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A COMPARATIVE *IN VITRO* CYTOTOXICITY STUDY OF COLLOIDAL SILVER AND CHITOSAN STABILIZED SILVER NANOPARTICLES ON MCF-7 AND HEPG2

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ABSTRACT

Recently nanotechnology research was discussed for the generation of nanoparticles as anticancerous agents. It is mandatory to elaborate the application of colloidal silver and chitosan stabilized silver nanoparticles in general and anticancerous property in particular. The present study was aimed to investigate the *in vitro* cytotoxicity effect of colloidal silver and chitosan stabilized silver nanoparticles against human breast cancer cells (MCF-7) and liver cancer cells (HepG2) towards the development of anticancer drugs. The colloidal silver and chitosan stabilized silver nanoparticles were fabricated by using sodium borohydride as novel reducing agent. It was well characterized by UV, SEM, EDS, XRD, and AFM studies showed spherical shaped nanoparticles in the size range of 20-40nm and in slightly agglomerated form. Surprisingly it also showed cytotoxic effect against MCF-7 and HepG2 cell lines were confirmed by MTT and cell viability assays. There was an immediate induction of cellular damage in terms of loss of cell membrane integrity, oxidative stress and apoptosis were found in the cell which treated with colloidal silver and chitosan stabilized AgNPs. This may be a first report on anti-MCF-7 and anti-HepG2 property of colloidal silver and chitosan stabilized AgNPs in the fourth generation of nanoparticle research. It is necessary to study the formulation and clinical trials to establish the nano drug to treat cancer cells.

KEYWORDS: colloidal silver, chitosan stabilized AgNPs, MCF-7 and HepG2.

INTRODUCTION

Design, fabrication and manipulation of nano sized materials have found tremendous interest in recent times because of its wide application in biology and medicine. Fabrication of nanoparticles using chitosan has described in earlier studies, where it was elaborated into first, second and third generations of nanotechnological research. Although the use of colloidal silver as an antimicrobial agent is recognized[1], there are scarce reports on its use as anticancer agent; among these, there is a recent report on the anti-proliferative effect of silver nanoparticles on human glioblastoma cells (U251) i*n vitro*.[2] 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 cells.[3] Cancer is an important cause of mortality worldwide and the number of people who are affected is increasing, being the breast cancer one of the major causes of death in women.[4] Although there is a wide range of cytotoxic agents used in the treatment of breast cancer, such as doxorubicin, cisplatin, and bleomycin, they have shown drawbacks in their use and are not as efficient as expected.[5] Therefore, 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.[6] Many attempts have been made to use silver nanoparticles as an anticancer agent and they have all turned up positive. Hence, it is of great interest to find novel therapeutic agents against cancer, we have evaluated the effects of colloidal silver and chitosan stabilized AgNPs on MCF-7 (human breast cancer) and HepG2 (liver cancer) cells growth.

MATERIALS AND METHODS

Chemicals

Silver nitrate ($AgNO_3$), Trisodium citrate ($C_6H_5O_7Na_3$), Sodium borohydride ($NaBH_4$), Ascorbic acid ($C_6H_8O_6$), Acetic acid (CH_3COOH), Chitosan were purchased from Himedia (P) Ltd, Mumbai, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) was purchasesd from Sigma-Aldrich (St.Louis, USA) were used as starting materials without further purification. Milli-Q water was used for the fabrication of nanoparticles throughout the experiment.

METHODS

Fabrication of Silver Nanoparticles (AgNPs)

Silver nanoparticles were prepared by chemical reduction method.[7] Freshly prepared $AgNO_3$ solution (50 mL of 1×10^{-3} M) was heated to boiling. To this solution, 5 mL of 1% trisodium

citrate was added drop by drop under constant stirring. The reaction mixture was heated until the solution color changed from colorless to yellow. Then, it was removed from heating element and stirred until cooled to room temperature.

Mechanism of reaction for the formation of silver nanoparticles could be expressed as follows.

$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag^{\circ} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2 \uparrow$

The resultant silver colloids were centrifuged at 1×10^{4} rpm under ambient temperature and washed three times in Milli-Q water and once in ethanol solution. The obtained particles were dried overnight in hot air oven at 60^{o}C. The dried particles were used for further studies.

Fabrication of chitosan stabilized silver nanoparticles (CH-AgNPs)

Chitosan stabilized silver nanoparticles were prepared via seed-mediated growth.[8] Aqueous solution of silver nitrate (10 mL, 2.9×10^{-4} M) was mixed with trisodium citrate (10 mL, 2.5×10^{-4} M) and cooled in an ice-bath under vigorous stirring. To this mixture, aqueous solution of sodium borohydride (0.6 mL, 1×10^{-1} M) was added dropwise which results in the formation of a bright yellow solution. The freshly prepared seed solution was stored in the dark condition for 2-3 hours before use in order to prevent any excess borohydride reacts with water. Then, a mixture of trisodium citrate (35 mM, 200 µL), ascorbic acid (1×10^{-1} M, 50 µL), chitosan in 1% acetic acid (2 mg/mL, 10 mL), seeds (200 µL), and $AgNO_3$ solution (1×10^{-1} M, 300 µL) was added dropwise under continuous magnetic stirring. The formation of chitosan stabilized silver nanoparticles was completed after 5 minutes at 35 ± 2^{o}C.

Characterization of colloidal AgNPs and CH-AgNPs

The reduction of metal ions was roughly monitored by visual inspection of the reaction solution by color change. UV-Vis spectra of all the nanoparticles suspensions were recorded on a Shimadzu dual beam spectrophotometer (UV-1650 PC) operated at a resolution of 1 nm in the wavelengths ranging between 200 – 800 nm. The crystallographic analysis of the samples was performed by powder X-ray diffraction in the scanning mode on an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation (λ=1.54060 Å). The diffraction intensities were compared with the standard JCPDS files. Morphology of the synthesized silver nanoparticles was observed with the scanning electron microscope (JSM 35 CF JEOL) operated at a resolution of 60 Å at 15 kv. The quantitative information and distribution of CH-AgNPs was investigated by EDS analysis (JSM 35 CF JEOL) in a resolution of 60 Å, magnification of 5 k. AFM of CH-AgNPS was measured the

exact particle size in the sample was characterized using Atomic Force Microscopy (Nanonics imaging MN1000) which measures the atomic range of particles using tapping mode.

In vitro anticancer studies of synthesized AgNPs and CH-AgNPs

Cell culture

The human tumoral HepG-2 (hepatocellular carcinoma) and MCF-7 (breast carcinoma) were obtained from National Centre for Cell Science (NCSS), Pune, India. The cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin-streptomycin solution. The cells were incubated at 37^{o}C with 5% carbondioxide (CO_2) atmosphere and were grown in 25 cm^2 tissue culture flasks (Tarson Products Limited, Kolkata, India). The medium was changed every 3 days until the cells reached confluence, at which point the cells were sub cultured.

Preparation of penicillin-streptomycin solution

0.61 g (one vial) of penicillin was weighed and dissolved in 1 ml of sterile phosphate buffered saline. It was stirred for 5 minutes and then filtered using sterilized syringe by passing through 0.22 µ filters. Finally, it was stored at -20^{o}C until use. Similarly, streptomycin solution was prepared by dissolving 10 g (one vial) of antibiotic streptomycin in 10 ml of sterilized phosphate buffer saline (PBS).

Evaluation of cytotoxicity assay

Cytotoxic effect of colloidal silver nanoparticles and CH-AgNPs was evaluated using MTT assay.[9] Briefly, the cultured HepG-2 and MCF-7 cells (1x10^5 cells/mL) were plated separately on 96 flat bottom well plates. Then, the cells were treated with different concentrations of AgNPs (1 – 50 µg/mL) in DMSO solution such that the final concentration of DMSO in media is not more than 0.5%, so that it did not affect cell survival. The plates were then kept in CO_2 incubator in the presence of 5% CO_2 at 37^{o}C for 24 hours. Blank contain only cell suspension and control well contain 0.5% DMSO plus cell suspension. After incubation period, MTT (5 µg/mL) was added to the incubated cells, then further it was incubated for another 4 hours at 37^{o}C in the presence of 5% CO_2 atmosphere. The plates were covered with aluminium foil to protect it from light. MTT was reduced in metabolically active cells to yield an insoluble purple formazan product. Cell viability was noticed by the conversion of the tetrazolium salt MTT to a colored formazan by the mitochondrial dehydrogenases. Color development was measured using microplate (ELISA) reader at 570

nm (Biorad 680). The readings were averaged and viability of the tested samples was compared with DMSO control. The cell viability was calculated using the following formula:

$$\text{Percentage growth inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Measurement of cytomorphological changes

MCF-7 and HepG2 cell lines were treated with different concentrations of synthesized silver nanoparticles and chitosan stabilized silver nanoparticles (0 – 50 µg/ml) in DMSO and incubated for 24 hours at 37^{o}C in 5% CO_2 atmosphere. After the incubation period, the cells were rinsed with PBS, stained with 0.2% trypan blue for 5 minutes and observed under an inverted phase contrast microscope (Nikon, Japan).

Statistical analysis

Statistical analyses of data for all experiments are expressed as means and standard deviations. The data were analysed using correlation co-efficient (Microsoft excels, Microsoft Corporation). The correation co-efficient value" r" = +1 or -1 was considered as statistically significant when the P value was <0.05.

RESULT AND DISCUSSION

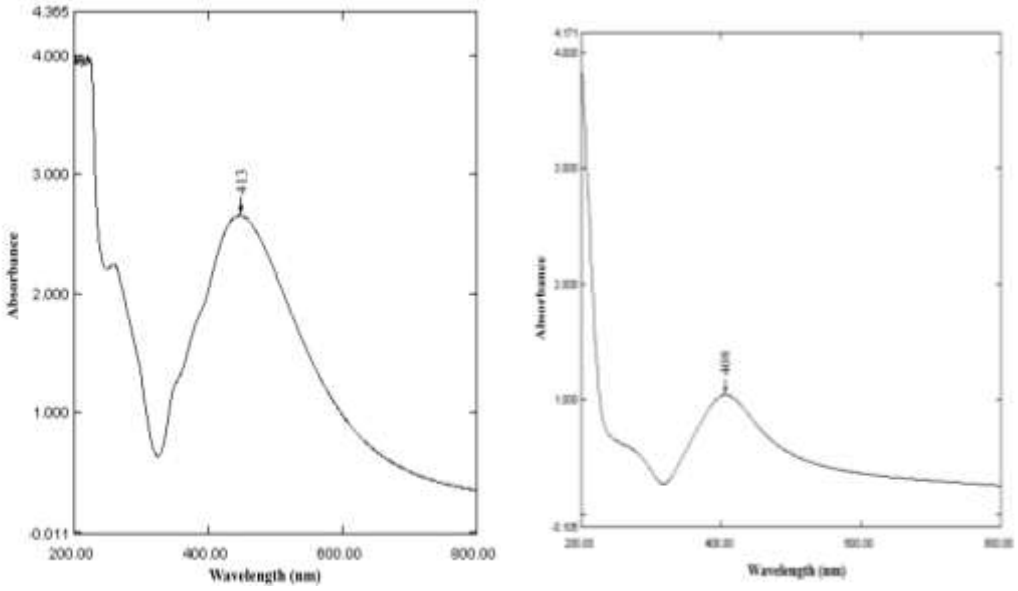
Characterization of colloidal AgNPs and CH-AgNPs

Nanodrug particles and development of nanoformulations for drug delivery were intensively studied, which includes the production, stability, characterization, formulation, delivery and biological fate.[10] The appearance of colourless to yellow and deep brown colloidal solution for colloidal silver and chitosan stabilized silver in the reaction mixture indicates the formation of AgNPs (Fig.1). The silver nanoparticle with and without stabilizer was synthesized.

a) Colloidal silver **b) CH-AgNPs**

Fig. 1: Visual inspection of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

successfully and confirmed with the formation of yellow and deep brown colour due to the excitation of surface Plasmon resonance.[11] The characteristic brown color arises due to the excitation of plasmon vibrations in the silver nanoparticles.[12,13] reported that the brown color exhibited by silver nanoparticles is due to the coherent excitation of the entire free electrons within the conduction band, leading to surface plasmon resonance. Colloidal silver nanoparticles exhibit adsorption at wavelength from 380-420 nm due to Mie scattering theory.[14] The UV-Vis spectrum of colloidal silver and chitosan stabilized silver nanoparticles showed strong absorbance peaks at 413nm and 408 nm respectively (Fig. 2). UV-Vis absorption spectrum of Silver nanoparticles showed an intense absorption peak due to its surface plasmon excitation which represents the collective excitation of conduction electron in metal.[15] The interactions of silver with chitosan in the present study are in consistent with earlier report[16], who stated that the attachment of silver to nitrogen atoms, which reduces the vibration intensity of the N-H bond due to the molecular weight becoming greater after binding of silver. Likewise, the interaction between silver nanoparticles and amino group of chitosan was noticed earlier.[17]

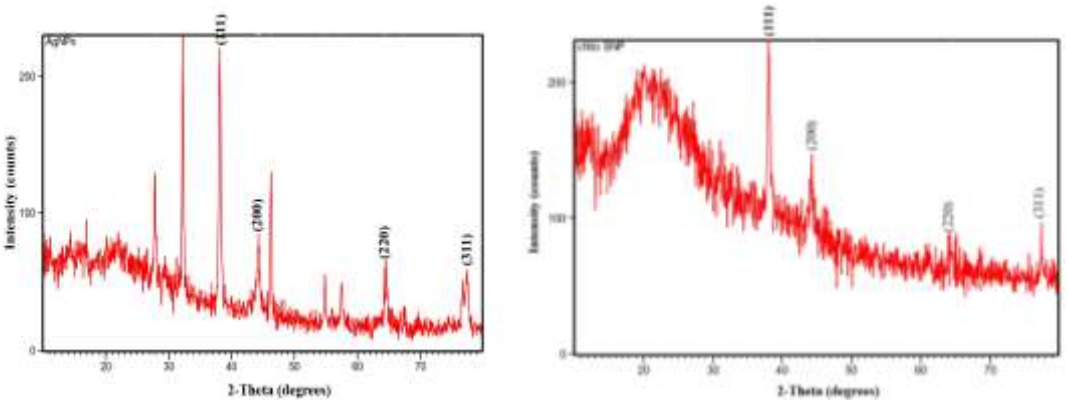


a) Colloidal silver **b) CH-AgNPs**

Fig. 2: UV-Vis absorption spectrum of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

Four well-defined characteristic diffraction peaks for chemically fabricated silver nanoparticles were appeared at $2\theta = 38.11^{o}$, 44.00^{o}, 64.40^{o}, and 77.40^{o} corresponding to (111), (200), (220), and (311) planes of face centered cubic (fcc) crystal structure of metallic

silver. Similarly the intensive diffraction peaks at 2θ value of 38.11°, 38.33°, 38.06°, and 38.04° indexed as (111) lattice plane of face centered cubic (fcc) form for chitosan stabilized AgNPs respectively unequivocally indicated that the particles are made of pure silver (Fig.3).



a) Colloidal silver **b) CH-AgNPs**

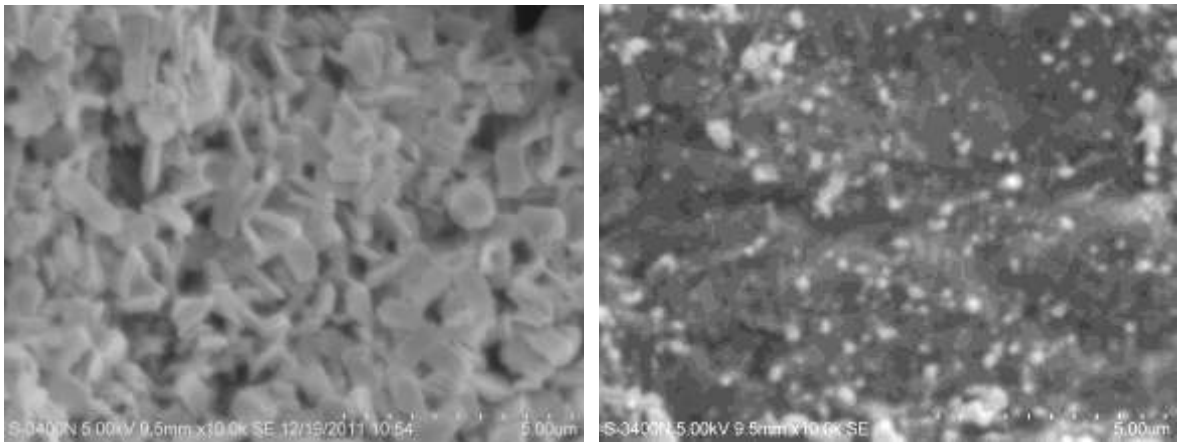
Fig. 3: X-ray diffraction spectrum of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

In the obtained spectrum, the Bragg's peak position and their intensities were compared with the standard JCPDS files (JCPDS File No. 04-0783). The diffraction peaks of XRD appearing at 2θ values of silver in the present study exactly matched with previous reports.[18, 19]

The size of the colloidal silver and CH-AgNPs was found to be 43.92 nm and 21.95 nm respectively. The calculated crystalline sizes of the resultant nanoparticles are coincide with the previous report.[20, 21]

The scanning electron microscopy of synthesized colloidal silver nanoparticles and CH-AgNPs revealed that the particles are spherical and hexagonal in nature (Fig. 4). The micrograph shows that the AgNPs do not appear as discrete particles but form much larger dendritic flocs whose size could reaches micron scale.

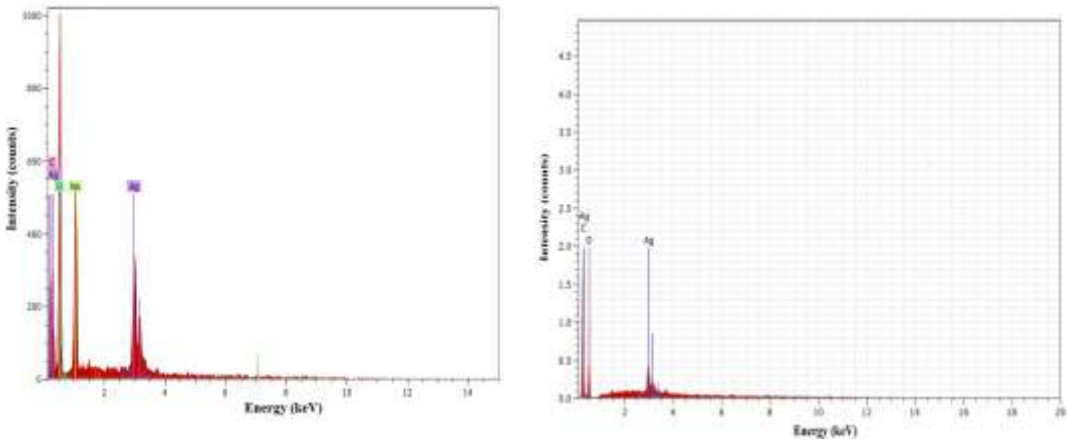
The aggregation is attributed due to the interparticular interactions among them. The spherical shaped carbohydrate-stabilized silver nanopaticles were observed by other researchers.[22, 23] The size and shape of the nanoparticles depend on the many parameters such as choice of reduction technique, concentration of metal precursor, reductant and capping agent used.[24]



a) Colloidal silver **b) CH-AgNPs**

Fig. 4: Scanning electron micrograph of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

EDS exhibited strong signals from the atoms in the respective nanoparticles and the weakersignals from N, Na, O and Cl atoms as shown in the Fig. 5. The elemental analysis of chemically fabricated silver nanoparticles revealed that silver was in major constituent (70.34%) followed by sodium, oxygen and carbon (26.25%, 2.39% and 1.02%) (Table 1). The appearance of additional peaks for sodium, oxygen and carbon can be attributed due to the chemicals used for the synthesis of nanoparticles. The elemental analysis of CH-AgNPs revealed that silver was in highest proportion (66.24%) followed by carbon and oxygen (30.75% and 3.01%) in nanoparticles mass (Table 1). In the CH-AgNPs, peaks of Ag were observed along with significant peaks of C, and O reflecting the presence of elements constituting chitosan. Polysaccharides stabilized silver nanoparticles displayed an emission energy at 3.0 keV in the resultant EDS spectra indicated the reduction of Ag ions to elemental Ag. Same type of elemental Ag signal was observed earlier[25], who reported that the EDX spectrum of silver nanoparticles has the strongest peaks at 3.0 keV, confirming the presence of elemental Ag in the nanoparticles. The surface morphology and size of the synthesized silver nanoparticles was analysed by AFM. The obtained particles are spherical in nature (Fig.6). The particle size measured by AFM was ranged from 20 nm to 40 nm for silver and chitosan stabilized silver nanoparticles.

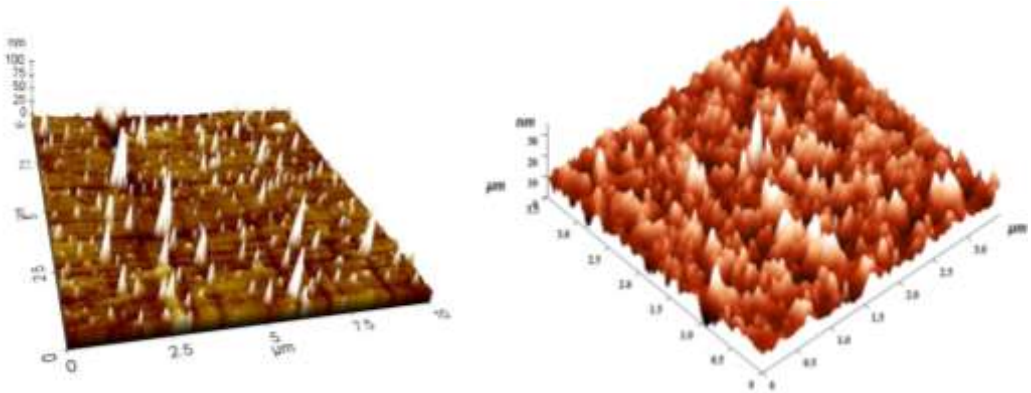


a) Colloidal silver **b) CH-AgNPs**

Fig. 5: Energy Dispersive Spectroscopy of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

Table 1: EDS elemental analysis of silver nanoparticles and chitosan stabilized silver nanoparticles

Nanoparticles	Element	Weight (%)	Atomic (%)
Silver	Ag	70.34	58.17
	Na	26.25	32.48
	O	2.39	6.50
	C	1.02	2.85
	Total	**100**	**100**
Chitosan stabilized silver	Ag	66.24	70.59
	C	30.75	28.44
	O	3.01	0.97
	Total	**100**	**100**



a) Colloidal silver **b) CH-AgNPs**

Fig. 6: Atomic Force Microscopy of colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

The SEM and AFM results correlate with each other. Earlier reports[26] suggested that the bright spots on the surface topology of AFM micrograph indicated that the nanoparticles were spherical in shape. The size and shape of the silver nanoparticles depend on the concentration and type of reducing as well as stabilizing agents.[27]

Cytotoxic effect of silver nanoparticles on human cancer cell lines

The cytotoxic effect of colloidal silver and chitosan stabilized AgNPs was evaluated *in vitro* against human breast carcinoma (MCF-7) and hepatocarcinoma (HepG-2) cell lines at five different concentrations (1, 5, 10, 25, 50 µg/mL) by MTT assay and the results are depicted in Figs.7 and 8. The *in vitro* screening of the synthesized colloidal silver and CH-AgNPs exhibited.

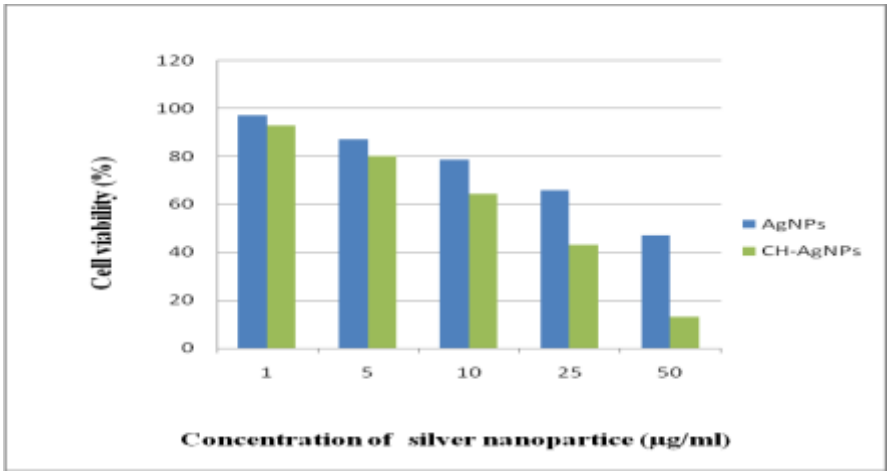


Fig. 7: Cell viability (%) of synthesized silver nanoparticle and chitosan stabilized silver nanoparticle against MCF-7 cell line

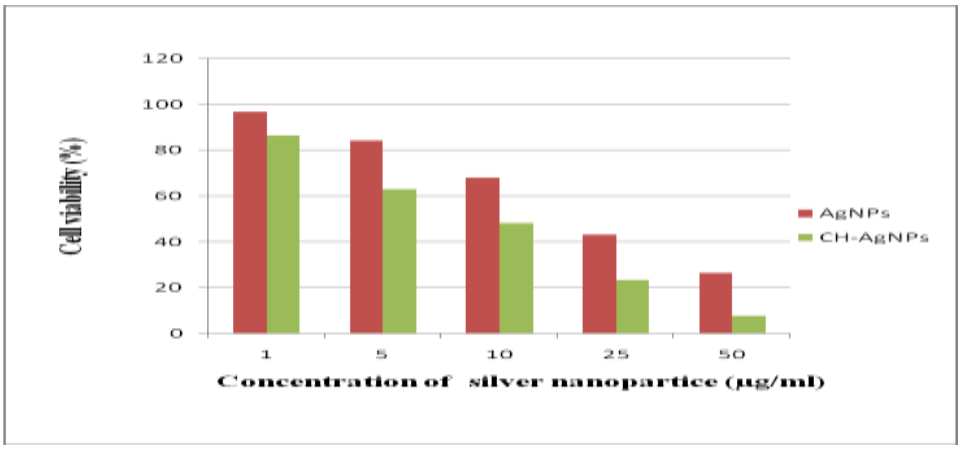


Fig. 8: Cell viability (%) of synthesized silver nanoparticle and chitosan stabilized silver nanoparticle against HePG2 cell line

potential cytotoxic effect against both the tested cell lines. The percentage viability of MCF-7 and HepG-2 tumor cell lines decreased with increased concentration of colloidal silver abd CH-AgNPs in a dose dependent manner. The decrease in percentage viability of MCF-7 and HePG2 tumor cell lines can be fitted with correlation coefficient. The correlation coefficient between nanoparticles and tumor cell lines revealed that there is a strong negative correlation of silver nanoparticle and chitosan stabilized silver nanoparticle against MCF-7 and HePG2 cell lines (r = -0.981 and -0.926 respectively). This study clearly indicated that the size and dose of AgNPs as well as cells used in the experiment play a crucial role in the cytotoxic effect of AgNPs. The CH stabilized AgNPs exhibited 50% tumor cell death at 25.00 µg/mL concentration. It showed potential distinct anticancer activity on the tested tumor cell lines. Table 2 represents the two way ANOVA test for the cytotoxic effect of types and concentration of silver nanoparticles on MCF-7 cell line were statistically significant (F = 17.95 and 9.39; P < 0.05). Similarly, the cytotoxic effect exhibited by different types of silver nanoparticles on HepG-2 cell line and the variance due to concentration of nanoparticles were statistically significant (F = 192.38 and 36.39; P < 0.05).

Table 2: Two way analysis of variance for the data on cytotoxic effect exhibited by the selected human cancer cell lines as a function of types and concentration of nanoparticles

Cancer cell lines	Source of Variation	Sum of Squares	Factor Df/ Total Df	Mean Square	'F' value	'P' value
MCF-7	Variance due to nanoparticles Variance due to concentration	5188.36 678.48	4/9 4/9	1297.09 678.48	17.95 9.39	< 0.05 < 0.05
HepG-2	Variance due to nanoparticles Variance due to concentration	7215.08 810.00	4/9 4/9	1803.77 810.00	192.38 36.39	< 0.05 < 0.05

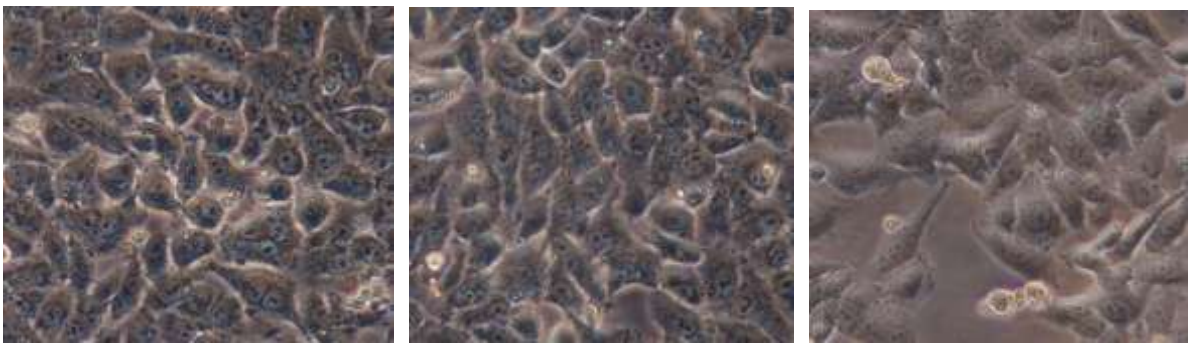
P < 0.05 is statistically significant

The enhanced cytotoxicity of CH-AgNPs may be due to their size which facilitates their subsequent penetration in tumor cells.[28] This is due to smaller nanoparticles usually having larger surface area and high reactivity results in stronger cytotoxic effect.[29] Owing to their small size, SNPs impair the sulfur and phosphorus containing essential macromolecules such as protein and DNA.[16] Previous studies[30] stated that silver nanoparticles may interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular

chemistry. Furthermore, there was a considerable direct dose response relationship between the concentration of silver nanoparticles and cytotoxic effect has been seen in the present study. Increasing concentration of nanoparticles resulted in decreased cell viability. In addition to that, size of the nanoparticles played a crucial role in cytotoxicity. The cytotoxic effect is inversely proportional to the size of nanoparticles.[31]

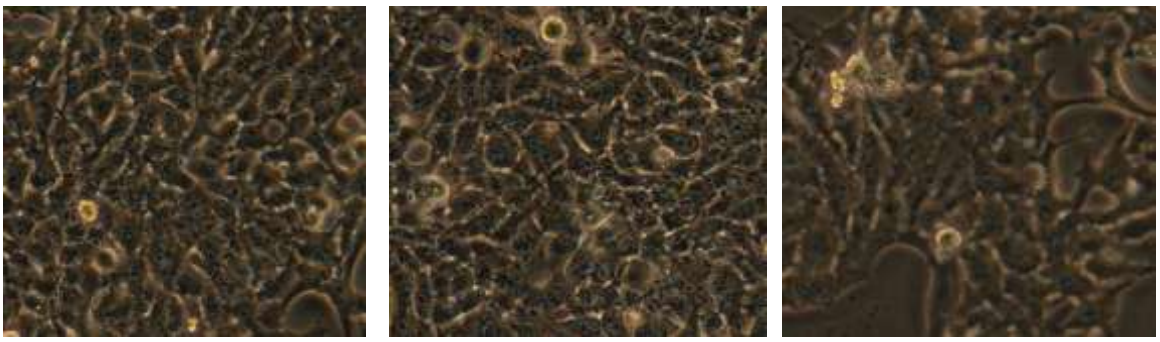
Observation of cytomorphological changes on human cancer cell lines

The morphological changes of the human cancer cell lines treated with colloidal silver and chitosan stabilized silver nanoparticles (50 µg/mL) were compared with the untreated cells (Figs.9 and 10) showed that there were diverse morphological alterations observed in nanoparticles treated MCF-7 and HepG-2 cells, however no such effects were noticed in untreated cells. In the control cells, all the cells appeared to be normal and they were regular in morphology. Images of the cells treated with CH-AgNPs showed distinct morphological changes indicating abnormal characteristics such as cell shrinkage, nuclear condensation etc.



Control cell **a) Treated with AgNPs** **b)Treated with CH-AgNPs**

Fig. 9. Cytomorphological changes of MCF-7 cell line treated with colloidal silver (a) and chitosan stabilized (b) silver nanoparticles



Control cell **a) Treated with AgNPs** **b)Treated with CH-AgNPs**

Fig. 10. Cytomorphological changes of HepG2 cell line treated with colloidal silver (a) and chitosan stabilized (b) silver nanoparticles

The experimental results in the present study indicated that the colloidal silver has antitumor activity through induction of apoptosis in human cancer cell line, suggesting that CH-AgNps might be a potential alternative therapeutic agent for human cancer. The morphological changes of Hep-2 cells were observed in their study when they were treated with AgNPs.[32] They suggested that generation of reactive oxygen species plays key role in the induction of apoptosis in Hep-2 cells.

CONCLUSION

Nanobiotechnology is an upcoming and developing field with potential application of human welfare owing to its small size and volume ratio to fight against cancerous cell growth. The synthesized AgNPs and CH-AgNPs exhibited excellent cytotoxicity against MCF-7 and HepG-2 cell lines. The present study explored the potential anticancer activity of silver nanoparticles and CH-AgNPs by using MCF-7 and HepG2 cell lines through induction of apoptosis and by inhibiting cell proliferation. Hence, the findings in the present study might provide a novel approach for the treatment of the disease in future.

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