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Green Synthesized Silver Nanoparticles Using Anise (*Pimpinella anisum L.*) have Antibacterial Effects

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ABSTRACT

Introduction: Silver nanoparticles are particles of silver with a size of 1 to 100 nm. These agents have various applications and particularly have received much attention for their antibacterial activity and their use in vaccine production. Among the various methods of synthesizing nanoparticles, using plants due to their high reducing capabilities and also their eco-friendliness is of interest. **Methods:** Silver nanoparticles (AgNPs) were synthesized using plant anise (*Pimpinella anisum L.*) and validated using UV-spectrophotometer, transmission electron microscopy and Fourier transform infrared spectroscopy. The produced AgNPs were used against *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Enterococcus faecalis* to examine their antibacterial activities via agar well diffusion, disk diffusion and minimum inhibitory concentration methods. Furthermore, AgNPs were used in combination with three antibiotic disks, namely, Ceftriaxone, Tetracycline and Gentamicin to seek any cooperative effect. **Results:** Antibacterial effects due to the synthesized AgNPs were observed toward *E. coli*, *S. aureus*, *S. typhimurium* in this order; however, *E. faecalis* showed the highest resistance to the synthesized AgNPs. **Conclusion**: AgNPs synthesized using anise had similar antibacterial effects as conventional antibiotics; however with potentially less side effects.

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INTRODUCTION

Nanotechnology has gained importance in many fields of science and technology in recent years [1]. Nanoparticles are particles with a size of 1 to 100 nanometers [2]. Some metal nanoparticles such as silver, gold and platinum are broadly applied in medicine and pharmaceutical industries as well as common consumables such as detergents and cosmetics [3, 4]. Nanoparticles are also used in the production of new generation of vaccines as both antigen nanocarriers and adjuvants[5-7]. Nowadays, nanoparticle-based vaccines have attracted a lot of attention due to their high-efficiency in stimulating the humoral and cellular immune responses as well as their low risks for human consumption [8-10].

Silver nanoparticles (AgNPs) are widely used in recent years due to their low toxicity compared to alternative chemical compounds [11]. Moreover, using biological methods for nanoparticle synthesis such as by microorganisms and plants with their high potential of reducing metal are considered as eco-friendly, and cost-effective compared to conventional means of synthesis [12, 13]. Although chemical methods are easier to perform than green synthesis and have higher efficiencies, their applications for nanoparticles synthesis are deemed more toxic and detrimental to the environment. Alternatively, plant extracts can be suitably scaled up for large scale biosynthesis of AgNPs in a controlled manner, according to their size, shape, and sensitivity [14-16]. So far, green synthesis of nanoparticles using plant extract of *Andrachne cordifolia* [17], *Azadirachta indica* [18], *Medicago sativa*[19], *Gliricidia sepium [20]*, *Aloe vera* [21], *Chenopodium album* [22], *Capsicum annuum* [23], *Citrus sinensis* [4], *Cinnamon zeylanicum* [24] have been reported, to name a few.

Pimpinella anisum L. (Apiaceae), also known as aniseed or anise is an annual aromatic herb and a grassy plant with white flowers and small green to yellow seeds [25]. It is native to the eastern Mediterranean region while it has been used in traditional Iranian medicine as a remedy for carminative, neurologic, anticonvulsant, respiratory disorders, disinfection,

epilepsy, galactagogue, anti-asthma, and dyspnea [26]. Anise extracts have flavonoids, phenols and proteins while they also have antioxidant activities [26, 27]. The antioxidant activity of anise and the presence of agents such as flavonoids, proteins and phenols in it lead to the reduction of Ag^+ ions to nanoparticles [16, 28]. The biosynthesis of silver nanoparticles is a complex process during which their quantity and quality are affected by many factors, such as pH, temperature and time [29]. Here, we used anise extract as a reducing agent to produce AgNPs and evaluated its antibacterial activity against 4 bacterial pathogens of human, namely *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and *Enterococcus faecalis*.

MATERIALS AND METHODS

Anise Extract Preparation

Anise plants were collected from a farm pasture at Bu-Ali Sina University (Hamedan, Iran). Seeds and stems of anise were weighed carefully and rinsed with tap water to remove excess dirt, dust, and mud, and then dried at room temperature. After adding 100mL of deionized double-distilled water, to 5 g anise seeds and stems and boiling them at 100˚C for 3 min, the mixture was cooled down at room temperature and then filtered with Whatman filter paper No. 2. The filtered extract was stored at 4˚C [20].

AgNPs Biosynthesis

Pure $AgNO_3$ was purchased from Titran Co. (Tehran, Iran). A solution of 1 mM $AgNO_3$ was prepared for the biosynthesis of AgNPs. To complete the biosynthesis process, 5 mL of the anise extract were added to 250 ml $AgNO_3$ (1 mM) in an Erlenmeyer flask, incubated in dark at room temperature on a shaker (140 rpm) for 96 h [20].

UV-Vis Spectra Analysis

The formation and stability of AgNPs were determined with PerkinElmerLamda-45 UV-Vis spectrophotometry. The calibration of UV-Vis spectrophotometry was carried out with distilled water. To test the accuracy of the device, $K_2Cr_2O_7$ was used. The absorptions were monitored every 6 h for 96 h.

Transmission Electron Microscopy (TEM) Analysis

The synthesized AgNPs were centrifuged 4 times (13'000 rpm, 10 min) and washed with deionized distilled water and then put in a dark place at room temperature overnight to dry it out and obtain its powder form. Inside an Eppendorf vial, ethanol was added to the nanoparticles and the vial was placed in an ultrasonic device for 10 min. Fifty µl of the sample was put on a copper grid, coated by carbon. The excessive sample was removed by a cone of a blotting paper and kept in a grid box to dry out. TEM (Zeiss, EM900, Germany) was used to analyze the formation and sizes of the nanoparticles.

Fourier-Transform Infrared (FTIR) Spectroscopy

To study the structure and formulation of the synthesized AgNPs and the extract, FTIR (PerkinElmer65) was used in the range of 4000-400 cm^{-1} with resolution of 0.01 cm^{-1} [30]. The powder of AgNPs and the extracts were prepared as described above for TEM. The obtained powders were then mixed with KBr with a ratio of 2/50 to achieve a relatively homogenized solution. The solutions were then examined with FTIR spectrometer.

Antimicrobial Activities

Antibacterial assays were carried out on 4 bacterial human pathogens (i.e. *E. coli*, *S. aureus*, *S. typhimurium* and *E. faecalis*) by standard agar well diffusion and minimum inhibitory concentration (MIC)[31]. The unfrozen bacteria were incubated on brain heart broth (BHB) for 24 h at room temperature and then were cultured on brain heart agar (BHA) for 24 h at 37˚C. Densities of 0.5 Macfarlane were produced from each bacterial culture. The bacteria were swabbed uniformly onto separate Muller-Hinton agar (MHA) plates by sterile cotton swabs. For agar well diffusion method, aqueous AgNPs was produced from the powdered AgNPs in 50 and 100 µg/ml densities. A sterile micropipette was used and 50 µl of each density was poured onto each well in all plates, and also 20 µl and 50 µl of the direct non-centrifuged aqueous AgNPs were poured onto each well. For the disk diffusion method, 50 µl aqueous from the prepared powdered AgNPs and non-centrifuged AgNPs were added to blank disks. Three antibiotic disk papers, containing Ceftriaxone, Tetracycline, and Gentamicin were used as controls and cooperative effects of these antibiotics and AgNPs were examined by addition of 10 µl AgNPs to each antibiotic paper [32, 33]. Examination of the antibacterial activity of MHA plates were also done. It should be noted that using 10 µl of AgNPs solution was more compatible with our antibiotic paper absorption capacity. All the plates were incubated for 24 h at 37˚C, except for *E. faecalis* that were cultured on BHA. After the incubations, the inhibition zones by the bacteria were measured in mm. All the experiments were carried out for at least 4 replicates.

MIC of the AgNPs for evaluation of their antimicrobial activity was assessed by using standard 96-wells microplates [31]. MHB medium (50 µl) was added to all wells except for *E. faecalis* in which BHI was used. AgNPs homogenized solutions (50 µl) with 100µg/ml densities were added to the first well and a serial dilution followed. Then, 10 µl of each bacterial suspension was added onto all wells of a row to be incubated for 18 h at 37˚C. Finally, 50 µl tetrazolium was added to the wells and re-incubated for additional 2 h.

RESULTS

The Reduction Process

The observation of the first stage of AgNPs synthesis was carried out with naked eyes. After 15 min of adding the anise extract to AgNPs, the color changed from yellowish to brown and within 96 h, the reduction was completed, making the color a stable clear yellow (Fig. 1). The reduction process was also detected by UV-Vis spectroscopy. AgNPs had absorption spectrum at different wavelengths ranging from 300 - 600nm, revealing a peak at 423nm. Fig. 2 shows an absorption graph of the synthesized AgNPs

TEM Analyses

The images obtained from TEM (Fig. 3) showed that the synthesized nanoparticles had different shapes such as circular, spherical, oval, and hexagonal as well as different sizes, ranging from 10 to 60 nm with an average size of 35 nm in diameter. As depicted in Fig. 1A, the AgNPs were capped by the reducing agents of the anise extract. These confining agents had stopped the growth of the AgNPs by shaping and stabilizing them.

Fig. 1. Right: A flask containing AgNPs, 1 min after adding the anise extract. **Left:** Complete reduction, after 96 h of adding the anise extract

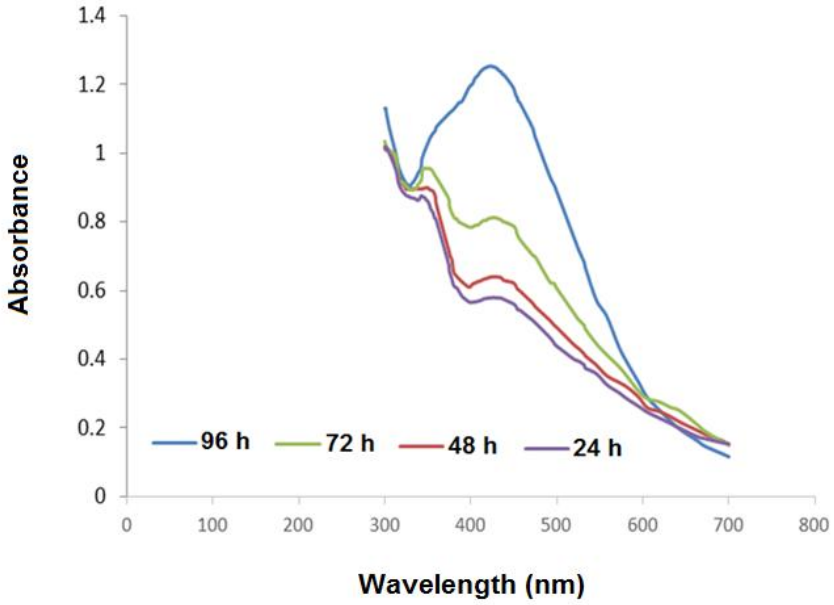

Fig. 2. The plot of UV-Vis spectrum of the synthesized AgNPs reduction process at 4 different time points (i.e. 24, 72, 48 and 96 h)

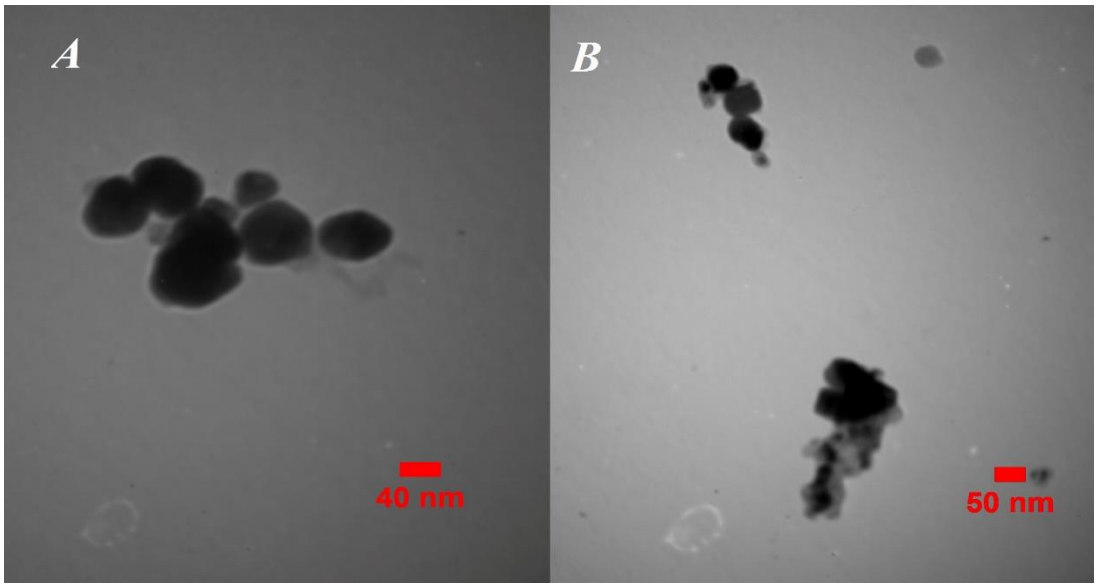

Fig. 3. TEM image of AgNPs recorded various sizes (scale bars: A: 40nm; B: 50nm)

FTIR Spectroscopy Analyses

FTIR analyses of the extract before and after the addition of AgNPs showed strong bands at 1376.50, 1393.67, 1560.40, 1624.94, and 1636.60 cm^{-1}. A strong band at 1636.60 is attributed to carbonyl groups involved in nanoparticle production. Table 1, Fig.4 and Fig.5 show the FTIR spectra of anise before and after the reduction.

Table 1. FTIR absorption of Ag-NPs and their presumptive functional groups before and after the reduction.

Absorption	Extract	Ag-NPs	Band	Functional groups	Reference
1030	Appear	Decrease	C-N Stretch	Aliphatic amines	Jacob et al. 2011 [34]
1323	Appear	Disappear	C-N Stretch	Aromatic amines	----------
1376	Appear	Disappear	C-H Bend	Alkenes	Kaviya et al. 2011 [4]
1463	Appear	Disappear	C-H Bend	Alkenes	Kaviya et al. 2011 [4]
1636	--------	Appear	C=C Stretch	Alkenes	Ajitha et al. 2015 [28]
1648	Appear	Increase	C=C Stretch	Alkenes	Jain et al. 2009 [35]
2850	Appear	Decrease	C-H Stretch	Alkenes	-
2919	Appear	Decrease	N-H Stretch	Secondary amines	Sadeghi and Gholamhoseinpoor 2015 [1]

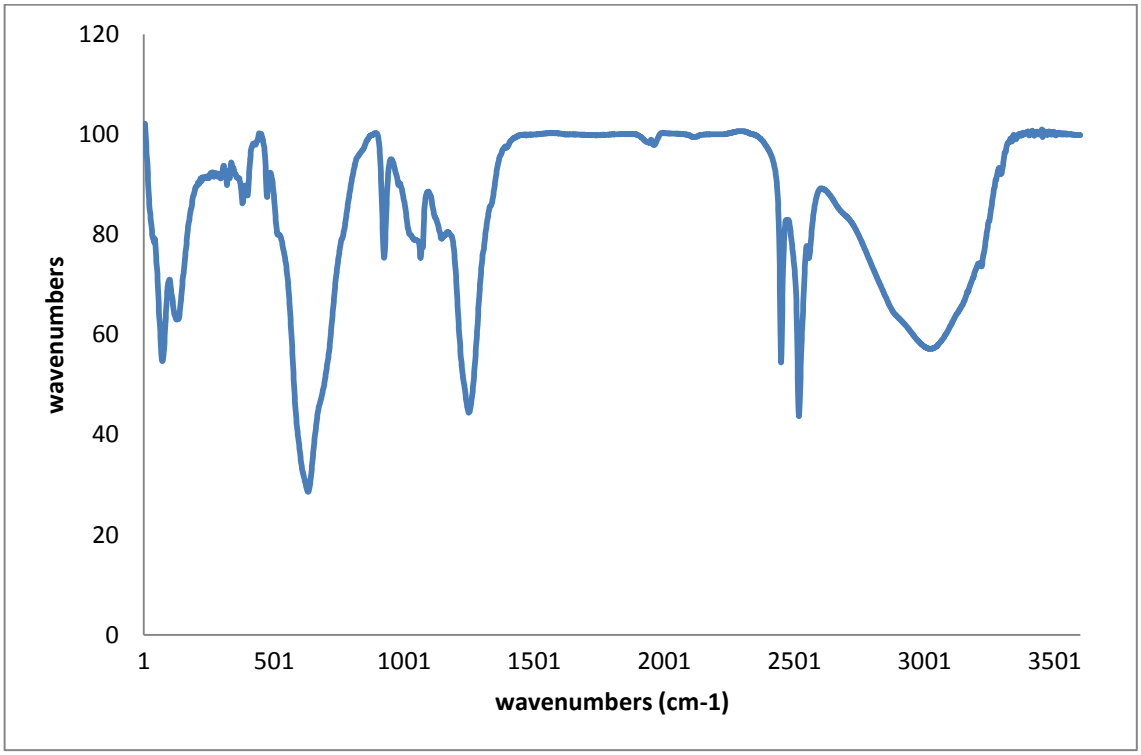

Fig. 4. FTIR spectra of the powdered anise extract

Fig. 5. FTIR spectra of the powdered AgNPs after the reduction

Antimicrobial Assays

The antimicrobial assays of the biosynthesized AgNPs against 4 bacterial human pathogens are shown in Table 2 and Fig. 6. These results indicated that the biosynthesized AgNPs had antimicrobial activities. The susceptibility to the antimicrobial property was observed for *E. Coli*, *S. Aureus*, *S. Typhimurium* in this order while no inhibitory effect was seen for *E. faecalis*. The MIC showed the lowest concentration of AgNPs for inhibition of *E. coli*, *S. aureus* and *S. typhimurium* as shown in Table 3. The combination of AgNPs and three types of antibiotics (i.e. Gentamicin, Erythromycin, and Ceftriaxone) against the same bacteria was examined which their results are indicated in Table 3. The result showed that using AgNPs with antibiotic disk paper increased the antibiotic activity. This antibiotic activity was especially more prominent against *E. faecalis* which was resistant to Ag-NPs

Table 2. Zone of inhibition (mm) of produced AgNPs against bacterial pathogens.

	Well diffusion				**Disk diffusion**				**MIC** (µg/ml)
	Centrifuging		Non-centrifuging		Centrifuging		Non-centrifuging		
	100ppm	50ppm	50µl	20µl	100ppm	50ppm	50µl	20µl	
E. coli	10±0.0	8.66±2.08	10.33±0.57	8±2.00	10±1.00	8.66±0.57	11.33±1.52	7.33±2.08	50±00
S. aureus	10±1.0	8.66±1.15	10.33±2.08	8.66±3.05	10.33±0.57	7±1.00	6±1.00	7±1.00	50±00
S. typhi	8.6±0.57	8.33±1.52	10±1.73	7±1.00	8.66±0.57	7.66±0.57	8.66±0.57	7±1.00	12.5±00
E. faecalis	0	0	0	0	0	0	0	0	------

Table 3. Zones of inhibition (in mm) of the combination of AgNPs and three types of antibiotic

	Water	**Gentamicin**	**Erythromycin**	**Ceftriaxone**	**Gentamicin +AgNPs**	**Erythromycin +AgNPs**	**Ceftriaxone +AgNPs**
E. coli	0	27±1.73	11±1.00	37±2.64	30.33±1.52	22±1.00	37.33±57
S. aureus	0	25±0.00	27.66±0.57	24.33±0.57	28.66±1.52	36.33±1.52	37.33±1.52
S. typhi	0	22.66±0.57	9.66±1.52	31.33±1.52	23.33±0.57	13±1.00	34.33±1.52
E. faecalis	0	10.66±1.15	14.66±0.57	18±2.00	23.66±1.52	19.33±1.52	26.66±1.52

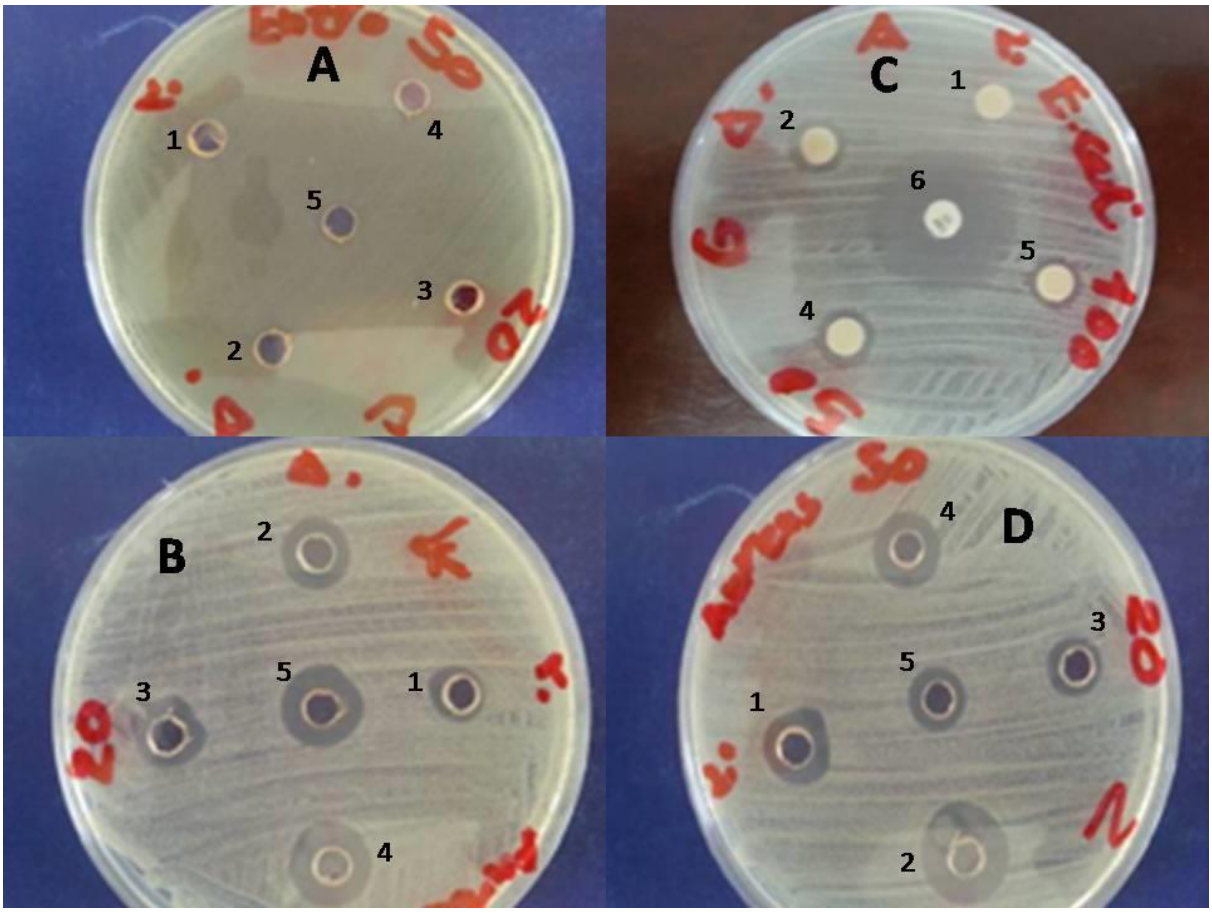

Fig. 6. Antibacterial activities of the synthesized AgNPs in varies densities and volumes on **A:** *E. faecalis*, **B:** *S. typhimurium*, **C:** *E. coli*, **D:** *S. aureus* (1: 20µl; 2: 50 µl; 3: 20 ppm; 4: 50ppm; 5: 100 ppm; 6: Gentamicin)

DISCUSSION

In this study, we presented the green synthesis of AgNPs, produced by anise as an environmentally-friendly method and examined its antibacterial activity. Aqueous extracts from anise seed have a potential capacity toward silver nitrate reduction and production of AgNPs. The study of the synthesis process by UV-Vis at different time points and absorption intervals confirmed the production of AgNPs. The FTIR graph pointed out the reducing agents of the extract. These results also showed that the FTIR graph of anise extract had paramount differences from the graph of nanoparticles biosynthesized. These differences can be described as an increase of absorption, an increase in the length of peaks, and elimination of some peaks in the graph that may be resulted from the centrifugation, washing of the nanoparticles, or breaking of the chemical bonds of the extract after reduction of the nanoparticles. The band at 1376.50 and 1393.67 corresponded to C-H of alkane's. The band at 1560.40 can be assigned to the N-H at secondary amides while the band at 1648.39 can be attributed to C=C alkenes.

The spherical, circular, and hexagonal nanoparticles exhibited antibacterial activities. These nanoparticles are assumed to adhere to the bacterial cell wall and penetrate through the cell membrane. This can cause the destruction of the bacterial cell wall and disruption of the membrane permeability, leading to the cell death. Considering that the antibacterial efficacy has a significant relationship with nanoparticle size and concentration, the small size and high concentration of AgNPs was proved to be more effective. This ability was increased by cooperation with antibiotics such as Ceftriaxone, Erythromycin, and Gentamicin and this capability was more pronounced during the cooperation of AgNPs with Ceftriaxone. *E. coli* was more sensitive compared to other bacteria and *E. faecalis* was strongly resistant to AgNPs. The reason behind the resistance of *E. faecalis* to silver particles is still unclear [36]. On the other hand, these organisms have shown resistance to many other conventional antimicrobial substances [37].

Collectively, the metallic nanoparticles could be considered as novel alternatives for antibiotics with potentially lower side effects and more safety over the conventional chemical antibiotics. Moreover, using these nanoparticles in preparation and production of the new generation of vaccines has gained more attention. For instance, Manaf et al [38], have worked on a vaccine against *S. aureus* tested in mice. This vaccine which was made from inactivated *S. aureus* in combination with $AgNO_3$, has been shown to be more effective than a vaccine with S. aureus alone and has led to an increase in T-cell and B-cell counts while raising the rate of phagocytes and antibodies in the mice blood serum. Also, the positive effects of such nanoparticles on the respiratory viruses have been demonstrated [39, 40]. Furthermore, it has been shown that when nanoparticles are used as adjuvants in vaccine production, they lead to increased antigen presentation and stronger immunity than common adjuvants [40, 41]. It should

be noted that the use of nanoparticles causes the vaccine to be released at a slower rate, which in turn results in a stronger humoral response with lower toxicity [42, 43]. Therefore, aside from the observed antibacterial effects of the produced AgNPs against both Gram-positive (e.g. *S. aureus*) and Gram-negative (e.g. *E. coli*) bacteria, it is envisaged that these AgNPs could have potentially vaccine adjuvant properties which requires further studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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