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Efficiency of Silver Nanoparticles against Bacterial Contaminants Isolated from Water in Basra

Hassanein HASSOON
University of Baghdad

Mais AHMED
University of Baghdad

Yasir QADDOORI
University of Baghdad

Abstract: The increased rates of morbidity and mortality due to contamination with pathological microorganisms that continuously develop antibiotic resistance has re-emphasized the need for alternative antimicrobial methodologies. Previous evidence showed such activity of *Punica* nanoparticles. The sources of microorganisms and plants are playing a major role in reduction of metallic nanoparticles such as silver, as it emerges as an eco-friendly and exciting approach in nanotechnology. The purpose of the present work is to investigate the potential antibacterial efficiency of silver nanoparticles obtained from pomegranate peel. Silver *Punica* nanoparticles with a size of 42 nm were prepared. The synthetized nanoparticles were analyzed by different techniques including UV-VisSpectrophotometer, atomic force microscopy and transmission electron Microscope. The potential of the silver *Punica* nanoparticles for water disinfection was examined on *Enterobacter* spp. isolated from Basra river. The results have shown that silver nanoparticles display a high antibacterial activity against bacterial contamination in the water samples of Basra river. In conclusion, nanotechnology provides an alternative solution to clean germs in water.

Keywords: Nanoparticles, *Punica grantum*, Antibacterial

Introduction

Waterborne diseases are major drive of mortality in the present world, especially in less developed communities. Data from international agencies indicates that a minimum of 15% of the world population do not have access to healthy water (Bohn et al., 2009). As a result, an annual mortality of approximately 760.000 deaths in children under the 5 years of age was recorded (Griffiths, 2016), which is an amelioration as compared to 2.2 million deaths majorly in children during 1998 due to diarrhea (Prüss-Üstün & Corvalän, 2007). Due to this un-negligible significance of water contamination, measures of microbial control were strengthened and permitted levels of drinking water contamination with coliforms/ *E. coli* should not exceed 0/100ml (Barrell, Hunter, & Nichols, 2000).

To avoid environmental contamination with wastewater, providing a suitable strategy to remove these contaminants could be of great importance in maintaining a healthy environment. For efficient removal of toxicants from water sources, different physiochemical mechanisms have been extensively used, including precipitation, adsorption, ion exchange, reverse osmosis, treatments, membrane filtration, flotation, oxidation and biosorption (Amin, Alazba, & Manzoor, 2014; Rasalingam, Peng, & Koodali, 2014).

The use of nanoparticles is among currently interesting applications for water-detoxification purposes, mainly due to their remarkably small size of 1 to 100nm, and hence their larger surface area per unit mass. Nanomaterials commonly exert remarkably improved physicochemical and biological characteristics in

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comparison with macro-sized particles (Morose, 2010). Microorganisms are of major significance to environmental systems as they form the ground floor for the provision of various functions such as primary productivity, nutrient cycling and waste decomposition, therefore a comprehension of nanoparticles toxicity effects on microorganisms is a matter of concern, which requires an estimation of these particles influence on the environment (Klaine et al., 2008).

In order to get an environmentally friendly nanotechnology, delicate experimental design should be performed in order to provide best conditions that take into consideration nanoparticle physical and chemical features such as size, shape and stability. Such conditions involve dynamics of reaction between metal ions and the used reducing agents, as well as the adsorption reactions between the stabilizing agents and the nanoparticles(Guzmán, Taylor, & Banfield, 2006) .

Punica granatum is a fruit whose trees are extensively cultivated around the Mediterranean areas. Previous reports have indicated the presence of several active compounds species (e.g. flavonoids and anthocyanidins) isolated from this plant with powerful anti-oxidant activity (Abdelmonem & Amin, 2014).

Pomegranate peel, the most essential constituent of the plant, contains several phenolic compounds, gallic acid and other fatty acids, flavonols. flavones and flavanones. Recent research has focused on finding alternative environmentally-safe antimicrobial approaches, among which the employment of plant extracts in the biosynthesis of nanoparticles. Particles produced by plant extracts have been shown to ensure higher level of biocompatibility as compared to those chemically synthesized (GnanaJobitha, Shanmugam, Gurusamy, & Kannan, 2013).

Methods

Samples Collection

Three water samples were collected from river, tap water and RO (reverse osmosis installation) units. Water was sampled from different places within the same region of Basra city (Iraq) during the period from October to December 2019. For sampling, sterile bottles and caps were first rinsed three times with the source water and then filled to 1-2 inches below the top of the bottle aseptically in sterile 500 mL Duran Schott glass bottles from different sampling points by directly dipping the bottles into the surface of the water.

Cultivation of Bacteria

Samples were processed as previously described (Forbes, Sahm, Weissfeld, & Bailey, 2007). Briefly, 10ml of water sample was diluted in 90ml sterile enrichment peptone water and incubated at 37°C for 18- 24 hrs. Using a sterile standard loop (1ml), a loopful of the incubated culture was then successively cultured on mannitol salt agar and MacConkey agar plates to differentiate between Gve+ and Gve- bacteria. Following incubation at 37°C for 24 hours, the bacteria were identified by Gram's stain and standard biochemical tests.

Antibiotic Sensitivity Test

Antibiotics and antimicrobial susceptibility tests were performed by Bauer-Kirby method (Hoelzer et al., 2011) for Both Gve+ and Gve- bacteria detected by antibiotic disk.

Molecular assay

DNA extraction

Genomic DNA was extracted from the detected bacterial isolates according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Quantus Florometer was used to detect the concentration of extracted DNA.

Primers Selection

Sets of primers 27F (AGAGTTTGATCTTGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT) were used for amplification of 16s rRNA and so identification of bacteria at gene level (Srinivasan et al., 2015).

Preparations of *Punica granatum* peel extract

Punica granatum obtained from local Baghdad markets was washed, the peel was scrapped and brought to room temperature (24℃) for two days. Thereafter, 40 grams of dried *Punica granatum* peel powder was soaked in 300 ml of deionized water. In this process, extraction was performed by incubating the mixture-containing flask on an orbital shaker (Genex. Cat.No. 60HZ, USA) at room temperature with agitating at 100 rpm for 48 hours. After the extraction process was accomplished, the aqueous solution was filtrated twice by using Whatman filter paper No.1 (Whatman. Cat.No. Q/FML61-2004, England) (Jurenka, 2008)(11).

Preparation of 1mM Silver Nitrate

Silver nitrate was purchased from Lobachemie and a weight of 0.0169gm was dissolved in 100mL of distilled water in an amber colored bottle.

Synthesis of Silver nanoparticles under Room temperature

9 ml of 2mM silver nitrate solution was prepared. 1 ml of freshly prepared pomegranate peel extract was added separately to the silver nitrate solution. The bio-reduction of AgNO3 ions occurred within 3hrs (figure1). Color of peel extract solution has slowly changed from gray to brown indicating the formation of silver nanoparticles (figure1) (G. Gnana Jobitha, S. Rajeshkumar, G. Annadurai, & C. Kannan, 2013).

Figure 1. Preparation of *Punica granatum* peel extract – nanosilver

Preparation of Silver Nitrate

Silver nitrate was brought from Lobachemie. Weigh 8.49 gm of silver nitrate and dissolve in 1L of distilled water to obtain three concentration 50 mM and prepration another concentration (25, and 75) mM in amber coloured bottle.

Transmission Electron Microscope (TEM Analysis)

For transmission electron microscopy (TEM) imaging, the AgNP suspensions (approximately 7 µL for each sample) in the absence or in the presence of anesthetics were deposited on the specimen grids and adsorbed for 1 minute. The excess solution was removed with filter paper, and the samples were air-dried (Sadowski, 2010).

Optical, Size and Morphological Characterization of Silver nanoparticles

Surface plasmon resonance of the samples was studied with a UV-VisSpectrophotometer (Cary 300 Conc spectrophotometer) at wavelength range of 250-600 nm with a resolution of 1 nm (Abimbola, Kolawole, Ajanaku, & Adeyemi, 2019) .The size of nanoparticles was examined with Atomic Force Field Microscopy (AFM). Preparation of the sample was performed through mounting one drop of the colloidal solution of the nanometal on a carbon-coated copper grid, with complete dryness being achieved by using the vacuum desiccators. Images of atomic force microscopy (AFM) were taken on the nanostructured film of AgNPs obtained by vertical adsorption from the colloid solution on glass plates for 5 minutes and dried out in air (Mocanu et al., 2013).

Well Diffusion Method

Well diffusion method (WDA) then carried out to test the antimicrobial activity of the synthetized sliver nanoparticles (Balouiri, Sadiki, & Ibnsouda, 2016; Charannya et al., 2018).100µL of the suspension was spread on the test plate (Muller Hinton Agar). And put(10 µL) of the essential oils and placed on well in agar. Plates were incubated at 37° C for 24 hours and the zone of inhibition was measured. The experiment was performed in triplicate and the average diameter of inhibition was calculated.

Results and Discussion

Isolation and identification of Bacteria

Isolation and Identification of Gram positive: (Staphylococcus. epidermidis, Staphylococcus. aureus) and Enterobacter spp.

The microscopic examination of the cultured bacteria showed that gram positive cocci appear as single cells, pairs, tetrads and chains. The macroscopic examination of the isolates on Mannitol Salt Agar showed that they have the ability to ferment mannitol and turn the color of medium from red to yellow. The isolates were on MacConkey agar as a presumptive *S. aureus and S. epidermidis* isolates (Figure2) and Enterobacter spp as pale colonies.

The isolates on blood agar showed yellow-gray colonies which are 4-3mm in diameter on the zones of β-hemolysis (Figure3).The result showed (Figure4) *S. aureus* colony on milke agare yellow while *S. aureus* appeared white color this medium using differential between *staphylococcus* spp.

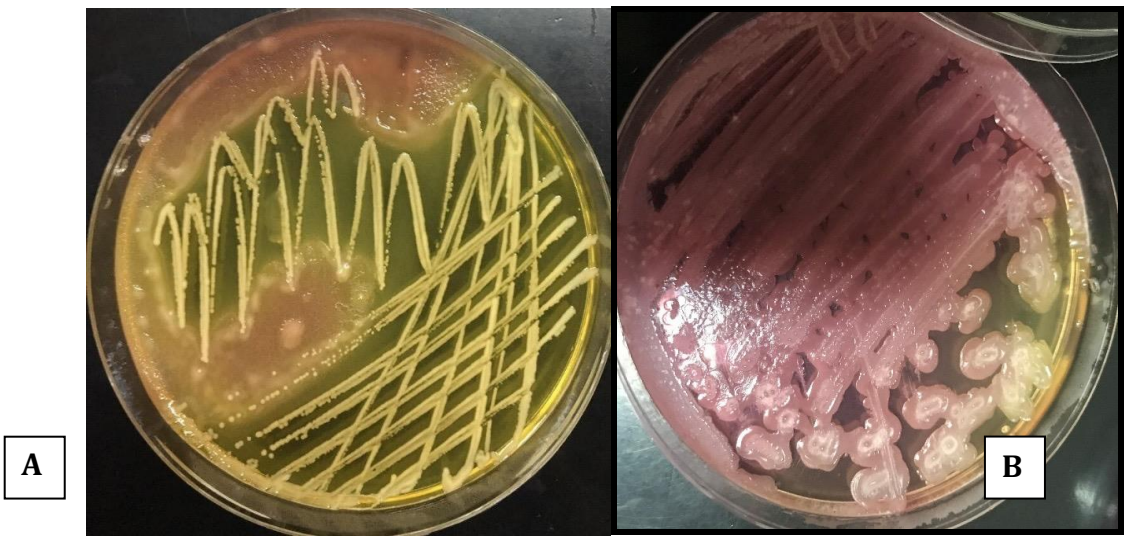

Figure 2. A presumptive of **A**: *S. aureus* **B:** *S. epidermidisS. epidermidis* on mannitol salt agar **C**:*Enterobacter* spp

Figure 3. A presumptive of *S. aureus* on blood Agar

Figure 4. A presumptiveof **A**: *S. aureus* **B**: *S. epidermidis* on Milk agar

Isolation and Identification of Gram Negative Bacteria

The macroscopic examination of presumptive (*E. coli*, and *Klebsiella* spp) isolates gave large flat colonies that produced zones of -haemolysis with odor on the blood agar and pale colonies on MacConkey agar (lactose fermenting and non fermenting).

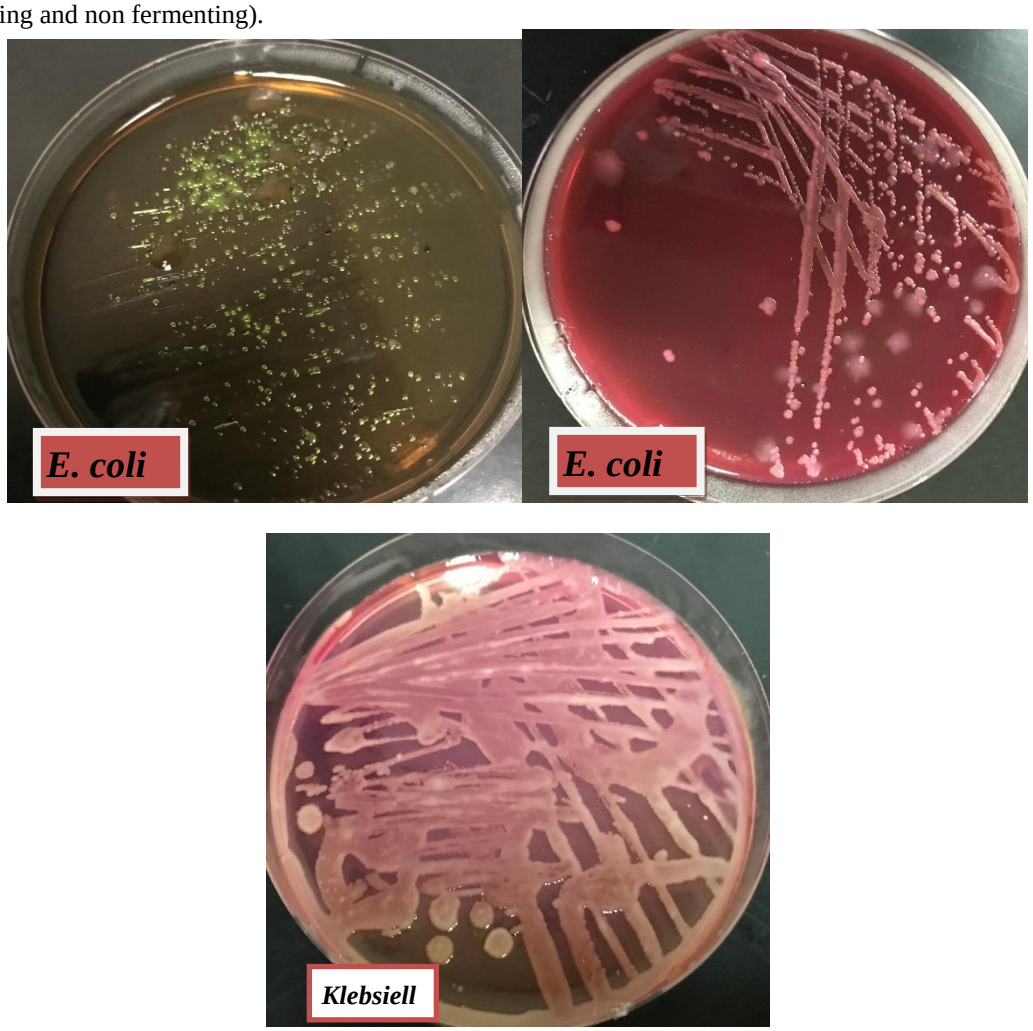

Figure 5. Bacterial isolation from Water sample *E. coli* on MacConkey and Eosin methicillin blue agar , while ***Klebsiell*** on MacConkey agar for 24hrs at 37°C.

Antibiotic susceptibility test (AST)

Resistance patterns for the isolated bacteria against different antibiotics demonstrated that the Gr-ve isolates had the highest resistance to Ampicillin, Amikacin and Ceftazidim, whereas for Gr+ve isolates they were Methicillin, and Gentamycin, with sensitivity was shown to Vancomycin (figure 6).

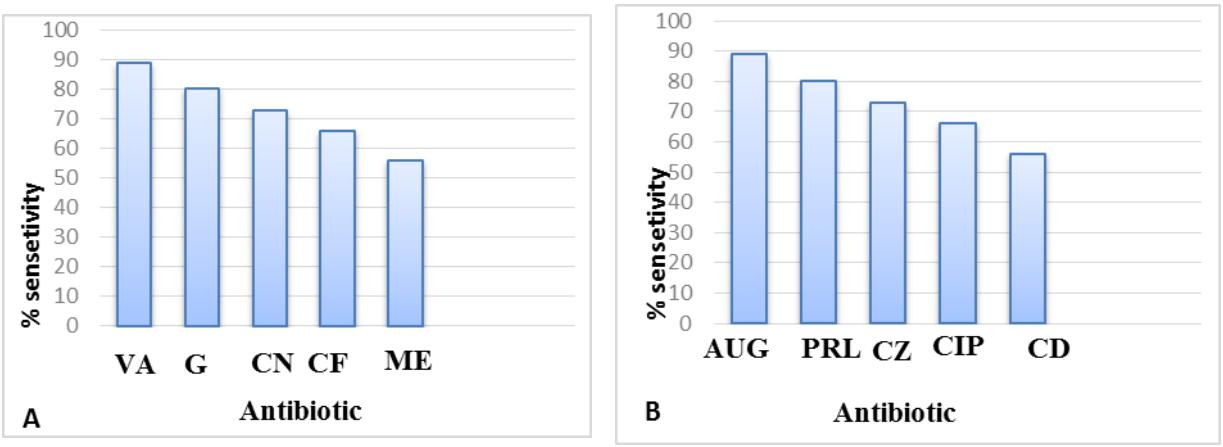

Figure 6. Antibiotic susceptibility test of **A**: Gram positive. **B**: Gram negative

Genomic DNA Extraction

In this study, the genomic DNA of bacterial isolate was successfully extracted of Gram positive and the extracted DNA had an appropriate quality to perform PCR (10 ng/µl). 16S rDNA was amplified by PCR using specific primers that gave a distinct amplicon pattern with a size of 1500 bp when analyzed by gel electrophoresis (Figure7).

Figure 7. PCR product the band size 1500bp. The product was tested by electrophoresis on 2% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100). *Enterobacter* spp

Study of Silver Nanoparticles compounds characterization

Spectral Properties of the Silver Nanoparticles

The strong interaction of the silver nanoparticles with light occurs because the conduction electrons on the metal surface undergo a collective oscillation when excited by light at specific wavelengths Surface .Plasmon resonance of the samples was studied with a UV-Vis Spectrophotometer (Figure 8).

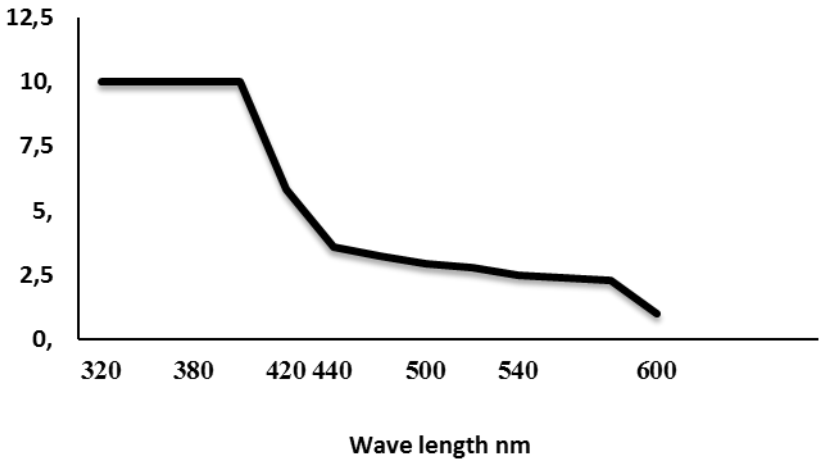

Figure 8. Absorption spectra of -silver nanoparticles

Atomic Force Electron Microscopy (AFM)

The AFM micrograph obtained for the silver nanoparticles (Figure 9) indicates the surface roughness changes as identified by the root mean square (Rp) values. For the sample, the roughness value was 56 nm and the section analysis of the sample's grain size resulted in a value of 42 nm.

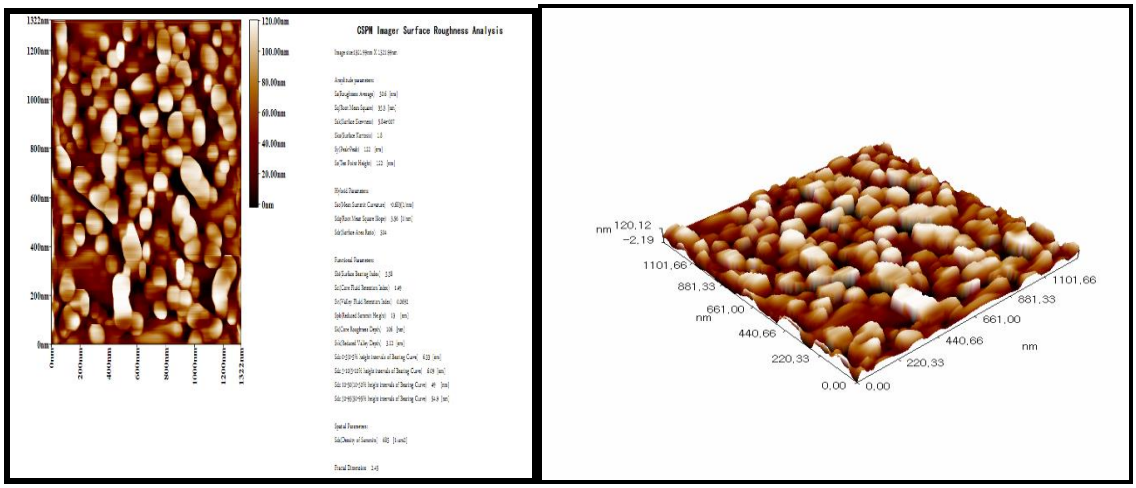

Figure 9. AFM for -silver nanoparticles

Transmission ElectronMicroscopy analysis (TEM)of silver nanoparticles:

TEM micrographs for Silver *Punica* nanoparticles was shown in Figure (10), the diameter of nanoparticles was measured to be 42 nm.show figure (10-A).

The figure showed *Enterobacter* spp before treatment appeared as cocci and had regular arrangement of cell wall figure (10-B), while after treatment with Silver *Punica* nanoparticles the bacteria swelled and had irregular cell wall Figure (10-C).

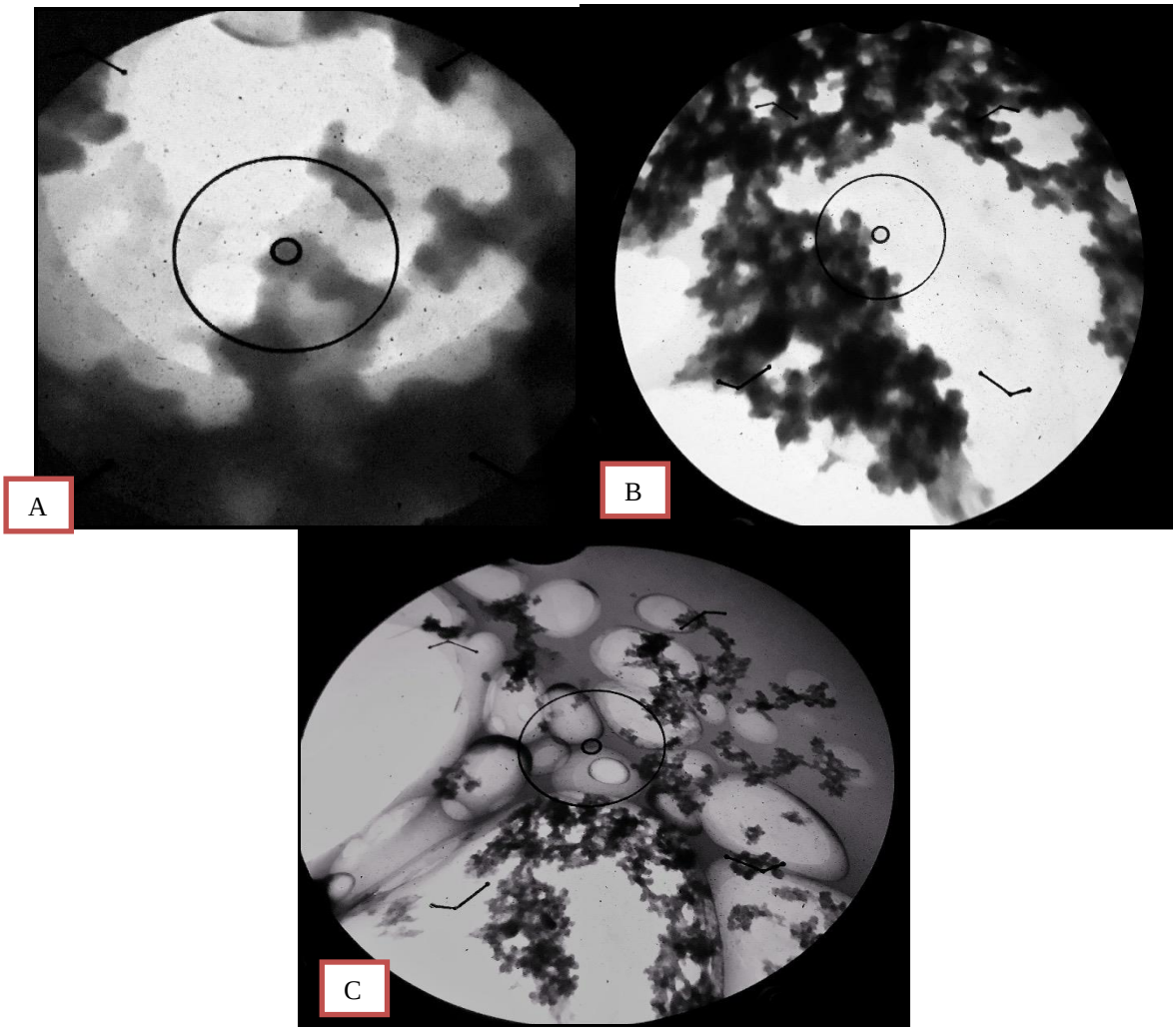

Figure 10. TEM of **A**: Sliver nanopartical **B**: *Enterobacter* spp before treatment -Silver Nanoparticles
C: *Enterobacter* spp after treatmentwith Nisin-Silver Nanoparticles

In the present study, we have successfully used the peel (exocarp and mesocarp) as well as the Peel pomegranates as a source to green-senthesize silver nanoparticles from silver nitrate.

We have also investigated the antimicrobial potential for these particles using various Gr-ve and Gr+ve bacteria as targets (Figure11). The results showed that ***S.****aureus* and *Enterobacter* spp were sensitive for the extractions of pomegranate as compared to the antibiotic zones nearer to standard antibiotic.

The present study concludes the obvious potential of nanoparticles synthesized using pomegranate to be invested as alternative antimicrobial agents against waterborne microorganisms.

Figure 11. Antibacterial activity of Sliver nanopa pomegranate nanoparticles against *Enterobacter* pp by WDA for 24-48 hrs at 37°C on Muller Hinton agar 1: Sliver nanoparticles 2: Sliver pomegranate nanoparticles

The present results shows the antimicrobial activities as compared with a previous study (Haniff Nisha, Tamileswari, & Jesurani, 2015), although with different microorganisms. In that study, antibiotic zones for peel demonstrated 16mm inhibition for Pseudomonas and resistance for Proteus. By using the seed extracts, they obtained the antibiotic zones against Pseudomonas and Proteus as 13 mm and resistant respectively, while using the leaf extracts (Haniff et al., 2015).

Conclusion

Introducing nanotechnology to various health fields has brought clear advantages that can be observed in the areas of nano-medicine and alternative medicine. In line with this, the present work could provide the evidence that pomegranate can be successfully used as a source of silver nanoparticles which in turn showed a remarkable antimicrobial potential against different bacteria. This work provides the basis for further investigations on the bio-medical activities of those pomegranate-synthesized silver nanoparticles.

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Author Information

Hassanein Hassoon
Department of Biology, College of Science,
University of Baghdad, Baghdad 10071, Iraq
Contact E-mail:hassanainabbood@yahoo.com

Mais Ahmed
Department of Biology, College of Science,
University of Baghdad, Baghdad 10071, Iraq

Yasir Qaddoori
Department of Biology, College of Science,
University of Baghdad, Baghdad 10071, Iraq

Raghad Mohammed
Department of Biology, College of Science,
University of Baghdad, Baghdad 10071, Iraq