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Original article

Extracellular directed ag NPs formation and investigation of their antimicrobial and cytotoxic properties

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ABSTRACT

The use of microbial cell culture a valuable tool for the biosynthesis of nanoparticles is considered a green technology as it is eco-friendly, inexpensive and simple. Here, the synthesis of nanosilver particle (AgNP) from the yeast, *Saccharomyces cerevisiae*, gram (+), *Bacillus subtilis* and gram (−), *Escherichia coli* was shown. In this field we are the first to study their the antimicrobial effects of the microorganisms mentioned above against pathogens and anticancer activity on MCF-7 cell line. Silver nanoparticles in the size range of 126–323 nm were synthesized extracellularly by the microorganisms, which have different cell structures. Optical absorption, scanning electron microscopy, and zetasizer analysis confirmed the silver nanoparticles formation. Antimicrobial activity of AgNPs was evaluated the minimum inhibition concentration and disc diffusion methods. AgNPs inhibited nearly 90% the growth of Gram-positive *Listeria monocytogenes, Streptococcus pneumoniae* and Gram-negative *Haemophilus influenzae, Klebsiella pneumoniae, Neisseria meningitidis* bacterial pathogens. Anticancer potentials of AgNPs were investigated by MTT method. The synthesized AgNPs exhibited excellent high toxicity on MCF-7 cells and had a dose-dependent effect on cell viability. Especially AgNP 2 eliminated 67% of the MCF-7 cells at the concentration of 3.125 μg/mL. We found that extracellular synthesis of nanoparticles from microbial culture may be 'green' alternative to physical and chemical methods from the point of view of synthesis in large amounts and easy process.

1. Introduction

Nanoparticles are materials with nanoscale structural features, ie, under 100 nm. Recent years, these nanoparticles have become more prominent in many field including medical, pharmaceutical and biological applications. The benefits of using nanotechnology are numerous depending on the nanoparticles features. In general, nanoparticles are considered a green technology as it is eco-friendly, inexpensive and simple.

Silver nanoparticles (AgNps), are among the most attractive nanomaterials, and have been widely used in a range of biomedical applications, including diagnosis, treatment, drug delivery, medical device coating, and for personal health care. The major methods used for AgNp synthesis are the physical and chemical methods. The problem with the chemical and physical methods is that the synthesis is expensive and can also have toxic substances absorbed onto them. Biosynthesis (green synthesis) of nanosilver has received extensive attention due to the growing need for environmentally friendly synthesis methods that use eco-friendly reducing agents, such as protein, peptides, carbohydrate, various species of bacteria, fungi, yeast, algae and plants. The major biological systems involved in this are bacteria, fungi, and plant extracts. The major applications of silver nanoparticles in the medical field include diagnostic applications and therapeutic applications. In most of the therapeutic applications, though it is the antimicrobial property that is being majorly explored, it has the anti-carcinogenic properties Fu et al. (2000); Allsopp et al. (2007); Parashar et al. (2009); Narayanan and Sakthivel (2010); Sintubin et al. (2012). The most important advantage of the biosynthesis compared with chemical production of AgNPs is that it does not contain toxic agents. Moreover, the biological production of these

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nanoparticles provides recycling of waste containing silver. Recycling, recovery and regeneration of silver are important for economy due to limited resources Veglio and Beolchini (1997); Nakiboglu et al. (2003); Kononova et al. (2007); Nowack (2010); Sukumaran and Eldho (2012). Antimicrobial activity of AgNPs depends on particle size. The smaller nanoparticle the higher specific surface area and hence nanoparticle has the higher antimicrobial effect. In addition, it was reported that nanoparticle shape is effective on the antimicrobial activity Pal et al. (2007). Therefore, each microbial silver particle size, shape and interaction with other biomolecules is unique. The most important parameter for bioactivity studies of nanoparticles is to formulate the most effective concentration via characterization of these particles.

There are a considerable number of study reports demonstrate that the onset and progression of many disease is caused by microbial activities of Gram (+) and Gram (−) bacteria. Meningitis is an inflammation of the meningeal membranes surrounding the brain and spinal cord. Meningitis continues to be the cause of serious mortality and morbidity despite advances in vaccination and antimicrobial treatment. Over 170.000 death per year because of meningitis have been reported by the World Health Organization, and the shekels have been reported as 19.9%. This situation has heightened the need for novel antimicrobial therapies as alternatives to traditional antibiotics. *Streptococcus pneumoniae, Neisseria meningitidis* and *H. influenzae* type B are responsible for over 80–85% of the cases therefore treatment and control efforts have concentrated on these factors of meningitis.

Breast cancer affects one in eight women in their lifetime. It is the second cause of death in women's deaths, resulting in more than 40.000 deaths per year. Despite the 5.5 billion dollars spent on breast cancer research in the last two decades, the source of the majority of breast cancer cases is still unknown. Obesity, colon cancer and colitis are associated with the microbes. Recent research has shown that it is becoming increasingly clear that community composition and discrete bacterial species may either exhibit pathogenic effects that promote disease development (Xuan et al., 2014).

In this study, AgNPs synthesis using culture supernatant of *E. coli*, *B. subtilis* and *S. cerevisiae* was intended. These microorganisms possess the ability to reproduce of large-scale and inexpensive broth. Besides these microorganisms can be easily inoculated and have the potential for rapid growth. Since these properties AgNPs were produced in a short time and large amount. The main purposes of this study are (1) the extracellular synthesis of AgNPs using the different cell structure microorganisms, (2) characterization of those AgNps by using ZT, SEM and UV–vis to evaluate their quality, morphology and size, (3) evaluation of antimicrobial activity and anticancer potentials of AgNPs.

2. Experimental

2.1. Chemical and materials

Silver nitrate, ACS reagent, 99+% ($AgNO_3$) for AgNP synthesis were obtained from Sigma-Aldrich, for appropriate microorganisms culturing Muller Hinton (MHB), Sabaroud Dextrose Broth (SDB) and Brain Heart Broth (BHB) were obtained from Merck. All microorganism strains were kept at −20C in the appropriate medium containing 10% glycerol and regenerated twice prior to the manipulations. For anticancer analyses all chemicals were purchased from Sigma Aldrich, Germany. Freshly prepared doubly distilled water was used throughout the experimental work.

2.2. Preparation of microorganisms

B. subtilis ATCC 6633, *E. coli* ATCC 8739, and *S. cerevisiae* ATCC 9763 were used for the synthesis of silver nanoparticles. All microorganism isolates were obtained from American Type Culture Collection (ATCC). Bacterial cultures were grown in MHB at 37 ∘C in shaker incubator at 220 rpm. Yeast was grown in SDB at 30 ∘C in shaker incubator at 220 rpm. After 48 h, when the culture OD at 600 nm was in the range of 1.9–2.2, the culture supernatant was used for the production of AgNPs. Microorganisms used for the antimicrobial activity were *Listeria monocytogenes* (Murray et al.) Pirie (ATCC® 19115™), *Streptococcus pneumoniae* (Klein) Chester (ATCC® 49619™), *Klebsiella pneumoniae* subsp. *pneumoniae* (Schroeter) Trevisan (ATCC®33495), *Neisseria meningitidis* (Albrecht And Ghon) Murray (ATCC® 13077™), *Haemophilus influenzae* (Lehmann And Neumann) Winslow et al. (ATCC®49766™) were obtained from the ATCC. Each microorganism was incubated at the appropriate environment and condition for development.

2.3. Synthesis of silver nanoparticles

B. subtilis and *E. coli* were inoculated into flasks containing sterile MHB and then incubated at 37 °C for 24 h in 220 rpm. S. cerevisiae was inoculated in SDB at 30 ∘C for 48 h at 220 rpm. After the incubation period the culture was centrifuged for 15 min. at 8000*g* and the supernatant was used for the synthesis of silver nanoparticles. Three erlenmeyer flasks, one containing 100 mL supernatant with silver nitrate (Merck, Germany, 99.9% pure) at a concentration of 5 mM and the second containing only the supernatant and the third containing only $AgNO_3$ solution, were incubated for 24 h. The diluted AgNP solution was prepared for recording the absorption spectrum of AgNPs via Uv/vis spectrophotometer (Perkin Elmer).

2.4. Instrumentation and characterization

Scanning electron microscopy (SEM) images were obtained using a (ZEISS EVO LS10) scanning electron microscope with a working voltage of 25 kV. 50 μL of the concentrated AgNP aqueous solution was deposited on the adhesive carbon tape coated stubs and were dried overnight to generate clear images. The sample on stub has to be dried well to obtain clear SEM images. The effective diameter and surface charge of AgNPs were measured using Zetasizer (Malvern).

2.5. Minimum inhibitory concentration

Antibacterial properties of AgNPs were tested by the microdilution technique of Clinical and Laboratory Standards Institute (CLSI, 2012). The study was performed with a microorganism concentration of 1×10^8 in each milliliters of the MHB and SDB, in which 24 and 48 h incubated microorganisms were activated, and at 0.5 McFarland standard turbidity for bacteria and at 1.0 McFarland standard turbidity for yeast. AgNPs, which will be tested, were fully dissolved in distilled water and dilutions were conducted from 500 to 4 μg/mL. 96-well microplates were used for the experiment. Minimum inhibitory concentration (MIC), the lowest concentration that prevents microorganism growth after incubation, was defined as μg/mL. At the concentration in which reproduction is not observed, cultivation was spread on petri dishes again and the accuracy of the MIC value was tested. The disc diffusion method; 100 μL of suspension of the test microorganisms were spread on agar plates. Filter paper discs [6mmindiameter] were impregnated with 20 μL of the AgNPs, then placed on the inoculated plates and stored at +4 °C for 2 h. After that they were incubated for 24 h. The diameters [mm] of the inhibition zones were measured after 24 h of incubation. The tests were carried out as replicates of three and results were given as mean ± SD.

2.6. Cell culture

MCF-7 ATCC HTB 22 cells (human breast adenocarcinoma cell line) were obtained from the ATCC. For MCF-7 cells; 10% solution of Dulbecco's Modified Eagle Medium (DMEM), inactivated fetal bovine serum, medium containing the antibiotic mixture and L-glutamine were used. Readily inactivated serums were applied in this study. Additionally; antibiotic 100× mixture, containing 10.000 units penicillin and 10.000 μg streptomycin in 1 mL, were used after dilution with sterile water (1×). Prior to the application, the medium was sterilized by using a sterile filter with 0.22 mm of diameter.

2.7. Cell viability assay

Cultured MCF-7 cells (1×10^5 cells/mL) were cultivated in 96-well microplate with a ratio of 15.000 cells per well and developed. The growing cells were treated with 100 μL Phenol red-free DMEM and various concentrations of AgNPs (0–200 μg/mL); and left for 24 h incubation. After incubation, the fluids on the cells were

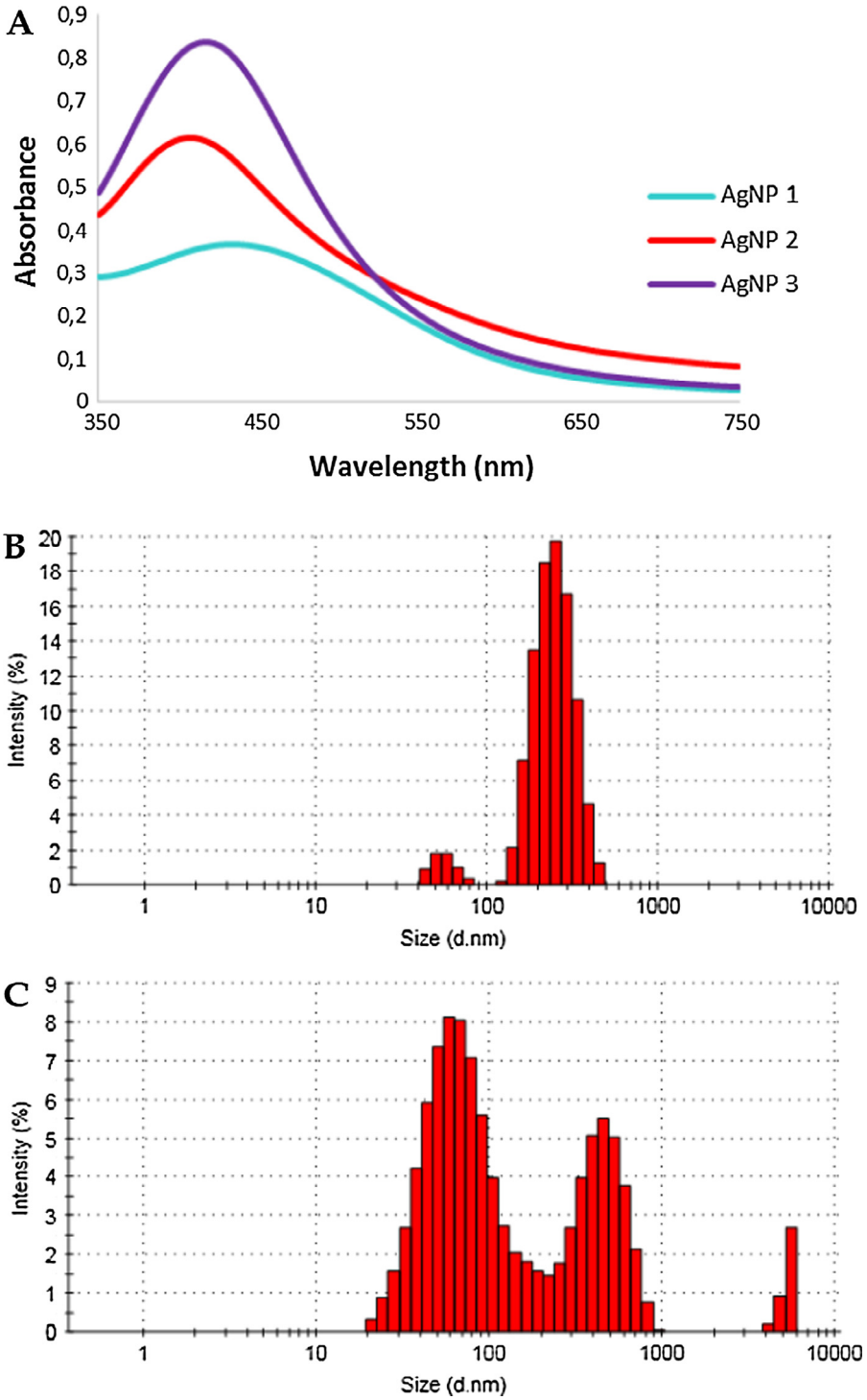

Fig. 1. UV–vis absorption spectra of (A) AgNP 1, AgNP 2 and AgNP 3. Dynamic size of (B) AgNP 1,(C) AgNP2 and (D) AgNP 3. SEM images (E) AgNP 1, (F) AgNP 2, (G) AgNP 3.

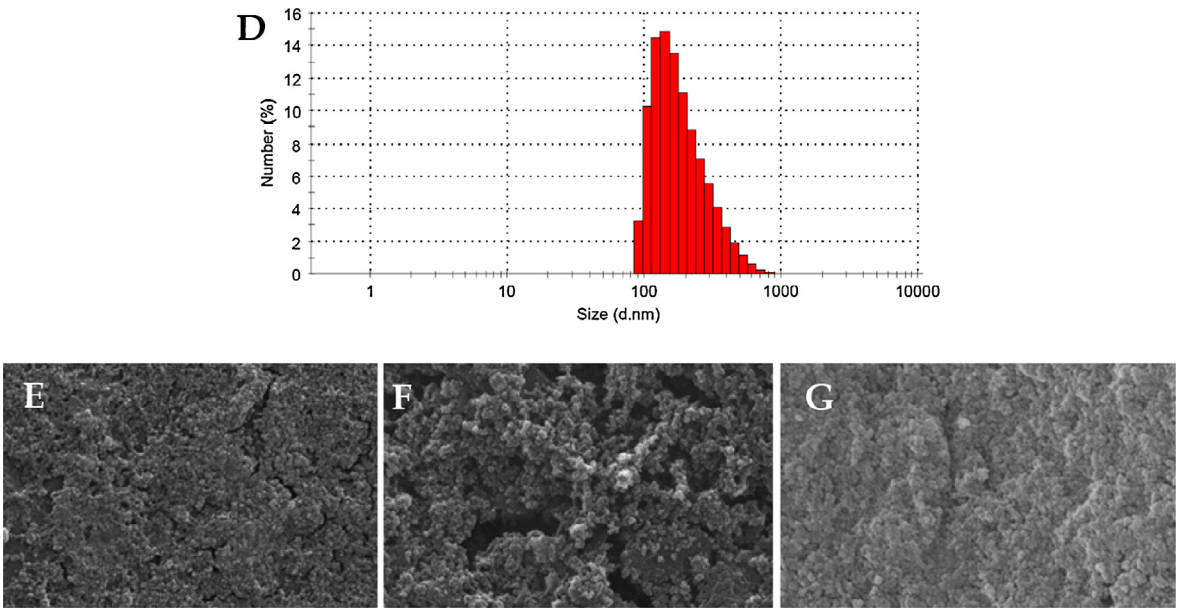

Fig. 1 (*continued*)

thrown and 100 μL fresh medium and 13 μL MTT solution (5 mg/mL) was added into each well. After 2 h of incubation at 37 °C, the medium containing MTT solution was removed. 100 μL DMSO was added into each well with the goal of providing the dissolution of the purple MTT formazan crystals. Finally; the absorbance was read by using ELISA (Biotek Synergy HT) at 570 nm wavelength. Experiments were repeated as three times and results were given as mean ± SD.

2.8. Statistical analysis

The experimental data are shown as mean ± SD. Statistical analysis was done by ANOVA. Range of identification of the statistical difference was adoted as $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of AgNPs

The effects on particle formation were examined with the UV–Vis (ultra violet visible) spectrometer after the 24-hour incubation of $AgNO_3$ salt with microorganisms. The results of UV–Vis spectrum demonstrated the formation of silver nanoparticles after adding $AgNO_3$ to the cell extract. Values given in Fig. 1 illustrate the formation of nanoparticles in 24 h period. The nanoparticles synthesis was observed with a dark brown colored appearance for 3 microorganism extracts in the study. It is characterized by using B. subtilis extract, with a peak given at 433 nm for the obtained silver nanoparticle AgNP 1, by using *E. coli* extract with a peak at 405 nm for the obtained silver nanoparticle AgNP 2, and by using *S. cerevisiae* extract with a peak at 415 nm for the obtained silver nanoparticle AgNP 3 (Fig. 1A).

The results of AgNPs size analysis are given in Fig. 1(B)–(D). Maximum density was found to be 220–295 nm for AgNP 1, 50–70 nm for AgNP 2 and 300–400 nm for AgNP 3, respectively. The sizes of AgNP 1 (288 nm), AgNP 2 (126 nm), and AgNP 3 (323 nm) are effective diameters measured by dynamic light scattering. These large sizes can be attributed to the presence of entities of extracellular component attached on surface of AgNPs and the partial aggregation of AgNPs. However, real size was measured by scanning electron microscopy (SEM) in nanoparticles based research. The SEM image indicates that AgNPs have spheric shape and the sizes of AgNP 1, AgNP 2 and AgNP 3 were ~53, ~41 and ~63 nm, respectively. Images received with the SEM analysis are given in Fig. 1(E)–(G). Spherical particles formation was observed according to the analysis results.

It has been reported in literature that these nanoparticles might have some negative impact on environment and people 14–16. AgNP's physical, chemical and biological synthesis methods are described many studies. The use of hazardous chemicals which can pose potential environmental and biological risks in most of the physical and chemical methods for the silver nanoparticles synthesis is the main argument against choosing these methods (Chandran et al., 2006). Furthermore, in most cases chemical synthesis methods may obstruct the medical use of some toxic substances by causing the absorption on their surface. Therefore biological methods used by microorganisms, biomolecules and plant extracts represent an applicable alternative way for silver nanoparticles synthesis. Nanoparticles biosynthesis generally depends on the reduction/oxidation reactions. Microbial enzymes provide the desired nanoparticles with antioxidant and reduction properties (Huang et al., 2007; Ocsoy et al., 2013; Ocsoy et al., 2013; Strayer et al., 2016; Duman et al., 2016; Demirbas et al., 2016; Şeker Karatopraka et al., 2017; Ocsoy et al., 2017).

The process of nanoparticles preparation through biological methods contains 3 major components including solvent, environmentally friendly solvent and non-toxic stabilizing agent (Clinical and Laboratory, 2012). The most important advantage of biosynthesis in comparison to the chemical production of AgNP is the occurence of synthesis far from toxic agents. Moreover, biological production of these nanoparticles provides the recycling of silver-containing waste (Jain et al., 2009; Bar et al., 2009; Feng et al., 2008; Matsumura et al., 2003; Morones et al., 2005; Hatchett and Henry, 1996; Shrivastava et al., 2007). Silver recycling, recovery and regeneration are important in economic terms due to the limitedness of resources. In addition, recycling of silver-bearing wastes provides the products' formation with added values like biocide, catalyst and biosensor (Kirsner et al., 2001). Another advantage of the biological production is that, when microbial cells

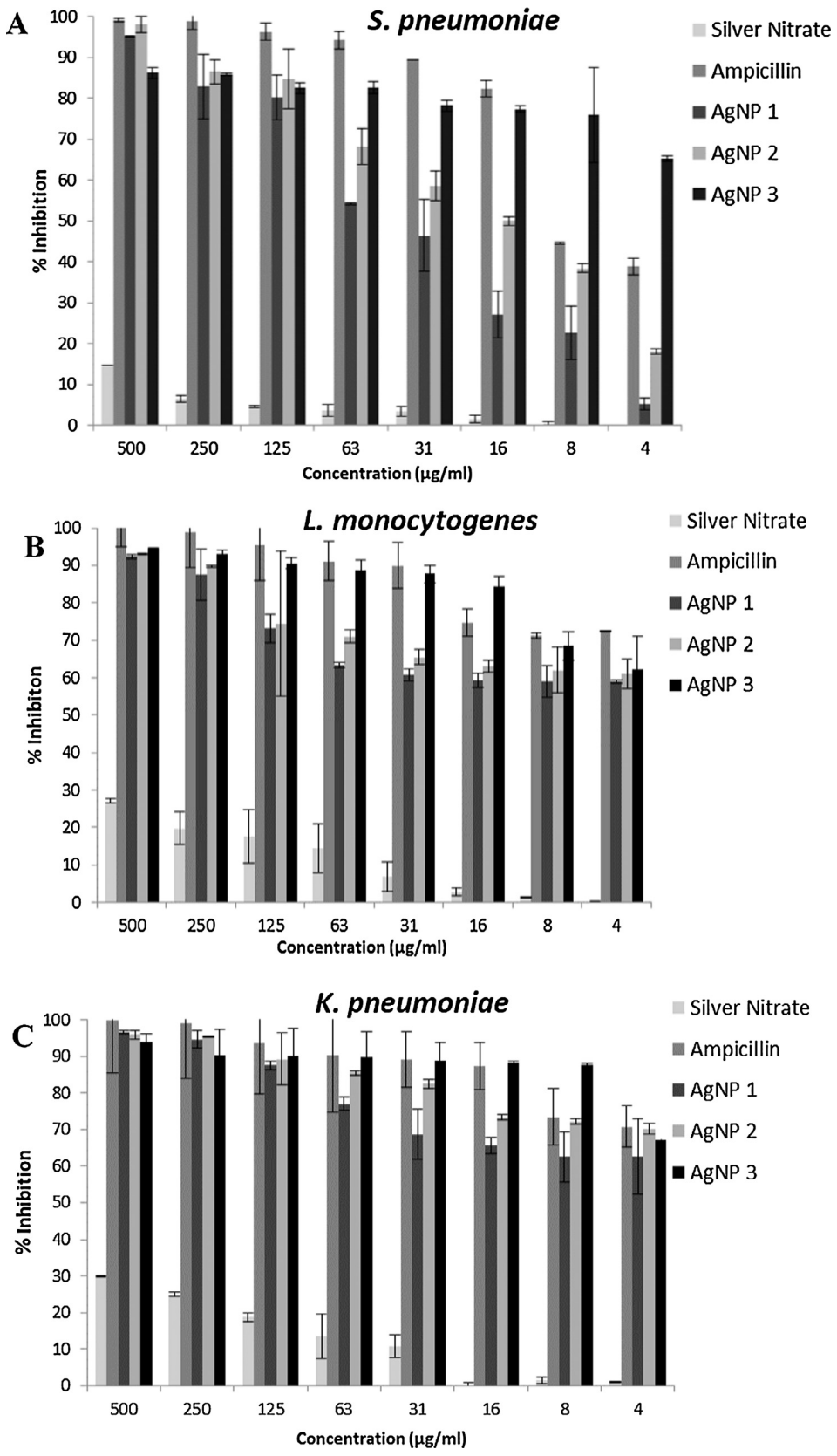

Fig. 2. Inhibitory effect of AgNP 1, AgNP 2 and AgNP 3 with respective concentrations towards (A) *S. pneumoiae*, (B) *L. monocytogenes,* (C) *K. pneumoniae*, (D) *H. influenzae* and (E) *N. meningitidis*. n = 3.

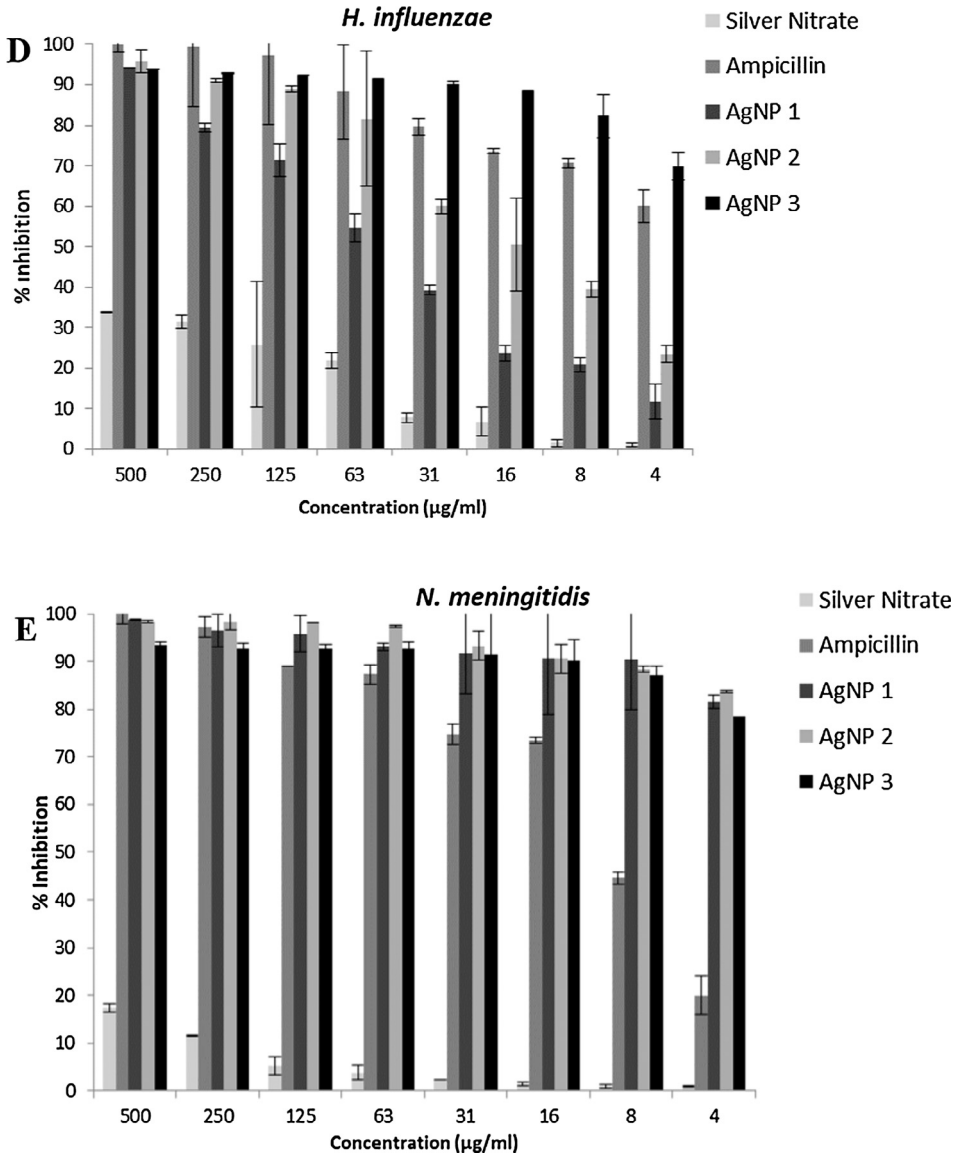

Fig. 2 (*continued*)

Table 1
Disc diffusion results of samples for 500 μg/mL concentration.

	S. pneumoniae	*L . monocytogenes*	*K. pneumoniae*	*H. influenzae*	*N. meningitidis*
$AgNO_3$	ND	ND	ND	ND	ND
Ampicillin	19 ± 0.5	22 ± 2.0	23 ± 1.5	21 ± 0.5	18 ± 0.5
AgNP 1	17 ± 0.5	19 ± 1.0	20 ± 0.5	19 ± 1.5	17 ± 1.5
AgNP 2	19 ± 1.0	22 ± 0.5	21 ± 1.0	21 ± 1.5	19 ± 0.5
AgNP 3	16 ± 1.0	20 ± 1.0	19 ± 0.5	19 ± 0.0	16 ± 0.5

ND: Not Determined, Mean ± Standart deviation (n = 2).

and non-toxic concentrations are used in the synthesis, these cells would synthesize AgNP before dying and thus they could form a continuous production process (Tian et al., 2007).

3.2. In vitro antimicrobial activity of AgNPs

AgNP is being widely used in multiple spheres, with a prevalence in physical, biological and pharmaceutical applications (Shankar et al., 2004). Especially due to the AgNPs quickness to pass the cell membrane, antimicrobial and antivirus properties, AgNp became an excellent candidate that can be used for many purposes in medicine.

The antibacterial activities of synthesized AgNPs at different concentrations (500–4 μg/mL) were tested against three Gram negative bacteria (*H. influenzae, K. pneumoniae, N. meningitidis*) and two Gram positive bacteria (*L. monocytogenes, S. pneumoniae*) and the percentage of inhibition was compared with a positive control (Ampicillin) and a negative control ($AgNO_3$). Antimicrobial activity results were detected by the disc diffusion method and MIC (minimum inhibitory concentration) tests were shown in Fig. 2 and

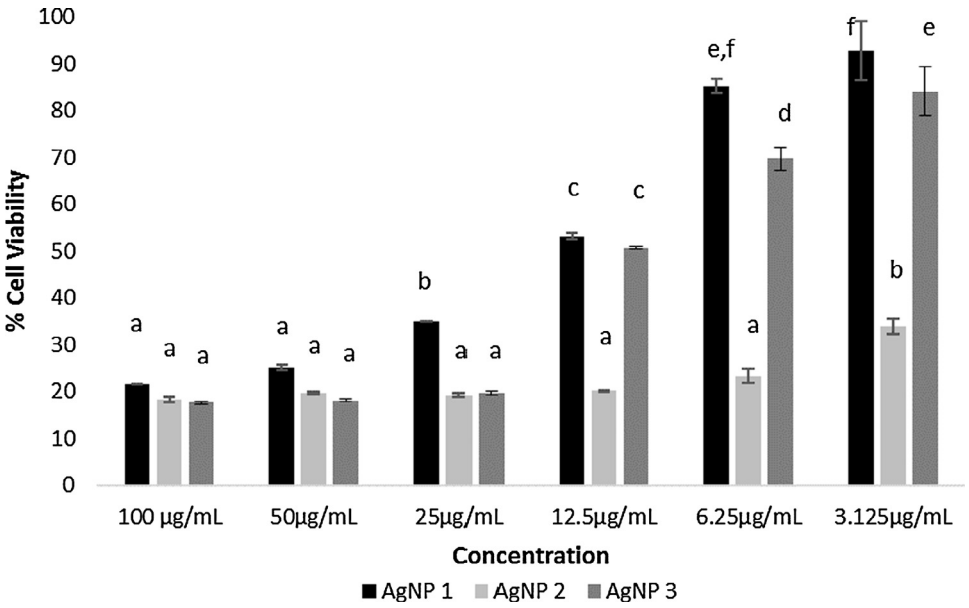

Fig. 3. Cytotoxicity of the AgNPs assessed by MTT reduction assay in MCF-7 cell line. Bars with the same lower case letter (a-f) are not significantly ($p < 0.05$) different. n = 3.

Table 1. According to the MIC results, silver nanoparticles showed the inhibition effect of more than 85% against all microorganisms studied at the concentration of 500 µg/mL. The AgNPs inhibitory effect of over 60% was observed, particularly against *L. monocytogenes* and *K. pneumoniae* strains with the concentration of 3.9 mg/mL. AgNPs demonstrated 80% antimicrobial activity against N. meningitidis strain. It was seen that the antimicrobial effect of AgNP 2 tends to be higher than the one of AgNP 1 and AgNP 3 according to the disc diffusion test. By showing the larger diameter zone, these nanoparticles have demonstrated higher antimicrobial activity against microorganisms Table 1.

AgNP's antimicrobial activity depends on the size of the particle. The smaller the nanoparticle, the higher the specific surface area and hence its the better antimicrobial activity. Besides that, nanoparticle's shape was also reported to have an effect on its activity (Alt et al., 2004). That is why each biogenic silver's particle size, shape and interaction with other biomolecules are unique. Therefore, the characterization of these particles is important in order to formulate the most effective concentration in studies for the nanoparticles bio-activities (Chandran et al., 2006).

3.3. Cytotoxicity of AgNPs in MCF-7 cell line

According to the results obtained, silver nanoparticles synthesized by using a microbial cell extract were observed to have an anticancer effect against the breast cancer cell line MCF-7. AgNP 2 and AgNP 3, at the concentration of 100 and 50 µg/mL respectively and showed approximately 83% of the lethal activity against MCF-7 cells. It was noticed that the anti-cancer effect of AgNP 1 was lower than the one of AgNP 2 and AgNP 3. AgNP 2 particularly eliminated 67% of the MCF-7 cells at the concentration of 3.125 µg/mL. The cytotoxic effect against MCF-7 cells was shown in Fig. 3.

According to the general opinion on nanoparticles, nano-sized particles increase their penetration potential and metal activities by providing the larger surface area for the silver particles. NPs are capable of penetrating into circulatory system and even into the blood-brain barrier based solely on the size factor. There are studies indicating the applicability of AgNP with such a quality in diverse areas: from the antimicrobial agent role in medical field to the anti-cancer agent's feature (Morley et al., 2007; Cohen et al., 2007).

4. Conclusion

Herein, we synthesized three microbial AgNPs and analyzed characterization by UV–Vis spectroscopy analysis confirmed the synthesis of nanoparticles. The SEM and ZT analysis showed that the particle size varied between ~41 and ~63 nm as well the face cubic centered structure of the nanoparticles. The synthesized AgNPs exhibited significant antibacterial activity against pathogenic bacteria and also high toxicity on MCF-7 cells findings its application as potential antimicrobial and anticancer agent.

As a result, AgNPs microbial synthesis' ability of being an alternative for AgNP synthesized by chemical and physical methods and the potential antimicrobial activity's demonstration can make an important contribution to scientific knowledge in this field. According to the present chemical and physical methods AgNP might also contribute to the various industrial branches providing easier supply, high efficiency, better quality, ability to remain stable for a long time and less costly obtaining process. Thus, it may be possible to reduce the toxic effect of silver by byosynthesis from microorganisms. Being indispensable in the pharmaceutical field, is quite important in the development of new medicines. Plus, production through biological methods instead of chemical ways, which cause major pollution, may lead to significant gains for the environment.

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Conflict of interest

The authors declare no conflict of interest.

Ethical issue

Not applicable.

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