

Green Synthesis of Silver Nanoparticles Using the Extract of *Lonicera Nummulariifolia* and Investigating Its Antioxidant, Antimicrobial and Anticancer Effects Against Lung Cancer Cell Line A549

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ABSTRACT

BACKGROUND AND OBJECTIVE: Due to the increasing incidence of cancer-related deaths and the deficiencies of chemotherapy and radiotherapy in advanced forms of cancer, new approaches are needed to control cancer, and one of these techniques is the use of nanoparticles, especially silver nanoparticles. Nowadays, the use of plants for the synthesis of silver nanoparticles has attracted the attention of researchers due to their low cost. Therefore, the aim of this study was to investigate the green synthesis of silver nanoparticles using the extract of *Lonicera nummulariifolia* and to study its antioxidant, antimicrobial and anticancer effects against lung cancer cell line A549.

METHODS: In this experimental study, silver nanoparticles were synthesized using the extract of *Lonicera nummulariifolia* as a regenerative agent. The antioxidant effects of synthesized silver nanoparticles were evaluated by DPPH assay and finally its antimicrobial and anticancer activity were respectively evaluated by Broth Microdilution and MTT assays at concentrations of 3.125, 6.25, 12.5, 25, 50, 100 µg/ml on lung cancer cell line within 24 h.

FINDINGS: The results of DPPH assay showed that the synthesized silver nanoparticles at 100 µg/ml had an antioxidant effect of 33.77 ± 0.83 . Antimicrobial test results showed that the antimicrobial effects of silver nanoparticles were greater on gram negative bacteria. MTT results also showed that cell viability was 70.33 ± 0.21 ($p > 0.05$), 51.66 ± 0.24 ($p < 0.05$), 35.75 ± 0.35 ($p < 0.01$), 20.66 ± 0.28 ($p < 0.001$), 13.5 ± 0.31 ($p < 0.001$), and 7.6 ± 0.37 ($p < 0.001$), respectively. Results of DPPH assay showed that silver nanoparticles has significant antioxidant effects ($p < 0.05$).

CONCLUSION: Considering the antimicrobial and anticancer effects of synthesized silver nanoparticles, it can be used as a drug candidate.

KEY WORDS: *Lonicera nummulariifolia*, Silver nanoparticles, Antioxidant effects, Antimicrobial effects, Anticancer effects, Lung cancer.

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Introduction

Nanotechnology is a field of applied knowledge and technology that covers a wide range of sciences such as pharmacy, drug design and biology (1). Materials smaller than one micrometer are used in nanotechnology, typically from 1 to 100 nanometers (2, 3). There are different physical and chemical methods to synthesize nanoparticles, including chemical reduction, lithography, electrochemistry, lasers and microwave radiation (4). One of the disadvantages of chemical methods that act as reducing agents as well as stabilizing agents is that they remain undecomposed in the environment and ultimately cause environmental pollution (5).

Another disadvantage of these methods is the low production and the need for high pressure, temperature and energy during the reaction process (6). The biological synthesis of silver nanoparticles using natural and biological agents such as bacteria, fungi and plants has recently been the focus of researchers. One of the biological methods is the green synthesis method, in which the metal ions are converted to silver nanoparticles by using plant compounds without the need for surfactants and other stabilizing compounds (7, 8). One of the native plants of Iran is *Lonicera nummulariifolia*. *Lonicera nummulariifolia* is a climbing and spiral shrub native to East Asia (China, Taiwan, Japan and Korea). *Lonicera nummulariifolia* has high medicinal value in traditional Chinese medicine. It is used to treat fever, flu, headache, cough, thirst, and sore throat. One of the most important applications of silver nanoparticles is their use in destroying cancer cells (9).

In recent years, due to the increasing prevalence of cancer-related deaths and the deficiency of chemotherapy and radiotherapy in advanced forms of cancer, there is a need for finding new ways to control cancer, one of which is the use of nanoparticles, especially silver nanoparticles (10–12). In recent years, various plant extracts have been used to synthesize silver nanoparticles. Kavaz et al. showed that silver nanoparticles synthesized by *ficus ingens* extract have antimicrobial and anticancer effects (13).

Given the cost-effectiveness and absence of environmental toxic effects of plant extracts for the synthesis of silver nanoparticles, as well as the anticancer and antimicrobial effects of silver nanoparticles, the present study was conducted to investigate the biological synthesis of silver nanoparticles using the extract of *Lonicera nummulariifolia* and to investigate its antioxidant,

antimicrobial and anticancer effects on lung cancer cell line (A549) so that its results can be used in decision-making for using or not using silver nanoparticles in the treatment of microbial infections and lung cancer.

Methods

Plant Collection and Extraction: In this experimental study that was performed with Code of Ethics IR.IAU.TMB.REC.22.34, first the *Lonicera nummulariifolia* plant was obtained from the Iranian Biological Resource Center (Tehran, Iran) with herbarium number 1342. To prepare extract from the aerial parts of the plant, ethanol solvent and maceration method were used. The prepared extract was stored at 4°C until the time of synthesizing silver nanoparticles.

Silver Nanoparticle Synthesis: The silver nanoparticles were synthesized through sedimentation by reduction of silver ions. Ten milliliter extract was mixed with 90 ml of 1 mM silver nitrate solution and place for 2 hours at laboratory temperature. Two hours after the reaction, the sediment was washed three times with distilled water.

All washing steps were done through centrifuge at 13,000 rpm for 20 minutes. Finally, the final wash was performed with ethanol and the resulting product was placed at 75 °C for 2 hours (14).

Investigation of the physical and chemical properties of nanoparticles: Ultraviolet–visible spectrophotometry of synthesized silver nanoparticles was done using UV-Visible spectrophotometer (Agilent, USA) between 200 and 700 nm. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to evaluate the morphology and size of silver nanoparticles.

X-ray diffraction (XRD) patterns of the samples were used to determine the crystalline phases of the synthesized silver nanoparticles and to measure the crystalline constants of silver nanoparticles. X-ray diffraction provides the understanding of the crystallographic structure of silver nanoparticles. In this study, X-ray diffraction (XRD) was done by CuK α radiation in the range of 0 to 110 degrees and commercial silver was used as positive control. In addition, to identify the chemical bonds formed in the silver nanoparticle structure originating from the extract of *Lonicera nummulariifolia* plant, the fourier-transform infrared spectroscopy (FTIR, Thermo Nicolet Model Nexus 870, USA) in the range of 400 to 4000 cm⁻¹ was used.

Antioxidant properties of DPPH: DPPH (1,1-diphenyl-2-picrylhydrazyl) compound was used to test the antioxidant activity; 0.1 mM solution of DPPH was prepared in methanol and 1 ml of it was added to 3 ml of nanoparticle suspension at concentrations of 5, 25, 50, 100 µg/ml. The resulting suspension was incubated at room temperature for 30 min and finally adsorption was read by spectrophotometer at 517 nm wavelength. The adsorption rate of free radical was calculated by the following formula:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

A₀ was the adsorption rate of the control sample, and A₁ was the adsorption rate of the treated sample (15).

Evaluation of antimicrobial effects: Minimum Inhibitory Concentration (MIC) method was used to determine the minimum inhibitory concentration of silver nanoparticles. The MIC test was performed by dilution in microplate and was repeated three times at concentrations of 3.125 to 100 µg/ml.

In this test, standard pathogenic strains of *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 39327 at 0.5 McFarland were used. MIC value is considered as the lowest concentration of bacterial growth inhibition (16). It should be noted that ampicillin was used as positive control.

Anticancer effects: MTT (Sigma Aldrich, Germany) was used to evaluate the apoptotic effects of silver nanoparticles against lung cancer cell line. Concentrations of 3.125, 6.25, 12.5, 25, 50, 100 µg of nanoparticles were treated on A549 cell line for 24 hours. After this period, the contents of the 96-well plate were carefully extracted and added to the tetrazolium dye (MTT) and kept for four hours in 5% CO₂ exposure at 37 °C.

The MTT dye was then separated and the formazan crystals produced by living cells were dissolved in isopropanol. Finally, the absorption rate of the samples was measured using an ELISA reader (ELISA reader, Organon Teknika, The Netherlands) at a wavelength of 570 nm and the cell apoptosis was calculated by the following formula (17):

$$100 \times (\text{Optical Absorption of Control Cells on Optical Absorption of Treated Cells}) = \text{Cell Survival Rate}$$

Statistical analysis: The collected data were analyzed using SPSS ver. 20 and one-way ANOVA statistical test, while P<0.05 was considered significant.

Results

Results of changes in solution color and UV-Vis spectroscopy: During the process of synthesis of silver nanoparticles, Ag⁺ ions are exposed to reducing agents of the extract and thereby the reduction of silver nitrate salt is initiated. Complete reduction of Ag⁺ ions to silver nanoparticles was performed with changes in the ambient color and spectroscopy. The color of the solution changed from pale to brown by adding the herbal extract to the silver nitrate solution for 120 minutes. The changes in color indicate the reduction of silver nitrate and the formation of silver nanoparticles in solution. The peak at 339 nm for silver nanoparticles was also confirmed by UV-Vis spectroscopy at different reaction times (Fig 1). The pattern of XRD peaks related to the face centered cubic structure (FCC) in (111), (200), (220), (311) regions can be seen in the silver sample spectrum (Figs 2 a and b).

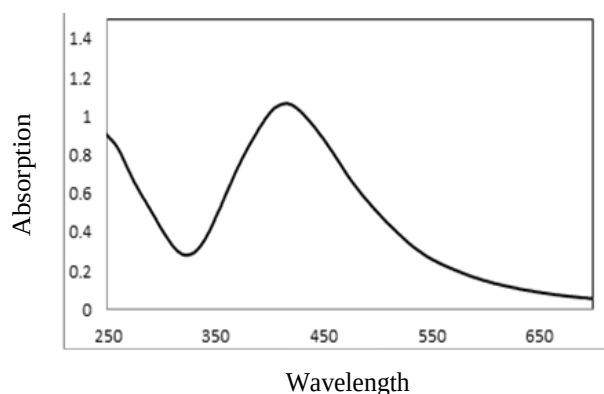


Figure 1. UV-Vis spectroscopy of synthesized silver nanoparticles. According to the diagram, the maximum UV absorption of silver nanoparticles is at 438 nm. X-ray diffraction (XRD) results of silver nanoparticles

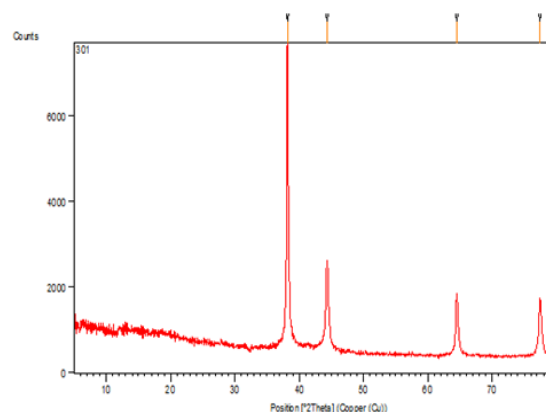


Figure 2 a. XRD pattern of synthesized silver nanoparticles. The pattern of XRD peaks related to the face centered cubic structure (FCC) in (111), (200), (220), and (311) regions

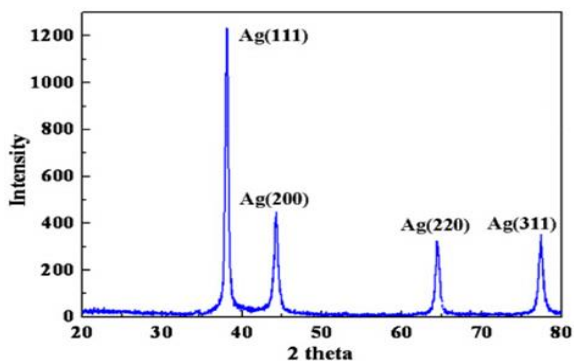


Figure 2 b. Standard sample spectrum of silver nanocrystals

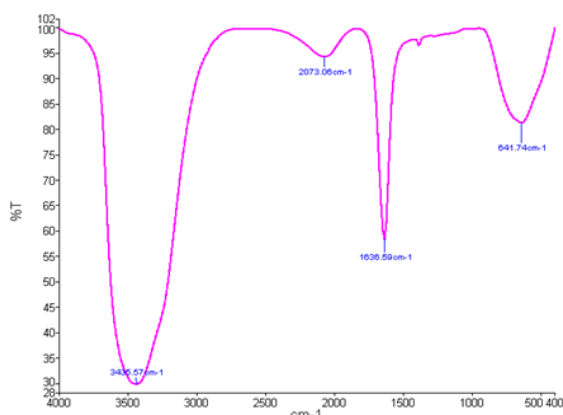


Figure 3. Fourier Transform-Infrared Spectroscopy (FTIR) of synthesized silver nanoparticles. The absorption bands in the 2973 cm^{-1} and 2948 cm^{-1} regions are related to the alkynes and 1630 cm^{-1} is related to alkenes, which can be created through reaction with the herbal extract. The absorption band of 3421 cm^{-1} in the silver nanoparticles was related to the hydroxide compounds of the plant extract with silver nanoparticles

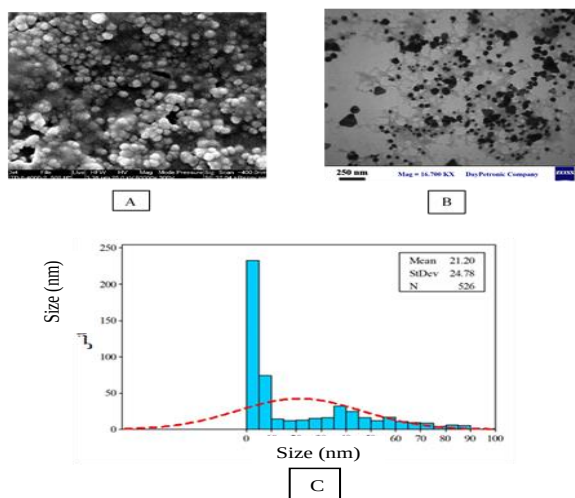


Figure 4. SEM (A) and TEM (B) electron microscopy and diagram of nanoparticle size (C). As can be seen, the synthesized nanoparticles have a spherical shape with an average size of 21.20 nm

DPPH results: The antioxidant property of the synthesized silver nanoparticles was determined by DPPH test, in which the target nanoparticles convert free radical diphenylpicrylhydrazyl (DPPH), which is a purple compound, to diphenylpicrylhydrazine compound (yellow compound) by reducing the radical capacity. The results showed that silver nanoparticles at 100 $\mu\text{g}/\text{ml}$ had antioxidant activity (33.77 ± 0.83); as the concentration of silver nanoparticles increases, their antioxidant power increases (Table 1).

Antimicrobial Test Results: The results showed that silver nanoparticles had antibacterial effects on all the studied bacteria; the minimum inhibitory concentration (MIC) of the nanoparticles was related to *Pseudomonas aeruginosa* and the maximum was related to *Bacillus cereus* (Table 2). The results also showed that ampicillin had the highest effect on *Escherichia coli* with the lowest MIC concentration.

Table 1. Measurement of antioxidant power of silver nanoparticles synthesized by DPPH method. Results are repeated three times

Silver nanoparticle concentration ($\mu\text{g} / \text{ml}$)	Silver nanoparticle inhibition percentage
5	18.25 ± 0.55
25	67.25 ± 0.29
50	52.26 ± 0.37
100	77.37 ± 0.83

Table 2. Antimicrobial effects of silver nanoparticles synthesized by MIC and its comparison with ampicillin antibiotics

Bacteria name	MIC($\mu\text{g}/\text{mL}$) Silver nanoparticles	MIC($\mu\text{g}/\text{mL}$) ampicillin
<i>S. aureus</i> ATCC 25923	25	12.5
<i>Bacillus cereus</i> ATCC 14579	50	50
<i>E. coli</i> ATCC 25922	6.25	3.125
<i>P. aeruginosa</i> ATCC 39327	12.5	6.25

Cytotoxicity results: A549 cells were treated with different concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 $\mu\text{g}/\text{ml}$ of silver nanoparticles for 24 hours. Treatment of A549 cells with mentioned concentrations showed that cell viability was 70.33 ± 0.21 ($p > 0.05$), 51.66 ± 0.24 ($p < 0.05$), 35.75 ± 0.35 ($p < 0.01$), 20.66 ± 0.28 ($p < 0.001$), 13.5 ± 0.31 ($p < 0.001$), and 7.6 ± 0.37 ($p < 0.001$), respectively (Fig 1).

The results of treatment of cancer cells with silver nanoparticles showed that these nanoparticles induce genomic DNA fragmentation of A549 cancer cells. Genomic DNA fragmentation, which is a characteristic of apoptotic cells, was confirmed on 2% agarose gel (Fig 5).

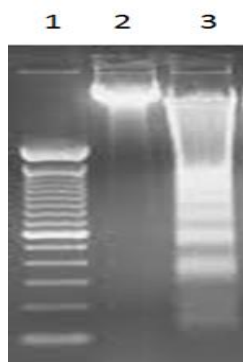


Figure 5. Gel electrophoresis image of DNA fragmentation test to show apoptosis. 1: DNA marker, 2: Control DNA sample, 3: Nanoparticle-treated DNA sample. As can be seen, the DNA sample treated with nanoparticles was fragmented, indicating the induction of the apoptosis process

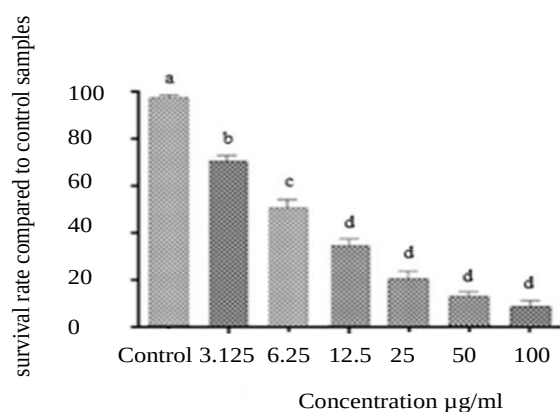


Figure 6. Survival rate of A549 cells against different concentrations of silver nanoparticles over 24 h; results are reported as survival rate compared to control samples (mean±SD)

Discussion

The results of this study showed that the synthesized silver nanoparticles were 20.22 nm in size and had a spherical structure. Studies show that *Lonicera nummulariifolia* extract has amino acids, phenolic acid and flavonoids that can reduce metal silver to silver nanoparticles, and many studies show that proteins and enzymes present in this extract play an important role in the synthesis of silver nanoparticles (18, 19). Varghese et al. synthesized silver nanoparticles using *Trigonella foenum-graecum* extract and investigated its

antimicrobial and anticancer effects. The results of this study showed that the lowest MIC of silver nanoparticles was found to be related to *Staphylococcus aureus* at a concentration of 62.5 µg/ml (20).

Behravan et al. synthesized silver nanoparticles using *Berberis vulgaris* extract through green synthesis and the results showed that the synthesized silver nanoparticles had a size between 30 and 70 nm and that the silver nanoparticles had significant antimicrobial effects on the extract (21).

The similar point between all these studies and the present study is that silver nanoparticles can be easily synthesized using plant extracts, but the difference between all these studies is the size of the synthesized silver nanoparticles, which may vary depending on the phytochemical content of the plant. Silver nanoparticles produced in this study had significant antimicrobial effects on gram-negative bacteria compared to gram-positive bacteria. This is due to the lower cell wall thickness of gram-negative bacteria and its negative charge (22).

In this study, DPPH test results showed that the percentage of free radical scavenging increased with increase in the concentration of silver nanoparticles. One of the reasons for the antioxidant property of the produced silver nanoparticles could be the presence of bioactive compounds in it (23).

The results also showed that the synthesized nanoparticles had dose-dependent cytotoxicity and had significant cytotoxic effects. Satpathy et al. showed that the synthesized silver nanoparticles had a spherical structure and had a 50% apoptotic concentration (IC₅₀) of 3.859 µg/ml against the breast cancer cell line (MCF-7) (24). Behboodi et al. showed that silver nanoparticles synthesized by *cichorium intybus* extract have significant cytotoxic effects on breast cancer cell line and are capable of inducing apoptosis (25). The results of all researchers are consistent with the results of our study; silver nanoparticles have significant cytotoxic effects on cancer cell lines and can induce apoptosis. In general, the results of this study showed that the extract of *Lonicera nummulariifolia* has a high potential for the reduction of silver ions and their conversion to silver nanoparticles.

The results also showed that silver nanoparticles have dose-dependent anticancer effects. Considering the results of this study, it can be concluded that synthesized silver nanoparticles can be considered as a drug candidate for antimicrobial and anticancer therapeutic purposes.

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