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Anticancer and antibacterial potential of MDR *Staphylococcus aureus* mediated synthesized silver nanoparticles

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ABSTRACT

The increasing cases of resistance, low efficacy and high toxicity of antibiotics and anticancer agents have led to the discovery of nanoparticles as potent antimicrobial and anticancer agents to combat the threat of Multi Drug Resistance (MDR) and to minimize the side effects of drugs. The synthesized silver nanoparticles (SAgNPs) (synthesized using MDR *Staphylococcus aureus*) were characterized to confirm synthesis, shape, size, hydrodynamic diameter, colloidal stability and surface functionalization, by UV-visible spectroscopy, TEM, DLS, zeta potential test and FTIR respectively. SAgNPs were found to have spherical shape with a size of 15 nm. Hydrodynamic diameter of nanoparticles was found to be 88.65 nm and the value of zeta potential was recorded to be -24.03 mV. The antimicrobial and anticancer potential of SAgNPs was performed against normal & MDR strains of *S. aureus* and human colon cancer cell line (HCT-116), respectively.. The MICs of SAgNPs against normal and MDR *S. aureus* strains were found to be 0.025 µg/ml & 0.053 µg/ml, respectively. Similarly, IC50 against HCT-116 cell line was found to be 0.069 µg/ml by MTT Assay. DAPI analysis confirmed the interaction of SAgNPs with DNA in order to initiate apoptosis for killing cancer cells.

KEY WORDS: ANTICANCER; DAPI; DLS; MDR; MTT; SAGNPS

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INTRODUCTION

Toxicity, high cost of the drugs used in the treatment of cancer and microbial diseases, rise in the cases of antibiotic resistance due to frequent use of antibiotics have forced researchers to look for alternatives in medicine and therapeutics. Side effects of chemotherapeutic drugs due to poor specificity towards targets and high dose requirements have also made it necessary for nano-medicine to address the issue and look for other efficient and cost effective molecules bearing high degree of efficacy for the target cells **(Riehemann *et al.*, 2009)**.

Inorganic nanoparticles are being widely used throughout the world due to their incredible yet, potential applications. Among the metal NPs, Silver nanoparticles (AgNPs) in particular have attracted considerable attention in various fields and have been used as therapeutic agent (Shrivastava *et al.*, 2009), as catalyst (Christopher *et al.*, 2011) antimicrobial and anti-inflammatory agents (El-Chaghaby, and Ahmad, 2011; Veerasamy *et al.*, 2011), as a strong cytotoxic agent against cancer cell lines (Jacob *et al.*, 2012) . Silver and silver ions have been used since a long time as strong antimicrobial and anti-inflammatory agents. Nano-silver or silver nanoparticles (AgNPs) have shown promising results against the antibiotic resistant bacteria (Yah and Simate, 2015). AgNPs have also been investigated for their antimicrobial potential against Multi Drug Resistant MDR strains of several pathogenic microbes (Alshaye *et al.*, 2017). The role of AgNPs in diagnostic and probing of cancer has also been reported earlier in a research study (Huang *et al.*, 2017).

In literature, several chemical and physical methods have been used for the synthesis of AgNPs, but these methods utilize large amount of toxic chemicals and extreme conditions like high temperature and are not economical. The green synthesis approaches are a solution to this problem and are more ecofriendly, simple, reliable, reproducible and non-toxic. Recently, researchers have exploited microorganism as a production tool for the biosynthesis of inorganic metallic nanoparticles, such as silver, cadmium, gold and sulfide (Hassaan *et al.*, 2018; Elsalam *et al.*, 2018).

The bacterial synthesis seems to be advantageous because of minimal utilization of toxic chemicals and sustainability of large scale production. Many reports have shown that numerous bacterial strains of Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Bacillus licheniformis, Enterobacter cloacae, Klebsiella pneumonia, Lactobacillus acidophilus, and Pseudomonas aeroginosa, are capable of synthesizing nanoparticles (NPs) (Ghiuą *et al.*, 2018; Muthulakshmi *et al.* 2018; Fatemi *et al.*, 2018).

Biosynthetic methods can be categorized into two groups, on the basis of location of synthesis i.e. intracellular and extracellular synthesis. The extracellular synthesis of nanoparticles is simple and economical because of the simplicity in its procedure for not only synthesis but also of recovery and purification as well, under large scale production **(Singh *et al.*, 2018).** All these reasons make the bacteria a potential source for the extracellular synthesis of AgNPs avoiding the use of toxic and hazardous chemicals.

Novel technologies are being developed to overcome the challenges imposed by bacteria and cancer cells, the resistance associated with the regular use of antibiotics through MDR phenomenon and to minimize the side effects of the drugs used in chemotherapy **(Mohammed *et al.*, 2018).**

The current study aims to address the issues of MDR resistance of bacteria *S. aureus* an uses AgNPs as a tool against cancer cells. Here, AgNPs were synthesized extracellularly from bacteria MDR *S. aureus*, characterized by UV-vis spectroscopy, Transmission Electron Microscopy (TEM), Zeta potential value, Dynamic Light Scattering (DLS), Fourier Transformed Infra Red (FTIR), and subsequently tested against normal and MDR *S. aureus* as well as against cancer cell lines HCT-116

MATERIALS & METHODS

All the chemicals and media were purchased from Sigma Aldrich (St. Louis, USA) and HiMedia, India. The multidrug resistant (MDR) and normal strain of *Staphylococcus aureus* (NCIM 2079) were obtained from NCIM, Pune, India.

Synthesis of AgNPs

The MDR strain of *S. aureus* (NCIM 2079) was used for the synthesis of AgNPs. Bacterial strain was maintained on nutrient agar at 37° C. The bacterial cultures (OD600- 0.60) were transferred to Erlenmeyer flasks containing 250 ml nutrient broth and incubated at 37°C on a rotary shaker (180 rpm) for 6-8 hrs. The bacterial cells were collected by centrifugation (6000 g, 10 min at 10°C), washed extensively with sterile distilled water under aseptic conditions and used for further studies. Two different reactions were carried out by incubating *S. aureus* (NCIM 2079) cells (wet weight-5gm) in 100ml sterile distilled water containing 1mM AgNO3 in two different 500ml Erlenmeyer flasks for 6-8 hrs at rotatory shaker (180 rpm) at 37°C. The monitoring of the bacteria mediated reduction of silver ions was accomplished by the visual color change and UV–visible spectrum analysis for the reaction mixture.

At the end of synthesis, the unbound proteins were removed by Precipitation with 100% (v/v) volumes of absolute ethanol and the AgNPs were collected for further characterization.

Characterization Of AgNPs

The AgNPs synthesized using *S.aureus* (NCIM 2079) were characterized by UV-Vis Spectroscopy, Dynamic Light Scattering, Zeta potential analysis, TEM and FTIR for the confirmation of synthesis, size, shape, stability, surface functionalization and identification.UV-Vis spectrophotometer measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1nm.

Transmission Electron Microscopy (TEM) measurements were performed to analyze the size and morphology. Samples were prepared by drying a drop of AgNPs solution on carbon coated TEM copper grids followed by measurements on (TEM) FEI Company, TecnaiTM G2 Spirit BioTWIN operated at an accelerating voltage of 80kV. The study was done Indian Institute of Toxicological Research (IITR), Lucknow, India. Dynamic Light Scattering (DLS) was used to analyze the mean particle size (MPS) of biosynthesized AgNPs using dynamic light scattering particle size analyzer (Zetasizer Nano-ZS, Model ZEN3600, Malvern Instrument Ltd, Malvern, UK). The samples were taken in a DTS0112-low volume disposable sizing cuvette of 1.5 ml capacity. The sample powder was dissolved to a concentration of 0.5% (w/v) in deionised water and sonicated for 1 min before analysis. After filtering through a syringe filter of 0.4µm pore size, solution was centrifuged at 5000 rpm for 30min and measured for its particle size. Mean particle size was the average of triplicate measurements for a single sample. Zeta potential of AgNPs was determined to measure the charge, as the metal nanoparticles are normally charged or carry charge of capping agents. This analysis was done in Zetasizer Nano-ZS, (Malvern Instrument Ltd. and Malvern, UK).Fourier-transform infrared spectroscopy analysis (FTIR) was used to identify capped biomolecules over the surface of as synthesized AgNPs was done by FT-IR (Perkin Elmer Spectrum, Jasco- 6100). For the analysis, silver nanoparticles were dried, grounded with KBr pellets and analyzed in the wavelength range of 4000 to 400 cm^{-1}.

The antibacterial activity of *AgNPs* was determined against MDR strain of *Staphylococcus aureus* (NICM 2079), and Normal Strains of *Staphylococcus aureus* (NICM 2079). The bacterial cells ($OD_{600\text{-}0.60}$) were allowed to grow in nutrient broth for 24 hours and kept in a shaker incubator at 180 rpm for 24 hrs at 37ºC. Bacterial lawns were prepared using 100 ml of cultured broth. Agar well diffusion method (Khan *et al.*, 2011) was used to determine preliminary antibacterial activity of biosynthesized AgNPs. Plates were prepared by pouring around 25 ml of sterile Mueller Hinton Agar (MHA) media in sterilized petridishes. Different strains of bacterial culture chosen for the study was swabbed using sterilized cotton swab. Sterilized gel puncture was used to make wells of 5 mm diameter. Two wells were made in each plate, with each one containing distilled water (for control) and the other was loaded with synthesized silver nanoparticles. The plates were left for 24 hr incubation at 37ºC. The inhibition was examined by identifying the clear zone around the well.

The minimum inhibitory concentration (MIC) of the synthesized AgNPs was determined by double diffusion method (Sarker *et al.*, 2007). The MIC_{50} was determined by broth dilution, performed on a 96 flat-bottom well plate. The medium used in the plates were prepared at double the final strength to allow for a 50% dilution once the inoculum is added. This approach allowed the inoculum to be prepared in distilled water, which permitted the absorbance to be determined using a spectrophotometer without interference from colored media. All the bacterial strains were allowed to grow till mid-logrithmic phase. Culture was subsequently harvested by centrifugation, washed with 1mM sodium Phosphate buffer (SPB) at pH 7.4, and diluted to 2×10^5 colony forming units (CFU)/ml in Saline phosphate buffer. About 90 µL of Mueller-Hinton broth was used in 96 well- microtitre plates to serially dilute the silver nanoparticles in desired concentrations. Bacterial suspension of 95×10^4 CFU/ well was used as inoculum. Inoculated Microtitre wells were incubated overnight at 37º C. The MIC_{50} was determined by taking lowest concentration of AgNPs at which the bacterial growth was inhibited. The numbers of colonies were determined by agar plate count method as discussed by Morones *et al.*, (2005).The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium. It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by sub culturing to agar plates that do not contain the test agent.

Cell viability and IC_{50} value determination of AgNPs against cancer line HCT-116

The biosynthesized silver nanoparticles were tested against the Human colon cancer cell line HCT-116. The anticancer activity of synthesized silver NPs was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay and nuclear degradation was checked by DAPI (4', 6-diamino-2-phenylindiole) toxicology assays. For this, the cells were treated with varying concentration of AgNPs and the effect was analyzed by MTT assay and DAPI.

To determine the effect of Ag nanoparticles on cancer cells, MTT assay was performed. For this the HCT 116 cells were plated in 96 well plate with each well seeded with 1 X 10^4 cells. The plate was incubated for 24 hours in CO_2 incubator. After incubation, cells were treated with Ag NP of various concentrations in triplicate with

untreated cells as the positive control and incubated for 48 hours. After this, media was removed and wells were loaded with 50µl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) dye (5mg/ml in PBS) and fresh culture media. Then the plate was incubated for 4 hours. Formosan crystals formed during the process were dissolved by adding 100µl of DMSO (Dimethyl sulfoxide) and incubated for one hour. The reduced MTT was quantified by measuring optical density at 570 nm in ELISA reader.

The Percentage inhibition of the cells was calculated using the formula

$$X = 100 - (A\ test - A\ blank) / (A\ control - A\ blank) \times 100$$

Where, X- Percentage inhibition, A test – absorbance of the test sample, A blank – absorbance of blank and A control – absorbance of the control sample. The IC50 value was calculated from the data obtained.

The Apoptotic effect of Ag NP on the cell line was determined by nuclear fluorescent staining by DAPI. For this the cells were seeded in 96 well plate and treated with the Ag NP as mentioned above. Then media was removed and cells were washed with PBS and fixed with 4% para formaldehyde for 10 minutes. Then the staining with DAPI was performed along with permeabilizing buffer to fix the stain into the cells. After staining, the cell imaging was obtained under fluorescence microscope. The cells showing fragmented and condensed nuclei were considered as the apoptotic cells.

RESULTS AND DISCUSSION

Nanoparticles synthesis was observed as the color of reaction mixture changed from light yellow to dark brown, a characteristic color change associated with formation of silver nanoparticles. UV-Vis, spectroscopy is simple and sensitive techniques for characterization of colloidal suspension and requires very short period of time for measurement (Tomaszewska *et al.*, 2013). In UV-Vis, spectroscopy, the measurement of intensity of light passing through the sample is done, as the optical properties of metal nanoparticles are due to collective oscillation of conduction electrons, excited by electromagnetic radiation (Singh *et al.*, 2018).

The UV-Vis spectrum in our results shows the maximum absorbance peak around 430 nm, thereby confirming the synthesis of AgNPs in the sample.

Transmission Electron Microscopy (TEM) was used to visualize the shape and to determine the size distribution of synthesized AgNPs (fig.2.). TEM images were obtained using JEOL 3010, operating at 200 kV accelerating voltage. The TEM analysis was performed at Indian Institute of Toxicological Research (IITR), Lucknow, India. The high resolution images for topographical studies revealed the structure and morphology of synthesized silver nanoparticles (AgNPs) and average size was found to be 15 nm.

Gomaa and Zakaria (2017) have reported similarly in the synthesis of spherical shaped AgNPs with an average size of 17 nm using *Staphylococcus aureus* and *Escherichia coli*.

Dynamic light scattering (DLS) based on the laser diffraction method with multiple scattering technique was chosen to determine the size of AgNPs. DLS is a method which is dependent upon the interaction of light with particles in suspension. It is typically used to measure the particles size distribution in the range of 2 nm to 500 nm (Tomaszewska *et al.*, 2013). In DLS measurement the scattered light passing through the colloidal suspen-

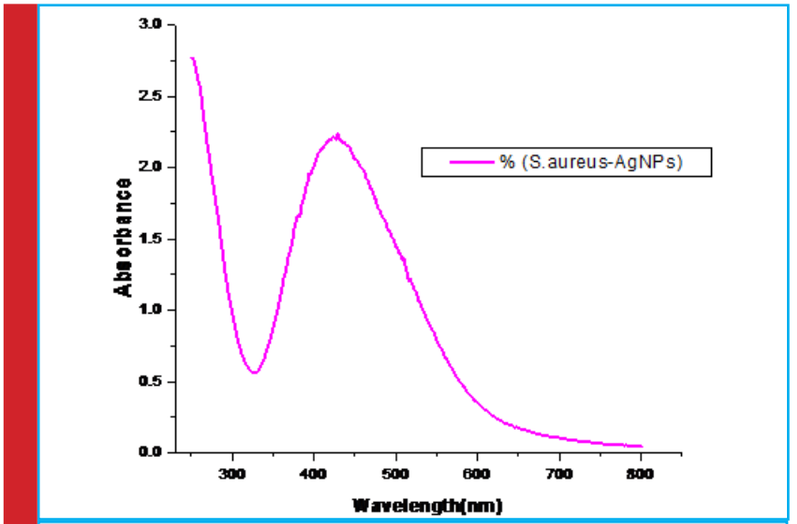

FIGURE 1. UV-Visible Spectrum of Synthesized Silver Nanoparticles

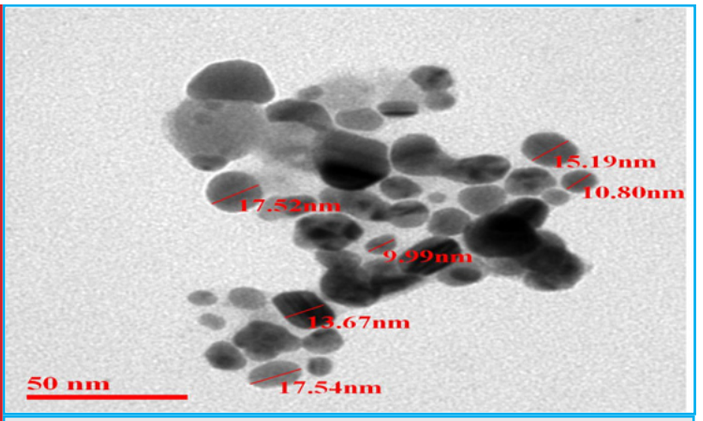

FIGURE 2. TEM image (taken at magnification of 110000x) Showing the Shape, Internal Structure and Size of AgNPs

sion relies on Rayleigh scattering from the particles in suspension (Fissan *et al.*, 2014). Next, the hydrodynamic size of the particles is determined by analyzing the modulation of intensity of scattered light as function of time (Dieckmann *et al.*, 2009). DLS revealed the Z-average value to be 88.65 nm. Similar results were obtained in another research in which average diameter silver NPs was found to be 87.46 nm in the aqueous colloidal suspension of AgNPs. (Singh *et al.*, 2018).

The determination of Zeta potential is considered as an effective simplest, and most straight forward method to predict the stability and understand surface properties of the nanoparticles. Zeta potential is a measure of the colloidal stability of the nanoparticles in a solution (Saeb *et al.*, 2014). The shielding or exposure of charged groups or concentration, distribution, ionization and adsorption of nano-particles can also be estimated from zeta potential (Guilatt *et al.,* 2004).

Information with reference to the concentration, distribution, exposure or shielding of charged moieties; ionization and adsorption could be inferred from the analysis of zeta potential. In the present study, the zeta potential of the synthesized AgNPs was found to be -24.3 mV, which confirmed that the AgNPs were highly stable in colloidal suspension. Similar observation were also recorded in which zeta potential value was measured to be -25.5 mV (Singh *et al.*, 2018).

FTIR of the synthesized AgNPs is performed to spot the position of various functional groups of the capping agent and their vibration patterns. The FTIR results of the sample states that the position of Amide I(C=O) bond is 1637 cm-1 with transmittance of 10.75%, O-H(Stretch) is 3583.13 cm-1 with transmittance of 9.19%, C-O(Stretch) is 1082.41 cm-1 with transmittance of 16.19% and Alkyne (Stretch) bond 2097.88 cm-1 with transmittance of 33.17%.

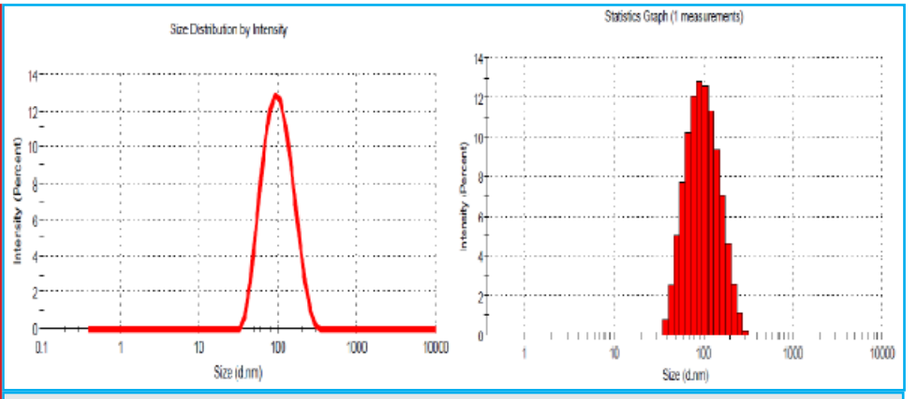

FIGURE 3. DLS Curve (Z-value-88.65 nm) of the AgNPs synthesized using MDR *S.aureus* bacteria.

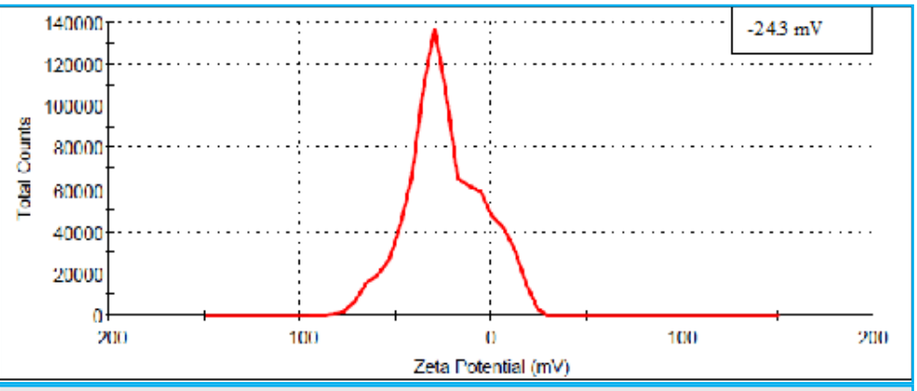

FIGURE 4. Curve representing the Zeta potential (-24.3 mV) of the AgNPs synthesized

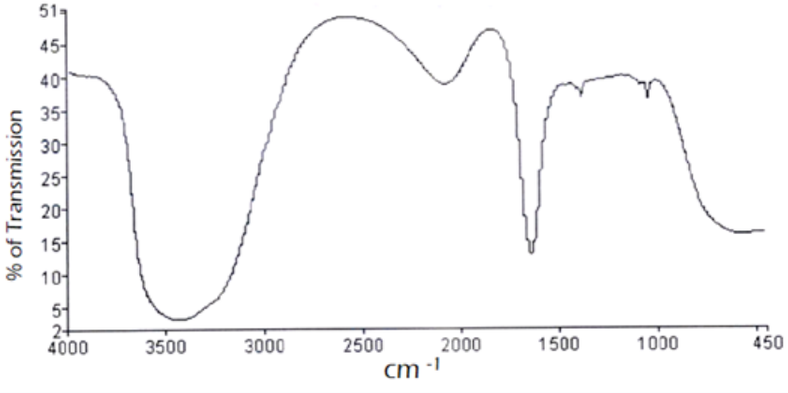

FIGURE 5. The FTIR Spectraof the AgNPs synthesized using MDR *S.aureus* bacteria.

Antibacterial activity of AgNPs against normal and MDR strains

The AgNPs synthesized using MDR *S.aureus* was tested for its antibacterial potential, against normal strains of *S.aureus*. The same was tested against strains of MDR *S.aureus* as well. The antimicrobial potential was tested by agar well diffusion method, MIC and MBC.

In general MIC is defined as the minimum amount of drug required to inhibit the growth of bacteria to 70%-80%. MIC_{50} is defined as the minimum amount of drug required to inhibit the growth of bacteria to 50%. The clear zone of inhibition around the inoculated area confirmed the antibacterial potential of as the synthesized AgNPs. MIC and MBC concentrations were calculated

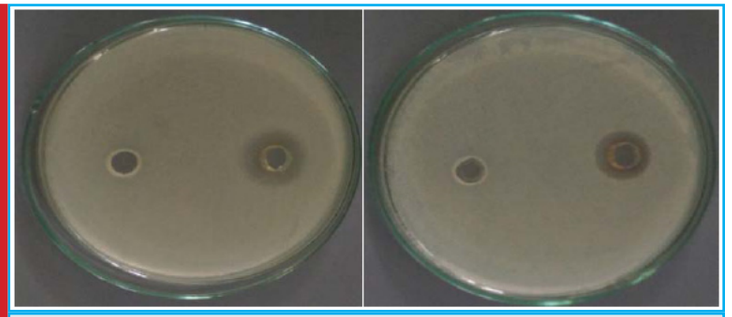

FIGURE 6. Plates showing the zone of inhibition of AgNPs against- a) Normal strain of *S.aureus*, b) MDR *S. aureus*

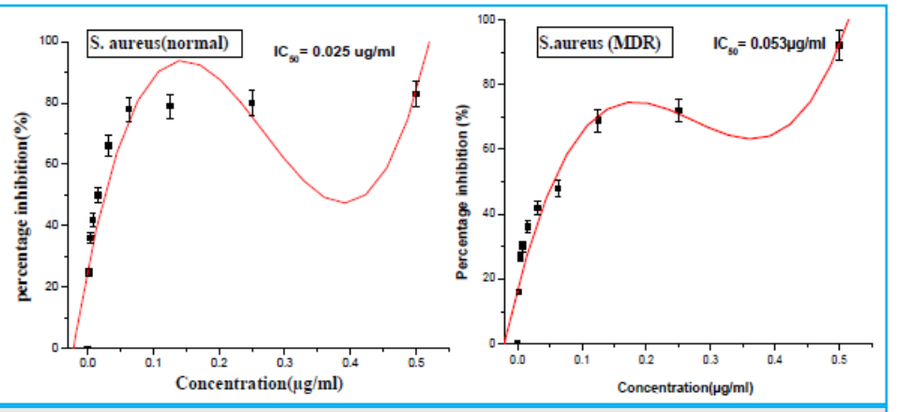

FIGURE 7. Curve representing the MIC of AgNPs against a) Normal strain of *S.aureus*. b) MDR *S.aureus*

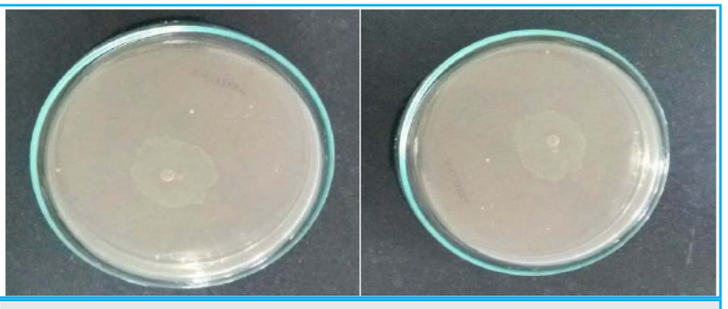

FIGURE 8. Plates showing MBC of AgNPs against: a) MDR strain of *S.aureus* and b: *S.aureus* (Normal strain)

under varying concentrations of the AgNPs synthesized using MDR *S.aureus*. The Zone of inhibition of AgNPs against normal strain and MDR strains of *S.aureus* were found to be 2.8 cm and 2.5 cm respectively (figure no. 6a and 6b). The Minimum inhibitory concentration of AgNPs against normal strain and MDR strains of *S.aureus* were found to be 0.025 µg/ml and 0.053 µg/ml for respectively. The lowest concentration with no visible growth indicating 99.5% killing of the inoculum was found to be double the value of MIC, (see figure 8 a and b).

Anticancer activity against HCT-116

To evaluate the sensitivity of colon cancer cells to the AgNPs, human colon cancer cell line HCT-116 was treated with different doses (0.01–10 µg/ml) of AgNPs

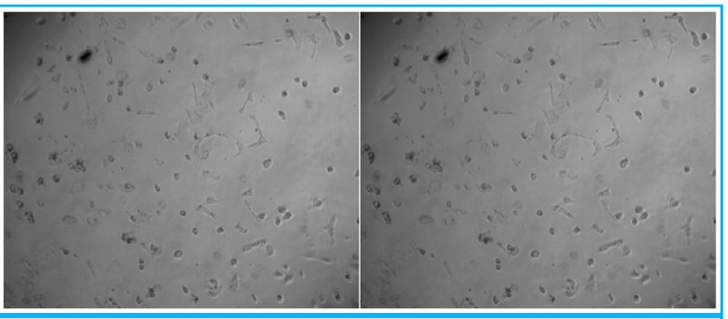

FIGURE 9. Imagesof human colon cancer cell line HCT-116 (Taken at magnification of 10x): a) untreated cells b) cells treated with AgNPs.

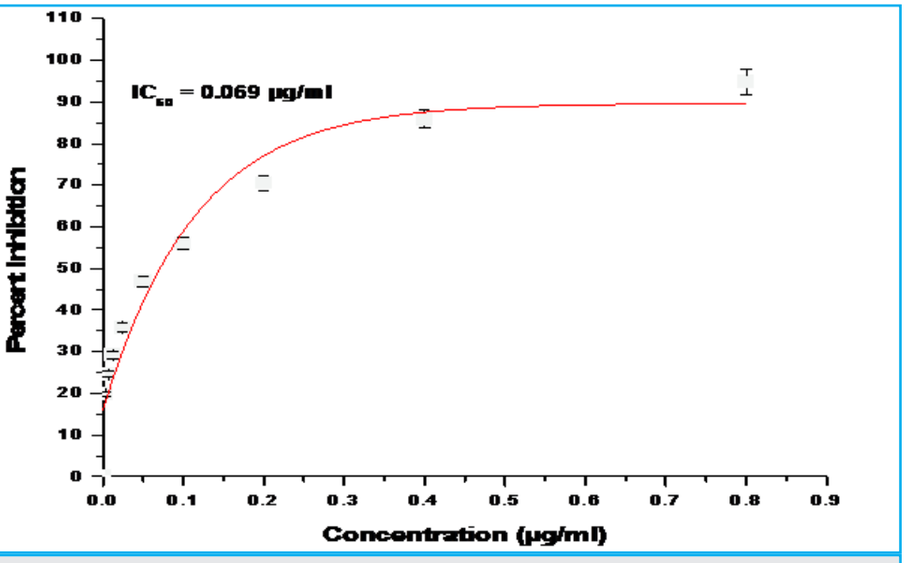

FIGURE 10. Graph showing the IC_{50} of AgNPs against Human coloncancer cell lines HCT-116

synthesized using MDR *S.aureus* for 24 and 48 h followed by MTT assay (Fig. 9 a and 9b). MTT is a dye which is reduced to formazon crystals during metabolism, cellular mechanism of MTT reduction into formazan is understood, involving reaction with NADH or similar reducing molecules that transfer electrons to MTT Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. So, the intensity of the colored product is measured which is directly proportional to the number of viable cells in the culture **(Präbst *et al.*, 2017).** The MTT analysis was done to assess the activity of mitochondrial succinate dehydrogenase **(Singh *et al.*, 2018).**

Here, the decrease in the MTT reduction could be due to decrease in cell viability which in turn can be attributed to cytotoxic activity of silver nanoparticles. Our results shows that AgNPs at a concentration of 0.069 µg/ml reduced growth of human colon cancer cells by 50%, after 24 h of treatment. Similar pattern of results with more pronounced cytotoxic effect was observed after 48 h of treatment thus proving AgNPs to be more anti-proliferative and cytotoxic for colon cancer cells. Similar findings have been reported in earlier studies, (Singh *et al.*, 2018; Patra *et al.*, 2018).

AgNPs induced nuclear condensation in HCT-116 cells: Apoptosis is characterized by changes in cellular morphology such nuclear fragmentation, degradation of DNA, condensation of chromatin, apoptotic body formation and membrane blabbing (Sheikh *et al.,* 2018). In order to confirm apoptosis in AgNPs treated colon cancer cells, DAPI nuclear staining was performed. DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent stain which preferably binds to AT rich regions of DNA.

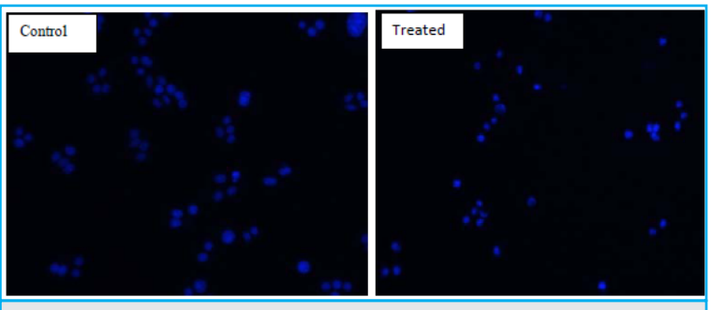

FIGURE 11. Representing DAPI Images of Human Colon cancer cell line HCT-116 (taken at a magnification of 20x): Control cells, on left and Cells treated with AgNPs, on right.

The fluorescence intensity of DAPI increases to approximately 20 times when DAPI is bound to DNA. As the ell permeability is compromised due to apoptosis; DAPI enters easily inside the cell where its binding to DNA produces a strong blue color (Baharara *et al.,* 2018).

After treatment with AgNPs for 24 h, significant nuclear changes in colon cancer cells were observed via DAPI staining. As apparent from photomicrographs, AgNPs induced nuclear condensation and fragmentation in colon cancer cells in a dose dependent manner, whereas the control cells exhibited normal cell morphology. The results were evident that AgNPs induced apoptosis in colon cancer cells in a time and dose-dependent manner. Our results are in agreement with several other researches published earlier (Gurunathan *et al.,* 2018; Baharara *et al.,* 2018; Kishore *et al*., 2018).

CONCLUSION

In this study, MDR strain of *S. aureus* was used to synthesize silver NPs, with the goal to inhibit the MDR strains of *S.aureus* as well as normal strain of *S.aureus* to assess its anticancer potential.The synthesized NPs were found to be spherical in shape and approximately 15 nm in size. The NPs were found to form a stable suspension with a zeta potential value of -24.03 m. These particles were found to be capped by extracellular bacterial proteins (confirmed by FTIR). The minimum inhibitory concentrations were found to be 0.025 µg/ml for *S.aureus* (normal) and 0.053 µg/ml for *S.aureus* (MDR), respectively. These nanoparticles were also found to be substantially effective against human colon cancer cell lines HCT-116, with an IC_{50} value of 0.069 µg/ml. Further optimization is needed in order to assess the anticancer properties of as synthesized AgNPs.

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