Letter to the Editor

Stable Silver Nanoparticles Synthesis by *Citrus Sinensis* (Orange) and Assessing Activity Against Food Poisoning Microbes

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Silver nanoparticles are considered as good antimicrobial agent. AgNPs were synthesized by mixing silver nitrate solution with *citrus sinesis* extract for 2 h at 37 °C and analyzed by UV-visible spectra, SEM, XRD, and FTIR. AgNPs were tested against *B. subtilis, Shigella, S. aureus, and E. coli*. Minimum inhibitory concentration of AgNPs was 20 μg/mL for *B. subtilis* and *Shigella* and 30 μg/mL for *S. aureus* and *E. coli*. Antibiofilm activity (80% to 90%) was observed at 25 μg/mL. AgNPs were stable for five months with sustained antimicrobial activity. Biosynthesized AgNPs can be used to inhibit food poisoning microbial growth.

Noble metals are very important in medical and industrial sciences due to their distinctive physical properties, size, shape and as carrier for drug delivery. Among them, silver nanoparticles (AgNPs) have attained attraction due to its broad spectrum antimicrobial activity[1]. This antimicrobial activity is due to its surface structure, small size and interaction with proteins[2]. Another beneficial effect of AgNPs is the production of oxygen derived free radicals such as O_2^-, $OH^·$ radicals under aerated conditions[3]. AgNPs are also used as disinfectant, coating agent to prevent biofilm formation and to treat the drinking water[4].

Chemical and physical methods such as laser radiation, gamma rays, combination of different toxic hazardous capping and stabilizing agents are available for the synthesis of AgNPs. These toxic chemicals may leads to pathological conditions. There is a need to develop non-toxic and ecofriendly procedures for the synthesis of AgNPs. Bio-inspired synthesis procedure is non-toxic and cost effective[5]. It is observed that some health hazardous pathogenic microbes are resistant to antibiotics. These antibiotic resistant microbes have capabilities to survive by making biofilm which leads to pathogenic conditions and infection. The synergistic effects of AgNPs with antimicrobial medicines indicated that AgNPs can play significant role in the treatment of many bacterial infections[6]. AgNPs can be synthesized by pure citric acid but these are not cost effective. Due to the presence of high concentration of citric acid in *Citrus sinensis* (orange) juice, it may be used as a bio-reductant for the synthesis of AgNPs. Our objective in this work was to bioinspired synthesis of AgNPs for testing as an antimicrobial agent against clinically important food poisoning bacteria such as *Staphylococcus aureus, Bacillus subtilis, Shigella,* and *E. coli*. A good bactericidal activity was observed by using bioinspired AgNPs.

Fresh orange (*Citrus sinensis*) juice was extracted, filtered through nylon mesh and used within 24 to 48 h. The concentration of citric acid in orange juice was estimated (0.35% to 0.4%). Two different concentrations of $AgNO_3$ solution (0.01 mol/L and 0.001 mol/L) were treated with orange juice in different ratios (V/V, 1:1, 1:3, 1:4, 1:6, 1:8) in 50 mL final reaction volume of deionized water. The treated silver nitrate solutions were incubated for different time periods (half hour, 1, 2, and 3 h) at 37 °C. AgNPs pellet was collected, washed with deionized water and lyophilized. It was optimized that the best preparation can be made by mixing of 0.01 mol/L silver nitrate solution with orange juice (1:4 ratios) for 2 h at 37 °C. The prepared AgNPs were stored at 4 °C to check the shelf life. The stability of AgNPs was checked after every month by measuring the UV-visible absorption specrta at 200-700 nm. AgNPs were further analyzed by X-ray diffraction (XRD) and scanning electron microscopy (SEM). Whereas, the capping ability of citric acid was characterized by Fourier transform infrared spectroscopy. Antimicrobial activities of AgNPs were checked against food poisoning bacteria (mentioned above). Different assays such as well

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diffusion assay, determination of minimum inhibitory concentration (for 10^6 CFU), effect on microbial growth kinetics (for 10^7 CFU), bactericidal activity (for 10^6 CFU), biofilm inhibition (for 10^8 CFU) and effect of antioxidant (ascorbic acid) on AgNPs activity were conducted.

The synthesis of AgNPs was carried out for various time periods and it was concluded that two hours incubation of silver nitrate solution with orange extract (1:4 ratios) is sufficient to form AgNPs and the absorbance intensity steadily increased without shifting wave length maximum during different time periods (Figure 1A). The stability of the prepared AgNPs was also confirmed by UV-visible spectra after every month (Figure 1B). XRD analysis further confirmed the crystalline structure of AgNPs and its spectrum is shown (Figure 1C). The peaks at 2θ scale 38.285°, 46.367°, 64.562°, 77.539° was observed and could assigned to (111), (200), (220), and (311) planes of faced, center, cubic lattice of silver. The sharp diffraction peaks may be due to caping agent that stabilizes the AgNPs. SEM indicated that AgNPs havee oval shape.

Anti-microbial activity assays of AgNPs were evaluated against four clinically important food poisoning bacteria such as, *S. aureus, Shigella, B. subtilis,* and *E. coli.* In broth dilution method, the minimum inhibitory concentration (MIC) of biosynthesized AgNPs for *S.aureus*, *E. coli*, *Shigella, B.subtilis* was observed 40, 30, 20, and 20 μg/mL respectively. In well diffusion assay, a zone of inhibition approximately 4 mm and 5 mm was observed in plate spreaded with *S.aureus* and *shigella* respectively. While in case of *B. subtilis* and *E. coli,* the zone of inhibition was approximately 7 mm and 6 mm respectively. It was also observed that the zone of inhibition increased with increasing amount of AgNPs.

The bactericidal effect of various concentrations of AgNPs was checked with 1.4×10^6 CFU of microbes. It was observed that lower concentration (5 and 10 μg/mL) of AgNPs was not effectively bactericidal. The concentration of AgNPs at 20 μg/mL was found bactericidal for *B. subtilis,* 30 μg/mL for *Shigella* and *E. coli* and 40 μg/mL for *S. aureus*. A 92% to 95% biofilm inhibition was observed in the growth of *S. aureus* and *E. coli* at 25 μg/mL AgNPs. The inhibition of biofilm formation of *B. subtilis and E. coli* was observed nearly 80% at 25 μg/mL (Figure 2C). The reduced antibacterial activity of AgNPs in the presence of antioxidant (pure ascorbic acid) was observed (Figure 2D). In this assay, the bacterial growth was observed on plates supplemented with ascorbic acid (10 mmol/L) and AgNPs (30 μg/mL). The plates containing AgNPs only (30 μg/mL) inhibited the growth successfully. It indicated that ascorbic acid alongwith AgNPs reduces the generation and effect of oxygen derived free radicals. The growth kinetics of above mentioned microorganisms was also checked (Figure 3). It was observed that 40 to 50 μg/mL concentration of AgNPs completely inhibited the growth of *S. aureus* and *E. coli* for more than 12 h. AgNPs at 10-30 μg/mL caused growth delay of *S. aureus* and *E. coli.* It was further observed that 30-50 μg/mL of AgNPs completely inhibited the growth of *B. subtilis* and *Shigella*. A moderate dose of AgNPs (10 and 20 μg/mL) delayed the growth of *B. subtilis* and *Shigella*.

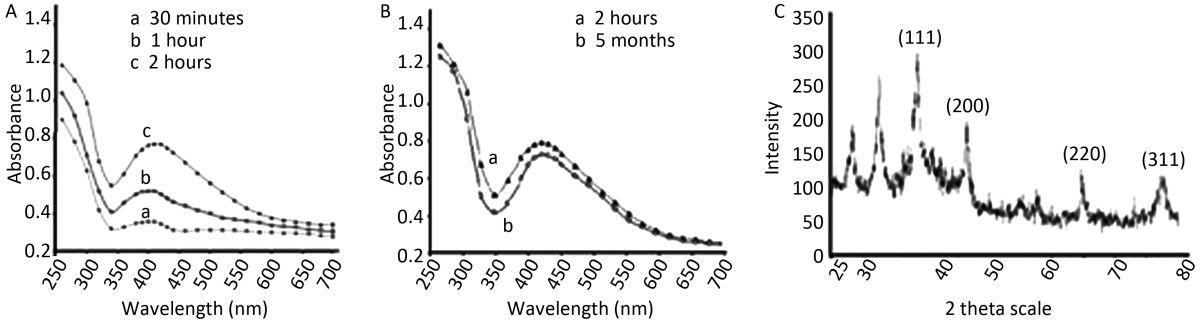

Figure 1. Synthesis and stability of AgNPs. (A), Synthesis of AgNPs was confirmed by measuring the UV-Visible spectra at 260-700 nm wavelengths. (a): 30 min, (b): 1 h, and (c): 2 h. (B), The stability of AgNPs was checked under same conditions (a), after 2 h of synthesis (b), storage for 5 months at 4 °C. (C), XRD spectrum of AgNPs.

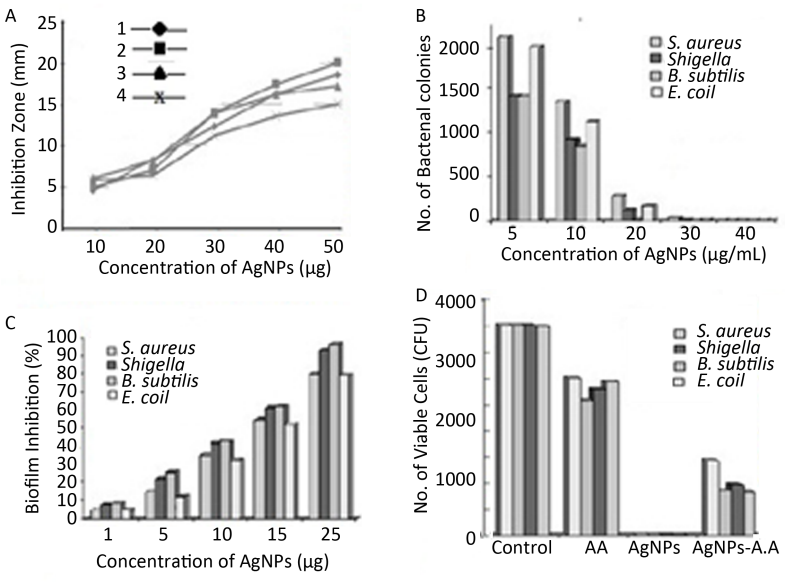

Figure 2. Bacterial growth in the presence of AgNPs. (A), Well diffusion assay to check the antibacterial activity of AgNPs with different amounts (10, 20, 30, 40, 50 µg per per well. (1), *S. aureus* (2), *Shigella* (3), *B. Subtilis* (4), *E. coli*. Zone of inhibition was increased with increasing concentration of AgNPs. (B), MH agar dilution method was used to determine the bactericidal activity of different concentration of AgNPs. A good inhibitory effect was observed at 30 µg/ mL. (C), Antibiofilm activity of AgNPs. A good inhibition was observed at 25 µg/mL. Antibiofilm activity of AgNPs was observed 80% to 96% in all tested microbes. (D), Effect of antioxidant on bactericidal activity of AgNPs. Effect of ascorbic acid (anti-oxidant) on bactericidal activity of AgNPs against *S. aureus, Shigella, B. subtilis* and *E. coli*. All bacterial strains survived in the presence of ascorbic acid. The number of viable cells in the presence of antioxidant (10 mmol/L ascorbic acid) and AgNPs (30 µg/mL) are shown.

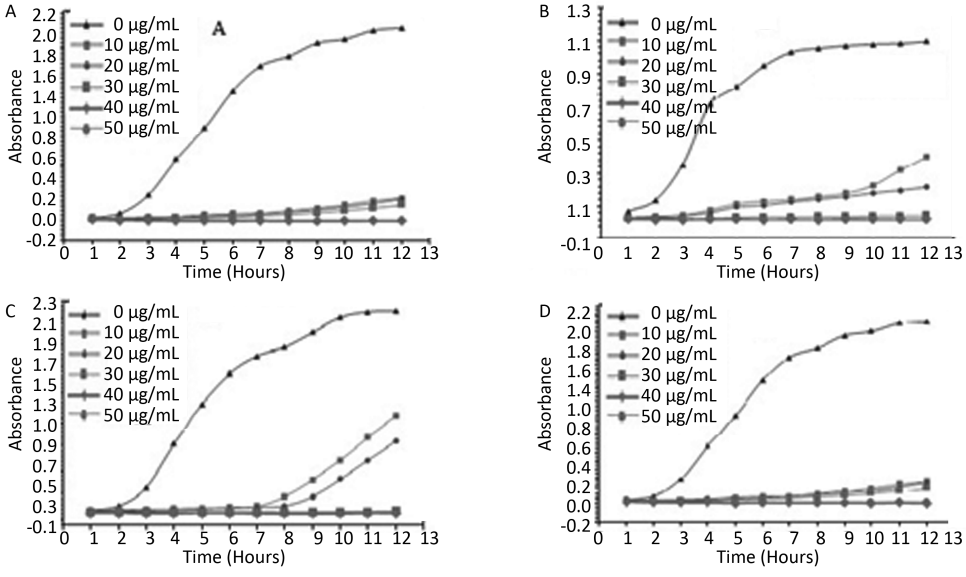

Figure 3. Microbial growth in the presence of AgNPs. Growth curve of microbes (107 CFU/mL) supplemented with different concentration of AgNPs. Control assays were conducted without AgNPs. (A), *S. aureus* (B), *Shigella* (C), *B. Subtilis* (D), *E. coli*. Inhibitory effect of AgNPs is discussed in the text section.

The growth inhibition of bacteria depends on the concentration of AgNPs and initial CFU. The data suggested that AgNPs have good bactericidal effect for practical applications. In addition, it is known that silver ions bind with functional groups of proteins or enzymes and denatured it. The bactericidal activity is due to the ionic interaction between charged bacterial cell membrane surfaces and AgNPs. The binding of AgNPs also depends on the particle size. Decoration of small nanoparticle of AgNPs on bacterial surfaces increases the permeability[8-9]. The outer membrane of Gram negative bacterial cells is fabricated with protective barrier of lipopolysaccharide (LPS) and may be destroyed by AgNPs. The metal attachment on the surface of bacteria aids in the formation of irregular-shape or pits on the outer surface membrane and alter the permeability. This permeability causes the progressive release of cellular proteins by disrupting LPS surface[10]. Here, we consider the similar mechanism that explains the degradation of the membrane structure of clinically important food poisoning microbes during incubation with prepared AgNPs.

Many methods are reported for the synthesis of AgNPs but green synthesis method is non-toxic, cost effective and ecofriendly. In this preparation, citric acid not only reduces the silver ions into AgNPs but also behave as caping and stabilizing agent. The pure citric acid can be used for the preparation of AgNPs but it is not cost effective. The fresh orange extract is available for many months locally and can be used for synthesis. These AgNPs have an excellent antibacterial activity against food poisoning bacteria. In summary, our results indicate that the synthesized AgNPs can be used for the development of antimicrobial coatings for different biomedical materials as well as for drinking water treatment.

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