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Green Synthesis of Silver Nanoparticles Using Garlic Extract with Enhanced Antibacterial Activities against *Lactobacillus Acidophilus*

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Abstract **-** In this research, due to the antibacterial nature of silver nanoparticles (AgNPs), these metal nanoparticles (NPs) are prepared by chemical and green synthesis methods using garlic extract as a reducing and stabilizing agent. The antimicrobial activity of prepared AgNPs against *Lactobacillus acidophilus* in comparison with garlic extract and chlorhexidine (CHX) was evaluated by measuring the minimum inhibitory and bactericidal concentrations (MIC and MBC) by microdilution method. The UV-Vis spectrum showed the absorbance maximum at peak at 425, and 457nm for green and with a blue shifting in wavelength for chemical synthesis methods respectively, which is a characteristic of surface plasmon resonance of silver for both synthesis methods. The zeta potential value confirmed the high stability, good colloidal nature, and high dispersity of the AgNPs prepared by the green method (−31.7mV). The average particle size of AgNps was 94.71 and 146 nm with a polydispersity index (PDI) of 0.62 and 0.19 in green and chemical synthesis methods respectively indicating that AgNPs were monodispersed. Microdilution results also showed that the MIC was 76 ppm and 15 ppm for chemically and green synthesized AgNPs respectively. The MBC values were obtained the same as MIC values. The results confirmed that garlic extract can be used to produce AgNPs with a significant amount of antimicrobial activity against *Lactobacillus acidophilus*. Also, we established a higher antimicrobial effect of NPs synthesized by the green method than NPs synthesized chemically, which would allow for achieving important clinical effects. Bacteriostatic experiments showed that the obtained AgNPs had a promising future in bacterial infections.

Keywords**:** green synthesis, antibacterial activity, *Lactobacillus acidophilus*, silver nanoparticles, *Allium sativum*

1. Introduction

Dental caries is a biofilm-mediated, sugar-driven, multifactorial, dynamic disease that causes the phasic demineralization and remineralization of dental hard tissues, which, if net demineralization occurs over adequate time, results in the initiation of specific caries lesions [1]. Caries and associated infections can create some burdens such as pain, discomfort, and reduction in intake of food and hemoglobin, therefore affecting growth and as well as the quality of life [2]. Cariogenic bacteria, fermentable carbohydrates, a susceptible tooth, the host, and the time are the main players in the etiology of this disease. Previous studies suggested that the main pathogenic bacteria are *Streptococcus mutans* and *Lactobacillus*. Consequently, *Lactobacillus* is considered the second most cariogenic bacteria of oral flora. It is a gram-positive, rod shape facultative anaerobic, non-spore-forming bacilli, which plays a significant role in dental caries by producing lactic acid after carbohydrate fermentation [3]. Caries preventive therapy includes behavioral management, dietary modification, and fluoride in various forms such as toothpaste or mouthwash [4-5].

CHX is the most commonly used mouthwash and is considered the gold standard in bacterial plaque control [6]. Although CHX can be non-destructive and has high antimicrobial properties in the presence of liquids, it has side effects like brown discoloration of teeth, bitter taste, change in sense of taste, and erogenous oral mucosa [7]. Considering the side effects associated with mouthwashes, especially CHX, as well as the emergence of antibiotic-resistant pathogens, the use of other compounds with antimicrobial properties with lower side effects has become very important [8]. Studies have shown that AgNPs exhibit high antibacterial activity and are used in antimicrobial applications since the antimicrobial effect of Ag ions is well known. Moreover, recent findings show that AgNPs inhibit the development of the gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* strains [9]. However, the conventional methods of developing NPs are costly and environmentally incompatible due to the use of toxic chemical-reducing agents such as citrate, so the need of the hour is to decrease the risk of toxicity in the environment from the different chemicals used in the physical and chemical methods. The alternate approach found to develop NPs is "green synthesis" [10]. Recently, green synthesis has been considered a simple, cost-effective, biocompatible, stable, and eco-friendly nature method for the synthesis of NP; further, unlike traditional methods, green synthesis reduces the toxicity of NPs [11-12]. In the green synthesis plant metabolites and natural substances, mainly plant extracts and microorganisms, are utilized as reducing and stabilizing agents to orchestrate the NPs for the pharmaceutical and other applications [13].

Garlic (*Allium sativum*) is one of the plants used for the green synthesis of NPs [14-15]. Studies have proved that garlic extract shows antimicrobial activity against both gram-positive and gram-negative bacteria [16-17]. In addition, AgNPs green synthesized by Various Medicinal Plant Extracts also exhibited significant antimicrobial activity against gram-positive and gram-negative bacteria [18]. Therefore, considering the importance of the prevention of dental caries, the first aim of this study was to develop a simple, cost-effective, biocompatible, and eco-friendly approach for the extracellular biological synthesis of AgNPs using garlic extract. The various characterization methods such as UV visible spectrophotometry, Transmission electron microscopy (TEM), Infra-red spectroscopy (IR), and dynamic light scattering (DLS) characteristics are employed to evaluate the structure, performance, and polydispersity properties of AgNPs.The second aim of our study involved the analysis of the antibacterial activity of both chemically and biologically prepared AgNPs against *Lactobacillus acidophilus*. The green-synthesized AgNPs exhibited significant antibacterial activity.

2. Experimental

2.1. Preparation of garlic alcoholic extract

Fresh garlic bulbs were purchased (East Azarbaijan, Iran), peeled, weighed (500 g), thoroughly washed with plenty of distilled water, dried in the shade at 30◦C for 3 h under the hood, and powdered with a blender. The powder was loaded in a glass percolator, then 1 lit. ethanol 70% was poured into the material as a solvent and capped tightly closed to avoid any evaporation. Then the mixture was allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then was opened and the liquid contained therein was allowed to drip slowly. The solvent was evaporated till dryness with the help of a rotary evaporator. Finally, a thick yellow extract with a pungent garlic odor was obtained [19]. The extract was stored in the dark at 4°C until use.

2.2. Green and Chemical synthesize of the AgNPs

Fig.1 shows the apparatus scheme for synthesizing the AgNPs, including the position of the initial source materials. The Turkevich method was applied for the green synthesis of the AgNPs. In this method, 50 mL of a 0.001 mol silver salt (AgNO3) solution was boiled as an initial material, and 5 ml of garlic extract was rapidly added to the boiling solution under vigorous stirring. In this case, a color change from pale yellow to light green was rapidly observed in the solution. Then the solution was further boiled for 10 min, after which rotation on the stirrer was continued for 15 minutes and the solution cooled down to room temperature. Finally, the solution containing the synthesized NPs was filtered with a Gelman membrane filter with a pore size of 0.8 μm [20]. Before use in microbial experiments, NPs were diluted 200 times. The final concentrations of the solutions used in susceptibility tests were 152 and 960 ppm for chemically and green synthesized NPs respectively.

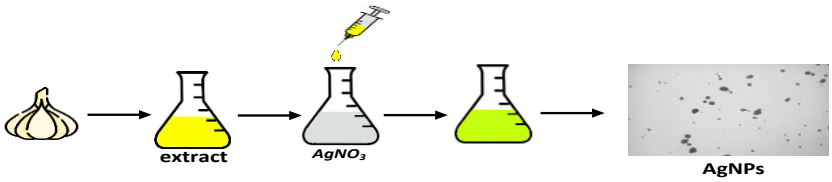

Fig. 1. Schematic diagram for the green synthesis of AgNPs using garlic extract.

To synthesize AgNPs chemically, first Sodium borohydride (as the chemical reducing agent) was dissolved in distilled water and mixed with $AgNO_3$ (0.01 mol) with a ratio of 2.5 to 1 respectively. During a nucleophilic chemical reaction, AgNPs were obtained from silver salt [21]. Eventually, the solution was placed on the stirrer and passed through the filter paper to collect NPs. The chemical reaction is the sodium borohydride reduction of silver nitrate as indicated by the following reaction [22]:

$$AgNO_3 + \ NaBH_4 \rightarrow Ag \ + \ {}^{1}\!/_{2}\, H_2 \ + \ {}^{1}\!/_{2}\, B_2H_6 + \ NaNO_3$$

2.3. Standard bacterial culture and Microdilution method

To investigate the antimicrobial effects, first, a reference microbial strain of *Lactobacillus acidophilus* (ATCC 4356) was prepared in lyophilized form from the microbial collection of the Iranian Biological Resource Center (IBRC). MRS (Man, Rogosa, Sharpe) medium was used as a growth medium to determine the sensitivity of *Lactobacillus acidophilus*. A standard microbial suspension with a concentration equivalent to 0.5 McFarland's of *Lactobacillus acidophilus* was prepared to perform Susceptibility tests [23].

Susceptibility tests with AgNPs were carried out in two 96-well microtiter plates in MRS, following the Clinical and Laboratory Standards Institute (CLSI) guidelines [24]. To examine AgNPs MICs, a standard bacterial solution with a concentration of 0.5 McFarland's was diluted 100 times to obtain a concentration of 10^6 bacteria per mL. Therefore, 990 µl of physiological serum was mixed with 10 µl of bacterial solution with a concentration of 0.5 McFarland's. After pipetting the resulting mixture, 15 µl of it was poured into each well of both 96-well plates. Then, 150 µl culture medium was added to wells No. 1 to 9 of each plate. Next, 150 µl of solution containing green and chemical AgNPs were added to wells number 1 of the first and the second plate respectively. Through up and down, the AgNPs and bacteria were well mixed. Then in each plate, 150 µl of the mixture was removed and added to the side wall. Again, the culture medium was mixed well, and 150 µl was removed from it and added to the next well. This process continued until well No. 9. In well No. 9, 150 µl was taken and discarded. Using this method, descending dilutions of NPs were created in the wells. In the next rows, serial dilutions were made of other investigated solutions and CHX. Well No. 10 was empty. Wells No. 11 and 12 were used as control samples. Only 150 µl culture medium was poured (negative control) in well No. 11, and in well No. 12, 150 µl culture medium and 15 µl bacterial solution were poured (positive control). Finally, the plates were incubated at 37°C for 24 hours. The MIC was determined as the lowest concentration that inhibited the visible growth of the bacteria or the lowest concentration at which no turbidity was observed. The wells in which no growth of bacteria was observed at the end of the MIC test were selected, and 10 µl of it was cultured on the solid culture medium. The culture medium was incubated again for 24 hours at 37°C. The plate on which bacteria did not grow and had the lowest concentration of antimicrobial substance was considered MBC.

3. Result and Discussion

Fig.2 depicts the UV-visible absorption curves in the range of 300 to 700nm and IR-spectroscopy spectra of the that were synthesized by green and chemical methods. As shown by Fig.2(a) both synthesized methods present an approximately wide band with a maximum single and strong peak at 425, and 457nm for green and chemical synthesis methods respectively. All samples prepared by green and chemical methods present the surface plasmon resonance of free electrons in the silver nanoparticles [25]. However, a similar behavior has been observed for every two curves in the visible light area but there is a shift (redshift) in the maximum absorption peak of about 32 nm toward the higher wavelength for AgNPs which has been prepared by the green method. It is reported that the absorption spectrum of spherical silver nanoparticles presents a maximum between 420 and 450 nm with a blue or red shift when particle size diminishes or increases, respectively [26-27]. For this reason, the Nanosilver synthesized by the chemical method that presents plasmon is red-shifted compared that silver nanoparticles prepared by the green method [27]. Fig.2(b) presents the IR-spectroscopy of the AgNPs synthesized by green and chemical methods in the range of 4000- 400 cm^{-1}. IR spectrum shows absorption bands at 3460, and 1635, indicating the presence of a capping agent with the nanoparticles. The bands at 3460 cm^{-1} in the spectra correspond to O-H stretching vibration indicating the presence of hydroxyl group [28-29]. The band at 1635 cm-1 in the spectra corresponds to C--N and C-C stretching indicating the presence of proteins [30].

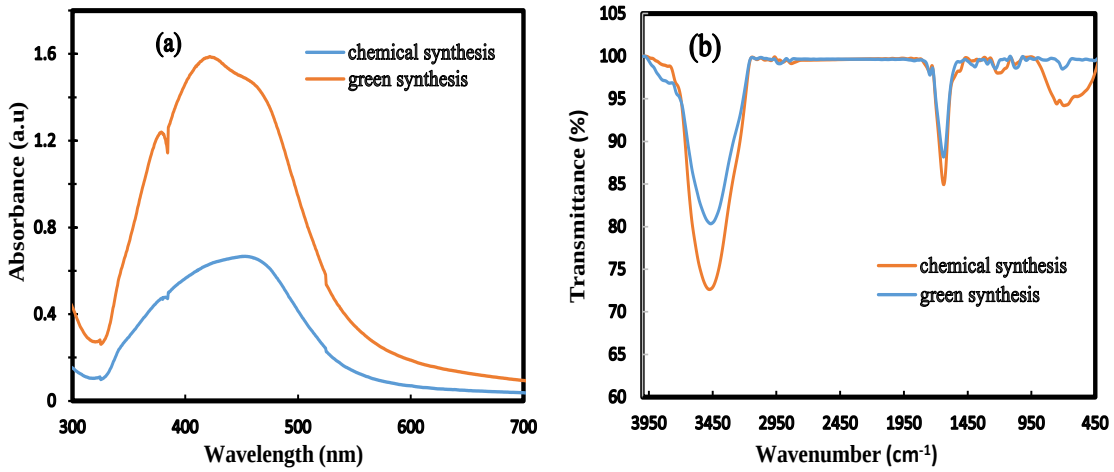

Fig. 2. a) UV-visible absorption spectra of the Argentum (Silver) synthesized, and b) IR-spectroscopy spectra of the Ag NPs synthesized by green and chemical methods.

To understand the surface morphology and to provide more information about the size of colloidal AgNPs prepared by two synthesis methods, TEM investigation was conducted under similar conditions, as shown in Fig.3. It can be seen from the shape of Ag NPs obtained from both synthesis methods are mostly spherical. The TEM image of the sample using the green method (Figures 3(c) and 3(d) gave corroborative evidence on the findings that silver colloids prepared by the green method did not aggregate in solution, confirming the stability of AgNPs with high zeta potential. In contrast, as shown in Figures 3(a) and 3(b) the Ag nanoparticles were shown to be aggregated in the case of using chemical method.

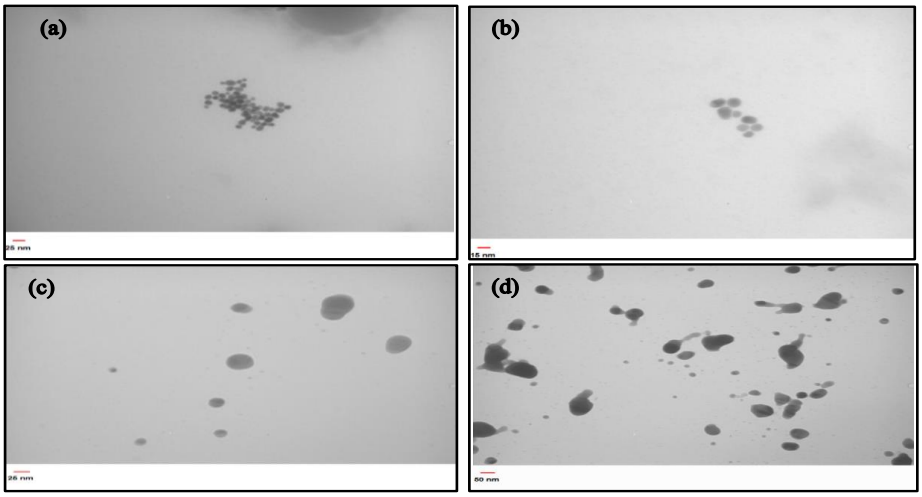

Fig. 3. TEM image of Ag NPs synthesized by a, b) chemical method in the scale of 25 and 15 nm respectively, and c, d) green method in the scale of 25 and 50 nm respectively.

The DLS analysis measures the hydrodynamic radius of dispersed nanoparticles, therefore the size of AgNPs obtained by the chemical method is slightly bigger than that by the green method. The PDI of the AgNPs for both synthesized methods is in the range of 0.01–0.7 indicating that AgNPs were mono dispersed [31]. It is known that colloids with high zeta potential are electrically stabilized and they do not show any disposition to come together. Nevertheless, in the case of low absolute zeta potential values, these particles aggregate and flocculate due to the absence of repulsive force which prevents such agglomeration [32]. Here in Fig.3(a, b), the Zeta values varied in the range from −13.30 mV to −31.70 mV, depending upon the type of synthesis methods. Thus, the different stability of colloidal AgNPs prepared by the two methods is also reflected by the changes in Zeta values. According to Fig.3(a, b), the average surface zeta potential of -31.70 mV for green synthesized silver nanoparticles while this potential value is -13.30 for AgNps that are prepared chemically. Interestingly, the zeta potential value confirmed the high stability, good colloidal nature, and high dispersity of the fresh AgNPs prepared green method, whereas the less stable particles for AgNPs were obtained by the chemical method [33-34].

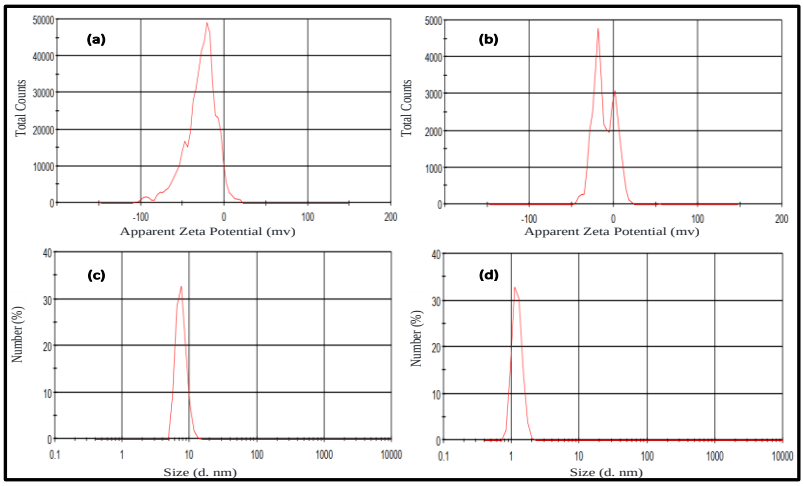

Fig. 4. Zeta Potential Distribution of Ag NPs synthesized by a) green, and b) chemical methods respectively, Dynamic Light Scattering (DLS) of Ag NPs synthesized by c) green, and d) chemical methods respectively.

Table. 1 presents the MIC, MBC, and Average size of AgNPs for green and chemical synthesis methods in Standard bacteria culture. According to microdilution results, CHX inhibited bacterial growth in all wells which means all the wells were clear, and no turbidity was observed in any of them. Compare the antimicrobial performance of AgNPs prepared by two methods, MIC, MBC, and the average size of AgNPs are summarized in Table (1). Accordingly, The MIC value was 76

ppm and 15 ppm for chemically and green synthesized AgNPs respectively. The MBC values were obtained the same as MIC values. By comparing the results. AgNPs synthesized by the green method exhibited higher antimicrobial properties than AgNps prepared chemically.

Table 1. The MIC, MBC, and Average size of AgNPs for green and chemical synthesis methods.

Group	MIC (µg/ml)	MBC (µg/ml)	AgNPs Average size(nm)
AgNPs $_{\text{green synthesis}}$	15	15	94.71
AgNPs $_{\text{chemical synthesis}}$	76	76	146.4

As summarized in Table. 1, the NPs synthesized by the green method have smaller particle sizes. Considering the important role of NPs size in their antimicrobial activity, the presence of antioxidant compounds in the plant extract may cause the creation of NPs with a smaller size. As a result, the surface-to-volume ratio of NPs increases [35]. However, Reda et al. showed that using different plant extracts produces NPs with different antimicrobial properties, which are not related to the size of the NPs [36]. They concluded that the type of extract and products present in the extract play a more important role than the size of NPs. In such a way that the compounds in the extract, such as tannins, flavonoids, and other secondary metabolites, are attached to silver NPs during synthesis, then they are attached to the bacterial cell wall. This makes them exert their antimicrobial effects [36]. Secondary metabolites are attached to the surface of the synthesized NPs and improve the biological function of the NPs (Biofunctionalization). A similar process is observed in binding antibiotics to the surface of NPs. However, there were no such metabolites in the chemical method. This makes NPs synthesized by the chemical method have fewer biological activities than NPs synthesized by the green method [37]. In addition, these metabolites prevent the aggregation and clumping of NPs by covering the surface of NPs and also reduce their toxicity [37].

In our study, CHX showed much more inhibitory effects than AgNPs on *Lactobacillus acidophilous*, but it should be noted that these antimicrobial compounds - CHX and AgNPs - were used with different concentrations. AgNPs were diluted 200 times after synthesis. This was while CHX was examined without dilution. The reason for not diluting CHX was the investigation of this compound in the concentration used in conventional mouthwashes. As a result, the antimicrobial effects of AgNPs were compared to the concentration of CHX in mouthwashes. Certainly, if the AgNPs were examined in an undiluted form, different results would be obtained, and perhaps the antimicrobial effect of AgNPs would be greater than that of CHX. Every study has limitations, and our study is no exception. We investigated the antimicrobial effects in vitro. Because the conditions in the mouth (in vivo) are very different, the antimicrobial effects of the studied compounds and solutions may be affected by the temperature of the mouth, as well as pH, compounds, and saliva flow.

4. Conclusion

In this paper, we report a reproducible green approach for the synthesis of AgNPs by the reduction of $AgNo_3$ – ions using garlic extract, which provides a simple and efficient way for the size-controlled synthesis of AgNPs. The results have indicated that the green synthesized AgNPs had smaller sizes compared with AgNPs synthesized by chemical method. For each green and chemical method, AgNPs were spherical, also the bands at 3460 cm-1 and 1635 cm-1 in the IR spectra showed the presence of a hydroxyl group and proteins respectively. The low value of PDI for both synthesized methods confirmed that AgNPs were monodispersed. The antibacterial activity of AgNPs against *Lactobacillus acidophilus* preserved the antibacterial efficiency, although these antimicrobial effects were less than CHX. Also, AgNPs synthesized by the green method indicated more antimicrobial effects than AgNPs synthesized chemically. Hence, green synthesized AgNPs represent antibacterial properties that could be proven promising candidates for future applications. However, more studies regarding the toxicity of AgNPs on human tissues and cells are needed to reach

this conclusion with certainty. In this regard, it is suggested to study the cytotoxic effects of AgNPs synthesized by the green synthesis method on human cells. Investigating the antimicrobial effects of AgNPs synthesized by the green synthesis method on other microorganisms causing oral and dental diseases can also provide valuable results in future studies.

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