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Biosynthesis, Characterization, Antibacterial Activity and Anticancer Effect of Silver Nanoparticles Using *Anethum Graveolens* Leaf Extract

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Abstract

Nanoparticles are used in various fields of science, especially medicine. Advent of nanotechnology has led to significant development in disease diagnosis, treatment and drug delivery. Silver nanoparticles (Ag-NPs) play an important role in medical application, which makes them a viable alternative to common antibiotics. Amongst various methods, synthesis of Ag-NPs *via* green method has the advantage of being cost effective with no toxic agent. In this study, Spherical shape Ag-NPs with average size of 30 nm were synthetized using *Anethum graveolens* leaf extract as a green, cost-effective, non-toxic and environment-friendly source. Transmission electron microscopy (TEM), particle size analysis (PSA) and Fourier transform infrared (FT-IR) were performed to characterize synthesized Ag-NPs. The antibacterial activity of the synthetized Ag-NPs was evaluated against gram positive and negative bacterial pathogens. The minimum inhibitory concentration (MIC) at different concentrations of Ag-NPs were used to evaluate their antibacterial properties against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* pathogens. The results exhibited a desirable antibacterial property of Ag-NPs, suggested its usage as putative antibacterial agents. Moreover, the anticancer effect of green synthesized Ag-NPs was evaluated against MCF-7 lines and results showed that the cell viability is depended on the concentration of Ag-NPs. In short, this method provides a simple, cost effective and eco-friendly way to synthesis Ag-NPs which can be used as a suitable alternative to common antibiotics that use hazardous chemical agents, additionally, with anticancer effects against MCF-7 cells.

Keywords: Silver nanoparticles, Nanotechnology, Antibacterial agents, Anticancer effect

1 Introduction

Emergence of nanotechnology has attracted researchers in various fields of science and industries (1). Nanoparticles bridge the gap between bulk material, atomic and molecular structures. They have the potential to be used in medicine, hygiene, pharmacy, dentistry and etc., which is due to their interesting and unique physical and chemical properties such as optical, magnetic, mechanical and conductivity properties (2). Excessive use of antibiotics has led to microbial resistance; thus, many researchers have focused on development of novel and effective antimicrobial agents (3, 4). Several studies showed the antibacterial activity of Ag-NPs against a wide range of microorganisms (5, 6). Hence, they can be a promising compound in development of novel antimicrobial agents (7).

Nowadays, a great deal of efforts has been put into synthesis of Ag-NPs (8, 9). The Ag-NPs are being applied in cosmetic and hygiene products such as shampoos, soaps, toothpastes, anti-septic gels and pomades. Ag-NPs are generally synthesized using physical and chemical methods, but these approaches use toxic chemicals, organic solvents and non-biodegradable materials that are dangerous to human health and environment. Therefore, non-toxic, biocompatible, and eco-friendly methods to synthesize Ag-NPs are warranted (10, 11). Recent developments showed the critical role of plant extracts in production of Ag-NPs (12). Plants extracts contain several phenolic and antioxidant compounds that reduces Ag to Ag-NPs (13). There are several studies on the synthesis of Ag-NPs using various plants, such as *Cupressus sempervirens* Leaf

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(10) Givotia moluccana leaf (4), Syzygiumcumini fruit (14), Azadirachta indica aqueous leaf (15), Eriobotrya japonica leaf (16), aloe vera (17), Nigella sativa (18), Pistacia atlantica (13), Nyctanthes arbortristis (19), Orange and pineapple (20), and Pinus densiflor (21). Anethum graveolens (dill) is an annual herb and since antient time it has been exploited in ayurvedic medicines. Anethum graveolens is a member of Apiaceae family and rich in polyphenols, anti-oxidants and minerals (22). It is helpful in patients with hyperlipidemia (23) and as a component in gripe water, it can alleviate colic pain in babies and flatulence in young children (24). We used Anethum graveolens aqueous leaf extract to synthesize and evaluate antibacterial activity of Ag-NPs.

The main purpose of this study was to green synthesis of Ag-NPs, using *Anethum graveolens* leaf extract as an inexpensive, simple and non-hazardous BioSource. The antibacterial properties of resultant Ag-NPs were investigated against *Staphylococcus* aureus, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* pathogens. Moreover, the anticancer effect of green synthesized Ag-NPs were evaluated against MCF-7 cells.

2 Materials and methods

2.1 Chemicals

Silver nitrate (AgNO3, 99%) was purchased from Sharlo (Spain). Before usage, the glassware was washed with HCl and deionized water. Ultrapure deionized water was used for synthesis reactions and leaves extraction.

2.2 Leaf extract preparation

A. graveolens leaves were collected from the countryside of Shiraz, Fars, Iran and an herbalist confirmed the species. The fresh plant leaves were washed with distilled water for several times (diH2O) to remove any dusts and mods. The leaves were shade dried at room temperature. To prepare leaf extract, 5 g dried leaf powder was boiled in 100 ml deionized water for 15 min. Then mixture was filtered through a Whatman filter paper No.1. The filtered extract was stored at 4oC, which was used for synthesis reaction.

2.3 Ag-NPs synthesis

For green synthesis, 1 ml silver nitrate (AgNO3·6H2O, 0.1 M) was added to 9 ml leaves extract under vigorous stirring at room temperature. After 24 h, the final mixture was centrifuged at 12000 rpm for 5 min. The obtained Ag-NPs was washed with deionized water and dried in an oven at 50°C.

2.4 Ag-NPs characterization

Ag-NPs size and morphology were evaluated by TEM (Philips, CM 10; HT 100 Kv) and PSA. Functional groups of the phytochemicals, involved in the formation of Ag-NPs were analyzed using FTIR (Perkin- Elmer, Two).

2.5 Antibacterial activity

The synthesized Ag-NPs were tested for antimicrobial activity by evaluating MIC minimum inhibitory concentration (MIC) against gram-positive (Staphylococcus aureus ATCC 25923 and Enterococcus faecalis ATCC 29212) and gram negative (Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853) pathogens. The bacteria were cultured in Muller-Hinton agar and fresh colonies were suspended in sterile water to reach their tarnish equivalent to 0.5 McFarland. MIC with different concentration of Ag-NPs (4, 8, 16, ..., 2048 $\mu gr/ml)$ was evaluated against 50 μl of 1.5 $\times 106$ CFU/ml bacterial suspension and cultured in Muller-

Hinton Broth medium. One Ag-NPs sample was used as the positive control and another with no bacteria as negative control

2.5 Anticancer effect

To evaluate the anticancer effect of synthesized synthetized nanoparticles, five different concentration of Ag-NPs (6.25, 12.5, 25, 50, 100 µgr/ml) were exposed to MCF-7 cells for 24 hours. MCF-7 cell line which is a breast cancer cell line was purchased from Pasteur institute of Iran. At first, cancerous cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. Optimum condition for cell growth were considered as 5% CO2, 95% humidified atmosphere air and 37 $^{\circ}$ C temperature. After subculture and cell count, we added 10000 cells to each well of a 48-well plate, additionally, cells were treated with different concentration of Ag-NPs. In addition to samples treated with silver nanoparticles, an untreated cell sample was considered as a control. After 24 hours of incubation (MTT) assay was performed to evaluate the effect of Ag-NPs on MCF-7 cells. Therefore, supernatant of each wells was extracted and while cells are attached at the bottom of wells, MTT was added to each well. After 4 hours of incubation in 37 °C and 5% CO₂ contents of each wells were removed and 50 µl of DMSO was added to the wells. Consequently, after 30 min of incubation, the plate was read at 570nm with an ELISA reader and the percentage of cell cytotoxicity was calculated using the optical density and the below formula:

% Cell viability = (OD value for test/OD value for control) \times 100

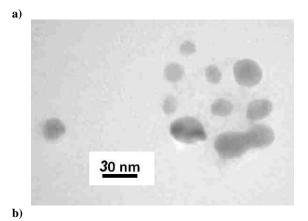
3 Results

3.1 synthesis of Ag-NPs

After adding AgNO3 solution to the *A. graveolens* leaf extract, the color of mixture changed to dark brown. This alteration indicates the silver ions reduction to Ag-NPs.

3.2 Characterization of synthetized Ag-NPs

The TEM micrograph and PSA data of the synthesized Ag-NPs by A. graveolens extract are depicted in figure 1. TEM micrograph showed spherical Ag-NPs was surrounded by a thin layer of biological matrix from A. graveolens leaf extract. The particle size distribution of the biosynthesized Ag-NPs was in the range about 30 nm. The PSA analysis showed that most Ag-NPs had a size less than 100 nm. It is worth noticing that PSA measures hydrodynamic diameter of nanoparticles, always reported larger size than TEM image. The FTIR analysis are exhibited in figure 2. The band at 3417 cm⁻¹ indicates the combined peaks of the amine or amide and -OH group stretching vibration in the plant extract. The methyl group gives its symmetric tensile vibrations of about 2863 cm⁻¹ and asymmetric traction in 2922 cm⁻¹. The peak at 1100 cm⁻¹ was related to C-O stretching. The peak at 1628 cm⁻¹ (sp 2 hybridized C = C) can be assigned to the skeletal vibrations of the plant extract. Likely, the peak intensities in 1383 cm⁻¹ is due to C-N stretching in the extract of the plant capped silver nanoparticles. The peaks at 2322 and 2355 cm⁻¹ are related to N-H amine and amide group. This indicates the binding of Ag with O and N of -OH and -NH2 groups in the extract of the plant.



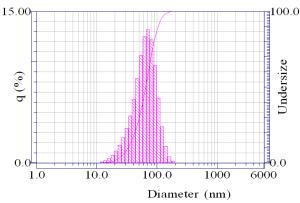


Figure 1: a) The approximate size and shape of synthetized Ag-NPs are shown in TEM micrograph. b) The PSA analysis of the Ag-NPs showed that the most of Ag-NPs have size less than 100 nm

3.3 The antibacterial activity of obtained Ag-NPs

The antibacterial activity of the synthesized Ag-NPs against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* photogenes were evaluated. The lowest concentration of Ag-NPs (µgr ml⁻¹) which prevented the visible growth of bacteria are reported as MIC in table 1. The results showed noticeable effect of synthesized Ag-NPs on the growth of these pathogens. The MIC of Ag-NPs against Escherichia coli and three other organisms were evaluated 64 µgr ml⁻¹ and 512 µgr ml⁻¹, respectively. Consequently, it can be concluded that *Escherichia coli* was more sensitive than the other tested pathogens against synthetized Ag-NPs.

3.4 The anticancer effect Ag-NPs on MCF-7 cell line

The anticancer effect of five concentration (6.25, 12.5, 25, 50, 100 $\mu gr/ml)$ of the synthesized Ag-NPs were evaluated against MCF-7 cell line using cell culture and MTT assay. After exposing each concentration of Ag-NPs with MCF-7 cells for 24 h, cell viability was calculated using the optical density. MCF-7 cell treatment with Ag-NPs at 6.25, 12.5, 25, 50, 100 $\mu gr/ml$ concentration in 24 h reduced cell viability 60.40 \pm 0.83 %, 48.70 \pm 0.49 %, 35.54 \pm 0.78 %, 21.78 \pm 0.39 %, 11.14 \pm 0.13 % respectively and cell viability for control sample calculated 100 % after 24 hours. (Figure 3).

4 Discussion

The study results indicated that *A. graveolens* leaf extract can efficiently reduce the silver ions to Ag-NPs in a diameter range of 30 nm, and prevented the aggregation of nanoparticles. Srinithya et al. (25) biosynthesized spherical Ag-NPs with a

size of 10-30 nm. Raj et al. (26) synthesized Ag-NPs in a diameter ranging 2-20 nm, and Arunachalam et al. (27) reported the biosynthesis of spherical Ag-NPs with a size range of 9-12 nm.

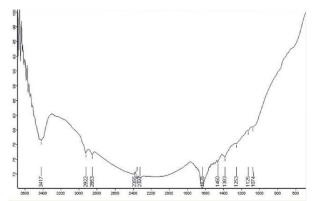


Figure 2: The FTIR spectra of synthesized Ag-NPs is illustrated. The standard peaks related to amine, amide and –OH groups appear at 3417 cm⁻¹. The peaks at 2863 cm⁻¹ (methyl), 2922 cm⁻¹ (methyl), 1100 cm⁻¹ (C-O), and 1628 cm⁻¹ (C = C) can be observed. The C-N stretching caused an intensive peak at 1383 cm⁻¹. The N-H amine and amide group give the peaks at 2322 and 2355 cm⁻¹

Table 1: Minimum inhibitory concentrations of Ag-NPs (μgr ml⁻¹⁾ are evaluated against candidate bacteria

| Strains | MIC |
|---------------|-----|
| E. coli | 64 |
| E. faecalis | 512 |
| P. aeruginosa | 512 |
| S. aureus | 512 |

This study is a report about the synthesis of Ag-NPs by *A. graveolens* extract, their antibacterial activity and anticancer effect against some pathogenic bacteria and MCF-7 cell line respectively. In our study, TEM micrograph and PSA data were used to investigate the morphology, size and shape of Ag-NPs.

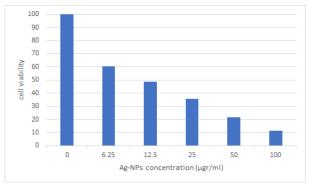


Figure 3: Cell viability of MCF-7 cells against different concentration of Ag-NPs after 24 h. results are reported as % cell viability compared with control sample

According to PSA analysis, the spherical nanoparticles with average size of 30 nm have desirable size distribution. The FTIR analysis was performed to identify the functional groups of biological compounds in the *A. graveolens* leaf extract that was involved in the reduction of Ag ions to Ag-NPs. FTIR analysis indicates the binding of Ag with O and N of –OH and –NH2 groups in the plant extract.

Various studies showed antimicrobial activities of Ag-NPs against both Gram-negative and Gram-positive bacteria (7). In the present study, the antibacterial effect of Ag-NPs was evaluated against pathogenic strains such as E. coli, E. faecalis, P. aeruginosa and S. aureus. According to the results, synthesized Ag-NPs exhibited excellent antibacterial effect against these pathogenic bacteria. Based on MIC value reported in table 1, it can be concluded that Escherichia coli (64 µgr ml⁻ 1) was more sensitive than the other tested pathogens (512 µgr ml⁻¹) against synthetized Ag-NPs. These results are in line with previous studies. For example, Singhal et al. investigated antimicrobial properties of synthesized silver nanoparticles by Ocimum sanctum leaf extract. The MIC of biosynthesized silver nanoparticles was also measured using pathogenic bacteria such as E. coli (0.314 µgr ml⁻¹) and S. aureus (1.25 µgr ml⁻¹). Hence, results showed that *Escherichia coli* was more sensitive than S. aureus (28). Martinez-Castanon et al. (29) Synthetized 29 nm silver nanoparticles and reported MIC 13.02 and 16.67 µgr ml⁻¹ for E. coli and S. aureus, respectively. It seems these differences might be due to particle size and bacterial strains (29, 30).

This inhibitory effect was due to different mechanisms. For example, Ag-NPs can be attached to the bacterial cell wall and infiltrate it. Therefore, these nanoparticles can produce reactive oxygen species (ROS) and free radicals in the bacterial cell, causing apoptosis and cell death (28). Anticancer effect of green synthesized silver nanoparticles with different leaf extracts have been reported recently (31, 32). Thus, we prepared five different concentration of synthesized Ag-NPs (6.25, 12.5, 25, 50, 100 µgr/ml) to evaluate their anticancer effect against MCF-7 cell line. Cell culture and MTT assay were used for this purpose. MTT assay is a colorimetric assay and reduction of MTT (yellow) to formazan (purple blue) is the basis of test. This reaction is mediated by a mitochondrial enzyme termed succinate dehydrogenates. The results of cell exposure to various concentration of Ag-NPs after 24 h indicated that the cell viability is depended on the concentration of silver nanoparticles. Highest concentration of Ag-NPs (100 μ gr/ml) caused lower cell viability (11.14 \pm 0.13 %). A key strength of the present study was synthetizing Ag-NPs, using green synthesis, which is eco-friendlier and more cost effective in comparison to commonly used methods. However, there are some drawbacks and limitation when using this method to produce metal nanoparticles. Due to plant impurities, the size and size distribution was not controllable. The nanoparticles produced using green synthesis are sometimes less stable than the ones synthetized using chemical methods (33).

5 Conclusions

In this study, A. graveolens leaves extract is presented as a beneficial capping and reducing agent to synthesize Ag-NPs. The synthetized nanoparticles were stable even after several months and no change was observed visually. The obtained Ag-NPs were characterized using TEM, PSA, and FT-IR spectroscopy. The results showed spherical nanoparticles with approximately 30 nm in size and also desirable size distribution. The FT-IR analysis showed adding functional group to Ag-NPs. The analyses confirmed that the proposed green synthesis could be effective in synthetizing Ag-NPs. Also, the antibacterial activity of the Ag-NPs was analyzed against gram positive and negative pathogens. The results showed an intensive antibacterial property and bio-synthetized Ag-NPs can be used as a putative antibacterial agent. Moreover, we showed the anticancer effect of Ag-NPs is depended on the concentration of nanoparticles. For further researches, it is suggested to evaluate anticancer effects of green synthesized Ag-NPs against different cell lines, and anti-inflammation studies can be implemented. Moreover, the antibacterial activity of the synthetized Ag-NPs should be investigated against other pathogenic bacterial strains.

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Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing.

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