Introducing a novel chemotherapeutic drug for the treatment of oral squamous cell carcinoma: Silver nanoparticles green-formulated by Matricaria chamomilla

**Type**
Research paper

**Keywords**
Silver nanoparticles, Matricaria chamomilla, chemotherapeutic drug, oral squamous cell carcinoma

**Abstract**

**Introduction**
In the present research, we formulated a modern chemotherapeutic drug by silver nanoparticles (AgNPs) containing Matricaria chamomilla aqueous extract for the treatment of oral squamous cell carcinoma.

**Material and methods**
Characterization of AgNPs was done by UV–Visible Spectroscopy (UV-Vis), Fourier Transformed Infrared Spectroscopy (FT-IR), Transmission Electron Microscopy (TEM), and Field Emission Scanning Electron Microscopy (FE-SEM). For investigating the antioxidant properties of AgNO₃, M. chamomilla, and AgNPs, the DPPH test was used in the presence of butylated hydroxytoluene as the positive control. To survey the cytotoxicity and anti-oral squamous cell carcinoma effects of AgNO₃, M. chamomilla, and AgNPs, MTT assay was used on the HSC-4, Ca9-22, and HSC-3 cell lines.

**Results**
DPPH test revealed similar antioxidant potentials for M. chamomilla, AgNPs, and butylated hydroxytoluene. Silver nanoparticles had very low cell viability and anti-oral squamous cell carcinoma properties dose-dependently against HSC-4, Ca9-22, and HSC-3 cell lines without any cytotoxicity on the normal cell line. The best result of anti-oral squamous cell carcinoma properties of AgNPs against the above cell lines was seen in the case of the HSC-3 cell line.

**Conclusions**
According to the above findings, the silver nanoparticles containing M. chamomilla aqueous extract can be administrated in humans for the treatment of several types of oral squamous cell carcinoma.
Introducing a novel chemotherapeutic drug for the treatment of oral squamous cell carcinoma: Silver nanoparticles green-formulated by *Matricaria chamomilla*

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ABSTRACT

In the present research, we formulated a modern chemotherapeutic drug by silver nanoparticles (AgNPs) containing Matricaria chamomilla aqueous extract for the treatment of oral squamous cell carcinoma. Characterization of AgNPs was done by UV–Visible Spectroscopy (UV-Vis), Fourier Transformed Infrared Spectroscopy (FT-IR), Transmission Electron Microscopy (TEM), and Field Emission Scanning Electron Microscopy (FE-SEM). For investigating the antioxidant properties of AgNO₃, M. chamomilla, and AgNPs, the DPPH test was used in the presence of butylated hydroxytoluene as the positive control. To survey the cytotoxicity and anti-oral squamous cell carcinoma effects of AgNO₃, M. chamomilla, and AgNPs, the MTT assay was used on the HSC-4, Ca9-22, and HSC-3 cell lines. DPPH test revealed similar antioxidant potentials for M. chamomilla, AgNPs, and butylated hydroxytoluene. Silver nanoparticles had very low cell viability and anti-oral squamous cell carcinoma properties dose-dependently against HSC-4, Ca9-22, and HSC-3 cell lines without any cytotoxicity on the normal cell line. The best result of anti-oral squamous cell carcinoma properties of AgNPs against the above cell lines was seen in the case of the HSC-3 cell line. According to the above findings, the silver nanoparticles containing M. chamomilla aqueous extract can be administrated in humans for the treatment of several types of oral squamous cell carcinoma.

KEYWORDS Silver nanoparticles; Matricaria chamomilla; chemotherapeutic drug; oral squamous cell carcinoma.
1. Introduction

Common cancer treatments, including chemotherapy, radiation and surgery, may reduce the size of the tumor, but the effect of these methods is transient and has no positive effect on patient survival [1,2]. There are many types of nanoparticles available and choosing the right carriers according to demand is a key issue [2-4]. Nanoparticles are very close in size to biological molecules in terms of size and can easily penetrate into the cell, for this reason, one of the goals of nanotechnology is to mount molecules and drugs on nanoparticles and transfer them to the target cell [1-4]. It is also possible to create different surface properties for nanoparticles by attaching protective ligands to increase the nanoparticles' resistance to the immune system and increase their presence in the bloodstream, and even binding ligands to specifically bind the nanoparticles to the target tissue [3-5]. Replacing more effective, more specific therapies with fewer side effects with higher anti-cancer activity is a dominant issue in clinical oncology [1-3]. The gradual maturation of nanotechnology has been considered not only for treating cancer but also for a wide variety of applications, especially for drug delivery and diagnostic and imaging cases [4].

Nanomaterials can be created from the engineering of multiple or single molecules which are engineered and have several functional groups. The field of synthetic nanotechnology has developed the self-sufficiency of materials with specific sizes and shapes at the nanometer scale environmentally friendly. Biocompatible nanocarriers include peptides, engineered antibodies, micelles, polymers, and liposomes [6-9]. Nanomaterials can be designed to gain the desired chemical and physical characteristics and purposefully direct drugs to dynamic tumor environment with high therapeutic effects and low toxicity. Features to be controlled when designing nanoparticles are surface to volume ratio, shape, size, molecular charge, and drug release. For example, nanoparticles, after binding to functional chemical components such as antibodies and ligands, bind to the surface receptors of the cancer cell and prevent its clearance by the blood [5]. Drug delivery by nanoparticles using biodegradable polymers solves many problems. For cytotoxic drugs to be efficiently delivered to cancer cell tissue, first, cytotoxic agents must be removed from the bloodstream and after leaving the extracellular space, the cancerous tissue must pass through the membrane surface and be targeted inside the cancer cell [11-13]. The ideal properties of nano foundations that should be considered when designing are: 1- Continuous drug release 2- Inactive accumulation of the drug in cancerous tissue 3- Targeting antigens or surface
receptors of cancer cells with a controlling effect on endosomal uptake and membrane destruction. 4- Drug release into the cytoplasm and protection from enzymatic degradation [14-17].

The composite of AgNPs nanoparticles could be promising material with good potential in biological and pharmaceutical sciences. Therefore, we interested to describe the synthesis, characterizations and applications of novel AgNPs decorated Matricaria chamomilla extract for the first time. The biomolecules of extract afford additional stability to the AgNPs from unwanted oxidation and corrosion. The modified surface can easily have capped the Ag ions and in situ reduced and stabilized them without aggregations. In the recent study, we extended our material in the bio-assay against human oral squamous cell carcinoma cell lines i.e., HSC-4, Ca9-22, and HSC-3 for the first time.

2. Experimental
2.1 Material
All materials used in the recent manuscript were achieved from Sigma-Aldrich Company of USA.

2.2 Synthesis of silver nanoparticles
First, the leaves of the Matricaria chamomilla plant, after drying in the air, are pulverized using an electric grinder (model, Moulinex AR1066Q). 25 g of the leaves were soaked by maceration with distilled water and kept for at least three days with repeated stimulation to dissolve the solvent at room temperature. After three days, the mixture of extract and water was filtered using filter paper to separate the solids from the liquid. Finally, the excess solvent was evaporated and concentrated using a rotary evaporate. With the help of a freezer dryer (Scientific LTE UK, Ltd), it was completely dried and turned into a powder. Finally, the dry powder was stored in a sealed glass container and refrigerated and used to prepare different concentrations. The green synthesis of the silver nanoparticles was initiated with a reaction mixture of 100 mL of silver salt (AgNO₃) in the concentration of 1 × 10⁻³ M and 200 mL of aqueous extract solution of Matricaria chamomilla 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. To analyze
the 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 210-710 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.

2.3. Cytotoxicity and anti-oral squamous cell carcinoma properties

Investigation of cell proliferation and survival is one of the most important and basic techniques in cell laboratories. This study requires accurate quantification of the number of living cells in the cell culture medium. Therefore, cell survival calculation methods are necessary to optimize cell culture conditions, evaluate cell growth factors, detect antibiotics and anticancer drugs, evaluate the toxic effects of environmental pollutants, and study apoptosis. Many methods can be used for such purposes, but indirect methods using fluorescent or dye (chromogenic) markers provide very fast large-scale methods. Among these methods, measurement of cell survival by MTT method is the most widely used method. This method is a colorimetric method to study cell proliferation and survival, introduced in 1983 by Mossman. The method basis is based on mitochondrial activity. Mitochondrial activity in living cells is stable and therefore a raise or reduce in the living cells number is linearly related to mitochondrial activity [18]. Mitochondrial dehydrogenases in living cells break the tetrazolium ring and yield NADPH and NADH, leading to forming a purple insoluble deposit called formazan. This precipitate can be dissolved by dimethyl sulfoxide or isopropanol. Dye formation is used as a marker of living cells. The color intensity produced is measured at a 540 to 630 nm wavelength and is directly proportional to the living cells number. High safety and providing a colorimetric and non-radioactive system are important advantages of this method. This kit is very easy to use, has high sensitivity and accuracy and can detect less than 950 cells. On the other hand, it has high efficiency for measuring cell proliferation, survival and mortality, and its implementation method does not require time-consuming washing steps and transfer from one plate to another. Examples studied in this method are adhesive or suspended cells and proliferating or non-proliferating cells [14].

In this research, we used Human Umbilical Vein Endothelial Cells (HUVECs) to determine the cytotoxicity potentials of AgNPs using MTT. Also, the in vitro anti-oral squamous cell carcinoma
effects of biologically synthesized AgNPs against HSC-4, Ca9-22, and HSC-3 cancer cell lines were evaluated. These cell lines were plated on DMEM media using 96-well plates with antimycotic solution, 10% FBS, streptomycin and penicillin. Cells number in each plate-based well was 10,000, which were incubated at 37 °C for 24 h and treated with AgNPs with different dilation sizes, (0-1000 µg/mL) and incubated for 24 h. Then, 5mg/mL of MTT were added to all wells, which were finally incubated at 37ºC for 4h. After the absorbance evaluation at 540 nm, the cell viability percentage was calculated from the following equation [18]:

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

2.4. Antioxidant capacities of AgNPs

Free radicals are unstable atoms that have one or more unpaired electrons. These active species are very harmful due to their high reactivity. They are most often formed when oxygen molecules in the body split into separate unstable atoms. This process can turn into a chain reaction. Free radicals excessive production in the body causes cell damage and oxidative stress. Genetics and the environment affect the extent of free radical damage in individuals. These active molecules are produced as part of the body's natural biological processes. One of the most important free radicals is DPPH. DPPH is widely used to study the antioxidant activities of natural compounds and nanoparticles [18].

In the recent study, DPPH was used to test antioxidant activities of AgNPs [18]. At the beginning, 100 mL of CH₃OH were added to 39.4 g of DPPH to prepare its solution which was added to different concentrations of AgNPs (0-1000 µg/mL). Then we added DPPH solution to samples, as explained above, and incubated it at 37 °C for 30 min. Mixture absorbance were clarified at 570 nm. BHT and CH₃OH (50 %) were considered positive and negative controls. Samples antioxidant capacities were computed according to the following formula:

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

Then, based on the absorption rate of each well and its comparison with the control, the inhibitory concentration of 50% (IC₅₀) was obtained [18].
3. Results and discussion

The AgNPs, being prepared following a green approach by using *Matricaria chamomilla* extract, was analyzed over different techniques like scanning electron microscopy (SEM), transmission electron microscopy (TEM), and UV-Vis spectroscopy techniques. To have a detailed idea of its physical and chemical features.

**UV–visible spectroscopy analysis**

The UV-Vis. spectra of AgNO₃, the plant extract, and AgNPs are shown in Figure 1. The synthesis of AgNPs was confirmed by UV-Vis. spectra. Peak at 451 nm are found in plant extract and AgNPs.

![Figure 1. The image of UV-Vis of AgNPs.](image-url)

**SEM analysis**
Scanning Electron Microscope (SEM) was used to recognize morphology and size of AgNPs. The results indicated the formation of homogeneous and relative capping of AgNPs with spherical shape possesses an average diameter size of 24.16 nm. Aggregation of the nanoparticles usually cause to increase of the NPs size, this is a common phenomenon during biosynthesis of metallic nanoparticles especially when the plant's extracts are used. This occurrence was also observed for AgNPs. However, the aggregated particles of the synthesized AgNPs in this research were showed the size around 60 nm (Figure 2).

![Figure 2. The SEM image of AgNPs.](image)

**TEM analysis**

To record the TEM images, drop coated films of the AgNPs, obtained via reaction of 2.5mL plant extract with AgNO₃, were used. Figure 3 shows the size and shape of the AgNPs using TEM techniques. The obtained Ag NPs found spherical and the size ranges of 10.1 to 28.7 nm. In rare cases, particles with larger sizes were also seen in the sample, but their numbers were rather low. The fast reduction of the silver ions by the plant extract allowed homogeneous nucleation of Ag metals which cause to form AgNPs with small size. As can be seen in Figure 3, the AgNPs revealed a very excellent dispersion inside the bio-reduced aqueous solution without any agglomeration. In
other words, the AgNPs are confined within a plant extract matrix, likely comprised of biomolecules which are acting as a capping or stabilizer agent during synthesis.

Figure 3. The TEM image of AgNPs.

**Therapeutic effects of AgNPs**

The most important problem of cytotoxic drugs is their distribution in healthy and cancer cells, which causes drug toxicity and harmful side effects on healthy cells. It also reduces the dose of drug delivery to cancer cells. In addition, rapid excretion and widespread distribution into the organs and tissues require the administration of drugs in large quantities that are not economically viable [1-4]. Nanoparticles as pharmaceutical carriers have solved some of the current drug delivery limitations in cancer treatment. These structures are known as a very effective drug delivery system due to the protection of the drug molecule, reduction of toxicity and side effects, ability to cross biological barriers to deliver the drug to the target site and increase the shelf life of the drug in the bloodstream, which increases pharmacotherapy efficiency [5].

The most important feature of nanoparticles is particle size and particle distribution. Particle size and drug particle distribution play important roles in the biological fate, toxicity, and targeting of
drug delivery systems. They affect the loading, release and stability of particle tattoos. Many studies have shown the advantages of nanoparticles compared to microparticles [20,21]. One of the advantages of nanoparticles over micro-particles is their high cellular uptake. Due to their small size, the particles enter various cells and intracellular space. Nanoparticles cross the blood-brain barrier following the opening of hard endothelial junctions by mannitol, which provides stable drug release systems to treat severe brain diseases [20]. Studies have shown that Tween 80-coated nanocomposites are able to cross the barrier of blood-brain [21]. Particles smaller than a micron are removed by a large number of cells [22]. The cellular uptake of 100 nm nanoparticles is 2.5 times that of 1 μm particles, and the cellular uptake of 100 nm nanoparticles is 6 times that of 10 μm particles by 2-caco cells [23]. Drug release is also controlled by particle size. Smaller particles have a lower volume-to-surface ratio. Therefore, if the drugs are placed on or near the surface of the nanoparticles, it will lead to the rapid release of the drug. But, larger particles are annealed at the center of the nanoparticles, which are released more slowly [24]. Therefore, particle size control is effective in determining drug release. Smaller particles may form during transport, disrupting their distribution. The decomposition of polymers is affected by particle size. For example, as the particle size of polylactic-co-glycolic acid polymer increases, its decomposition rate increases. Therefore, it is hypothesized that larger particles cause the polymer to decompose faster and the drug to be released faster [25].

In this research, the treated cells with different concentrations of the present AgNPs were assessed by MTT assay for 48h about the cytotoxicity properties on normal (HUVEC) and human esophageal malignancy cell lines i.e. HSC-4, Ca9-22, and HSC-3. The absorbance rate was evaluated at 540 nm, which represented viability on normal cell line (HUVEC) even up to 1000μg/mL for AgNPs (Table 1 and Figure 4). The viability of malignant esophageal cell line reduced dose-dependently in the presence of AgNPs. The IC₅₀ of AgNPs were 235, 377, and 361 μg/mL against HSC-4, Ca9-22, and HSC-3 cell lines, respectively (Table 1 and Figure 4).
Fig. 4. The anti-oral squamous cell carcinoma properties of AgNPs against HSC-4 (I), Ca9-22 (II), HSC-3 (III), and HUVEC (IV) cell lines.

Table 1. The IC50 of AgNPs in the anti-oral squamous cell carcinoma tests.

| Cells     | AgNPs (µg/mL) |
|-----------|---------------|
| HSC-4     |               |
| Ca9-22    |               |
| HSC-3     |               |
| HUVEC     |               |
Free radicals are a number of single-electron atoms produced during the reaction of oxygen with certain molecules. If many free radicals are suddenly produced in the body, it triggers a series of specific reactions in a row. Free radicals react with certain parts of the cell, such as DNA and cell membranes, causing cell damage or even death [2-4]. Normally, the body's immune system neutralizes these free radicals. However, destructive environmental agents such as environmental, ultraviolet rays and alcohol pollution make the body unable to fight these free radicals [1-5]. As a result, the structure and function of body cells are destroyed by free radicals, leading to premature aging and diseases such as cancer and heart disease. Free radicals react with certain parts of the cell, such as DNA and cell membranes, causing cell damage or even death. In this case, these radicals are harmful and dangerous to the health of the body [6-10]. To prevent these atoms from working, the body must have a defense barrier against antioxidants. Antioxidants block the free radicals action and prevent the vital cells destruction. Preventing cell damage inhibits diseases such as cardiovascular disease, cancer, and skin aging [4-8]. Consumption of more antioxidants provides the basis for the body to easily eliminate harmful free radicals. Recent studies have shown that metal nanoparticles have unique antioxidant properties [10-14].

The scavenging capacity of AgNPs and BHT at different concentrations expressed as percentage inhibition has been indicated in Table 2, Figure 5. In the antioxidant test, the IC$_{50}$ of AgNPs and BHT against DPPH free radicals were 173 and 123 µg/mL, respectively (Figure 8).
Fig. 5. The antioxidant properties of AgNPs and BHT against DPPH.

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

| IC50 against DPPH | AgNPs (µg/mL) | BHT (µg/mL) |
|-------------------|---------------|-------------|
| 173               | 123           |

4. Conclusion

Finally, we like to conclude to introduce a green method by plant extract for modification of magnetic nanomaterial that then could be used as magnetic reducing absorbent to Ag NPs being decorated over its surface. Follow by this way, we synthesized AgNPs for the first time using *Matricaria chamomilla* extract. The structural features were analyzed through a wide range of analytical techniques. The viability of malignant esophageal cell lines reduced dose-dependently in the presence of AgNPs. The IC\textsubscript{50} of AgNPs were 235, 377, and 361 µg/mL against HSC-4, Ca9-22, and HSC-3 cell lines, respectively. The AgNPs showed the best antioxidant activities against
DPPH. So, the findings of the recent research show that biologically synthesized AgNPs might be used to cure oral squamous cell carcinoma.

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