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Biofabrication of silver nanoparticles using *Lactobacillus casei subsp. Casei* and its efficacy against human pathogens bacteria and cancer cell lines

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ABSTRACT

Bio-fabrication of metallic nanoparticles is interesting research field because of its different potential applications in nanomedicine. In the present work, human strain probiotic *Lactobacillus casei subsp. Casei* was used as potential bioreductant for the synthesis of silver nanoparticles (AgNPs), its antimicrobial and anticancer properties were evaluated. The synthesized nanoparticles were characterized using UV–visible spectroscopy, Fourier-transform infrared spectroscopy (FT- IR) and Transmission electron microscopy (TEM). Biosynthesized silver nanoparticles were confirmed by color changes and were found to be spherical in shape and well dispersed, with no agglomeration. Antimicrobial and anticancer activities of silver nanoparticles were evaluated against selected pathogens (Staphylococcus aureus, Pseudomonas aeruginosa) and human gastric carcinoma cells (*AGS*) and Human Colon Carcinoma cells (*HT-29*). Results showed that biosynthesized AgNPs had excellent antibacterial activities against *Staphylococcus aureus and Pseudomonas aeruginosa* which were observed by well diffusion, minimum inhibitory concentration (*MIC*) methods. These nanoparticles were able to inhibit proliferation of human cancer cell lines. Ag NPs were shown to promote apoptosis as seen in the acridine orange/Propidiom Iodide staining and increase nitric oxide (NO) secretion determined by NO production assay. Our results are promising for potential use of the biosynthesized Ag NPs as an antibacterial and anticancer agent. *Lactobacillus casei subsp. Casei* can act as a novel source for AgNPs biosynthesis. This method can be employed for development of new nano-drug formulations in cancer therapy.

Keyword: Silver nanoparticles, biosynthesis, *Lactobacillus casei subsp. Casei,* anticancer activity, antibacterial activity.

1. INTRODUCTION

Recently, researchers have focused on fabrication and application of metal nanoparticles (MNPs), which are potentially safe toward human welfare. Herein Silver nanoparticles (AgNPs) are topical consider as top-grade carriers' metal in the field of material science due to their extravagant properties in various fields of technology, healthy, drug delivery and medicine (Bindhu, 2015; Cao, 2010; Panacek, 2006; Escosura Muniz,2009; Kumar, 2016; becker, 2014). A Range of chemical, physical and biological methods can be used for synthesis of nanoparticles. Among these methods, biological synthesis method is proven to be simplicity of synthesis, cost effective, highly safe, non toxic, solvent-free and ecofriendly alternative to chemical and physical methods (Pui so, 2014; Yu, 2007; Shahverdi , 2007). Various Microorganisms including bacteria (Klaus, 1999; Gurunathan, 2009), fungi (Ahmad, 2003), yeasts (Mourato, 2011; Mugdha, 2013) as well as plant extracts (Ahmad, 2015) have been employed as biological mediators for the synthesis of nanomaterials. Among the groups of bacteria, probiotics are live microorganisms that confer a health benefit on the host when governed in adequate amounts. (Korbekandi, 2012; Nair, 2002) Biosynthesis of AgNPs using probiotics bacteria as capping and reducing agents has been beneficial to plant extract or other methods due to their less biohazard. In addition, the rate of reduction of metal ions using these bacteria has been found to be much faster than to plant extract, and the stable formation of metallic NPs has been studied (Iravani, 2014). Several reports are available in the literature on the biosynthesis of AgNPs using various microorganisms including, biosynthesis of AgNPs has been successfully conducted using Plectonemaboryanum UTEX 485, a filamentous cyanobacterium and among the first reports of intracellular semiconductor nanoparticle synthesis (Lengke, 2007). Sweeney et al. demonstrated that *Escherichiacoli* formed semiconductor nanocrystal. Interestingly, Bharde et al. have demonstrated magnetite nanoparticle synthesis by *Actinobacter*, a non-magneto tactic bacterium (Bharde, 2005; Thakkar, 2010). On the other hand nowadays, increased incidence of multiple drug resistance (MDR) and high drug toxicity has caused existing tumor chemotherapy methods to evolve. Similarly, many of the bacteria and fungi in developed countries cause infectious diseases and affect the people in several abnormal conditions. Hence, there is an urgent need to improve the current statues of antimicrobial and antitumor drugs with novel antimicrobial and anticancer properties. Earlier studies have demonstrated the antibacterial, anticancer, antifungal and antiviral properties of AgNPs. Therefore in this study Ag NPs was synthesis using *Lactobacillus casei subsp. Casei* that is a strain of probiotics that can be considered as a good candidate for the fabrication of nanoparticles in laboratory and even industrial scale due to their ecofriendly structure, high growth rate, proliferation and non-pathogenicity. Accordingly the aim of this work is to investigate the antibacterial and cytotoxicity effects of silver nanoparticles biosynthesized by *Lactobacillus casei* ATCC 39392 on *Staphylococcus aureus and Pseudomonas aeruginosa* and human gastric carcinoma cells (*AGS*) and Human Colon Carcinoma Cell Line (*HT-29*) respectively.

2. MATERIALS AND METHODS

2.1. Materials

$AgNO_3$ and MTT powder were purchased from Sigma-Aldrich Chemical Co. (Steinheim, Germany). Müller-Hinton broth was purchased from Carlo, Italy. The lyophilized bacteria of *Lactobacillus casei* subsp. *Casei* (ATCC 39392) was obtained from Persian Type Culture Collection (PTCC). MRS broth was purchased from MERCK (Darmstadt, Germany). Distilled water was used in all experiments. The HT-29 and AGS cell lines were purchased from the cell bank of the Pasteur institute (Tehran). The antibacterial activity of treatment synthesized AgNPs was evaluated on gram-positive and gram-negative bacterial strains. *Staphlococcus aureus* ATCC 25923 was evaluated as gram-positive strain and Gram negative strain was *Pseudomonas aeruginosa* ATCC27853. Bacterial strains were aerobically cultivated in Mueller Hinton Agar (MHA) or Mueller Hinton broth (MHB, Scharlau, Spain) for 24h at 37°C. Antibacterial analysis on the AgNPs was achieved against standard strains in different methods.

2.2. Biosynthesis of silver nanoparticles

Lactobacillus casei (ATCC39392) was cultivated in a round bottom flask containing MRS broth for 24 hours at 30 °C. After the incubation period pH of the broth culture was adjusted to 6 using 0.8 M NaOH in order to delay the process of transformation. Aqueous solution of $AgNO_3$ (1mM) was added to the mixture and incubated for 48h in 37 °C. The medium turned from yellow to dark brown at the bottom of the flask indicating the formation of silver nanoparticles. 12, 24, 48 h after the reaction, the mixtures were monitored by UV–Vis spectrophotometer. The product was filtered and washed with deionized water and then dried at 40 °C in a hot air oven for 4 h.

2.3. Characterization of silver nanoparticles

Bio-fabrication of Ag NPs was monitored with UV–Vis spectroscopy (Perkin Elmer dual beam spectrophotometer, Model Lambda 25) at the scanning region from 300 to 700 nm. Fourier Transform Infrared Spectrometer spectra (FTIR) analysis of the Ag NPs was performed by the potassium bromide pellet method under identical conditions in the 4000 500 cm^{-1} region using FT-IR Spectrometer (Thermo Nicolet Nexus 670 Model) that demonstrated the chemical nature of the synthesized Silver NPs. The morphological structure of the silver nanoparticles was characterized via transmission electron microscopy (TEM) at an accelerating voltage of 100 KV coater and then the material was analyzed (Zeiss, EM10C model, Germany).

2.4. Determination of antimicrobial activity by Well-diffusion method

The antimicrobial activity of AgNPs was tasted against *P. aeruginosa* and *S. aureus* by well diffusion method as the primarily screening test. The turbidity of the suspension was adjusted at 590nm to obtain turbidity equal to 0.5 McFarland standards. 1.5×10^6 colony forming units (CFU) of each strain were spread over on entire MHA surface. Wells of 6-mm diameter were made on Müller-Hinton agar plates using gel puncture. The wells were loaded with 40 µL AgNPs. Further, the plates were incubated at 37 °C for 24 h. After the incubation period, the zone of the diameter of inhibition was measured around the wells (millimeter) using meter ruler. The experiments were done three times and the mean values are considered.

2.5. Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations of Ag nanoparticls

The MIC study was conducted to investigate the lowest concentration of the examined antimicrobial agent which noticeably inhibited the growth of the tested microorganisms and the obtained values were reported in µg/ml or mg/ml. The most recognized standards are provided by CLSI and the European committee on antimicrobial susceptibility testing (EUCAST). MIC values of the silver nanoparticles synthesized using *L.casei sub.Casei,* were analyzed against *P.aeruginosa* , *S. aureus* as gram negative and gram positive bacteria respectively. Following Clinical and Laboratory Standards Institute (CLSI) guidelines the test was carried out in 96-well microtiter plates. The inoculums contained 1.5×10^{-6} C.F.U /ml bacterial strain that was adjusted by optical density at 590 nm (OD590) in comparison with *0.5 McFarland turbidity standards.**100 µL Mueller-Hinton-agar. Each wells serially aliquot with five concentrations of Ag NPs (5, 2.5, 1.25, 0.625, 0.304, 0.154 and 0.075 mg/ml). A tube containing growth medium without AgNPs and an un-inoculated tube were used as positive and negative growth controls respectively. After incubation at 37°C for 24 h, the turbidity of the medium was measured by a microplate reader (Awareness Technologinc, American) at 630 nm. The MIC values were determined as the concentration in which the absorbance was equal to or lower than those of the negative control. For determining MBC 10 microliters from each well was inoculated onto Nutrient Agar (NA) and were incubated under the same conditions. The highest dilution that inhibited 99.9% of bacterial growth on nutrient agar after overnight incubation was taken as MBC. Three wells were applied for each concentration of AgNPs and the modal values were considered.

2.6. MIC determination by MTT method

To compare the MTT method with routinely operation method of broth microdilution, the similar densities of all strains were seeded in culture media. The identical concentrations of AgNPs which are used in the former method were provided for MTT technique as well. The culture medium alone and medium containing bacteria without AgNPs were used as negative and positive controls respectively. MTT was dissolved in PBS (pH 7.2) for preparing the final concentration of 5 mg/ml. Each 96 well plate contained 100 µl broth medium, 100 µl tested concentration of Ag NPs (in the range of 0.156-5 mg/ml) and 1 µl bacteria. The plates were incubated for 18 h at 35°C in aerobic conditions. Following the incubation time (18 h), 20 µl MTT solution was added to each well and then the 96-well micro-titer plates were incubated for 30 min at 35°C. After 30 min incubation with MTT solution, to avoid the removal of the formed formazan granules, 4/5 of the MTT solution was carefully removed and the insoluble purple formazan granules were solubilized with MTT lyses buffer (0.5% sodium dodecyl sulphate, 36 mM HCl, and isopropanol acid). After that, the absorbance (Ab) was measured at 540 nm. The ultimate absorbance for each well was calculated as: Ab (540 nm) of the sample–Ab (540 nm) of the control which contains only the culture medium and test compound solvent (Malekinejad, 2011).

2.7. Cell culture

The cells were cultured in DMEM (Sigma) containing 10% (w/v) fetal bovine serum (Gibco), and 90 µg/ml penicillin/streptomycin. All cells were maintained at 37°C in air supplemented with 5% CO_2 in a humidified atmosphere. The adherent cells were exposed to the prepared particles in the concentration range of 0.156- 5 mg/ml. Each experiment included a negative control (nanoparticles-free cell culture medium). To determine the interactions of the synthesized nanoparticles with different assay reagents, each experiment included nanoparticles control, which consisted of cell culture.

2.8. MTT assay

Cancer cells were seeded into 96-well culture plates at the density of 1×10^4 cells per well in complete medium. 24 h after incubation, the cells were treated with the indicated concentrations of silver oxide nanoparticles and growth rates were evaluated by MTT assay. Following each incubation period the MTT solution (5 mg/ml in Phosphate-buffered saline; PBS) was added to each well and incubation continued for 4 h at 37 °C. The culture medium of the wells was removed and replaced with 200 µL dimethyl sulfoxide (DMSO) to dissolve the MTT formazan crystals. The absorbance of each well was then measured with a microplate reader (State fox, USA) at 492 nm. Growth rate was calculated as $(A_{sample}-A_{blank}) / (A_{control}-A_{blank}) \times 100\%$. Then, the results were expressed as the percentages of non-treated control cells. All experiments were repeated at least 2 times with triplicate assays.

2.9. Acridine Orange/ Propidium Iodide dual staining

Acridine Orange/Propidium Iodide (AO/PI) dual staining was carried out to detect the morphological evidences of apoptosis in silver nanoparticle treated cells. Cancer cells were seeded into 6-well culture plates at a density of 2.5×10^5 per well in complete medium. After 24 h incubation, the cells were treated with different concentrations of silver nanoparticles. After 48 hours, twenty five microliters of treated and untreated cell suspensions (5×10^6 cells/mL) were stained with 1 ml Acridine Orange and Propidium Iodide dye mix (100 mg/ml Acridine Orange and Propidium Iodide prepared in PBS separately). Samples were then examined under fluorescent microscopy (Nikon Eclipse TS 100).

2.10. Nitric Oxide (NO) production

NO production was quantified indirectly by measuring nitrites (NO_2) and nitrates (NO_3) (Bryan, 2007), according to Griess method (Espey, 2001). Briefly, 100 µl aliquots of culture medium were sampled into 96 well plate and were incubated for 30 min in the presence of Griess reagent (1% Sulphanilamide, 0.1% Naphthyl ethylenediamine dichloride and 3% phosphoric acid), 10 ml NEDD (0.1%, w/v), 10 ml Sulf (2%, w/v) and 80 ml vanadium (III) chloride (50 mM) at 37◦C. After incubation, the absorbance of each sample was measured with plate Chameleon microplate reader (Hidex Co., Turku, Finland) with an emission filter set at 560 nm. NO_2/NO_3 concentration was calculated using NO_2 standard curve. Media without cells in the presence of different concentrations of silver NPs were used as background controls and their absorbance were subtracted from the readings of the samples.

3. RESULTS AND DISCUSSION

3.1. UV–visible spectral studies

Formation of Nano-silver was monitored using UV-visible absorption spectroscopy (Perkin Elmer dual beam spectrophotometer Model Lambda 25), which is one of the important techniques to verify the formation of metal nanoparticles, provided that the metal has Surface Plasmon Resonance (SPR). During incubation in a dark room, the colorless reaction mixture turned into dark brown that

corroborated the synthesis of Ag NPs (Darroudi, 2014; Mulvaney, 1996). This color transitions which is shown in Fig.1 (A) was due to the molecular and structural changes in the substances being examined, leading to corresponding changes in the ability to absorb light in the visible region of the electromagnetic spectrum (Ramar, 2015). In the present study, aliquots of 2 ml samples were withdrawn from flasks containing colloidal Ag NPs at predetermined time intervals, and their absorbance were measured with the resolution of 1 nm in the range of 250–800 nm at three time intervals (12 h-24 h-48 h). Fig.1(B) shows UV–visible spectra of these solutions which can be seen that the peak area and the height of the spectra of the biomass-free medium were considerably higher after 48h of incubation (i.e. the early stationary phase).

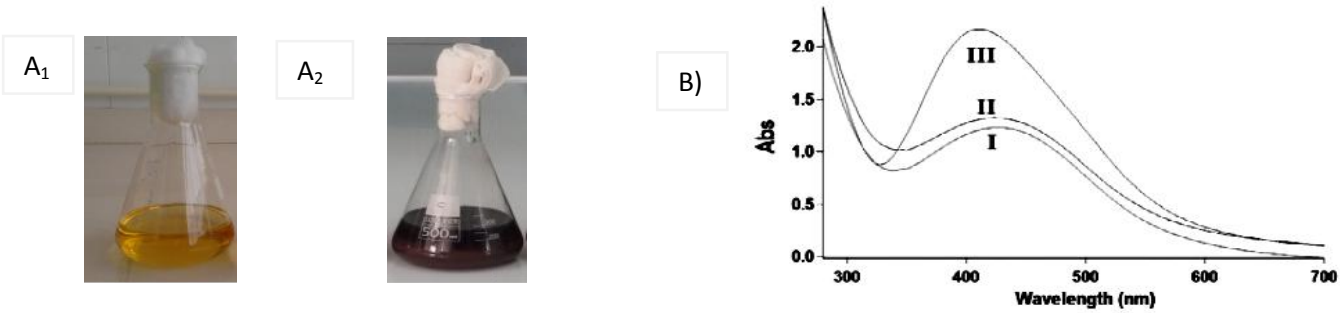

Figure 1

Color **(A_1)** change to dark brown after AgNPs synthesis **(A_2) (B)** UV–visible spectra of colloidal AgNPs originated from *L.casei* media culture after 12 h (I), 24h (II) and 48h (III).

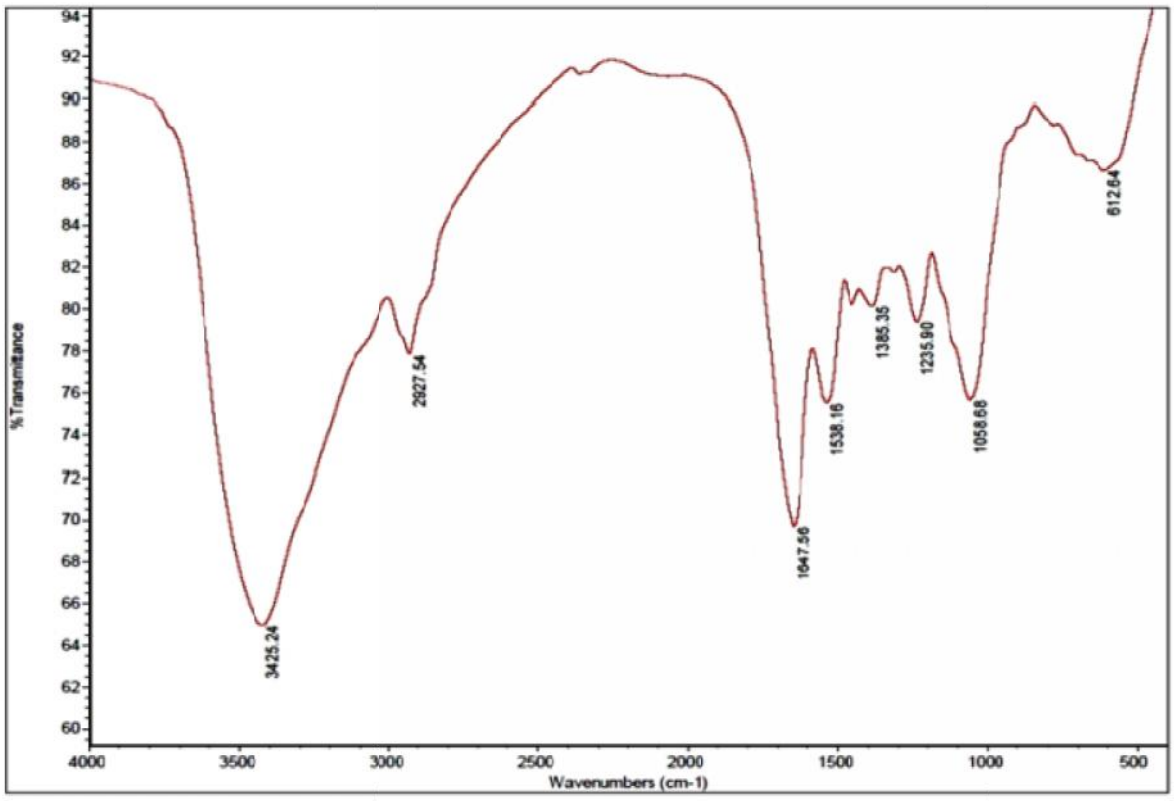

Figure 2

FT-IR spectra of AgNPs synthesis by *L.casei*

3.2. FT- IR analysis

The main goal of IR spectroscopic analysis is to determine chemical functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation. Thus, the characterization of functional groups on the surface of AgNPs was

performed by fourier-transform infrared spectroscopy (FT- IR) (FT-IR Thermo Nicolet 670). The spectra were scanned in the range of 500-4,000 cm^{-1}. Fig.2. shows strong peaks at 3100-3680 cm^{-1} corresponding to N-H stretching vibrations of primary and secondary amines or amide linkages in the protein. The small peak at 2921 cm^{-1} was characteristic of C-H stretching vibration of the alkenes group. The bands at 1647 cm^{-1} and 1520 cm^{-1} correspond to the binding vibrations of amide I and amide II bands of proteins molecules respectively. The bands observed at 1385 and 1235, cm^{-1} could be assigned to C-N stretching vibrations of aromatic and aliphatic amines respectively. The weaker band at 1034 cm^{-1} corresponds to the C-O stretching vibrations due to carboxylate and alcoholic groups respectively. IR spectroscopic study has confirmed that the carbonyl group from amino acid residues and peptides of proteins had stronger ability to bind metal, so the proteins could most possibly form a coat covering the metal nanoparticles and prevent agglomeration of the particles and thus their stabilization in the medium. This evidence suggested that biological molecules could possibly form and stabilize AgNPs in the aqueous medium.

3.3. Transmission electron microscopy (TEM)

The characterization of silver nanoparticles was done by TEM (Zeiss-EM10C-100 KV- German). TEM micrographs indicated the spherical morphology of the particles with diameter in the range of 20–90 nm with an average diameter of 20 nm (Fig 3). Samples were prepared by drop coating the silver nanoparticle solution onto carbon coated copper grid and were subjected to vacuum desiccation before loading onto the specimen holder. TEM micrographs were taken by analyzing the prepared grids.

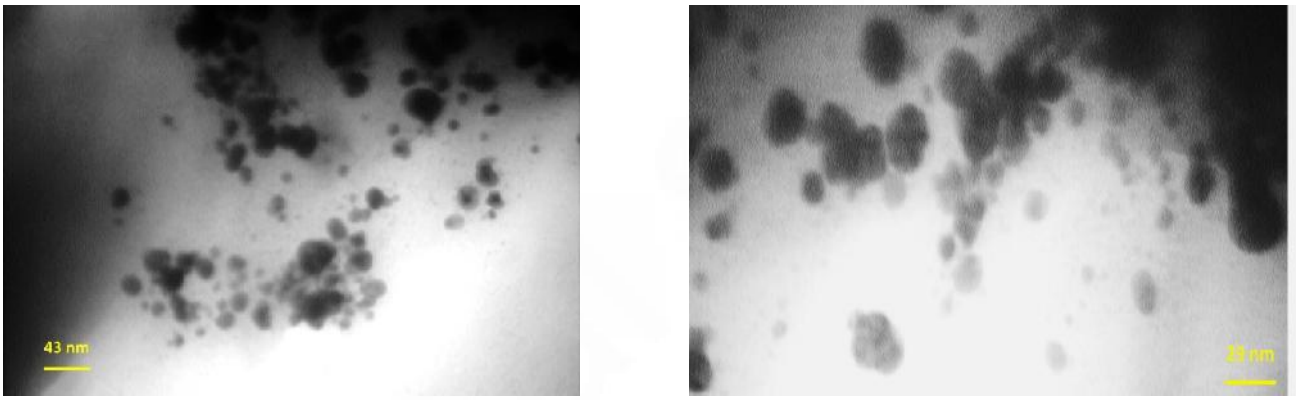

Figure 3
TEM Image shows AgNPs, synthesized by L.casei

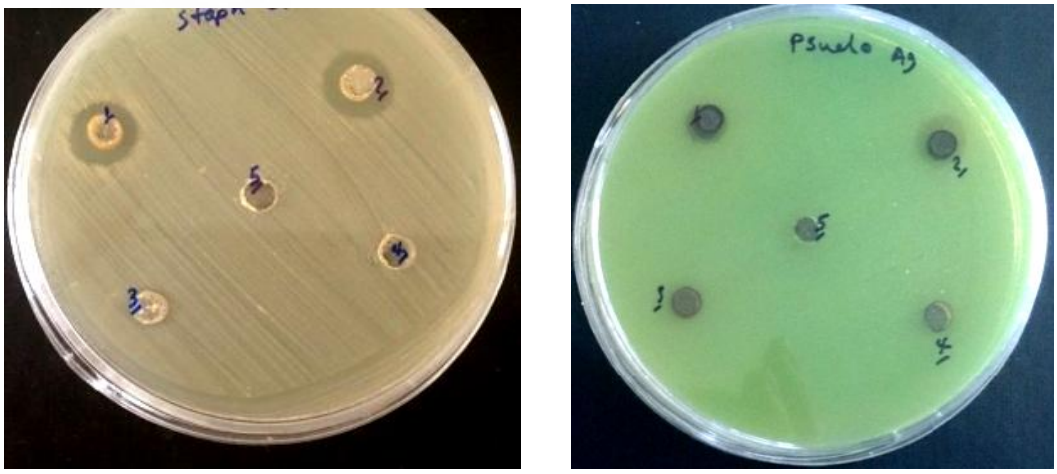

Figure 4
Antibacterial activity of biosynthesized AgNPs against gram negative and positive bacteria by well diffusion method

3.4. Antimicrobial activity

The antibacterial activity of silver and its compounds is well documented. The mechanism of bactericidal effect of colloidal silver particles against bacteria is only partially understood (SStoimenov, 2002; Feng, 2000). It has been demonstrated that silver ions strongly interact with thiol groups of vital enzymes and inactivate them (Thakkar, 2010). The inhibitory effect of silver nanoparticles is accomplished via two possible mechanisms: 1) the electrostatic attraction between the negatively charged cell membrane of microorganisms and Ag^{+}ion, 2) by anchoring and penetrating the bacterial cell wall related to Ag concentration which causes further damage by possibly interacting with sulfur and phosphorus containing compounds such as DNA (Sondi, 2004). Ivan Sondi and Branka Salopek-Sondi studied silver antibacterial activity against *E-coli* as a model of gram-negative bacteria. They reported that the antibacterial activity was probably originated from the electrostatic attraction between negatively charged cell membranes of bacteria and positively charged nanoparticles. In the present study, the antibacterial effect of AgNPs biosynthesized by *L. casei* was tested on two standard bacterial strains namely *S. aureus* (gram positive) and *P. aeroginosa* (gram negative), using three different methods. Zone of inhibition obtained from agar well diffusion method for individual bacteria is shown in Fig.4. Among the tested bacteria, the zone of inhibition of *S.aureus* was bigger than that of *P. aeroginosa* (Table 1). The differences observed in the susceptibility of gram negative and gram positive bacteria may be partly due to the differences in the cell wall or membrane structures, physiology, metabolism and their interaction with charged silver nanoparticles. The results of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) obtained from microdilution of the prepared AgNPs are given in (Table 2). It seems that antibacterial properties of AgNPs affect *S.aureus and P. aeroginosa* which could be seen in our estimated MIC values by MTT method at that concentration of 2.5 mg/ml (Fig. 5).

Table1 Diameter of zone inhibition by AgNPs against human pathogenic bacteria

Bacteria	inhibition Zone (mm)	
	Taste Control negative	Ag- NPS Water
S.aureus **(gram positive)**	**13±0.5**	**NA**
P. aeroginosa **(gram negative)**	**10±0.2**	**NA**

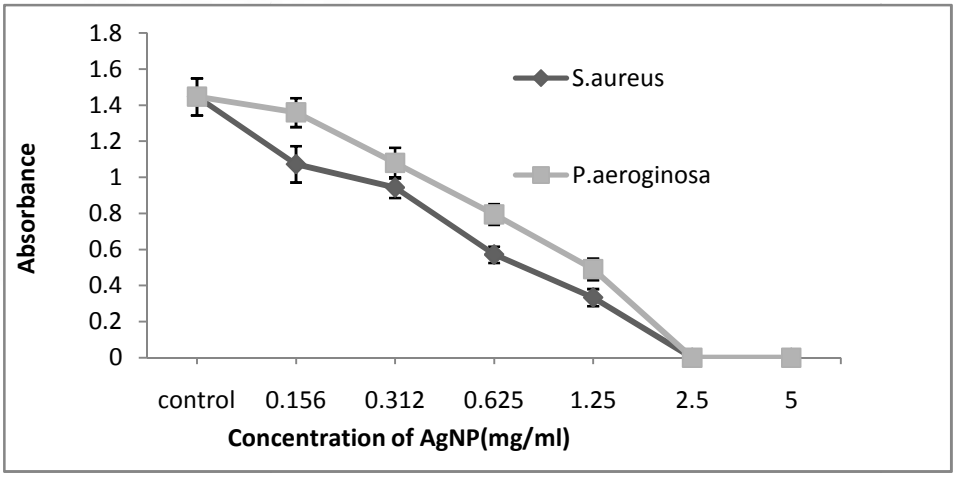

Figure 5

Antibacterial effect of AgNPs against *S.aureus and P. aeroginosa* incubation with MTT solution, result showed significant decrease in bacteria numbers in a dose-dependent manner ($p < 0.05$).

Table 2 MIC and MBC valve of AgNPs against tested microorganisms (mg/ml)

AgNPs originated from *L.casei* (mg/ml)		
Bacteria	**MIC**	**MBC**
S.aureus **(gram positive)**	0.625	1.25
P.aeroginosa **(gram negative)**	1.25	>1.25

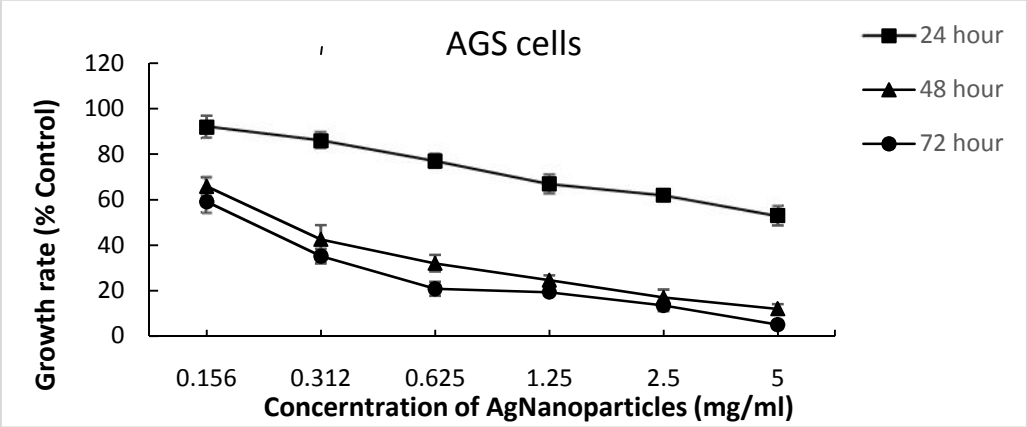

Figure 6a
Cell viability of AgNPs by MTT assay. Data represent at least three independent experiments. * P < 0.05

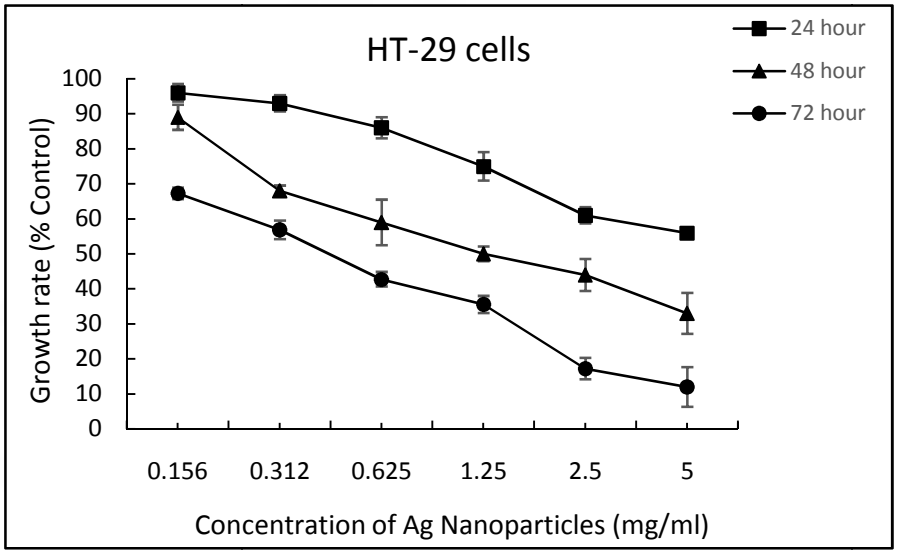

Figure 6b
Cell viability of AgNPs treated HT-29 cells (0.156-5 mg/ml). Data represent at least three independent experiments.* P < 0.05.

3.5. Cytotoxic effects of Ag NPs on cancerous cell lines

The MTT assay was used to determine the effect of Ag nanoparticles on the mitochondrial activity of the cancerous cells as an indicator of viable cells. As shown in Fig. 6, treatment of cancerous cells with Ag NPs exhibited significant effects in cell viability. Treatment of cells with increasing concentrations of Ag NPs significantly diminished cell growth in a dose- and time-dependent manner (Fig. 6). As shown in Fig. 1a, Ag NPs at 0.153 and 5 mg/ml concentrations inhibited growth rate by 11±1 and 67±2.5 %

respectively after 48 h treatment in HT-29. Furthermore we found that AGS cell line showed 25±1.4 and 89 % growth rate inhibition after 48 h treatment with 0.153 and 5 mg/ml Ag NPs respectively (Figs. 6a and 6b).Unlike the commonly used broth micro dilution method, in which the turbidity of the bacteria density (live or dead) is evaluated by either eye or machine In MTT method, only the density of live microorganisms is measured. Therefore, any false positive evaluation due to dead bacteria is excluded. The MTT method has advantages such as time saving and bacteria concentration lowering.

3.6. Increased NO level

To investigate the relation between inflammation and cytotoxicity caused by Ag NPs, we measured secreted nitric oxide, which acts as a second messenger in inflammatory signaling. As shown in Fig. 7 , NO levels of cells were remarkably enhanced after treatment with 0.156-5 mg/ml Ag NPs in both cell lines. The detected levels after treating with Ag NPs of 5mg/ml for 72 h were 3 ± 0.03 and 3.5±0.03 M in the control group, and 25 ± 0.9 and 20.7±0.45 M in the HT-29 and AGS cell lines, respectively (Figs. 7a and 7b).

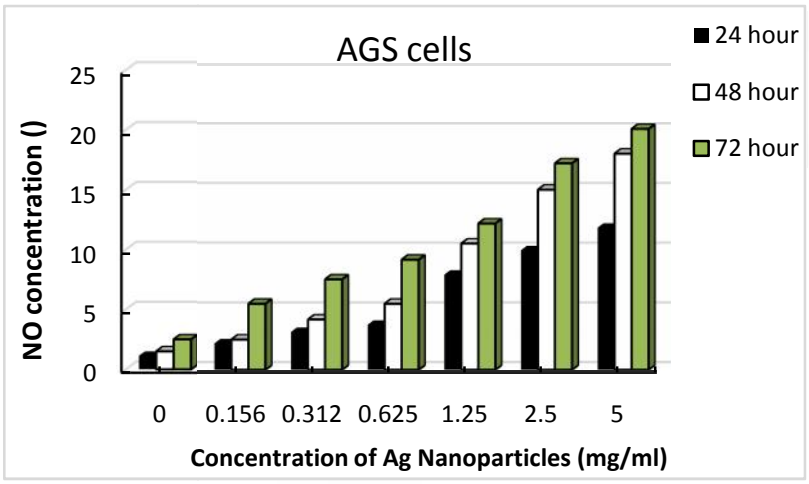

Figure 7a

Effect of Ag NP on NO production in AGS cells. After 24, 48 and 72h indication by Griess test method.. Data represent at least three independent experiments.* P < 0.05

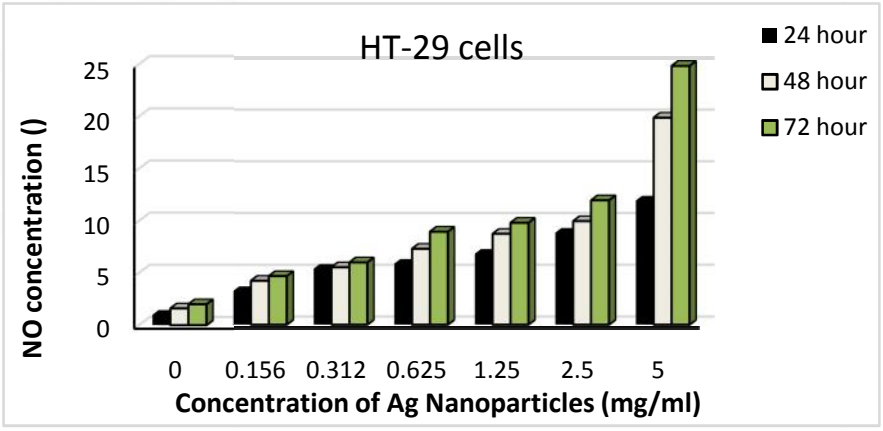

Figure 7b

Effect of Ag NPs on NO production in HT-29 cells. Cells were treated with indicated concentrations of NPS for 24, 48 and 72h and production of NO were determined by Griess test. Data represent at least three independent experiments.* P < 0.05.

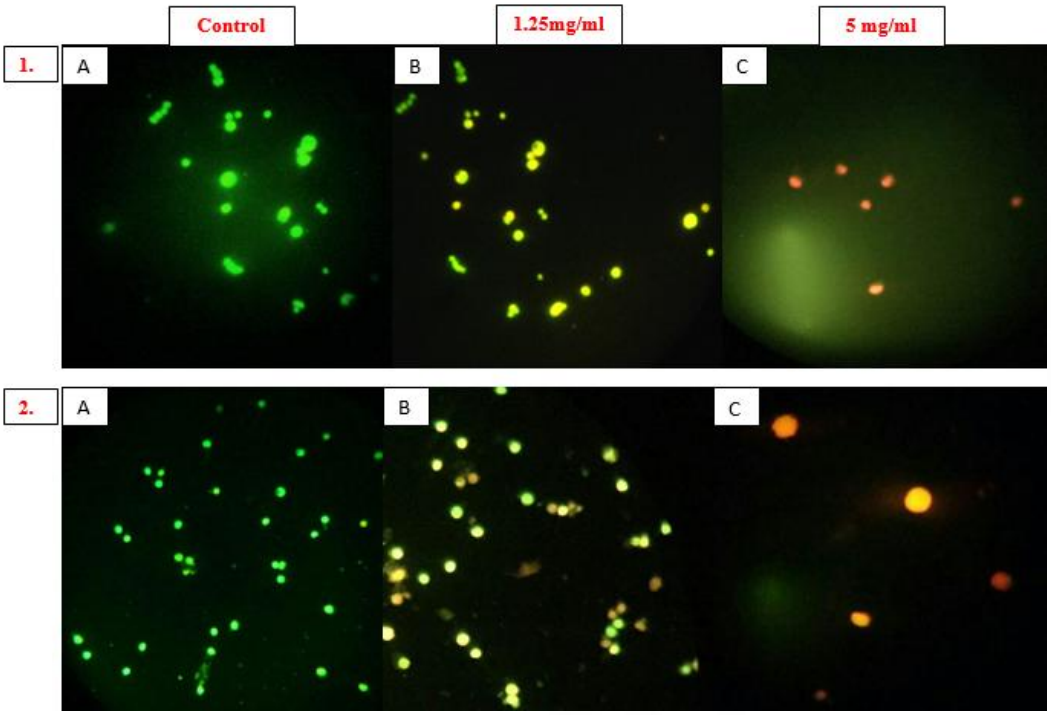

Figure 8

Morphological evidence of apoptosis by AO/PI dual staining. Fluorescent microscopic images of 1) AGS human gastric cancer cells 2) HT-29 human colorectal cancer cells treated for 48 hours. (A) Untreated cells; (B) cells treated with biosynthesized AgNPs (1.25mg/ml); (C) cells treated with biosynthesized AgNPs (5mg/ml).

3.7. Effects of Ag NPs on apoptosis

In order to evaluate the effects of Ag NPs on the induction of apoptosis, Ag NPs-treated cancerous cells were stained with Acridine Orange/Propidium Iodide. After treatment for 48 h, majority of the cells in control group exhibited green fluorescence while diffused or orange-colored nuclei were raisedin Ag NPs-treated cells by increasing Ag NPs concentration. The cells treated with 5 mg/ml Ag NPs developed orange and orange-red fluorescence, indicating membrane disruption (Fig. 8). AGS cells presented similar results. These results supported the theory that Ag NPs could induce both apoptosis and necrosis at high concentrations.

4. CONCLUSION

Bio-fabrication of Ag nanoparticles using *L.Casei subsp. Casei,* was successfully conducted. The silver nanoparticles were characterized by FTIR, UV–Vis spectroscopy with single SPR band at 430 nm as well as TEM that revealed the spherical nanoparticles formation in nanoscale. Silver nanoparticles showed improved antibacterial activity against *S. aureus and P. aeroginosa* at different concentrations. The cytotoxic effects of AgNPs against human gastric carcinoma cells (*AGS*) and human colon carcinoma cell line (*HT-29*) showed good anticancer response in a dose dependent manner with significant IC50 value. Results of our study proposed that biosynthesis of AgNPs can be employed as a promising method for industrial and biomedical applications.

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