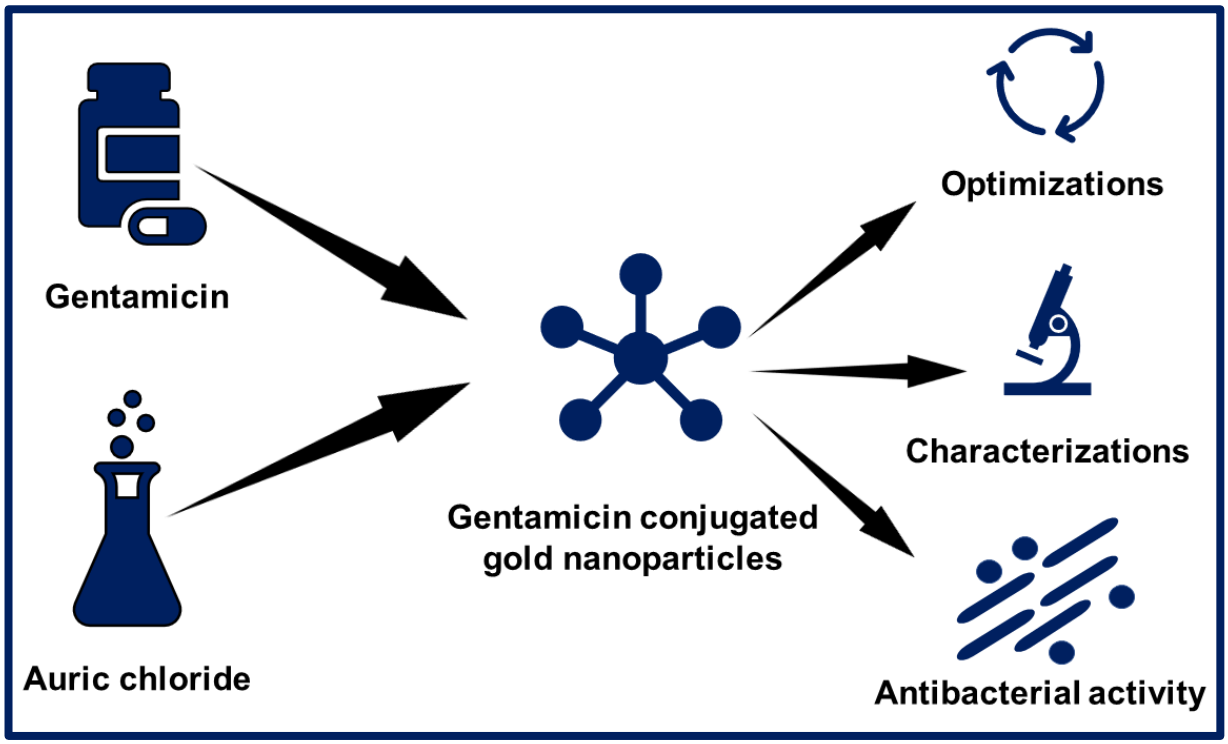
Chapter 3

Development of Gentamicin Conjugated Gold Nanoparticles and Evaluation of their Antimicrobial Potential

GRAPHICAL OVERVIEW OF THE CHAPTER



HIGHLIGHTS OF THE CHAPTER

- **Synthesis of gentamicin conjugated gold nanoparticles (G-AuNPs).**
- **Characterization of G-AuNPs.**
- **Antibacterial activity of G-AuNPs.**
- **Gentamicin release analysis of G-AuNPs.**
- **Cytotoxicity analysis of G-AuNPs on C2C12 cell line.**

CHAPTER 3

DEVELOPMENT OF GENTAMICIN CONJUGATED GOLD NANOPARTICLES AND EVALUATION OF THEIR ANTIMICROBIAL POTENTIAL

3.1 INTRODUCTION

Resistance to antibiotics in pathogenic microbes has been one of the contemporary medicine's possible challenges, that must be tackled in order to benefit humanity across the globe. The resistance mechanisms in these microbes are emerging and spreading, including enzymatic modification, reduced cell permeability, target shielding, alterations in target sites/enzymes, and enhanced efflux owing to overexpression of efflux transporters [375], [376]. Curable microbial infections, such as tuberculosis, pneumonia, and foodborne diseases, have become fatal or nearly fatal as a result of antimicrobial resistance. Antibiotic-resistant microbes kills 23,000 individuals as per CDC in United States annually. Furthermore, it is projected that through 2050, antibiotic resistance would kill 300 million people prematurely, resulting in a worldwide economic loss of up to $100 trillion dollars [377]. Despite the issues raised above, antibiotics remain a crucial tool in the fight against bacterial illnesses. Because of its inexpensive cost and widespread availability, gentamicin is extensively used to treat a variety of microbial illnesses. However, a large number of microorganisms have evolved resistance to gentamicin through various strategies and *Escherichia fergusonii* ATCC 35469 is one of them. *Escherichia fergusonii* ATCC 35469 have 64 % of its genomic similarity with *Escherichia coli*. *Escherichia fergusonii* ATCC 35469 can cause infections in both humans and animals [378], [379]. Nanomedicine or nano-therapy is an effective way to fight resistance. Compared with free drug molecules, nano-drug systems enhance the pharmacokinetics, pharmacodynamics, physicochemical characteristics, and efficacy of drug molecules [380]. The production of drug-conjugated nanoparticles typically involves three steps: nanoparticle synthesis, functionalization of nanoparticle surfaces, and finally, drug molecule loading on the functionalized surfaces [381], [382]. Furthermore, the majority of procedures include the use of hazardous chemicals, and the product must be purified, which increases the cost of the final product. Additionally, if these substances stay in nanoparticles, might have a negative impact on health. In present work we have tried to produce gentamicin-conjugated gold nanoparticles

(G-AuNPs) using one-pot synthesis approach. Our technique has the benefit like it does not need additional functionalizing agents or hazardous compounds in the synthesis process. An agar well diffusion experiment was used to examine the antibacterial activities of G-AuNPs towards gram-positive, gram-negative, and gentamicin-resistant microorganisms. Dialysis membrane approach was used to evaluate the release of gentamicin from the drug conjugate system. The MTT test was also used to investigate the cytotoxicity of the produced G-AuNPs on cell line (mouse myoblast C2C12).

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

Alfa Aesar, United Kingdom, provided the hydrogen tetrachloroaurate (III) trihydrate. HiMedia Laboratories Private Limited, Mumbai, India, provided gentamicin sulphate, potassium hydroxide (KOH), potassium dihydrogen phosphate (KH_2PO_4), sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH), potassium chloride (KCl), sodium chloride (NaCl), Muller Hinton broth (MHB), Luria Bertani broth (LB), Muller Hinton agar (MHA), dialysis membrane-70, MTT, and disodium hydrogen phosphate (Na_2HPO_4). Loba-Chemie in India provided the thiobarbituric acid (TBA), while S D Fine Chemical Limited in India provided the nitro blue tetrazolium chloride (NBT). Super Religare Laboratories, India provided the bovine serum albumin (BSA). Thermo Fisher, India, provided trypsin-ethylenediaminetetraacetic acid (EDTA) solution, foetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), and Dulbecco's phosphate-buffered saline (PBS). Merck India provided the dimethyl sulfoxide (DMSO) and methanol. Water for the experimentation was taken from the institution's Merck Millipore assembly.

3.2.2 Microorganisms and their maintenance

Microbes such as *Escherichia coli* DH5, *Escherichia. coli* ATCC 25922, *Escherichia fergusonii* ATCC 35469, and *Staphylococcus aureus* MTCC 3160 were taken from the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology's, Solan, India. LB was used to keep all of the cultures alive, and Luria agar plates and slants were used to preserve them. Cell line (C2C12) was purchased from The National Centre for Cell Science in Pune, India and sub-cultured in DMEM media.

3.2.3 G-AuNPs synthesis

G-AuNPs were synthesised by reacting 0.1 mM gentamicin with 2.5 mM auric chloride. The reactants ratio for synthesis was optimized by varying ratio viz. 1:3, 1:1, 3:1, and 2:3. Similarly, the pH for the synthesis was optimized by varying pH from 7-14. The reaction temperature for synthesis was optimized by varying condition from 35 °C to 75 °C. The optimum time for synthesis was determined by taking out samples at various time intervals (0 min to 300 min) and monitoring UV-vis spectra. UV-vis spectrophotometric measurement of different time samples was used to test the storage stability of G-AuNPs. All of the reagents were made prepared instantly and brought to room temperature (RT) before use.

3.2.3 Characterizations of synthesized G-AuNPs

The shape and size of G-AuNPs was studied using transmission electron microscopy (TEM, FP 5022/22-Tecnai G2 20 S-TWIN, FEI company of USA). Dynamic light scattering (DLS, Nano ZS, Malvern, U.K.) revealed the G-AuNPs hydrodynamic diameter and distribution. The UV-spectroscopy analysis was done using Thermo Scientific's TM Evolution 201 to record absorbance spectra. The cytotoxic assay was carried out using a microplate reader (Bio-Rad) and a fluorescence microscope (Nikon).

3.2.4 G-AuNPs antibacterial activity evaluation

Agar plate diffusion assay employing gram-negative bacteria viz. *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and gram-positive *S. aureus* MTCC 3160 were used in the current study to evaluate G-AuNPs antibacterial property. The assay procedure briefly involves the cultivation of all the bacterial cultures in Mueller Hinton broth for overnight. 0.5 McFarland standard concentration of bacterial cell was adjusted to as per Clinical and Laboratory Standards Institute instructions for antibacterial activity. A 50 µl of the each of bacteria was aseptically dispersed on MHA plates. A gel puncture having 3 mm internal diameter was used for making wells in MHA plates. For each culture on MHA, 15 µl of G-AuNPs, gentamicin equivalent to 0.5 µg concentration (working concentration), and gentamicin equivalent to 6.0 µg concentration (control) were introduced to the respective wells followed by 16-18 h incubation at 37 °C. The experiment was done in triplets. Results were analysed through measuring the inhibition zone on MHA plates.

3.2.5 Minimum inhibitory concentration (MIC) determination

The CLSI methodology was used to determine the gentamicin, G-AuNPs, and auric chloride MIC [383]. Methodology briefly involves overnight culturing of microorganisms (*E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and *S. aureus* MTCC 3160) in MHB. The cultures were subjected to dilution for achieving cell count of 1.5×10^8 cells/mL to meet requirements of CLSI guideline. A sterile microtiter plate (96-well plate) was introduced with MHB, gentamicin drug from 0 µg/mL - 200 µg/mL concentration, auric chloride solution from 0 µg/mL - 250 µg/mL concentration and colloidal G-AuNPs from 0 nM - 0.46 nM concentration, respectively. A 10 µl culture of each bacterium was incorporated into respective wells. The 96-well plate was incubated at the 37 °C temperature for a duration of 24 h followed by the monitoring of optical density (O.D.) at 600 nm to find out the MIC concentrations [382].

3.2.6 Determination of oxidative stress caused by G-AuNPs

3.2.6.1 Reactive oxygen species (ROS)

Metal nanoparticles have been fully proven to kill microorganisms by generating active oxygen. To determine the enhanced degree of reactive oxygen species by G-AuNPs, the nitro blue tetrazolium (NBT) assay was employed [384]. The experimental procedure for the assay involves incorporation of 150 µl G-AuNPs having 2.3 nM concentration to over-night grown 3 mL of all the bacterial cultures viz. *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and *S. aureus* MTCC 3160. The culture with inoculated G-AuNPs were allowed to incubate at 37 °C temperature for a duration of 6 h. The reaction mixture after incubation was subjected to centrifugation at 10,000 rpm speed and 10 min time. After centrifugation the pellet obtained was suspended in 2 % solution of NBT, and incubated in dark for 1 h at RT condition. The incubated reaction mixture was subjected to centrifugation (10,000 rpm) for a duration of 10 min. The pellet after centrifugation was gently washed with the buffer solution of sodium phosphate (0.1 M). The washed reaction mixture was again centrifuged at speed of 10,000 rpm for time period of 10 min. The obtained pellet after centrifugation was mixed with 2 M KOH solution (500 µl), and equal amount of DMSO (50 %) solution. All the components of reaction were mixed well and again incubated at RT conditions for a period of 10 min followed by again centrifugation at 10,000 rpm speed for a duration of 10 min. After the centrifugation supernatant was collected gently and was further used measure O.D. at wavelength of 620 nm. In the current

experiment MHB was used as a blank, while the culture lacking G-AuNPs treatment was utilised as a control.

3.2.6.2 Evaluation of membrane lipid peroxidation (LPO) caused by G-AuNPs

The effects of oxidative stress on LPO in microbial cells have been extensively researched. Cell membrane lipids, according to observations in the literature, are the best substrate for oxidative damage. The detection of lipid peroxidation in diverse systems is frequently done by measuring magnitude of thiobarbituric acid reactive compounds [385]. Malondialdehyde is a hazardous by-product of lipid peroxidation, which is triggered by enzymatic and oxygen free radicals. Malondialdehyde produces a compound with thiobarbituric acid (TBA) in the thiobarbituric acid reactive compounds test, that may be recognized and measured using a spectrophotometer at 535 nm. Briefly, to 3 mL of overnight grown bacterial cultures (*E. fergusonii* ATCC 35469, *E. coli* DH5α, *E. coli* ATCC 25922, and *S. aureus* MTCC 3160) G-AuNP (150 µl of 2.3 nM concentration), followed by incubation at 37 °C temperature in shaking conditions for a period of 6 h. After the incubation the cultures were centrifuged at speed of 10,000 rpm for a duration of 10 min. The pellets obtained after centrifugation were treated with 500 µl volume of 10 % SDS solution followed by addition of acetic acid (20 %). The suspensions were left for incubation at RT condition for 10 min of time. After incubation period the suspension mixtures were incorporated with 250 µl of 0.8 % TBA solution made in NaOH (2 M) solution. All the contents were again incubated at 95 °C temperature for a period of 1 h. Reaction mixtures were subjected to centrifugation (10,000 rpm) for 10 min. The supernatant after the centrifugation was collected and was utilized to measure the lipid peroxidation through recording absorbance value at 532 nm wavelength. The MHB was employed as a blank, while the cultures without any treatment were used as control.

3.2.7 Determination of bacterial membrane disruptions by G-AuNPs

The damage of G-AuNPs to the membrane was analysed as per the protocol explained by Zhao et al. [386]. Procedure of experiment briefly involves treatment of bacterial cultures (*E. fergusonii* ATCC 35469, *E. coli* DH5α, *E. coli* ATCC 25922, and *S. aureus* MTCC 3160) with 150 µl of 2.3 nM G-AuNPs for a duration 12 h. After the incubation period, all the bacterial cultures subjected to centrifugation at speed of 6000 rpm for a period of 5 min, and then washed twice with PBS. After the centrifugation period all the bacterial culture were mixed with 100 µM propidium iodide solution for a period of 30 min, followed by again centrifugation at speed

of 6000 rpm for a 5 min duration. The centrifuges bacterial cultures after the centrifugation were thoroughly washed thrice with PBS solution. After washing procedure 10 µl volume of each bacterial cell suspensions (in PBS) were placed on a neat and clean glass slides fooled by covering with cover slips. Bacteria that had not been treated with G-AuNPs were served as controls. Using a Nikon fluorescent microscope, cells with red fluorescence were recorded as the average of 3 individual observations.

3.2.8 Analysis of gentamicin release from G-AuNPs

Gentamicin release from the G-AuNPs was measured in PBS at 37 °C and pH 7.3. Briefly, a 1 mL colloidal G-AuNPs were taken to a dialysis membrane and was submerged in phosphate buffer saline. All the components were kept in incubation at 37 °C and samples were taken out certain intervals to determine the drug's release as described by Perni et al. [387]. In summarized way, 100 µl volume of o-phthalaldehyde (0.8 mg per mL) and absolute isopropanol were combined with a 100 µl volume of test sample followed by incubation at RT (dark conditions) for a period of 30 min. The fluorescence of samples was measured against a standard calibration plot using excitation and emission wavelength of 340 nm and 450 nm, respectively. The experiment was repeated three times.

3.2.9 Cytotoxicity analysis of G-AuNPs

The MTT assay has been used to test the toxicity of G-AuNPs on the C2C12 cell line [388]. In a 96 well plate, C2C12 (1×10^4 cells/well) cells were introduced in DMEM having 10 % FBS concentration for a period of 24 h at 37 °C in the CO_2 incubator. After the incubation period, a fresh DMEM was added to wells after decanting old medium followed by treatment of cells with G-AuNPs (150 µl volume of 2.3 nM), DMEM (150 µl), and PBS (150 µl). The cell plate was then cultured for another 24 h time at 37 °C in the incubator. The culture plate was once more incubated at 37 °C for 3-4 h supplemented with a volume of 10 µl MTT reagent (5mg/mL) prepared in PBS. Each well received 50 µl of DMSO after incubation to dissolve formazan crystals, and tissue culture plate was again kept for incubation at temperature of 37 °C for a duration of 30 min in CO_2 incubator.

3.2.10 Statistical analysis of data

All the experiments in the current study were performed in triplets. The data obtained from experimentation was statistically assessed with IBM SPSS version 20 through ANOVA and Tukey's multiple comparison test ($p>0.05$) (SPSS Inc., Chicago, IL, USA).

3.3 RESULTS AND DISCUSSIONS

3.3.1 Synthesis, optimization and characterization of G-AuNPs

One-pot synthesis of G-AuNPs in the current study was achieved using amino-glycosidic drug gentamicin. Gentamicin drug have both hydroxyl and amino groups in its structure. The hydroxyl groups of drug molecule have been believed to take part in the reduction of gold ions (Au^{+}) while amino groups acted as capping agents for G-AuNPs synthesis [389], [390]. Primarily, the synthesis of G-AuNPs was observed visually by monitoring colour change from yellowish colour of auric chloride and gentamicin mixture to red wine colour (Figure 3.1 inset). However, the synthesis of G-AuNPs was validated through UV-vis spectrophotometric analysis, where G-AuNPs exhibited surface plasmon resonance band at 520 nm (Figure 3.1) as there was no absorption peak was noticed in the spectrums of gentamicin and auric chloride in the 400-800 nm range.

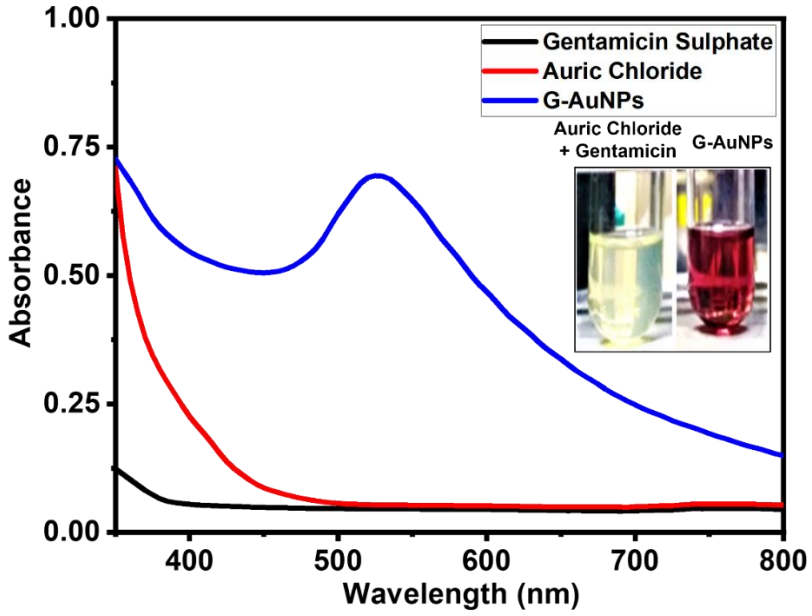


Figure 3.1: UV-vis absorption spectrum of auric chloride solution (1.25 mM), gentamicin sulphate drug (0.05 mM) and G-AuNPs (2.3 nM), inset auric chloride + gentamicin sulphate solution before synthesis and synthesised G-AuNPs.

The reactant solutions were mixed in various ratio (volume/volume) to optimized best combination for synthesis of G-AuNPs. The result obtained from UV-vis spectroscopy revealed that 1:1 ratio was optimum for the synthesis of G-AuNPs (Figure 3.2a). Visually also 1:1 ratio combination displayed red wine colour of G-AuNPs. In contrast, the 1:3 and 2:3 combinations exhibited light red colour while 3:1 displayed purple colour of synthesised G-AuNPs (Figure 3.2c). After ratio optimization, the pH of reaction mixtures was varied (7-14) to obtain an optimum pH for the synthesis of G-AuNPs. The finding obtained highlighted that pH 10 was optimum for the synthesis as there was heavy aggregation of G-AuNPs at 12 and 14 pH while incomplete or less synthesis yield at lower pH (7-9) observed through UV-spectroscopy (Figure 3.2b). There were also variations in the colour of G-AuNPs with respect to change in pH (Figure 3.2d).

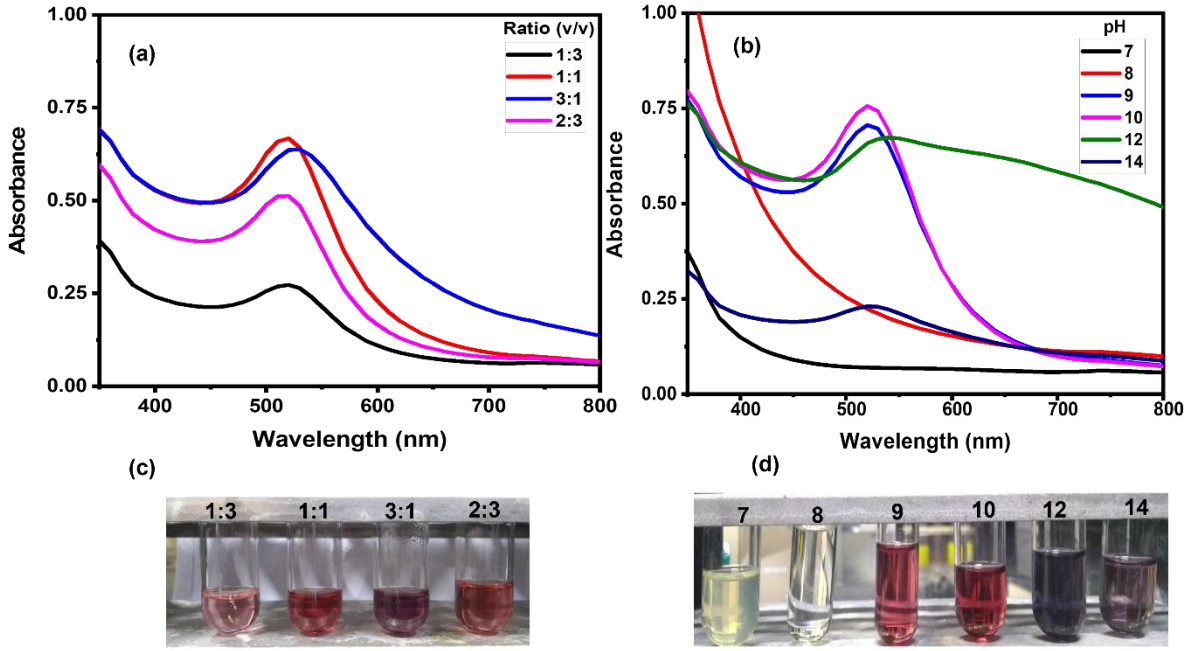


Figure 3.2: (a) UV-vis spectrum of G-AuNPs synthesized at various ratios (v/v), (b) UV-vis spectrum of G-AuNPs synthesised at various pH, (c) Variation in G-AuNPs colour synthesized at various ratio, (d) Variation in G-AuNPs colour synthesized at various pH.

Furthermore, we optimized temperature for G-AuNPs synthesis. UV-vis spectra of G-AuNPs synthesized at various temperature revealed that 55 °C was optimum for synthesis (Figure 3a) while at higher temperature (75 °C) there was aggregation in nanoparticles (Figure 3.3a). At lower temperature (35 °C) the synthesis process was slow (Figure 3.3a). After temperature optimization we analysed the effect of time on G-AuNPs synthesis. Results indicated that there was gradual increase in the intensity of G-AuNPs from 0-200 min while from 200-300 min

there was not significant difference in the intensity (Figure 3.3b). Hence, 200 min was found to be optimum time for the G-AuNPs synthesis. After optimization, the concentration of G-AuNPs was calculated using UV-vis spectroscopy data as described by by Haiss et al [391]. The concentration of G-AuNPs was found to be 2.3 nM.

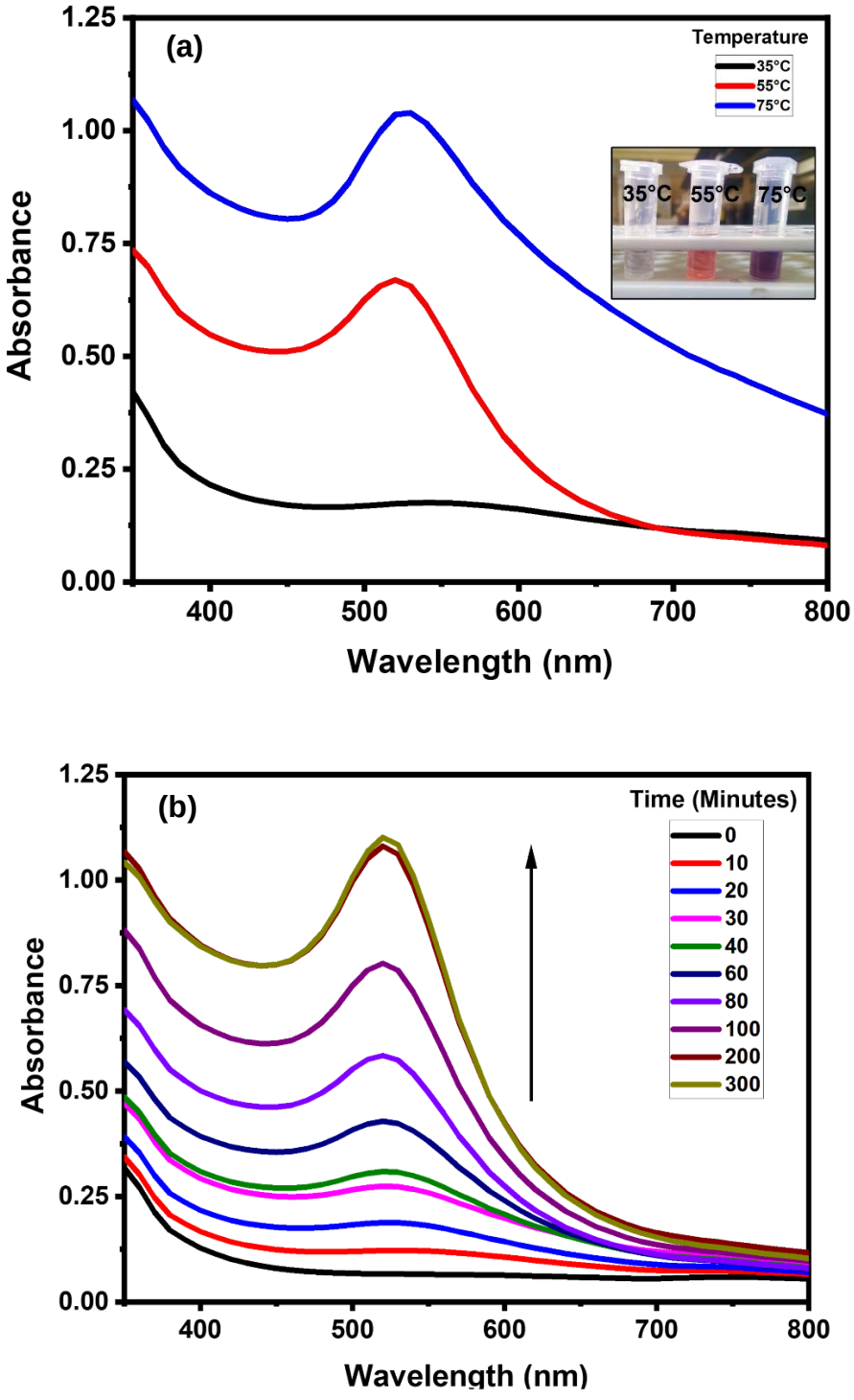


Figure 3.3: (a) UV-vis spectrum of G-AuNPs synthesized at various temperatures, inset variation in G-AuNPs colour synthesized at various temperature, (b) UV-vis spectrum of G-AuNPs at various time intervals.

The stability of nanoparticles is an important parameter. Hence, we also analysed the storage stability profile of G-AuNPs at refrigerator (4 °C) conditions for 75 days. The results indicated that G-AuNPs were enough stable for 35 days with small change in intensity of absorption spectrum of G-AuNPs as shown in Figure 3.4. Furthermore, on 75th days a second hump started appearing on the UV-vis spectrum of G-AuNPs indicating initiation of aggregation of particles due to loss of stability (Figure 3.4).

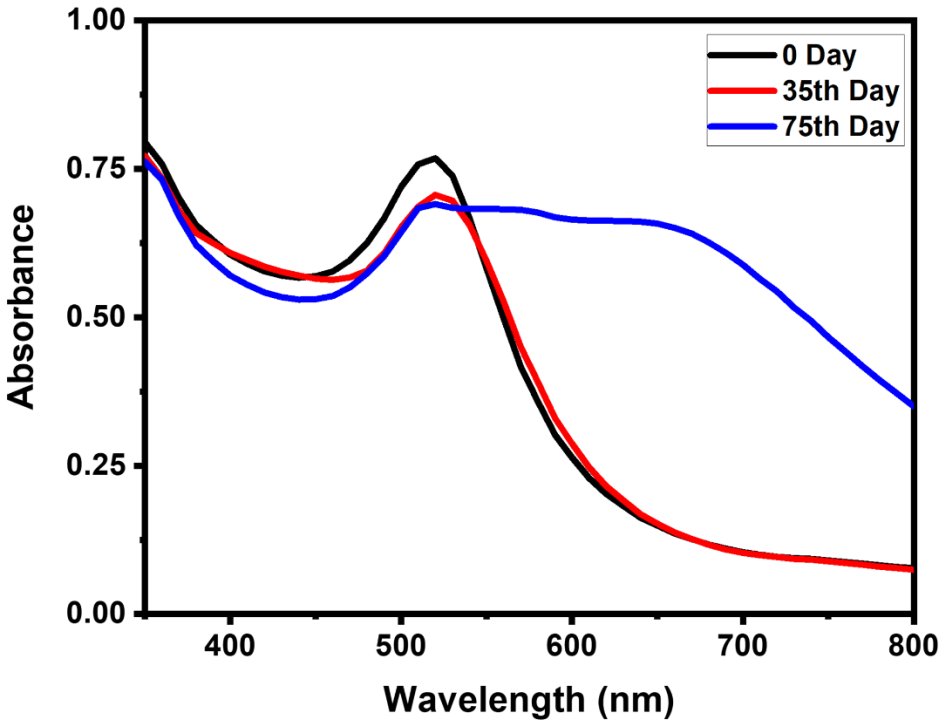


Figure 3.4: Analysis of G-AuNPs stability at storage conditions (4 °C).

The morphology of G-AuNPs was determined through TEM. TEM analysis revealed that synthesized G-AuNPs were spherical in morphology (Figure 3.5a). The average size of G-AuNPs through TEM was found to be 13±3 nm while distribution of nanoparticle was in narrow range as revealed by histogram (Figure 3.5a inset). Furthermore, the G-AuNPs size and distribution was also analysed through DLS. The findings of DLS revealed that most of G-AuNPs exhibited hydrodynamic size of approximately 15±3 nm with the distribution of size was found from 8 to 30 nm and polydispersity index of 0.34 (Figure 3.5b).

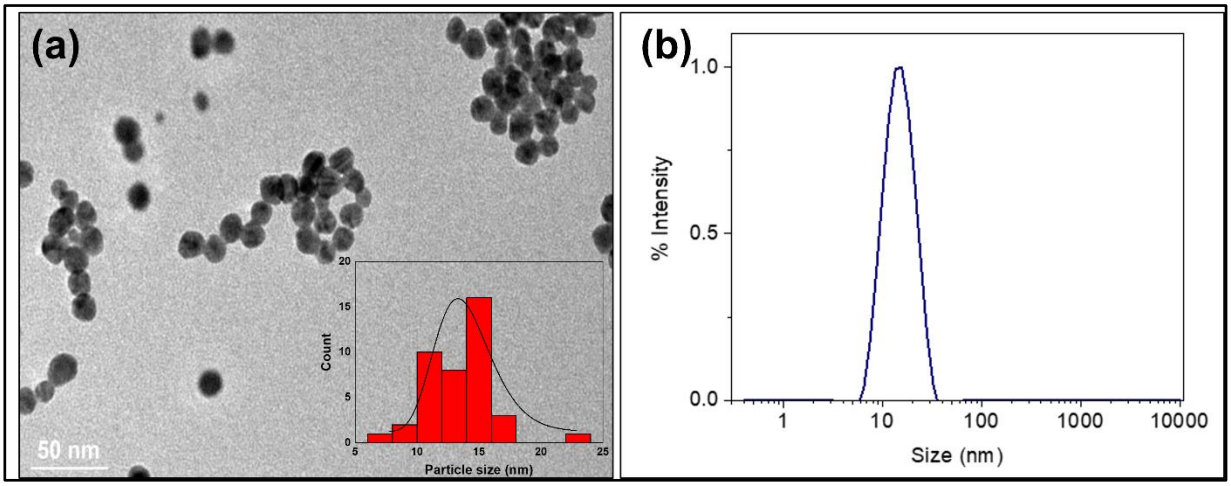


Figure 3.5: (a) TEM image of G-AuNPs (inset histogram of G-AuNPs showing distribution of particles), (b) DLS analysis of G-AuNPs.

3.3.2 Determination of antibacterial activity and MIC of G-AuNPs

Gram-negative *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5, and gram-positive *S. aureus* MTCC 3160 bacterial were used to measure the antibacterial activity of G-AuNPs. The activity was performed though agar plate (well) diffusion assay. Synthesized G-AuNPs were found to exhibit synergistic antibacterial activity (Figure 3.6). The zone of inhibitions by G-AuNPs against *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and *S. aureus* MTCC 3160 observed to be 14 mm, 15 mm, 11 mm, and 11 mm ($p>0.05$) respectively (Figure 3.6 and Table 3.1). The pure gentamicin sulphate (6 µg equivalent) exhibited 11 mm, 10 mm, and 9 mm zone of inhibition ($p>0.05$) towards *S. aureus* MTCC 3160, *E. coli* ATCC 25922, and *E. coli* DH5α, while no zone of inhibition against *E. fergusonii* ATCC 35469 (Figure 3.6 and Table 3.1). The working concentration of gentamicin sulphate (0.5 µg equivalent) did not display any zone of inhibition against any of the bacterium (Figure 3.6 and Table 1). The G-AuNPs were observed to had MIC of 0.0046 nM, 0.046 nM, 0.01 nM, and 0.0046 nM, ($p>0.05$) towards *S. aureus* MTCC 3160, *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, and *E. coli* DH5α, respectively (Table 3.2). The MIC of gentamicin sulphate was 1.0 µg/mL against *S. aureus* MTCC 3160 and *E. coli* ATCC 25922, 0.13 µg/mL against *E. coli* DH5α, and >16.0 µg/mL *E. fergusonii* ATCC 35469 (Table 3.2). Auric chloride solution exhibited MIC of 17 µg/mL against *E. coli* ATCC 25922 and *E. coli* DH5α, and 68 µg/mL towards *S. aureus* MTCC 3160 and *E. fergusonii* ATCC 35469 (Table 3.2).

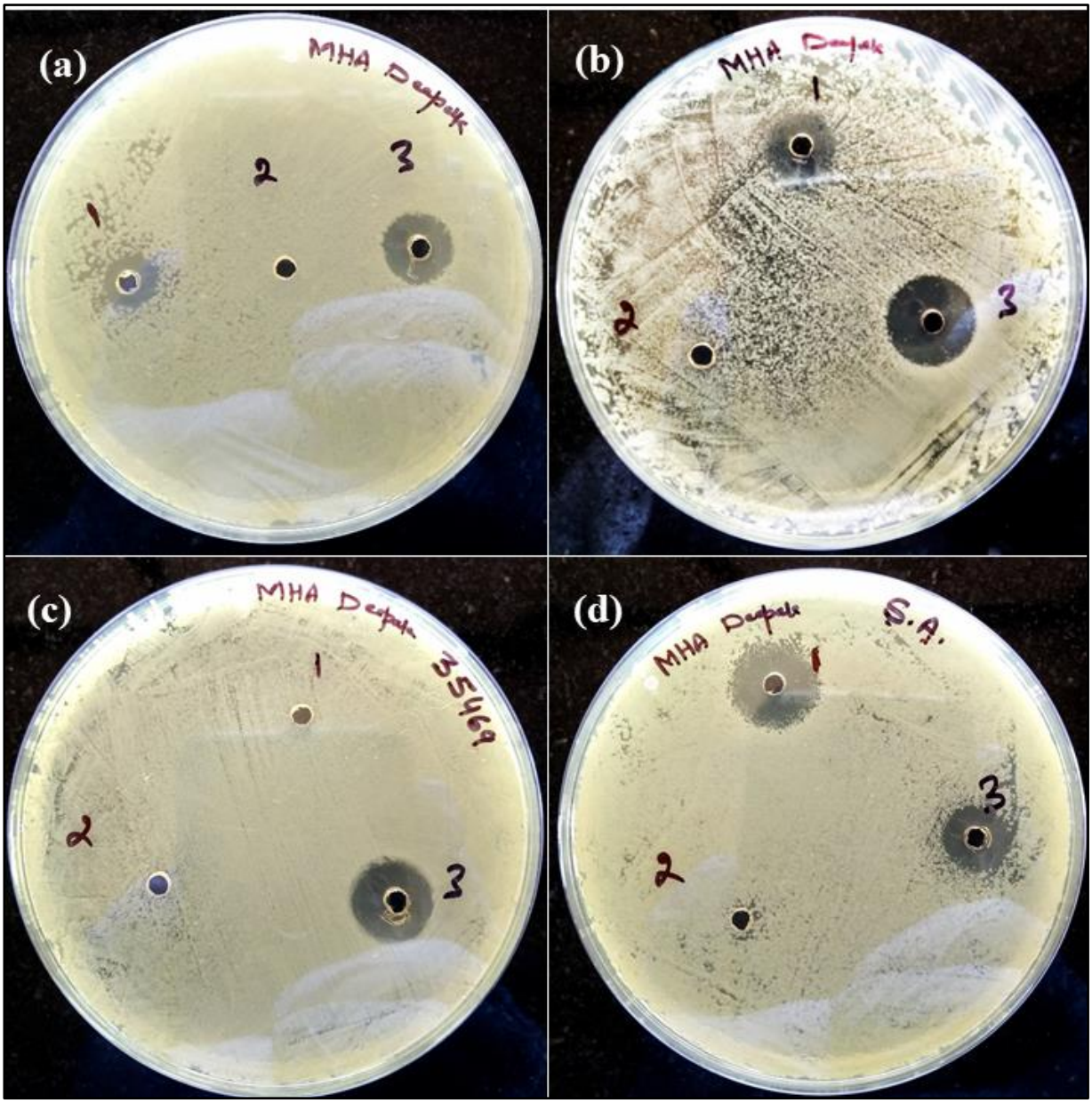


Figure 3.6: Evaluation of antibacterial activity by G-AuNPs against (a) *E. coli* DH5α, (b) *E. coli* ATCC 25922, (c) *E. fergusonii* ATCC 35469, and (d) *S. aureus* MTCC 3160.

**Well-1: 6 µg gentamicin sulphate equivalent, Well-2: Working conc. of gentamicin sulphate (0.5 µg equivalent), and Well-3: G-AuNPs.*

Table 3.1: Measurement of inhibitory zones (diameter in mm) produced by G-AuNPs and gentamicin sulphate drug molecules.

Well constituents	***E. coli* DH5α**	***E. coli* ATCC 25922**	***E. fergusonii* ATCC 35469**	***S. aureus* MTCC 3160**
Gentamicin sulphate (6 µg equivalent)	$9^{a,x}$	$10^{a,y}$	0	$14^{a,z}$
Working conc. of gentamicin sulphate (0.5 µg equivalent)	0	0	0	0
G-AuNPs	$11^{b,x}$	$15^{b,z}$	$14^{a,y}$	$11^{b,x}$

[a-b] Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. [x-z] Means in the row with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. However, there was no significant difference for all the culture against working conc. of gentamicin sulphate.

Table 3.2: Evaluation of MIC against gram-positive and gram-negative bacteria.

Microorganisms	**Auric chloride**	**Gentamicin**	**G-AuNPs**
E. coli DH5α	17 µg/ml^{a,x} (56 µM)	0.13 µg/ml^{a,y} (0.19 µM)	0.0046 nM^{a,z}
E. coli ATCC 25922	17 µg/ml^{a,x} (56 µM)	1.0 µg/ml^{b,y} (6.14 µM)	0.011 nM^{b,z}
E. coli ATCC 35469	68 µg/ml^{b,x} (224 µM)	>16.0 µg/ml^{c,y} (>98.25 µM)	0.046 nM^{c,z}
S. aureus MTCC3160	68 µg/ml^{b,x} (224 µM)	1.0 µg/ml^{b,y} (6.14 µM)	0.0046 nM^{a,z}

[a-c] Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications. [x-z] Means in the row with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

3.3.3 Evaluation of ROS and LPO production in microorganisms due to G-AuNPs

The treatment of test microorganisms with G-AuNPs was observed to have increase in ROS levels. *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and *S. aureus* MTCC 3160 displayed 9.0±0.25 %, 8.0±0.3 %, 4.0±0.27 %, and 9.0±0.25 %, respectively increase in ROS level (Figure 3.7). Nanoparticles have reported to exhibit antimicrobial activity due to production of ROS that results in creating oxidative stress in microorganisms [375], [392]. Furthermore, there was 9.0±0.25 % 6.0±0.3 %, 5.0±0.25 %, and 8.0±0.3 % increase in LPO level in *E. fergusonii* ATCC 35469, *E. coli* ATCC 25922, *E. coli* DH5α, and *S. aureus* MTCC 3160, respectively (Figure 3.8). Increased levels of ROS and LPO have been associated with membrane disintegration in microorganisms due to antibacterial action of metal nanoparticles [385], [393].

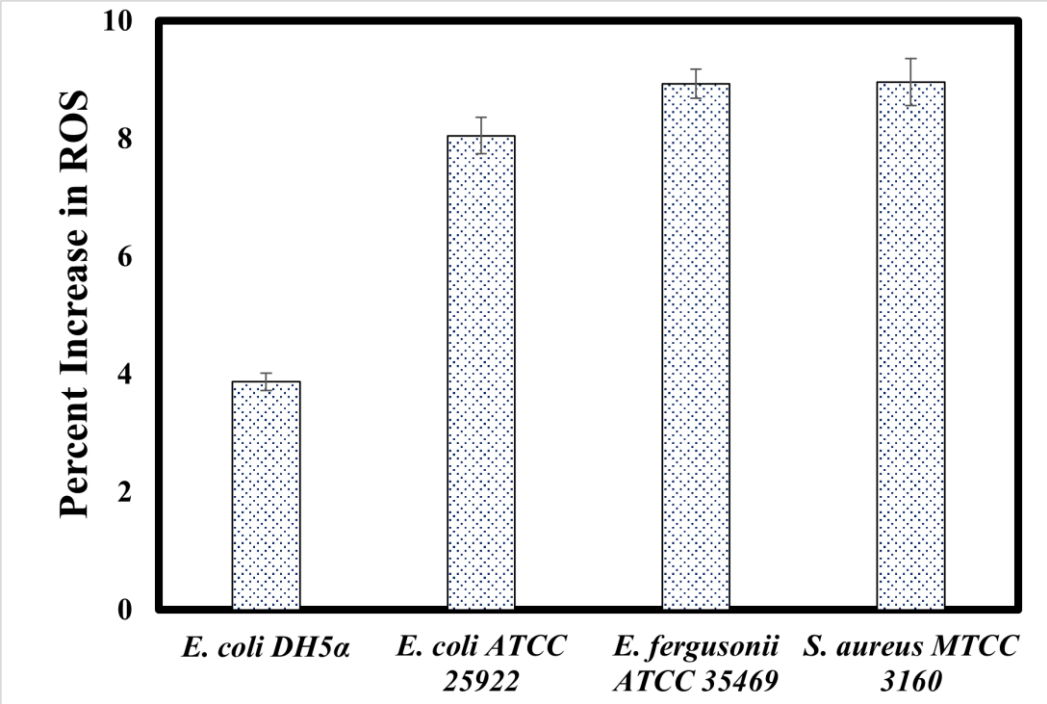


Figure 3.7: Effect on ROS levels due to G-AuNPs treatment on microorganisms.

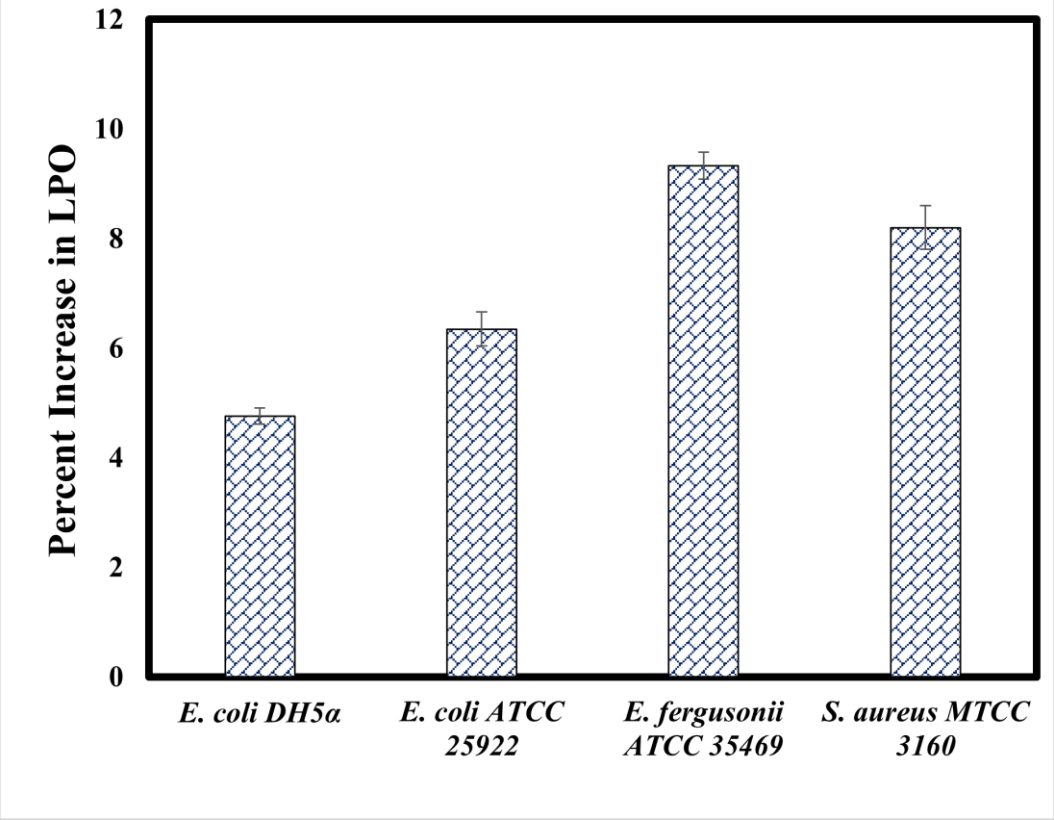


Figure 3.8: Effect on LPO levels due to G-AuNPs treatment on microorganisms.

3.3.4 Analysis of G-AuNPs effect on membrane permeability of microorganisms

The damage caused by G-AuNPs to bacterial cells was investigated through labelling bacteria with a fluorescent dye i.e., propidium iodide. The fluorescent images were analysed by Image J software to predict the death of bacteria in the presence of G-AuNPs. Fluorescence intensity results revealed that the bacterial cells treated with G-AuNPs exhibited 13.0 %, 19.0 %, 24.0 %, and 35.0 % more intensity for *E. coli* ATCC 25922, *E. coli* DH5α, *S. aureus* MTCC 3160, and *E. fergusonii* ATCC 35469 respectively as compared to untreated cultures (Figure 3.9). Figure 3.10 shows the fluorescence microscopic images of microorganism with and without G-AuNPs treatments. The intensity (red fluorescence) of propidium iodide was supposed to be linked with dead or impaired bacterial cells due to G-AuNPs treatment [394]. The results clearly revealed that treatment with G-AuNPs enhanced membrane permeability in all of the tested strains, confirming the bactericidal action of G-AuNPs.

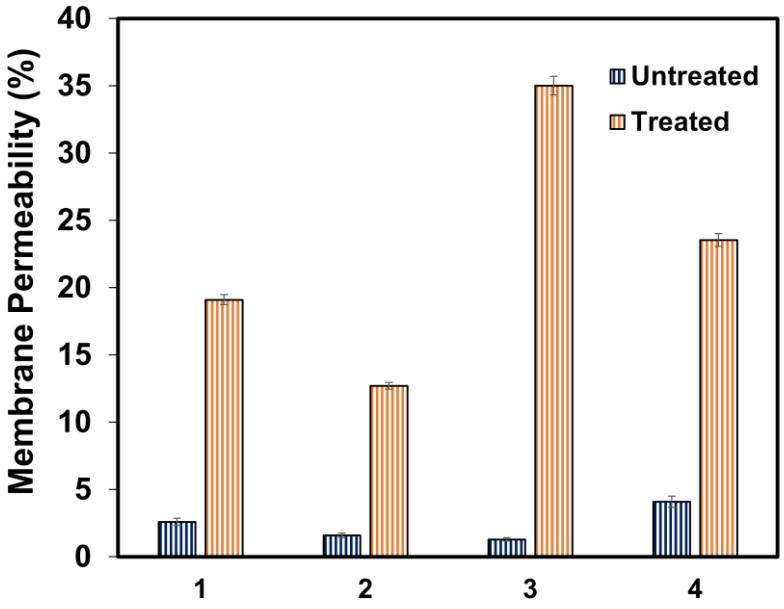


Figure 3.9: Measurement of G-AuNPs effect on the permeability of bacterial membrane

(1) E. coli DH5α, (2) E. coli ATCC 25922, (3) E. fergusonii ATCC 35469, and (4) S. aureus MTCC 3160

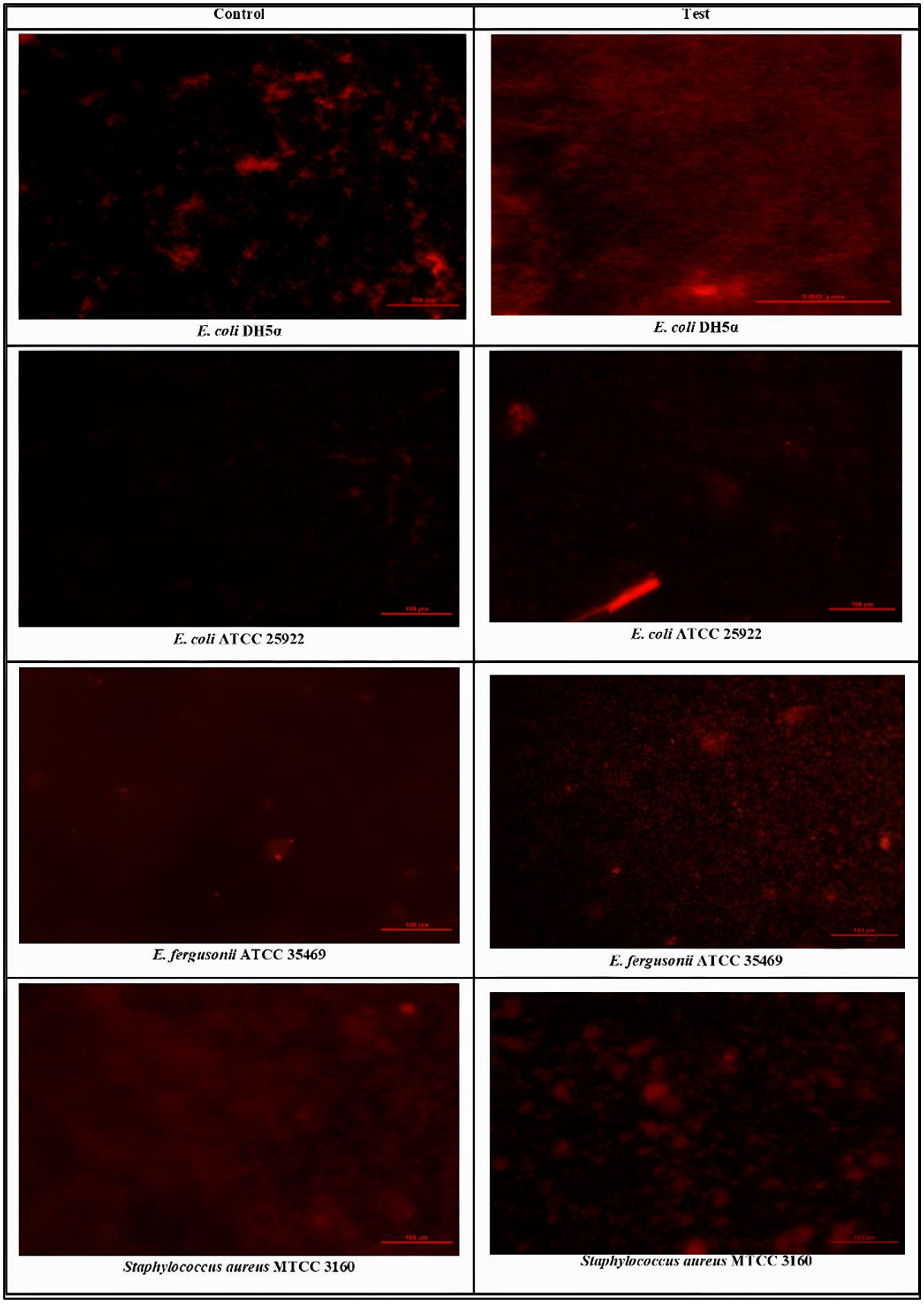


Figure 3.10: Fluorescent imaging of microorganisms using propidium iodide dye with and without G-AuNPs treatment (scale 100µm).

3.3.5 Analysis of gentamicin release from G-AuNPs

Dialysis membrane method was used to study gentamicin drug molecules release from G-AuNPs and determined by fluorescence measuring. Drug release studies was performed for a duration of 72 h at pH 7.4 (physiological pH). The results obtained from fluorescence analysis revealed in the initial 8 h the drug release was maximum (37.0 %). However, after 8 h the release of drug molecules was persistent as only 53.0 % of drug was released at 72 h of time (Figure 3.11). The release of drug molecules may be triggered by a stimulus such as pH. The pH of the generated G-AuNPs after synthesis was alkaline (8.6), which was thought to operate as a trigger in the current investigation to start the release of the gentamicin drug molecules from G-AuNPs. This pH difference can cause the bulk charge affect and protonation of gentamicin to cause the release of gentamicin molecules from the G-AuNPs. The change in the pH conditions of solution like alkaline to physiological or acidic conditions increases the magnitude of protons in the drug solution and promotes the breaking of electrostatic connections among the drug molecule and nanoparticles surface to start up drug release [395].

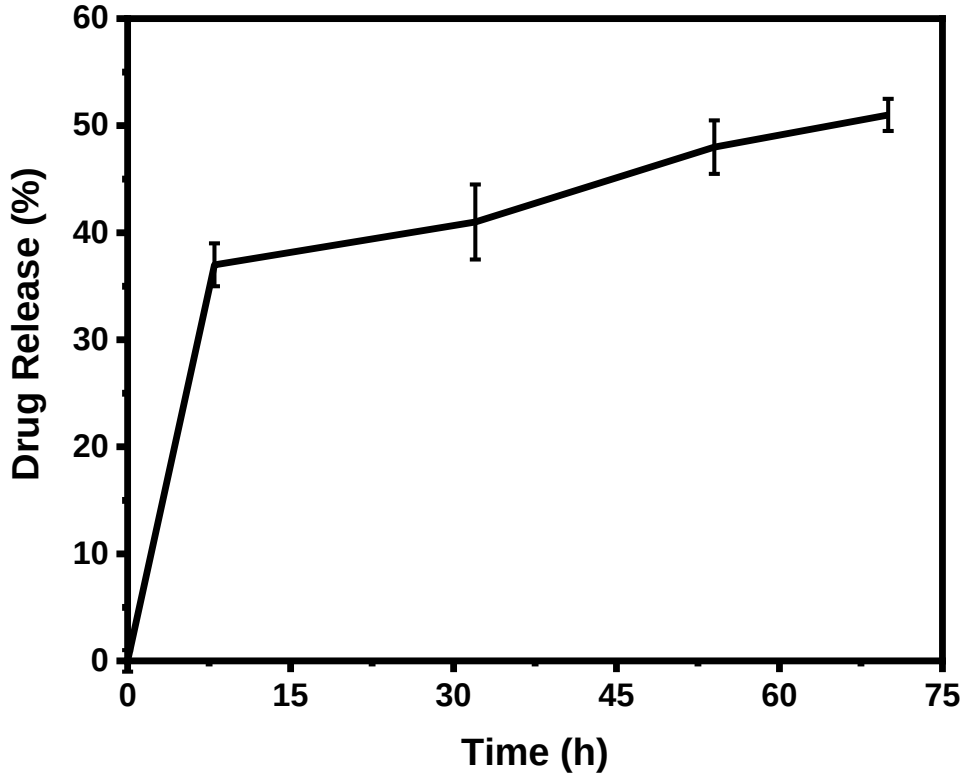


Figure 3.11: Analysis of gentamicin drug release from G-AuNPs at cell physiological pH 7.4.

3.3.6 Analysis of G-AuNPs cytotoxicity on C2C12 cell line

The cytotoxicity of G-AuNPs was investigated on the C2C12 mouse myoblast cell line. The MTT assay analysis revealed that at greater concentrations of G-AuNPs, there was negligible cytotoxicity and a survival of 92.4 percent cells was recorded (Figure 3.12). Higher survival of C2C12 cell line suggests that the G-AuNPs don't have toxic effects. The reason for higher survival of C2Cl2 cell line may be the presence of cholesterol in the cell membrane maintains the cell membrane and renders it less sensitive to attack by antibacterial G-AuNPs [396]. The finding from the current study highlights that synthesised G-AuNPs are biocompatible and can be employed as carriers for drug molecules [396], [397].

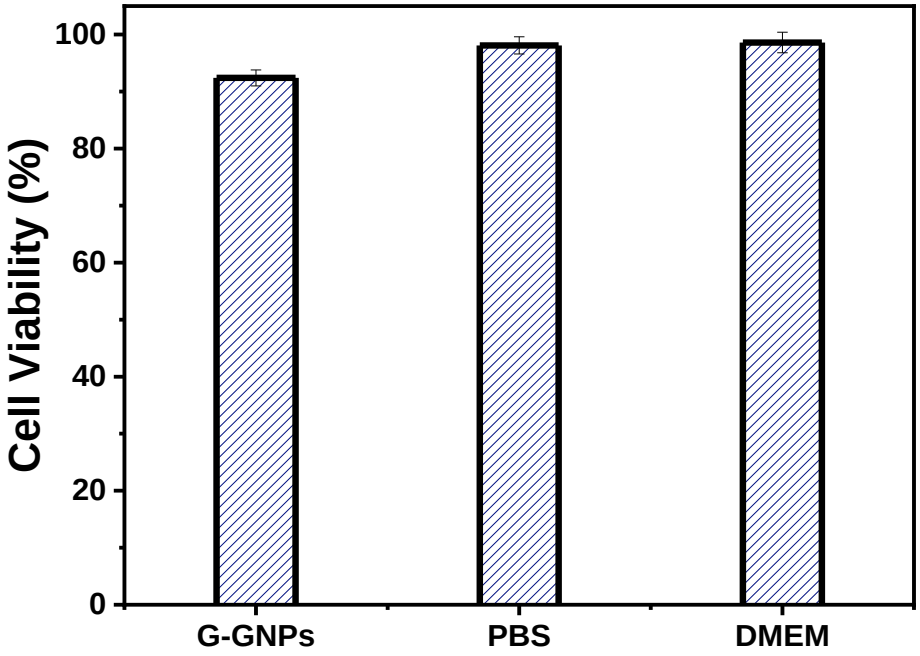


Figure 3.12: Investigation of cytotoxic effects of G-AuNPs, PBS and DMEM on C2C12 cell line.

3.4 SUMMARY

The conventional approach for the development of drug conjugated nanoparticles involves multiple steps. Furthermore, these methods also involve use of harsh chemicals at any stage of the process and that have severe adverse health effects [397]. One-pot synthesis of nanoparticles has emerged as alternate method due to some advantages over existing methods. In the current research, a one-pot method for the synthesis of G-AuNPs was developed. The synthesised G-AuNPs were observed to have absorption maxima at 520 nm confirming the synthesis of G-AuNPs. Visual appearance of G-AuNPs was wine red colour. The results obtained from UV-vis spectroscopy were in correlation with the findings of Amendola and Meneghetti [398]. The red colour imparted by G-AuNPs was well supported by the work of Philip [399] who reported the various red colour intensities of gold nanoparticles as a factor of size. G-AuNPs synthesized were found to have spherical shape and 15 nm size as revealed by TEM and DLS analysis. Similarly, Amendola and Meneghetti [398] have shown synthesis of gold nanoparticle and estimated 4-25 nm size with spherical morphologies using TEM.

G-AuNPs were found to have strong antibacterial activities towards gram-positive *S. aureus* MTCC 3160, gram-negative *E. coli* ATCC 25922 and *E. coli* DH5α, and gentamicin drug resistant *E. fergusonii* ATCC 35469. The damage to cell wall by physical interaction between G-AuNPs and the cell wall of gram-negative bacteria and the hydrophilic lipopolysaccharide layer promotes the intake of G-AuNPs. However, the entrance of G-AuNPs into gram-positive bacteria may be facilitated by physical breakdown of bacterial membranes, electrostatic interactions, hydrophobic interactions, and hydrogen-bonding interactions between G-AuNPs and gram-positive bacteria [393], [394], [396]. Furthermore, G-AuNPs generate oxidative stress in bacteria by producing ROS, which further causes lipid peroxidation in bacterial cells and ultimately results in bacterial cell death. Further evaluation of G-AuNPs' cytotoxicity was conducted using mouse fibroblast cells (C2Cl2). The C2C12 cell line displayed greater than 90% vitality at higher G-AuNP concentrations. As a consequence of this study, the produced G-AuNPs may be used as therapeutic agents against gram-positive, gram-negative, and gentamicin-resistant bacteria. The research's conclusions will contribute to the development of nanotechnology-based antibacterial and other medications, as well as drug delivery systems.

Chapters 3 of the study represent work of 1- 2 objectives and the work has been published in Journal of Cluster Science (https://doi.org/10.1007/s10876-020-01864-x).

Several studies have demonstrated that the amount of cysteine varies when individuals are infected by microbes. Microbes demands more cysteine for various metabolic activities hence the level of cysteine gets decreased and can be used as marker for determining microbial infections. However, level of cysteine also gets fluctuated during various disease conditions. Therefore, we aimed to develop M-NPs based colorimetric sensor for the detection of cysteine in aqueous and other complex medium. The work for the same has been described in chapter 4.