



CYTOTOXIC, APOPTOTIC AND ANTI-ANGIOGENIC EFFICACY OF SILVER NANOPARTICLES, SYNTHESIZED USING TANNIC ACID AS REDUCING AGENT UNDER CONTROLLED TEMPERATURE.

SUDHIR YADAV, HEMRAJ SEER, RAYASA RAMACHANDRA MURTHY*

I.S.F. College of Pharmacy, Firozpur G.T. Road, Moga, Punjab, India.

Abstract

Accepted Date:

26/05/2012

Publish Date:

27/10/2012

Keywords

Silver nanoparticles

Anticancer activity

Apoptosis

Anti-angiogenesis,

DNA Fragmentation assay

Corresponding Author

Mr. Rayasa Ramachandra Murthy

I.S.F. College of

Pharmacy, Firozpur G.T.

Road, Moga, Punjab,

India.

Silver nanoparticles has been previously used as antimicrobial and antiviral agent .The present study reveal the cytotoxic, apoptotic and anti-angiogenic efficacy of temperature dependent synthesized silver nanoparticles by using tannic acid as reducing agent. MCF-7 cancerous [breast cancer cell] were treated with silver nanoparticles (ranged from 100nm to 800nm/ml) for different time period at 37°C and 5% CO₂ atmosphere. Cell Viability was evaluated by MTT assay and the mechanism of cell death [Apoptosis] through Ethidium bromide/Acridine orange and DNA laddering assay. Anti-angiogenic properties were determined by cell migration assay. Silver nanoparticles showed dose-dependent cytotoxic, apoptotic and anti-angiogenic effect in MCF-7 breast cancer cells at an IC₅₀ of 400nM/ml. The present study showed that silver nanoparticles may be an alternative chemotherapeutic agent for treatment of cancer.

INTRODUCTION

Nanobiotechnology, an emerging field of nanoscience, utilizes nanobased-systems for various biomedical applications. This rapidly developing field of nanoscience has raised the possibility of using therapeutic nanoparticles in the diagnosis and treatment of human cancers¹. Nanoscale particles and molecules are a potential alternative for treatment of disease because they have unique biologic effects based on their structure and size, which differ from traditional small-molecule drugs². In the last few years, FDA has approved several pharmaceutical companies for the development of nanotechnology-based drugs. The global market for medical nanotechnology is expected to reach more than \$3 billion within the next five years³.

Silver was known only as a metal until the recent advent of the nanotechnology era, when it became recognized that silver could be produced at the nanoscale using recent engineering technologies. These ultrafine particles, the sizes of which are measured in nanometers (nm) possess distinctive morphologies and characteristics^{4, 5}. Silver

nanoparticles (AgNPs) have also become a common component in clothing, food containers, wound dressings, ointments, and implant coatings^{6,7} and some have already received approval from the FDA⁸.

In the recent years, AgNPs are among the emerging nanoproducts that have gained increasing interest in the field of nanomedicine due to their unique properties and obvious therapeutic potential in treating a variety of diseases, including retinal neovascularisation^{9, 10} and acquired immunodeficiency syndrome due to human immunodeficiency virus (HIV)^{11, 12}. AgNPs are also known for their antimicrobial potential against several other viruses, including hepatitis B¹³ respiratory syncytial virus¹⁴ herpes simplex virus type^{1, 15} and monkey pox virus¹⁶.

AgNPs are synthesized by means of various physical, chemical, and biological methods. Even though the chemical methods involve a very simple procedure, they employ chemical reducing agents, such as citrate, borohydride, or other organic compounds^{17, 18} which pose problems of toxicity due to

residual contamination of these agents. Kemp et.al¹⁸ demonstrated a clean method of synthesizing gold and silver nanoparticles by reducing AuCl₄ and AgNO₃ using heparin and hyaluronan as both reducing and stabilizing agents. The particles show stability under physiological conditions and narrow size distributions. These nanoparticles not only exhibited biological functions but also avoided problems of contamination incorporated in typical synthesis of gold and silver nanoparticles reported earlier. In the present study, we investigated tannic acid as a possible reducing agent to prepare and stabilize silver nanoparticles and to offers better control over the shape and size of the AgNPs produced.

Silver nanoparticles inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in bovine retinal endothelial cells¹⁹. Similar studies have proven their inhibitory effect on vascular permeability induced by VEGF. Kalishwaralal, et al.(2009)¹⁹ demonstrated that silver nanoparticles are potent anti-angiogenic molecule that inhibit the cell-survival signal. Anti- proliferative activity of silver

nanoparticles has also been reported by several workers^{20,21}

The aim of present study is to investigate the possibility of synthesizing AgNPs by reduction method using tannic acid as the reducing agent and to study the efficacy of nanoparticles as potent anti-angiogenic, apoptogenic and anti-proliferative agent under in vitro conditions.

MATERIALS & METHODS

Materials

Silver Nitrate and tanic acid are of reagent grade procured from HiMedia, Mumbai. **Reagents:** Penicillin-streptomycin solution, trypsin-EDTA solution, RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM/F-12), Fetal bovine serum (FBS) were purchased from Himedia [India]. DNA laddering kit was purchased from Genex bio [India]. **Cell Culture:** MCF-7 human breast cancer cell line was purchased from National Center for Cell Sciences [NCCS, Pune India] and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution. Cells were

grown to confluence at 37°C, and 5% CO₂ atmosphere.

Methods

SYNTHESIS OF SILVER NANOPARTICLES:

Tannic acid solution (0.25 to 1.0 M) and silver nitrate solution (4 mM) was prepared in distilled water. Silver nitrate solution (5ml) was added drop wise into 20 ml tannic acid solution in a beaker and heated up to 90°C under continuous magnetic stirring until colour changes to pale yellow indicating the formation of silver nanoparticles. The conditions of preparation were optimized to obtain the nanoparticles of required size and shape by varying the molar ratio of silver nitrate to tannic acid during the synthesis. Silver nanoparticles synthesized using silver nitrate and tannic acid in 1:0.5 molar ratios was found optimum. The nanoparticles obtained were recovered by centrifugation and purified by repeated washing with water and centrifugation.

CHARACTERIZATION OF SILVER NANOPARTICLES:

Purified AgNPs was characterized for Morphology, Particle size, zeta potential and polydispersity index (PDI) and

concentration of nanoparticles by established methods reported previously²². Morphology was determined by Scanning Electron Micrograph (SEM), particle size (Photon Correlation Spectroscopy), Zeta potential and PDI using zeta sizer (Delsi Nano,C, Beckman Coulter).

DETERMINATION OF NANOPARTICLES CONCENTRATION:

Accurate determination of the size and concentration of nanoparticles is essential for the biomedical application of nanoparticles²³. The concentration of AgNPs to be administered at an nM level was determined by a method which has been previously reported²⁴. The calculation was as follows

Initially the average number of atoms per nanoparticles was calculated using the formula:

$$N = \frac{\pi \rho D^3}{6M} N_A$$

Where,

N = Number of atoms per nanoparticle,

π = 3.14,

ρ = Density of cubic silver = 10.5 g/cm³,

D = Average diameter of nanoparticles =
18.6 nm = 18.6×10^{-7} cm,

M = Atomic mass of silver = 107.868 g,

N_A = Number of atoms per mole
(Avogadro's number = 6.023×10^{23}).

From the above calculation, assuming 100% conversion of all silver ions to silver nanoparticles, $N = 197780.89$.

Then, the molar concentration of the nanoparticles (C) was calculated using the formula:

$$C = \frac{N_T}{NVN_A}$$

Where,

C = molar concentration of the nanoparticles,

N_T = total number of silver atoms added as $\text{AgNO}_3 = 1 \text{ M}$,

N = number of atoms per nanoparticles (from above calculation),

V = volume of the reaction solution in L,

N_A = Avogadro's number (6.023×10^{23})

$$C = \frac{1 \times 6.023 \times 10^{23}}{197780.89 \times 1 \times 6.023 \times 10^{23}}$$

Hence, $C = 5.065 \times 10^{-6} \text{ M/L}$

The concentrations required for any experimentation were prepared thereafter from the above stock solution.

STABILITY OF SILVER NANOPARTICLES

In order to study the effect of the storage condition on the shape and size of Ag NPs, the samples were stored in amber colour bottles, covered with aluminium foil at ambient temperature. The samples withdrawn at periodic intervals were tested for absorbance, size and zeta potential.

IN VITRO ANTICANCER ACTIVITY OF SILVER NANOPARTICLES

In vitro anti-cancer activity of prepared silver nanoparticles was carried out on MCF-7 cell line (human breast carcinoma). The studies included morphological assay [using phase contrast microscopy], cell viability assay [using MTT assay], and anti-angiogenic property [using cell migration assay]. The mechanism of cell death was determined using Ethidium bromide/Acridine orange assay and DNA fragmentation assay.

CELL CULTURE AND MAINTENANCE

The cell lines MCF-7 cell line (breast carcinoma) was obtained from National Center for Cell Sciences, Pune, India. Cell

lines were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum. Cells were maintained in 5% CO₂ humidified incubator at 37°C. During subculture, cells were detached by trypsinization when they reached 80% confluency and split (1:4). Growth medium was changed every 3 days.

CELL MORPHOLOGY

Phase contrast microscopy

When cells reached 60% confluence silver nanoparticles were added to the cells at different concentrations. After 24 h incubation, cell morphology was assessed with Olympus inverted phase-contrast microscope.

CELL VIABILITY

MTT assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye reduction assay was performed to determine the cytotoxic effect of the AgNPs at various concentrations. The assay depends on the reduction of MTT by mitochondrial dehydrogenase, an enzyme present in the mitochondria of viable cells, to a blue formazan product. Briefly, the MCF-7 cells

were at a concentration 3×10^4 cells/mL plated onto 96-well flat bottom culture plates with various concentrations of AgNPs. All cultures were incubated for 24 hours at 37°C in a humidified incubator. After 24 hours of incubation (37°C, 5% CO₂ in a humid atmosphere), 5 µl of MTT (5 mg/mL in DMSO) was added to each well, and the plate was incubated for a further four hours at 37°C. The resulting formazan was dissolved in 200 µl dimethyl sulphoxide and absorbance of the solution was read at 595 nm using a ELISA plate reader (Biorad, Model 680, Japan). All determinations were carried out in triplicate. Concentrations of AgNPs showing 50% reduction in cell viability (ie, IC₅₀ values) were then calculated²⁵.

MECHANISM OF CELL DEATH [APOPTOSIS]:

Acridine Orange / Ethidium Bromide assay:

MCF-7 cells grown in 24 well plates (12000cells/well) were treated with and without formulation for 16 h. After washing once with PBS, the cells were stained with 100 µl of a mixture of acridine orange and ethidium bromide (1:1, 4 mg/ml) solutions. The cells were immediately washed once with PBS and viewed under a Olympus inverted fluorescent microscope. Acridine

Orange/Ethidium Bromide Staining uses combination of two dyes to visualize cells with aberrant chromatin organization. To achieve differentiation between viable and non-viable cells, a mixture of Acridine Orange and Ethidium Bromide was used. The differential uptake of these two dyes allows the identification of apoptotic and non apoptotic cells²⁶.

DNA Fragmentation assay:

DNA laddering experiments were initiated in 25 cm² flasks by seeding MCF-7 cells [1x10⁵] in 5 mL growth medium. After overnight growth, cells were treated with silver nanoparticles (400nM/mL) for 24 h. DNA was isolated using a commercially available kit (Genex bio, India). In brief, after incubating with silver nanoparticles, the cells were rinsed twice with PBS (0.1 M, pH 7.4). Cells were detached from substratum using 0.1% Trypsin Versene Glucose solution and recovered by centrifuged at 4000 rpm for 5 min. The cell pellet was washed with PBS (0.1 M, pH 7.4), resuspended in 1 mL of DNA extraction solution (Genex bio, India) and lysed the cells by repeated pipetting. Homogenate obtained was centrifuged at 8000 rpm for 10 min. Supernatant was transferred to a

fresh eppendorf tube and equal volume of 100% ethanol was added to precipitate DNA. Precipitated DNA was sediment by centrifugation at 8000 rpm for 5 minutes and washed twice with 1 mL of 95% ethanol. Final wash was given with 70% ethanol. The washed DNA pellet was dried completely and suspended in 50 µl TE buffer (10mM TRIS-HCl, 0.1mM EDTA, pH 7.4) supplemented with 0.1 µg/mL ribonuclease A for 1 h at 37°C. The DNA was subjected to electrophoresis on a 1.5% agarose gel (containing 3 µg/mL ethidium bromide in 1 X TAE buffer of pH 8.5) at 90 V for 1.5 h and visualized using a UV transilluminator. The sizes of DNA fragments were analyzed by comparing their mobility with 100 bp DNA ladder²⁷.

ANTI-ANGIOGENESIS

Cell migration assay

Cell migration assay was performed in 6 well plates. Each well was coated with 3ml of fibronectin solutions [10µg/ml] then incubate the dishes overnight at 4°C without shaking and rotation. Approximately 1x10⁵ MCF-7 cells were plated in previously coated 6 well plate and incubated at 37°C humidified 5% CO₂ incubator for 24hours to create a confluent monolayer, after that

wounds were created manually by scraping the cell monolayer with the help of a surgical blade and cell were washed with 1 ml of desired medium. Treatment was given with optimized dose [400nM] of silver nanoparticles in duplicate, then images were collected at 0 hr, 6hr and 12hr under inverted fluorescent microscope at 40x. The image analysis was determined by the number of cells migrate into wound area in comparisons to untreated¹⁹.

RESULTS AND DISCUSSION

CHARACTERIZATION OF SILVER NANOPARTICLES

Prior to the study of the anti-cancer effect of AgNPs, characterization of synthesized AgNPs was performed. UV-spectroscopy of silver nanoparticles showed characteristic peak at 409 nm that shows clear indication of formation silver nanoparticles. SEM showed that the nanoparticles were spherical [Figure 1].

STABILITY STUDIES OF SILVER NANOPARTICLES:

The UV-VIS spectra, particle size and zeta potential of the optimized formulation have been recorded during the time of this study

are shown in table 1. The changes observed in the absorption maxima, particle size and zeta potential with time is an indicative of the extent of aggregation of nanoparticles on storage.

IN VITRO ANTICANCER ACTIVITY OF SILVER NANOPARTICLES

CELL MORPHOLOGY

Phase contrast microscopy

Phase contrast microscopic images (200x) of MCF-7 cells treated with varied concentrations of silver nanoparticles compared with that of untreated control are shown in figure 2. MCF-7 cells (control) appeared polyhedral or stellate showing slender lamellar expansions that joined neighboring cells. With increasing concentration of silver nanoparticles (from 200nM to 800 nM/mL), cells were seen as less polyhedral; and more fusiform, shrunken and rounded.

CELL VIABILITY

MTT assay

The effect of AgNPs on viability of MCF-7 cells was checked using the MTT assay. The AgNPs were able to reduce viability of the MCF-7 cells in a dose-dependent manner, as shown in Figure 3. After 24 hours of

treatment, the AgNPs were found to be cytotoxic to MCF-7 cells at concentrations of 400 nM and higher. AgNPs at 400 nM decreased the viability of MCF-7 cells to 50% of the initial level, and this was chosen as the IC₅₀. Because a 400 nM concentration of AgNPs was found to be the IC₅₀, further experiments were carried out using this concentration, to show the effect of AgNPs.

CELL DEATH STUDIES [APOPTOSIS]

Acridine Orange / Ethidium Bromide assay

The induction of AgNPs mediated cell apoptosis was observed by Acridine Orange/Ethidium Bromide double staining of treated cell nuclei with optimized dose of silver nanoparticles. Microscopic images of the dual stained cells, presented in figure 4[A], show that the live cell nuclei stained green due to Acridine Orange uptake and their numbers gradually decreased with time owing to more cell death figure 4[B], which correlates with the SEM data. One can observe progressive nuclear uptake of Ethidium Bromide due to cell membrane perforation during apoptosis, which stained nuclei red, and such an effect was prominent from 6 h onwards. Microscopic images of treated as well as untreated cells at 6 h, indicates that live untreated cells had

well organized chromatin structures, whereas the treated cells had fragmented or condensed chromatin consisting of apoptotic nuclei. Therefore, the nuclear staining experiment shows that apoptosis started between 6 and 12 h after addition of Ag NPs to the culture medium.

DNA fragmentation assay

DNA ladders of the corresponding treated samples confirmed apoptosis (Figure 5) and showed that the Ag NPs treated MCF-7 cells exhibited extensive double strand breaks, thereby yielding a ladder appearance (Lane 3), while the DNA of control MCF-7 cells supplemented with 10% serum exhibited minimum breakage (Lane 2). The 1 kb ladder (Lane 1) was used to find the molecular weight of cleaved DNA fragments.

ANTI-ANGIOGENESIS ACTIVITY

Cell migration assay

Upon treatment with proper cell culture media maximal migration of MCF-7 was observed at different time interval figure 6[B, C]. Whereas, the treatment of silver nanoparticles (400 nM) resulted in the reduction in the number of cells and a

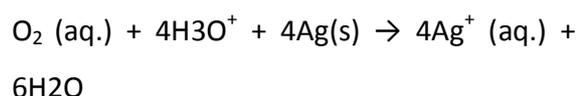
lesser amount of cell migration as shown in Figure. 6[E,F].

DISCUSSION

Cancer is the most important cause of mortality in the world⁹. All most all anticancer agents approved for the therapy of different types of cancer show several side effects such as myelosuppression, anemia, Alopecia, Secondary neoplasm and most importantly the generation of cellular resistance. A need for an alternative therapies or drugs to prevail over these drawbacks¹³ is always felt. Silver is in use for several ailments and most studies are focused on its antimicrobial and anti viral activities. The present study is focussed on its possible anticancer activity particularly when used as nanoparticles.

Silver nanoparticles are generally prepared by the reduction of silver salts using reducing agents like citrates or borohydrides. A clean method of synthesizing gold and silver nanoparticles by using heparin and hyaluronan as both reducing and stabilizing agents was reported¹⁸. These organic agents have been demonstrated to produce silver nanoparticles of bigger particle size^[28] but

with narrow size distributions than conventionally used reducing agents like citrates and borohydrides. such nanoparticles prepared using “green synthesis” not only exhibited biological functions but also avoided problems of high reactivity due to the release of silver ion from the nanoparticles (Generally less than 20nm) in presence of dissolved oxygen (O₂(aq.)) in water. They release silver ions according to the following equation:



Silver nanoparticles prepared using tannic acid as a reducing agent, in the present study, were found to be stable and the process offered better control over the shape and of the AgNPs without agglomeration. Stability study conducted over 8 weeks showed insignificant change in particle size up to 6 weeks but showed sudden increase in next two weeks while no appreciable change in zeta potential is recorded over the period of the study.

Control MCF-7 cells were found polygonal with extensive and almost continuous intercellular contacts during the phase

contrast microscopic study. Silver nanoparticles treated cells exhibited bipolar, spindle-type cell morphology with lesser intercellular contacts. At concentrations >200 nm/mL numbers decreased significantly and the cells appeared rounded and refractile after 24hr incubation.

In vitro cytotoxicity studies showed that the silver nanoparticles induced a dose-dependent cytotoxic effect and cell death in MCF-7 cancer cell line through apoptosis. AgNPs have been shown to have important anti angiogenic properties so are attractive for study of their potential antitumor effects. In this study, AgNPs of size 18.6 nm at a concentration of 400 nM had cytotoxic effects on MCF-7 cancer cells under *in vitro* conditions. Initially, a dose-dependent effect of AgNPs on cell lines assessed by MTT assay showed an IC₅₀ value of about 400 nM that induced partial reduction in cell viability in comparison with controls. The cytotoxic effect of AgNPs on cell viability has a major role in anticancer activity, thereby reducing disease progression. This is consistent with the effect of AgNPs on cell viability during VEGF induced angiogenesis in retinal endothelial

cells thereby inhibiting the development of angiogenic retinal disorders or reduced the complications. The cytotoxic effects of silver are the result of active physicochemical interaction of silver atoms with the functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA. The DNA fragmentation experiments subsequently confirmed induction of apoptosis.

Therefore, it can be suggested that silver nanoparticles treatment may be used as an alternative treatment against cancer. However, the mechanism and pathways by which silver nanoparticles induced cytotoxic activity on MCF-7 human breast cancer cell line need further investigation.

CONCLUSION

In conclusion, the great advantage of silver nanoparticles described in the present study is the simple and fast preparation and the excellent tissue tolerance, relatively free of side effects with low toxicity profile. Use of silver nanoparticles should emerge as one of the novel approaches in cancer therapy and, when the molecular mechanism of targeting is better understood, the applications of silver

nanoparticles are likely to expand further. The present study explores the potential anti-cancer activity of silver nanoparticles by using MCF-7 [Breast cancer cells] in vitro through induction of apoptosis and by inhibiting cell proliferation and angiogenesis. Thus, a study of the exact mechanism by which silver nanoparticles inhibit signaling cascades responsible for

the development and progression of the disease would be a tremendous breakthrough in the field of nanomedicine and make these agents an effective alternative in tumor and angiogenesis-related diseases.

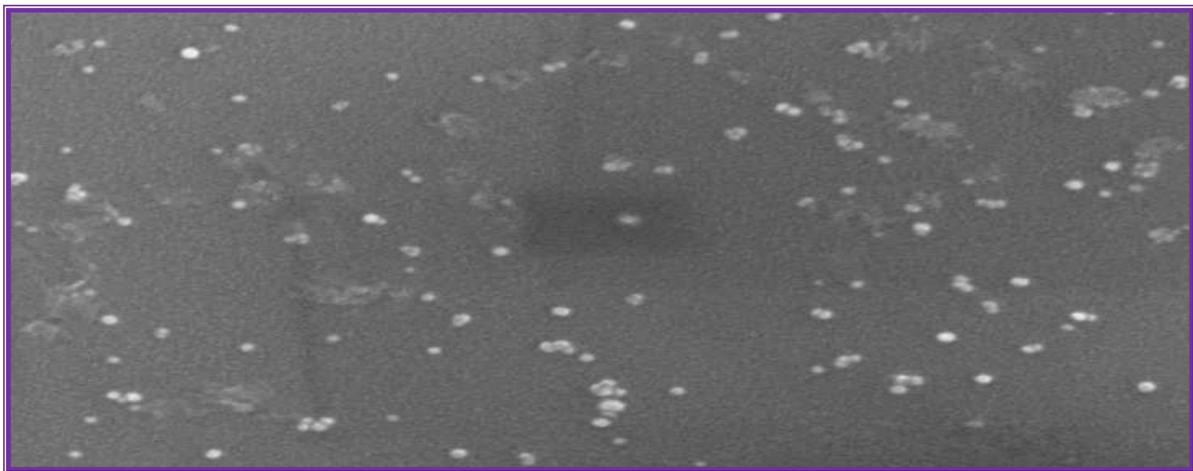


Figure 1 SEM Photograph of silver nanoparticles

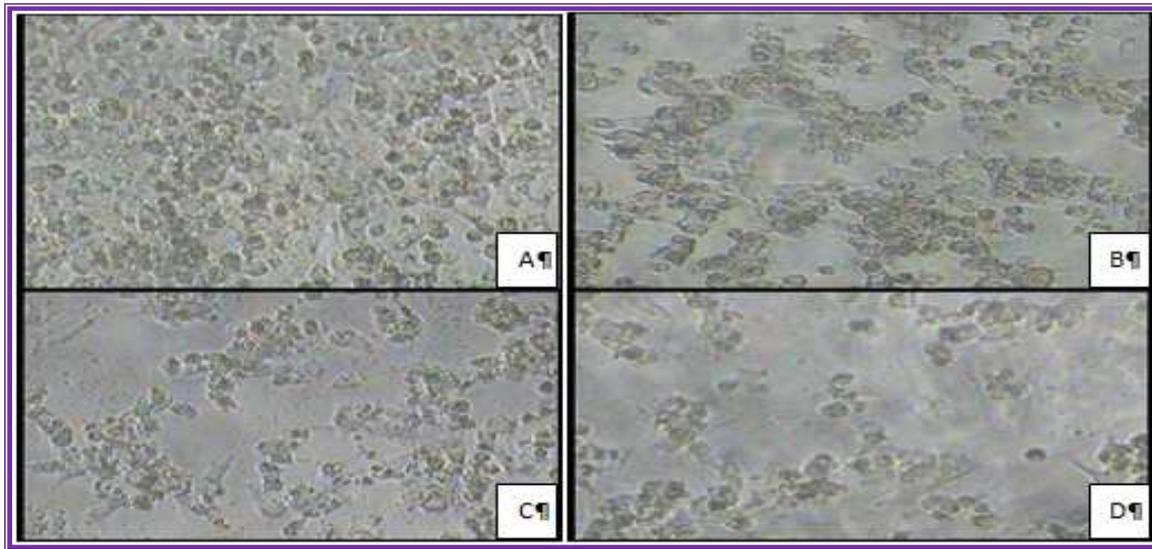


Figure 2 Dose dependent phase contrast microscopic images (200x) of MCF-7 cell lines treated with silver nanoparticles as compared to control. [A] Control [B] 200nM [c] 400nM [D] 800 nM

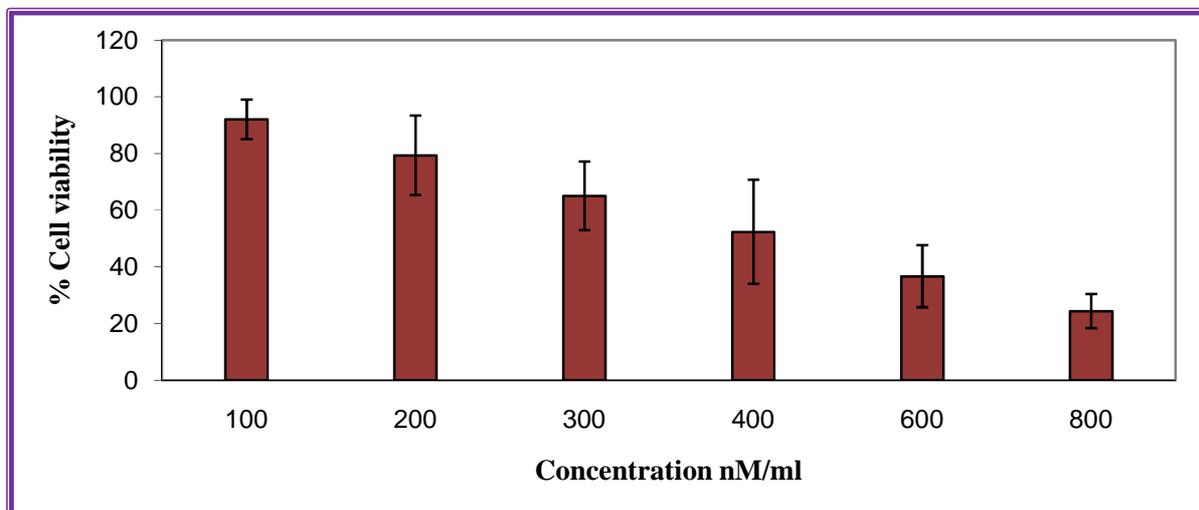


Figure 3 Dose-dependent effect of silver nanoparticles over cell viability using MTT assay. Results are presented in relative units compared with controls. Data represent the mean \pm standard error of the mean of three individual experiments. $P < 0.05$ compared with the control group.

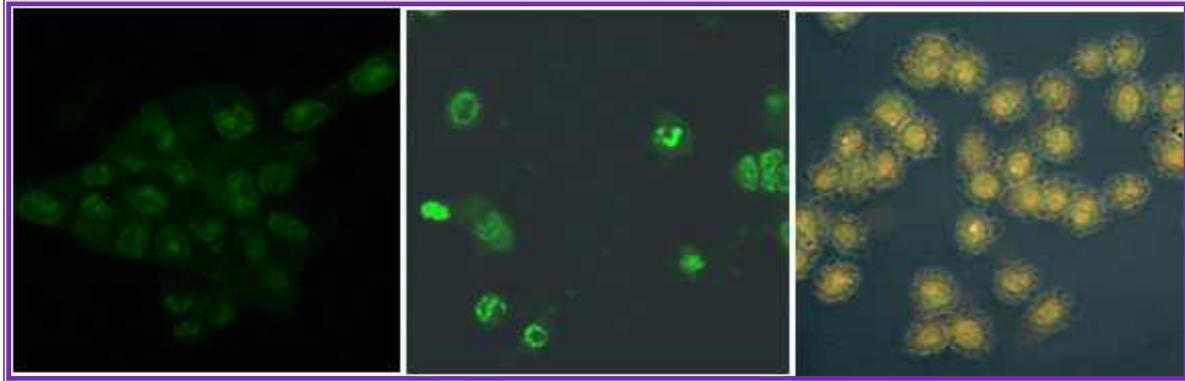


Figure 4 Time dependent micrographs of Acridine Orange/Ethidium Bromide stained cells. A–C are the representative images of MCF-7 cells respectively at 0, 6, and 12 h of Ag NP (400 nM) treatment. Magnification micrographs of MCF-7 shows that the untreated cell nuclei (A) stained green, whereas treated nuclei (B) consist of early apoptotic (EA) nuclei that stained green and (C) consists of late apoptotic (LA) fragmented nuclei that stained red.

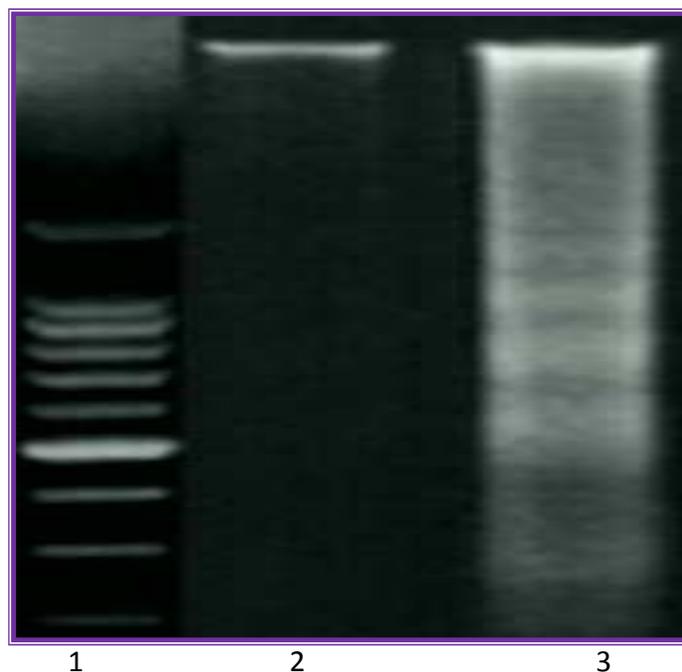


Figure 5 DNA Fragmentation assay: Lane 1 (1 kb ladder); Lane 2 (10% serum), and Lane 3 [treated with silver nanoparticles]

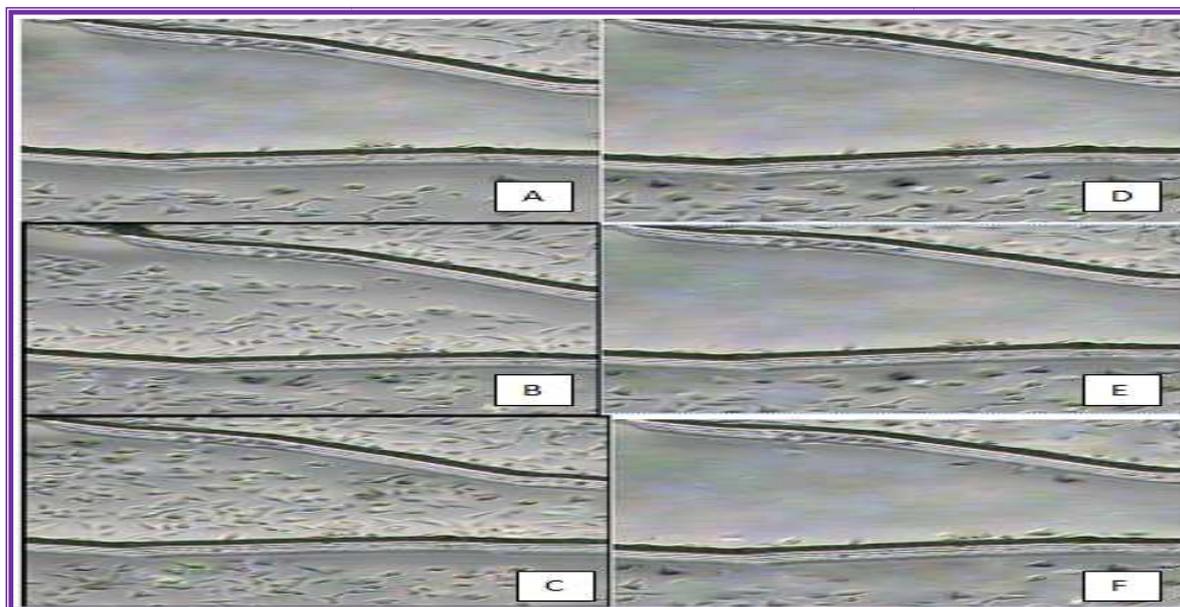


Figure 6 Confluent treated and untreated monolayer of MCF-7 at various time interval photographed using digital camera. Control cultures at 0 h (A). After 6 h [B] and after 12 h [C] when proper culture media is added, more cells migrated from the margin (B) and the wounded area was closed by 12 h [C] while significant area of wound is uncovered in plates treated with 400 nM of silver nanoparticles after 6 hours (E) and 12 h [f].

Table 1
Stability studies of silver nanoparticles

TIME	Particle size[nm]	Zeta Potential	UV absorbance [nm]
Initial data	18.6	-27.13	409
After 2 weeks	19.4	-27.13	409
After 4 weeks	20.2	-29.84	411
After 6weeks	21.8	-25.37	412
After8 weeks	25.3	-26.75	419

REFERENCES

1. Yezhelyev MV, Gao X, Xing Y, Hajj AA, Nie S and Regan RMO: Emerging use of nanoparticles in diagnosis and treatment of breast cancer *Lancet Oncol.* 2006; 7: 657–667.
2. Wagner V, Dullaart A, Bock AK and Zweck A: The emerging nanomedicine landscape, *Nat Biotechnol.* 2006; 24: 1211–1217.
3. Sahoo SK, Dilnawaz F and Krishnakumar S: Nanotechnology in ocular drug delivery. *Drug Discov Today* 2008; 13: 144–151.
4. Silver S, Phung LT and Silver G: Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds, *J Ind Microbiol Biotechnol.* 2006; 33: 627–634.
5. Klasen HJ: Historical review of the use of silver in the treatment of burns. I. Early uses, *Burns.* 2000; 26: 117–130.
6. Arora S, Jain J, Rajwade JM and Paknikar KM: Cellular responses induced by silver nanoparticles: In vitro studies, *Toxicol Lett.* 2008; 179: 93–100.
7. Kumari A, Kumar P, Ajayan PM and John G: Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil, *Nat Mater.* 2008; 7: 236–241.
8. Dunn K and Edwards-Jones V: The role of Acticoat with nanocrystalline silver in the management of burns, *Burns.* 2004; 30: S1–S9.
9. Bhattacharya R and Mukherjee P: Biological properties of “naked” metal nanoparticles, *Adv Drug Deliv Rev.* 2008; 60: 1289–1306.
10. Kalishwaralal K, BarathManiKanth S, Pandian SRK, Deepak V and Gurunathan S: Silver nano – a trove for retinal therapies. *J Control Release* 2010; 29: [Epub ahead of print].
11. Lara HH, Ayala-Nuñez NV, Ixtapan-Turrent L and Rodriguez-Padilla C: Mode of antiviral action of silver nanoparticles against HIV-1, *J Nanobiotechnology* 2010; 8: 1.
12. Sun RW, Rong C, Chung NPY, Ho CM, Lin CLS and Che CM: Silver nanoparticles

fabricated in Hepes buffer exhibit cytoprotective activities toward HIV-1 infected cells, *Chem Commun (Camb)* 2005; 28: 5059–5061.

13. Lu L, Sun RW, Chen R, et al.: Silver nanoparticles inhibit hepatitis B virus replication. *Antivir Ther.* 2008; 13: 253–262.

14. Sun L, Singh AK, Vig K, PillaiPR and Singh SR: Silver nanoparticles inhibit replication of respiratory syncytial virus, *J Biomed Biotechnol.* 2008; 4: 149–158.

15. Baram-Pinto D, Shukla S, Perkas N, Gedanken A and Sarid R: Inhibition of herpes simplex virus type 1 infection by silver nanoparticles capped with mercapto ethane sulfonate, *Bioconjug Chem.* 2009; 20: 1497–1502.

16. Rogers JV, Parkinson CV, Choi YW, Speshock JL and Hussain SM: A preliminary assessment of silver nanoparticle inhibition of monkeypox virus plaque formation, *Nanoscale Research Letters* 2008; 3: 129–133.

17. Hu R, Yong KT and Roy I: Metallic nanostructures as localized plasmon resonance enhanced scattering probes for multiplex dark field targeted imaging of

cancer cells, *J Phys Chem C Nanomater Interfaces* 2009; 113: 2676–2684.

18. Kemp MM, Kumar A and Mousa S: Synthesis of gold and silver nanoparticles stabilized with glycosaminoglycans having distinctive biological activities. *Biomacromolecules* 2009; 10: 589–595.

19. Kalishwaralal K, Banumathi E, Pandian SBRK, Deepak V, Muniyandi J, Eom SH and Sangiliyandi G: Silver nanoparticles inhibit VEGF induced cell proliferation and migration in bovine retinal endothelial cells. *Colloid Surf B: Bioint* 2009; 73(1): 51-7.

20. Hussain SM, Hess KL, Gearhart JM, Geiss KT and Schlager JJ: In vitro toxicity of nanoparticles in BRL 3A rat liver cells, *Toxicology in vitro* 2005; 19(7): 975–983.,

21. Jain J, Arora S, Rajwade JM, Omray P, Khandelwal S and Paknikar KM: Silver Nanoparticles in Therapeutics: Development of an Antimicrobial Gel Formulation for Topical Use, *Mol. Pharmaceutics* 2009; 6 (5): 1388–1401.

22. Gurunathan S, Kalishwaralal K and Vaidyanathan R: Biosynthesis, purification and characterization of silver nanoparticles

using Escherichia coli, Colloid Surf B Biointerfaces 2009; 74: 328–335.

23. Khlebtsov BN, Khanadeev VA and Khlebtsov NG: Determination of the size, concentration, and refractive index of silica nanoparticles from turbidity spectra., Langmuir The Acs Journal Of Surfaces And Colloids 2008; 24 (16): 8964-8970.

24. Liu X, Atwater M, Wang J and Huo Q: Extinction coefficient of gold nanoparticles with different sizes and different capping ligands. Colloids and Surfaces B: Biointerfaces 2007; 58(1): 3-7.

25. Moaddab S, Ahari H, Shahbazzadeh D, Motallebi AA, Anvar AA, Rahman-Nya J and Shokrgozar MR: Toxicity Study of Nanosilver (Nanocid) on Osteoblast Cancer Cell Line. Int Nano Lett. 2011; 1:11-16.

26. Babykutty S, Padikkala J, Sathiadevan PP, Vijayakurup V, Karedath T, Azis A, Srinivas P, and Gopala S: Apoptosis Induction of Centella Asiatica on Human Breast Cancer Cells, Afr J Tradit Complement Altern Med. 2009; 6(1): 9–16.

27. Muthu IS, Selvaraj BM, Kalimuthu K and Sangiliyandi G: Antitumor activity of silver nanoparticles in Dalton's lymphoma ascites

tumor model, Int J Nanomedicine 2010; 5: 753–762.

28. Sharma VK, Yngard RA and Lin Y: Silver nanoparticles: green synthesis and their antimicrobial activities. Advances in Colloid and Interface Science 2009; 145: 83-96.