

Research Article



Synthesis, Physicochemical Properties, and Cytotoxicity of Silver Nitrate Nanoparticles of *Achillea filipendulina* L. Extract in Lung Cancer Cells

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Abstract

Background: Silver nanoparticles (AgNPs) with varied physicochemical properties have garnered substantial attention in recent years for their applications in imaging, diagnosis, treatment, and drug delivery within nanopharmaceuticals. This study synthesized AgNPs using *Achillea filipendulina* (A.F) extract as a reducing and stabilizing agent.

Methods: The synthesized nanoparticles underwent characterization via UV-Vis spectroscopy, FTIR, FESEM, DLS, and zeta potential analysis. The total phenolic content (TPC) method was also employed to estimate the quantity of *A. filipendulina* extract loaded onto the AgNPs. The antioxidant capacity of A.F-AgNPs was evaluated using the FRAP and DPPH methods and compared to crude extract. The study also investigated the impact of process variables, such as the mixing ratio of reactants, type of solvent, and use of polymers, on the size of A.F-AgNPs to attain optimal sizing. Finally, the cytotoxicity of the nanoparticles was assessed via the MTT assay on the lung adenocarcinoma cell line (A549) and the regular lung fibroblast cell line (MRC-5), comparing it with silver nitrate (AgNO₃) and extract.

Results: The findings indicated a 24% loading of the extract in the synthesized nanoparticles, which mitigated the toxicity of silver nitrate in both cancer and regular cell lines. However, the resulting formulation exhibited more significant toxicity on the cancer cell line. In addition, the in vitro antioxidant assay demonstrated that the extract's antioxidant potency was four times higher than that of A.F-AgNPs at a concentration of 3.2 mg/mL, which was directly in relationship with drug loading in synthesized nanoparticles.

Conclusion: The results suggested that polyphenolic compounds or pure phenolic structures act as capping and reducing agents, with more selective effects on cancer cells to minimize side effects on normal lung cells.

Introduction

Nanotechnology represents a significant facet of contemporary research within materials science, characterized by the strategic manipulation and resizing of particles. This manipulation results in products with altered chemical, physical, and biological properties, which, due to their enhanced characteristics, have garnered interest across various scientific disciplines, notably in drug delivery applications.^{1,2} Historically, noble metals and their derivatives have been utilized for their medicinal properties in treating diverse infections. Recent advancements in nanobiotechnology have led to the development of various nanomaterials, including those with extensive applications.³ In particular, silver nanoparticles (AgNPs) stand out in the research landscape due to their unique physicochemical properties and a spectrum of biological activities such as anti-inflammatory, antimicrobial, and anticancer effects.¹

These nanoparticles, with a size range of 1-100 nm, exhibit significant alterations in their physical, chemical, and electrical properties, influencing their melting temperature, magnetic behavior, redox potential, and color.^{4,5}

Various methodologies, encompassing both physical and chemical approaches—such as electrochemical techniques,⁶ microwave irradiation,⁷ gamma irradiation,⁸ chemical reduction,^{9,10} and thermal evaporation¹¹ have been developed to synthesize AgNPs. However, these methods often present industrial and environmental drawbacks, including using toxic chemicals that contribute to environmental pollution and physical techniques that are energy-intensive, costly, and yield low production volumes.¹²⁻¹⁸ The biosynthesis of nanoparticles emerges as an innovative method to circumvent these limitations, offering a cost-effective, environmentally friendly, and straightforward alternative. This method is characterized

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by its scalability and simplicity, utilizing capping agents as crucial stabilizers to prevent the formation of oversized nanoparticles and inhibit their aggregation and coagulation during colloidal synthesis.^{1,2,13-16,18}

For the biosynthesis of AgNPs, plant extracts, and microorganisms serve as sources of capping and stabilizing agents.¹ Plant extracts, known as green synthesis, are preferred over microorganisms due to their non-toxic, rapid, simple, and cost-effective nature.^{2,12,14,19} Various plant extracts, mainly medicinal herbs, have recently been explored for the green synthesis of AgNPs, attributed to their biological and therapeutic metabolites.²⁰⁻²² Within this domain, water is recognized as the ideal solvent for its non-toxic and environmentally safe properties.⁵

Achillea filipendulina L. (Fernleaf Yarrow), belonging to the Asteraceae family, comprises approximately 130 flowering and perennial species native to Central Asia. This plant has traditionally been employed to treat gout, malaria, arthritis, congestion, and cardiovascular diseases. Investigations into the chemical composition of essential oils and hydro-alcoholic extracts derived from the flowers and leaves of *A. filipendulina* have revealed a higher concentration of oxygenated monoterpenoids in essential oils. In contrast, hydro-alcoholic extracts are rich in phenolic acids, flavonoids, and bioactive metabolites like tannins and saponins.^{23,24} Prior research indicates that phenolic acids and flavonoids act as effective capping agents in the synthesis of AgNPs, positioning hydro-alcoholic extracts of this plant as a viable candidate for AgNP synthesis.²⁵

Given the medicinal and therapeutic properties of *A. filipendulina*, this study pioneers the biological synthesis of AgNP utilizing extracts from the aerial parts of *A. filipendulina*. Furthermore, the cytotoxic effects of this new formulation were evaluated in the A549 lung cancer cell line.

Methods

Materials

Standard laboratory-grade chemicals/reagents such as silver nitrate (AgNO_3), absolute ethanol, gelatin type A, gallic acid, Folin-Ciocalteu indicator, sodium carbonate powder, and dimethyl sulfoxide (DMSO) were obtained from Merck. Fetal bovine serum (FBS), trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMEM (Dulbecco's Modified Eagle Medium), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich. Dr. Mojallali provided methanol. All other chemicals used were of analytical grade and obtained from local suppliers.

Plant Extract Preparation

A. filipendulina was gathered from the Tabriz-Marand roadway, East Azerbaijan, Iran, and identified and deposited at the Tabriz Faculty of Pharmacy herbarium (Tbz-fph-136). The extraction method used in this study was maceration. Maceration at room temperature (25 °C)

was carried out three times in actinic glass for 24 hours with 70% ethanol as the solvent. Dried extract powder was obtained by freeze-drying after filtration.²⁶

Biosynthesis of *A. filipendulina* Silver Nanoparticles (A.F-AgNPs)

Firstly, the solubility of the extract in different solvents, including distilled water, absolute ethanol, and 70% ethanol, was evaluated, and distilled water was selected as the best solvent. In addition, various concentrations of the obtained plant extract were used to synthesize the desired sizes and shapes of A.F-AgNPs. Samples included: (I) 5 mL of the aqueous solution of extract (1.2 mg/mL) was added drop by drop to 22 mL of 1 mM silver nitrate solution, (II) 6 mL of 1.2 mg/mL aqueous solution of extract was added drop by drop to 22 mL of 1 mM silver nitrate solution, (III) 4 mL of 1.56 mg/mL 70% hydro-alcoholic extract solution was added dropwise to 22 mL of 1 mM silver nitrate solution, (IV) 3 mL of absolute ethanol solution of extract (1.96 mg/mL) was added drop by drop to 22 mL of 1 mM silver nitrate solution, (V) 6 mg of gelatin powder was completely dissolved in 10 mL distilled water at 40°C and added to 22 mL of 1 mM silver nitrate aqueous solution, then 6 mL of aqueous extract solution was added drop by drop to them. All the mixtures were continuously stirred (700 rpm) for 15 minutes at room temperature. The mixtures were kept undisturbed until the colorless solution turned brownish-red, revealing the reduction process of Ag^+ to Ag^0 NPs. The particles were then separated by centrifugation at 12000 rpm for 20 minutes. Then, the A.F-AgNPs were freeze-dried prior to characterization. The dry powder of AgNPs was stored at -80 °C for the next steps.^{2,27}

UV-Visible Spectroscopy

UV-Vis spectroscopy was employed to confirm the formation of biosynthesized AgNPs. It was utilized to observe simultaneous changes in the color of aqueous dispersion and the UV-Vis spectrum. By gradually adding the extract over time, the color shifted from yellow to brownish-red, and a UV-Vis spectrum was acquired, indicating the biological reduction of silver ions and the formation of AgNPs.^{14,17} To accomplish this, 1 mL of suspension samples were periodically collected during biosynthesis. Spectroscopy ranged from 300 to 800 nm at different time intervals, such as 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours. The operation was carried out using a UV-Vis spectrophotometer (Agilent, USA).

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR was conducted to identify functional groups formed during biosynthesis. Samples were prepared by mixing dried A.F-AgNPs with potassium bromide (KBr) in a 1:100 ratio and pressing them into a disc.²⁸ The investigated samples included dried powder of extract, silver nitrate, and A.F-AgNPs. This analysis was performed using the

potassium bromide pellet method (FTIR grade).¹⁷ and the spectrum was recorded using an infrared spectrometer (Bruker, Germany). IR spectra were collected in the wavelength range from 400-4000 cm^{-1} and averaged over 9 scans for each measurement with a resolution of 2 cm^{-1} .

Dynamic Light Scattering (DLS) Analysis

DLS is a rapid and straightforward method capable of determining particle size and size distribution based on the Brownian motion of particles. The sample can be a solution or suspension.²⁹ Particle size distribution of NPs was analyzed using Nano-Zeta Sizer (Malvern, UK).

Zeta Potential Measurement

Zeta potential indicates the electrostatic resistance between particles, which determines their surface charge. It is necessary to prepare the sample as a suspension using an ultrasonic device.³⁰ In this study, AgNPs were washed and centrifuged three times, then dispersed in water using ultrasonic dispersion. Analysis was performed using Nano-Zeta Sizer (Malvern, UK).

Scanning Electron Microscopy (SEM) Analysis

SEM is a method used to scan a sample with an electron beam to produce an exaggerated image for determining its surface characteristics and morphology.^{12, 31} This study investigated the surface morphology and exact size of AgNPs using SEM (Tescan, Czech Republic) with an accelerating voltage of 15 kV. An operating pressure of 7×10^{-2} bar and a current of 20 mA were applied for two minutes during operation.

In-vitro ANTIOXIDANT ASSAYS

The synthesized silver nitrate nanoparticles of *A. filipendulina* hydro-alcoholic extract were subjected to antioxidant activity evaluation using two different methods: DPPH (2,2-diphenyl-1-picryl hydrazyl) free radical scavenging assay and FRAP (Ferric Reducing Antioxidant Power).^{32,33}

DPPH Free Radical Scavenging Assay

A series of concentrations (7.8–250 $\mu\text{g/mL}$) of A.F-AgNPs and extract were separately prepared using a serial dilution method. Then, 1 mL of each sample was mixed with 1 mL of DPPH solution (final concentration = 10 $\mu\text{g/mL}$). The samples were incubated in darkness for 30 minutes. To calculate the reduction of DPPH radical, UV-Vis absorption of all samples was recorded using an Agilent UV-Vis spectrophotometer at 517 nm. The reduction capacity (RC_{50}) was measured according to the following equation:

$$\text{RC}_{50} = (\text{Absorbance of control} - \text{Absorbance of the sample}) \times 100 / \text{Absorbance of control}$$

FRAP Assay

The method is based on the ability of antioxidants to reduce

Fe^{3+} to Fe^{2+} in the presence of TPTZ (2,4,6-tripyridyl-s-triazine), forming an intense blue Fe^{2+} -TPTZ complex.³⁴ 3 mL of the FRAP solution mixed with 100 μL extract and A.F-AgNPs aqueous solution, both at a concentration of 3.2 mg/mL. The mixtures were incubated in the dark at room temperature for 10 minutes. The absorbance of samples at a wavelength of 593 nm was measured. This operation was carried out by an autoanalyzer (Abbott, USA). The samples' absorbance was compared to the Ferrous Sulfate standard curve, and the FRAP values were expressed as mmol Fe (II)/mg extract.

Determination of Total Phenolic Content (TPC)

The TPC of the extract and AgNPs was measured by the Folin-Ciocalteu method, and the outcomes were expressed in terms of mg gallic acid equivalent per gram of dry weight of samples (mg GAE/g sample dry weight). Aqueous solutions of A.F-AgNPs and extract were prepared at 0.25 mg/mL concentrations. 1 mL of each sample was mixed with 0.2 mL of Folin-Ciocalteu and 1 mL of 2% Na_2CO_3 . After 30 minutes of incubation, the absorbance of samples and standards was recorded by a UV-Vis spectrophotometer at 750 nm.³⁴

Cytotoxic Activity

Cytotoxic activity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay on A549 lung tumor cell line and MRC-5 lung regular cell line according to the international standards for biological evaluation of medical device (ISO 10993-5:2009). In brief, after cells were counted, they were transferred to 96-well plates at a density of 12,000 cells per well for A549 in columns 6, 7, 8, and 8000 cells per well for MRC-5 in columns 1, 2, and 3. Both cell lines were treated with a series of A.F-AgNPs aqueous suspensions ranging from 6.25 to 400 $\mu\text{L/mL}$, extract aqueous solutions ranging from 3.125 to 100 $\mu\text{L/mL}$, and silver nitrate solutions ranging from 4.6875 to 300 $\mu\text{L/mL}$. A stock concentration (5 mg/mL in phosphate-buffered saline) of MTT solution was prepared, and 20 μL of the solution was added per well and incubated in the dark for 3 hours. After dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan crystals, the plate was shaken in a Heidolph shaker incubator for 20 minutes. The absorbance was recorded by an ELISA reader (Epoch microplate spectrophotometer) at 570 nm. The analysis obtained from the MTT test was performed using GraphPad PRISM (version 2.01) and Excel software.

After calculating the percentage of cell growth using Microsoft Excel software, the percentage of cell growth was calculated with the following formula:

$$\% \text{ growth inhibition} = (1 - \text{Test V} / \text{Max V}) \times 100$$

Test V is the number of live cells after loading the sample, and Max V is the number of live cells in the control (negative control). The value of IC_{50} , the sample

concentration required for the growth of 50% of the cells, was calculated using the sigmoid dose-response curve by PRISM software and nonlinear regression analysis.³⁵

Results

UV-Vis Spectroscopy

When the reduction of silver ions to A.F-AgNPs occurs, the color starts to change in the solution to brownish-red, indicating the transformation of silver ions into elements. Due to the surface plasmon resonance of A.F-AgNPs, the UV-Vis spectrum shows a peak near 450 nm after 24 hours of incubation, indicating the formation of AgNPs and completion of the reaction (Figure 1).

FTIR Analysis

The absorption peaks of the extract spectra displayed at 3413 cm^{-1} , 2926 cm^{-1} , 1620 cm^{-1} and 1516 cm^{-1} are attributed to the -OH stretching band of alcohols and phenols, C-H of aliphatic chains or aromatic rings, -C=O stretching band of carbonyl, and C=C stretching band of aromatic rings. The strong band at 3413 cm^{-1} in *A. filipendulina* extract was attributed to the -OH stretching band of alcohols and phenols, which shifted to 3420 cm^{-1} in A.F-AgNPs. The absorption peaks of A.F-AgNPs displayed at 3420 cm^{-1} , $2963\text{--}2852\text{ cm}^{-1}$ and $1621\text{--}1638\text{ cm}^{-1}$ are attributed to the O-H stretching band of alcohol and phenol, C-H of aliphatic chains or aromatic rings, and -C=O stretching band of carbonyl, demonstrating that these bands were formed in biosynthesized NPs (Figure 2).

DLS Analysis

NPs A

Based on the number of nanoparticles (NPs), the particle size distribution diagram is a crucial and informative tool for analysis. Only one peak was observed after 24 hours of incubation, indicating a relatively narrow range. The sharper the peak, the closer the particle sizes are to each other. All NPs exhibited a diameter of $48.94 \pm 18.93\text{ nm}$, with an average size of 129.7 nm . The polydispersity index

(PDI) was calculated as 0.262, suggesting a narrow size distribution and indicating proximity to the optimal value (Figure 3A). PDI ranges from 0.0 (for a sample exhibiting complete uniformity in size) to 0.1 (for a highly dispersed sample with multiple particle size populations). Values of 0.2 and below are typically deemed optimal for NP materials in size analysis.^{36,37}

NPs B

Based on the number of NPs, the particle size distribution diagram exhibited only one peak after 24 hours of incubation, indicating a uniform size distribution. All nanoparticles measured a diameter of $87.64 \pm 30.53\text{ nm}$, with an average size of 145.1 nm . The PDI was 0.283, approaching the optimum value (Figure 3B).

NPs C

Insufficient nanoparticles were synthesized using absolute ethanol as a solvent. After 24 hours of incubation, there was no discernible color change.

NPs D

Based on the number of NPs, the particle size distribution diagram exhibited a single peak after 24 hours of incubation. All nanoparticles measured a diameter of $141.7 \pm 66.03\text{ nm}$, with an average size of 214.9 nm . The PDI was 0.196, representing the optimum value (Figure 3C).

NPs E

Based on the number of NPs, the particle size distribution diagram exhibited a single peak after 24 hours of incubation. All nanoparticles measured a diameter of $163.9 \pm 66.70\text{ nm}$, with an average size of 196.1 nm . The PDI was 0.222, closely approaching the optimum value (Figure 3D).

NPs A After Two Weeks to Estimate the Stability of NPs in Water

Based on the number of NPs, the particle size distribution

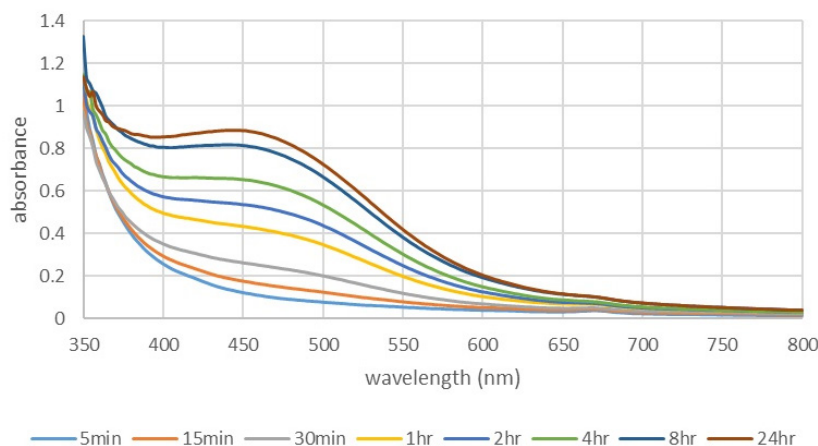
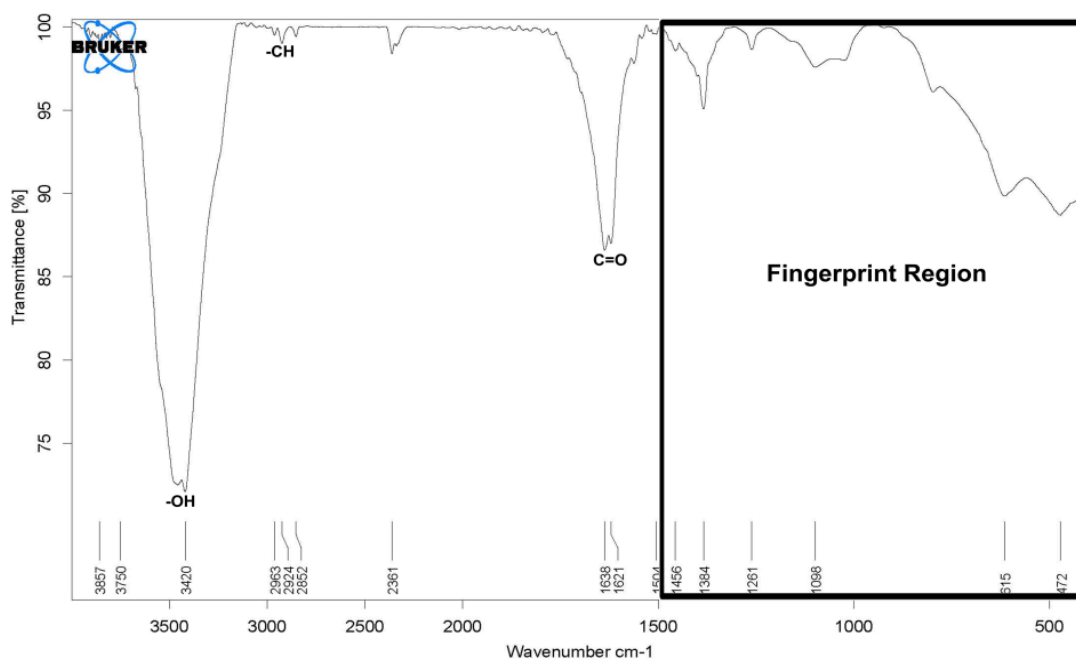


Figure 1. UV-Vis absorption spectra of A.F-AgNPs at different time intervals. UV-Vis spectrum shows a peak near 450 nm after 24 hours of incubation, indicating the formation of AgNPs.

(A)



(B)

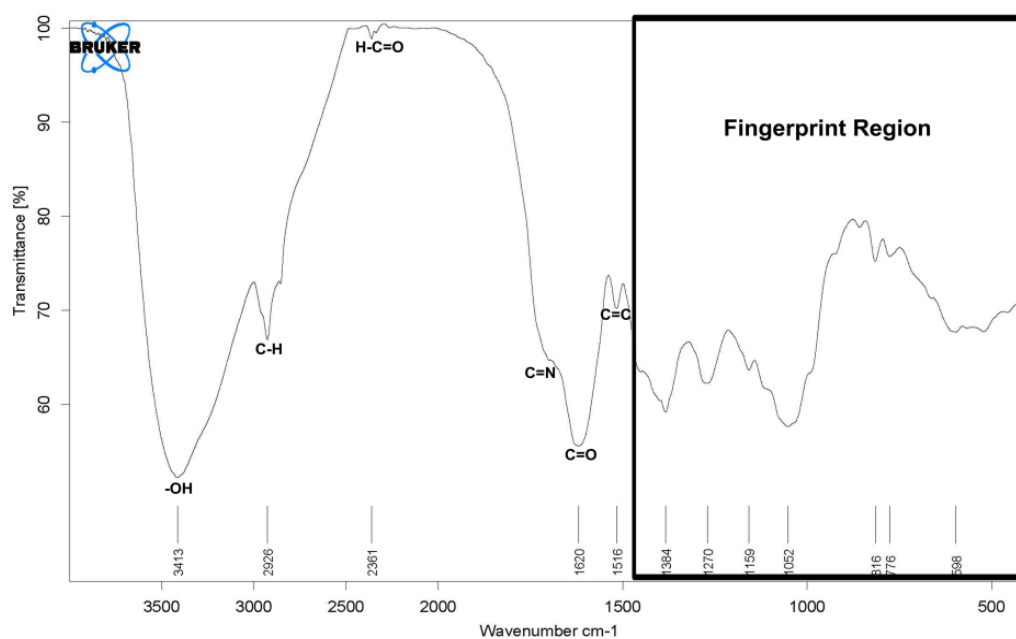


Figure 2. FTIR spectra of A.F-AgNPs (A) and *A. filipendulina* extract (B). IR spectra were collected in the wavelength range from 400-4000 cm^{-1} and averaged over 9 scans for each measurement with a resolution of 2 cm^{-1} .

diagram exhibited a single peak after two weeks of dispersion in water. All nanoparticles measured a diameter of 165.8 ± 61.59 nm, with the average size increasing from 129.7 nm to 200.9 nm. The PDI was 0.245, closely approaching the optimum value.

Zeta Potential of NP A

The zeta potential value determined the surface charge of the NPs. A negative zeta potential of approximately

-32.2 mV was recorded in the current study, indicating the high stability of the synthesized NPs (Figure 4). The cell lines used in this study are A549 tumor lung cells and MRC-5 normal lung cells, both non-phagocytic cells. Cellular uptake and cytotoxicity were higher for positively charged surface NPs compared to negatively charged ones in non-phagocytic cells.³⁸⁻⁴⁰ Particles with surface charge exhibited more cytotoxicity than neutral nanoparticles.⁴¹ The surface charge of A.F-AgNPs is negative, possibly

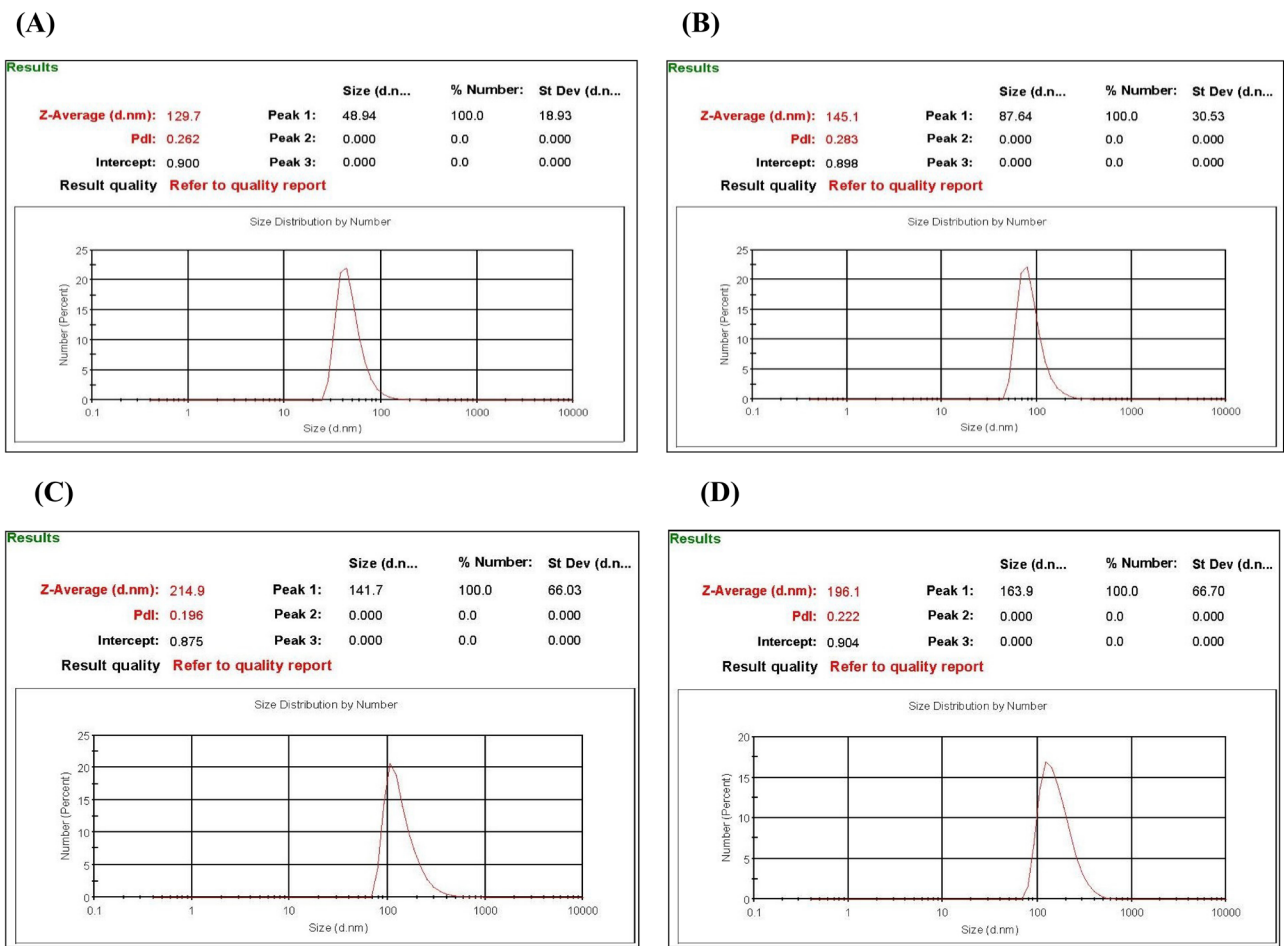


Figure 3. Particle size analysis of A.F-AgNPs mediated by a 1.6x weight ratio of extract to silver nitrate (A), 2x weight ratio of extract to silver nitrate (B), 70% ethanol extract solution (C), and gelatin (D).

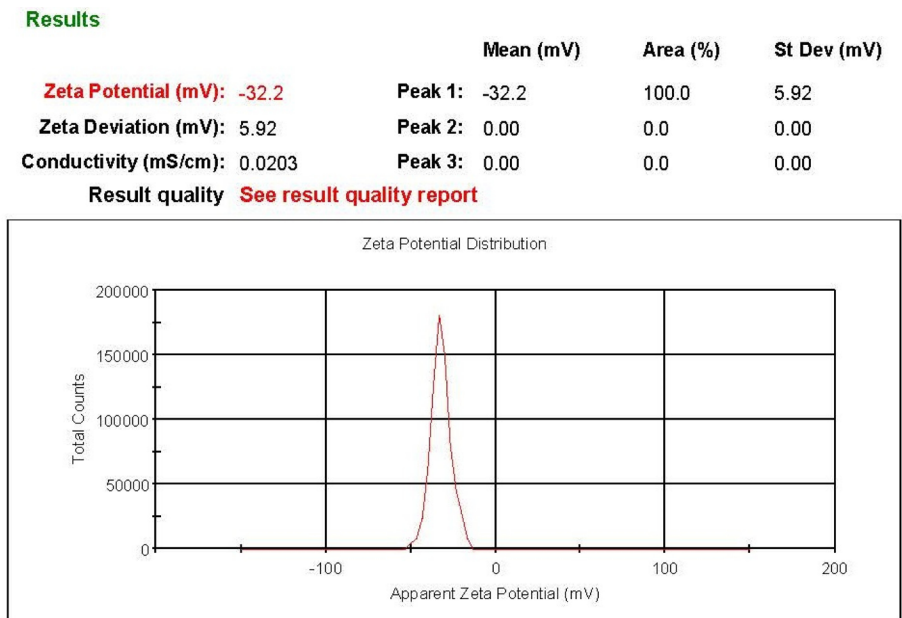


Figure 4. The Zeta potential of A.F-AgNPs is mediated by a weight ratio of 1.6x.

attributed to functionally effective components acting as capping agents in the hydro-alcoholic extract of *A. filipendulina*.

FESEM Analysis

The SEM images revealed that biosynthesized A.F-AgNPs exhibited a spherical morphology with diameters ranging

from approximately 38.23 to 55.38 nm, displaying a remarkably narrow diameter distribution (Figure 5).

In-vitro Antioxidant Assays

DPPH is a stable free radical compound that readily accepts electrons or hydrogen from AgNPs. The results of free-radical scavenging assays indicate that the reduction percentage increases with the rising concentrations of AgNPs and crude extract.²

The findings demonstrated that the aqueous solution containing A.F-AgNPs and the extract exhibited radical scavenging and antioxidant activities. The RC_{50} (concentration of antioxidant necessary to scavenge 50% of free radicals) of quercetin, used as the standard compound, was 4 $\mu\text{g/mL}$, indicating that a lower RC_{50} value signifies higher antioxidant potency.

Furthermore, the FRAP assay revealed that 3 mL of A.F-AgNPs and extract (3.2 mg/mL) can reduce 1.01 mmol and 4.68 mmol of Fe^{3+} to Fe^{2+} , respectively. Hence, the extract's antioxidant potency was four times higher than that of A.F-AgNPs at a concentration of 3.2 mg/mL (Table 1).

Total Phenolic Content (TPC)

According to the TPC assay results, the extract's TPC was determined to be 78.57 mg/g DW (dry weight), while in the AgNPs, it was 18.91 mg/g DW. The FTIR spectra of both the extract and the AgNPs exhibited firm absorption peaks corresponding to the stretching vibration of the hydroxyl (-OH) functional group, indicating the presence of phenolic groups in both. The TPC of the extract was four times that of A.F-AgNPs, corresponding to the proportion of extract loaded onto nanoparticles (24% loading). The results of the TPC assay corroborate those of the antioxidant assay, suggesting that *A. filipendulina* extract serves as a rich source of phenolic compounds for the biosynthesis of A.F-AgNPs.

Cytotoxic Activity

The cytotoxic activity of A.F-AgNPs, *A. filipendulina* extract, and silver nitrate was evaluated by assessing the cell viability of MRC-5 normal lung fibroblast cells and A549 lung adenocarcinoma cells following 48 hours of incubation. Figure 6A illustrates that silver nitrate exhibited high cytotoxicity, approaching 100%, even at low concentrations in both cell lines, rendering the calculation of the IC_{50} (half maximal inhibitory concentration) unfeasible. Conversely, as depicted in Figure 6B, the extract demonstrated no lethality at the tested concentrations, precluding an assessment of its cytotoxic potency. However, the IC_{50} cannot be computed.

A.F-AgNPs displayed dose-dependent cytotoxicity against both cell lines, as illustrated in Figure 6C. The IC_{50} values of A.F-AgNPs against A549 and the MRC-5 cells were $10.48 \pm 1.90 \mu\text{g/mL}$ and $15.75 \pm 1.44 \mu\text{g/mL}$, respectively. These results indicated that the cytotoxicity of A.F-AgNPs against A549 tumor cells surpassed that against MRC-5 normal cells, with the cytotoxicity ratio on A549 cells compared to MRC-5 cells estimated at approximately 1.5 times.

Discussion

Today, the prevention and treatment of cancer stand as paramount concerns within the medical sciences. In recent years, extensive research has delved into natural products, notably plants, to discern bioactive compounds

Table 1. Antioxidant results of silver nitrate, AgNPs with extract (A.F-AgNPs), and crude extract

Samples	DPPH assay (RC_{50} : $\mu\text{g/mL}$)	FRAP assay (m mol)
Silver nitrate	N/A	N/A
Silver nanoparticles with extract (A.F-AgNPs)	181.871	1.01
Crude extract	49.878	4.68

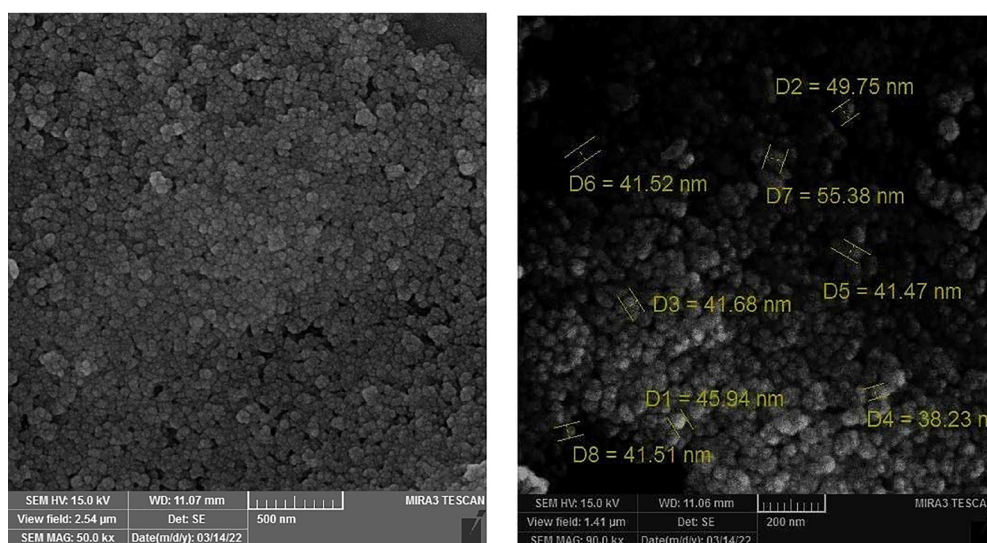


Figure 5. FE-SEM images of A.F-AgNPs

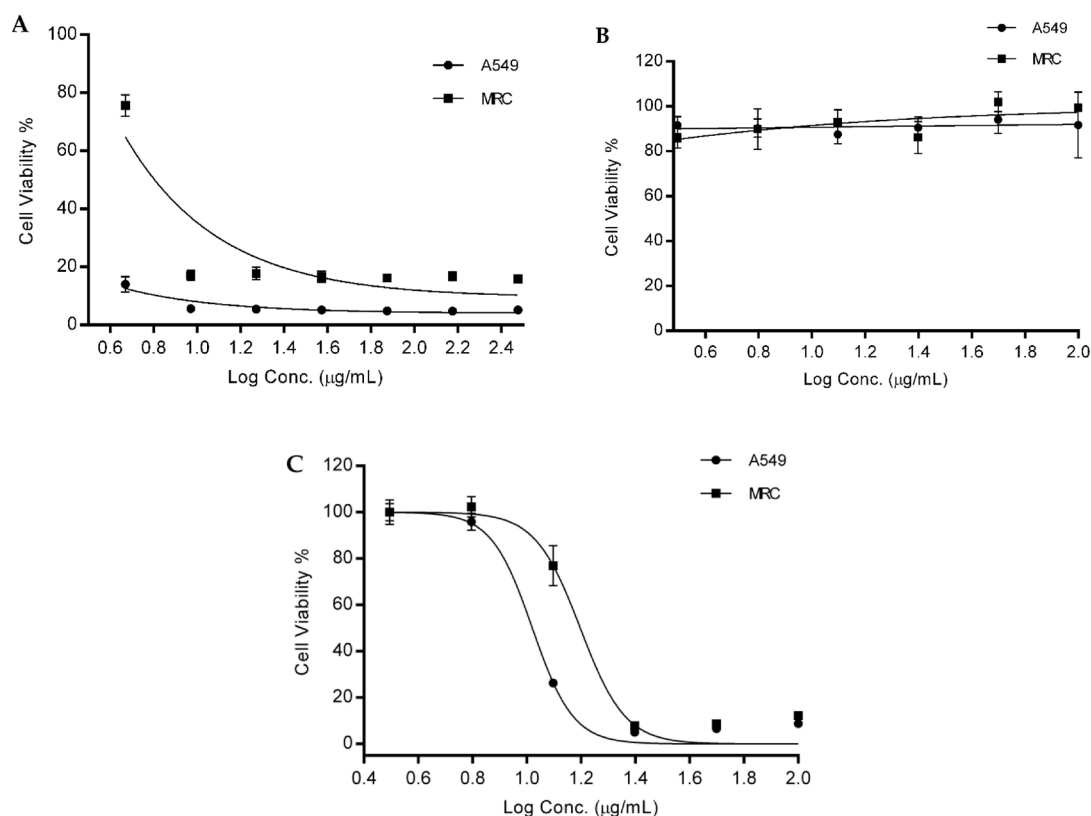


Figure 6. The percentages of cell viability of MRC-5 normal lung fibroblast cell lines & A549 lung adenocarcinoma cell lines at the presence of various concentrations of silver nitrate (A), *A. filipendulina* extract (B), and A.F-AgNPs (C)

and their potential medicinal properties in combating malignancies.^{12,42} Prior investigations have elucidated the pivotal role of secondary metabolites in plants, augmenting the efficacy of conventional drugs while mitigating their adverse effects.^{43,44} With advancements in drug delivery systems and the interdisciplinary utilization of biological sciences, including traditional and herbal medicine, alongside cutting-edge biotechnology and nanotechnology tools facilitating the production of green synthesized nanoparticles, a novel strategy has emerged for preventing and treating diseases, including cancer.¹²

The red-brown color produced by *Achillea* nanoparticles under the influence of electromagnetic waves occurred due to the collective oscillations of the conduction band electrons of A.F-AgNPs because of surface plasmon resonance. According to UV visible spectrum of different concentration of A.F-AgNPs (Figure 1), the appearance of the surface plasmon resonance band with UV-Vis spectrum near 450 nm, absolutely established the successful synthesis of AgNP using the *A. filipendulina* extract as a reducing agent. This absorbance peak could be used to predict the synthesized A.F-AgNPs.

Previous studies have shown that the silver reduction reaction was attributed to phytochemicals hydroxyl groups.⁴⁵ FTIR analysis (Figure 2) and TPC assay outcomes confirmed the presence of hydroxyl groups in *A. filipendulina* extract, which can be responsible for the silver ions reduction reaction in the current report. Moreover, the presence of phenolic structures can be the

major classes of plant phytochemicals with antioxidant activities.⁴⁶ Our studies on the antioxidant properties of the hydroalcoholic extract of *A. filipendulina* using DPPH and FRAP tests indicated significant antioxidant capacity of extract. In addition, comparing the antioxidant effects of the crude extract and prepared nano-formulation designated the loaded amount of hydroalcoholic extract in prepared nano-formulation (Table 1). A 2020 published study reported significant antioxidant activities of the ethanolic extract of flowers and leaves of *A. filipendulina* with potent radical scavenging effects equal to 53.93 and 51.70 mg trolox equivalents/grams of sample for DPPH assay and 43.47 and 35.03 mg trolox equivalents/grams of sample for ABTS assay, respectively.²⁴ In addition, a recently published study demonstrated remarkable antioxidant effects of ethanolic extract $18.13 \pm 1.53 \mu\text{g/mL}$ and $432.32 \pm 3.43 \text{ mg/g}$ using DPPH and FRAP assays, respectively.⁴⁷

The particle size and surface charge determination of nanoparticles are essential for suitable characterization. DLS and zeta potential measurements have been considered as easy, simple, and reproducible tools for determining particle size and surface charge.⁴⁸ According to the DLS results in Figure 3, the particle size distribution diagrams showed the prepared A.F-AgNPs with a weight ratio of 1.6 (extract to silver nitrate) water solution was the best nano-formulation for the next cytotoxic studies based on intensity, number, and volume, respectively. The PDI value of the nanoparticles was 0.262 indicating

uniformity in the size of the nanoparticles. Although the particle size uniformity (PDI) of prepared nanoparticles with hydroalcoholic solution was slightly better, but the size of them was not optimum. The particle size of A.F-AgNPs prepared with aqueous solution was much smaller (48.94 ± 18.93 nm) and the particle size distribution diagram based on its number is more symmetrical. Moreover, the particle size of A.F-AgNPs prepared in the presence of gelatin was greater and it was not suitable for further studies. As the next step, the prepared A.F-AgNPs with aqueous solution with 1.6 ratio were stored in aqueous solution for two weeks to measure the changes in nanoparticle size and physical stability. The results demonstrated the particle size distribution diagram (based on number) that only one peak at size of 165.8 nm, Z-average of 200.9 nm, and PDI value of 0.245, which showed that after 2 weeks of water exposure of nanoparticles, they have increased in size approximately 2-fold. The average size has increased from 129.7 nm to 200.9 nm. The PDI has decreased from 0.262 to 0.245. Therefore, the uniformity of particle size has increased slightly.

Various nanoparticles with a zeta potential among -10 and $+10$ mV are approximately neutral, whereas nanoparticles with zeta potentials of higher than $+30$ mV are cationic and the nanoparticles with zeta potentials less than -30 mV are anionic.⁴⁹

Most cell membranes are negatively charged, therefore zeta potential can affect the tendency of a nanoparticle to penetrate membranes, with cationic particles generally exhibiting greater toxicity in relation to cell wall disruption.⁴⁹ In addition, one of the general applications of zeta potential data is its relationship to colloidal stability. According to nanoparticle-dispersions guidelines, the zeta potential values of more than $+30$ mV and less than -30 mV introduce as highly stable particles in drug delivery literature.⁴⁹ The result of the evaluation of the zeta potential of prepared A.F-AgNPs (1.6 ratio in aqueous solution) showed the -32.2 ± 5.92 mV (Figures 4 and 5). As mentioned above, the zeta potential value of -32.2 mV confirmed the stability of the nanoparticles. In addition, the uptake of A.F-AgNPs with a negative zeta potential of -32.2 ± 5.92 mV depends on various factors for these particles to be absorbed into lung cells. The negative zeta potential indicates a negative charge on the surface of the particles and they may not be directly absorbed by the cells, but this interaction may be influenced by factors such as particle size. The greater negative zeta potential value may be attributed to the effective functional constituents as capping agents present in the *A. filipendulina* extract. In this study, the hydro-alcoholic extract of *A. filipendulina* synthesized AgNPs, investigating their cytotoxic properties on normal and cancerous lung cells. AgNPs have garnered significant attention due to their remarkable physicochemical properties, particularly their potent antimicrobial activity against a spectrum of bacteria, viruses, and fungi.^{50,51}

Moreover, these nanoparticles find application in numerous medical and consumer products.^{52,53} While it was estimated that approximately 30% of consumer goods contained AgNPs by 2011, these nanoparticles exhibit various toxicities in humans, particularly affecting vital organs such as the lungs.⁵⁰ Chairuangkitti et al reported that AgNPs induce toxicity in A549 cell lines through ROS (reactive oxygen species)-dependent and ROS-independent pathways. The ROS-dependent pathway, associated with cytotoxicity, involves the generation of ROS within cells, leading to various toxic effects, including disruption of normal MMP and mitochondrial function and induction of cell death, potentially through apoptosis. ROS-independent pathways, manifesting as anti-proliferative activity, operate through cell cycle inhibition with S-phase arrest.⁵⁰ Despite numerous in vitro and in vivo studies on the therapeutic potential of green synthesized AgNPs for various disorders, many overlook silver nitrate's toxicity and side effects on lung tissues, commonly used in silver nanoparticle synthesis protocols.⁵⁴ Fehaid et al. investigated the effects of PVP (polyvinylpyrrolidone)-coated AgNPs and silver nitrate on the inflammatory response following a single intratracheal instillation in rats. Their findings demonstrated that silver ions exhibited more significant toxicity than PVP-coated AgNPs in lung tissue, albeit with higher stability. This discrepancy stemmed from the rapid absorption of silver ions into the bloodstream, leading to acute toxicity dispersed across various tissues, including pulmonary epithelial cells, within 24 hours. In contrast, PVP-coated AgNPs displayed slower absorption and distribution, resulting in subchronic toxicity.⁵⁴

The cytotoxicity assay results (Figure 6) revealed nearly 100% cytotoxicity for AgNO₃, even at low concentrations, in both A549 cancer cells and MRC-5 normal cells, while the crude plant extract proved safe for both cell lines. Notably, A.F-AgNPs exhibited dose-dependent cytotoxic activity on both cell lines, with an IC₅₀ values of 10.48 ± 1.90 µg/mL for A549 cells and 15.75 ± 1.44 µg/mL for MRC-5 cells. Consequently, A.F-AgNPs demonstrated more significant toxicity toward A549 cancer cells than MRC-5 normal cells. Thus, the *A. filipendulina* extract utilized in silver nanoparticle synthesis served as a safe capping and reducing agent, attenuating the toxicity of silver nitrate on normal lung cells (MRC-5). It is conceivable that employing plant extracts rich in polyphenolic contents or pure phenolic structures as capping agents may enhance therapeutic effects on cancer cells while minimizing side effects on normal lung cells.

Limitations of Study

The study had several limitations that suggested the necessity of further studies to evaluate the therapeutic potential of A.F-AgNPs more comprehensively:

1. Limited in vitro testing: The cytotoxicity was assessed only on two cell lines (A549, a cancer cell line, and MRC-5, a normal lung fibroblast cell line).

Expanding the range of cell lines to include other cancer types and normal tissues could provide a broader understanding of the nanoparticles' effects.

2. Lack of in vivo evaluation: The study was confined to in vitro methods, which may not fully replicate the complexities of a living organism. In vivo studies in animal models would be necessary to confirm the biological activity, biodistribution, and safety of the synthesized AgNPs.
3. Absence of long-term toxicity and stability data: The study primarily focused on immediate cytotoxicity and antioxidant potential. Long-term studies on the stability of the nanoparticles in biological systems and their possible accumulation or clearance would help assess their viability for therapeutic use.

Conclusion

This study demonstrates the green biosynthesis of AgNPs facilitated by *A. filipendulina* extract, presenting an uncomplicated, cost-effective, and eco-friendly approach. The metabolites inherent in the extract serve as both capping and reducing agents. The morphology and dimensions of the nanoparticles were characterized using FE-SEM and DLS. AgNPs exhibited significantly lower cytotoxicity in normal and tumor cell lines than silver nitrate. This reduction in cytotoxicity can be attributed to the presence of bioactive compounds from the extract encapsulated within the nanoparticles. FTIR analysis, DPPH scavenging assay, and TPC evaluation indicate that A.F-AgNPs possess antioxidant activity and phenolic functional groups. The higher cytotoxicity observed in tumor cells compared to normal cells is a favorable outcome, suggesting promising potential for further cellular-level research on AgNPs derived from *A. filipendulina* extract. At concentrations below 10 µg/mL, the cytotoxicity of A.F-AgNPs decreased compared to silver nitrate. However, it is noteworthy that at higher concentrations, the cytotoxicity of A.F-AgNPs approaches that of silver nitrate (100%). Biosynthesized AgNPs hold promise as potential anticancer agents in the biopharmaceutical industry.

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Competing Interests

None declared.

Ethical Approval

All experimental procedures were carried out in consistent with the guideline of the Ethics committee of the Tabriz University of Medical Sciences (code: IR.TBZMED.VCR.REC.1400.011).

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