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Research Article
Characterization of Biogenic Silver Nanoparticles by *Salvadora persica* Leaves Extract and its Application Against Some MDR Pathogens *E. coli* and *S. aureus*

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Abstract
Background: Now a days, the multidisciplinary scientific research conception in the field of nanotechnology has witnessed development with regard to the numerous applications and synthesis of nanomaterials. **Objective:** The current investigation has been conducted with the main focus on the green synthesis of silver nanoparticles from the leaves of *Salvadora persica* and its antibacterial activity against MDR pathogens *E. coli* and *S. aureus*. **Methodology:** Silver nanoparticles (AgNPs) were prepared after addition of aqueous extract of *Salvadora persica* leaves. The UV-Vis spectrophotometer, Transmission Electron Microscopy (TEM), zeta potential and Scanning Electron Microscopy (SEM) were employed to detect the particle size and morphology, besides Fourier transform infra-red spectrometer (FTIR) analysis was performed to determine the capping and stabilizing agents in the extract. Antibacterial assay for the biogenic AgNPs was conducted against *E. coli* and *S. aureus*. **Results:** Color change of the mixture from yellow to dark brown is the first indication to AgNPs formation. Furthermore, 420 nm was the peak value for UV-Vis spectroscopy absorption of the mixture. Besides, TEM and SEM micrographs showed wide variability in the diameter of smaller NPs aggregated together with spherical shapes and zeta sizer showed about 153.3 nm as an average size of nanoparticles. Microbial suppression was noticed for the tested microorganisms. Furthermore, with the help of FTIR analysis the biomolecules that act as capping and stabilizing agents of AgNPs are proteins and phenols present in the plant extract. **Conclusion:** *Salvadora persica* leaves extract act as a reducing and stabilizing agent for the synthesis of AgNPs keeping its ability to suppress the MDR pathogen.

Key words: Green synthesis, AgNPs, SEM, TEM, FTIR, MDR pathogen, *Salvadora persica*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Resistance of human pathogens to antibiotics has become an immense challenge in the field of pharmaceutical research. The ability of bacteria to develop resistance to antimicrobial agents such as Multi Drug Resistant (MDR) pathogens has arisen from the treatment of bacterial infection[1]. For the treatment of these pathogens a wide-spectrum of antibiotics is used, which is expensive and less effective[2]. Therefore, seeking adequate antimicrobial compounds with bactericidal potential is the highest apriority in the modern research area due to the development of microbial resistance to antibiotics and metal ions[3]. From the applied bioscience and nanotechnology area the nanobiotechnology studies were developed focusing on the synthesis and handling of the particles with smaller size of about[4] 100 nm. The components of any nanotechnology are the nanoparticles that possess unique and distinctive characteristics due to their size, distribution and morphology[5]. Conveniences of the metal nanoperticles in medicine, biology, material science, physics and chemistry have recently gained them much attention and concern from the scientific community due to their antibacterial activity which is related to their large surface area to volume ratio[3,6]. Among such metal nanoparticles silver nanoparticle (AgNPs) is generally considered the best regarding its antimicrobial abilities against bacteria, viruses and eukaryotic microorganisms through the disturbance of the unicellular membrane and therefore, interrupt their enzymatic activities[7,8]. Moreover, DNA cells are likely the superior part for binding AgNPs since Ag has high affinity to react with phosphorus-containing biomolecules[5]. Further biomedical applications for AgNPs are now a days adopted successfully, among these applications is the usage of AgNPs as an important tool in cancer therapy due to its integration with biological molecules, being added to wound dressing, topical creams and antiseptic spray and fabrics[7,9]. Nanoparticles can be prepared by different chemical and physical methods which might be a main reason for various biological hazards since the use of toxic chemical is involved. The green synthesis of AgNPs as a biological method is the best method for synthesis since they have slower kinetics and provide a good handling, control and stabilization[9]. Different biological methods for the synthesis of AgNPs which are ecofriendly, cost effective easily and scaled up for large scale synthesis of nanoparticles, were applied including plant extract, microorganisms and enzymes for the synthesis of AgNPs[7,10-14]. Less biohazard and easiness to maintain cell cultures are the main advantages for using plant extract over the microorganism in the biosynthesis of AgNPs[15]. Plant phenolic compounds, flavonoids, alkaloids and terpenoids are the mainly metabolites constantly involved in redox reactions that are responsible for the conversion of metal ions to metal nanoparticles[5,16]. Approximately 2-4 h are the time needed for the conversion of 90% of Ag ions to AgNPs[17]. In a recent study Abubacker and Sathya[18] used *Salvadora persica* chewing sticks for the synthesis of AgNPs but using *Salvadora persica* green leaves is not reported so far. Therefore, the current investigation is an attempt to find out the ability of *Salvadora persica* leaves extract as a reducing and stabilizing agent in silver nanoparticles formation in order to study the antibacterial ability against MDR pathogens. The miswak (*Salvadora persica* L.) is a medium size shrub belonging to family Salvadoraceae, commonly found in India, Africa, Saudi Arabia, Iran, Israel and Pakistan. Glycosides, proteins, saponins, tannins, phenolic compounds, flavonoids, alkaloids, steroids and vitamin C were well documented for the aqueous extracts of *Salvadora persica*, miswak[19]. Benzyl nitrile, isotymol, thymoleugenol β-caryophyllene eucalyptol isoterpinolene are the *Salvadora persica* secondary metabolities reported from GC-MS analysis[20].

MATERIALS AND METHODS

Materials: Arak, *Salvadora persica* green leaves were collected from Jazan region, Saudi Arabia. Silver nitrate ($AgNO_3$) and nutrient agar media were purchased from Wateenalhyaa company (Riyadh, Saudi Arabia) for the antibacterial assays.

Biogenic synthesis of silver nanoparticles (AgNPs): The amount of 10 g powder of *Salvadora persica* leaves was mixed with 100 mL of highly purified water of aqueous extract preparation. For enzyme denaturation the mixture was heated for 10 min at 80°C. To obtain pure extract, the mixture was filtered twice via a Whatman filter paper (pore size 125 μm) and (pore size 25 μm). Furthermore, 10 mL of aqueous extracts was added to 90 mL of a 1 mM $AgNO_3$ solution in an Erlenmeyer flask at room temperature. The mixture was stored under dark conditions for 24 h after that kept at 4°C until further analysis[11].

Characterization of AgNPs: Monitoring of conversion of silver ions to AgNPs in the mixture was detected by means of a spectrophotometer with the aid of Ultraviolet-visible spectrum, UV 2450 double-beam spectrophotometer (Shimadzu, Tokyo, Japan) at a resolution of 2 nm in the range[19] from 400-500 nm.

Transmission Electron Microscopy (TEM): Transmittance through an ultra-thin specimen of an electron beam is the concept of using TEM for determination of AgNPs size and shape. The JEOL microscope (JEM-1011) at 80 kV voltage was used. A drop of AgNPs was properly dried and placed on the carbon-coated copper TEM grids then loaded on the sample holder.

Scanning Electron Microscopy (SEM): For detection of morphology, surface properties, shape and dispersion of biogenic AgNPs, SEM technique (Quanta 250, FEI) was applied. Images were obtained at 10 kV.

Dynamic Light Scattering (DLS) and zeta potential measurements: Zetasizer, nano series, HT laser, ZEN 3600 (Malvern Instruments Ltd., Malvern, UK) was used to detect the Dynamic Light Scattering (DLS) and zeta potential measurements for the biogenic AgNPs. Measurements were performed at a wide range between 0.1 and 10,000 nm.

Fourier Transform Infrared Spectroscopy (FTIR): FTIR (Shimadzu FT-IR Prestiage 21) analysis in the range of 500-5000 cm^{-1} was used for the biogenic AgNPs. At diffuse reflectance mode the possible biomolecules that act as reducing, capping and stabilizing agents for the AgNPs were recorded using FTIR spectrophotometer.

Antibiotic susceptibility test: To study the antibiotic susceptibility test, antibiotic discs such as norfloxacin (NF), pefloxacin (PF), ciprofloxacin (CI) were used against *E. coli* and ciprofloxacin (CI), penicillin-G (PG), chloramphenicol (CK) discs were used against *S. aureus*. The tested bacterial cultures were sub-cultured and incubated at 37°C for 7-8 h. Thereafter, pathogenic bacteria was swabbed on nutrient agar and antibiotic disc was used and incubated at 37°C for 24 h. Results were recorded as an inhibition zone (mm).

Evaluation of antibacterial activity of AgNPs: Antimicrobial ability of the biosynthesized AgNPs was detected against MDR pathogens, Gram-negative *E. coli* and Gram-positive *S. aureus* using agar well diffusion methods. Clinical microbes were obtained from the Biology Laboratory, Faculty of Science, PNU. Stock cultures of *E. coli* and *S. aureus* were prepared and sub-cultured in a nutrient agar media for 24 h at 37°C. Sterile swabs were used for each strain to be swabbed uniformly onto individual agar plates. Consequently, three spaced wells of 4 mm diameter were made per plate using sterile metal cup borer. In each well, 0.2 mL of the biosynthesized AgNPs extract and $AgNO_3$ were put under aseptic conditions, kept at room temperature for 1 h to allow the extracts to diffuse into agar medium and incubated accordingly. Sterile distilled water was used as the reference negative control. All the plates were incubated at 37°C for 24 h. The plates were examined for sign of inhibition zone, which appears as a clear space around the wells. The diameter of inhibition zones were measured using a ruler. Experiments were performed in three replicates and mean values were calculated.

Minimum bactericidal concentrations (MBCs): Broth dilution method was used to determine the Minimum Bactericidal Concentrations (MBCs) of the biogenic AgNPs against the tested bacterial strains, namely, *E. coli* and *S. aureus*. Different concentrations (1000, 500, 250 and 125 $\mu g\ mL^{-1}$) of AgNPs were used to determine the lowest concentration required for inhibiting the bacterial growth. Concentrations of 5×10^5 CFU mL^{-1} from the live cells of each bacteria were inoculated with the different AgNPs concentrations for 24 h. Three replications were applied.

Statistical analysis: All results were computed and expressed as Mean$\pm$Standard Deviation (SD) from triplicates. Statistical analysis was performed using JMP software (version 18.0) with analysis of variance (One-way ANOVA).

RESULTS AND DISCUSSION

The present results in this study showed the green synthesis of AgNPs using *Salvadora persica* leaves extract as capping and stabilizing agents. For confirmation of NPs development different techniques were carried out as indicated.

Biogenic synthesis of silver nanoparticles (AgNPs): Following the incubation of the mixture of *Salvadora persica* leaves extract and 1 mM $AgNO_3$ for 24 h, the color of the mixture was changed from pale yellow to dark brown which is a clear indication of AgNPs formation. The distinct color in the mixture was due to the excitation of surface plasmon vibrations and the minute dimensions of silver nanoparticles[11,21,22]. Furthermore, it was observed that the intensity of the brown color increased as the incubation period increased. This observation is in well agreement with Singh *et al.*[23].

Characterization of AgNPs: The U-Vis spectrophotometer showed a maximum absorbance peak at 410 nm for the synthesized AgNPs which is mostly related to plasmon

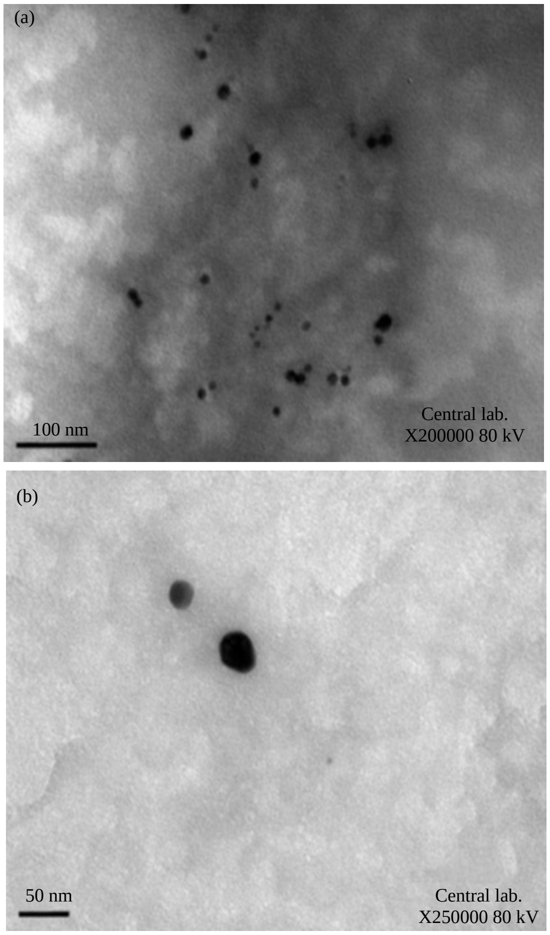

Fig. 1(a-b): TEM images of the biogenically synthesized AgNPs prepared from the *Salvadora persica* leaves extract at 100 and 50 nm scales

resonance of silver nanoparticles. The SPR absorption band is related to the metal nanoparticles free electrons as a result of the combined vibration of electrons in resonance with light wave[22].

Transmission Electron Microscopy (TEM): TEM image (Fig. 1) shows that the morphology of biogenic AgNPs is almost spherical in shape with 50 nm average size. Several studies have reported spherical shape for the biosynthesized silver nanoparticles ranging from 20-100 nm when TEM images were taken[23-25]. It is quite obvious from the image that the AgNPs were capped with phyto constituents of *Salvadora persica* leaves extract. Similar observations were also detected when *Senna siamea* seeds and *Cassia javanica* where used for AgNPs[24,26].

Scanning Electron Microscopy (SEM): White and spherical particles was observed from SEM analysis (Fig. 2). Small size aggregated nanoparticles were clear from the image (Fig. 2). Same observation was also recorded by Tahir *et al.*[27], when using *Salvadora persica* stem extract for AgNPs preparation.

Dynamic Light Scattering (DLS) and Zeta potential measurements: Dynamic light scattering measurement is a technique used to determine the particle size of the synthesized AgNPs. Size distribution of the biogenic AgNPs is represented in Fig. 3. It was observed that the particles sizes obtained were in the range 50-300 nm. The average size of the biogenic AgNPs using *Salvadora persica* leaves extract is around 153 nm. Same trend of observation was also detected by Umoren *et al.*[28] when he used red apple (*Malus domestica*) fruit extract for AgNPs preparation. Furthermore, to recognize the state of the AgNPs surface and to expect its stability, zeta potential was applied. Zeta potential of -22 mV was detected showing stable AgNPs in the solution since NPs with zeta potential greater than 20 mV or less than -20 mV were stated to remain stable in solution[29]. Furthermore, negative zeta potential values might be due to the potential capping ability of phytoconstituents present in the extract[30]. Zeta potential of -22.9 mV was achieved[26] when the extract of *Senna siamea* plant seed was used as biomediator in AgNPs formation where negative zeta potential has been speculated to be the main factors leading to biogenic AgNPs stability.

Fourier transform infrared spectroscopy (FTIR): Detecting of the bioactive compounds, which might be responsible for synthesis and stabilization of AgNPs is the goal of using FTIR spectra for the NPs synthesized using *Salvadora persica* leaves extract. The presence of two bands located at 1634.20 and 3283.62 cm^{-1} (Fig. 4) might be due to the amide 1 band protein since absorbance between 1600 and 1700 cm^{-1} might indicate the amide 1 vibration for proteins which is most likely due to the C=O stretching vibration of the amide groups coupled with little in-plane NH bending. Furthermore, the absorption peak at 1634.20 cm^{-1} is close to that reported for native proteins[31]. On the other hand, FTIR bands appeared at 3283.62 cm^{-1} might be due to indole NH[32]. In a recent study by Kumar *et al.*[33] it was suggested that the FTIR peak at 3283 cm^{-1} is corresponding to the bounded hydroxyl (-OH) or amine groups. The findings from the current study confirmed the presence of proteins in the biogenic AgNPs solution that may act as reducing and stabilizing agents for the nanoparticles. Strong affinity to bind metal ions is accessible by the phytoconstituents from which carbonyl groups of amino acids and peptides of proteins which may encapsulate nanoparticles leading to their stabilization and prevent them from aggregation in the medium[26,34].

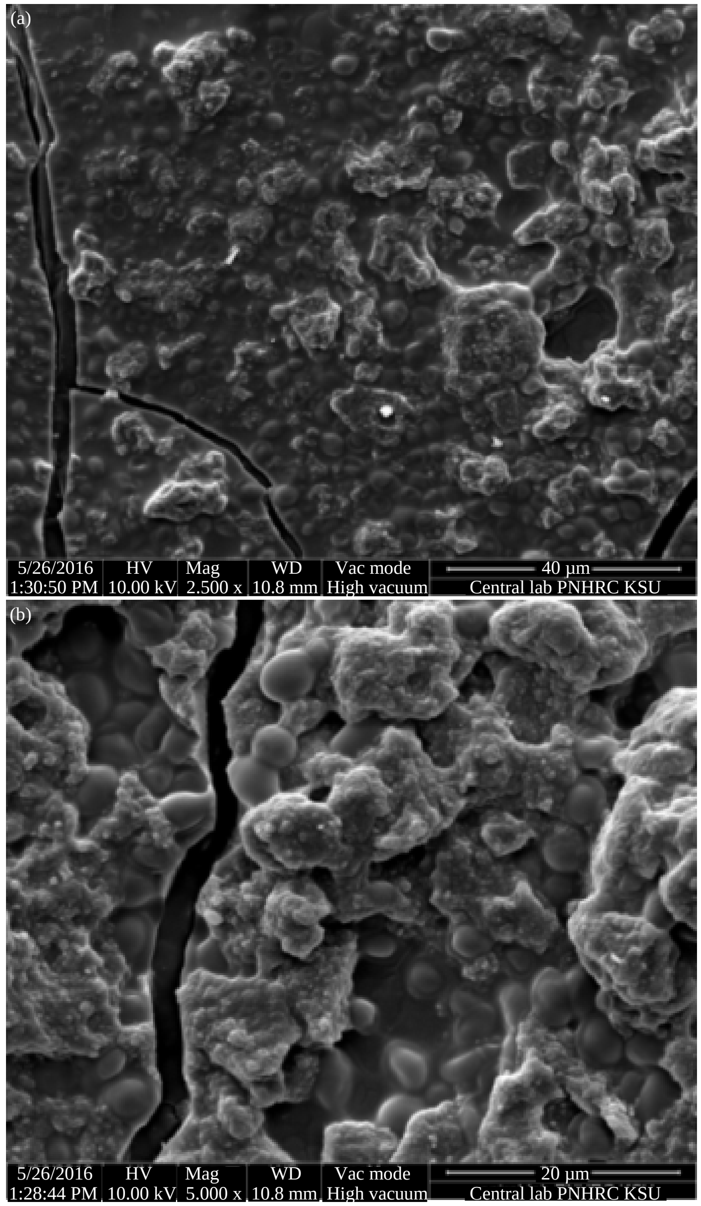

Fig. 2(a-b): SEM images of the biogenically synthesized AgNPs prepared from the *Salvadora persica* leaves extract at 2500 and 5000 magnification

Antibiotic susceptibility test: Antibiotic susceptibility test was accomplished using discs of different antibiotic for both *E. coli* and *S. aureus*. The *E. coli* showed resistance for all tested antibiotics whereas *S. aureus* showed sensitivity against chloramphenicol (CK) with an inhibition zone of 22 mm. A similar effect of antibiotics was also detected by Singh *et al.*[23] against *E. coli* and *S. aureus*, only chloramphenicol (CK) showed sensitivity against *S. aureus* with an inhibition zone of 18 mm.

Evaluation of antibacterial activity of AgNPs: Antimicrobial ability of the biosynthesized AgNPs was detected against clinical isolate of the MDR pathogens, *E. coli* and *S. aureus* using agar well diffusion methods with different concentrations of 25, 50, 75 and 100 μL AgNPs. Results from this study showed that AgNPs were effective against the tested bacterial species (Table 1). The higher the concentration applied the higher inhibition zone detected for both tested MDR pathogens (Table 1). The AgNPs with 100 μL were more effective against MDR *S. aureus* strain (21 mm) when compared with MDR *E. coli* (18 mm) at the same concentration (Table 1). Same observation was also recorded by Paredes *et al.*[35] when AgNPs were prepared using cysteine and cetyl-tri-methyl-ammonium bromide as a reducing and

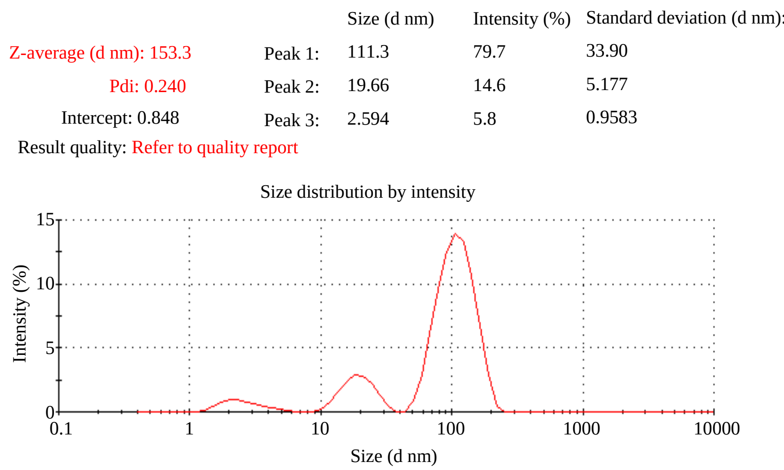

Fig. 3: Histogram of particle size distribution of biogenic silver nanoparticles biogenically synthesized AgNPs prepared from the *Salvadora persica* leaves extract

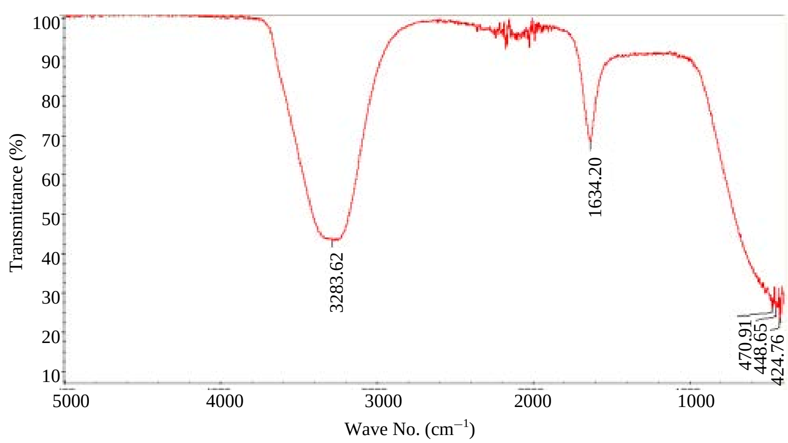

Fig. 4: FTIR spectrum recorded for biogenically synthesized AgNPs prepared from the *Salvadora persica* leaves extract

Table 1: Diameter of inhibition zones (mm) by AgNPs against MDR *E. coli* and *S. aureus*

	Inhibition zone (mm)					
	AgNPs concentrations					
MDR bacterial isolates	25 μL	50 μL	75 μL	100 μL	$AgNO_3$	SP extract
Staphylococcus aureus	15	18	19	22	16	14
Escherichia coli	13	15	17	14	14	13

AgNPs: Silver nanoparticle, SP: *Salvadora persica* leaves extract, $AgNO_3$: Silver nitrate

stabilizing agents, respectively despite some reports showed that *S. aureus* are more resistant to AgNPs than *E. coli*[23]. The actual action of these AgNPs could be due to its interactions with the cell components that may lead to change of cell DNA or membrane permeability[36]. The DNA are superior sites for binding AgNPs since silver has high affinity to react with sulfur or phosphorus-containing biomolecules in the cell-5. Differences in of microbial response to AgNPs might be due to variation in membrane structures of Gram-negative and Gram-positive bacteria, the lipopolysaccharide (LPS) layer in the Gram-negative bacteria has an ability to eliminate the macromolecules and hydrophilic compounds[37] and therefore reduce their susceptibility to AgNPs compared with Gram-positive bacteria which lack this LPS layer[35]. Resistance of microorganisms to many antibiotics has been developed recently and therefore AgNPs could be a promising alternative since they have noble bactericidal and inhibitory effects. AgNPs showed better antibacterial ability compared with $AgNO_3$ which might be due to the smaller size of AgNPs.

CONCLUSION

Nanotechnology offers a noble stand to develop nanomaterial that can be applicable in different fields. *Salvadora persica* was found to have good ability to convert $AgNO_3$ to AgNPs which had high powerful to suppress MDR *S. aureus* and *E. coil.*

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