RESEARCH ARTICLE

Characterization of herbal synthesized Ag doped ZnO nanoparticles as a potent cytotoxic agent on glioblastoma cell line

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ABSTRACT

The green synthesis of nanoparticles (NPs) can be achieved through the use of eco-friendly and readily available herbal extracts. In this particular study, the aqueous root extract of *Biebersteinia multifidi (B. multifidi)* plant was used to prepare pure zinc oxide (ZnO) nanoparticles as well as Ag-doped ZnO NPs (Ag/ZnO NPs) at concentrations of 1%, 5%, and 10%. The physicochemical features of NPs were characterized by field emission scanning electron microscopy (FESEM) and energy-dispersive X-ray spectroscopy (EDX), powder X-ray diffraction (PXRD), and UV-Vis spectrophotometer techniques. The findings exhibited that Ag ions were effectively doped in the ZnO structure based on PXRD and EDX analyses, while FESEM indicated that the obtained NPs were spherical with an increase in particle size as silver was introduced into the ZnO structure. To assess their cytotoxicity performance, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed on brain glioblastoma cells (U87) using both pure ZnO NPs and Ag-doped ZnO NPs. The findings indicated that Ag-doped ZnO NPs had a higher toxicity on U87 cells compared to pure ZnO NPs, suggesting that doping can enhance the cytotoxic performance of ZnO NPs.

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INTRODUCTION

Nanoparticles are tiny substances with the size less than 100 nm. Their tiny size results in large surface area per unit volume with a significant proportion of their constituent atoms located on or near the surface [1]. Polymers, metals, metal oxides, carbon materials, semiconductors, organics, and biological based NPs exist in various chemical forms. They also have a wide range of morphological diversity, including spheres, hollow spheres, platelets, tubes, disks and cylinders [2-4]. Metal nanoparticles (MNPs) and metal oxide nanoparticles (MONPs) have been used in different areas such as antibacterial, anticancer, cosmetics, drug delivery, anti-catalytic, and diagnosis agents [5-10]. Because of their remarkable chemical, electrical, and optical features, ZnO and Ag NPs have been considered for improving cancer treatment. ZnO nanoparticles may be a promising anticancer agent due to their unique biocompatibility, high selectivity, increased cytotoxicity, and ease of manufacturing. Zinc is considered as a vital trace element in the human body, acting as a cofactor for more than 300 mammalian enzymes and facilitating cellular processes such as DNA replication, oxidative stress, apoptosis, cell cycle progression, and DNA repair. As a result, it is clear that a change in zinc levels in cancer cells might have a negative impact. Low levels of zinc in cells have been linked to cancer development and progression, while high concentrations have been associated with toxicity. This cytotoxic effect may be due to an imbalance in protein activity caused by zinc or oxidative stress from reactive oxygen species (ROS) [11, 12]. Ag NPs also exhibit anti-cancer

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properties by inducing oxidative stress that result in higher lipid peroxidation and ROS levels and a lower glutathione level. The increased intracellular ROS production eventually led to mitochondrial membrane destruction and disruption of cell cycle. Ag NPs have been also demonstrated to induce apoptosis and necrosis in cancer cells via Sub-G1 cell cycle arrest in studies [13]. Studies exhibited that Ag doping can significantly increase the anti-cancer activity of ZnO NPs [14-18].

Chemical reduction, gamma radiation, microemulsions, electrochemical procedures, laser ablation, autoclave, microwave, and photochemical reduction are among several physical and chemical techniques used to make these nanoparticles. Although effective, the utilization of harmful chemicals, high running expenses, and high energy requirements are limitations of conventional techniques. However, emerging alternatives such as reducing and masking agents based on microorganisms, plant extracts, and natural polymers are novel, cost-effective, and efficient. Therefore, they have the potential to replace traditional physicochemical approaches for NP production. Green synthesis is an efficient way to make NPs on a large scale via plant extracts as reducing agents to decrease particle size and increase surface area. The integration of nanotechnology and green chemistry can lead to the discovery of metal nanoparticles that are safe for physiological and cytological use [19]. Green synthesis techniques are preferred because of their low cost, biocompatibility, and eco-friendliness [17]. So far, several studies have been performed in which nanoparticles have been synthesized using green chemistry methods and used against cancer cells [20-23]. For instance, Pandiyan et al [19] used Justicia adhatoda plant extract to synthesize Ag-Au/ZnO nanostructure and found antibacterial effects against E.coli and S.aureus bacteria and considerable anticancer activity in human cervical cancer cells (HeLa). Also, Nithya et al [24] used Justicia adhatoda plant extract to synthesize Ag and Au doped CeO2 NPs and investigated their antibacterial and anticancer properties. They have discovered that bimetal loaded cerium oxide nanoparticles, synthesized using ionic liquid functionalization, have the potential to be effective against bacteria and cancer cells. Biebersteinia multifida DC, a plant native to Iran but also found in Afghanistan, Armenia, Lebanon, Syria and Central Asia under the names Chele Daq, and Adamak contains active substances such as polypeptides, polysaccharides, alkaloids and flavonoids (e.g. , Luteolin, Apigenin and Tricetin) that possess antioxidant and antibacterial properties [25].

Several studies have focused on the green synthesis of Ag/ZnO NPs [26-30] and the cytotoxicity of these NPs. However, to our knowledge, this study is the first to use B. multifida root extract for the green synthesis of Ag/ZnO NPs. The cytotoxic effect of these NPs on U87 cell lines was also investigated.

EXPERIMENTAL

Preparation of the aqueous extract of B. multifidi root

NPs were synthesized using the powdered root of B. multifidi. The root was mixed with distilled water in a 1:10 ratio and shaken for 10 hrs at 150 rpm. The resulting suspension was filtered and the extract obtained was applied for synthesising NPs.

Synthesis of pure ZnO NPs and Ag/ZnO NPs

NPs were synthesized as described by Yadav et al [31] with modifications using B. multifidi root. 10 mL of the root extract was poured to four Erlenmeyer flasks. Each flask was then filled with distilled water to a volume of 50 mL. The flasks were placed in an 80 ˚C water bath. Solutions containing zinc nitrate ($Zn(NO_3)_2.6H_2O$, 99.99%, Millipore Sigma) and silver nitrate ($AgNO_3$, 99.0%, Millipore Sigma) were prepared based on the $Ag_{1-x}Zn_xO$ formula with varying concentrations of AgNO3 (0%, 1%, 5%, and 10%). These solutions were mixed with the root extract and stirred for three hrs. The resulting solutions were then dried at 70 ˚C for twelve hrs before being calcined into a furnace at 600˚C for two hrs (see Fig.1).

Characterization of NPs

The pure ZnO NPs and Ag-doped ZnO NPs were analysed through PXRD (Netherlands, PANalytical X'Pert PRO MPD system, Cu Kα) at a scan speed of 2°/min with a lower angle of 20°–80°, UV-Vis spectrophotometer (Rayleighuv-2100, Chine), and FESEM (MIRA3 TESCAN, Czech).

Cytotoxic assay

Cell culture

In this research, the cytotoxic impact of prepared samples on U87 cells was evaluated. The U87 cells were purchased from the Pasteur Institute (Iran) and were thawed before being centrifuged

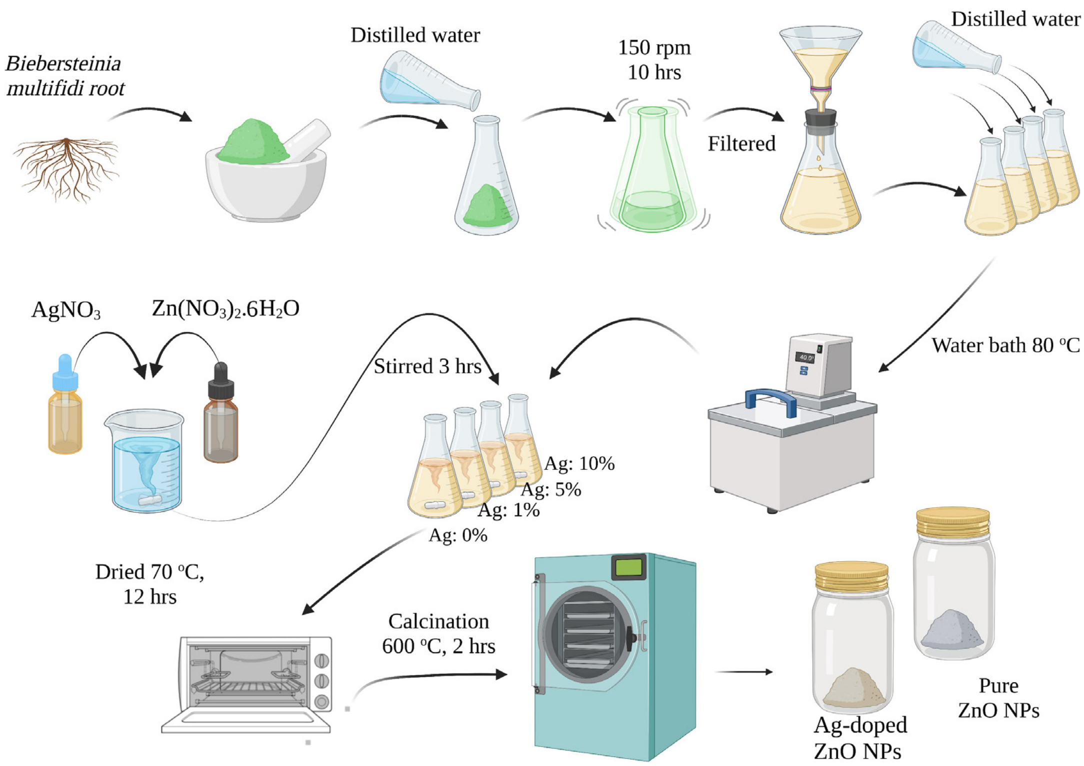

Fig. 1. Schematic of synthesis steps of Ag/ZnO NPs (Created with BioRender.com).

at 830 rpm for 9 min in Falcon tubes. The cells were mixed with a complete culture medium after removing the supernatant and then transferred into flasks for incubation at 37 °C under 5% CO2. The DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

MTT Test

To evaluate the potential harm of pure ZnO NPs and Ag-doped ZnO NPs, a MTT test was conducted. U87 cells with a density of 10^4 cells per well were seeded in a 96-well plate and maintained in a 5% CO_2 environment at 37°C. After 24 hrs, the cells were examined under a microscope, and the culture medium was removed. Then, 100 µL of 1%, 5%, and 10% Ag-doped ZnO NPs (1, 5, 10, 50, 100, 250, 500 and 1000 µg/mL) were added to wells (n=3). Doxorubicin and cell culture medium alone were considered as positive and negative controls, respectively. The treated cells were incubated for 24 hrs before adding MTT solution (10 µL) and keeping it at a temperature of 37°C for four hrs. At last, an ELISA reader measured the optical density (OD) of wells with a wavelength of 490 nm.

RESULTS AND DISCUSSION

PXRD analysis

The patterns of pure ZnO NPs and 1%, 5% and 10% Ag/ZnO NPs depicted in Fig. 2. The pattern of pure ZnO NPs shows the diffraction peaks at 2θ positions of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004) and (202) which implied the hexagonal wurtzite structure of ZnO [32]. The graphs of 1%, 5% and 10% Ag/ZnO NPs included additional diffraction peaks, which placed in position of 2θ = 38.14, 43.26 and 63.82˚ related to face-centered-cubic (fcc) phase of Ag [16]. In Fig. 2, the intensity of silver's peak enhanced by increasing the concentration of doped metal. The crystallite size of NPs was estimated with Scherrer's equation [16], and 39±0.5, 43±0.3, 46±0.2 and 52±0.6 nm sizes were obtained for pure ZnO NPs and 1%, 5% and 10% Ag/ZnO NPs, respectively. By increasing the Ag concentration, the crystal size of NPs was increased as well, which could be due to the higher ionic radius of Ag (1.26 Å) with respect to ZnO (0.74 Å).

FESEM/EDX analysis

FESEM is a device to identify the morphology

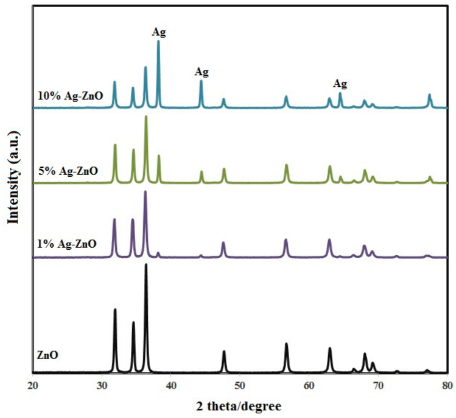

Fig. 2. PXRD patterns of pure ZnO NPs and 1%, 5% and 10% Ag/ZnO NPs.

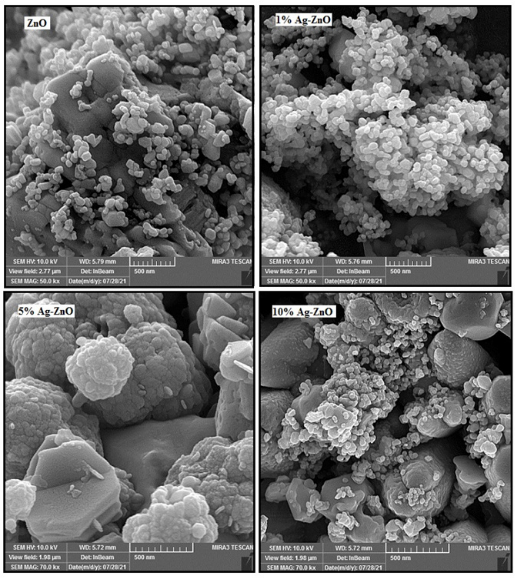

Fig. 3. FESEM images of pure ZnO NPs and 1%, 5% and 10% Ag/ZnO NPs.

and size of a sample. Based on the findings in Fig. 3, it was determined that the size of pure ZnO NPs was approximately 45-50 nm, while the particle size of Ag/ZnO NPs was larger than that of pure ZnO NPs, which confirmed the results obtained from PXRD. The EDX results also indicated that silver had been successfully incorporated into the zinc oxide structure. As shown in Fig. 4, the amount of Ag in pure ZnO NPs and 1%, 5%, and 10% Ag/ZnO NPs was found to be 0%, 0.89%, 4.25%, and 8.92%, respectively. Additionally, no impurities were detected in the structural compound of the synthesized NPs through EDX analysis.

UV-Vis analysis

UV-Vis spectroscopy is a valuable technique for determining the stability and formation of samples in aqueous solutions. Typically, light wavelengths ranging from 250-500 nm are used to analyze various metal and metal oxides at the nanoscale [33]. In this study, the electron spectra of pure ZnO NPs and 1%, 5%, and 10% Ag/ZnO NPs were examined (Fig. 5). These wavelengths are associated with the wurtzite hexagonal phase of bulk ZnO [34]. The red shift observed in the adsorption peak of doped NPs is due to silver ions being incorporated into the ZnO NPs. The absence of any additional absorption peaks in the electron spectra confirms that there were no impurities present in the synthesized NPs, which supports their optical properties.

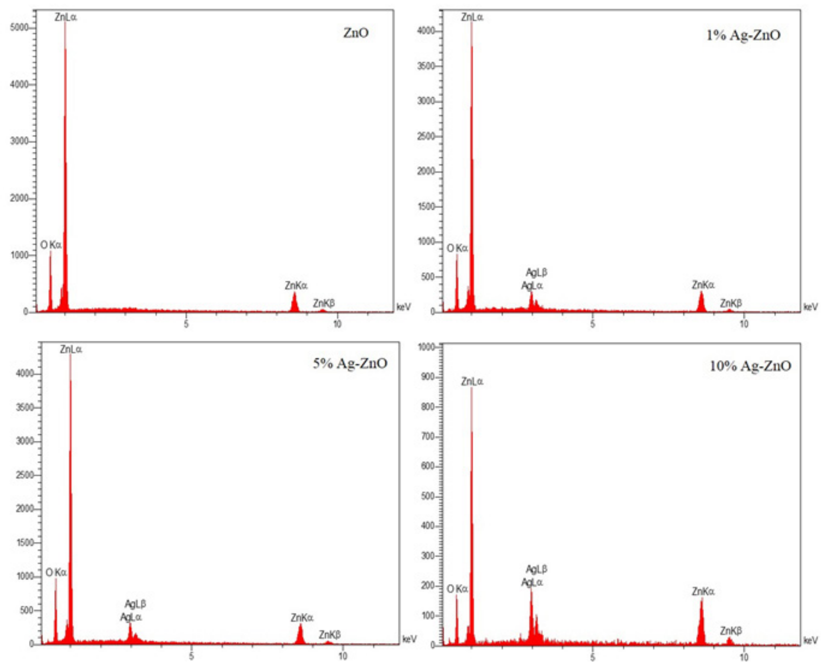

Fig. 4. EDX spectra of pure ZnO NPs and 1%, 5% and 10% Ag/ZnO NPs.

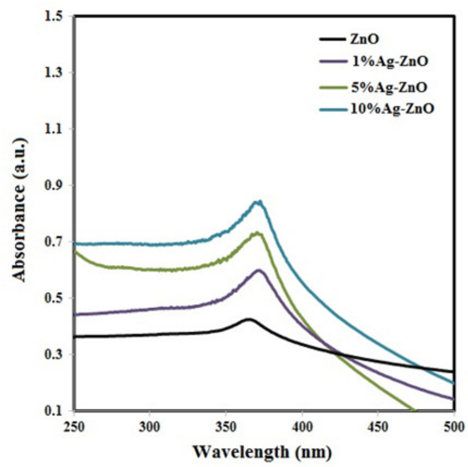

Fig. 5. UV-Vis spectra of pure and 1%, 5% and 10% Ag/ZnO NPs

Cytotoxicity performance

The cytotoxic effect of pure ZnO NPs and 1%, 5%, and 10% Ag/ZnO NPs on U87 cells was assessed by MTT. The results, shown in Fig. 6, indicate that cell viability decreased with increasing nanoparticle concentration. Therefore, the toxicity of the NPs against U87 cells is concentration-dependent. Additionally, doping silver into ZnO NPs enhanced their toxicity compared to pure NPs.

The toxic effect of ZnO NPs on cancer cells is attributed to their ability to produce ROS, which creates a redox system in the cells and results in the production of chemical species and oxidative stress. This oxidative stress is a major factor in cell death [35]. In a study by R. Wahab et al [36] the cytotoxic effect of ZnO NPs on U87, HEK, and HeLa cells was investigated using MTT assay. The findings indicated that NPs were more effective against U87 and HeLa cells and non-toxic against HEK cells, suggesting that ZnO NPs could be

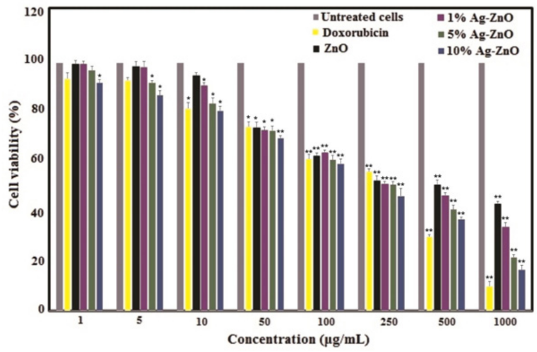

Fig. 6. Cell viability of pure and 1%, 5% and 10% Ag/ZnO NPs on U87 cell line after 24 hrs incubation (* ; $p < 0.05$, ** ; $p < 0.01$).

potent anti-cancer agents. Additionally, ZnO NPs enhanced apoptosis and cytogenetic damage in HeLa and U87cells.

In another study by Rajendran et al [35], the treatment of HaCaT cells with Ag/ZnO NPs caused the changes in cell morphology and apoptosis, which were due to an increase in ROS levels produced by the NPs. The other study indicated that the inhibitory effects of un-doped ZnO NPs on U87 cells were similar to those of doxorubicin at a concentration of 100 μg/mL [16].

As a result, this study showed that Ag-doped ZnO NPs had a greater toxic effect on U87 cells compared to pure ZnO NPs, and higher concentrations of Ag increased the toxicity of doped NPs. Therefore, Ag-doped ZnO NPs exhibited effective anti-cancer properties on U87 cells.

CONCLUSION

This work demonstrates the successful preparation of pure ZnO NPs and Ag-doped ZnO NPs using *B. multifidi* aqueous root extract. According to the obtained results, the synthesized NPs had consistent and approximately spherical shape. The size of pure ZnO NPs is 45-50 nm and with the addition of Ag to ZnO NPs, the size of doped NPs becomes larger. Achieved results indicated that Ag/ZnO NPs had high inhibitory effect on glioblastoma cell growth compared to pure ZnO NPs. Also, the higher amounts of Ag in the doped NPs increased the potency of their cytotoxicity. Therefore, the synthesized NPs can be proposed as a therapeutic agent for biological applications such as cancer treatment.

Studies have previously been conducted on the green synthesis of Ag/ZnO NPs [26-28] and their cytotoxicity, but this work is the first to use *B. multifida* root extract for the green synthesis of Ag/ZnO NPs for investigating the cytotoxicity on glioblastoma cell line. Overall, this research provides important information for developing safe and effective nanomedicines using natural extracts for various biomedical applications. In addition, future studies should focus on in vivo studies and proposed molecular mechanisms.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest regarding the publication of this paper.

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