Evaluation of antioxidant and anticancer properties of zinc oxide nanoparticles synthesized using Aspergillus niger extract

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Abstract
Microorganisms and plants have grabbed great attention as potential biological sources for ecofriendly synthesizing nanoparticles. In this study, zinc nitrate and Aspergillus niger were applied to synthesize stable spherical zinc oxide nanoparticles (ZnO-NPs). The FTIR, DLS, SEM, TEM and XRD methods were utilized to characterize the synthesized nanoparticles in terms of structure, morphology, and optic features. Electron microscopic images revealed poly dispersed nanoparticles with the length of 30 to 70 nm. Regarding morphology, the majority of the particles were spherical. Regarding antioxidant capacity, the synthesized ZnO-NPs showed the IC50 of about 1000 μg ml⁻¹. The synthesized ZnO-NPs also induced apoptosis and inhibited cellular growth in neoplastic MCF-7 cells.

Abbreviations

ZnO-NPs  Zinc oxide nanoparticles
NPs  Nanoparticles
FTIR  Fourier-transform infrared spectroscopy
DLS  Dynamic light scattering
SEM  Scanning electron microscope
TEM  Transmission electron microscopy
XRD  X-ray diffraction
MCF7  Breast cancer cells
PDA  Potato Dextrose Agar
DMEM  Dulbecco’s Modified Eagle Medium
BHA  Butylated hydroxyanisole
MTT  3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS  Phosphate-Buffered Saline
PI  Propodium Iodide
AO  Acridine-Orange
DPPH  1, 1-Diphenyl-2-picrylhydrazyl

1. Introduction
Nanotechnology is the science of engineering nanoscale (1–100 nm) materials (i.e. nanoparticles-NPs), characterizing their physiochemical features, and finally divulging their effects on living cells [1–4]. Various medicinal diagnostic (e.g. imaging techniques, biosensing) and therapeutic applications (e.g. drug targeting,
In this study, cancer therapy has been noted for metal oxides as sources for synthesizing inorganic NPs [5–8]. Zinc oxide (ZnO) NPs are commonly utilized as solar cells, biosensors, and photocatalysts in various industrial and pharmaceutical fields [9–13]. Also, ZnO-NPs have been applied as safe antimicrobial agents in biomedicine [14–17]. In addition, ZnO-NPs are resistant against degradation by microorganisms delivering them as stable sources for developing antimicrobial agents [18]. The functional properties of ZnO-NPs largely depend on their structural (e.g. size, morphology, direction, and surface ratio) and physiochemical (e.g. electrical and thermal conductance) features [19, 20]. Although physicochemical methods which are commonly used to synthesize NPs can deliver large volumes of these particles in a relatively short period of time, trace depositions of toxic elements on the surface of physiochemically synthesized materials limit their clinical application [21]. As alternatives to physicochemical biosynthesis methods, green-synthesis approaches have increasingly been used to safely and eco-friendly produce NPs [22, 23].

Anti-apoptotic mechanisms of cancer cells are important for their proliferation and propagation [24]. Therefore, apoptosis induction is one of the main therapeutic goals of any cancer therapeutic agent [25]. In the present study, we aimed to ascertain the cytotoxicity effects of ZnO-NPs against MCF-7 breast cancer cell line. Furthermore, the antioxidant capacity of the biosynthesized ZnO-NPs was also studied.

Fungi have notable metal binding and bioaccumulating abilities and produce a wide variety of enzymes. They are also ease in the scale-up process, handling the biomass, and cost-effective to be grown [26, 27]. In this study, we used Aspergillus niger to synthesize ZnO-NPs and evaluate their antioxidant and anticancer properties.

### 2. Materials and methods

#### 2.1. ZnO-NPs synthesis

In this study, A. niger (PTCC: 5012) was prepared from the Iranian Bank of industrial fungi and bacteria. After activation, 2 ml of the fungus suspension was transferred to plates containing Potato Dextrose Agar (PDA). The plates were incubated for 7 to 10 days at 25 °C. The fungal biomass was cultured in a fluid culture medium while shaking. After that, 10 ml of 0.1 M sterile nitrate solution was prepared and added to 50 ml of the bacterial culture medium. The solution was heated in a hot water bath at 80 °C for 10 to 5 min. The turning to white of the precipitate marked the beginning of the production of ZnO-NPs.

#### 2.2. Characterizing biosynthesized ZnO-NPs

The synthesized ZnONPs were characterized using particle size analyzer, as well as TEM, SEM, XRD, and FTIR analyses. The physical properties (i.e. size and shape) of the ZnO-NPs were characterized by TEM (JEOL, Japan) and FESEM (JEOL, Japan). The crystal structure and purity of the ZnO-NPs were also determined using Philips PW1800 x-ray diffractometer (XRD) (Almelo, Netherlands). Meanwhile, FTIR was carried out (Perkin Elmer, Walthman, MA, USA) to further characterize the NPs.

#### 2.3. DPPH test

DPPH method was utilized to determine the radical scavenging capacity of the synthesized NPs. Equal volumes of various concentrations of ZnO-NPs were admixed with 0.1 mM methanolic DPPH solution. The solutions were then incubated at room temperature for 30 min. The absorbance of the sample was finally read at 517 nm. The butylated hydroxyanisole (BHA) was used as a reference antioxidant compound [28].

#### 2.4. Cell cytotoxicity

The MCF-7 breast cancer cell line was cultured in DMEM and incubated at 37 °C and 5% CO₂. These cells (5 × 10⁴ cells/well) were treated with different concentrations of ZnO-NPs (0, 15.6, 31.2, 62.5 and 125 μg ml⁻¹) for 24, 48 and 72 h. Finally, MTT assay was utilized to determine cell viability by reading the absorbance at 570 nm (Plate Reader Spectrophotometer, Epoch, Biotek, UK) [29].

#### 2.5. Apoptosis assay using flow-cytometry and AO/PI staining

The MCF-7 cells were cultured in 6-well plates and then treated with different concentrations of ZnO-NPs for 48 h. After incubation and washing with Phosphate-buffered saline (PBS), 1 mg ml⁻¹ PI was added to the wells. Finally, the plates were placed in incubator for 30 min, and then the cells were separated and analyzed using flow-cytometry. To perform acridine-orange (AO) test, cell suspensions (5 ml) were cultured and incubated for 24 h. At the next step, the culture medium was discarded, and the cells were treated with different concentrations of ZnO-NPs for 48 h. After trypsinization and washing cells with PBS, 10 μl acridine orange and 10 μl PI (Propodium Iodide) were added to 10 μl of the cell suspension. The mixture was then incubated for 5 min. In the next step, 20 μl of the mixture was placed on a slide to be photographed and examined by fluorescence microscopy [30].
3. Results and discussion

3.1. Characterizing of the biosynthesized ZnO-NPs

The x-ray diffraction (XRD) pattern was acquired using a x-ray diffractometer (Panalytical X’PERT) equipped with a Ni filter applying Cu K\(\alpha\) (\(\lambda = 1.540 \text{ Å}\)) radiation as an x-ray source at room temperature. The crystalline structure of the ZnO-NPs has been shown in figure 1. The peaks at 2\(\theta\) = 31.67°, 34.31°, and 36.14° were assigned to (100), (002), and (101) indicating the polycrystalline wurtzite structures of the NPs (Zincite, JCPDS 5-0664).

The field emission (JSM-7600F, JEOL Inc., Akishima, Japan) and transmission (JEM-2100F, JEOL Inc.,) electron microscopies with accelerating voltages of 15 kV and 200 kV, respectively were used to determine the structural properties of the NPs. The TEM images showed that the diameters of the ZnO-NPs ranged from 30 to 70 nm (average of about 40 nm). The NPs also revealed normal length distribution (figure 2).

The dynamic light scattering (DLS) (Nano-ZetaSizer- HT, Malvern Instruments, Malvern, UK) was performed to determine the average hydrodynamic size and zeta potential of the ZnO-NPs in water and complete cell culture (DMEM) medium (figure 3). At the 15 \(\mu\)g ml\(^{-1}\) concentration, ZnO-NPs were dispersed in water and DMEM medium for 24 h. Finally, the suspension was sonicated (room temperature, 15 min, 40 W), and the DLS method was conducted (figure 4).

The FTIR spectroscopy was performed to determine the effects of bioactive compounds in A. niger extract in the synthesis of ZnO-NPs (figure 5). The peaks appeared around 3478, 2083, 1638, 1075 and 566 cm\(^{-1}\) probably
Figure 3. Zeta potential of ZnO-NPs synthesized using *A. niger*.

Figure 4. Size dispersion of ZnO-NPs synthesized using *A. niger*.

Figure 5. The FTIR spectra of ZnO-NPs synthesized using *A. niger* extract.
representing flavonoid and phenolic (i.e. aromatic) groups in the structure of biosynthesized ZnO-NPs. Another study showed the peak at 1637.56 cm$^{-1}$ showing $-$C$=$C– aromatic structure [31]. Thus, the strong aromatic ring identified in FTIR analysis seems to participate in the biosynthesis of ZnO-NPs.

3.2. DPPH antioxidant assay

The capacity of ZnO-NPs to scavenge free radicals was investigated by the DPPH assay which is an easy, rapid, and amenable (requiring only an UV–vis spectrophotometer) method widely used to determine antioxidant activity of plant extracts [32]. The results showed that the synthesized ZnO-NPs dose-dependently scavenged free radicals (figure 6, $p < 0.001$). The IC$\text{_{50}}$ of the NPs was obtained as 1000 $\mu$gm l$^{-1}$. The BHA was used as a reference antioxidant.

3.3. Cytotoxicity activity of ZnO-NPs

The cytotoxicity of the synthesized ZnO-NPs against MCF-7 breast cancer cells was investigated by MTT assay at 0, 15.6, 31.2, 62.5 and 125 $\mu$gm l$^{-1}$ concentrations for 24, 48, and 72 h (figure 7). Cell viability of cancer cells depend on time and concentration. The IC$\text{_{50}}$ of 24, 48 and 72 h were measured in 50, 48 and 38 $\mu$gm l$^{-1}$ respectively.

3.4. Apoptosis assessment using flow cytometry and acridine orange test

The results of flow cytometry showed a significant elevation in the ratio of cells at the sub-G1 phase of cell cycle in cells treated with the synthesized ZnO-NPs compared with control. The ratio of cells at the sub-G1 phase increased dose-dependently up to 60 $\mu$gm l$^{-1}$ concentration of the synthesized ZnO-NPs (figure 8). The results of fluorescence microscopy indicated a green appearance in control cells indicating their viability. However, the cells treated with the synthesized ZnO-NPs had orange to brownish colors indicating apoptotic cells. An increase in the ratio of orange cells at higher concentrations of the ZnO-NPs showed a dose-dependent apoptotic effect (figure 9).
4. Conclusion

Fungi have gained attention as beneficial sources for synthesizing nano-materials. We here utilized *A. niger* extract to introduce a simple and fast approach to synthesize ZnO-NPs. The synthesized ZnO-NPs were revealed as spherical particles with an average length of 40 nm (the range of 30 to 70 nm). Various biological compounds in *A. niger* extract (e.g. phenols, flavonoids, and proteins) can contribute to the development of ZnO-NPs. The biosynthesized ZnO-NPs showed potent antioxidant activity and also inhibited the growth of MCF-7 cancer cells.
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Conflicts of interest

The authors declare no conflict of interests.

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