In-vitro study on genotoxicity of Silver Nanoparticles coated with herbal extract

A Thesis Submitted to

NIRMA UNIVERSITY

In Partial Fulfillment for the Award of the Degree of

Master of Science

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April-2018

Declaration

I declare that the thesis entitled "**In-vitro study on genotoxicity of Silver Nanoparticles coated with herbal extract"** has been prepared by **Ayushi Srivastava (16MMB003), Marcellin Mecwan (16MMB027), Prakash Chaudhary (16MBC004)** and **Sandhya Kasundra (16MBC027)** under the guidance of **Dr. Sonal Rajiv Bakshi,** Assistant Professor of Institute of Science, Nirma University, Ahmedabad. No part of this thesis has been formed the basis for the award of any other degree or diploma.

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Acknowledgement

Acknowledgement

This thesis is the end of our journey in obtaining our M.Sc. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people including our well wishers, friends, colleagues and various institutions. At the end of our thesis we would like to thank all those people who made this thesis possible and an unforgettable experience for us.

First and foremost, we would like to thank our mentor **Dr. Sonal Bakshi** for her guidance and all useful discussion that we had during our dissertation period. We are very grateful to her to teach us the value of time and discipline in Science. We would like to thank **Prof. Sarat Dalai, Director in-charge, Institute of Science, Nirma University** for all support, we thank Nirma Education and Research Foundation (NERF), Nirma University for the infrastructure and facilities that help us to complete our dissertation work.

We have been very lucky throughout most of our M.Sc, this is due in a large part to the gracious support of **Dr. Shalini Rajkumar, Dr. Shiram Sheshadri, Dr.Amee Nair, Dr. Vijay Kothari and Dr. Nasreen Munshi Dr. Rajiv Tiyagi, Dr. Pranati Sar**, and **Dr. Kirti Verma**. We would like to express our thanks to **Mr. Sachin Prajapati** and **Mrs.Sweta Patel**, laboratory attendees and **Mr. Hasit Trivedi** non teaching staff of the department who had provided us all the requirements that we need even if they had to go side tract to help us.
PhD guide **Mrs Shikha Tiwari** and **Krupali Parmar**, under whose guidance we worked who allowed us to use the facility under their guidance and also gave valuable suggestions which helped us reframe our objectives.

We also owe special thanks to,

Dr. Sonal Thakor, faculty at M.S University Baroda, Chemistry Dept., for giving valuable time, feedback, ideas and allowing us to observe work carried out in their lab at M.S University, Baroda.
Ms Manita, pursuing PhD at M.S University, for synthesizing and providing the information on silver Nano-particles

We would like to thank our Group members, Friends from the M.Sc batch of 2017, Microbiology, Biochemistry, Biotechnology who made the time amazing, jolly, and cheerful.
Finally, we would like to dedicate this work to our Parents, without whose unending support and love from childhood to now, we would never have made it through this process or any of the tough times in our life. Thank you

CONTENTS

Abstract

Abstract

Nanotechnology is a popular technique used in wide range throughout world in many different formulations of nano-particles. Nano-particles are particles between 1 to 100nm in size and can be classified into different classes based on their shapes, size or properties. Due to their broad range of properties and uses in industrial, domestic, cosmetic and biomedical products they are used on a large scale.

Since these particles are used in bulk they are realized in environment where they stay in suspended form and does not degrade easily, causing risk to human exposure and intake. Nano-particles cause an alteration of inherent material alteration which is responsible for death of the cell, expansion of cancer, undesirable effect on reproductive health and fertility disorder. In the human body, skin serves a major route for entry of nano-particles. Nano-particles can also enter into the body via respiratory ways.

Recent studies showed preparation of silver nano-particles by green synthesis approaches that have advantages over conventional methods. This involves chemical agents associated with environmental toxicity.

These processes include capping of NPs along with herbal extract. Studies have proven plant extracts to be an effect source of capping and reducing agent which are required in the field of nano formulations, to keep the particles in dispersed form and prevent its agglomeration. Therefore using the plant extract can be a preventive measure towards the green synthesis and preventing the environmental pollution caused by the accumulation of nano-particles which are in the suspended form.

Our approach is to check the genotoxic dose dependent level as any nano-particle administered at high dose will induce toxicity. Here Silver NPs are coated with cinnamon extract.

Characterization of silver nanoparticles done by Malvern zeta sizer, shows AgNPs size range between 1-100nm and zeta potential range is -9.1mV which shows the particle is in stable form.

To check the cytotoxicity level of AgNPs, we performed MTT assay which shows CHO cell line was viable in response to this particles. For the interaction between nanoparticles and DNA, UV visible & fluorescence spectroscopy were performed. This shows AgNPs were not interacting with DNA.

Chromosomal aberration assay was carried out to check the genotoxicity which shows the toxicity of AgNPs at higher concentration as compared to lower concentration.

List of abbreviation

AgNP: Silver Nanoparticle

ASTM: American Society for Testing and Materials

CA: Chromosome Aberration

DNA: Deoxyribonucleic acid

DPX: Mixture of Distyrene, Plasticizer Xylene

EDTA: Ethylene diamine tetra acetic acid

ENM: Engineered Nanomaterial

ENP: Engineered Nanoparticles

FBS: Fetal Bovine serum

KCL: Potassium Chloride

Mg: Miligram

mM: milli Molar

mm: millimeter

MMC: Mitomycin C

OECD: Organization for Economic Co-operation & Development

PBL: Peripheral Blood Lymphocytes

PHA: Phytohemagglutinin

RBC: Red Blood Cells

RPM: Rotation per minute

RPMI: Roswell Park Memorial Institute

SD: Standard Deviation

SE: Standard Error

µg: microgram

Introduction

Introduction

In this 21st century, nanotechnology is one of the rapidly growing technologies. The use and success of this technology is because of its multipurpose usage. In last decades NPs are found in our day to day life, while at the same time the awareness towards risk of NPs for human and environment are continuously increasing since last decade [11].

Nanotechnology is an emerging field in research, which ranges from 1 to 100nm according to the standard definition of ASTM (American Society for Testing and Materials). Nano-materials are important as the physico-chemical properties of metals are changed and it imparts nano-size which is completely different than bulk metal. These NPs are used in several fields such as electronics, cosmetics, paintings, packaging, and in biotechnology [10].

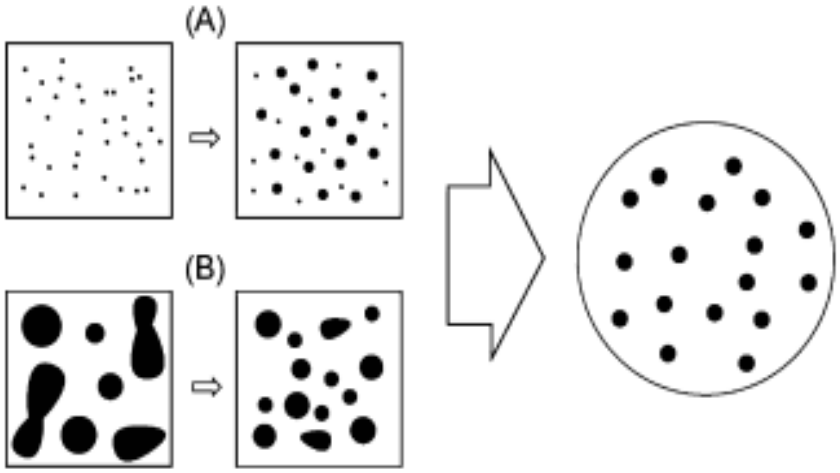
NPs can be manufactured by two methods:

1. **Bottom up :**

These approaches used to produce nanoparticles from atoms which are chemical processed based on transformation in solution.

2. **Top down**:

Top down approaches use larger primary structures which can be externally controlled in the processing of nano-structure.



A= Bottom up B= Top down

Figure.3.1 Bottom up & top down approaches

[Yuliang Wang et al., 2004]

Nanoparticles can be classified into different categories depending on their size, morphology and chemical properties. NPs can be classified as metal NPs, carbon based NPs, ceramic NPs, polymeric NPs and semiconductor NPs. Among all these NPs, metal nanoparticles are focus of interest because of their huge potential in nanotechnology.

Among several metallic nanoparticles, silver nanoparticles (AgNPs) are one of the most vital and fascinating nanomaterials. Because of their unique properties such as anti-microbial agents, they have been used for biomedical applications, in industrial, household, and healthcare related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopedics, drug delivery, as anti-cancer agents, and have ultimately enhance the tumor-killing effects of anti-cancer drugs. Recentl, AgNPs have been used in many textiles, keyboards, wound dressings, and biomedical devices.

AgNPs are widely used in the house disinfectants/medical devices, water purification, wound treatment, sterilization, food sanitation, drug delivery and cosmetics. Silver was effectively used during the 1st world war to prevent microbial growth in wounded soldiers **[20].** Nanosized metallic particles are unique and can considerably change physical, chemical & biological properties due to their surface-to-volume ratio; therefore, these nanopaticles have been adopted for synthesis**.**

Generally, conventional physical and chemical methods seems to be very expensive and hazardous. Where biologically-prepared AgNPs show high stability, high yield & solubility. In our study, AgNPs were synthesized by green synthesis method to reduce the effect of AgNPs in environment, which is found to be eco-friendly. Green synthesis approaches are simple, less expensive and less toxic as compared to synthetic approaches. Green synthesis of NPs by biological methods using micro-organisms, enzymes, plant extract and panchakavya are suggested eco-friendly.

Synthesis of NPs can be done by three methods**:** 1) physical method,

2) Chemical method &

3) Biological method

- The green synthesis of NPs involves following 3 steps:
    1. Selection of environment friendly solvent medium
    2. Use of less toxic reducing agent
    3. Nontoxic material for stabilization **[22]**.

The plant extracts act as a stabilizing agent for synthesis of NPs using biological methods. We have used *Cassia cinnamon* extract as a capping agent for synthesis of AgNPs. It is

available in local market. We have synthesized AgNPs by green synthesis method to check the genotoxic effect on human exposure [16].

Properties and applications of cinnamon:

- Cinnamon extract contain sugars, terpenoids, polyphenols, alkaloids, phenolic acid and protein which are excellent reducing agents in synthesis of NPs.
- They are mainly responsible for the conversion of Ag^{+} to Ag^{o}.
- This bio-reduction of metal ions provide stability of the size and morphology of NPs[6].
- Cinnamon is a spice obtained from the inner bark of several tree species from the genus *Cinnamomum*.
- The aroma and flavor of cinnamon derive from its essential oil and principal component, cinnamaldehyde, as well as numerous other constituents, including eugenol. Cinnamon *Cassia* and *Ceylon* are the two types of cinnamon generally obtained. In our experiment we have used *Cassia.*

Exposure routes of nano-particles:

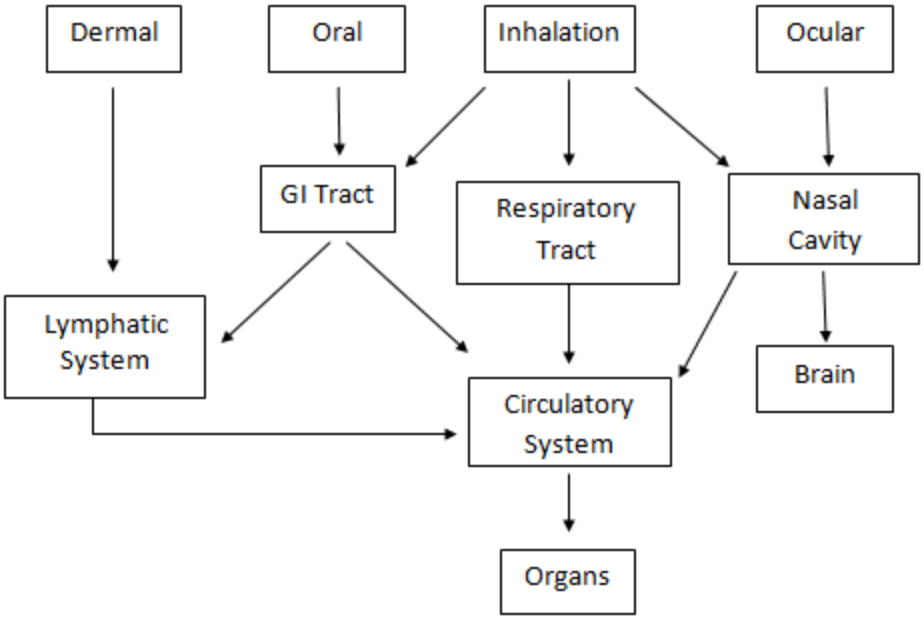


Figure 3.2 Schematic representation of exposure routes

The route of exposure becomes a primary determinant of potential toxicity. The exposure may occur via the following portals of entry: 1. Inhalation via the respiratory tract

2. Dermal exposure via skin

3. Oral exposure via the gastrointestinal tract

The toxicological assessments of NPs in general are questioned due to the questionable dosimetry which is unique to nano form of chemicals. There have been coordinated efforts reported to form standardized and harmonized dosimetry for NPs, so that data can be comparable between labs and results are reproducible.

Special consideration regarding laboratory study of NPs and Dosimetry:

The unique properties of NPs necessitate that special care regarding following to be taken while doing experiments with AgNPs.

A. Storage:

AgNPs are stored away from light at 4-6˚C in a sealed container. Short periods at room temperature are acceptable, however lower temperature storage prolongs the shelf life of AgNPs.

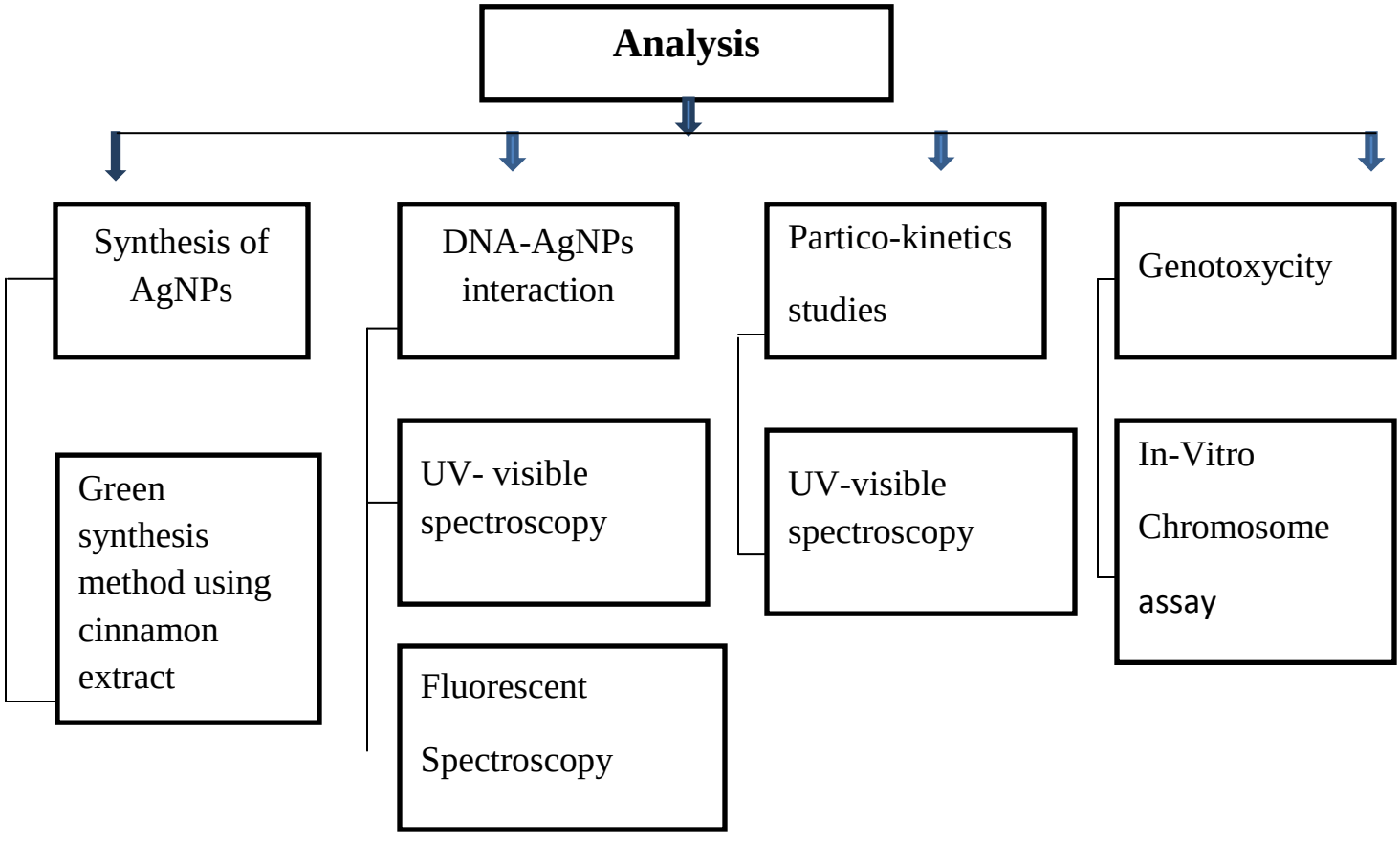
B. Handling:

Resuspend the settled AgNPs by sonication until a homogenous suspension is obtained.

C. Experimental design:

Due to relationship between toxicology and nano-particle size, shape and agglomeration state, it is critical that experiments account for the state of the nano-particles as well as residual chemicals (such as capping agents) in solution

Figure 3.3 Schematic representation of the experimental protocol



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The present study aimed to assess the potential genotoxicity of nano-particles of silver in cultured human blood lymphocytes in terms of induced level of chromosomal aberrations.

- The possible effect of NPs on genetic material by CA assay would reflect genotoxicity of NPs which can be either directly by DNA binding or indirectly by increasing oxidative stress in cellular microenvironment. It was planned to study the interaction of DNA with AgNPs by assessing the degree of fluorescence/absorbance of DNA in presence and absence of NPs which can support the hypothesis that chromosomal damage if any could be due to DNA interaction capacity.

GENOTOXICITY ASSESSMENT:

Genetic toxicity assessment is the evaluation of agent for their ability to induce genetic changes i.e. aberration in the genetic material (DNA) that can be detected at various levels via, molecular level or chromosome level. In humans, the genes are composed of DNA made up of individual units of nucleotide bases. The genes are arranged in discrete physical structures called chromosomes.

Genetic toxicology is the study of genetic damage, agents that induce the damage, its consequences, and mechanisms involved. Genotoxicity can result in significant and irreversible effect upon human health.

Genotoxicity is a critical step in the induction of cancer and it can also be involved in the induction of birth defects and fetal death. The knowledge that many environmental agents are associated with human cancer development and the genetic alterations are the basis for neoplasia scored under the need for testing genotoxic potential of chemicals.

As a result many short term assays are developed. Assays that measure gene mutation are those that detect the substitution, addition or deletion of nucleotides within a gene. Assays that measure chromosomal aberrations are those that detect breaks or chromosomal rearrangements involving one or more chromosomes and also numerical changes i.e., aneuploidy. Thus, the chromosome alterations are of two types:

1. Structural chromosome aberrations (chromosome or chromatid breaks, rearrangements)
2. Numerical chromosomal aberrations (aneuploidy, polyploidy).

Numerous in vivo and in vitro cytogenetic alterations can result from exposure to chemicals, and ionizing or non-ionizing radiations. Cell lines with defects in DNA repair have been exploited to increase the sensitivity for detecting effects of chemicals and radiations.

The association between specific cytogenetic alteration and tumor genesis is strong and it is this relationship that is used as a justification for including cytogenetic endpoints in toxicological evaluations of industrial chemicals and new pharmaceuticals including ecological and environmental monitoring, assessment and clean up and for work place hazard evaluations. Cytogenetic assays have the important advantage that they enumerate damage at the level of individual cell.

Cytogenetic assays are divided into two types:

1. ***In vivo:***
   Chromosomal aberration in rodent bone marrow cells (metaphase analysis)
   In which rats are used as model system or the mouse bone marrow micronucleus test.

2. ***In vitro****:*
   Mammalian cells in culture. There are four genetic endpoints most frequently used in hazard identification assays for risk assessment process. These are structural chromosome aberrations, micronuclei, and aneuploidy and sister chromatid exchanges.

Review of Literature

Review of Literature:

Sr.no	*Reference*	*Synthesis with extract*	*Test*	*Assay*	*Inference*
1	Ali H Saliem et al.,(2016)	*C. zeylanicum* Cinnamon	–	Spectrophotometery Electron Microscopy	• Successful conventional methods of silver nanoparticles synthesis • Nanoparticles were roughly spherical or circular in shape, the no. of particles increased with increasing time due to the variation in the amount of reductive molecules.
2	Khalid H. Abdalla et al.,(2015)	*Cinnamomum cassia*	–	UV-Vis spectroscopy Transmission electron microscopy Antimicrobial activity	• The size distribution histogram of AgNP's for all three methods of preparation. It can be seen that the microwave heating has more narrow distribution with a high yield of size 2nm compared with room temperature with a high yield of size 6nm • The antibiotic disks were applied with aseptic precautions. After incubation, disk was observed; the formation of the clear zone around the LB agar is an indication of antibacterial activity.
3	Xi-Feng Zhang et al.,(2016)		–	UV-Visible Spectroscopy X-ray Diffraction Dynamic Light Scattering FTIR Spectroscopy	• The stability of silver nanoparticles was seen at same wavelength in UV- vis spectroscopy. • XRD technique identifies crystalline nature at the atomic scale

				X-ray Photoelectron Spectroscopy Scanning Electron Microscopy Transmission Electron Microscopy Atomic Force Microscopy Localized Surface Plasmon Resonance	• DLS is mainly used to determine particle size and size distributions in aqueous or physiological solutions • FTIR is frequently used to find out whether biomolecules are involved in the synthesis of nanoparticles

Sr. no	***Reference***	***Cells***	***Test***	***Assay***	***Inference***
5	Asharani et al.,(2008)	Zebrafish embryos *(Daniorerio)*	Genotoxicity	Mortality assay TEM EDS	• AgNPs induce a dose-dependent toxicity in embryos, which hinders normal development
6	Lsima et al.,(2013)	Human lymphocyte and *Allium cepa*	Genotoxicity	Comet assay	• Human cells had greater resistance to the toxic effects of Ag NP in comparison with other cells

7	Hackenberg et al.,(2011)	Human mesenchymal stem cells	Cytotoxicity Genotoxicity	Trypan blue exclusion test Comet assay Chromoosomal aberration test	• Cytotoxic effects were seen at all test exposure period • Comet assay and chromosomal aberration test showed DNA damage
8	Ghosh et al., (2012)	Human lymphocytes	Cytotoxicity	MTT assay WST assay	• Good correlation was seen between the in vitro and in vivo experiments

Table 4.1 Details of review literature

Materials & Methods

5.1 Equipments:

INSTRUMENT	MANUFACTURER
Autoclave	Yorco
Blood Collection Vials	B.D.Bioscience
Blood Collection Needles	Greniar bio-one
Bench top Centrifuge	CM 101 REMI
Electrophoresis Unit	GENEI
Gloves	Kimberley Clark\
Glass wares	Borosil
Incubator	EIE Instruments
Magnetic Stirrer	Tanco
Microscope	Labomed
Microwave Oven	Intellocook (LG)
Nanodrop	Jenway, Geneva Nano
Quartz Cuvette	Sigma Aldrich
Serological Bath	Wiswo
Sonicator	Ningbo Haishu-Sklon
UV-visible Spectroscopy	Agilent
Weighing Balance	Simbadzu
Zeta Sizer	Malvern

5.2 materials:

Material	Catalogue Number	Manufacturer
Acetic Acid	1000632500	MERCK
Agarose Low, EEO	9012-36-6	HIMEDIA
BrdU	B5002	Sigma
Bisbenzimide	B1155-100mg	Sigma
Chromic Acid	1048640500	MERCK
Cinnamon	-	Local market
Colchicine	TCL062-20ml	HIMEDIA
Deionised Water	3363	Mono Quartz
DNA Loading dye	R0611	Thermo Scientific
DPX	GRM655-500G	HIMEDIA
EDTA	TLC099	HIMEDIA
EDTA Vacuettes	455036	BD Biosciences
ETBR	1239-45-8	HIMEDIA
Falcon tubes (15ml)	P10402	ABDOS
Filter paper no. 40		Whattman
Giemsa stain	G9641-5G	Sigma
Glacial Acetic acid	1.93402.0521	MERCK
Isopropanol	1.94524.2521	MERCK
Methanol	1.94516.2521	MERCK
Mytomycin-C	50-07-7	Sigma
Potassium Chloride (KCl)	7447-40-7	MERCK
Qaigen DNA Blood midi Kit	51104	Qaigen
RPMI-1640 Culture Medium	AL165A	HIMEDIA
Silver Nitrate	1.93654.0521	MERCK
Sodium Chloride (NaCl)	1064040500	MERCK
Sodium Citrate	1.93619.0521	MERCK
Sodium Dihydrogen Phosphate	10049-21-5	MERCK
Sodium Hydrogen Phosphate	1065860500	MERCK
Sodium Hypochloride	6184201000	MERCK
Syringe Filter(0.2 µm)	SFNY25R	AXIVA
TrisCl	1185-53-1	HIMEDIA
UV Bulb	-	OSRAM

6.1 Reagent Preparation:

- **KCL:** 0.56 gm of KCL was dissolved in 100 ml of distilled water
- **Fixative:** methanol and acetic acid freshly prepared in 3:1 ratio respectively and chilled
- **Stock Giemsa Stain:** 1 gm Giemsa Powder was added in 54 ml glycerol, mixed and kept in 60°C water bath overnight, 84 ml methanol was added, filtered, and kept in dark bottle
- **Working Giemsa stain:** 4 ml Giemsa stain was added in 25ml Sorenson's buffer with pH 7 and 25ml water, mixed well and prepared freshly whenever required
- **Sorenson's buffer:** 0.345 gm sodium phosphate (NaH_2PO_4) and 0.454gmdibasic sodium phosphate(Na_2HPO_4) was added in 250ml of sterile distilled water for obtaining pH 7.0
- **Chromic acid:** 10% $K_2Cr_2O_7$ was added with 25% H_2SO_4 for slide washing
- **Colchicine:** 10mg absolute powder was dissolved in 10ml autoclaved deionized water and aliquots were dispensed (0.3mg/ml). it was used to block the cells at metaphase stage
- **Hoechst dye (Bisbenzimide) :**
  Stock: 10 mg of Hoescht dye was dissolved in 100ml of Sorenson's Buffer
  Working: 0.1 ml of stock Hoescht dye was taken and mixed with 9.9 ml of distilled water.
- **TAE buffer(stock):** 24.2 gm Tris base (100mM) in 100 ml distil water, 37.22gm EDTA (10mM) in 100 ml water.
- **Working TAE buffer:** 10mM TrisCl and 1mM EDTA

Methods

7.1 Green Synthesis of Silver Nanoparticles:

Principle: The main aim of green synthesis of AgNPs is to reduce the use of toxic compound and prevent the environment from pollution. Therefore biological routes of NPs become more popular. In the green synthesis of NPs, three main conditions are applied:

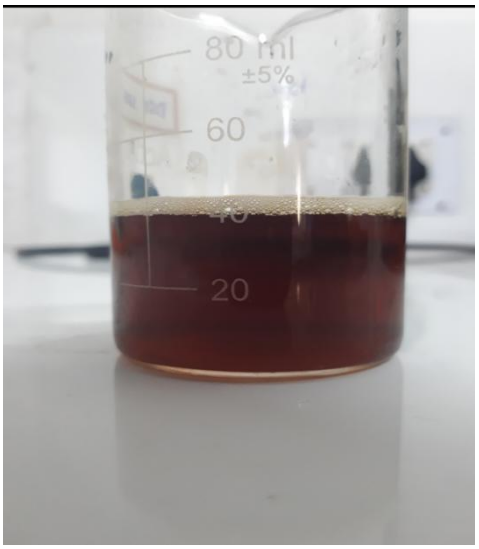
1) Choice of environment friendly solvent medium,

2) Selection of reducing agent &

3) A nontoxic material for stabilization

Here, deionized water used as a solvent medium. Sodium citrate used as a reducing agent, that prevent the metallic form to turned into ionic form. Cinnamon cassia used as stabilizer agent, which prevents the agglomeration of NPs. Addition of reducing agent and $AgNO_3$ in cinnamon extract and giving continuous heating and shaking, color of cinnamon extract turned yellow to brown, that indicate synthesis of AgNPs.

Cinnamon extracts



Cinnamon capped NPs

Figure 7.1 Synthesized AgNPs from cinnamon extract

Protocol :

7.1.1 Extract preparation:

- Take 2000mg of cinnamon powder
- Add into 100ml of deionized water
- And Sonicate it for 30 minutes
- Keep it on stirring for overnight
- Filter it with Whatmann filter paper no. 40

7.1.2 Reducing Agent preparation:

- Take 1gm of Trisodium citrate dehydrate
- Add into 10ml of deionized water

7.1.3 $AgNO_3$ solution preparation:

- Take 0.0084g of $AgNO_3$
- Add into 5ml of deionized water

7.1.4 Synthesis of AgNPs:

- Take 50ml of cinnamon extract
- Stir for 10 – 15 minutes
- Add 1ml of $AgNO_3$ during stirring
- Keep stirring for 10 more minutes
- Add 2.5ml of reducing agent
- Now provide alternate treatment of stirring and heating till the color of extract changes from yellow to brown

7.2 MTT Assay:

Principle:

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells.

- Chinese hamster ovary (CHO) cell line were seeded with DMEM media to 96 well plate and incubated overnight.
- Silver NPs were added and incubation was continued for 48 hour.
- After 48 hrs, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (0.5 mg/ml; Sigma Aldrich).
- Plates were maintained at 37°C for 2 h.
- The medium was then discarded, and DMSO was added to each well to lyse the cells.
- Absorbance was measured using a multiwall spectrophotometer after 24, 48 and 72hours of incubation.

7.3 Isolation of genomic DNA from blood (As per QIAamp DNA blood midi kit)

Human genomic DNA was isolated from whole blood. Blood was collected from a healthy donor. Human genomic DNA isolation was carried out as per the manufacturer protocol:

- blood was obtained in EDTA containing vaccutainer tubes from healthy donors not exposed to any drug, radiation or infection.
- 200 µl QIAGEN protease was pipette into the bottom of a 15 ml centrifuge tube.
- 2 ml blood was added and assorted for a transitory period followed by buffer addition.
- The contents were mixed properly by inversion for at least a minute and incubated at 70°C for 10 minutes.
- 2 ml of ethanol was added and mixed by inversion. Half of this mixture was added to the QIAamp column placed in a 15 ml centrifuge tube, and centrifuged at 3000 rpm for 3 minutes.
- The QIAamp column was then removed, the filtrate discarded and placed back into the 15ml centrifuge tube.
- 2 ml AW1 buffer was then added to the QIAamp midi column and centrifuged at 5000 rpm for 1 min, followed by addition of 2 ml AW2 buffer.
- This column was centrifuged at 5000 rpm for 15 minutes.
- Post centrifugation, the column was transferred and placed in a clean 15 ml centrifuge tube and the filtrate was discarded.
- 300µl AE buffer was added into the column and incubated at room temperature for 5 minutes.
- The column was then centrifuged at 5000 rpm for 2 minutes.
- Pure human genomic DNA was obtained, that was quantified by the Nano drop (Jenway, Geneva Nano).

Quantification by Nanodrop

Sample: Result

Ratio: 1.9 (260/280nm)

Concentration: 85.0ng/µl

7.4 DNA binding studies:

7.4.1 DNA binding study by UV-Vis measurements using PBS buffer

UV absorption spectroscopy is used to determine structural changes of DNA and examine DNA-ligand complex formation [23]. The complex formation is found based on their absorption maxima in the UV visible spectroscopy. Each molecule shows its maximum absorption at a particular wavelength, and when these molecules interact with another molecule, they show maximum absorption at different wavelength or a change in maximum absorbance is observed [4].

Method:

- Take a 10µg of human genomic DNA in 3ml of Phosphate buffered saline (PBS) and mixed well.
- It was titrated with successive additions of a 2µl of AgNPs in a quartz cuvette with 1cm path length in Agilent Cary 60 UV-Visible spectrophotometer.
- Titrations of Manual addition of AgNPs in series of 2µl, 12µl, 22µl, 32µl, 42µl, 52µlcarried out using micropipette.
- The UV absorption spectra for all titrations were recorded in the range of 230–300nm.

7.4.2 DNA binding study by Fluorescence measurements

Fluorescence quenching measurements have been widely used to recognize the Drug-DNA interactions. It also gives information about types and binding mechanisms of metal ions to the DNA. High sensitivity and selectivity is one of the major advantages of Fluorescence spectroscopy [23].

Binding of AgNPs to human DNA was studied using extrinsic fluorescence quench titration method.

To study the interaction between the NPs and DNA, we performed a competitive binding experiment by fluorescent Ethidium Bromide (EtBr) displacement assay. The experiment was performed using EtBr, a common DNA binding and sensitive fluorophore with increasing concentrations of AgNPs, to determine the extent of binding of nanoparticles and human genomic DNA. EtBr consisting of a planar phenenthridinium ring strongly fluoresces in presence of DNA due to intercalation between the adjacent DNA base pairs. It has been previously reported that this enhanced fluorescence can be quenched by the addition of a second molecule [23]. With the addition of a second molecule, there would be a competition between EtBr and the second molecule to bind to human genomic DNA, leading to a decrease in fluorescence intensity. The extent of fluorescence quenching of EtBr bound to human genomic DNA can be used to determine the extent of binding of second molecule with human genomic DNA.

Method:

- Fluorescence measurements were performed on a Cary Eclipse fluorescence Spectrophotometer (Agilent Technologies, USA) equipped with thermo stated cell holder for temperature control.
- A fluorescence cuvette having 1cm path length was used for all experiments and measurements were recorded at 25˚C. EtBr was used as fluorescence stain.
- Intercalation of EtBr between the base pairs of DNA leads to significant increase in fluorescence.
- 20µl sample of human DNA in 2965µl deionized water containing 15µl EtBr was titrated with increasing concentrations of AgNPs. The mixture was kept for 2- 3 minutes before each measurement.
- Manual addition of AgNPs in series of 2µl, 12µl, 22µl, 32µl, 42µl, 52µl.
- Fluorescence emission spectra were recorded at 605nm excitation wavelength, 570-650 nm emission wavelength.
- The fluorescence quenching data were analyzed according to the Stern-Volmer equation 1 (Ranjbar, et al. 3) .
- F0/F= 1 + KSV [Q]
- Where F0 and F are the fluorescence intensities in the absence and presence of different concentrations of quencher [Q]; TiO2 and ZnO NPs, respectively. KSV is the Stern-Volmer quenching constant, which was obtained from the slope of the plots F0/F versus [Q].

7.5 Assessment of Genotoxicity

Short-term human whole blood culture for Chromosome Aberration Assay

Principle:

In vitro chromosomal aberration test was performed to assess whether NPs cause structural chromosomal aberrations (Chromatid or Chromosome) in metaphase cells. Human whole blood lymphocyte cultures were exposed to the test chemical at an appropriate predetermined interval and treated with a metaphase-arresting agent colchicine, at a specific hour. The culture was then harvested, exposed to hypotonic treatment, treated with Carnoy's fixative (fixation) followed by preparation of slides by the air-dry method and Giemsa staining. Metaphase cells were analyzed microscopically for the presence of chromatid type and chromosome type aberrations (OECD, 473).

Protocol:

7.5.1. blood sample collection:

- Blood sample were collected from an healthy individuals
- Blood was collected in sodium heparin evacuates
- Blood was stored at room temperature for not more than 2 hours

7.5.2 NP suspension preparation:

- NPs were sonicated for 10 min
- For sterilization UV exposure to NP was given for 15 minutes before addition in complete culture media RPMI-1640
- Further NPs were added to culture

Culture setting plan:

1) Positive control (whole blood with MMC)

2) Negative control (whole blood with PHA)

3) Vehicle control (whole blood with 1000µl cinnamon extract)

4) Three sample tubes (whole blood treated with 350µl, 650µl, 1000µl AgNPs.)

7.5.3. Initiation of culture:

Day 1 (0 hour):

To 10 ml RPMI-1640 complete growth culture medium (with FBS), 1ml of whole blood was added to each. The culture vials were incubated for 48 hours at 37°C in incubator.

Day 2 (24 hours):

The cultures were mixed well and continued to incubate at 37°C.

7.5.4. Treatment of Culture:

Day 3 (48 hours):

The cultures were exposed to AgNPs. From the stock solution of NPs, 350µl, 650µl, 1000µl NPs were added into culture. For the positive control, 5µlMitomycin-C was added to the culture vial. The culture vials were mixed well and incubated at 37°C for 22 hours.

Day 4 (70th hour):

At the 70th hour of the commencement of blood culture, all the culture vials were mixed with Colchicine at a concentration of 0.5µg/ml, followed by incubation at 37°C for 2 hours.

7.5.5 Harvesting of Cells:

Day 4 (72nd hour):

The culture tubes were centrifuged at 3000 rpm for 10 minutes and supernatant was discarded. Cells were subjected to hypotonic treatment with 7 ml, 0.56% KCl at 37°C for 20 minutes. Around 2 ml chilled Carnoy's fixative (3:1 Methanol: Glacial Acetic acid) was added to each tube and centrifuged again at 3000 rpm for 10 minutes, the supernatant was discarded and the Fixation procedure with chilled fixative was carried out and centrifuged repeatedly until clear pellet was obtained.

7.5.6. Slide Preparation:

Two slides were prepared for each culture by dropping few drops of cell suspension on a pre-cleaned, chilled slide. Slides were then dried and stained with 4% Giemsa solution, followed by mounting using DPX. Each slide was identified using sample name, date, concentration and coded for blind scoring.

7.5.7. Scoring of Slides:

The slides were examined under a microscope and at least 100 well spread metaphases per experimental group were scored under 100X oil immersion objective for the structural changes. The structural chromatid and chromosome aberrations (gaps and breaks) were recorded.

7.5.8. Criteria for selection of Metaphase:

A well spread metaphase containing number of centromeres equal to modal no ± 2. A well spread metaphase containing clearly seen chromosomes without considerable over lapping and free from non-chromosomal material (e.g. dirt, stain particles).

7.6 SCE assay (sister chromatid exchange):

Principle:

sister chromatid exchange(SCE) is the exchange of genetic material between two identical sister chromatid. 5-bromo deoxyuridinie (BrdU) is thymidine analogue; on exposure of UV light in the presence of Hoechst dye, BrdU gets incorporated in the newly synthesizing DNA strand which replaces thymidine appears lighter when stained with Giemsa. The sister chromatid exchange assay is used for testing many mutagenic products and detects the ability of chemical to enhance the exchange of DNA between two sister chromatids of duplicating chromosome. This assay is performed in vitro.

Protocol:

DAY 1(0^{th} hour)

- Under sterile condition (i.e. LAF) 10ml of RPMI-1640 complete media was transferred to a sterile 15ml falcon tube. 1ml of whole blood was added to it.

DAY 3 (48^{th} hour)

- Cell culture were allowed to replicate in the presence of 5-Bromo deoxyuridine and cell culture tube exposed to Different concentration of AgNPs (350µl, 650µl, 1000µl and 1000µl of extract) and mitomycin C 48^{th} hour.

DAY 4 (72th hour)

- The cells were then arrested with colchicines at 72th hour to areesr cells in a metaphase (stage of mitosis). This step is followed by harvesting and chromosomal slide preparation.

Slide preparation:

- Placed the prepared slides and cover with 10mg Hoechst dye under dark condition.
- Incubate for 4 hours under exposure of UV light (when slide becomes dry, flood it again with Hoechst dye repeatedly).
- Rinsed the slide with distilled water.
- Kept the slides in coplin jar containing sodium saline citrate at 60º C for 20 minutes in water bath.
- Rinse it with distilled water and kept for dry.
- Staining was done with Giemsa for 8-10 minutes.
- Observed under oil immersion lens (100X) and scoring was carried out.

RESULT AND DISCUSSION

8.1 Characterization of AgNPs by Zeta sizer:

Zeta size:

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	107.9 nm	35.5 nm	98.6 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	107.9 nm	35.5 nm	98.6 nm

Histogram Operations

% Cumulative (1)	:	10.0 (%) - 67.6 (nm)
% Cumulative (2)	:	50.0 (%) - 100.9 (nm)
% Cumulative (3)	:	90.0 (%) - 159.4 (nm)
Mean	:	107.9 nm

Cumulant Operations

Z-Average	:	185.2 nm
PI	:	0.367

Table 8.1.1 Details of AgNPs size

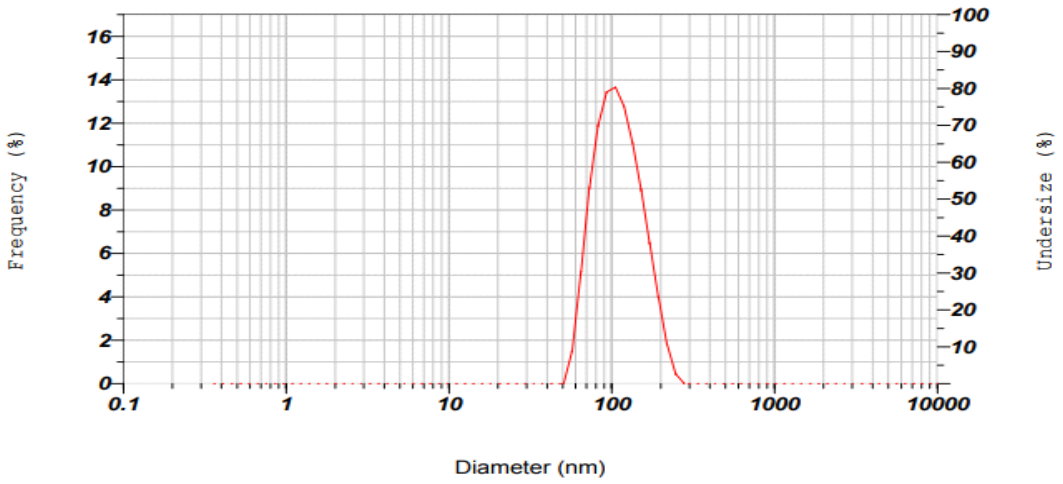


Figure 8.1.1 Graphical representation of AgNPs size

Result:

The Zeta sizer Nano range of instrument gives the ability to measure three parameters like particle size, zeta potential and molecular weight. In zeta sizer three different angles are available. Light passes from all three different angles and give three sizes of NPS. From three different dimension of size, one dimension should be between 1-100nm. In our study, NPs sizes were 67.6nm, 100.9nm, 159.4nm from three different angles of zeta sizer.

Zeta potential:

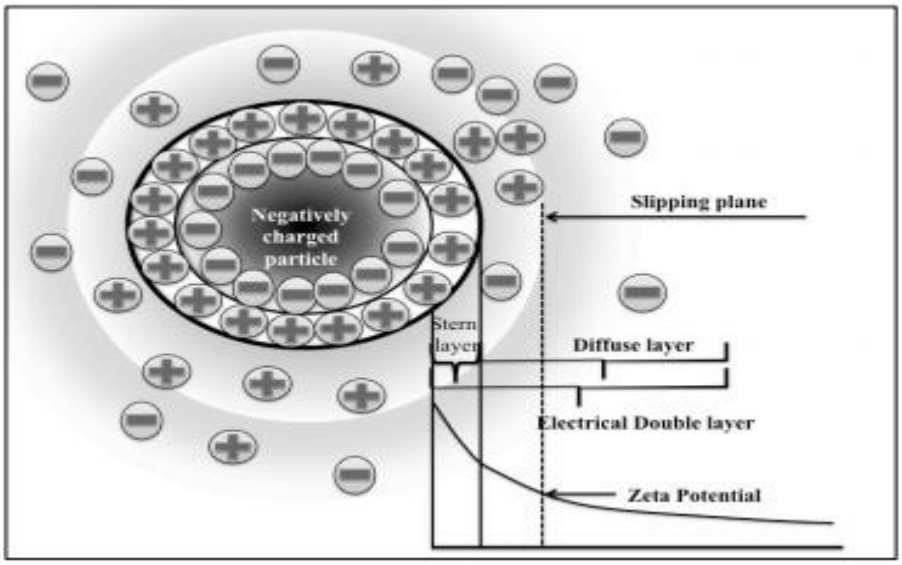


Figure 8.1.2 www.biophysics.bioc.cam.ac.uk/files/Zetasizer_Nano_user_manual_Man0317-1.1.pdf (Adapted and modified from Zeta potential Zeta sizer nano series user manual)

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	-9.1 mV	-0.000070 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : -9.1 mV

Electrophoretic Mobility Mean : -0.000070 cm^2/Vs

Table 8.1.2 Details of AgNPs zeta potential

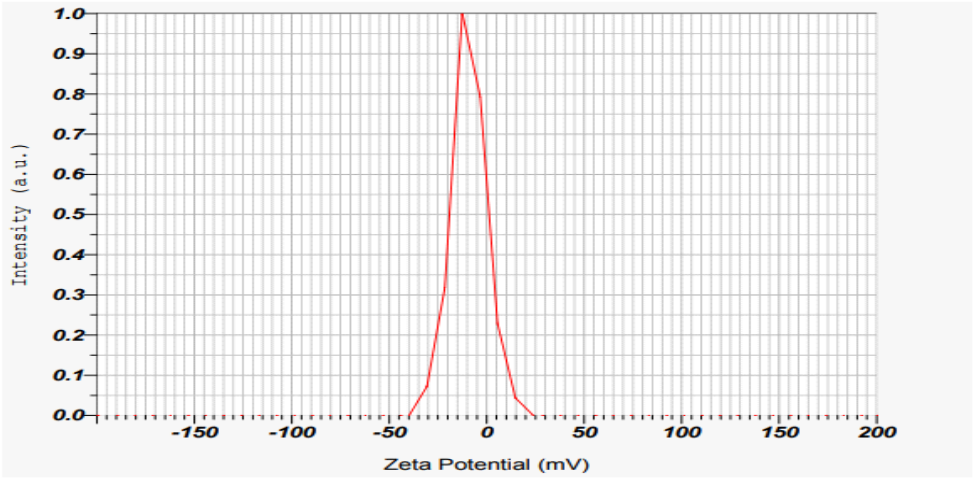


Figure 8.1.3 Graphical representation of zeta potential (AgNPs)

Result:

Zeta Potential analysis is a technique to determine the surface charge of nanoparticles in solution (colloids). Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticle surface. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to -100 mV. Nanoparticles with Zeta Potential values greater than +25 mV or less than -25 mV typically have high degrees of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal interparticles attractions. In this our study of NPs Zeta potential range was -9.1mV, this range between +25mV to -25mV. So this result indicates our NPs sample stability was very good.

8.2 MTT assay:

Result:

Sample	Cell viability			Mean			Std Dev		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
Negative	100	100	100	0.47198	0.487667	0.496543	0.0149	0.026297	0.010214
Vehicle	161.2356	92.19057267	100.6962	0.761	0.449583	0.5	0.036742	0.042286	0.04542
169.8µl	158.5872	97.11134852	99.01794	0.7485	0.47358	0.491667	0.192874	0.058996	0.042343
Positive	52.89631	25.49547129	7.988566	0.24966	0.124333	0.039667	0.032892	0.034033	0.00692

Table: 8.2.1 Analysis of cell viability of CHO cell line in response to AgNPs

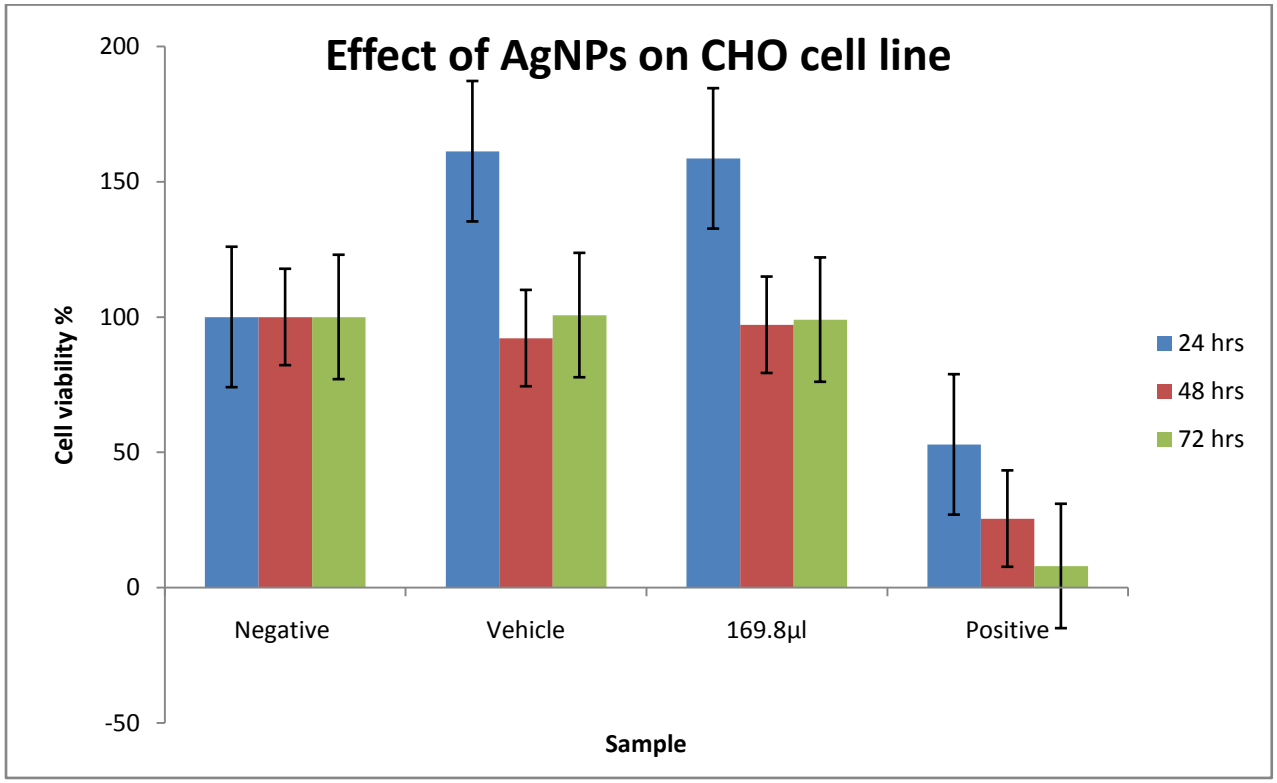


Figure 8. 2.1: Effect of AgNps on CHO cell line at 24, 48 and 72 hours of incubation

Result:

MTT assay is widely used to screen the possible toxicity of various nanoparticles on mitochondrial functions. This assay was peformed to study the nanoparticles activity on human cell lines (CHO) to find out its safety profile. The cell viability is shown in the figure 8.2.1. The morphology of the cells treated with the positive control was compared with the cells treated with AgNPs. In CHO cells, we identified less cytotoxic effects after 24 hours as compared to 48 and 72 hours. The assay did not show any significant changes suggesting that there is no cellular damage after treatment with AgNPs. These results suggest no alterations in the cellular integrity and mitochondrial activity in the CHO cell line, thus indicating that the biocompatible nanoparticles studied herein is non toxic. These results further justify the non toxic nature of AgNPs.

(i) Before addition of MTT (ii) **After addition of MTT**

Figure 8.2.2: Color changes after addition of MTT dye

8.3 DNA binding assay:

DNA binding assay can be carried out by two methods:

A. U.V visible spectroscopy
B. Fluorescent spectroscopy

A. U.V visible spectroscopy:

Experiment: 1

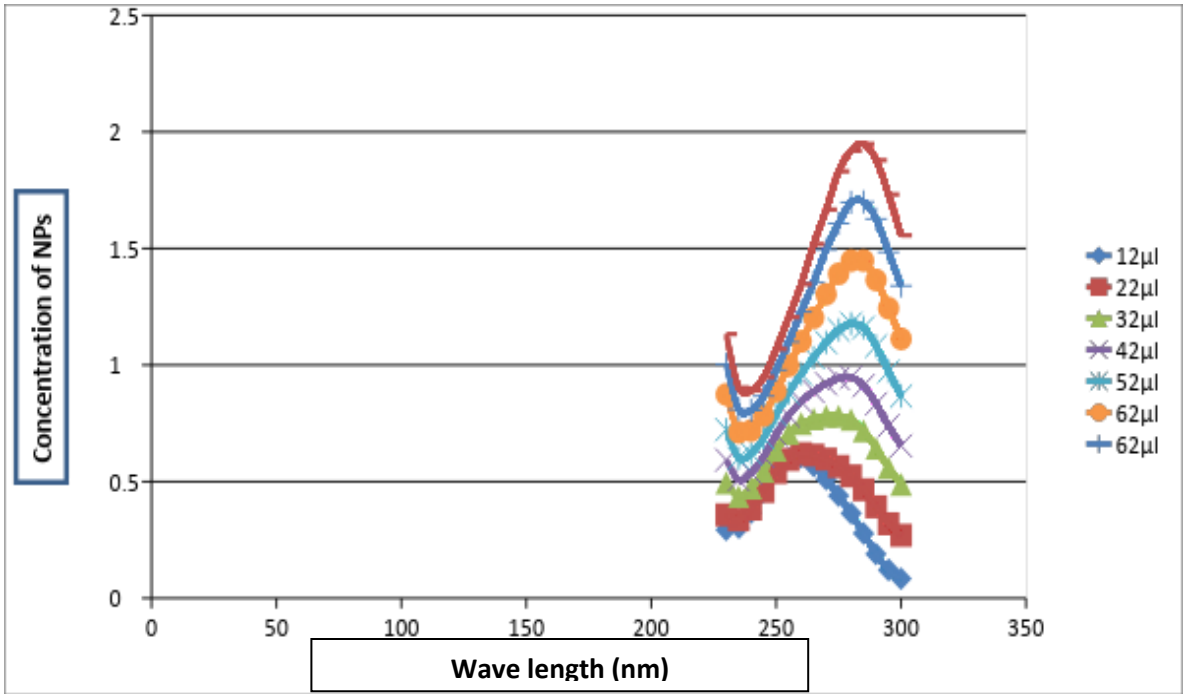


Figure 8.3.1 U.V. visible spectra of genomic DNA in the presence of different concentration of AgNPs 2, 12, 22, 32, 42, 52, 62, 72µl

Experiment: 2

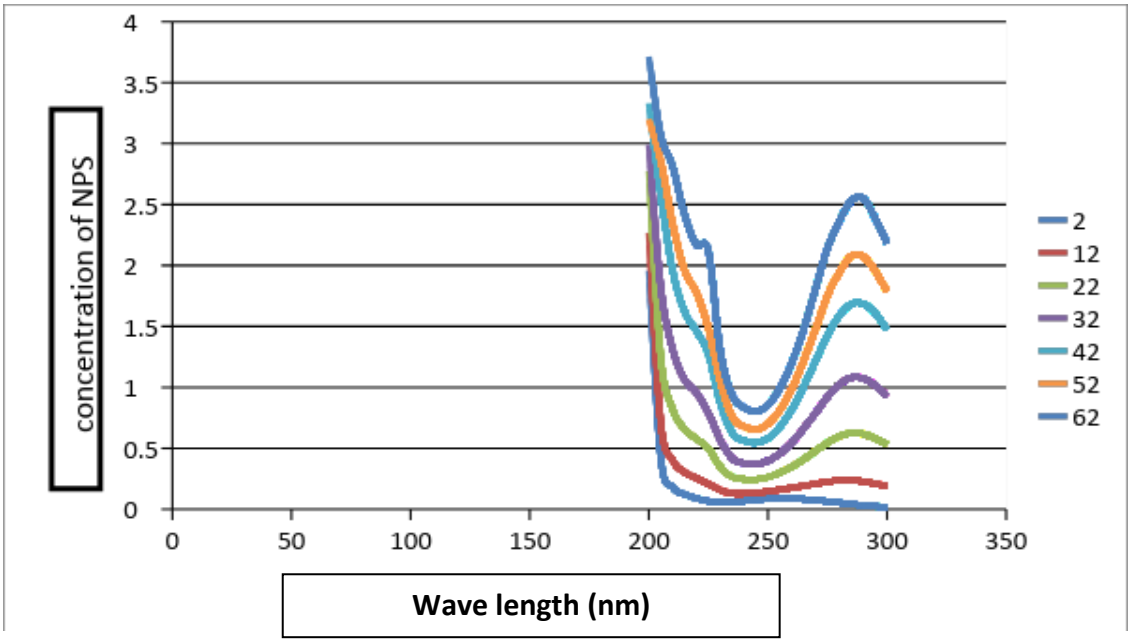


Figure 8.3.2 UV visible spectra of genomic DNA in the presence of different concentration of AgNPs 2, 12, 22, 32, 42, 52, 62, 72µl

RESULT:

The interaction of AgNPs with human genomic DNA has been analyzed using UV absorption spectroscopy technique. The nitrogenous bases in nucleotides have absorption maxima around 260-280 nm. If absorption maxima of DNA molecule change, it indicates interaction between NPs and DNA molecule. The fig. shows the UV absorption spectra of human genomic DNA in deionized water in presence of AgNPs. When AgNPs particles were added to the DNA, the maximum absorption was found at the same range i.e. (260- 280nm), suggesting there may be no AgNPs interaction with the DNA.

B. Fluorescence spectroscopy:-

Experiment:1

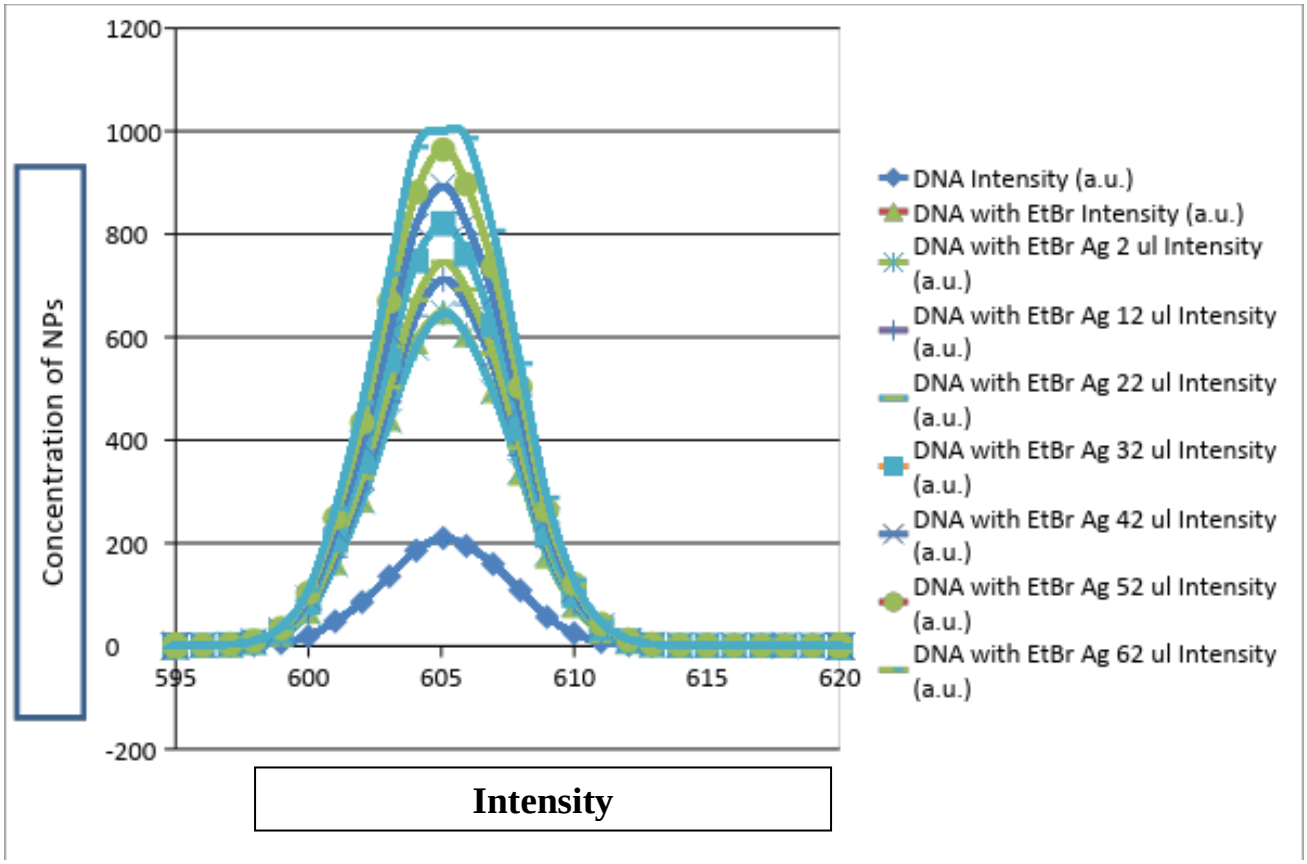


Figure 8.3.3 Graphical representation of fluorescence emission spectra of intercalated ethidium bromide incubated with genomic DNA by increasing concentration of AgNPs

Experiment: 2

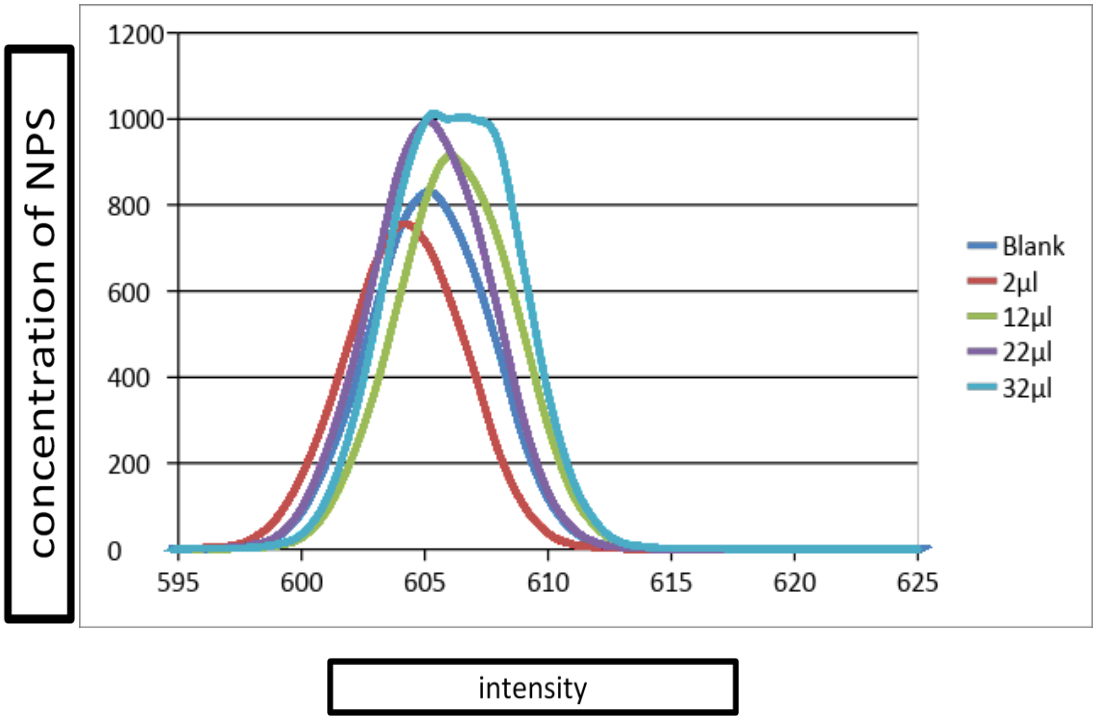


Figure 8.3.4 Fluorescence emission spectra of intercalated ethidium bromide incubated with genomic DNA by increasing concentration of AgNPs

Result:

Fluorescence quenching measurements have been widely used to understand the Drug-DNA interactions. It also gives information about types and binding mechanisms of metal ions to the DNA. High sensitivity and selectivity is one of the major advantage of Fluorescence. Binding of AgNPs to human DNA was studied using extrinsic fluorescence quench titration method. To study the interaction between the NPs and DNA, we performed a competitive binding experiment by fluorescent Ethidium Bromide (EtBr) displacement assay. The experiment was performed using EtBr, a common DNA binding and sensitive fluorophore with increasing concentrations of AgNPs, to determine the extent of binding of nanoparticles and human genomic DNA. EtBr consisting of a planar phenenthridinium ring strongly fluoresces in presence of DNA due to intercalation between the adjacent DNA base pairs. It has been previously reported that this enhanced fluorescence can be quenched by the addition of a second molecule [23]. With the addition of a second molecule, there would be a competition between EtBr and the second molecule to bind to human genomic DNA, leading to a decrease in fluorescence intensity. The extent of fluorescence quenching of EtBr bound to human genomic DNA can be used to determine the extent of binding of second molecule with human genomic DNA.

In our study, on addition of AgNPs with human geome DNA the result showed no significant decrease in the fluorescence, this may be due to no interaction of AgNPs with DNA.

8.4 Chromosomal aberration assay

Experiment : 1

No	Treatment group	No of well spread metaphase scored	No of aberration observed with cells
1	Positive control	100	35
2	Negative control	100	3
3	Vehicle control	100	8
4	1000µl	100	13
5	650µl	100	4
6	350µl	100	5

Table 8.4.1 Effect of Ag NPs on short term cultured peripheral blood lymphocytes

Analysis:

Exp 1	Mean	Std Dev	Std Error	P Value	P value summary
Negative	0.03	0.170587	0.017058	0.001	***
Vehicle	0.08	0.365513	0.036551	0.0005	****
1000ul	0.13	0.467959	0.046796	0.01	**
650ul	0.04	0.241661	0.024166	0.01	**
350ul	0.05	0.259808	0.025981	0.0005	****

Table 8.4.2 P value summary of chromosomal aberration assay

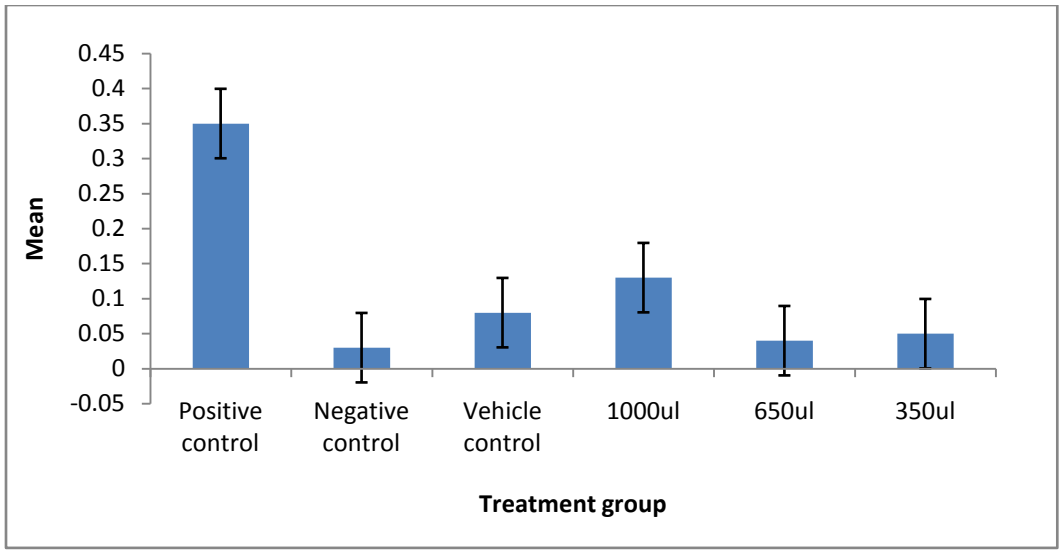


Figure 8.4.1 Graphical representation of effect of Ag NPs on short term cultured peripheral blood lymphocytes

Experiment 2:

No	Treatment group	No of well spread metaphase scored	No of aberration observed with cells
1	Positive control	100	27
2	Negative control	100	2
3	Vehicle control	100	6
4	1000µl	100	16
5	650µl	100	7
6	350µl	100	4

Table 8.4.3 Effect of Ag NPs on short term cultured peripheral blood lymphocytes

Analysis:

Exp 2	Mean	Std Dev	Std Error	P Value	P value summary
Negative	0.02	0.14	0.014	0.0005	****
Vehicle	0.06	0.341174	0.0341174	0.05	*
1000ul	0.16	0.484149	0.048415	0.01	**
650ul	0.07	0.38092	0.038092	0.01	**
350ul	0.04	0.241661	0.024166	0.01	**

Table 8.4.4 P value summary of chromosomal aberration assay

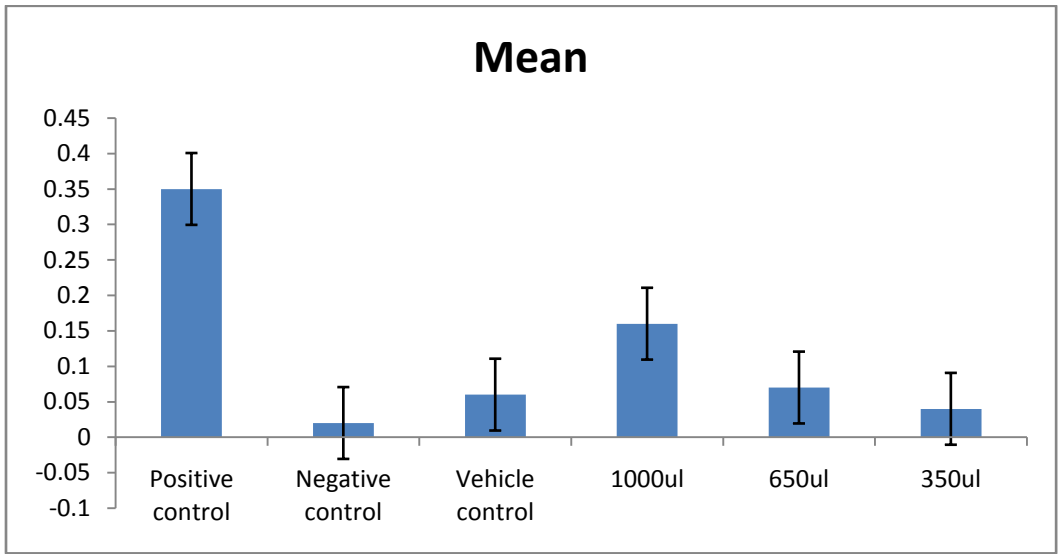
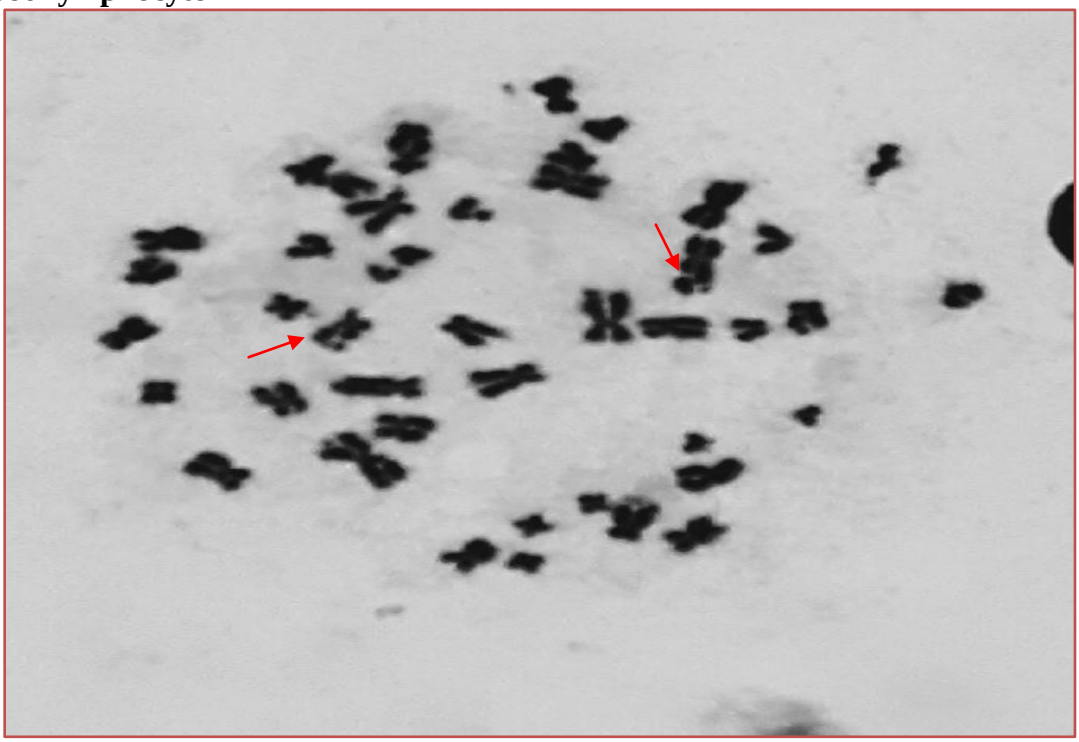


Figure 8.4.2 Graphical representation of effect of Ag NP on short term cultured peripheral blood lymphocyte



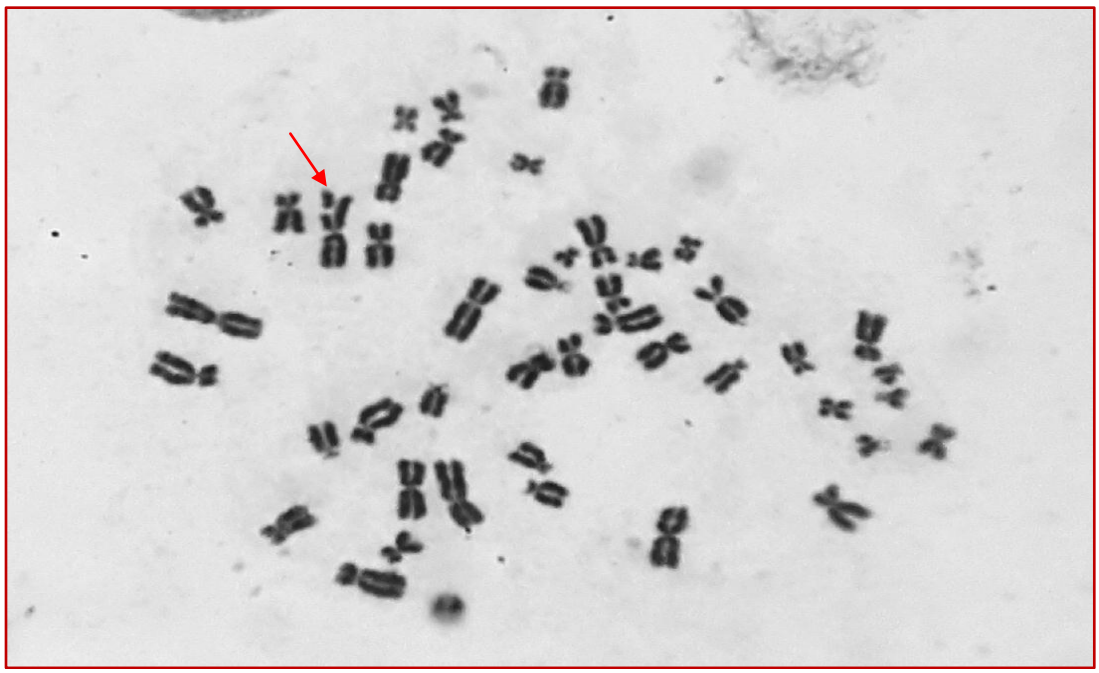


Figure 8.4.3 Photomicrographs of chromosomal aberration assay (a) Single chromatid gap (b) Single chromatid break

Result:

In this study we have assessed the genotoxic potential of AgNPs in vitro short term human peripheral blood lymphocytes following exposure for 24 hours. A minimum of 100 metaphases per sample per replicate were scored for the Chromosomal aberration assay.

Figure 8.4.1.shows the mean no. of aberrations of set 1 when exposed to 350µl, 650µl and 1000µl AgNPs. It may indicate a high concentration ratio shows more aberration as compared to minimum concentration ratio. Figure 8.4.2 shows aberration increase in dose dependent manner.

Discussion:

One of the in vitro methods recommended by OECD guidelines for novel drug molecule and any compound for human safety assessment. In our study, human peripheral blood lymphocyte cultures were exposed to AgNPs at the 48th hour, and harvested at the 72nd hour. Considering the average cell cycle duration 18 hours, NPs were added at 48th hour after culture initiation, when cells divide asynchronously. After the addition of NPs, cells need to undergo S phase, to express aberrations. Since asymmetrical structural chromosome aberrations prevent unlimited division, harvesting cells when they are in first division post exposure of test substance is crucial [3]. A harvesting time of 1.5 cell cycles is considered to be optimum for detecting

clastogens [8]. Therefore, at the time of harvesting, most cells will be in their first cell division after exposure to NPs. The type of structural chromosome aberrations observed at metaphase reflects the duplication status of chromosomes in the treated cell.

8.5 SCE (Sister Chromatid Exchange)

Control	PRI	AGT
Negative	1.952	0.027
Positive	1.7904	0.024
1000µl	0	0
650µl	1.4	0.019
350µl	2.08	0.028

Table8.5.1 PRI and AGT value

Control	Mean	Std Dev	Std Error	P Value	P value summary
Negative	0.6	1.232883	0.246577	0.05	*
1000µl	0	0	0	0.5	NS
650µl	3.2	2.74226	0.54845	0.2	NS
350µl	4	2.33238	0.46648	0.05	*

Table8.5.2 P value of samples of SCE assay

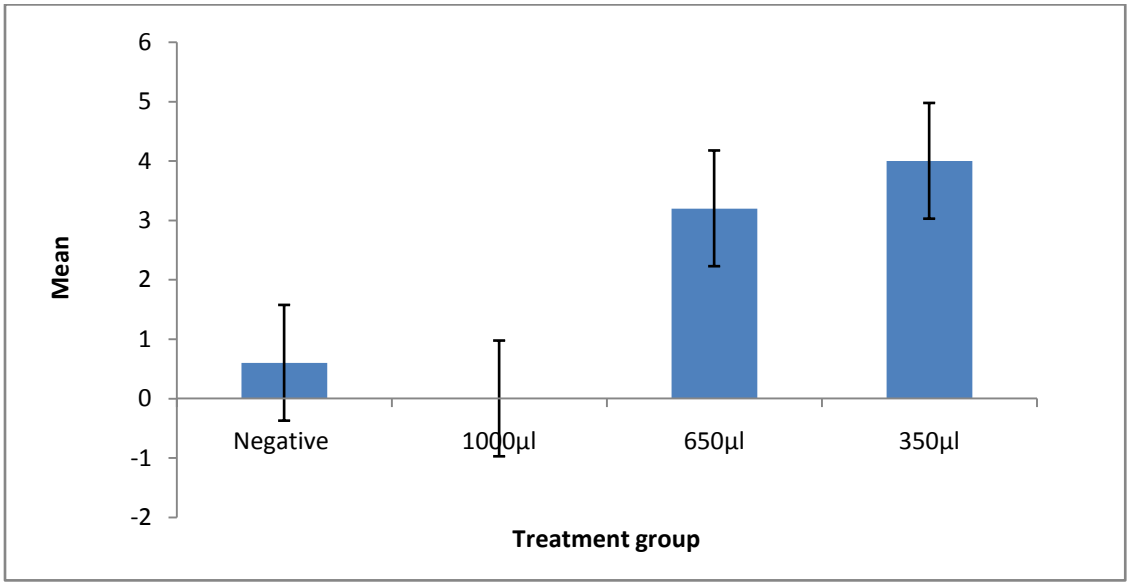


Figure8. 5.1 Graphical representation of effect Ag NPs on short term cultured peripheral blood lymphocytes

Result:

The BrdU was added in the cultures as Thymidine analogue for differential staining of first, second, and third cell division.The CA scoring is recommended in first division cell metaphases in order to avoid in vitro effects on the CA yield.

Figure 8.5.2 Photomicrographs depicting SCE differentially stained metaplates in M2 phase

Summary:

The characterization data of Malvern zetasizer suggested that size of synthesized AgNPs were between 1 to 100nm. Zeta potential range of AgNPs was -9.1mV suggesting our nanoparticles were in stable form. Green synthesized AgNPs could remain stable for a month.

The cytotoxic effect of silver nanoparticles is checked by MTT assay which did not show any significant changes suggesting that there is no cellular damage after treatment with AgNPs, thus indicating that the biocompatible nanoparticle is non-toxic and cells was viable in response to AgNPs.

DNA damage can be in two possible ways; 1) Direct DNA damage &

2) Indirect DNA damage.

Direct DNA damage deals with breakage of chromatids or chromosomes that are observed in the form of gaps and breaks, while in-direct damage of DNA deals with generation of ROS species and oxidative stress enzyme.

To confirm the effect of direct interaction AgNPs with the DNA, DNA binding and fluorescence assay was performed. In the UV visible spectroscopy, when AgNPs were added to the DNA, it showed the maximum absorption at the same range (260- 280nm), suggesting no DNA interaction with AgNPs. In the fluorescent spectroscopy, a competitive binding experiment was performed using EtBr, shows the unbinding of Ag NPs with the DNA. This assay shows that our NPs do not bind directly to the DNA.

To check whether our NPs could do any indirect damage, we decided to performed in vitro chromosomal aberration assay. This assay showed more aberration in higher concentration as compared to low concentration of NPs. Thus AgNPs showed in direct genotoxicity by chromosomal aberration assays.

Previous study with the synthetic AgNPs shows genotoxicity at 0.04mg/L concentration where with the green approaches, AgNPs shows minimum genotoxcity at 0.05mg/L which suggest that the green approaches are better than synthetic ones.

List of Figures

List of Tables

Referencing:

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