Cytotoxic and antifungal studies of biosynthesized zinc oxide nanoparticles using extract of Prosopis farcta fruit

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ABSTRACT
Zinc oxide nanoparticles (ZnO NPs) are one of the most widely used mineral particles. These nanoparticles are biocompatible and safe which can be used in medical-related industries. In this study, ZnO NPs were synthesized through an aqueous extract of Prosopis farcta fruit. Biosynthesized nanoparticles were identified through Ultraviolet–visible (UV–Vis), Powder X-Ray Diffraction (PXRD), Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-ray analysis (EDX), and Transmission Electron Microscopy (TEM) methods. The results of electron imaging showed that biosynthesized nanoparticles are hexagonal in shape with range size between 40 and 50 nm. Electron spectroscopy results showed that the bandgap of biosynthesized nanoparticles is 3.9 eV. The results of antifungal assay against Candida albicans showed 32–64 and 128–512 µg/ml values for minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), respectively. Cytotoxicity of nanoparticles against breast cancer cells (MCF7) was performed using WST-1 assay and its IC50 was obtained 500 µg/ml.

1. Introduction
ZnO is one of the hardest materials in the family of II–VI compound that due to its high bandgap (3.37 eV), great excitation energy (60 mev) and high thermal conductivity have numerous applications in glass, plastic, dyeing, food, cosmetics, and drug industries (1). There are many studies on the synthesis of ZnO nanostructures in various forms (2–5). Green synthesis is one of the most common methods for the synthesis of zinc oxide nanoparticles (ZnO NPs). High purity, low contamination, economically, biocompatibility are some of the advantages of this method (6–9). So far, many studies have been carried out on synthesis methods with various conditions for the formation of ZnO NPs, which in most cases are required high temperatures and pressures, or expensive complex equipment, or using active martial in surface (10).

Candida albicans, as a pathogen, has been introduced as the most abundant strain compared to the other C. albicans. Wide range of infections involves by C. albicans such as superficial skin and mucosal infections to deep infections of tissue (11). According to its cell wall structures, it can connect to the surface of organic and inorganic bodies and accumulate on it in a short time. Previous studies have shown that cumulative masses of Candida are strongly resistant to drugs; therefore, to prevent the accumulation, growth and proliferation of these fungi at tissue surface or inorganic levels, it is necessary to identify and introduce antifungal agents that control or inhibit the growth of fungal elements.
ZnO NPs are highly active against bacteria and can be used as antifungal agent (13, 14).

In general, metal oxide nanoparticles have different toxicity effects and its toxicity depends on their nanostructure, surface-to-volume ratio, and nature of metal part (15). Previous studies show that the concentration of released zinc ions from zinc oxide is usually considered as the toxicity of ZnO NPs. Zinc ions induce oxidative stress and cell destruction by producing reactive oxygen species (ROS) (16).

**Prosopis farcta** belongs to Papilionaceae that grows as a perennial shrub; with a height of 30–100 cm. It grows in a wide geographical regain from northern such as Russia to eastern India and Algeria. Its fruit is a dark brown pod, curved oval and leathery. In traditional medicine, *P. farcta* fruit is used as a diuretic, for constipation, hemorrhoids, toothache, diabetes, kidney stones and skin problems (17–19). In this study, ZnO NPs were synthesized using the aqueous extract of *P. farcta* fruit and then their cytotoxicity and antifungal activity were investigated.

**2. Materials and methods**

**2.1. Preparation of ZnO NPs**

Collected *P. farcta* fruit was washed, dried, and crushed. The crushed sample was weighed and shaken for 1 day after adding water as solvent (1:10 ratio). The mixture was filtered and 5 ml of obtained extract was added into 45 ml of zinc sulfate solution (3 mM) and then it was stirred at 80°C for 5 h. The resulting solution was dried at 90°C and calcined at 500°C, 600°C, and 700°C separately, in order to find optimum temperature, for 2 h. The milky resulting powder is ZnO NPs.

**2.2. Characterization of ZnO NPs**

Biosynthesized ZnO NPs were identified through PXRD, model PANalyticalX’Pert PRO MD system (Cu Kα) made in the Netherlands; UV–vis, model UV-1800 (Shimadzu) made in Japan; FESEM, model MIRA3 TESCAN made in Czech; and TEM, model CM120 made in the Netherlands. For doing cellular experiments used Invert Microscope, model HUND made in Germany; and Micro Plate Reader, model BioChrom Anthos 2020 MicroPlate Reader made in England.

**2.3. Antifungal activity**

For study antifungal activity of synthesized ZnO NPs was evaluated ten clinical isolates of *Candida albicans*. The isolates were maintained at the Kerman Medical Mycology Laboratory collection, Kerman, Iran. All strains were preliminarily identified using conventional methods including colony characteristics on CHROM agar *Candida* medium, germ tube production and confirmed by PCR-RFLP (Identification of Candida spp. isolated from oral mucosa in patients with leukemias and lymphomas in Iran).

Antifungal effects of ZnO NPs were performed according to the Clinical and Laboratory Standards Institute (CLSI) M38-A2 protocol. Various concentrations in the range of 0.03–512 μg/ml for the ZnO NPs were prepared by serial twofold dilutions in RPMI 1640 (Sigma Aldrich, USA) culture medium. The strains were subcultured on sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, USA) at 35°C for 48 h. The yeast suspensions were prepared according to the CLSI protocol. To obtain final yeast cells inoculum, suspensions were adjusted spectrophotometrically at 530 nm. The final inoculum was containing 2.5 × 10^3 CFU/ml of *C. albicans* yeast cells with dilution 1:50 in RPMI 1640 medium. In 96-well tissue culture plates ZnO NPs solutions serial twofold dilutions were prepared in RPMI 1640. In the next step, each well of the microplates containing nanoparticles was inoculated with 10 μl of the final yeast cells inoculum of candida isolates. After well-mixing, the 96-well microdilution plates were incubated at 35°C for 24 h. Voriconazole and Fluconazole were utilized with RPMI1640 culture medium as the negative control. Fungal suspension with RPMI 1640 culture medium was used as the control to determine the maximum fungus growth. All the experiments were performed in triplicates. MICs were measured as the lowest concentration of nanoparticle that resulted in >90% decrease in growth inhibition of Candida isolates compared with the control growth levels (wells without ZnO NPs). The strains *C. parapsilosis* (ATCC 22019) and *C. albicans* (ATCC 10231) were chosen as quality controls to assess every batch of MIC plates. Finally, 10 μl of the clear or invisible growth wells were inoculated on SDA plates to survey the minimum fungicidal concentration (MFC) of nanoparticles. The lowest concentration of ZnO NPs that inhibited ≥99.9% of yeast cell growth was considered as minimum fungicidal concentration (MFC).

**2.4. Evaluation of ZnO NPs cytotoxicity**

The breast (MCF7) cell line was purchased from the Pasteur Institute, Iran. In first, cells were incubated in RPMI culture medium supplemented by 10% FBS, Streptomycin (100 μg/ml) and Penicillin (100 U/ml) with the environmental condition including 37°C temperature,
CO₂ atmosphere and 5% moisture for 24 h. The cytotoxic activity of synthesized ZnO NPs was surveyed using water-soluble tetrazolium-1 (WST-1). 200 µl of cell suspension was poured in marked wells (1 × 10⁵ cell/well) and 96-well plate incubated for 24 h. Then, 50 µl of synthesized ZnO NPs (0–500 µg/ml, separately) was added to each well with 24 h incubation. In the next step, 10 µl of WST-1 solution was poured to all of the wells, and then the 96-well plate was incubated at 37°C temperature for 4 h. Finally, for the determination of dead and live cells, 100 µl of DMSO was added to all of the wells, and the optical absorbance of each well was measured at 450 nm by using ELISE reader. The cell viability of MCF7 cells was stated as a percent relative to the control.

2.5. Statistical analysis

Statistical analysis was carried out by GraphPad Prism 5. The statistical comparisons of multi-group data were surveyed using two way ANOVA. Values of *p < 0.01 were considered as statistically significant. Results were stated as mean ± SD, and tests were done in triplicates.

3. Results and discussion

ZnO NPs have been biosynthesized by using the aqueous extract of P. farcta fruit. The fruit of P. farcta includes phenolic compounds specially Flavonoids and Alkaloids. The scheme of biosynthesis of ZnO NPs was presented in Figure 1.

Figure 2(a) presented UV–Vis spectrum of biosynthesized ZnO NPs using fruit extract of P. farcta, which shows the formation of nanoparticles (20, 21). The peak in 383 nm is related to an electron transfer from valence to the conduction band of ZnO NPs (Zn₃d → O₂p) (21). The bandgap of biosynthesized ZnO NPs was calculated by using Tauc equation \((αhv) = A (hv-E_γ)^n\), where \(hv\) = photon energy, \(E_γ\) = energy bandgap, \(A\) = band tailing parameter and \(α\) = absorption coefficient (22), which is estimated as 3.9 eV (Figure 2b), that is larger than bandgap of its bulk (3.37 eV). The cause of this phenomenon is confinement effect and reduction of particle size is relative to its bulk state in biosynthesized nanoparticles (23).

![Figure 1. Schematic plan of biosynthesis process of ZnO NPs using fruit extract of P. farcta.](image)

![Figure 2. (A) UV–vis spectrum and (B) bandgap of biosynthesized ZnO NPs using fruit extract of P. farcta at 600°C.](image)
PXRD is a powerful tool for survey structure and chemical the compound of nanomaterials. The PXRD patterns of ZnO NPs at temperatures of 500°C, 600°C and 700°C are demonstrated in Figure 3. The diffraction peaks were found in levels of (100), (002), (101), (102), (110) at the range of 10–60° (24). All of the peaks suggested a hexagonal structure for the biosynthesized nanoparticles. By using the Scherer equation \( D = \frac{0.9\lambda}{\beta\cos\theta} \), where \( \lambda \) is wavelength of the utilized X-ray (1.5406 Å), \( \beta \) is the angular peak width at half maximum in radians and \( \theta \) is the Bragg’s diffraction angle, the particle size of nanoparticles was estimated as 44.39, 47.32 and 49.32 nm for biosynthesized nanoparticles at 600°C, 500°C and 700°C, respectively (22). As seen in Figure 3, by increasing sintering temperature, peaks become sharper and particles grow.

Figure 3. PXRD pattern of biosynthesized ZnO NPs using fruit extract of *P. farcta* at different temperatures.

Figure 4. (A) FESEM image, (B) TEM image, and (C) EDX spectrum of biosynthesized ZnO NPs using fruit extract of *P. farcta* at 600°C.
TEM and SEM are used for particle analysis. The SEM is a tool in nanotechnology that produces images or objects as little as 10 nm through electron bombardment. Shape, size, and placement of particles can be studied by using SEM. Figure 4(a) shows that biosynthesized ZnO NPs by using fruit extract of *P. farcta* are uniform, hexagonal, and sheet form. According to the TEM image of nanoparticles, the average particle size is 40–50 nm (Figure 4b). The graph of synthesized ZnO NPs was presented oxygen and zinc with 13.51 and 74.20 of elemental percentage, respectively (Figure 4c).

The MIC and MFC of ZnO NPs against *C. albicans* have been surveyed via turbidimetric antifungal assay, which has been calculated to be 32–64 and 128–512 µg/ml, respectively (Table 1). In agreement with previous available studies, our result showed ZnO NPs synthesized using green method possesses good antifungal activity against *C. albicans* strains. TEM and SEM are used for particle analysis. The SEM is a tool in nanotechnology that produces images or objects as little as 10 nm through electron bombardment. Shape, size, and placement of particles can be studied by using SEM. Figure 4(a) shows that biosynthesized ZnO NPs by using fruit extract of *P. farcta* are uniform, hexagonal, and sheet form. According to the TEM image of nanoparticles, the average particle size is 40–50 nm (Figure 4b). The graph of synthesized ZnO NPs was presented oxygen and zinc with 13.51 and 74.20 of elemental percentage, respectively (Figure 4c).

The MIC and MFC of ZnO NPs against *C. albicans* have been surveyed via turbidimetric antifungal assay, which has been calculated to be 32–64 and 128–512 µg/ml, respectively (Table 1).

ZnO NPs have received considerable attention because they can be produce high surface areas and unusual crystal structures (25). These nanoparticles are known as multifunction with their unique antibacterial and antifungal activities (26).

Previous studies have demonstrated the antifungal effect of ZnO NPs. Lili He et al. investigated the antifungal activities of ZnO NPs against *Botrytis cinerea* and *Penicillium expansum*. They observed that ZnO NPs at concentrations >0.3 mmol can significantly inhibit the growth of *B. cinerea* and *P. expansum*. They showed that ZnO NPs inhibited the growth of *B. cinerea* and *P. expansum* by affecting cellular functions and prevented the development of conidiophores and conidia respectively, which eventually led to the death of fungal hyphae. They concluded that ZnO NPs could be used as an effective fungicide (25).

Sawai and Yoshikawa evaluated the antimicrobial efficiency against *Saccharomyces cerevisiae, Candida albicans, Aspergillus niger* and *Rhizopus stolonifer* (25). They observed that ZnO NPs have good antifungal effects. Sangeetha Gunalan et al. compared the antimicrobial efficacy of green and chemical synthesized ZnO NPs against various bacterial and fungal pathogens. According to their study, the green ZnO NPs show more enhanced biocidal activity against various pathogens when compared to chemical ZnO NPs (26).

In agreement with previous available studies, our result showed ZnO NPs synthesized using green method possesses good antifungal activity against *C. albicans* strains.

The antimicrobial activity of the ZnO NPs closely correlates to generate various other reactive oxygen species (ROS), such as the hydrogen peroxide, superoxide radicals, hydroxyl radicals, and singlet oxygen. ROS could cause damage to DNA and proteins and led to inhibitory effects in yeast cells (27).

The cytotoxic effect of synthesized ZnO NPs was a survey against breast cancer cells (MCF7) by using WST-
1 assay. The assay is based on the cleavage of tetrazolium salts to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases, and the formation of formazan is directly proportional to the number of metabolic active cells in the culture.

Our results showed that cytotoxic activity of synthesized ZnO NPs is dependent on NPs concentration. Therefore, cell viability was decreased by increasing NPs concentration. As shown in Figure 5, fifty percent of inhibitory concentration (IC₅₀) of synthesized NPs was observed in 500 ± 5 μg/ml concentration (*p < 0.01, that inhibitory concentration is significant rather than control). Studies on the possible cytotoxic effects of ZnO NPs after exposure with cell lines have shown that they are able to damage the cell membrane, increase oxidative stress, destroy cellular organelles, and ultimately cell death (28).

Yijuan Song et al. studied the cytotoxic effect of ZnO NPs on human epithelial colorectal adenocarcinoma (Caco-2) cells. They suggested that the cytotoxicity activity of ZnO NPs is due to cellular oxidative stress (29). Studies of Gazaryan et al. and Osmond et al. on cytotoxic activity of ZnO NPs showed that Zn²⁺ ion causes mitochondrial damage due to the inhibition of mitochondrial breathing, and then lead to apoptosis and cellular death (30).

In other study, potential toxic effects of ZnO NPs evaluated against human periodontal ligament fibroblast cells (hPDLFs) and mouse dermal fibroblast cells (mDFs) by Şeker et al. Their results showed that increase in ZnO NP concentrations causes decrease of mitochondrial dehydrogenase activity of fibroblasts, and cytotoxic activity of NPs is depended to concentration of NPs and treatment time (30).

4. Conclusion
Zinc oxide is one of the materials with unique properties. It is used in energy storage devices, sensors, ceramics, and cosmetics industries. The synthesis of ZnO NPs improves the properties of this material and provides more applications. Therefore, in this study, ZnO NPs were synthesized using the aqueous extract of P. farcta fruit. Synthesized NPs were in hexagonal shape with 40–50 nm of particle size. The antifungal activity of synthesized NPs was studied on C. albicans. Our result showed that synthesized ZnO NPs possess good antifungal activity against C. albicans. In the following, cytotoxic activity of synthesized NPs is surveyed by using WST-1 assay on MCF7 cell line. Cell viability of synthesized NPs was observed 50.23% in 500 μg/ml concentration of NPs.
Disclosure statement
No potential conflict of interest was reported by the authors.

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