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# Cytotoxic and Antibacterial Activity Evaluation of MDR Bacteria Mediated Synthesized Silver Nanoparticles

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## ABSTRACT

In the current study cytotoxic and antibacterial activities of MDR bacteria *S.aureus* and *E.coli* mediated synthesized AgNPs (Silver Nanoparticles) were determined. The Antibacterial activities of silver nanoparticles were determined against *L. monocytogenes* and *S. abony*. The cytotoxicity of silver nanoparticles was determined against SH-SY5Y cell lines. MTT assay and DAPI staining were used to determine the cytotoxic potential. DCFH-DA was used to determine ability of ROS generation of synthesized silver nanoparticles. The MIC values of MDR S.aureus mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were found to be 36.22 µM and 35.65 µM respectively. Whereas MIC values of MDR E.coli mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were calculated to be 41.44 µM and 31.62 µM, respectively. Similarly, IC50 values of MDR S.aureus and MDR E.coli mediated synthesized AgNPs against SH-SY5Y cell line were reported to be 148.25 µM & 152.1 µM. DAPI results suggest that synthesized silver nanoparticles were able to cause nuclear condensation in the treated cell. Qualitative ROS generation via DCFH-DA assay suggest that MDR bacteria *S.aureus* & *E.coli* mediated synthesized Silver Nanoparticles have the capacity to generate significant amount of Reactive Oxygen Species(ROS), the higher amount of ROS generation was observed at a concentration of 300 µM AgNPs.

**KEY WORDS:** ANTIBACTERIAL, CYTOTOXIC; MDR; SH-SY5Y; ROS.

## INTRODUCTION

Increase in microbial and cancer resistance is on a continuous rise and has now become a global problem. The primary cause of this increased resistance is due to rapid misuse of antimicrobial and chemotherapeutic agents (Skladanowski et al., 2016). In order to address this fast rising problem, scientific fraternity is in continuous search of alternative treatment. Researchers are now concentrating more on unconventional and alternative approaches such as use of antimicrobial peptides, nanoparticles and quorum quenching nanomaterials (Ma et al., 2018).The rapid emergence of nanotechnology and specifically nanoparticles has led to the discovery and recognition of nanoparticles as a potent antimicrobial and anticancer agent. Silver ions in particular have shown their extraordinary potential as antimicrobial and anti-inflammatory agent, and are being used widely since ancient times. Silver NPs (AgNPs), due to their unique characteristic properties and high therapeutic potential have found great application. The AgNPs are

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now used in diagnosis and probing of cancer (Alshaye et al., 2017), biomolecular detection (Karim et.al., 2018), catalysis (Burdusel et al., 2018), wound healing (Orlowski et al., 2018). The antimicrobial potential of these silver nanoparticles (AgNPs) against MDR strains of a variety of pathogenic bacteria and cytotoxic potential against cancer cells has been well established (Abalkhil et al., 2017; Huang et al., 2017).

The bactericidal potential of the AgNPs can be attributed to large surface area to volume ratio, a consequence of smaller size, which permits the AgNPs to interact closely with the bacterial membrane (Helmlinger et al., 2016). Several studies propose that silver nanoparticles disturb the function of their target cells by getting attached to the cell membrane surface (Yan et al., 2018). Other studies have suggested that silver nanoparticles could induce cell death in gram negative bacteria *E. coli*, by causing the formation of small ''pores'' in the bacterial cell wall, which increases the permeability and ultimately inactivates the respiratory chain and electron transport system. In the present work the cytotoxic and antibacterial potential of previously synthesized silver nanoparticles from MDR *S.aureus* and *E.coli* was determined (Mohd Haseeb et al., 2019). Through this study we have aimed to address the challenges associated with cancerous cells and bacterial infections. The synthesized silver nanoparticles were characterized by various techniques such as UV-Vis Spectroscopy, Zeta potential, Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM), and Fourier Transformed Infra Red (FTIR) Spectroscopy (Mohd Haseeb et al; 2019). The synthesized silver nanoparticles were subsequently tested for their antibacterial activity against *L. monocytogenes* and *S. abony*. The cytotoxic activity and the ability of these silver nanoparticles to generate ROS was determined against SH-SY5Y.

## MATERIAL AND METHODS

All the Media and chemicals used in the experiment were purchased from Sigma Aldrich (St. Louis, USA) and HiMedia, India.

**Antibacterial Activity:** The antibacterial potential of MDR *S.aureus* and *E.coli* synthesized Silver nanoparticles was determined against *L. monocytogenes and S. abony*. These bacteria (OD600-0.60) were allowed to grow in Nutrient broth (NB) for 24 hours then incubated for 24 hrs at 37$^0$C kept at 180 rpm in a rotary shaker incubator. 100 ml of cultured broth was used to prepare bacterial lawns. The antibacterial potential of MDR *S. aureus and E.coli* mediated synthesized silver nanoparticles was determined by method discussed elsewhere (Mohd. Haseeb et al., 2019). 25 milliliters of sterile Mueller Hinton Agar (MHA) medium was poured in sterilized petriplates to prepare culture plates. Sterilized cotton swab was used to swab the bacterial strains from culture plates. Three wells of 5 mm diameter each were made in every plate by using Sterilized gel puncture. One of the well from each plate was filled with sterilized dH2O (for control) and the other two were added with MDR *S.aureus* and MDR *E.coli* mediated synthesized AgNPs. The inoculated plates were incubated 24 hours at 37$^0$C temperature. Clear zone of inhibition around the wells were examined in order to determine the antibacterial potential of as synthesized AgNPs.

**Minimum Inhibitory Concentration (Mic) of AgNPs:** MIC50 is the amount of drug needed to inhibit the bacterial growth to 50%. A flat-bottom, 96 well plates was employed to determine the MIC50 by broth dilution method. This enabled the inoculum to be prepared in distilled water allowing the determination of absorbance by UV- spectrophotometer and thereby eliminating the need of interference from colored media. Double diffusion method, was used to determine the MICs of the E.coli synthesized silver nanoparticles. The bacterial strains were grown till mid-log phase, culture harvesting was achieved by centrifugation, 1mM sodium Phosphate buffer (SPB) was used to wash the harvest at pH 7.4, and finally diluted to 2 × 105 CFU/ml in same buffer. AgNPs were serially diluted in desired concentrations by taking 90 microtitre (µL) of Mueller-Hinton broth in 96 well- microtitre plates. Bacterial suspension of $95\times10^4$ CFU/ well was taken as inoculum. After inoculation, wells were incubated for overnight at 37o C. The lowest concentration of silver nanoparticles inhibiting the bacterial growth was used to determine MIC50 of biosynthesized AgNPs.

**Evaluation of Cytotoxic Potential Against SH-SY5Y Cell Line:** The synthesized silver nanoparticles were analyzed for their anticancer activities against SH-SY5Y Cell line. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was used to determine the Cytotoxic activity of synthesized AgNPs whereas DAPI (4', 6-diamino-2-phenylindiole) toxicology assays was used to assess the nuclear degradation. In order to assess the same, the cells were treated with varying doses of silver nanoparticles. SH-SY5Y cells were grown in 96 well plates with each well having 1 X 104 cells, followed by 24 hours incubation in CO2 incubator. After a successful incubation period, silver nanoparticles of varying concentrations were used to treat the cells. The experiment was repeated in triplicate with untreated cells as the positive control and incubated for 48 hours. Spent medium was removed, followed by loading of wells with 50µl of MTT dye (5mg/ml in PBS) and freshly prepared culture media. Then the plate was incubated for 4 hours. Formazon 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 of cells Atest - absorbance of the test sample at 570 nm.

Ablank - absorbance of blank at 570 nm Acontrol - absorbance of the control sample at 570 nm The IC50 value was calculated by the obtained data. Nuclear condensation in silver nanoparticles treated SH-SY5Y cells was determined by using nuclear fluorescent stain 4', 6-diamino-2-phenylindiole (DAPI) dye. SH-SY5Y Cells were seeded in 96 well plates, treated with varying concentration silver nanoparticles were maintained in CO2 humidification incubator at 37 °C for 4 hrs. It was followed by careful removal of medium at appropriate times and washing the cells twice with saline phosphate buffer (SPB). The treated cells were washed again, stained with DAPI for 20 minutes at 37 °C, along with a permeabilizing buffer that allows the fixing of the stain into treated cells. Methanol and phosphate buffer saline was used to wash the stain respectively. Fluorescence microscope was used to visualize and capture the images of the stained cells and cells with fragmented and condensed nuclei were considered to be the dead cells.

**Assessment of Intracellular ROS (Reactive Oxygen Species) Generation:** The intracellular reactive oxygen species (ROS) level was measured by DCFH-DA method as per standard protocol. It is based on ROS-induced formation of the highly fluorescent dye 2'7'- dicholorofluorescin diacetate (DCFH-DA). Shortly, Human neuroblastoma cell lines SH-SY5Y were seeded in a 12 well plate at a density of 5 x 104 per well and incubated for 24 hr at 37 $^0$C. After treatment with AgNPs (0, 50, 100, 200, and 300 µM) for 12 hrs, the cells were incubated with 10 µM DCFH-DA for 30 minutes at 37 $^0$C. Cells were washed to remove the excessive amount of DCFH-DA. Images were captured by using an inverted fluorescence microscope) Nikon ECLIPDE Ti-S, Japan).

## RESULTS AND DISCUSSION

The antimicrobial potential of silver nanoparticles from MDR *S.aureus* and *E.coli* was determined against *L. monocytogenes and Salmonella abony.* The antibacterial potential of as synthesized silver nanoparticles were confirmed on the basis of the clear zone of inhibition observed around the area inoculated with NPs. Different concentration of silver NPs synthesized MDR *S.aureus* and MDR *E.coli* was used to calculate and determine the minimum inhibitory concentrations (MIC).

The zone of inhibition of MDR *S.aureus* silver nanoparticles on *L. monocytogenes* and *Salmonella abony* was found to be 18 mm and 19 mm respectively. The zone of inhibition of MDR *E.coli* synthesized silver nanoparticles on *L. monocytogenes and Salmonella abony* was found to be 17 mm and 18 mm respectively. The MIC values of synthesized silver nanoparticles against treated bacteria are given in table 1. The exact mechanism of action of Ag+ ions on microorganisms is not fully known. It is believed that the DNA of Ag+ treated microbial cells loses the ability of replication and protein inactivation occurs (Divya et.al. 2016). It has also been shown that silver ions cause protein denaturation by binding to functional groups on the protein. In a study involving *E.coli* and Metal oxide nanoparticles, treated bacterial cells were observed to have a significant increase in membrane permeability, making cells incapable of regulating the proper transport across the cell membrane and ultimately leading to cell death (Gomaa, 2017).

It has earlier been reported that antimicrobial potential is dependent upon dose and size, which makes gram-negative bacteria an obvious target as compared with gram positives bacteria. AgNPs may interact directly with microbial cells, may inhibit the enzymes of respiratory chain and affect the permeability of protons and phosphates for example; by halting the transmembrane transfer of electrons, disrupting the cell envelope thereby making it susceptible to the ROS (Reactive Oxygen Species) (Rajeshkumar and Malarkodi, 2014). Additionally, Silver nanoparticles can cause damage to bacterial cells by permeating the cell and interacting with proteins, DNA and some other cell constituents containing sulfur and phosphorus (Nayak and Chitra, 2015). In a similar research experiment where *E.coli* and *K. pneumonia* were treated with AgNPs the highest antibacterial effect was recorded on E.coli with a zone of inhibition of 13 mm and the lowest was examined on *K. pneumonia* with a zone of inhibition of 7mm (Feng et.al., 2000).

**Figure 1: Plates showing the zone of inhibition of MDR *S.aureus* and *E.coli* mediated synthesized Silver NPs against- *L. monocytogenes***

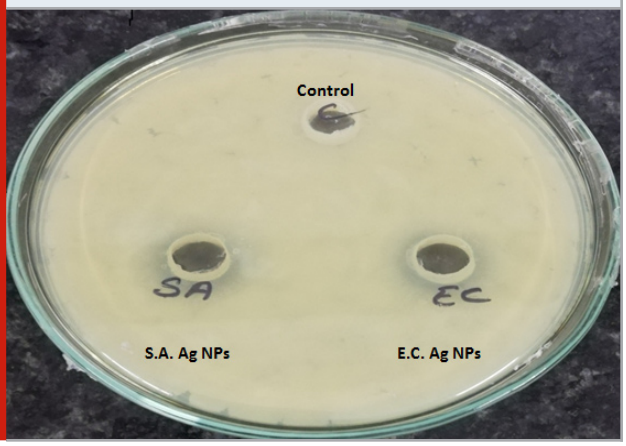
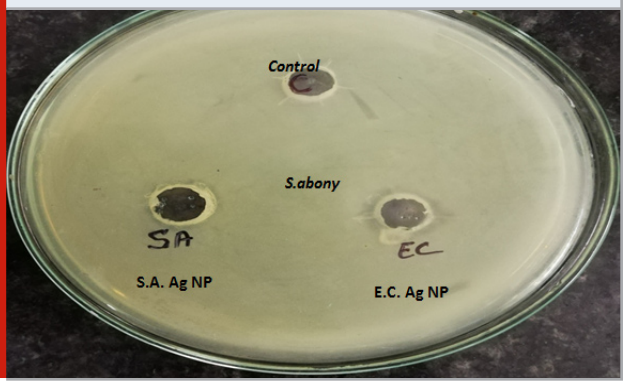



**Figure 2: Plates showing the zone of inhibition of MDR *S.aureus* and *E.coli* mediated synthesized Silver NPs against- *S.abony***



**Cytotoxic Potential Against SH-SY5Y:** The synthesized silver nanoparticles froms MDR *S.aureus* and *E.coli* were assessed for their cytotoxic potential on SH-SY5Y neuroblastoma cancer cell line. The cell viability or cytotoxic activity was assessed via MTT assay and nuclear fragmentation was studied via DAPI staining.

**Cytotoxicity of Mdr MDR S.aureus and MDR E.coli Synthesized Silver Nanoparticles:** The vey aim of cell viability assays is to determine the cellular response of any toxic material that has the potential to influence the metabolic activity of the target cells. MTT analysis is used to analyse the mitochondrial succinate dehydrogenase activity as metabolically active viable cells have the capacity to convert the MTT to purple color formazon crystal which gives maximum absorbance near 570 nm. The cellular mechanism behind this reduction of MTT probably involves NADH or a similar reducing agent from which electrons are transferred to tetrazolium, which is then reduced to formazon. The dead cells lose the ability

Table 1. Representing MIC values of synthesized silver nanoparticles

| Name of sample | MIC (µM) L. monocytogenes | *S. abony* |
|---|---|---|
| MDR *S.aureus* AgNPs | 36.22 µM | 35.65 µM |
| MDR *E.coli* AgNPs | 41.44 µM | 31.62 µM |

Figure 3: Curve showing the MIC of silver NPs synthesized from MDR *S. aureus* and MDR *E.coli* against L. monocytogenes

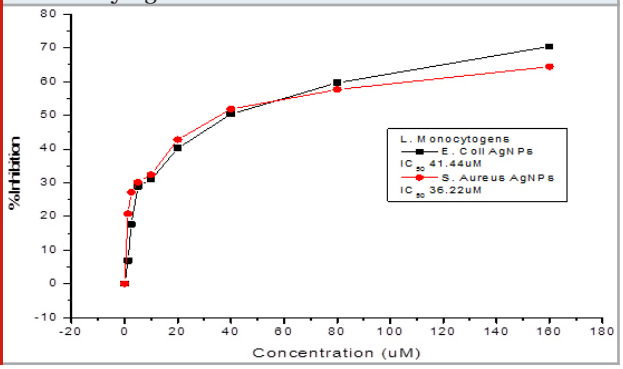
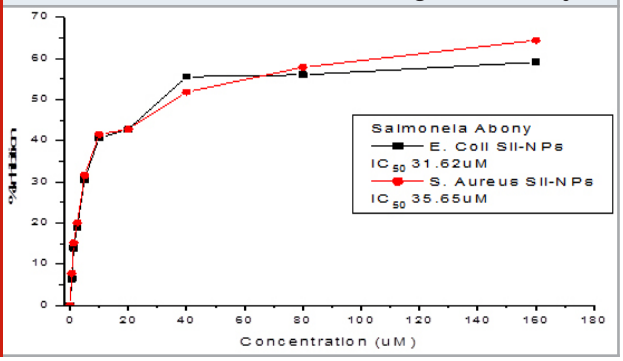



Figure 4: Curve showing the MIC of silver NPs synthesized from MDR S. aureus and MDR E.coli against S. abony




colored product decreases with decrease in cell viability hence the intensity of colored product which is directly proportional to number of viable cells in culture, is measured to determine the level of apoptosis (Präbst et al., 2017).

Here in our study, the cytotoxicity of silver nanoparticles in human neuroblastoma cancer cells SH-SY5Y was determined by treating the cells with varying dose of (10 µM to 300 µM) for 24 hr and 48 hr respectively, followed by MTT reduction assay. After a successful incubation period of 24 hr, 50% reduction in cell viability was noticed. The IC50 values were found to be 148.25 µM & 152.1 µM for MDR S.aureus and MDR E.coli mediated synthesized Silver nanoparticles. Almost same patterns of results with strong cytotoxic effects were observed after 48 hrs of treatment period, proving silver nanoparticles to be more cytotoxic and of anti-proliferative nature. Earlier studies have predicted similar findings in which the cytotoxic effects of silver nanoparticles have been reported (Singh et al., 2018). The cytotoxic efficacy of silver nanoparticles in affecting the survival of various cell types by disturbing the mitochondrial activity, structure and metabolism have been also predicted by several types of research (Suliman et al., 2015). In several previous studies, smaller sized Ag-nanoparticles have been shown to have more toxicity than the NPs with a larger size (Gurunathan et al., 2015). It has also been demonstrated that the type of capping agent dictates the potency of silver nanoparticles (Patra et.al., 2018). In order to determine the apoptotic cell death induced by as synthesized silver NPs, DAPI nuclear staining method was used. The apoptotic cells are characterized by cell shrinkage, extensive blabbing in the plasma membrane, and condensation of chromatin and formation of apoptotic bodies from separated cellular fragments (Sheikh et al., 2018).

The cells treated with AgNPs, were stained with DAPI, a DNA staining dye. DAPI is a fluorescent staining dye which binds preferentially to the AT-rich regions of double stranded DNA. When DAPI binds DNA its fluorescence intensity increases around 20 folds as compared with unbound DAPI (Baharara et.al., 2018). The intensity of stain is less in the viable cells as the membrane permeability is intact, DAPI fails to pass through but when cell permeability is compromised due to apoptosis; DAPI enters inside the cell with ease and a strong blue fluorescence is produced as it binds to DNA (Baharara et.al., 2018). After treatment with AgNPs for 24 h, significant nuclear changes in SH-SY5Y neuroblastoma cancer cells were observed (Fig. 7 & 8). As apparent from pictures of DAPI staining, AgNPs induced nuclear condensation and fragmentation in treated cancer cells in a dose dependent manner whereas the control cells exhibited normal cell morphology (Fig. 7 & 8). The apoptotic effect of silver nanoparticles can be attributed to excessive ROS generation which leads to DNA and protein damage (Foldbjerg et al., 2009; Gurunathan et al., 2018). The findings of our current research are in agreement with previously published reports that exposure of cells to NPs induces apoptotic

cell death ( Kishore et al., 2018; Mohammed et al., 2018). The results were evident that AgNPs induced apoptosis in neuroblastoma cells in a time dependent and dose-dependent manner.

The ability to generate ROS by AgNPs from MDR strains of *E.coli* and *S.aureus* was analyzed in human neuroblastoma cell lines SH-SY5Y. It was measured by the H2DCF-DA assay. After 24 h of exposure, increasing concentrations of AgNPs significantly increased ROS levels at various concentrations used in this study. In general, nanoparticles are known to kill cancer cells by producing ROS. Ag ions play an important role in catalyzing ROS production in the presence of oxygen species and AgNPs can induce oxidative stress in a variety of cellular systems by generating ROS, including in human cancer cells. To examine the effect of MDR S. aureus and E.coli mediated synthesized AgNPs on ROS generation, human neuroblastoma cell lines SH-SY5Y were treated with various concentrations (0, 50, 100, 200, and 300 µM) of AgNPs for 24 h, and then, ROS generation was measured in an H2DCF-DA assay. After 24 h of exposure, increasing concentrations of AgNPs significantly increased ROS levels at various concentrations used in this study. ROS levels generated in response to AgNPs treatment were significantly higher than those in untreated cells.

Marked differences were evident in the production of ROS between treated and untreated control cells. Treated cells emitted bright fluorescence and displayed deformed morphologies because of the loss of integrity of plasma membrane due to ROS generation. The cytotoxicity effects may have been due to the induction of oxidative stress and apoptosis upon ROS overproduction. Cancer cells generate high levels of ROS that leads to a state of increased basal oxidative stress. Since this state of oxidative stress makes cancer cells vulnerable to agents that further augment ROS levels, the use of pro-oxidant agents is emerging as an exciting strategy to selectively target tumor cells (Cordero et al., 2012). However, the induction of ROS formation plays an important role in the chemotherapeutic activity of several anticancer drugs and a large number of anticancer compounds (Fruehauf et al., 2007; Trachootham et al., 2009). Our data for the qualitative analysis of ROS generation suggested that AgNPs induced ROS-mediated induction of apoptosis in SH-SY5Y cells in a concentration- dependent manner.

Figure 5: Showing the IC50 of MDR *E.coli* mediated synthesized AgNPs against human neuroblastoma cell lines SH-SY5Y

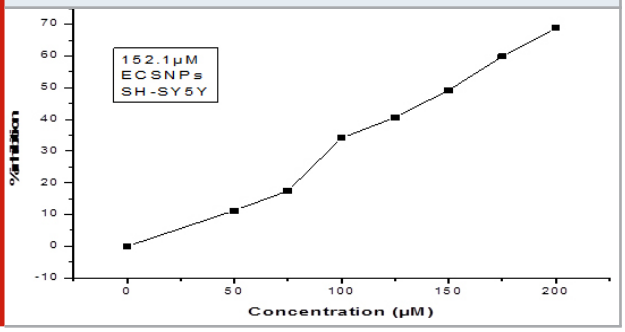



Figure 6: Graph showing the IC50 of S.aureus mediated synthesized AgNPs against Human neuroblastoma cell lines SH-SY5Y.

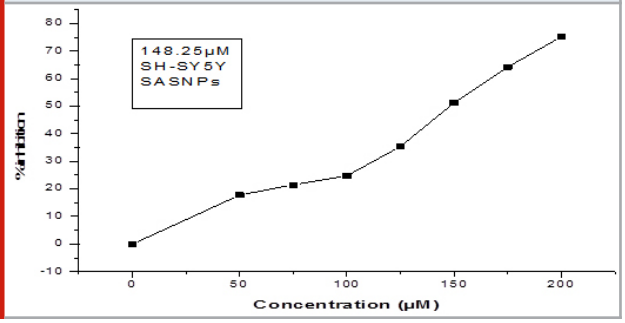



Figure 7. DAPI Images of SH-SY5Y (taken at a magnification of 40x): a) Control cells, on top left and b), c) & d) Cells treated with S.aureus mediated synthesized AgNPs, on right

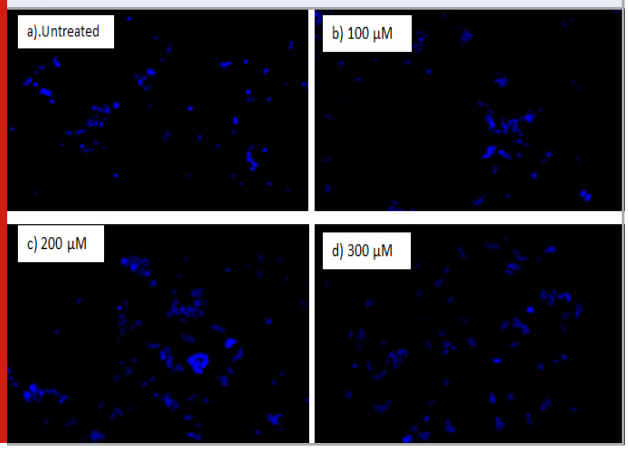



Figure 8: DAPI Images of SH-SY5Y (taken at a magnification of 40x): a) Control cells, on left and b), c) & d) Cells treated with *E.coli* mediated synthesized AgNPs, on right n clockwise.

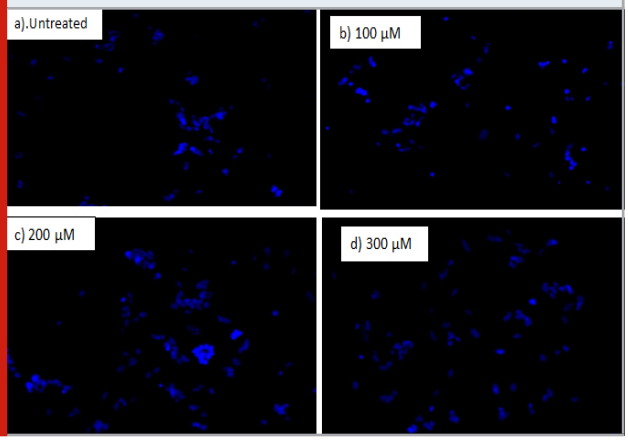


Figure 9: Effect of MDR *E.coli* mediated synthesized AgNPs on reactive oxygen species (ROS) generation. SH-SY5Y cells were treated with or without AgNPs for 24 h, and ROS generation was measured using DCFH-DA.

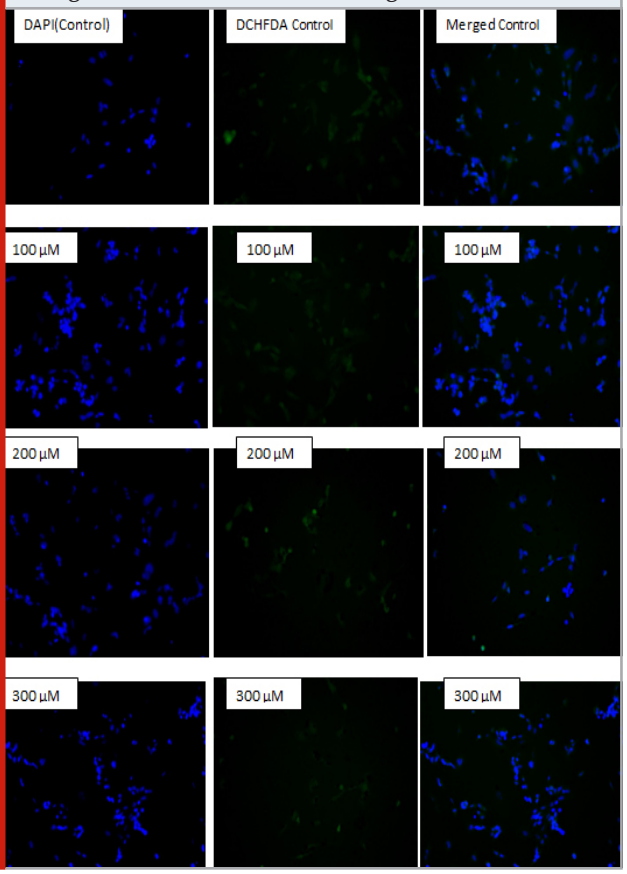
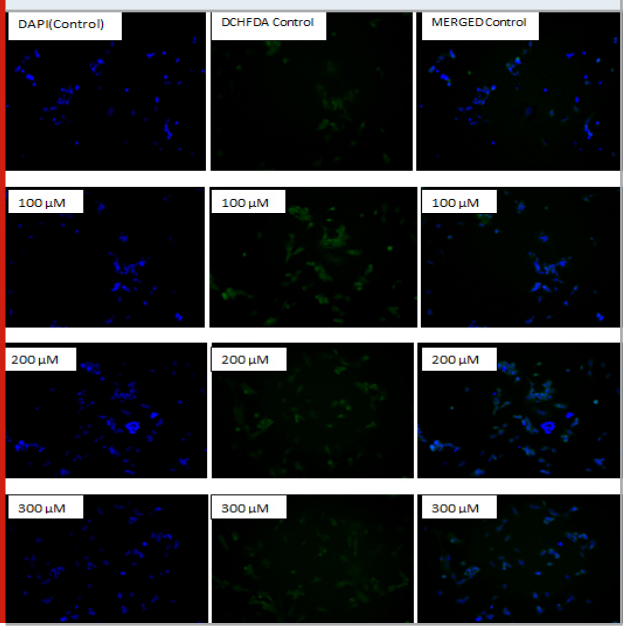



Figure 10: Effect of MDR S.aureus mediated synthesized AgNPs on reactive oxygen species (ROS) generation. SH-SY5Y cells were treated with or without AgNPs for 24 h, and ROS generation was measured using DCFH-DA.At all tested concentrations, the most pronounced effects were observed at higher concentrations, showing up to 2–3-fold higher ROS levels (Fig- 9 & 10).




## CONCLUSION

In the present work, the cytotoxic and antibacterial activities of MDR *S.aureus* and *E.coli* mediated synthesized silver nanoparticles (AgNPs) were determined. The MIC values of MDR *S.aureus* mediated synthesized silver nanoparticles against *L. monocytogenes* and *S. abony* were found to be 36.22 µM and 35.65 µM respectively. Whereas MIC values of MDR *E.coli* mediated synthesized AgNPs against *L. monocytogenes* and *S. abony* were calculated to be 41.44 µM and 31.62 µM, respectively. Similarly, IC50 values of MDR S.aureus and MDR *E.coli* mediated synthesized AgNPs against SH-SY5Y cell line was reported to 148.25 µM & 152.1 µM. DCFH-DA results show that silver nanoparticles are capable of generating large amount of ROS in their target cells; the ROS generation was recorded to be increasing on the increase of silver nanoparticle concentration. It was for the first time that MDR bacteria mediated synthesized were tested for their cytotoxicity against SH-SY5Y cell lines. Further research on various other cell lines is inevitable to establish these nanoparticles as a potent anticancer agent, also, being of MDR bacteria origin; these nanoparticles must also be assessed for any kind of associated antigenic challenge in animal models in order to determine the safety of these synthesized Silver nanoparticles.

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