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RESEARCH PAPER

Antibacterial activity of green synthesized silver nanoparticles using *Pistacia* hull against multidrug-resistant clinical isolates

Zary Nokhodian [1], Mohammad Mousavi-Khattat [2], Shima Gharibi [3], Soodabeh Rostami [4]*, Parisa Zeraati [1], Mehdi Rahimmalek [5], Antoni Szumny [6]

[1]Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[2]Department of Biotechnology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran
[3]Core Research Facilities, Isfahan University of Medical Sciences, Isfahan, Iran
[4]Nosocomial Infection Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
[5]Department of Horticulture, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
[6]Department of Food Chemistry and Biocatalysis, Wrocław University of Environmental and Life Sciences, Wroclaw, Poland

ABSTRACT

Objective(s): Silver nanoparticles (AgNPs) can be considered as the new antibacterial agents. The antibacterial effects of synthesized AgNPs from Iranian pistachio hulls on several antibiotic-resistant bacteria were assessed in this study.

Materials and Methods: In an experimental study, AgNPs were synthesized by reducing Ag+ ions using pistachio hulls. Several methods characterized the qualities of AgNPs. Antibacterial activities of the AgNPs against six gram-positive and gram-negative standard bacteria and 30 multidrug-resistant (MDR) clinical isolates were investigated by well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) methods.

Results: The aqueous extract of pistachio hulls had an acceptable potential to synthesize AgNPs, and the formed nanoparticles displayed suitable size and acceptable stability in solutions. Antibacterial activities of the AgNPs were detected against two standard strains, *Escherichia coli*, and *Staphylococcus aureus*, with growth inhibition zones of 13 and 11 mm, respectively. MIC were 10 mg/ml for *E. coli* and 20 mg/ml for *S. aureus*. MBC for both bacteria was the same as MIC. MIC and MBC AgNPs against 15 methicillin-resistant *S. aureus* (MRSA) isolates ranged from 40 to 10 mg/ml. In extended-spectrum beta-lactamase (ESBL) *E. coli* isolates, 11 and 3 isolates have MIC equal to 20 and 10 mg/ml, respectively. Three ESBL *E. coli* isolates had 10, 5 and 2.5 mg/ml MBC; in other isolates, MBC and MIC were the same.

Conclusion: The green synthesis of AgNPs using pistachio hull can replace common chemical and physical methods. AgNPs displayed antibacterial activities, and they could replace some antibiotics.

Keywords: *Anti-bacterial agents, Drug resistance, Green chemistry technology, Nanoparticles, Pistacia*

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INTRODUCTION

Antibiotic resistance is a serious public health challenge and a threat to human health. It is not limited to specific geographical areas, races, age groups, and socioeconomic status [1]. A group of bacteria, such as *Acinetobacter baumannii, Pseudomonas aeruginosa, Enterococcus faecium, and Staphylococcus aureus,* have been considered resistant pathogens to the drug by the World Health Organization (WHO) [2, 3]. Increasing medical costs, adverse effects on the economic situation and patient's life, prolonged hospitalization, and increased mortality and morbidity rates are some consequences of antibiotic resistance [3, 4]. Using new bactericides could help humans to overcome the problems caused by this phenomenon. One of the newest strategies to fight and prevent diseases is nanotechnology.

* *Corresponding author: Email: srostami1876@gmail.com*
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Antimicrobial agents and nanoparticles (NPs) have been interesting to researchers during the last decades. NPs have broad and specific functional properties as compared to bulk materials and potential applications in different industries, such as catalysis and biosensors [5]. Metal nanoparticles (MNPs) with excellent physicochemical properties are critical NPs. Parameters such as size, shape, electrical charge, and composition have a significant role in determining the physical and chemical properties of the elements. So controlling these parameters, especially their shape, is very important [6-9]. Silver nanoparticles (AgNPs) are attractive metal nanoparticles with antimicrobial properties and are widely used in different fields such as medicine, health, agriculture, and food industries [10, 11]. They, with various sizes and shapes, can be synthesized by chemical and physical methods. However, these methods are relatively expensive and have environmental hazards due to toxic and hazardous chemicals [12].

Green synthesis is an inexpensive and eco-friendly method for the synthesis of MNPs. Green Synthesis uses biological microorganisms such as bacteria, fungi, yeast, seaweed, protein, carbohydrates, enzymes, or plant extracts. Using plants is more accessible, less dangerous, and more cost-effective than other methods of green synthesis. In addition, Plant-mediated synthesis can produce nanoparticles on a large scale [12, 13].

Pistacia vera, a small tree belonging to the Anacardiaceae family, is widely found in western and northern Iran. It has anti-inflammatory, antibacterial, and antiviral properties with compounds such as flavonoids, α-pinene, triterpenoids, terpinolene, fatty acids, and phenolic compounds [13- 15]. Several studies have used different pistachio plant parts to produce MNPs, such as silver nanoparticles [16]. Although thousands of tons of pistachios are produced annually in Iran, their hull or leaves are not used, and their elimination has become a serious environmental problem for the industries. Proper use of pistachio waste solves this problem and generates profit.

The mass production of pistachios in Iran, the possibility of producing AgNPs from its shell, and the high bactericide activity of AgNPs made us think about this project. The study aimed to investigate the antibacterial effects of the synthesized AgNPs from Iranian pistachio hulls on antibiotic-resistant *S. aureus, A. baumannii, E. coli, Klebsiella pneumonia, E. feacalis,* and *P. aeruginosa.*

MATERIALS AND METHODS

Silver nanoparticle

In an experimental study conducted in the infectious diseases and tropical medicine research center at Isfahan University of Medical Sciences in 2022, the antibacterial effect of Nanosilver was investigated. We synthesized pure AgNPs by the reducing of Ag^+ ion using the Fandoghi pistachio hull cultivar. The hulls were collected in September 2021 from Kerman province, Iran. They were washed with distilled water, dried in the shade at room temperature, and then powdered. The aqueous extract of pistachio hulls was prepared by adding 4 gr dried powder with 100 ml distilled water in a glass beaker. The beaker was placed on a shaker for 15 min at 100 RPM at 80 °C. The beaker contents were filtered, and pH was adjusted using NaOH (8.5 <PH<9). 20 ml of the clear hull extract sample was added with 980 ml $AgNO_3$ 1 mM in a 1000-mL Erlenmeyer. Then, the Erlenmeyer was placed on a shaker for 6 hours at 150 RPM at 80 °C. The colorless $AgNO_3$ solution became brown or reddish, demonstrating the production of Ag-NPs.

Characterization of silver nanoparticles:

Ultra-violet visible (UV-vis) spectroscopy, X-ray diffraction (XRD), transmission electron microscope (TEM), scanning electron microscopy (SEM), Fourier transform infrared (FTIR), spectroscopy[13], dynamic light scattering (DLS), and atomic force microscopy (AFM), were used to study the characters and qualities of AgNPs.

HPLC analysis

The pistachio hull extracts were analyzed using HPLC (model Agilent 1090). All phenolic and flavonoid standards were from Sigma-Aldrich with high purities (≥95% purity). The 1000 mg/L stock solutions were applied in HPLC-grade methanol. The elution was performed using the Gharibi et al protocol [17]. Accordingly, 20 µl of the hull extract was injected into the analytical column (250 mm × 4.6 mm (5 µm) Symmetry C18 column (Waters Crop., Milford, MA, USA) (10 mm × 4 mm I.D.). The mobile phase was 0.1% formic acid in acetonitrile (flow rate of 0.8 mL min-1). The detection wavelengths were between 200–400 nm. Solvents A and B were applied in the mobile phase. 0.1%

of water-formic acid was used as solvent A, while solvent B included 0.1% of formic acid in acetonitrile. A linear step from 10% to 26% solvent B (v/v) for 40 min, 65% solvent B for 70 min, and finally to 100% solvent B for 75 min was used as the gradient program. The amount of polyphenolic compounds was calculated according to the peak areas and their retention times. The results were reported as mg /100 g of the sample dry weight.

Antibacterial activity of AgNPs

Bacterial strains

Antibacterial activities of AgNPs by agar well-diffusion and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) techniques were assessed against six bacteria: *Escherichia coli* (ATCC: 25922), *Klebsiella pneumonia* (ATCC: 13883), *Pseudomonas aeruginosa* (ATCC: 27853), *Acinetobacter baumannii* (ATCC: 13305) as gram-negative bacteria and *Staphylococcus aureus* (ATCC: 25923) and *Enterococcus feacalis* (ATCC: 29212) as gram-positive bacteria.

If the nanoparticle has antibacterial properties against one of the standard bacteria, 15 multidrug-resistant (MDR) clinical bacteria isolated from patients with different infections were evaluated using the micro-dilution method.

Agar well-diffusion technique

Mueller-Hinton agar (MHA) plates were inoculated with 18-24-hour-old cultures of standard bacteria (1-2 ×107 CFU ml-1 0.5 McFarland Standard). Two wells (6 mm in diameter) were cut into the agar media with a sterilized cork borer. Then, 40 µl of AgNPs containing 40 mg/ml was poured into one well, and 40 µl sterile distilled water was poured into another as a negative control. Inoculated plates were then incubated at 37 °C for 24 hr, and inhibition zones were measured in mm. Three replicates were prepared for each microorganism.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The micro-dilution method was used to determine the MIC of the AgNPs. Two-fold serial concentrations of the nanoparticle of AgNPs containing 40 mg/ml were prepared and poured into the sterile 96-well microplate. Muller-Hinton broth culture medium and microbial suspension (equal to 0.5 McFarland Standard) were also added to the wells. After shaking for a few seconds, the microplate is incubated at 37 °C for 24 hr. The lowest concentration of AgNPs that inhibit 50% of cell growth was considered as MIC. 10 microliters of the MIC dilution and several higher dilutions were cultured on Mueller Hinton agar medium and incubated for 24 hr at 37 °C. After examining the plates, the lowest concentration, 99.9% of the bacteria did not grow, was considered the minimum lethal concentration. Each experiment is repeated three times.

Ethical aspects

This study was approved by the ethical committee of Isfahan University of Medical Sciences with code IR.MUI.MED.REC.1400.056.

RESULTS

Characterization of silver nanoparticles and HPLC analysis

The first sign of AgNP formation during exposure to $AgNO_3$ with the aqueous extract of pistachio hulls was detected by a color change from colorless to brown (Fig. 1). In Fig. 2, the results of the UV-Vis

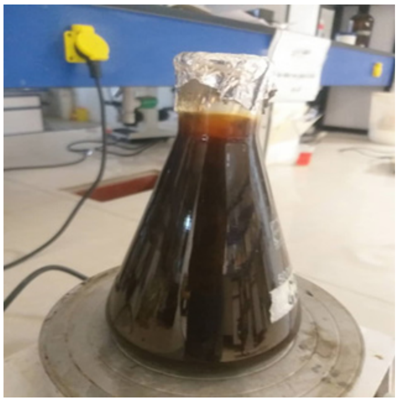

Fig. 1. The change in the color confirmed the formation AgNPs

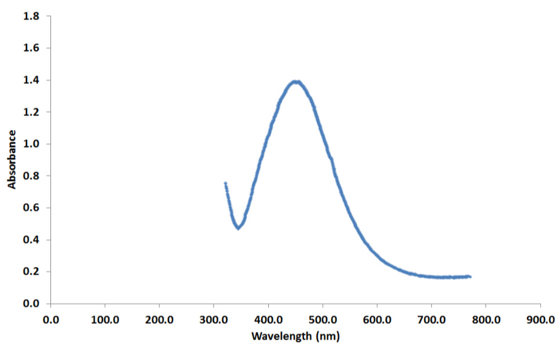

Fig. 2. UV- visible spectroscopy of green synthesized silver nanoparticles

spectroscopy of the AgNPs synthesized by the green method can be seen. Since the extract concentration used in the reaction solution was first made, the spectrophotometer device was standardized with it, and absorbance was equal to zero; the absorption obtained from the resulting solution after the synthesis reaction only shows the absorption of nanoparticles. A peak of absorption equal to 1.38 at a wavelength of 430 nm indicates the formation of AgNPs because their maximum absorption is at this wavelength.

As can be seen from the results of DLS and TEM, most of the AgNPs are in the nanometer size (10 nm-200 nm), and their morphology is generally spherical, rod-shaped, or triangular (Fig. 3 A and B).

The XRD patterns showed the diffraction peaks in the 2θ range from 30° to 80° (Fig 4).

The pattern of peaks shown in the XRD results indicates the presence of silver in the synthesized nanoparticles. The surface charge of nanoparticles, which is expressed by potential zeta, is one of the indicators of the stability of nanoparticles. After conducting the zeta potential test on AgNPs, it showed a value equal to -50 mV (Fig. 5).

According to HPLC results, cyanidin-3-O-galactoside, gallic acid, and catechin were the most

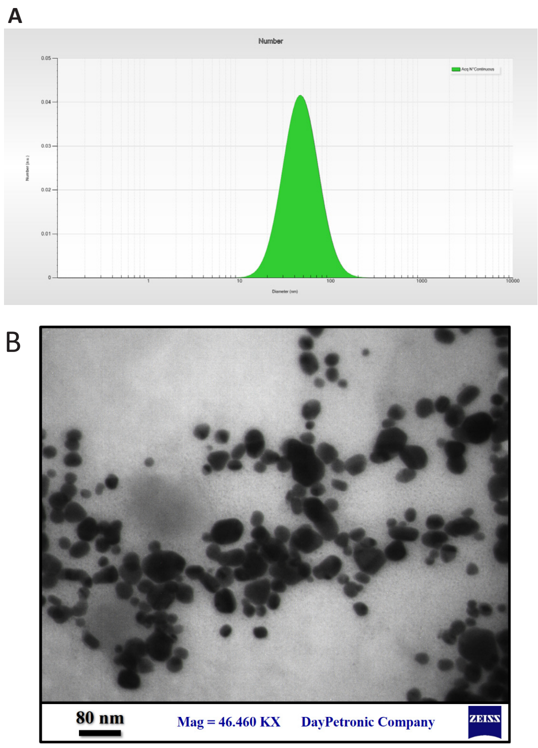

Fig. 3 A: Particle size distribution of synthesized AgNPs using aqueous extract of Pistacia vera, B: The morphology of AgNPs was imaged by transmission electron microscope (TEM)

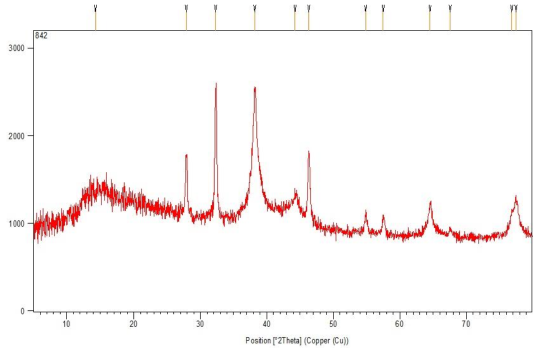

Fig. 4. XRD pattern of the synthesizes AgNPs in green synthesis

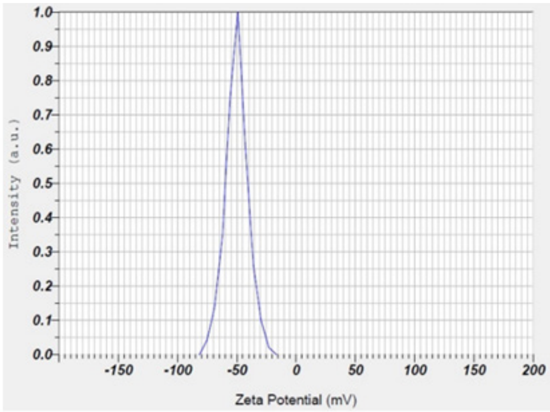

Fig. 5. The image of Zeta potential of AgNPs

abundant compounds in the studied pistachio cultivar (Table 1).

Table 1. HPLC analyses of the polyphenolic compounds content (mg/100 g dried hulls) in Fandoghi pistachio hull cultivars

Compounds	RT[1] (min)	Concentration (Mean±SD[2])
Gallic acid	5.7	42.6±0.11
Cyanidin-3-*O*-galactoside	22.75	186.7±0.15
Catechin	28.8	7.41±0.07
Epicatechin	40.2	5.23±0.08
Eriodictyol-7-*O*-glucoside	50.7	14.02±0.14
Naringin	58.1	3.23±0.05
Eriodictyol	63.2	1.62±0.08
Naringenin	70.4	0.76±0.04
Quercetin	68.1	0.82±0.04
Kaempferol	79.6	0.02±0.01
Luteolin	72.7	0.55±0.05

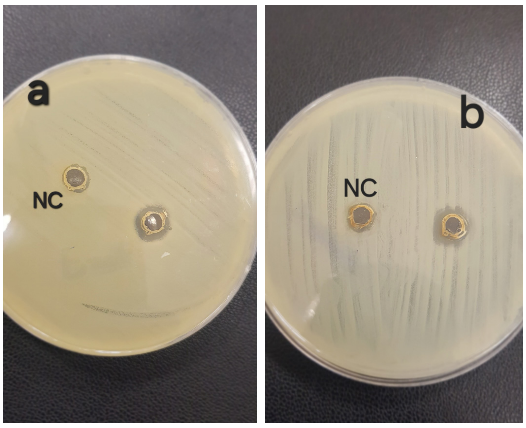

Fig. 6: Zone of inhibition for a: *E. coli* (ATCC: 25922) and b: *S. aureus* (ATCC: 25923)
NC: Negative control

Antibacterial activity of AgNPs

The AgNP's antibacterial activities were evaluated against several pathogenic gram-positive and gram-negative bacteria at 40 mg/ml concentrations by agar well diffusion method. The zone of inhibition for *E. coli* (ATCC: 25922) and *S. aureus* (ATCC: 25923) was 13 and 11 mm, respectively. The AgNPs were inactive against other gram-negative and gram-positive bacteria (Fig. 6).

Antibacterial activity results against six bacterial strains using MIC and MBC methods are shown in Table 2. The most sensitive strains were *E. coli* (ATCC: 25922) and *S. aureus* (ATCC: 25923); other bacteria were resistant.

The MIC and MBC were evaluated for 15 methicillin-resistant *S. aureus* (MRSA) and 15 extended-spectrum beta-lactamase(ESBL) producer *E. coli* clinical isolates. From 15 MRSA, 12 isolates showed 40 mg/ml MIC, and 3 isolates 20 mg/ml. Except for one isolate with MBC=10 mg/ml, in other MRSA isolates, MIC and MBC were the same. In ESBL-positive *E. coli* isolates, 11 have MIC= 20 mg/ml, three have MIC=10 mg/ml, and one isolate was completely resistant. Three ESBL-positive *E. coli* isolates had 10,5 and 2.5 mg/ml MBC; in other isolates, MBC and MIC were the same.

DISCUSSION

One of the most critical challenges in disinfection is the resistance of bacteria to common antibiotics, which is increasing daily. The main reason for this is the indiscriminate and uncontrolled use of antibiotics [18]. Nanotechnology has largely overcome this problem by providing various nanoparticles with antibacterial properties because these particles can destroy bacteria with different mechanisms and prevent them from developing resistance [19].

Silver ion has been used as a bacterial growth inhibitor and bacteriocidal compound in medical science and industry for years. The effects of silver metal particles on bacteria are different compared to their nanometer size. Silver ion has effects such as reacting with thiol groups of enzymes, preventing DNA replication, and changing the cell membrane structure. If silver is in the form of nanoparticles, it works differently. According to the studies, silver nanoparticles can have effects such as severe disturbance in the permeability and respiration of bacteria in the size of 1 to 10 nm, penetration into the bacterial cell and damage to DNA, and the release of silver ions, which have their own effects [1].

In this study, silver nanoparticles, which have higher antibacterial properties than other nanoparticles, were produced by pistachio hull extract and the green synthesis method. Considering that these nanoparticles showed acceptable stability after characterization and their size was in the nanometer scale range, the green synthesis of silver nanoparticles using pistachio hull can replace common chemical and

Table 2. Determination of MIC and MBC of six bacterial strains

Microorganism	Strain	MIC (mg/ml)	MBC (mg/ml)
Escherichia coli	ATCC: 25922	10	10
Klebsiella pneumonia	ATCC: 13883	-	-
Pseudomonas aeruginosa	ATCC: 27853	-	-
Acinetobacter baumannii	NCTC: 13305	-	-
Staphylococcus aureus	ATCC: 25923	20	20
Enterococcus feacalis	ATCC: 29212	-	-

ATCC: American Type culture collection; NCTC: The National Collection of Type Cultures; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration physical methods. Also, in addition to creating a stronger antibacterial effect due to the presence of pistachio hull extract, which has antibacterial properties, it could prevent the addition of toxic chemical substances to nature and reduce the cost and time of making these nanoparticles [13].

Gallic acid, cyanidin galactoside, and catechin were the most polyphenolic compounds in the Fandoghi hull cultivar that were used for AgNP production. Gharibi et al. compared the polyphenolics in different pistachio hull cultivars, and the Fandoghi cultivar was the best in terms of most non-volatiles esp. cyanidin-3-O-galactoside [20]. This compound was also the most frequent one in the Bellocco et al. report [21]. Fandoghi's hull cultivar revealed a relatively high amount of flavonoids, such as catechin, that are consistent with those obtained by Gharibi et al. [20].

The present study exhibited that AgNPs have antimicrobial activity against two bacteria: *E. coli* and *S. aureus*. Our results were supported by several studies conducted on nanoparticles synthesized by using Iran's pistachio. The inhibition of the growth of *S. aureus* was observed by synthesized AgNPs using an aqueous extract of Pistacia atlantica [12]. The green AgNPs had antimicrobial properties on *P. aeruginosa, S. aureus, Streptococcus pyogenes, E. coli, Salmonella paratyphi, and K. pneumonia* [13]. Antimicrobial activities against *E. coli* were seen in the Kashi et al. study [22]. Nanoparticles could create a structural change in the bacterial cell membranes, disrupting membrane integrity and leading to cell death [23]. Due to their small size and large surface area, some papers believe they can pass through the bacteria membrane pores and affect DNA and protein synthesis [24]. Also, it has been demonstrated that nanoparticles inhibit the respiratory process of the bacterial cell, and cell death occurs [1].

In 2017, Word Health Organization (WHO) published a list of bacteria that were a serious threat to human health, and there was an urgent need for new antibiotics to destroy them. The list was grouped according to their critical, high, and medium priority. A significant part of the list consists of gram-negative bacteria. Due to their spectfic structure, Gram-negative bacteria are more resistant to antibotics than Gram-positive bacteria. According to the WHO list of priority pathogens, ESBL-positive *E.coli* is one of the members of priority one and is considered a critical bacteria to encourage researchers to develop new antibiotics. In this study, the green synthesized AgNPs had lower MIC and MBC on ESBL *E.coli* as a resistant gram-negative and were more effective. In this list, MRSA is placed at the level two, which is a high priority. Therefore, the antimicrobial activity of AgNPs against *S.aureus* could be a significant achievement, although it needs further investigation [25, 26].

On the other hand, the antibacterial properties of the studied nano-samples can be attributed to poly-phenolic compounds in pistachio hull. The antimicrobial effect of polyphenolics has been mentioned in various studies around the world. For instance, catechin and kaempferol have antibacterial effects on *S. aureus*, and catechin and epicatechin have antibacterial effects on Enterobacteriaceae [27]. In the study by Bhattacharya et al. on the catechin extracted from Kombucha tea, the size of the inhibition zone for *E. coli* ATCC 25922 was almost 21 mm, and MIC and MBC were equal to 6.25 mg/ml [28]. Also, in another study on catechin extracted from *Libidibia ferrea* and *Parapiptadenia rigida*, the size of the inhibition zone against *E. coli* ATCC 25922 was 7 mm and 8.3 mm; respectively [29]. In the current study, the effect of polyphenolic compounds was not investigated separately, but the antibacterial effect of AgNP could originate from these compounds. In addition to the concentration and composition of polyphenols, the type of solvent is also effective in their antibacterial effects. In this study, aqueous extract of pistachio hull was used, and since the best nanoparticles were obtained with aqueous extract, only this solvent was used. Further studies are needed to investigate the effect of other solvents.

In this study, the antibacterial activities of AgNPs against six pathogens were tested, and only 2 showed antimicrobial properties. The growth of 6 bacteria (*S. pyogenes, S aureus, S. paratyphi B, K. pneumonia, E. coli, and P. aeruginosa)* was inhibited by AgNPs in the Golabiazar et al. study [13]. This difference might be due to the size and nature of the AgNPs used in various studies.

Our data showed silver synthesized nanoparticles had a higher zone of inhibition and lowered MIC and MBC on *E. coli* compared to *S. aureus*. Behravan et al. studied the antibacterial effect of synthesized AgNPs using Berberis vulgaris leaf and root on *E. coli* and *S. aureus*. They reported that the effect on *E. coli* was higher than on *S. aureus* [30]. In a study in Iran, the antibacterial activity of the aqueous extract of saffron and purchased and biosynthesized AgNPs on six bacteria, including *E. coli* and *S. aureus,* was tested. The results showed that only biosynthesized AgNPs could inhibit the growth of all bacteria. The control strain of *E. coli* was more sensitive (MIC =16 µg/ml) than the tested *S. aureus* strains (MIC= 32 µg/ml) [31]. The cell walls of gram-negative and gram-positive bacteria have structural differences, and the higher susceptibility of E. coli as a gram-negative could be related to the difference.

The synthesized AgNPs showed antibacterial effects against ESBL-positive *E. coli* and MRSA in clinical isolates collected from a hospital in Isfahan. Antibiotic resistance is increasing in the world and in Iran. A review article assessed the antibiotic resistance of *E. coli* isolates in Iran from 2007 to 2016, revealing that it is on the rise [31]. Also, high resistance to different antibiotics was reported for *S. aureus* in Iran [32]. For this reason, preparing materials with suitable antibacterial properties can be an excellent solution for overcoming antibiotic resistance. In a similar research in Tehran, Niakan et al. evaluated the antibacterial effects of AgNPs and 18 different antibiotics on isolated *A. baumannii* from clinical specimens. They concluded that the nanoparticle could be a bactericidal agent on the MDR pathogen [33]. In this way, it seems that the production of nanoparticles can help treat some infectious diseases.

CONCLUSIONS

The present investigation revealed an eco-friendly, cost-effective method for a synthesized nanoparticle using pistachio waste that can replace common chemical and physical processes. The prepared nanosilver showed antimicrobial activities against *E. coli, S. aurous*, and their MDR clinical isolates, and they could replace some antibiotics.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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