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Introducing a Novel Chemotherapeutic Drug for the Treatment of Lung Adenocarcinoma: Silver Nanoparticles Green-formulated by *Cinnamomum verum*

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Abstract: In this study we report the green synthesis of nontoxic, stable, and small size silver nanoparticle by *Cinnamomum verum* with reducing/capping ability without any toxic reducing agents. The *in situ* prepared AgNPs were characterized by advanced physicochemical techniques like FE-SEM, TEM, and UV-Vis study. It has been established that AgNPs have a spherical shape with a mean diameter from 10 to 45 nm. In the antioxidant test, the IC50 of AgNPs and BHT against DPPH free radicals were 191 and 242 µg/mL, respectively. In the cellular and molecular part of the recent study, the treated cells with AgNPs were assessed by MTT assay for 48 h about the cytotoxicity and anti-human lung adenocarcinoma properties on normal (HUVEC) and lung adenocarcinoma cell lines i.e. PC-14, LC-2/ad, and HLC-1. The IC50 of AgNPs were 259, 291, and 395 µg/mL against PC-14, LC-2/ad, and HLC-1 cell lines, respectively. The viability of malignant lung cell line reduced dose-dependently in the presence of AgNPs.

Key words: *Cinnamomum verum*, silver nanoparticles, antioxidant, lung cancer

1 Introduction

Cancer is recognized as one of the leading causes of death in today's society and several drugs have been introduced to treat this disease, but, most common cancers are not yet controllable and this disease imposes huge costs on the patient and society[1-4]. The main factor in the development and progression of cancer has not yet been precisely identified; however, the available data suggest that metabolic disorders in the tissue and immune disorders may be involved in the development and exacerbation of this disease. In addition, metabolic disorders in the production and excretion of oxygen free radicals are important factors affecting cancer cells[4, 5]. Free radicals are destructive compounds that are produced as a by-product by the body's chemical reactions and are destroyed by the body's defense system and enzyme system and antioxidants. However, in cases where the body's metabolic disorders and the production of free radicals are high and they are not destroyed by the neutralizing system, due to their instability, these compounds have a strong tendency to react with a variety of molecules in the body[6-8]. It is estimated that each cell in the human body is exposed to free radicals 10,000 times a day and DNA strands 5,000 times a day. Damage to cell components includes proteins (genetic disorder), fats (lipid oxidation), and cell membranes (permeability disorder) that if the damage is not repaired, it leads to disruption of the chemical reaction and normal proteinization of the cell and the formation of harmful compounds and sometimes cancer cells in the body [8-10]. It is reported that thousands of cancer cells are produced daily in the human body that are killed by the body's defense system. In some cases, due to dysfunction of the above systems, cancer cells proliferate and conditions for cancer development in different tissues[7-9]. According to the above, antioxidants play a vital role in preventing disorders caused by the effects of free radicals and thus the prevention and treatment of cancer. Antioxidants are a wide range of molecular compounds with complex properties that combine with and neutralize free radicals. The results show that more than 60,000 types of molecular antioxidants have

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been identified so far. Antioxidants can be effective in three known ways to prevent and treat cancer; 1. Destruction of free radicals 2. Strengthen the immune system to destroy cancer cells. Prevent the adhesion of cancer cells to other cells and prevent their proliferation[6-10].

Today, nanoparticle technology has made great strides in the production of many drugs, and the production of nanoparticles is one of the hopes in the effective treatment and diagnosis of many diseases, including cancer[11-14]. Metallic nanoparticles have long been considered as a candidate for cancer treatment. Because natural metal oxides are present in large quantities in nature, the processing and synthesis of these nanoparticles can be one of the least expensive synthesis protocols[14-17]. Metallic nanoparticles are one of the new types of widely used mineral particles that have been considered by researchers due to their suitable physical and chemical properties and at the same time, it has more adsorption power than other metallic nanoparticles-containing compounds[12-15]. Metallic nanoparticles are one of the therapeutic compounds recognized by the US Department of Food and Drug Administration as a safe substance. Metallic nanoparticles are biocompatible and non-toxic and have also been used as medical fillers, cosmetics and drug carriers[11-13]. The special properties of metallic nanoparticles are high catalytic activity, low dielectric constant, high chemical stability, absorption of infrared and ultraviolet light and most importantly its antibacterial properties. If the therapeutic and anticancer effects of these compounds are confirmed, this could be a significant step in advancing cancer therapies[14-17].

In the present work, we report a simple, environmentally friendly, cost-effective and highly reproducible green synthetic approach for producing AgNPs using *Cinnamomum verum* as the stabilizing and reducing agent and water as the dispersion medium. The prepared AgNPs were characterized and applied as efficient anti-human lung adenocarcinoma agent.

2 Experimental

Dimethyl sulfoxide (DMSO), Antimycotic antibiotic solution, hydrolysate, decamplmaneh fetal bovine serum, Ehrlich solution, 4-(Dimethylamino) benzaldehyde, 2,2-diphenyl-1-picrylhydrazyl (DPPH), carbazole reagent, borax-sulphuric acid mixture, Dulbecco's Modified Eagle Medium (DMED), and phosphate buffer solution (PBS) all were achieved from Sigma-Aldrich company of USA.

2.1 Preparation of the AgNPs

Fresh leaves of *Cinnamomum verum* were collected, washed several times with distilled water to eliminate the dirt particles present on the leaves. The cleared leaves were dried in the oven at 60℃ for 12 h to remove the remaining moisture, and then ground into powder. The *Cinnamomum verum* leaf aqueous extract was prepared by adding 20 g of dried leaves powder in 500 mL of boiling distilled water for 5 min. Then the mixture was centrifuged at 5000 rpm for 15 min in a Jouan-type centrifuge, and the supernatant was filtered in flasks to obtain a cell aqueous extract and stored at 4℃ in refrigerator for further use[18].

The green synthesis of the silver nanoparticles was initiated with a reaction mixture of 100 mL of silver salt ($AgNO_3$) in the concentration of 1×10^{-3} M and 200 mL of aqueous extract solution of *A. sativum* leaf (20 μg/mL) in the proportion 1:10 in a conical flask.

The reaction mixture was kept under magnetic stirring for 12 h at room temperature. At the end of the reaction time, the black colored colloidal solution of Ag was formed. The mixture was centrifuged at 10000 rpm for 15 min. The precipitate was triplet washed with water and centrifuged subsequently[18].

For analyzing silver nanoparticles, the common techniques of organic chemistry, i.e. UV-Vis. spectroscopy, FE-SEM, and TEM were used.

Silver nanoparticles were primarily confirmed using UV-Vis spectroscopy at a scan range from 250-700 nm wavelength (Jasco V670 Spectrophotometer).

The morphological features of silver nanoparticles in terms of surface, shape and sizes were exactly analyzed by common morphological testes i.e., FE-SEM (Fe-SEM ZEISS EVO18) and TEM (TEM FEI-TECNAI G2-20 TWIN) microscopic techniques.

2.2 Anti-human lung adenocarcinoma properties of AgNPs

The process of the controlled culture of prokaryotic or eukaryotic cells in a filtered or unfiltered flask or cell culture plate by a suitable culture medium is called. This term is mostly used for culturing multicellular cells. Special culture media are used to culture cells. The cells are usually cultured at 37℃ in equipment such as CO_2 incubators. Cell culture should be performed under aseptic (disinfected) conditions because the growth of these cells is much slower than the growth of bacteria and yeasts and there is a possibility of contamination of the culture medium. Antibiotics such as penicillin, streptomycin, or gentamicin are sometimes used to stop the growth of bacteria. In order for cells to proliferate well in culture medium, their density in culture medium must be low. For this purpose, the cells should be passed to the fresh culture medium from time to time. One of the goals of cell culture is to study cells in terms of how they grow, their nutritional needs, and the reasons they stop growing, each of which can have a profound effect on the morphology of the cells we see under a microscope. Therefore, to study the cell growth cycle, develop methods to control the growth of cancer cells and modulate the expression of genes, it is

necessary to cultivate these cells in the external environment[18].

With the help of cell culture, cells can be prepared that are in different stages of differentiation and can be differentiated into other cells with the help of hormones and growth factors. With the help of cell culture, homogenous cells can be prepared and intracellular activities such as DNA replication, DNA transcription synthesis, RNA and protein synthesis and other details related to metabolism can be studied. It is also possible to examine the subsequent events and intracellular currents, such as the displacement of these complexes, the type of intracellular messages, and how the messages are transmitted, after connecting different molecules to the corresponding membrane receptor. The cultured cells can be stored frozen at very low temperatures. Such conditions will maintain the growth rate or genetic composition of these cells and can be thawed and used again at the appropriate time. This prevents the aging of cells, while it is currently not possible to prevent the aging of animals. When working with laboratory animals, systemic changes due to the effect of the animal's natural homeostasis or the stress of the experiments on the results should be considered. While the use of cell culture eliminates this problem. In addition, standardizing laboratory tests is easier and more practical than tests on living organisms. In laboratory environments, it is much easier to control the physical and chemical factors in the living environment of cells, including acidity, heat, osmotic pressure, and the pressure of gases such as oxygen and carbon dioxide. Cells that are taken directly from the individual are known as primer cells and have a limited lifespan. Most cells have a limited lifespan, except for those taken from a tumor. An immortal cell line can proliferate indefinitely by creating a random or targeted mutation (such as artificial expression of the genus and be established as a representative of specific cell types[18].

In this study, PC-14, LC-2/ad, and HLC-1 cells were used to evaluate the anticancer effect of AgNPs on cell culture.

In the recent study, the cells were cultured in medium (RPMI1640 = Roswell Park MemoryL Institute1640) with 10% FBS combined with penicillin and streptomycin antibiotics in an incubator containing 5% CO_2 in a flask (T25). After three passages for purification, the cells were used to perform the next steps. Cell count and the number of viable cells were performed with a hemocytometer slide using trypan blue. Evaluation of the cytotoxic effect of the AgNPs was performed by the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric test. In this method, MTT, which is yellow, is converted to insoluble and formazan purple dye by the dehydrogenase enzymes in the mitochondria of active cells. The adsorption of this compound can be measured after dissolving at 570-540 nm. After two days and covering the flask bottom with cell, the cell layer adhering to the flask bottom was isolated enzymatically using trypsin-EDTA (5%) (Tetraacetic acid ethylenediamine), after transfer to sterile test tubes, it was centrifuged at 2000 rpm for 10 minutes. The cells were then resuspended in a fresh culture medium with the help of a pasteur pipette and cell suspension (10^6 mL/μg) was prepared from them. 40 μL of this cell suspension (equivalent to $10^4 \times 4$ cells) was poured into 96-well plate flat-bottomed wells (for cell culture). Then the final volume of each well with 10% FBS medium reached 200 μL. The first-row containing cell suspension was considered as negative control (control). After incubation for 18-24 hours to remove cells from the stress caused by trypsinization, the supernatant was removed slowly and carefully, a new medium was added to all rows with different concentrations of the AgNPs (only a new medium was added to the negative and positive control rows), so that the diluted AgNPs with concentrations of 1-1000 μg/mL was added to the third to sixth rows, respectively, the plate was incubated in CO_2 for 48, 24 and 72 hours. After the incubation time, the plate was taken out of the incubator and 20 μL of MTT (Sigma) was added to all wells, and incubated for 3 hours. The supernatant was then gently removed and 100 μL of DMSO was added to the wells and pipetted to dissolve the formazan crystals. The amount of light absorption (OD) according to the intensity of the blue color of formazan at 540 nm was read by Eliza reader. To convert OD to the percentage of living cells, the following formula was used and the percentage of cell life at each concentration was calculated after 48, 24 and 72 hours[18].

$$\text{Cell viability}(\%) = \frac{\text{Sample A}}{\text{Control A}} x100$$

The concentration of the tested compounds that reduced the percentage of cell life by half was considered as IC50 (The half maximal inhibitory concentration)[18].

2.3 Antioxidant activities of AgNPs

The DPPH molecule has a stable free radical in which delocalization of the spare electron over the molecule, results the molecules do not dimerise. This delocalization results in the deep violet color with a characteristic absorption peak in ethanol solution at 517 nm. When the DPPH solution was mixed with electron donating substance, which leads to the reduced form with color loss. Later, the solution undergoes further reactions and control the stoichiometry, hence reduced (decolorized) the number of molecules of the DPPH by one molecule of the reductant[18].

In this experiment, 3 mL 0.004 % DPPH solution was mixed with several concentrations of AgNPs for determining antioxidant properties. A group containing 3 mL ethanol and 3 mL DPPH was considered as the control group. According to the international standards, the samples absorption rate was determined at 517 nm and the antioxidant potentials of AgNPs were assessed according to the following formula[18]:

$$\text{Inhibition}(\%) = \frac{\text{Sample A}}{\text{Control A}} x100$$

2.4 Qualitative Measurement

At least three independent replications were performed for each data and the result was presented as mean ± SD. Data statistical analysis was done with SPSS software version 19 and Anova Way One and Duncan's test. Significance was considered at the level of $p \leq 0.05$.

3 Results and Discussion

3.1 Structural characterization of synthesized AgNPs

FE-SEM analysis is one of the common chemistry tests for determining the morphology and size of several materials such as metallic nanoparticles. In the present study, the FE-SEM image of silver nanoparticles synthesized using *Cinnamomum verum* leaf aqueous extract is shown in Fig. 1. The Ag nanoparticles appeared as an agglomerated structure. The hydroxyl groups present in *Cinnamomum verum* could be responsible for agglomeration[18]. Also, FE-SEM images indicated the range size of 16-44 nm and the shape of spherical for gold nanoparticles.

TEM is the other test for determining the morphology and size of metallic nanoparticles. In our study, the range size of the nanoparticles (12-41 nm) calculated through TEM images (Fig. 2). Furthermore, the histogram plot from the TEM image showed the particle size distribution of biosynthesized silver nanoparticles ranges of 10 to 45 nm. In the previous studies, the size of silver nanoparticles formulated by aqueous extract of medicinal plants had been calculated in the ranges of 10-45 nm with the shape of spherical[18]. These reports support the results of the current work.

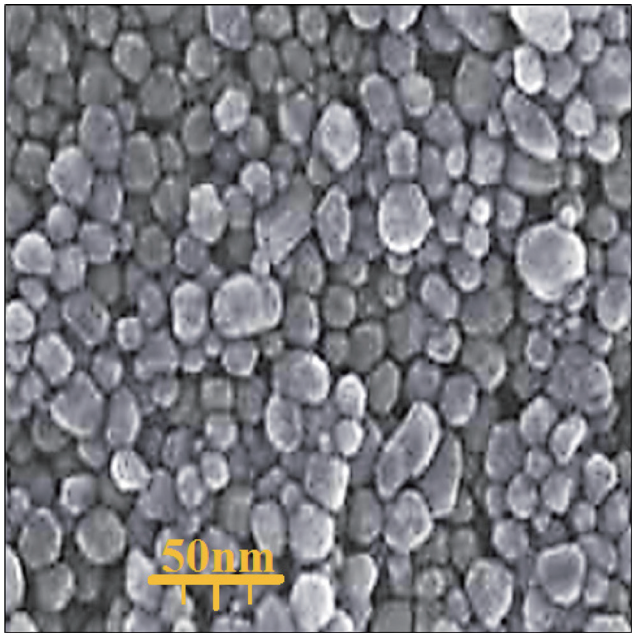


Fig. 1 FE-SEM image of AgNPs.

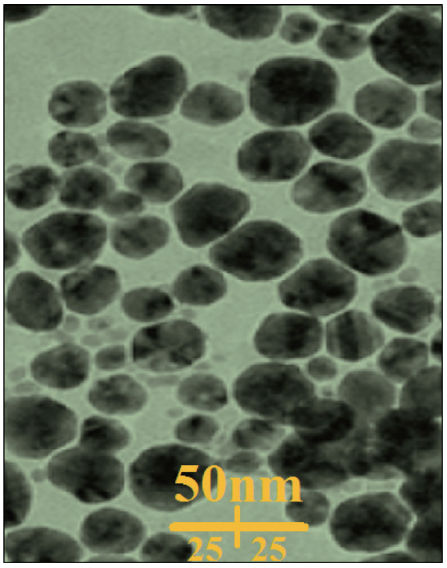


Fig. 2 TEM image of AgNPs.

Ultraviolet–visible spectroscopy or ultraviolet–visible spectrophotometry (UV–Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in part of the ultraviolet and the full, adjacent visible regions of the electromagnetic spectrum. This means it uses light in the visible and adjacent ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the spectrum, atoms and molecules undergo electronic transitions. Absorption spectroscopy is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions of electrons from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state[18]. UV-Vis spectroscopic analysis showed the presence of an absorption peak at 446 nm which confirmed the formation of the silver nanoparticles (Fig. 3).

3.2 Analysis of the anti-human lung adenocarcinoma potentials of AgNPs

Cancer, one of the deadliest causes of death in the world, kills more than 7.5 million people annually. Genetic factors and environmental factors are among the most important factors involved in the development of cancer. On the other hand, environmental factors such as diet, lifestyle, geographical conditions, stressors, age and obesity are involved in the incidence of this disease[19–23]. Clinicians use a variety of methods to diagnose and treat a variety of cancers, the most important of which are chemotherapy, radiation therapy, surgery, and hormone therapy, but the most important way to fight cancer is chemotherapy, which causes many side effects for the patient, including fatigue,

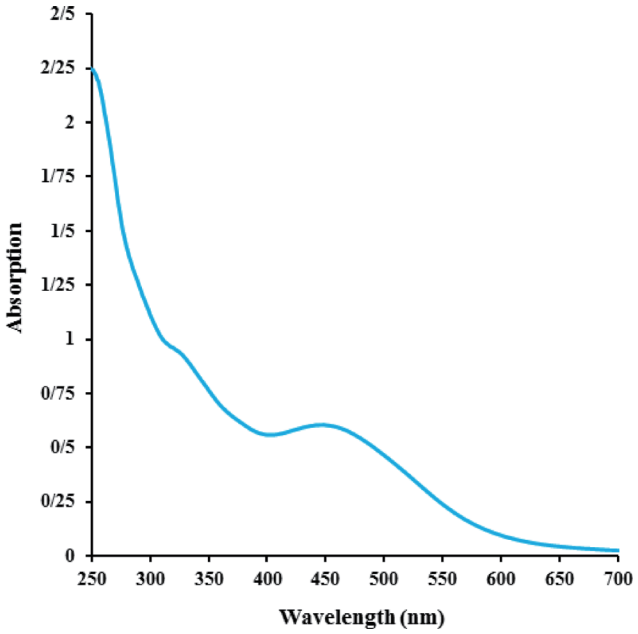


Fig. 3 UV-Vis analysis of AgNPs.

nausea and hair loss, etc[22–26]. These complications can have a great effect on the patient's life quality. Because many of the chemical drugs used to treat cancer cause gastrointestinal disorders, kidney damage, etc[27, 28]. Scientists are looking for drugs with fewer side effects than chemical materials, in which natural compounds and molecules have received much attention. Natural compounds and molecules have fewer side effects than chemical materials due to the combination of other compounds with a specific drug effect. Many natural compounds and molecules contain anti-cancer agents that can exert their effects at different stages of the onset and growth of cancer cells[29–32]. The main goal in preventing cancer with natural or chemical substances is to slow down or inhibit the carcinogenic process. This approach focuses purposefully on abnormal intracellular pathways that lead to abnormal cellular function[29, 33].

In this investigation, the treated cells with different concentrations of the present AgNPs were assessed by MTT assay for 48 h about the cytotoxicity properties on normal (HUVEC) and lung malignancy cell lines i.e. PC-14, LC-2/

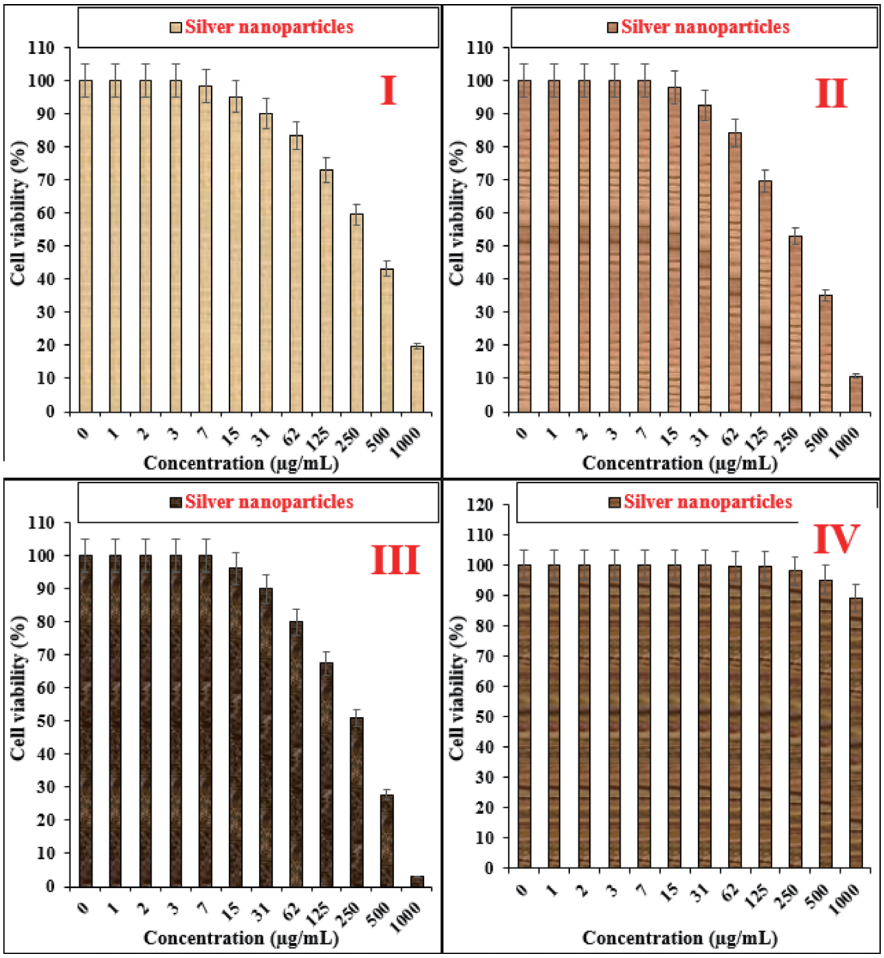


Fig. 4 The cytotoxicity and anti-lung adenocarcinoma properties of AgNPs against HLC-1 (I), LC-2/ad (II), PC-14 (III), and HUVEC (IV) cell lines.

Table 1 The IC50 of AgNPs in the anti-lung adenocarcinoma test.

	Silver nanoparticles (μg/mL)
IC50 against HUVEC	–
IC50 against HLC-1	395 ± 0[b]
IC50 against LC-2/ad	291 ± 0[a]
IC50 against PC-14	259 ± 0[a]

ad, and HLC-1 (Fig. 4).

The absorbance rate was evaluated at 570 nm, which represented viability on normal cell line (HUVEC) even up to 1000 μg/mL for AgNPs (Table 1 and Fig. 4).

The viability of malignant lung cell line reduced dose-dependently in the presence of AgNPs. The IC50 of AgNPs were 259, 291, and 395 μg/mL against PC-14, LC-2/ad, and HLC-1 cell lines, respectively (Table 1).

3.3 Analysis of the antioxidant potentials of AgNPs

Free radicals are molecules with a free electron ready to react, and oxygen is produced with some molecules. If many of them are suddenly produced in the body, they react with some parts of the cell, such as DNA and cell membranes, and cause cell damage or even death[34–39]. Normally, the body's defense system neutralizes these harmless free radicals. Antioxidants prevent the spread of oxidation chain reactions. Thus, the strength of an antioxidant formed by the contact of an H atom with a free radical is due to the effect of an antioxidant on the ease with which this H atom separates from it. Thus, antioxidants can protect cell membranes and various living compounds against oxidants in small amounts[33, 34]. Numerous biochemical and physiological processes may cause the production of free radicals. Reactive oxygen species (ROS) include free radicals and radical-free forms. When the concentration of ROS increases, it can oxidize macromolecules such as proteins, nucleic acids, and membrane lipids, resulting in cell damage and possibly "cell and tissue destruction"[31–34]. Natural compounds and molecules have two main mechanisms for reducing the concentration of ROS, in other words, natural compounds and molecules reduce the concentration of ROS by producing antioxidants and thus prevent cell damage[34, 35]. Recently, many researchers have paid close attention to natural compounds and molecules and their relationship to their antioxidant properties, and many natural compounds and molecules have been studied for their antioxidant activity[31–37].

The scavenging capacity of AgNPs and BHT at different concentrations expressed as percentage inhibition has been indicated in Fig. 5. In the antioxidant test, the IC50 of AgNPs and BHT against DPPH free radicals were 191 and 242 μg/mL, respectively (Table 2).

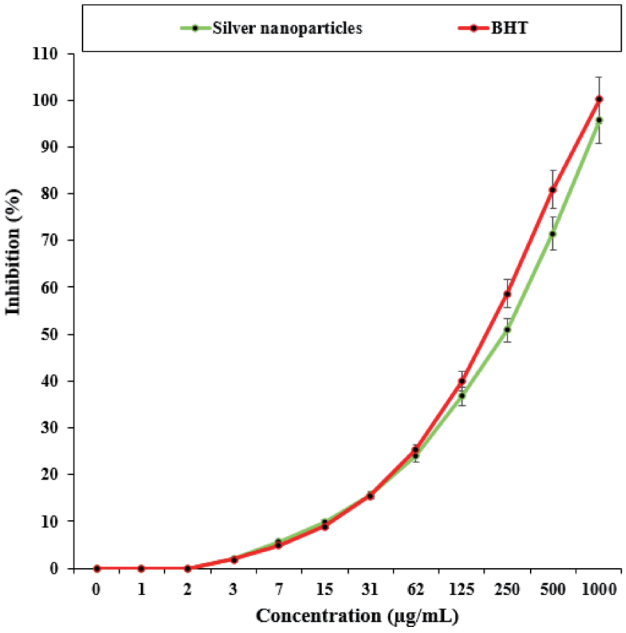


Fig. 5 The antioxidant properties of AgNPs and BHT against DPPH.

Table 2 The IC50 of AgNPs and BHT in antioxidant test.

	Silver nanoparticles (μg/mL)	BHT (μg/mL)
IC50 against DPPH	242 ± 0[a]	191 ± 0[a]

4 Conclusion

In our research, the gold nanoparticles were successfully obtained from the bioreduction of $AgNO_3$ solutions using an aqueous extract of *Cinnamomum verum* leaf. The Ag nanoparticles have been appropriately characterized and confirmed using FE-SEM, TEM, and UV-Vis. The viability of malignant lung cell line reduced dose-dependently in the presence of AgNPs. The IC50 of AgNPs were 259, 291, and 395 μg/mL against PC-14, LC-2/ad, and HLC-1 cell lines, respectively. The AgNPs showed the best antioxidant activities against DPPH. After clinical study, AgNPs can be utilized as an efficient drug in the treatment of lung adenocarcinoma in humans.

Author Contributions

Jianzhong Zhou, Xiaogang Zheng, Qigui Cai, and Chunlin Song had equal roles in designing research and performing research, contributing analytic tools, analyzing data, and writing the manuscript.

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