Green Synthesis and Comparison of Biological Properties of Ag-NPs as a Metal Nanoparticle with ZnO-NPs as a Metal Oxide Nanoparticle and their Applications

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Research Article

Keywords: Zinc-oxide nanoparticle, Silver nanoparticle, Green-synthesize, Antioxidant, Antiangiogenesis

Posted Date: February 7th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1324734/v1>

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Abstract

Aim

The aim of study was synthesized both silver- and zinc-oxide nanoparticles utilizing the *Peganum harmala* smoke extract (PHSE) bio-platform to evaluate their cytotoxicity on different types of human cancer cell lines and study their antioxidant, and antiangiogenic potentials.

Material and Methods

The Ag and ZnO nanoparticles were produced utilizing the green-synthesize method applying PHSE bio-platform. After characterization by XRD, DLS, FESEM, and FTIR methods, MTT assay was used for evaluation toxicity of nanoparticles. ABTS, DPPH, FRAP and ROS for antioxidant capacity, CAM and qPCR for antiangiogenesis effects of nanoparticle's were used

Results

Ag-NPs (82.42 nm) and ZnO-NPs (163.05 nm) were inhibited prostate, ovarian and liver malignant cells. Inhibition ABTS^{•+} and DPPH^{•+} and increasing the rate of intracellular ROS exhibited the anti and pro-oxidant capacity of Ag and ZnO-NPs out and inside of malignant cells. Also, their anti-angiogenesis impact was verified by significant dose-dependent VEGF and VEGFR down-regulation and the decreased blood vessels in the chicks' chorioallantoic membrane (CAM).

Conclusion

The antioxidant, cytotoxicity and anti-angiogenesis effects of Ag and ZnO-NPs synthesized from Pecan smoke extract make it possible to use these nanoparticles in cancer chemotherapy.

1. Introduction

Nanotechnology is one of the emerging branches of science with wide application in the field of biomedicine[1]. Size-dependent quantum effects alter the physicochemical and biological properties of nanomaterial relative to their bulk counterparts. The small size of nanoparticles and the presence of a higher percentage of atoms on their surface make it possible to interact with biomolecules both on the surface and inside the cells of the body and can leading to achieve methods for diagnosing and treating cancer [2].

Nanotechnology has taken an important step in improving the quality of anti-cancer drugs by producing nano drugs and targeting tumor cells [3]. One of the important fields of study in biomedical sciences is the evaluation of pharmacological properties of nanomaterial [4]. Silver and ZnO-NPs have recently

attracted considerable attention due to their beneficial and potential effects on human health. The use of plant extracts and natural products as reducing and stabilizing agents in the synthesis of these nanoparticles (green synthesis) can increase clinical efficiency and reduce the effects of toxicity and inefficiency of nanoparticles synthesized by chemical and physical methods [5]. In various studies, the antioxidant [6, 7], anti-cancer [8, 9], antimicrobial [10, 11] and anti-angiogenesis [12, 13] activities of these nanoparticles have been investigated and confirmed.

NPs can lead to cell death by increasing the intracellular reactive oxygen species (ROS), followed by increased oxidative stress and inflammatory products such as cytokines [14, 15]. ROS are produced as byproducts of oxygen metabolism in the body and play several physiological roles, such as cellular signaling. However, some factors such as environmental stressors help increase ROS production and subsequently cause an imbalance in production and its elimination by the antioxidant system. Antioxidants play an important role in the body's defense against the damaging effects of free radicals and ROS. An imbalance between antioxidants and ROS causes oxidative stress and subsequent cell damage [16]. Although an increase in ROS in healthy cells can cause carcinogenesis, however, the increase in ROS and subsequently damage to biological macromolecules and the initiation of apoptosis is one of the most important mechanisms for the removal of cancer cells in chemotherapy [17]. Despite the inhibitory effect of ZnO and Ag nanoparticles on free radicals in vitro [6, 7], their role in the production of oxidative stress following the increase in ROS in cancer cells has been shown in various studies [18, 19]. Cancer cells have a negative charge on their surface due to the high percentage of anionic phospholipids and certain groups of charged proteins and carbohydrates on their surface, and this feature can cause electrostatic interaction of nanoparticles with a positive surface charge to cancer cells as target cells [20, 21]. Exposure of cancer cells to nanoparticles activates cellular defense mechanisms to minimize damage, and if the stimulation of ROS production within the cell by nanoparticles is greater than the cell antioxidant defense capacity, the cells undergo apoptosis [14, 15]. Other effective mechanisms of nanoparticles in inhibiting cancer cells include inhibition of angiogenesis and their anti-cancer effects. Studies have shown that cells in tumor tissue create new blood vessels, by secreting stimulatory factors. Therefore, the initial growth of the tumor and its continued growth depends on adequate blood supply to the tumor tissue, and inhibition of blood flow can prevent further tumor growth [22]. Various studies have confirmed the inhibitory effect of silver [13] and ZnO-NPs [12] on angiogenesis. However, some studies have reported the pro-angiogenesis effects of ZnO-NPs [23].

In current survey, for the first time, Pecan smoke extract was used to synthesize ZnO and Ag-NPs by green way. After characterizing and confirming the synthesis of nanoparticles, their antioxidant, cytotoxic and anti-angiogenesis effects were investigated and compared.

2. Material And Methods

2.1. Materials

ABTS, DPPH, MTT, ferric tripyridyl triazine (Fe^{3+} TPTZ), Dimethyl sulfoxide (DMSO), Zinc acetate, silver nitrate were purchased from Merck Co. The human cancer cell lines including PC3, A2780, HepG2, and HFF were purchased from Ferdowsi University of Mashhad, Iran. All the materials needed for cell culture were bought from Sigma Aldrich.

2.2. PHSE extraction

The dried *Peganum harmala* seeds (1000 g) were burned at 80 °C in a 1000-mL distilling flask. The oxygen gas was injected 1 puff per 5 minutes during burning. Then, the smoke components were collected by cooling and distilling by a condenser tube. N-Hexane was used as the collector solvent then, the *Peganum harmala* smoke extract (PHSE) was dried.

2.3. Zn-oxide nanoparticles synthesis

Zinc acetate was added at 9 g concentration in 20 mL distilled water (DW) and mixed for 1 h at 40 °C. After adding the PHSE (20 mg), 1M NaOH solution was added drop wise to the mixture. In order to form a white precipitate of nanoparticles, the resulting mixture was placed on the heater for 2 to 3 hours. Sediment (ZnO-NPs) was collected by centrifugation (8000 rpm, 20 min) and then dried at 80 °C for 2 hours.

2.4. Ag-NPs synthesis

In order to synthesize silver nanoparticles, 0.85 g of silver nitrate was added to 500 mL of DW and after adding 20 mg of the PHSE, it was placed on the stirrer (3 h at 37 °C). The NPs were centrifuged and dried at 80 °C for 2 [24, 25].

2.5. Identification of Ag and ZnO-NPs

The size and dispersion index (PSI) of NPs were evaluated by DLS method and then XRD assay was used to evaluate the crystal structure and phase of pure Ag and ZnO-NPs. The FTIR spectroscopy was applied to verify the NPs' formation, features, and interactions [26]. The size and morphology of NPs were evaluated by FESEM. For this purpose, a drop of nanoparticles (1mg / 10mL distilled water) was sprayed on a special grade and after drying and coating, it was examined microscopically.

2.6. Cell Culture

The PC3, A2780, and HepG2 as cancer cells and HFF as normal cells were seeded at the 8×10^4 cells/cm² density in special cell culture media (RPMI or DMEM) at 37 °C. The media was supplemented by FBS, streptomycin, and penicillin in the required proportion.

2.7. MTT assay

For this purpose, 5×10^3 cells in each well of 96-well plates were cultured for 24 h and after that, the cells were exposed with serial doses of NPs. After 48 h the old culture media of each treating group was refreshed with a fresh media supplemented with MTT (0.5 mg/mL). The cells were incubated (3 h at 37 °C) and next, the MTT solution was replaced with 100 µL of DMSO. The OD of sample was recorded at

570 nm and used for calculating the viability of the cells: Cell viability (%) = (OD sample/OD control) × 100.

2.8. Antioxidant assay

2.8.1. ABTS assay

The antioxidant potential of Ag and ZnO nanoparticles was assessed by determining their ability to reducing the ABTS free radicals [27]. Briefly, ABTS solution was made by adding potassium persulfate and ABTS in a proportion of 2.45 mM: 7 mM in distilled water (1:1). The solution was incubated for 16 h at room temperature (RT) and diluted with DW. Absorbance of the diluted solution was recorded at ~ 0.700 in 734 nm wavelength. Then, the diluted solution of ABTS was mixed with a range of nanoparticles' concentrations with equal volume and incubated for 1h in dark conditions at RT; finally, the absorbance of ABTS was read at 734 nm.

2.8.2. DPPH assay

For this, an ethanolic solution of DPPH (1mg/16ml) was made and mixed with a range of nanoparticles' different concentrations in equal volume. Then, the solution was incubated for 30 min in dark at room temperature and, the samples absorbance was read at 517 nm. The antioxidant activity of NPs was measured by using the following equation:

$$\text{Antioxidant activity} = \frac{\text{Sample OD} - \text{Control OD}}{\text{Control OD}} \times 100$$

2.8.3. FRAP assay

The antioxidant potential of Ag and ZnO nanoparticles was studied by detecting their reductive potential for producing ferrous from ferric ions. According to the Benzie and Strain protocol [28], the solution called FRAP was prepared by mixing tripyridyltriazine (TPTZ), HCL, acetate buffer, and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a proportion of 10 ml: 40 mM: 300 mM: 20 mM at 37°. Finally, different doses of nanoparticles (1, 2, 3, 4, 5, and 6 mg/mL) were mixed with the FRAP solution in a ratio of 20: 280 µl. The standard curve was prepared regarding the FeSO_4 absorbance of the several standard doses at 593 nm [29]. Then, after 30 min incubation in FRAP solution, the absorbance of both ZnO- and Ag-NPs was recorded. The FRAP values were expressed as the produced ferrous ions (mg) for each concentration of nanoparticles (mg). All the measurements were carried out in triplicate manner.

2.8.4. ROS assay

ROS is naturally produced in cells as a byproduct of oxygen metabolism. Increasing the amount of ROS due to environmental pressures causes damage to macromolecules and cellular structures and causes oxidative stress. In this study, due to the high sensitivity of A2780 cells to both nanoparticles, this cell line was used to evaluate the amount of intracellular ROS. For this, cells were cultured in 96-well plates and

48 h after treatment with various doses of NPs were prepared according to the protocol of the ROS kit, and finally the fluorescence intensity was measured (excitation 480-500 nm and emission 510-550 nm) [30].

2.9. Anti-angiogenesis activity of Ag and ZnO-NPs

2.9.1. CAM assay

The anti-angiogenesis potential of NPs was analyzed by performing the CAM assay. Briefly, 64 fertilized Ross chicken eggs were divided into 8 group (N=8). The eggs were stored at 37°C for 48 hours in 55-70% humidity. Then, a 1-cm² window was cut on the shell of eggs and sealed by sterilized paraffin. Samples were treated with various doses of NPs on the eighth day as following treatment plan:

- Control group: no treatment
- Lab control: treating with PBS (0.1 M)
- Experimental groups: treating with different doses of both NPs

Finally, the eggs were resealed and incubated for 72 h. The number and length of the CAM blood vessels were measured utilizing the stereomicroscopic photos and image J software. Calipers and scales were used to measure the height and weight of the fetuses [31].

2.9.2. Gene expression profile

The antiangiogenic activity of both NPs was measured on the chorioallantoic membrane tissue of the treated eggs. For this purpose, chorioallantoic membranes were separated from the eggs and pulverized in separate groups with liquid nitrogen. Then, using the bioteck kit, their total RNA was extracted and the amount of RNA in each group was evaluated by Nano drop method. Then a certain volume of RNA was used to synthesize cDNA. The resulting cDNA with sybergreen, specific primer and deionized distilled water to evaluate the expression of VEGF and VEGFR as angiogenesis genes was examined by qPCR device (Bio-Rad CFX96).

2.10. Statistical study

For the cam method, image J software was used and then the data obtained from different experiments were analyzed with SPSS software version 21. One way ANOVA and LSD test was used to evaluate the significance level of the results. The less than 0.05 p-values were assigned as the significant levels.

3. Findings

3.1 Identification of Ag-NPs

The Z-average and PDI values of Ag-NPs were estimated 82.42 nm and 0.25, respectively, which make Z-average as a reliable size index regarding Stetefeld's theory (Fig. 1A). The theory demonstrates that less than 0.7 PDI values refer to the mono-distribution condition [32]. Also, the XRD algorithms show the

crystal features Ag-NPs (Fig. 1B). The individual peaks of Ag-NPs crystals are observable at 32 and 54 2θ -degrees. FE-SEM micrographs of Ag-NPs indicate its pseudo spherical morphology, support the DLS data and approve their estimated D mean number values (Fig. 1C). FTIR spectrum of Ag-NPs expressed visible bands at 3314.84 (O–H and N–H stretching), 2923.14 and 2847.67 (C–H stretching), 1691.26 and 1596.60 (N–H stretching), 1454.26 and 1357.90 (C–H stretching), 1245.86 (C–C(=O)–O stretching), 1156.0 (asymmetric stretching of the C–O–C), 1066.18 (C–O stretching), and The peak at 602.96 cm^{-1} is attributed to the bonding of oxygen from the O–H groups [33]. The FTIR data (Fig. 1D) indicate that the biological molecules could possibly be involved for synthesis and stabilization of Ag-NPs.

3.2. Characterization of ZnO-NPs

The DLS data exhibited the formation of ZnO-NPs with a size of 163.05 nm and PDI of 0.25. Since colloidal particles with dispersion index of 0-0.7 are introduced as particles with uniform dispersion, so ZnO-NPs can be considered as monodisperse particles (Fig. 1A). FESEM results confirm the presence of spherical particles with a rough appearance and size of about 100 nm, which is consistent with the data from DLS (Fig. 2B). The XRD algorithm of ZnO-NPs (the specific peaks for 2θ values in the range of 30 to 40 degrees) (Figure 2C) verified their synthesis. The chemical features of ZnO-NPs were defined by two specific lowest and highest wavenumbers at 435.18 cm^{-1} and 3436.97 cm^{-1} , respectively (Fig. 2D). The sharp peak at 435.18 cm^{-1} is the corresponding to the Zn–O stretching bond. The absorption peaks at the range of 1700 to 600 cm^{-1} attributed to C=O, C–O and C–H vibrations respectively. Other peaks are due to the hydroxyl stretching vibrations and bending modes of the adsorbed water [34].

3.3. The cytotoxicity of Ag-NPs

The effect of concentration-dependent toxicity of silver nanoparticles against cancer cell lines compared to normal is shown in Fig. 3. As shown in the diagram (Fig. 3A), prostate cancer cells show the highest resistance to treatment, while ovarian and liver cancer cells are highest sensitive to treatment. Ag-NPs reduced only HepG2 and A2780 cancer cells in lower than its IC_{50} doses for normal HFF cells (Fig. 3B). Therefore, the safe toxic doses (STD) of Ag-NPs are defined at lower than $19.23\text{ }\mu\text{g/ml}$.

3.4. The cytotoxicity of ZnO-NPs

The results MTT assay showed that ZnO-NPs are able to inhibit all three cancer lines in dose-dependent way (Fig. 4A). No inhibitory effect against normal cells was reported at the concentrations studied. Examination and comparison of IC_{50} showed that nanoparticles inhibited A2780, HepG2 and PC3 cells with moderate concentrations of 11.65, 47.36 and $49.96\text{ }\mu\text{g/mL}$, respectively (Fig. 4B). Thus, A2780 cell showed the highest sensitivity to ZnO-NPs treatment compared to other cells.

3.5. Antioxidant capacity of Ag-NPs

Figure 5A demonstrates the potential of Ag-NPs to reduce iron ions compared to Feso4 as a standard antioxidant. According to the Fig, the FRAP value increases with increasing concentration of Ag-NPs and its highest value (0.14) is observed at a concentration of 4 mg / mL Ag-NPs. Therefore, it can be said that nanoparticles at a concentration of 4 mg / mL 0.1 Feso4 have the ability to reduce iron ions. The

concentration-dependent inhibitory effects of Ag-NPs against ABTS and DPPH free radicals are shown in Fig. 5B. As can be seen, nanoparticles are able to inhibit 50% of ABTS free radicals at a concentration of about 200 $\mu\text{g} / \text{mL}$, while the median concentration for inhibiting DPPH free radicals is about 707.3 $\mu\text{g} / \text{mL}$. Therefore, the results show higher inhibitory effects of nanoparticles on ABTS free radicals compared to DPPH. Evaluation of changes in intracellular ROS in Ag-NPs treatment was measured by adsorption method (5C). The results showed a significant increase in the amount of intracellular ROS in the treated cells with a concentration of 2 mg / mL. As the concentration of the treatment increases, the amount of intracellular ROS increases dramatically, this confirms the role of Ag-NPs in inducing cell death by increasing intracellular ROS and causing oxidative stress damage [30].

3.6. Antioxidant capacity of ZnO-NPs

The reducing power of iron ions in the presence of ZnO-NPs compared to FeSO_4 was evaluated by FRAP method. The results showed that nanoparticles at the highest concentration are able to inhibit iron ions with a frap value of about 0.1 (Fig. 6A). The inhibitory effect of ZnO-NPs on ABTS and DPPH free radicals was evaluated to evaluate the antioxidant power of ZnO-NPs. As shown (Fig. 6B), increasing the concentration of ZnO-NPs increases the inhibition rate and the highest percentage of inhibition for ABTS free radicals is reported at a concentration of 2 mg / mL ZnO-NPs of about 4%. While at similar concentrations, about 34.25% of DPPH free radicals were inhibited. These results confirm the moderate inhibitory potency of ZnO-NPs against DPPH free radicals. One of the most important features of anti-cancer compounds is their ability to induce intracellular ROS, followed by cell damage and induction of cell death. In this study, intracellular ROS changes were evaluated in cells treated with different concentrations of ZnO-NPs. The results, showed a significant increase in ROS in treated cells with concentration of 8, 16 and 32 $\mu\text{g}/\text{mL}$ (Fig. 6C).

3.7. Anti-angiogenesis potential of Ag-NPs

Figure 7A shows the images of the treated area in the experimental groups compared to the control. As can be seen, the images clearly show the reduction of blood vessels in the treated groups with 250 and 500 $\mu\text{g}/\text{mL}$ of Ag-NPs. Examination of the mean number of blood vessels in control laboratory control and samples treated with a concentration of 125 $\mu\text{g}/\text{mL}$ Ag-NPs not show a statistically significant difference ($P>0.05$). However, with increasing the concentration to 250 ($*P<0.05$) and 500 $\mu\text{g}/\text{mL}$ ($***P<0.001$), a significant decrease in the number of blood vessels is observed. Significant reduction in blood vessel length was also observed only in treated samples with concentrations of 250 ($**P<0.01$) and 500 $\mu\text{g}/\text{mL}$ ($***P<0.001$). Examination of fetal height and weight in experimental groups compared to control shows that only in samples treated with a concentration of 500 $\mu\text{g}/\text{mL}$ there is a significant reduction ($*P<0.05$) (Fig. 7B). Examination of alterations in the expression of angiogenesis-related genes in Ag-NPs-treated samples showed that the VEGFR gene was significantly reducing in all concentrations studied, while the expression of the VEGF gene at both concentrations of 125 and 250 $\mu\text{g}/\text{mL}$ is approximately constant and similar to the control group, while in the samples treated with a concentration of 500 $\mu\text{g}/\text{mL}$, its expression was significantly reduced (Fig. 7C).

3.8. Anti-angiogenesis properties of ZnO-NPs

Microscopic changes of vessels in nanoparticle-treated samples compared to the control are shown in Fig. 8A. As can be seen, the amount of blood vessels in the experimental groups is almost similar to the control sample. To further evaluate the effect of nanoparticles on angiogenesis, mean length and number of blood vessels were evaluated with Imag J software and the results exhibited an increase in the average number of blood vessels in the two treated groups 500 (*P<0.05) and 1000µg/mL (**P<0.01). While the average length of blood vessels showed an increase only in 1000µg/mL (**P<0.01) treated group. An increase in the mean length (**P<0.01) and weight (*P<0.05) of the embryos during the increase in vascular factors was observed only in samples treated with 1000µg/mL (Fig. 8B). In order to evaluate the NPs effect on angiogenesis at the molecular level, the expression of VEGF and VEGFR genes was evaluated by QPCR method. As can be seen, at two concentrations of 250 and 500µg/mL, a significant decrease in the expression of genes associated with angiogenesis is observed, but with increasing the concentration of treatment to 1000 µg / mL, the expression of genes increases significantly (Fig. 8C). Considering the alteration in the expression of angiogenesis-related genes and the results of CAM test, it can be said that the effect of ZnO-NPs on angiogenesis was dose-dependent. In such a way that at low concentrations at the molecular level, it inhibits angiogenesis and by increasing the concentration, its effect on angiogenesis is reversed. Therefore, in order to use these nanoparticles in studies related to cancer treatment, it is important to pay attention to the concentration used.

4. Discussion

The genotype complexity and poor prognosis of cancer are considered as the main barriers in its treatment. The use of nanotechnology as a practical field to develop effective methods in the diagnosis and treatment of cancer has been considered by many researchers today [35]. Metal and metal oxides NPs such as Ag and ZnO-NPs play a prominent role in biomedical applications [36]. In current study, Pecan smoke extract (PHSE) was used as a biological platform for the green synthesis of ZnO and Ag-NPs, and then the biological effects of nanoparticles were investigated and compared.

The use of biological systems such as plant extracts and metabolites as reducing and stabilizing agents for the fabrication of NPs is called the green synthesis method, which has been successfully used in cancer treatment approaches and is an effective step in eliminating the disadvantages of physical and chemical methods [37].

In this study, smoke from burning *Peganum Harmala* seeds was used to green synthesize of silver (ps: 25.99 nm and PDI: 0.25) and zinc oxide (ps: 55.72 nm and PDI: 0.25) nanoparticles. Harmala is a native plant of Iran and belongs to the Zygophyllaceae family [38, 39]. In the roots and seeds of this plant, alkaloid compounds, beta-carbolines such as harmin, Harman, quinazoline and etc. are found [40, 41] and its anti-cancer, antimicrobial, anti-inflammatory and antioxidant effects have been reported in previous studies [42]. Due to the growing burden of cancer worldwide, different types of metal NPs have been fabricated through the green approach. For example, Zinc oxide-nanoparticles have been fabricated by various types of bio-platforms such as *Laurus nobilis* [43], *Deverra tortuosa* [44], *Garcinia mangostana* fruit [45], and *Cucumis melo inodorus* rough shell extracts [46]. Also, there are several types

of plant extracts that have been utilized for synthesizing Ag-NPs including *Amphipterygium adstringens* [47], *Centella Asiatica* [48], and *Ficus benghalensis* prop root extracts [49]. In a study in 2015, Ag-NPs with a size of 5 to 25 nm from the aqueous extract of *Caulerparacemosa* were prepared by green method [50]. In another study, Ag-NPs were green synthesized using the sumac aqueous extract with an average size of 35nm [51] which is comparable to the Ag-NPs synthesized in the present study.

Similar to the present study, in a study conducted in 2021, globular nanoparticles with 55 nm were fabricated by the green method. In this study, *Arthrospira platensis* was used as a reducing agent for the formation of NPs [52].

In this study, the toxicity of Ag-NPs and ZnO-NPs against three cancer cell lines was compared with HFF cells. The most sensitive and resistant cell lines to treatment with Ag-NPs were HepG2 and PC3 with IC_{50} about 1.95 and above of 40 $\mu\text{g/mL}$, respectively, while in the treatment with ZnO-NPs the highest and lowest resistance to treatment in HFF and A2780 cells with IC_{50} higher than 62 $\mu\text{g/mL}$ and 11.65 $\mu\text{g/mL}$ (respectively) was observed. Since ZnO-NPs did not show any toxic effect on normal cells at the studied concentrations, it can be said that ZnO-NPs are safer compared to Ag-NPs. Many studies similar to our study have shown the toxicity of various NPs including Ag-NPs and ZnO-NPs on cancer cells.

A study in 2018 showed higher cytotoxic effects of Ag-NPs synthesized from *Artemisia tournefortiana* extract against HT-29 (IC_{50} =40.71 mg/mL) compared to the HEK293 (IC_{50} =61.38 mg/mL) as a normal cells [53]. In another study was reported the strongly cytotoxic effects of Ag-NPs synthesized by aqueous extract of *Rubia tinctorum* against HepG-2 cells (IC_{50} : 6 $\mu\text{g/mL}$) compared to the HFF (IC_{50} : 100 $\mu\text{g/mL}$) as a normal cells [54].

In a study was conducted in 2021 on the fabricated ZnO-NPs by *Swertia chirayita* leaf extract revealed the toxic effects against HCT-116 and Caco-2. The nanoparticles showed a strongly cytotoxic potency against HCT-116 and Caco-2 cell lines, but less so against the normal HEK-293 cell line [55]. Also, in a study in 2019, Mahdizadeh et al. have reported the toxicity of ZnO-NPs from *Cucumis melo* on MCF7 (40 $\mu\text{g/mL}$) and Tubo (20 $\mu\text{g/mL}$) cells after 24 h [46].

ROS increasing, although under natural conditions causes diseases related to oxidative stress such as cancer, but in the treatment of cancer is used as one of the applied strategies in chemotherapy [17]. Studies show that increasing the amount of ROS can lead to the release of cytochrome C and activation of apoptotic pathway by changing the permeability of the mitochondrial membrane [56]. Our findings show the antioxidant effect of ZnO and Ag-NPs in vitro and its pro-oxidant effects inside the cancer cell. Our results exhibited a significant antioxidant capability for Ag-NPs for ABTS (IC_{50} : 150.07 $\mu\text{g/mL}$) and DPPH (IC_{50} : 707.33 $\mu\text{g/mL}$) free radicals (Fig. 5). In other words, ZnO-NPs appeared as a weak radical scavenger compared with Ag-NPs and glutathione antioxidant potential.

In 2019, Ahmadi et al reported the inhibitory effect of ZnO-NPs against ABTS and DPPH free radicals with IC_{50} about of 31.2 and 60 $\mu\text{g/mL}$ [57], which shows a higher antioxidant power compared to our study.

Similar to the present study, in a 2015 study, the inhibitory effects of Ag-NPs from *Cassia roxburghii* on ABTS free radicals (IC_{50} : 140 $\mu\text{g/mL}$) and their reducing power on iron ions (FRAP of 2.64 (Mg^{-1})) were investigated and confirmed[58]. Also, in a study in 2017, 23-95% of ABTS free radical scavenging capacity and 8.8 Mg^{-1} FRAP activities of Ag-NPs were reported [59]. The results of many studies show that NPs increase ROS production in various cancer cells [22]. Similar to the present study, in a 2020 study, the anti-proliferative and pro-oxidant effects of Ag-NPs from *Beta vulgaris L* were demonstrated in human hepatic cancer cells (HUH7) by ROS induction[60]. Lee et al. reported the potential to stimulate ROS production in human keratinocytes treated with ZnO-NP. They showed that the mechanism of nanoparticle toxicity is related to ROS production, oxidative stress and apoptosis [61]. In another study in 2017, Bai et al. have exhibited the increasing intracellular ROS in SKOV3 treated with ZnO-NPs [62].

The results of this investigation showed the wide range of safe toxic doses of ZnO and Ag-NPs indicate their pro-oxidant activity and thus approve their ROS-mediated cytotoxicity [63].

Angiogenesis is one of the most important features of tumor cells that help their survival and reducing angiogenesis plays the main role in preventing the growth of cancer cells and inflammation [22]. The results of this survey confirmed the reduction of angiogenesis in the treatment with different concentrations of Ag-NPs in CAM and qPCR assay. Investigation of the effect of ZnO-NPs on angiogenesis showed that, they suppress angiogenesis by down-regulating VEGF and VEGFR gene expression at 0 to 500 $\mu\text{g/mL}$ concentrations and act as a pro-angiogenic compound at >500 $\mu\text{g/mL}$ doses. In many studies consist with the present investigation, the anti-angiogenesis effects of nanoparticles have been investigated and confirmed [64, 65] .

For example, a reduction in angiogenesis by the CAM method was reported in a 2015 study by examining the mean number and length of blood vessels in samples treated with Ag-NPs[13]. Also, the inhibitory effect of Ag-NPs on VEGF expression in the mouse matrigel plug [64] and retinal endothelial cells [66] has been reported in previous studies.

Various investigations have shown the anti and pro angiogenesis potential of ZnO-NPs. In a study in 2019, the anti-angiogenesis effects of ZnO-NPs from *Hyssops officinalis L* were reported using the CAM assay [12]. Ahtaz et al reported the pro-angiogenic effects of ZnO-NPs in 2017 using the CAM method [23]. In a study by Oikawa et al., The inhibitory effect of ZnO-NPs on the formation of blood vessels by reducing the expression of VEGF and VEGFR was reported [67] which is comparable to our results.

5. Conclusion

According to the results, Ag-NPs were exhibited stronger antioxidants capacity than ZnO-NPs, also, the higher cytotoxic effect of Ag-NPs have been showed compared to ZnO-NPs by MTT method. In addition, Ag-NPs showed significant anti-angiogenesis effects by reducing VEGF and VEGFR genes expression. Considering the survival of cancer cells and suppression of angiogenesis, higher anti-cancer activity of Ag-NPs was detected compared to ZnO-NPs.

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Figures

Figure 1

The Ag-NPs characterization results. (A) The Z-average and PDI indices were defined at 82.42 nm and 0.25, respectively. (B) The XRD pattern of Ag-NPs. (C) The FESEM image of Ag-NPs. (D) The FTIR diagram indicating chemical characteristic of Ag-NPs.

Figure 2

The ZnO-NPs characterization data. (A) The Z-average and PDI indices were defined at 163.05 nm and 0.25, respectively. (B) The XRD pattern of ZnO-NPs. (C) The FESEM image of ZnO-NPs. (D) The FTIR diagram indicating chemical properties of ZnO-NPs crystal structure.

Type of Cells	IC ₅₀
HFF	19.23 μg/ml
PC-3	> 40 μg/ml
A2780	8.01 μg/ml
HepG2	1.95 μg/ml

Figure 3

Cytotoxic effects of Ag-NPs. (A) the diagrams indicate the cancer cells' viability following to different Ag-NPs concentrations. (N= 3), **P<0.01 and ***P<0.001. (B) Medium concentrations (IC₅₀) of Ag-NPs against different cell lines.

Figure 4

The cytotoxic activity of ZnO-NPs. (A) the diagrams indicate the cancer cells' viability following to different ZnO-NPs concentrations (N= 3), ***P<0.001. (B) Medium concentrations (IC₅₀) of ZnO-NPs against different cell lines.

Figure 5

The Ag-NPs antioxidant activity. (A) The FRAP assay data. (B) Scavenging of ABTS and DPPH free radicals with nanoparticle exposure (C) Improved intracellular ROS production in Ag-NPs-treated cells. (N= 3) and ***P<0.001.

Figure 6

The ZnO-NPs antioxidant properties. (A) Ferric Reducing Antioxidant Power (FRAP) of the ZnO-NPs. (B) Scavenging of ABTS and DPPH free radicals with ZnO-NPs exposure (C) Enhanced intracellular ROS production in ZnO-NPs-treated cells. (N= 3) and ***P<0.001.

Figure 7

CAM assay (A) The stereomicroscopic micrograph of CAM following the treatment with different doses of Ag-NPs, (B) Decrease in mean number and length of blood vessels and also changes in fetal growth factors (height and weight) following the treatment with different doses of Ag-NPs (N= 8); (*P<0.05, **P<0.01, ***P<0.001). (C) Change in VEGF and VEGF-R gene expression in Ag-NPs-treated samples compared to control. Data are significant at the level of *P<0.05 and ***P<0.001 (N=3).

Figure 8

CAM assay (A) The stereomicroscopic micrograph of CAM following the treatment with different doses of ZnO-NPs, (B) Change in mean number and length of blood vessels and embryonic growth factors in ZnO-NPs treatment (N= 8); (*P<0.05, **P<0.01, ***P<0.001). (C) Change in VEGF and VEGF-R gene expression in ZnO-NPs-treated samples compared to control. *P<0.05 and **P<0.01 (N=3).