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SYNTHESIS OF SILVER NANOPARTICLES DISCOURAGE THE GROWTH OF ISOLATED BACTERIA INVADING THE BLOOD STREAM

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Silver Nanoparticles (Ag NPs) have strong antibacterial activities, for that it is very useful for medical applications. Stable silver nanoparticles were synthesized using sodium borohydride (NaBH4) and PVP as reducing and stabilizing agent respectively. Their shape and size distribution characterized by UV-vis spectrophotometer, scanning electron microscopy (SEM) equipped with the energy dispersive spectroscopy (EDS)and (TEM) transmission electron microscopic study their shape and size(< 20nm).The activity of Ag nanoparticles was investigated against isolated bacteria invading the blood` stream by agar diffusion method. The synthesized AgNPs show the ability to discourage the growth of the tested bacteria, which show high activities against most of the tested isolate of G^{+}ve bacteria and G^{-}ve bacteria that invading the blood stream. The current approach described rapid synthesis of silver using NaBH4 and PVP as reducing and stabilizing agents, respectively. This would be suitable for developing a process for discouraging the pathogenic bacteria activities.

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1. Introduction

The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology, which provide solutions to technological and environmental challenges in many areas [1-2]. The chemical reduction methods are involved in the preparation of silver nanoparticles with well-controlled size in which silver ions are reduced by reductants and stabilizing or protecting agents to prevent these nanoparticles from agglomeration [3-4]. The new approach for the functional silver nanoparticles can be used as agents for the selective killing of pathogenic bacteria, however, topical silver have gained popularity once again, principally in the management of open wounds [5].There are many theories on the mechanism of anti-bacterial action of silver nanoparticles. It is widely believed that silver nanoparticles penetrate into the bacterial cell membrane, causing leakage and removal of intracellular material, ultimately causing bacteria death. It has also been reported that the impact

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and effectiveness of antibacterial properties of silver nanoparticles are also influenced by the type of microorganisms [6].

The blood is richest environment in the food, which leads to the possibility of microbial infection and serious blood diseases [7], so the detection of bacteria in the blood (most commonly with blood cultures) is always abnormal. Bacteria can enter the bloodstream as a severe complication of infections (like pneumonia or meningitis), during surgery (especially when involving mucous membranes such as the gastrointestinal tract), or due to catheters and other foreign bodies entering the arteries or veins [8].

The aims of this study synthesis of silver nanoparticle and to evaluate their effects to kill or discourage the growth of pathogenic bacteria from blood pathogenic isolates from community and hospitalized patients at a Security Forces Hospital in Riyadh, Saudi Arabia.

2. Experimental

2.1 Synthesis of Silver Nanoparticles

The formation of silver nanoparticles can be observed by the change of color since small nanoparticles of silver are yellow. 30mL of sodium borohydride (NaBH4) (Sigma-Aldrich, ST. Louis, MI) was added to an Erlenmeyer flask. Then a magnetic stir bar was used and the flask was placed in an ice bath on a stir plate. The aqueous of NaBH4 was stirred and cooled for about 20 minutes. 2mL of silver nitrate (AgNO3) (Sigma-Aldrich, ST. Louis, MI) was dripped into the stirred NaBH4 solution at approximately one drop per second. Stirring was stopped as soon as all of the AgNO3 was added. Then a few drops of 1.5M sodium chloride (NaCl) (Sigma-Aldrich, ST. Louis, MI) solution was added which caused change of the suspension color to darker yellow and then to gray as the nanoparticles aggregates . A small portion of the solution was transfer to a test tube. A drop of 0.3% polyvinyl pyrrolidone (PVP) was added to prevented aggregation [9].

2.2 Samples Collection

Data as well as isolates of bacteria were collected from the bacteriology laboratory in Security Forces Hospital in Riyadh, Saudi Arabia (This hospital for patients Saudis only).

Special bottles of BACTEC / ALERT PF microbial detection System (BIOMÉRIEUX brand) were used, each bottle contains suitable nutritional and environmental conditions for organisms commonly encountered in blood infections, and each bottle was spiked with 10 ml of patients' blood, to determine if microorganisms were present in blood taken from a patient suspected of having bacteremia / fungemia. Bottles were mixed and loaded onto their respective instruments as per the manufacturer's instructions. Antimicrobial removal was evaluated on the basis of time to detect organism growth for up to 7 days of incubation.

A total of 50 blood samples were collected from patients during the three months within the study period. These organisms were identified on blood agar, chocolate agar and MacCkonky [10] in carbon dioxide, oxygen incubator and anaerobes incubator. The plates were incubated overnight at 37°C. Bacterial species were identified using standard biochemical test beside the microscan. All the isolates were tested for their sensitivity by means of the disc diffusion method by Kaçmazand Aksoy [11].

2.3 Bacteria Test Confirmation

Bacterial isolates included the Gram-Positive Bacteria (G^+ve), Methecillin Resistant *Staphylococcus aureus* (MRSA), *Staph.aureus* (MSSA) and Gram-Negative Bacteria isolates (G^- ve) 3 isolates of *Klebsiella pneumonia,* 2 isolates of *E. coli, 2 isolates of Citrobacter freundii complex, 2 isolates of Pseudomonas aeruginosa and 1 isolate of K. oxytoca, Acinetobacter baumanni/haemolyticus, Salmonella* sp., *Pseudomonas fluorescens, Stenotrophomonas maltophilia and Enterobacter cloacae.* The bacteria were grown in the Nutrient Broth at 37°C, sub cultured on Nutrient Agar slants and preserved in the refrigerator at 4°C until required for the study [12].

2.4 Bacterial Identification

All isolates were identified using the standard method. G-ve and G+ve bacteria were identified by cultural, morphological and biochemical tests. Moreover the oxidase, catalase and Indole tests were performed, and to support the identification process, an automated system microscan **(**Analytical Profile Index 20 Enterobacteriaceae (API-20E) was used [13, 14, 15].

2.5 Agar well diffusion method

Antibacterial activity of silver Nanoparticles was determined by using the agar well diffusion method [16] on Mueller-Hinton agar medium. A small volume of sterile water was poured inside a test tube to which general colonies of the test bacteria, taken directly from the plate were emulsified by adding distilled water the suspension was adjusted to match the 0.5 McFarland's standard which had a similar appearance of an overnight broth culture. Half ml volume of the suspensions were spread over the plates containing M.H. agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The sterile cork borer were placed alternatively on the Petri plates, two wells of 11 mm diameter were made into previously seeded M.H. agar plates, to which 500 µl of solution of Silver Nanoparticles was introduced in each hole of the medium and was allowed to put inside the refrigerator for about two hours for proper diffusion. Each extract and control was incubated at 37°C for 24 hrs. The same quantity of sterile distilled water was taken as control. The sensitive bacteria grew everywhere except in areas around the holes in the medium. Then, the resulting inhibition zones obtained were measured in millimeters. Studies were performed in triplicate.

2.6 Preparation of Isolated Bacteria

Some bacterial tested isolates (1 isolate of *E. coli* (11161644), 1isolate of *K. pneumoniae* (11176977), 1 isolate of *P.aeruginosa (11093627*) as Gram Negative bacteria, and (MRSA) (11182821) as Gram Positive bacteria were prepared to be examined using Scanning Electron Microscope, using a method of Afrikian et al [17] as follows:

1- Inoculate N.B. medium tube with bacteria isolated from blood stream as a control.

2- Incubate inoculated tubes for 18 hours at 37°C.

3- Re cultivate isolated bacteria in the same medium for two hours to have bacteria in log phase.

4- Centrifuge the medium at 224× *g* for 3 minutes in order to have the bacterial cells and get rid of the medium.

2.7 Preparation of isolated bacteria after Growing in silver Nanoparticles

Fixation:

Place bacterial cells in the initial fixation solution (2% Sodium, 2.5% Glutaraldehyde, Para-formaldehydehyde pH 7.4 - 0.1 M, Cacodylate Buffer) for 24 hours at a temperature of 4°C. Wash the bacterial cells for three times in a buffer solution for 15 min. in each time. Place bacterial cells in a secondary fixation solution (1% of Osmium Tetroxide) for an hour at a temperature of 4°C.

Dehydration:

Pass the bacterial cells in an ascending series of a gradual concentration ethanol (30%, 50%, 70%, 90%, and 95%). Move the bacterial cells to an absolute ethanol for 30 min. during that ethanol was changed twice. After each addition for solutions the centrifugation for bacterial cells with different solutions was done at 224× *g* for 3 minutes. Use millipore filter in diameter 0.2 mm to hold the bacterial cells and get rid of the solution.

Drying:

Use the critical point drying method by using (SAMDRI-PVT-3B) equipment. Move the bacterial cells which were dried by the critical point method to the samples (bacterial cells) on filter paper and then stick attach them to the holder by the conducting silver paint. Cover the bacterial cells that aimed to be examined by a thin layer of gold as a conducting material using the vacuum evaporation machine (Fine Coat Ion Sputter JCF-1100), at 1200 volt.

Observation:

The bacterial cells were examined by JEOL JSM-6360LV Scanning Electron Microscope at magnification (15.000 X).

2.8 Characterization of biogenic silver nanoparticles

Silver nanoparticles were characterized spectrophotometrically using UV-vis spectroscopy analyses using Perkin Elmer UV-Vis spectrometer, Scanning electron microscopy (SEM) has been employed to characterize the shape and morphologies of formed synthesized of AgNPs, JEOL-FE-SEM, and Energy dispersive spectrometer (EDS) analysis for the confirmation of elemental silver was carried out for the detection of elemental silver. The samples were dried at room temperature and then analyzed for samples composition of the synthesized nanoparticles. Elemental analysis on single particles was carried out using Oxford Instrument, Incax-act, equipped with SEM. Transmission electron microscopy (TEM) has been employed to characterize the size, shape and morphologies of formed synthesized of AgNPs.

3. Results and Discussion

3.1 Visual Observation and UV-visible analysis

When the NaBH4 aqueous solution was quickly added to aqueous solution of AgNO3 the suspension immediately turned a light yellow color which indicates that formation of the corresponding nanoparticles.

In metal nano particles such as in silver, the conduction band and valence band lie very close to each other in which electrons move freely. Small spherical nano particles (< 20nm) exhibit a single surface plasmon band. The absorption peak (SPR) is obtained in the visible range at 410 nm.

3.2 TEM images of Silver Nanoparticles

Images of the synthesized silver nanoparticles shown in Fig. 1 (a, b) were taken by (TEM) and used in measuring the size of spherical AgNPs nanoparticles 7.11 nm. The images show spherical AgNPs deposited onto a carbon-coated copper grid, and shows that the particles are in the range of 1 to 10 nm.

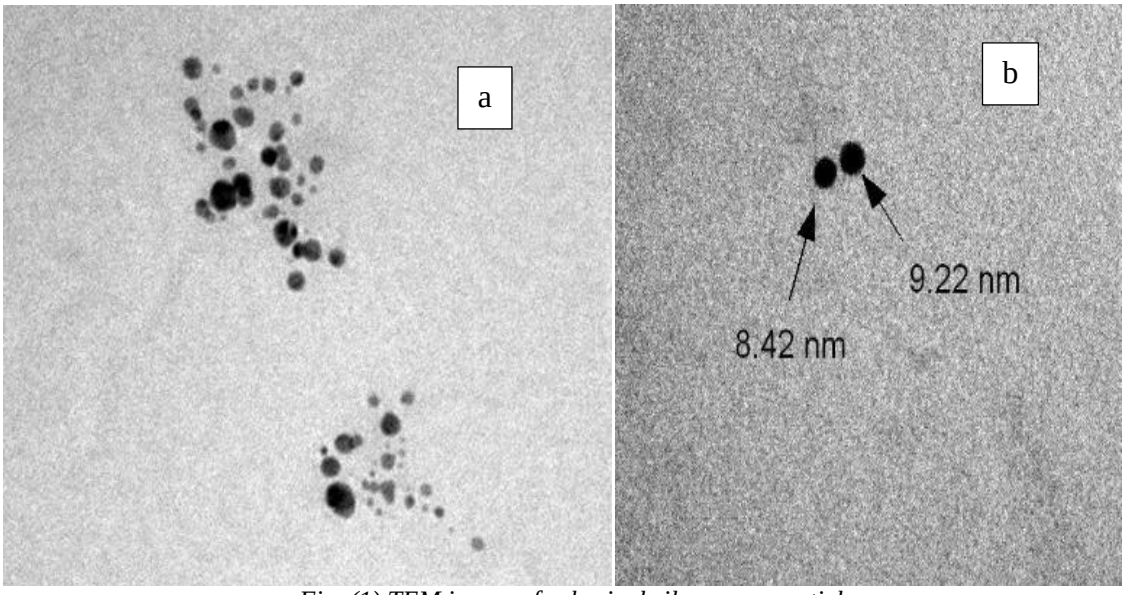


Fig. (1).TEM image of spherical silver nanoparticles

3.3 SEM images of Silver Nanoparticles and EDS analysis

Fig.2. showed the SEM image and EDS analysis. SEM was employed to analyze the structure and morphology of the nanoparticles to give further insight into the features of the synthesized AgNPs, the image showed relatively spherical shape of the formed nanoparticles Fig.2a. The EDS microanalysis is shown in Fig. 2b and confirms the presence of AgNPs which is known to provide information on the chemical analysis of the elements. The spectrum analysis reveals signal in the silver region and then confirms the formation of AgNPs.

Element	Weight %	Atomic %
Ag L	100.00	100.00
Totals	100.00	

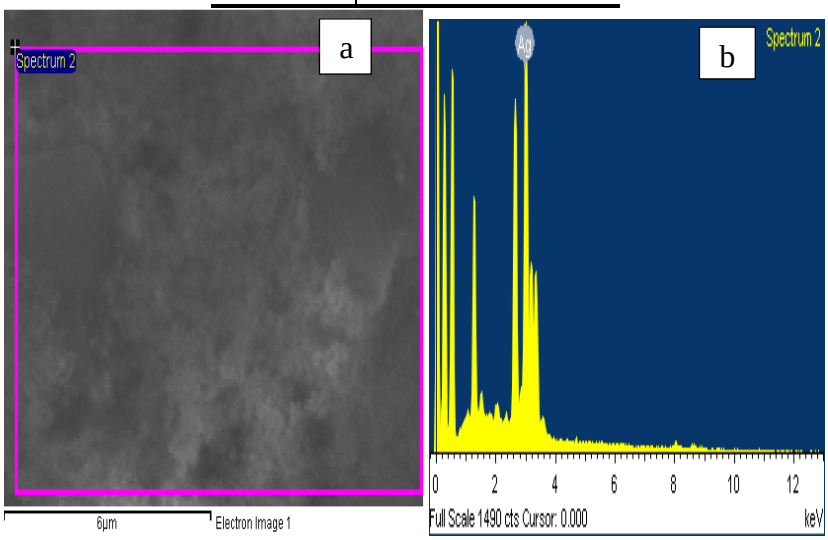


Fig2. (a) SEM image, (b) EDS pattern of spherical AgNPs prepared.
Table .EDS elemental micro-analysis of the AgNPs

3.4 Evaluation of the antibacterial potential of silver Nanoparticles

All tested nanoparticles were effective against all tested G^+ve bacteria and were different in their antibacterial activity against tested G^-ve bacteria except one isolate of *K. pneumoniae* (11176977) as shown in tables 2 and 3. This result was disagreement with Helmstetter and Nicholas (2010) [19] who revealed that the silver nanoparticles they used were active against to G^+ve bacteria and G^-ve ones.

The isolated of *K. pneumoniae* (11176977) as G-ve bacteria exhibited sensitivity to silver nanoparticles in this study. The result obtained was agreed with [20] who found that *K. pneumoniae* was most sensitivity to all tested nanoparticles.

The difference between tested nanoparticles in their antibacterial activity against either G^- ve or G^+ve bacteria (Table.4) in the present study may be due to general physiological differences in the cell wall membrane constitution of G^-ve and G^+ve bacteria [21, 22] confirmed that the G^-ve bacteria, perhaps due to their external lipopolysaccharide structure, the peptidoglycan layer is a specific membrane feature of bacterial species, were more resistant, which can explain why G^+ve bacteria were more sensitive to studied silver nanoparticles than G^-ve bacteria [19,20]. The mechanism of the inhibitory effects of Ag ions on microorganisms is partially known. Some studies have reported that the positive charge on the Ag ion is crucial for its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticle[23,24]Ag nanoparticles accumulated in the bacterial membrane caused the permeability, resulting in cell death., we expect that there is another possible mechanism. Amro *et al.* [25] suggested that metal depletion may cause the formation of irregularly

shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins [25].

Table 1: Sensitivity pattern and determination of bacteriostatic/bacteriocidal effects of silver nanoparticles against Gram Negative Bacteria isolated from bloodstream

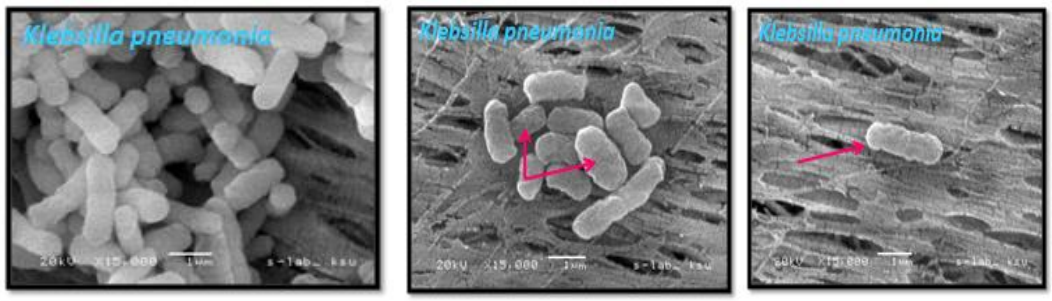
No. of Specimen	Gram negative Bacteria	Zone of inhibition (diameter mm)	bacteriostatic/ bacteriocidal effects
11159053	*En. cloacae*	0.0 R	NT
11093623	*E. coli*	0.0 R	*NT
11161644	*E. coli*	0.0 R	NT
11109045	*A. baumanni*	0.0 R	NT
11132667	*P. aeruginosa*	0.0 R	NT
11176991	*C. freundii*	0.0 R	NT
11193865	*P. fluorescens/putida*	0.0 R	NT
11102476	*K. oxytoca*	0.0 R	NT
11176977	*K. pneumonia*	**19** **S**	NT
11179803	*K. pneumonia*	0.0 R	NT
11120629	*Salmonella* sp.	0.0 R	NT
11182565	*C. freundii*	0.0 R	NT
11108778	*S. maltophilia*	0.0 R	NT
11232940	*K. pneumonia*	0.0 R	**BS**
11093627	*P. aeruginosa*	0.0 R	NT

*S= > 15 mm in diameter, R= ≤ 4-8 mm in diameter, According to PRIMAXIN® I.M (2009) [26].
**BS=Bacteriostatic effect, BC= Bactericidal effect, NT= not tested where no inhibition zone appeared

Table 2: Sensitivity pattern and determination of bacteriostatic/bacteriocidal effects of silver nanoparticles against Gram Positive Bacteria isolated from bloodstream

No. of Specimen	Gram negative Bacteria	Zone of inhibition (diameter mm)	bacteriostatic / bacteriocidal effects
11182821	Methecillin Resistant *Staphylococcus aureus* (MRSA)	**19** **S**	**BS**

According to BARRY, *et al.,* 1986) ≤ 10 for weekly effect (S)



a b c

Fig 3. scanning electron micrograph of (11176977) K. pneumonia mounted on filter paper from blood stream (SEM), (a) Non treated bacterial cell (NT) of K. pneumonia showed straight rods arranged singly; (b,c) bacterial cells of K. pneumonia after treated silver nanoparticles and which illustrated some shortness, decreasing number of cells and crease on some cells walls.

4. Conclusion

The NaBH4 and PVP were good as reducing and stabilizing agents in silver nanoparticles formation process. The formation of AgNPs was determined by UV-vis spectroscopy, where surface plasmon absorption maxima can be observed at about 410 nm. The synthesized AgNPs showed the ability to discourage the growth of the tested bacteria, which showed highly activities against most of the tested isolate of G^+ve bacteria and G^-ve bacteria that invading the blood stream. These findings suggest that the nanoparticles may be a promising candidate for a wide variety of applications in medical fields and nanomedicine.

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