

INVITRO ANTICANCER ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING THE EXTRACT OF *GELIDIELLA Sp.*

J. SARANIYA DEVI^{1,2}, B. VALENTIN BHIMBA², KRUPA RATNAM³

¹Department of Microbiology, Asan Memorial College of Arts and Science, Chennai-100, ²Department of Biotechnology, Sathyabama University, Chennai, ³Marine Biotechnology, National Institute of Technology, Chennai. Email: bvbhimba@yahoo.co.in

Received: 18 Apr 2012, Revised and Accepted: 28 May 2012

ABSTRACT

The eco-friendly synthesis of nanoparticles through various biological means helps us to explore various seaweeds for their ability to synthesize silver nanoparticles (AgNPs). It was found that aqueous silver ions when exposed to aqueous extract of *Gelidiella sp.* are reduced in solution, thereby leading to the formation of the AgNPs within 10 min. at 121°C. These AgNPs were characterized by means of several techniques. The nanoparticles show absorbance at 435nm on UV-Vis spectroscopy. The presence of proteins was identified by Fourier transform-infra red spectroscopy (FT-IR). The presence of elemental silver was characterized by Energy dispersed spectroscopy (EDS). The morphology of AgNPs was characterized by Scanning electron microscopy (SEM). The synthesized AgNPs are very stable in aqueous solution for about 1 month without any sign of precipitation. The nanoparticles were assessed for their cytotoxic activity on Hep-2 (Human laryngeal) cell lines.

Keywords: Ecofriendly, Silver nanoparticles, *Gelidiella sp.*, UV-Vis, FT-IR, EDS, SEM, Cytotoxic activity, Hep-2 cell lines.

INTRODUCTION

Nanotechnology deals with processes that take place on the nanometer scale, that is, from approximately 1 to 100 nm. Nanoparticles with controlled size are of fundamental and technological interest as they provide solutions to technological and environmental challenges in the areas of medicine, solar energy conversion, catalysis and water treatment. Thus, production and application of nanomaterials from 1 to 100 nanometers (nm) is an emerging field of research (1). Nanoparticles are collection in aggregate of atoms in the range of 1-100 nm with unique structure and properties, which are widely used in an increase amount of applications. Silver nanoparticles (Ag-NPs) in particular, provide effective growth inhibition of various microorganisms in suspension and on solid medium.

Although chemical and physical methods may successfully produce pure, well-defined nanoparticles, these methods are quite expensive and potentially dangerous to the environment. Use of biological organisms such as microorganisms, plant extract or plant biomass could be an alternative to chemical and physical methods for the production of nanoparticles in an eco-friendly manner (2). In recent years, plant-mediated biological synthesis of nanoparticles is gaining importance due to its simplicity and eco-friendliness. Green synthesis of nanoparticles is an easy, efficient and eco friendly approach, where most researchers are looking at the eco-friendly and green synthesis of nanoparticles paves the way for the researchers around the world to explore the ability of various herbs in synthesizing nanoparticles. Nanoparticles synthesized by chemical method are not eco-friendly (3).

AgNPs have several characteristics that make it currently among the most widely used nanoparticle in science. One highly useful characteristic is its antimicrobial property (4). Silver in its pure form was known as a great material to keep microbes at bay. If silver is transformed into a nanoparticle, this anti-microbial property is intensified, making it useful in effectively eliminating fungus, bacteria, and viruses. As a natural material, silver is known to be safe to man and produce little to no allergic reactions when tested for curing various diseases.

Silver is an effective antibacterial agent with low toxicity which is important especially in the treatment of burn wounds. Given its broad spectrum activity, AgNPs have been the focus of increasing interest and are being used as an excellent candidate for therapeutic purposes (5).

Cancer is an abnormal type of tissue growth in which the cells exhibit an uncontrolled division, relatively in an autonomous

fashion, leading to a progressive increase in the number of dividing cell (6). There is increasing demands for anticancer therapy (7). *In vitro* cytotoxicity testing procedures reduces the use of laboratory animals (8) and hence use of cultured tissues and cells have increased (9).

The discovery and identification of new antitumor drug with low side effects on immune system has become an essential goal in many studies of immuno-pharmacology (10). With this aim, many attentions have been paid to natural compounds in plants, marine organism and microorganisms.

Many medically relevant nanoparticles such as AgNPs were investigated for their cytotoxicity aspect. AgNPs showed different degrees of *in vitro* cytotoxicity (11). The Apoptotic Effect of Nanosilver is mediated by a ROS- and JNK-Dependent Mechanism Involving the Mitochondrial Pathway in NIH3T3 Cells (12).

The present study was carried out to verify the possible cytotoxic action of AgNPs synthesized using *Gelidiella sp.* on HEp2 cells, evaluating morphology and the number of viable cells after incubation with the plant extract in different concentrations and duration of exposure.

MATERIALS AND METHODS

Collection of seaweed samples

Seaweeds were collected from the intertidal regions of the Mandapam coastal regions (Lat.09° 17.417 N; Long.079° 08.558 E) of Gulf of Mannar. Samples were washed with freshwater to remove adhering debris and associated biota and identified as *Gelidiella sp.* by CAS Botany, Madras University. Collected samples were transferred to the lab in a cool bag, lyophilized, powdered and stored in -4°C.

Seaweed extraction

About 5gm of the powdered material were extracted with 1000ml of sterilized double distilled water. Then the crude extract was blended thoroughly and filtered through Whatmann filter paper No.1 (42 µm) and the residue was again blended with water and filtered. The filtrate was stored and used for further analysis.

Synthesis of silver nanoparticles

900ml filtrate was treated with 100ml of aqueous 1mM silver nitrate solution in an Erlenmeyer flask slowly under the magnetic stirring conditions. The bio reduction of silver nitrate occurred within 10 min. at 121°C. The color change to dark brown of the medium was noted by visual observation indicating the formation of AgNPs,

which remain stable for more than 3 months without any changes in the absorption spectrum. The reduction of pure Ag⁺ ions was monitored by measuring the UV-Vis spectrum of the reaction medium after diluting a small aliquot of the sample into distilled water. Aliquots of the reaction solution were measured using a UV-1601 Shimadzu spectrophotometer operated at a resolution of 1nm.

Fourier transform infrared spectroscopy

The interaction between protein-silver nanoparticles were analyzed by Fourier transform infrared spectroscopy (FTIR) in the diffuse reflectance mode at a resolution of 4cm⁻¹ in the KBr pellets and the spectra were recorded in the wavelength interval of 4000 to 400nm⁻¹. FTIR measurements were carried out to identify the possible biomolecules responsible for the reduction of the Ag⁺ ions and the capping of the bioreduced AgNPs synthesized by seaweed extract. For comparison, the seaweed filtrate was mixed with KBr powder and pelletized after drying properly and subjected to measurement.

X-ray diffractometry

X-ray diffraction (XRD) measurement of the seaweed reduced AgNPs was carried out using powder X-ray diffractometer instrument (PXRD-6000 SCHIMADZU) in the angle range of 10°C-80°C at 2θ, scan axis: 2:1 sym. The size of the AgNPs was calculated from the PXRD peak positions using Bragg's law.

Extended Dispersive analysis X-ray Spectroscopy

The presence of elemental Silver was confirmed through EDS. Energy dispersive analysis X-ray spectrometer takes advantage of the photon nature of the light. In the X-ray range the energy of a single photon is just sufficient to produce a measurable pulse X ray; the output of an ultra low noise pre-amplifier connected to the low noise is a statistical measure of the corresponding quantum energy. A semiconductor material is used to detect the X-rays together with processing electronics to analyses the spectrum. The EDX observations were carried out in STIC, CUSAT, Kerala (JOEL Model JED-2300).

SEM observation of silver nanoparticles

The sample was prepared by placing a drop of AgNPs on carbon coated copper grid and subsequently drying in air, before transferring it to the microscope operated at an accelerated voltage of 120 KV (JOEL Model JSM-63 90 LV).

In vitro assay for Cytotoxicity activity (MTT assay).

Hep-2 (Human Epidermoid larynx carcinoma) cell lines were obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37 °C.

The Cytotoxicity of synthesized AgNPs on Human Epidermoid Larynx cancer cells was determined by the MTT assay (Mosmann et al., 1983). Cells (1 × 10⁵/well) were plated in 100 µl of medium/well in 96-well plates. After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570nm with reference at 655nm. Measurements were performed in 3 times, and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically. The absorbance at 570 nm was measured with a UV spectrophotometer, using wells without sample containing cells as blanks. All experiments were performed in triplicate. The effect of the AgNPs on the proliferation of Human Epitheloid larynx cancer cells was expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = A570 \text{ of treated cells} / A570 \text{ of control cells} \times 100\%.$$

Data analysis

The IC₅₀ values (concentration at which 50% of cells were death) are reported as mean± standard deviation of three independent experiments. The IC₅₀ values against the Human laryngeal Hep-2 cancer cell lines were calculated for the synthesized AgNPs inhibiting at least 50% inhibition when tested at a concentration. One-way analysis of variance (ANOVA) and Student t-tests were used to compare data using Statistica version 5.0 software at a 95% confidence limit.

RESULTS AND DISCUSSIONS

Reduction of silver ion into Ag particles during exposure to the seaweed extract could be followed by color change. AgNPs exhibit dark yellowish-brown color in aqueous solution due to the surface Plasmon resonance phenomenon (Fig.1). UV-Vis spectrograph of Ag nanoparticles has been recorded as a function of time (Fig.2). Absorption spectra of Ag nanoparticles formed in the reaction media at 72 hrs has absorbance peak at 435 nm, broadening of peak. The position and the number of peaks in the absorption spectra are dependent on the shape of the particles: for an ellipsoidal particle there are two peaks whereas for spherical particle there is only one peak centered at 420 nm, indicating the formation of silver nanoparticles (13). The absorption maximum at 420 nm is attributed to the Mie scattering by silver metal (14).

The appearance of the yellow color indicated the formation of AgNPs in the reaction mixture, as it is well-known that AgNPs exhibit striking colors (light yellow to brown) due to excitation of surface plasmon vibrations in the particles (15). It was reported that some amount of OH- groups tended to promote the reduction of silver ions in some chemical methods (16).

FT-IR spectrum of AgNPs is shown in Fig.3. This spectrum shows the presence of bands at 3419, 1650, 1489, 1059 cm⁻¹. The bands at 3419cm⁻¹ corresponds to primary amine O-H band, 1650 cm⁻¹ corresponds to primary amine N-H band, the band at 1489 cm⁻¹ is assigned to methylene scissoring vibration from the protein in the solution, and the band at 1033 cm⁻¹ were assigned to C-N stretching vibration of the proteins (17). The positions of these bands were close to that reported for native proteins (18). This evidence suggests that the protein molecules could possibly perform the function of the formation and stabilization of AgNPs in the aqueous medium.

XRD pattern taken using powder X-ray diffractometer instrument (PXRD-6000 SCHIMADZU) in the angle range of 10°C-80°C of the AgNPs at 2θ, scan axis: 2:1 sym is shown in Fig.4. A number of Bragg reflections corresponding to (111), (200), (220), (311) sets of lattice planes are observed, which can be indexed to face-centered cubic silver. The peaks matches with the Joint Committee on Powder Diffraction Standards (file No. 04-0783), which further proves the formation of crystal AgNPs (19). The peaks were identified as AgNPs according to PCPDFWIN software (PDF#030921). The XRD pattern thus clearly shows that the AgNPs are crystalline in nature. Furthermore, the average diameter of the silver nanoparticles is calculated as 6.4nm by the Scherrer formula

$$D = \frac{0.89\lambda}{\beta \cos\theta}$$

Where D is mean grain size, λ is the wavelength for Cu target, β is the FWHM of diffraction peak and θ is the diffraction angle. Thus XRD is commonly used for determining the chemical composition and crystal structure of a material. The line broadening of the peaks is primarily due to small particle size. The X-ray diffraction results clearly show that the AgNPs formed by the reduction of silver ions by the extract of *Gelidiella sp.* are crystalline in nature (20).

EDS micro-analysis is performed by measuring the energy and intensity distribution of X-ray signals generated by a focused electron beam on a specimen. Fig.5 shows the EDS spectrum recorded in the spot-profile mode. The optical absorption peak is observed at 3KeV, which is typical for the absorption of metallic AgNPs (21). Strong signals from the silver atoms are observed, while weaker signals from C, O, P and Cl atoms are also recorded. Those

weaker signals are likely to be due to X-ray emission from the *Gelidiella sp.* From the EDS spectrums, it is clear that AgNPs reduced by *Gelidiella sp.* have the weight percentage of silver as 30.15%.

SEM technique was employed to visualize the size and shape of AgNPs. The formation of AgNPs as well as their morphological dimensions in the SEM study demonstrated that the average size was from 40-50 nm. The shapes of the AgNPs were proved to be spherical (Fig.6.). Similar phenomenon was reported by Chandran *et al* (22).

Cytotoxicity of silver nanoparticles

The *invitro* cytotoxicity of the AgNPs was evaluated Hep-2 cell lines at different concentrations. Our cytotoxicity analysis of the sample

shows a direct dose-response relationship; cytotoxicity increased at higher concentrations. The IC_{50} value was plotted by taking the concentration of AgNPs on X-axis versus percentage of cell viability on Y- axis (Fig.7). The samples demonstrated a considerable cytotoxicity against the Hep-2 cell lines. The result showed that Hep2 cells proliferation were significantly inhibited by AgNPs with an IC_{50} value of 31.25 $\mu\text{g/ml}$ of the concentration. As shown in Table.No.1, in the lowest tested concentration (3 $\mu\text{g/ml}$), AgNPs were able to inhibit the cell line's growth by less than 10%. In contrast the presence of 15.62 $\mu\text{g/ml}$ of AgNPs significantly inhibited the cell line's growth (> 60%). Previous study shows that phytochemicals depletes intracellular antioxidants thereby induced cancer cell death (23).

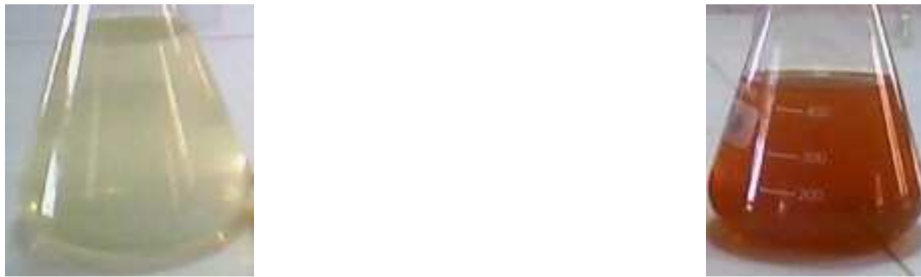


Fig. 1: Photography of a) *Gelidiella sp.* extract. b) Formation of AgNPs

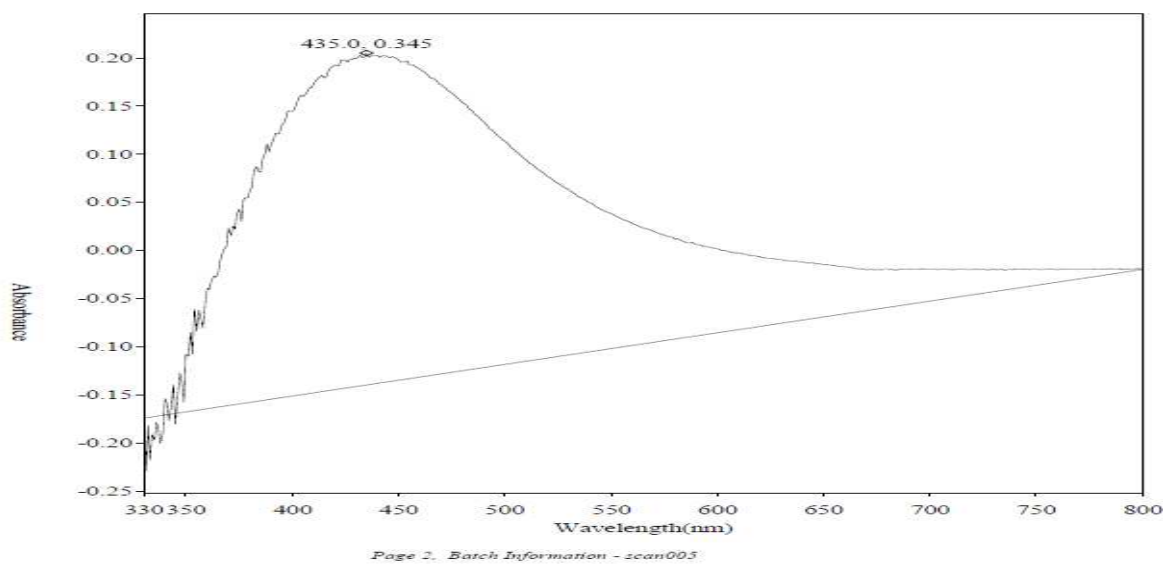


Fig. 2: UV-VIS absorption spectra of Ag nanoparticle synthesized from *Gelidiella sp.* at 1mM silver nitrate.

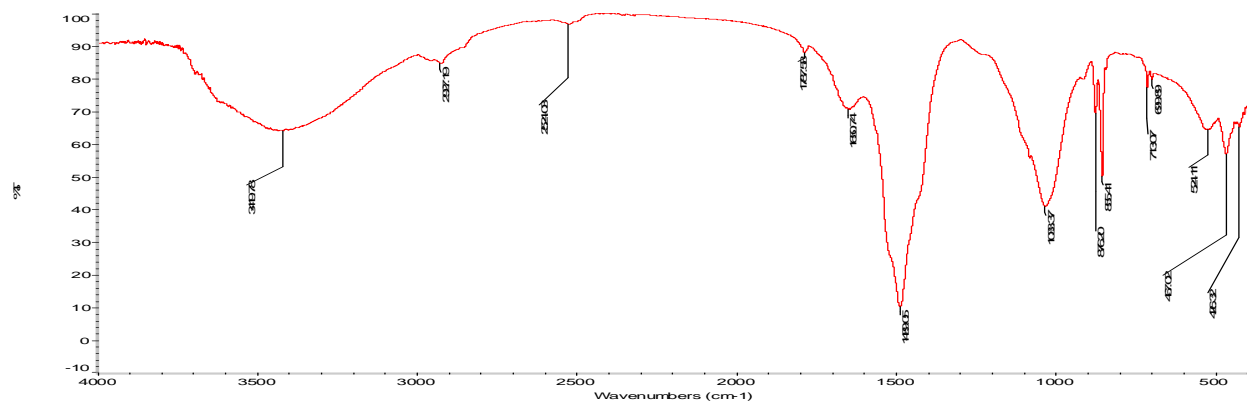


Fig. 3: FTIR spectra of capped silver nanoparticles synthesized using aqueous extract of *Gelidiella sp.*

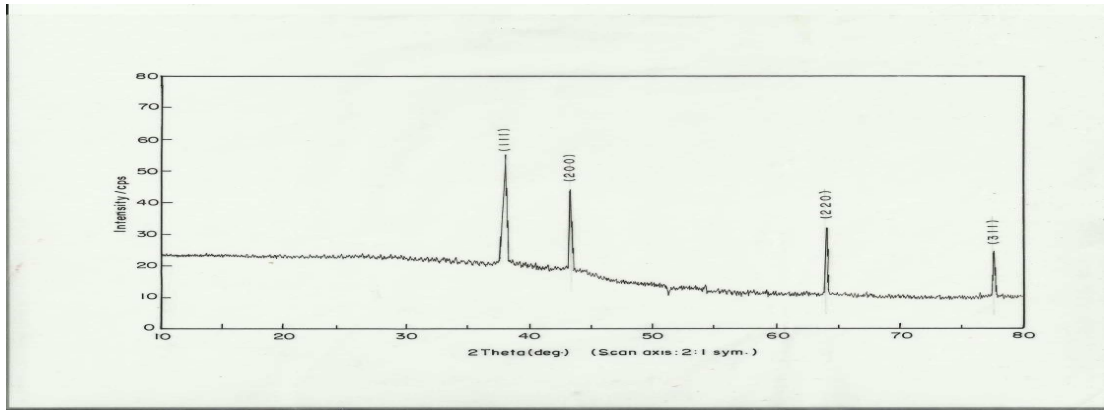


Fig. 4: XRD pattern of synthesized silver nanoparticles at 20

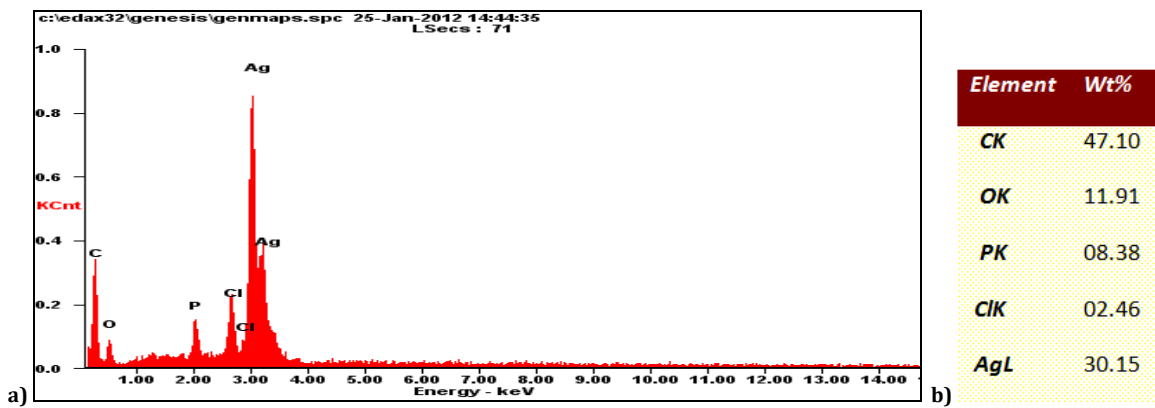


Fig. 5: a) EDS spectra of AgNPs. Silver X-ray emission peaks are labeled. Strong signals from the atoms in the nanoparticles are observed in spectrum and confirms the reduction of silver ions to AgNPs and b) the elemental composition of the AgNPs

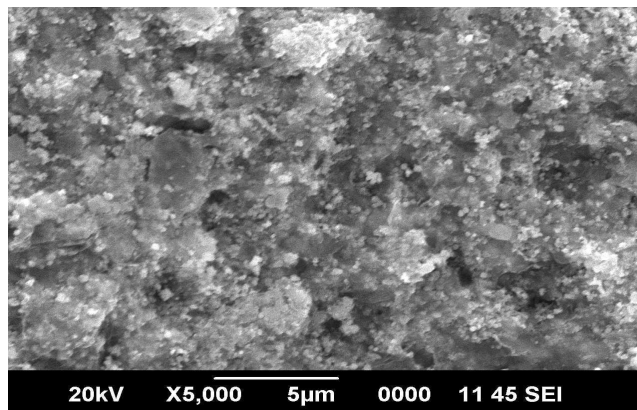


Fig. 6: Scanning Electron Microscopy image of AgNPs

Table 1: Tumor cell proliferation inhibitory activity of synthesized AgNPs using aqueous extract of *Gelidiella sp.* on Hep-2 cell lines

S. No.	Concentration (µg/ml)	Dilutions	Absorbance at 570nm	%Cell viability	%Cell inhibition
1	1000	Neat	0.05	9.80±0.41	90.20
2	500	1:1	0.11	21.56±0.13	78.44
3	250	1:2	0.14	27.45±0.26	75.55
4	125	1:4	0.19	37.25±0.82	62.75
5	62.5	1:8	0.23	45.09±0.35	54.91
6	31.25	1:16	0.28	54.90±0.91	45.10
7	15.625	1:32	0.33	64.70±0.73	35.30
8	7.8125	1:64	0.37	72.54±0.56	27.46
9	3.906	1:128	0.46	90.19±0.18	09.81
10	Cell control	-	0.51	100	0.00

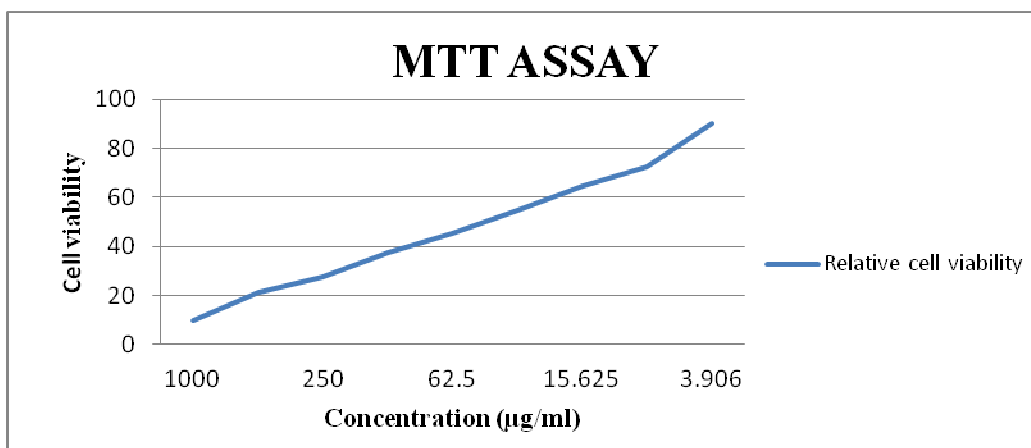


Fig. 7: Effect of AgNPs on Hep2 cell viability



Fig. 8: Photography of a) Cytotoxic changes observed Vs b) Control

CONCLUSION

In conclusion, the bio-reduction of aqueous silver ions by the aqueous extract of *Gelidiella sp.* has been demonstrated. This approach towards the synthesis of AgNPs can be easily scaled up economically and eco-friendly. Applications of the synthesized AgNPs in bactericidal, fungicidal and cytotoxic applications, makes this method potentially for in vivo method.

ACKNOWLEDGEMENT

We are grateful to Dr. Rengaswami, Director, CAS Botany, Madras University for identifying the seaweed. The authors thank, Dr. Sugumaran, Department of Animal Biotechnology, Veterinary College, Vepery, Chennai for TEM analysis, Chemistry Lab for XRD analysis. We also thank Mr. Magesh Peter, NIOT, Chennai for helping us in the initial progress of work.

REFERENCES

- Hutchison JE. "Greener nanoscience: a proactive approach to advancing applications and reducing implications of nanotechnology," ACS Nano 2008; vol. 2, no. 3, pp. 395-402.
- Mohanpuria P, Rana NK, Yadav SK. Biosynthesis of nanoparticles: technological concepts and future applications. J Nanopart Res 2008; 10, 507.
- Savithamma N, Rao ML, Devi PS. Evaluation of antibacterial efficacy of biologically synthesized silver nanoparticles using stem barks of *Boswellia ovalifoliolata* Bal. and henry and *Shorea tumbuggaia*. Roxb. J. Bil. Sci. 2011; (In Press).
- Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramirez JT, Yacaman MJ. The bactericidal effect of silver nanoparticles. Nanotechnology 2005; 16:2346-2353.
- Bellantone M, Coleman NJ, Hench LL. Bacteriostatic action of a novel four-component bioactive glass. J. Biomed. Mater Res 2000; 51:484-490.
- Kanchana A, Balakrishna M. Anti-cancer effect of saponins isolated from *solanum trilobatum* leaf extract and induction of apoptosis in human larynx cancer cell lines. International journal of pharmacy and pharmaceutical sciences 2011; 3(4):356-364.
- Unno Y, Shino Y, Kondo F, Igarashi N, Wang G, Shimura R, Yamaguchi T, Asano T, Saisho H, Sekiya S, Shirasawa H. Oncolytic viral therapy for cervical and ovarian cancer cells by sindbis virus AR339 strain. Clin. Cancer Res. 2005; 11(12):4553-4560.
- Abraham SA, McKenzie C, Masin D, Harasym TO, Mayer LD, Bally MB. *In-vitro* and *in-vivo* characterization of doxorubicin and vincristine coencapsulated within liposomes through use of transition metal ion complexation and pH gradient loading. J. Clin. Cancer Res. 2004; 10:728-738.
- Byrd JC, Lucas DM, Mone AP, Kitner JB, Drabick JJ, Grever MR. A novel therapeutic agent with *in-vitro* activity against human B-cell chronic lymphocytic leukemia cells mediates cytotoxicity via the intrinsic pathway of apoptosis. J. Hematol. 2000; 101(11): 4547-4550.
- Xu H, Yao L, Sung H, Wu L, Chemical composition and antitumor activity of different polysaccharides from the roots *Actinidia eriantha*, Carbohydr. Pol. 2009; Vol.78, 316-322.
- Hsin, YH, Chen CF, Huang S, Shih TS, Lai PS, Chueh PJ. The Apoptotic Effect of Nanosilver is mediated by a ROS- and JNK-Dependent Mechanism Involving the Mitochondrial Pathway in NIH3T3 Cells. Toxicol. Lett. 2008; 179:130-139.
- Park S, Lee YK, Jung M, Kim KH, Chung N, Ahn EK, Lim Y, Lee KH. Cellular Toxicity of Various Inhalable Metal Nanoparticles on Human Alveolar Epithelial Cells. Inhal. Toxicol. 2007; 19:59-65.
- Creighton JA, Eadont DG. Ultraviolet-visible absorption spectra of the colloidal metallic elements. J. Chem Soc., Faraday Trans. 1991; 87:3881-3891.

14. Aoki K, Chen J, Yang N, Nagasava H. Nanochemistry. Langmuir. 2003; 19:9904.
15. Kapoor S. Preparation, characterization, and surface modification of silver particles. Langmuir, 1998; 14:1021- 1025.
16. Huang Z, Mills G, Hajek B. Spontaneous formation of silver particles in basic 2-propanol. J. Phys. Chem. 1993; 97:11542-11550.
17. Gole A, Dash C, Ramakrishnan V, Sainkar SR, Mandale AB, Rao M, Pepsin-gold colloid conjugates: preparation, characterization and enzymatic activity. Langmuir. 2001; 17:1674-9.
18. Macdonald IDG, Smith WE. Orientation of cytochrome C adsorbed on a citrate-reduced silver colloid surface. Langmuir. 1996; 12:706-713.
19. Swanson, Tatge, Natl. Bur. Stand. (U.S.), Circ Vol. 539, No.1, 1953, 23.
20. Udayasoorian C, Vinoth Kumar R, Jayabalakrishnan M. Extracellular synthesis of silver nanoparticles using leaf extract of *Cassia auriculata*. Dig J Nanomater Bios. 2011; 6(1) 279-283.
21. Magudapathy P, Gangopadhyay P, Panigrahi BK, Nair KGM, Dhara S. Electrical transport studies of Ag nanocrystallites embedded in glass matrix. Physics B, 2001; 299:142-6.
22. Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M. Synthesis of Gold Nanotriangles and Silver Nanoparticles Using *Aloevera* Plant Extract. Biotechnol Prog., 2006; 22:577.
23. Bhosle SM, Huilgol NG and Mishra KP. Enhancement of radiation induced oxidative stress and cytotoxicity in tumor cells by ellagic acid. Clinica Chimica Acta. 2005; 359: 89-100.