Novel green formulation, characterization, anti-human oral cancer, antioxidant and cytotoxicity effects of plant extract based silver nanoparticles

Huang Fang¹

¹Huazhong University of Science and Technology

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Abstract

Rheum ribes L. is one of the popular fruits in Asian culture. R. ribes has many pharmaceutical applications in folklore medicine. In the present study, the extract of the dried leaf of R. ribes was used as a reducing agent for the green synthesis of silver nanoparticles. Various chemical methods such as FE-SEM, XRD, and FT-IR were used to characterize AgNPs. The results of XRD showed 28.19 nm for the crystal size of AgNPs. A spherical morphology was confirmed for the nanoparticles using FE-SEM images with an average size of 26.11 nm the nanoparticles. In the cellular and molecular part of the recent study, the treated cells with AgNPs were assessed by MTT assay for 48h about the cytotoxicity and anti-human oral cancer properties on normal (HUVEC) and oral cancer cell lines i.e. HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22. The IC50s of AgNPs were 220, 192, 174, 142, 125, 131, 250 and 219 μ g/mL against HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 cell lines, respectively. The viability of malignant oral cell lines reduced dose-dependently in the presence of AgNPs. It seems that the anti-human oral cancer effect of recent nanoparticles is due to their antioxidant effects.

1. Introduction

Rheum ribes L. well known as a medicinal plant with wide variety of applications in traditional medicine. The whole plant of R. ribes has laxative, diuretic, and expectorant properties [1-3].R. ribes effective agent to reduce gastric acidity [4]. Diaphoretic, antiseptic, healing of wounds are the other medicinal properties of the plant [5]. The leaves volatile oil of the plants an efficient agent to cure rheumatism [6]. According to the previous studies, phenolic compounds, glycosides, steroids, resins, alkaloids, flavonoids, fatty acids, coumarins, and vitaminsare the most important secondary metabolites in R. ribes [7-10]. Indeed, this variety of compounds in R. ribes are responsible to wide spectrum of pharmaceutical uses of the plant [11-14]

Today, the use of nanomaterials is increasing widely, so it has been introduced in all aspects of life, and in the meantime, the use of nanocomposites in medical processes has also found an increasing use. Nanoparticles have various applications in medicine, including prevention and treatment of diseases, nano robots for diagnosis, various medical sensors, imaging and drug delivery system [11-13]. One of the practical aspects of nanotechnology that has received attention today is the use of nanocomposites as anticancer compounds and their use as a drug delivery system in cancer treatment. Encapsulation of drugs used in cancer treatment on nanoparticles allows the drug to be delivered to cancer cells with higher efficiency, and on the other hand, its toxicity and side effects on healthy cells are reduced [13-17]. Recently, Because of the deaths caused by increasing prevalence of cancers and the failure of radiotherapy and chemotherapy ways, the need to invent modern methods to treat cancer is felt. Targeting anticancer drugs so that they are effective only on cancer cells and also using the minimum concentration of drugs in such a way that the toxic effects on normal cells are reduced is also considered necessary in this regard [18-22]. Therefore, to specifically deliver the drug to the cancerous tissue and reduce its side effects, we can take help from new delivery methods and to the tissue with the nanoparticles help as a carrier. The used nanoparticles with a size of 10-100 to target medicinal and diagnostic agents are very widespread [23-25]. In recent years, many nanoparticles have been used in a targeted manner to destroy cancer cells by reducing the systemic toxicity of anticancer supplements/drugs. Today, the use of catalysts that are activated by light (photocatalysts) have become very common [26-30]. One of the photocatalysts that has been used in the industry since the past is silver nanoparticles, and it has also been proven that this material is harmless to humans and the environment. Also, from the economic point of view, the preparation of this nanoparticle is cost-effective, and studies conducted since the beginning of the 20^{th} century on Ag nanoparticles have proven two different roles of this compound [31-34]. The first role is the oxidation-regeneration ability and the second role is changing the properties of the surface of the particles to a hydrophilic state when Ag is placed on that surface [35-38]. Also, this nanoparticle has high biocompatibility, resistant to human body fluids and very resistant to corrosion. Today, AgNPs are an important product in nanotechnology as a medicinal compound and also an attractive candidate for delivering many small medicinal molecules or large biomolecules such as RNA, DNA and proteins. Ag nanoparticles are resistant to heat and due to light radiation, they can produce 0_2 and OH ions, which can affect membrane lipids and cause their destruction [37-40].

In the recent study, the properties of silver green-medicated by R. ribes leaf aqueous extract against common oral cancer cell lines i.e. HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 were evaluated.

2. Methods and Materials

2.1. Preparation and extraction of aqueous extract

The desired plant was individually powdered by a home mill. Pour 50 gr of each of the powders separately into a flask, add 400 ml of distilled water to shake it in a shaker at 160 rpm for one day. The extract from this step was filtered and added again to the remaining amounts of distilled water and the previous step was repeated. The extracts collected in these two stages were distilled in a vacuum distillation apparatus at 45° C and 70 rpm until the remaining volume reached one-fifth of the initial volume. After that, the contents of the tank were transferred into the petri dish and dried completely in the oven at 50°C within a few days. Different dilutions were prepared from the obtained extract with phosphate buffer saline and sterilized using 0.22 μ filters. Finally, the diluted extracts were stored in sterile microtubes in the freezer until use.

2.2. Green synthesis of AgNPs

The synthesis of AgNPs was carried out according to a previous report [20]. A 50 mL of R. ribes extract with a concentration of 0.02 g/mL was poured into a flask containing AgNO₃ (100 mL, 0.1 M). For the next step, the reaction mixture was stirred at 40 °C for 24 h. The nanoparticles were formed during the reaction time as brown precipitates. The nanoparticles were washed with water four times and centrifuged at 12000 rpm for 12 min. Afterward, AgNPs were dried at room temperature.

2.3. Anti-human oral cancer properties of AgNPs

HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 cells were used to evaluate the anticancer effect of AgNPs on cell culture.

The cancer cell lines were cultured in 1640-RPMI culture medium enriched with 10% FBS and 1% streptomycin and penicillin antibiotics in 5% CO_2 in an incubator at 37°C.

To determine the cytotoxicity effects of nanoparticles against cancer cell lines, the MTT assay was used. At first, 10^6 cells were planted in 96 plates. Then the cancer cells were received the nanoparticles at the 0-1000 µg/ml concentrations for 24, 48 and 72 hours, and then after the mentioned times, the content of the 96-well plate was emptied. Then the added MTT dye was incubated at 37°C temperature and 5% CO₂ for 5 hours. Finally, the samples absorbance was read and recorded by an ELISA reader (Bio Tek, USA) at a wavelength

of 570 nm, and the lethality of the cells was computed by the following formula [41]:

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

Also, the concentration at 50% lethality (Concentration half maximal inhibitory) or IC_{50} was checked. Also, after the treatment of cells with nanoparticles, the morphology of cancer cells was also compared to the control group (untreated).

2.4. Statistical analysis

The statistical analysis of all the tests in this study was repeated 3 times and the statistical analysis of this study was done using Graph pad Prism version 8 software. Cytotoxicity data were analyzed by One Way ANOVA.

3. Results and Discussion

3.1. Chemical characterization of AgNPs

EDX analysis:

The qualitative analysis of EDX was run to screen the elemental analysis of silver nanoparticles. The EDX diagram of silver nanoparticles is shown in Figure 1. The findings approved the appearance of silver (by the peaks at 3 keV for AgL α and peak at 3.1 keV for AgL β), oxygen (by the peak around 0.5 keV for OL α), and carbon (by the peak around 0.3 keV for CL α) in silver nanoparticles. The presence of oxygen and carbon approved the linkage between silver nanoparticles and organic compounds of the plant extract.



Figure 1. EDX analysis of silver nanoparticles.

XRD analysis:

The analysis of the XRD diffraction pattern is known as a method to study different compounds including metallic nanoparticles. The XRD pattern of the green synthesized silver nanoparticles is exhibited in Figure 2. The result has approved the crystallinity of AgNPs with a small size. The data for 2ϑ values has been matched to the standard database of JCPD card 04–0783. The signals at 2ϑ of 37.85, 65.11, and 77.49 belonged to the planes 111, 220, and 311 respectively. The crystal size of 28.19 nm was measured for the NPS using Scherer's equation. A range of 5 up to 60 nm has been reported by other research groups for the green synthesized in the literature [40-43].



Figure 2. XRD Pattern of silver nanoparticles.

FE-SEM and **TEM** analysis:

FE-SEM and TEM analysis are members of the scanning electron microscope family and are used to examine the surface characteristics and morphology of different samples. In FE-SEM, electron beams with specific energy and wavelength sweep the sample surface. By the detector data that have collected the return sample surface electrons, benefits data is obtained from the sample surface [40-42]. It should be noted that image quality and high resolution in the images have a direct relationship with the structure of the sample and the quality of synthesis and the absence of contamination and unwanted particles, and samples with a specific structure provide acceptable images [43-45].

The morphology investigation of silver nanoparticles was carried out using the FE-SEM and TEM imaging technique (Figures 3 and 4). The images show a spherical morphology for NPs with an average size of 26.11 nm. Furthermore, aggregation, which is a property of the metallic nanoparticles, is well seen for the green synthesized silver nanoparticles [40,41,44,45]. The reported size of the green synthesized of silver nanoparticles is beginning of 5 nm to 200 nm [40-43,46].



Figure 3. FE-SEM image of silver nanoparticles.





FT-IR analysis:

In laboratories, a large part of the measurements is based on absorbance reactions. The activity of most cholesterol, triglycerides, enzymes, lipoproteins, creatinine, urea, sugar and a wide range of analytes with research and clinical applications, drugs and metabolites can be measured by spectrophotometry. Investigating the molecular structure, identifying compounds, comparing structures and finding the maximum absorption wavelength are other spectrophotometry applications in research problems [45-47].

The FT-IR spectrum of AgNPs is presented in Figure 5. The bands at 433, 471, and 534 belong to the Ag-O bond. The peaks in the range of 400-600 cm⁻¹ have been reported for silver nanoparticles [47]. In addition, the peaks for organic compounds at 3217, 2919, 1429 to 1675, and 1009 cm⁻¹, which are belonged to O-H, C-H, C=C, C=O, and C-O bond, confirm the linkage of *R. ribes* secondary metabolites such as phenolic and flavonoids compounds to the surface of nanoparticles [44-47].



Figure 5. FT-IR Spectrum of silver nanoparticles.

FTIR analysis was performed to investigate possible organic compounds involved in the synthesis of nanoparticles. According to Figure 5, after the reaction with Ag salt, there is some shift in the location and height of the peaks. This shift is related to the breaking of the bonds of hydroxyl and carbonyl groups, the release of hydrogen and carbon, and their role in reducing the charge and regenerating iron ions. The results of this research showed that the OH and CO groups in the water extract of the plant are possible compounds for reducing iron salt to silver nanoparticles. The definitive mechanism of the nanoparticle formation during green formulation has not yet been determined. Despite this, some researchers believe that the active surface of terpenoid molecules causes the regeneration of metal ions and stabilizes the synthesized nanoparticles [43,44]. Probably, these molecules with or without other reducing agents (sugars) are effective in this process. Terpenoids are a large and diverse group of metabolites that are made of five carbon isoprene structural units and have different types. Since these substances are the largest group of natural products that exist in almost all living organisms, it is likely that many plant extracts can be used in the metal nanoparticle synthesis because of the presence of terpenoids and reducing sugars in them [45].

UV-Vis analysis:

In this study, silver nanoparticles were formulated at a temperature of 80 °C and a duration of 30 minutes. The reaction solution color changed from dark red to black due to the interaction between the plant extract and the silver salt solution, and it is the first sign of producing silver nanoparticles, was quite evident. After that, the absorption spectrum of the nanoparticle solution using a spectrophotometer showed a peak wavelength of about 442 nm, which shows the silver nanoparticle presence (Figure 6). The above findings in the present study proved that the color change is a function of time and with the passage of time and the progress of the reaction, the concentration of silver nanoparticle increases. By measuring the optical density of the reaction solution in different time intervals, we came to the conclusion that the rise in the silver nanoparticle concentration causes the color of the reaction solution to change to black.



Figure 6. UV-Vis analysis of silver nanoparticles.

3.2. Analysis of the anti-oral cancer properties of AgNPs

The anti-cancer properties of nanoparticles have been known for several years, and for this reason, nanoparticles are of great importance. Because several genetic changes are needed to create a cancerous form of a cell and because the development of many cancers is hereditary, in some types of cancer, after treatment and surgery, it is possible to create a primary mass [48-51]. Apoptosis, in addition to metastasis, is the most important form of cell suicide. In this process, dangerous, damaged, and unwanted cells are removed without damaging the surrounding tissues or cells. Cancer cells escape from programmed death, one of the reasons for which is the convert in the gene expression that is involved in the process regulation. Most anticancer agents exert their remedial activities by inducing apoptosis [52-55]. Programmed death induction is a main important method to kill tumor cells without complications. In recent research, by the MTT colorimetric method, it was indicated that nanoparticles have a concentration-dependent lethal activity on tumor cells. In addition, it was found that nanoparticles increased apoptosis in cancer cells. Today, the focus of cancer studies is the search for anti-cancer agents with a higher safety factor and greater acceptability for patients. In addition, nanoparticles can act synergistically or redundantly in combination with radiation therapy or chemotherapy for anticancer activity [56-59]. With the advent of nanotechnology, the paradigm of drug delivery systems has been developed, which can carry many drugs with chemical properties and actively target specific cells. Also, it can pass through biological barriers and transfer its cargo to the target location without pharmaceutical interventions. Today, the development of nanoparticles as effective drug carriers is at the center of attention. In these effective carriers, the drug is at the center of attention [58-60]. In this study, nanoparticles were used for cytotoxic purposes by the MTT method. One of the reasons that nanoparticles had a significant effect on cancer cells is that this phenomenon is due to their direct effect on the cell's respiratory system in the mitochondria. Therefore, due to the high level of mitochondrial activity in the respiration process of cancer cells compared to normal cells, a suitable substrate is provided for nanoparticles to destroy cancer cells. Another reason is the morphological differences between the membranes of cancer cells in terms of the size of their pores [61-63]. Various researches have been conducted to investigate the cytotoxic activities of nanoparticles on cancer cell lines [58-60]. The results of this study showed that metal nanoparticles in low concentrations do not have significant cytotoxic effects, but in higher concentrations, they have significant cytotoxic effects. The results of this research showed that the cytotoxicity of nanoparticles is dependent on dose and time and suggested that this nanoparticle can have biomedical applications. In general, the results of our study are consistent with other studies in the field of cytotoxicity of nanoparticles. Another mechanism of cytotoxicity of nanoparticles is the toxic oxygen radical's generation [58-61]. Another mechanism of nanoparticles cytotoxicity is the toxic oxygen radicals (ROS) generation that disturb the cellular redox balance, which is called oxidative stress. Oxidative stress destroys cellular antioxidant enzymes, destruction of cellular DNA structure, oxidation of cellular proteins and membrane lipids, and finally cell death. One of the important issues in the field of using metal nanoparticles in cancer treatment is the non-toxicity of nanoparticles on normal cells [59-64]. Studies show that nanoparticles have a greater effect on cancer cell lines, which is due to their direct effect on the cell's respiratory system in the mitochondria. Therefore, due to the high level of mitochondrial activity in the respiration process of cancer cells compared to normal cells, a suitable platform is provided for nanoparticles to destroy cancer cells [64-67]. Another reason is the morphological differences between the membrane of normal and cancer cells in terms of the difference in the size of their pores. Also, the difference in the shape, size and surface charge of nanoparticles is another factor in the difference in toxicity of nanoparticles between cancer and normal cells [67-69]. It is important to identify the importance of the apoptotic genes expression pattern in response to the anticancer drugs metastasis activity. Therefore, more investigations are necessary to prove that the mRNA expression profile of this gene can be in response to treatment [66-69]. The results obtained from this study showed the therapeutic use of nanoparticles in cancer cells. According to these reviews, clinical studies on human and animal models are necessary to prove the effect of nanoparticles and also the effect of these nanoparticles on healthy and normal cell lines. So, the nanoparticle's use can be efficient in enhancing the expression of some proliferative and apoptotic genes [62-66]. According to this study and previous research, it can be concluded that nanoparticles have powerful anticancer effects on cancer cells and derivatives of this compound can be used in the treatment of cancer. Therefore, if the clinical process of this nanoparticle is confirmed, these nanoparticles can be used in clinical cases for cancer patients in the future.

In this investigation, 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 oral malignancy cell lines i.e. HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 (Table 1;Figures 7-11).

In the recent study, the absorbance rate was evaluated at 570 nm, which represented viability on normal cell line (HUVEC) even up to $1000\mu g/mL$ for AgNPs (Table 1 and Figure 7).



Fig. 7. The cytotoxicity effects of AgNPs against normal (HUVEC) cell line.

The viability of malignant or al cell lines reduced dose-dependently in the presence of AgNPs. The IC50s of AgNPs were 220, 192, 174, 142, 125, 131, 250 and 219 μ g/mL against HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 cell lines, respectively. (Table 1; Figures 8-11).



Fig. 8. The anti-oral cancer activities (Cell viability (%)) of AgNPs (Concentrations of 0-1000 μ g/mL) against HSC-2 (a) and HSC-3 (b) cell lines.



Fig. 9. The anti-oral cancer activities (Cell viability (%)) of AgNPs (Concentrations of 0-1000 $\mu g/mL)$ against HSC-4 (a) and KB (b) cell lines.



Fig. 10. The anti-oral cancer activities (Cell viability (%)) of AgNPs (Concentrations of 0-1000 µg/mL) against BHY (a) and HN (b) cell lines.



Fig. 11. The anti-oral cancer activities (Cell viability (%)) of AgNPs (Concentrations of 0-1000 μ g/mL) against OECM-1 (a) and Ca9-22 (b) cell lines.

Table 1.	The IC50	of AgNPs	in the	anti-oral	cancer	test
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	Σιλερ νανοπαρτιςλες $(\mu\gamma/\mu\Lambda)$
IC50 against HSC-2	$220 \pm 0^{\rm b}$
IC50 against HSC-3	$192\pm0^{\mathrm{ab}}$
IC50 against HSC-4	$174\pm0^{\mathrm{ab}}$
IC50 against KB	142 ± 0^{a}
IC50 against BHY	125 ± 0^{a}
IC50 against HN	131 ± 0^{a}
IC50 against OECM-1	$250 \pm 0^{\rm b}$
IC50 against Ca9-22	$219\pm0^{\mathrm{b}}$
IC50 against HUVEC	-

4. Conclusion

In conclusion, AgNPs were synthesized using an aqueous extract of *R. ribes* leaves. The techniques of FT-IR, EDX, UV-Vis, XRD, TEM and FE-SEM AgNPs were applied for the nanoparticle's characterization. The obtained results confirmed the green synthesis of silver nanoparticles with an average size of 26.11 nm and spherical morphology. The viability of malignant oral cell lines reduced dose-dependently in the presence of AgNPs. The IC50 of AgNPs were 220, 192, 174, 142, 125, 131, 250 and 219 µg/mL against HSC-2, HSC-3, HSC-4, KB, BHY, HN, OECM-1, and Ca9-22 cell lines, respectively.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author

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