR-TEM). This microscope enables to view lattice resolution of 0.14 nm and point-to-point resolution of 0.19 nm at 200 kV acceleration voltages. HR-TEM is equipped with Gatan Orious CCD camera (2K x 2K) for image recording. SAED analysis was carried out during HR-TEM analysis. A drop of an aqueous solution of silver nanoparticles was placed on the carbon-coated copper grid and allowed to dry at ambient temperature for 12 hours and then analyzed. The size of silver nanoparticles in HR-TEM image was measured by using the imageJ software. The FT-IR analysis, UV-visible spectroscopy, scanning electron microscopy (SEM) and EDAX spectrum were reported earlier [20](Prabhu et al., 2015).

In Vitro Cytotoxicity Activity

Cell line and Conditions

MCF-7 (Human, Breast cancer) and A549 (Human lung cancer) cell cultures were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma-Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell Viability by Trypan Blue Dye Assay

Trypan blue dye assay was carried out to evaluate the viability of *S.Verticillata* mediated silver nanoparticles against MCF-7 and A549 cells. The trypan blue exclusion assay is a rapid to screen the efficiency of nanoparticles [21] Both the cells (1.0 x 10^5 cells/ml) were grown in complete medium, these cells were treated with *S.Verticillata* mediated silver nanoparticles at concentrations of 32.5, 125, 250, 500 and 1000 µg/ml in PBS. The cell cultures were incubated for 24 h. After incubation, the cell cultures were treated with 0.4 % trypan blue dye. The dead cells were stained and living cells were translucent. The cells were quantitatively measured by using hemocytometer.

Cell Viability by MTT Assay

The colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water-soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble purple coloured formazan product which is measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the cells. The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using a medium containing 10% FBS and were used for the determination of cell viability by MTT assays as described by Francis and Rita (1986) respectively. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

DNA Ladder Assay

DNA fragmentation was measured by DNA ladder assay. It was used to detect and differentiate apoptosis from necrosis [22]. The treated cells were harvested by centrifugation for 2,500 rpm for 5 min and cell pellets were washed with PBS and lysed. The lysates were finally treated with 10 µl of 50 mg/ml RNase A and incubated for 2 h at 56 °C. Then 10 µl of 25 mg/ml Proteinase K (final 2.5 µg/µl) was added and incubated for 2 h at 37 °C. It is mixed with 1/2 vol. (65 µl) of 10 M ammonium acetate and 2.5 vol. (500 µl) of ice-cold ethanol thoroughly and stand for 1 h in a freezer at 80 °C. After precipitation pellets were centrifuged for 20 min at 12,000 rpm, then pellets were washed with 200 µl 80% ice-cold ethanol and air-dry for 10 min at room temperature. The pellets were dissolved with 50 µl of TE buffer. DNA electrophoresis was performed in 2% agarose gel electrophoresis of the same concentration of DNA (about 4 µg) staining by 1 µg/mL of ethidium bromide at 70 V and DNA fragments were visualised by exposing to UV light radiation.

RESULTS AND DISCUSSION:

Characterization by Electron Microscopy

The Electron microscopy represents the morphology of silver nanoparticles. In this study HR-TEM of silver nanoparticles appears to be polydispersed and spherical in shape [20]. There are many ultra fine nanoparticles less than 5 nm are present as shown in HR-TEM images (Fig. 1) and histogram (Fig. 2)[23]. The average grain size of silver nanoparticles is 12±4 nm. The grain size of nanoparticles depends upon physiochemical parameters such as pH, temperature and plant extract and concentration of silver nitrate solution. In this study evaluation of seteria verticillata mediated silver nanoparticles against MCF-7 and A549 cell lines. The average size of synthesized silver nanoparticles was found to 12±4 nm. The variation in the size of nanoparticles was determined by using DLS, TEM and BET. In this study, we preferred TEM to calculate the variation in size of nanoparticles. The effectiveness of nanoparticles depends on several factors such as dose, time and size of nanoparticles. Previous reports evidence nanosized particles are effective against cancer cells than larger ones. The optical property of nanoparticles were determined by SAED (Fig. 3), The bright spots of selected area electron diffraction (SAED) pattern shows the silver nanoparticles are crystalline in nature. The bright concentric circles indexed at (111), (200), (220) and (311) planes d-spacing was estimated to be 0.22 nm correlates with the orientation of Face centered cubic structure similar to metallic silver (JCPDS card no.04-00783)[24].

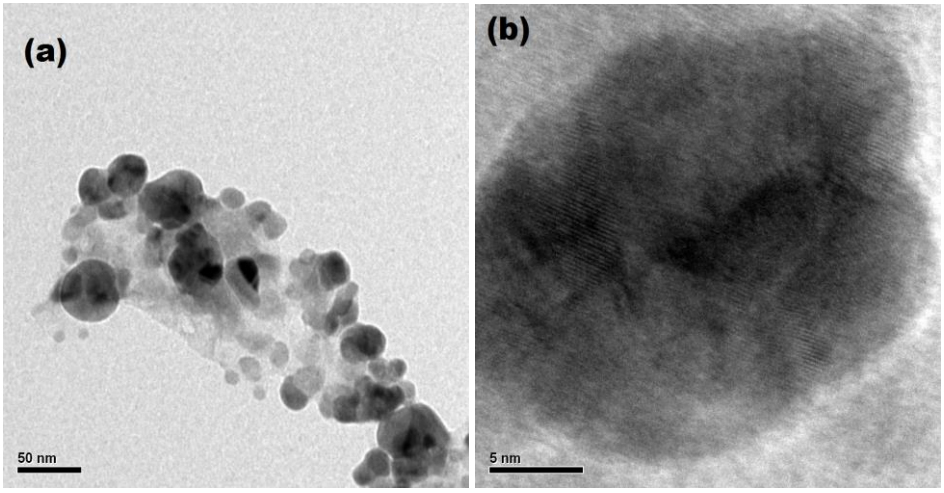

Fig. 1: HR-TEM image of *S.Verticillata* Mediated Silver Nanoparticles at (a) 50 nm and (b) 5 nm

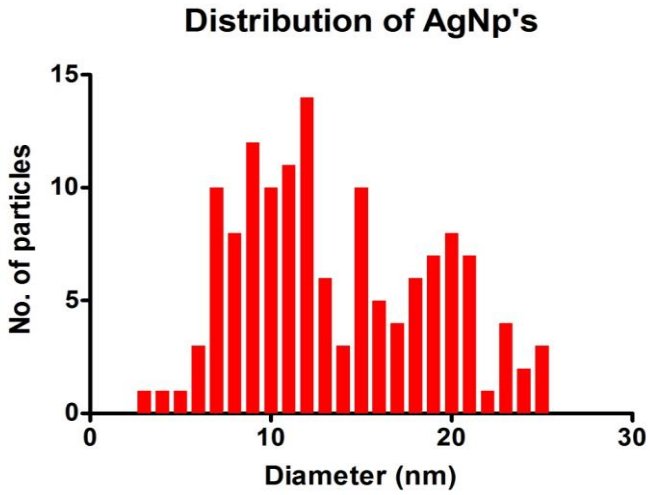

Fig. 2: Size Distribution of *S.Verticillata* Mediated Silver Nanoparticles

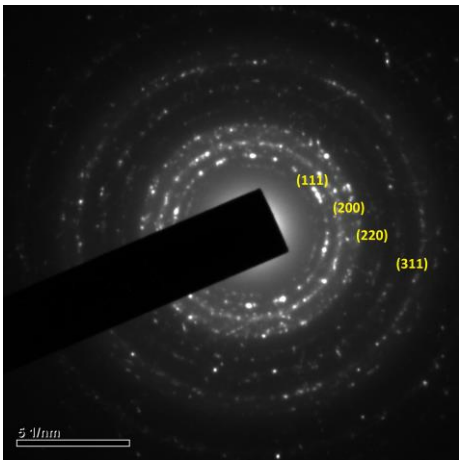

Fig. 3: SAED Pattern of *S.Verticillata* Mediated Silver Nanoparticles

Trypan Blue Dye Assay

In this study, the synthesized silver nanoparticles were tested for cytotoxic effects against MCF-7 and A549 cell lines. The cytotoxic effect increases with increase in the concentration of silver nanoparticles upto 1000 µg/ml (Fig. 4). When the concentration was increased from (32.5, 125, 250, 500 and 1000 µg/ml) cytotoxic effect also increases as it depends only on the dosage of silver nanoparticles [25] (Fig. 2) shows the cytotoxic effect of silver nanoparticles by trypan blue dye assay.

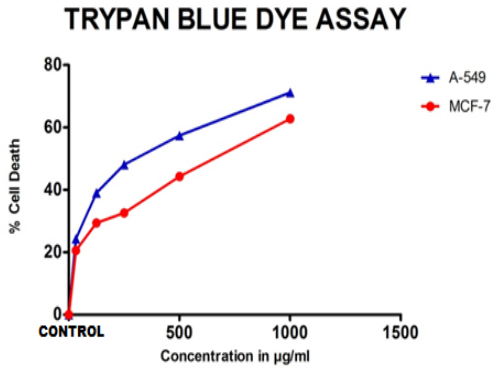

Fig. 4: *In Vitro* Cytotoxicity of *S.Verticillata* Mediated Silver Nanoparticles against MCF-7 Cell Line and A549 Cell Line by Trypan Blue Dye Assay

MTT Assay

In vitro anti cancer activity of nanoparticles was analysed against MCF-7 (Human, Breast cancer) and A- 549 (Human lung cancer cell line) cell cultures by MTT assay. In earlier studies cellular intake of nanoparticles leads to reactive oxygen species (ROS) creates oxidative stress in the cell [26]. Silver nanoparticles show the cytotoxic effect against MCF-7 and A549 cells. A reduction in mitochondrial function of A549 cells exposed to silver nanoparticles was observed in a dose-dependent manner [27]. The graph (Fig. 5) shows % of cell death against the concentration of silver nanoparticles. Fig. 6 shows the effect of silver nanoparticles against MCF-7 cell lines at different concentrations. The MTT assay shows the CTC_{50} values are 897.05µg/ml for Breast cancer cell (MCF-7) and 402.75 µg/ml for lung cancer cell (A549) respectively. An MTT assay result reveals that the silver nanoparticles have better anti cancer potentials against MCF-7 and A549.Further the results confirm that low concentration of *setaria verticillata* synthesized silver nanoparticles shows the high percentage of cell death against A549 cells.

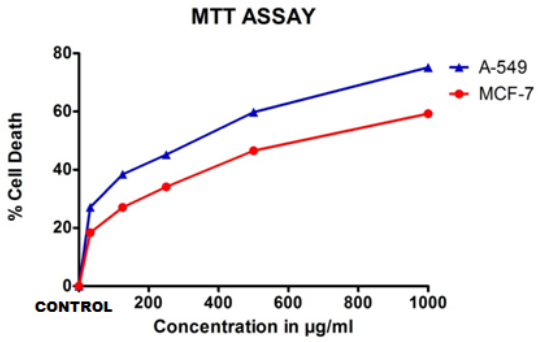

Fig. 5: *In Vitro* Cytotoxicity of *S.Verticillata* Mediated Silver Nanoparticles against MCF-7 Cell Line and A549 Cell Line by MTT Assay

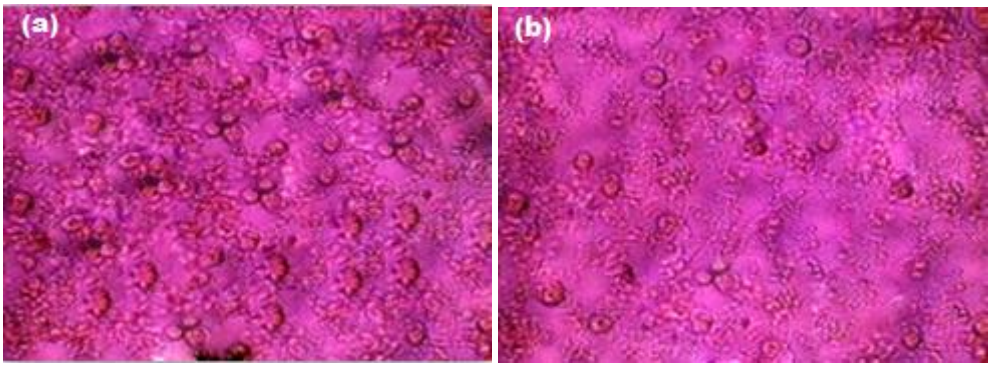

Fig 6: *In Vitro* Cytotoxicity of *S.Verticillata* Mediated Silver Nanoparticles against MCF-7 Cell Line
a) 32.5 µg/mL b) 1000 µg/mL

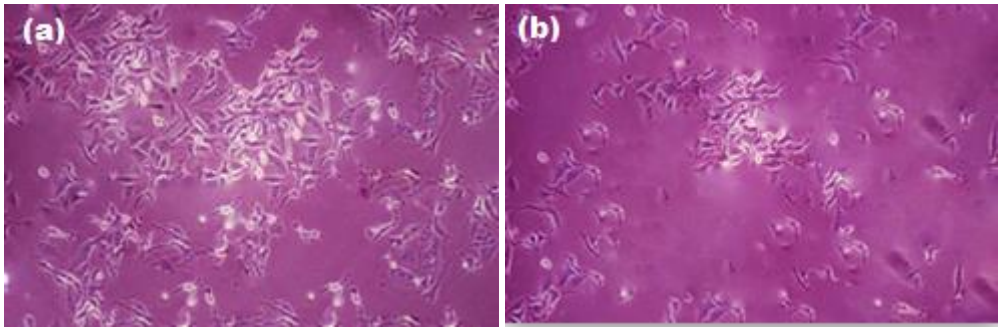

Fig. 7: *In Vitro* Cytotoxicity of *S.Verticillata* Mediated Silver Nanoparticles against A549 Cell Line a) 32.5 µg/mL b) 1000 µg/mL

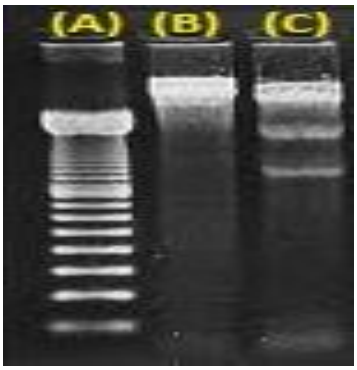

Fig 8: DNA Fragmentation Assay (A) Marker (B) MCF-7 Cells Treated with *S.Verticillata* Mediated Silver Nanoparticles (C) A549 Cells Treated with *S.Verticillata* Mediated Silver Nanoparticles

DNA Ladder Assay

silver nanoparticles with MCF-7 and A549 cells shows double strand breaks and formation of DNA ladders on agarose gel represents the characteristics of apoptosis. In this study silver nanoparticles treated with MCF-7 and A549 cells yields a continuous spectrum of DNA (Fig. 8). The 1 kb ladder was used to find the molecular weight of cleaved DNA fragments. Inorganic nanoparticles interact directly with DNA and enzymes in the cell [28]. Seteria verticillata mediated silver nanoparticles induce cell damage by ROS leads to cell membrane damage and apoptosis.

CONCLUSION:

The silver nanoparticles were synthesized by economical and eco-friendly method. The HR-TEM images show that the average size of the particle is 12±4 nm. Further, SAED pattern reveals the crystalline nature and face centered cubic structure of silver nanoparticles. The synthesized *setaria verticillata* mediated silver nanoparticle was effective against Breast cancer cell (MCF-7) and lung cancer cell (A549). The CTC_{50} value of lung cancer cell (A549) is less when compared to Breast cancer cell (MCF-7). The silver nanoparticles were the best therapeutic agent against cancer cells. The *setaria verticillata* synthesized silver nanoparticles can be used in many bio medical applications.

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