Original Article

Green Synthesis of Silver Nanoparticles Using the Plant Extracts of Vitex Agnus Castus L: An Ecofriendly Approach to Overcome Antibiotic Resistance

Sepideh Ghani, Behnam Rafiee[1], Samira Bahrami[2], Azam Mokhtari[1], Shahin Aghamiri[3], Fatemeh Yarian[3]

Student Research Committee, Department of Medical Biotechnology, School of Advanced Technology in Medicine, Shahid Beheshti University of Medical Sciences, [3]Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, [1]Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, [2]Acquired Immunodeficiency Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: These days, silver nanoparticles (Ag NPs) have been given considerable attention and applied in medical technology due to their great antimicrobial and antioxidant features. In the present study, we aimed to synthesize Ag NPs through the reduction of silver nitrate in the presence of *Vitex agnus castus L* fruit extract. **Methods:** After collecting fruits, their extract was prepared and added to Ag NO_3 to produce Ag NPs. The effect of different parameters like AgNO3 concentration (0.5, 1, 3, and 5 mM), sunlight exposure, and sunlight irradiation time (10, 20, 30, and 40 min) was investigated in the synthesis of Ag NPs. The features of Ag NPs were characterized using UV-visible spectroscopy, scanning electron microscope (SEM), X-ray diffraction (XRD) analysis, and dynamic light scattering analysis. Moreover, antimicrobial function of Ag NPs was evaluated using *Escherichia coli* and *Bacillus cereus* bacteria species and minimal inhibitory concentration (MIC) of Ag NPs against these two pathogens was measured. **Results:** The results showed that the synthesized nanoparticles had a spherical shape and the range size of 30-60 nm. For the first time, the antimicrobial activity of synthesized Ag NPs of *Vitex agnus castus L* fruit extract was shown. **Conclusions:** It can be stated that the biosynthesis of Ag NPs using fruit extract of this plant is an environmentally friendly, economic and harmless method without any use of poisonous substances and no side effects. These Ag NPs can be considered as suitable antibacterial agents and replacements for antibiotics.

Keywords: *Antibacterial activity, green synthesis, silver nanoparticles, Vitex agnus castus L*

Introduction

Nanotechnology is the science of making particles in the range of 1-100 nm and exploring the relation of different features of synthesized nanoparticles with their dimensions.[1] Nanoparticles have various applications in packaging food materials, making personal care, and delivering drugs to improve treatment efficacy.[2] They have unique optical features because of their large surface area to mass ratio.[3] These days, silver nanoparticles (Ag NPs) have been given considerable attention and applied in medical technology due to their great antimicrobial and antioxidant features. Moreover, they act as a great catalyst in several chemical reactions.[4]

Ag NPs are produced through various methods such as chemical, physical, and biological methods. Chemical method as the most common production one is usually expensive. The toxic and noxious materials that are used in this procedure leads to low yield of synthesis and intricate purification downstream methods.[5] Therefore, biological methods as the cleaner, safer, cheaper, and more environmentally compatible methods have been developed.[6,7]

The most major sources in biological methods are bacteria, fungi, and plants.[6] Among them, plants or their extracts are the most advantageous source. They omit the downstream process of cell culture and can develop for extensive production of nanoparticles. Moreover, plant extracts contain different metabolites playing as reducing and capping agents during the green synthesis of nanoparticle.[8] Therefore, pharmaceutical plants as valid and safe sources for medicinal applications are widely applied in controlled size and shape production of Ag NPs.[9]

Various parts of plants include seeds, roots, leaves, and fruits are applied to prepare plant extracts for the green synthesis of NPs. In this method, the extract is mixed

Address for correspondence:
Dr. Fatemeh Yarian,
SBUMS, Arabi Ave, Daneshjoo Blvd, Velenjak, Tehran, Iran.
E-mail: f.yarian@sbmu.ac.ir

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with a silver solution. Then, the reaction was performed quickly at room temperature.[10] Different factors such as the kind of plant and concentrations of the plant extract and silver salt temperature, time of reaction, and pH can affect the speed, quantity, and other features of Ag NPs.[11]

Vitex agnus-castus L. (agnus castus) is a deciduous shrub belonging to the Verbenaceae family. This plant is native to the Mediterranean countries and spread in Asia, Europe, and North America. It has been used as a medicinal plant, and its fruits have traditionally been used in Iran, Italy, Greece, and Egypt to treat gynecological disorders for over 2500 years.[12] Furthermore, Vitex agnus-castus L. Contains numerous bioactive metabolites (flavonoids, ketosteroids, iridoids, essential oils, etc.) with high reducing features and great activity in the green synthesis of Ag NPs.[13]

In the present study, we aimed to synthesize Ag NPs through the reduction of silver nitrate in the presence of *Vitex agnus castus* L fruit extract. Then, effective synthesis of Ag NPs was evaluated through different parameters. The synthesized Ag NPs were characterized using UV-visible spectroscopy, scanning electron microscope (SEM), X-ray diffraction (XRD) analysis, and dynamic light scattering analysis. The antimicrobial function of Ag NPs was evaluated using two typical human pathogenic species of bacteria (*Escherichia coli (E. coli)* as Gram-negative and *Bacillus cereus* (*B. cereus*) as Gram-positive bacteria). The minimal inhibitory concentration (MIC) of Ag NPs against these two pathogens also was measured.

Methods

Preparation of fruit extracts of *Vitex agnus castus L*

The fruits of *Vitex agnus castus L* were gathered in spring from around Qom city (Iran). Fruits were washed thoroughly with 1 Liter of distilled water and maintained at room temperature within two days to dry completely. Then, dried fruits were powdered with the grinder. The aqueous extract was prepared by mixing 10 g powder of *Vitex agnus castus L* fruit with 100 mL sterilized distilled water (ratio: 1:10). Then, the extract was boiled in the Bain-marie for 10 minutes. After cooling, the extract was filtered with Whatman filter paper and stored at 4°C for later experiments.

Green synthesis of silver nanoparticle

For the green synthesis of Ag NP, 10 ml of prepared extract was added to 90 mL of 1 mM AgNO3 (MW: 169.87, 99.99% purity, Merck, Germany) at room temperature. The appearance of brown color in the solution indicated the Ag NPs formation.[14]

Exploring the effect of different parameters on the green synthesis of Agnanoparticles

The effect of concentration of $AgNO_3$ salt

To explore and optimize the concentration of $AgNO_3$, 90 mL of different $AgNO_3$ concentrations (0.5, 1, 3, and 5 mM) were added to 10 mL of Vitex *agnus castus L*. fruit extract. UV-visible spectra of all solutions were measured and the optimal concentration was selected.

The effect of sunlight exposure

Two solutions of optimal concentration of $AgNO_3$ were prepared to explore the effect of sunlight on the synthesis of Ag NP. They were separately located at room temperature and sunlight exposure. Then, UV-visible spectra of both solutions were measured, and the optimal condition was selected.

The effect of sunlight irradiation time

To explore and optimize the effect of sunlight irradiation time on the synthesis of Ag NPs, considering the optimal conditions (concentration and light), solutions were prepared at various sunlight irradiation times (10, 20, 30, and 40 min). Then, UV-visible spectra of each solution were measured separately and the optimal sunlight irradiation time was selected. For all conditions, brown color in the solution was the indicator of Ag NP formation, and the UV-visible spectra for measuring by spectroscopy were in the range of 300-700 nm.

Characterization of Ag nanoparticles

Absorption spectra of nanoparticles were measured using a Uv-vis SERIES8000CECIL spectrophotometer at 300-700 nm. The SEM device, HITACHI S-4500 model, was used to assay the shape and size of Ag nanoparticles. Dynamic light scattering (DLS) analysis was performed (Zetasizer, Malvern-England) to determine the average size distribution and zeta potential (an indicator of nanoparticle stability) of produced nanoparticles. Moreover, to verify the structure of synthesized Ag nanoparticles, X-ray diffraction analysis (XRD) was performed. For this purpose, the solution containing Ag NPs was centrifuged at 12,000 rpm for 15 min three times. Then, the obtained pellets were air-dried thoroughly and used for XRD analysis.

Antimicrobial assays

Disk diffusion assay

Antimicrobial activity of synthesized Ag NPs against the gram-positive and gram-negative bacteria was investigated using the Disk diffusion assay. Based on the standard, bacterial suspensions were prepared equivalent to 0.5 McFarland. Applied bacteria included *B. cereus* (PTCC: 1015) and *E. coli* (PTCC: 1399). Both bacteria were separately cultured on Mueller-Hinton agar. 8 µL of each sample was poured on the disk to prepare the disks. The disks were dried under sterile conditions. Then, on each plate were put eight disks (6 mm diameter) containing 0.5-, 1-, 3-, and 5-mM concentrations of Ag nanoparticles, negative control (water and fruit extract), and positive control (antibiotics Gentamicin and Ciprofloxacin). They were incubated in the incubator at 37°C for 24 hours. Finally, the area where bacteria have not grown was observed and measured.[6]

Minimum inhibitory concentration (MIC) assay

Different concentrations of Ag NP (0.5, 1, 3, and 5 mM) were used to measure the MIC of Ag NPs. For this assay, negative control was considered water and fruit extract and positive control was considered Gentamicin and Ciprofloxacin antibiotics. The assay was performed on a sterile 96-well plate. The columns were as follows: column A for the 0.5 mM concentration, column B for the 1 mM concentration, column C for the 3 mM concentration, column D for the 5 mM concentration, column E for Gentamicin antibiotics, column F for ciprofloxacin antibiotics, column G for fruit extract control, and column H for water control.

Briefly, first Muller Hinton Broth culture media (100 µL) was added to all wells. Then, 200 µL of 0.5 mM Ag NP concentration stock was added to first well. After it, 100 µL of the first well was added to the second well. Similarly, various dilutions of this stock were prepared. Also, this act was performed for other rows. Finally, 100 µL of diluted microbial suspension, equivalent to 0.5 McFarland, was poured into each well. After 24 hours of incubation at 37°C, the degree of turbidity indicates the bacteria's growth. The final concentration that was clear and without turbidity is introduced as MIC. All actions were similarly performed for *S. aureus.*[6]

Results

Optimization of effective parameters on the Ag NP synthesis

The optical properties of nanoparticles relating to their shape and size are among the most important properties in their identification.[15] In the synthesis of Ag NPs, the brown color indicates the formation of the nanoparticles in the aqueous fruit extract of *Vitex agnus castus L* [Figure 1a].

The absorption spectrum was analyzed to investigate the effect of metal ion concentration on the synthesis of Ag NPs. This spectrum showed that a gradual increase in the concentration of silver ions leads to an increase in the NPs adsorption. In other words, it can be said that at higher concentrations of AgNO3, the conversion rate of Ag^+ to Ag is more elevated and consequently, the concentration of nanoparticles is higher [Figure 1b]. This increase continued up to a concentration of 3 mM but, at a higher concentration (5 mM) no significant increase in the amount of NPs adsorption was observed. As a result, 3 mM silver nitrate concentration was selected as the optimal concentration.

To investigate the effect of light on the synthesis of nanoparticles, 10 ml of fruit extract of *Vitex agnus castus L* was added to 90 ml of 3 mM AgNO3 and exposed to both room temperature and sunlight. The color changed from light green to light brown at room temperature and from light green to dark brown under sunlight, indicating the formation of Ag NPs [Figure 2a]. Absorption of samples under both conditions was investigated using UV-Vis spectroscopy in the range of 300-700 nm. Fruit extract and silver nitrate were considered as controls [Figure 2b]. Synthesis of Ag NPs using fruit extract of *Vitex agnus castus L* under sunlight exposure showed a sharper absorption peak at about 450 nm.

The effect of sunlight irradiation time (10, 20, 30 and 40 minutes) on the formation of Ag NPs was investigated under optimal conditions (concentration of 3 mM silver nanoparticles and sunlight exposure). The maximum absorption was observed at 450 nm, which is the characteristic of Ag NPs. The results showed that the increase of irradiation time enhanced the interaction between reactants until 30 min. After this time, no significant change was observed in the amount of absorption, which proves the stability of the obtained NPs. As a result, 30 min was chosen as the optimal time [Figure 3].

Characterization of Ag nanoparticles

SEM analysis

Biosynthesized Ag NPs using the fruit extract of *Vitex agnus castus L* were analyzed by SEM to determine their morphology and size [Figure 4a]. The spherical shape and the range size of 30-60 nm of synthesized Ag NPs were confirmed by SEM micrograph.

DLS analysis

Using DLS analysis, the average size distribution of the synthesized Ag NPs was obtained by measuring the dynamic change of light scattering intensity resulting from the brown motion of the synthesized particles. The diameter of the synthesized Ag NPs in the optimal conditions was obtained at 50-70 nm. The polydispersity index (PDI) and the zeta potential were measured at 0.251 and78.77, respectively, which indicates the high and suitable uniformity of the synthesized Ag NPs [Figure 4b].

XRD analysis

XRD analysis was performed to study the crystal structure of Ag NPs. The obtained XRD pattern from X-ray diffraction of these NPs at 2 θ angle showed 4 sharp peaks in (38.3 º, 44.1 º, 63.9 º, and 77.2 º), which were assigned to points (111), (200), (220), and (311), respectively [Figure 4c]. Therefore, the XRD pattern indicated the crystalline nature of Ag NPs and confirmed the formation of Ag NPs from the fruit extract of *Vitex agnus castus L*. These findings were consistent with the results of other researchers.

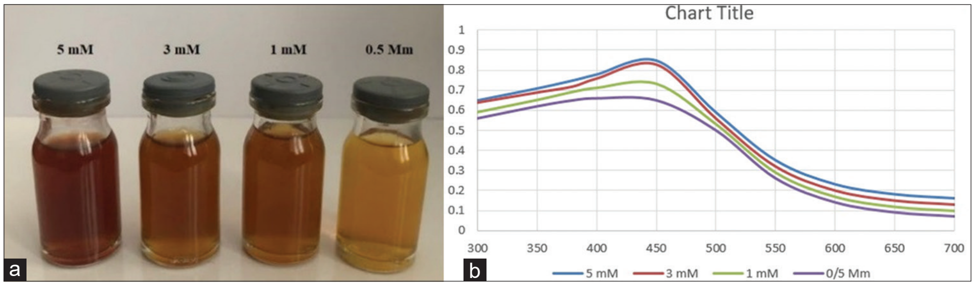

Figure 1: The effect of concentration of $AgNO_3$ salt. (a): The appearance of brown color in different concentrations of Ag nanoparticles; (b): evaluating the effect of different concentrations of $AgNO_3$ salt on the synthesis of Ag NPs

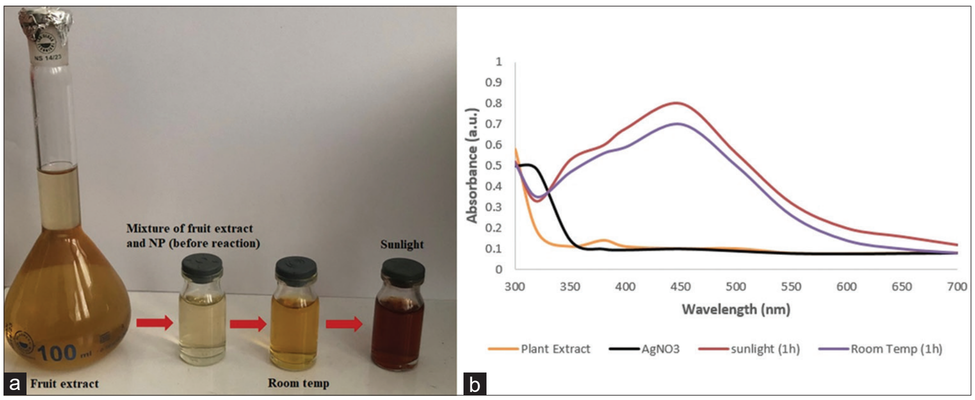

Figure 2: (a): Ag NP biosynthesis; (b): Biosynthesis of Ag NP under room temperature and sunlight exposure after 1 hour. The fruit extract and silver nitrate salt were used as controls

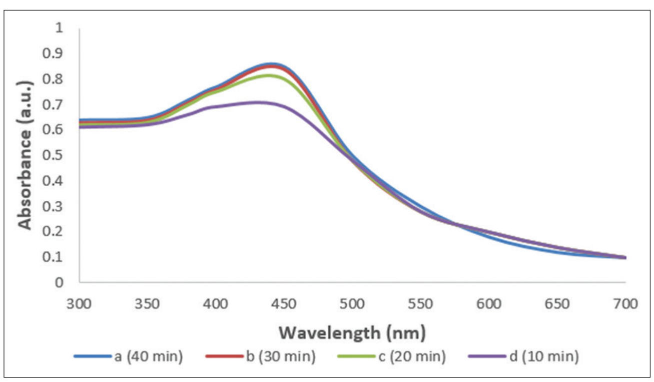

Figure 3: Uv–vis spectra of synthesized Ag NPs at different sunlight irradiation times (10, 20, 30, and 40 min)

Antimicrobial assays

Disk diffusion analysis

Antimicrobial activity of synthesized silver nanoparticles was performed using the disk diffusion method against two different pathogens including, *B. cereus* (Gram-positive) and *E. coli* (Gram-negative). Based on measuring the diameter of the cloud's lack of growth, the antimicrobial effect of Ag NPs enhanced by increasing their concentration. Therefore, the highest antimicrobial effect was observed at 5 mM concentration of Ag NP in both bacterial strains. Also, *Vitex agnus castus L* fruit extract had no antimicrobial effect on both strains [Figure 5].

MIC analysis

The MIC of synthesized Ag NPs was determined using the MIC test on *B. cereus* and *E. coli*. Different concentrations of AgNP (0.5, 1, 3, and 5 mM), water and extract (negative controls) and gentamicin and ciprofloxacin antibiotics (positive controls) were assayed in this test.

MIC results showed the highest antibacterial effect for *E. coli* at 5 mM with MIC: 3/12 µg/ml and the lowest at 1 mM with MIC: 25 µg/ml. Also, at a concentration of 0.5 mM, silver nanoparticles had no antibacterial effect on *E. coli*.

In the case of *B. cereus*, the highest antibacterial effect was observed at a concentration of 5 mM with MIC: 12/5 µg/ml and the lowest at a concentration of 3 mM with MIC: 25 µg/ml. It should be noted that silver nanoparticles at concentrations of 0.5 mM and 1 mM had no antibacterial effect on *B. cereus*. MIC was obtained for gentamicin and ciprofloxacin antibiotics for *E. coli* 3/12 and 6/25 µg/ml, respectively, and for B. cereus 6/25 and 1/56 µg/ml respectively. Also, no antibacterial activity was observed for water and fruit extract. In general, the results of the MIC test were consistent with the results of the Disk diffusion test.

Discussion

Recently, the use of Ag NPs in medical sciences has significantly increased due to the advancement of

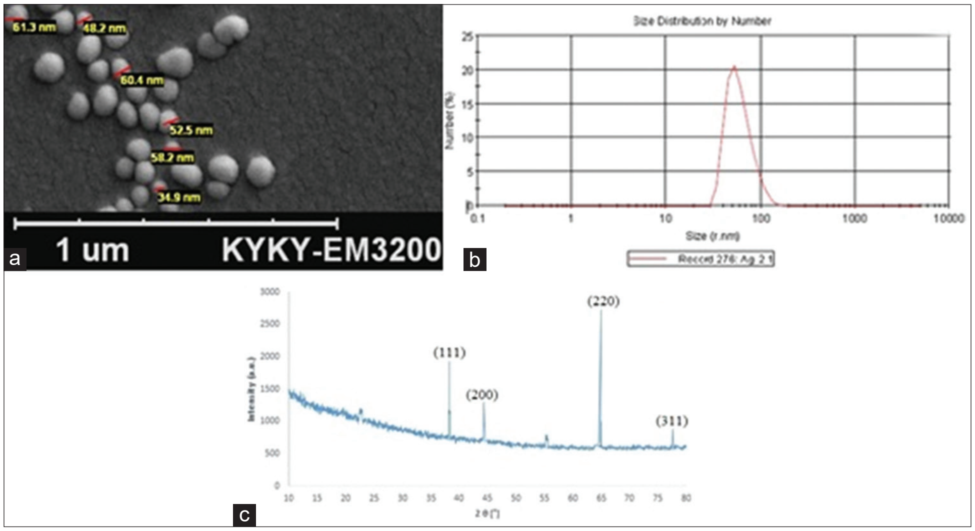

Figure 4: Characterization of Ag nanoparticles. (a): SEM image of synthesized silver nanoparticles by fruit extract of *Vitex agnus castus L*; (b): DLS analysis showing diameter of synthesized silver nanoparticles by fruit extract of *Vitex agnus castus L*; (c): X-ray diffraction patterns (XRD) of biosynthesized silver nanoparticles

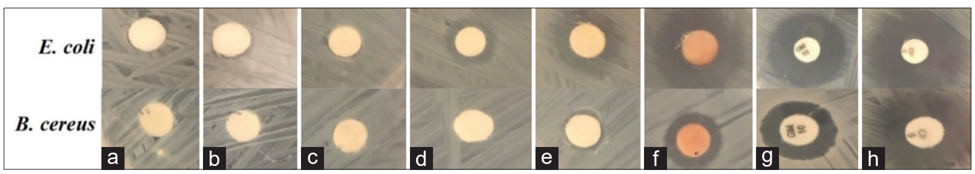

Figure 5: Disk diffusion assay on different concentrations of Ag NP on *E. coli* and *B. cereus* strains; (a): negative control (water), (b): negative control (extract), (c-f): 0.5 mM, 1 mM, 3 mM, and 5 mM concentrations of Ag NPs respectively, (g): positive control (gentamicin), (h): positive control (ciprofloxacin)

nanotechnology, and the high potential of antimicrobial properties of silver nanoparticles.[16] In principle, green nanoparticle synthesis methods have priority over physical and chemical synthesis methods due to lower cost, energy, and time. Moreover, green synthesis methods do not require toxic solvents and hazardous substances for the environment and are considered as environmentally and biocompatible methods.[17-20] Biosynthesized Ag NPs have significantly excellent medicinal properties. For example, they have antioxidant, anticancer, anti-inflammatory, anti-diabetic, anti-parasitic, and antimicrobial potentials.[21-23]

The present study showed that the aqueous extract of *Vitex agnus castus L* fruit could produce Ag NP from $AgNO_3$ through green synthesis method and it was further determined that these particles have antibacterial activity against *E. coli* and *B. cereus*.

Ag NPs contain four known antimicrobial effects: 1) adhesion to the surface membrane of microbes 2) penetration into bacterial cells and disruption of intracellular structures and biomolecules damage 3) production of reactive oxygen species (RoS) causing oxidative stress and cytotoxicity in cells 4) disruption of cell signal transduction pathways.[24]

Ag NPs have a high tendency to sulfur and phosphorus in the bacterial cell membrane leading to the development of their antibacterial properties. Since Ag NPs react with proteins containing these two elements and affect bacterial survival.[25] The antibacterial effect of Ag NPs on gram-negative bacteria is much more significant than that of gram-positive bacteria, which is related to the three-dimensional structure of their cell wall.[26] Since Ag NPs attach to the membrane surface of microorganisms, they can enter the cell through a water channel called purine in the outer membrane of gram-negative bacteria. After their entrance, they bind to cellular structures and biological molecules such as proteins, lipids and DNA and damage them[27,28] so that Ag NPs can react with the DNA of bacteria and cause its denaturation and finally stop the growth of bacterial cells.[29]

Therefore, nanotechnology is applied to solve a significant problem in microbiology, namely the fight against antibiotic-resistant microorganisms.[30] Wei *et al*.[31] in 2020, made Ag NPs using *Berry* extract of sea buckthorn and studied its antimicrobial effects on *S. aureus* and *E. coli*. The obtained results were promising, and the synthesized NPs had a spherical shape and a size of 10 to 20 nanometers.

In 2018, Shaik *et al*.[32] produced Ag NPs using *Origanum vulgare L* plant extract and its antimicrobial effects on a variety of bacterial and fungal strains, including gram-positive (*S. aureus*, *S. epidermidis*, *micrococci Luteus* and methicillin-resistant *Staphylococcus aureus* (MRSA)) and gram-negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Shigella sonei*, *Salmonella typhimurium*) and pathogenic fungi (*Aspergillus flavus*, *Alternaria alternate*, *Phialophora alba*, *Paecilomyces variotii*) were studied. The antimicrobial activity was observed in all strains. The synthesized NPs in this study had a spherical shape and a size of 20 to 25 nanometers.

Carson *et al*.[33] in 2019, produced Ag NPs using *Phyla dulcis* extract and its antibacterial effects on two gram-positive bacteria (*Listeria monocytogenes* and *S. aureus*) and two gram-negative bacteria (*E. coli* and *Salmonella Typhimurium*) were studied. The crude extract of the plant had no antibacterial effect, but the synthesized Ag NPs had antimicrobial properties on all bacterial strains.

Moreover, in another study in 2020, Rajkumar *et al*.[34] produced Ag NPs using *Gymnema Sylvestre* leaf extract. The green synthesized NPs had antibacterial activity against *E. coli* and *S. aureus*, and they had a spherical shape and a size of 20-30 nm.

Conclusions

In this study, the fruit extract of *Vitex agnus castus L* was used for the green synthesis of Ag NPs and their antibacterial activity was investigated. It is the first report on discovering of antimicrobial activity of synthesized Ag NPs of fruit extract of this plant. It was observed that the synthesized nanoparticles had a spherical shape and their shape and stability were affected by various parameters such as $AgNO_3$ concentration, light irradiation and time of irradiation. In general, it can be stated that the biosynthesis of Ag NPs using fruit extract of *Vitex agnus castus L* is an environmentally friendly, economical, and harmless method without any use of poisonous substances and no side effects. Moreover, the present findings showed that biosynthesized nanoparticles have good antibacterial activities that can be considered suitable antibacterial agents and replacements for antibiotics.

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Conflicts of interest

There are no conflicts of interest.

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