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Type
Research paper

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Introduction
The present work demonstrates the synthesis of Ag nanoparticles (Ag NPs) by aqueous extract of Thymus capitatus as green reductant and capping agent without any toxic reagent.

Material and methods
Physicochemical characteristics of the said nanocomposite were elucidated by field emission scanning electron microscopy (FE-SEM), fourier-transform infrared spectroscopy (FTIR), and UV-Vis Spectroscopy.

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The biogenic Ag NPs are uniformly globular. The Ag NPs has been explored biologically in the anticancer and antioxidant assays. In the cellular and molecular part of the recent study, the treated cells with Ag NPs were assessed by MTT assay for 48h about the cytotoxicity and anti-human lung adenocarcinoma properties on normal (HUVEC) and lung adenocarcinoma cell lines i.e. lung well-differentiated bronchogenic adenocarcinoma (HLC-1), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung poorly differentiated adenocarcinoma (PC-14). The viability of malignant lung cell line reduced dose-dependently in the presence of Ag NPs. The IC50 of Ag NPs were 209, 185, and 106 µg/mL against HLC-1, LC-2/ad, and PC-14 cell lines, respectively. In the antioxidant test, the IC50 of Ag NPs and BHT against DPPH free radicals were 86 and 76 µg/mL, respectively.

Conclusions
After clinical study, Ag NPs containing Thymus capitatus leaf aqueous extract may be used to formulate a new chemotherapeutic drug or supplement to treat the several types of human lung adenocarcinoma.
Fabrication of Ag nanoparticles mediated by extract of plant and determination of the anti-human lung cancer effects

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Fabrication of Ag nanoparticles mediated by plant extract and determination of the anti-human lung cancer effects

Abstract. The present work demonstrates the synthesis of Ag nanoparticles (Ag NPs) by aqueous extract of *Thymus capitatus* as green reductant and capping agent without any toxic reagent. Physicochemical characteristics of the said nanocomposite were elucidated by field emission scanning electron microscopy (FE-SEM), fourier-transform infrared spectroscopy (FTIR), and UV-Vis Spectroscopy. The biogenic Ag NPs are uniformly globular. The Ag NPs has been explored biologically in the anticancer and antioxidant assays. In the cellular and molecular part of the recent study, the treated cells with Ag NPs were assessed by MTT assay for 48h about the cytotoxicity and anti-human lung adenocarcinoma properties on normal (HUVEC) and lung adenocarcinoma cell lines i.e. lung well-differentiated bronchogenic adenocarcinoma (HLC-1), lung moderately differentiated adenocarcinoma (LC-2/ad), and lung poorly differentiated adenocarcinoma (PC-14). The viability of malignant lung cell line reduced dose-dependently in the presence of Ag NPs. The IC50 of Ag NPs were 209, 185, and 106 µg/mL against HLC-1, LC-2/ad, and PC-14 cell lines, respectively. In the antioxidant test, the IC50 of Ag NPs and BHT against DPPH free radicals were 86 and 76 µg/mL, respectively. After clinical study, Ag NPs containing *Thymus capitatus* leaf aqueous extract may be used to formulate a new chemotherapeutic drug or supplement to treat the several types of human lung adenocarcinoma.

Keywords: *Thymus capitatus*; Ag nanoparticles; Antioxidant; Anti-human lung cancer; Cytotoxicity.
1 INTRODUCTION

Lung is the last part of the respiratory tract that participates in the transferring of respiratory gases such as O₂ and CO₂. The normal function of the lung is necessary for the routine activities of the body.¹ The main diseases that affect the normal function of the lung are included cold, flu, and cough, tuberous sclerosis, cystic fibrosis, chronic obstructive pulmonary disease, allergies, asthma, bronchitis, pulmonary hypertension, tuberculosis, pneumonia, emphysema, and lung cancer. Among the above diseases, the mortality rate of lung cancer is more.¹²

Lung cancer is the most common cancers in men, also one of the most common cancers in the woman. The symptoms of the metastasis of lung cancer are symptoms of stroke such as weakness, headaches, seizures, and blurred vision.¹³ The common signs of lung cancer are weakness, weight loss, hoarseness, fatigue, coughing up blood, wheezing, cough, shoulder pain, shortness of breath, chest pain, and dysphagia. The history and physical examination, sputum cytology, bronchoscopy, needle biopsy, thoracentesis, blood tests, molecular testing, and medical imaging such as chest X-ray, computerized tomography, low-dose helical computerized tomography scan, magnetic resonance imaging, and positron emission tomography are used for diagnosis of lung cancer.⁴ The main risk factors of lung cancer are radiation therapy, smoking, exposure to asbestos fibers, exposure to radon gas, exposure to diesel exhaust, air pollution, and familial predisposition.³

For the treatment of lung cancer, surgery, chemotherapy, radiation therapy, targeted therapy, immunotherapy, and EGFR-targeted therapy are used. The main anti-lung cancer chemotherapeutic drugs are included ceritinib (Zykadia), alectinib (Alecensa), brigatinib (Alunbrig), and crizotinib (Xalkori).⁵ According to the high side effects of chemotherapeutic drugs such as weight loss, mouth sores, vomiting, diarrhea, hair loss, fatigue, and nausea, the formulation of modern chemotherapeutic drugs is necessary.⁵ From past to now, scientists have understood that metallic nanoparticles have excellent anticancer properties.⁶,⁷

Recently, silver nanoparticles synthesized by plants have been formulated due to the antibacterial, anti-parasitic, antifungal, antiviral, antioxidant, wound healing, anti-inflammatory, and anti-cancer properties.⁸⁻¹⁶ The results of many studies have indicated significant antibacterial properties of silver nanoparticles green-synthesized by plants in the treatment of Staphylococcus, Salmonella, Streptococcus, Pseudomonas, and Bacillus infectious and their antifungal effects in the treatment of candida diseases.¹⁶,¹⁷

Silver nanoparticles have a great interest because of low cost, availability and its known remedial activities.⁸⁻¹¹ Among all nanoparticles, silver nanoparticles made fastidious attention because of its wide applications in chemical, electrical, optical, bioremediation, sensor, and biological field.¹²,¹³ Silver nanoparticles synthesized by plants have been abundantly used in the biomedical sciences for the treatment of many diseases. Due to the low cost and high availability of the medicinal plants, the green synthesis of silver nanoparticles by medicinal plants significantly have increased.¹⁴⁻¹⁶ Every year, the notable applications of silver nanoparticles green-synthesized are being gained and this trend is continuing.¹⁴,¹⁵

The silver nanoparticles have excellent potentials in the treatment of several cancers.⁶,⁷,¹⁷ In this regard, in the research of Zangeneh and Zangeneh (2019) showed that metallic nanoparticles synthesized using Camellia sinensis leaf aqueous extract had excellent anti-acute myeloid leukemia in the in vivo and in vitro conditions. In the previous study, metallic nanoparticles significantly removed the acute myeloid leukemia cell lines i.e., Murine C1498, 32D-FLT3-ITD, and Human HL-60/vcr and regulated the biochemical and hematological parameters in the animals.⁶ Also, Hemmati et al. (2019) introduced a chemotherapeutic drug formulated by metallic nanoparticles containing Thymus vulgaris leaf aqueous extract for the treatment of acute myeloid leukemia.⁷ In the study of Peng et al. (2009) was reported that metallic nanoparticles as useful metallic nanoparticles in medicine, can be used for the diagnosis of several types of lung cancers.¹⁶

Recently, scientists have used the anticancer effects of medicinal plants in several traditional medicines for synthesizing the silver nanoparticles containing natural compounds. So far, the anticancer effects of Tinospora cordifolia, Sophora subprostrata, Euphoria hirta, Barleria prionitis, Lubinus perennis, Maytenus boaria,
Cephaelis acuminate, Phyllanthus niruri, Solanum seaforthianum, Boswellia serrate, Lavendula officinalis, and Cephalotaxus harringtonia drupacea have been proved.\textsuperscript{19,20}

In the recent research, we decided to investigate the anti-human lung cancer effects of silver nanoparticles formulated by \textit{Thymus capitatus} against well-differentiated bronchogenic adenocarcinoma, moderately differentiated adenocarcinoma of the lung, and poorly differentiated adenocarcinoma of the lung cell lines.

2 EXPERIMENTAL

2.1 Material

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

2.2 Synthesis of AgNPs

Fresh leaves of \textit{Thymus capitatus} 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 °C for 12 h to remove the remaining moisture, and then ground into powder. The \textit{Thymus capitatus} 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°C in refrigerator for further use.\textsuperscript{[6,7]}

The green synthesis of the silver nanoparticles was initiated with a reaction mixture of 100 mL of silver salt (AgNO\textsubscript{3}) in the concentration of $1 \times 10^{-3}$ M and 200 mL of aqueous extract solution of \textit{Thymus capitatus} 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.\textsuperscript{[6,7]}

For analyzing silver nanoparticles, the common techniques of organic chemistry, i.e. FT-IR and UV-Vis. spectroscopy, and FE-SEM were used. The biomolecules involved in the reduction of silver nanoparticles were detected by the FT-IR spectrophotometer (Shimadzu IR affinity.1). Silver nanoparticles were primarily confirmed using UV-Vis spectroscopy at a scan range from 200-800 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) microscopic techniques.

2.3 Assessment of the antioxidant potential of AgNPs by DPPH

At the beginning of the study, 100 mL of methanol (50 %) was added to the 39.4 g of DPPH. Also, several concentrations of AgNPs i.e., 0-1000 µg/mL were considered. The above DPPH was added to the various concentrations of AgNPs and all samples were transfer to an incubator at the temperature of 37 °C. After 30 min incubating, the absorbances were measured at 517 nm. In this study, methanol (50 %) and butylated hydroxytoluene (BHT) were negative and positive controls, respectively. Acceding to the following formula, the antioxidant properties of AgNPs were determined:\textsuperscript{21,22}

$$\text{Inhibition (\%)} = \frac{\text{Sample A}}{\text{Control A}} \times 100$$

2.4 Measurement of cell toxicity of AgNPs

In this experiment, the following cell lines have been used for investing the cytotoxicity and anti-human lung cancer effects of the AgNPs using an MTT assay:
1) Normal cell line: HUVEC.
2) Well-differentiated bronchogenic adenocarcinoma cell line: HLC-1.
3) Moderately differentiated adenocarcinoma of the lung cell line: LC-2/ad.
4) Poorly differentiated adenocarcinoma of the lung cell line: PC-14.
For culturing the above cells, penicillin, streptomycin, and Dulbecco’s modified Eagle’s medium (DMEM) were used. The distribution of cells was 10,000 cells/well in 96-well plates. Then, all samples were transferred to a humidified incubator with 5% CO$_2$ at the temperature of 37 °C. After 24 h incubating, all cells were treated with several concentrations of AgNPs, then incubated for 24 h. Finally, 5 mg/mL of MTT was added to all wells and all samples were transfer to an incubator at the temperature of 37 °C for 4 h. The percentage of cell viability of samples was measured at the absorbance of 570 nm and according to the following formula:

\[
\text{Cell viability (\%)} = \frac{\text{Sample A}}{\text{Control A}} \times 100
\]

2.5 Statistical analysis
The obtained results were fed into SPSS-22 software and analyzed by one-way ANOVA, followed by Duncan post-hoc test ($p \leq 0.01$).

3 RESULTS AND DISCUSSION

The unique physical and chemical properties of metallic nanoparticles have attracted the attention of the scientific community due to their high plasmonic properties, heat transfer, chemical stability and antibacterial effects. Using metallic is nothing new; it dates back to Hippocrates, who used it as an antibacterial to control wounds $^{[24-27]}$. Today, metallic nanoparticles are used in many commercial products, including soap, food, plastics, catheters, textiles, and bandages. However, their mechanism of action is still unknown. Many factors (shape, size, surface chemistry, morphology, density, charge, and purity) affect the biological activity of metallic nanoparticles $^{[26-29]}$. Another important feature of metallic nanoparticles is their role in treating cancer. Au nanoparticles are a promising tool as an anti-cancer factor in evaluation and diagnosis $^{[28-30]}$. They have so benefits with strong properties against various cancer cell lines. Their better ability and penetration to detect Au nanoparticles in the body make them a more effective tool in treating low-risk cancers compared to standard treatments $^{[24-27]}$. The unique effects of Au nanoparticles, such as their high surface-to-volume ratio, easy synthesis, and optical properties, make them suitable for treating cancer. Au nanoparticles can also be conjugated to several molecules, including RNA and DNA, to target several cells and polymers or antibodies $^{[25-29]}$. These important factors are important for increasing the half-life for circulating in vivo, which is very important in drug and gene delivery applications $^{[26-28]}$.

According to the above explanations, we tried to prepare and formulate metallic nanoparticles in aqueous medium using Thymus capitatus leaf aqueous extract as a modern chemotherapeutic drug for the treatment of several types of human lung cancer including well-differentiated bronchogenic adenocarcinoma, moderately differentiated adenocarcinoma of the lung, and poorly differentiated adenocarcinoma of the lung.

3.1 UV-visible spectroscopy of metallic nanoparticles synthesized using Thymus capitatus

UV-Vis spectroscopic analysis showed the presence of an absorption peak at 421 nm which confirmed the formation of the silver nanoparticles (Figure 1).
3.2 FT-IR analysis of silver nanoparticles synthesized using *Thymus capitatus*

In FT-IR test, the antioxidant and secondary compounds are determined based on several peaks in special wavelengths. The IR spectra investigated for the silver nanoparticles revealed the absorption peaks at (I) 3287 cm\(^{-1}\) (OH group of alcohols and phenols); (II) 1623 cm\(^{-1}\) (C=O group of carboxylic acid group); (III) 1383 cm\(^{-1}\) (C=O stretching of carboxylic acid group); (IV) 1038 cm\(^{-1}\) (C-OH vibrations of the protein/polysaccharide).\(^{6,7,14,15}\)

In the recent study, the analysis of the IR spectra of the silver nanoparticles revealed the peaks at 3479, 1611, 1341, 1093, and 531 cm\(^{-1}\) related to the OH, C-O, C=O, C-OH, and Au-O respectively (Figure 2).
3.3 FE-SEM analysis of silver nanoparticles synthesized using *Thymus capitatus*

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 *Thymus capitatus* leaf aqueous extract is shown in Figure 3. The silver nanoparticles appeared as an agglomerated structure. The hydroxyl groups present in *Thymus capitatus* could be responsible for agglomeration. Also, FE-SEM images indicated the range size of 11-28 nm and the shape of spherical for silver nanoparticles. Many similar observations are noted by Zhaleh *et al.* (2019), Zangneh *et al.* (2019), Hemmati *et al.* (2019), and Shahriari *et al.* (2019).
3.4 Antioxidant properties of silver nanoparticles synthesized using *Thymus capitatus*

In the present experiment, the antioxidant effects of the silver nanoparticles synthesized using *Thymus capitatus* leaf aqueous extract were evaluated by DPPH assay revealed concentration-dependent effects i.e., an increase in the concentration of the silver nanoparticles leads to an increase in antioxidant activities. In the concentrations of studied, the best result was seen in the high concentration or 1000 µg/mL (Figure 4). Comparative analysis of the individual antioxidant assays showed significant variations in the exertion of radical scavenging effects. In contrast, standard (butylated hydroxytoluene) demonstrated higher antioxidant effects compared to the silver nanoparticles.

The IC50 of butylated hydroxytoluene and AgNPs were 76 and 86 µg/mL, respectively (Table 1).
Fig. 4. The antioxidant properties of AgNPs and BHT against DPPH.

Table 1. The IC50 of AgNPs and BHT in antioxidant test.

| IC50 against DPPH | AgNPs (µg/mL) | BHT (µg/mL) |
|-------------------|----------------|--------------|
|                   | 86             | 76           |

Usually, many free radicals such as Reactive oxygen species (ROS) are produced in the procedure of mitochondrial respiration which is responsible for oxidative stress in the human body. This oxidative stress damages human body DNA and develops several oxidative diseases like nephrotoxicity, hepatotoxicity, and hematotoxicity. The oxidative stress can be efficiently reduced with the help of antioxidant materials such as metallic nanoparticles. The synthesized silver nanoparticles exhibit higher antioxidant activity for the formation of free radicals into the living system. The silver nanoparticles have redox properties and play a significant role in deactivating free radicals in the living system. In recent years, researchers evaluated plants and bio mediated synthesized nanoparticles for antioxidant activity. The reason behind the antioxidant activity of green or biosynthesized nanoparticles could be due to the presence of metabolites compounds such as phenolic compounds, flavonoids, carbohydrates, and other sugar substances. Also, many researchers reported phenolic and flavonoids attached to the nanoparticles exhibited the antioxidant activity. Previously has been indicated that Thymus capitatus is rich in antioxidant compounds. Several studies were carried out in the nanotechnology field.
using various medicinal plants, but still, no report is available on silver nanoparticles synthesized using *Thymus capitatus* leaf aqueous extract.

### 3.5 Cytotoxicity and anti-human lung cancer potentials of silver nanoparticles synthesized using *Thymus capitatus*

In the present experiment, the treated cells with several concentrations of the present AgNPs were examined by MTT test for 48h regarding the cytotoxicity properties on normal (HUVEC) and human lung cancer cell lines i.e., well-differentiated bronchogenic adenocarcinoma (HLC-1), moderately differentiated adenocarcinoma of the lung (LC-2/ad), and poorly differentiated adenocarcinoma of the lung (PC-14) (Figures 5,6; Table 2). The absorbance rate was determined at 570 nm, which indicated extraordinary viability on normal cell line (HUVEC) even up to 1000μg/mL for AgNPs.

In the case of human lung cancer cell lines, the viability of them reduced dose-dependently in the presence of AgNPs. The anticancer of silver nanoparticles was found to be highly dependent on a range of factors related to their physical characteristics, such as surface coating, shape, and size. About the size, it has been reported that silver nanoparticles with small size can transfer of cell membrane of tumor cells and remove them. In the larger size, the above ability significantly is confined. As can be observed in Figure 3 of our study, silver nanoparticles had uniform spherical morphology in the range size of 11-28 nm. The size of silver nanoparticles at lower than 50 nm is very suitable for the killing of tumor cell lines in vivo and in vitro. About the anticancer properties of silver nanoparticles, they have used for the treatment of several cancers including human lung cancer, mammary carcinoma, uterus cancer, lung epithelial cancer, Lewis lung carcinoma, colon cancer, and human glioma.
Fig. 5. The anti-human lung cancer properties of AgNPs against HLC-1 (A), LC-2/ad (B), and PC-14 (C) cell lines.
Fig. 6. The cytotoxicity effects of AgNPs against Normal (HUVEC) cell line.

Table 2. The IC50 of AgNPs in the anti-human lung cancer test.

|                | AgNPs (µg/mL) |
|----------------|--------------|
| IC50 against HUVEC | -            |
| IC50 against HLC-1    | 209          |
| IC50 against LC-2/ad  | 185          |
| IC50 against PC-14    | 106          |

Probably the anti-human lung cancer properties of silver nonparties against well-differentiated bronchogenic adenocarcinoma (HLC-1), moderately differentiated adenocarcinoma of the lung (LC-2/ad), and poorly differentiated adenocarcinoma of the lung (PC-14) are related to their antioxidant activities. The previous researches have revealed that antioxidant compounds such as medicinal plants and silver nanoparticles as single electron donors can stabilize and scavenge the free radicals, which in conditions of oxidative stress may begin angiogenesis or carcinogenesis.\(^{38,39}\) High levels of free radicals are observed in various cancerous cells and several accumulating evidence suggests that free radicals function as key signaling molecules stimulate different growth-related responses that finally begin tumorigenesis and angiogenesis.\(^{38-40}\) In detail, Free radical-induced development of cancer involves malignant transformation due to DNA mutations and changed gene expression through epigenetic mechanisms which in turn causes the uncontrolled proliferation of cancerous cells.\(^{39,40}\) Many researchers reported a remarkable role of antioxidant compounds such as medicinal plants and silver nanoparticles in growth inhibition of prostate, ovary, breast, endometrial and lung, and colon cancer cells with removing free radicals.\(^{41,42}\)
4 CONCLUSIONS

In the present study, *Thymus capitatus* leaf collected was applied for biosynthesizing silver nanoparticles as a safe and suitable material. After silver nanoparticles synthesizing, they were characterized by FE-SEM, UV Vis., and FT-IR. The above analyses revealed that silver nanoparticles were synthesized as the best possible form. In the FT-IR test, the presence of many antioxidant compounds with related bonds caused the excellent condition for reducing of silver in the silver nanoparticles, so that the antioxidant properties of silver nanoparticles was the better than the BHT as a positive control. Silver nanoparticles showed significant anti-human lung activities against well-differentiated bronchogenic adenocarcinoma (HLC-1), moderately differentiated adenocarcinoma of the lung (LC-2/ad), and poorly differentiated adenocarcinoma of the lung (PC-14) cell lines. It looks these nanoparticles may be administrated as a chemotherapeutic drug for the treatment of several types of lung cancers especially well-differentiated bronchogenic adenocarcinoma, moderately differentiated adenocarcinoma of the lung, and poorly differentiated adenocarcinoma of the lung.

Conflict of interest

The authors declare that they have no conflict of interest.

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