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SYNTHESIS OF SILVER NANOPARTICLES AND ITS CYTOTOXIC EFFECT AGAINST THP-1 CANCER CELL LINE

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ABSTRACT

Nanotechnology is a field of science which involves the synthesis and applications of nanoparticles. Silver nanoparticles (AgNp) have applications in various fields due to their unique optical, physical, electrical and medicinal properties. In this study, silver nanoparticles were synthesized by biophysical method. Silver nanoparticles were characterized by using UV-Visible absorption spectroscopy (Surface Plasmon absorption at 418-420 nm), Energy Dispersive Spectroscopy (EDX), Scanning Electron Microscopy (SEM) and X- ray diffraction (XRD). The cytotoxic effect of silver nanoparticles on acute myeloid leukemia cell line (THP – 1 cell line) was studied *in vitro*. The activity of chemotherapeutic drugs Cyclophosphamide and Busulfan was enhanced in presence of silver nanoparticles whereas Mercaptopurine was found to be more effective when used individually against the THP- 1 cell line.

KEYWORDS : Silver nanoparticles, Anti-cancerous property, Cyclophosphamide, Mercaptopurine, Busulfan

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INTRODUCTION

Nanotechnology is a promising arena and has exposed a panorama of applications in industrial, mechanical and medical field. In general nanoparticles are defined as particles with size < 100nm. Various methods are used for the synthesis of metallic silver nanoparticles like chemical [1], electrochemical [2], laser ablation [5], photochemical [4] and biological, γ-radiation [3]. Among all metallic nanoparticles, silver (Ag) nanoparticles are used in diverse fields because of their unique physical, chemical as well as their anti cancerous property [6] and antimicrobial [7].Smilarly nanoparticles of zinc oxide, iron oxide, gold and other metals of nanoparticles exhibits unique physical, chemical as well as biological properties. Silver nanoparticles are generally produced by chemical methods including reductions of silver salts such as silver nitrate, silver sulphate by different reducing agents such as hydrazine hydrate [8], ethanol [9], Isopropyl alcohol [10], Polyvinyl Alcohol [11] and also by using other methods such as the sol-gel route [12] and chemical precipitation[13]. Ag nanoparticles were produced by physical methods including, γ-radiation [3], microwave irradiations [9], and ultra sonication vibrations [10].

In recent years, the approach of production was shifted towards less harmful and eco friendly biological methods which involve extracts from plants such as *Carica papaya* [14], *Ocimum*[15] and *Capsicum annuum*[16]. Synthesis of nanoparticles can be brought about by using some microorganisms such as *Bacillus subtilis* [17] and fungi like *Penicillium sp.*containing extracellular or intracellular enzymes[18]. Biosynthetic methods utilize either biological microorganisms or plant extracts which have emerged as a simple and possible alternative to chemical synthesis procedures and physical methods[17]. Some bacteria and fungus secrete minute, diffusible redox compounds that can operate as electron shuttle linking the microbes and the insoluble iron substrate. It was evident that such compounds or reducing agents (*e.g.*Glycosyltransferase, Reductase, and Hydroquinones) reduce the metallic ions to nanoparticles[19]. Such reduction takes place within minutes and is very rapid[20].Microwave radiation[9], γ-radiation[3] raise the temperature of system which supports the synthesis of Ag nanoparticles[19].

Silver Nanoparticles displays a synergistic effect[21] and also a cytotoxic effect on cell viability which have a chief role in antitumor and anticancer effect[22]. Silver nanoparticles aid in gathering and transporting drug into the cancer cells[21] and they also obstruct with metabolism of cancer and tumor proliferation[6]. There are diverse types of leukemia like chronic lymphocytic leukemia, acute lymphocytic leukemia, chronic myeloid leukemia and acute myeloid leukemia[23]. Several drugs such as Cyclophosphamide, Mercaptopurine and Busulfan are used in leukemia treatment. Cyclophosphamide and Busulfan are alkylating agents[24, 25] and Mercaptopurine is analogue of Purines which is also known as 6-Mercaptopurine[26]. Cyclophosphamide is a precursor of the alkylating agent nitrogen mustard, an antineoplastic and an immunosuppressive agent which activates active aldophosphamide in the liver[24]. Busulfan has been used in the analgesic treatment of chronic myeloid leukemia[25]. In this study we evaluate the role of silver nanoparticles in enhancing the cytotoxic effect of Cyclophosphamide, Mercaptopurine and Busulfan against THP-1 (human acute myeloid leukemia) cell lines.

MATERIALS AND METHODS

All the chemicals used in the experiment were of analytical reagent (AR) grade. Silver nitrate ($AgNO_3$), Sodium Chloride (NaCl) and Tryptone was purchased from SRL. DMEM medium and DMSO were purchased from HIMEDIA R. Cyclophosphamide Injection I.P (Endoxan™-N) and Mercaptopurine was purchased from Cadila Healthcare Ltd. Busulfan was purchased from Celon Laboratories.

(1) Synthesis of Silver nanoparticles (Biophysical method):

24 hr old culture of *Bacillus subtilis* was inoculated in Luria broth (peptone 1g/L, yeast extract- 0.5%, trypton-1%, and pH – 7) and incubated for 36 hours. After incubation, the broth was subjected to centrifugation at 10,000 rpm for 10 minutes. The supernatant was added to equal volume of 3.5mM aqueous $AgNO_3$ and pH was adjusted to 9. The sample was placed in a domestic microwave and further subjected to microwave irradiation at 2.45 GHz in cyclic mode (on 15 seconds, off 15 seconds) at power output of about 100 W to prevent overheating. At 5, 7, 9, 12 and 15 cycles samples were collected and subjected to UV-Vis spectra. The spectra of these samples were recorded at room temperature.

Characterization of AgNp by UV-Vis spectroscopy, SEM, EDX and XRD

The silver nanoparticles were characterized by using UV-Vis spectroscopy (Shimadzu UV 1600). The sample were obtained after 5^{th}, 7^{th}, 9^{th}, 12^{th} and 15^{th} cycle of microwave irradiation and then subjected to U-Vis spectroscopy. The morphology of the obtained nanoparticles is characterized by using JSM 6360, an analytical Scanning electron microscope of JEOL .The image was obtained (Scanning mode is in vacuum at PHA mode - t3 at Acc Voltage 20.0 kV). Chemical composition of obtained nanoparticles was characterized by Energy dispersive spectroscopy (EDX) spectrum of sample at Acc Voltage 20.0 kV. The X-ray diffraction (XRD) of obtained nanoparticle powder was performed using P W1840 diffractometer control, Philips with scan speed 0.1, scale 20 ^{0} to 80 ^{0} C. The average crystallite size (t) was calculated by the Scherrer's relation: $t = 0.94/\beta \cos\theta$, where l is the wavelength of X-ray and β is the half maximum line width.

(2) In vitro cytotoxicity study of Ag NPs

The THP-1 cell culture was purchased from National Centre for Cell Sciences, Pune. They were cultured in Roswell Park Memorial Institute 1640 medium (Himedia®) with 10% Fetal Calf Serum (FCS). THP-1 (Acute Myeloid Leukemia cell line) cells were seeded in 96-well plates at a density of 1.0×10^4 cells/well. It was then incubated overnight at 37°C in a 5% CO_2 humidified environment. The cells were then treated with 10 µg/mL Ag nanoparticles and 10 µg/mL Drugs (Cyclophosphamide, Mercaptopurine and Busulfan) for 24 h. The final concentration of Ag nanoparticles and Drug was 100 µg ml^{-1} (dissolved in Phosphate Buffered Saline) respectively. Control group was cultivated without Ag nanoparticles and Drug on the same conditions. After all samples were cultured for 24 hours, 20 µL of 5 gL^{-1}3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was added. It was then incubated for additional 4 hours. Then, the plates were centrifuged at 1000 rpm for 10 minutes, the supernatant was discarded lightly, after which 150 µL of DMSO (Dimethyl sulphoxide) was added into each well followed by gentle shaking in the shaker incubator at 37°C for 10 min. The optical density (OD) was recorded at 492 nm on micro plate reader (i mark™). The experiment was carried out in triplicates.

The Cell viability was expressed as follows

Cell viability (%) = [A]test/ [A] Control × 100%,

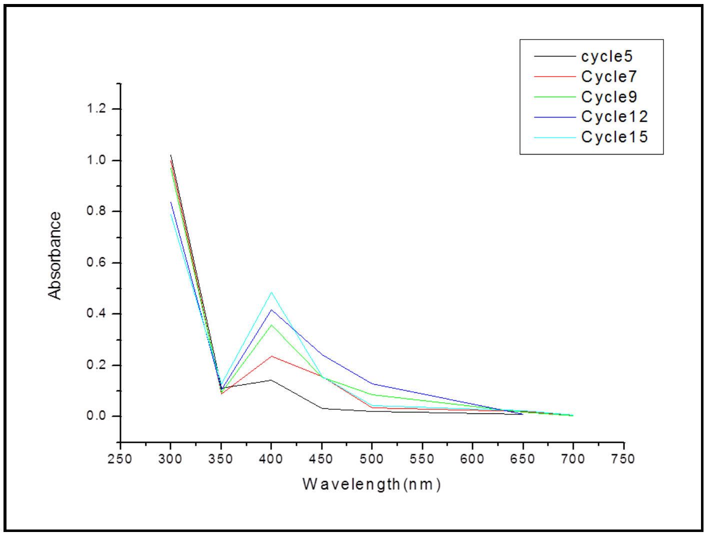
[A] Represents the absorbance value noted at 492 nm.

RESULTS AND DISCUSSION

1. Synthesis of silver nanoparticles

Bio-physical method consists of the combination of a biological element i.e. bacterial supernatant (*Bacillus subtilis*)[20] and physical element i.e. microwaves making it very quick[16]. Microwaves provide consistent heating which decreases aggregation. The dielectric constant of metal plays a key role in the creation of stable size and uniform nanoparticles[19].

Graph 1
UV Vis spectrometer obtained from bio-physical method



From graph no. 1 it can be observed that the silver surface plasmon band occurs at around 410 nm and a steady raise in the intensity of particles as the number of cycles are increased.

It can be noticed that surface plasmon absorption band of Ag particles was observed just after 5 cycles of irradiation and enlarged with further cycles of irradiation. The surface Plasmon bands of the aqueous solution include isolated silver nanoparticles with no evidence for aggregation remains close to 410 - 415nm during the reaction period. It suggests the presence of roughly spherical silver nanoparticles which was confirmed by SEM imaging (Fig.1). Speedy microwave heating also offers uniform nucleation and growth conditions, leading to homogeneous nanomaterial with smaller sizes [9, 19].

2. Characterization of silver nanoparticles

Scanning Electron Microscopy (SEM) of silver nanoparticles

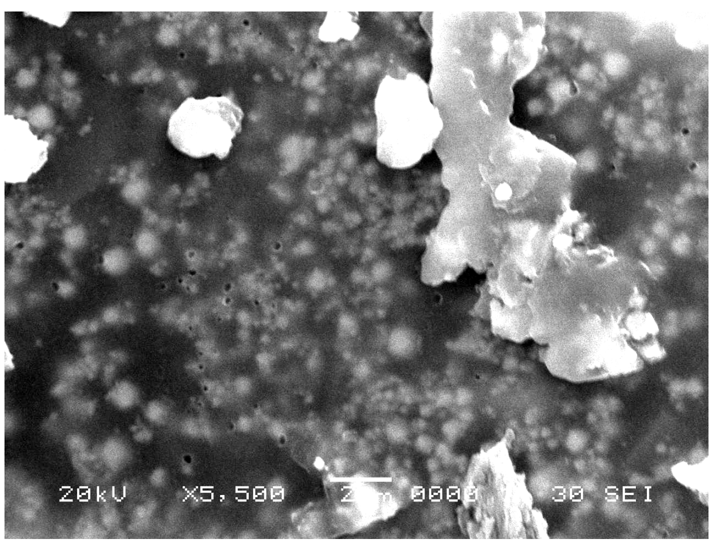
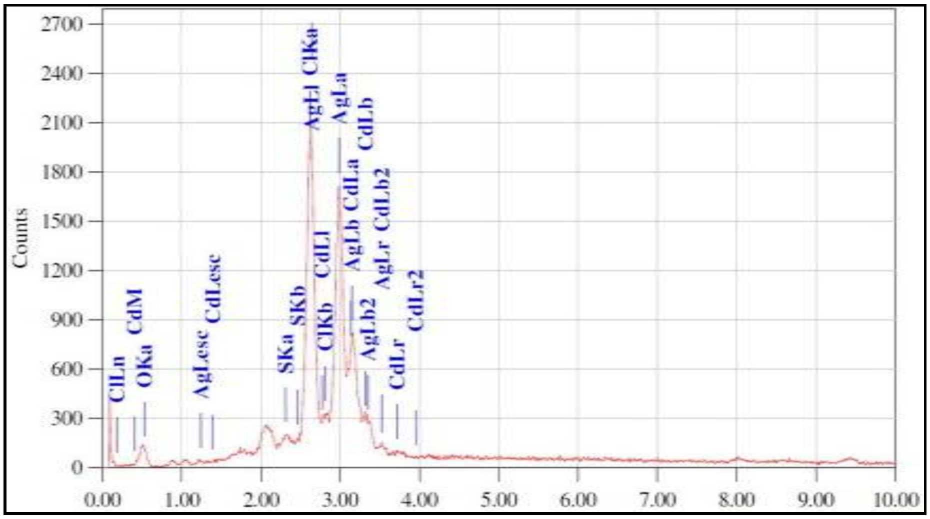


Figure 1
Scanning Electron Microscopy (SEM) of silver nanoparticles

The above figure clearly shows that the size of particles was in nano range. It can also be observed that the nanoparticles produced by the biophysical method have a spherical morphology.

Graph 2

Energy dispersive X-ray (EDX) spectroscopy of silver nanoparticles

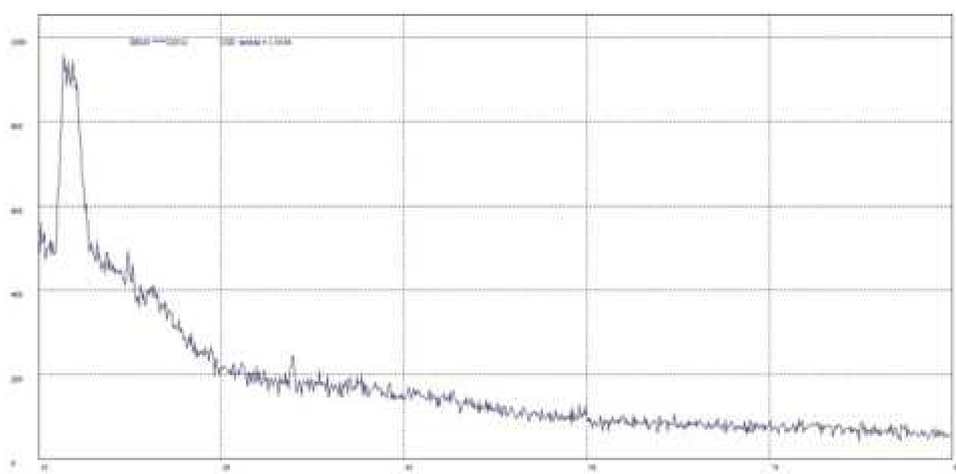


EDX spectra of spherical nanoparticles produced by Bio-physical method.

The nanoparticles have the peak around 1.3 ke V, 2.2 ke V, 3 ke V, 3.20 ke V that are corresponding to binding energy of Ag L_{sec}, AgLa, $AgLb_{1,}AgLb_2$. While binding energy around 3.3 ke V and 2.6 ke V belongs to Cd k_a and Cl k_a respectively. The result indicates that the synthesized product is composed of Ag nanoparticles.

Graph 3

X-ray Diffraction spectroscopy (XRD) of silver nanoparticles



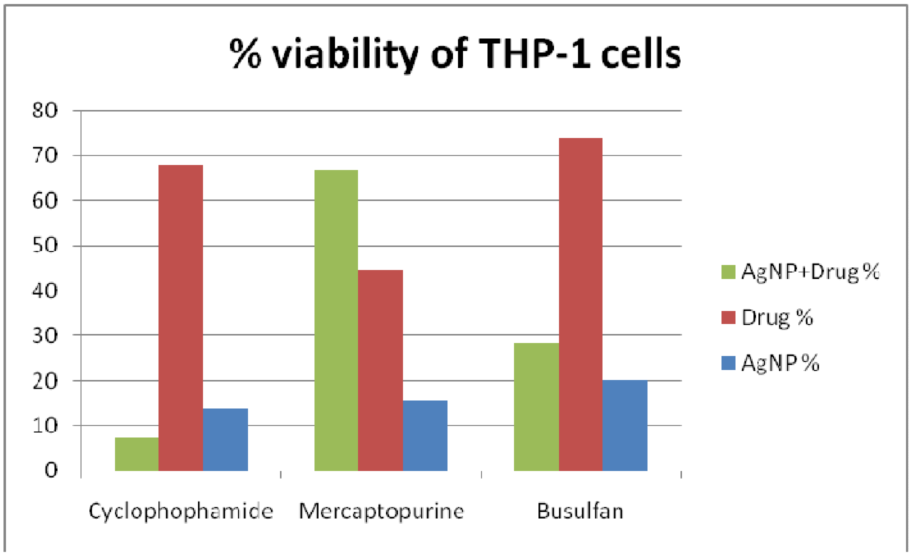
XRD graph

XRD (manual mode) was used to characterize the AgNp. The 2d angle is converted into the diameter using Scherrer formula. The size of silver nanoparticles synthesized by biophysical method was found to be around 20 nm.

3. *Invitro cytotoxicity study of Ag NPs*

The Cytotoxicity of AgNp was assessed against cancerous cell lines. Chemotherapeutic drugs like Busulphan, Cyclophosphamide and Mercaptopurine were used to compare the cytotoxic effect of AgNp by the MTT assay.

Graph 4
Comparative study of Ag NP, Drug and AgNP +Drug showing the percentage viability of THP-1 cell line.



It is observed from graph no. 4 that silver nanoparticles and chemotherapeutic drugs (Cyclophosphamide, Mercaptopurine and Busulfan) show cytotoxic result against THP-1 cells *i*n vitro. The combination of drugs (Cyclophosphamide or Busulfan) and silver nanoparticles exhibits a extensive cytotoxic effect against THP-1 cells as over that of with Mercaptopurine. The cytotoxic effect of Silver nanoparticles because of the nano size, surface area and surface functionalisation are chief factors that control bio - kinetics and thus toxicity leading to lesser cell viability. A possible mechanism of toxicity is projected which involves disruption of the mitochondrial respiratory chain by Ag-nanoparticles leading to creation of Reactive Oxygen Species and disturbance of ATP synthesis, which in turn leads to DNA damage [7]. It is estimated that DNA damage is increased by deposition, followed by interactions of Ag-nanoparticles to the DNA leading to cell cycle arrest in the G2/M phase [23]. The high sensitivity of THP-1 could be explored more for evaluating the probable use of Ag-nanoparticles in cancer therapy. The main effect of Cyclophosphamide is because of its metabolite phosphor amide mustard. This metabolite is only produced in cells that have small levels of ALDH. Phosphor amide mustard forms DNA interstrand, crosslinkages and also intrastrand crosslinkages at guanine N-7 positions. This process is irreversible and leads to cell death [24]. The cytotoxic result of Busulfan is because Busulfan on hydrolysis releases methanesulfonate groups and which undergoes an intermolecular displacement to yield the final products such as methanesulfonic acid and tetrahydrofuran, alkylate the DNA resulting into obstruction of DNA replication and RNA translation leading to the disruption [27]. This alkylation results in DNA- DNA cross linking i.e. Guanine-Adenine or Gaunine interstrand or DNA – Protein crosslinking [25]. Such crosslink takes place by SN2 reaction mechanism [27]. These kinds of damages cannot be repaired by cellular machinery and hence cell undergoes apoptosis mostly via p53 pathway[25]. Mercaptopurine is an analogue of the Purine bases. It exhibits Cytotoxicity through inhibiting *de novo* purine synthesis by incorporating the active metabolites into DNA and RNA of cell [26]. In case of the mixture of AgNP and Cyclophoshoamide or Mercaptopurine or Bulufan, AgNp may have damaged to cell membrane leading to disruption in permeability that may have raised the uptake of the drug into cells. The nanoparticle may affect factors that control bio – kinetics, DNA synthesis or cell growth. Among Cyclophosphamide, Mercaptopurine and Busulfan; Cyclophosphamide shows more promising results than Mercaptopurine and Busulfan when used in combination with AgNp.

CONCLUSION

Nanoparticles were synthesized by Bio-Physical method and were characterized by UV-spectroscopy SEM, EDX and XRD. They have spherical shape and have size in the range of 17 nm. The combination of Ag nanoparticles and Cyclophosphamide or Busulfan was found to be more effective than activity of the cancer drug individually, whereas Mercaptopurine is not effective when used in combination with silver nanoparticles. With use of Ag nanoparticles and the drugs in combination the required amount of drug will be reduced which may lead to lesser the side effects of the chemotherapeutic drug.

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