Araştırma Makalesi – Original Article

Investigation of *in-vitro* biological activities of silver nanoparticles synthesized by green synthesis method using wild edible mushroom *Macrolepiota procera*[*]

Yenilebilir mantar *Macrolepiota procera* kullanılarak yeşil sentez yöntemiyle sentezlenen gümüş nanopartiküllerinin *in-vitro* biyolojik aktivitelerinin araştırılması

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ABSTRACT

Aim: Mushrooms known that to be used in traditional treatment among the people as they can generate a large diversity of secondary metabolites. In the present study, it was aimed to synthesized silver nanoparticles (AgNPs) mediated *Macrolepiota procera*, which is known to have diverse biological activities such as anticancer, antioxidant, antimicrobial in previous studies, and to investigated various in-vitro biological activities of these AgNPs.

Materials and Methods: Synthesized Mp-AgNPs were characterized using transmission electron microscopy (TEM). Various biological activities including antimicrobial, biofilm inhibition and cell viability inhibition, DNA cleavage, DPPH activity of synthesized AgNPs were investigated *in-vitro*.

Results: The highest DPPH scavenging activity of Mp-AgNPs was found as 92.72%, at 200 mg/L concentration. Mp-AgNPs caused single strain break in the *E. coli* pBR322 plasmid DNA. Mp-AgNPs showed moderate antimicrobial activity against tested microorganisms. Furthermore, the biofilm inhibition activity of Mp-AgNPs toward *P. aeruginosa* and *S. aureus* was 65.80% and 72.60% at 200 mg/L, respectively. Inhibition activity of *E. coli* cell viability of Mp-AgNPs was found as 99.99% at 500 mg/L.

Conclusion: From the findings obtained in the study , the use of *M. procera* in the AgNPs synthesis it is important private regard due to its low cost, eco-friendly, high yield and non-toxicity human health. In addition, newly synthesized AgNPs can be used effectively for different applications after further studies.

Keywords: Nanoparticles, Antioxidant, Antimicrobial activity, Biofilm inhibition, Microbial cell viability.

ÖZ

Amaç: Mantarlar, çok çeşitli sekonder metabolitler üretebilmeleri nedeniyle halk arasında geleneksel tedavide kullanıldıkları bilinmektedir. Bu çalışmada, daha önce yapılan çalışmalarda anti-kanser, antioksidan, antimikrobiyal gibi çeşitli biyolojik aktiviteleri olduğu bildirilen gümüş nanopartiküllerin (AgNP'ler) *Macrolepiota procera* aracılı sentezlenmesi ve bu AgNP'lerin çeşitli *in-vitro* biyolojik aktivitelerinin araştırılması amaçlanmıştır.

Yöntem: Sentezlenen Mp-AgNP'ler, transmisyon elektron mikroskobu (TEM) kullanılarak karakterize edildi. Sentezlenen AgNP'lerin antimikrobiyal, biyofilm inhibisyonu ve hücre canlılığı inhibisyonu, DNA bölünmesi, DPPH aktivitesi gibi çeşitli biyolojik aktiviteleri *in-vitro* olarak incelenmiştir.

Bulgular: Mp-AgNP'lerin en yüksek DPPH süpürme aktivitesi 200 mg/L konsantrasyonda %92.72 olarak tespit edildi. Mp-AgNP'ler, *E. coli* pBR322 plazmid DNA'sında tek zincir kırığına neden oldu. Mp-AgNP'ler, test edilen mikroorganizmalara karşı orta düzeyde antimikrobiyal aktivite gösterdi. Ayrıca, Mp-AgNP'lerin *P. aeruginosa* ve *S. aureus*'a karşı biyofilm inhibisyon aktivitesi, 200 mg/L'de sırasıyla %65.80 ve %72.60 idi. Mp-AgNP'lerin *E. coli* hücre canlılığının inhibisyon aktivitesi, 500 mg/L'de %99,99 olarak tespit edildi.

Sonuç: Çalışmada elde edilen bulgulara göre, *M. procera*'nın AgNP sentezinde kullanılması, düşük maliyetli, çevre dostu, yüksek verime sahip olması ve insan sağlığına toksik olmaması nedeniyle özel önem taşımaktadır. Ayrıca yeni sentezlenen AgNP'ler yapılacak ileri çalışmalardan sonra farklı uygulamalar için etkin bir şekilde kullanılabilirler.

Anahtar Kelimeler: Nanopartikül, Antioksidan, Antimikrobiyal aktivite, Biyofilm inhibisyonu, Mikrobiyal hücre canlılığı.

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Introduction

Nanotechnology is a scientific, technological and engineering branch that has become a major field worldwide in recent years to study the application of tiny things between 1-100 nm. Nanotechnology is a branch the study of nanoscience which indicates various implementation in many area including biology, physics, and chemistry.[1] In recent years, studies on metallic NPs, which are an indispensable part of nanotechnology, are progressing rapidly. NPs show the significant physical and chemical features when compared to their bulk form, which is referred to their high surface area/volume ratio as well as electronic features.[2]

Nanoparticles are mainly synthesized via three approaches: physical method, chemical method, and biological method.[3] An alternative method is needed due to the use of toxic and very precious chemicals in addition to high temperature in the synthesis phase of NPs by chemical and physical methods.[4] The green synthesis method of NPs worths special regard owing to its low cost, eco-friendly, high yield and non-toxicity.[3,4]

Among the metal NPs, AgNPs one of the most notable nanomaterial specially due to their biological features. In numerous studies by different researchers demonstrated the antibacterial, antifungal, anticancer, antimutagenic action, antiviral, antioxidant, and the wound healing properties of AgNPs.[5-11]

For thousands of years, people have used mushrooms for the treatment of various diseases.[12] Moreover, though mushrooms are regarded as nutrients for the first time, it has been detected by many studies that they have medicinal features including antioxidant, antimicrobial, immune modulation and antitumor properties so in folkloric-medicine some of them have been used as drug.[12-14] It has been reported in studies that the medicinal properties of edible mushrooms have been attributed to the high polysaccharides they contain, especially β-glucans.[12] In addition, according to research, in relation to β-glucans found in edible mushrooms are believed to strengthen the immune system by influencing cellular activities and secondary production of chemical compounds, and to repair diseases and to restore damaged cellular immunity caused by radiation and chemotherapy.[13] Also, mushrooms can be utilized for therapeutic aims due to they can generate various secondary metabolites including steroids, terpenoids, organic acids, phenolic compounds, and alkaloids.[15]

M. procera includes lepiotan, mannitol, trehalose, glucose, glycerol, and almost 20 amino acids. Since *M. procera* has been noticed that to indicate antitumor activity to human body and show an antimicrobial activity, the fruiting bodies have been broadly utilized to fabrication conventional foods and medicine.[14]

Considering the above information, in our study, we used *Macrolepiota procera*, an edible fungus species, as a stabilizing and reducing agent in the production of AgNPs. The synthesized NPs were characterized by TEM. Moreover, we aimed to investigate various *in-vitro* biological activities including antimicrobial, antioxidant, and DNA cleavage activities of synthesized AgNPs.

Material and Methods

Materials

Silver nitrate ($AgNO_3$) to be used in the synthesis of silver nanoparticle was obtained from Merck. All used chemicals were of analytical reagent grade. The distilled water (DW) used in the study was obtained from Millipore Direct-Q 3 UV.

Extraction procedure

Washed mushroom material was dried in oven set at 25 °C for five days at laboratory condition and produced a fine powder with an electric grinder. Later, 5 g of powdered dried mushroom *Macrolepiota procera* was soaked in 100 mL of methanol at room temperature for 48 h. It was then filtered using by Whatman filter paper. Then, they were kept in an oven set at 65°C for three days to remove methanol from the filtered samples.

Green synthesis of AgNPs

To obtain AgNPs using *Macrolepiota procera* metanol extract, silver nitrate solution was prepared at 10 mM.[16] Then, 300 mL of the prepared solutions was added to 100 mL of *Macrolepiota procera* metanol extract and left to react at 70 °C overnight. Finally, the prepared AgNPs were washed a few times with distilled water and left to dry in an oven set at 80 °C for 24 h.

Nanoparticles characterization methods

The morphological and topographical analysis of synthesized MP-AgNPs were performed via TEM. The information about the device used for characterization was Jeol JEM-1011.

DPPH activity

The DPPH scavenging activity of Mp-AgNPs was studied as reported by Ağırtaş with some modifications.[17] Briefly, 250 µL AgNPs in concentrations range of 12.5–100 mg/L (12.5, 25, 50, 100, and 200 mg/L) and standards (Ascorbic acid and Trolox) were taken and 1 mL of newly prepared 1 mM DPPH methanol solution was added. Later, this mixture was vortexed thoroughly and incubated in dark place for 30 min. After 30 min, the DPPH free radical scavenging activity was detected at 517 nm by a spectrophotometer. The radical scavenging activity was calculated using the following equation. (1):

$$Capacity\ (\%) = \left(\frac{Abs(control) - Abs(sample)}{Abs(control)} \right) \times 100 \quad (1)$$

DNA cleavage ability

DNA cleavage study was realized to test the effect of AgNPs synthesized from *Macrolepiota procera* on DNA, and *E. coli* pBR 322 plasmid DNA was used as a model target substance for this aim. The experiment was based on the principle of treating NPs at varied concentrations range from 50-200 mg/L with plasmid DNA for 45 min at 37 °C. Then, to observe the effect of newly synthesized Mp-AgNPs on DNA, the reaction mixture was loaded to the gel and agarose gel electrophoresis was performed at 90 min, 80 V, and 120 mA.

Antimicrobial activity

Minimum inhibitory concentration (MIC) of synthesized AgNP was researched against Gr (+), Gr (–) and fungal strain using serial dilution method. The strains used for this purpose were as follows; *Staphylococcus aureus, Enterococcus faecalis*, *Enterococcus hirae*, *Escherichia coli*, *Legionella pneumophila* subsp. *pneumophila*, and *Pseudomonas aeruginosa*, *Candida tropicalis*, and *Candida parapisilosis*. The microorganisms used in our research were grown overnight before serial dilution. For the work, two-fold serial dilutions of Mp-AgNP were made. Then, the microorganisms we used in our study were inoculated to the microplate wells. Subsequent plates were kept in the oven for incubation at 37 °C for 24 h. After 24 h, antimicrobial activity was evaluated with MIC, described as the lowest concentration that inhibits microbial growth.

Bacterial cell viability activity

E. coli bacteria was used to microbial cell viability procedure. First, the bacterium was inoculated into Nutrient Broth (NB) medium. It was then incubated at 37 °C on a shaker for 24 h. When the incubation time was over, the growth medium was centrifuged to collect *E. coli* cells. Afterwards, sterile saline solution was added to clean the bacterial pellet and with centrifugation was washed several times. When washing step was finished, *E. coli* strains were suspended with sterile NaCl. This prepared suspension was used in the cell viability method afterwards. *E. coli* was mixed homogenously with different concentration (range from 125-500 mg/L) of Mp-AgNPs for 60 min at 37 °C. Later 60 min, it was diluted in different ratios and inoculated on NB agar medium and incubated at 37 °C for 24 hours. The same procedure was also studied with the control group that did not contain green synthesized AgNPs. Eventually, microbial cell viability was calculated by counting colonies and using equation (2) given below.

$$Cell\ viability\ (\%) = (A_{control} - A_{sample} / A_{control}) \times 100 \quad (2)$$

Biofilm inhibition activity

The two microorganisms were selected Gr (+) and Gr (-) (*P. aeruginosa* and *S. aureus*), to reveal the biofilm inhibition activity of newly synthesized AgNPs. Well plates containing different concentrations of AgNPs were inoculated with *P. aeruginosa* and *S. aureus* and left to incubate at 37 °C for 72 h. After 72 h, the well plates were emptied slowly and washed twice with distilled water. The plates were kept in an oven set at 70 °C for 20 min to dry. Then, crystal violet (CV) dye was added to the wells and left for 30 min. At the end of the time, the CV was poured and the contents of the wells were gently washed two times. Subsequently, ethanol was then added. It was waited for 15 min for the absorbed CV to recover the CV. The biofilm inhibition activity was defined with spectrophotometer at 595 nm. Wells with only bacteria were considered as positive control and the activity of biofilm inhibition was calculated using the equation (3) given below.

$$Biofilm\ Inhibition\,(\%) = \left(\frac{Abs(control) - Abs(sample)}{Abs(control)} \right) \times 100 \quad (3)$$

Result and Discussion

Nanoparticles characterization

TEM was applied to detect Mp-AgNPs' surface morphology. It was determined that the particle size of the Mp-AgNPs obtained as a result of TEM was in the range of 22.67-25.99 nm. Moreover, based on the data obtained from the TEM images, the synthesized AgNPs were in spherical morphology (***Figure 1***).

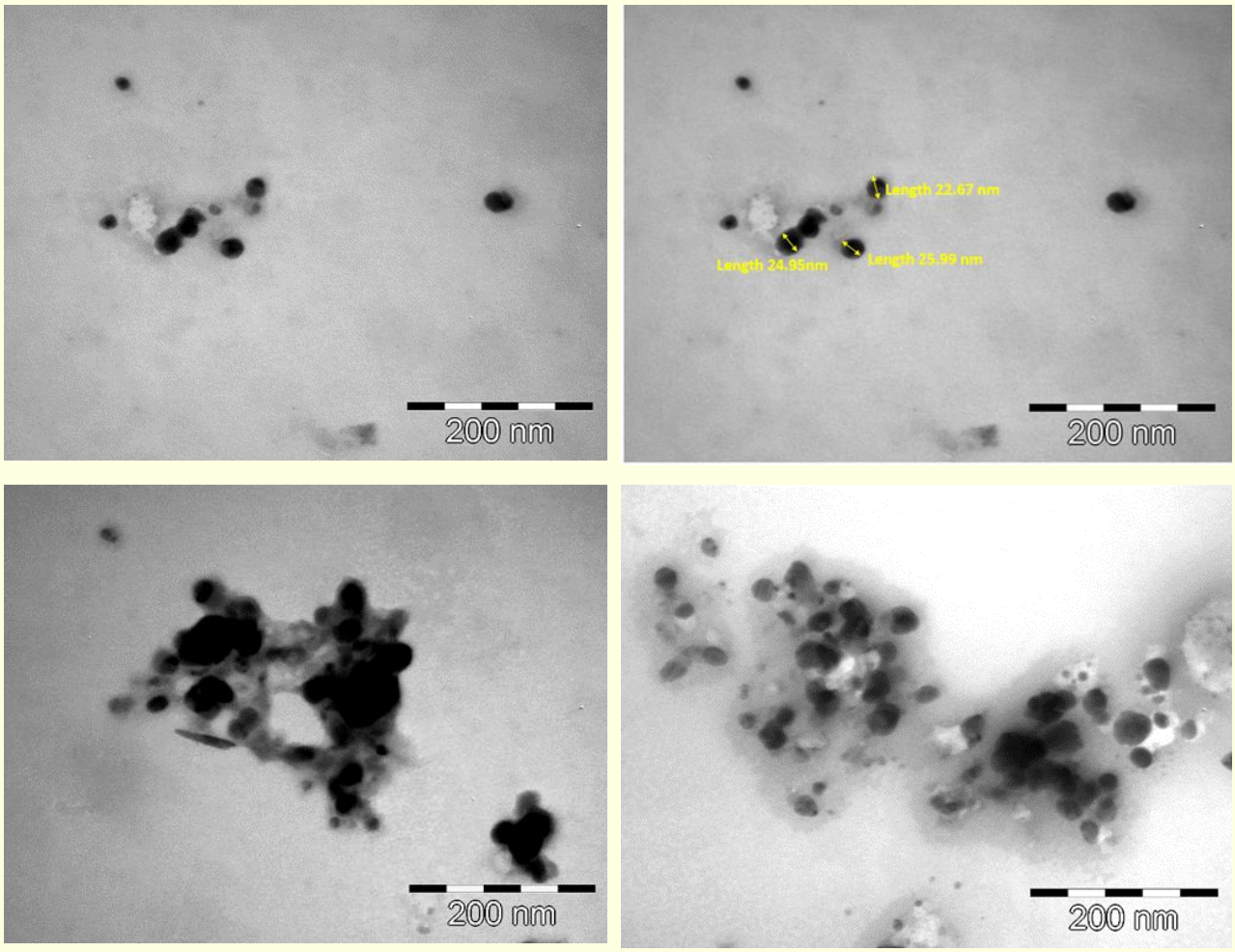

Figure 1. Characterization of AgNPs – TEM image

DPPH activity

An unbalance between the antioxidant and pro-oxidants results in formation of oxidative stress in biological systems with reduce in antioxidant enzymes causing to harm of crucial biomolecules and other cellular components.[18] In addition, ROS, which is released as a result of oxidative stress, contributes to the formation of various diseases including cardiovascular and neurodegenerative diseases. Antioxidants play a protective role in diseases caused by oxidative stress.[19] Therefore, it is important to discover new products that are natural and environmentally friendly with antioxidant properties. In the study, the antioxidant activity of AgNPs synthesized from *Macrolepiota procera* was investigated by DPPH method. The results of the study are presented in ***Figure 2***. As seen in the Figure 2, DPPH activity increased with the increase in concentration as mentioned by other researchers.[20,21] In addition, the DPPH scavenging effect of Mp-AgNPs was compared with Ascorbic acid and Trolox, which were used as standard. According to the results of the study, the DPPH scavenging activity of Mp-AgNPs was found to be 38.64%, 49.30%, 56.83%, 81.80%, and 92.72% at 12.5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L concentrations, respectively. As the concentration increased from 12.5 mg/L to 200 mg/L, the DPPH scavenging activity of Ascorbic acid, Trolox and Mp-AgNPs also increased from 84.12% to 100%, from 86.39% to 100% and from 38.64% to 92.72%, respectively. The efficient free radical scavenging activities of the newly synthesized AgNPs could be due to the combined impact of both AgNPs as well as of the bioactive compounds present in the *Macrolepiota procera* extract. In previous studies with AgNPs, it has been reported that AgNP had antioxidant activity. A study, Palanisamy synthesized silver nanoparticles using *Sargassum polycystum* and evaluated its *in-vitro* antioxidant activity via DPPH method.

They found that AgNPs exhibited the high DPPH radical inhibition with 78.2% at concentration of 500 µg/mL.[22] Ravichandran reported that they synthesized AgNPs using *Atrocarpus altilis* and DPPH radical scavenging activities was 79.79% at 100 µg/mL concentration.[23] This results are lower than the results of present study. Wang synthesized silver NPs using *Psidium guajava* leaf aqueous extract and it was determined that the DPPH scavenging activities was found 83.59% at 100 µg/mL concentration which was compatible with our results.[24] In another study conducted by Khorrami, the newly synthesized AgNPs inhibited 77% of DPPH free radicals.[25] As a result, the antioxidant activity results of the synthesized Mp-AgNPs are quite good and the usage of synthesized Mp-AgNPs as an antioxidant agent will open new opportunities for the improvement of medical treatment after further studies.

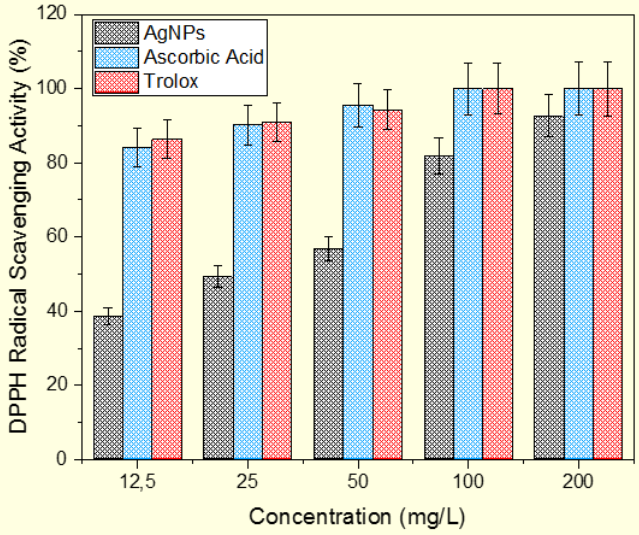

Figure 2. DPPH scavenging ability

DNA cleavage activity

In the presented study, the effect of *Macrolepiota procera* mediated silver nanoparticles on DNA was also examined by the agarose gel electrophoresis method. DNA cleavage activity results are shown in ***Figure 3*** Under normal conditions, *E. coli* plasmid DNA is in circular form and is observed as Form I in the gel, as seen in line 4, and is the fastest migrating form in the gel compared to other forms. As seen in line 1, 2, and 3 a single-strand DNA breaks occurred as a result of the effect with Mp-AgNPs and a transition from Form I to Form II occurred. Different studies were done to define the DNA cleavage activity of silver nanoparticles. Mousavi-Khattat investigated the DNA cleavage activity of AgNPs synthesized by chemical and green synthesis method and ultimately used the genomic DNA extracted from *E. coli* as a target and they reported that it caused DNA damage.[26] Begum synthesized AgNPs by using *Ficus carica* leaf extract and studied DNA cleavage activity of them using as a target extracted pure DNA from *S. aureus*. They finally reported that AgNPs exhibited DNA cleavage ability.[27] Our DNA cleavage activity results showed similarity with the investigations mentioned. In the present study, DNA cleavage efficiency could be regarded as an antimicrobial mechanism for Mp-AgNPs depending upon the results acquired from DNA cleavage activity test. In the pharmaceutical industry, the DNA molecule is the most significant objective molecule in both anticancer and antimicrobial studies. Therefore, molecules that can interact straight with DNA become even more significant in pharmacology as alternative solutions. As it is known, it has been shown in studies that *Macrolepiota procera* has anticancer activity.[14] Due to, we can say that the active compounds in the *Macrolepiota procera* extract will also contribute to the DNA cleavage activity of Mp-AgNPs. As a result, in

the study, the effects of AgNPs synthesized mediated *M. procera* on DNA promise a future in terms of use in anticancer studies and Mp-AgNPs can be applied as DNA nuclease agents after further studies in nanomedicine.

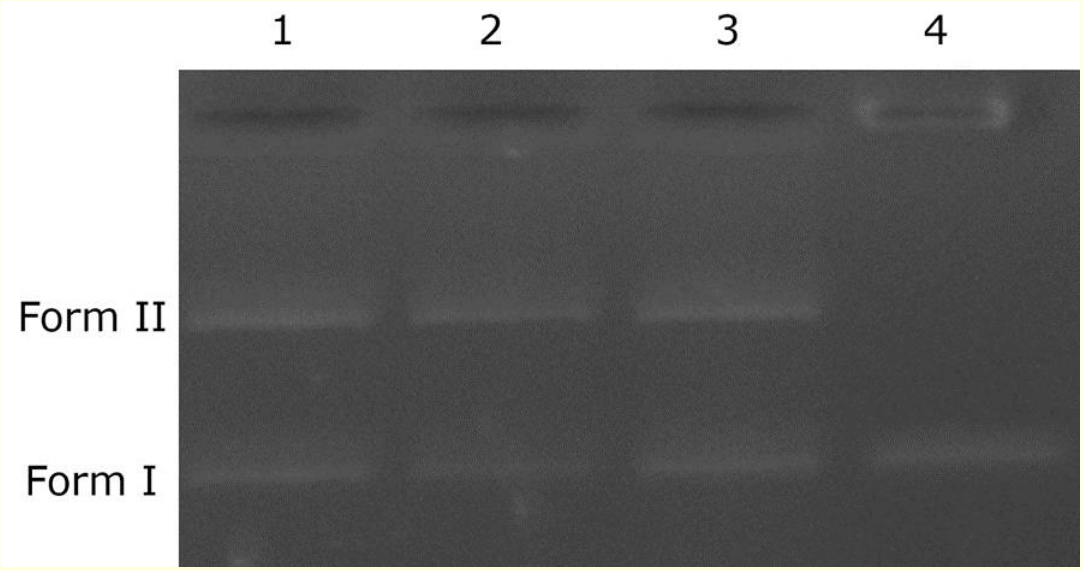

Figure 3. DNA Cleavage activity of Mp-AgNPs. Lane 1, pBR 322 DNA + 50 mg/L Mp-AgNPs; Lane 2, pBR 322 DNA + 100 mg/L of Mp-AgNPs; Lane 3, pBR 322 DNA + 200 mg/L Mp-AgNPs; Lane 4, pBR 322 DNA.

Antimicrobial activity

Biosynthesis methods used to obtain AgNPs are a suitable alternative to reduce the toxic effect of reagents used in chemical synthesis, which is one of the traditional methods.[28] Taking this information into account, in the study the antimicrobial activity of the green synthesized Mp-AgNPs was determined using microdilution method. MIC results of the study are presented in the ***Table 1***. As can be seen in the Table 1, the synthesized Mp-AgNPs showed antimicrobial activity against all studied microbial strains with varying degrees. Accordingly, the MIC value of Mp-AgNPs for was determined as 256 mg/L *E. coli*, *L. pneumophila*, *C. tropicalis*, 128 mg/L for *P. aeruginosa*, *E. hirae*, *E. fecalis*, *C. parapisilosis* and 64 mg/L for *S. aureus*. Moreover, among the strains studied, the strain most susceptible to Mp-AgNPs was *S. aureus* with a MIC value of 64 mg/L. The antibacterial activity of newly synthesized AgNPs may be attributed to the formation of oxidative stress, deterioration in replication of DNA, and also AgNPs can directly reason lysis of bacterial cell via damaging the cell membranes.[29] As mentioned above, newly synthesized AgNPs had also DNA cleavage activity. This results from present study can be caused the antimicrobial activity against studied microorganisms. Antimicrobial activity of AgNPs has also been reported in previous studies. Such as; Morales-Lozoya synthesized AgNPs using different parts of *Morinda citrifolia* and reported that silver nanoparticles demonstrated antibacterial activity toward *S. aureus* and *E. coli*.[28] Sathishkumar reported that the synthesized AgNPs using *Trichodesmium erythraeum* showed antimicrobial activity against various clinical and drug resistant bacterial strains.[30] Sangaonkar and Pawar synthesized AgNPs mediated *Garcinia indica* and indicated that newly synthesized AgNP displayed antibacterial activity against various Gr (+) and Gr (-) bacteria.[31] Mehwish synthesized AgNPs using *Moringa oleifera* and demonstrated its antimicrobial activity against Gram +ve (*S. aureus*) and Gram -ve (*E. coli*, *Salmonella enterica typhimurium*, *P. aeruginosa*) bacteria.[32] In conclusion, in the presented study, Mp-AgNPs showed varying degrees of antibacterial activity against the test microorganisms studied. When these results are combined with other studied parameters, it can be highlighted that the newly synthesized AgNPs can be used as an antimicrobial agent after further studies.

Table 1. The minimum inhibition concentration (MIC) of test microorganisms

Microorganisms	Mp-AgNPs*
E. coli	256
P. aeruginosa	128
L. pneumophila subsp. *pneumophila*	256
E. hirae	128
E. fecalis	128
S. aureus	64
C. parapisilosis	128
C. tropicalis	256

* mg/L

Bacterial cell viability test

In the study, *in-vitro* cell viability inhibition activity of AgNPs synthesized from *Macrolepiota procera* by green synthesis method was also tested against *E. coli*. The results of the study are shown in ***Figure 4***. Cell viability inhibition activity results of Mp-AgNPs at 125 mg/L, 250 mg/L, and 500 mg/L concentrations were as follows; 94.37%, 99.18%, and 99.99%, respectively. It is known that silver Np's show antimicrobial activity through different mechanisms. One of these mechanisms is the effects of AgNPs through ROS production. Under normal conditions, ROS produced in cells can be exterminated by antioxidant compounds (for example glutathione), but in the existence of AgNPs, the expression of antioxidant enzymes is braked. Thus, an increase in the amount of ROS causes some damage including reduced ATP, DNA damage, lipid peroxidation, breathing inhibition, and apoptosis-like response.[33] The Mp-AgNPs showed excellent antibacterial activity against *E. coli* at all studied concentrations. Therefore, NP synthesized after further studies may find use in different fields in nanomedicine.

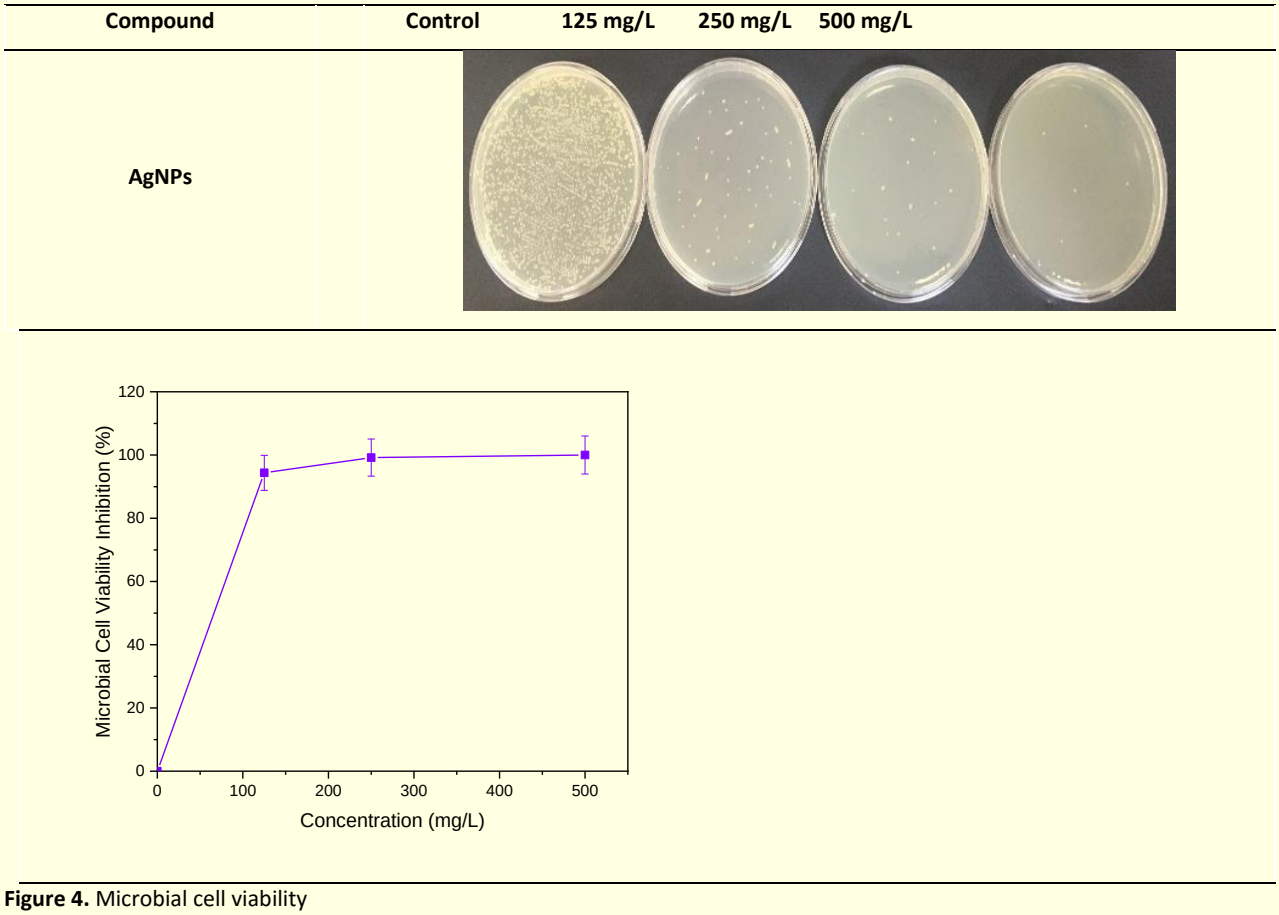

Figure 4. Microbial cell viability

Biofilm inhibition activity

The assessment of the biofilm inhibition action of synthesized AgNPs is beneficial to establish their probable implementations in the therapy of many diseases caused by biofilm formation microorganisms. The biofilm inhibition activity of the green synthesized Mp-AgNPs was evaluated by *in-vitro* method against *S. aureus* and *P. aeruginosa*. The results of the study are given in ***Figure 5***. According to these findings; biofilm inhibition activity of synthesized Mp-AgNPs was determined as 43.18%, 58.51%, 72.60% for *S. aureus* and 40.64%, 48.58%, 65.80% for *P. aeruginosa* at 125 mg/L, 250 mg/L, 500 mg/L concentrations, respectively. Moreover, *S. aureus*, which is one of the strains used in the evaluation of biofilm inhibition activity against NP, was found to be more sensitive than *P. aeruginosa*. In previous studies by different researchers, it has been reported that AgNPs inhibited biofilm formation. Such as; Lara investigated the inhibition effect of AgNPs on biofilm formation of *Candida auris* after treating silicone elastomer and bandage fibers with silver NP. As a result, they reported that silicone elastomers functionalized with silver NPs demonstrated with >50% biofilm inhibition and bandage dressings loaded with silver NPs inhibited growth of *C. auris* biofilms formation by more than 80%.[34] In another study, Singh reported that synthesized AgNPs using *Cannabis sativa* inhibited biofilm formation such bacteria including *P. aeruginosa* and *E. coli*.[35] The results of this research suggest that the newly synthesized AgNPs using *Macrolepiota procera* extract could be considered as alternating antimicrobial compounds useful to create new pharmaceutical products.

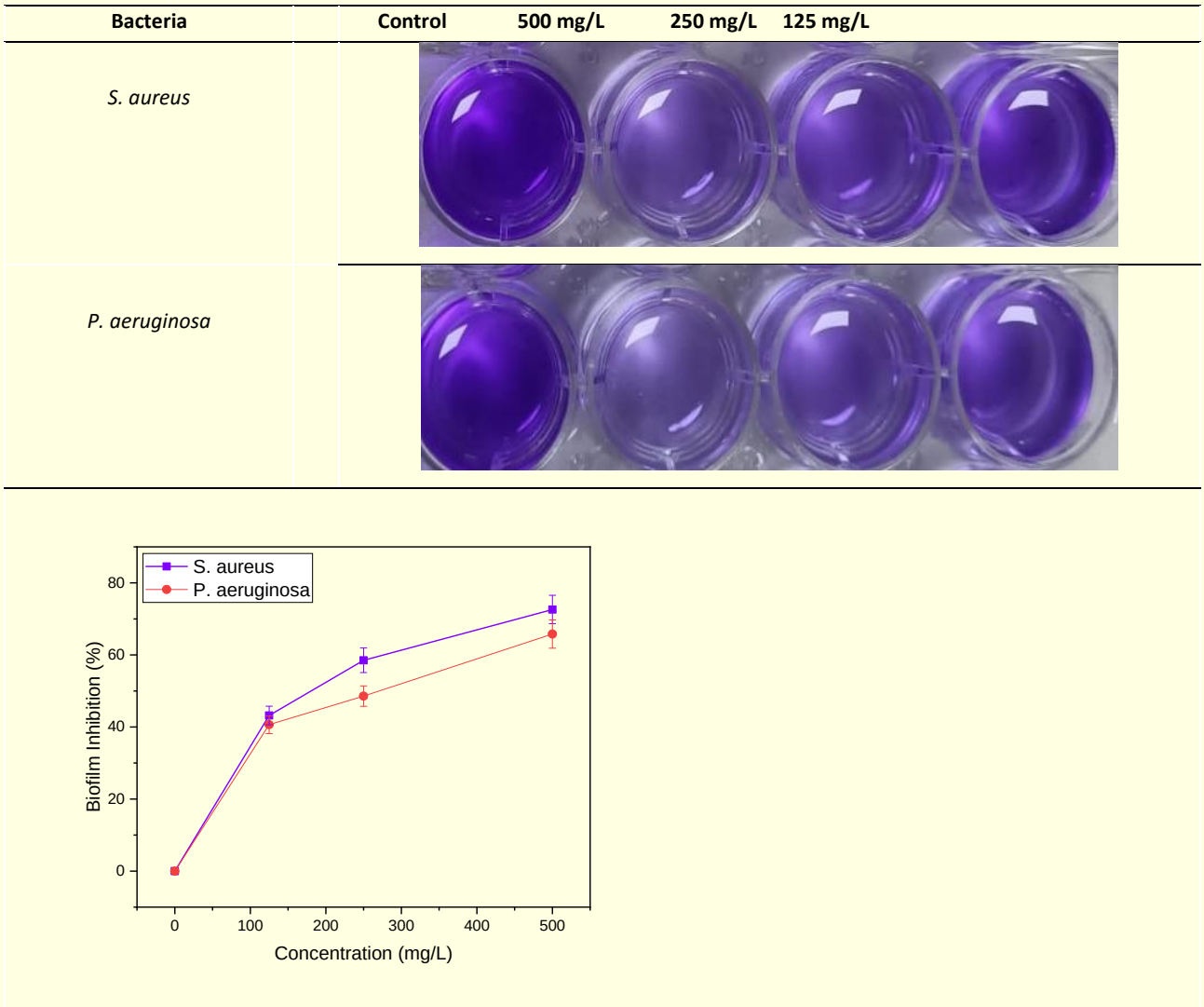

Figure 5. Biofilm Inhibition of *S. aureus* and *P. aeruginosa*

Conclusion

Nanotechnology is the most considerable area for developing new medical implementations. The use of *Macrolepiota procera* for AgNPs synthesis would let for lower toxicity and greater biocompatibility, than those synthesized by other methods (such as chemical methods), which makes them a promising alternative in various applications including bio-indicators, sensing, nanomedicine growth and targeted drug deliver. Moreover, in the potential biological activity of the synthesized AgNPs can be contribute to the presence of the polysaccharides and secondary metabolites the surface of the AgNPs which is present in *Macrolepiota procera*. The present study is very important as it sheds light upon the *in-vitro* biological activities of Mp-AgNPs synthesized by the green synthesis method. Moreover, the fact that the synthesized NPs showed good biological activities such as 92.72% DPPH scavenging activity, biofilm inhibition activity 72.60% for *S.aureus* and 65.80% for *P. aeruginosa*, and showed 99% cell viability inhibition activity is another important part of the study. According to the present study, it has been determined that the use of *Macrolepiota procera* is important in the synthesis of silver nanoparticles. As a results, this molecule is promising, further *in-vivo* examination, pre-clinical and clinical trials are required in this respect.

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