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Biologically Fabricated Silver Nanoparticles from Mesophilic Bacteria *(E. coli)* Produces Cytotoxicity Through Apoptotic Signaling in Breast Cancer MCF-7 Cells

[1]Thirumalpur Neelakandan Baskaran, [1]Murali Santhosh, [1]Kalaimani Saravanan, [1]Sivaji Sathiyaraj, [1]Gunasekaran Suriyakala, [1]Babujanarthanam Ranganathan*

[1]Department of Biotechnology, Thiruvalluvar University, Serkadu, Vellore - 632 115.

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***Corresponding Author Email:** babukmg@gmail.com

Abstract

The most exclusive properties of silver nanoparticles are very small with optical frequency when the propagation of surface-plasmon, non-toxic with significant electro-thermal conductivity. Moreover, it has been exhibiting several broad spectra of highly resistant antimicrobial activity and anticancer properties. There are various methodologies have been adopted to synthesis the AgNPs which includes electrochemical, microwave, chemical reduction, biochemical, ultra-sonication. This method implies highest energy consumption, usage of chemical may produce toxicity and highly hazardous. Therefore, use of simple and low-cost methodology with bio-compatible are considered crucial approach for synthesis of AgNPs.

Keywords

Nanotechnology, MCF-7 Cells

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1. INTRODUCTION:

Nanotechnology concern as very emerging field in the subject of biotechnology, commerce with the enterprise and manipulation of newly synthesise particles with estimated sizes from 1 to 100 nm. Nanoparticles (NPs) have been used in several aspects such drug delivery, biomedical sciences, space industries, preparation of cosmetics, chemical industries etc., (Jha R.K *et al* 2014). Metal nanoparticles considered as crucial one to shows size depending physico-chemical properties suggestively dissimilar from their majority of counterpart (Hochell, *et al* (2012). The metal nanoparticles are potentially using in several kind of purposes such as medicine for drug delivery, catalysis, cosmetics, industries and environmental remediation etc., Silver nanoparticles (Ag-NPs) belongs to metal particles exhibit a comprehensive spectrum with eco-friendly and environmentally safe. Besides, AgNPs are most familiar due to their exclusive physio-chemical and biological properties when related to other particles (Salem & Amr Fouda (2021).

The most exclusive properties of silver nanoparticles are very small with optical frequency when the propagation of surface-plasmon, non-toxic with significant electro-thermal conductivity. Moreover, it has been exhibiting several broad spectra of highly resistant antimicrobial activity and anticancer properties. There are various methodologies have been adopted to synthesis the AgNPs which includes electrochemical, microwave, chemical reduction, biochemical, ultra-sonication. This method implies highest energy consumption, usage of chemical may produce toxicity and highly hazardous. Therefore, use of simple and low-cost methodology with bio-

compatible are considered crucial approach for synthesis of AgNPs.

The synthesis of silver nanoparticles by the source of bacteria is very easiest method to handle and it can be modified genetically with ease. This provides a means to develop biomolecules that can synthesize AgNPs of varying shapes and sizes in high yield, which is at the forefront of current challenges in nanoparticle synthesis.

The scientific evidence stated that synthesis of nanoparticles from bacteria was first established by using with *Pseudomonas stutzeri.* This microorganism can significantly survive metal ion under those conditions, and this phenomenon can exhibit resistance to that metal. *Escherichia coli* (E. coli) has been concerning as the family of gram-negative with rod-shape structured anaerobic mesophilic bacterium. The bacterium produces enterotoxins, hence it is thermo stable in nature. In this current study, we evaluated biologically fabricated AgNPs were synthesized from mesophilic bacteria (E. coli) and it can be further examined anticancer activity against breast cancer MCF-7 cells.

2. MATERIALS AND METHODS:

2.1. Chemicals

Silver nitrate ($AgNO_3$) were procured from Sigma Merck Ltd, USA. Mesophilic bacteria's was collected from S. V. Veterinary Dairy University, Tirupathi, Chittoor district, Andhra Pradesh, India. All the solvents were used for this study is molecular grade.

2.2. Isolation of Mesophilic bacteria from water samples

Mesophilic bacteria samples (Water) (1ml) were taken suspended in 9ml sterile saline solution in a test tube and vortexed. Then 1ml of water was taken from the test tube and mixed with 9 ml of sterile double distilled water in order to reduce the microbial load in the sample and marked as 10^{-2}. This procedure was followed up to 10^{-7} dilution enumerated by pour plate technique using Nutrient agar (NA) medium for isolating bacteria. 1ml of sample was taken in a petriplate and Nutrient agar for bacteria was added thoroughly by rotating the plate in clockwise and anti-clock wise direction and allowed to solidify. Then the inoculated plates were incubated at 37ºC triplicates were maintained for 2-3 days incubation Petri plates with 30-300 colonies were selected and the total viable counts/gm was made based on the following formula (Maheshwari et al., 2016).

$$\text{CFU} = \frac{\text{Average number of colony (av) X 10 ml}}{\text{Dilution factor X volume of sample added X weight of scale sample}}$$

(av) = average for triplicate samples.

10ml = volume of diluents

Many bacterial sp were isolated fresh bacterial growth from the plated samples was then transferred on NA. Finally, every isolate was further purified by single- colony culture on NA. Nutrient broth (NB) medium was used for the harvesting of bacteria.

2.3. Preparation of Mesophilic bacteria (*E.Coli*) aqueous extract

The pure culture of the *E.Coli* was isolated from nutrient agar medium by using quaternary streaking method. The pure colony of the *E.Coli* was taken and sub cultured on separate nutrient agar medium and the Fresh culture of the *E.Coli* was stored in refrigerator at 4ºC nutrient broth for further analysis. Extract was filtered by using Whatman No. 1 filter paper and collected in plastic bottle and stored at 4±C for further characterization and experimentation (Twum-Danso et al., 2013).

2.4. Preparation of Mesophilic bacteria (*E.Coli*) extract mediated silver (Ag) nanoparticles

Silver nitrate (>99% pure) was purchased from Sigma-Aldrich, India. Nutrient broth, nutrient agar plate, was supplied by Hi-Media, India. To prepare the AgNPs, a 90-mL aqueous solution of $1.0x10^{-3}$M silver nitrate was mixed with a 10-mL of 5% aqueous solution of *E.Coli* extract. The *E.Coli* Ag solution was yellow in color and the solution was stirred repeatedly for an hour, and it was observed that the color of the solution has been changed to brown which visually confirms the formation of Nanoparticles. These *E.Coli* silver Nanoparticles were characterized by using the techniques such as UV–Vis spectrophotometry, Fourier transform infrared spectrophotometry (FT-IR), X-ray Diffractometry (XRD), Dynamic light scattering (Particle size), zeta potential, Transmission electron microscopy (TEM) and Energy dispersion X-ray Spectrum (EDX). The AgNPs formed were stored for further characterization and bioactive assays (Alekshun and Levy, 2007).

2.5. UV-Spectroscopy

AgNPs synthesized by mesophilic bacteria source (E. coli) was confirmed the color reduction form by the method of UV-Visible Spectrophotometer.

2.6. Particle size and zeta potential (DLS)

The suspension of AgNPs have been filtered through a 0.22-µm syringe filter, and the size and distribution of the nanoparticles were measured using dynamic light scattering technique (Nanopartica, HORIBA, SZ-100).

2.7. FTIR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) analysis was conducted to studied the functional groups exhibits in synthesized Ag nanoparticles from mesophilic bacteria *(E.coli).* The nanoparticles were placed to FTIR analysis at a range of 4000–500 cm^{-1} spectra using FTIR Spectrum 2000, Perkin Elemer Waltham, USA.

2.8. X-ray diffraction (XRD)

The Ag nanoparticles from mesophilic bacteria *(E.coli)* were characterized to confirm the crystal nature structure was studied by X-ray diffraction technique. The XRD pattern have been recorded by computerized regulating XRD-system (JEOL, JPX-8030) with CuKa radiation (Ni filtered = 13418 A°) in the range of 40 kV, 20 A. The built-in software (syn master 7935) program was used for the documentation of XRD peaks agreeing to the Bragg's reflections.

2.9. Scanning electron microscopy (SEM)

The Ag nanoparticles from mesophilic bacteria *(E.coli)* were characterized to confirm the crystal nature structure and size of the structure was studied by SEM. The samples were prepared in well grinded with Morton piston and it subjected for analysis in SEM (Hitachi model S-3000H).

2.8. Cell culture

The breast cancer cell line MCF-7 were gained from the National Centre for Cell Sciences, Pune, India. This cell line was culture in the sterile equipped microbiological lab. The cultured cells were supplemented with sterilized DMEM medium with addition 10 % FBS and 1 % penicillin/streptomycin in the CO_2 incubator.

2.9. MTT assay

The cytotoxicity result of nobiletin and docetaxel was deliberate by MTT-based colorimetric assay (Nilamberlal Das et al., 2019). MCF-7 cells were equally seeded in 96-well plates (5000 - 6000 cells/ each well) and kept incubation for 24 h at 37°C and then cells have been treated with rang of concentrations of nobiletin allow keep incubation for 24 h at 37°C. After that, the cells were kept incubation with docetaxel and nobiletin (different concentrations) were added into selected wells. Later than 24 hr incubation (at 37°C), MTT solution (100 µL from 5 mg/mL) was added to all the wells and the plates were kept 4 hr incubation at 37°C. DMSO (100-200 µL) was then added in all the wells for dislodging the crystal formazan. Finally, the absorbance was measured via microplate reader at 570 nm.

2.10. DCFH-DA staining

MCF-7 cells were placed in the 6 well plates uniformly distributed and it has been treated with AgNPs, then kept incubation for CO2 incubator in 24 and 48 hrs respectively, After the treatment termination, cells were subsequently washed with saline based PBS and it allow for DCFH-DA fluorescence staining for 40 mins in the dark environment. Finally, the intensity of DCFH-DA was measured by Multimode reader.

2.11. Fluorescence microscopic examination for cell death

The Acridine Orange (AO)/ ethidium bromide (EB) fluorescent probe staining assay was executed for the observation of apoptosis via morphological studies [19]. MCF-7 cells were placed in the 6 well plates uniformly distributed and it has been treated with AgNPs, then kept incubation for CO2 incubator in 24 and 48 hrs respectively, after the treatment termination, cells were subsequently washed with saline based PBS. Then, the dye mixture (20 µL) was supplemented to the treated cells and instantaneously cells were visualized using a fluorescent microscope.

2.12. Statistical Examination

In this study, mean ± standard deviation have been used in the existing results and three experiments were done separately. Duncan's Multiple Range Test (DMRT) using in the software package of SPSS 11.0 have been used. The value $p < 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION:

There are numerous scientific data demonstrated that use of bacteria for the synthesis of metal nanoparticles are crucial event. Previously, green synthesis of silver NPs using a mixture of cultured *Bacillus subtilis* and microwave irradiation was used and successfully characterized. This kind of synthesized nanoparticles shows stable with five-month period of storage at 37 C in the dark environment. This kind of nanoparticles activity against clinically resistant microorganisms.

Therefore, in this study, we analysed the synthesis of silver nanoparticles (AgNPs) from the source of mesophilic bacteria (E.coli) and its characterization.

3.1. Biological fabricated of Ag-NPs from Mesophilic bacteria (*E.Coli*) and its characterization

The biological synthesis of AgNPs from mesophilic bacteria (E. coli) characterized by numerous techniques such as UV absorbance, FTIR, XRD, SEM and DLS analysis. These methods were highly adopted to confirm the nanoparticles size, structure,

and active molecules presence. When the synthesis of AgNPs, mesopillic bacteria *(E.coli*) suspension have been added to the flask which containing $AgNO_3$ reagents resulted in conversion colour from yellow to brown within 15 min of incubation time. These changes were initially we conform the solution are AgNPs. Moreover, the synthesized nanoparticles and its colour density were measured by UV spectrometer. Fig. 1 shows that the UV-vis absorption spectra of Ag nanocomposite from mesophilic bacteria (*E.Coli*) were recorded at room temperature. The solution of UV-vis spectra has been recorded at wavelength between 200 and 800 nm consequently. The spectra absorption peak of Ag nanocomposite material exhibits at 420 nm (Salam et al., 2013). This result confirmed that the AgNPs present in the solution.

Particle size and zeta potential evaluation have been crucially conforming the size of the nanoparticles when the synthesize of AgNPs and it has been technically analyzed by Dynamic light scattering (DLS) (Skandalis *et al*., 2017). It have been used to measurement or confirm the hydrodynamic particle suspension present in the synthesized nanomaterial. Mesophilic bacteria (*E.Coli*) AgNPs was found to be 181.8 nm and the recorded value of zeta potential of the silver nanoparticles from mesophilic bacteria (*E.coli*) was exhibits -18.3 mV, which resulted in the agglomerated state of the formed AgNPs (Manjula et al., 2019).

FT-IR spectrum have been concerning as crucial instrument which studied the active biological functional groups are present in the synthesized nanoparticles (Zhang *et al*., 2016). In this study, silver nanoparticles synthesized by mesophilic bacteria (*E.Coli*) has subjected to evaluates the bio-organic functional groups through FTIR measurement (Fig. 3). The peaks value of 3387cm^{-1} correspondence to primary, secondary amines groups and hydroxyl group respectively. These observed peaks were scientifically equivalent to proteins, enzymes and polysaccharide molecules presence from cell biomass. The peak shows 2945 and 2868cm^{-1} were owed to symmetric and asymmetric outspreading shaking of sp3 hybridization. The peak at 2308 and 1612cm^{-1} denotes to C=O widening vibrations of the carbonyl active functional molecules in ketones, aldehydes and carboxylic acids. The peak at 1300, 1290, 1201, 1170, 1064 and 1035cm^{-1} were allotted to widening vibration of –C=C– aromatic ring. The peaks at 846cm^{-1} corresponded to C=O, C-N widening vibrations of aromatic and aliphatic amines, respectively. In addition to this band at 559 and 518cm^{-1} corresponds to metal binding carboxylic (M↔C≡O) groups, this functional group May acts template, reducing and capping of nanocrystals. (Bindhu and Umadevi, 2015).

The crystal-based structure of biologically fabricated AgNPs from mesophilic bacteria was confirmed by the studies of X-ray diffraction (XRD). The X-Ray diffraction pattern of Ag nanocomposite material are wurtzite hexagonal phase and was shown in (Fig. 4) which indicates the well indexed XRD peaks corresponding to the planes (100), (002), (111) and (103) indicates the presence of silver (Ag) and no other impurities observed. In addition, biologically fabricated AgNPs by the source of mesophilic bacteria (E. coli) was subjected to examine the surface morphology nature of particles, size and shape of particles through investigation of scanning electron microscope (SEM). It is observed that the AgNPs were spherically exhibited with irregular in shape and poly-dispersed. In this study, we used the average size have 20 µm.

3.2. Biologically fabricated of Ag-NPs from Mesophilic bacteria (*E.Coli*) produces cytotoxicity in breast cancer MCF-7 cells

Breast cancer have been concerning as the utmost communal kind of malignancy in the women and also occupies more deadliest cancer afterward lung cancer (Sharma *et al*., 2010). The treatment of breast cancer patients was taken standard protocol in both radio and chemotherapy. If these methods are failure, patients go for surgery (Hanan Ahmed Wahba, 2015). Moreover, chemotherapy and radiotherapy-based treatments are frequently failed due to the numerous side effects in the normal cells and organs (Baskar, *et al.,* 2012). Therefore, numerous nanoparticles were synthesised to selectively targets for the treatment strategies for breast cancer. In this current study, we biologically fabricated of Ag-NPs from mesophilic bacteria (*E.Coli*) produces cytotoxicity in breast cancer MCF-7 cells.

After synthesis of AgNPs from the source of mesophilic bacteria (*E.coli*) has initially analysed for breast cancer cell cytotoxicity. The AgNPs mediated cytotoxicity was assessed by colorimetric based MTT assay. In this study, MCF-7 cells were treated with different concentration of AgNPs (10-80 µg/ml) from mesophilic bacteria (*E. coli*) exhibits significant cell death (Fig. 6). Moreover, we have observed the IC 50 value is 50 µg/ml. Hence, AgNPs (50 µg/ml) has been selected for maximum dose of this study for 24 hrs and 48 hrs incubation time. Previously, AuNPs from plant source impedes cell proliferation in both A549 and Hep-2 cell lines (Rajeshkumar *et al*., 2015).

Over production of reactive oxygen species (ROS) involved in the redox homeostasis resulted in the oxidative stress mediated apoptosis.

The overproduction ROS in the MCF-7 cells were analysed by DCFH-DA fluorescent staining method. The 2',7'-Dichlorofluorescin Diacetate (DCFH-DA) (non-fluorescent probe) which can liberally pierce into intra-cellular matrix wherever been oxidized into fluorescent dichlorofluorescein (DCF) by ROS (Dikalov, S. I. *et al*., (2014). Methods for Detection of Mitochondrial and Cellular Reactive Oxygen Species. Antioxidants & Redox Signaling, 20(2), 372–382. doi:10.1089/ars.2012.4886 Dikalov, S. I. *et al*., (2014). As a result, the fluorescence green intensity is straightly relatively into the quantity of ROS production (Wu, D., & Yotnda, P. (2011). In this study, we observed that AgNPs from mesophilic bacteria (E. coli) produces significant level of ROS in MCF-7 cells thereby stimulates oxidative injury in MCF-7 cells (Fig.7). AgNPs (50 µg/ml) for 48 hrs incubation showed more ROS production in the MCF-7 cells. This activity was compared with standard doxorubicin drug in the MCF-7 cells. Previous research has been decided to demonstrate the oxidative and nitro-oxidative stress in AgNPs mediated toxicity and ROS production were highly observed in human pancreatic adenocarcinoma cells (Barcińska *et al.,* 2018).

Apoptosis concerned as crucial programmed cell death that can eliminating unwanted harmful cells (Elmore, S. (2007). Moreover, the apoptotic morphological changes of AgNPs mediated MCF-7 cells were assessed by acridine orange and ethidium bromide staining. Here, AgNPs significantly increases the level of apoptotic cells by observing red coloured fluorescent cells in the MCF-7 cells. AgNPs (50 µg/ml) for 48 hrs incubation showed highly increases the apoptotic MCF-7 cells. Previous report also supported that ROS- associated apoptosis were observed when the treatment with AgNPs against human breast cancer cells (Gurunathan et al. 2013).

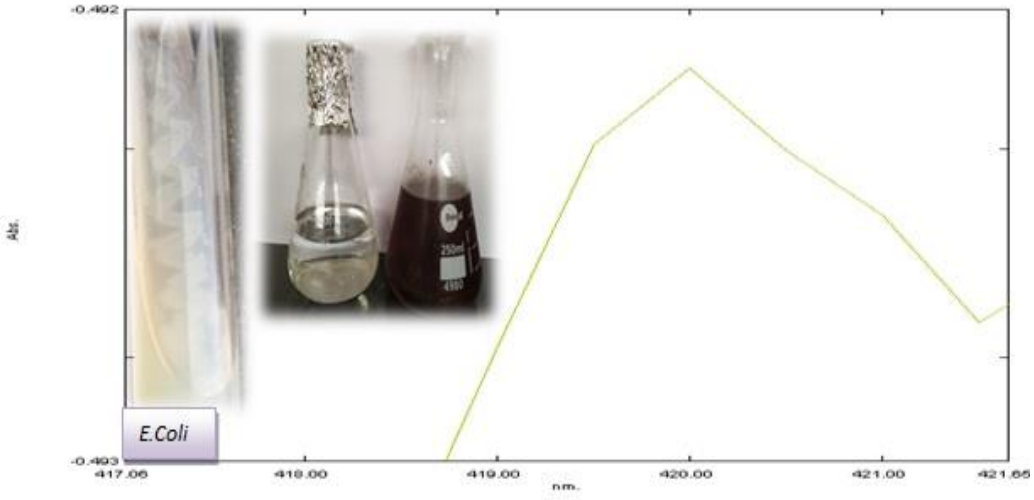

Fig. 1. UV-visible spectrum of Mesophilic bacteria (*E.Coli*) mediated synthesized AgNPs

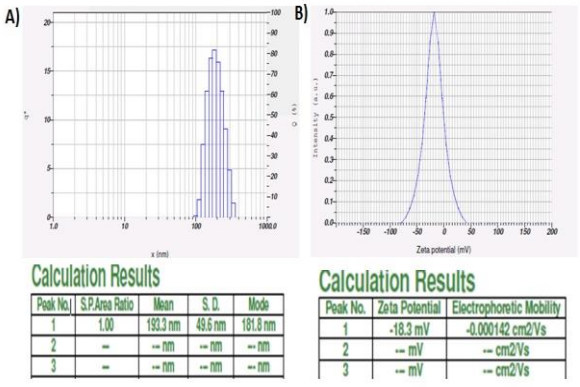

Fig. 2. Particle size analysis of Mesophilic bacteria (E.Coli) mediated synthesized AgNPs. A) Particle size analysed dynamic light scattering (DLS). B) Zeta potential measured by Nanopartica SZ-100

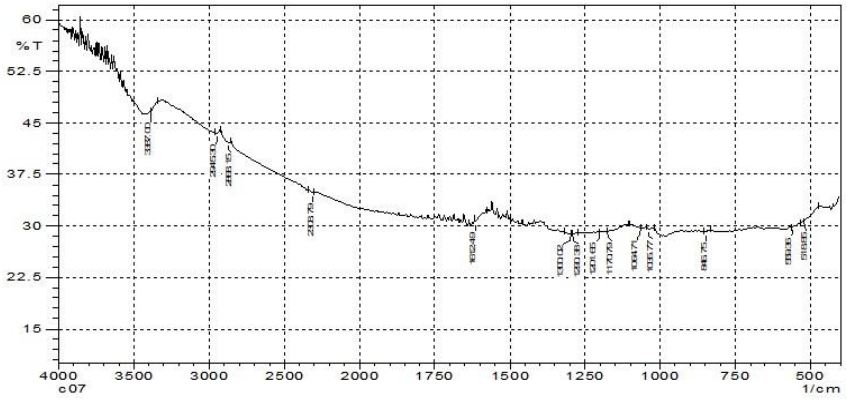

Fig. 3. FT-IR spectrum of Mesophilic bacteria (E.Coli) mediated synthesized AgNPs

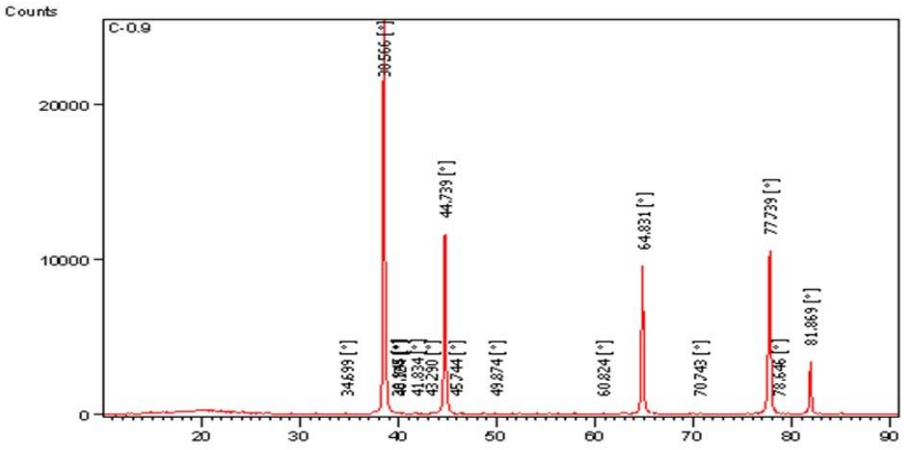

Fig. 4 XRD spectrum of Mesophilic bacteria (*E.Coli*) mediated synthesized AgNPs

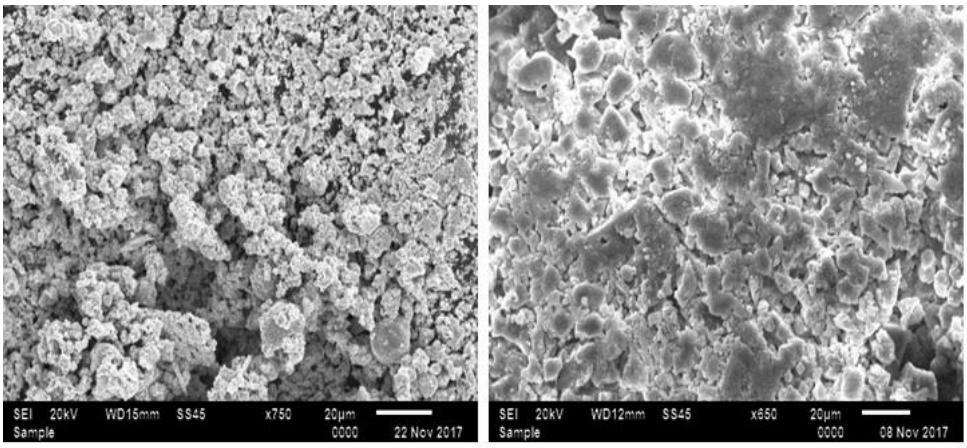

Fig. 5. SEM images of Mesophilic bacteria (*E.Coli*) mediated AgNPs

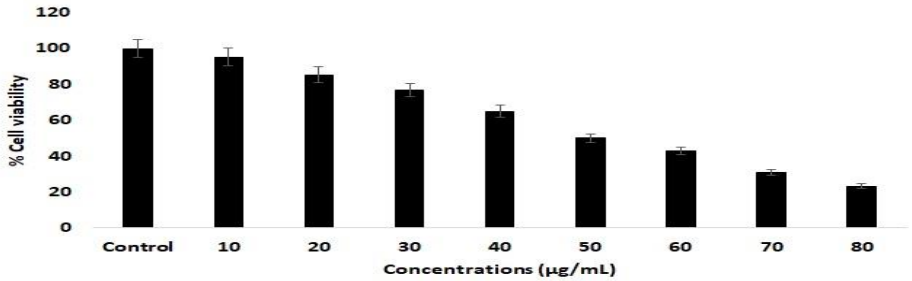

Figure 6. Cytotoxicity effect of AgNPs synthesised from mesophilic bacteria

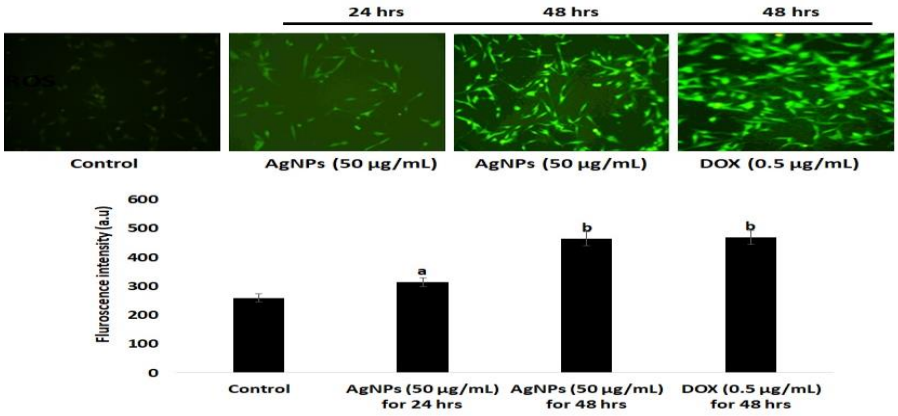

Figure 7. Reactive oxygen species measurement of AgNPs synthesised from mesophilic bacteria (E.coli) against MCF-7 cells. A) Microscopic images represent DCFH fluorescent staining 20 X. B) Data representing as mean ± standard deviation has been used in the existing results and three experiments were done separately. Duncan's Multiple Range Test (DMRT) using in the software package of SPSS 11.0 have been used. The value $p < 0.05$ was considered as statistically significant.

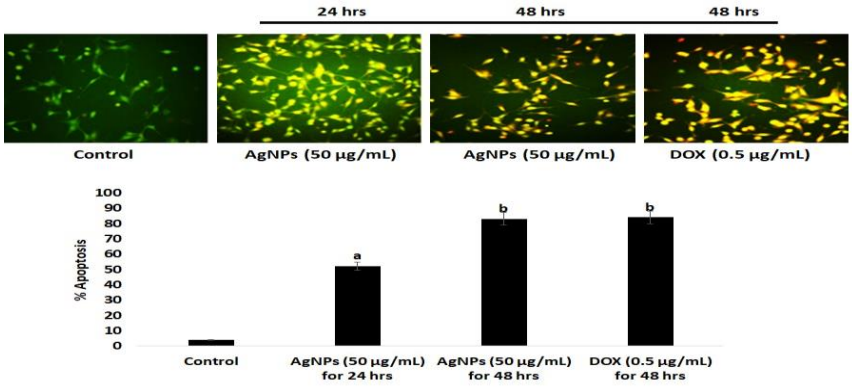

Figure 8. Apoptotic morphological changes evaluation of AgNPs synthesised from mesophilic bacteria (*E. coli*) against MCF-7 cells. A) Microscopic images represent acridine orange and ethidium bromide fluorescent staining 20 X. B) Data representing as % of apoptotic cells and three experiments were done separately. Duncan's Multiple Range Test (DMRT) using in the software package of SPSS 11.0 have been used. The value $p < 0.05$ was considered as statistically significant.

4. CONCLUSION:

In this study, we concluded that biologically fabricated AgNPs from mesophilic bacteria (E. coli) was characterized by UV spectroscopy, FTIR, SEM, DLS and XRD. Based on the characterized studies synthesised AgNPs from mesophilic bacteria were confirmed by size, morphology and crystal-based structure. Moreover, AgNPs produces cytotoxicity, ROS production mediated apoptosis in human breast cancer cells. Our results might be helpful to developing new sights for the nonotherapy mediated treatment of breast cancer.

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