

Toxicity Study of Silver Nanoparticles Synthesized from *Suaeda monoica* on Hep-2 Cell Line

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Abstract

Recently there has been fabulous excitement in the nano-biotechnological area for the study of nanoparticles synthesis using some natural biological system, which has led the growth advanced nanomaterials. This intention made us to assess the biologically synthesized silver nanoparticles from the leaf of *Suaeda monoica* (*S.monoica*) using 1 mM silver nitrate. The leaf extract of *S.monoica* incubated with 1 mM silver nitrate solution and characterized by UV- spectrometer and AFM. The effect of synthesized silver nanoparticles on Human Epidermoid Larynx Carcinoma cell line was evaluated by the MTT colorimetric technique. As a result we observed gradual change in the colour of extract from greenish to brown. The synthesized silver nanoparticles confirmed by UV at 430 nm and spherical shape identified in the range of 31 nm under AFM. The effect of silver nanoparticles on Human Epidermoid Larynx Carcinoma cell line exhibits a dose-dependent toxicity for the cell tested and the viability of Hep-2 cells decreased to 50% (IC₅₀) at the concentration of 500 nM. Further findings will be determined the exact mechanisms of this cost effective Nano-treatments.

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Introduction

Suaeda monoica is a salt marsh herb growing in hyper saline soils that belongs to *Chenopodiaceae* and is distributed throughout the East and West coast mangroves in India. Traditionally, the leaf from *S.monoica* is known to be used as a medicine for hepatitis and scientifically it is reported to be used as ointment for wounds and possess antiviral activity because of the presence of triterpenoids, sterols⁽¹⁾.

Rapidly developing field of nanoscience had raised the possibility of using therapeutic nanoparticles in the diagnosis and treatment of human cancers⁽²⁾. Nanoscale particles and molecules are a potential alternative for treatment of disease because they have unique bio-

logical effects based on the structure and size, which differ from traditional small molecule drugs⁽³⁾.

In the last few years, several pharmaceuticals companies had obtained approval from the US Food and Drug Administration (FDA) for the development of nanotechnology based drugs. Silver had long been recognized as having an inhibitory effect towards many bacterial strains and micro organisms commonly present in medical and industrial processes⁽⁴⁾. The most widely used and well known applications of silver and silver nanoparticles includes topical ointments and creams containing silver to prevent infection of burns and open wounds⁽⁵⁾.

Production of nanoparticles can be achieved through different methods. Chemical approaches are the most popular methods for the production of nanoparticles. However, some chemical methods cannot avoid the use of toxic chemicals in the synthesis protocol. Since noble metal nanoparticles such as gold, silver and platinum nanoparticles are widely applied to human contacting areas, there is a growing need to develop environmentally friendly processes of nanoparticles synthesis that do not use toxic chemicals. Biological methods of nanoparticles synthesis using microorganisms⁽⁶⁾, enzyme⁽⁷⁾, and plant or plant extract have been suggested as possible ecofriendly alternatives to chemical and physical methods. Using plant for nanoparticles synthesis can be advantageous over other biological processes by eliminating the elaborate process of maintaining cell culture⁽⁸⁾.

The role of silver nanoparticles as an anti-cancer agent should open new door in the field of medicine. Silver nanoparticles should serve as one of the best ways of treating diseases that involve cell proliferation and cell death⁽⁹⁾. In the present study, we screened salt marsh plant species *S.monoica* leaf extracts for extracellular silver nanoparticles synthesis and to determine the cytotoxicity threshold in this Human Epidermoid Larynx Carcinoma (Hep-2) cell line.

Materials and Methods

Plant material

S.monoica leaves freshly were collected from the Kollidam coast (Tamil Nadu) India. The specimen was certified by Botanical Survey of India (BSI) Coimbatore, and documented in the Herbaria of C.A.S. in Marine Biology (Voucher No. AUCASMB15), Annamalai University, India.

Synthesis of silver nanoparticles

The analytical grade silver nitrate was purchased from Sigma Chemicals (Mumbai). The leaves weighing 25 g were thoroughly washed in distilled water, dried, cut into fine pieces and were crushed into 100 ml sterile distilled

water and filtered through what man No.1 filter paper. The filtrate was further filtered through 0.6 μm sized filters and stored at 4 °C. Ten ml suspension of leaf extract was added to 90 ml aqueous solution of silver nitrate (1 mM) solution separately for reduction in to Ag⁺ ions and incubated at room temperature (35 °C) for 5 hr. After 5 hr of incubation the silver nanoparticles were isolated and concentrated by repeated (4-5 times) centrifugation of the reaction mixture at 10,000 \times g for 10 min. The supernatant was replaced by distilled each time and suspension stored as lyophilized powder. The optical measurements, was carried out by UV-Vis spectrophotometer (UV- 2450 (Shimadzu) and scanned the spectra between 200-700 nm at the resolution of 1nm.

Atomic force microscope

Purified SNP (Silver Nanoparticles) in suspension also characterized their morphology using a VEECO diNanoscope 3D AFM (Atomic Force Microscope). A small volume of sample was spread on a well-cleaned glass cover slip surface mounted on the AFM stub, and was dried with nitrogen flow at room temperature.

Images were obtained in tapping mode using a silicon probe cantilever of 125 μm length, resonance frequency 209-286 kHz, spring constant 20-80 nm⁻¹ minimum of five images for each sample were obtained with AFM and analyzed to ensure reproducible results.

Cell culture

The Hep-2 cell line was purchased from National Cell Centre, Pune (India). Cancerous cells were seeded in flask with MEM (Minimal Essential media) medium with 2-10% Fetal Calf Serum (FCS) and incubated at 37°C in a 5% CO₂. After 24 hr incubation period the attached cells were trypsinized for 3-5 min and centrifuged (1,400 rpm, 5 min). The cells were counted and distributed in 96 well ELISA plate with 10,000 cells in each well. The plate was incubated 24 hr at 37 °C in a 5% CO₂ atmosphere to allow the cells attach to the bottom of the well⁽¹⁰⁾.

Cell treatment with silver nanoparticles

Silver nanoparticles reduced ATP content of the cell, caused damage to mitochondria and increased production of Reactive Oxygen Species (ROS) in a dose-dependent manner⁽¹⁰⁾. Hence we determined the toxicity of silver nanoparticles at different concentrations (15, 30, 62, 125, 250, 500, 1000 $\mu\text{g/ml}$) and duplicates added to the wells were incubated in to as grown cell (1×10^4 cells/well) and the cell population was determined by optical microscopy at 24 and 48 *hr*.

MTT assay

Cell viability was evaluated by the MTT colorimetric technique with slight modification⁽¹¹⁾. Briefly, in each well 200 μl of MTT [3-(4, 5-dimethylthiazol-2)-2, 5 diphenyl tetrazolium bromide] without phenol red, yellowish in color solution (5 mg/ml in PBS), was added to each well. The plates were incubated for 6-7 *hr* in 5% CO_2 incubator for reduction of MTT by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan solubilized the MTT crystals by adding and quantified by spectrophotometric mean and then the supernatants were removed.

For solubilization of the MTT crystals, 100 μl of isopropanol or DMSO was added to the wells. The plates were placed on a shaker for 15 *min* for complete solubilization of crystals and then the optical density of each well was determined. The quantity of formazan product, as measured by the amount of 595 *nm* using a scanning Multiwell spectrophotometer (Biorad, Model 680, Japan) and DMSO, served as a blank. The absorbance is directly proportional to the number of living cells in cultural.

Each experiment was done in duplicate. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles as a vehicle was calculated by $(A)_{\text{test}} / (A)_{\text{control}} \times 100$; where (A)

$(A)_{\text{test}}$ is the absorbance of the test sample and $(A)_{\text{control}}$ is the absorbance of control sample.

Statistical analysis

All experiments were done in duplicate, and the results were presented as mean \pm standard deviation. The experimental data were analyzed by using SPSS. Statistical significance was accepted at a level of $p < 0.05$ to calculate IC_{50} values.

Results

The leaf extract of *S.monoica* was used for the synthesis of silver nanoparticles. The reaction started within first *hr* of the incubation with silver nitrate (1 *mM*). The primary detection of synthesized silver nanoparticles was carried out in the reaction mixture by observing the colour change of the medium from greenish to dark brown. The reaction rate was maximum after 25 *hr* of incubation as indicated by the formation of silver nanoparticles.

Our findings showed resemblance to the results already reported by in the case of extract of *Capsicum annum*⁽¹²⁾ and in case of extract of *Aloe vera*⁽¹³⁾. They reported that when the extracts of their respective test plants were challenged with silver nitrate (1 *mM*), it turned brown and the intensity of colour was increased with the time of incubation.

In order to verify the synthesis of silver nanoparticles, the test samples were subjected to the UV-Vis spectrophotometer analysis after 5 *hr* of incubation. A peak specific for the synthesis of silver nanoparticles was obtained at 430 *nm*. AFM is used for morphological characterization of SNP⁽¹⁴⁾. The shape of the SNP synthesized by leaf extract was spherical and was found to be in the range of 31 *nm* (Figure 1).

The *in vitro* cytotoxicity effects of silver nanoparticles were screened against human epidermoid larynx carcinoma cell lines by means of MTT assay. The silver nanoparticles were able to reduce viability of the Hep-2 cells in a dose-dependent manner, as shown in

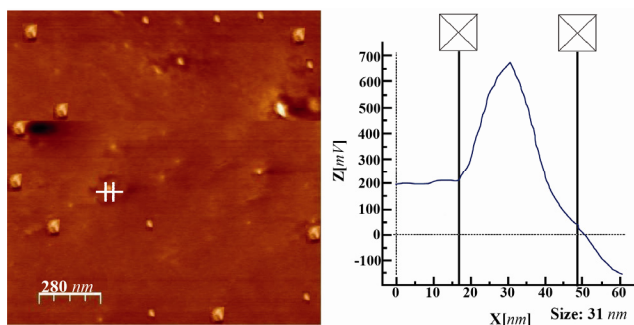


Figure 1. Tapping mode of AFM micrograph of silver nanoparticles synthesized from leaf of *Suaeda monoica* using silver nitrate solution (1 mM)

figure 2. After five hr of treatment, the silver nanoparticles were found to be cytotoxic to tumour cells at a concentration of 500 nM and higher. Silver nanoparticles at 500 nM decreased the viability of Hep-2 cells (Figure 3) to 50 % of the initial level, and this was chosen as the IC₅₀. Longer exposures resulted in additional toxicity to the cells.

These results demonstrate that silver nanoparticles mediate a dose and time dependent increase in toxicity. The result suggested that the plant mediated synthesized silver nanoparticles possess great selectivity to cancer cell and can display potential application in cancer chemoprevention and chemotherapy. Silver nanoparticles revealed to have important anti angiogenic properties⁽¹⁵⁾; so are attractive for study of their potential antitumor effects. Compounds possessing antiangiogenic properties are known for their potential ability to block the activity of abnormally expressed signaling proteins⁽¹⁶⁾.

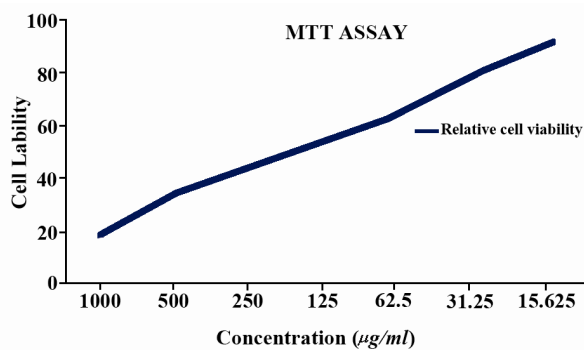


Figure 2. Cytotoxic study of silver nanoparticles synthesized from leaf of *Suaeda monoica* against Hep-2 cell line on a dose dependent manner

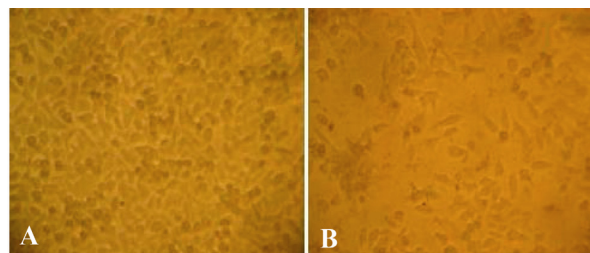


Figure 3. Cytotoxicity of Silver Nanoparticles (SN) synthesized from leaf of leaf of *Suaeda monoica* on Hep2 cell line (A) Normal cells (B) SN treated cells (500 µg/ml) decreased the viability of HeP-2 cells

Discussion

In the present study, it has been shown a simple, rapid and green synthesise of silver nanoparticles from *S.monoica* of size 31 nm, at a concentration of 500 nM had cytotoxic effects on Hep-2 tumor cells under *in vitro* conditions. Similar reports was observed by Bilberg et al, in male zebra fish with a semi-static 48 hr exposure LC₅₀ of 84 µg L⁻¹ and LC₁₀ of 57 µg L⁻¹⁽¹⁷⁾. Initially, a dose dependent effect of SNP on Hep-2 cell lines assessed by MTT assay showed an IC₅₀ value of about 500 nM that induced partial reduction in cell viability in comparison with control.

The cytotoxic effect of SNP on cell viability has a major role in antitumor activity, thereby reducing disease progression. The cytotoxic effects of silver are the result of active physiochemical interaction of silver atoms with functional groups of intracellular proteins, as well as with the nitrogen bases and phosphate groups in DNA⁽¹⁸⁾.

Conclusion

In conclusion, the silver nanoparticles synthesized from *S.monoica* serve as an anti-tumor agent by decreasing progressive development of tumor cells. Our result suggests that SNP can induce cytotoxic effects on Hep-2 cells, inhibiting tumour progression and thereby effectively controlling progression without toxicity to normal cells. This may be due to their inhibitory activities in several signaling cascade responsible for the development and pathogenesis of disease which are as yet not understood.

Although, some of the researchers pointed out that the nanosilver toxicity is caused by chemical interactions, the toxic portion of nanoparticles must originate either from silver ions dissolved from the particle or from the exposed silver atoms on the particle surface⁽¹⁹⁾. While the mechanism(s) by which AgNPs are toxic are unclear, their increasing use raises the concern that its release into the environment could lead to environmental toxicity.

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References

1. Bandaranayake WM. Traditional and medicinal uses of mangroves. *Mangroves Salt Marshes* 1998; 2(3):133-148.
2. Yezhelyev MV, Gao X, Xing Y, Al-Hajj A, Nie S, O'Regan RM. Emerging use of nanoparticles in diagnosis and treatment of breast cancer. *Lancet Oncol* 2006;7(8):657-667.
3. Wagner V, Dullaart A, Bock AK, Zweek A. The emerging nanomedicine landscape. *Nat Biotechnol* 2006;24(10):1211-1217.
4. Murphy CJ. Sustainability as an emerging design criterion in nanoparticle synthesis and applications. *J Mater Chem* 2008;18:2173-2176.
5. Schultz S, Smith DR, Mock JJ, Schultz DA. Single-target molecule detection with non bleaching multi-color optical immunolabels. *Proc Natl Acad Sci USA* 2000;97(3):996-1001.
6. Nair B, Pradeep T. Coalescence of nanoclusters and formation of submicron crystallites assisted by *Lactobacillus* strains. *Cryst Growth Des* 2002;2(4): 293-298.
7. Willner I, Baron R, Willner B. Growing metal nanoparticles by enzymes. *Adv Mater* 2006;18(9): 1109-1120.
8. Taleb C, Pettai M, Pileni P. Nanoparticles and nanostructured films: preparation, characterization and applications. *Chem* 1998;22:1203.
9. Vaidyanathan R, Kalishwaralal K, Gopalram S, Gurunathan S. Nanosilver-the burgeoning therapeutic molecule and its green synthesis. *Biotechnol Adv* 2009;27(6):924-937.
10. AshaRani PV, Low Kah Mun G, Hande MP, Valiyaveetil S. Cytotoxicity and genotoxicity of silver nanoparticles in human cells. *ACS Nano* 2009;3(2): 279-290.
11. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65(1-2):55-63.
12. Gurunathan S, Lee KJ, Kalimuthu K, Sheikpranbabu S, Vaidyanathan R, Eom SH. Antiangiogenic properties of silver nanoparticles. *Biomaterials* 2009;30(31):6341-6350.
13. Li S, Han Q, Qiao C, Song J, Lung Cheng C, Xu H. Chemical markers for the quality control of herbal medicines: an overview. *Chin Med* 2008;28;3:7.
14. Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M. Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* plant extract. *Biotechnol Prog* 2006;22(2):577-583.
15. Song JY, Kim BS. Rapid biological synthesis of silver nanoparticles using plant leaf extracts. *Bio-process Biosyst Eng* 2009;32(1):79-84.
16. Martins D, Frungillo L, Anazzetti MC, Melo PS, Duran N. Antitumoral activity of L-ascorbic acid-poly-D,L-(lactide-co-glycolide) nanoparticles containing violacein. *Int J Nanomedicine* 2010;5:77-85.
17. Bilberg K, Hovgaard MB, Besenbacher F, Baatrup E. In vivo toxicity of silver nanoparticles and silver ions in Zebrafish (*Danio rerio*). *J Toxicol* 2012; 2012:293784. Epub 2011 Dec 1.
18. Blagoi Yu P, GalkinVL, Gladchenko GO, Kornilova SV, Sorokin VA, Shkorbatov AG. Metal complexes of nucleic acids in solutions. *Naukova Dumka* 1991:272. (Russian)
19. Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramirez JT, et al. The bactericidal effect of silver nanoparticles. *Nanotechnology* 2005;16 (10):2346-2353.