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Research Article

# **GREEN SYNTHESIS OF SILVER NANOSTRUCTURES AGAINST**

# HUMAN CANCER CELL LINES AND CERTAIN PATHOGENS

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#### ABSTRACT

The exploitation of various plant materials for the biosynthesis of nanoparticles is considered a green technology as it does not involve any harmful chemicals. The present study reports the synthesis of silver (Ag) nanoparticles from silver precursor using the powder of novel *Heliotropium indicum*. Water-soluble organics present in the plant materials were mainly responsible for the reduction of silver ions to nanosized Ag particles. The average sizes of the silver nanoparticles were 80 nm to 90 nm as determined through scanning electron microscopy (SEM). Energy-dispersive X-ray spectra (XRD) of silver nanoparticles revealed that the silver was in pure form. The antimicrobial effect of silver nanoparticles were compared with positive control based on inhibition zone in disk diffusion tests microbial sensitivity to nanoparticles was found to vary depending on the microbial species. All the microbial strains depict higher sensitivity to the higher concentration (30  $\mu$ L) for the test sample when compared to the positive control except B3, B4 and B6. The higher (30  $\mu$ L/disc) concentration of sample got greater sensitivity than (15  $\mu$ L/disc) lower concentration in all the tested microorganisms. The anticancer effects of Ag nanoparticles were challenged with HeLa cancerous cells with ten different concentrations (0.6, 1.2, 2.5, 5, 7.5, 10, 15, 20, 25 and 30  $\mu$ g/ml). The Ag nanoparticles inhibited the growth of the cancer cells significantly, in a dose and duration dependent manner.

Keywords: Heliotropium indicum, Silver nanoparticles, anticancer activity, antimicrobial activity.

#### INTRODUCTION

As the Indian medicinal plants are a treasurehouse of Phytochemicals principles remains to be investigated or untapped it is proposed to investigate potential medicinal plants. The first generations of plant drugs were usually used as crude form but after the industrial revolution, a plant based drugs were emerged and their active constituents will be isolated and purified. Now a day, however most of the clinically used antibiotics are obtained from the microorganisms and plants. But it takes much long time to synthesize in the commercial aspects because the least level of bioactive compounds are available in the plants. Hence, the field of nanotechnology is one of the most active research areas in modern material/medical science (Ankamwar et al 2005). There have been impressive developments in the field of nanotechnology in the recent past year, with numerous methodologies developed to synthesize nanoparticles of particular shape and size depending on specific requirements (Beevi et al 2012; Pandiyarajan et al 2013). Nanotechnology can be termed as the synthesis, characterization, exploration and application of nanosized (1-100 nm) materials for the development of science. The intrinsic properties of metal nanoparticles are mainly determined by size, shape, composition, crystallinity and morphology.

It is also being utilized in medicine for diagnosis, therapeutic drug delivery and the development of treatments for many diseases and disorders. Nanotechnology is an enormously powerful technology, which holds a huge promise for the design and development of many types of novel products with its potential medical applications on early disease detection, treatment, and prevention. The development of reliable green process for the synthesis of silver nanoparticles is an important and alternative aspect of alternative

antibiotic research (Koperuncholan and Ahmed 2011a). Development of nanoparticles forms green chemistry as they are free from pollution and efficient in activity (Wagner et al, 2005). Silver in its ionic form (Ag<sup>+</sup>) is an environmentally friendly antimicrobial that is commonly used against many bacterial and fungal pathogens (Koperuncholan et al 2010). The silver-based inorganic antimicrobial/ anticancer agents were produced in the forms of silver-supported inorganic powders, silver colloids, metal silver powders (Anitha, 2011). The main object of the present study is to synthesize the Ag nanoparticles by using green plants against certain pathogens and cancerous cells. To understand the antimicrobial/ anticancer efficacy of biosynthesized Ag nanoparticles by using Heliotropium indicum plant in the medicinal fields.

#### MATERIALS AND METHODS Study area and sampling

The *Heliotropium indicum* plant materials were collected from Thanjavur, Tamil Nadu during monsoon 2013. The present study, all the chemicals, solvents, antibiotic/standard/ sterile disks and media at AR grade (S.D. Fine-Chemicals Ltd., Qualigens Chemicals Ltd., and Hi-Media Laboratories Pvt. Ltd., Mumbai, India) were used.

#### Preparation of Plant Extraction Aqueous Extraction

The plant materials were collected individually, washed thoroughly thrice with distilled water, shade-dried up to 5 days and prepared fine powder by grinding. The fine powder of the plant material was sterilized at 121°C for 15 min and weighed. Sterilized fine powder, 20 g each was taken, mixed with 200 ml of Milli Q water and kept in boiling water bath at 60°C for 10 min. The extracts were filtered with Whatman 1 filter paper and the filtered extracts were stored in a refrigerator at 4°C for further studies to avoid microbial contamination.

#### **Biosynthesis of nanoparticles**

For the biosynthesis of silver nanoparticles, silver nitrate prepared at the concentration of 10<sup>-3</sup> M with pre-sterilized Milli Q water was used respectively. A quantity of 1.5 ml of each extract was mixed with 30 ml of 10<sup>-3</sup> M of silver nitrate for the synthesis of silver nano particles. Silver nitrate has taken in similar quantities of 1.5 ml each without adding plant extracts to main respective controls. The saline bottles were tightly covered with aluminium foil in order to avoid photo reduction of silver ions, incubated at room temperature under dark condition and observations were recorded at 15, 30, 60, 120 mins.

# Characterization of Nanoparticles UV-VIS Spectroscopy

The Ag nanoparticles were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda-19 to know the kinetic behavior of Ag nanoparticles. The scanning range for the samples was 200-800 nm at a scan speed of 480 nm/min. Base line correction of the spectrophotometer was carried out by using a blank reference.

#### Fourier Transform-Infra Red (FT-IR) Spectroscopy

The analysis of bio-reducing agent present in each of the extracts was measured by FT-IR. After the reaction, a small aliquot of the concentrated reaction mixture was measured in the transmittance mode at 400 to 4000 cm<sup>-1</sup>. The spectra of the extracts taken after the biosynthesis of nanoparticles were analyzed.

#### Dynamic Light Scattering Particle size analyzer

In order to find out the particles size distribution the Ag powder was dispersed in water by horn type ultrasonic processor [Vibronics, model: VPLP1]. Then experiment was carried out in computer controlled particle size analyzer [ZETA Sizers Nanoseries (Malvern Instruments Nano ZS)] to find out the particles size distribution.

## Dynamic Light Scattering Zeta Potential Measurement

Zeta potential describes the electrical potential in the double layer of ions surrounding a particle at the boundary of the particle surface and the adsorbed ions in the diffuse layer (Ives, 1956; Henderson, 2008). Zeta potentials were determined with a Zetaphorementer IV (CAD, France).

#### Scanning electron microscopy (SEM)

In this research work, Jeol JSM-6480 LV SEM machine were used to characterize mean particle size, morphology of nanoparticles. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM. The EDX analysis of Ag sample was done by the SEM (JEOLJSM 5800) machine. The EDX normally reveals the presence of phases.

#### X-ray Diffraction method

The phase evolution of calcined powder as well as that of sintered samples was studied by X-ray diffraction technique (Philips PAN analytical, The Netherland) using Cu Ká radiation. The generator voltage and current was set at 35 KV and 25 mA respectively. The Ag samples were scanned in the 2è ranges 15 to 700C range in continuous scan mode. The scan rate was 0.04o/sec.

# Antimicrobial screening Microorganisms

The test strains were: Aeromonas liquefaciens MTCC 2645 (B1), Enterococcus faecalis MTCC 439 (B2), Klebsiella pneumonia NCIM 2883 (B3), Micrococcus luteus NCIM 2871 (B4), Salmonella typhimurium NCIM 2501 (B5), Vibrio cholerae MTCC 3906 (B6), Candida albicans MTCC 1637 (F1), Cryptococcus sp. MTCC 7076 (F2), Microsporum canis MTCC 3270 (F3), Trichophyton rubrum MTCC 3272 (F4). The cultures were obtained from MTCC, Chandigarh and NCIM, Pune, India. Microbial strains were tested for antimicrobial sensitivity using the disc diffusion method (Bauer et al 1966). This method was used to evaluate in vitro antibacterial and antifungal activity of test sample against certain human pathogenic microorganisms on muller hinton agar (MHA) and potato dextrose agar (PDA), respectively. A sterile cotton swab was used to inoculate the standardized bacterial suspension on surface of agar plate. The 15 and 30  $\mu$ L of test solutions were poured in each disc (6 mm diameter), separately. One separate disc was used for control study by taking sterile triple distilled water (without test sample). The plates were incubated at 37±1°C for 24–48 h (for bacteria) and 25 ±1°C for 48-72 h (for fungus). After incubation, the zone of inhibition was measured with ruler/HiAntibiotic ZoneScale-C. The assays were performed in triplicate and the average values are presented. Methicillin - 10mcg (for bacteria) and Itraconazole - 10mcg (for fungus) was used as positive control. All the media, standard discs and HiAntibiotic ZoneScale-C were purchased from Hi-Media (Mumbai, India).

#### Anticancer screening Cell line and cell treatment

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity. Maintenance cultures were passage weekly, and the culture medium was changed twice a week. The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1x10<sup>5</sup> cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. An aliquot (200 µl) of the sample solution was diluted to 1 ml with serum free medium and additional four serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37°C, 5% CO<sub>2</sub>, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

# Cytotoxicity/ MTT assay

The biosynthesized Ag nanoparticles was dissolved in DMSO, diluted in culture medium and used to treat the chosen cell line (HeLa) over a sample concentration (10 different concentrations - 0.6, 1.2, 2.5, 5, 7.5, 10, 15, 20, 25 and 30 µg/ml) range of 0.6 - 30 µg/ml for a period of 24 h and 48 h. DMSO solution was used as the solvent control. A miniaturized viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetra-zolium bromide (MTT) was carried out according to the method described by Mosmann 1983. To each well, 20 µl of 5 mg/ml MTT in phosphate-buffer (PBS) was added. The plates were wrapped with aluminum foil and incubated for 4 h at 37°C. The purple formazan product was dissolved by addition of 100 µl of 100% DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for four replicates. Each and used to calculate the respective means. The percentage of inhibition was calculated, from this data, using the formula:

#### <u>Mean absorbance of untreated cells (control) – mean absorbance of treated cells (test)</u> x 100 Mean absorbance of untreated cells (control)

The  $IC_{50}$  value was determined as the complex concentration that is required to reduce the absorbance to half that of the control.

# **RESULT AND DISCUSSION**

# Green synthesis of Ag nanoparticles

The plant aqueous solution and silver nitrate solutions were prepared separately. A quantity of 1.5 ml of plant extract was mixed with 30 ml of 10-3 M of silver nitrate for the synthesis of silver nano particles. During silver nanoparticles synthesis, the change of color from pale green to brownish color suggested the formation of silver nanoparticles.

#### **UV-VIS spectral analysis**

The UV-VIS spectroscopic studies revealed the presence of beard peaks at 420 nm Figure 1. The absorption spectra of Ag nanoparticles formed in the reaction media has absorbance maxima at 421 nm. A remarkable broadening of peak at around 350 nm to 480 nm indicates that the particles are polydispersed. During each time interval, the peak became distinct and rising. This peak rising clearly denoted the increasing nanoparticles synthesis as the time increases. Huang et al., (2007) reported formation of silver nanoparticles when constant aqueous AgNo3 at 50 ml, 1 mM with 0.1 g biomass produced silver nanoparticles as indicated by sharp absorbance at around 440 nm in Cinnamomum camphora.

# Fourier Transform Infra-Red Spectroscopy

The FTIR spectrum of the Heliotropium indicum extract was given Figure 2. Wherein some pronounced absorbance was recorded in the region between 4000 and 400 cm<sup>-1</sup>. They include 3435(secondary amine, free, N-H asymmetric stretching), 2368 (Tertiary amine salt, -NH+ stretching), 2076 (Diazo, RCH=N=N Stretching), 1636 (Nitrate, O-NO<sub>2</sub> Stretching asymm) and 687 (C-S, R-C-CH<sub>3</sub> stretching for sulphur compounds), cm<sup>-1</sup> (Table 1). The FTIR spectrum of the Heliotropium indicum extract with Silver nitrate solution was given Figure 2b. Wherein some pronounced absorbances were recorded in the region between 4000 and 400 cm<sup>-1</sup>. They include 3434(secondary amine, free, N-H asymmetric stretching), 2371 (Tertiary amine salt, -NH+ stretching), 2076 (Diazo, RCH=N=N Stretching), 1636 (Nitrate, O-NO<sub>2</sub> Stretching asymm), 1365

(Monametric, O-H plane bending),1229 (Formats, Acetates, propionate and higher ester, C-O-C stretching) and 687 (C-S, R-C-CH<sub>3</sub> stretching for sulphur compounds),  $cm^{-1}$ .

# Scanning Electron Microscope (SEM)

SEM absorption of the products was recorded as synthesis of nanoparticles spherical in structure of about 90 nm in diameter in the case of silver nanoparticles (Figure 3).

# Energy Dispersive Spectroscopy (EDS)

EDS revealed the presence of pure silver (Figure 4) nanoparticles in higher percentages. Silver peak is higher than other peak. The EDX reading proved that the required phase of silver (Ag) is present in the sample.

# Dynamic Light Scattering of Particle Size Analyzer

The Figure 5 shows the particle size of the nanoparticles samples. After analyzing data, it was found that Ag nanoparticles size were in the range of 80-120nm. However, beyond 100 nm range the percentage of nanoparticles present is very less. The highest fraction of Ag NP present in the solution was of 73nm.

# Dynamic Light Scattering of Zeta Potential Measurement

The Figure 6 shows the the zeta potential ( $\zeta$ ) is a measure of the electrostatic potential on the surface of the nanoparticle and is related to the electrophoretic mobility and stability of the suspension of nanoparticles of the nanosilver. The overall absorbance of Zeta Potential revealed the incipient instability nature occurred in this sample.

# X-Ray Diffraction study

In this result, peaks were observed at  $2\theta$  of 38, 44, 65 and 77 are corresponding to the Bragg's reflections such as (111), (200), (220) and (311). This may be due to the crude nature of the extracts containing other metabolites and salts. These components would have reacted with the ionic silver during the synthesis reaction Figure 7.

# Antibacterial and Antifungal screening

The antimicrobial activity of test sample was examined with various pathogenic

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microorganisms using the (measure the inhibition zone) disc diffusion test. Anitha et al (2011) found that the Ag nanoparticles have exhibited considerable activity against some human pathogens. The antimicrobial property of silver is found to be the best among different metals in the following order Ag >Hq > Сп >Cd>Cr>Pb>Co>Au>Zn>Fe>Mn>Mo>Sn (Petica 2008). The results of the antimicrobial activities are summarized in Figure 8. In the present study, higher (30  $\mu$ L/disc) concentration of sample got greater sensitivity than (15  $\mu$ L/disc) lower concentration in all the tested microorganisms. In this study, all the pathogens were fairly affected and nil effect was not observed in the test samples. The silver nanoparticles not only interact at the surface of cell membrane, but also enter inside the bacteria and cause damage of the cells by interacting with phosphorus/ sulfur containing DNA and its replication (Feng 2000). In bacteria, the test sample was most effective against B5 while smaller effect was noticed from B4. In fungi, this was effective against F4 whereas smaller effect was observed in F2. All the microbial strains depict higher sensitivity to the higher concentration (30  $\mu$ L) for the test sample when compared to the positive control except B3, B4 and B6 (Koperuncholan and Ahmed 2010). studied the increasing use of silver based products as antimicrobial agents and he concluded that the silver materials are an efficient alternative to antibiotics for the treatment. This nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Sondi 2004 and Morones 2005). There is no antimicrobial

activity in solution devoid of sample used as a vehicle control (sterile triple distilled water), reflecting that antimicrobial activity was directly related to the sample.

# MTT assay

The cytotoxic effect of the biosynthesized Ag nanoparticles was examined on cultured HeLa cells by exposing cells for 24 h and 48 h to medium containing the complex at 0.6 – 30 g/ml concentration (Figure 9). The Ag nanoparticles inhibited the growth of the cancer cells significantly, in a dose- and duration dependent manner. The cytotoxic activity was determined according to the dose values of the exposure of the complex required to reduce survival to 50% (IC50), compared to untreated cells. The IC50 values are given in Table 2. The Ag nanoparticles showed highly effective cytotoxic activity against Hela cells at 48 h than 24 h in the treatment group. The cytotoxic effect of the sample may be interpretable as due to its amphiphilic nature and, hence, would penetrate the cell membrane easily, reduce the energy status in tumors and also alter hypoxia status in the cancer cell micro environment, which are factors that would influence the antitumor acidity. It is known that biosynthesized Ag nanoparticles have a wide range of biological activities such as antitumor, antifungal, apoptosis (Jeyaraj, 2013 and Jeyaraj, 2013a), interaction with DNA thereby inhibiting replication, transcription, and other nuclear functions and arresting cancer cell proliferation so as to arrest tumor growth.

Plant Extract			Phyto derived Nanoparticles		
F	Туре	Vibration Mode	F	Туре	Vibration Mode
3435	Secondary amine (free)	N-H asymmetric stretching	3434	Secondary amine (free)	N-H asymmetric stretching
2368	Tertiary amine salt	-NH+ stretching	2371	Tertiary amine salt	-NH+ stretching
2076	Diazo	RCH=N=N Stretching	2076	Diazo	RCH=N=N Stretching
1636	Nitrate	O-NO <sub>2</sub> Stretching asymm	1636	Nitrate	O-NO <sub>2</sub> Stretching asymm
-	-	-	1365	Monametric	O-H plane bending
-	-	-	1229	Formats, Acetates, propionate and higher ester	C-O-C stretching
682	Sulphur compounds	C-S stretching	687	Sulphur compounds	C-S stretching
F - Frequency					

Table 1: FTIR analysis of vibration modes and function groups of Heliotropium indicum



Fig. 2a: FTIR analysis of vibration modes on Heliotropium indicum



REF 4000 99.96 2000 92.65 600 3434.87 15.88 2371.31 98.16 2076.64 90.40 1636.94 38.94 1365.53 94.88 1229.14 96.95 687.96 69.68





Fig. 3: SEM –microscopic view of silver nanoparticles





Fig. 4: EDX reading of silver nanoparticles



Fig. 7: Nanoparticles characterized by Powder XRD Data



Fig. 9: In vitro cytotoxicity of AgNPs by Heliotropium indicum



Fig. 10: *In-vitro* Cytotoxicity of biosynthesized silver nanoparticles by *Heliotropium indicum* (The Aero mark (2) indicated that the HeLa cells damages)

#### CONCLUSION

Nanotechnology is a most promising field for generating new applications in medicine. The present investigation is highly warranted to through more light upon the Ag nanoparticles from medicinal plants will helpful to investigate the active principle action for biochemical and molecular studies. At nanoscale, silver exhibits remarkably unusual physical, chemical and biological properties. Effective green synthesis of nanoparticles will have greater implication and application in biomedical research. In this study nanoparticles of 80  $\pm$  90 nm were synthesized by using Heliotropium indicum, as confirmed by SEM TEM. These nanoparticles showed and characteristic absorption peak at 420 nm in UV spectra. The possibility of protein as a stabilizing material in silver nanoparticles is revealed by FTIR analysis. The crystalline structure of silver nanoparticles was confirmed by XRD. The antimicrobial and anticancer study was confirmed that Ag biosynthesized nanoparticles will act as an alternative antibiotic in future.

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